Modulation of Toll-Like Receptor Signalling as a New Therapeutic Principle

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Editorial

Modulation of Toll-Like Receptor Signalling as a New Therapeutic Principle

Philipp M. Lepper, 1 Martha Triantafilou, 2 Luke A. O’Neill, 3 Natalija Novak, 4 Hermann Wagner, 5 Andrew E. Parker, 6 and Kathy Triantafilou 2

1 Department of Internal Medicine V—Pneumology, Allergology and Respiratory Intensive Care Medicine, Saarland University Hospital, 66421 Homburg, Germany
2 Infection and Immunity Group, Department of Child Health, School of Medicine, University of Cardiff, University Hospital of Wales, Heath Park, Cardiff CF14 4XN, UK
3 School of Biochemistry and Immunology, Trinity College Dublin, Dublin 2, Ireland
4 Department of Dermatology and Allergology, University of Bonn, 53012 Bonn, Germany
5 Institute for Medical Microbiology, Immunology and Hygiene, Technical University of Munich, 80333 Munich, Germany
6 Institute of Molecular Medicine, Trinity Centre for Health Sciences, St. James’ Hospital, Dublin 8, Ireland

Correspondence should be addressed to Philipp M. Lepper, philipp.lepper@gmx.de

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In the 20th century, immunologists focused much of their attention in deciphering the mechanisms of adaptive immunity. As a consequence, tremendous progress was made in the field of adaptive immunity, including tolerance, mechanisms of MHC restriction, the structure and function of MHC receptors, and development and activation of B- and T-cells.

The reason why adaptive immunity was the major focus of immunologists is because adaptive immunity gives vertebrates immunity a memory and is most probably an important aspect of human evolution—responsible not only for enabling cognitive abilities but also general cultural accomplishments.

In contrast, innate immunity was sidelined. It was viewed as the most archaic of the two branches of immunity, simple and unsophisticated. Since the discovery of phagocytes by Ilya Mechnikov in 1882 and the investigations of Richard Pfeiffer, a fellow worker of Robert Koch, on “endotoxin,” the concept of innate immunity for almost 100 years was that of a static and nonspecific apparatus. It was seen as an undifferentiated system, engulfing and digesting invaders in contrast to the sophisticated framework of T- and B-cells that have elaborate clonal mechanisms to form a plethora of highly specific antibodies by DNA rearrangement.

Almost twenty years ago, Charles Janeway changed our view of the innate immune system, by publishing “Approaching the asymptote” as part of the Cold Spring Harbor Symposium on immune recognition. In this publication, he predicted that there would be molecules that were encoded in the germ line which would recognize the presence of molecules produced by broad classes of pathogens. He called these molecules pattern recognition receptors (PRRs) and the ligands that they recognise, pathogen-associated molecular patterns (PAMPs).

Janeway’s view was justified in the late 1990s with the discovery of Toll-like receptors (TLRs) and the verification that the innate immune system is actually highly specific, relying on germline-encoded pattern-recognition receptors (PRRs) that have evolved to detect components of foreign pathogens referred to as PAMPs. Over the years, research studies have shown that this system is highly specific in recognising microbial signatures; it has virtues that are equally specific and elaborate as the features of adaptive immunity; for example, TLR4 was found to recognise bacterial lipopolysaccharide (LPS) or endotoxin; TLR2 was found to recognise lipoteichoic acid (LTA) and peptidoglycan; TLR3 was able to sense double-stranded viral RNA; TLR5...
was found to recognise bacterial flagellin; TLR7 and TLR8
to sense single stranded viral RNA, whereas TLR9 was found
to delicately distinguish between methylated DNA from
host DNA and unmethylated DNA from microorganisms.
Subsequently, TLRs have been identified as operational
centers for both innate and adaptive immunity.

Cancer, infection, autoimmune, and allergic disorders
involve various complex signaling pathways; however, data
accumulates that TLRs are involved in all of these seemingly
different entities.

Their ability to initiate and propagate inflammation
makes them attractive therapeutic targets. By understanding
TLR-induced mechanisms, we can design more targeted
therapeutic interventions for inflammatory disorders in the
future. The ancient system of host defense of our cells
is an outstanding target for intervention in the relentless
efforts in finding cures. Pharmacological intervention in TLR
pathways may potentially hold great therapeutic promise.
Although for the past decades corticosteroids and in some
instances antibodies were the mainstay of anti-inflammatory
treatment, we are now at the stage of evaluating TLR path-
way modifying molecules in human diseases; this targeted
approach will bring more specificity to the treatment of a
wide panel of disorders.

Given the body of excellent literature on TLRs in disease,
we can be very optimistic that targeting them will prove
useful in human disease. In this special issue, we compiled 24
interesting papers on various aspects of human disease rang-
ing from autoimmune disorders via dermatologic disease to
cardiovascular and respiratory problems, demonstrating the
impact of TLRs in a vast number of disorders and the as-
et unrealized therapeutic potential that is awaiting to be
translated from the bench to the clinic.

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Philipp M. Lepper
Martha Triantafilou
Natalija Novak
Hermann Wagner
Andrew E. Parker
Kathy Triantafilou
Review Article

Toll-Like Receptor Signaling Pathways—Therapeutic Opportunities

Jiankun Zhu¹ and Chandra Mohan¹,²

¹ The Department of Internal Medicine (Rheumatology), University of Texas Southwestern Medical School, Dallas, TX 75390, USA
² Department of Internal Medicine/Rheumatology, UT Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-8884, USA

Correspondence should be addressed to Chandra Mohan, chandra.mohan@utsouthwestern.edu

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Toll-like receptors (TLRs) are transmembrane proteins acting mainly as sensors of microbial components. Triggering TLRs results in increased expression of multiple inflammatory genes, which then play a protective role against infection. However, aberrant activation of TLR signaling has a significant impact on the onset of cancer, allergy, sepsis and autoimmunity. Various adaptor proteins, including MyD88, IRAKs, TIRAP, TRIF, and TRAM, are involved in specific TLR signaling pathways. This article reviews the role of these molecules in TLR signaling, and discusses the impact of this pathway on various disease scenarios. Given their important role in infectious and non-infectious disease settings, TLRs and their signaling pathways emerge as attractive targets for therapeutics.

1. Introduction—The TLR Pathway

The immune system consists of two closely related systems known as the innate and adaptive immune systems. The adaptive immune system responds to specific “nonself” antigens and generates immunological memory. In contrast, the innate immune system provides an immediate first line of defense against a diverse repertoire of invading microbial pathogens. The key components of innate immunity, cognate pattern recognition receptors (PRRs), are considered to act as sentinels against both invading organisms bearing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern molecules (DAMPs). Toll-like receptors (TLRs) are transmembrane proteins acting mainly as sensors of microbial components. The first TLR to be characterized was TLR4 and the family has now been expanded to include 10 members in humans and 12 members in mice [2, 3]. Apart from the highly conserved pathogenic components of bacteria, viruses, fungi, and parasites, TLRs can also be activated by endogenous ligands such as chromatin-IgG complexes [4].

Varying TLR domain-containing adaptors such as MyD88, TIRAP, TRIF, and TRAM become engaged or activated upon DAMPs. DAMPs are exogenous molecules derived from both pathogenic and nonpathogenic microbes. In contrast, the vast majority of DAMPs are endogenous molecules released from dying host cells molecules upon cellular stress or tissue damage [2–5]. The TLRs are a family of evolutionarily conserved PRRs that play a key role in sensing the microbial world. Different TLR members are reported to recognize and respond to different PAMPs and some endogenous DAMPs [6], thus initiating innate immune responses and priming antigen-specific adaptive immunity, both in infectious and noninfectious disease scenarios.

TLRs are type I transmembrane glycoproteins which are structurally characterized by extracellular leucine-rich repeats (LPRs) and Toll/IL-1 receptor (TIR) signaling domains. The first TLR to be characterized was TLR4 and the family has now been expanded to include 10 members in humans and 12 members in mice [2, 3]. Apart from the highly conserved pathogenic components of bacteria, viruses, fungi, and parasites, TLRs can also be activated by endogenous ligands such as chromatin-IgG complexes [4].

Innate immune responses are triggered mainly by a spectrum of “danger” signals referred to as PAMPs and DAMPs. PAMPs are exogenous molecules derived from both pathogenic and nonpathogenic microbes. In contrast, the vast majority of DAMPs are endogenous molecules released from dying host cells molecules upon cellular stress or tissue damage [2–5]. The TLRs are a family of evolutionarily conserved PRRs that play a key role in sensing the microbial world. Different TLR members are reported to recognize and respond to different PAMPs and some endogenous DAMPs [6], thus initiating innate immune responses and priming antigen-specific adaptive immunity, both in infectious and noninfectious disease scenarios.

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ligation of TLRs, as diagrammed in Figure 1. TLR1, TLR2, TLR4, and TLR6 recruit TIRAP, which serves as an adaptor between the TIR domain of TLRs and MyD88, while TLR5, TLR7, TLR9, and TLR11 can recruit MyD88 directly. Binding of TLR3 and TLR4 ligands results in the recruitment of TRIF. However, the recruitment of TRIF by TLR4 needs the participation of TRAM (Figure 1). The recruitment of these adaptors triggers a cascade of signaling molecules and ultimately activates the transcription factors, NF-κB and IRFs (Figure 1). These transcription factors induce the expression of various inflammatory cytokines, type I interferons, and chemokines. NF-κB is a central regulator of immune responses involved in cell proliferation and survival and induces the expression of many cytokine and chemokine genes including IL-2, IL-6, IL-12, MCP-1, and TNF-α [1].

Except for TLR3, all TLR ligation events recruit the adaptor MyD88, followed by the IRAK family of protein kinases, leading to the activation of TRAF6. TRAF6 induces the activation of TAK1 through K63-linked polyubiquitination. NF-κB or AP-1 is then activated by the IKK complex or MAP kinases, respectively (Figure 1). On the other hand, TLR3 initiates a TRIF-dependent pathway that dictates the expression of inflammatory cytokines and type I IFNs via two independent pathways. The N-terminal domain of TRIF interacts with TRAF6 while the C-terminal domain of TRIF interacts with RIP1 and activates TAK1, both of which can activate NF-κB, resulting in the expression of inflammatory cytokines [5]. TLR3 engagement induces the expression of type I IFNs via IRF3 [7]. IRF3 is phosphorylated and activated by the IKK-related kinase, TBK1, facilitated by the recruitment of TRAF3 [8]. TLR7 and TLR9 engagement induces the secretion of inflammatory cytokines through the activation of NF-κB via MyD88. However, TLR7 and TLR9 can also induce the expression of type I IFNs through the activation of IRF7, which is phosphorylated by IRAK1, IRAK4, or IKKα, and then translocated to the nucleus where it induces the transcription of IFN-α (Figure 1) [9].

As is evident from Figure 1, the IRAK family of molecules plays a pivotal role in mediating almost all TLR-mediated functions. The IRAK family has four members: IRAK1, IRAK2, IRAK3, and IRAK4. All IRAK family members contain an amino-terminal death domain and a serine-threonine kinase domain. IRAK4 is known to be essential for TLR-IL-1R-mediated cellular responses. After TLR ligation, IRAK4 phosphorylates IRAK1 [10]. The IRAK1 gene is composed of 14 exons and is located on the X chromosome [11]. IRAK1 is the first member of this kinase family that was identified as a key component of the IL-1R signaling pathway and plays a specific and essential role in IFN-α induction downstream of TLR7 and TLR9 engagement. IRAK1 deficiency abrogates the production of IFN-α, IL-6, IL-12, and TNF-α when stimulated with TLR7 or TLR9 ligands [12, 13].

2. TLR Signaling and Disease

TLRs constitute a primary defense mechanism in both infections and some noninfectious disease settings in mammals. Activation of TLRs and the MyD88 signaling pathway plays a protective role during infection with several pathogens, including protozoan parasites [14] and pyogenic bacteria [15]. Patients with autosomal recessive MyD88 deficiency have been reported to suffer from life-threatening, often recurrent pyogenic bacterial infections. Interestingly, however, their clinical status improved in later life, alluding to the compensatory effect of adaptive immunity [15]. Consistent with their vital role in fighting infections, downregulation of TLR-related molecules or signaling has been associated with sepsis and autoimmune disease, as discussed below. On the other hand, upregulation of these molecules has been linked to cancer, allergy, other autoimmune diseases and immune abnormalities in HIV [16, 17], as detailed below.

Sepsis is a serious medical condition that is characterized by a systemic inflammatory state and the presence of a known or suspected infection, which can lead to rapid tissue damage. The recognition of LPS by TLR4 during an acute Gram-negative bacterial infection plays a key role in the pathogenesis of sepsis. Weighardt and colleagues found that MyD88-deficient mice were protected from developing sepsis in a polymicrobial septic peritonitis disease model [18]. In the IRAK4-deficient model, it was found that mice were severely impaired in their responses to viral and bacterial challenge [19]. Recently, Ferwerda and coworkers reported that polymorphisms in human Mal/TIRAP allele showed association to the pathogenesis of sepsis [20]. On the other hand, others have provided proof that Mkp-1, a key negative regulator of TLR-induced inflammation, plays a critical role in the inhibition of innate immunity during Gram-negative bacteria sepsis [21, 22]. MyD88 also plays a pivotal role in infectious diseases, as demonstrated by Naiki et al. in another model of bacterial pneumonia [23]. Collectively, these studies revealed that the TLR pathway plays a pivotal role in the pathogenesis and control of sepsis.

The importance of TLR expression and function in cancer cells and its association with tumorigenesis and tumor progression have recently been examined. TLR4 expression is linked to several cancers such as gastric cancer and human epithelial ovarian cancer. Huang et al. reported that TLR4 was expressed in murine tumor cells and that the activation of TLR4 in these cells by LPS induced the expression of various soluble factors including interleukin-6, inducible nitric oxide synthase, interleukin-12, B7-H1, and B7-H2 and rendered tumor cells resistant to CTL attack. The factors in LPS-stimulated tumor cell supernatants also had the capacity to inhibit T cell proliferation and natural killer cell activity [24]. Kelly et al., have reported that human epithelial ovarian cancer cells (EOC) ubiquitously express TLR4 and that the proliferation of EOC cells or enhanced cytokine/chemokine production by EOC cells was dependent upon expression of MyD88 [25]. MyD88-dependent signaling controls the expression of several key modifier genes in tumorigenesis and has a critical role in both spontaneous and carcinogen-induced tumor development [26]. Others have reported that gene expression differences in the TLR gene cluster (TLR10-TLR1-TLR6) were associated with a statistically significant reduced risk of prostate cancer [27].
Figure 1: The TLR signaling pathway and downstream effector molecules. Depicted are key TLR molecules, their signaling adaptors and downstream mediators that are essential for TLR signaling and function. The specific molecules in this network that are presently being interrogated as potential therapeutic targets include TLR7/9, MyD88 and IRAK1/4, as discussed in the text.

Functionally active TLR9 is also known to be expressed on several human tumors, including lung cancer, Burkitt lymphoma, cervical neoplasia, breast cancer, and prostate cancer [28–32]. Although the role of TLR9 in the development of these cancers is not fully understood, TLR9 appears to be playing an important modulatory role in several of these tumors [32–35]. Based on these findings, several TLR9 agonists, including CpG oligonucleotides, are currently in development for the treatment of cancer. These agents have the capacity to upregulate the innate immune response, as well as a humoral immune response against the tumor, as reviewed elsewhere [36]. Thus, CpG-ODN can activate plasmacytoid DCs (pDCs) to secrete type I interferon (IFN) and also promote the expression of costimulatory molecules such as CD80 and CD86, on the DCs as well as on TLR9-expressing tumors. This can then lead to the secretion of various cytokines/chemokines, and also activate natural killer (NK) cells, T<sub>H</sub>1 cells, and cytotoxic T lymphocytes (CTLs). Engaging these diverse mechanisms, TLR9 agonists have been found to be therapeutically effective in various cancer trials [36–40].

The link between TLR and autoimmune diseases has also become apparent. It was reported that inhibition of TLR4 suppressed the severity of experimental arthritis and resulted in lower IL-1 expression in arthritic joints [41]. Roelofs found that a TLR4 variant (Asp299Gly) could reduce its potency to mediate signaling in rheumatoid arthritis patients [42]. A TLR2 R753Q polymorphism has been reported to be more prevalent among reactive arthritis patients [43]. Likewise, polymorphisms in TLR2 and TLR4 may also be important in the pathogenesis of SLE in both patients [44] and mouse models [45]. Moreover, TLR7 has been suggested to be functionally involved in autoantibody production, and hence closely linked to the pathogenesis of SLE [46]. Indeed, the translocation of the X-linked TLR7 gene to the Y-chromosome has been documented to facilitate the development of fatal lupus in mice with numerous immunological aberrations, hence constituting disease genes for murine lupus [47, 48].

The impact of MyD88 and IRAK1 deficiency on autoimmunity has been quite well documented. Harada and coworkers reported that MyD88-deficiency protected MRL/lpr mice from the development of autoimmune nephritis, marked by lower levels of serum anti-double-stranded DNA (anti-dsDNA) antibodies and reduced cytokines, including interferon-α, interleukin-12, IL-6, and IFN-γ [49]. Similarly it was reported that MyD88-deficient mice were completely resistant to experimental autoimmune uveitis (EAU) and had reduced Th1, but not Th2, responses upon immunization with retinal Ag [50]. In all of the above studies of sepsis, cancer, and autoimmunity, a caveat should be kept in mind when interpreting findings derived using MyD88-deficient mice. It has been documented that MyD88-deficient mice show a loss of IL-1- and IL-18-mediated
functions indicating that the phenotypes observed in Myd88-deficient mice might arise in part because of defects in signaling downstream of the IL-1 and IL-18 receptors [51].

Autoimmunity also appears to be contingent upon the expression of another TLR signaling molecule, IRAK1. It has been reported that IRAK1-deficient mice are resistant to experimental autoimmune encephalomyelitis (EAE), exhibiting little or no inflammation in the central nervous system [52]. IRAK1-deficient mice exhibited impaired Th1 cell development with minimal IFN-γ secretion during disease induction following immunization with myelin antigen [52]. The authors suggested that the mechanism underlying this observation might relate to an impaired adjuvant effect on antigen-presenting cells as a result of suboptimal TLR activation. Our recent study has revealed that the IRAK1 gene was highly associated with both adult- and childhood-onset SLE [53]. Using the IRAK1-deficient mice, we found that in mice bearing the lupus susceptibility loci, Sle1 or Sle3, IRAK1 deficiency abrogated all lupus-associated phenotypes, including IgM and IgG autoantibodies, lymphocytic activation, and renal disease. In addition, the absence of IRAK1 reversed the dendritic cell “hyperactivity” associated with lupus [53].

Heme oxygenase-1 (HO-1), a key cytoprotective, antioxidant, and anti-inflammatory molecule, was reported to be involved in the activation of IRF3 after TLR3 or TLR4 stimulation, or viral infection. HO-1 is necessary for the expression of primary IRF3 target genes encoding RANTES, IP-10, and MCP-1. In the experimental autoimmune encephalomyelitis model, mice with myeloid-specific HO-1 deficiency developed severe disease correlating with hyperactive antigen-presenting cells, enhanced infiltration of Th17 cells, and nonregressing myelin-specific T cell reactivity [54]. Interestingly, IFN-β could alleviate these defects. The overproduction of IFN-α and IFN-α-dependent genes, such as IRF5, are additional factors that play a key role in the pathogenesis systemic lupus erythematosus [42].

### 3. Potential Therapeutic Targets in the TLR Signaling Pathway

Since the bulk of the data suggests that the TLR pathway plays a key role in multiple pathogenic processes, the targeting of either the TLRs themselves or the signals they generate is of great interest, as reviewed by O’Neill [55]. As MyD88 is clearly involved in infectious disease, cancer, and autoimmune diseases, it is obviously an attractive target for intervention in these diseases. Loiarro et al. have reported a MyD88 inhibitor ST2825, a heptapeptide analog specifically designed to inhibit MyD88 dimerization, which could control TLR-mediated inflammatory responses [56]. ST2825 is reported to inhibit MyD88 dimerization in co-immunoprecipitation experiments, and is specific for homodimerization of the TIR domains but does not affect homodimerization of the death domains. They tested ST2825 in experimental animal models of autoimmune, and inflammatory diseases, such as lupus, inflammatory bowel disease, and multiple sclerosis, and found that ST2825 could interfere with the recruitment of IRAK1 and IRAK4 by MyD88, resulting in the inhibition of IL-1β-mediated activation of NF-κB and IL-6. They also observed that ST2825 suppressed B cell proliferation and differentiation into plasma cells in response to CpG-induced activation of TLR9. Bartfai et al. have also designed a low molecular weight MyD88 mimic, “Compound 4a”, which could interfere with the interaction between MyD88 and IL-1R at the TIR domains [57]. Compound 4a inhibited IL-1β-mediated phosphorylation of the mitogen-activated protein kinase p38 in EL4 thymoma cells and in freshly isolated murine lymphocytes, and significantly attenuated IL-1β-induced fever response in vivo. These data suggest that MyD88 might be a potential target for pharmaceutical usage in several disease settings where increased TLR signaling is driving disease pathogenesis.

Modulation of another signaling molecule in the TLR pathway, IRAK4, has also been suggested to be an attractive therapeutic approach for the treatment of cancer, autoimmune and inflammatory diseases. A series of small-molecule compounds that inhibit IRAK4 has been developed by Wang et al. [58]. However, the effectiveness of these IRAK4 inhibitors in vitro and in vivo remains to be documented. Another group has reported that a dual inhibitor of IRAK1 and IRAK4 (RO0884) could efficiently inhibit both IL-1β and TNF-α induced p38 MAP kinase, c-Jun N-terminal kinase activation, and IL-6 production in human cells [59]. Through silencing IRAK1 or IRAK4 gene by siRNA, they also found that IRAK4 was essential for response to IL-1β but not to TNF-α, whereas IRAK1 was essential for cellular responses to TNF-α but not IL-1β. These data suggested that inhibition of both IRAK1 and IRAK4 kinases would be necessary to block proinflammatory cytokine production [59]. Collectively, these findings raise hope that IRAK1/IRAK4 inhibitors may be of therapeutic benefit in autoimmune and inflammatory diseases.

Administration of synthetic oligodeoxynucleotides with immunoregulatory sequences (IRS) that specifically inhibit both TLR7 and TLR9 signaling (IRS 954) has been shown to decrease the production of IFN-α by human PDCs after stimulation with DNA or RNA viruses [60]. It has been reported that (NZB×NZW) F1 mice showed decreased serum levels of antinuclear autoantibodies, proteinuria, and glomerulonephritis, with increased survival upon IRS 954 treatment [61]. Another oligodeoxynucleotides inhibitor IRS 661, which is specific to TLR7, had the capacity to significantly reduce disease in MRL/lpr mice, particularly the weights of spleen and lymph nodes and serum levels of TNF-α as compared with saline-treated control [62]. Injection of IRS 661 but not IRS 954 can significantly decreased anti-dsDNA and anti-Sm antibodies in lupus-prone mice. Most interestingly, both IRS 661 and IRS 954 could be detected in the kidneys and were found to be taken up by glomerular cells, tubular epithelial cells, and intrarenal macrophages. The mRNA levels of inflammatory chemokines CCL2 and CCL5 and their respective chemokine receptors CCR2 and CCR5 were also decreased in the kidneys. Consistent with these findings, the activity and chronicity scores for antibody-mediated nephritis in IRS
661- or IRS 954-treated mice were also reduced significantly [62]. Besides lupus, these inhibitors have also shown efficacy in other autoimmune diseases including arthritis and multiple sclerosis [63–65]. Based on a recent report suggesting [66], antagonists of this pathway are expected to confer therapeutic benefit in patients as well.

4. Conclusion

TLR molecules and their downstream signaling pathways play a critical role in activating innate immune cells and selected cell types in the adaptive arm of the immune system. Given that this pathway is aberrantly expressed or activated in several diseases, this axis constitutes an attractive target for therapeutic intervention. There is mounting evidence documenting that the crippling of this pathway at the level of TLR, MyD88, or IRAK1/4 may confer therapeutic efficacy in autoimmunity and auto-inflammatory diseases. On the other hand, the total crippling of these pathways may compromise immune defense against invading infections and immune surveillance against cancers. In fact, agonists of these pathways appear to be useful in some of these disease settings. Hence there is a need to carefully select the therapeutic target in the TLR signaling cascade, and closely regulate the degree of pathway activity so as to attain the desired therapeutic end-point. Like many other molecules in biology, the TLR signaling axis emerges as yet another “double-edged sword”.

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Review Article

DAMPening Inflammation by Modulating TLR Signalling

A. M. Piccinini and K. S. Midwood

Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College of Science, Technology and Medicine, 65 Aspenlea Road, Hammersmith, London W6 8LH, UK

Correspondence should be addressed to K. S. Midwood, k.midwood@imperial.ac.uk

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Damage-associated molecular patterns (DAMPs) include endogenous intracellular molecules released by activated or necrotic cells and extracellular matrix (ECM) molecules that are upregulated upon injury or degraded following tissue damage. DAMPs are vital danger signals that alert our immune system to tissue damage upon both infectious and sterile insult. DAMP activation of Toll-like receptors (TLRs) induces inflammatory gene expression to mediate tissue repair. However, DAMPs have also been implicated in diseases where excessive inflammation plays a key role in pathogenesis, including rheumatoid arthritis (RA), cancer, and atherosclerosis. TLR activation by DAMPs may initiate positive feedback loops where increasing tissue damage perpetuates pro-inflammatory responses leading to chronic inflammation. Here we explore the current knowledge about distinct signalling cascades resulting from self TLR activation. We also discuss the involvement of endogenous TLR activators in disease and highlight how specifically targeting DAMPs may yield therapies that do not globally suppress the immune system.

1. The Danger Hypothesis

Both infection and sterile tissue injury generate strong immune responses. This paradox was first resolved by Matzinger in 1994 who proposed that our immune system is designed to combat danger, rather than mediate recognition of non-self over self [1]. Pathogen-associated molecular patterns (PAMPs) and endogenous molecules created upon tissue injury, since called damage-associated molecular patterns (DAMPs), signal the threat of either infection or injury to the organism, independently of their non-self- or self-identity [2–5]. Among the cellular receptors that sense these danger signals, Toll-like receptors (TLRs) represent a key molecular link between tissue injury, infection, and inflammation. In the last decade, a number of endogenous molecules specifically generated upon tissue injury that activate TLRs have been identified. Some are intracellular molecules normally inaccessible to the immune system that are released into the extracellular milieu as a result of cell necrosis or activation following injury. Others are extracellular matrix (ECM) molecule fragments that are released upon tissue damage or ECM molecules that are specifically upregulated in response to tissue injury [6].

In addition to playing a key role in host defence against danger, activation of TLRs has been linked to the pathogenesis of many inflammatory and autoimmune diseases including sepsis, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), type 1 diabetes, and multiple sclerosis (MS). Hence, in recent years TLRs and associated signalling molecules have become attractive targets for their treatment and a number of inhibitors are currently in development or have progressed to clinical trials. Aberrant TLR activation is also thought to contribute to diseases with a strong association with inflammation such as cancer and atherosclerosis (reviewed in [7–11]).

One of the key questions to emerge from these studies is what factors drive TLR activation during the progression of disease. There is an increasing body of evidence to suggest that DAMP-mediated inflammation plays a vital role. It is also becoming apparent that PAMPs and DAMPs act in quite a different manner in order to stimulate an immune response. Here we review the mechanisms of DAMP recognition by TLRs, the signalling cascades, and the biological outcomes resulting from self TLR activation, focusing on the differences to non-self TLR activation.
We also discuss the evidence that implicates endogenous molecules in pathological TLR activation and examine how blockade of DAMP action may be therapeutically beneficial. Understanding more about the differences between PAMP- and DAMP-induced inflammation may enable us to specifically target inappropriate, pathogenic inflammation whilst leaving the host defence intact.

2. Endogenous Activators of TLRs

The first report of a putative endogenous activator of TLRs dates back to 2000, when heat shock protein 60 (HSP60) was shown to induce cytokine synthesis through TLR4 activation [12]. In the same year necrotic cells were found to induce pro-inflammatory and tissue repair gene synthesis and cause DC maturation in a TLR2 dependent manner, as a result of the release of their intracellular contents [13, 14]. The list of endogenous TLR2 and 4 activators has expanded quickly and encompasses other intracellular molecules such as heat shock proteins including HSP70, Gp96 [15–17], HSP22, and HSP72 [18, 19] and high-mobility group box-1 protein (HMGB1) [20–22] as well as ECM molecules such as biglycan [23], tenasin-C [24], versican [25], and fragments of ECM molecules including oligosaccharides of hyaluronic acid (HA) [26] and heparan sulfate (HS) [27].

Notably, the list of TLRs activated by endogenous molecules is also expanding. For instance, TLR1 was shown for the first time to be required, along with TLR2, for the activation of professional antigen-presenting cells by β-defensin-3, a host-derived antimicrobial peptide [28]. Self-nucleic acids have also been described as endogenous danger signals, namely, mRNA recognised by TLR3 [29], single-stranded RNA (ssRNA) sensed by TLR7 and 8 [30], and IgG-chromatin complexes recognised by TLR9 [31]. Interestingly, emerging data support the activation of TLR7 and 8 by antiphospholipid antibodies (APL) isolated from patients with APL syndrome [32, 33], as has been also shown previously for TLR2 and 4 [34–36]. A more complete list of DAMPs and their cognate TLRs can be found in Figure 1.

Given the use of E. coli to produce many of these endogenous molecules recombinantly, and the fact that most endogenous proteins activate TLR2 and 4, originally described as sensors of microbial products such as lipopolysaccharides (LPSs) and lipoproteins, the question of whether microbial contamination can partially or wholly account for DAMP activity remains a key issue. Erridge and Samani recently showed that apparent stimulation of TLR4 by saturated fatty acids was due to microbial contamination in their preparations of BSA [37]. In contrast, professional antigen-presenting cells that are not responsive to LPS were shown to be activated by necrotic cells indicating that LPS independent TLR4 activation does occur in response to endogenous ligands [38]. Similar to TLR2, TLR3 was also shown to recognize cells undergoing necrosis during acute inflammatory events, independently of viral infection [39]. Indeed, as details of the mechanisms of endogenous TLR ligand recognition emerge, it becomes clear that there are significant differences between PAMP and DAMP activation of TLRs. We discuss these differences in detail in the following sections of this review. In addition, the phenotype of mice with targeted deletions in a number of endogenous TLR activators confirms that removal of endogenous danger signals correlates with the effects of addition of exogenous DAMPs. Together these data indicate that DAMP activity is not reliant on the presence of contaminating PAMPs.

Recent data indicate that endogenous danger signals and microbial products can also cooperate in the induction of immune responses. Neither highly purified HSP preparations nor LPS alone at concentrations corresponding to those found in contaminated HSP preparations could induce pro-inflammatory cytokine production (reviewed in [40–42]). Further studies showed that HSP60 and Gp96 can tightly bind to LPS in a saturable manner and enhance its biological activity, as well as that of the TLR2 ligand Pam3Cys [43–45]. In the light of these results, the function of HSPs has been proposed to modulate early immune responses during infection by mediating a synergy between PAMPs and DAMPs. Similarly, HSP90 has also been implicated in the recognition of CpG DNA by TLR9 and the binding of HMGBs to nucleic acids is required for efficient recognition by TLR3, 7, and 9 [46–48].

3. Mechanisms of TLR Activation by DAMPs versus PAMPs

There is an increasing body of evidence that demonstrates how exogenous and endogenous activation of TLRs is mediated and this reveals that, whilst there is some overlap in molecular machinery, DAMPs possess distinct mechanisms of action to PAMPs. These similarities and differences emerge below where we explore the mechanisms of PAMP and DAMP recognition by TLRs and the subsequent TLR signalling and biological outcomes.

3.1. Ligand Recognition by TLRs

3.1.1. Exogenous Ligand Recognition. TLRs interact with a wide variety of ligands ranging from proteins and lipoproteins to nucleic acids and saccharides, all of which are different in size and chemical properties. The extracellular domains (ECDs) of TLRs contain leucine-rich repeat (LRR) motifs that are responsible for PAMP recognition [49]. The crystal structures of three TLR ECD-ligand complexes have been solved. One structure shows that TLR3 interacts with hydrophobic double-stranded RNA (dsRNA) via surface-exposed sites [50]. A second structure shows TLR1-TLR2 heterodimers bound to the hydrophobic Pam3CSK4 lipopeptide that fits in an internal hydrophobic pocket [51]. Finally, the structure of the TLR4-MD-2-LPS complex shows that TLR4 employs the co-receptor MD-2 to recognise LPS and that no direct contacts between the receptor and the ligand take place [52–54]. The latter structure also provided insights into the structure-activity relationship of the lipid A moiety of LPS. Not only the number of lipid chains [55, 56] but also the phosphate groups and their positioning in the lipid A are important factors affecting the immunological activity
of LPS [53]. This suggests that even minor modifications to ligands may cause significant changes in the responses they generate.

These three crystal structures highlight three diverse modes of exogenous ligand recognition by TLRs involving TLR homo- and heterodimerization as well as direct TLR-ligand interactions or the use of co-receptors and accessory molecules. A number of accessory molecules have been shown to assist microbial recognition by TLRs. For instance, LPS is extracted from the bacterial membrane by the LPS-binding protein (LBP) after which it is transferred to CD14. Subsequent transfer from CD14 to an additional accessory molecule MD-2 then allows TLR4-mediated LPS recognition [57]. Interestingly, in the absence of MD-2, the LPS-dependent TLR4 signalling can be reconstituted by the mite dust allergen Der p 2, which has structural and functional homology with MD-2 and mimics the activity of MD-2 by presenting LPS to TLR4 [58, 59]. HMGB1 can also mediate LPS transfer to CD14 to initiate a TLR4-mediated pro-inflammatory response [60]. In B cells, the TLR-like molecule radioprotective 105 (RP105) forms a complex with the MD-2 homolog MD-1 and is essential for regulating TLR2 and 4-dependent antibody production to the ligands lipoproteins and LPS. Conversely, in macrophages and DCs, RP105/MD-1 acts as a TLR4 decoy receptor that, by interacting directly with the TLR4 signalling complex, inhibits the ability of the receptor to bind microbial ligands [61, 62]. Other accessory molecules bind directly to TLR ligands. CD14 facilitates LPS transfer to TLR4/MD-2 and, accordingly, in the absence of CD14 rough LPS cannot initiate the TRIF/TRAM pathway and smooth LPS cannot be detected at all [63, 64]. CD14 binds also to triacylated lipoproteptides facilitating their recognition by TLR2/TLR1 complexes [65] and can enhance dsRNA-mediated TLR3 activation [66]. CD36 is a sensor of diacylated lipopeptides recognised by TLR2/TLR6 [67]. NAD(P)H oxidase 4 (Nox4) modulates the production of LPS-induced reactive oxygen species (ROS) by interacting with the cytoplasmic TIR domain of TLR4 [68, 69]. TLRs also cooperate with other families of receptors to recognise microbial ligands. TLR2 was shown to collaborate with dectin-1 in zymosan recognition [70] or with the macrophage receptor with collagenous structure (MARCO) in addition to CD14 to respond to TDM, a cell wall glycolipid from Mycobacterium tuberculosis [71]. Collectively these data point to specific and complex mechanisms at the basis of PAMP recognition, highlighted by the requirement of a number of distinct co-receptors and accessory molecules for individual ligands.

**Figure 1**: *Endogenous TLR activators*. TLRs are activated by damage-associated molecular patterns (DAMPs) including intracellular molecules released in the extracellular milieu by activated or necrotic cells and extracellular matrix molecules either upregulated upon injury or degraded following tissue damage. Known endogenous TLR activators are listed based on their biochemical nature.
3.1.2. Endogenous Ligand Recognition by TLRs. No crystal structures of TLR-endogenous ligand complexes have been reported so far. Most of the proposed endogenous TLR activators have been shown to form complexes with TLRs in vitro by means of immunoprecipitation assays and functional cell-based assays or in vivo, taking advantage of mice deficient in TLRs or their adaptor proteins. Recently, FRET confocal microscopy and GFP fragment reconstitution have been proposed to study TLR interaction and measure distances between receptors in the range of molecular interactions [72]. This technique might be of great benefit in demonstrating and characterising endogenous ligand recognition by TLRs.

There exists circumstantial evidence that DAMPs and PAMPs may occupy the same or neighbouring binding sites on TLRs. For instance, surfactant protein A was shown to downregulate peptidoglycan and zymosan induced NFκB activation and TNFα secretion by binding to the extracellular domain of TLR2 in RAW 264.7 and alveolar macrophages [73, 74]. However, some DAMPs may utilize different binding sites; whilst the TLR4 mutations D299G and T399I prevent activation by LPS, these polymorphisms confer enhanced ability of TLR4 to respond to fibrinogen [75].

There is also evidence that DAMPs require different co-receptors and accessory molecules to PAMPs. Reviewing the proposed modes of endogenous ligand recognition leads to a rational classification of endogenous molecules based on the receptor, co-receptor(s), and accessory molecule(s) requirement for recognition by TLR(s) and subsequent cellular activation that is summarized in Figure 2. A first group of DAMPs requires both CD14 and MD-2. This includes both TLR2 and 4 agonists, such as HSP60, HSP70, and biglycan, as well as TLR4 activators such as oxidized LDL and S100 proteins [15, 23, 69, 76, 77]. A second group of DAMPs requires only CD14 as an accessory molecule and these are surfactant protein A and D and lactoferrin [78–80]. A third group comprises DAMPs that have been shown to involve only MD-2 in their recognition by TLRs. Among these, Gp96 and HMGB1 activate TLR2 and 4, whereas fibronectin EDA (FNEDA) and saturated fatty acids activate TLR4 [17, 20, 22, 81–86]. A fourth group includes DAMPs that use co-receptors or accessory molecules different from CD14 and MD-2. Biglycan was recently shown to induce the NLRP3/ASC inflammasome through activation of TLR2/4 and purinergic P2X4/P2X7 receptors [87]. Versican-induced responses require TLR2, TLR6, and CD14 [25]. HA dependent TLR4 activation involves CD44 in addition to MD-2 [88]. Autoantibodies against dsDNA and nucleosomes from SLE patients induce DC activation through TRIF complexed to bound to HMGB1 [89, 90]. Similarly, HMGB1 mediates the activation of plasmacytoid DCs and B cells through TLR9 by DNA-containing immune complexes through a mechanism involving the immunoglobulin superfamily member RAGE [46]. IgG2a-chromatin immune complexes require the synergistic engagement of IgM and TLR9 to activate B cells [31], TLR7, 8, and TLR9 expressed by pDCs respond to self-RNA and -DNA respectively when coupled with the endogenous antimicrobial peptide LL37 [91, 92]. Furthermore, CD32 delivers DNA-containing immune complexes found in serum from SLE patients to intracellular lysosomes containing TLR9, leading to DC activation [89, 90]. Finally, B cells are activated by DNA- or RNA-associated autoantigens by combined B cell antigen receptor (BCR)/TLR9 or TLR7 engagement [93, 94]. This is a provisional list of endogenous activators and their accessory molecules that will certainly expand as we learn more about DAMP-TLR interactions. Collectively, these data indicate that several co-receptors and accessory molecules required for ligand recognition by TLRs are employed by both DAMPs and PAMPs. Further detailed investigation of how DAMPs are recognised by the cell is required to elucidate the precise structural organization of these receptor complexes. A signalling competent conformation of the receptor is required for TLRs to function; however it is not known whether the conformation induced by DAMPs is similar or distinct to that produced by microbial structures where sequential changes in receptor conformation occur upon ligand binding (reviewed in [95]).

3.2. TLR Signalling and Biological Outcomes. Ligand-induced receptor homo- or heterodimerization leads the cytoplasmic signalling domains of TLRs to dimerize. Despite diverse mechanisms of ligand interaction, PAMP-TLR complex crystal studies showed striking similarities in the organization of ligand-TLR dimer complexes that may apply to all TLRs. All three structures feature an “m”-shaped TLR dimeric architecture in which the C-terminal ends of the TLRs converge and, presumably, cause dimerization of the intracellular domains for signal initiation (reviewed in [97]). The resulting TIR-TIR complex initiates downstream signalling through recruitment of specific adaptor molecules. Five adaptors have been described so far: myeloid differentiation factor 88 (MyD88), MyD88-adaptor like (Mal), TIR domain-containing adaptor inducing IFN-beta (TRIF), TRIF-related adaptor molecule (TRAM), and sterile alpha and HEAT-Armadillo motifs (SARM) [98].

Depending on the adaptors recruited to the TLRs, two major intracellular signalling pathways can be activated by TLRs. The first, a MyD88-dependent pathway, is activated by all TLRs except TLR3. It involves the IL-1R-associated kinases (IRAK), IRAK-1 and IRAK-4, TNF receptor-associated factor 6 (TRAF-6), and mitogen-activated kinases (MAPK) and it culminates in the activation of the transcription factor NFκB via the IkB kinase (IKK) complex. In turn, NFκB mediates the transcription of pro-inflammatory cytokine genes. The second pathway, known as TRIF pathway, is independent of MyD88 and can be activated upon stimulation of TLR3 or 4. It leads to activation of the interferon-regulated factors (IRF) family of transcription factors via recruitment of TRIF and results in the synthesis of interferon (IFN).

TLR signalling pathways induced by endogenous molecules in different cell types are poorly investigated, but recent studies report usage of distinct adaptor molecules and induction of distinct signalling pathways downstream of TLRs when stimulated with exogenous or endogenous.
molecules. Activation of TLR4 by LPS can induce both TRIF and MyD88-dependent pathways. In contrast, we have shown that the endogenous TLR4 agonist tenascin-C signals via MyD88 [24]. Similarly, biglycan has been shown to signal through TLR2 and 4 in a MyD88-dependent manner [23].

TLR signalling results in the activation of transcription factors regulating the expression of specific genes whose products trigger various cellular responses. For example, NFκB, AP-1, and IRF5 control the expression of genes encoding inflammatory cytokines, whereas IRF3 and IRF7

Figure 2: Endogenous ligand recognition by TLRs. The co-receptor(s) and accessory molecule(s) required by DAMPs for recognition by TLR(s) and subsequent cellular activation are shown. (a) TLRs localised on the plasma membrane; (b) TLRs resident in intracellular compartments. (*) HMGB1 may require MD-2 and CD14 for TLR4 activation (see [96]).
induce the expression of type I IFN and IFN-inducible genes. Thus a large number of proteins are synthesised that mediate inflammatory and immune responses and include inflammatory cytokines such as IL-1, IL-6, TNFα, IL-12, IFNs, chemokines, adhesion molecules, costimulatory molecules, growth factors, tissue-degrading enzymes such as metalloproteinases, and enzymes that generate inflammatory mediators such as cyclo-oxygenase 2 and inducible nitric oxide synthase (iNOS).

Different microbial agents trigger multiple pathways in different cell types and induce the expression of distinct subset of genes [99–103]. A detailed comparative analysis of the biological outcomes induced by different endogenous versus exogenous TLR molecules has not been performed. However, some crucial differences between host responses to endogenous versus microbial agents are emerging. HMGBl and LPS were shown to induce distinct patterns of gene expression in neutrophils. For instance, expression of monoamine oxidase B and the anti-apoptosis protein Bcl-xl was increased in neutrophils by HMGBl but not by LPS. Furthermore, whilst the cytokine expression profile induced by HMGBl versus LPS was similar, a slower induction of TNFα mRNA occurred upon LPS stimulation compared to HMGBl [20, 104, 105]. HSP60 and LPS, in addition to synergistically enhancing IL-12p40 and IFNγ production in murine macrophages and in mHSP60-expressing COS1 cells, were shown to differentially activate APC function. Indeed, only HSP60 was able to stimulate the production of IFNα in peritoneal macrophages and bone marrow-derived DCs and IFNα release was not further increased by HSP60/LPS complexes [43]. Hyaluronan fragments generated following injury were reported to induce inflammatory responses distinct from LPS. A microarray analysis performed on the mouse alveolar macrophage cell line (MH-S) generated a list of genes that respond differently to hyaluronan and LPS. For instance, MMP13, TGF-β2, SOCS3, and other genes were induced exclusively by hyaluronan. There were also major differences in the cytokine profile induced. While some cytokines including TNFα, MCP-1, and RANTES were equally induced by both ligands, others, such as granulocyte macrophage-colony stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and IL-1α, were significantly different [88]. We have shown that tenascin-C stimulated pro-inflammatory cytokine synthesis in primary human macrophages and synovial fibroblasts in a cell type specific manner, which was significantly different from LPS. Tenascin-C dose dependently induced TNFα, IL-6, and IL-8 production in human macrophages. However, it only induced IL-6 synthesis in synovial fibroblasts, whereas LPS induced both IL-6 and IL-8 [24]. Further investigation is required to fully define the differences in signalling pathways and gene expression induced by endogenous versus exogenous TLR activators.

4. DAMPs and Disease

DAMPs are key danger signals that alert the organism to tissue damage and initiate the process of tissue repair. However, in addition to this physiological role in the response to tissue injury, there is evidence which indicates that endogenous TLR activators also contribute to the pathogenesis of many inflammatory and autoimmune diseases that are characterized by aberrant TLR activation.

4.1. High Levels of DAMPs Are Associated with Human Inflammatory Disease. The etiology of many inflammatory and autoimmune diseases is unclear; the initiating stimuli are often not well defined and the reasons why the mechanisms that ordinarily control the immune response fail are not known. However, it is clear that these diseases are characterized by an extremely destructive tissue environment. Accordingly, high levels of DAMPs occur locally and/or systemically in many of these conditions. For example, a wide range of endogenous TLR activators, including heat shock proteins, HMGBl, host DNA, fibrinogen, FNEDA, and tenascin-C, are observed in synovia of RA patients but not in synovia from normal joints or non-inflamed synovia from osteoarthritis (OA) patients [106–112]. High levels of HMGBl and tenascin-C circulate in the serum of septic patients [113, 114], and high serum concentrations of DNA-containing immune complexes are associated with SLE [46], including nucleosome-HMGBl complexes [90, 115]. In addition, elevated levels of low MW HA fragments are reported in the bronchial alveolar lavage fluid and serum of patients with inflammatory lung diseases [116–118]. In many cases levels of endogenous TLR activators are indicative of disease activity; elevated levels of extracellular HMGBl localize specifically to active lesions of multiple sclerosis (MS) patients and correlate with active inflammation [119]. Furthermore, the S100 family of calcium binding proteins have long been reliable biomarkers of inflammation in a wide variety of diseases; for example, both MRP8 and MRP14 levels in the RA synovium and synovial fluid correlate with disease activity to a degree greater than levels of C-reactive protein (reviewed in [120]). Many endogenous TLR activators are also overexpressed in tumor cells. Figure 3 summarises some of the diseases with which endogenous TLR activators are associated.

4.2. Administration of Exogenous DAMPs Induces Inflammation In Vivo. Further support of a role for endogenous TLR activators in driving disease derives from in vivo studies using experimental models of inflammatory disease. Levels of many DAMPs are elevated during the pathogenesis of numerous diseases in rodent models. In addition, delivery of exogenous DAMPs promotes inflammation in vivo via activation of TLRs. Intra-articular injection of the TLR4 activators FNEDA or tenascin-C induces joint inflammation in wild type but not in TLR4 null mice [24, 86]. Systemic injection of HS causes lethal sepsis, similar to that induced by LPS or zymosan, in wild type but not in TLR4 null mice [121]. Furthermore, DNA released from necrotic hepatocytes stimulates cytokine synthesis via activation of TLR9 during murine acetaminophen-induced liver injury [122]. These and other studies are summarized in Table 1. In addition,
many DAMPs can act as adjuvants; this has recently been comprehensively reviewed by Kono and Rock [6]. For example, purified genomic dsDNA boosted both antibody and CD8+ T cell responses in mice when injected with antigen [123]. Likewise lactoferrin, defensins, low MW HA, and HMGB1 all exhibit adjuvant properties in vivo [124–127]. Together these data show that many endogenous TLR activators exhibit pro-inflammatory properties in vivo.

4.3. Inhibition of DAMP Action In Vivo Ameliorates Disease. Whilst many DAMPs can induce TLR dependent inflammation in vivo, this does not necessarily demonstrate that these molecules are important in the progression of disease. This evidence has come from mice that do not express specific endogenous TLR activators (Table 2) and studies showing that inhibition of DAMP function can ameliorate disease in vivo (Table 3) and we review these data below.

4.3.1. Targeted Deletion of DAMPs Protects from Inflammatory Disease. Biglycan null mice have a considerable survival benefit in LPS-induced sepsis due to reduced TLR2 and 4 dependent cytokine synthesis, cellular infiltration into tissues [23], and lower levels of active caspase-1 and mature IL-1β in the kidney, lung, and circulation [87]. We have shown that tenascin-C null mice are protected from persistent joint inflammation and tissue destruction during antigen-induced arthritis [24]. In addition, mice lacking MRPL8/MRP14 complexes are protected from endotoxin-induced lethal shock and E. coli-induced abdominal sepsis [140, 143] and exhibit reduced lesion volume, brain swelling, and inflammatory cell infiltration during cerebral ischemia [142]. Furthermore, consistent with their enhanced expression during myocardial infarction, mice that lack MRPL-8/14 complexes exhibited reduced inflammatory cell infiltration upon experimental arterial injury and attenuated atherosclerotic lesions and macrophage accumulation in plaques compared with mice deficient in apolipoprotein E alone [141].
**Table 1: DAMPs induce disease in vivo.** Administration of DAMPs to rodents either intra-articularly (i.a.), intracerebroventricularly (i.c.), intraperitoneally (i.p.), intratracheally (i.t.), or intravenously (i.v.) can provoke pathological inflammation in vivo.

<table>
<thead>
<tr>
<th>Pathology</th>
<th>DAMP</th>
<th>Effect</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atherosclerosis</td>
<td>Apo CIII-rich VLDL (i.v.)</td>
<td>Stimulated TLR2 dependent monocyte activation and adhesion</td>
<td>[128]</td>
</tr>
<tr>
<td>Brain injury</td>
<td>HMGB1 (i.c.)</td>
<td>Increased cytokines, taste aversion, fever, mechanical allodynia, promotes severity of infarction</td>
<td>[129–131]</td>
</tr>
<tr>
<td>Gut inflammation</td>
<td>HMGB1 (B box) (i.p.)</td>
<td>Increased ileal mucosal permeability and bacterial translocation to mesenteric lymph nodes</td>
<td>[132]</td>
</tr>
<tr>
<td>Joint disease</td>
<td>FNEDA (i.a.)</td>
<td>Induced TLR4 dependent transient ankle swelling, cytokine synthesis, synovial inflammation</td>
<td>[86]</td>
</tr>
<tr>
<td></td>
<td>HMGB1 (i.a.)</td>
<td>Induced synovial inflammation, some pannus formation</td>
<td>[133]</td>
</tr>
<tr>
<td></td>
<td>Tenascin-C (i.a.)</td>
<td>Induced TLR4 dependent joint inflammation and tissue erosion</td>
<td>[24]</td>
</tr>
<tr>
<td>Liver injury</td>
<td>DNA</td>
<td>During acetaminophen induced cell death induced TLR9 dependent tissue injury</td>
<td>[134]</td>
</tr>
<tr>
<td></td>
<td>HMGB-1 (i.p.)</td>
<td>Aggravated ischemic reperfusion injury</td>
<td>[135]</td>
</tr>
<tr>
<td>Lung injury</td>
<td>HMGB-1 (i.t.)</td>
<td>Stimulated acute inflammatory injury, neutrophil accumulation, edema, cytokine production</td>
<td>[136–138]</td>
</tr>
<tr>
<td>Sepsis</td>
<td>HS (i.p.)</td>
<td>Induced TLR4 dependent lethality</td>
<td>[121]</td>
</tr>
<tr>
<td></td>
<td>HMGB1 (i.p.)</td>
<td>Induced partially TLR4 dependent lethality</td>
<td>[114]</td>
</tr>
</tbody>
</table>

**Table 2: Targeted deletion of DAMPs protects from experimental disease.** Mice which do not express certain DAMPs exhibit reduced symptoms of inflammatory disease or tumor metastasis in vivo.

<table>
<thead>
<tr>
<th>DAMP</th>
<th>Disease Model</th>
<th>Effect of deletion</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biglycan</td>
<td>Renal inflammation (unilateral ureteral obstruction)</td>
<td>Reduced kidney damage</td>
<td>[87]</td>
</tr>
<tr>
<td></td>
<td>Sepsis (LPS or zymosan)</td>
<td>Protected from lethality</td>
<td>[23, 87]</td>
</tr>
<tr>
<td>FNEDA</td>
<td>Atherosclerosis (Apo E deficient, high fat diet)</td>
<td>Reduced lesion size, number and macrophage infiltration</td>
<td>[139]</td>
</tr>
<tr>
<td>MRP8/MRP14</td>
<td>Abdominal sepsis (<em>E. coli</em>)</td>
<td>Reduced bacterial dissemination, systemic inflammation, liver damage</td>
<td>[140]</td>
</tr>
<tr>
<td></td>
<td>Arterial injury (femoral wire insertion)</td>
<td>Reduced inflammatory cell infiltration and neointimal formation</td>
<td>[141]</td>
</tr>
<tr>
<td></td>
<td>Atherosclerosis (Apo E deficient, high fat diet)</td>
<td>Attenuated atherosclerotic lesions and macrophage accumulation in plaques</td>
<td>[141]</td>
</tr>
<tr>
<td></td>
<td>Cerebral ischemia (cerebral artery occlusion)</td>
<td>Reduced lesion volume, brain swelling and inflammatory cell infiltration</td>
<td>[142]</td>
</tr>
<tr>
<td></td>
<td>Lethal sepsis (LPS)</td>
<td>Protects from lethality</td>
<td>[143]</td>
</tr>
<tr>
<td></td>
<td>Vasculitis (cytokine induced)</td>
<td>Reduced neutrophil accumulation and lesion severity</td>
<td>[141]</td>
</tr>
<tr>
<td>Tenascin-C</td>
<td>Destructive joint inflammation (antigen induced arthritis)</td>
<td>Protected from joint erosion and tissue destruction</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>Tumorigenesis (cross with MMTV/PyV mice)</td>
<td>Induced smaller tumor nests</td>
<td>[144]</td>
</tr>
</tbody>
</table>
Table 3: Manipulation of DAMP function ameliorates experimental disease. Therapeutic blockade of DAMP function, for example, using monoclonal or polyclonal antibodies (mAb, pAb) or specific inhibitors or silencing DAMP expression by siRNA can reduce disease progression in vivo.

<table>
<thead>
<tr>
<th>DAMP</th>
<th>Disease Model</th>
<th>Mechanism of blockade</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>Lung inflammation (bleomycin)</td>
<td>Over expression of HA synthase improved survival and decreased apoptosis</td>
<td>[145]</td>
</tr>
<tr>
<td></td>
<td>Sepsis (LPS)</td>
<td>High MW HA reduced lung neutrophil infiltration and cytokine synthesis</td>
<td>[146]</td>
</tr>
<tr>
<td></td>
<td>Brain injury (transient ischemia)</td>
<td>mAb reduced infarct size and severity, locomotor function, cytokine synthesis</td>
<td>[130]</td>
</tr>
<tr>
<td></td>
<td>Colitis (DSS, TNBS)</td>
<td>pAb, ethyl pyruvate ameliorated disease, reduced cytokine synthesis, associated tumors</td>
<td>[147, 148]</td>
</tr>
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<td></td>
<td>Lung inflammation (LPS)</td>
<td>pAb decreased neutrophils, lung edema</td>
<td>[136, 138]</td>
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<td></td>
<td>Lung injury (ventilator)</td>
<td>Ab improved oxygenation, neutrophil influx, cytokine synthesis</td>
<td>[149]</td>
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<td>Hepatic ischemia reperfusion injury</td>
<td>pAb decreased liver damage</td>
<td>[135]</td>
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<td>Acute pancreatitis (duodenal loop closure)</td>
<td>pAb improved pancreas and lung damage, aggravated bacterial translocation to pancreas</td>
<td>[150]</td>
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<td>Hemorrhagic shock (blood withdrawal)</td>
<td>pAb improved survival, ameliorated ileal permeability, decreased serum cytokines</td>
<td>[151]</td>
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<td></td>
<td>Hemorrhagic lung injury (cyclo-phosphamide)</td>
<td>pAb reduced pulmonary edema, neutrophil accumulation, lung permeability</td>
<td>[152]</td>
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<td></td>
<td>Sepsis (LPS, E. Coli, cecal ligation and puncture)</td>
<td>Ethyl pyruvate, stearoyl lysophosphatidylcholine, nicotine (inhibit secretion) protected from lethality</td>
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<td>Neural ant-inflammatory peptides vasoactive intestinal peptide and urocortin rescue lethality</td>
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<td>Cisplatin (nuclear sequestration of HMGB1) protected from lethality</td>
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<td>SNX-7081 (inhibitor) ameliorated disease, joints returned to normal</td>
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<td>SNX-7081 (inhibitor) ameliorated disease, joints returned to normal</td>
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<td>BX-2819 (inhibitor) inhibited tumor growth</td>
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<td>Rheumatoid arthritis (Collagen)</td>
<td>SNX-7081 (inhibitor) ameliorated disease, joints returned to normal</td>
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<td>ONO-5046 (inhibitor) reduced disease severity</td>
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<td>ONO-5046 (inhibitor) reduced incidence and severity of disease, ablated cartilage destruction</td>
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<td>GW311616A (inhibitor) ameliorated liver damage, decreased neutrophil infiltration</td>
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<td>pAb, DNA binding box A reduced severity of established joint disease</td>
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<td>SNX-7081 (inhibitor) ameliorated disease, joints returned to normal</td>
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<td>GW311616A (inhibitor) ameliorated liver damage, decreased neutrophil infiltration</td>
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<td>pAb reduced lung metastasis</td>
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<td>siRNA reduced lung metastasis</td>
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4.3.2. DAMP Antagonists Ameliorate Disease. The fact that blockade of DAMP function ameliorates disease \textit{in vivo} further supports a role for endogenous TLR activators in inflammatory disease. The best example of how this can be achieved is with HMGB1 (reviewed in [170–174]), although manipulation of other DAMPs including HA, neutrophil elastase (NE), and versican can all protect against disease (Table 3). DAMPs comprise an enormously diverse subset of molecules. As such there exists a number of different mechanisms to prevent their inflammatory action, some of which are described below.

(i) Blockade of TLR Activation. One strategy that has proved effective is to manipulate the function of individual DAMPs by preventing TLR activation at the cell surface. Administration of polyclonal anti-HMGB1 antibodies or the DNA-binding A box of HMGB1, a competitive inhibitor of the pro-inflammatory B box, can reverse the lethality of established sepsis [114, 153, 154] and ameliorate collagen-induced arthritis in rodents (reviewed in [161]). However, whilst some reports demonstrate that monoclonal anti-HMGB1 antibodies are efficacious preventing organ damage in experimental models of sepsis [155], others suggest that monoclonal antibodies are not effective in suppressing arthritic disease \textit{in vivo} [175]. This may be due to the multivalent nature of the mode of action of HMGB1. One alternative approach may be to use synthetic, bent oligonucleotides which have a high affinity for HMGB1, and suppress HMGB1-induced proliferation and migration of smooth muscle cells \textit{in vitro} [176]. Another approach may be the use of an engineered mutant fragment, HMGB1 Mut (102–105) carrying two glycine substitutions, that decreased TNFα release induced by the full-length HMGB1 protein in human monocyte cultures [177]. In addition, the N-terminal domain of thrombomodulin, an endothelial anticoagulant cofactor, exerts anti-inflammatory effects in a model of lethal endotoxemia partly by binding to and sequestering HMGB1 [178].

(ii) Prevention of DAMP Accumulation. DAMPs can be generated by release from necrotic cells, secretion from activated cells, cleavage of larger molecules, or specific upregulation upon tissue injury. Manipulation of tissue levels of DAMPs may provide another window of therapeutic opportunity. Indeed, ethyl pyruvate, stearoyl lysophosphatidylcholine, and nicotine have been shown to be efficacious in ameliorating experimental sepsis by preventing HMGB1 release during experimental sepsis [156–158]. However, the mechanism by which they do so is unclear and these compounds are likely also to affect numerous other cell processes. HMGB1 is released from cells by two distinct mechanisms: it is liberated from cells undergoing necrosis [179], or it is hyperacetylated and then actively secreted from stimulated cells. This non-classical secretion pathway is distinct from the passage through the ER and Golgi taken by signal tagged proteins, instead requiring the microtubule cytoskeleton [180]. Other DAMPs including the S100 proteins are also secreted in the same way [181] and targeting this pathway therefore may potentially offer a means to modulate the release of intracellular DAMPs.

One class of DAMPs comprises ECM fragments generated by release from intact matrices. Inhibition of this process has been demonstrated; for example, release of immune-stimulatory HS fragments from the ECM \textit{in vivo} can be mediated by the proteolytic action of elastase [182]. Injection of elastase into the peritoneal cavity of mice caused the release of HS and induced sepsis, nearly as effectively as direct injection of HS or LPS [121]. Thus therapeutic measures aimed at blocking elastase could reduce the production of endogenous TLR4 activators. Indeed, pre-treatment with NE inhibitor before induction of hepatic ischemia-reperfusion injury ameliorated liver damage [168]. HS fragments are also generated upon ECM oxidation by reactive oxygen species (ROS). Extracellular superoxide dismutase (EC-SOD) is an antioxidant enzyme that protects the lung from oxidant-mediated inflammation. One way in which this is to protect HS from oxidative fragmentation; bronchoalveolar lavage fluid from EC-SOD knockout mice after asbestos exposure showed increased HS shedding from the lung parenchyma [183]. An alternative strategy may be to alter the balance of immune-silent intact ECM molecules versus immune-stimulatory fragments either directly or indirectly. Specific over expression of high MW HA in the lung has been achieved using transgenic mice that constitutively express HA synthase. These mice showed that improved survival and decreased apoptosis during bleomycin induce lung inflammation [145].

Finally, for DAMPs whose expression is specifically upregulated during inflammation it may be possible to manipulate this induction of expression. Indeed, knockdown of versican expression in Lewis lung carcinoma cell lines (LLC) ablated their tumorigenic capability, promoting mouse survival and reduced metastasis, whilst overexpression of versican in LLC lines with low innate metastatic potential increased lung metastasis [25]. Together these data indicate that endogenous TLR activators significantly contribute to driving inflammatory disease \textit{in vivo} and suggest that targeting this method of TLR activation may potentially be of therapeutic value in combating disease.

5. Conclusions and Perspectives: Targeting DAMPs in the Clinic?

Current strategies in clinical development for TLR blockade include (1) global blockade of individual TLR function using neutralizing antibodies, soluble TLR extracellular domains (ECDs), natural antagonists, and small molecule inhibitors, (2) inhibition of signalling pathways-activated downstream of TLR stimulation using small molecules to target MyD88/TRAF1/IRAK complex formation, MAPK, or IKK activity, or (3) using PAMP antagonists such as LPS inhibitors. Some of these compounds have reached phase II clinical trials and the results are currently awaited, whilst others, particularly those targeting common signalling pathways such as MAPK, have proved to be of limited efficacy (reviewed in [11]).
Suppressing DAMP activation of TLRs offers a host of new potential targets for treating inflammatory diseases that may be viable alternatives to current approaches. Evidence that blockade of these mediators can ameliorate disease in human studies is beginning to emerge. Hyaluronate improves pain and prostaglandin E (PGE) levels in patients with RA [184], transfer of HSP-specific regulatory T cells inhibits inflammation in animal models of arthritis and exhibited promising results in preliminary clinical trials [185], HMGB1 antibodies prevent the activation of cells by serum from SLE patients [46], and the neutrophil elastase inhibitor sivelestat improves the mortality rate of patients with sepsis [186, 187]. By carefully choosing a target unique to the response to tissue damage, and not to pathogen mediated activation of the immune response, this strategy may have the additional advantage of leaving the host response to infection intact. Given the evidence that supports the idea that distinct molecular machinery is required for DAMP activation of TLRs, another strategy would be to block co-receptors or accessory molecules essential for DAMP activation. In addition a comparative analysis of adaptors, kinases, and transcription factors involved in signalling activated by DAMPs versus PAMPS may highlight key differences that, if selectively targeted, could lead to specific therapies engineered to silence danger signals without compromising the host immune defence.

We have highlighted here the possible levels of intervention in DAMP activation of TLR-mediated inflammation, namely, manipulating DAMP activation of TLRs or controlling tissue level of DAMPs. Whilst these strategies are efficacious in preventing experimental disease, there is also evidence that preconditioning with DAMPs can have the same effect. Administration of small doses of HMGB1 one hour prior to induction of hepatic reperfusion injury protected from liver damage and reduced serum TNFα and IL-6 levels via inhibition of TLR4 signalling [188, 189] and lactoferrin can protect from lethal E.coli injection [190].

It is apparent that low tissue levels of DAMPs are beneficial during tissue repair to induce a resolvable, physiological immune response. In contrast, high levels of DAMPs are generated during chronic inflammation. We propose a situation where a damage chain reaction occurs: increasing levels of pro-inflammatory DAMPs create more tissue damage which significantly amplifies the tissue levels of DAMPs which go on to create yet more tissue damage ad infinitum. These tissue levels of DAMPs become harmful and mediate a non-resolving perpetual inflammatory state (Figure 4). Thus targeting DAMP-mediated activation of TLRs may block this chronic inflammatory loop, although it will be important to assess whether total blockade of DAMP function will compromise tissue repair to any deleterious extent.

In addition, in the destructive milieu that occurs during inflammatory disease there are likely to be high levels of many DAMPs. Working out which are keys to disease pathogenesis may not be a trivial matter, and combinations of inhibitors may be needed to successfully dampen down endogenously driven inflammation. Alternatively, hierarchies may exist amongst DAMPs such as those that exist for inflammatory cytokines, for example, where TNFα induces a cascade of cytokine synthesis. Indeed, low MW HA induces β-defensin2 via TLR2 and 4 activation in murine keratinocytes ex vivo and in vivo [191]. These DAMPs may be key targets to prevent the induction of an autocrine loop of inflammation. Unravelling potential hierarchies amongst DAMPs represents a major challenge for future investigations. These may be aided by approaches such as microarray and deep sequencing technologies, as well as proteomewide screening, to enable the comparison of the global effects of different DAMPs on inflammatory gene expression. Likewise, examining the stimuli that induce DAMP expression or release upon tissue injury will be important in establishing a “chain of command” of DAMP action. In parallel, the development of validated reagents and tools which serve to ablate the expression or function of individual DAMPS will yield key information about redundancy and co-dependency.

The threshold of DAMP(s) required to induce disease may vary upon the duration and degree of host tissue damage. However, current knowledge about the kinetics of expression or release of DAMPs and their turnover during disease progression is limited. Indeed, on one hand, validated commercial assays for measuring various endogenous danger signals are often unavailable or prohibitively expensive. On the other hand, access to patient specimens and pathological data is in many cases restricted. Thus, the correlation between degree of tissue damage and levels of DAMP(s) is either unknown or limited to small sample size, often representative of end stage of disease. The threshold of
DAMPs required to trigger chronic inflammation may also depend on a variety of host genetic factors, including single-nucleotide polymorphisms (SNPs), which can affect how humans respond to injury and develop disease. Examining the role of DAMPs within the context of different genetic backgrounds will also be key to dissecting out their role in inflammatory disease. The use of larger patient sample sizes including diverse genetic populations and a befitting proportion of male and females will be vital. In addition, the development of mouse strains with much greater DNA diversity than strains traditionally employed may provide mice with combinations of different traits that more closely reflect the genomic variations of humans in preclinical studies. We expect that the next few years will provide a much more concrete picture of how DAMPs link tissue damage to chronic inflammation as an increasing number of tools become available.

Finally, a normal wound healing response does not typically lead to chronic inflammation. This is, in part, because a number of mechanisms exist to negatively regulate TLR activation. These include the release of soluble decoy TLR receptors, intracellular inhibitory molecules such as IRAK-M, SOCS1, Tam family kinases, and transmembrane regulators such as SIGIRR (reviewed in [5, 193]). Viruses have also evolved mechanisms to target adapters in TLR signalling: A46R from vaccinia virus, which sequesters MyD88, Mal, Trif, and Tram [194], and NS3/4A from hepatitis C virus, which degrades Trif [195]. In addition, recently, microRNA, mir-147, whose expression is induced upon stimulation of multiple TLRs, was shown to attenuate TLR stimulation-induced-inflammatory response in macrophages [196].

However, these pathways do not appear to discriminate between distinct methods of TLR stimulation and act on DAMP- and PAMP-mediated activation alike. Chen et al. recently identified one way in which specific activation of TLRs by DAMPs, but not PAMPs, may be inhibited (reviewed in [197]). CD24, or heat stable antigen, is a GPI anchored protein that binds to DAMPs such as HMGB1, hs70, and hsp90 in order to suppress their activation of inflammatory signalling pathways. CD24 null mice exhibit increased susceptibility to DAMP-, but not PAMP-, induced inflammation. This is mediated at least in part through CD24 association with Siglec-10/G causing activation of associated phosphatases which are proposed to repress DAMP-initiated signalling. Dysfunction of this pathway might contribute to the etiology of autoimmune diseases and likewise may offer a means to selectively inhibit DAMP activity [198]. In addition, sTLR2 can blunt immune responses without preventing microbial recognition: mice injected with Gram positive bacteria together with sTLR2 exhibited reduced inflammatory cytokine levels and cell migration but this did not compromise their ability to clear live Gram-positive bacteria-induced infection [199]. As such enhancing naturally suppressive mechanisms may also be a viable strategy for reducing inflammation.

Thus DAMPs appear to be a double-edged sword. While being vital for tissue repair, they also play a role in the pathogenesis of many inflammatory and autoimmune diseases that feature aberrant TLR activation. In these diseases harmful stimuli cause tissue damage; in an attempt of tissue healing, inflammatory responses are initiated and generate DAMPs that induce an autocrine loop of inflammation. Understanding why the natural mechanisms that keep DAMP-mediated inflammation in check fail in disease, as well as dissecting out which mechanisms of TLR activation and signalling are unique to DAMPs, may enlighten our approach to engineering targeted and efficacious therapies designed to dampen inflammation.

Acknowledgments

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Toll-Like Receptors, Tissue Injury, and Tumourigenesis

Savvas Ioannou and Michael Voulgarelis

Department of Pathophysiology, Medical School, National University of Athens, 75 Mikras Asias Street, 11527 Athens, Greece

Correspondence should be addressed to Michael Voulgarelis, mvoulgar@med.uoa.gr

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Toll-like receptors (TLRs) belong to a class of molecules known as pattern recognition receptors, and they are part of the innate immune system, although they modulate mechanisms that impact the development of adaptive immune responses. Several studies have shown that TLRs, and their intracellular signalling components, constitute an important cellular pathway mediating the inflammatory process. Moreover, their critical role in the regulation of tissue injury and wound healing process as well as in the regulation of apoptosis is well established. However, interest in the role of these receptors in cancer development and progression has been increasing over the last years. TLRs are likely candidates to mediate effects of the innate immune system within the tumour microenvironment. A rapidly expanding area of research regarding the expression and function of TLRs in cancer cells and its association with chemoresistance and tumourigenesis, and TLR-based therapy as potential immunotherapy in cancer treatment is taking place over the last years.

1. Introduction

Toll-like receptors (TLRs) are a family of transmembrane receptors that play a key role in the nonspecific or innate immune defense, particularly in inflammatory response against various invading exogenous pathogens, by recognising receptor-specific pathogen-associated molecular patterns of highly conserved pathogenic components of bacteria, viruses, fungi, and parasites [1]. Once these pathogens have breached physical barriers such as the skin or intestinal tract mucosa, they are recognized by TLRs which activate immune cell responses against their structurally conserved molecules. The most important role of TLRs in host defense is the regulation of innate and adaptive immune responses by epithelial cells, the first line of defense at mucosal sites such as the respiratory, genitourinary, and gastrointestinal tracts, and the skin. TLRs also have a crucial role in mediating leukocyte recruitment to infected tissues and the uptake of microorganisms by phagocytic cells [2, 3]. Activation of antigen-presenting cells (APCs) such as dendritic cells (DCs) and stimulation of both T- and B-cell-mediated immune responses are due mostly to ligation of TLRs [4].

Moreover, TLRs have an important role in maintaining tissue homeostasis by regulating tissue repair and regeneration. TLR ligands in this case can be either microbial (exogenous) or host derived (endogenous) [5]. In addition, TLR signalling has also been shown to regulate apoptosis with the expression of antiapoptotic proteins or inhibitors of apoptosis.

They derive their name from their similarity to the protein coded by the Toll gene identified in Drosophila in 1985 by Christiane Nüsslein-Volhard [6]. The human TLR family consists of currently ten members, which are structurally characterized by the presence of a leucine-rich repeat (LRR) domain in their extracellular domain and a Toll/Interleukin (IL)-1 receptor (TIR) domain in their intracellular domain [7]. Epidemiologic studies of the many single nucleotide polymorphisms that have been identified in various TLR genes have been studied in relationship to tumourigenesis. Studies of common genetic variants in various populations and growth stimulation of cancer cell lines suggest that TLR4 plays an important role in the development of H. pylori-associated gastric cancer [8, 9]. Other studies have found that common polymorphisms in TLR2 are associated with increased risk of colorectal
cancer [10], gastric cancer [11] and lymphoma [12], while a specific haplotype in TLR10 is associated with increased risk of nasopharyngeal cancer [13]. A recent study revealed a significant association of a common genetic variant within the TLR10-TLR1-TLR6 gene cluster with decreased risk of initial development of prostate cancer [14].

The relationship between inflammation and tumourigenesis and tumour progression is widely accepted. Numerous studies have provided convincing evidence that bacterial- and viral-induced inflammatory process can mediate tumourigenesis. Moreover, it is well known that regular intake of nonsteroidal antiinflammatory drugs lowers the risk of developing some types of cancer.

Due to the important role in inflammation and tissue regeneration, TLRs are likely candidates to mediate effects of the innate immune system on tumourigenesis. TLR expression and function in cancer cells and its association with the inflammation process and tumourigenesis will be discussed here.

2. Toll-Like Receptors Signalling Pathways

TLRs belong to a class of molecules known as pattern recognition receptors. The ligands for these receptors are components of pathogenic microbes and are often called pathogen-associated molecular patterns (PAMPs). The recognition of PAMPs by TLRs is a cornerstone of innate immunity and is associated with the inflammatory response to pathogens in both vertebrate and invertebrate species [15]. In addition to recognizing ligands derived from foreign microbes TLRs, in particular TLR2 and TLR4 have been reported to bind numerous endogenous ligands termed damage-associated molecular patterns (DAMPs) that are released from injured and inflamed tissue [5]. Thus, the TLR-mediated immune response may be activated in the absence of foreign microbes. Upon binding of a microbial ligand, TLRs activate signalling pathways that stimulate cytokine production and other parts of the innate immune response. Well-known TLR microbial ligands are lipopolysaccharide (LPS), a membrane component of Gram-negative bacteria, bacterial lipoproteins, lipoteichoic acid and fungal zymosan, bacterial flagellin, double-stranded RNA, and single stranded RNA. TLR4 senses Gram-negative bacteria by binding LPS. Bacterial lipopeptides and lipoteichoic acids (LTAs) stimulate TLR2 responses. TLR3 and TLR7 sense viral infections by recognizing double-stranded and single-stranded RNA, respectively. TLR9 recognizes nonmethylated CpG-containing DNA from bacteria and viruses.

TLRs that mainly serve to detect bacterial LPS and lipoproteins are located on the cell surface (TLR1, TLR2, and TLR4-6) whereas those that mainly recognize viral RNA and bacterial DNA are located in late endosome-lysosomes in which these materials are processed (TLR3, TLR7, TLR8, and TLR9). TLR1, TLR2, TLR4, and TLR6 initiate signalling by heterodimerization. TLR2 forms heterodimers with TLR1 or TLR6 which recognize bacterial triacylated and diacylated lipoproteins, respectively [7]. A wide range of DAMPs including heat shock proteins, high-mobility group box 1 (HMGB1), uric acid crystals, hyaluronan, heparin sulfate, messenger RNA, surfactant protein A, and various products of the extracellular matrix such as fibronectin and fibrinogen have been suggested to activate TLRs [16, 17].

After ligation of TLR ligands, TLRs dimerize and transmit signals throughout the cell through one or more of four adaptor proteins: myeloid differentiation primary response gene 88 (MyD88), toll/interleukin-1-receptor-domain-containing adaptor inducing interferon-β (TRIF), toll/interleukin-1-receptor-domain-containing adaptor protein (TIRAP), and TRIF-related adaptor molecule (TRAM). Whereas MyD88 is part of the signalling cascade of all TLRs except for TLR3, TRIF only interacts with TLR3 and TLR4 (Figure 1).

TLR2 or TLR4 agonists stimulate the MyD88 signaling pathway in APCs such as macrophages and DC, which leads to subsequent downstream activation of the major transcription factors, the nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) and mitogen-associated protein (MAP) kinase signaling pathways (such as the ERK-CREB pathway, the JNK-AP1 pathway, and the p38 pathways) [18]. This leads to the rapid expression of inducible nitric oxide synthase (iNOS) and a wide variety of proinflammatory cytokines, chemokines, and their receptors, including tumor necrosis factor alpha (TNF-α), interleukin (IL)-1α, IL-1β, IL-1ra, IL-6, IL-8, IL-10, IL-12p40, IL-23, and macrophage inflammatory protein (MIP)-1α, and MIP-1β (Figure 2) [19, 20]. These factors initiate the inflammatory response, increase vascular permeability, direct DC and macrophage migration from the periphery to the central lymphoid organs, and regulate various aspects of adaptive immunity development.

Recently, naturally arising regulatory T-cells (Tregs) have been shown to express TLRs. Tregs originate from the thymus and are characterized by the expression of Foxp3 as a key control gene for their development and function. Their pivotal role is maintaining immunological self-tolerance. Recent data suggest that the activation of TLRs on Tregs can increase or decrease their suppressive activity, thus providing an important link between innate and adaptive immune responses [21]. Milkova et al. suggested an important role of the NF-κB signalling pathway for the induction and modulation of suppressive function of Tregs, if they are confronted with TLR4 ligands such as LPS [22].

3. Toll-Like Receptors, Tissue Repair, and Fibrogenesis

The tissue repair and regeneration process has been reported to depend on MyD88 signalling. The importance of this signalling pathway has been demonstrated by a recent study which showed that wound healing was impaired in MyD88-deficient mice [23]. MyD88 is a critical signal adaptor for TLRs 2, 4, 5, 7, 8, 9, and 11 whereas TLR3 signals in a MyD88-independent manner through the TRIF adapter pathway.

TLR activation in wound healing appears to be mediated by two entirely different classes of ligands: (1) in organs that are in direct contact with microbiota (intestine, skin, liver),
Figure 1: Toll-like receptor (TLR) signalling pathways. Membranal TLRs (represented by TLR4) recognize external ligands (exogenous and endogenous), while cytoplasmic TLRs (TLR3) recognize intracellular signals. When activated, the majority of TLRs induce activation of NF-κB (early phase of NF-κB activation) and cytokine production in a MyD88-dependent manner; while TLR4, like TLR3, can also signal in a MyD88-independent manner and induce the expression of type I interferons (IFN) and IFN-inducible proteins in addition to a late phase NF-κB activation.

Tissue injury leads to a breakdown of protective barriers and subsequent TLR activation by bacterial PAMPs. (2) In many organs such as liver, heart, and kidney, tissue injury leads to release of DAMPs from dead and dying cells resulting in the activation of TLRs and “sterile inflammation.” The release of endogenous TLR ligands predominantly occurs after massive tissue injury, especially where a significant percentage of cells undergo necrosis such as in ischaemia-reperfusion injury. Activation of TLRs modifies tissue injury in positive or negative fashion either by recruiting inflammatory cells that release cytotoxic mediators or by activating cytoprotective signals. TLRs exert a cytoprotective role and prevent tissue injury in the lung and the intestine. In bleomycin-induced lung injury, TLR2-TLR4- and MyD88-deficient mice display an increased degree of lung injury despite reduced recruitment of inflammatory cells. Jiang et al. showed that blocking the endogenous ligand hyaluronan by a peptide-based approach, the pattern of lung injury was highly similar to that of MyD88 and TLR2-TLR4-deficient mice [24].

In contrast, ischaemia-reperfusion injury represents the scenario in which a profound injury-promoting role of TLR2 and TLR4 has been most thoroughly established. Several studies have shown the protection of TLR4-mutant or deficient mice after hepatic and cardiac ischaemia-reperfusion [25, 26]. DCs are the most likely candidate to mediate injury following ischaemia-reperfusion injury. After hepatic ischemia-reperfusion, wild-type but not TLR4-mutant dendritic cells displayed a strong increase of TNF-α production [27], a well-known mediator of hepatic ischemia-reperfusion injury that is rapidly and potently released from macrophages and DC following TLR activation [28, 29]. Therefore, the most likely scenario is that HMGB1 is released from necrotic parenchymal cells to activate TLR4 on DC which in turn releases TNF-α to promote tissue injury.

TLR receptors are also involved in the regulation of epithelial proliferation and angiogenesis following injury. TLR4- and MyD88-dependent signals following dextran sulfate sodium-(DSS-) mediated colonic injury, are required to induce cyclooxygenase 2-(Cox2-) mediated generation of prostaglandin E2 (PGE2) and to stimulate epithelial cell proliferation [30]. The MyD88-Cox2 signal that promotes regeneration is largely provided by macrophages which migrate toward the site of injury to stimulate the proliferation of epithelial progenitors. PGE2 is one crucial growth-promoting signal provided by stimulated macrophages. The mechanism for improved epithelial repair may be through...
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Figure 2: Toll-like receptor (TLR) signalling is mediated by at least two distinct pathways. After recognition of a pathogen-specific molecular pattern, TLRs are capable of differentially activating distinct downstream signalling events via different cofactors and adaptor proteins mediating diverse immune responses [1]. The MyD88-dependent TLR signalling pathway is activated via the conserved, cytoplasmic TIR domain, which provides a scaffold for recruitment of the adaptor molecule MyD88 and serine/threonine kinases of the IL-1R-associated kinase (IRAK) family. Following IRAK autophosphorylation, the TRAF6 adaptor protein interacts and induces translocation of the transcription factor NF-κB to the nucleus, resulting in transcriptional activation of genes encoding cytokines and chemokines (TNF-α, NO, COX-2, SOCS (for "suppressor of cytokine signalling"), IP-10, IFN-b and IL-1, 6, 8, 10, 12). In addition, expression of proteins involved in apoptosis and production of adherent and costimulative molecules such as ICAM1, occur. Moreover, TLRs bridge the signalling pathway via ECSIT (for "evolutionarily conserved signalling intermediate in Toll pathways") to TRAF6 for p42/p44 mitogen-activated protein kinase (MAPK) kinase (MKK), p38, and JNK in response to specific bacterial products [2]. The MyD88-independent TLR signalling pathway is activated via TIRAP and results in activation of the dsRNA-binding protein kinase PKR. This protein has been proposed to be a central downstream component of both the TIRAP- and MyD88-dependent signalling pathways and could mediate potential crosstalk between them. The MyD88-independent pathway appears to utilise both IFN-regulatory factor 3 (IRF3) and NF-κB, and results in the expression of IFN-g-inducible genes including IP-10.

PGE2-dependent activation of epidermal growth factor receptor (EGFR) [31]. The TLR4-MyD88 signaling axis is also involved in the regulation of angiogenesis to restoring blood flow to the site of injured tissue. Promotion of angiogenesis by TLR4 appears to be restricted to specific pathophysiological circumstances. In skin wounds, absence of MyD88 results in slower wound healing, decreased angiogenesis, and decreased vascular endothelial growth factor (VEGF) [23]. TLR4 activation alone is not sufficient for the induction of VEGF, but requires the presence of adenosine, and is largely mediated by a transcriptional upregulation of HIF1α which binds to a known hypoxia response element in the VEGF promoter [32].

Moreover, there is accumulating evidence that TLRs directly target fibroblasts to induce activating signals. One main mechanism by which TLRs modulate fibrogenic responses is through the transforming growth factor beta (TGF β) signaling pathway. In the liver, TLR4 and MyD88 are required for the development of fibrosis in chronic hepatitis. Activation of TLR4 sensitizes hepatic stellate cells, the main precursor of fibroblasts in the liver, toward the effects of TGFβ and thereby, promotes collagen production [33]. This effect is mediated by the downregulation of an inhibitory TGFβ pseudoreceptor, Bambi. Bambi down-regulation is mediated through a MyD88-dependent and TRIF-independent pathway [33]. In addition, TLRs promote proinflammatory and antiapoptotic signals in fibroblast populations through NF-κB [34, 35].

4. Toll-Like Receptors and Apoptosis

Toll-like receptors are potent activators of the NF-κB pathway [4]. NF-κB regulates the transcription of a number of antiapoptotic genes such as Bcl-2, iNOS, c-FLIP, inhibitor of apoptosis (IAP), and TRAF molecules [36]. There is increasing evidence that TLRs provide signals to promote the survival of epithelial cells under stress conditions. It has already been demonstrated that TLRs exert a cytoprotective role in the lung and the intestine [24]. Additionally, TLR4-MyD88-NF-κB-Cox2 axis is involved in protection from
apoptosis in normal as well as premalignant cells of the colon [30]. Cox2 is a known mediator of antiapoptotic, proliferative, and tumor-promoting effects in the colon as mentioned above [30].

Moreover, TLRs assist natural killers (NK) cells in the killing of infected cells either by direct stimulation on NK cells or the induction of type I interferons and IL-15. Becker et al. found that Leishmania lipophosphoglycan (LPG) activates NK cells through TLR2 [37].

5. Toll-Like Receptors and Tumourigenesis

Several studies indicate that TLR signalling contributes to the growth of tumours in numerous organs. The development of cancer has been associated with microbial infection, injury, inflammation, and tissue repair. The subsequent activation of TLRs in cancer cells and the ensuing signalling cascade with the cytokine/chemokine production may promote cancer cell survival, chemoresistance and therefore tumour progression. In a recent study, Huang et al. reported the expression of TLR4 in murine tumour cell lines and showed that the activation of TLR4 in these tumour cells by LPS induced tumour evasion from immune surveillance [38]. In another report it has been shown that in a subgroup of epithelial ovarian cancer cells that express MyD88, ligation of TLR4 by LPS induced cell proliferation and enhanced cytokine/chemokine production [39]. Huang et al. also showed that Listeria monocytogenes promotes cell growth through TLR2 [40]. In 2007, He et al. described the expression of TLR4 in human lung cancer cells [41]. The direct promotion of cancer cell survival and the inflammation-induced chemoresistance have been linked to the hyperactivation of NF-κB in cancer cells, which induces upregulation of antiapoptotic proteins such as c-FLIP and XIAP, and inhibition of proapoptotic proteins such as Bax and Caspase-9 [42, 43]. Another report showed that the ligation of TLR2 in lung cancer cells induced the activation of mitogen-activated protein kinases (MAPK) as well as NF-κB which were shown to prolong cancer cell survival [40].

A number of recent studies have investigated carcinogenesis in mice deficient in TLRs or TLR adapter molecules using models of inflammation-associated cancer. These models provide a physiological tumour environment thus accounting for a possible role of TLRs in stroma-tumour interactions, possibly mediated by endogenous TLR ligands released from necrotic tumour cells. TLR4-deficient mice display a profoundly reduced dysplasia, number and size of tumours [44]. Similar results were reported in a study that investigated the role of MyD88 in colon cancer. MyD88 deficiency leads to decreased carcinogenesis in noninflammatory model of colon cancer [45]. Additionally, the development of fibrosis-associated hepatocellular cancer in patients with chronic hepatitis, it is likely that is promoted by the TLR-MyD88 pathway [33].

Another interesting hypothesis is that cancer development might be an abnormal form of tissue repair in which the control mechanism loses its function. The presence of deregulated infection, inflammation and/or tissue injury as occurs during various stages of tumourigenesis, leads to unregulated TLR-regulated tissue repair response [46]. MicroRNAs (miRNAs) are a class of small RNA molecules that regulate gene expression at posttranscriptional level and may function as either oncogenes or tumour suppressors. Several studies have demonstrated a link between miRNAs and TLR function, and therefore potential association with inflammation and cancer formation. MiR-155, one of the most studied miRNAs related to inflammation and cancer, is highly expressed in B-cell lymphoma, breast and lung cancers, and pancreatic adenocarcinomas. A recent study showed that ligands for TLR2, TLR3, TLR4 and TLR9 could all induce the upregulation of miR-155 expression, through both MyD88- and TRIF-dependent pathways [47]. MiR-146 is highly expressed in breast, prostate, pancreatic, stomach and papillary thyroid carcinomas, and is a target of NF-κB, upregulated upon TLR2, TLR4 or TLR5 ligation [48].

6. Toll-Like Receptors and Tumour Microenvironment

The regulation of immune response within the tumour microenvironment might be another consequence of TLR activation. The infiltrating immune cells contribute to cancer growth and metastasis. In a study on oral epithelial squamous cell carcinoma it has been shown that the level of immune cell infiltration was directly correlated with the level of morphological and pathological transformation from normal to malignant phenotypes [49]. During both cancer development and tissue repair processes, the immune infiltrate is characterized by the presence of a high number of macrophages, which produce VEGF, IL-6, IL-10, prostaglandins, iNOS, and IDO. Inflammatory cells, primarily macrophages, are consistently regarded as critical mediators involved in cancer initiation and promotion [50]. They facilitate angiogenesis and extracellular matrix breakdown and remodeling and promote cancer cell invasion.

The tumor stroma is made up of diverse cellular populations including macrophages, lymphocytes, vascular cells, and carcinoma-associated fibroblasts. Versican, a large extracellular matrix proteoglycan, accumulates both in tumour stroma and cancer cells. It participates in cell adhesion, migration, and angiogenesis, all features of invasion and metastasis. A recent study has documented that versican can activate tumour-infiltrating myeloid cells through TLR2 and its coreceptors TLR6 and CD14 and elicit the production of proinflammatory cytokines including TNF-α that enhance tumour metastasis [51]. The interaction between versican and TLR2 links inflammation and metastasis.

As both resident fibroblasts and endothelial cells (ECs) also express functional TLR2 and its coreceptors, versican may trigger the activation of both fibroblasts and ECs, leading to a marked increase in interleukin-8 (IL-8) production [52]. IL-8 is a proinflammatory chemokine with leukocyte chemotactic, tumourigenic, and proangiogenic properties. It increases proliferation and survival of endothelial and cancer cells and enhances the migration of cancer cells, ECs, and infiltrating neutrophils at tumor sites [53]. Interestingly,
ligation of TLR2 by versican appears to be directly involved in the activation of multiple types of cells in tumour stroma, including macrophages, and the induction of inflammatory cytokine secretion to generate an inflammatory microenvironment hospitable for tumor progression [54].

7. Toll-Like Receptors and Cancer Treatment

It was the original observation that certain cancer patients who developed concurrent bacterial infections would also experience concomitant remission of their malignant disease [55]. It has been suggested that host molecules including TNF, have implicated in the mediation of the antitumour effects of endotoxin [56]. However, subsequent testing using recombinant TNF showed many of the toxicities of endotoxin without however equaling the significant antitumour efficacy in both clinical trials and laboratory studies. Although TNF failed to materialize the antitumour effect, the identification and production of several cytokines supplied the dream of cancer immunologists. However, almost all of several cytokines have been tested in animal tumor models, but none has demonstrated similar antitumour effects of endotoxin, except one: IL-12. Taking into consideration the close similarity of the antitumour characteristics between endotoxin and IL-12, and the fact that endotoxin is able to induce IL-12 production, it has been hypothesized that endotoxin exerts its antitumour effect through the induction and the biological activity of IL-12. Interestingly, exposure of DC to PAMPs like TLR ligands induces DC to produce high levels of IL-12p70 and promote efficient T-cell help [55]. Murine studies also showed that only maturation with TLR ligands generates mature DC able to produce IL-12 and promote optimal T-cell help [57]. In this regard, it has recently demonstrated that a maturation cocktail combining the TLR3 ligand polyinosinic-polycytidylic acid (poly(I:C)) and the TLR7/8 ligand R848 supplemented with PGE2 yields DC with both high migratory capacity and IL-12p70 production upon T-cell encounter [58]. Whether the vaccine-matured DC improves antitumour responses in vivo is yet unknown but all these findings open new roads concerning the role of TLRs in the treatment of neoplastic diseases.

A recent study suggests that TLR4 and MyD88 play an important role in antitumour responses following chemotherapy and irradiation. TLR4-deficient mice have significantly larger tumours after doxorubicin and oxaliplatin treatment or irradiation than wild-type mice [59]. Data from this study suggests that cell death induction by chemotherapy or irradiation induces the release of HMGB1 to subsequently trigger TLR4 activation in DC, enhance antigen presentation, and promote cytotoxic T-cell responses. The ability of TLR signalling to activate the adaptive immune system has led to attempts to harness this response against cancer cells through the use exogenous administration of TLR ligands. It has been shown that high doses of TLR agonists can lead to apoptosis and directly kill both tumour cells and ancillary cells of the tumour microenvironment, whereas low doses of TLR agonists promote cancer growth [60]. TLR activation may also cause tumours to regress by increasing vascular permeability and by recruiting leukocytes, resulting in tumour lysis by NK and cytotoxic T cells. In addition TLRs are important in the recognition of microbial pathogens such as Epstein-Barr virus, hepatitis B and C viruses, human papilloma virus, and Helicobacter pylori, all of which are important aetiological agents of human cancers.

TLR2/4 agonists are promising molecules against chemotherapy- or radiotherapy-relapsing tumours. TLR2/4 signalling produces TNF-α, which is required for inducing DC maturation and migration [61]. Mature DC that reach the lymph nodes induce a rapid and sustained congestion of lymphocyte traffic and their number determines the magnitude of T-cell proliferation and effector response. In this respect, helper T-cell 2 (Th2) immunity and antibody responses may not be desirable in cancer where Th1 and cytotoxic T-cell responses are necessary [62]. TLR-dependent cytokines play a pivotal role in the establishment and maintenance of the Th1/Th2 balance, with IL-12 and IFN-γ committing cells to Th1 lineage differentiation [52, 59]. An IFN-γ and IL-12 rich local environment attracts T-cells to the tumor where the rich cytokine milieu promotes the development of a CD4+ Th1 antitumor response that eventually gives rise to cytotoxic CD8+ antitumor cell response. Conversely, in the absence of IL-12 and IFN-γ, IL-4 promotes Th2 development.

TLR2 and TLR4 agonists may differ in their ability to influence Th-cell proliferation [19, 63]. TLR2 preferentially induces IL-10, a cytokine that inhibits the synthesis of several proinflammatory cytokines, and that belongs to the Th2 response in the mouse.

Conversely, the factors specifically induced by TLR4 (IP-10, IL-12, IL-15, and IFN-γ) are all associated with a Th1 lineage commitment. But the picture is somewhat complicated by the observation of Komai-Koma et al. that TLR2 is expressed on activated T-cells as a costimulator for antigen-specific T-cell development and participates in the maintenance of T-cell memory [64].

Moreover, TLR2/4 signaling promotes iNOS-dependent apoptosis of chemotherapy-resistant tumor cell clones. The TLR2/4 agonist, OM-174 is a promising molecule against cancer metastases. Studies in cancer patients showed that intravenous OM-174 induces well-tolerated TNF-α secretion, at plasma levels known to permeabilize neoangiogenic tumour vessels to the passage of cytotoxic drugs [65].

Mycobacterium bovis bacillus Calmette-Guerin (BCG) is another TLR2/4 agonist effective against superficial bladder tumours [65]. OK-432, a penicillin-killed and lyophilized preparation of a low-virulence strain of Streptococcus pyogenes (group A), is a TLR4 agonist which has been successfully used as an immunotherapeutic agent in many types of malignancies, including head and neck cancer and oral squamous cell carcinoma [66]. OK-432 polarizes the T-cell response to a Th1 dominant state. Tano et al. showed that OK-PSA, an active component of OK-432, induces apoptosis by the activation of caspases through p53-independent pathway via TLR4 signaling in head and neck cancer cells [67]. Imiquimod, a synthetic agonist of TLR7 has been proven very effective as monotherapy for basal cell carcinoma, when
applied topically as a 5 percent cream [68]. It is also used in the treatment of actinic keratoses and genital warts.

8. Conclusion

TLRs have emerged over the last decade as key regulators of innate and adaptive immune responses. In addition, the TLR pathways have been shown to play a critical role in the regulation of tissue injury and wound healing process as well as in the regulation of apoptosis. However, interest in the role of these receptors in cancer has been increasing. There is a large and growing body of literature exploring associations of TLR biology with tumourogenesis. Common polymorphisms in TLR genes have been associated with increased risk for several types of cancer. TLR activation and regulation of immune response within the tumour microenvironment and the ensuing signaling cascade with the cytokine/chemokine production may promote cancer cell survival, chemoresistance, and therefore tumour progression. Moreover, the unregulated TLR-regulated tissue repair response during various stages of tumourigenesis, and the upregulation of several miRNAs through TLR ligation, might explain the critical role of TLR pathways in cancer progression. Alternatively, increased TLR activation by either microbial or endogenous ligands may stimulate anticancer immunity.

Taken all the above into consideration, targeting the TLRs with TLR agonists might be promising as effective regimens to fight cancer and to prolong survival in cancer patients who relapse under chemotherapy. Thus, better understanding of the function and regulation of the TLR signalling pathways and further study of the effect of TLR activation on cancer cells are essential for the understanding of tumour initiation and progression.

References


Review Article

Targeting TLR/IL-1R Signalling in Human Diseases

Maria Loiarro, 1, 2 Vito Ruggiero, 3 and Claudio Sette 1, 2

1 Department of Public Health and Cell Biology, University of Rome “Tor Vergata”, 00133 Rome, Italy
2 Laboratory of Neuroembryology, Fondazione Santa Lucia, Istituto Di Ricovero e Cura a Carattere Scientifico (IRCCS), 00143 Rome, Italy
3 Department of Immunology (Bldg. LABIO), R&D Sigma-tau Industrie Farmaceutiche Riunite S.p.A, Via Pontina km 30.400, 00040 Pomezia (RM), Italy

Correspondence should be addressed to Vito Ruggiero, vito.ruggiero@sigma-tau.it and Claudio Sette, claudio.sette@uniroma2.it

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The members of Toll-like receptor/Interleukin (IL)-1 receptor (TLR/IL-1R) superfamily play a fundamental role in the immune response. These receptors detect microbial components and trigger complex signalling pathways that result in increased expression of multiple inflammatory genes. On the other hand, an aberrant activation of TLR/IL-1R signalling can promote the onset of inflammatory and autoimmune diseases, raising the interest in the development of therapeutic strategies for the control of their function. In this review, we illustrate the structural and functional features of TLR/IL-1R proteins and discuss some recent advances in the approaches undertaken to develop anti-inflammatory therapeutic drugs. In particular, we will focus on inhibitors, such as decoy peptides and synthetic mimetics, that interfere with protein-protein interactions between signalling molecules of the TLR/IL-1R superfamily. Given their central role in innate and adaptive immune responses, it is foreseen that pharmaceutical modulation of TLR/IL-1R signalling pathways by these drugs might yield clinical benefits in the treatment of inflammatory and autoimmune diseases.

1. Introduction

All living organisms are constantly exposed to pathogenic microorganisms that are present in the environment. To face this continuous challenge, evolution has selected mechanisms of immune defence to eliminate or counteract these invading pathogens [1]. In mammals, the immune response relies on complex strategies of defence consisting of two components: “adaptive immunity” and “innate immunity”. Adaptive immunity is a highly sophisticated system—observed only in vertebrates—characterized by an exquisite capacity to establish efficient memory responses to specific antigens. This system is able to anticipate subsequent encounters with pathogens and represents a potent defence against microbial infection [2]. Adaptive immunity is involved in the elimination of pathogens during the late phase of infection and is elicited by B and T lymphocytes, which utilize immunoglobulins and T cell receptors, respectively, as antigen receptors to recognize “non self” molecules. These receptors are generated through DNA rearrangement and respond to a wide range of potential antigens [3]. In contrast, the innate immunity, which was first described over a century ago, is phylogenetically conserved and is present in almost all multicellular organisms [4]. Innate immunity represents the first line of protection against the invading microbial pathogens and is mediated by phagocytes, such as macrophages and dendritic cells (DCs). Although it was initially viewed as a non specific response, innate immunity is indeed able to discriminate between “self” molecules and a variety of pathogens through the function of a small array of germline-encoded pattern-recognition receptors (PRRs). These receptors can specifically recognize conserved microbial components known as pathogen-associated molecular patterns (PAMPs) [4]. The PRRs include members of nucleotide oligomerization domain proteins, containing leucine-rich repeats (NLRs), retinoic acid inducing gene (RIG)-like helicases (RLHs), and toll-like receptors (TLRs) [5]. TLRs, which are one of the largest and best studied families of PRRs, and their signal transduction pathways are the focus of this review.
2. Structural Features of TLRs

TLRs are evolutionary conserved from plants to vertebrates. In mammals there are 12 identified TLRs [5]. These receptors undergo homo- or hetero-dimerization to detect a wide range of PAMPs including lipids, lipoproteins, proteins, glycans, and nucleic acids [6, 7]. Exhaustive reviews covering the specificity for different ligands recognized by TLRs [8, 9] as well as the structural features of these receptors have been recently published [10, 11]. Here we will focus on the domains that characterize these receptors, with a particular attention to the TIR domain.

TLRs are characterized by two conserved regions: the extracellular leucin-rich region (LRR) and the cytoplasmic Toll/IL-1 receptor (TIR) domain. The LRR, which is depicted to recognition of the ligand, is composed of 19–25 tandem repeats of 24–29 amino acids, folded in β-strands and in α-helices that are linked by loops. The structures of TLR1, TLR2, TLR3, and TLR4 have been recently solved, leading to models that predict the mechanism of interaction with their cognate ligands [11]. The TIR domain, which shares homology with the interleukin 1 receptor (IL-1R) [12], is instead responsible for the propagation of the signal within the cell through interaction with a complex signalling cascade [8, 9, 13]. Crystallographic analyses of the TIR domain of human TLR1, TLR2 [10], and TLR10 [14] revealed that they are composed of five β-strands alternated with five α-helices connected by eight loops (Figure 1). Moreover, this domain contains three highly conserved motifs denoted Box 1, 2, and 3 [10] (Figure 1). Box 2 forms a loop connecting the second β-strand and α-helix, referred to as the BB-loop. This loop is critical for proper signalling, as single residue substitutions abolish the ability to recruit targets without changing the overall fold of the TIR domain [10]. For instance, a naturally occurring point mutation (P712H) affecting a conserved proline within the BB-loop is required for TLR4-triggered downstream signals [15]. This mutation leads to pathological consequences and renders C3H/HeJ mice hyporesponsive to lipopolysaccharide (LPS) [15]. Notably, the homologous point mutation in human TLR2 (P681H) disrupts the signal transduction induced by Gram-positive bacteria [16], confirming the critical role of the BB-loop in TLR signalling.

3. The TLR/IL1-R Superfamily: A Central Role for the Intracellular Adaptor Proteins

In addition to the TLR subfamily, the presence of an intracellular TIR domain is the hallmark of a large group of proteins that belong to the TLR/IL-1R superfamily [17] such as the IL-1R subfamily and the TIR-domain-containing adaptor proteins [17].

The IL-1R subfamily encodes nine members that are characterized by the presence of extracellular immunoglobulin-like (Ig) domains and by an intracellular TIR domain. IL-1R contains three Ig domains and, together with the highly homologous IL-1R accessory protein (IL-1RαCP), forms a receptor complex for IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra) [18]. Similarly, the IL-18 receptor (IL-18R), following binding to IL-18, forms a complex with IL-18RαCP to initiate downstream signalling. IL-1Rrp2 is the receptor for the agonists IL-1F6, IL-1F8, and IL-1F9, which also uses IL-1RαCP as a second chain [19]. Thus, IL-1RαCP appears to be promiscuous since, in addition to IL-1RI and IL-1Rrp2, it also associates with ST2, which has recently been shown to bind IL-33 [20]. IL-1R2 and SIGIRR are two inhibitory receptors, the former lacks the TIR domain whereas the latter contains a single Ig domain for the extracellular segment. The only members that still remain without any identified function in this subfamily are IL-1RAPL and TIGIRR [21].

The third subfamily comprises several adaptor molecules: the Myeloid differentiation factor 88 (MyD88), the MyD88-adaptor-like (MAL, also known as TIRAP), the TIR-domain-containing adaptor protein inducing interferon-β (IFNβ) (TRIF; also known as TICAM1), the TRIF-related adaptor molecule (TRAM; also known as TICAM2) and the sterile α- and armadillo-motif containing protein (SARM) [12]. These adaptors bridge the TLR/IL-1R receptors to the intracellular molecules that transduce their signals into a biological response and play a central role in innate immunity. Among them, MyD88 is without doubts the most widely utilised adaptor molecule in TLR/IL-1R signalling. It was originally identified as a novel myeloid differentiation primary response gene in M1 monoleukemic cell lines [22]. MyD88 has a modular structure consisting of an N-terminal death domain (DD) separated by a short linker region from the C-terminal TIR domain [23]. Studies on MyD88-deficient mice have clearly demonstrated that this protein is an essential component in the responses to various TLR ligands, with the sole exception of TLR3 [24–26]. The second adaptor in the subfamily to be discovered was MAL/TIRAP. MAL/TIRAP has a binding domain to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2), required for its recruitment to the plasma membrane, at the N-terminus and a TIR domain at the C-terminus [27, 28]. MAL/TIRAP interacts with MyD88, and MAL-deficient mice have revealed that this adaptor is essential for MyD88-dependent signalling through TLR2 and TLR4 [29]. TRIF contains consensus TRAF6-binding motifs (T6BM) in the N-terminal region, a TIR domain and a receptor-interacting protein (RIP) homotypic interaction motif (RHIM) in the C-terminal region [30]. TRIF is the only adaptor used by TLR3, and TRIF-mutant mice display an impaired TLR3-mediated response being defective in the TLR4-mediated activation of the MyD88-independent pathway [31]. TRAM contains a TIR domain in the C-terminal region and functions exclusively in the TLR4 pathway. The N-terminus of TRAM undergoes constitutive myristoylation that mediates its association with membranes. TRAM interacts with TRIF, and TRAM-deficient mice demonstrated that this protein is also essential for the MyD88-independent pathway of TLR4 signalling [32]. Finally, SARM [33], which contains a TIR domain at C-terminus, two “sterile a” motif (SAM) protein-protein interactions domains, and an Armadillo repeat motif (ARM) [34], functions as an inhibitor of TRIF-dependent TLR signalling [35].
4. TLR/IL-1R Signalling Pathways

Upon recognition of their cognate ligands, TLR/IL-1R proteins homo- or hetero dimerize (TLR1/2, TLR2/6, IL-1R/IL-1RacP) and initiate a signalling cascade through recruitment of different combinations of TIR-domain-containing adaptor protein to their TIR domain, in order to turn on both common and unique pathways (Figure 2). All receptors of the superfamily, with the sole exception of TLR3, use MyD88 to initiate their signalling pathway. In some cases, MyD88 acts in concert with other adaptors, like MAL/TIRAP in the response triggered by stimulation of TLR4, TLR1/2, and TLR2/6 [12]. On the other hand, TLR3-mediated signalling requires only the adaptor molecule TRIF, which is also recruited by TLR4 in association with the other adaptor TRAM [12]. Based on the type of adaptor molecules involved, the TLR/IL-1R-induced pathways can be sub-grouped in two classes: MyD88-dependent and MyD88-independent responses.

5. MyD88-Dependent Signalling

The MyD88 TIR domain differs from the TIR domain of TLRs, as it contains five central $\beta$-strands surrounded by four $\alpha$-helices instead of five $\alpha$-helices [36] (Figure 3(a)). TLR/IL-1R receptors associate with MyD88 through homotypic interactions between their respective TIR domain. This interaction then allows MyD88 to recruit members of the interleukin-1 receptor-associated kinase (IRAK) family (IRAK1, IRAK2, and IRAK4) through homotypic interactions between their respective Death Domains (DDs) [38] (Figure 2). Notably, recent studies have identified critical residues in MyD88 DD required for association with either IRAK1 or IRAK4 (Figure 3(b)) [37]. Since their substitution impaired propagation of the downstream signaling response [37], it is likely that these interactions are necessary. The interaction between MyD88 and IRAK1/4 induce the formation of macromolecular complexes that ultimately impinges on TAK1 (transforming growth factor $\beta$-activated kinase 1) [39] and leads to activation of the transcription factor NF-kB (p50/p65) [8, 9, 17, 40] (Figure 2). However, MyD88-dependent activation of NF-kB can also be induced by TAK1-independent pathways, as shown by the incomplete suppression of IL-1 or LPS-induced NF-kB activation in TAK1-deficient murine embryonic fibroblasts [41]. Two candidates for this TAK1-independent pathway are the mitogen-activated protein kinase kinase kinase 3 (MEKK3) and Protein kinase C (PKC) [42, 43].
expression of type I IFN (IFN-α) and activates the transcription factor IRF-7 thereby inducing IFN-β and IFN-inducible genes. TLR7 and TLR9 recruit a complex consisting of MyD88, IRAK-4, IRAK-1, and TRAF6 [45], which binds to the TIR domains of TLR7 and TLR9 to activate the noncanonical IKKs, TBK1, and IKKε [46].

In summary, the small adaptor MyD88 is envisioned as a potential target to downregulate excessive immune responses. At the same time, its involvement in so many physiological responses is a challenge for the development of anti-inflammatory therapeutic agents devoid of potentially dangerous side-effects.

6. MyD88-Independent Signalling

Several observations have indicated the presence of MyD88-independent TLR/IL-1R signalling pathways. Although MyD88-deficient cells do not express several inflammatory cytokines upon LPS stimulation, activation of NF-κB and JNK is only delayed [25]. Furthermore, induction of type I IFNs is not impaired [47]. The best characterized MyD88-independent pathway is that triggered by TLR3, which requires only TRIF as adaptor [48]. On the other hand, recruitment of TRAM is required to bridge TRIF to TLR4 (Figure 2). Thus, TLR4 is capable of activating both MyD88-dependent and TRIF-dependent signalling pathways, in a...
7. Inhibition of TLR/IL1-R Function As a Therapeutic Approach

The central role of the members of the TLR/IL-1R superfamily in the immune response is highlighted by their implication in inflammatory and immune disorders such as sepsis syndrome, asthma, atherosclerosis, Alzheimer’s disease, rheumatoid arthritis (RA) [21, 51]. Moreover, on a susceptible genetic background, TLR signalling can also induce autoimmune diseases such as Systemic Lupus Erythematosus (SLE), Multiple Sclerosis (MS), and Inflammatory Bowel Diseases (IBD) [52]. For these reasons, therapeutic targeting of TLR/IL-1R signalling is gaining more and more attention as a potentially valuable approach for many diseases of the immune system. Herein, we illustrate some novel strategies utilized for the development of anti-inflammatory therapeutics based on interference with the function of the TIR domain of members of the TLR/IL-1R superfamily.

One approach to modulate the activity of TLRs is the inhibition of intracellular proteins involved in the signalling pathways triggered by multiple receptors [53]. This view has been criticized on the ground that global inhibition of TLR signalling might be deleterious, as it could lead to a reduction in the body’s defences against pathogens [54]. However, recent evidence suggests that the redundancy of mammalian host’s immune responses together with the high degree of cross-talk between TLR-initiated signalling pathways might allow to overcome a generalized block in the immune response [55]. For instance, children with recurrent pyogenic infections display inactivating mutations in either the DD or TIR domain of MyD88 that render the patients vulnerable to *S. pneumoniae*, *S. aureus*, and *P. aeruginosa*. However, these patients are normally resistant to most common bacteria, viruses, fungi, and parasites [56]. Thus, although MyD88 is involved in all TLR signalling pathways, suppression of its function does not cause a complete block in the immune response. Similar findings were reported with IRAK-4-deficient patients [57]. Albeit these deficiencies are life-threatening in the childhood—with about 40% mortality in the first eight years of life—they progressively become less severe with age. Indeed, no deaths or invasive infections were observed in patients over the age of 8 and 14 years, respectively [58]. The improved clinical status was not due to any leakiness in MyD88 and IRAK-4 deficiency, suggesting that the MyD88-dependent TLR/IL-1R signalling plays a vital role early in life, but becomes less important for survival during ageing. This is likely consequent to activation and/or maturation of TLR-independent innate immunity [9, 59–62]. Moreover, these findings seem to suggest that innate immunity is more important upon the very first encounter with a pathogen. Once adaptive immunity is generated, however, resistance to infection becomes quite efficient even in the absence of crucial functional components of TLR signalling [63].

TLR-mediated signalling is of paramount importance in eradicating microbial infections and promoting tissue repair. Nevertheless, it must be tightly regulated [67] in order to prevent a sustained, overzealous activation that might set the ground for autoimmune and inflammatory response.

Figure 3: Schematic representation of the TIR and Death Domains of MyD88. (a) A schematic representation of the human MyD88 TIR domain. The TIR domain of MyD88 consists of five central β-strands surrounded by four α-helices, connected by loops [36]. It lacks the α-helix αD in the region between βD and βE strands, this region has an helical coil conformation. (b) Surface of interaction of MyD88 Death Domain (DD) comprised by residues 27–72 (predicted α1, α2, α3 and N-terminal α4 helices) is required for the recruitment of IRAK1 with MyD88. The region MyD88 Death Domain (DD) comprised by residues 73–274 (predicted α1, α2, α3 and N-terminal α4 helices) is required for the recruitment of IRAK1 [37]. Residues E52 and Y58 of MyD88 DD are implicated in the interaction of IRAK1 with MyD88. Moreover, residue K95 in the predicted α3 helix is involved in the recruitment of IRAK4 by MyD88 DD.

sequential process that involves the endocytosis of the TLR4 complex [49]. In particular, TLR4 first induces MAL/TIRAP-MyD88 signalling at the plasma membrane. Then, following its endocytosis into early endosomes, TLR4 activates TRAM-TRIF signalling. Once recruited to the receptor, TRIF interacts with TRAF3 to activate the noncanonical IKKs TBK1 and IκKε resulting in activation of IRF3 and transcription of IFNβ and IFN-inducible genes [50] (Figure 2).

The more limited spectrum of action of these additional adaptors suggests that specific inhibitors of their function might exert a more selective anti-inflammatory response. On the other hand, the efficacy of such compounds might also be more limited than MyD88 inhibitors. Thus, it is at the moment unclear which member of the TLR/IL-1R superfamily is the most suitable target for pharmaceutical approaches.
disorders [68, 69]. Therefore, therapeutic agents targeting the TLR signalling must be able to antagonize the harmful effects resulting from TLR hyperactivation while sparing their properly operating functions in host-defence. In spite of these apparent obstacles, evidence is accumulating that drugs targeting TLRs and their signalling adaptors can provide new therapeutic opportunities to prevent or treat human inflammatory and autoimmune diseases [70–72]. Herein, we will focus on approaches aimed at developing rationally-designed inhibitors (Figure 4) that interfere with protein-protein interactions of adaptor-adaptor or adaptor-TLR complexes. The readers are referred to several recent reviews that discuss additional approaches that are currently under development to target TLR function [73–76].

8. Targeting the TIR BB-Loop for Development of Novel TLR/IL1-R Signalling Inhibitors

Protein-protein interactions are central to most biological processes, suggesting that interfering with specific interactions might affect cellular responses. Nevertheless, developing small molecules that modulate these interactions may not be an easy task, due to typical flatness of the interface of contact between proteins and because large surface areas are usually involved [77, 78]. However, despite this approach presents a major challenge in terms of therapeutic feasibility, initial steps have been taken with the design of peptide-based inhibitors.

The TIR domain of TLR/IL-1R proteins is a putatively suitable target. In particular, the BB-loop region may be regarded as a critical functional interface of TIR domain for its critical role in proper signalling [10, 15, 79].

9. BB-Loop Decoy Peptides

Decoy peptides are short amino acid sequences of a protein that are expected to mimic its interaction surface and to prevent interaction of the prototype proteins with their partners. Several reports have shown the successful realization of this concept, and a number of decoy peptides binding to BB-loops were found to inhibit TLR/IL-1R signalling.

A TIRAP decoy peptide consisting of the 14 amino acid-long sequence in the BB-loop (LQLRDAAPGGAIVS),
 fused to the *Drosophila* antennapedia homeodomain to facilitate the intracellular delivery [80], specifically blocked TLR4-induced activation of NF-κB without affecting the TIRAP-independent TLR9 response [27]. In vivo administration of TIRAP inhibitory peptide counteracted the lung inflammatory response in healthy C57BL/6 mice [81]. The peptide abolished LPS-induced TNF-alpha, IL-6, and IL-8 expression in alveolar macrophages, whereas it attenuated *E. coli*-induced expression of these cytokines and chemokines [81]. These results have suggested new therapeutic options for TIRAP inhibitors in the treatment of acute lung injury and acute respiratory distress syndrome.

Similarly, BB-loop heptapeptides derived from MyD88 and IL-1R inhibited homomeric interaction of MyD88 TIR domain or full-length MyD88 in vitro [82]. These peptides exerted a specific effect, because heptapeptides derived from BB-loop of other TLR/IL-1R proteins were either less effective (TLR1) or completely inactive (IL-1RaCP). Moreover, a cell permeable derivative of the MyD88 BB-loop decoy heptapeptide (RDVLPGGT) significantly reduced IL-1-induced NF-κB reporter activity and blocked MyD88 homomeric interaction in live cells [82]. A number of studies have confirmed the activity of this construct in different experimental settings. For instance, this MyD88 inhibitory peptide significantly suppressed HMGB1-induced IL-23 release in alveolar macrophages by significantly inhibiting IRAK4 activation [83]. It was also found that this decoy peptide diminished MyD88-dependent MMP-13 gene expression, phosphorylation of MAPKs, and AP-1 activity in normal human knee articular chondrocytes [84], suggesting a possible application of this approach to treatment of RA. Moreover, the MyD88 inhibitor peptide specifically reduced TNF-α production and Poly(g-Glutamic acid) nanoparticles (NPs)-induced DC maturation [85]. Other authors reported that preincubation of professional antigen-presenting cells (APCs) with this molecule almost completely inhibited induction of CD80 expression by either human β-defensin-3, an antimicrobial peptide, or LPS. Remarkably, the MyD88 inhibitory peptide had minimal and nonsignificant effects on costimulatory molecule induction by IFN-α, indicating its specific action in TLR-induced APC differentiation [86].

By following the same experimental approach, Toshchakov and colleagues performed systematic investigations of decoy cell permeable peptides containing TIR domain BB-loop sequences derived from the adaptor proteins MyD88, TIRAP, TRAM, and TRIF as well as the receptors TLR1, 2, 4, and 6 [87–89]. These decoy peptides were all able to inhibit, with varying activity, the TLR signalling pathways [88]. Although the TLR2 and TLR4 decoy peptides also showed some degree of cross-reactivity, they did not interfere with TLR3 signalling [89]. Notably, BB-loops of TLR4 and TLR3 share only five identical amino acids, with proline 712 present in TLR4 but not conserved in TLR3, hence providing a possible structural base for the lack of effects of TLR2- and TLR4- derived decoy peptides toward TLR3 signaling.

Thus, the studies reported above highlight the possibility to produce inhibitory drugs that interfere with protein-protein interactions in the TLR/IL-1R signalling pathways.

### 10. BB-Loop Peptidomimetics

The BB-loop decoy peptides may also represent a valuable starting point to produce synthetic small molecules that mimic the structure of target proteins, hence paving the way for developing novel therapeutic agents.

Bartfai and colleagues were the first to show that the BB-loop region of the TIR domain was amenable to development of selective synthetic inhibitors of protein-protein interactions. By focusing on TIR-domain interactions between IL-1R1 and MyD88, they synthesized a low-molecular-weight molecule mimetic, hydrocinnamoyl-L-valyl pyrrolidine. This molecule is based upon the protruding three amino acid residues of the MyD88 BB-loop, which mimic the (Phe/Tyr)-(Val/Leu/Ile)-(Pro/Gly) sequence [64], consensus for several TLR/IL-1R family members [90]. This mimetic compound blocked IL-1β-induced phosphorylation of the mitogen-activated protein kinase p38 in EL4 thymoma cells [64]. Moreover, sandwich ELISA assays demonstrated that this compound inhibits the IL-1β-mediated association of IL-1R1 and MyD88 in both EL4 cells and in freshly isolated lymphocytes from mouse spleen. The disruption of the IL-1R1-MyD88 interaction was also shown to be selective over other TLR members. Remarkably, the inhibitory effects on IL-1β-signaling were confirmed in vivo, as mice treated with 200 mg/kg of the compound exhibited significant attenuation of the IL-1β-induced fever response [64]. The same TIR BB-loop mimetic was investigated in vivo in a myocardial ischaemia model, and it was shown to decrease infarct size by ~33% and to improve ejection fraction and fractional shortening in treated mice [91]. These results suggested that modulation of the IL-1R/MyD88 interaction could be a strategy for reducing myocardial ischaemic injury, and additional recent investigations support this notion [92, 93].

Based on this compound, Bartfai and colleagues synthesized a novel series of bifunctional BB-loop mimetics. Their rationale stemmed from the assumption that bifunctional compounds might be more effective blockers of protein-protein interactions than monofunctional compounds [94]. They reported that two such mimetic compounds, EM77 and EM110, possessed antiinflammatory and neuroprotective properties. They inhibited MyD88-dependent proinflammatory action of IL-1β without affecting activation of the kinase AKT/PKB, which depended on PI3-kinase activation through binding to IL-1R [65]. The selectivity of action of the MyD88 BB-loop mimetics toward the two pathways activated by IL-1β in primary cultures of preoptic area (POA)/AH neurons allowed the authors to suggest that they may exert antiinflammatory effects while concomitantly promoting neuronal survival in the nervous system.

The MyD88 BB-loop heptapeptide [82] also served as a template for the design and synthesis of a peptidomimetic library [95]. The RDVLPGGT (Arg-Asp-Val-Leu-Pro-Gly-Thr) region was subdivided into three distinct portions: a charged portion (Arg-Asp amino acids), a hydrophobic portion (Val-Leu amino acids), and a β-turn portion (Leu-Pro-Gly-Thr amino acids). A peptidomimetic library consisting of 4368 direct and 234 retroinverse mimetics was designed by combining all these building blocks. For
practical reasons, a subset of 83 compounds selected from the library was prepared on solid phase by using a polymer supported (aminomethyl) polystyrene (Rink amide) resin [95]. All selected compounds for synthesis met the “rule of five” [96]. The ability of the peptidomimetics to inhibit protein-protein interaction was first assessed by a yeast 2-hybrid assay [95]. Active compounds were then further validated in a mammalian cell system by evaluating the inhibition of MyD88-dependent NF-κB activation. One of the most effective compounds, termed ST2825, inhibited homomeric interaction of MyD88 TIR domains [66]. This effect was specific for TIR domains and did not affect interaction of MyD88 DDs. Moreover, ST2825 blocked recruitment of IRAK1 and IRAK4 by MyD88, leading to inhibition of IL-1β-mediated NF-κB activation. ST2825 also blocked TLR9-elicited signalling pathways by suppressing B cell proliferation and differentiation into plasma cells in response to CpG. Additionally, oral administration of ST2825 in mice dose-dependently inhibited IL-1β-induced production of IL-6 [66]. Finally, ST2825 intraperitoneal administration significantly protected against left ventricular (LV) enlargement in a permanent ligation model of acute myocardial infarction in mice [97].

These findings [91, 97] suggest that MyD88 inhibition may represent a completely novel approach for future translational investigations for the prevention of heart failure following acute myocardial infarction [98]. Nevertheless, a generalised suppression of MyD88 function might cause unwanted side effects, especially in chronic diseases that require continuous treatments. Thus, a controlled suppression may prove to be a viable therapeutic approach in an anti-inflammatory therapy once an inflammatory condition is presented. This notion is supported by studies conducted using RDP58, a novel anti-inflammatory d-amino acid decapeptide that inhibits the MyD88 pathway by disrupting the formation of the MyD88/IRAK4/TRAF6 complex [99]. Indeed, early human trials have shown an improvement in mild-to-moderate ulcerative colitis [100], and RDP58 is currently being developed in clinical trials for IBD (http://www.genzyme.com/corp/licensing/genz_p_rdp58_login.asp).

11. Conclusions

A tremendous progress has been made over the past several years in elucidating signalling pathways involved in inflammatory disorders, pointing to NF-κB as the crucial downstream player. An immediate and transient activation of NF-κB is important for the normal physiological response to pathogenic damage, but its persistent and excessive activation is conducive to development and progression of cancer and chronic inflammatory disorders [101, 102]. Although much emphasis has been placed on the development of NF-κB inhibitors [103], generic inhibition of NF-κB may lead to undesired side effects. Hence, a challenging objective is to develop drugs that block its effects in specific pathways, while leaving its physiological functions in other contexts largely intact. TLR/IL-1R pathways seem to respond to these requirements. They represent attractive targets for anti-inflammatory drug discovery, because their inhibition may impair a subset of noxious inflammatory signals impinging on NF-κB, while sparing its normal physiological activation [104]. Blockade of adaptor proteins connected to these signalling pathways, such as MyD88, is expected to be more effective than inhibition of individual ligand activities, due to the mechanistic sharing of a common transduction pathway [53, 55]. In particular, the blockade of TIR-TIR interactions between various members of the TLR/IL-1R superfamily provides new opportunities in light of the highly conserved nature of the TIR domain.

Although several reports have shed light on the structures of TIR domains from human TLR/IL-1R proteins [10, 14, 105], Protein Data Bank (PDB) IDs: 2JS7; 2Z5V, their homomeric and heteromeric interactions have not been fully elucidated yet. Recent investigations suggest that following ligand-induced interaction of TIR-containing receptors a multi-TIR complex may form upon recruitment of multiple cytoplasmic adaptors [37, 106]. A major goal is the development of specific antagonists able to dismantle assembly of these signalling platforms. Despite such an approach presents daunting challenges in terms of therapeutic feasibility, initial steps have been taken by designing inhibitor decoy peptides that block the function of adaptors [81, 82]. However, it has to be underlined that these peptides will unlikely form per se the basis for new drugs, but chemists may use them as templates to develop peptidomimetics or other compounds. The recent identification [64, 66, 94] of a few TIR mimetics (Figure 4) allows to envision that design of further selective inhibitors of TIR-domain-containing proteins may be within reach. Yet, determining how to maintain the balance between host-defence functions and the undesired effects that may result from TLR inhibition remains a serious issue for those designing new therapeutics. Additional clinical experience with these novel molecules might allow to establish their relative safety and efficacy in human beings. Hopefully, these novel therapeutics may not only find application in acute settings, such as septic shock, but also in the treatment of autoimmune disorders characterised by recurrent episodes of inflammatory flares.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AP-1:</td>
<td>activator protein 1</td>
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<tr>
<td>IL-1R:</td>
<td>interleukin (IL)-1 receptor</td>
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<tr>
<td>IL-18R:</td>
<td>IL-18 receptor</td>
</tr>
<tr>
<td>IL-1RaCp:</td>
<td>IL-1 receptor accessory protein</td>
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<tr>
<td>IL-18RaCp:</td>
<td>IL-18 receptor accessory protein</td>
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<tr>
<td>IRAK:</td>
<td>IL-1-receptor-associated kinase</td>
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<tr>
<td>IRF:</td>
<td>interferon regulatory factor</td>
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<tr>
<td>JNK:</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>MyD88:</td>
<td>myeloid differentiation primary response protein 88</td>
</tr>
<tr>
<td>MD-2:</td>
<td>myeloid differentiation factor-2 (synonym of LY96, i.e., lymphocyte antigen 96)</td>
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<tr>
<td>IKK:</td>
<td>inhibitor of NF-κB kinase</td>
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<tr>
<td>NF-κB:</td>
<td>nuclear factor κB</td>
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<tr>
<td>NEMO:</td>
<td>NF-κB essential modulator</td>
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<tr>
<td>TAB:</td>
<td>TAK1 binding protein</td>
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</table>

Mediators of Inflammation
References


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Mediators of Inflammation 9


Review Article
The Battle between Virus and Host: Modulation of Toll-Like Receptor Signaling Pathways by Virus Infection

Shin-ichi Yokota, Tamaki Okabayashi, and Nobuhiro Fujii
Department of Microbiology, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060-8556, Japan
Correspondence should be addressed to Nobuhiro Fujii, fujii@sapmed.ac.jp
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In order to establish an infection, viruses need to either suppress or escape from host immune defense systems. Recent immunological research has focused on innate immunity as the first line of host defense, especially pattern recognition molecules such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs). Various microbial components are recognized by their vague and common molecular shapes so-called, pathogen-associated molecular patterns (PAMPs). PAMPs induce inflammatory reactions mediated by the activation of the transcription factor, NF-κB, and by interferons, which lead to an antiviral immune response. Viruses have the capacity to suppress or escape from this pattern recognition molecule-mediated antimicrobial response in various ways. In this paper, we review the various strategies used by viruses to modulate the pattern recognition molecule-mediated innate immune response.

1. Introduction
The host immune system recognizes and eliminates invading pathogenic microorganisms such as viruses, bacteria, and fungi. The first line of defense in mammals is the innate immune system. Recently, the mechanisms by which the innate immune system recognizes pathogen have been extensively studied. Pattern recognition molecules/pathogen recognition receptors (PRRs) are classified into three families: Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and nucleotide binding-oligomerization domain (NOD)-like receptors (NLRs) [1, 2]. Ten TLRs (TLR1 to 10) have been identified in humans. The RLR family contains retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5) [3]. The NLR receptor family contains NOD1, NOD2, NLRP3, NLRPC5, NLRP1, NAIP, and CIITA [4]. In addition, DNA-dependent activator of interferon regulatory factors (DAI) has been identified as a DNA sensor [5]. Various microbial components are recognized as their vague and common molecular shapes by PRRs. Early responses against virus infection are initiated on recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition molecules, triggering two responses. One is the production of interferons (IFNs) resulting in an antiviral state as part of the innate immune response, and the second is maturation of dendritic cells (DCs) to establish acquired immunity. In order to establish an infection within a host, viruses must escape from and/or suppress the immune system by various strategies. An important strategy used by viruses is modulation of PAMP-induced immune responses.

TLR signaling proceeds via two pathways: the myeloid differentiation factor 88 (MyD88)-mediated pathway, and the Toll-interleukin-1 receptor (TIR)-domain-containing adaptor inducing IFN-β (TRIF)-mediated pathway [1, 2]. The former causes activation of the transcription factor NF-κB, which activates various genes contributing inflammatory reactions. The latter causes induction of IFNs, whose stimulation leads cells to antiviral state. TLR3 only activates the TRIF-mediated pathway. TLR3 signaling activates IRF-3, an important transcription factor for IFN-β, and IFN production is induced. TLR2 only activates the MyD88-mediated pathway. However, TLR4 activates both pathways, so TLR4 agonists activate NF-κB and induce IFN production. Cytosolic PRRs, such as RIG-I, MDA5, and DAI, commonly activate IRF-3. The expression of PRRs differs depending on
the cell type. Importantly, it is different between cells derived from myeloid stem cells (myeloid dendritic cells (mDCs), monocytes, macrophages, Langerhans cells, and neutrophils) and cells derived from lymphatic stem cells (plasmacytoid dendritic cells (pDCs), T cells, and B cells). For example, TLR7 and TLR9 are rarely expressed on mDCs, whereas TLR3 and TLR8 are rarely expressed on pDCs. TLR4, on the other hand, is expressed at very low levels on both pDCs and mDCs.

2. Viruses and Innate Immunity

Initially, viruses invade the host epithelial tissues found in the oral cavity, respiratory tract, intestinal tract, and the urogenital apparatus (Figure 1). Lamina propria DCs, Langerhans cells, and stromal cells are resident in these tissues. In the connective tissues, fibroblasts resident, and capillary vessel and lymphatic vessel are expanded. Monocytes, macrophages, T cells, B cells, pDC, and mDC circulate within the blood vessels and lymphatic vessels, and patrol the interstitial spaces. All these cells are potential targets for virus infection. Virus-infected epithelial cells and fibroblasts produce IFNs, mainly IFN-β and IFN-λ, which provide surrounding uninjured cells with antiviral state. Furthermore, chemokines and cytokines, such as interleukin-1β (IL-1β), IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor-α (TNF-α) are also produced. These molecules promote chemotaxis of the resident DCs (lamina propria DCs and Langerhans cells) toward virus-infected and dead cells. Neutrophils, monocytes, macrophages, plasma cells, mDCs, and pDCs also migrate from the blood vessels to the site of infection. IFN-γ-inducible protein 10 (IP-10), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), MIP-3α, and MIP-3β largely contribute to the transmigration of blood DCs. However, these blood-borne immune cells can also be infected by viruses, which can then modulate the production of various cytokines and chemokines.

To establish an infection, viruses need to suppress a number of host immune responses, the antiviral activity induced by IFNs, the chemotaxis of immune cells induced by chemokines/ cytokines, the maturation and activation of DCs, activation of NK and NKT cells, transmigration of mature DCs to the lymph nodes, and the differentiation and activation of T cells and B cells in the lymph nodes, for example. When viruses infect immune cells, such as DCs, the infected cells frequently show suppression of maturation and differentiation, suppression of cytokine receptor and costimulatory molecule expression, secretion of molecules that mimic cytokines and cytokine receptors, and so on. Furthermore, infected cells often alter their cytokine profiles. These strategies are used by the virus to inhibit the acquired immune response. In addition, it has been suggested that virus infection induces regulatory T cells.

3. RNA Virus

3.1. Human T Lymphotropic Virus Type 1 (HTLV-1). HTLV-1, which is a retrovirus, infects CD4+ T lymphocytes, CD8+ T lymphocytes, DCs, B cells, macrophages, and astrocytes, and preferentially replicates in CD4+ T lymphocytes. HTLV-1 causes latent infection as a provirus, whose genome is integrated into the host DNA, and does not replicate in cells in G0 phase. When the infected T lymphocytes are stimulated with antigen presentation from DCs, they proliferate triggering HTLV-1 replication. During the replication stage, a viral protein, Tax, activates NF-κB and promotes the growth of infected cells via upregulation of IL-2 and IL-2 receptors [6, 7]. NF-κB also activates the long terminal repeat (LTR) of HTLV-1 genome, which further enhances viral replication [8]. The replicated virus induces a host immune response, and cells infected with virus are eliminated by the induction of cytotoxic T cells specific for HTLV-1 Tax. In patients with HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), the high proviral load induces a strong HTLV-1-specific immune response [9]. This leads to the rapid elimination of infected cells through the induction of proinflammatory cytokines and cytotoxic T lymphocytes. So, in order to escape from acquired immune responses, HTLV-1 needs minimum replication and latent infection.

Some virus proteins are known as negative regulators of replication. The HTLV-1 basic leucine-zipper factor (HBZ) protein suppresses Tax-mediated transcription activation of the viral LTR [10]. The p30 protein suppresses transcription of mRNAs encoding Tax and Rev [11]. p30 also contributes to the expression of TLR2, TLR4, and TLR9, and activation of IRF4. In monocytes, the TLR2 gene promoter is regulated by the transcription factors Sp1, Sp3, and PU.1 [12], and the TLR4 gene by ISRE and PU.1 [13]. p30 downregulates the expression of both TLR2 and TLR4, because it binds to PU.1 and prevents it from binding to DNA [14]. This leads to the suppression of DC maturation, and of their subsequent migration to the lymph nodes. Furthermore, p30 suppresses the enzymatic activity of glycogen synthetase kinase 3β (GSK3β) through promotion of the phosphorylation of nine serine residues. This leads to the induction of IL-10, which suppresses the function of macrophages, and also the maturation and activation of DCs. In fact, serum IL-10 levels are elevated in patients with adult T lymphocyte leukemia. These immunosuppressive properties of IL-10 indirectly contribute to the inhibition of virus replication and to the suppression of virus-induced immune responses. Activation of infected T lymphocytes by DCs in the lymph nodes and cell-to-cell transmission of virus are considered to be important for virus proliferation in human. The viral p12 protein suppresses cell surface expression of both MHC class I and the IL-2 receptor, and also suppresses linker for activation of T cells (LAT), which is an adaptor protein required for T cell activation [15]. This results in suppression of T cells and dysfunction of the stimulation/activation by DCs via the T cell receptor. HTLV-1 causes proliferation of infected cells rather than virus and suppression of host immune responses, which helps it to maintain a latent infection in order to survive.

3.2. Human Immunodeficiency Virus (HIV). HIV is another retrovirus that uses similar escape strategies to HTLV-1. HIV
can infect CD4+ T lymphocytes, monocytes, macrophages, and DCs, and preferentially replicates in activated T lymphocytes and activated macrophages. It replicates generally much more slowly in DCs regardless of maturation stage. HIV establishes a latent infection in resting T lymphocyte, and T lymphocyte activation by DCs or stimulation with IL-2 is thought to trigger active replication of the virus. Resting T lymphocytes show resistance to infection and proliferation of HIV. Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G (APOBEC3G) is important for this resistance [16, 17]. The antiviral mechanisms of APOBEC3G are considered to act via inhibition of viral reverse transcriptase (RT). It also produces a transition (G to A) in the DNA strand transcribed by RT due to its cytidine deaminase activity. However, a mutant lacking cytidine deaminase retains antiviral activity. Low molecular weight complexes of APOBEC3G (LMM: 70–100 kDa) have antiviral activity, but high molecular weight complexes (HMM: 700 kDa) do not. The Vif protein of HIV converts LMM to HMM, and promotes proteasome-dependent degradation of the complex, so HIV can counteract its antiviral activity [18]. T cell activation leads to conversion of LMM to HMM (proviral environment). On the other hand, IFNs induce LMM type (antiviral environment) [19, 20].

During the early stage of HIV infection, clinical symptoms show signs of immune system activation such as flu-like symptoms, rather than immunosuppression. Immunological activation is caused by RNA40, an oligonucleotide derived from HIV, which activates pDCs, mDCs, T lymphocytes, and monocytes via TLR7 and TLR8 [21]. The virus proteins, Tat and Vpr, induce proinflammatory cytokines via activation of NF-kB [22, 23], which then enhances the transcription of the virus genome via the LTR. IL-8 also contributes to the propagation of the virus via the accumulation of T lymphocytes.

Enhanced proliferation of HIV causes immune responses that attempt to eliminate the virus. So, HIV maintains a latent infection in order to survive within the host. The Vpu protein suppresses NF-kB activation by inhibition of proteasome-dependent degradation of IxIB [24]. The Nef protein suppresses phosphorylation of ERK through induction of a phosphatase MKP-1 [25]. This causes suppression of TNF-α production by macrophages via TLR4 signaling. Because TNF-α is necessary for maturation and translocation of DCs (including Langerhans cells) to the lymph nodes, downregulation of TNF-α leads to suppression of acquired immune responses, and so prevents inhibition of virus proliferation by activated T lymphocytes. Furthermore, Nef
### Table 1: Molecular mechanisms involved in modulation of host innate immune systems by viruses.

<table>
<thead>
<tr>
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<th>Host pathway</th>
<th>Function</th>
<th>Effect</th>
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<tr>
<td><strong>RNA virus</strong></td>
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<tr>
<td>Human immunodeficiency virus (HIV)</td>
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<tr>
<td>Nef</td>
<td>TCR-CD30</td>
<td>Inhibit NF-κB and AP-1 activation</td>
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<td></td>
<td>TLR4</td>
<td>Inhibit ERK activation (Dephosphorylation of ERK by induced MKP-1)</td>
<td>Suppress TNF-α production</td>
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<td>Vpu</td>
<td>TLRs</td>
<td>Inhibit NF-κB activation (Stabilize IκB)</td>
<td>Suppress cytokine production</td>
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<td>Tat</td>
<td>TLRs</td>
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<td>Enhance IL-6, IL-8, and IL-10 production</td>
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<td>Vpr</td>
<td>TLRs</td>
<td>Activate NF-κB</td>
<td>Enhance IL-6, IL-8, and IL-10 production</td>
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<td>RNA40</td>
<td>TLR7/8</td>
<td>Activate IRF-7 mediated by MyD88</td>
<td>Activate pDCs</td>
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<td>p30</td>
<td>TLR4</td>
<td>Suppress TLR4 expression (Suppress PU.1 function)</td>
<td>Suppress cytokine production</td>
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<td>Inhibit GSK3-β</td>
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<td>Tax</td>
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<td>Degrade TRIF</td>
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<td>TLR2</td>
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<td>Dysfunction of pDCs by enhanced production of IL-10 and TNF-α</td>
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<td>Suppress NF-κB and AP-1 activation (Upregulate host NF-κB negative regulator A20)</td>
<td>Suppress IL-12 production mediated by TLR4 in DCs (Suppress Th1 differentiation)</td>
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<td>Inhibit proinflammatory cytokine and chemokine production</td>
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<td>TLR2</td>
<td>Activate NF-κB</td>
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<td>Suppress of IRF3/7 activation</td>
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Table 1: Continued.

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<td><strong>Human respiratory syncytial virus (RSV)</strong></td>
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<td>G</td>
<td>TLR2/4/9</td>
<td>Suppress NF-κB activation</td>
<td>Induce IL-1, IL-6, IL-8, and RANTES</td>
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<td>F</td>
<td>TLR4</td>
<td>Activate NF-κB</td>
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<td>TLR7/9</td>
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<td>Suppress IFN production in pDC</td>
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<td>NS1, NS2</td>
<td>TLR3/4, RIG-I</td>
<td>Suppress IRF3 activation</td>
<td>Suppress IFN production in mDC</td>
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<td><strong>Rotavirus</strong></td>
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<td>VP4</td>
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<td>Activate NF-κB Suppress JNK (Bind to TRAF2)</td>
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<td><strong>DNA virus</strong></td>
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<td><strong>Vaccinia virus</strong></td>
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<td>A46R</td>
<td>TLRs</td>
<td>Suppress NF-κB activation (Bind to MyD88, TRAM, and TRIF)</td>
<td>Suppress cytokine production</td>
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<td>A52R</td>
<td>TLRs</td>
<td>Suppress NF-κB activation (Bind to IRAK2)</td>
<td>Suppress cytokine production</td>
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<td>Activate p38 MAPK and JNK (Bind to TRAF6)</td>
<td>Induce IL-10 production</td>
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<td>N1L</td>
<td>TLRs</td>
<td>Suppress NF-κB activation (Bind to IKKγ) Suppress IRF3 activation (Bind to TBK1)</td>
<td>Suppress cytokine production Suppress IFN production</td>
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<td>TLR4</td>
<td>Suppress NF-κB and AP-1 activation</td>
<td>Suppress MCP-1 and IL-8 production</td>
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<td>capsid</td>
<td>TNF pathway</td>
<td>Suppress TNFR1 expression</td>
<td>Suppress MCP-1 and IL-8 production</td>
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<td></td>
<td>Activate NF-κB, ERK, and MAPK</td>
<td>Induce RANTES and IL-10</td>
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<td><strong>Herpes simplex virus (HSV)</strong></td>
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<td>virion</td>
<td>TLR2</td>
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<td>Induce cytokine production</td>
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<td>viral DNA</td>
<td>TLR9</td>
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<td>Induce IFN production</td>
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<td><strong>Varicella-zoster virus</strong></td>
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<td>virion</td>
<td>TLR2</td>
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<td>Induce cytokine production</td>
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<td><strong>Human cytomegalovirus (HCMV)</strong></td>
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<td>late proteins</td>
<td>IL-1 system</td>
<td>Suppress NF-κB activation (Inhibit site located upstream of MAP3K)</td>
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<td></td>
<td>TNF system</td>
<td>Suppress TNFR1 expression</td>
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<tr>
<td>gB/gH</td>
<td>TLR2/1</td>
<td>Activate NF-κB</td>
<td>Produce proinflammatory cytokines</td>
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addition, HIV is thought to induce regulatory T cells [27] through their interaction with DCs and T lymphocytes. In upregulated in patients with HIV, and induce apoptosis such as B7-H1 on DCs and PD1 on T lymphocytes, are by HIV have also been reported. Cosuppressive molecules, of infection. Other immunosuppressive mechanisms used in the lymph nodes, and also inhibit the propagation of TNF-α, TNF-α signal transduction, and chemotaxis and maturation. Dysfunction of DCs, suppression of T lymphocyte activation, and a decrease in DC number due to apoptosis allow HCV to establish a chronic infection [32]. It has also been reported that HCV-specific cytotoxic T lymphocytes share upregulated expression of the coinhibitory molecule PD-1, and that signaling from PD-1 ligand on DCs results in suppression of HCV-specific cytotoxic T lymphocytes [33].

3.3. *Hepatitis C Virus (HCV)*. HCV does not only infect hepatocytes. It can also infect DCs, macrophages, monocytes, and T lymphocytes. However, the virus does not replicate efficiently in these cell types. Although, virus particles and virus proteins are found in the blood of HCV patients. HCV causes no cytopathic effects. Immune responses against HCV may be weak, but infected cells are attacked and eliminated. In order to survive in the host, HCV maintains a chronic infection by suppressing the host immune responses. Both HCV core and NS3 proteins activate NF-κB and AP-1 via stimulation of TLR2, which requires TLR1 and TLR6 as costimulators, in monocytes and Kupffer cells [28]. This activation leads to the production of IL-10 and TNF-α, both found in HCV patients at a high titer. IL-10 suppresses the maturation of pDCs and the activation of T lymphocytes, and induces apoptosis in pDCs. The NS3-NS4A protein complex, which is a serine protease, degrades TRIF and IPS-1/Cardiff/MAVS/VISA, which are essential for cellular signaling via TLR3, TLR4, and RIG-I [29]. This shutting off of TRIF- and IPS-1-dependent signaling results in the suppression of IFN-α and IFN-β production, and in dysfunction of mDCs.

The NS5A protein suppresses activation of IRAK-1 through its interaction with MyD88. This leads to the shutting off of TLR7 and TLR8 signaling, and to the suppression of maturation and differentiation of pDCs [30]. On the other hand, NF5A suppresses TRAF2 dependent NF-κB activation via interaction with NF5A and TRAF2, but suppress neither MEK1 activation nor IKKβ-dependent NF-κB activation [31]. TNF-α-dependent activation of JNK is inhibited. Infected DCs are thought to affect the production of TNF-α, TNF-α signal transduction, and chemotaxis and maturation. Dysfunction of DCs, suppression of T lymphocyte activation, and a decrease in DC number due to apoptosis allow HCV to establish a chronic infection [32]. It has also been reported that HCV-specific cytotoxic T lymphocytes share upregulated expression of the coinhibitory molecule PD-1, and that signaling from PD-1 ligand on DCs results in suppression of HCV-specific cytotoxic T lymphocytes [33].

3.4. *Measles Virus*. Measles virus infection causes strong immunosuppression. Measles virus wild strains (clinical isolates) recognize CD150/SLAM (signaling lymphocyte activation molecule) as a receptor. However, laboratory (vaccine) strains only recognize CD46. SLAM is strongly expressed on memory T cells and B cells, but is also expressed on monocytes, T cells, B cells, and mature DCs. SLAM is not expressed on immature DCs, epithelial cells, and endothelial cells. On the other hand, CD46 is expressed on variety of cell types. Infection of SLAM-negative mucosal epithelial cells with measles virus wild strains is thought to be mediated by an as yet unknown “third” receptor [34, 35]. The HA protein of wild virus strain, but not the laboratory strain, induces cytokines such as IL-1α, IL-1β, IL-6, IL-8, and IL-12 via the TLR2 signaling pathway [36]. These cytokines activate and recruit immune cells to the site of inflammation, where the activated immune cells are infected with measles virus via SLAM. The infection and cytokine production are thus propagated.

Interaction of the HA protein with SLAM suppresses TLR4-mediated IL-12 production, but not IL-6 and TNF-α production. However, IL-12 production mediated by other TLR signaling pathways (i.e., not via TLR4) is unaffected by the HA protein [37]. These observations suggest that SLAM is a coupling factor for TLR4, and that the HA protein inhibits this function. The HA protein also interacts with a C-type lectin, dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) on DCs, and activates the serine/threonine protein kinase Raf-1 via the Ras signaling

<table>
<thead>
<tr>
<th>Virus/Virus protein</th>
<th>Host pathway</th>
<th>Function</th>
<th>Effect</th>
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<tbody>
<tr>
<td>Epstein-Barr virus (EBV)</td>
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<tr>
<td>LMP-1</td>
<td>TLR2/CD21</td>
<td>Suppress NF-κB activation</td>
<td>Inhibit COX-2 activation</td>
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<td></td>
<td>Activate NF-κB</td>
<td>Induce MCP-1 production</td>
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<td></td>
<td></td>
<td>Activate p38 MAPK (TRAF6-dependent)</td>
<td>Suppress DC function by induced IL-10</td>
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<tr>
<td>Human herpesvirus 8 (HHV8)</td>
<td></td>
<td>Activate IKK (Bind to IKKγ)</td>
<td>Suppress IFN-α/β production</td>
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<td>Degradate IRF7 by proteasome system</td>
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<tr>
<td>Hepatitis B virus</td>
<td>IL-1 pathway</td>
<td>Activate NF-κB (Bind to mIL-1RAcP)</td>
<td>Induce cytokine production</td>
</tr>
</tbody>
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Table 1: Continued.
Figure 2: Modulation of TLR and other PAMP-induced signal transduction pathways by virus infection. Red line indicates suppression by virus. Blue line indicates activation by virus. Parentheses denote viral proteins or nucleic acids.

pathway. DC-SIGN-mediated Raf-1 activation induces phosphorylation of NF-κBp65 on Ser-276, and its subsequent acetylation. This leads to the enhanced transcription of IL-10 [38]. During infection stage that virus proteins are synthesized de novo, the host cells, such as monocytes and DCs, infected with measles virus show suppressed IL-12 production and TLR signaling, via TLR2, TLR4, TLR7, and TLR9. pDCs infected with the measles virus showed suppressed IFN production and dysfunction of maturation [39, 40].

Both monocytic cell lines U937 and THP-1 and human peripheral blood mononuclear cells infected with measles virus show markedly suppressed TLR2- and TLR4-mediated proinflammatory cytokine induction via NF-κB and AP-1 [41]. However, epithelial cells infected with measles virus show constitutive activation of NF-κB and proinflammatory cytokine production, and these are further enhanced by treatment with TLR agonists such as lipopolysaccharide (LPS). Monocytic cell lines infected with the mumps virus, which also belongs to the Orthomyxoviridae family, show constitutive activation of NF-κB and constitutively high levels of IL-8 production. In monocytic cells infected with the measles virus, LPS-induced ubiquitination (probably K63-linked type) of TNF receptor-associated factor 6 (TRAF6) is suppressed and does not form active complexes of TAK1, TAB2, and TRAF6. An ubiquitin-modifying enzyme A20,
which is a host NF-κB negative regulator, is upregulated in measles virus-infected monocyctic cells, but not in infected epithelial cells. The promoter region of the A20 gene shares two NF-κB binding sites and a negative regulatory motif, ELIE, which is located upstream of, and adjacent to the two NF-κB binding motifs. Measles virus P protein (phosphoprotein) interacts with the ELIE motif, and activates transcription of A20. P protein is thought to release the suppressed A20 transcription machinery independently of activated NF-κB [42]. The reason for cell-type specific A20 expression is unclear. However, the measles virus V protein, which is formed by RNA editing of the P genome and has an N terminal amino acid sequence identical to that of P protein, does activate NF-κB. The balance of the expression levels and time courses of the P and V proteins may also contribute to the cell-type specific suppression of TLR signaling pathways.

3.5. Influenza Virus. The NS1 protein of type A influenza viruses suppresses innate immune system signaling activated by the PAMPs, via TLR3, TLR4, RIG-1, and MDA5 system. The suppression should contribute to efficient replication of infected virus in respiratory epithelial. The mechanism of suppression is mainly via inhibition of IRF-3 phosphorylation [43], leading to suppressed induction of IFN-α/β, and IFN-λ1, 2, and 3. In addition, inhibition of NF-κB and AP-1 activation in influenza virus infected cells leads to suppressed proinflammatory cytokine production, for example, IL-8 and TNF-α. Influenza virus NS1 protein is known to be a multifunctional protein able to inhibit the type I IFN induction, the IFN-induced antiviral activity, the binding and sequestration of dsRNA, the interference with the host mRNA processing, the facilitation of preferential viral mRNA translation, and the inhibition of DC activation [44, 45].

3.6. Human Respiratory Syncytial Virus (RSV). RSV F protein causes TLR4-mediated NF-κB activation during the early infection stages of infection that is dependent upon virus replication. IL-1β, IL-6, and IL-8 are induced via NF-κB activated by the stimulation of TLR4. During the late stages, the viral G protein is produced, and secreted, which then suppresses TLR4-mediated signal transduction [46]. The cysteine-rich (GCCR) region of the G protein is also important for NF-κB suppression. Following the interaction of virus proteins with host cell surface proteins, the viral M2-1 protein inhibits the translocation of RelA, a component of NF-κB, to the cell nucleus [47]. Expression of the viral nonstructural proteins, NS1 and NS2 inhibit activation of IRF3 induced by TLR3, TLR4, and RIG-1 signaling, and also suppress IFN production [48]. However, NS1 and NS2 proteins have little effect on NF-κB or AP-1 activation, so the production of proinflammatory cytokines may be effectively induced in the RSV-infected cells.

4. DNA Virus

4.1. Vaccinia Virus. Orthopox viruses, including the vaccinia virus, produce proteins that mimic cytokine receptors, such as those for IFN-α, IFN-β, IFN-γ, IL-1β, IL-18, and TNF-α, and disturb the cytokine signal transduction system. Other viral proteins also disturb the intracellular signal transduction systems. The viral A46R protein has a TIR domain, and interacts with MyD88 and TRIF, suppressing both IL-1 and TLR signal transduction, but not TNF-α signal transduction [49]. The viral A52R protein binds to IRAK2, suppresses TRAF6-dependent IKK and NF-κB activation, and then inhibits production of IL-8 and RANTES. However, A52R also binds to TRAF6 and promotes polyubiquitination of TRAF6, TAK1 activation, MAPKK6 phosphorylation, and activation of the JNK-p38 MAP kinase pathway. The later leads to the induction of IL-10 production [50]. The viral N1L protein interacts with the IKK complex (IKKα-IKKβ-IKKγ), TBK1, and IKKe, and then suppresses the activation of NF-κB and IRF-3 [51]. Vaccinia virus not only suppresses proinflammatory cytokines, but also induces production of an immunosuppressive cytokine IL-10, which shifts the Th1 response and suppresses cellular immunity. Human monocytes infected with vaccinia virus produce IL-10, and this IL-10 is then further upregulated by stimulation with LPS [52].

4.2. Adenovirus. During the early stages of infection, adenovirus particles induce the MyD88-dependent production of RANTES, IP-10, and MIP-1 [53]. The cytokines produced enhance both sensitivity to LPS and the production of TNF-α. TNF-α suppresses the formation and maturation of virus particles, and induces apoptosis of infected cells. TNF-α also promotes the chemotaxis and maturation of dendritic cells. The induction of TNF-α is considered to be a host defense response. On the other hand, the adenovirus E1A and E3 proteins inhibit TNF-α-induced apoptosis. The receptor internalization and degradation (RID) complex, which consists two E3 products, E3(10.4 k)/RIDα and E3(14.5 k)/RIDβ, suppresses cell surface expression of Fas, TNF-related apoptosis-inducing ligand (TRAIL) receptor 1, TRAIL receptor 2, and TNF receptor 1 [54, 55]. This results in the shutting down of the TNF-α-mediated signaling pathways in the infected cells. RID also suppresses the TLR4 signaling pathway. LPS-induced MCP-1 and IL-8 production are suppressed through the inhibition of NF-κB and AP-1 activation [56]. RID does not alter the expression levels of TLR4, and so is thought to affect other components of the TLR signal transduction pathway.

4.3. Human Cytomegalovirus (HCMV). HCMV modulates NF-κB activity during the various stages of infection. During the early stages, the membrane glycoproteins gB and gH interact with TLR2 and activate NF-κB [57]. NF-κB then contributes to the induction of proinflammatory cytokines, to the expression of virus immediate early genes, and to the replication of the viral genome. The viral US28 protein, which is a HCMV-encoded chemokine receptor, constitutively activates both NF-κB and phospholipase C signaling pathways [58]. The activated NF-κB mediates the upregulation of the host serine/threonine protein kinase, receptor-interacting protein-like interacting caspase-like apoptosis regulatory protein kinase (rick).
4.4. Epstein-Barr Virus (EBV). EBV mainly infects B cells, although it can also infect T cells, NK cells, and epithelial cells. Both the attachment of EBV to receptor CD21 and the interaction of the virus glycoprotein gp350-gp250 with TLR2 activate NF-κB [62]. The virus gB and gH proteins are also candidates for TLR2 ligands. Immortalization of B cells by EBV infection is due to the activation of NF-κB by the latent membrane protein 1 (LMP1), and antigen stimulation-like signaling of the B cell receptor by LMP-2. TLR2 signaling suppresses the transcription of the TLR9 gene via activation of NF-κB containing p65 protein. So downregulation of TLR9 and upregulation of TLR7 and MyD88 are observed in EBV-infected cells [63]. Cell proliferation is thought to be driven by TLR7 signaling activated by virus RNA, because the TLR7 antagonist IRS661 suppresses cell division. Small RNA encoded by the EBV genome (EBV-EBERs) activates TLR7 signaling, and induces IL-10 production. TLR7 signaling also upregulates and activates IRF-5, and induces proinflammatory cytokines. However, activated IRF-5 is negatively regulated by EBV-induced IRF-4 and a splice variant of IRF-5 (V12IRF-5) [63]. This suggests that the TLR7 signaling system play a role in the cell division of EBV-infected cells, and in the establishment of persistent infection. Lytic infection and production of virus particles are observed during the late stage of EBV infection. Late proteins suppress NF-κB activation. This leads to the downregulation of proinflammatory cytokines, the upregulation of TLR9, and the suppression of TLR7 function through interaction of TLR7 and TLR9. So the TLR9 system should be important during late stage of EBV infection.

5. Concluding Remarks

Viruses are in a constant battle with the host immune system. Viruses modulate both the innate and acquired immune systems using a number of clever strategies. The goal appears to be to survive within the host for a long time, rather than efficient replication. Excessive replication would lead to detection and elimination by the host innate and acquired immune systems, thus bringing about the death of the virus. The virus needs to strike a balance between activation and suppression of host immune response. It is likely that each virus has developed various strategies to modulate the host immune response individually, and viruses that have succeeded in creating a good balance between host and parasite have survived.

Modification of TLR signaling is a promising strategy for treatment of cancer, allergy, and infectious diseases [64, 65]. Especially, the immunomodulators should not generate resistant virus to drugs compared to antiviral drugs targeting viral proteins. As an existing example of virus infection, imiquimod, which is a TLR7 agonist, have been applied to an infectious disease caused by human papilloma virus, namely, condylomata acuminata [66]. TLR4 antagonists are trying to be applied to treatment of sepsis. Several issues must be considered for the clinical application of TLR signaling. Among the greatest is assessment of what are keys of host defense or virus survival in view of a series of viral infection process. For example, human herpes simplex virus 1 (HSV-1) requires activated NF-κB for its efficient replication [67]. On the other hand, NF-κB has a key role in inflammatory reactions via transcription activation of proinflammatory cytokines, cell adhesion molecules, and MHC. Thus, activation of NF-κB is a double-edged sword for HSV-1. In the host side, TLR signaling varies according to organs and tissues. For example, intestinal epithelial cells express low levels of TLRs and high levels of negative regulators of TLR signaling, such as Tollip [68, 69]. Also, in intestinal immunity, NF-κB activation in a subset TLR signaling in DCs and macrophages is suppressed by a negative regulation IxBSNS [70]. The dysregulation of TLR signaling in the lumen of intestinal epithelial causes limit chronic inflammatory activation induced by commensal bacteria. Various TLR polymorphisms have been found, and some of them are shown to contribute to dysregulation of TLR signaling. The dysregulation has been suggested to be linked with a number of disease sensitivity and condition depending on individual differences [71, 72].

In conclusion, the molecular mechanisms involved in modulation of host immune systems, including TLR signaling, give us important hints on how to overcome infectious diseases caused by viruses.

References


Type I IL-1 Receptor (IL-1RI) as Potential New Therapeutic Target for Bronchial Asthma

Jyh-Hong Lee,1 Li-Chieh Wang,1 Hsin-Hui Yu,1 Yu-Tsan Lin,1 Yao-Hsu Yang,1 and Bor-Luen Chiang1,2

1Department of Pediatrics, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei 100, Taiwan
2Graduate Institute of Clinical Medicine, National Taiwan University College of Medicine, Taipei 100, Taiwan

Correspondence should be addressed to Bor-Luen Chiang, gicmbor@ntu.edu.tw

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The IL-1R/TLR family has been receiving considerable attention as potential regulators of inflammation through their ability to act as either activators or suppressors of inflammation. Asthma is a chronic inflammatory disease characterized by airway hyperresponsiveness, allergic inflammation, elevated serum total, allergen-specific IgE levels, and increased Th2 cytokine production. The discovery that the IL-1RI–IL-1 and ST2–IL-33 pathways are crucial for allergic inflammation has raised interest in these receptors as potential targets for developing new therapeutic strategies for bronchial asthma. This paper discusses the current use of neutralizing mAb or soluble receptor constructs to deplete cytokines, the use of neutralizing mAb or recombinant receptor antagonists to block cytokine receptors, and gene therapy from experimental studies in asthma. Targeting IL-1RI–IL-1 as well as ST2–IL-33 pathways may promise a disease-modifying approach in the future.

1. Introduction

Asthma is characterized by allergic inflammation of the airways with local infiltration of eosinophils, mast cells, and activated T helper lymphocytes [1]. The initial immune response responsible for this is the generation of allergen-specific CD4+ T helper-2 cells (Th2) that produce Th2 cytokines (IL-4, IL-5, IL-9, and IL-13), but not T helper-1 (Th1) cytokines (IL-2, IL-12, and interferon-γ [IFN-γ]). Overwhelming evidence in literature supports the concept that allergic inflammation is driven by an imbalance between Th1 and Th2 cytokines, favoring the Th2 immune response [1]. IL-4, together with IL-13, is required for Th2-cell development and is intimately involved in the regulation of immunoglobulin E (IgE) production by sensitized allergen-specific B cells, which is a fundamental mechanism in the pathogenesis of allergic asthma [2]. IL-5 is the principal cytokine involved in eosinophil growth, maturation, differentiation, survival, and activation [3, 4]. Cross-linking of allergen-specific IgE on mast cells and the activation of T cells and eosinophils during subsequent encounters with antigens stimulates the release of various preformed and newlysynthesized products, including histamines, cytokines, and chemokines. Together, these lead to characteristic airway changes that contribute to airway obstruction, airway hyperresponsiveness (AHR), goblet cell metaplasia, mucus overproduction, mucosal edema, and airway remodeling [5].

Pulmonary allergic inflammation can be induced in small rodents, such as BALB/c mice, is widely used as an experimental model for human asthma, and is central to the preclinical development of drug therapies [6]. One of the most common murine models of allergen-induced airway inflammation involves mouse sensitization using a small dose of a protein allergen followed by allergen challenge of the airways to induce pulmonary inflammation [7]. This is usually done by injecting the protein intraperitoneally along with an aluminum hydroxide as adjuvant to enhance the protein’s immunogenicity [8]. After the immune system has had a chance to mount a reaction against the antigenic protein (sensitization) over several days, the animal receives further antigen exposure either directly to the lungs in the form of an aerosol or via postnasal drip following nasal instillation (challenge). This characteristically leads to AHR, lung eosinophilia, mucus hypersecretion, and increased IgE...
levels, which are all features commonly associated with human allergic asthma [5].

The IL-1 family has been involved in inflammatory and immunologic responses [9]. Members contain activators and suppressors of inflammation [9]. Interleukin-1 receptors (IL-1Rs) and Toll-like receptors (TLRs) are members of a large superfamily of phylogenetically conserved proteins involved in innate immunity and inflammation [10]. The common characteristics of these two receptor families include their presence in the cytoplasmic region of a conserved sequence called Toll/IL-1R (TIR) domain [9, 11]. The IL-1R/TLR-driven immune response also has an essential role in the induction and/or regulation of allergic inflammation and disease exacerbations.

ST2, one member of the IL1 receptor family, was firstly identified as an orphan receptor in 1989 [12]. In 2005, the discovery of interleukin-33 (IL-33) as an ST2 ligand provided new insights into ST2 signalling pathway [13]. ST2/IL-33 signalling is involved in T-cell mediated immune responses, particularly Th2 cells and the production of Th2-associated cytokines [14].

Allergic asthma is used as an example of a chronic inflammatory disease to show how IL-1R/TLR-related pathway offer possibilities of therapeutic intervention. Due to similar roles in the pathogenesis of allergic inflammation, focus was on the IL-1RI-IL-1 and ST2-IL-33 pathways to review their potential as therapeutic targets for treating asthma [15]. Special attention was given to the experimental approach in validating the possibility of targeting IL-1R/TLR to explore emerging treatments.

2. IL-1RI-IL-1

IL-1 was first described and cloned in the family [11, 16–21]. The IL-1R type 1 (IL-1RI) contains three extracellular immunoglobulin (Ig) domains [9, 11]. A second chain, the IL-1 receptor accessory protein (IL-1RaP) [22] has been reported. It is essential for the signal transduction for IL-1 and IL-33 and is highly homologous to IL-1RI. IL-1RaP forms a heterodimer with either the IL-1RI or the IL-33Ra chain (ST2) [9]. IL-1RI and IL-1RaP form the receptor complex for IL-1 (both IL-1α and IL-1β) and binds naturally occurring IL-1 receptor antagonists (IL-1Ra) [23]. The Drosophila protein Toll has a cytosolic domain homologous in sequence to IL-1RI [24], which is called the TIR domain. It is also found in the cytoplasmic domains of each TLR, sometimes shortened to the Toll-IL-1 receptor domain [25]. The TIR domains of IL-1RI and the coreceptor IL-1RaP are necessary for signal transduction.

2.1. Signaling Pathway. Detailed structures for IL-1 bound to the IL-1RI/IL-1RaP complex have been discovered, as well as structures for IL-1RA bound to IL-1RI/IL-1RaP [26, 27]. In crystallization studies, IL-1RI undergoes conformational change when binding IL-1β and allows IL-1RaP to form the heterodimer [9]. The formation of an IL-1 receptor heterodimer complex results in the approximation of adjacent TIR domains. This complex recruits intracellular adapter molecules, including MyD88 (myeloid differentiation factor 88), IRAK (IL-1R associated kinase), and TRAF6 (tumor necrosis factor [TNF] receptor-associated factor 6), to activate signal transduction pathways such as nuclear factor-κB (NF-κB), AP-1 (activator protein-1), JNK (c-Jun N-terminal kinase), and p38 MAPK (mitogen-associated protein kinase) [21, 28].

In an animal model of asthma, persistent NF-κB activation in the bronchi is driven by granulocytes via IL-1β and TNF-α, which both induce β-β degradation, perpetuating the immune response in asthmatic airways [29, 30]. IL-1Ra binds tightly to IL-1RI and blocks the activity of either IL-1α or IL-1β. One of the binding sites of IL-1Ra binds to IL-1RI with high affinity such that the second binding site cannot recruit the IL-1RaP [9].

2.2. Studies in IL-1RI/IL-1 Pathway-Deficient Mice. The critical role of IL-1/IL-1RI in the development of allergic Th2 responses in both mild and more severe asthma has been studied [31]. The role of IL-1 in pulmonary immune responses in models of allergic asthma has been investigated using IL-1RI-deficient (IL-1RI−/−) mice. Pulmonary eosinophilic inflammation, goblet cell hyperplasia, priming of CD4+ T cells in bronchial lymph nodes and their recruitment to the lungs, and antibody responses, including IgG, IgE, and IgA, are strongly reduced in IL-1RI−/− compared to control BALB/c mice. In contrast, in a model of more severe asthma, eosinophilic inflammation, antibody responses, and CD4+ T cell priming in lymph nodes are comparable between IL-1RI−/− and wild-type mice. These results suggest an important role of IL-1/IL-1RI in developing allergic Th2 responses, but may not be necessary for severe allergic Th2 responses.

Another study demonstrates that IL-1 plays important roles in the development of AHR by validating IL-1α, IL-1β, and the natural inhibitor of both molecules [32]. It demonstrates that ovalbumin (OVA)-induced AHR, OVA-specific T cell proliferation, IL-4 and IL-5 production by T cells, and IgG1 and IgE production by B cells, in IL-1α/β-deficient [IL-1α−/−/β−/−] mice is significantly reduced from levels seen in wild-type mice, whereas responses seen in IL-1α−/− mice are profoundly exacerbated or enhanced. These observations indicate that IL-1 plays important roles in the development of AHR and in establishing an important balance between proinflammatory cytokines and their inhibitors in allergic airway disease [33].

2.3. Experimental Application in Targeting the IL-1RI/IL-1 Pathway. Using the asthma animal model, the effects of targeting the IL-1RI/IL-1 pathway are summarized in Table 1. Reagents used include recombinant adenovirus expressing human IL-1rA (Ad-hIL-1rA), recombinant human interleukin-1 receptor antagonist (rhIL-1rA), and neutralizing antibodies to both IL-1β and IL-1α. Following airway sensitization with ovalbumin, there is suppression of AHR, inflammatory infiltration, and IL-5 production after antigen challenge in mice expressing the IL-1rA adenovirus [34]. Using guinea pigs sensitized with different allergens, two
Table 1: Effect of therapeutic experiments Targeting IL-1RI/IL-1 pathway.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Mechanism</th>
<th>Animal model</th>
<th>airway hyper-reactivity (AHR)</th>
<th>Inflammatory infiltration (eosinophils and lymphocytes)</th>
<th>IgE</th>
<th>Th1 cytokine</th>
<th>Th2 cytokine</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant adenovirus expressing human IL-1ra (Ad-hIL-1ra)</td>
<td>receptor antagonist; gene therapy</td>
<td>Mice sensitized with ovalbumin (OVA)-immunized mice</td>
<td>↓</td>
<td>↓</td>
<td>N.A.</td>
<td>↑ (IFN-γ)</td>
<td>↓ (IL-5)</td>
<td>[34]</td>
</tr>
<tr>
<td>Recombinant human interleukin-1 receptor antagonist (rhIL-1ra);</td>
<td>receptor antagonist</td>
<td>guinea pigs sensitized with Ascaris antigen</td>
<td>↓</td>
<td>(pulmonary resistance)</td>
<td>↓</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>antibodies against IL-1β neutralizing monoclonal Ab</td>
<td></td>
<td>murine model of toluene diisocyanate-induced asthma</td>
<td>↓</td>
<td>adhesion-Molecules (sICAM-1 and VCAM-1) levels</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

studies using rhIL-1ra also demonstrate reduced airway symptoms induced by allergen challenge [35, 36]. Regarding the effect on inflammation, there is decreased expression of adhesion molecules only in one study [36]. In mice treated with neutralizing anti-IL-1β antibodies, AHR to inhaled antigen is partially reduced but with a concomitant decrease in the expression of other adhesion molecules, as well as the suppression of IL-4 [37].

3. ST2-IL-33

IL-33, another IL-1-like cytokine, drives the production of Th2-associated cytokines from in vitro polarized Th2 cells. In vivo, IL-33 induces the expression of IL-4, IL-5, and IL-13 and leads to severe pathologic changes in mucosal organs [13]. Mice injected with human IL-33 exhibit impressive pathologic changes in the arterial walls, lungs, and intestinal tissues [13]. Of particular relevance to the concept of IL-33-driven Th2 response is the prominent eosinophilic infiltration in lung tissue. Airway smooth muscle cells have IL-33 expression in both the protein and mRNA levels. IL-33 expression increases in bronchial biopsies in asthmatic subjects compared to controls, as well as subjects with severe asthma [38].

IL-33 mediates its biologic effects via ST2, an IL-1 receptor-related protein specifically expressed on mast cells and Th2 lymphocytes [15] that has been shown to function as an important effector molecule of Th2 responses in some experimental settings, including mouse asthma models. IL-33 administration induces AHR and goblet cell hyperplasia through the induction of IL-4, IL-5, and IL-13 entirely independent of the acquired immune system. Administration of IL-33 induces AHR and goblet cell hyperplasia in the lungs in the absence of an adaptive immune system [39]. It stimulates mast cells into producing IL-13 in an FcεRI-independent manner [40].

A prior study has shown ST2 to be highly expressed on Th2 cells and it appears to play a role in Th2 cell activation [41]. The ST2 gene is a member of the IL-1 receptor family, producing a secreted soluble form, soluble ST2 (sST2), and a transmembrane form, ST2L [42]. The structure of ST2L is similar to that of IL-1 receptor type I (IL-1RI), which consists of three extracellular immunoglobulin domains and an intracellular Toll/IL-1 receptor domain. Although the extracellular domain is common to sST2 and ST2L, sST2 lacks the trans-membrane and intracellular Toll-interleukin-1 receptor domains [15].

Beyond its role as a therapeutic target, sST2 has also emerged as a disease biomarker. Previous studies in human patients and animal models have shown that the level of sST2 in sera is elevated in asthma [43, 44]. Therefore, it is suggested that sST2 also plays a critical role in Th2 cell-mediated diseases. Administering a recombinant sST2-Fc fusion protein or a sST2 expression vector to asthmatic mice effectively attenuates inflammatory responses and
Table 2: Effect of therapeutic experiments Targeting ST-2/IL-33 pathway.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Mechanism</th>
<th>Animal model</th>
<th>airway hyper-reactivity (AHR)</th>
<th>Inflammatory infiltration (eosinophils and lymphocytes)</th>
<th>IgE</th>
<th>Th1 cytokine</th>
<th>Th2 cytokine</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>antibodies against IL-33</td>
<td>neutralizing monoclonal Ab</td>
<td>Mice sensitized with ovalbumin</td>
<td>N.A.</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>[53]</td>
</tr>
<tr>
<td>antibodies against T1/ST2</td>
<td>blocking monoclonal</td>
<td>Mice sensitized with ovalbumin</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>↓ (IL-4);</td>
<td>[54]</td>
</tr>
<tr>
<td>recombinant soluble ST2</td>
<td>soluble receptor</td>
<td>Mice sensitized with ovalbumin; OVA-stimulated splenocytes</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>↑ (IFN-γ) from stimulated splenocytes</td>
<td>↓ (IL-4, IL-5, and IL-13) from IL-33-stimulated splenocytes</td>
<td>[42]</td>
</tr>
<tr>
<td>plasmid expressing soluble ST2</td>
<td>soluble receptor; gene therapy</td>
<td>Mice sensitized with ovalbumin</td>
<td>N.A.</td>
<td>↓ (eosinophils)</td>
<td>N.A.</td>
<td>↑ (IFN-γ)</td>
<td>↓ (IL-4 and IL-5)</td>
<td>[44]</td>
</tr>
</tbody>
</table>

3.1. Signaling Pathway. The ST2 receptor is similar to the IL-1RI and IL-18Ra in that it is composed of three extracellular Ig domains and an intracellular Toll domain. T1/ST2-dependent IL-33 responses resemble classical IL-1-like signaling, consistent with IL-33 receptor signaling via the recruitment of a coreceptor, IL-1RaCP [28]. IL-33 forms a heterodimer complex with ST2 and IL-1RaCP for signal transduction [46, 47]. Thus, IL-1RaCP represents a shared co-receptor within the IL-1 family that is essential for IL-33 signaling via T1/ST2, aside from the IL-1 signaling. Binding of IL-33 to ST2 receptor activates NF-κB and MAPKs, induces Th2 cytokine expression, and leads to severe pathologic changes in mucosal organs. ST2 can sequester TLR adaptor molecules such as MyD88 [48], while TRAF6 is a critical signal transducer in the IL-33 signaling pathway [49].

3.2. Studies in ST2/IL-33 Pathway-Deficient Mice. ST2 (−/−) mice develop reduced allergic airway inflammation compared to wild-type (WT) mice. This is associated with reduced differentiation of IL-5+ T cells. However, IL-4 and IL-13 levels are similar in WT and ST2 (−/−) mice. There is a less pronounced increase in total cell, macrophage, and eosinophil accumulation in the BAL fluids of ST2 (−/−) mice compared to WT mice [50]. These indicate that IL-33/ST2 signaling is an important pathway in allergic airway inflammation. IL-33 may be involved in lung macrophage activation in clinical asthma and may play a significant role in the amplification of alternatively activated macrophage (AAM) polarization and chemokine production, which contribute to both innate and Ag-induced airway inflammation [51].

Using a primary pulmonary granuloma model induced with Schistosoma mansoni eggs, Townsend et al. have demonstrated that granuloma formation, characterized by eosinophil infiltration, is abrogated in T1/ST2-deficient mice [52]. Naive immune cell populations, cytokine levels, Th1 and Th2 cell development, and total Ig isotype production are normal in T1/ST2-deficient mice. In the absence of T1/ST2-expression, induction of primary synchronous pulmonary granuloma formation and Th2 cytokine (IL-4 and IL-5) production occurs in response to the formation of secondary pulmonary granuloma. Such data demonstrates clearly that T1/ST2 expression plays a role in the development of Th2-like cytokine responses.

3.3. Experimental Application in Targeting the ST2/IL-33 Pathway. Using the asthma animal model, the effects of targeting the ST2/IL-33 pathway is summarized in Table 2. Reagents used include neutralizing antibodies against IL-33, neutralizing antibodies against T1/ST2, recombinant sST2, and recombinant ST2-expressing plasmid. Treatment with anti-IL-33 significantly reduces serum IgE secretion, the number of eosinophils and lymphocytes, and concentrations of IL-4, IL-5, and IL-13 in bronchoalveolar lavage fluid compared to administering a control antibody, which indicate that blocking IL-33 may be a new therapeutic strategy for allergic asthma [53]. Blocking IL-33–T1/ST2 signaling using an antibody against T1/ST2 abrogates persistent AHR, suggesting that the IL-33–T1/ST2 pathway is necessary not only in the development of an allergic response but also for its maintenance. Anti-T1/ST2 antibody also significantly reduces the expression of IL-4. In contrast, IL-13 levels are unchanged [54].

In a murine model of asthma, pretreatment with sST2 reduces IL-4, IL-5, and IL-13 production from IL-33-stimulated splenocytes [42]. This indicates that sST2 acts as a negative regulator of Th2 cytokine production and allergic airway inflammation modulates the biological activity of IL-33 signaling. Soluble ST2 directly binds to IL-33 and suppresses the activation of NF-κB in EL-4 cells stably expressing ST2L, suggesting that the complex of sST2 and IL-33 fails to bind to ST2L. Enhanced expression levels of sST2 are also achieved by intravenous gene transfer, resulting in a
drastic reduction in the number of eosinophils and in IL-4 and IL-5 levels in BAL fluid [44].

4. Conclusions and Future Perspectives

Collectively, the IL-1RI—IL-1 and T1/ST2—IL-33 pathways are critical for immunologic control of allergic inflammation (Figure 1). From asthma animal studies, emerging strategies for asthma treatment are aimed at restoring the imbalance in cytokine (IL-1 and IL-33) and signaling pathways (IL-1RI and ST2) that mediate inflammatory and structural changes, and in skewing the cytokine profile away from a pro-inflammatory response towards a regulatory response. Potential therapeutic approaches may identify new strategies targeting key molecular mediators that drive inflammatory responses in asthmatic lungs. Such strategies include the depletion of cytokines using neutralizing mAb and soluble receptor constructs, receptor blocking via binding to recombinant receptor antagonists, small-molecule receptor antagonists or neutralizing mAb [55], and target receptors or cellular signal transduction pathways that are activated following cytokine receptor ligation, and gene therapy (expressing receptor antagonists or soluble receptor). Such approaches provide disease-modifying treatments.

Recently, there has been much focus on the signaling pathways involved in asthma. Among these, the MAPK pathway members JNK and p38 have attracted much interest, aside from NF-κB, AP-1, and signal transducer and activator of transcription (STAT)-6 [56]. These signaling pathways are all involved in the IL-1RI—IL-1 and T1/ST2—IL-33 pathways. With advances in knowledge of cellular and molecular mediators involved in inflammation underlying asthma, there is much promise the future development of potential new therapeutics.

Figure 1: IL-1RI—IL-1 and T1/ST2—IL-33 pathways involved in allergic inflammation in asthma. “+” or “−” denote enhanced/activated or attenuated/suppressed effects of modulating signaling pathways, respectively. IL-1RI, IL-1 receptor type I; IL-1RaCp, IL-1 receptor accessory protein; IL-1Ra, IL-1 receptor antagonist; TIR, Toll/IL-1 receptor domains; sST2, soluble ST2; ST2L, trans-membrane ST2; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase. Modified from [13, 21, 29, 30].

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Research Article

Inhibition of Toll-Like Receptor 2-Mediated Interleukin-8 Production in Cystic Fibrosis Airway Epithelial Cells via the \( \alpha 7 \)-Nicotinic Acetylcholine Receptor

Catherine M. Greene, Hugh Ramsay, Robert J. Wells, Shane J. O’Neill, and Noel G. McElvaney

Department of Medicine, RCSI Education and Research Centre, Beaumont Hospital, Dublin 9, Ireland

Correspondence should be addressed to Catherine M. Greene, cmgreene@rcsi.ie

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Cystic Fibrosis (CF) is an inherited disorder characterised by chronic inflammation of the airways. The lung manifestations of CF include colonization with *Pseudomonas aeruginosa* and *Staphylococcus aureus* leading to neutrophil-dominated airway inflammation and tissue damage. Inflammation in the CF lung is initiated by microbial components which activate the innate immune response via Toll-like receptors (TLRs), increasing airway epithelial cell production of proinflammatory mediators such as the neutrophil chemokine interleukin-8 (IL-8). Thus modulation of TLR function represents a therapeutic approach for CF.

Nicotine is a naturally occurring plant alkaloid. Although it is negatively associated with cigarette smoking and cardiovascular damage, nicotine also has anti-inflammatory properties. Here we investigate the inhibitory capacity of nicotine against TLR2- and TLR4-induced IL-8 production by CFTE29o- airway epithelial cells, determine the role of \( \alpha 7 \)-nAChR (nicotinic acetylcholine receptor) in these events, and provide data to support the potential use of safe nicotine analogues as anti-inflammatories for CF.

1. Introduction

CF is an autosomal recessive inherited disorder characterised by mutations in the gene encoding the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein. It is the most common inherited metabolic disorder among Caucasians of European descent, with the most common defect being the \( \Delta F508 \)CFTR mutation which causes the protein to fold aberrantly and accumulate in the endoplasmic reticulum of CFTR-producing cells. This leads to decreased apical expression of CFTR in airway epithelial cells, impaired Cl\(^-\) conductance, Na\(^+\) hyperabsorption, mucus hypersecretion, impaired mucociliary clearance, and colonization with microorganisms [1].

The lung manifestations of CF are characterised by chronic infection and neutrophil-dominated airway inflammation and are initiated by proinflammatory microbial stimuli culminating in increased airway epithelial cell production of proinflammatory mediators, including the neutrophil chemokine interleukin-8 (IL-8) [2]. Toll-like receptors (TLRs) play an important role in these events [3]. TLRs respond to microbial antigens and initiate signalling cascades that culminate in proinflammatory gene expression, principally via activation of the transcription factors NFkB and the IRFs [4–6]. TLRs are present on a variety of cell types, including both immune cells and epithelial cells within the lung [7]. The expression and function of ten members of the human TLR family have been partially or fully characterized to date. TLRs expressed by airway epithelial cells contribute to the pulmonary immune response by regulating the production and secretion of diffusible chemotactic molecules, mucins, antimicrobial peptides, and cytokines and by enhancing cell surface adhesion molecules expression [3, 8–23]. A plethora of proinflammatory cytokines is regulated by TLR activation in airway epithelial cells; TNF\( \alpha \) and IL-6 can be induced by TLR2, TLR4, and TLR9 agonists, for example, [3, 10, 21, 24]. IL-8 is a potent neutrophil chemoattractant. It
is a particularly important cytokine in the neutrophil-dominated CF lung. In the context of CF and airway epithelial cells, various TLR agonists have been shown to promote proinflammatory gene transcription (reviewed in [7]). Chronic activation of TLRs can lead to overproduction of these factors and ultimately have a deleterious effect on pulmonary function and homeostasis.

Of all the TLRs, TLR2 has emerged as the principal receptor responsible for orchestrating changes in proinflammatory gene expression in airway epithelial cells [11, 16, 17, 19, 20]. TLR2 is activated by the broadest repertoire of agonists including lipoteichoic acids, peptidoglycan, di- and tri-acylated lipopeptides from Gram-positive and/or Gram-negative bacteria, protozoans, mycobacteria, yeasts, and mycoplasma and is interesting amongst the TLR family in that it can heterodimerize with other TLRs to confer responsiveness to these diverse ligands. In conjunction with TLR1 it recognizes triacylated lipopeptides and Gram-positive lipoteichoic acid; whereas with TLR6 it can respond to diacylated lipopeptides such as MALP-2 from mycoplasma. Due to the presence of multiple potential TLR2 agonists in the CF lung, this environment represents a milieu where TLR2 is likely to be chronically activated [25]. Thus modulation of TLR2 function represents a therapeutic target for CF.

Nicotine is a naturally occurring plant alkaloid. Although it is negatively associated with cigarette smoking, addiction, and cardiovascular damage, nicotine also has therapeutic properties and is a promising new treatment for chronic inflammatory disorders. For example nicotine is prescribed to treat the overt inflammation of gut epithelial cells in ulcerative colitis [26] and is reported to have potential therapeutic benefit for neuroinflammatory conformational disorders including Alzheimer’s and Parkinson’s diseases [27]. Interestingly TLRs have been shown to play a role in the disordered inflammatory response in ulcerative colitis (UC) [28].

Nicotine exerts a variety of biological effects via the nicotinic acetylcholine receptors (nAChRs), for example, inhibiting LPS-induced TNFα, IL-1, and IL-6 in rat peritoneal macrophages, iNOS in murine macrophages or IL-18 in human monocytes [29–31]. nAChRs are ligand-gated cation channels that comprise a pentameric transmembrane complex of multiple α(1-10), β(1-4), γ, δ or ε subunits, each of which has four transmembrane spanning domains that form the ion channel [32]. α(2-6) and β(2-4) can form hetero-oligomeric nAChRs, whereas α(7-9) subunits form homo-oligomers. It is the α subunit that contains the ligand binding domain. The human α7 subunit is ~50 kDa and is composed of 502 amino acids and a 22-residue signal peptide [32]. Studies of the anti-inflammatory effects of nicotine implicate α7-nAChR as the receptor involved [27, 29, 31]. The α7-nAChR has been shown to be present on human bronchial epithelial cells [33]. However, it remains to be determined if the α7 receptor is present on CF airway epithelial cells.

In this study we investigate the effect of nicotine on IL-8 production by a CF airway epithelial cell line (CFTE290-) in response to a range of TLR2 and TLR4 agonists. We assess expression of α7-nAChR in these cells and use general and specific nAChR antagonists to determine the role of α7-nAChR in nicotine-mediated inhibition of TLR2-induced IL-8 expression.

2. Materials and Methods

2.1. Cell Cultures and Treatments. CFTE290- cells are a ΔF508 homozygous tracheal epithelial cell line. These were obtained as a gift from D. Gruenert (California Pacific Medical Center Research Institute, San Francisco, CA). The cells were cultured in EMEM (Invitrogen Life Technologies) supplemented with 10% foetal calf serum (FCS) at 37°C in a humidified atmosphere in 5% CO2. Twenty-four hours before agonist treatment, the cells were washed with serum-free EMEM and placed under serum-free conditions or in medium with 1% FCS for LPS treatments.

Stock nicotine (Sigma, 1 mg/mL or 6.2 mM in methanol) was diluted in serum-free EMEM. Pseudomonas LPS, peptidoglycan, zymosan, phorbol myristic acetate (PMA), d-tubocurarine, and α-bungarotoxin were from Sigma; triacylated lipopeptide (palmitoyl-Cys((RS)-2,3-di((palmitoyloxy)-propyl)-Ala-Gly-OH) (Pam3) was from Bachem.

2.2. IL-8 Protein Production. Cells (1 × 10⁵) were left untreated, or in some experiments pretreated with d-tubocurarine or α-bungarotoxin as indicated, prior to addition of nicotine at various concentrations for 1 hour at 37°C. Cells were then left untreated or stimulated with TLR2 or TLR4 agonists or PMA for 24 hours at 37°C as indicated. IL-8 protein concentrations in the cell supernatants were determined by sandwich ELISA (R & D Systems). All assays were performed in triplicate.

2.3. Cell Proliferation Assay. CFTE290- cells (1 × 10⁵/mL) were left untreated or stimulated with increasing doses of nicotine (in triplicate) for 24 hours. Following this, the supernatant in each well was replaced with 500 μL of serum free medium and 100 μL of proliferation assay reagent (CellTiter 96 Aqueous One Solution Cell Proliferation Assay) and the samples were incubated for a further 3 hours at 37°C. Samples (120 μL) were transferred from each well of the 24-well plates to a 96-well plate in duplicate. The plate was read at 490 nm. The effect of the blank well was subtracted and change in cell proliferation was measured as a percentage change from the untreated cells.

2.4. Laser-Scanning Cytometry. Cells (1 × 10⁵) were grown in a four-well chamber slide, washed with PBS, Fc-blocked for 15 minutes at room temperature with 1% BSA (Sigma-Aldrich), then labelled with anti-α7-nAChR primary antibody (Abcam) for 30 minutes at 4°C. Following three washes, cells were incubated with 10 μg/mL FITC-labelled secondary antibody (antirabbit F(ab)2 FITC (DakoCytomation)) for 30 minutes at 4°C. Cells were counterstained with propidium iodide (PI) (Molecular Probes), and laser-scanning cytometry (LSC) (Compucyte) was used to quantify cell surface α7-nAChR expression. LSC is slide-based cytometry which enables the detection and quantification of cell surface
expressed (or intracellular markers if a permeabilisation reagent is used) on cytospun or adherent cells without the need for trypsinization, a process which can potentially remove some receptors [3, 24, 34–40]. Cells are stained with PI enabling detection of all cell nuclei and an FITC-labelled antibody directed against the receptor of interest allows quantification of the target on the total cell population. FITC and PI cellular fluorescence of at least 2000 cells were measured. α7-nAChR expression was quantified using CompuCyte software on the basis of integrated green fluorescence. An appropriate rabbit antimouse isotype antibody was used as a control (DakoCytomation).

2.5. Statistical Analysis. Data were analysed with GraphPad Prism 4.0 software (GraphPad). Results are expressed as mean ± SE and were compared by Mann Whitney U-test. Differences were considered significant when the P-value was ≤ .05.

3. Results

3.1. TLR2 and TLR4 Agonists Induce IL-8 Production from CFTE29o- Cells. The effect of the TLR agonists zymosan, peptidoglycan (PTG), triacylated lipopeptide (Pam3), and Pseudomonas LPS on IL-8 production by CFTE29o- cells was quantified by ELISA (Figure 1). Each of the TLR2 agonists dose dependently increased IL-8 production by CFTE29o- cells compared to untreated cells after 24 hours treatment (Figure 1(a)). The zymosan preparation was found to be contaminated with intact yeast particles so for subsequent experiments only PTG or Pam3, at 5 μg/mL and 1 μg/mL, respectively, were used. LPS treatment (10 μg/mL, 24 hours) also significantly increased IL-8 expression by CFTE29o- cells (Figure 1(b)). PMA (50 ng/mL) is a known inducer of IL-8 and was used as a positive control.

3.2. Nicotine Inhibits Peptidoglycan- and Triacylated Lipopeptide-Induced IL-8 Production by CFTE29o- Cells. We next investigated the effect of nicotine on TLR2 agonist-induced IL-8 production (Figure 2). As before PTG treatment (5 μg/mL, 24 hours) led to a significant increase in IL-8 production from CFTE29o- cells compared to untreated controls. This response was significantly reduced in the presence of nicotine at concentrations of 10 and 50 μM. The vehicle control had no effect at these doses however at a dose equivalent to 100 μM nicotine, vehicle significantly impaired PTG-induced IL-8 production (data not shown). For this reason we carried out all subsequent experiments using nicotine at concentrations up to 50 μM.

Figure 3 shows that nicotine also significantly inhibited Pam3-induced IL-8 expression from CFTE29o- cells at 10 and 50 μM.

3.3. Nicotine Does Not Inhibit LPS-Induced IL-8 Production by CFTE29o- Cells. Next the effect of nicotine on IL-8 production induced by the TLR4 agonist Pseudomonas LPS was assessed. These assays were performed in the presence of 1% FCS to facilitate LPS-TLR4 signalling. Figure 4 shows that LPS-induced IL-8 production was not significantly inhibited by pretreatment with nicotine at concentrations of 1–50 μM.

3.4. Effect of Nicotine on CFTE29o- Proliferation. Nicotine has known antiapoptotic effects in a variety of cells [41–44]. However in order to determine that nicotine’s ability

![Figure 1](image1.png)
Figure 1: Effect of TLR2 and TLR4 agonists on IL-8 production in CFTE29o- cells. Triplicate samples of CFTE29o- cells (1 × 10^5/mL) were left untreated or treated with (a) 1–100 μg/mL zymosan, PTG and Pam3, or PMA (50 ng/mL) in serum-free media for 24 hours, or (b) Pseudomonas LPS (10 μg/mL) or PMA (50 ng/mL) in medium supplemented with 1% FCS for 24 hours. Levels of IL-8 in supernatants were measured by ELISA and values are expressed in pg/mL (* P ≤ .05 versus control) (n = 7).
3.5. CFTE29o- Cells Express the α7-nAChR. Nicotine is known to exert an anti-inflammatory effect through the α7-nAChR [45]. We used laser scanning microscopy to examine cell surface expression of α7-nAChR on CFTE29o-cells. Figure 6 illustrates that CFTE29o-cells express the α7-nAChR; the histogram in Figure 6(a) shows clear detection of α7-nAChR with an anti-α7-nAChR antibody (solid) compared to an isotype control antibody (clear). In Figure 6(b) the median channel fluorescence (MCF) emitted by the FITC-linked anti-α7-nAChR antibody is significantly greater than that of the isotype antibody (163,710 ± 31,788 versus 325,680 ± 55,554 MCF; *P = .0011).

3.6. α7-nAChR Mediates Nicotine’s Inhibitory Effect on TLR2-Induced IL-8 Production in CFTE29o- Cells. Finally we investigated whether nicotine mediates its anti-inflammatory effects via α7-nAChR in CF airway epithelial cells. To do this we employed the use of d-tubocurarine, a broad-range nAChR inhibitor, and α-bungarotoxin, a specific α7-nAChR inhibitor. For these experiments we used nicotine at 10 μM and as before this dose significantly inhibited Pam3-induced IL-8 protein production (Figure 7). Pretreatment with either antagonist for 1 hour had no effect on nicotine’s ability to inhibit the TLR2 response (data not shown). However pretreatment for 16 h with the broad range nAChR antagonist d-tubocurarine reversed the inhibitory effect of nicotine on Pam3-induced IL-8 expression, with IL-8 levels not significantly different from those induced by Pam3 alone. Similarly 16 h pretreatment of CFTE29o-cells with α-bungarotoxin (1 μM) abrogated nicotine’s ability to decrease expression of IL-8 in response to Pam3. These data implicate α7-nAChR in nicotine’s anti-TLR2 effect.

4. Discussion

Whilst inflammation in the CF lung is a neutrophil-dominated process, the airway epithelium plays a key role in the regulation of neutrophil recruitment via TLR-mediated changes in gene and protein expression [3]. Here we show that CF airway epithelial cells express α7-nAChR and respond to nicotine by inhibiting TLR2 agonist-induced IL-8 expression. This novel finding is of particular interest with respect to CF, as the CF lung is a milieu rich in potential TLR2 agonists and because TLR2 is the predominant TLR
cells express α7-nAChR. Our studies have detected α7-nAChR on CF tracheal epithelial cells for the first time and show that specific inhibition of α7-nAChR using α-bungarotoxin (a 75 amino acid peptide from Bungarus multicinctus venom) abrogates nicotine’s ability to impair Pam3-induced IL-8 protein production. Thus α7-nAChR may represent a new therapeutic target for CF. Agonists of α7-nAChR have previously been proposed for the treatment of inflammatory diseases via their ability to reduce TNFα release from macrophages. For example in vivo treatment with nicotine can inhibit TNFα-induced HMGB1 secretion and has a proven therapeutic benefit in models of sepsis [48]. In these studies nicotine did not affect levels of total or phosphorylated versions of ERK, JNK, or p38 MAPK, rather the observed effects occurred directly via α7-nAChR-mediated blockade of NFκB.

A major drawback to the potential use of nicotine as a therapeutic agent is its negative side effects which are associated with addiction, cardiovascular disease, hypertension, cancer, reproductive and gastrointestinal disorders. However, nicotine analogues exist that lack addictive or damaging side effects but retain desirable anti-inflammatory and cognitive-enhancing properties. Indeed the objective in developing nicotine analogues is the discovery of novel drugs that feature the beneficial actions of nicotine whilst eschewing its side-effect profile [56, 57]. The addictive properties of nicotine are mediated via the β2-containing nAChR subtypes, hence compounds that are selective for the α7-nAChR—the receptor that mediates nicotine’s anti-inflammatory effects—are attractive as potential therapeutic agents. Varenclline is a partial agonist of the αβ2 receptor and a full agonist of α7-nAChR that is currently used as a smoking-cessation therapy. Given its nAChR affinity, unlike nicotine, it lacks addictive effects but retains anti-inflammatory benefits [58]. Thus evaluation of the anti-inflammatory properties of varenclline for CF would be worthy of further study.
Notwithstanding the novelty of this study the observations are limited somewhat by the fact that only a single CF epithelial cell line was used, cytokines other than IL-8 were not measured and nicotine analogues were not tested. It will also be important to explore in greater detail the mechanism by which nicotine achieves its anti-inflammatory effect in CF epithelium. These questions will form the basis of future studies.

In conclusion the findings of this study indicate that nicotine and nicotine analogues have potential to inhibit TLR2-mediated inflammation in response to common agonists in the CF lung via α7-nAChR. These useful effects occur at dose levels that could be delivered to CF lungs through inhaled preparations.

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Review Article

Association of Toll-Like Receptor Signaling and Reactive Oxygen Species: A Potential Therapeutic Target for Posttrauma Acute Lung Injury

Meng Xiang, 1, 2 Janet Fan, 3 and Jie Fan 1, 4

1 Department of Surgery, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15260, USA
2 Department of Pathophysiology, Fudan University, Shanghai 200032, China
3 Department of Medicine, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15260, USA
4 Surgical Research, VA Pittsburgh Healthcare System, University Drive, Pittsburgh, PA 15240, USA

Correspondence should be addressed to Jie Fan, fanj2@upmc.edu

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Morphologically, ALI is manifested by alveolar and interstitial fluid accumulation, alveolar hemorrhage, fibrin deposition, and lung neutrophil sequestration. The accumulation of neutrophils (PMNs) in the lung vasculature, interstitium, and alveolar space is considered a critical event and has been the target of various preventative strategies. In our laboratory, a simplified animal model of the “two-hit” paradigm, as an example of human diseases, has been used to address the mechanisms of HS-primed PMNs migration and lung inflammation [4]. In this model, animals are subjected to a nonsevere resuscitated HS (hypotension at 40 mmHg for 1 h), followed by a small intratracheal dose of lipopolysaccharides (LPSs). While neither shock nor LPS alone induced injury, the combination caused lung PMNs accumulation and increased 125I-albumin transpulmonary flux [11], suggesting that the mechanisms underlying the HS-primed ALI involve cross talk between Toll-like receptors (TLRs) and interactions between PMNs and alveolar macrophages (AMφ) as well as endothelial cells (ECs), in which reactive oxygen species (ROSs) are a key mediator. The

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Acute lung injury (ALI) frequently occurs in traumatic patients and serves as an important component of systemic inflammatory response syndrome (SIRS). Hemorrhagic shock (HS) that results from major trauma promotes the development of SIRS and ALI by priming the innate immune system for an exaggerated inflammatory response [2]. The lung is an important target organ for systemic inflammatory mediators released after severe infection [3, 4] and trauma [5–7], and thus acute lung injury (ALI) frequently occurs in traumatic patients and serves as an important component of SIRS [8]. Over the past decade, despite improvements in supportive care, ALI is still associated with a high mortality rate ranging from 26 to 35 percent [9]. Resuscitated HS promotes the development of ALI by priming an amplified inflammatory response to a second stimulus, the so-called “two-hit hypothesis” [10]. However, the mechanism underlying HS-primed inflammation has yet to be fully determined.

1. Introduction

Trauma is the fifth leading cause of death among all age groups in the United States and is the leading cause of death among people less than 45 years of age [1]. Hemorrhagic shock (HS) that results from major trauma promotes the development of systemic inflammatory response syndrome (SIRS) by priming the innate immune system for an exaggerated inflammatory response [2]. The lung is an important target organ for systemic inflammatory mediators released after severe infection [3, 4] and trauma [5–7], and thus acute lung injury (ALI) frequently occurs in traumatic patients and serves as an important component of SIRS [8]. Over the past decade, despite improvements in supportive care, ALI is still associated with a high mortality rate ranging from 26 to 35 percent [9]. Resuscitated HS promotes the development of ALI by priming an amplified inflammatory response to a second stimulus, the so-called “two-hit hypothesis” [10]. However, the mechanism underlying HS-primed inflammation has yet to be fully determined.
TLR4 and TLR2 sit at the interface of microbial and sterile inflammation by selectively responding to both bacterial products and endogenous ligands [18], including hyaluronic acid [19], heparan sulfate [20], fibrinogen [21], heat shock proteins [22], and high-mobility group box 1 (HMGB1) [23, 24]. Both inflammation and injury responses in organs subjected to ischemia/reperfusion depend, at least partially, on TLR4 and TLR2 [25–26]. Previous studies from both our group and others have demonstrated that a low level expression of TLR2 in cells can be upregulated by TLR4 signaling [27, 28], suggesting a mechanism of inducible cellular sensitivity to both exogenous and endogenous stimuli. In this paper, we will focus on the role of TLR4 and TLR2 cross talk in the mechanisms of post-trauma ALI.

2. The Role of TLRs in ALI

Toll-like receptors (TLRs) are the first family of pattern recognition receptors (PRRs) discovered in mammals. They are now well accepted that in addition to recognizing pathogen-associated molecular pattern molecules (PAMPs), TLRs can also respond to endogenous molecules released in response to stress, trauma, and cell damage. These molecules have been termed damage-associated molecular patterns (DAMPs) [12]. Interaction between PAMPs and DAMPs enhances the inflammatory response through TLR signaling, as illustrated in Figure 1.

Activation of TLRs initiates two major pathways: the MyD88-dependent pathway, which is used by all TLRs except TLR3, resulting in the activation of NF-κB and activator protein-1 (AP-1); and the TRIF-dependent pathway, which is initiated by TLR3 and TLR4, resulting in the activation of type I interferons (IFNs) [13–15]. TLRs are expressed on a range of immune cells including PMNs, macrophages, dendritic cells (DCs), B cells, and certain types of T cells, as well as on certain nonimmune cells, such as endothelial cells, smooth muscle cells, and epithelial cells that lie at potential sites of entry, including the skin, respiratory tract, intestinal tract, and genitourinary tracts [16, 17].

TLR4 and TLR2 sit at the interface of microbial and sterile inflammation by selectively responding to both bacterial products and endogenous ligands [18], including hyaluronic acid [19], heparan sulfate [20], fibrinogen [21], heat shock proteins [22], and high-mobility group box 1 (HMGB1) [23, 24]. Both inflammation and injury responses in organs subjected to ischemia/reperfusion depend, at least partially, on TLR4 and TLR2 [25–26]. Previous studies from both our group and others have demonstrated that a low level expression of TLR2 in cells can be upregulated by TLR4 signaling [27, 28], suggesting a mechanism of inducible cellular sensitivity to both exogenous and endogenous stimuli. In this paper, we will focus on the role of TLR4 and TLR2 cross talk in the mechanisms of post-trauma ALI.

2.1. Role of TLR4

Expression of functional TLR4 has been found in many cell types in the lung [29]. LPS-induced lethal shock and ALI are TLR4 dependent [30–32]. Findings from our laboratory have demonstrated that LPS downregulates TLR4 expression in AMs, whereas antecedent HS prevents the decrease in TLR4 gene transcription in response to LPS; also, LPS-induced TLR4 mRNA destabilization is reduced in the AMs exposed to HS as compared to that in sham animals [33]. These findings suggest that sustained TLR4 expression following HS may contribute to an enhanced cell response to LPS [33].

Recent studies have shown that HMGB1 is a potent activator of TLR4 [34]. The activation of TLR4 signaling by HMGB1 has been verified in cell lines [35, 36]. HMGB1 was initially identified as a nuclear protein that stabilized nucleosome formation and acted as a transcription factor regulating the expression of several genes [37]. HMGB1 is now known to be as an early inflammatory mediator in ischemia [23, 24], trauma, HS, and noninfectious hepatitis [38, 39]. Marked increase in HMGB1 levels in serum, lungs, and liver was detected within 2 h after HS in mice [40]. Regarding how HS induces HMGB1 secretion, study has shown that epinephrine directly acts through Mβ-adrenergic receptor to stimulate HMGB1 secretion from the Mφ in an autocrine manner [41].

2.2. Role of TLR2

TLR2 is predominantly expressed in the cells involved in first-line host defense, including monocytes, macrophages, dendritic cells, and PMNs [27, 42]. In ECs and epithelial cells the TLR2 expression is low [27], but can be upregulated [43]. TLR2 senses a broad range of components from bacteria, mycoplasma, fungi, and viruses. These components include lipoproteins from a number of pathogens, PGN and LTA from Gram-positive bacteria, LAM from mycobacterium, glycosylphaptatidylinositol anchors from Trypanosoma Cruzi, a phenol-soluble modulin from Staphylococcus epidermis, zymosan from fungi, and glycolipids from Treponema maltophilum [44–47]. TLR2 recognizes its ligands by forming a heterodimer with either TLR1 or TLR6. The resulting TLR1/TLR2 and TLR6/TLR2 complexes recognize distinct ligands, triacyl and diacyl lipoproteins, respectively.

We have reported that in HS, HMGB1 through TLR4 signaling upregulates TLR2 in ECs, and this upregulation...
mediators of inflammation

primary pmn sequestration

alveolus

nucleus

af

nf-κb

induced

tlr2

induced chemokines cytokines

secondary enhanced pmn infiltration

shock-activated pmn

lps

pgn

ros

nf-κb signaling

+ +

induced tlr2

nf-κb

+ +

pmn

amφ

induced chemokines cytokines

figure 2: model of shock-activated pmn in mediating the tlr4-tlr2 cross talk in amφ and amφ priming. hemorrhagic shock-activated pmns primarily migrate into alveoli in response to a trivial inflammatory stimulus, such as lps, and interact with amφ. the interaction between pmn and amφ enhances lps-induced tlr2 expression (+) in the amφ, possibly mediated by pmns-derived oxidants and augmented nf-κb activation. the increased tlr2 expression results in the amplified response of amφ to the tlr2 agonist (pgn), thereby augmenting cytokines and chemokines expression (circled +) and promoting enhanced pmn transalveolar migration. thus, the shock-activated pmn-mediated tlr4-tlr2 cross talk activates a positive feedback signal leading to amφ priming and exaggerated lung inflammation in response to invading pathogens.

associates with an amplified ec function including augmented activation of nadh oxidase and expression of icam-1 in response to tlr2 activation by hmgb1 [48]. previous reports, using both in vivo hs mouse model and in vitro pmn-amφ coculture approaches, have also demonstrated that tlr4 upregulates tlr2 expression in amφ, and this upregulation is significantly augmented by hs-activated pmns [49]. the amplified tlr4-induced tlr2 expression in amφ serves as an important mechanism underlying hs-primed lung inflammation in response to a second challenge from bacterial products. the study shows that upregulated tlr2 markedly increases expression of macrophage inflammatory protein-2 (mip-2), cytokine migration inhibitory factor (mif), and tnf-α in the amφ and induces augmented pmn migration in response to tlr2 ligand pgn (figure 2) [49].

the inducible expression of tlr2 suggests an important physiological significance of tlr-tlr cooperativity, namely that as ligand activation of tlr4 signaling wanes, the signaling functions can be transferred to tlr2, and thus the tlr mediated cellular response can be maintained over a prolonged period of time [28, 50].

3. the role of ros in ali

ros is a collective term that includes a large variety of free oxygen radicals, for example, superoxide anion (o2−) and hydroxyl radicals (•oh), as well as derivatives of oxygen that do not contain unpaired electrons, such as hydrogen peroxide (h2o2), hypochlorous acid (hocl), peroxynitrite (ono), and ozone (o3) [51]. during normal cellular metabolism, ros are steadily produced. however, recent reports have demonstrated their involvement in signaling which affects cellular functions including gene expression, proliferation, cell death, migration, and inflammation [52]. ros are generated from various catalytic pathways mediated by enzymes which are differentially localized inside the cell, including no synthases, enzymes of the respiratory chain, cytochrome p450 monooxygenases, xanthine oxidase, and nadh oxidase.

studies have suggested that ischemia/reperfusion primes circulating pmns for increased ros production, thereby augmenting pmn-mediated lung injury once the pmns are sequestered in the lung [53, 54]. ros appear to participate in the regulation of tlr4 gene expression. the use of the antioxidant n-acetylcysteine (nac) supplementation during resuscitation markedly reduced levels of tlr4 mRNA and partially reverses the prolongation of tlr4 mRNA half-life observed following hs [33].

3.1. nadh oxidase is an important source of ros in hs. emerging evidence has shown that ros derived from nadh oxidase play an important role in mediating organ injury after hs [55–57]. the nadh oxidase complex
Activation in HS [48]. The study shows that oxidant signaling below, but also contribute to endothelial NADPH oxidase way involving HMGB1, TLR4, and Rac1, but independent of NADPH oxidase in response to HS through a signaling path-
By the PMN NADPH oxidase enhances the activation of EC
3.2. Role of ROS in TLR2 Upregulation in AMφ. We have reported an important role of PMN NADPH oxidase in mediating amplified LPS-induced TLR2 upregulation in AMφ [40]. Using both in vivo hemorrhage mouse model and in vitro PMN-AMφ coculture approaches, the studies demonstrated that the TLR4-dependent TLR2 upregulation in AMφ is significantly augmented by antecedent shock; and this effect of shock is particularly mediated by shock-induced ROS released from PMN. The endogenous NADPH oxidase in AMφ may also be involved in the signal transduction, however, the exogenous ROS from PMN NADPH oxidase are essential for inducing amplified TLR2 expression in AMφ in response to TLR4 signaling, because when the AMφ from NADPH oxidase-deficient gp91<sub>phox</sub><sup>−/−</sup> mice were cocultured with PMNs isolated from WT mice subjected to shock, the expression of TLR2 in the gp91<sub>phox</sub><sup>−/−</sup> AMφ was elevated to the same level as that in WT AMφ [40]. Figure 3 illustrates the physiological significance of the ROS-augmented TLR2 upregulation by TLR4 in AMφ.

3.3. Role of ROS in TLR2 Upregulation in Lung EC. ROS also contribute to LPS/TLR4 signaling induced TLR2 expression in lung EC [28]. LPS through TLR4-MyD88-dependent signaling activated NF-κB and induced TLR2 expression in ECs, and this process was enhanced by oxidant signal-
generating generated by PMN NADPH oxidase. The functional relevance of NADPH oxidase in mediating TLR4-induced TLR2 expression in ECs was evident by markedly elevated and stable ICAM-1 expression as well as augmented PMN migration in response to sequential challenge with LPS and PGN (Figure 4) [28].

Interaction of PMNs with ECs is important for the process of PMN sequestration into the lung [30, 60]. EC activation and expression of adhesion molecules are critical to initiate a firm ICAM-1-dependent PMN adhesion to EC and, thus, mediate the early-onset migration of PMNs across the endothelial barrier. In vivo PMN depletion and repletion experiments demonstrated that PMN NADPH oxidase is an important determinant of TNFα-induced NF-κB activation and ICAM-1 expression in lung EC [61]. In vitro PMN-EC coculture study also showed that WT PMNs induced a rapid and increased increase in ICAM-1 expression in lung ECs from WT and NADPH oxidase-deficient p47<sub>phox</sub><sup>−/−</sup> mice in response to TNFα stimulation; while, antioxidant GSH prevented the effect of WT PMNs in amplifying ICAM-1 expression in the ECs, indicating that the interaction between PMNs and ECs is mediated through PMN-derived ROS [62].

4. Targeting Both TLRs and ROS as a Novel Therapeutic Strategy for ALI

As described above, ROS derived from PMN NADPH oxidase through interaction between PMNs and AMφ or PMNs and lung ECs mediate an augmented upregulation of TLR2 in the AMφ and ECs following HS, and in turn, sensitize the cells to TLR2 agonists, exaggerate inflammatory response, and promote the development of ALI. Based on

![Figure 3: Model of HS-induced PMN NADPH oxidase activation. HMGB1 acts through TLR4 and MyD88-dependent signaling to mediate HS-induced NADPH oxidase activation. Akt and P38 MAP kinase are both involved in this event.](image-url)
these findings, targeting both TLRs and ROS simultaneously may present a novel therapeutic strategy for ALI.

Drugs targeting TLRs mainly include either agonists of TLRs to enhance immune responses against infectious agent, or antagonists designed to reduce inflammation due to infection or autoimmunity responses [63]. Since TLR4 deficiency displayed a beneficial effect in attenuating inflammation following trauma, HS, and ischemia/reperfusion [23, 48, 64, 65], TLR antagonists should be considered during the treatment of post-trauma ALI. The approaches to reduce TLR activity have focused on two aspects: (a) monoclonal antibodies, soluble receptors, and other accessory proteins and (b) signal transduction blockers. For instance, a natural soluble form of TLR2 has been found in mouse plasma and breast milk that acts to block TLR2 ligand stimulation [66]. In addition, a member of the TLR/IL-1 receptor family, TIR8 or SIGIRR, has been described to inhibit NF-κB signaling and suggested an endogenous inhibitor of the TLR system [67]. Many of the key molecules in the signaling pathways for each TLR have been identified and are considered druggable targets [43, 68, 69]. The structural basis of the TIR domain of TLRs and adapters, such as MyD88, Mal, TRAM, or TRIF, has been modeled, and small peptidic sequences based on the TIR domain BB loop or peptidomimetics of this region have been made that can block the interactions [69–71]. Although the drugs targeting TLRs have not yet been applied to the treatment of ALI, the critical role of TLRs in the development of ALI targeting of PRRs has opened up a productive area for the therapy of ALI.

ROS-induced injury was considered to occur by at least two major mechanisms: (a) a direct toxic effect of reactive oxygen species on cellular components including lipid peroxidation, oxidation of critical protein thiols on enzymes, and structural protein and nucleic acid damage [72]; (b) an indirect effect mediated via activation of cell signaling pathways culminating in the generation of a number of proinflammatory molecules [73]. The role of ROS in augmenting TLRs crosstalk is now a new addition to the mechanisms underlying ROS-induced organ injury. Moreover, ROS overwhelm the endogenous antioxidant mechanisms, thereby rendering tissues more susceptible to oxidant damage. Indeed, excessive oxidative stress has been shown to correlate with poor outcomes in patients with acute respiratory distress syndrome [74, 75]. Therefore, one potential strategy for obviating the effects of oxidative stress is to employ antioxidant strategies aimed at neutralizing oxidants or enhancing endogenous antioxidant mechanisms. NAC combines both of these properties. NAC can exert antioxidant activity through a direct scavenger effect by virtue of its reduced sulfhydryl group and also by entering the cell and releasing cysteine, which contributes to the synthesis of intracellular reduced glutathione, an important regulator
of the intracellular redox balance [76]. This drug is also attractive for use in humans, since there is long-standing experience with its use in the management of acetaminophen toxicity [77]. In fact, some human studies including those employing NAC as the antioxidant have shown antioxidant therapy to be beneficial [78–81]. Effective NADPH oxidase inhibitors also have been investigated in the attempt to salvage organs from oxidative injury. Two chemically distinct inhibitors of NADPH oxidase, namely diphenylene iodonium (DPI) and 4-hydroxy-3-methoxy-acetophenone (apocynin), have been found to reduce the organ injury associated with HS [55]. Apocynin in combination with TNF-α converting enzyme inhibitor (TACEI) can completely prevent lung from ischemic injury [61]. This system may represent an effective therapeutic approach for the delivery of antioxidants and other anti-inflammatory treatments into the lung after HS.

5. Conclusion

The current pharmacotherapy has not been highly successful in increasing survival during ALI. The role of TLR signaling in the development of ALI has now been well recognized. Recent studies have further demonstrated that ROS are important determinants for augmented TLR4-induced TLR2 upregulation in HS. Therefore, antioxidant strategies together with modification of TLR pathways are likely to be a logical therapeutic target for ALI.

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References

Mediators of Inflammation


Review Article

TLR2 and TLR4 in Ischemia Reperfusion Injury

F. Arslan,1 B. Keogh,2 P. McGuirk, and A. E. Parker

1 Laboratory of Experimental Cardiology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands
2 Opsona Therapeutics Ltd., Institute of Molecular Medicine, Trinity Centre for Health Sciences, St. James’ Hospital, Dublin 8, Ireland

Correspondence should be addressed to A. E. Parker, aparker@opsona.com

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Ischemia reperfusion (I/R) injury refers to the tissue damage which occurs when blood supply returns to tissue after a period of ischemia and is associated with trauma, stroke, myocardial infarction, and solid organ transplantation. Although the cause of this injury is multifactorial, increasing experimental evidence suggests an important role for the innate immune system in initiating the inflammatory cascade leading to detrimental/deleterious changes. The Toll-like Receptors (TLRs) play a central role in innate immunity recognising both pathogen- and damage-associated molecular patterns and have been implicated in a range of inflammatory and autoimmune diseases. In this paper, we summarise the current state of knowledge linking TLR2 and TLR4 to I/R injury, including recent studies which demonstrate that therapeutic inhibition of TLR2 has beneficial effects on I/R injury in a murine model of myocardial infarction.

1. Introduction

Our understanding of the molecular components that link dysregulation of innate immunity and human disease has led to a plethora of experimental evidence in support of Toll-like receptors (TLRs) as novel therapeutic targets for a range of inflammatory and autoimmune diseases including rheumatoid arthritis, systemic lupus erythematosus (SLE), multiple sclerosis, inflammatory bowel disease, cancer and diabetes [1].

TLRs, of which there are currently 10 described in humans, are a family of transmembrane proteins and are the major pattern recognition receptors (PRRs) binding to a range of microbial products, often termed pathogen-associated molecular patterns (PAMPs) [2]. TLR2 functions as a heterodimer with either TLR1 or TLR6 and senses lipopeptides from bacteria, with TLR1/2 dimers recognising triacylated lipopeptides, TLR2/6, and diacylated lipopeptides [2]. TLR4 binds to LPS from gram negative bacteria, which is presented to TLR4 by the accessory factor MD2 [2]. TLR4 can also recognise F protein from respiratory syncytial virus and glycerophosphatidylinositol anchors from parasites and TLR5 binds bacterial flagellin. TLR3 senses double-stranded RNA, TLR7/8 both recognise single-stranded RNA, and TLR9 senses CpG-rich hypomethylated DNA [2].

On activation of the receptor, adaptor molecules (MyD88, MAL, TRIF and TRAM) are recruited to the receptor through their respective TIR domains, which interact with the TIR domain of the TLR. This allows the recruitment and activation of a downstream family of kinases, IRAKs (IL-1 receptor-associated kinases) 1, 2, and 4. IRAK4 is recruited to the complex first, becomes activated, and phosphorylates IRAK1. These kinases lead to activation of further downstream kinases, including inhibitor of NF-κB (IκB) kinases (IKKs), resulting in the release of NF-κB from IκB, allowing NF-κB to translocate to the nucleus and mediate an increase in inflammatory cytokine gene expression, leading to pro-inflammatory responses [2, 3].

In addition to the recognition of PAMPs, TLR2, TLR4, and TLR9 have also been shown to recognise endogenous ligands, which have been termed danger-associated molecular patterns (DAMPs). TLR2 and TLR4 are extracellular TLRs and have a wide range of putative endogenous ligands which include heat shock proteins, high mobility group box 1 (HMGB1) and breakdown products of fibropectin, heparan sulfate, and hyaluronic acid. The broad expression profile of the TLRs and their ability to recognise many ligands that are released predominantly as a consequence of injury and stress positions TLR dependent signaling as a rapid response mechanism to local tissue damage.
2. Ischemia Reperfusion Injury

There is a growing body of evidence linking TLRs, particularly TLR2 and TLR4, to the deleterious inflammatory effects seen in ischemia/reperfusion injury associated with trauma, stroke, myocardial infarction, and solid organ transplantation. Ischemia reperfusion injury refers to the tissue damage caused when blood supply returns to tissue after a period of ischemia. Cessation of arterial blood flow with immediate oxygen deprivation of cells (ie, hypoxia with accumulation of metabolic products) is defined as ischemic injury. Tissue can be subjected to periods of either cold or warm ischemia depending on the clinical setting. Cold ischemia occurs typically in transplantation after organ harvesting and static cold preservation whilst warm ischemia occurs during vascular anastomosis or following organ trauma such as stroke or myocardial infarction. The resistance of various cell populations to different types of ischemia varies depending on the affected organ, for example, cardiac endothelial cells are quite resistant to warm ischemia, and major endothelial injury is only apparent during the reperfusion phase [4]. In contrast, hepatocytes and Kupffer cells in the liver and kidney proximal tubular cells are extremely sensitive to periods of warm ischemia [5, 6]. Nevertheless organs can tolerate prolonged cold ischemia periods or short periods of warm ischemia without significant deterioration of function. However, when ischemia is followed by reperfusion, significant cellular damage is caused [7] which in the case of solid organ transplantations has been associated with an increased incidence of delayed graft function (DGF) and primary graft nonfunction [8, 9].

The absence of oxygen and nutrients from blood creates a condition in which the restoration of circulation (reperfusion) through the ischemic tissue results in a set of reactions that can cause injury to vascular and parenchymal cells [10]. Although the cause of this injury is multi-factorial, increasing experimental evidence suggests an important role for the innate immune system in initiating the inflammatory cascade leading to detrimental/deleterious changes. Pathologically, reperfusion-induced inflammation is characterised by deposition of complement, upregulation of adhesion molecules, inflammatory cell infiltration, and cytokine release [11–14]. Neutrophils, monocytes and lymphocytes are the principal immune cells implicated in this process [15, 16] and facilitate tissue damage through secretion of proinflammatory cytokines, reactive oxygen species, and chemokines.

3. Cardiac Ischemia/Reperfusion

Cardiac ischemia/reperfusion is predominantly associated with myocardial infarction but can also be seen in transplant and coronary artery bypass graft (CABG) surgery. Early reperfusion of the ischemic myocardium is a prerequisite for cardiomyocyte salvage in myocardial infarction. The shorter the ischemic period, the better the clinical outcome. Postischemic reperfusion causes deleterious responses in both cardiomyocytes and circulating cells [17]. Myocardial I/R (MI/R) injury is the acceleration of both apoptosis and necrosis of cardiomyocytes at the onset of reperfusion therapy, resulting in an increase of infarct size, arrhythmias and contractile dysfunction [18–22]. Current reperfusion therapy remains suboptimal and necessitates adjunctive interventions to limit infarct size and enhance clinical outcome. Experimental studies have clearly demonstrated that infarct size can be reduced when MI/R injury is prevented [20, 22].

Reperfusion after myocardial ischemia is a typical “double-edged sword” that results in disease specific changes within cardiomyocytes and circulating cells [23, 24]. The fact that characteristic pathological changes occur in these two compartments (parenchymal, i.e., cardiomyocytes versus, hematopoiesis-derived cells) creates the opportunity to tackle MI/R injury in two ways; either enhance cardiomyocyte survival and/or disarm deleterious circulating cells. Interestingly, TLRs are expressed in both compartments; leukocytes, endothelial cells [25] and cardiomyocytes [26] express certain TLRs that can have different pathophysiological consequences for both the host cell as well as distant organs. Upon ligand binding (either PAMPs or DAMPs), cardiomyocytes and endothelial cells undergo the same TLR signal transduction compared to leukocytes. The main hurdle in MI/R injury is a positive feedback loop between inflammation and cardiomyocyte death: leukocyte-cardiomyocyte/endothelial cell interaction causes cardiomyocyte death, which in turn releases the same cytokines that activate and attract leukocytes [24]. Recent studies using murine MI/R injury models reveal that both TLR2−/− and TLR4−/− offer protection from this vicious circle resulting in a decrease of infarct size and improved cardiac function [27].

Within the first few minutes after reperfusion, NF-κB translocates to the nucleus to induce pro-inflammatory and proapoptotic gene expression promoting cell dysfunction and death [24]. Interestingly, experimental data indicate that parenchymal (i.e. myocardium, endothelium) and circulatory TLR2 are associated with different manifestations of MI/R injury. MI/R injury has four manifestations that are associated with worse cardiac function and clinical outcome. Lethal MI/R injury is directly related to cell death and responsible for infarct size increase during reperfusion. The so-called “no-reflow phenomenon” is the disturbance of coronary flow in the culprit coronary artery after reperfusion [28]. Stunning is contractile dysfunction of the myocardium in the presence of restored coronary flow [29] and reperfusion associated arrhythmias [30]. Investigators have observed the different manifestations of MI/R injury, either deliberately or by accident, by using several challenging experimental methods. TLRs are expressed by both compartments and therefore the relative contribution of parenchymal and circulating TLRs to a certain disease entity requires the use of chimeric mice (WT mice transplanted with knockout bone marrow and vice versa).

Sakata et al. were among the first to show a critical role for TLR2 in an ex vivo model for MI/R injury [31]. Without the detrimental effects of blood components (e.g. leukocytes) after reperfusion, they showed that infarct size did not differ between TLR2−/− and WT hearts. Nevertheless, contractile
performance was significantly impaired in WT hearts, and associated with increased levels of TNFα and IL-1β in the myocardium. These data indicate that in the presence of cardiac ischemic injury, loss of cardiac TLR2 signaling is beneficial for cardiac function. The direct effect of TLR2 activation on contractile performance has been confirmed in an in vitro setting using a murine cardiomyocyte cell line. Boyd et al. showed that stimulation of TLR2 resulted in decreased contractility of plated cardiomyocytes [26]. The finding that TLR2−/− mice have preserved contractile performance compared to WT mice in the setting of S. aureus induced sepsis, serves as a proof for the link between cardiac performance and depressed cardiac function.

Endothelial dysfunction as seen in “no-reflow” seems to be mediated by both endothelial and circulating TLR2. Impaired relaxation responses after MI/R injury were observed in isolated coronary arteries of WT mice and TLR2−/− mice with WT bone marrow [32]. The fact that both compartments play a role in endothelial dysfunction after MI/R injury indicates that, indeed, a vicious circle caused by the interaction of leukocytes and endothelial cells is critical in MI/R injury-related endothelial dysfunction.

We and others were the first to document decreased infarct size in TLR2−/− mice [32, 33]. Using chimeric TLR2−/− mice, we showed that circulating TLR2 completely mediated TLR2-dependent lethal MI/R injury. Infarct size in WT mice with TLR2−/− bone marrow was similar to that in complete knockouts. In addition, TLR2−/− mice with WT bone marrow were not protected at all against MI/R injury, suggesting that parenchymal (i.e. cardiac/endothelial) TLR2 signaling does not play a role in lethal MI/R injury. Systemic administration of a TLR2 antagonist just prior to reperfusion, thus inhibiting circulating TLR2 activation, decreased infarct size and improved cardiac function via downregulated inflammation and apoptotic signaling in mice [33].

Ischemic preconditioning (IPC) is a phenomenon in which brief episodes of repeated ischemia protects the heart against a more severe and prolonged period of ischemia and subsequent reperfusion [34]. In line with the above mentioned findings, TLR2 also appears to play a pivotal role in IPC. TLR2−/− hearts subjected to MI/R injury did not benefit from IPC, whereas TLR4−/− hearts did show improved contractile function after IPC and subsequent MI/R injury [35].

4. Renal Ischemia/Reperfusion

Renal ischemia/reperfusion is most commonly associated with either trauma or transplant. It is possible to detect mRNA for all TLRs in human kidney but TLR2 and TLR4 have been the TLRs primarily implicated in mediating renal ischemia/reperfusion injury. TLR2 and 4 are constitutively expressed in both proximal and distal tubules, the thin limb of the loop of Henle and the collecting ducts. Expression is upregulated in these areas post I/R [36]. Several studies using TLR2−/− and TLR4−/− mice have demonstrated a protective effect in models of renal I/R. Leemans et al. [37] used both KO mice and antisense oligonucleotides to show that blockade of TLR2 has a beneficial effect on renal I/R injury. Following I/R, TLR2−/− mice displayed less tubular epithelial apoptosis, a reduced cellular infiltrate and reduced dysfunction. Through the generation of chimeric mice, the authors also showed that TLR2 expressed on renal parenchyma was the key cell type involved in renal tissue injury which is in contrast to the findings in myocardial infarction model where neutrophils and monocytes were the key cell types mediating injury [33]. Wu et al. [38] demonstrated up-regulation of TLR4 in tubular epithelial cells in response to renal I/R and protection against kidney dysfunction in TLR2−/− and Myd88−/− mice. They also showed up-regulation of HMGB-1, hyaluronan and
brevican, all proposed ligands for TLR2 and TLR4. Pulskens et al. [39] carried out a similar study using TLR4−/− mice demonstrating a protective effect on renal function, chemokine production and cellular infiltration.

The molecular pathways involved in TLR2-mediated damage in renal I/R were investigated by Shigeoka et al. [40]. Using a range of transgenic mice, this study showed that TLR2−/− mice were better protected from I/R damage than those deficient in MyD88, indicating that pathways dependent on TLR2 but independent of MyD88 contribute to kidney injury. More recently Rusai et al. [41] compared TLR2−/− and TLR4−/− mice with the double knockout TLR2/4−/− demonstrating protective effects with both single knock-outs but surprisingly no increased protection when both TLR2 and TLR4 are deleted. This may indicate that TLR2 and TLR4 prime each other in the presence of endogenous ligands, such as during reperfusion injury.

There is also a body of evidence suggesting that TLR2 plays a role in transplantation tolerance. Wang et al. have demonstrated the important role of TLR and TLR signalling pathways in the pathogenesis of kidney chronic allograft dysfunction [42]. In this paper, TLR2 and MyD88, deficiencies significantly improved the excretory function of chronic kidney grafts by 65% and 290% respectively, deficiencies significantly improved the excretory function, and histopathologic signs of chronic allograft damage were significantly ameliorated. T cells, dendritic cells (DCs), and macrophages were reduced in grafts by up to 4.5-fold and intragraft concentrations of IL-6, IL-10, monocyte chemokine production and cellular infiltration.

In a cardiac transplantation study, Chen et al. have demonstrated that TLR2 ligation by the TLR1/2 ligand Pam3CSK4 prevents heart allograft acceptance in mice cotreated with anti-CD154 costimulation therapy. In contrast, mice receiving anti-CD154 treatment alone were observed to display allograft acceptance [43]. In a further study by Jiang et al., innate immunity-mediated cardiac allograft rejection was not prevented by cyclosporine A (CsA) treatment [44]. In this study, mice co-treated with CsA and an anti-inflammatory compound called Serp-1 exhibited a significant down-regulation of TLR2 and TLR4, reduced graft infiltration of macrophage and T lymphocytes posttransplantation and associated indefinite graft survival. In contrast, CsA monotherapy did not prevent TLR2 and TLR4 down-regulation and was ultimately unsuccessful in preventing graft rejection [44]. Interestingly, the chronic application of agents such as cyclosporine, which are currently under investigation in transplant biology, has been associated with renal injury, which has been correlated with upregulation of TLR2 expression on renal tubular cells [45]. Furthermore, increased angiotensin II levels following CsA administration have also been shown to directly upregulate TLR2 expression [46].

Delayed Graft Function (DGF) is a frequent consequence of reperfusion injury of the transplanted donor organ. Furthermore, organ shortages and increased patient waiting times for transplants has necessitated the use of extended criteria and nonheart beating donors. Organs from these donors are exposed to periods of both warm and cold ischemia and as such are significantly more susceptible to DGF than organs from heart beating donors which are only exposed to periods of cold ischemia. Krüger et al. [47] looked at expression levels of TLR4 in human kidney transplants. Whilst TLR4 is constitutively expressed in donor organs, the level of expression was significantly higher in non-heart beating donor kidneys which also correlated with increased levels of HMGB-1. They also genotyped the organs for known TLR4 loss of function mutations which alter signalling in response to HMGB-1 and other ligands. Those organs carrying TLR4 mutations exhibited reduced levels of cytokines and a higher rate of immediate graft function. This argues that there is a significant donor TLR4 effect contributing to inflammation and graft function following cold ischemia and transplantation. The role of HMGB-1 has also been explored in a murine model of heart transplant where administration of a neutralising antibody to HMGB-1 reduced levels of circulating cytokines [48].

Jiang et al. have recently shown that in a rodent model of liver transplantation the expression of mRNA and protein of TLR2 and TLR4, CD14 and MD-2 mRNA as well as endogenous ligands of TLR2 and TLR4 such as HSP60 and HSP70 were quickly and significantly increased after reperfusion, and reached a peak at 3 h after reperfusion. The appearance of TLR2 and TLR4 mRNA was accompanied by increased HSP 60 and 70 mRNA within 24 h after reperfusion [49]. In addition to these studies, CD14+TLR2+ monocytes have been demonstrated to be significantly upregulated in patients with acute liver transplant rejection but not in those with normal liver function post transplantation [50]. Stimulation of TLR2 has also been associated with acute skin graft rejection in a murine co-stimulation blockade model where successful skin grafts were observed with anti-CD154 treatment alone but not in the presence of a TLR2 agonist [51]. In contrast, TLR2-defective animals exhibit prolonged skin graft acceptance [52]. In the latter model, it is worth noting that it is now well-established that lung, intestine and skin are more susceptible to acute rejection episodes posttransplantation than kidney, heart, and pancreas [33]. These studies serve to illustrate further the potential of TLR2 blockade in solid organ transplantation and predict a successful outcome in the amelioration of organ dysfunction post transplant.

6. Other I/R Settings

Several studies have indicated a role for TLR2 in ischemia of other organs such as brain [54–56], liver [57–59], bowel [60], and kidney [36, 61–63]. These studies highlight the complexity of the role of TLR signaling in ischemia/reperfusion.
Zhang et al. [59], showed that TLR2 mRNA was increased in the ischemic lobes of mice that underwent partial hepatic I/R. This was associated with an increase in TNF-α in the portal vein, and was independent of endotoxin as portal vein endotoxin did not increase. This indicates a potential role for endogenous TLR2 ligands in liver I/R. Shen et al. [58] used TLR2−/− and TLR4−/− mice to elucidate the role of TLRs in liver ischemia. They showed that in warm I/R in the liver, hepatocellular injury in WT and TLR2−/− mice was equally severe. This was associated with increased TNF-α and TLR4. However, when TLR4 signaling was prevented, hepatic injury was ameliorated and this was associated with reduced TNF-α levels. There was no effect on expression of TLR2. These effects appeared to be dependent upon intracellular expression of heme oxygenase 1 (HSP32). The role of HSPs, in this case HSP72, was further investigated by Galloway et al. [57]. This study looked at ex vivo hepatocytes from TLR2 and TLR4−/− mice stimulated with HSP72. HSP72 induced MIP-2 in WT cells, this was ablated in TLR2−/− and TLR4−/− mice. Interestingly, no effect on the concentration of either IL6 or TNF was seen. The importance of TLR2 and TNF-α was also shown by Zhang et al. [64]. However, while this study showed that a decrease in TNF-α concentration was beneficial, they also showed that a decrease in TLR2 expression correlated with a benefit on ischemic outcome. Hui et al. used a model of hepatic I/R and chimeric bone marrow TLR4−/− mice to demonstrate that protective effects were provided by both parenchymal and circulating cells [65]. Jin et al. [66] used N-acetylcysteine (NAC) to prevent the generation of reactive oxygen species (ROS) in a mouse model of hepatic I/R. They showed that TLR2 and 4 were activated in the liver and lung. It appears that ROS increases NF-κB activity and causes its translocation to the nucleus. NAC inhibited the activation of TLR2 and 4, and the associated induction of TNF-α.

TLR2 and TLR4 both appear to play critical but opposing roles in cerebral ischemia. Tang et al. [55] recently showed that both TLR 2 and 4 are expressed in cerebral cortical neurons and that selective elimination of their function suppresses activation of JNK. This protects neurons against death by energy deprivation and stroke. This indicated that TLRs also play a role in cerebral ischemia. Ziegler et al. [54] compared the response of TLR2−/− and TLR4−/− mice to cerebral ischemia. They confirmed earlier findings that TLR2 is indeed up-regulated in cerebral ischemia. However, contrary to Hua et al. [67], they showed that TLR2−/− mice had a smaller infarct size. In an earlier study, Hua et al. [68] showed that preconditioning with Pam3CSK4, a TLR2 agonist, 24 hours prior to 1 hour of cerebral ischemia significantly reduced brain infarct size, possibly via an effect on blood brain barrier integrity. In a later study [67], the same authors used knockout mice to show differential roles for TLR2 and TLR4 in cerebral ischemia. This study showed that brain infarct size was significantly less in TLR4−/− mice but was increased in TLR2−/− mice. This was associated with activation of the PI3K/AKT pathway in TLR4−/− mice. This pathway was inhibited in TLR2−/− mice. The difference between this study and that of Ziegler et al. [54] may be explained by the fact that Ziegler et al., occluded the middle cerebral artery, whereas Hua et al. [67] occluded the common carotid artery and the internal carotid artery or alternatively it may be a consequence of the differing genetic backgrounds of the transgenic mice.

7. Conclusions and Perspective

TLRs play complex roles in I/R injuries and the wealth of data generated using transgenic mice is not always in agreement. Both TLR2 and TLR4 appear to be key regulators on the outcome of ischemic damage in many organs. The relative contribution of parenchymal cells and leukocytes to I/R injury and subsequent inflammation appears to differ between organs and manifestations of reperfusion injury. Where double knockouts have been generated, it is perhaps surprising that there is no synergistic effect where each single knockout provides improvement in function and inflammation. There are also conflicting reports of the relative importance of Myd88-dependent and -independent signaling in conferring protection from I/R injury.

The specific benefit of inhibiting TLR responses appears to be governed by the organ however it remains to be established what the triggers and/or activators are for I/R injury. From a “danger model” perspective [69], it is postulated that molecules released during cell stress or cell death may serve as endogenous ligands for TLRs in ischemia [33]. In essence, the entire disrupted milieu within apoptotic cells represent potential candidate ligands when one considers that all hydrophobic portions (so called “Hyppos”) of molecules may initiate a danger signal [70]. So far, a few specific danger-associated molecules (e.g. HMGB1, HSP60, cardiac myosin) have been postulated as TLR ligands in ischemia largely based upon increased levels in damaged tissue but direct in vivo evidence that these drive the inflammatory response is still lacking. Studies on knockout animals have been very informative and clearly established a central role for TLR2 and TLR4 in mediating I/R injury. Nevertheless, therapeutic intervention studies in both small and large animal models are required to fully understand the potential of TLR signaling as pharmaceutical targets. The tools are now available to inhibit TLR2- and TLR4- dependent signaling in mice as well as neutralizing antibodies to postulated ligands such as HMGB-1. In addition, further work is required in other organs such as liver and brain to better understand if agonism or antagonism is required to confer the benefit.

Recent studies clearly demonstrate that inhibition of TLR2 has beneficial effects on I/R injury in a murine model of myocardial infarction [33] offering the first evidence that therapeutic benefit can be derived from targeting TLR2. Further studies are required to expand upon these observations and determine the potential for both TLR2 and TLR4 antagonists in treating I/R injury in multiple organs.

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References


Review Article
The Expression and Functions of Toll-Like Receptors in Atherosclerosis

Jennifer E. Cole, Ektoras Georgiou, and Claudia Monaco

Kennedy Institute of Rheumatology, Faculty of Medicine, Imperial College, 65 Aspenlea Road, London W6 8LH, UK

Correspondence should be addressed to Claudia Monaco, c.monaco@imperial.ac.uk

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Inflammation drives atherosclerosis. Both immune and resident vascular cell types are involved in the development of atherosclerotic lesions. The phenotype and function of these cells are key in determining the development of lesions. Toll-like receptors are the most characterised innate immune receptors and are responsible for the recognition of exogenous conserved motifs on pathogens, and, potentially, some endogenous molecules. Both endogenous and exogenous TLR agonists may be present in atherosclerotic plaques. Engagement of toll-like receptors on immune and resident vascular cells can affect atherogenesis as signalling downstream of these receptors can elicit proinflammatory cytokine release, lipid uptake, and foam cell formation and activate cells of the adaptive immune system. In this paper, we will describe the expression of TLRs on immune and resident vascular cells, highlight the TLR ligands that may act through TLRs on these cells, and discuss the consequences of TLR activation in atherosclerosis.

1. Introduction

Atherosclerosis is the principal cause of coronary artery and cerebrovascular disease, which together comprise the leading cause of death, accounting for a fifth of all deaths worldwide [1]. Over the past decade, a major change has occurred in the understanding of the mechanisms responsible for the development and progression of atherosclerosis, leading to an increasing recognition of atherosclerosis as an “inflammatory disease” [2]. Similarities in cellular and molecular mediators of disease can be found between atherosclerosis and other classical chronic inflammatory diseases, such as rheumatoid arthritis (RA) [3]. Similar to other inflamed tissues such as rheumatoid synovium, the atherosclerotic plaque is characterised by the migration into tissue of bloodborne inflammatory cells, followed by interactions with vascular endothelial cells and connective-tissue cells, leading to a chronic inflammatory response. In support of a strong link between inflammation and cardiovascular disease, RA, is associated with an increased risk of cardiovascular events, which account for 35% to 50% of excess premature mortality in RA patients [4].

Endothelial dysfunction/activation, is the earliest step in the pathogenesis of atherosclerosis [2]. Endothelial dysfunction can be induced by numerous factors including cytokines, free radicals, lipids, and bacterial or viral infection. In addition, endothelial cells may be primed for activation by haemodynamic forces. Activated endothelial cells upregulate adhesion molecule expression, promoting the recruitment of monocytes into the subendothelial space. Recruited monocytes ingest modified lipid and become foam cells, hallmarks of early atherosclerosis, trapped in the vessel wall. Progressive lipid accumulation and leucocyte recruitment leads to the gradual formation of an atheroma that protrudes into the lumen of the vessel wall, narrowing the artery. In addition to monocytes, other leucocyte populations including T lymphocytes, dendritic cells and mast cells have been implicated in the pathogenesis of atherosclerosis. As lesions progress, smooth muscle cells proliferate and migrate into the intima where they deposit extracellular matrix components and form a fibrous cap over the lesion. Rupture of unstable lesions causes thrombus formation, which may lead to myocardial infarction. These processes are now recognised to involve components of both the innate and adaptive immune systems [5].
Innate immunity constitutes the first line of defence against invading pathogens, and it is programmed to detect highly conserved molecular motifs called pathogen-associated microbial patterns (PAMPs) via specialised receptors. Amongst several families of pattern-recognition receptors (PPRs), Toll-like receptors (TLRs) are the most characterised so far. Although the exact gene numbers may differ between species, at least 13 different TLRs have been identified in mammals, each one with a certain degree of specificity for a range of ligands (reviewed later).

The members of the TLR family share the same cytoplasmic domain with the interleukin (IL)-1 receptor, known as the Toll/IL-1R (TIR) homologous domain. As a result, TLRs activate signalling pathways shared with IL-1. The TIR domain recruits the adaptor protein myeloid differentiation primary response gene 88 (MyD88), which activates a family of IL-1R associated kinases (IRAKs). IRAKs in turn activate tumour necrosis factor receptor associated factor 6 (TRAF6), and elicit downstream signalling via the nuclear factor \( \kappa \)B (NF\( \kappa \)B) pathway. NF\( \kappa \)B translocation to the nucleus activates transcription of proinflammatory genes, including tumour necrosis factor (TNF)-\( \alpha \), IL-1, and IL-12. The MyD88-dependent pathway is shared by all TLRs, with the exception of TLR3. TLR4 signalling encompasses both the MyD88- and independent pathway. The MyD88-independent pathway, engaged by TLR3 and -4, relies on TIR-domain-containing adaptor protein inducing interferon \( \beta \) (TRIF) to mediate interferon regulatory factor (IRF)-3 and NF\( \kappa \)B activation. TLR4 utilises Trif-related adaptor molecule (TRAM) to interact with TRIF and engage the MyD88-independent pathway [6].

In this paper, we will describe the expression, ligands, and functions of toll-like receptors in particular in reference to atherosclerosis. The aspects of TLR signalling will be treated in detail in other reviews in this series. We will consider toll-like receptors in both the human and murine systems highlighting important differences between the two organisms with regard to inflammatory mechanisms and TLR biology, which may hinder extrapolation of murine data into human systems.

2. Expression of Toll-Like Receptors

Cells of the innate immune system including monocytes/macrophages and dendritic cells are the main cellular expressers of toll-like receptors. However cells of the adaptive immune system and nonimmune cells have also been shown to express TLRs. Studies attempting to detail TLR expression on different cell types have some common shortcomings. Firstly, they tend to rely on expression at the mRNA level, due to limitations of existing antibodies. Secondly, discrepancies between TLR gene expression and responsiveness to TLR ligands are often observed. Thirdly, gene expression may be influenced by contamination with other cell types when, for example, purified populations of leucocytes are studied.

Notwithstanding these limitations, it is important to fully discern the expression patterns of TLRs in both health and disease as their knowledge may influence the choice of receptor or signalling pathway for therapeutic interventions targeting TLRs. This is particularly pertinent in atherosclerosis, a complex disease involving multiple inflammatory and noninflammatory cells. Within atherosclerotic lesions monocytes/macrophages, B and T lymphocytes, dendritic cells, smooth muscle cells and endothelial cells have all been described as expressing TLRs (Figure 1), or increasing their expression during disease development. Increased expression of TLR-1,-2, and -4 is found in inflammatory cells (including CD68-positive macrophages), smooth muscle cells and adventitial fibroblasts in human atherosclerotic vessels [7–10]. Consistent with human atherosclerosis, both TLR2 and TLR4 expression is increased in low-density lipoprotein receptor deficient (LDLR\(-/-\)) and apolipoprotein E deficient (ApoE\(-/-\)) mice, murine models of atherosclerosis [10, 11]. This increase in TLR expression by cells during atherogenesis may result in enhanced signalling through the TLRs and thus an exacerbation of cell activation and proatherogenic downstream pathways.

2.1. Monocytes/Macrophages. Monocytes and macrophages are present at all stages during atherogenesis and, due to their heterogeneity, have numerous functions through which they affect atherosclerotic plaque initiation and development. Monocytes comprise 5–10% of peripheral blood leukocytes in both humans and mice. Interestingly, this is the only similarity between human and mouse blood. In humans, neutrophils comprise 50–70% and lymphocytes the remaining 30–50% of blood leucocytes. In contrast, lymphocytes are the main leucocyte component of murine peripheral blood comprising 75–95%, with neutrophils only accounting for 10–25% of peripheral blood leucocytes (reviewed in [12]). It has been shown recently that blood is not the only compartment where monocytes reside. Swirski et al. observed that the spleen can act as a reservoir of undifferentiated monocytes, which, upon ischemic myocardial injury, can relocate in injured tissue, and participate in wound healing [13].

Two major subsets of monocytes have been described in both humans and mice [14–16]. These subsets can be delineated on the basis of size, granularity and the differential expression of chemokine receptors and adhesion molecules. In humans, “classical” monocytes, which represent 90–95% of the total population of circulating blood monocytes, can be identified by high expression of CD14 and by a lack of CD16 (F\( \gamma \)RIII) expression. These monocytes also express CCR2, CXCR2, CD62L, and CD64 [15]. In contrast, the other major subset of human monocytes, which have been shown to be similar to tissue macrophages, are CD14++CD16+ and express high levels of HLA-DR (MHCIi) and CX3CR1 but do not express CCR2 or CD62L [17–19]. An intermediate subset of human monocytes that expresses high levels of CD14 and that are CD16 positive has also been described [20].

The two major subsets of murine monocytes resemble those that have been described in humans. The “inflammatory” subset of murine monocytes can be defined by their high expression of Ly6C/Gr1 and CCR2. In addition, these monocytes express CD62L and low levels of CX3CR1, which makes them similar in phenotype to the “classical” CD14+CD16– human monocyte subset [14].
Mediators of Inflammation

Figure 1: Toll-like receptor expression in atherosclerotic lesions is cell-type specific. Endothelial cell activation leads to increased expression of adhesion molecules, promoting the infiltration of monocytes into the subendothelial space. Recruited monocytes differentiate into macrophages, ingest lipid and become foam cells that are retained within the lesion, promoting plaque growth. Smooth muscle cells proliferate and migrate into the intima where they form a fibrous plaque over the necrotic core of the lesion. In addition, myeloid dendritic cells, plasmacytoid dendritic cells and lymphocytes are observed in lesions. Both immune and resident vascular cells in atherosclerotic arteries express a variety of toll-like receptors or increase their expression during disease development. Each cell type expresses a specific combination which might dictate its ability to respond to exogenous or endogenous ligands and the consequences of such stimulation. Ligation of toll-like receptors on cells within atherosclerotic plaques can lead to numerous downstream effects including; promoting monocyte recruitment, activation of plaque cells, induction of foam cell formation and activation of adaptive immune responses, which can all affect lesion development.

other major murine monocyte subset expresses low levels of Ly6C/Gr1 and high levels of CX3CR1. These monocytes do not express CCR2 or CD62L and thus resemble CD14highCD16+ human monocytes [14]. In contrast to humans, the two main monocyte subsets appear to be equally represented in murine blood. High-cholesterol feeding leads to an altered balance of the two major circulating monocyte subsets in ApoE−/− mice. Both Świrski et al. and Tacke et al. have demonstrated that high-cholesterol feeding of ApoE−/− mice leads to monocytosis of the Ly6Chigh monocyte subset [21, 22]. These “inflammatory” monocytes are preferentially recruited into murine atherosclerotic plaques [22]. Monocytes are continuously recruited to atherosclerotic lesions, and their recruitment is proportional to lesion size [23].

Human blood monocytes express TLR1, TLR2, TLR4, TLR5, TLR6, TLR8, and TLR9 mRNA with TLR2 and TLR4 being the most highly expressed [24–27]. Surface expression of TLR2 and TLR4 has been confirmed by flow cytometry [26] and both the TLR2 ligand peptidoglycan and the TLR4 ligand LPS induce monocytes to secrete proinflammatory cytokines [25]. Circulating monocytes from patients with arterial disease exhibit increased expression of TLR4 and TLR2 compared to healthy controls [28–31]. However, such increases in expression do not always result in enhanced TLR signalling [32–34]. Analogous with human coronary artery disease, ApoE−/− mice with advanced atherosclerotic disease also display increased surface expression of TLR2 and TLR4 on circulating monocytes [35]. TLR4 expression in atherosclerotic lesions in ApoE−/− mice has been shown to colocalise with macrophage staining [10]. Increased expression of TLR2 and TLR4 in lesions may be a consequence of exposure to oxidised low-density lipoproteins in the plaque as expression of both receptors has been shown to increase in vitro following oxidised LDL stimulation and foam cell formation in monocyte-derived macrophages and THP1 cells [10, 36]. As we will discuss later, oxidised LDL can also act as a ligand engaging TLR-4, inducing a vicious circle of cell activation.

Despite the description of two subsets of human CD14+ peripheral blood monocytes with different LPS responsiveness [37], to our knowledge, no study has examined the differential expression of TLRs on different monocyte subsets. The varied and key functions of monocytes/macrophages in all phases of atherogenesis highlights the need for better understanding of the innate immune receptors expressed by these cells and their activation, in particular in relation to the different subsets of monocytes that have been described.
2.2. Dendritic Cells. Two broad subsets of dendritic cells have been described: myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs), although DC heterogeneity is greater than this basic simplification (reviewed in [38]). These two subsets, however, are relevant as they significantly differ in terms of TLR expression.

In the intima of normal arteries, networks of dendritic cells have been described [39–41]. According to Wick and colleagues, these dendritic cells form part of a vascular-associated lymphoid tissue (VALT), which also comprises T lymphocytes, macrophages and mast cells [42]. The function of VALT is postulated to be to monitor potential danger signals in arteries [42]. Dendritic cells in VALT are similar to skin Langerhan cells and are CD1a⁺S-100⁺CD31⁺CD83⁺CD86⁺CD11c⁻ [43]. These intimal DC networks have also been observed in wild-type mice [44, 45]. In particular, dendritic cells have been identified in the arterial intima at atherosusceptible sites such as branch-points [41, 43, 45], suggesting that DCs may play a role in the initiation of atherosclerosis. Whether this role is beneficial or deleterious is not yet known.

mDCs express numerous TLRs at the mRNA level including TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, and TLR8 [46–48]. Furthermore, mDCs secrete cytokines and upregulate costimulatory molecule expression in response to stimulation with the TLR ligands Poly(I:C), LPS and R848 [46–48]. Monocyte-derived dendritic cells (MoDCs) may be obtained by in vitro culture of monocytes in the presence of IL-4 and GM-CSF [49]. Similar to mDCs, monocyte-derived dendritic cells express mRNA for TLR2, TLR3, TLR4, and TLR5 and additionally express TLR1 mRNA [24]. Monocyte-derived DCs exhibit a strong response to LPS stimulation [50] and also respond to TLR3 stimulation with Poly(I:C) [48] by producing cytokines.

In contrast to mDCs, pDCs strongly express both TLR7 and TLR9 mRNA and only weakly express TLR2 and TLR4 mRNA [47, 50, 51], which may allow these cells to be particularly responsive to viruses. pDCs are activated, mature and secrete cytokines following stimulation with the TLR9 ligand CpG [25, 27, 47, 50, 51]. Similar to mDCs, pDCs also functionally respond to stimulation with the TLR9 ligand R848. However, the engagement of TLR-7 in mDCs and pDCs leads to different functional outcomes: pDCs express IFNα, while mDCs express IL-12 in response to R848 stimulation [46].

In contrast to human mDC and pDC subsets, TLR1, TLR2, TLR4, TLR6, TLR8, and TLR9 have been shown to be expressed at the mRNA level by both murine DC subsets [52]. Further dividing the murine splenic mDCs into CD8⁺ and CD8⁻ populations reveals that CD8⁺ mDC lack TLR5 or TLR7 expression but express more TLR3 in comparison to CD8⁻ mDCs. Functionally, murine pDC and CD8⁻ mDCs respond to ligands for TLR7 and TLR9 and CD8⁺ mDC respond to a TLR9 ligand only, by producing cytokines and increasing surface expression of co-stimulatory molecules [52]. Interestingly, dyslipidemia has been shown to functionally inhibit the CD8α⁻ subset, and their ability to respond to TLR ligands [53]. As the CD8 subsets have not been identified in humans [12], it is unclear whether such differences are relevant in terms of human disease.

Both pDCs and mDCs have been observed in human carotid atherosclerotic plaques, particularly in shoulder-regions, which are areas of plaque growth and instability, and at the base of the plaque [54, 55]. CD11c⁺ dendritic cells are recruited to atherosclerotic lesions via the chemokine fractalkine in murine models [44]. The precise role of dendritic cells in atherosclerosis is not yet clear. Different expression patterns of TLRs have been described for pDCs and mDCs and thus both subsets may contribute to atherogenesis differentially through recognition and response to different TLR ligands. pDCs in human plaques are high-producers of IFNα following TLR-9 stimulation [55]. Patients with acute coronary syndromes have lower levels of circulating mDCs, probably due to increased recruitment at the lesion site and secondary and tertiary lymphoid organs [56].

2.3. T Lymphocytes. T lymphocytes (both CD4+ and CD8+) are present in human and murine atherosclerotic lesions [57–60]. T cell clones isolated from human atherosclerotic plaques have been shown to be immunospecific for self-antigens including oxidised LDL [61]. Furthermore, the transfer of CD4⁺ T lymphocytes from ApoE⁻/⁻ mice into ApoE⁻/⁻ SCID mice has been shown to aggravate atherosclerotic lesion development and T lymphocyte accumulation in lesions [62]. In contrast, regulatory T cells have an athero-protective role in lesion development [63, 64].

At the mRNA level, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, and TLR9 have been detected in human peripheral blood T lymphocytes [27, 65] and flow cytometry has confirmed protein expression of TLR1, TLR2, TLR4, and TLR9 [66]. Differences in TLR expression patterns between T lymphocyte subsets and locations has been described, which may reflect specialised immune functions. Surface expression of TLR2 has been shown to increase following T cell receptor (TCR) activation [67] and memory T cells display enhanced responses to TLR-2, TLR-5 and TLR-7 activation compared to naïve T cells [65]. Tonsillar CD4⁺ T cells express more TLR1 and TLR9 than CD8⁺ T cells whereas CD8⁺ cells express more TLR3 and TLR4 than CD4⁺ cells [68]. In conjunction with TCR activation, ligands for TLR2, TLR3, TLR5, TLR7/8 and TLR9 act as costimulators for promoting proliferation and cytokine production by human T lymphocytes [65, 67, 69–71]. Interestingly, although both αβ and γδ T lymphocytes express TLR3, only stimulation of γδ T lymphocytes with PolyIC, in association with TCR activation, leads to increased IFNγ secretion [71].

Murine T lymphocytes also express TLRs and respond to their ligands although discrepancies in the literature exist. Gelman et al. reported that activated splenic CD4⁺ T cells express and respond to ligands for TLR3 and TLR9 but not TLR2 and TLR4 [72]. However, Sobek and colleagues
showed splenic murine T cells to express and respond to TLR2 following activation [73]. Different mouse strains were used in these studies, which may account for the differences observed. In addition to mRNA expression of TLR1, TLR2, TLR6, TLR7, and TLR9, murine CD8+ cells have also been shown to be responsive to TLR2 ligands with receptor ligation lowering the threshold for activation by antigen-presenting cells [74].

Murine CD4+CD25+ Tregs express mRNA for TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, and TLR8. Exposure of murine CD4+CD25+ regulatory cells to LPS leads to increased expression of activation markers, enhanced proliferation and augmented suppressor activity [75]. Costimulation of human CD4+CD25+ regulatory T cells with the TLR5 ligand flagellin increases the suppressive capacity of these cells [70]. The suppressor function of murine CD4+CD25+ cells is also increased following TLR7 stimulation [76].

2.4. B Lymphocytes. B lymphocytes express numerous TLRs at the mRNA and protein level. Human B cells express TLR1, TLR6, TLR7, TLR9, and TLR10 [27, 68, 77, 78] and secrete cytokines such as IL6 and TNFα in response to stimulation with CpG oligonucleotides [27, 78, 79] although discrepancies in the literature exist. Different patterns and levels of TLR expression have been described depending on the location and maturity of B lymphocytes [79]. For example, TLR2 is functionally expressed by a small subset of circulating B cells with intermediate CD19 expression and most tonsillar B cells [68, 80]. Naïve B cells express low levels of most TLRs but expression is increased upon activation and on memory B cells [79].

Naïve murine B cells express a wide repertoire of TLRs and proliferate in vitro in response to ligands for TLR2, TLR7, TLR9 and TLR4 [81–83]. In contrast to human B lymphocytes, murine B cells express TLR4 and respond to stimulation with LPS [82]. Furthermore, TLR expression levels on naïve murine B cells and memory B cells does not appear to differ as has been reported for their human counterparts [82].

2.5. Mast Cells. Mast cells are long lived tissue resident cells that derive from progenitors in the bone marrow, and circulate as progenitors in the peripheral blood until they are recruited to specific tissues where they undergo maturation [84]. Mast cells have important roles in host defence to helminth, bacterial and viral infections, and in allergic reactions. Upon activation, they release a variety of preformed mediators such as histamine, cytokines and proteases. Increased numbers of mast cells are observed at sites of plaque erosion, rupture, and haemorrhage in human atherosclerotic plaques, suggesting a role in the pathogenesis of thin cap fibroatheroma (TCFA) or vulnerable plaques [85]. Crossing mast cell deficient mice (KitW<sup>sh</sup>/W<sup>sh</sup>) with LDLR<sup>−/−</sup> mice identified the requirement for mast cells in plaque development and inflammatory cell infiltration via mast cell IL-6 and IFN-γ induced protease production by endothelial and smooth muscle cells [86]. Human and rodent mast cells have been shown to express TLR-1 to -7 and TLR-9 [87].

2.6. Resident Vascular Cells. Expression of several TLRs can be found on normal human vessels. However primary arterial endothelial and smooth muscle cells have been shown to respond to a wider range of TLR ligands than these cell types from venous tissues [88]. In addition, differential expression of TLRs in vessels occurs across different vascular beds. For example, TLR3 mRNA is expressed in the aorta whereas the temporal and iliac arteries do not express TLR3 but instead express TLR8 mRNA. The carotid artery, however, expresses mRNA for both TLR3 and TLR8 [89]. In contrast to normal human vessels, which express relatively low-levels of TLRs, protein expression of TLR1, TLR2, and TLR4 is increased in human atherosclerotic vessels [7]. TLR-2 is expressed on endothelial cells in atheroprone regions [11], as we will discuss later in more detail.

Human vascular smooth muscle cells constitutively express TLR1, TLR3, TLR4, and TLR6 at the mRNA level [90]. In addition, murine aortic SMCs constitutively express TLR2 mRNA [91]. However, TLR2 expression is inducible on human SMCs following exposure to *Chlamydia pneumoniae*, TLR3 and TLR4 ligands [92]. Expression of TLR4 on human vascular SMCs at the protein level has been shown [93, 94] and more importantly, functional expression of TLRs on smooth muscle cells has also been described. Exposure of aortic SMCs to the TLR4 agonist LPS induces MCP-1, IL6 and IL-8 production [91, 94, 95]. Stimulation of SMCs with the synthetic dsRNA ligand Poly:IC results in MCP1 and IL6 release [90] and exposure of SMCs to *Chlamydia pneumoniae* leads to TLR2-dependent MCP-1 release [92].

3. Toll-Like Receptor Ligands

A wide-repertoire of both exogenous and endogenous TLR ligands have been described (Table 1). TLRs 1, 2, 4, 5, and 6 specialise in the recognition of mainly bacterial products. TLRs 3, 7, 8, and 9, in contrast, specialise in the detection of viral and bacterial nucleic acids. For instance, TLR-2 is essential for the recognition of bacterial lipopolysaccharide, and lipoteichoic acid. TLR-3 is implicated in recognition of viral double stranded (ds) RNA. TLR-4 is predominantly activated by lipopolysaccharide (LPS), while TLR-5 detects bacterial flagellin, and TLR-9 is required for responses to unmethylated CpG DNA typically of bacterial origin [6]. Viruses can also be recognized by TLR2 and TLR4. Very recently, it has been reported that TLR2 activation by viruses led to the production of type 1 interferon only in response to viral ligands in Ly6Ch<sup>+</sup> inflammatory monocytes [96].

Many exogenous TLR ligands are expressed in atherosclerotic lesions. Infectious agents, such as *Chlamydia Pneumoniae*, have been detected in atherosclerosis [97, 98]. Human atherosclerotic plaques contain numerous bacterial signatures, including nucleic acids [99], peptidoglycan [100], and exogenous heat shock proteins (HSP) [101]. Viruses have also been detected (reviewed in [102]). It is worth noting, however, that peptidoglican—derived molecules are
<table>
<thead>
<tr>
<th>TLR Receptor</th>
<th>Exogenous Ligand</th>
<th>Endogenous Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Mycoplasma tri-acyl lipopeptides [184], N. meningitides soluble factors (with TLR2) [185]</td>
<td>Necrotic cells [186–189],</td>
</tr>
<tr>
<td></td>
<td><em>Pam3CSK4</em> (synthetic TLR2/TLR1 agonist) [127], Mycobacterial lipoprotein (with TLR1) [190], Bacterial lipoproteins (with TLR6) [191, 192], Yeast carbohydrates, [192] Borrelia burgdorferi lipoprotein (with TLR1) [194]</td>
<td><em>Apolipoprotein CIII</em> [120], <em>Oxidised LDL</em> [36], Serum amyloid A [193],</td>
</tr>
<tr>
<td>TLR2</td>
<td>Staph epidermidis phenol-soluble modulin [196] Viral envelope glycoproteins [197, 198] <em>Peptidoglycan</em> (Gram + bacteria) [199] Glycoinositolphospholipids (Trypanosoma cruzi), Glycolipids (Treponema maltophilum), Porins (Neisseria), Zymosan (fungi), Atypical LPS (Leptospira interrogans and <em>Porphyromonas gingivalis</em>) [200–202]</td>
<td><em>Versican</em> [110],</td>
</tr>
<tr>
<td>TLR2/TLR4</td>
<td>HSP60 [203], <em>Chlamydia pneumoniae</em> [138], HSP60 from <em>Chlamydia pneumoniae</em> [106], <em>Porphyromonas gingivalis</em> [215]</td>
<td><em>HSP60, HSP70, Gp96, HMGB1</em>, [204–211] <em>Hyaluronan fragment</em> [109, 212, 213] <em>Biglycan</em> [214]</td>
</tr>
<tr>
<td>TLR3</td>
<td>Viral <em>dsDNA</em> [48, 90, 216]</td>
<td><em>mRNA</em> [217]</td>
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<tr>
<td>TLR5</td>
<td>Bacterial flagellin [237, 238]</td>
<td>Lung surfactant protein-A, [225]</td>
</tr>
<tr>
<td>TLR6</td>
<td>Mycoplasma di-acyl lipopeptides [239], Group B Strep heat-labile soluble factor, Staph phenol-soluble modulin [200]</td>
<td>Lung surfactant protein-A, [225]</td>
</tr>
<tr>
<td>TLR7</td>
<td>Various synthetic compounds including imidazoquinoline, loxoribine and bropirimine [200]</td>
<td>Lung surfactant protein-A, [225]</td>
</tr>
<tr>
<td>TLR7/TLR9</td>
<td></td>
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<tr>
<td>TLR9</td>
<td>Hypomethylated Cpg motifs in microbial DNA [241, 246, 247], <em>HSV-2</em> [241]</td>
<td></td>
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</tbody>
</table>

Table 1: Exogenous and Endogenous TLR ligands. Ligands in italics represent ligands for which a link to atherosclerosis has been identified.
also sensed by nucleotide oligomerization binding domain (Nod)-like receptor family members [103].

There is growing evidence that TLR signalling may be elicited in the absence of infection though “endogenous” ligands generated at sites of tissue remodelling and inflammation, as reviewed in [104]. The atherosclerotic plaque is characterised by accumulation of lipoproteins, extracellular matrix turnover during tissue remodelling, and finally formation of debris from necrotic cells in the necrotic core. As such, the atherosclerotic plaque is likely to contain many endogenous ligands (Table 1). For example, HSPs induce the production of proinflammatory cytokines through TLR4 [105, 106]. Degradation products of extracellular matrix macromolecules are generated during tissue injury, or remodelling, and have been found to function as TLR ligands. The alternative splice of fibronectin, extra domain A (EDA) that has been shown to signal through TLR4 is detected in atherosclerotic plaques [107]. Tenascin C has recently been identified as a TLR-4 ligand with relevance in chronicity of inflammatory arthritis and given the similarities between RA and atherosclerosis, may also be relevant in atherosclerosis [108]. Hyaluronan (HA), one of the major glycosaminoglycans of the extracellular matrix that undergoes rapid degradation at sites of inflammation, is another ligand for TLR2 and TLR4 [109]. A recent study has documented that versican, a large extracellular matrix proteoglycan, can activate tumour-infiltrating myeloid cells through TLR-2 and its coreceptors TLR-6 and CD14 and elicit the production of proinflammatory cytokines including TNF-alpha that enhance tumor metastasis [110]. A similar mechanism may occur in infiltrating atherosclerotic plaque monocytes/macrophages.

Lipids are also putative ligands for TLR-2 and 4. Saturated fatty acids display the capacity of delivering a TLR4 signal and to induce inflammatory gene expression, while polyunsaturated fatty acids block the activation of TLR4 [111]. However, the ability of saturated fatty acids to directly induce TLR signalling has recently been questioned [112]. Minimally modified (mm) low-density lipoproteins (LDL) have been shown to induce cytokine production via TLR-4/MyD88 signalling [113] and reactive oxygen species via TLR4/MyD88-independent signalling [114] in murine macrophages. Very recently, oxidised LDL and amyloid-β peptide have been shown to initiate inflammatory responses through a TLR-4 and -6 heterodimer in association with CD36 [115]. Amongst phospholipids relevant to innate immunity, particular attention has been given to phosphorylcholine—a universal prokaryotic and eukaryotic membrane molecule, also represented in the phospholipid quota within lipoproteins. Watson et al. identified oxidised products of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (oxPAPC) as the major bioactive lipid in mmLDL [116]. Other oxidized phospholipid moieties contained within the fatty acid side chains, have been shown to elicit signalling through CD36 [117]—a class B scavenger receptor that mediates platelet aggregation and adhesion after injury, dendritic-cell recognition and uptake of apoptotic cells. Interestingly, CD36 acts as a coreceptor of TLR-2/6 heterodimers during recognition of microbial diacylglycerides [118]. In addition, the scavenger receptor lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1) cooperates with TLR2 during cellular responses to klebsiella pneumoniae [119]. ApoCIII, a component of very-low-density lipoprotein (VLDL), was also found to be recognised by TLR2 and to induce proinflammatory signals in monocytes [120].

4. Functional Consequences of Toll-Like Receptor Activation in Atherosclerosis

4.1. TLRs Regulate Leukocyte Subset Recruitment and Activation in Atherosclerosis. Interestingly the first cells that display TLR expression in early atherosclerosis appear to be resident vascular cells such as the endothelium. Atherosclerotic lesions do not develop uniformly throughout the arterial system. Instead, lesions preferentially occur at sites of disturbed blood flow such as curvatures, branches, and bifurcations such as the lesser curve of the aortic arch [121]. TLR-2 expression is increased in endothelial cells placed at regions of susceptibility of atherosclerosis, such as the inner curvature, and it is associated with areas of monocyte recruitment in atherosclerosis-prone LDLR−/− mice [11]. However, whether endothelial cell expression of TLR2 precedes temporally the migration or is a consequence of monocyte recruitment and production of proinflammatory mediators, is unknown.

The recruitment of cells belonging to innate and adaptive immunity from the circulation into the subendothelial space is a critical step in atherosclerotic lesion development. Over the last decade this process has been extensively studied and the sequence of events that lead to leucocyte recruitment have been termed “the adhesion cascade” [122]. Leucocytes tether and roll along the endothelium through low-affinity interactions that are mediated by the selectin family of adhesion molecules. Integrin activation via interactions between chemokines on the apical surface of endothelial cells and chemokine receptors on the leucocyte results in the firm adhesion of leucocytes to the endothelial cells and their arrest from flow. Firmly adherent leucocytes then migrate across the endothelial cell (EC) layer towards a chemotactic gradient by either passing through the borders between adjoining ECs (paracellular pathway) or by passing directly through the cytoplasm of ECs (transcellular pathway). The specific expression patterns of adhesion molecules and chemokines on both endothelial cells and leucocytes coupled with the dynamic regulation of these molecules allows highly regulated recruitment of different leucocyte subsets, that results in specific tissue responses. Activation of toll-like receptors induces the expression of adhesion molecules including selectins, chemokine and chemokine receptor genes and thus TLR signalling can regulate cell migration to sites of inflammation [88, 123–125].

Crossing of MyD88−/− mice with ApoE−/− mice has been shown to reduce the development of atherosclerotic lesions by approximately 60% and macrophage infiltration by 75% [126]. Whole body deficiency of TLR4 or TLR2 in ApoE−/− mice resulted in a 55% reduction of atherosclerotic lesion development [126, 127] and a 65% reduction in
macrophage infiltration in ApoE−/−TLR4−/− mice [126]. In these studies, decreased lesion size is associated with a reduction in serum CCL2/MCP-1 levels [126–128]. Mulllick et al. have shown that bone marrow transfer from TLR2−/− to LDLR−/− mice was effective in preventing exogenous TLR2 ligand-induced disease amplification, but not baseline atherosclerotic lesion formation [127]. Interestingly, C3H/HeJ mice, which carry a missense mutation affecting the cytoplasmic portion of TLR4, are resistant to diet-induced atherosclerosis [129, 130]. However, bone marrow transplantation from C3H/HeJ to Apolipoprotein E (ApoE)−/−, did not alter atherosclerosis development [131]. This finding points towards a key role for TLR expression in vascular cells [11]. Of relevance, only endothelial cells, but not myeloid cells, express TLR2 in murine lesions [11]. However, in human lesions TLR2 expression was detected in macrophages, endothelial cells and smooth muscle cells [7]. Differences in expression of TLR2 could result either from differences between early versus late disease stage, or a difference between murine and human atherosclerosis.

Recruited macrophages can be activated by a large number of signals within an atherosclerotic lesion, including innate activation through TLRs. The nature of macrophage activation plays a key role in determining the phenotype and development of an atherosclerotic plaque. Plaque macrophages display features of activation and can exert numerous effects on other vascular cells via release of a host of proinflammatory mediators including tumour necrosis factor (TNF)-α, which leads to the engagement of the proinflammatory cytokine cascade, resulting in interleukin (IL)-1, and IL-6 production. In addition, activated macrophages play key roles in lipid uptake and plaque stability. All of these functions may be initiated or enhanced by toll-like receptor engagement. Indeed, we have recently shown in human atherosclerosis that TLR-2 and MyD88 play a predominant role in NFκB activation, and in the production of inflammatory mediators, and matrix degrading enzymes in human atherosclerosis [132], suggesting that TLR-2 signaling influences the plaque vulnerability to rupture. In contrast, signalling though TLR-4 and the downstream TLR-4 signaling adaptor TRAM was not rate-limiting for cytokine production in human atherosclerotic plaques, but may have a role in MMP production.

4.2. TLR Engagement Influences Foam Cell Formation. Toll-like receptor pathways can influence lipid uptake by macrophages and thus foam cell formation. Stimulation of murine macrophages with TLR2, TLR4 and TLR9 ligands promotes lipid uptake and foam cell formation [110, 133–135]. *Chlamydia pneumoniae* stimulation of macrophages can induce foam cell formation via MyD88-dependent and MyD88-independent pathways downstream of TLR2 and TLR4 [136–138]. Expression of the scavenger receptors SRA, macrophage receptor with collagenous structure (MARCO) and lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1) are upregulated by macrophages following TLR3, TLR4 or TLR9 stimulation [134, 139], which is one potential mechanism of enhanced foam cell formation following TLR stimulation. Almeida et al. recently showed a role for TLR2 in increased lipid body formation in mycobacterium bovis bacillus Calmette-Guerin infection [140]. In addition, TLR4-dependent fluid phase uptake (macropinocytosis) of lipids has been shown to occur in differentiated macrophages [141].

Fatty acid binding proteins including aP2 (FABP4) and Mal1 (FABP5) facilitate the uptake of fatty acids by cells. Activation of TLR2, TLR3, and TLR4 on murine macrophages leads to increased expression of aP2 [142] and TLR2 and TLR4 agonists increase murine macrophage Mal1 expression [143]. However, increased expression of aP2 and Mal1 are not observed in human macrophages following TLR stimulation [143], suggesting different mechanisms of regulation of these molecules. Agonists of TLR2, TLR3, TLR4 and TRL7 have also been shown to increase ADRP/ADFP expression, which is associated with the formation of lipid droplets [143, 144]. Overexpression of ADRP/ADFP has been shown to increase macrophage cholesterol ester storage [145].

TLRs and their ligands may also interfere with cholesterol efflux mechanisms. Cholesterol efflux may be achieved through genes including ATP-binding cassette transporter A1 (ABCA1) and G1 (ABCG1), which are regulated by lipid-X receptors (LXRs). Signalling pathways involving IRF3 downstream of TLR3 and TLR4 activation can lead to inhibition of LXR transcriptional activity and thus reduced expression of LXR target genes and consequently reduced cholesterol efflux [146]. Interestingly, LXRs can inhibit inflammatory signalling pathways following TLR stimulation in a MyD88-dependent mechanism [147]. Thus, TLRs may affect lipid uptake and accumulation in macrophages through several mechanisms.

4.3. TLRs May Control Antigen Presentation and T Cell Activation in Atherosclerotic Plaques. Proposed antigens in atherosclerotic plaques include oxidised LDL, oxidised phosphatidylcholine, heat shock proteins, beta-2-glycoprotein-I and antigens of infectious organisms such as herpes virus, cytomegalovirus, and *Chlamydia pneumoniae*. The generation of an adaptive immune response starts with the encounter between an antigen presenting cell (APC) and an antigen in the peripheral tissues. This process requires the acquisition by DCs of a mature phenotype through upregulation of costimulatory molecules such as CD80, CD86 and CD40. TLR ligation typically induces expression of these cosstimulatory molecules (reviewed in [148]) in all DC subsets regardless of their differential TLR expression profiles.

DC maturation is followed by their migration to the draining lymph nodes. This migration is also mediated by TLR-induced downregulation of inflammatory chemokine receptors and upregulation of the receptors for lymphoid chemokines. CCR6 downregulation and CCR7 upregulation, is observed in experimental models of atherosclerosis [149]. This change in chemokine receptor expression is crucial for dendritic cells to migrate from the peripheral tissues to the T lymphocyte areas of draining lymph nodes. Besides secondary lymphoid organs, antigen presentation can happen in other sites in atherosclerosis. A variety of antigen
presenting cells might perform antigen presentation within an atherosclerotic plaque, including professional dendritic cells and lesional macrophages. Recently, tertiary lymphoid organs are proposed to be alternative sites of antigen presentation within atherosclerotic vessels [150, 151].

The next step is the differentiation of naive CD4+ T lymphocytes into either Th_1 or Th_2 or Th_17 cells [152]. The direction of differentiation is influenced by both the concentration of presented peptide and the presence of specific cytokines. For instance IL-12 and IL-18 tend to promote the generation of a Th_1 response. Th_1 responses appear to dominate in both humans and mice during atherogenesis and has been shown to be proatherogenic [153]. Exogenous treatment with either IL-12 or IL-18 accelerates atherosclerotic lesion development [154, 155] whereas deficiency of either IL-12 or IL-18 results in decreased lesion formation in ApoE−/− mice [156, 157]. Lesions in ApoE−/−IL-12−/− mice also display a more stable phenotype. Furthermore, both ApoE−/−IL-12−/− and ApoE−/−IL-18−/− mice exhibit a switch from Th_1 to Th_2 immunoglobulin subclass [157]. Th_1 cells may exert proatherogenic actions in part through secretion of proinflammatory cytokines such as interferon gamma (IFNγ) and tumour necrosis factor (TNF-α) that can then activate macrophages, induce protease and inflammatory cytokine production and inhibit smooth muscle cell proliferation and collagen production [158]. Indeed, genetic deletion of IFNγ in LDLR−/− mice leads to decreased atherosclerotic lesion size [155, 159] as does deletion of its receptor IFNγR in ApoE−/− mice, which also results in a more stable lesion phenotype [160]. Interestingly, genetic deficiency of MyD88, known to be associated with a decrease of atherosclerosis development [128], leads to impaired Th_1 differentiation and a switch towards Th_2 responses [161, 162]. Conversely, Th2 responses are broadly considered antiatherogenic. Extreme hypercholesterolemia itself in ApoE−/− mice has been shown to skew T cell responses towards a Th_2 phenotype [163]. Examining atherosclerotic lesion development in ApoE−/− mice on either a C57BL/6 or BALB/c genetic background, which display opposing Th_2 responses revealed that ApoE−/− mice on a C57BL/6 genetic background, which have predominantly Th_1 responses, develop significantly more atherosclerosis than ApoE−/− mice on a BALB/c genetic background, which have predominantly Th_2 responses [164]. Furthermore, ApoE−/− mice on a BALB/c genetic background display decreased CD4+ T cell accumulation and reduced MHCI expression in atherosclerotic lesions compared to ApoE−/− mice on a C57BL/6 genetic background [164]. The Th_2 cytokine interleukin (IL)-10, produced both by lymphocytes and macrophages, is antiatherogenic. IL-10-deficient (IL-10−/−) mice or LDLR−/− mice in which the leucocytes are IL-10−/− develop larger atherosclerotic lesions than matched controls [165, 166]. Lesions in IL-10−/− mice also exhibit increased accumulation of activated T cells, increased IFNγ secretion and decreased collagen production [165, 166]. Paradoxically, TLR-2 signalling, known to be proatherogenic, has been shown to promote Th_2 differentiation [167, 168].

TLR-2 has also been linked to regulatory T cell responses. The synthetic bacterial lipoprotein Pam3Cys-SK4, a TLR-2 ligand, leads to expansion of regulatory T cells and a temporal inhibition of their suppressive activity [169, 170]. Recently, Mazicassamy et al. have shown that TLR-2 stimulation of dendritic cells leads to an induction of T regulatory cells [171]. An athero-protective role for regulatory T cells in murine atherosclerosis, in part through suppression of Th_1 responses has been described [64, 153, 172].

Very recent evidence is presenting a complex role of Th_17 in atherosclerosis, which encompasses both modulatory [173] and proatherogenic functions of IL-17 [174]. Interestingly, SIGIRR (Single Ig IL-1-related receptor), a negative regulator of IL-1 receptor and Toll-like receptor signaling, has been shown to govern Th17 cell differentiation and expansion [175]. It also emerged recently that pDCs are capable of promoting Th17 differentiation in response to TLR7 stimulation [176].

Although few B lymphocytes have been observed in human atherosclerotic plaques [58], studies in mice have revealed a potential protective role for B lymphocytes in atherogenesis [177, 178]. B lymphocytes express both antigen-specific B-cell receptors and pattern-recognition receptors, including TLRs (described above). Ligation of TLRs on B lymphocytes has been shown to induce polyclonal activation and secretion of immunoglobulin M (IgM) antibodies [179, 180]. In addition, it has recently been shown that activation of TLR-2 and TLR-4 on murine B1 cells results in enhanced production of IgM antibodies against oxidation-specific epitopes [181]. Interestingly, serum IgM has been described as atheroprotective in LDLR−/− mice as LDLR−/− mice deficient in serum IgM exhibit larger lesions with increased cholesterol crystal formation [182].

4.4. TLRs as Therapeutic Targets in Atherosclerosis. Given the large body of data suggesting that TLRs can contribute to several atherosclerotic mechanisms key to the initiation and development of lesions such as leucocyte recruitment and foam cell formation (discussed above), these molecules may be important targets for the development of novel antiatherogenic therapeutics. To date, TLR-2 and TLR-4 have been the best characterised in terms of their contribution to atherosclerotic lesion development. Antagonism of TLR-2 or TLR-4 signalling is currently perceived as the most attractive target for development of therapeutics for the treatment of atherosclerosis. Indeed, deletion of either TLR-2 or TLR-4 confers a similar degree of protection from lesion development in murine models of atherosclerosis [126, 127]. However, as discussed, there are many differences between the human and murine immune systems, including the cellular expression patterns of TLRs. This may hinder the extrapolation of targets from murine studies into human targets. Indeed, we have recently demonstrated that a TLR-2 blockade can inhibit cytokine, chemokine and MMP production in human atherosclerosis, while interruption of TLR-4 signalling did not have a significant impact on the production of proatherogenic mediators [132]. Additionally, in a murine model of myocardial ischemia/reperfusion injury, TLR-2 blockade has recently been shown to reduce...
infact size and maintain heart function through reduction of proinflammatory mechanisms [183]. Together these studies support the idea that TLR-2 blockade may be beneficial in cardiovascular disorders. It is possible to envisage that blockade of TLR-2 during acute phases of the disease could be preferred to its chronic use. As TLRs are essential components of both the innate and adaptive immune responses and as they are expressed on both resident vascular and leucocyte populations, further studies are needed to ascertain the most effective timing of TLR inhibition in cardiovascular disease.

5. Conclusions

The role of TLRs in the development of atherosclerosis has just started to be unravelled. The key immune and resident vascular cells in the initiation and development of atherosclerosis all express various TLRs, suggesting these receptors and their ligands are critical players in atherogenesis. So far, it appears that TLR-2 and –4 activation has profound consequences on the recruitment of monocytes and foam cell formation in murine models of atherosclerosis. TLR-2 signalling appears to be a predominant event for activation of inflammation and matrix degradation in human atherosclerotic lesions. The consequences of activation and blockade of other TLRs in atherosclerosis remains to be explored. Due to the intricate outcomes of activation of TLRs on adaptive immunity, further studies need to explore the relationship between innate and adaptive responses in atherosclerosis.

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References

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Review Article

Toll-like Receptor 4 Modulation as a Strategy to Treat Sepsis

X. Wittebole, D. Castanares-Zapatero, and P. F. Laterre

Critical Care Unit, Acute Medicine Department, St Luc University Hospital, Université Catholique de Louvain, Avenue Hippocrate 10, 1200 Bruxelles, Belgium

Correspondence should be addressed to X. Wittebole, xavier.wittebole@uclouvain.be

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Despite a decrease in mortality over the last decade, sepsis remains the tenth leading causes of death in western countries and one of the most common cause of death in intensive care units [1]. Between 1979 and 2000, there was an annualised increase in the incidence of sepsis of 8.7 percent, reaching 240.4 per 100000 people in 2000 [2]. Despite progress in better recognition and improved standard of care, mortality still ranges from 30 to 50% in patients with septic shock [3]. Hence, unmet needs for those patients are still present.

About 12 years ago, the discovery of the Toll-like receptor (TLR) unravelled the missing link between endotoxin recognition by LBP and CD14 and the intracellular signalling pathway, leading to the activation and translocation of NFkB to the nucleus, and the subsequent production of proinflammatory cytokines [4–6]. TLRs were first described in Drosophila melanogaster where it functions as a key receptor for dorsoventral polarity during development and is required for immunity against fungal infections [7]. The toll-signalling pathway was shown to have major similarities with the mammalian IL-1 receptor pathway. To date, 10 TLR or pattern-recognition receptors (PRRs) are identified in human and a series of studies have revealed their respective ligands [8–10]. TLRs recognize essential structures expressed by pathogen (collectively referred to as Pathogen Associated Molecular Patterns or PAMPS) as well as endogenous mediators released during tissue damage, independently of infectious state (these mediators referred to as alarmins or Danger Associated Molecular Patterns or DAMPS). The role of TLR and TLR signalling in the pathogenesis and development of sepsis was recently reviewed [11–13].

In order to prevent an overwhelming activation of TLR, and its potential side effects, many natural substances modulate TLR expression and signalling. For instance, RP105, initially discovered in murine B cells [14], displays several similarities with TLR; it has an extra cellular leucin-rich domain and a TLR-like pattern of juxtamembrane cysteins; its surface expression depends on the cosecretion of a secreted helper protein, in this case, MD1 [15]. However, unlike TLR, RP105 lacks an intracellular domain. Furthermore, the extra cellular domain is a specific TLR4 homologue [16]. It therefore acts as a physiological inhibitor of TLR4 signalling. This was elegantly demonstrated and reviewed elsewhere [16, 17]. In summary, the complex RP105-MD1 interacts directly with the TLR4 signalling complex, preventing its ability to bind LPS. It regulates TLR4 signalling in various immune cells as well as in mice challenged with intraperitoneal E. coli LPS. Modulation of the RP105-MD1 complex could help

1. Introduction

Despite a decrease in mortality over the last decade, sepsis remains the tenth leading causes of death in western countries and one of the most common cause of death in intensive care units [1]. Between 1979 and 2000, there was an annualised increase in the incidence of sepsis of 8.7 percent, reaching 240.4 per 100000 people in 2000 [2]. Despite progress in better recognition and improved standard of care, mortality still ranges from 30 to 50% in patients with septic shock [3]. Hence, unmet needs for those patients are still present.

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abolish TLR4 overstimulation. Further clinical development is warranted to evaluate a potential role in the treatment of sepsis and associated clinical states. Some of the other natural molecules aimed at controlling TLR4 effects are listed in Table 1.

Since lipopolysaccharide (LPS) or endotoxin is a specific ligand for TLR4, and because TLR4 expression is increased on human monocytes in healthy volunteers undergoing LPS challenge [18], and in patients with sepsis [19, 20], particular attention has been made to this receptor and signalling pathway. The purpose of this paper is to focus on various drugs interfering with TLR4 expression or TLR4-related intracellular pathway and their potential role as adjunctive therapy in severe sepsis and septic shock, or as modulator of the TLR4-induced inflammatory response (Table 2).

### 2. Antibodies Directed against TLR4 and the TLR4-MD2 Complex

Soluble decoy receptors provide important negative regulatory mechanisms for cytokines and chemokines, and their interaction with their membrane-bound receptor. For instance, increased levels of soluble TNFα receptor (sTNFR) are present up to 24 hours after an LPS challenge in healthy volunteers and correlate with the severity of the insult in critically ill patients where low level of sTNFR predicts higher mortality [21]. In mice, Iwami et al. were able to clone a splice TLR4 mRNA that encodes a soluble 20-kDa protein [22]. When expressed in Chinese ovary (CHO)-K1 cells, this protein is secreted in the culture medium. It inhibits LPS-mediated TNFα secretion and NFκB activation in a mouse macrophage cell line. Interestingly, LPS stimulation increased the sTLR4 mRNA expression, suggesting a negative feedback to inhibit excessive cytokine production. Any compound able at increasing this natural soluble TLR4 would thus be of potential interest in treating patients with sepsis.

A specific antibody raised against the ectodomain of TLR4 was recently described [23]. In summary, a chimeric protein composed of the N-terminal half of the mouse TLR4 ectodomain was fused to the Fc domain of human IgG1. In the presence of soluble MD2, this protein binds LPS and inhibits LPS-induced TNFα release in whole blood. It was then used to generate high titres of rabbit antimouse TLR4 antibody. These antibodies were able to inhibit response of immune cells exposed to LPS or Gram-negative bacteria in vitro and in vivo. Furthermore, this antibody protects from lethality in mice exposed to endotoxemia or live *E. coli* [23].

Another TLR4 antibody was developed [24]. The extra cellular portion of mouse TLR4 was fused with mouse MD-2 via a 15-amino-acid flexible linker. IgG Fc fragments were added to the molecule. This molecule dose-dependently inhibits IL-6 production in RAW 264.7 cells exposed to LPS, and, binds to the surface of Gram-negative bacteria. Depending on the IgG isotype, it also modulates phagocytosis and complement activation. Hence, this molecule could act through 2 distinct mechanisms: on one hand, LPS binding and decreased inflammatory response, and, on the other hand improved bacterial phagocytosis and complement mediated killing [24].

Further development is required before these molecules could undergo clinical evaluation.

---

**Table 1: Some of the natural inhibitors of TLR4 signalling.**

<table>
<thead>
<tr>
<th>Level of action</th>
<th>Natural TLR4 signaling inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular</td>
<td>Soluble CD14 (sCD14)</td>
</tr>
<tr>
<td></td>
<td>Soluble MD2 (sMD2)</td>
</tr>
<tr>
<td></td>
<td>Soluble TLR4 (sTLR4)</td>
</tr>
<tr>
<td>Membrane</td>
<td>Receptor RP105 (RP 105)</td>
</tr>
<tr>
<td></td>
<td>TNF-Related Apoptosis Receptor (TRAIL-R)</td>
</tr>
<tr>
<td></td>
<td>Receptor ST2 (ST2)</td>
</tr>
<tr>
<td>Intracytoplasmic</td>
<td>Short form of MyD88 (MyD88s)</td>
</tr>
<tr>
<td>(i) MyD88</td>
<td>Single Immunoglobulin (SIGIRR)</td>
</tr>
<tr>
<td>(ii) IRAK</td>
<td>Interleukin-1 receptor associated kinase (IRAK M)</td>
</tr>
<tr>
<td>(iii) TRAF-6</td>
<td>Monarch 1 (TOLLIP)</td>
</tr>
<tr>
<td>(iv) P38 and JNK kinases</td>
<td>Phosphatidyl inositol 3 kinase (PI3K)</td>
</tr>
<tr>
<td>(v) NFκB</td>
<td>A20-binding inhibitor of NFκB activation (ABIN-3)</td>
</tr>
<tr>
<td>(vi) Cytokine secretion</td>
<td>Suppressor of cytokine secretion (SOCS1)</td>
</tr>
</tbody>
</table>

**Table 2: Mode of action of various molecules targeting TLR4.**

<table>
<thead>
<tr>
<th>Molecules interfering with TLR4 and TLR4-mRNA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Chloroquine</td>
</tr>
<tr>
<td>(2) Ketamine</td>
</tr>
<tr>
<td>(3) GTS21 (nicotinic analogue)</td>
</tr>
<tr>
<td>(4) Statins</td>
</tr>
<tr>
<td>(5) Vitamin D3 (?)</td>
</tr>
<tr>
<td>(6) Lidocaine</td>
</tr>
<tr>
<td>(7) Glycine</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Molecules interfering with TLR4-related intracellular signalling pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Eritoran (E5564)</td>
</tr>
<tr>
<td>(2) Resatorvid (TAK242)</td>
</tr>
<tr>
<td>(3) Ketamine</td>
</tr>
<tr>
<td>(4) Opioids</td>
</tr>
<tr>
<td>(5) Vitamin D3 (through its action on LL37)</td>
</tr>
<tr>
<td>(6) Lansoprazole (through its action on SOCS1)</td>
</tr>
</tbody>
</table>
3. Eritoran or E5564

E5531 is a first generation lipid A analogue, derived from the lipid A structure from the endotoxin of Rhodobacter capsulatus. It blocks LPS in cell culture without any endotoxin-like activity [25]. E5531 protects mice from lethal doses of LPS, and viable E. coli infections in combination with antibiotics [25]. It also blocks the endotoxin response in human healthy volunteers exposed to intravenous LPS [26]. Some issues on E5531, such as decreased activity over time in human blood due to interaction with plasma lipoproteins [27, 28], led to the search for second generation LPS antagonist (reviewed in [29]).

Like E5531, E5564, or eritoran is a synthetic molecule, derived from the nonpathogenic Rhodobacter sphaeroides [30]. The crystal structure of the TLR4-MD2 complex with bound eritoran was recently described, suggesting that eritoran mechanism of action lies within its binding in a large hydrophobic internal pocket in MD2 [31]. Hence, it acts as a LPS antagonist, since it is unable to trigger the intracellular signalling cascade leading to NFκB translocation to the nucleus.

Consequently, eritoran blocks the in vitro production of cytokines in human whole blood [32] and induces a down-regulation of intracellular generation of pro-inflammatory cytokines [33]. Pharmacodynamic studies demonstrated a continuous efficacy with every 12 hours intermittent infusion of the drug [34].

As for E5531, eritoran reportedly decreases clinical signs, biological parameters, and inflammatory response induced by endotoxin in healthy volunteers [35]. Efficacy of various doses of eritoran, ranging from 50 mcg to 250 mcg, was assessed, while subjects were challenged with 4 ng/kg LPS. All eritoran doses achieved statistically significant reductions in elevated temperature, heart rate, C-reactive protein levels, white blood cell count, TNFα and IL-6 levels. In the higher doses groups (>100 mcg/kg), eritoran also statistically blunted the LPS-induced clinical signs such as fever, chills, headache, myalgia, and tachycardia.

A trend toward decreased mortality was observed in a phase II randomised controlled trial [36]. This study, conducted in North America, recruited 293 patients who were randomised to 3 groups: Eritoran high dose (105 mg), Eritoran small dose (45 mg/d), or placebo. Actually, eritoran at a dose of 105 mg/d administered every 12 hours for 6 days, decreased mortality from 56.3% to 33.3% in patients with high risk of mortality, as assessed by the Acute Physiology and Chronic Health Evaluation II (Apache II) Score. A large ongoing phase III randomised, double-blind, placebo-controlled study is therefore recruiting patients with suspected or proven infection, criteria for the systemic inflammatory response syndrome and at least 1 sepsis-related organ dysfunction. Baseline APACHE II score must range between 21 and 37. Treatment has to be started within 12 hours after the onset of organ failure. We expect the trial to be completed by the end of 2010.

Eritoran could also modulate sepsis driven organ dysfunction such as cardiac depression and vasodilation, 2 frequent symptoms encountered in severe sepsis and septic shock. Indeed, while the expression of TLR4 on cardiac myocytes is known for years [37], the use of eritoran recently helped identify, in animal models, the role of TLR4 and intra-cellular signalling as one of the mechanism involved in sepsis-related cardiac dysfunction [38]. After 6 hours exposure to LPS, isolated cardiac myocytes from C3H/HeN mice (a normally LPS susceptible strain) develop a reduced sarcomere shortening amplitude and prolonged duration of relaxation. The addition of 2 μg/mL eritoran to the cultured medium leads to a reduced effect of LPS on all monitored contractile parameters. Eritoran further prevents attenuation of contractility observed in LPS treated isolated aortic rings from these mice [39]. Taken together, those data reinforce the idea that this molecule could help treating patient with severe sepsis, beyond its role in preventing cytokine production by immune cells.

Eritoran could also modulate other noninfectious disease processes, using the TLR4 pathway. Actually, in a model of myocardial ischemia-reperfusion syndrome in C57BL/6 mice, the use of eritoran resulted in smaller infarct size, decreased IFN phosphorylation, NFκB translocation, and cytokine production [40]. Because of the well-described increased level of endotoxemia in patients undergoing cardio-pulmonary bypass and the just-mentioned effects of eritoran on the heart and large vessels, eritoran efficacy was assessed in a double-blind, randomised, ascending dose, placebo-controlled trial in patients undergoing cardiac surgery [41]. While no statistically relevant difference could be observed in various inflammatory parameters, no significant safety concern was identified.

4. Resatorvid or TAK 242

TAK 242, or ethyl-(6R)-[N-(2-chloro-4-fluorophenyl) sulfamoyl] cyclohex-1-hene-1-carboxylate, identified by Takeda pharmaceuticals, is a small compound developed to inhibit inflammatory mediators production [42]. It initially was demonstrated to decrease NO and various cytokines production in LPS stimulated mouse macrophages, as well as in a mouse endotoxin model [42]. A further study demonstrated its ability to inhibit intracellular signalling, with decreased MAPkinases phosphorylation and IκB degradation, without any interference with LPS binding to TLR4 [43]. Since the effects of ligands to other TLR were not affected, this effect was specific for TLR4. Similar results were obtained using human peripheral blood mononuclear cells (PBMCs), monocytes and macrophages. While the action of TAK 242 in the intracellular domain of TLR4 is known for some times [44], Takashima et al. only recently demonstrated TAK 242 to inhibit TLR4 signalling by direct binding to a specific amino acid (Cys747) in the TLR4-intracellular domain [45].

In a mice intraperitoneal endotoxin model, intravenous TAK 242 inhibits the pro-inflammatory response and prevents lethality in a dose-dependent manner [46]. Of importance, treatment up to 2 hours after the LPS challenge results in similar benefits. In an intravenous endotoxia model using conscious guinea pig, the use of TAK 242 allows better hemodynamic control, decreased level of HMGB-1 and a dose-dependent improved survival [47].
In phase 1 clinical studies in normal healthy subjects given concomitant endotoxin, TAK 242 inhibited the production of cytokines TNFα, IL-6 and IL8. Nonclinically significant haemolysis and increases in methemoglobin levels were occasionally observed. A large, multicentre, multinational, randomised, double-blind, placebo-controlled study was initiated in September 2005 (http://clinicaltrials.gov/ NCT00143611). 18-year-old or older subjects with severe sepsis and related respiratory or cardiovascular failure were eligible. The study was ended prematurely after the DSMB determined there was insufficient cytokine suppression in the 150-subject analysis within stage 1 of the study. Another study was planned but unfortunately never started based on business decision (http://clinicaltrials.gov/. NCT00633477). While further development in sepsis patient is unlikely, the potential benefit of TAK 242 in other TLR4 related diseases, such as autoimmune diseases, has to be assessed.

5. Chloroquine and Other TLR 9 Antagonists

While the major signalling pathway of LPS lies within its binding to the MD2/TLR4 complex, several reports have indicated that endotoxin may enter immune cells [48] and localize in the Golgi apparatus and other vesicles [49]. This was further confirmed in human PBMC [50]. Therefore, intracellular receptors and medication interfering with those receptors or with intracellular trafficking could be of importance.

Actually, TLRs that recognize nucleic acids, such as TLR3, 7, 8, and 9, are confined to endocytic compartment where they encounter ligands internalised through receptor-mediated endocytosis or phagocytosis. Upon stimulation of cells, TLR9, for instance, appears to be trafficking from endosome to lysosome where it undergoes proteolytic maturation in an acidic environment to become competent [51]. Asparaginase endopeptidase looks critical for this phenomenon [52].

Recently, Plitas et al. demonstrated in a TLR9−/− mice model of cecal ligation and puncture-(CLP-) related peritonitis, an increased bacterial clearance, decreased serum cytokine production and increased granulocytes influx in the peritoneum as compared to wild type animals [53]. Using an inhibitory CpG sequence that blocks TLR9 just before the CLP, they also demonstrated an improved survival in wild type animals.

Taken together, those data suggest that medication able at blocking TLR9 maturation or signalling could be of interest in sepsis. Actually, chloroquine, a drug used in infectious (malaria) and inflammatory (SLE) diseases, blocks trafficking and, or acidification of the endosome. It is known for years that chloroquine decreases the in vitro response to various pro-inflammatory stimuli such as LPS [53] or CpG oligonucleotide. In vivo, chloroquine protected mice from lethal doses of LPS or CpG through a decrease of proinflammatory cytokine release [54]. Using murine macrophage ANA-1 cells, the authors further demonstrated with chloroquine a decreased expression of TLR4 and 9 mRNA expressions as well as a blockade of NFκB and API activation. Chloroquine demonstrates its positive effects when used prior to the induction of CLP but also up to 6 hours after [55]. In this experiment, decreased splenic apoptosis was observed, suggestive of a mechanism that improves sepsis-induced immune paralysis. Renal function was also improved.

Hence, chloroquine may act at 2 different levels: down-regulation of TLR4 expression and interfering with the intracellular trafficking of LPS through its action on TLR9. Of notice, chloroquine also interferes with other TLRs that are internalised and function through endosomal pathway. Whether its actions in polymicrobial sepsis are TLR9 specific or nonspecific has still to be elucidated.

Because of its excellence tolerance, further clinical development in sepsis and critical care looks promising.

6. Ketamine

Because of its effects on hemodynamic, ketamine, an intravenous anaesthetic agent is widely applied in critical care for induction of anaesthesia or even for maintenance of sedation. Anti-inflammatory effects of ketamine are widely demonstrated in various in vitro animal and human models. The ketamine effects on TLR expression are less known. In a rat model of intravenous LPS stimulation, TLR4 expression and NFκB activation were decreased in the intestine of ketamine-treated animals [56]. Using the same model, the authors demonstrated identical results in the lungs [57]. In a rat model of CLP, treatment with ketamine after the procedure decreased intestine levels of pro-inflammatory cytokines, as well as NFκB activation and TLR4 and 2 mRNA expression, when compared to rats treated with saline [58]. Again, similar results were observed in the lungs, with decreased secretion of pro-inflammatory cytokines, decreased activation of NFκB and decreased TLR2, and 4 mRNA expressions [59]. Doses of ketamine used in these various experiments (up to 10 mg/kg) are far beyond doses used in clinical settings. Mechanisms of action of ketamine were studied in cultured murine macrophage cell line Raw264.7. Not only does ketamine interfere with LPS binding to LBP, but it also decreases phosphorylation of various kinases involved in the TLR4 intracellular signalling [60]. Likewise, ketamine-treated macrophages, stimulated with lipoteichoic acid, a TLR2 agonist, produced less TNFα and IL-6. This results from decreased phosphorylation of ERK1/2, an upstream protein kinase for activating inhibitor of NFκB (IkB) kinase (IKK), leading to decreased NFκB translocation to the nucleus [61].

Clinical relevance of those results has to be assessed for patients with sepsis or for patients sedated with ketamine.

7. Nicotine

Since the description of the so-called cholinergic anti-inflammatory pathway [62], nicotine and analogues were studied in various cultured cells and animal models of sepsis, pancreatitis, and ischemia-reperfusion syndrome. In humans, transcutaneous nicotine exposure alters the
LPS-induced inflammatory response in healthy volunteers [63]. While the nicotinic acetyl-choline receptor, specifically those comprised only of alpha-7 subunits, on myeloid cells are required for this effect [64], the precise intra-cellular mechanism of action is not fully elucidated. Activation of the JAK2-STAT3 pathway and suppression of the NFκB activity at the transcriptional level are implied [65, 66]. Recently, Kox et al. confirmed the reduced cytokine production in human PBMC treated with nicotinic analogues, whatever the stimulated TLR [67]. This effect is likely mediated by JAK2/STAT3 signalling. Interestingly, they also demonstrated with GTS-21, a potent α-7 selective partial agonist, modulation of TLR expression after LPS stimulation; TLR2 up-regulation was decreased while TLR4 up-regulation was completely abolished. This further confirms an earlier experiment where nicotine induced a downregulation of TLR4 expression on human monocytes, with or without concomitant LPS stimulation [68].

All those data strongly support a potential role for nicotinic agonists to modulate cytokine production as well as toll like receptor expression in severe sepsis and septic shock. Further investigations are required.

8. Opioids

For years, we know that TLRs are expressed in the central nervous system (CNS): while microglia express a wide range of TLRs, astrocytes and oligodendrocytes mainly express TLR2 and 3 [69]. Interestingly, enhanced TLR expression is observed in inflamed CNS tissues. We also know that morphine and opioid derivates display, beyond their role in pain control, important immunomodulatory effects, characterized in animal as well as in human studies (reviewed in [70]).

TLRs are a key link between the innate immune system and the CNS. Furthermore, several reports demonstrate the involvement of TLR in various types of pain (chronic, neuropathic and inflammatory) as well as in morphine tolerance (reviewed in [71]). Very interestingly, TLR4 was demonstrated to be of particular importance, since select opioids may nonstereoselectively influence its signalling, while having no effects on classical morphine receptors [72]. Indeed, morphine-3-glucuronide, a morphine metabolite with no opioid receptor activity, displays significant TLR4 activity.

Those data raise at least 2 hypotheses; first, modulation of TLR, in particular TLR4, could be a strategy in the management of chronic pain. Secondly, the use of morphine and other opioids in the critical care setting could interfere with the response to inflammatory stimuli such as LPS. Again, clinical consequences of this warrant further investigation.

9. Statins

Beyond their well-demonstrated lipid lowering effects resulting in clinical benefits in cardiovascular diseases, 3-hydroxy3-methylglutaryl-(HMG-) coenzyme A inhibitors, or statins, display pleiotropic effects. Statins inhibit NF-κB activation and the subsequent pro-inflammatory cytokines such as TNFα and IL-6 production. They also blunt endotoxin related activation of cultured human coronary endothelial cells and human PBMC. While these effects are known for years, Methé et al. only recently reported an effect of statins on TLR4 expression [73]. They demonstrated a dose dependent decrease of TLR4 mRNA and protein expression in CD14+ human monocytes incubated in vitro with simvastatin or atorvastatin. They observed a similar effect in vivo, in 12 normocholesterolemic healthy volunteers. Four weeks treatment with atorvastatin 20 mg/d resulted in a 36.2% reduction in TLR4 expression on CD14+ monocytes. Intracellular mechanism of action could include inhibition of protein geranylgeranylation and farnesylation leading to the hypothesis that proteins of the Ras family and the phosphoinositide 3-kinase/protein kinase Akt-pathway are of importance in mediating the TLR4 expression [73]. In accordance with those results, simvastatin 80 mg/d for 4 days decreased the endotoxin-related upregulation of TLR2 and 4, in 20 healthy volunteers exposed to 2 ng/kg intravenous LPS [74]. This expression modulation was also demonstrated in moderate chronic heart failure patients [75]. Interestingly, statins were demonstrated to be most active in reducing the risk of cardiovascular diseases in patients carrying the G allele for TLR [76]. In human embryonic kidney (HEK) 293-CD14-MD2 cell transfected with various TLR4 variants, Hodgkinson and Yee demonstrated that TLR4 variations and statins have an additive inhibitory effect on TLR4-mediated response to LPS, in term of NFκB activation and cytokine production [77]. They further emphasize the role of geranylgeranyltransferase and Rho-kinase inhibition to explain the statin intracellular mechanism of action.

Another hypothesis on the effect of statin on TLR expression is its potential influence on TLR4 membrane trafficking in treated subjects because of altered cholesterol rich membrane domains, as observed in brain plasma membranes [78].

The potential of statins as an adjunctive therapy for severe sepsis is currently evaluated in various clinical trials. Actually, a recent meta-analyse suggests that statin treatment may be associated with a beneficial effect in treating and preventing various infections [79]. Because of the presence of heterogeneity and publication bias further randomised trials are required.

10. Vitamin D3 and Analogues

Beyond its important role as a regulator of the calcium-phosphate homeostasis, the hormonally active form of vitamin D displays numerous effects on the immune system [80]. Vitamin D3 and analogues were demonstrated to be protective in a mice model of intraperitoneal endotoxin shock [81]. This positive effect was also demonstrated on coagulation parameters in a rat model of CLP-related sepsis model [82]. Regulation of thromboxane A2 and free radical formation were initially proposed as mechanisms of action [83]. We now know that TLR activation in human monocytes and macrophages leads to an upregulation of...
the vitamin D receptor (VDR) and the vitamin-D-1 hydroxylase gene expression [83]. These authors also demonstrate that, in presence of vitamin D, this up-regulation leads to increased expression of the cathelicidin mRNA. This cationic antimicrobial peptide is stored in secretory granules and is processed during or after secretion into its mature form, LL-37 [84]. This α-helical peptide displays several functions, including killing of pathogens, neutralizing LPS, or acting as a chemo attractant [85].

The effect of vitamin D on TLR surface expression is inconsistently reported. On one hand, vitamin D3 was shown to decrease TLR2 and 4 mRNA and protein expression in a time- and dose-dependent fashion in human monocytes [86]. This led to a decreased production of cytokines and tissue factor production as well as a decreased NFκB translocation to the nucleus after LPS or LTA stimulation. Interestingly, the effect of vitamin D3 on TLR expression was VDR-dependent. On the other hand, TLR expression and MD2 expression were not affected by vitamin D3 in cultured human endothelial cells (HMEC) [87]. Nonetheless, vitamin D3 pretreatment resulted in decreased LPS-induced IL-6 and IL-8 production and blocked NFκB activation.

Taken together, all these data suggest a clear relationship between vitamin D and the TLR pathway and its biological outcomes. Actually, compared to healthy controls, critically ill patients with sepsis had lower levels of serum hydroxyl-vitamin D and LL-37 [88]. It looks therefore logical to assure a normal vitamin D serum level in patients with severe sepsis or septic shock. This should however further be assessed in a randomised trial.

11. Other Molecules

Because of their strong antisecretory effects, proton pump inhibitors (PPI) are widely used to treat gastric and duodenal ulcer as well as reflux oesophagitis. They were also reported to display anti-inflammatory and immune properties. For instance, they attenuate polymorphonuclear-dependent gastric mucosal inflammation by interfering with NFκB activation in gastric epithelial cells and vascular endothelial cells [89]. They also modulate the cytosolic concentration of calcium in polymorphonuclear cells. In a study using endotoxin-stimulated 293hTLR4/MD2-CD14 cells, lansoprazole modulated intranuclear transfer of NFκB and stimulated the expression of Suppressor of cytokine signalling-1 (SOCS1), a negative feedback gene involved in excessive LPS stimulation [90]. Therefore, the use of PPIs could modulate the intracellular cascade after TLR4 stimulation. However, those results warrant further investigations in other cell types as well as in vivo.

Lidocaine, a widely used local anaesthetic, has been reported to attenuate cytokine-induced cell injury and inhibit iNOS expression in activated murine macrophage [91]. To further investigate this latter effect, Lee et al demonstrated that it attenuates the up-regulation of TLR4 expression, NFκB, and some MAPKineses, in murine macrophages stimulated with LPS [92]. Voltage sensitive sodium channels, if present in macrophages, could be involved in the modulation of TLR and downstream signals modulation. While the use of lidocaine was demonstrated to attenuate acute lung injury in rats exposed to intraperitoneal doses of endotoxin [93], clinical relevance in humans remains to be established.

Glycine, a α-amino acid that acts as an inhibitory neurotransmitter in the central nervous system, also exerts immune-modulating actions via stimulation of glycine-gated chloride channels in immune cells. Glycine inhibits LPS-binding protein (LBP) mRNA expression in the liver mice challenged with LPS, also decreases TLR4 mRNA expression, and decrease activity of NFκB in kupffer cells of those animals [94]. Because of inconsistent result in various animal models, the clinical use of glycine as an immune-modulating agent in sepsis remains to be elucidated.

12. Conclusions

The TLR4 signalling pathway leading to lipopolysaccharide-mediated NFκB activation constitutes an important therapeutic target for sepsis therapy. Various molecules are involved in TLR4 membrane regulation and could behave as new adjuvant therapies able to weaken the deleterious effects of exaggerated host response to infection. Most of those are not yet exploited and additional laboratory and clinical investigations are required to confirm their expected influence. Many studies have documented capacities of new drugs to regulate TLR4 signalling and expression. For most of them, mechanisms underlying this action still need to be straighten out. Moreover, clinical implications remain to be corroborated, especially for those medications already used for other indications, such as ketamine. A better knowledge of TLR4 regulation molecules will be crucial to control host to infection reaction and avoid the detrimental consequences of sepsis. Among the drugs looking promising, eritoran, a lipid A analogue, is undergoing a large phase III clinical trial.

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Clinical Study
Upregulation of TLR2/4 Expression in Mononuclear Cells in Postoperative Systemic Inflammatory Response Syndrome after Liver Transplantation

Ziqing Hei, Xinjin Chi, Nan Cheng, Gangjian Luo, and Shangrong Li

Department of Anesthesiology, Third Affiliated Hospital, Sun Yat-sen University, Guangzhou 510630, China

Correspondence should be addressed to Ziqing Hei, heiziqing0530@hotmail.com

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Background. To explore the relationship between Toll-like peripheral blood mononuclear cells (PBMC) and systemic inflammatory response syndrome (SIRS) in postoperative patients of liver transplantation (LT).

Methods. Blood samples of 27 patients receiving LT were collected at T1 (after induction of anaesthesia), T2 (25 minutes after the beginning of anhepatic phase), T3 (3 hours after graft reperfusion), and T4 (24 hours after graft reperfusion). The expression of TLR2/4 on PBMC and serum concentration of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-8 were measured. The patients were divided into SIRS group (n = 12) and non-SIRS group (n = 15) for analysis.

Results. Blood loss and transfusion were higher in the SIRS group than in the non-SIRS group. Both the preanhepatic and anhepatic phase were significantly longer in the SIRS group. The TLR2/4 expression on PBMC as well as serum TNF-α, IL-1β, and IL-8 were significantly higher at T3 and T4 than that at T1 and T2 in the SIRS patients. The expression of TLR4 on PBMC is positively correlated to serum TNF-α, IL-8. Expression of TLR2/4 on PBMC and serum concentrations of TNF-α, IL-1β, did not differ among the 4-time points in non-SIRS patients.

Conclusions. Upregulation of TLR2/4 expression on PBMC may contribute to the development of postoperative SIRS during perioperative period of LT.

1. Introduction

Orthotopic liver transplantation (OLT) is the most effective and the best therapeutic solution for final stage liver diseases. More than 60,000 patients receive OLT every year worldwide [1]. Systemic inflammatory response syndrome (SIRS) often accompanies sepsis, trauma, hypoxia, and ischemia-reperfusion injury (IRI) [2–4]. The activation of mononuclear phagocytes, and consequently release of massive amount of proinflammatory cytokines may lead to multiorgan dysfunction syndrome (MODS) [3, 4]. SIRS is a common feature after major surgery [5, 6]. In recent years, SIRS has been interpreted as a warning sign for postoperative complications and organ failure [7–9]. Specifically, longer SIRS duration has been associated with poor outcomes after surgery [5, 6, 8, 9].

Toll-like receptors (TLRs) play an important role in many pathophysiological processes such as inflammation and IRI [10–14]. TLR2 and TLR4 are members of the TLRs family [13, 14], and could initiate inflammatory responses to various stimuli [2, 15].

A previous study in this laboratory revealed increased expression of TLR2/4 in mononuclear and proinflammatory cytokines in liver transplantation [16]. Based on this finding, we speculate that TLR2/4 may also contribute to the development of SIRS during OLT. In the current study, we examined the expression of TLR2/4 on PBMC in a group of OLT patients with SIRS, and compared the results to that in a group of patients without SIRS.

2. Patients and Methods

This study was approved by the Research Ethics Board of The Third Affiliated Hospital, Sun Yat-sen University. Written informed consent was obtained from all patients prior to the enrollment.
2.1. Study Population. Twenty-seven patients (24 males and 3 females) with end-stage liver diseases undergoing modified piggyback liver transplantation were enrolled. Among these patients, 12 had hepatitis B cirrhosis, 8 had small liver cancer (tumor diameter < 3 centimeter), 4 had chronic severe hepatitis B, and the remaining 3 had drug-related acute liver failure. Physical status of the patients was III or IV according to the American Society of Anesthesiologists (ASA) classification (Table 1).

The status of organ donors was cardiac death in 8 cases, brain death in 10 cases, and living relatives in 9 cases. Warm ischemia for donation after cardiac death was 3 to 4.5 minutes (Table 2). There was no warm ischemia in other cases.

2.2. Anesthesia. Anesthesia was induced with intravenous (i.v.) fentanyl and propofol. Tracheal intubation was facilitated with rocuronium. The lungs were mechanically ventilated with oxygen (50%). Partial pressure of carbon dioxide (PawCO2) was maintained at 30–35 mmHg. Anesthesia was interrupted by a satinskys clamp from the back of the liver. V ena cava of second hepatic hilum was blocked by a Klintmalm liver clamp. The liver was then removed. The openings of hepatic veins on the anterior wall of the vena cava were connected to form an open inverted triangular cuff. The posterior wall of the donor inferior vena cave (IVC) was incised to fashion a wide-open inverted triangular cuff that matched the IVC opening in the recipient. The openings were closed with 4–0 Prolene suture. The graft was flushed with 400 to 800 mL cold FFP. Donor infrahepatic vena cava was ligated, and the portal vein was anastomosized. The clamps were then removed to allow reperfusion. Hepatic artery and bile duct anastomoses were completed [18–20].

The entire procedure consisted of an anhepatic phase (from vascular clamping to reperfusion of portal vein and inferior vena cava) and a neohepatic phase (from reperfusion of donor liver to the end of operation).

2.4. Collection of General Data. The demographic data as well as the Child-Turcotte-Pugh (CTP) scores, ASA classification, duration of the operation, volume of blood loss and input were collected. Duration of postoperative mechanical ventilation, alanine aminotransferase (ALT), aspartate aminotransferase (AST), prothrombin time (PT), blood urea nitrogen (BUN), and serum creatinine (SCr) were also recorded.

2.5. Collection of Blood Samples. Whole blood (4 mL) was collected at T1 (after induction of anaesthesia), T2 (25 minutes after the beginning of anhepatic phase), T3 (3 hours after graft reperfusion), and T4 (24 hours after graft reperfusion). Two mL of blood sample was collected in EDTA tubes for analysis of TLR2/4 immediately with flow cytometry. The remaining two mL of blood sample was collected in dry tubes for TNF-α, IL-1β, and IL-8 assay.

2.6. Analysis of TLR2/4 Expression. Twenty μL al phylococyanin (APC) antihuman CD14 (eBioscience) plus 20 μL fluorescein isothiocyanate (FITC) antihuman Toll-like receptor 2 (eBioscience, San Diego, California, USA) or phycoerythrin (PE) antihuman Toll-like receptor 4 (eBioscience) were added to 100 μL of EDTA treated blood. The mixture was incubated for 20 minutes in the dark at ambient temperature. After which, they were mixed with 2 mL of RBC Lysis Buffer (eBioscience) in the dark for 15 minutes and then centrifuged for 5 minutes at 300 g. After rinsing twice, the supernatant was discarded and sample was preserved at 4°C in the dark. Samples were quantified with FACs Calibur flow cytometry (Becton Dickinson, Franklin Lakes, New Jersey, USA). The isotype controls were FITC mouse IgG2α and PE mouse IgG2α (eBioscience).

2.7. Cytokine Assay. TNF-α, IL-1β, and IL-8 were measured with ELISA (Rapidbio, West Hills, California, USA).

2.8. Perioperative SIRS Monitoring. Patients’ temperature, heart rate, respiratory rate, and white blood cell count were assessed every 6 hours for 7 days after the operation. The diagnosis of SIRS was based on the presence of two or more of the following criteria [21], verified by an ICU physician as well as an anesthesiologist: (1) temperature >38°C or <36°C,
P<.05 is considered statistically significant. All data were processed by SPSS12.0 for windows (SPSS Inc., Chicago, Ill, USA).

2.9. Data Analysis. The data are expressed as mean ± standard deviation. One-Way ANOVA was used to analyze the difference between the different phases in the same group. Independent-samples t-test was used to analyze the difference between the SIRS and non-SIRS groups. Data of nonnormal distribution are expressed as median (interquartile range) [Median (Q)], and were analyzed by Wilcoxon signed ranks test. Spearman correlation analysis was used to determine the relationship between different measures. P < .05 is considered statistically significant. All data were processed by SPSS12.0 for windows (SPSS Inc., Chicago, Ill, USA).

3. Results

3.1. General Data. Twelve out of 27 patients developed SIRS after OLT (at 6 to 78 hours), and two died of lung infection. There were no significant differences between two groups on graft origination, duration of cold or warm ischemia (Table 2). Blood loss and concentrated red blood cell (RBC) transfusion during the operation were larger in the SIRS group than in the non-SIRS group. The pre-anhepatic phase and anhepatic phase lasted longer in SIRS patients (Table 3). CTP score, age, gender, body weight, ascites, urinary production, the length of neohepatic phase or the entire operation did not differ between the SIRS and non-SIRS groups (Table 3).

Duration of postoperative mechanical ventilation in the SIRS group was significantly longer than non-SIRS group. Hepatic function, renal function, and infection (respiratory tract) after the surgery also did not differ between the 2 groups (Table 4).

3.2. Difference of TLR2/4 Expression on PBMC between SIRS and Non-SIRS Groups. The baseline TLR2 on PBMC was 74% (interquartile range: 25%) and 80% (interquartile range: 28%) in SIRS and non-SIRS groups (P > .05, Figure 1). Baseline TLR4 was 12% (interquartile range: 8%) and 18% (interquartile range: 21%) in SIRS and non-SIRS groups (P > .05, Figure 2). TLR 2 expression was significantly higher at T3 and T4 in comparison to T1 and T2 in the SIRS patients but not in the non-SIRS group.

### Table 2: Postoperative SIRS in patients receiving different categories of transplant.

<table>
<thead>
<tr>
<th>Features</th>
<th>Total</th>
<th>SIRS</th>
<th>non-SIRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold ischemia times (min)</td>
<td>0 (3)</td>
<td>1.8 (2)</td>
<td>1.5 (3)</td>
</tr>
<tr>
<td>Cold ischemia times (h)</td>
<td>6.4 ± 0.5</td>
<td>6.4 ± 0.5</td>
<td>6.4 ± 0.5</td>
</tr>
<tr>
<td>Warm ischemia times (min)</td>
<td>4.3 ± 0.6</td>
<td>5.0 ± 2.6</td>
<td>4.4 ± 0.4</td>
</tr>
</tbody>
</table>

### Table 3: Clinical characteristics in SIRS versus non-SIRS during OLT.

<table>
<thead>
<tr>
<th>Features</th>
<th>Total</th>
<th>SIRS</th>
<th>non-SIRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>47 ± 11</td>
<td>47 ± 12</td>
<td>47 ± 12</td>
</tr>
<tr>
<td>Weight (kilogram)</td>
<td>64 ± 10</td>
<td>61 ± 8</td>
<td>66 ± 11</td>
</tr>
<tr>
<td>Pre-anhepatic phase (min)</td>
<td>10 ± 5</td>
<td>4 ± 2</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>Ascites (mL)</td>
<td>622 ± 1305</td>
<td>775 ± 983</td>
<td>500 ± 1538</td>
</tr>
<tr>
<td>Warm ischemia times (min)</td>
<td>2527 ± 800</td>
<td>2527 ± 800</td>
<td>2527 ± 800</td>
</tr>
<tr>
<td>Cold ischemia times (h)</td>
<td>44 ± 125</td>
<td>44 ± 125</td>
<td>44 ± 125</td>
</tr>
<tr>
<td>Volumes of blood loss (mL)</td>
<td>3011 ± 1286</td>
<td>3617 ± 1380</td>
<td>2527 ± 1004</td>
</tr>
<tr>
<td>Concentrated red blood cell (mL)</td>
<td>989 ± 536</td>
<td>1225 ± 554</td>
<td>800 ± 453</td>
</tr>
<tr>
<td>Neohepatic phase (min)</td>
<td>252 ± 46</td>
<td>242 ± 26</td>
<td>259 ± 57</td>
</tr>
<tr>
<td>Total operation time (min)</td>
<td>402 ± 65</td>
<td>422 ± 65</td>
<td>386 ± 63</td>
</tr>
</tbody>
</table>

Mean ± SD or median (Q). *P < .05, compared with non-SIRS.

(2) heart rate >90 per minute, (3) respiratory rate >20 per minute or PaCO2 < 32 mmHg, and (4) white blood cell count >12,000/mL, <4,000/mL, or >10% immature (band) forms.

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Mediators of Inflammation
3.3. Difference of the Serum Levels of TNF-α, IL-1β and IL-8 between SIRS and Non-SIRS Groups. Baseline TNF-α, IL-1β, and IL-8 was 90 (interquartile range: 118), 34 (interquartile range: 239), and 163 (interquartile range: 181) pg/mL in SIRS group, and 96 (interquartile range: 488), 38 (interquartile range: 161), and 64 (interquartile range: 173) pg/mL in the non-SIRS group, respectively. Serum TNF-α, IL-1β, and IL-8 was significantly higher at T3 and T4 in comparison to T1 and T2 in the SIRS group, but not in the non-SIRS group (Figures 5, 6, and 7).

3.4. Correlation Analysis. There was no relationship between TLR2/4 and CTP score. The expression of TLR4, but not TLR2, was positively correlated to serum TNF-α.
4. Discussion

The current study demonstrated that expression of TLR2/4 on PBMC and concentration of inflammatory cytokines after liver transplant reperfusion were significantly higher in patients with SIRS than those without.

SIRS is an inflammatory state caused by serious trauma, and infection [2–4, 21]. A cardinal feature of SIRS is the activation of inflammatory cells such as monocyte-macrophages, neutrophils, and massive release of proinflammatory cytokines [2, 4]. SIRS is common in OLT due to surgical trauma, hemorrhage, and ischemia-reperfusion injury. Incidence of postoperative SIRS in our study is 44%.

TLR2/4 on immune cells can activate nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) in response to a variety of pathological conditions, which in turn initiate or amplify inflammation, and ultimately, organ injury [11–14]. Lipopolysaccharide (LPS) can induce TLR4 gene expression in granulocyte and endothelial cells, and activate NF-κB and the production of TNF-α, IL-6, and IL-8 [22]. TLR4 antibody can inhibit activation of NF-κB and production of inflammatory cytokines [23]. Previous studies also demonstrated that high expression of TLR4 is positively correlated with ischemia-reperfusion injury [24]. Importantly, the transcriptional and translational signal of TLR2/4 in mononuclear cell was upregulated significantly in SIRS patients [25].

Although there was no significant difference in CTP scores between the SIRS and non-SIRS groups, the expression of TLR2/4 on PBMC and serum proinflammatory cytokines at T3 and T4 were significantly higher in the SIRS group, suggesting that high expression of TLR2/4 in OLT patients is associated with SIRS.

Previous studies indicated that cytokine, endotoxin are involved in the regulation of TLR2/4 expression [26–30]. Within the context of liver transplantation, factors that could
upregulate TLR2/4 may include prolonged surgery, massive blood loss and transfusion, liver ischemia/reperfusion, translocation of enteric microbes during portal vein occlusion and reopening, and ischemia-reperfusion injury of graft [31–33]. Specifically, blood loss, the length of prehepatic phase and anhepatic phase may be the most important factors for upregulation of TLR2/4 expression in PBMC after OLT.

In conclusion, our findings suggest that upregulation of TLR2/4 on PBMC could initiate SIRS after major surgery such as OLT.

Acknowledgments

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[17] G. Tarantino, V. Citro, and P. Esposito et al., “Blood ammonia levels in liver cirrhosis: a clue for the presence of portosystemic


Review Article

Biliary Innate Immunity: Function and Modulation

Kenichi Harada and Yasuni Nakanuma

Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan

Correspondence should be addressed to Kenichi Harada, kenichih@med.kanazawa-u.ac.jp

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Biliary innate immunity is involved in the pathogenesis of cholangiopathies in patients with primary biliary cirrhosis (PBC) and biliary atresia. Biliary epithelial cells possess an innate immune system consisting of the Toll-like receptor (TLR) family and recognize pathogen-associated molecular patterns (PAMPs). Tolerance to bacterial PAMPs such as lipopolysaccharides is also important to maintain homeostasis in the biliary tree, but tolerance to double-stranded RNA (dsRNA) is not found. In PBC, CD4-positive Th17 cells characterized by the secretion of IL-17 are implicated in the chronic inflammation of bile ducts and the presence of Th17 cells around bile ducts is causally associated with the biliary innate immune responses to PAMPs. Moreover, a negative regulator of intracellular TLR signaling, peroxisome proliferator-activated receptor-γ (PPARγ), is involved in the pathogenesis of cholangitis. Immunosuppression using PPARγ ligands may help to attenuate the bile duct damage in PBC patients. In biliary atresia characterized by a progressive, inflammatory, and sclerosing cholangiopathy, dsRNA viruses are speculated to be an etiological agent and to directly induce enhanced biliary apoptosis via the expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Moreover, the epithelial-mesenchymal transition (EMT) of biliary epithelial cells is also evoked by the biliary innate immune response to dsRNA.

1. Introduction

Clarification of the molecular mechanisms of innate immunity and significance of innate immune responses to the pathogenesis of immune-mediated diseases as well as to the defense against infections has progressed steadily since the cloning of Tolls in drosophila and Toll-like receptors (TLRs) in mammals including humans [1, 2]. Innate immunity was initially thought to be limited to immunocompetent cells such as dendritic cells and macrophages, but epithelial cells also possess TLRs and proper innate immune systems. Liver and extrahepatic bile ducts consisting of hepatic tissue and biliary epithelial cells (BECs) are also exposed to microorganisms and their components originating from the intestines via portal blood and duodenum. In the gastrointestinal tract, TLRs expressed in intestinal epithelial cells may be involved in innate immunity to maintain mucosal homeostasis and also the development of enterocolitis by producing inflammatory molecules [3]. Similar processes using TLRs may operate in the biliary tree.

Human bile is sterile under normal conditions, but bacterial components such as lipopolysaccharide (LPS), lipoteichoic acid, and bacterial DNA fragments, known as pathogen-associated molecular patterns (PAMPs), are detectable in normal and pathologic bile [4–7], and cultivable bacteria are also detectable in bile of patients with inflammatory biliary diseases [8–11], indicating that BECs are exposed to bacterial components under physiological as well as pathological conditions (Table 1). Although hepatocytes are usually infected by the hepatitis virus, no microorganisms showing BEC-specific tropism have been identified. The participation of microorganisms, however, in the etiology or pathogenesis of various cholangiopathies and biliary diseases has been reported or speculated. In this paper, we describe the biliary innate immune system, its association with the pathogenesis of cholangiopathy and biliary diseases, and finally a strategy for the attenuation of cholangiopathy, particularly cholangitis, by the regulation of innate immune responses.
2. Association with Biliary Innate Immunity in Biliary Diseases

Infectious agents have been implicated in the etiology or progression of cholangiopathies including cholangitis, bile duct loss, and lithiasis as a trigger or aggravating factor. Notably, several enterobacteria and viruses are speculated to be primary or secondary factor for primary biliary cirrhosis (PBC), primary sclerosing cholangitis, biliary atresia, hepatolithiasis, and chronic cholecystitis [4, 5, 12–16] (Table 1).

### 2.1. Primary Biliary Cirrhosis (PBC)

PBC patients have an increased incidence of recurrent urinary tract infections compared to patients with other chronic liver diseases [17–19]. Recent findings also support an association between vaginal or urinary tract infections and PBC [20]. Furthermore, endotoxin and lipoteichoic acids abnormally accumulate in or around the intrahepatic bile ducts [7, 16, 21] and DNA of *Propionibacterium acnes* (*P. acnes*) was detected as a major clone in the granulomas of PBC patients (Table 1) [22]. Because these bacterial components, whether proteins or nucleic acids, act as PAMPs, the presence of PAMPs in bile or around bile ducts is known to induce a variety of inflammatory reactions and speculated to underlie the etiopathogenesis of the cholangiopathy in cases of PBC.

The major autoantigens against antimitochondrial antibodies (AMAs) in PBC are members of the 2-oxo-acid dehydrogenase complex (2-OADC), which includes the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) [23]. An immune response to intrahepatic BECs through 2-OADC-specific CD4+ helper T cells and CD8+ cytotoxic T cells is thought to be the major mechanism responsible for the immunological destruction of BECs in PBC and these T cells show molecular mimicry between human and bacterial PDC-E2 [24, 25]. Therefore, environmental factors such as microorganisms and xenobiotics are speculated to disrupt the self-tolerance to autoantigens as a specific intrahepatic BEC malfunction, supporting the aforementioned role of PAMPs in the etiopathogenesis of PBC.

### 2.2. Biliary Atresia

Biliary atresia consists of a fetal type affecting 10–25% of patients and the more common perinatal type. The perinatal type is characterized by a progressive, inflammatory, and sclerosing cholangiopathy. The presence of several viruses including Reoviridae (type 3 reovirus and type C rotavirus) (Table 1) in liver tissue or affected bile duct specimens obtained from patients with biliary atresia during the Kasai procedure or a liver transplantation, has been demonstrated [13, 26–31], though conflicting results have been reported [13, 26, 28–30, 32]. Immunostaining for Mx proteins, which mediate an early innate immune response and are highly sensitive markers for type I interferon (IFN) activity, revealed that hepatocytes and intrahepatic bile ducts in biliary atresia are positive for Mx, suggesting the presence of viruses in hepatocytes and biliary epithelial cells of patients with biliary atresia [33]. Among these viruses, Reoviridae having a double-stranded RNA (dsRNA) genome, in particular, are characterized by epithelial tropism, and rotavirus type A is the most frequent etiological agent in cases of acute infantile diarrhea in young children. Moreover, the infection of newborn Balb/c-mice with Reoviridae including type A rhesus rotavirus (RRV) and type 3 reovirus (Abney) leads to a cholestasis and biliary obstruction resembling human biliary atresia [34, 35]. Therefore, it is likely that BECs are a target of these viruses which directly cause the cholangiopathy in cases of biliary atresia.

### 2.3. Hepatolithiasis

Hepatolithiasis is not a rare disease in East Asian countries including Japan and is characterized by the formation of stones and histologically “chronic proliferative cholangitis”. Bacterial infections in the biliary tree and cholestasis have been implicated as the major etiopathogenic factor for lithogenesis in patients with calcium-bilirubinate stones. *Escherichia coli* (*E. coli*) is the bacterium most frequently isolated, followed by several species shown to have β-glucuronidase (Table 1). Moreover, the presence of *Campylobacter* species-specific DNA has been demonstrated in bile samples and biliary mucosa specimens in cases of hepatolithiasis, by PCR (Table 1) [9]. These bacteria in the

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**Table 1: Bacteria and viruses possible etiological of biliary diseases.**

<table>
<thead>
<tr>
<th>Primary biliary cirrhosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Detection of microorganisms</td>
</tr>
<tr>
<td>(a) lipopolysaccharide (LPS)</td>
</tr>
<tr>
<td>(b) lipoteichoic acid</td>
</tr>
<tr>
<td>(c) <em>Helicobacter</em></td>
</tr>
<tr>
<td>(d) β-retrovirus</td>
</tr>
<tr>
<td>(e) <em>P. acnes</em></td>
</tr>
<tr>
<td>(ii) Molecular mimicry between human and microbial PDC-E2</td>
</tr>
<tr>
<td>(a) <em>E. coli</em></td>
</tr>
<tr>
<td>(b) <em>Mycobacterium</em></td>
</tr>
<tr>
<td>(c) <em>Novosphingobium</em></td>
</tr>
<tr>
<td>(d) <em>Lactobacillus</em></td>
</tr>
<tr>
<td>(e) <em>Chlamydia</em></td>
</tr>
<tr>
<td>(iii) Biliary atresia</td>
</tr>
<tr>
<td>(a) Reovirus</td>
</tr>
<tr>
<td>(b) Rotavirus</td>
</tr>
<tr>
<td>(c) cytomegalovirus (CMV)</td>
</tr>
<tr>
<td>(d) adenovirus</td>
</tr>
<tr>
<td>(e) enterovirus</td>
</tr>
<tr>
<td>(f) <em>Ebstein-Barr virus</em></td>
</tr>
<tr>
<td>(iv) Primary sclerosing cholangitis</td>
</tr>
<tr>
<td>(a) <em>Helicobacter</em></td>
</tr>
<tr>
<td>(b) α-hemolytic streptococcus</td>
</tr>
<tr>
<td>(v) Hepatolithiasan</td>
</tr>
<tr>
<td>(a) <em>Escherichia coli</em> (<em>E. coli</em>)</td>
</tr>
<tr>
<td>(b) <em>Klebsiella</em></td>
</tr>
<tr>
<td>(c) <em>Streptococcus</em></td>
</tr>
<tr>
<td>(d) <em>Pseudomonas</em></td>
</tr>
<tr>
<td>(e) <em>Bacteroides</em></td>
</tr>
<tr>
<td>(f) <em>Clostridium</em></td>
</tr>
<tr>
<td>(g) <em>Campylobacter</em></td>
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</tbody>
</table>

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biliary epithelium are speculated to influence the occurrence and development of cholangitis and lithogenesis, though the mechanism of such an effect is still unclear.

3. Basic Mechanisms of Biliary Innate Immunity

BECs are immunologically potent cells. The BECs of inflamed bile ducts actively participate in the inflammation by secreting cytokines and expressing immune receptors. In addition to immunocompetent cells, epithelial cells including BECs recognize microbes and their constituents via a set of receptors, referred to as pattern-recognition receptors (PRRs). TLRs are the major epithelial PRRs recognizing PAMPs. Ten TLRs (TLR1 to TLR10) have been identified in humans, with TLR4 known to mediate inflammatory responses to LPS. In immunocompetent cells, the response to LPS is mediated by interaction with the TLR4 in conjunction with TLR4 accessory proteins MD-2 and CD14, triggering transduction of intracellular signals followed by the activation of TLR-associated adapter proteins, myeloid differentiation factor 88 (MyD88), and IL-1 receptor-associated kinase (IRAK)-1, leading to the activation of nuclear factor-κB (NF-κB) and then to the synthesis of antibiotics and proinflammatory cytokines. In contrast to bacterial PAMPs, dsRNA viruses such as Reoviridaes (reovirus and rotavirus) are recognized by TLR3, IFN-inducible helicase retinoic acid-induced protein 1 (RIG-I), and melanoma differentiation-associated gene-5 (MDA-5). The stimulation of these receptors by dsDNA transduces signals to activate the transcription factor interferon regulatory factor 3 (IRF3) as well as NF-κB. Human and murine BECs possess at least TLR1-TLR5, related molecules (MD-2, MyD88, and IRAK-1), RIG-I, and MDA-5 [4, 36–38]. Moreover, immunohistochemistry has confirmed that TLR1-TLR5, MyD88, and IRAK-1 are distributed diffusely in the epithelium of several organs, constituting an important barrier at mucosal surfaces. So far, human β-defensins (hBD-1 to -6) have been identified. hBD-1 is constitutively expressed in cultured BECs and diffusely distributed in the cytoplasm of intrahepatic bile ducts irrespective of anatomical levels [42] (Figure 2). Moreover, because hBD-1 is constantly detectable in bile samples, hBD-1 is believed to play a role in the constitutive antimicrobial defense of the hepatobiliary system [42]. This may be why biliary tract infections are rare and bile is sterile under physiological conditions, though the biliary tree is potentially exposed to enteric bacteria. In contrast, hBD-2 is not detected in BECs cultured without a stimulant, but de novo expression is found in LPS- or E. coli-treated BECs. In vivo, hBD-2 expression is restricted to the intrahepatic large bile ducts and peribiliary glands, in particular, showing cholangitis in extrahepatic biliary obstruction and hepatolithiasis (Figure 2) [42]. Because in these diseased livers, enteric bacteria are mostly cultivable in bile, the participation of bacteria-related cholangitis is closely associated with the hBD-2 expression in BECs. Therefore, hBD-1 plays a constitutive role in biliary antimicrobial defense, while hBD-2 expression is induced in response to local infections and may play a role in additional antimicrobial defenses.

4. Chemical Mediators Produced by a Biliary Innate Immune Response

Innate immunity provides defense against bacterial and viral infections. Therefore, as part of an innate immune response, several antibiotics are produced. Cytokines and chemokines are also produced in immunocompetent cells and play an important role in subsequent acquired immunity. Moreover, BECs have been shown to secrete polymeric immunoglobulin A, several antibiotics against bacteria (lactoferrin, lysozyme, and defensins) and viruses (IFN-β1 and MxA), cytokines, and chemokines on treatment with PAMPs, thereby contributing to biliary mucosal defense and subsequent acquired immunity [39–41].

4.1. Defensins. Defensins are antimicrobial peptides identified as key elements in innate immunity. Structurally, they are divided into α- and β-defensins. The β-defensin family is distributed in the epithelium of several organs, constituting an important barrier at mucosal surfaces. So far, human β-defensins (hBD-1 to -6) have been identified. hBD-1 is constitutively expressed in cultured BECs and diffusely distributed in the cytoplasm of intrahepatic bile ducts irrespective of anatomical levels [42] (Figure 2). Moreover, because hBD-1 is constantly detectable in bile samples, hBD-1 is believed to play a role in the constitutive antimicrobial defense of the hepatobiliary system [42]. This may be why biliary tract infections are rare and bile is sterile under physiological conditions, though the biliary tree is potentially exposed to enteric bacteria. In contrast, hBD-2 is not detected in BECs cultured without a stimulant, but de novo expression is found in LPS- or E. coli-treated BECs. In vivo, hBD-2 expression is restricted to the intrahepatic large bile ducts and peribiliary glands, in particular, showing cholangitis in extrahepatic biliary obstruction and hepatolithiasis (Figure 2) [42]. Because in these diseased livers, enteric bacteria are mostly cultivable in bile, the participation of bacteria-related cholangitis is closely associated with the hBD-2 expression in BECs. Therefore, hBD-1 plays a constitutive role in biliary antimicrobial defense, while hBD-2 expression is induced in response to local infections and may play a role in additional antimicrobial defenses.

4.2. Interleukin 8. IL-8 is a major cytokine of neutrophils, and functions not only as a chemoattractant of neutrophils, basophiles, and some populations of T cells, but also as an activator of neutrophils for releasing leukotrienes, activated oxygen, and neutrophil defensins. Bacteria or their products have been reported to induce the secretion of
IL-8 from intestinal or gingival epithelial cells, and such cytokines and chemokines are speculated to be involved in epithelial cell damage during bacterial or fungal infections. Cultured human BECs express and release IL-8 in response to bacterial PAMPs including LPS [43]. IL-8 expression is found in proliferating bile ductules in various diseased livers and closely associated with neutrophilic infiltration [43]. Particularly cholangitis lenta defined as bile ductular proliferation, ductular cholestasis, and ductular epithelial damage, is also accompanied by a prominent neutrophilic infiltration [44]. Cholangitis lenta is usually encountered in septic conditions, so circulating infectious reagents such as bacterial toxins or products, cytokines, or chemokines are speculated to be involved in its pathogenesis. In a state of sepsis, particularly endotoxemia, BECs may secrete IL-8 and attract neutrophils to reactive bile ductules. IL-8 produced in bile ductular biliary epithelia is a potential target in the prevention of liver and biliary damage in diseased livers such as septic liver.

5. Tolerance

The luminal surface of the biliary tree is continually exposed to PAMPs via bile and/or portal blood, but no inflammatory response is elicited in BECs. This lack of response to PAMPs, especially LPS, could be due to “endotoxin tolerance,” an important mechanism of maintaining the homeostasis of organs such as the intestines which have commensal bacterial flora and to avoid excessive tissue damage [47]. In addition to intestinal epithelial cells, BECs possess similar tolerance; treatment with LPS for 24 hours significantly induced tolerance to a subsequent exposure to LPS as assessed by measuring levels of NF-κB activity and TNF-α mRNA production in cultured BEC cells (Figure 3) [48]. Moreover, pretreatment with Pam3CSK4 (TLR1/2 ligand) effectively induced tolerance to subsequent stimulation with LPS (TLR4 ligand) (Figure 3) [48]. This cross-tolerance has been demonstrated in monocytes and intestinal epithelial cells [47]. However, treatment with poly(I:C) (TLR3 ligand) significantly enhanced NF-κB activity in fresh cultured BECs and pretreatment did not lead to tolerance to poly(I:C) (Figure 3) [49]. Levels of production of MxA and TRAIL were also preserved. Therefore, tolerance to a TLR3 ligand (poly(I:C)) is not found in BECs.
In response to LPS, the structural complex formed by myeloid differentiation factor 88 (MyD88), IL-1 receptor-associated kinase (IRAK)-1, IRAK-4, and TNF receptor-associated factor 6 (TRAF6) induces a series of phosphorylation events, leading to the activation of nuclear transcription factors. IRAK-M plays a critical negative regulatory role in the signaling between MyD88 and IRAK-1 [50]. In LPS-tolerant cultured BECs, levels of the IRAK-M mRNA and protein were increased, implying that the expression of IRAK-M interferes with the association between IRAK-1 and MyD88 and is crucial to the LPS-induced tolerance of endotoxin in BECs [48]. Moreover, Pam3CSK4 as well as LPS induced IRAK-M expression in BECs [48], suggesting that the tolerance caused by the upregulation of IRAK-M expression is also associated with cross-tolerance to LPS induced by Pam3CSK4. Immunohistochemically, IRAK-M was constitutively expressed in the cytoplasm of BECs, irrespective of intrahepatic biliary levels (Figure 4). This finding suggests that the expression of IRAK-M is associated with hypo- or unresponsiveness to bacterial PAMPs in bile and/or portal flow. In contrast, although IRAK-M mRNA expression was upregulated by stimulation with dsRNA (TLR3 ligand), no tolerance to the dsRNA was found in cultured BECs. This is reasonable because the intracellular signaling of dsRNA-related receptors is a MyD88-independent pathway, that is, the dsRNA-related response is not affected by IRAK-M [51]. Moreover, the upregulation of IRAK-M expression on treatment with poly(I:C) is speculated to cause dsRNA-stimulated BECs to become tolerant to TLR2- and TLR3-related PAMPs including LPS. However, BECs are speculated to be in an entirely virus-free state and at the time of infection of a dsRNA virus, an innate response to dsRNA develops and continues in the presence of virus in the biliary tree, suggesting that the infection likely causes progressive destruction in the biliary epithelium without inducing tolerance.

6. Cholangiopathy Associated with Biliary Innate Immunity

6.1. Cell Death. BECs lining the biliary tree are tolerant of non-pathogenic commensal bacterial PAMPs so as to maintain the homeostasis of biliary innate immunity under physiological conditions. Even though biliary innate immunity is activated by pathological PAMPs and provides ‘danger signals’ to the biliary tree, more effective negative mechanisms occur to avoid tissue damage.

However, because innate immune tolerance of dsRNA is lacking in BECs, cell and tissue damage is found in the biliary innate immune response via TLR3 [49]. Stimulation with poly(I:C) induced the activation of NF-κB and IRF-3, followed by the production of antiviral IFN-β1 [37] and also enhanced apoptosis via production of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [37]. Moreover, in biliary atresia in which Reoviridae are speculated to be an etiological agent, BECs lining extrahepatic bile ducts diffusely and constantly expressed TLR3 and showed an enhancement of TRAIL and single-stranded DNA (ssDNA)-positive apoptosis as well as the activation of NF-κB and IRF-3 and increased expression of an antiviral product, MxA [33, 37, 38]. Therefore, BECs not only directly participate in the antiviral innate immune response through the production of antiviral effectors to prevent viral replication by secreting antibiotics in response to dsRNA, but also play a role in the generation of apoptotic responses to infected cells. This additional mechanism concerning cell death in biliary innate immunity is directly associated with the pathogenesis of cholangiopathies in biliary atresia.

6.2. Epithelial-Mesenchymal Transition (EMT). Fundamentally, EMT is a loss of normal epithelial features such as cell-to-cell adhesion molecules and the gain of a mesenchymal phenotype [52]. Recently, the EMT of BECs has been speculated to be associated with periductal fibrosis and portal fibrosis in several chronic hepatobiliary diseases [53–56]. In biliary atresia, in particular, the mesenchymal markers vimentin, and S100A4 (also known as fibroblast-specific protein 1), and an essential transcription factor for EMT, Snail, are expressed but the biliary-type cytokeratin
CK19 and the common epithelial marker E-cadherin are not, in BECs of extrahepatic bile ducts and peribiliary glands. The occurrence of EMT in the BECs of these anatomical biliary components is closely associated with the pathogenesis of sclerosing cholangiopathy in biliary atresia [56, 57]. As mentioned, although the biliary innate immune response to dsRNA reduces the viability of cultured human BECs via TRAIL-mediated apoptosis, the rate of cell death is approximately 30% [37]. The cells that evade apoptosis show a gradual loss of CK19 and E-cadherin, and increased expression of S100A4 and Snail, demonstrating the occurrence of biliary EMT. Because EMT confers resistance to apoptotic effects in fetal rat hepatocytes [58], biliary EMT is thought to be a survival mechanism and associated with an incomplete induction of apoptosis caused by the biliary innate immune response.

TGF-β1 and basic fibroblast growth factor (bFGF) are the major inducers of EMT and TGF-β1 plays an important role in the initiation and progression of liver fibrosis [59, 60]. Moreover, because expression of the TGF-β1 pseudoreceptor, bone morphogenetic protein and activin membrane-bound inhibitor (Bambi), is decreased by an innate immune response and consequently, susceptibility to TGF-β1 is increased, loss of Bambi and upregulation of the TGF-β receptor are also speculated to be inducers of EMT [61]. Cultured human BECs constantly express TGF-β1, its receptor TGFβR1, and the bFGF receptor (FGFR1) [54, 57]. However, because Bambi is also expressed in BECs, the induction of EMT is likely inhibited by the effect of Bambi. Treatment with poly (I:C) gradually decreases and increases the expression of Bambi and bFGF, respectively, and stimulation with bFGF quickly induces a reduction in the level of Bambi. Therefore, the biliary innate immune response to dsRNA could increase susceptibility to TGF-β1, and both TGF-β and bFGF play important roles in the biliary EMT induced by a dsRNA-related innate immune response.

6.3. Chronic Inflammation. Recently, in addition to Th1 and Th2 cells, a third type of pathogenic helper T cell, the Th17 cell, and its association with the chronic inflammation of autoimmune diseases, has been noted [62]. Human Th17 cells are characterized by the production of interleukin (IL)-17 (IL-17A and IL-17F) and differentiate from naïve T cells (Th0). Th17 cells are part of the mucosal host defense system and have a major role in protection against bacterial infections. Moreover, with the discovery that Th17 cells are also involved in the pathogenesis of chronic inflammatory disorders including models of some autoimmune diseases, there has been intense interest in the relative contributions of Th17 and Th1/Th2 cells to the pathogenesis of these diseases. In liver, IL-17-positive cells identified as Th17 cells are mainly present at the interface of inflamed portal tracts in cases of PBC and CVH-C, and also, in PBC, accumulated around damaged interlobular bile ducts [63]. Th17 cells are associated with interface hepatitis in chronic liver diseases. Moreover, the Th17-related peribiliary cytokine milieu is enhanced in PBC and implicated in the histogenesis of the sustained cholangitis of PBC.

In human Th17 cells, IL-6 and IL-1β are required for differentiation [64], while IL-23 is necessary for maintaining or stabilizing cellular functions and survival, but not differentiation [62]. Bacterial PAMPs including LPS cause the production of Th17-inducible cytokines (IL-6 and IL-1β) and a Th17-maintaining cytokine (IL-23) in cultured BECs [63]. Moreover, BECs lining damaged bile ducts in PBC, express IL-6, IL-1β, and IL-23 p19 [63]. The biliary innate immune response to bacterial components involves the production of Th17-inducible and -maintaining cytokines in BECs and also the differentiation into Th17 cells of periductal dendritic cells and macrophages. Biliary innate immunity plays a role in the induction and maintenance of Th17 cells in the periductal area in cases of PBC.

7. Modulation of Biliary Innate Immunity as a Therapeutic Strategy

Therapeutic strategies have been proposed for the modulation of hepatic innate immunity, but rarely for biliary innate immunity. Peroxisome proliferator-activated receptor y (PPARy) is a nuclear receptor involved in regulating adipocyte differentiation and also antiinflammatory activities [65]. The activation of PPARy by its ligands is shown to inhibit the expression of proinflammatory cytokines such as TNF-α, the induction of which is mediated via NF-κB [66]. In liver, PPARy is constitutively expressed in intrahepatic bile ducts, irrespective of the anatomical level (Figure 5), and may relate to the maintenance of biliary homeostasis and absence of inflammatory reactions by attenuating inflammatory signals in BECs to PAMPs [67]. In PBC liver, PPARy expression is significantly downregulated in the affected bile ducts (Figure 5), indicating an increased susceptibility to PAMPs. IL-4 (Th2 cytokine) and IFN-γ (Th1 cytokine) up and downregulate PPARy expression, respectively, in cultured human BECs. An unique cytokine milieu is associated with the reduction in PPARy expression in the affected bile ducts of PBC liver [67].

Several PPARy ligands have been identified, including the prostaglandin D metabolite 15-deoxy-D12,14-prostaglandin J2 (15d-PGJ2) and thiazolidinedione derivatives. 15d-PGJ2 functions as an endogenous ligand for PPARy and attenuates the activation of NF-κB by preventing the phosphorylation of its inhibitor protein (I-κB). In cultured human BECs, 15d-PGJ2 treatment attenuates PAMP (LPS or peptidoglycan)-induced NF-κB activation and also TNF-α production (Figure 6). PPARy ligands provide protection against biliary inflammation in PBC, but 15d-PGJ2 inhibited NF-κB’s activation independent of PPARy [67, 68]. In fact, a PPARy antagonist (GW9662) partially blocked the inhibitory effects of 15d-PGJ2 on NF-κB activity. Therefore, 15d-PGJ2 is expected to be effective in the antiinflammatory treatment of bile ducts with reduced as well as preserved PPARy expression in PBC. Because PPARy is a key immunomodulatory molecule, a reduction in its expression in the bile ducts of PBC liver may be important to the immunopathogenesis of chronic cholangitis. Therefore, immunosuppression using PPARy ligands may help to reduce bile duct damage in PBC.
Figure 5: Immunohistochemistry for peroxisome proliferator-activated receptor γ (PPARγ). (a) Normal liver. PPARγ is expressed in the cytoplasm of bile ducts (arrow). (b) Primary biliary cirrhosis (PBC). Damaged bile ducts (arrowhead) show reduced expression of PPARγ, though evidently positive biliary cells (arrow) also remain.

Figure 6: Effect of the peroxisome proliferator-activated receptor γ (PPARγ) ligand, 15d-PGJ2, on lipopolysaccharide (LPS, TLR4 ligand)- and peptidoglycan (PGN, TLR2 ligand)-induced NF-κB activation in cultured human biliary epithelial cells (BECs). BECs are pretreated in the presence or absence of 15d-PGJ2 (20 μM) before stimulation with LPS or peptidoglycan. Pretreatment with 15d-PGJ2 significantly prevents PAMP-induced NF-κB activation (* < .05).

8. Conclusion

Biliary innate immunity consisting of an organ-specific system is important for the mucosal immunity in intrahepatic and extrahepatic bile ducts. Biliary innate immunity is surely associated with the pathogenesis of biliary diseases as well as the defense against microbial infections. The molecular mechanisms involved have recently been clarified. Targeting of NF-κB is thought to be a potential therapeutic strategy in various cells, but TLR signaling-specific molecules are also likely to be suitable molecular targets. Further translational research concerning the regulation of biliary innate immunity is needed.

References


Review Article

Toll-Like Receptors: Role in Dermatological Disease

Aswin Hari,1,2 Tracy L. Flach,1,2 Yan Shi,1,2 and P. Régine Mydlarski1,3

1 Immunology Research Group, University of Calgary, Calgary, AB, Canada T2N 4N1
2 Department of Microbiology & Infectious Diseases, University of Calgary, Calgary, AB, Canada T2N 4N1
3 Department of Medicine, University of Calgary, Calgary, AB, Canada T2N 4N1

Correspondence should be addressed to Aswin Hari, haria@ucalgary.ca

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Toll-like receptors (TLRs) are a class of conserved receptors that recognize pathogen-associated molecular patterns (PAMPs) present in microbes. In humans, at least ten TLRs have been identified, and their recognition targets range from bacterial endotoxins to lipopeptides, DNA, dsRNA, ssRNA, fungal products, and several host factors. Of dermatological interest, these receptors are expressed on several skin cells including keratinocytes, melanocytes, and Langerhans cells. TLRs are essential in identifying microbial products and are known to link the innate and adaptive immune systems. Over the years, there have been significant advances in our understanding of TLRs in skin inflammation, cutaneous malignancies, and defence mechanisms. In this paper, we will describe the association between TLRs and various skin pathologies and discuss proposed TLR therapeutics.

1. Introduction

Toll receptors were first discovered in Drosophila where they are involved in embryogenesis [1]. They were later shown to assist in innate immunity where their activation resulted in the production of antimicrobial peptides. Thus, structurally similar receptors found in other species were named toll-like receptors (TLRs) [1, 2]. In mammals, TLRs represent a family of pattern recognition receptors (PRRs) that recognize distinct, conserved microbial components and permit cells to recognize self from nonself in immune activation [1, 3]. The TLR family represents a known group of at least 10 human transmembrane proteins which are essential for innate immunity [4]. Immune cells such as monocytes, macrophages, dendritic cells, granulocytes, and nonimmune cells like keratinocytes express TLRs. Predictably, TLRs are mostly found on cells that initiate the primary immune response. TLRs detect a variety of PAMPs which include Lipopolysaccharide (TLR 4), double-stranded (DS) RNA (TLR 3), and single-stranded (SS) RNA (TLR 7) [5, 6]. These receptors are located on the cell surface, the endocytic vesicle membrane, or intracellular organelles [6]. TLRs have an ectodomain composed of leucine-rich repeats (LRRs) that may bind directly to ligands [2]. Alternately, accessory molecules may also be involved in ligand binding in addition to a cytoplasmic toll/interleukin-1 (IL-1) receptor (TIR) domain, which interacts with TIR-domain-containing adaptor molecules [6]. In the case of human TLR 4, two accessory molecules CD14 and MD2 are essential for LPS recognition [2, 7, 8]. TLRs have the ability to initiate a rapid and potent response upon ligand engagement. While TLRs are single-membrane spanning noncatalytic receptors; different TLRs are able to pair up with each other to expand their range of recognition targets. For instance, TLR 1 and 2 pair up and sense peptidoglycans. While most TLRs signal through the Myd88 pathway (Myeloid differentiation factor-88), TLR 3 and 4 utilize the TRIF pathway (TIR domain-containing adapter protein that induces IFN-β), and TRAF 6 (TNF receptor-associated factor 6) was found to be the additional transducer for TLR 7 and 9 [9, 10]. Ensuing these signalling intermediates, TLRs eventually trigger nuclear factor kappa-light-chain-enhancer of activated B cells-(NF-kB) dependent and interferon regulatory factor (IRF-) dependent activation events [9]. Activation of these transcription factors results in induction of immune and inflammatory genes, namely, tumor necrosis factor alpha (TNF-α) and type I interferons (IFNs) [9]. At these events, TLR-mediated activation is quite similar to that of another important inflammatory cytokine receptor, interleukin-1 receptor (IL-1R). TLRs and IL-1R share the Myd88 adaptor molecule.
and promote the production of proinflammatory cytokines such as leukotrienes (LTs), prostaglandins, and chemokines [4]. Once activated by TLRs, immune cells initiate phagocytosis/killing of pathogens, cytokine, and chemokine production, leukocyte activation, and antigen presentation to T cells, thereby, initiating an adaptive immune response. An in-depth insight into different TLRs and their associated characteristics such as ligands, cytokines induction profile and functions have been previously explained by others [11]. TLRs can modulate adaptive response with respect to Th1 and Th2 as reported in study, by Medzhitov et al. [2, 12]. The study found that the mice lacking Myd88 were defective in giving rise to antigen-specific Th1 T helper cells but could initiate a normal Th2 response. All TLR ligands trigger Th1 response which seem essential in defence against microbial antigens that are viral, bacterial, and fungal in nature, in contrast to specific interaction against antigens such as helminths that require a Th2 response [2].

The skin is the first line of defence against a variety of physical and biological assaults. It shields the body from harmful chemicals, physical trauma, and ultraviolet (UV) radiation. In addition, it is essential in maintaining temperature and homeostasis and in our sensing of the environment. The skin has evolved into a complicated, yet tightly regulated system appropriate for the complex functionalities associated within it. The outermost layer of the skin is the epidermis and is composed of four main types of cells: keratinocytes, Langerhans cells, melanocytes, and Merkel cells (Figure 1(a)). Keratinocytes are capable of proliferating and maintaining the outer layer which is composed of a stratified epithelial zone and a water resistant layer of lipids on the outmost surface. The melanocytes residing in the skin provide the melanin pigment that is essential for protection against UV radiation. Interspersed between keratinocytes and melanocytes are the Langerhans cells (which function as cutaneous antigen presenting cells (APCs)) and intraepithelial T lymphocytes. Merkel cells are oval sensory cells found in the skin. They play a role in light touch discrimination and may have a neuroendocrine function. The epidermis is anchored to the dermis below via connective tissues. The dermis is rich in blood vessels, nerves and has abundant fibroblasts, dendritic cells, macrophages, and lymphocytes.

The repertoire of TLRs found on each of these three epidermal cell types varies (Figure 1(a)). Even though the precise role against pathogens is poorly understood, in general, TLRs in the skin respond to their ligands by activating NF-κB and producing cytokines [13]. Keratinocytes are reported to express TLRs 1, 2, 3, 5, 9, and 10 [14–21]; the evidence supporting TLR 4 expression in keratinocytes is conflicting [14, 18–20] (Figure 1(b)). As an example of TLR 2 engagement, human keratinocytes mainly signal through TLR 2 once activated with Staphylococcus aureus (S. aureus) or Candida albicans [15, 20]. Keratinocytes, treated with S. aureus, transcribe NF-κB controlled genes (cyclooxygenase-2, nitric oxide synthetase, and IL-8) and produce enhanced levels of IL-8, nitric oxide, and chemokines [15]. Ligands for TLR 2, 3, and 5 stimulate production of matrix metalloproteases (MMPs) 1 and 9, along with activation of the NF-κB pathway [22]. The expression of these cytokines is needed for proper recruitment, inflammation, and damaged tissue remodelling [22]. Translocation of NF-κB and the associated events permits keratinocytes to stimulate dendritic cell maturation and enhance antigen presentation [23]. Activated keratinocytes are also important epidermal cytotoxic producers which mobilize leukocytes, signal other cutaneous cells and attract neutrophil granulocytes and professional killer cells [20]. Langerhans cells express significant levels of TLRs 2, 3, 4, 8, and 10 and low levels of TLRs 1, 5, 6, 7, and 9 [24] (Figure 1(c)). DS RNA, present in some viruses, induces a particularly robust response in Langerhans cells, implicating its role in anti-viral immunity via TLR 3 [25]. Additionally, Langerhans cells can be activated indirectly by activated myeloid derived dendritic cells and keratinocytes (Figure 1(d)). For example, they were found to mature and initiate a Th1 response in the presence of keratinocytes that secreted IFNα and IL-18 upon exposure to antigen, such as DS RNA or polyinosinic-polycytidylic acid (poly IC) stimulation [23]. Lastly, human melanocytes have been shown to express TLRs 2, 3, 4, 7, 9 (at the protein level), and respond to TLR 4 ligands by MMP induction [25, 26] (Figure 1(e)). When stimulated by TLR ligands, human melanocytes can: (1) release IL-6 and IL-8 cytokines, (2) enhance chemokine (CCL2, CCL3, and CCL5) mRNA production, (3) upregulate phosphorylated IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), and (4) promote translocation of NF-κBp65 to the nucleus [25].

TLRs are increasingly being implicated in many immune and inflammatory diseases, cancer, and wound healing. For example, TLR 2, TLR 4, TLR 5, and TLR 7 are implicated in tumor metastasis, sepsis, radioprotection, and systemic lupus erythematosus, respectively [83]. Wound healing was found to be affected in Myd88−/− mice suggesting a synergistic role for TLRs in this process [84]. That study revealed interplay between purinergic receptor signalling and Myd88−/− pathways but did not explore IL-1 cytokine profile. The involvement of Myd88 hints at an important function of associated TLRs, especially TLR 2 and 4, in skin tissue remodelling. Lai et al. revealed a novel mechanism by which bacterial products modulate local inflammation in a TLR 2-dependent manner [85]. Understanding TLR signalling pathways, their structural interaction with ligands, and inhibition strategies may provide avenues for potential clinical intervention [83]. In order to clinically manipulate TLRs, pharmacologics should alter TLR activity through receptor antagonists, receptor agonists and signal transduction inhibitors. Moreover, neutralizing antibodies, monotherapies and adjuvancy may also be used to target TLRs. E5564 (Eritoran) is a good example of a current pharmacological (TLR 4 antagonist) agent which is being clinically tested for treatment of Gram-negative endotoxemia and sepsis [83]. TLR-mediated activation or dysfunction has been attributed to exacerbation of different diseases. The manipulation of specific TLRs may therefore lead to the development of novel therapies for autoimmunity, cancer, and inflammatory disease. Next we will discuss how
Keratinocyte (TLR-1, 2, 3, 4, 5, 9, 10)

Melanocyte (TLR-2, 3, 4, 7, 9)

Langerhans cell (TLR-2, 3, 4, 8, 10)

Merkel cell

**Figure 1:** Continued.
Figure 1: Continued.
TLRs have been linked to dermatological disease and their proposed therapeutic role.

2. TLRs in Dermatologic Disease

2.1. Atopic Dermatitis and Allergic Contact Dermatitis.

Atopic dermatitis (AD), which affects up to 20% of the pediatric population, is a chronic inflammatory skin disease characterized by pruritus, eczematous lesions, xerosis, and lichenification. It often forms part of the atopic triad composed of allergic rhinitis, asthma, and eczema [86, 87]. Patients suffering from AD have greater susceptibility to bacterial, viral, and fungal infections; in fact, *S. aureus* has been associated with its flares and severity [71, 88, 89]. In addition to *S. aureus*, the best characterized infectious agent of AD, strains of *Candida* species have a strong ability to colonize atopic skin, and viruses such as herpes simplex virus can aggravate the infection and exacerbate the disease [90]. These pathogens express microbial products that stimulate TLR 1, 2, 6, and 9. Recent studies show a strong association between TLR 2 and the symptoms of severe AD in some populations [29, 30]. The presence of a single nucleotide polymorphism (SNP) R753Q in the TLR 2 allele has been reported to be associated in patients with severe AD and whose skin was prone to *S. aureus* infection [11, 91]. The role of R753Q in AD has been confirmed in the cytokine-based profiling of patients where stimulation of TLR 2 induced altered production of IL-6 and IL-12 [92]. Conversely, a study in a German population found no significant effect of TLR 2 and 4 polymorphisms in relation to susceptibility for AD [93]. On the other hand, polymorphism C-1237T in the TLR 9 gene has been attributed as the cause for impairment of immunity in some cases of AD [31]. Taken together, these studies suggest that there is a defect in TLR 2/9. These defects may be genetic (dysfunctional proteins) or functional (attenuation of regulatory pathways) [31, 32, 87]. This defective recognition of pathogenic antigens by TLRs renders greater susceptibility of AD lesions to various bacterial and viral infections [1]. However, there has been no significant correlation between TLRs 1 and 6 (recognize peptidoglycans, and lipoproteins resp.) and AD [30, 94, 95]. Although correlation between TLRs 2, 9 and AD has been reported, another study using monocyte-derived DCs...
found no relation between polymorphism, function of TLR 2-4 with respect to AD [96]. Allergic contact dermatitis (ACD), another eczematous process, is a type IV delayed hypersensitivity reaction seen in the skin typically after sensitization by haptons [97]. The bacterial infections and haptons binding with self-proteins in the skin leads to activation of DCs stimulating a specific autoreactive CD8+ T cell proliferation, T cell activation leading to psoriatic skin lesions, in a self-sustaining feedback loop. Concurrently, Th17 cells produce IL-22 and IL-17 which stimulate epidermal hyperplasia and neutrophil recruitment and further enhance inflammation. IFN-α (type 1 IFN) in addition to ssRNA (a simulator of viral infection) also acts as an important trigger for DC activation in psoriasis [109]. Another important growth factor expressed during psoriasis is TGF-α, which upregulates TLRs 5 and 9 expression and function in human keratinocytes [110]. Heat shock proteins (i.e., HSP60) are suspected immunogenic proteins that are heavily expressed by epidermal keratinocytes of guttate and plaque psoriasis in comparison to normal skin [111]. HSP60 may subsequently trigger TLR 2 and TLR 4 to stimulate innate and adaptive immunity which develops and/or aggravates psoriasis. The A domain of fibronectin (keratinocyte derived) may act on the TLR 4 pathway in APCs (Langerhans cells) to cause maturation, TNF-α and IL-12 secretion, and antigen presentation to autoreactive T cells [112]. Interestingly, anti-keratin 16 antibodies are highly concentrated in the serum of psoriasis patients and may be involved in chronic inflammation. Keratinocytes incubated with mouse antikeratin 16 monoclonal, antibodies have increased levels of TLR 2, TLR 4, involucrin, and NF-κB nascent polypeptide-associated complex mRNA [113]. As implied earlier, psoriasis is a T cell-mediated disease caused in part by activated T cells interacting with antigen-presenting cells (APCs) in addition to IFN-γ, IL-1, and TNF-α effects [114, 115]. T cells are distinctly compartmentalized in the different skin layers in psoriatic lesions [116]. CD4+/Th1 T cells are, for example, located in the upper dermis unlike CD8+/Th1 T cells that are found in the epidermis [117]. T cells are functionally important since cyclosporine A (an immunosuppressant which binds the cyclophilins of T lymphocytes), as well as CTLAflg, anti-CD4 antibodies, DAB389IL-2, and alefacept (all T cell-based immunomodulators) are highly effective therapies for psoriasis [118–122]. Further support is provided in rare instances of psoriasis transfer to recipients of bone marrow transplants from donors with the disease and similar episodes in xenotransplantation models [123, 124].

In regard to TLR expression, keratinocytes in the epidermis constitutively express TLR 1, 2, and 5. In psoriasis lesions, the expression of TLR 1 and TLR 2 on keratinocytes is further upregulated [62]. Keratinocytes in human psoriatic skin, activated by TLR 2, 3, and 4 ligands exhibited NF-κB nuclear translocation and release of TNF-α and IL-8 [63]. An immunohistochemistry-based profile of psoriatic skin also demonstrates the overexpression of TLR 2 in epidermal and dermal DCs and the enhanced TLR 2 expression in basal layer keratinocytes. It enhanced TLR 4 expression in epidermal and dermal DCs, in addition to accumulation of CD14-positive macrophages [125]. TLRs 7 and 8 signalling have also been implicated in psoriatic exacerbations [112] since imiquimod (a TLR 7/8 agonist) aggravates pathological symptoms.
Topical immunosuppressive therapies such as topical corticosteroids (i.e., budesonide), pimecrolimus, and tacrolimus are currently used for treating inflammatory skin diseases like atopic dermatitis. They function by suppressing IL-8 and TNF-α mRNA expression and either induce (budesonide) or suppress (tacrolimus) TLR 2 mRNA expression in human keratinocytes [126, 127]. Although further studies are required, it appears that the clinical use of systemic and topical retinoids helps to control psoriatic inflammation through TLR 2 inhibition [50]. Monomethyl fumarate (fumaric acid ester) is also used as an immunotherapy for psoriasis; markedly, it interferes with LPS signalling through TLR 4 in dendritic cells, inhibits NF-κB activation, decreases IL-12p40 and IL-10 production, and modulates monocytes-derived DC polarization [128]. Furthermore, the TLR 7/8 agonist imiquimod, which was considered a potential therapy, resulted in exacerbated psoriatic lesions, increased DCs infiltration, and elevated IFN-α production. Another example of potential treatment for psoriasis is the use of anti-inflammatory pharmacologics such as salicylates (as well as other nonsteroidal anti-inflammatory drugs) and parthenolide, which block IkappaB kinase (IKK-1 and IKK-2) activity [123]. IKK-1 and IKK-2 are part of the TLR signalling cascade [129]. Although advances are being made and compounds are being clinically tested to target the common signaling pathways of the TLR superfamily to prevent p38 mitogen-activated protein, NF-κB activity, and TNF production, caution must be exercised when testing these TLR therapies [129].

2.1.2. Acne Vulgaris. Sebaceous glands are a holocrine gland that secretes sebum [130] formed by the breakdown of glandular cells. Full blown acne manifests in the pilosebaceous follicle and is attributed to increased sebum excretion, sebum lipid alteration, androgen and neuuropeptide activities, follicular hyperkeratinization, dysregulation of cutaneous steroidogenesis, and growth of anaerobic, gram positive Propionibacterium acnes (P. acnes) [131, 132]. P. acnes helps sustain inflammation by TLR-mediated induction of cytokines, upregulation of adhesion molecules, and chemokine mediated recruitment of immune cells [133]. Immune cells such as TLR 2 expressing macrophages (perifollicular and peribulbar) tend to surround pilosebaceous follicles in acne lesions; subsequent TLR 2 triggering due to P. acnes results in IL-6, IL-8, and IL-12 cytokine production [4]. The pro-inflammatory response in acne lesions seems to be largely initiated by TLR 2 since the lipid components from the cell wall of P. acnes also trigger TLR 2 activation in monocytes [4]; almost all of the TLR 2-expressing cells in acne lesions are derived from CD14 monocytes, suggesting active monocyte recruitment. Monocytes are, in turn, stimulated to produce TNF-α, IL-12, IL 1-β, and IL-8 [4, 133], which are all major pro-inflammatory cytokines/chemoattractants. Consequently, neutrophils and lymphocytes are attracted to the follicle and exaggerate the observed inflammation. Acne inflammation may also develop because P. acnes stimulates the expression of TLR 2, 4 and CD14 in human keratinocytes [27]. Keratinocytes and sebocytes, located near the pilosebaceous unit, may be capable of detecting either pathogens or abnormal lipids [27]. Human SZ95 sebocytes, in particular, express innate immune molecules such as TLR 2, TLR 4, IL-1β, IL-6, IL-8 [27], CD11d, and CD14 [131]. As a result of TLR stimulation due to P. acnes, pro-inflammatory cytokines, chemokines, antimicrobial lipids [132] and antimicrobial peptides (i.e., cathelicidin, defensin-1, defensin-2, and psoriasin) [134–137], and human B-defensin-2 are produced by these cells [138].

In acne vulgaris, TLR 2 activation can induce robust inflammation as well as cellular apoptosis and tissue injury [4, 28]. Pharmacological agents have been designed to target inflammation through downregulation of TLR expression and function. Retinoids (a class of vitamin A-derived compounds) bind retinoic acid receptors (RAR) and modulate keratinocyte maturation [139–141]. When primary human monocytes are treated with ATRA (all-trans retinoic acid), TLR 2 and CD14 are downregulated without any change in expression of TLR 1 and 4 [142]. Further, ATRA pre- and cotreatment of monocytes inhibited the ability of TLR 1/2 ligands to trigger cytokine production [142]. In response to P. acnes, ATRA-treated monocytes result in cytokine downregulation of IL-12p40, TNF-α, and IL-6 [142]. Other antiacneic drugs such as retinoic acid, ZNS04, doxycycline, nicotinamide, nitroimidazole, retinol, and isotretinoin prevent O2•– production, IL-8 release and keratinocyte apoptosis [143]. Nicotinamide, through interaction with TLR 2 on primary keratinocytes and immortalized HaCaT cells, significantly depresses IL-8 production (both at mRNA and protein levels) in a dose-dependent manner [144]. It appears that nicotinamide’s downregulation of IL-8 is accomplished at the level of transcription by reducing promoter activity. It also inhibits IxB degradation (reduced NFkB) and phosphorylation of Jun N-terminal (JNK) MAP kinases and extracellular receptor kinases (ERK) [144]. Current acne therapies exert their effects, at least in part, via TLRs, and newly developing TLR therapies show great potential.

2.2. Nonmelanoma Skin Cancers. Non-melanoma skin cancer (NMSC) is the most common form of cancer worldwide. The two major types of NMSC are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Risk factors for the development of NMSC include exposure to high doses of UV and ionizing radiation, chemical carcinogens, immunosuppression, oncogenic viral strains, and genetic predisposition.

BCCs, typically occur in areas of chronic sun exposure are slow growing and rarely metastatize [145–147]. Current therapies consist of surgical removal, radiotherapy, photodynamic therapy, cryotherapy, and intralesional injections of IFN-α [148]. Imiquimod has been administered successfully as a topical immune modulator which induces production of IFN-α and IL-2, leading to a heightened immune response [33, 145, 146]. The effect is therefore expected to be mediated by TLR 7/8. Topical application of 5% imiquimod has demonstrated efficacy in several clinical trials [33–35, 145, 149–151]. Stary et al. reported observations where topical imiquimod treatment leads to induction of a specific subset of DCs which mediated destruction of BCC lesions [36, 37]. CpG ODNs are composed of repeating unmethylated
CG regions causing them to resemble bacterial DNA. PF-3512676 (a synthetic CpG ODN) is also considered safe and effective in treating BCC [38], which presumably targets TLR 9.

The prevalence of SCC is second only to that of BCC. It is found more commonly in elderly Caucasian men with significant cumulative UV damage [147, 152–154]. Unlike BCCs, SCCs have higher rates of metastasis and tend to be more aggressive in immunosuppressed patients [152]. SCCs are typically treated by surgery, radiation therapy and other chemotherapeutic modalities [154]. Combination therapy consisting of immunosuppressive agents (methotrexate, bleomycin supplemented with 5-bromouracil) did not yield tangible results [155]. A similar method using interferon instead of bromouracil was reported to be much more effective [154, 156]. As in the case of many skin diseases, imiquimod has been tested for SCC mitigation. TLR 7 and 8 have been manipulated to activate the pro-inflammatory machinery against SCC [154]. The modulation of IL-1, IL-6, IL-8, and IL-12 along with promotion of a Th1 response promotes antitumor and antiviral behaviour [154]. Several studies have shown imiquimod to be a viable option to test for efficacy in treatment of SCC [68–70].

2.3. Melanoma. Melanoma is a malignant tumor of melanocytes which causes the majority of skin cancer-related deaths worldwide [58, 157, 158]. Exposure to UV radiation is the main risk factor for the disease [58, 157, 159]. Melanocytes have the ability to help tumor progression in melanoma by responding to hyaluronic acid fragments through TLR 4 by inducing MMP and cytokine production [59]. Although melanocytes have been shown to express other TLRs [25], TLR 9 has been targeted for modulating immune response. A study on a murine lymphoma model which revealed the potential of CpG ODNs in enhancing immunogenicity of the tumours resulted in exploitation of TLR 9 ligands for melanoma [60, 160].

Many TLR ligands are considered good adjuvant candidates as they can activate dendritic cells. Different TLR 9 agonists are now included in vaccine formulations for human trials. In earlier studies, a vaccine with antigenic peptide and IFA (incomplete Freund’s adjuvant) was reported to induce a weak T cell response. On the contrary, studies with CpG ODNs produced a strong immune response against the Hepatitis B virus [161]. In clinical studies using CpG 7909 (a variant of CpG ODNs) in combination with a melanoma peptide, the vaccine resulted in 10-fold greater specific CD8 T cells compared to peptide alone [60, 161, 162]. Recently a variant of CpG 7909 named PF-3512676 was used [163] in a phase II clinical trial which yielded promising results and had a moderate safety profile but displayed some very rare adverse events. In all, TLR 9 targeting appears to be a viable option in melanoma treatment [164]. In addition, imiquimod (a TLR 7 agonist) was used to harness the potential TLR 7-based treatment for melanoma. Imiquimod is effective in both activating dendritic cells and producing tumor-specific cytotoxic T cells that arrest disease progression [165].

2.4. Therapeutic Drugs

2.4.1. Imidazoquinolinamines—Immune Modulators. Imidazoquinoline compounds (ICs) have similar structure to nucleosides found in all living organisms. They were initially produced and tested as antiallergic drugs in rats, and were reported to be reasonably effective [166]. After extensive studies, the potential of ICs to stimulate secretion of pro-inflammatory cytokines was finally discovered [167, 168]. Toll like receptor 7/8 directs a cascade of events which leads to the release of specific cytokines [169]. Imiquimod and resiquimod are two compounds that are efficient at activating the immune system [170]. Both compounds induce secretion of IFN-α, TNF-α, IL-6, and other pro-inflammatory cytokines from APCs such as resident B cells, pDCs, and monocytes [170–173]. They achieve this response by activating TLR pathways and promoting nuclear transmigration of transcription factors like NFκB and activator protein 1 (AP1). Imiquimod, the more extensively studied analogue, is known to enhance apoptotic pathways in cancer cells. It binds to the high affinity adenosine receptors and suppresses its ability to regulate negative feedback [168]. Resiquimod, a chemically related compound, exhibited 100-fold greater ability to stimulate apoptosis in preliminary studies [170]. Unfortunately, unlike imiquimod, resiquimod has not been studied extensively [50]. At the time of their discovery, imidazoquinolines were hypothesized to effectively treat Kaposi’s sarcoma, human papillomavirus (HPV) and other infections [170]. Initially generated to be an antiviral agent, it was quickly utilized for its expanding therapeutic potential. Imiquimod was one of the first of such analogues to be authorized for treatment of genital warts caused by HPV [6, 26]. Imiquimod has also been used to activate the immune system in many diseases like actinic keratoses [50], BCC [35, 149–151], SCC [70, 154], and melanoma [58, 60, 165, 174–176]. Two other immunomodulators which function through TLR 7, loxoribine, and bropirimine have also been used [6]. Loxoribine, a guanosine ribonucleoside, enhances production of IFN and activates NK cells and B cells [50, 177, 178]. Bropirimine, an aryl pyrimidinone class of antineoplastic compounds, has a similar effect to loxoribine and is currently in clinical studies for treatment of carcinomas [50, 179].

2.4.2. Calcineurin Inhibitors—Immune Suppressors. Calcineurin inhibitors are potent suppressors of the T cell mediated immune response due to their ability to inactivate the serine protease calcineurin. This prevents nuclear translocation of (NFAT nuclear factor of activated T cells) thereby inhibiting the synthesis of pro-inflammatory cytokines in T cells [104, 171]. Pimecrolimus and tacrolimus are ascomycin macrolactam derivatives that are categorised as inhibitors of calcium-dependent phosphatase. In the early stages of AD, the topical application of 1% pimecrolimus was deemed more effective than topical steroids [102, 171]. Different formulations of the drug are being tested for efficacy in psoriasis, vitiligo, and other skin diseases [127, 171, 180]. As previously mentioned, pimecrolimus has been shown to enhance the ability of keratinocytes to fight infection.
Table 1: Toll like Receptors in dermatologic disease.

<table>
<thead>
<tr>
<th>Disease/infection</th>
<th>Toll like receptor associated</th>
<th>Associated function with the disease</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acne vulgaris</td>
<td>TLR 2, 4, CD14</td>
<td>TLR upregulation with eventual exacerbation of the disease</td>
<td>[4, 27, 28]</td>
</tr>
<tr>
<td>Atopic dermatitis</td>
<td>TLR 2, 9</td>
<td>TLR polymorphism leading to increased susceptibility</td>
<td>[29–32]</td>
</tr>
<tr>
<td>Basal cell carcinoma</td>
<td>TLR 7, 8, 9</td>
<td>Exacerbation of disease, target for therapy</td>
<td>[33–38]</td>
</tr>
<tr>
<td>Behçet’s disease-vasculitis</td>
<td>TLR 4, 6</td>
<td>TLR 4 polymorphism leads to increased susceptibility, differential regulation of TLR 6 helps in progression of disease</td>
<td>[39, 40]</td>
</tr>
<tr>
<td>Borrellosis/Lyme disease</td>
<td>TLR 1/2 (heterodimers), 4, 6</td>
<td>TLR upregulation with eventual exacerbation of the disease</td>
<td>[41, 42]</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>TLR 2, 4-CD14, 6</td>
<td>TLR function and signalling leads to disease progression</td>
<td>[43–45]</td>
</tr>
<tr>
<td>Cutaneous graft versus host disease</td>
<td>TLR 4</td>
<td>Exacerbation disease in response to LPS</td>
<td>[46]</td>
</tr>
<tr>
<td>Herpes simplex/Varicella zoster</td>
<td>TLR 2, 3, 9</td>
<td>Select TLR 2 polymorphism associated with disease severity, TLR 3 and 9 help in viral clearance</td>
<td>[47–49]</td>
</tr>
<tr>
<td>Leprosy</td>
<td>TLR 1, 2</td>
<td>TLR function and signalling lead to disease progression</td>
<td>[21, 50–52]</td>
</tr>
<tr>
<td>Lichen planus</td>
<td>TLR 9</td>
<td>TLR upregulation with eventual exacerbation of the disease</td>
<td>[53, 54]</td>
</tr>
<tr>
<td>Lupus erythematosus</td>
<td>TLR 3, 7, 9</td>
<td>TLR 7 upregulation and TLR 3*, TLR 9 function helps to create autoreactive cells</td>
<td>[55–57]</td>
</tr>
<tr>
<td>Melanoma</td>
<td>TLR 4, 7, 9</td>
<td>TLR 4 exacerbates disease TLR 7,9-targetted for immune modulation</td>
<td>[25, 58–60]</td>
</tr>
<tr>
<td>Psoriasis</td>
<td>TLR 1–4, 5, 9</td>
<td>TLRs upregulated and help in creation of autoreactive T cells</td>
<td>[17, 61–63]</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>TLR 2, 4</td>
<td>TLR polymorphism associated with disease severity</td>
<td>[64–66]</td>
</tr>
<tr>
<td>Scleroderma</td>
<td>TLR 4</td>
<td>TLR function and signalling lead to disease progression</td>
<td>[67]</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>TLR 7, 8</td>
<td>Exacerbation of disease, being studied to target for therapy*</td>
<td>[68–70]</td>
</tr>
<tr>
<td>Staph. Aureus</td>
<td>TLR 2, 6</td>
<td>TLR 2 polymorphism associated with severity*, TLR 2/6 function and signalling lead to disease progression</td>
<td>[71, 72]</td>
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<tr>
<td>Stevens-Johnson syndrome/Toxic epidermal necrolysis</td>
<td>TLR 3</td>
<td>TLR polymorphism linked to disease severity</td>
<td>[73]</td>
</tr>
<tr>
<td>Syphilis</td>
<td>TLR 2, 4/5 (heterodimer)</td>
<td>TLRs activate the immune system TLRs help in viral clearance but are targeted by viral products which suppress host defense</td>
<td>[26, 50, 74]</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>TLR 2, 3, 4</td>
<td>TLR 3, 9 help in immune activation, TLR 7* possible association with disease exacerbation, proposed target for therapy</td>
<td>[77–80]</td>
</tr>
<tr>
<td>Verruca and Molluscum Contagiosum</td>
<td>TLR 3, 7, 9</td>
<td>Pathogen exploits TLR pathway to survive</td>
<td>[50, 81, 82]</td>
</tr>
</tbody>
</table>

* proposed association subject to further investigation.
when coadministered with TLR 2/6 ligands [104]. Similar to pimecrolimus in structure and function, tacrolimus is utilized for identical purposes, albeit at lower concentrations (0.03% to 0.1%). Tacrolimus appears safe for administration at 0.1% for treatment of AD but with certain adverse side effects, such as burning sensation and pruritus observed in a small minority [103]. The drug is under trial for treatment of other diseases such as seborrheic and contact dermatitis, systemic lupus erythematosus, and bullous pemphigoid [171, 180]. Sirolimus or rapamycin is a macrolactam-like compound which inhibits IL-2 synthesis and cell cycle pathways [181]. Sirolimus, if injected along with cyclosporine A, prevents the progression of UV-mediated skin cancer [182]. The drug is under consideration in combination with other drugs for treatment of skin malignancies, hepatocarcinomas, lupus, psoriasis, dermatomyositis, graft rejection, and a genetic disorder, pachyonychia congenital [180, 183–188].

Table 1 lists a selected group of skin diseases and the different TLRs affiliated with each disease. The TLRs associated can either activate the immune system or promote disease progression as indicated.

3. Conclusion

Toll like receptors represent a conserved group of receptors which help the immune system to function properly. Different TLRs are associated with an array of skin diseases (Table 1). TLR agonists and antagonists have great potential for the treatment of allergic and inflammatory diseases. More research must discern the relationship between specific TLRs and the corresponding disease in order to harness the therapeutic potential of TLR ligands. Although studies have proven that TLR agonists like CpG can induce a robust immune response, the efficacy of the vaccines, optimization of dosage, long-term effects, and augmentation requires further study [162, 172].

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12 Mediators of Inflammation


Review Article

Targeting Toll-Like Receptors for Treatment of SLE

Christopher G. Horton, 1, 2 Zi-jian Pan, 2 and A. Darise Farris 1, 2

1 Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA
2 Arthritis and Immunology Program, Oklahoma Medical Research Foundation, 825 NE 13th Street, Oklahoma City, OK 73104, USA

Correspondence should be addressed to A. Darise Farris, farrisd@omrf.org

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Toll-like receptors (TLRs) are important innate immune receptors for the identification and clearance of invading pathogens. Twelve TLRs that recognize various conserved components of microorganisms are currently known. Among these, the endosomal TLRs 3, 7/8, and 9 recognize dsRNA, ssRNA, and CpG DNA, respectively. Nucleic acid-sensing TLRs, TLR 7 in particular, have been implicated in systemic lupus erythematosus (SLE) and are thought to exacerbate disease pathology. Activation of these TLRs results in the production of inflammatory cytokines and type I interferon. Genome-wide association studies, single nucleotide polymorphism analyses as well as experimental mouse models have provided evidence of TLR signaling involvement in SLE and other autoimmune diseases. Since activation of these receptor pathways promotes autoimmune phenotypes, inhibitory drugs that target these pathways constitute important new therapeutic strategies for the treatment of systemic autoimmunity.

1. Introduction

Toll-like receptors (TLRs) are pattern recognition receptors of the innate immune system that recognize specific pathogen-associated molecular patterns (PAMPs) conserved among microorganisms. There are currently twelve known TLRs in mammals, which identify common constituents of invading pathogens including double-stranded and single-stranded RNA, unmethylated CpG DNA, bacterial lipopolysaccharide (LPS), lipoproteins, and flagellin [1]. Upon interaction with their ligands, TLRs signal through adapter proteins, most commonly Myeloid Differentiation Primary Response Gene 88 (MyD88), though TLR 3 alternatively signals via the TIR-Domain Containing Adaptor Inducing Interferon-β (TRIF) adapter [2]. These adapters recruit other molecules to initiate signaling cascades ultimately leading to the production of proinflammatory cytokines and type I IFN [3]. These TLR responses are important in the functioning of both the innate and adaptive arms of immunity [4, 5].

Although TLRs are important for antimicrobial immunity, they have also been implicated in the pathogenesis of autoimmune diseases. For example, TLRs 2 and 4 have been identified as factors involved in the onset of type 1 diabetes mellitus [6–9], and TLRs 1–6 are expressed by rheumatoid arthritis (RA) synovial fibroblasts and are thought to provoke joint inflammation in RA [10, 11]. Moreover, the nucleic acid binding TLRs 7 and 9 have been connected to both human and mouse models of systemic lupus erythematosus (SLE) [12–17]. Because of these links between TLRs and autoimmunity, a great deal of work has been directed toward understanding how these receptors act in disease progression. Two major possibilities arise in describing how TLRs might work in autoimmunity; either they are stimulated by exogenous antigens, like viral ssRNA, which then stimulate resident immune cells, or the TLRs recognize endogenous self-antigens to initiate and propagate inflammation and autoimmunity.

The accumulation of evidence pointing towards TLRs in autoimmunity has opened the door for potential therapeutic interventions directed towards the modulation of Toll-like receptors and their signaling pathways. Since TLRs are normally responsive to microbial pathogens, there has been some speculation as to the application of TLR agonists as vaccine adjuvants to stimulate more robust immune responses [18, 19]. On the other side of the spectrum, for autoimmunity, disease can result from aberrant hyperactive signaling; therefore, the application of inhibitory, or
antagonistic, TLR therapeutics is of considerable interest. A few inhibitors have been developed already and show some promise for potential human therapeutics. The focus of this paper is to summarize the present literature documenting how modulation of Toll-like receptor pathways may be used as potential methods of treatment for autoimmunity, particularly SLE.

2. TLR Function and Signaling

TLRs are localized to either the cell surface or endosomes of several cell types, most notably of antigen-presenting cells (APCs) such as dendritic cells [20, 21] and B cells [22]. Under normal circumstances, TLRs aid in the identification and removal of materials that may be detrimental to the host organism; these are usually of bacterial, viral, fungal, or protozoan origin [1, 23]. Because TLRs recognize common molecular motifs instead of specific peptide sequences, they maintain capacity for recognition of a broad repertoire of microbes. We know, with relative certainty, what most of the TLRs recognize and that the end result is inflammation, but how does this occur? The cascade by which TLRs induce an inflammatory environment varies depending on the particular TLR that is stimulated. Since we are more concerned with SLE, we will concentrate on the signaling that takes place upon activation of nucleic acid-binding TLRs 3, 7/8, and 9.

TLRs exist as dimers or heterodimers with the capacity to engage their respective ligands. Ligand binding is thought to induce a conformational change resulting in interaction or close juxtaposition of the two cytosolic Toll/IL-1 receptor (TIR) domains, thus providing an interface for adaptor protein binding and subsequent signal transduction [24]. As described by several groups, MyD88 is the most common of these adaptors and has been shown to be involved in signaling through all TLRs except TLR 3 [24–27]. This adapter protein provides a scaffold for further interaction with IL-1R-associated kinase (IRAK) 1 and 4 to propagate the signal to downstream effectors via phosphorylation. Once recruited to the adaptor protein, IRAK4 is activated. IRAK4, in turn, activates IRAK1 via phosphorylation [28]. These activated kinases recruit tumor necrosis factor receptor-associated factor (TRAF) 6, which is an E3 ubiquitin ligase required for activation of NFκB by freeing it from its inhibitor, IκB [29]. In addition to this, interferon regulatory factors (IRFs) 5 and 7 are also recruited to the MyD88/IRAK/TRAF6 complex, where they can become phosphorylated and activated [30]. Ultimately, the activation of transcription factors NFκB and IRFs 5 and 7 results in their translocation into the nucleus where they initiate gene transcription and production of proinflammatory cytokines and type I IFN (Figure 1) [3, 31–35].

TLR 3 signaling is distinct from the previously described TLR 7 and 9 signaling pathways. Instead of utilizing MyD88 as an adaptor protein, TLR 3 uses TRIF [2]. As with MyD88, TRIF also recruits additional proteins necessary for downstream signaling, including TRAF-family-member-associated NFκB-activator-binding kinase 1 (TBK1), TRAF6, and receptor-interacting protein 1 (RIP1) [24]. TRIF interaction with TBK1 is necessary for the activation of IRF3, which is a transcription factor involved in the production of IFN-β [37]. TLR 3 can also activate NFκB by the interaction of TRIF with TRAF6 or RIP1, which allow the transcription factor to migrate to the nucleus to induce target gene transcription [24, 28].

The multiple proteins involved in TLR signaling yield many opportunities to inhibit this process. Several endogenous inhibitors have been identified that halt, or at least impair, the signaling cascade, dampening the TLR-mediated production of inflammatory cytokines and type I IFN. The ability to modulate TLR signaling helps to maintain a homeostatic balance important for antimicrobial immunity while also preventing collateral damage to self-tissues. The inhibitory proteins important in this process target the receptors themselves, adapter molecules, and key kinases, as well as transcription factors to diminish the TLR-mediated production of inflammatory cytokines and type I IFN. Triad3A acts as an E3 ubiquitin ligase promoting the degradation of TLR 4 and 9. The degradation of these TLRs was shown to impair NFκB activity upon stimulation with LPS and CpG DNA whereas siRNA knockdown of Triad3A enhanced LPS and CpG DNA-mediated NFκB activity [38] indicating a prominent role of Triad3A in modulating TLR response. Although Triad3A appears to act specifically on TLR 4 and 9, other inhibitors provide a more global impact on TLR signaling. As mentioned earlier, MyD88 is an adapter protein downstream of all TLRs except TLR 3. Upon stimulation
with LPS or TNF, a splice-variant of MyD88, MyD88s, can be induced which prevents activation of NFκB [39]. This short form of MyD88 fails to recruit IRAK4, impairing the ability to phosphorylate IRAK1, therefore preventing the activation of NFκB [39], although maintaining the ability to activate the transcription factor AP-1 [40]. In addition, recently, An et al. found that the phosphatase, SHP-1, selectively impairs activation of NFκB and subsequent production of proinflammatory cytokines while also inhibiting IRAK1 resulting in an increase in IFN-γ [41]. Several other factors have inhibitory roles directed to the active IRAK kinases. Kobayashi et al. showed that IRAK-M, which lacks the kinase activity of its counterparts IRAK1 and IRAK4, suppresses the production of proinflammatory cytokines and proposed a model whereby IRAK-M prevents the dissociation of the IRAK1/IRAK4/MyD88 complex to inhibit downstream NFκB activation [42]. Likewise, the splice variant IRAK1c acts in a similar manner by interacting with MyD88 and IRAK4. Like IRAK-M, IRAK1c cannot be phosphorylated by IRAK4 and thereby inhibits the dissociation of the complex, preventing activation of NFκB [43]. Two other inhibitors have been identified downstream of the IRAK kinases: tumor necrosis factor-α-induced protein 3 (TNFAIP3, or A20) and IRAF. A20 is a ubiquitin-editing enzyme that deubiquitinates TRAF6 to inhibit the release of NFκB from IκB, thus inhibiting subsequent NFκB-mediated gene transcription [3, 44]. IRAF inhibits TLR signaling by competing for the same binding site on MyD88 with IRF5 [45]. IRF5 engages MyD88 downstream of TLR stimulation in order to inhibit reductions or alterations in antichromatin antibodies; by IRAF competing with IRF5 for binding, it inhibits the activation of IRF5 thus impairing IRF5-mediated cytokine production.

3. TLRs in Autoimmunity

We know that TLRs are involved in protective immunity to invading microorganisms, but what are the consequences of misregulated TLR activation—meaning situations in which the TLR pathways are turned on too easily or activated by self-stimuli? The work of several groups suggests that in such instances, autoimmunity ensues. The resulting phenotype is characterized by the production of autoantibodies and tissue destruction. TLRs have been identified as instigators in type I diabetes, rheumatoid arthritis, and systemic lupus erythematosus.

The nucleic acid binding TLRs are particularly implicated in SLE, an exceedingly complex and variable disorder. SLE is a systemic autoimmune disease characterized most commonly by antinuclear antibodies (ANAs). This disease is believed to affect at least 5 million individuals worldwide. Modern, effective treatment options are lacking for SLE, in that the primary treatment methods are currently corticosteroids, antimalarial, or anti-inflammatory drugs. There has not been an FDA-approved lupus treatment in over 40 years. The issue with this is the lack of identification of a “common denominator,” so to speak, for all lupus patients, which relates back to the complexity of the disease. However, the relatively new idea that Toll-like receptor pathways play crucial roles in lupus pathogenesis shows promise for potential drug targets.

The role of nucleic acid binding TLR 7 has become quite apparent in both animal models of the disease and human patients. This receptor promotes autoantibodies and cytokines responsible for chronic inflammation [14, 46, 47]. One particularly important animal model at the forefront of these observations has been the BXSB mouse. This model was derived from a cross between C57BL/6 and SB/Le inbred strains, which resulted in male mice expressing an accelerated, lupus-like autoimmune disease phenotype characterized by production of ANAs and circulating immune complexes causing severe glomerulonephritis [48, 49]. Several subsequent studies have shown that the reason for the male bias in these mice was due to an X-to-Y chromosomal translocation of a gene cluster known as Y autoimmune accelerator (Yaa) and that the primary contributor to this accelerated autoimmunity is TLR 7 overexpression [16, 17]. FcγRIIB<sup>−/−</sup> mice have a characteristic lupus-like phenotype with autoantibodies directed towards chromatin. However, FcγRIIB<sup>−/−</sup> mice that also expressed Yaa exhibited a shift in autoantibody specificity from an antichromatin to an antinucleolar ANA pattern, consistent with an observed shift from chromatin to RNA-binding specificities [50]. This work further indicates a role for TLRs, in particular TLR 7, in the development of a specific autoimmune phenotype. Other groups have also described nucleic acid-binding TLR involvement in the production of autoantibodies. In one case in particular, Kono and colleagues ablated TLR 3, 7, and 9 signaling in B6-Fas<sup>−/−</sup> and BXSB mouse models and showed that these mice expressed decreases in autoantibody production [51].

Additional work has shown that defective apoptotic cell clearance is common among SLE patients, and this leads to development of antinuclear antibodies [52–54]. Our laboratory hypothesized that inefficient clearance of apoptotic debris triggers nucleic acid-binding toll-like receptors, which confer the B-cell response and subsequent ANA production. Injection of syngeneic late apoptotic thymocytes into wild type B6 mice led to anti-dsDNA and antihistone antibody production whereas injection into MyD88<sup>−/−</sup> mice had no effect, suggesting that TLR stimulation is important in development of anti-dsDNA antibodies in situations of late apoptotic cell excess. Further studies using TLR 7- and TLR 9-deficient recipients of late apoptotic thymocytes showed that TLR 9 had no bearing on the development of anti-dsDNA and antihistone antibodies in this model, but TLR 7 did [55]. Moreover, the evidence suggested that TLR 7 promoted deposition of immune complexes in the renal glomeruli of these mice, possibly by influencing antichromatin antibody isotype. These studies suggest an important role for TLR 7 in the development of autoreactive antibodies and promotion of early events leading renal pathogenesis.

The DNA-binding TLR 9 has also been heavily studied in connection with murine lupus. TLR 9 deficiency in some lupus models including MRL<sup>1pr/1pr</sup> mice can variably lead to reductions or alterations in antichromatin antibodies; however, TLR 9 deficiency paradoxically leads to disease.
exacerbation in multiple models [13, 14, 56–58]. In contrast, TLR3 deficiency failed to modify disease in MRL\(^{1pr/lpr}\) mice [14].

Considering endogenous self-stimuli, then, murine studies indicate a central pathogenic role for TLR 7 in lupus pathogenesis and a complex overall protective role for TLR 9. Although endogenously triggered TLR 3 does not appear to drive lupus in the murine models investigated thus far, this particular TLR may play important roles in the promotion of lupus by environmental stimuli such as certain viral infections [59].

Most importantly, in addition to studies using mouse models, connections between TLRs and human lupus have also been identified. One of the most striking connections is the presence of elevated IFN-\(\alpha\) as well as a Type I IFN gene signature in lupus patients [60, 61]. IFN-\(\alpha\) is a key player in disease progression and severity and has even been shown to induce the production of autoantibodies when administered to nonautoimmune patients [62]. Another interesting finding was remission of SLE in a patient, which was attributed to unresponsiveness to both TLR 7 and 9 stimulation after development of common variable immunodeficiency- (CVID-) like disease [63]. Several groups have identified SNPs in the TLR 9 gene but have discovered that there is no correlation between these polymorphisms and SLE [64–67]. Although there was no correlation with particular SNPs, other groups showed that there was an upregulation of TLR 9 expression in B cells of lupus patients lending credence to the idea that TLR 9 could be involved in autoantibody production [68–70]. This finding led to the notion that the signaling proteins may be at fault in disease. Recent advances in genetic analysis techniques have allowed for the identification of a large number of genes (more than 25) that are associated with human lupus. At least three of the lupus-associated genes are involved in TLR signaling, including \(\text{IRF5}, \text{IRAK1}\), and \(\text{TNFAIP3}\) [71–74]. The implication of these genes in lupus patients further indicates a role of TLRs in the disease phenotype. Whether enhanced stimulation of the TLRs or genetic factors altering the signaling cascade are at fault, the accumulation of evidence implies that TLR pathways do, in fact, play a role in the pathogenic process of SLE and remain excellent candidates for therapeutic targets to alleviate disease.

Although most studies related to Toll-like receptors in SLE have focused primarily on the nucleic acid-binding TLRs, there is still a potential role for other TLRs in autoimmune disease. Komatsuda et al. looked at mRNA expression of several TLRs in peripheral blood mononuclear cells (PBMCs) from SLE patients. These studies indicated a slight increase in mRNA of TLR 2, 7, and 9 in the lupus patients compared to healthy controls [75]. Additionally, others have reported an important role for TLR 2 and 4 in the production of autoantibodies in the B6\(^{1pr/lpr}\) autoimmune mouse model [76]. In these studies, TLR 2- and TLR 4-deficient B6\(^{1pr/lpr}\) mice expressed lower titers of autoantibodies, excluding those directed towards nucleosome proteins. This shows that other TLRs could be candidates for targeted therapy in autoimmunity as well.

### 4. TLRs as Therapeutic Targets

Significant evidence supports the involvement of TLRs in multiple disease processes, including Type I diabetes, RA, and SLE. The assertion that TLRs play a role in disease pathogenesis, with lupus in particular, indicates potential for therapeutic intervention by targeting these molecules or their signaling pathways. Since there are several proteins involved in TLR signaling, there are a number of targets that may be utilized for potential drugs. Some of the possibilities include, but are not limited to, development of TLR antagonists, inhibitors of downstream signaling events, activation of natural inhibitory molecules, or blockade of the effector molecules produced. Here we will discuss reports that highlight the potential usefulness of these different types of drugs.

The development of TLR antagonists for the nucleic acid-binding TLRs has proven to be a tedious process due to the similarity between eukaryotic and noneukaryotic nucleic acids. Despite this difficulty, Barrat and colleagues have developed immunoregulatory DNA sequences (IRS) that can bind TLR 9 but inhibit its activation and downstream effects. They have shown that these inhibitory ODNs can relieve inflammation in multiple scenarios. In one instance, mice injected with immunostimulatory sequences (ISSs) and D-galactosamine developed severe inflammation and died within days. However, when coinjected with the IRS, inflammation was decreased and mouse survival was prolonged [77]. Similar experiments demonstrated the same effect with TLR 7 as well. In addition to these studies, the same group developed a dual TLR 7/9 inhibitor. This oligodeoxynucleotide (ODN) was sufficient for inhibition of both TLR 7 and 9 signaling and protection against inflammation. These IRS sequences were also demonstrated to inhibit the production of IFN-\(\alpha\) by human plasmacytoid dendritic cells (pDC), indicating the effectiveness of these inhibitors in human cells [78]. Because of the effectiveness in inhibiting TLR function, Barrat et al. also investigated the capacity of their ODNs to treat a lupus-prone mouse model. Several studies had previously established a potential role of IFN-\(\alpha\) in the progression of autoimmunity in (NZB × NZW) F1 mice [79, 80]. Since IFN-\(\alpha\) appears to play a central role in human SLE [81], this strain was selected as an ideal model for the application of IRS. IRS injections twice weekly in (NZB × NZW) F1 mice resulted in decreased ANAs, reduced glomerulonephritis at nine months, and increased rate of survival among the treated mice compared to untreated controls [82].

In addition to the studies by Barrat, two other groups used other inhibitory ODNs for targeting TLR signaling in autoimmunity. The ODNs designed by Dong et al. were injected into (NZB × NZW) F1 mice that were subsequently analyzed for kidney function and autoantibody production characteristic of lupus. Their results suggested a similar capacity of these inhibitory ODNs to minimize glomerulonephritis and reduce autoantibodies directed towards DNA [83]. Pawar et al. used IRS sequences as TLR 7 and 9 inhibitors in MRL\(^{1pr/lpr}\) mice and showed a reduction in inflammatory cytokine levels and autoantibody titers, as...
well as a decrease in resulting tissue damage [84]. These experiments using inhibitory ODNs in treating lupus-prone animal models suggest strong potential for treatment of human lupus and other autoimmune diseases by targeting TLRs.

Other potential therapeutic targets include the downstream signaling proteins involved in the TLR signaling cascade. There are a number of potential targets for this intervention including MyD88, TRAF6, and IRAK1 and 4. MyD88 is the common adaptor protein in most TLR signaling [85]. Since both TLR 7 and 9 utilize this protein, it is an excellent target to decrease the signaling that takes place in lupus. Two independent groups have shown that inhibiting MyD88 impairs the phosphorylation/activation of downstream kinases [86] and NFκB activity [87]. Bartfai and colleagues developed a chemical mimic to the three amino acid sequence at the conserved BB-loop of the TIR domain of MyD88. The results of this mimic showed an inhibition of MyD88 and 1-IRI interaction by ablating phosphorylation of MAP kinases [86]. Loiarro et al. describe a similar mimic; however, they employ a peptide sequence instead of a chemical mimic. This inhibitor showed similar inhibition of MyD88 signaling indicated by impaired NFκB activity [87]. They also went on to show that not only do these peptides inhibit dimerization of MyD88, but also inhibit the recruitment of IRAK1 and IRAK4 [88]. In view of the fact that MyD88 is a central mediator in TLR 7 and 9 signaling, these inhibitors of MyD88 dimerization and recruitment of essential kinases could prove to be an effective treatment option in lupus by abolishing the aberrant TLR signaling that induces the elevated type I IFN levels and persistent inflammation.

Another potential target for minimizing damaging TLR signaling is IRAK4. Briefly, IRAK4 is a kinase that interacts with MyD88 and TRAF6 and is associated with the activation of the downstream transcription factors in TLR signaling. Based on IRAK4-deficient animal models, this kinase has been shown to be indispensable in TLR signaling [89, 90]. Because of this idea that IRAK4 is necessary for production of effectors in a TLR-dependent manner, it has been subjected to targeted therapy. However, IRAK4 inhibitors developed to date have yet to be analyzed for efficacy in animal models [91–94].

The last actively studied therapeutic approach we will discuss targets the end-product effectors of TLR signaling as opposed to the signaling cascade itself. An important target that is now accepted to be characteristic of SLE is IFN-α. This cytokine is found in elevated levels among lupus patients, and high levels associate with worsened measures of disease activity [81]. Due to the apparent pathogenic nature of IFN-α, inhibitory monoclonal antibodies have been developed against the cytokine as a treatment for SLE [95]. One such drug is currently in phase II clinical trials and has shown promise thus far in relieving symptoms of lupus [96]. Among the TLR and TLR-related targets for therapy in lupus, this antibody directed towards IFN-α appears to be the most outstanding thus far.

Currently, active research on this topic is concerned with negatively regulating molecules that activate TLR-dependent signaling, but what about activating some of the natural inhibitory proteins, such as Triad3A, MyD88s, SHP-1, IRAK-M, IRAK1c, IRF4, and A20? These naturally occurring inhibitors of the signaling cascade could be targeted for agonistic drugs. Xie et al. reported that IRAK-M-deficient mice injected with tumor cells develop increased CD4+ T-cell differentiation and upregulation of costimulatory molecules and other activation markers on B cells, compared to wild-type controls, indicating an inhibitory role for IRAK-M [97]. Although IRAK-M deficiency appears to have a positive role in cancer models, the inverse is likely to be true for autoimmune situations. Stimulation of production or activity of IRAK-M may provide another alternative therapy in SLE. Similarly, promoting the production or activity of the other endogenous TLR signaling inhibitors, Triad3A, MyD88s, SHP-1, IRAK1c, IRF4, or A20, could provide additional options for effective treatment for systemic autoimmunity. Utilizing these endogenous proteins could provide a new avenue to decrease TLR-mediated inflammation in patients with autoimmune diseases, with each of these targets allowing a different route to decreasing production of inflammatory cytokines and subsequent risk of tissue damage.

Although the approaches presented here are in the context of nucleic acid-binding TLRs, the proposed strategies for TLR signaling inhibition could actually act more globally. Since most TLRs can signal through the adaptor protein MyD88, these potential therapeutic targets could prevent signaling through all TLRs with the exception of TLR 3. The specific TLR 7 and TLR 9 inhibitors discussed earlier would be ideal as a treatment method to avoid a total inhibition of the first line of microbial defense; however, with evidence revealing potential roles for other Toll-like receptors, namely, TLR 2 and 4, in SLE, a more widespread inhibition may be an important aspect worth investigating.

5. Conclusions

Although TLRs are extraordinarily important in pathogen recognition and normal immune function, including that of both innate and adaptive arms of the immune system, they have also been ascribed causative roles in autoimmunity. Various studies ranging from lupus-prone mouse models, to genome-wide association studies in human lupus patients, to in vitro studies with patient cells have indicated a significant role for nucleic acid-binding TLRs in the progression and severity of lupus and other autoimmune diseases. From these studies, we have learned about the involvement of TLRs in lupus-prone mouse models, the association of polymorphisms in IRF5, IRAK1, and TNFAIP3 with human disease, and the upregulation of TLRs in SLE patients. Due to the accumulation of these data suggesting a detrimental role of TLR signaling in lupus, efforts are currently underway to ascertain reliable treatment methods based on the targeting of TLRs and their signaling counterparts. Inhibition of TLRs and their signaling pathways have proven to be efficacious in lupus-prone mouse models and successfully inhibit IFN-α production by human pDC in vitro. Although the direct
TLR antagonists have not been studied in human patients, inhibitors of IFN-α, a primary downstream effector of TLR signaling and important disease mediator in SLE, have been developed and are currently undergoing clinical trials. Monoclonal antibody treatments have been successfully utilized in the setting of rheumatic diseases for some time now and are likely to comprise a new armament in the realm of lupus treatment. Although there has not been a new drug approved for the treatment of lupus in many years, current investigation regarding the targeting of TLRs and their downstream effectors is showing some promise and warrants high expectations.

References


Review Article

Classification, Mechanisms of Action, and Therapeutic Applications of Inhibitory Oligonucleotides for Toll-Like Receptors (TLR) 7 and 9

Petar S. Lenert

Department of Internal Medicine, Division of Rheumatology, Carver College of Medicine, The University of Iowa, 200 Hawkins Drive, Iowa City, Iowa 52242, USA

Correspondence should be addressed to Petar S. Lenert, petar-lenert@uiowa.edu

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Our immune defense depends on two specialized armed forces. The innate force acts as an alarm mechanism that senses changes in the microenvironment through the recognition of common microbial patterns by Toll-like receptors (TLR) and NOD proteins. It rapidly generates an inflammatory response aimed at neutralizing the intruder at the mucosal checkpoint. The innate arm also communicates this message with more specialized adaptive forces represented by pathogen-specific B cells and T cells. Interestingly, B cells also express some innate sensors, like TLR7 and TLR9, and may respond to bacterial hypomethylated CpG motifs and single-stranded RNA viruses. Intracellular nucleic acid sensing TLRs play an important role in the pathogenesis of Systemic Lupus Erythematosus (SLE). In this review, we describe recent achievements in the development of oligonucleotide—(ODN)-based inhibitors of TLR9 and/or TLR7 signaling. We categorize these novel therapeutics into Classes G, R, and B based on their cellular and molecular targets. Several short ODNs have already shown promise as pathway-specific therapeutics for animal lupus. We envision their future use in human SLE, microbial DNA-dependent sepsis, and in other autoinflammatory diseases.

1. Overview

In this review, we present multiple lines of evidence that short oligonucleotides (ODN) containing stretches of 3–5 guanine nucleotides may act as TLR9-specific antagonists. We define their optimal sequence requirements, discuss the importance of secondary structures, present evidence of their efficacy in animal models of lupus and sepsis in vivo, and offer a new classification based on their mechanisms of action and cellular selectivity. We further discuss the ability of phosphorothioate-modified ODNs to act as TLR7 antagonists.

2. Toll-Like Receptor 9 as an Immune Sensor of Unmethylated CpG-DNA

Cells of our innate immune system can be activated by bacterial DNA, but not by our own DNA [1]. When unmethylated CpG sequences flanked with two purines at the 5’ end and with two pyrimidines at the 3’ end (so-called CpG motif) were found to be necessary for bacterial DNA-induced immune activation [2–5], the whole field of oligonucleotide research exploded culminating in the discovery of the TLR9 as a receptor responsible for CpG-ODN (and bacterial DNA) action [6, 7]. This effect was recently found to be heavily dependent on DNA sugar backbone recognition by TLR9 [8]. Even though additional DNA recognition molecules and TLR9-independent pathways were recently discovered [9–15], TLR9 itself appears to be both necessary and sufficient for observed immunostimulatory effect of CpG-containing ODNs (reviewed in [3]). Interestingly, TLR9 has relatively limited distribution and in humans is found exclusively in Type I interferon-producing plasmacytoid dendritic cells and in B cells [16]. In mice, macrophages and myeloid dendritic cells also express high levels of TLR9 and respond to CpG-ODN stimulation [17, 18].

Toll-like receptors, including TLR9, warn us of the presence of infection, and the ligand-receptor interaction
mobilizes cellular resources to promote an early inflammatory response and to initiate robust adaptive immune response. For example, TLR9-activated B cells enter cell cycle and proliferate, upregulate cell-surface molecules involved in antigen presentation/collaboration with cognate T cells (e.g., CD40, MHC Class II and CD86), and secrete multiple chemokines and proinflammatory cytokines (e.g., IL-6 and TNF-α) ([19], reviewed in [3]). B cells also secrete polyclonal IgM and IgG3 [2, 20] and with the T cell help can undergo class switching to other Ig isotypes. Once the immediate danger is neutralized, certain TLR(9)-primed B cells and dendritic cells start making regulatory cytokines, such as IL-10 and TGF-β ([20, 21] and Lenert et al., unpublished observation) limiting the ongoing inflammation [21]. In dendritic cells, TLR9 (and TLR7) activation induces among others high levels of type I IFN [22], a cytokine heavily implicated in the pathogenesis of Systemic Lupus Erythematosus and Sjögren’s syndrome [23–26]. Thus, innate activation through TLRs stands at the cross-roads between innate and adaptive immunity, and if left unchecked may cause chronic immune stimulation and autoimmunity. For example, expansion of transgenic rheumatoid factor-specific B cells in lupus-prone MRL-Fas<sup>lpr/lpr</sup> mice is directly dependent on MyD88/TLR expression, but not on T cells [27]. However, the role of TLR9 in the pathogenesis of lupus in this strain of mice remains controversial as some reports suggest that TLR9 may be actually protective rather than pathogenic via induction of regulatory T cells [28, 29].

In contrast to the LPS receptor TLR4/MD2, TLR9 is not localized on the cell surface but signals from an interior compartment as first discovered by Wagner’s group [30, 31]. In concord with this observation, CpG-ODN— but not LPS-induced intracellular signaling is sensitive to inhibitors of endosomal acidification (e.g., chloroquine) [32]. Cationic peptides such as LL-37 or polymixin may facilitate the uptake of CpG-DNA (including self-DNA) into early endosomes [33]. Once CpG-ODN enters cells, TLR9 undergoes relocation from endoplasmic reticulum to CpG-ODN-containing endosomes [34]. This travel requires a help from the UNC93b1 shuttle protein [35, 36], as mice having a mutation in UNC93b1 fail to respond to intracellular TLR ligands (TLR3, 7 and 9) [37]. After reaching endosomes, TLR9 undergoes its final proteolytic cleavage into a functional receptor [38, 39]. TLR9 exists as a preformed homodimer and CpG-ODN binding promotes its conformational change, bringing the cytoplasmic TIR-like domains close to each other [40]. This allows a recruitment of the key adapter protein MyD88 which initiates a signaling cascade. Following further recruitment of IRAK1/TRAF6 [41, 42], two major signaling pathways are initiated: first through the MAPK/SAPK pathway resulting in AP1 nuclear translocation and second causing NF-κB activation [30, 42, 43], reviewed in [3, 44]. In IFN-α producing cells, PI3K, IRF5, and IRF7 are also implicated in CpG-ODN-induced cellular activation [45, 46]. Once these transcription factors bind to their DNA targets, rapid induction of early inflammatory and survival genes follows.

3. Discovery of TLR9 Inhibitors

During the course of experiments designed to understand what makes bacterial DNA, but not mammalian DNA, immunostimulatory [1, 4], Pisetsky’s group discovered that synthetic oligonucleotides containing poly-G sequences could block bacterial DNA-induced activation [47, 48]. The inhibition was seen at relatively high micromolar concentrations and required that inhibitors were made with the nuclease-resistant phosphorothioate (PS) backbone instead of the natural phosphodiester (PO) backbone. However, these effects were not specific for bacterial DNA-induced activation, as these ODNs could also block other forms of immune stimulation [49]. Others have observed that Poly G-ODNs could suppress tumor cell growth with an IC50 at 7 micromoles. The inhibition was due to direct binding of Poly-G ODNs to STAT3 preventing its nuclear translocation and interaction with target DNA sequences [50, 51]. As a consequence, the level of survival genes Bcl-2 and Bcl-XL dropped, promoting apoptotic cell death. Thus, poly-G ODNs may represent a new class of chemotherapeutic agents capable of blocking immune activation nonspecifically and promoting apoptotic cell death in tumor cells.

Envisioning application of TLR9 ligands as potential vaccine adjuvants and boosters of antitumor immunity, Krieg’s group noticed that certain CpG sequences, like CCGG and methylated CG sequences [52, 53], were not only non-stimulatory, but inhibitory when added to bacterial DNA-stimulated cultures [53]. These inhibitory CpG-sequences were overexpressed in certain strains of adenoviruses (e.g., serotype 2, but not serotype 12) [52]. Therefore, a concept of neutralizing or suppressive CpG-sequences was born [52].

Our contribution to the field was to clarify exact sequence requirements for TLR9 inhibition by inhibitory oligonucleotides in mouse and human settings and to study their mechanism of action both in vitro and in vivo. Contribution from other groups will also be mentioned and a modified classification of INH-ODNs will be presented.

We decoded sequence requirements for inhibitory ODN action in TLR9-activated cells by systematically altering the shortest active 15-mer stimulatory CpG-ODN 2084 (TCCT GACGGT GAAGT) by mutating one or two nucleotides at the time [54, 55]. A CpG to GpC flip created a 100-fold less potent TLR9 agonist compared to the parental molecule; however, the resulting ODN had no TLR9 inhibitory activity by itself. Interestingly, a simple switch from CpG to GpC created an ODN that was capable of blocking both experimental autoimmune encephalomyelitis and spontaneous lupus in mice, as shown by Ho et al. [56, 57]. However, a similar ODN failed to specifically block TLR9-induced stimulation in mouse B cells [55]. Exchanging pyrimidines for guanine nucleotides at the 3′ flank of the CpG motif completely abrogated the stimulatory activity of the prototypic CpG-ODN, creating an inhibitor of the TLR9 signaling. On the other hand, a 5′ flank change from GACGGT to GCGGT was tolerated quite well in regard to the stimulatory activity. However,
when these 3 substitutions were combined in a single ODN, a powerful TLR9 inhibitor—CpG-ODN 2088 (TCCTGGAGGGAAGT) was generated [58, 59]. CpG-ODN-2088 was not only unable to induce TLR9-dependent activation by itself, but could block TLR9-ligand-induced activation at very low nanomolar concentrations. All TLR9-induced biologic outcomes were completely inhibited not only in mouse B cells [59], but also in macrophages and dendritic cells [54]. There was a potency difference of 100–1000-fold between control PS-ODNs and INH-ODNs [59]. At the signaling level, the earliest steps in NF-κB [58] and SAPK/MAPK/AP1 activation were promptly and equally inhibited [60] suggesting a proximal mechanism of INH-ODN action, possibly at the level of TLR9 receptor itself [61].

Further mapping studies in mouse B and non-B cells [54, 55] have established the following rules for TLR9-inhibition: (1) CpG motif, either methylated or unmethylated, is not required for inhibition; (2) three consecutive G nucleotides are necessary for inhibition; (3) 5′ end of an ODN is important for both stimulation and inhibition; TCC is optimal for stimulation, while CC(T) triplet is required for optimal inhibition; (4) the distance between the 5′ CC(T) and downstream GGG triplet should optimally be 3–5 nucleotides long; (5) the order of 5′ CC(T) → GGG-3′ is critical as ODNs with the reverse order, for example, 5′GGG → CC(T)-3′, or with the reverse sequence are non-inhibitory; (6) the intervening sequence between the CC(T) and GGG elements contributes minimally to the overall ODN activity and can accept multiple modifications. (7) At the 3′ end of an INH-ODN, it is not the primary sequence but the length that contributes to the activity; (8) specific inhibition of TLR9-induced activation does not require intrachain and/or interchain Hoogsten hydrogen bonding between adjacent Gs, as deaza-substituted ODNs [59] and linear INH-ODNs incapable of making these bonds are equally effective TLR9 inhibitors [62].

ODNs containing the canonical mouse inhibitory motif for TLR9 (e.g., 2088, 2114, and 4024) were also active in human B cells, B cell lines, pDCs [63, 64], and in TLR9-transfected HEK cells [65]. However, extending INH-ODNs for 4-5 bases at the 5′ end significantly enhanced their activity for human cells. The activity in human cells does not depend on the ability of INH-ODN to either self-aggregate or directly bind to a stimulatory ODN. As in mouse TLR9-expressing cells, primary base sequence and backbone determine INH-ODN activity [65]. Even though TLR9 binds INH-ODNs as well as CpG-ODNs, the affinity for TLR9 does not correlate with the biologic activity (Ashman and Lenert, submitted for publication).

Indeed, recent studies have shown that the sugar backbone 2-deoxyribose determines DNA recognition by TLR9, and base-free deoxyribose homopolymers may act as TLR9 agonists [66]. Phosphorothioate-modified deoxyribose has much higher affinity for both TLR7 and 9 compared to PO-deoxyribose, transforming these molecules into TLR7 and TLR9 agonists [66]. Therefore, we hypothesize that some other molecule, not TLR9, must mediate sequence-specific recognition of INH-ODNs (unpublished data and [65]).

4. Concept of Class R and Class B INH-ODNs: Less Is Sometimes More

For our mapping studies, we initially used INH-ODN-2114 with the following sequence: 5′TCCTGGAGGGAAGT-3′ ([59]; Table 1). This ODN is a very potent TLR9-antagonist in both human and mouse settings in vitro [54, 59, 65] as well as in the MRL-Fas/br/br strain of lupus in vivo [67]. The PO variant of this ODN is active against PO-CpG-ODNs and bacterial DNA in B cells and mouse macrophages [68]. As PS ODNs bind to TLR9 with much higher affinity than PO-ODNs [69, 70], not surprisingly, PO-INH-ODN 2114 is at least 100-fold less potent against PS-CpG-ligands. Interestingly, a genomics search has shown that the optimal inhibitory sequence is severalfold more prevalent in mammalian DNA compared to bacterial E.coli DNA, suggesting a physiologic relevance of these findings [68]. Our INH-ODNs and similar ODNs developed by Stunz et al., Barrat et al., and Peter et al. have no inhibitory activity on TLRs 2, 3, 4, 5, and BCR-induced activation when used at concentrations up to 1 micromol [59, 71, 72]. The effects on TLR7/8 will be discussed below.

Since INH-ODN-2114 can make (some) G4-stacks as poly-G ODNs [73] (Figure 1), the interpretation of these results may be complicated by nonspecific effects of G4 stacks on immune activation. As a matter of fact, TLR9-independent effects of ODN-2114 were seen in the model of intracellular S. typhimurium infection in TLR9-deficient bone marrow derived macrophages [74]. Therefore, in order to avoid any contribution from G4 aggregates to TLR9 inhibition, we created INH-ODN 4024 (TCCTGGAGGGAAGT) [63]. This ODN contains both CC(T) and GGG triplets and is as potent as INH-ODNs 2088 and 2114 for CpG-ODN-stimulated mouse B cells and macrophages [55, 63]. Further truncation of ODN-4024 resulted in the shortest active 12-mer INH-ODN 4084-F with the sequence 5′CCTGGAGGGAAGT-3′ [62].

In order to better understand the role of secondary structures, for example, ability to make DNA duplexes or hairpins, we used INH-ODN 4084F as a template. We created 24 mer-ODNs in which the 4084F sequence was either at the 5′ or the 3′ end of the molecule, and was followed (or preceded) by 12 nucleotides complementary to the 4084F, making a complete palindrom (Table 1, INH-1, INH-4) [62]. We named these new TLR9-antagonists: Class R INH-ODNs (where “R” stands for restricted activity, [75]) as they showed similar inhibitory potency for TLR9-activated IFN-α producing dendritic cells (and macrophages/macrophage cell lines) as their linear analogues (Class B, broadly-active, Table 1, INH-18, INH-13), but were between 10–30-fold less active in resting mouse splenic (follicular) B cells irrespective of the outcome tested. These ODNs were also less potent in human peripheral blood B cells and in B cell lines [62]. Interestingly, even bigger potency difference (∼100 fold) was observed when these ODNs were made with the natural phosphodiester backbone [62]. Similar to complete palindromes, ODNs having short 5′ or 3′ overhangs (up to 6 nucleotides long) were less active in B cells when compared to their linear analogues [62]. The difference in activity
Table 1: Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequence</th>
<th>Class</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2088</td>
<td>TCCTGGCGGGGAAGT</td>
<td>B/G</td>
<td>[58, 59]</td>
</tr>
<tr>
<td>2114</td>
<td>TCCTGGAGGGGAAGT</td>
<td>B/G</td>
<td>[58, 59]</td>
</tr>
<tr>
<td>4024</td>
<td>TCCTGGATGGGAAGT</td>
<td>B</td>
<td>[54, 55]</td>
</tr>
<tr>
<td>4084F</td>
<td>CCTGGATGGGAA</td>
<td>B</td>
<td>[62]</td>
</tr>
<tr>
<td></td>
<td>G4-stacks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>INH-1</td>
<td>CCTGGATGGGAATTCCATCCAGG</td>
<td>R</td>
<td>[62]</td>
</tr>
<tr>
<td>INH-4</td>
<td>TTTCCATCCAGGCTTGAGGGA</td>
<td>R</td>
<td>[62]</td>
</tr>
<tr>
<td>INH-13</td>
<td>CTTACGCGTCGACCTGGATGGGA</td>
<td>B</td>
<td>[62]</td>
</tr>
<tr>
<td>INH-18</td>
<td>CTTACGCGTCGACCTGGATGGGA</td>
<td>B</td>
<td>[62]</td>
</tr>
<tr>
<td>Poly-G</td>
<td>GGGGGGGGGGGGGGGGGGGGGGGG</td>
<td>G</td>
<td>[47]</td>
</tr>
<tr>
<td>A151 (telomeric)</td>
<td>TTAGGTTAGGGTTAGGGTTAGGG</td>
<td>G</td>
<td>[61]</td>
</tr>
<tr>
<td>GpG</td>
<td>TGACTGTGAGGTAGAGATGA</td>
<td>B/G</td>
<td>[72]</td>
</tr>
<tr>
<td>G-ODN</td>
<td>CTCCTATTGGGGGTTCCTAT</td>
<td>B/G</td>
<td>[72]</td>
</tr>
<tr>
<td>IRS-869</td>
<td>TCCCTGGAGGGTTTGT</td>
<td>B/G</td>
<td>[64]</td>
</tr>
<tr>
<td>IRS-661</td>
<td>TGCTTGCAAGCTTGCAAGCA</td>
<td>R/TLR7 specific</td>
<td>[71]</td>
</tr>
<tr>
<td>IRS-954</td>
<td>TGCTCTGGAGGGTTTGT</td>
<td>B/TLR7/9 specific</td>
<td>[71]</td>
</tr>
</tbody>
</table>

![Figure 1](image-url)  
**Figure 1:** Class B INH-ODN 2114 can undergo G4-stacking in the presence of potassium ions. INH-ODN 2114 (1 μg) was dissolved in a buffer containing potassium ions at 65 degrees for 10 minutes and then slowly cooled down to room temperature for 2 hours. For comparison, the same ODN was dissolved in Tris-EDTA, boiled for 10 minutes and rapidly cooled on ice. Electrophoretic mobility on 20% native PAGE gel is shown. Gel was stained with Stains All overnight.

between Class R and B INH-ODNs in B cells could not be explained by differences in the uptake, but could depend on the ability of these ODNs to reach different TLR9-expressing compartments, for example, early versus late endosomes [62, 76–78]. We hypothesized, that in B cells, Class R INH-ODNs, similar to mammalian DNA, may have restricted access to late endolysosomes. Interestingly, similar to differences between Class R and B INH-ODNs, human naïve B cells and resting mouse follicular B cells poorly respond to complex TLR9 agonists, for example, double-stranded bacterial DNA and type A(D) CpG-ODNs which have a palindromic center and G-rich tails [20, 79–81]. Since the signal through the B cell receptor for antigen allows B cells to respond to a wider range of TLR9 ligands including complex TLR9-agonists [78, 82–86], we wondered whether the same principal holds true for Class R TLR9-antagonists. We studied this hypothesis in both autoimmune and nonautoimmune settings. We used autoreactive rheumatoid factor-specific AM14 B cells as a model for BCR/TLR9 cross talk [87]. AM14 B cells proliferate upon recognition of DNA/or RNA containing-immune complexes by their B cell receptor for antigen only if co-stimulated through the TLR7 or TLR9 [88, 89]. When AM14 B cells were stimulated with linear CpG-ODN ligands (e.g., with CpG-ODN 1826), similar to non-autoreactive B cells, Class R INH-ODNs were at least 10-fold less potent inhibitors compared to Class B INH-ODNs [62]. However, when DNA-containing immune complexes were used for stimulation, the potency of Class R INH-ODNs increased for at least 10-fold equalizing that of Class B INH-ODNs [62]. When AM14 B cells were stimulated with linear CpG-ODN ligands (e.g., with CpG-ODN 1826), similar to non-autoreactive B cells, Class R INH-ODNs were at least 10-fold less potent inhibitors compared to Class B INH-ODNs [62]. However, when DNA-containing immune complexes were used for stimulation, the potency of Class R INH-ODNs increased for at least 10-fold equalizing that of Class B INH-ODNs [62]. Since in AM14 B cells, INH-ODNs fail to inhibit signaling through the BCR, or by LPS, we concluded that the increased potency of Class R INH-ODNs for BCR/TLR9 coactivated autoreactive B cells could be advantageous for selective targeting of autoimmune B cells in lupus. Indeed, contrary to our expectations, our in vivo studies in the MRL-Fas<sup>−/−</sup> strain showed that potent linear TLR9-specific antagonist (Class B INH-18) was surprisingly ineffective while treatment with palindromic Class R INH-1 resulted in improved survival and less renal pathology [62]. Furthermore, levels of anti-dsDNA and anti-Sm/RNP antibodies were significantly reduced and abnormal lymphoproliferation was halted. These results could be explained by the fact that TLR9 may have some protective, rather than pathogenic, effects in the MRL-Fas<sup>−/−</sup> strain of lupus mice. TLR9 may be critical for the induction of regulatory T cells in this strain as hypothesized by Wu and Peng [29]. Moreover, the principal cytokine involved in the pathogenesis of lupus in this strain appears to
be IFN-γ, not IFN-α, as MRL-Fas\(^{lpr/lpr}\) mice deficient in IFN-α receptor have more severe disease [90–92]. Other explanations are also possible, including selective effects of palindromic INH-ODNs on TLR7 activation, as TLR7 plays a well-proven role in the pathogenesis of MRL-Fas\(^{lpr/lpr}\) lupus [28].

5. Telomeric TTAGGG Repeats as Immune Modulating Agents

Oligonucleotides containing repetitive TTAGGG motifs were developed by Klinman’s group and were shown to have multiple effects on immune activation [61]. TTAGGG repeats are found in telomeric ends and physiologically protect mammalian chromosomes from degradation [93]. It appears that when our own DNA is released from cells, these telomeric regions are responsible for inhibitory effects of mammalian DNA [53, 61]. Indeed, DNA from telomerase-deficient mice is much less suppressive than the control DNA (reviewed in [94]). Synthetic ODNs containing TTAGGG repeats were capable of blocking the production of proinflammatory and TH1 cytokines induced not only with TLR9 ligands, but also with a variety of polyclonal activators and antigens [61, 94, 95]. For example, they were active against double-stranded RNA, peptidoglycan, and even against lipopolysaccharide (LPS) when IFN-γ production was measured as an outcome (reviewed in [94]). Interestingly, others have shown that similar G-rich ODNs can bind IFN-γ directly and act as aptamers [96]. In vivo, these ODNs showed a remarkable potential to prevent pathology in animal models of inflammatory arthritis induced by intra-articular injection of CpG-ODNs [97], spontaneous SLE in NZB/W mice [98], experimental uveitis [94], acute silicosis [99], and LPS-induced toxic shock [100]. Interestingly, while TTAGGG-ODNs were capable of preventing the development of nephritis in NZB/W mice, treatment of animals with established lupus nephritis did not stop the progression of the disease [98]. Authors concluded that these ODNs may be promising agents for treatment of a variety of autoimmune and inflammatory diseases, particularly when administered early in the course of the disease [94].

While the mechanism of action of these ODNs is incompletely understood, immunosuppressive ability of these ODNs was found to be heavily dependent on their ability to make complex structures, for example, G4-stacks. TTAGGG motifs may act, at least in part, by selectively binding to STAT1 and STAT4 and by blocking their subsequent phosphorylation [95, 100]. Interestingly, we were not able to observe any (inhibitory) effects of linear (non-G4-stack forming) Class B INH-ODNs on STAT signaling suggesting that different classes of INH-ODNs may act through different signaling pathways (data not shown). Others have shown that G-rich ODNs, similar to TTAGGG repeats, can also target another member of the STAT family, a STAT3 oncogene, with an IC(50) of 7 micromol [50, 51]. Other cellular targets of G-rich ODNs have also been identified, including scavenger receptors [73], nucleolin [101], and interestingly, a lupus autoantigen-Ku [102].

6. Combined TLR7/TLR9 Antagonists

Barrat-Coffman’s group at Dynavax used our INH-ODN 2114 [54, 59] as a template for creating novel TLR9 inhibitors, such as IRS 869 (TCTTGAGGGGTGTGT). They studied their effects in human and mouse B cells and in IFN-α-producing plasmacytoid dendritic cells [64]. Noticeably, the ODN variant they used (IRS 869) differed from INH-ODN 2114 only by two A → T substitutions at the 3’ end where the number of nucleotides, but not the primary sequence, matters [54, 55]. They found that 4 contiguous G residues were essential for TLR9 inhibition [64]. Since IRS 869 could make G4-stacks, they also studied the contribution of primary sequence versus G4-aggregates to the inhibitory activity. Similar to our results, they observed that linear sequence, not the G-aggregate, was responsible for TLR9 inhibition in human B cells [64]. They further showed that these ODNs were efficacious in vivo in a model of d-galactosamine + CpG-ODN-induced sepsis. INH-ODNs prevented massive systemic inflammation and cytokine release responsible for sepsis in this model [64]. This effect was confirmed in a recent study by Plitas et al. in a model of polymicrobial sepsis [103].

Barrat’s group subsequently developed short INH-ODNs that preferentially block TLR7-induced innate activation. The prototypic TLR7 antagonist IRS 661 contained 5 GC motifs equally spaced within the complete palindrome [104]. This ODN specifically blocked small TLR7/8 agonist (R848)-induced splenocyte IL-6 secretion, but was ineffective against TLR9-ligand induced activation. In their hands, TLR9-specific antagonists (e.g., IRS 869) failed to block TLR7 (R848)-dependent activation. The same group also developed IRS 954 (TGCTCCTGGAGGGGTGT) which was capable of simultaneously blocking both TLR7- and TLR9-dependent activations. Combined TLR7 and TLR9 inhibitors suppressed IFN-α induction by either ultraviolet-light irradiated HSV (DNA), inactivated influenza virus (ss RNA virus), or by RNA-containing immune-complexes. IRS 954 also slowed down the progression of spontaneous lupus in the NZB/W-F1 strain of lupus mice and reduced the production of multiple autoantibodies (e.g., anti-dsDNA, antinucleosome, anti-Sm, and anti-RNP antibodies) [104]. Interestingly, the control ODN used in their study lacked the TLR9 motif but contained the TGC motif which was buried in the interior of the molecule. Since this control ODN was apparently ineffective, one may wonder which pathway (TLR7 or 9?) was a primary target of INH-ODNs in this model [104]. Interestingly, NZB/W-F1 mice, similar to SLE patients, constitutively express high levels of IFN-α regulated genes. Moreover, treatment with IFN-α accelerates disease, while mice deficient in the IFN-α receptor develop less severe disease with delayed onset [105, 106].

In contrast to the results from Barrat’s group, several groups, including our own, have shown that PS ODNs including INH-ODNs (but not PO INH-ODNs) have backbone-dependent and sequence-independent effects on TLR7 activation induced by either RNA-containing immune complexes, or by small TLR7 agonists like R837 and CL075 [57, 62, 89, 107–109]. However, in contrast to RNA-immune...
complex-induced activation of pDCs, R848-induced B cell activation is relatively difficult to inhibit with TLR9-specific antagonists, but remains sensitive to TGC-containing ODNs, clarifying this controversy (Lenert, unpublished data).

7. Proposed Mechanisms of INH-ODN Action

Diversity of published sequences for TLR9 inhibition suggests a possibility of different sites and mechanisms responsible for their inhibitory action. For example, INH-ODNs may act as nonsequence specific competitors for receptor-mediated endocytosis or phagocytosis. This effect may depend on a cell type, presence of scavenger receptors (e.g., CXCL16, SR-A, CD36, MARCO) and on the overall length of INH-ODNs, as well as on their ability to make G4 stacks [73, 110, 111]. In general, longer and G-rich ODNs are better taken up by macrophages than shorter ODNs. The opposite is true in B cells [111]. A second possibility is inhibition of TLR9-trafficking or TLR9-processing into functionally active product [38]. A third mechanism may involve competitive antagonism at the level of TLR9-expressing endosome. For example, INH-ODNs may bind TLR9 and prevent it from undergoing a conformational change critical for recruiting MyD88 [40]. Further mechanism may include inhibition of endolysosomal acidification (similar to chloroquine action) or pharmacologic inhibition of various proteases, for example, cathepsins [112] or asparaginyl endopeptidases. Recently discovered cysteine protease asparaginyl endopeptidase is important for TLR9 processing in DCs, but not in macrophages [39]. There is also a possibility that certain INH-ODNs may work downstream of the TLR9 (and TLR7) for example, at the level of STATs 1, 3, and 4. They can additionally block stimulation of other immune cells, for example, T cells, nonspecifically. Table 2 depicts the most important characteristics of these three categories of TLR9 antagonists.

9. Conclusions

At least three different classes of INH-ODNs have recently been developed. While all these ODNs can block TLR9-dependent activation, and exhibit backbone-dependent effects on TLR7 stimulation, depending on their size and ability to make G4-stacks, they may have additional cellular targets. For example, telomeric TTAGGG repeats and poly-G ODNs can be classified as Class G INH-ODNs. Compared to other classes they are relatively TLR9-nonspecific. They can block phosphorylation and nuclear translocation of multiple members of the STAT family, for example, STAT 1, 3, and 4. They can additionally interact with scavenger receptors on macrophages, Ku-autoantigen, and with nucleolin. They showed potent immune-modulatory effects in animal models of lupus in the NZB/W-F1 strain [98], and in various experimental models of arthritis, sepsis, uveitis, and silicosis (reviewed in [94]). Because of their cellular and target promiscuity, they can be more immunosuppressive than other classes of INH-ODNs. Thus, chronic treatment with Class G INH-ODNs may potentially lead to enhanced susceptibility to infection, even though the phenotype of mutated mice including those lacking the functional transporter molecule UNC93B1 is relatively mild [37]. Class B INH-ODNs are strictly linear ODNs unable to make significant secondary structures. They require a 5′CC(T) → GGG-3′ motif to block TLR9-induced activation in all responding cells, both in humans and in mice. Interestingly, they are less protective in the MRL-Fas<sup>lpr/lpr</sup> strain when compared to Class R INH-ODNs. They may find applications for prevention/treatment of TLR9-dependent microbial sepsis and chronic inflammation. When the number of consecutive Gs in a linear INH-ODN is increased from 3 to 4-5, this increases a chance for G4 stacking and for nonspecific effects on immune activation. Finally, Class R INH-ODNs are longer (20–28 mer) ODNs capable of either dimerizing or making hairpins. This property of Class R INH-ODNs depends on ODN-concentration, presence of ions, and on temperature. They are very potent suppressors

<table>
<thead>
<tr>
<th>Class</th>
<th>Characteristics</th>
<th>Prototype</th>
<th>TLR9 inhibition in B cells</th>
<th>TLR9 inhibition in DC/Mφ</th>
<th>TLR7 inhibition (backbone effect)</th>
<th>Inhibition of other signaling pathways</th>
<th>Reference</th>
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<tr>
<td>G</td>
<td>G4-stacking</td>
<td>TTAGGGn</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>[61]</td>
</tr>
<tr>
<td>R</td>
<td>Palindromic, Short 5′ or 3′ overhangs</td>
<td>INH-1</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>—</td>
<td>[62]</td>
</tr>
<tr>
<td>B</td>
<td>Linear</td>
<td>INH-18</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>—</td>
<td>[62]</td>
</tr>
</tbody>
</table>

Table 2: Classification of inhibitory oligonucleotides.
Mediators of Inflammation

of TLR9-induced activation in pDCs and macrophages, but are 10–30-fold less potent in human naïve B cells and mouse follicular B cells [62]. This cell selectivity of palindromic INH-ODNs is independent of the G4-stacking. BCR cross-linking increases their potency for TLR9-activated B cells for at least 10-fold making them ideal candidates for targeting dsDNA-, nucleosome-, or RF-specific autoreactive B cells.

All three classes of TLR9-antagonists have sequence-independent backbone-dependent effects on TLR7 (and possibly TLR3?) stimulation. TGC triplets may additionally increase the potency of an INH-ODN for the TLR7 pathway [71]. Literature search shows that classes B and G INH-ODNs and combined TLR7/9 inhibitors are effective in animal models of lupus [62, 67, 98, 104, 113]. We envision their future use as therapeutic agents for human lupus.

**Abbreviations**

TLR: Toll-like receptor
INH-ODN: Inhibitory oligonucleotide
BCR: B cell receptor for antigen
SLE: Systemic lupus erythematosus.

**Acknowledgments**

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**References**


Research Article

Normal Human Gingival Epithelial Cells Sense *C. parapsilosis* by Toll-Like Receptors and Module Its Pathogenesis through Antimicrobial Peptides and Proinflammatory Cytokines

Raouf Bahri,¹, ² Sèverine Curt,¹ Dalila Saidane-Mosbahi,² and Mahmoud Rouabhia¹

¹ Groupe de Recherche en Écologie Buccale, Faculté de Médecine Dentaire, Pavillon de Médecine Dentaire, Université Laval, QC, Canada G1K 7P4
² Laboratoire d’Analyse, Traitement et Valorisation des Polluants de l’Environnement et des Produits, Faculté de Pharmacie, Rue Avicenne, 5019 Monastir, Tunisia

Correspondence should be addressed to Mahmoud Rouabhia, mahmoud.rouabhia@fmd.ulaval.ca

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This study was designed to investigate the interaction between *C. parapsilosis* and human epithelial cells using monolayer cultures and an engineered human oral mucosa (EHOM). *C. parapsilosis* was able to adhere to gingival epithelial cells and to adopt the hyphal form in the presence of serum. Interestingly, when cultured onto the engineered human oral mucosa (EHOM), *C. parapsilosis* formed small biofilm and invaded the connective tissue. Following contact with *C. parapsilosis*, normal human gingival epithelial cells expressed high levels of Toll-like receptors (TLR)-2, -4, and -6, but not TLR-9 mRNA. The upregulation of TLRs was paralleled by an increase of IL-1β, TNFα, and IFNγ mRNA expression, suggesting the involvement of these cytokines in the defense against infection with *C. parapsilosis*. The active role of epithelial cells in the innate immunity against *C. parapsilosis* infection was enhanced by their capacity to express high levels of human beta-defensin-1, -2, and -3. The upregulation of proinflammatory cytokines and antimicrobial peptide expression may explain the growth inhibition of *C. parapsilosis* by the gingival epithelial cells. Overall results provide additional evidence of the involvement of epithelial cells in the innate immunity against *C. parapsilosis* infections.

1. Introduction

Fungal infections caused by *Candida* species are increasing, particularly in immunocompromized individuals [1]. *C. albicans* is the most common opportunistic fungal pathogen involved in health problems encountered in humans. However, infections by other *Candida* species are gaining ground worldwide, particularly in nosocomial settings. These emerging candidiases involve various *Candida* species, including *C. tropicalis*, *C. krusei*, *C. glabrata*, and *C. parapsilosis* [2].

In comparison to other *Candida* species, *C. parapsilosis* is largely distributed in nature. Unlike *C. albicans* and *C. tropicalis*, *C. parapsilosis* is not an obligate human pathogen, having been isolated from nonhuman sources [3] such as domestic animals, insects, soil, and marine environments [4]. *C. parapsilosis* is also a normal human commensal, and it is one of the fungi most frequently isolated from the subungal space of human hands. *C. parapsilosis* is a commensal of human skin, and its pathogenicity is limited by intact integument. *C. parapsilosis* is notorious for its capacity to grow in total parenteral nutrition and to form biofilms on catheters and other implanted devices, for nosocomial spread by hand carriage, and for persistence in clinical units [5]. *C. parapsilosis* is of special concern in critically ill neonates leading to high morbidity and mortality [6]. Furthermore, *C. parapsilosis* has been isolated from the oral cavity of HIV-infected [7] and diabetic individuals [8]. Although these studies document *C. parapsilosis* pathogenicity, further investigations are warranted to determine how human tissues can interact with, and prevent *C. parapsilosis* infection.
It is well known that the first line of defense against exogenous microorganism infections lies in the tissues, such as the skin and the oral mucosa [9, 10]. Both of these tissues are layered with an epithelial structure containing over 95% epithelial cells that not only have an active defensive role as a mechanical barrier but are also key players in antimicrobial innate immunity [7, 10]. The attachment of microorganisms such as C. parapsilosis to host cells is the initial stage of the infection process, enabling candida to survive and eventually colonize the host tissue during the development of candidiasis. Recognition of adherent C. parapsilosis by epithelial cells may involve specific receptors, including Toll-like receptors (TLRs) which play an active role by recognizing pathogens of considerable target specificity [11]. This recognition leads to a series of signaling events that result in the necessary acute host responses required to kill the pathogens [12]. Recent studies have demonstrated the involvement of TLRs in the recognition of fungal pathogens such as C. albicans, although less is known regarding the function of these receptors following Candida infection [13, 14]. The Candida-killing activity of keratinocytes involves TLR-2 and TLR-4 through NF-kB activation [15]. The host’s response to infection is most likely due to the different recognition/activation patterns of these receptors and the release of several proinflammatory cytokines [16, 17].

In response to C. albicans infection, oral epithelial cells were shown to produce significant amounts of IL-6, IL-8, and TNFα [18, 19], which suggests that cytokines have a significant role in controlling oral infections. Controlling Candida pathogenesis also involves the defensin family (human β-defensins, HBDs) of antimicrobial peptides that display broad-spectrum antimicrobial activity against a large array of pathogens, such as Candida species [20]. Expressed by different cell types, including epithelial cells, these peptides have been suggested as playing an active role in the commensal-to-pathogenic microorganism activity in epithelial cells [21]. While the antifungal activity of HBDs has been characterized to some extent [22], their exact role in the host’s defense mechanism against fungal infection remains unclear. Recent studies showed that the salivary levels of HBD-1 and -2 were lower in patients with oral candidiasis than in healthy individuals [23], whereas HBD-2 peptide expression was greater in human oral epithelia with candidiasis than in normal oral epithelia [24].

Given that C. parapsilosis can be acquired from different sites including soil and marine environments, and that C. parapsilosis is considered as an emerging pathogen in humans, we proposed to study (1) the virulence of C. parapsilosis (transition from blastospore to hyphal form) when cultured under the appropriate conditions (neutral pH in the presence or absence of serum), and its susceptibility to an antifungal agent (amphotericin B), (2) the ability of C. parapsilosis to attach to normal human epithelial cells in a monolayer culture and to form biofilm when used to infect EHOM in vitro, and (3) epithelial cell defenses against C. parapsilosis infection by investigating the gene activation of TLRs, antimicrobial peptides (HBD-1, -2, -3, and -4), and pro-inflammatory cytokines (IL-1β, TNFα, and IFNγ).

2. Materials and Methods

2.1. Candida Species. C. parapsilosis was isolated from water and sediment samples taken from various sites in the Mediterranean Sea near the city of Gabes, Tunisia (North Africa). The collected samples were immediately stored on ice and transported to the laboratory. To isolate and identify C. parapsilosis, a suspension of 50 g of sediment from each sediment sample was prepared in 100 mL of saline solution. The water samples were used undiluted. Both the sediment suspensions (1 mL) and the water samples (1 mL) were laid over Sabouraud dextrose agar (Difco; Becton-Dickinson, Sparks, MD) enriched with yeast extract and were incubated at 30°C [25]. Following microorganism growth, separate colonies were resuspended and seeded on Sabouraud dextrose agar gel and incubated at 30°C for 24 hours. To obtain homogeneous yeast suspensions, separate colonies were used to inoculate different tubes containing Sabouraud dextrose broth (Difco; Becton-Dickinson) supplemented with 0.1% glucose and antibiotics (streptomycin/penicillin at 20 μg/mL) to inhibit potential bacterial growth. The yeasts were grown to the stationary phase overnight at 30°C in a shaking water bath. Bactoconidia were then collected, washed with PBS, and subjected to specific identification. C. parapsilosis was selected following a rapid identification method based on the colorimetric detection of carbon assimilation (Api ID 32C, BioMèrieux SA, Marcy l’Etoile, France). This identification step was performed three times using Candida subculture from the same single colony used for the initial identification test. We were thus able to confirm that the Candida species involved was indeed C. parapsilosis. In some tests, we used C. albicans (ATCC 10231) as a reference species. Following cultures, C. parapsilosis and C. albicans were collected, washed with phosphate-buffered saline (PBS), counted using a hemocytometer [9], adjusted to 10^5/mL, and set aside for testing.

2.2. C. parapsilosis Susceptibility to Amphotericin-B. C. parapsilosis (10^4/well) was seeded in a round-bottom 96-well plate with or without various concentrations of amphotericin-B (1,000-0 ng/mL). The Candida cells in the presence or absence of varied concentrations of antifungal agent were incubated at 37°C for 24 hours. C. parapsilosis susceptibility to amphotericin-B was compared to that of C. albicans (ATCC-10231). Candida growth was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, which measures cell growth as a function of mitochondrial activity [18]. Standard growth curves were used to determine the concentrations of viable C. parapsilosis and C. albicans following treatment with amphotericin-B and the minimum inhibitory concentration (MIC), referring to the lowest concentration of amphotericin-B leading to a total inhibition of Candida growth.

2.3. C. parapsilosis Virulence through the Blastospore-Hyphal Transition. The transition from blastospore to hyphal form is considered to be a virulence factor that enables Candida to deeply penetrate the tissue and infect the host. We
therefore investigated the capacity of isolated \textit{C. parapsilosis} to adopt the hyphal form under favorable conditions. It has been shown that temperature, proteins, and neutral pH promote \textit{Candida} transition [26]. We therefore cultured \textit{C. parapsilosis} under three different conditions to assess the presence/absence of hyphae. \textit{C. albicans} (ATCC 10231) was used as a reference. The germ tube formation of both \textit{Candida} strains was examined in the presence of 10% FCS, at neutral pH (6 and 7), and finally at 30°C and 37°C. To do so, \textit{Candida} (10⁵ cells) was grown in 2 mL of Sabouraud dextrose broth under one of the above mentioned conditions for 3 and 6 hours. At the end of each culture period, the \textit{Candida} cultures were observed microscopically, and the germ tubes and total cells were counted and used to determine the transition percentage [18].

2.4. \textit{C. parapsilosis} Adherence and Growth following Contact with Gingival Epithelial Cells

2.4.1. Isolation and Culture of Oral Mucosal Cells. Small pieces of palatal mucosa were biopsied from gingival graft patients following their informed consent and with the approval of the Université Laval Ethical Committee. The epithelium was separated from the lamina propria using thermolysin treatment (500 µg/mL), after which time the epithelial cells were isolated with a 0.05% trypsin-0.01 EDTA solution. We used 0.125 U/mL of collagenase-P (Boehringer Mannheim, Laval, Québec, Canada) to extract fibroblasts from the lamina propria. Oral epithelial cells (9 × 10⁵ cells/cm²) were cultured with 1.3 × 10⁴/cm² irradiated mouse 3T3 fibroblasts in Dulbecco’s modified Eagle-Ham’s F₁₂ (3 : 1) (DMEM) supplemented medium [27]. Oral fibroblasts (1 × 10⁵ cells/cm²) were grown in Dulbecco’s modified Eagle (DME) supplemented medium. When the cultures reached 70%–80% confluence for the epithelial cells and 100% for the fibroblasts, the cells were detached and used for various tests.

2.4.2. Contact between \textit{Candida} and Epithelial Cells. Cells were seeded in six-well tissue culture plates and grown at 37°C in an 8% CO₂ atmosphere. At 80% confluence, epithelial cell monolayers were pulsed with \textit{C. parapsilosis} (10⁵ cells/cm²) for 3, 6, 12, and 24 hours. At the end of each contact period, the monolayers were used to assess \textit{C. parapsilosis} adhesion and growth. To assess \textit{C. parapsilosis} adhesion, monolayer cultures were washed six times with PBS and fixed with 40% methanol and 60% acetic acid for 10 minutes. The cells were washed twice with PBS and stained with crystal violet. The stained monolayers were then examined under an optical microscope and photographed. To assess the growth of \textit{C. parapsilosis} following contact with the epithelial cells, culture medium was used to collect nonadherent \textit{C. parapsilosis} as well as those adhering to the cells. This collection was performed by treating the monolayer epithelial cells with 1% (v/v) Triton X-100 in PBS to lyse the epithelial cells with no side effect on the \textit{Candida}. A pellet of \textit{C. parapsilosis} collected from the culture medium was then mixed with those adhering to and entering the epithelial cells. \textit{C. parapsilosis} viability and numbers under each condition were determined by trypan blue dye exclusion [9].

2.4.3. Effect of \textit{C. parapsilosis} on Epithelial Cell Viability. Immediately after each contact period, cells were detached from the culture flasks using a 0.05% trypsin-0.01 M EDTA solution for 20 minutes at 37°C. The cells were washed twice with DMEM-supplemented culture medium. The pellet was resuspended in 500 µL of DMEM and 50 µL of each epithelial cell suspension were withdrawn to assess the percentage of viable cells using the trypan blue exclusion technique [19].

2.5. Effect of \textit{C. parapsilosis} on Tissue Structure

2.5.1. Preparation of Engineered Human Oral Mucosa. Using normal primary oral epithelial cells and oral fibroblasts, we produced engineered human oral mucosa (EHOM) [28]. Briefly, oral fibroblasts were mixed with bovine skin collagen (BD Biosciences, Mississauga, ON, Canada) to produce the lamina propria. Tissue was grown in Dulbecco-Vogt modified Eagle’s medium supplemented with 5% fetal calf serum for 4 days. The oral epithelial cells were then seeded onto the lamina propria, and the tissue was grown in the presence of 10% fetal calf serum. Once the epithelial cells reached confluence, the EHOM were raised to the air-liquid interface for 5 days to enable the epithelium to stratify. Following this air-liquid interface culture, the EHOM were used to study the tissue interaction of \textit{C. parapsilosis}.

2.5.2. Tissue Structure following \textit{C. parapsilosis} Infection. To test the effect of \textit{C. parapsilosis} on tissue structure, the EHOM tissue was infected with 1 × 10⁶ cells/cm² of \textit{C. parapsilosis}. Tissue untreated with \textit{Candida} cells served as the control. Following a 24-h infection period, the tissue was collected and used for histological analyses. We collected multiple biopsies from each infected EHOM and stained them with hematoxylin and eosin. Each slide was assigned a number and blindly analyzed for tissue structure and the presence of \textit{C. parapsilosis} at regular intervals using a calibrated image analysis system [29].

2.6. Effect of \textit{C. parapsilosis} on the Expression of TLRs, β-Defensins, and Pro-Inflammatory Cytokines by Gingival Epithelial Cells

2.6.1. Stimulation of Epithelial Cells with \textit{C. parapsilosis}. Epithelial cells were detached from 75-cm² culture flasks using trypsin, washed twice in culture medium, counted, seeded into six-well tissue culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ) at 2.5 × 10⁵ cells/well, and finally incubated in an 8% CO₂ atmosphere at 37°C. After 5 days of culture, the epithelial cells reached approximately 80% confluence. They were then stimulated or not with \textit{C. parapsilosis} (10⁵/cm²) and were cultured again for 6 and 24 hours. Total RNA was extracted from the cells following each culture period.
Figure 1: Sensitivity of *C. parapsilosis* to amphotericin-B. The yeast was cultured in the presence of various concentrations of amphotericin-B for 24 hours and its growth was assessed by MTT assay. Quantification of live *C. parapsilosis* was obtained using a standard live curve. The effect of the antifungal agent on *C. parapsilosis* was compared to that on *C. albicans*. Results are presented as means ± SD of six different experiments.

2.6.2. RNA Extraction and Quantification. Total cellular RNA content was extracted using the Illustra RNAspin Mini (GE Health Care UK Limited, Buckingham, UK), while RNA concentration, purity, and quality were determined by means of the Experion system and RNA StdSens analysis kit according to instructions provided by the manufacturer (Bio-Rad, Hercules, CA).

2.6.3. Quantitative Real-Time RT-PCR. RNA (1 μg of each sample) was reverse transcribed into cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen Life Technologies, Mississauga, ON, Canada) and random hexamers (Amersham Pharmacia Biotech, Inc., Baie d’Urfé, QC, Canada). RT conditions were 10 minutes at 65°C, 1 hour at 37°C, and 10 minutes at 65°C. Quantitative PCR was carried out as previously described [18]. Amounts of mRNA transcripts were measured using the Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, Mississauga, ON, Canada). Reactions were performed using a PCR supermix from Bio-Rad (iQ SYBR Green supermix). Primers (Table 1) were added to the reaction mix at a final concentration of 250 nM. Five microliters of each cDNA sample was added to a 20 μL PCR mixture containing 12.5 μL of iQ SYBR Green supermix (Bio-Rad) and 0.5 μL of specific primers (TLR-2, TLR-4, TLR-6, TLR-9, HBD-1, HBD-2, HBD-3, HBD-4, GAPDH, IL-1β, TNFα, and IFNγ) (Medicorp, Inc., Montréal, QC, Canada) and 7 μL of RNase and DNase free water (MP Biomedicals, Solon, OH). Each reaction was performed in a Bio-Rad MyCycler Thermal Cycler (Bio-Rad). For the qPCR, the CT was automatically determined using the accompanying Bio-Rad CFX manager. Thermocycling conditions for the TLRs were 5 minutes at 95°C, followed by 45 cycles of 95°C for 15 seconds, and 60°C for 30 seconds and 72°C for 30 seconds, with each reaction done in triplicate. For the HBDs, the thermocycling conditions were 95°C for 3 minutes, followed by 45 cycles of 95°C for 10 seconds, 63°C for 10 seconds, and 72°C for 30 seconds, with each reaction performed in triplicate. For the cytokines, the conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds, and 72°C for 30 seconds, with each reaction done in triplicate.

Figure 2: Blastospore-to-hyphal transition of *C. parapsilosis*. Following culture at different temperatures and neutral pH with or without proteins (serum), the percentage of *C. parapsilosis* germ tubes was determined by dividing the number of hyphae over the total number of cells (blastospore and hyphae) in each culture condition. The mean relative values for six separate experiments are shown. *C. albicans* was used as a reference strain to *C. parapsilosis*.
Figure 3: *C. parapsilosis* adhesion to the gingival epithelial monolayer culture. Human gingival cells were grown up to 80% confluence, pulsed with *C. parapsilosis* or *C. albicans*, and incubated for 6 hours with or without serum. Following this culture period, the supernatant containing nonattached *Candida* was discarded, and the cultures were washed and stained with Cristal violet. Representative photographs are presented. Magnification: 250×.

Figure 4: Effect of *C. parapsilosis* on tissue structure and the formation of a biofilm on the engineered human oral mucosa. Histological features of EHOM following exposure to *C. parapsilosis*. EHOM tissue was infected with (a) no *Candida* cells (uninfected control) and (b, c) *C. parapsilosis* strain. Note the formation of a biofilm on the tissue (b) and the presence of *C. parapsilosis* in the connective tissue (c). Representative photographs of four different experiments are shown (two EHOMs per experiment). Magnification ×250 for parts (a) and (b), and ×600 for part (c). e: epithelium.
Table 1: Primer sequences used for the q-RT-PCR.

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<tr>
<td>TLR-9</td>
<td>sense: 5′-GACCTCTGACTGTCC-3′ antisense: 5′-AAGCTCGTTACCCAGTCT-3′</td>
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<tr>
<td>TLR-1β-defensin</td>
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<td>TLR-2β-defensin</td>
<td>sense: 5′-TGTTGTCCTCCCTGAA-3′ antisense: 5′-GTCGCAGTGCTTTGGAAGG-3′</td>
<td>105</td>
</tr>
<tr>
<td>TLR-3β-defensin</td>
<td>sense: 5′-CTTCTGTTGTTGCTCTCC-3′ antisense: 5′-CTGTTCCTCCTTTGGAAGGCA-3′</td>
<td>138</td>
</tr>
<tr>
<td>TLR-4β-defensin</td>
<td>sense: 5′-CAGCTACCAAACGAA-3′ antisense: 5′-CGCAAAGTGGAA-3′</td>
<td>133</td>
</tr>
<tr>
<td>IL-1β</td>
<td>sense: 5′-CTGCTCTGGTGGATGAG-3′ antisense: 5′-TTGGATGCTCTTGGGATCTACA-3′</td>
<td>69</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>sense: 5′-GCGATTGTCGAA-3′ antisense: 5′-TGGATGCTCTTGGGATC-3′</td>
<td>111</td>
</tr>
<tr>
<td>TNFα</td>
<td>sense: 5′-CAGCTCTTCTCTGCTTGAT-3′ antisense: 5′-GCCAGAGGGCTGATTAG-3′</td>
<td>122</td>
</tr>
<tr>
<td>GAPDH</td>
<td>sense: 5′-GGTATCGTAGGACTCATGAC-3′ antisense: 5′-ATGCCAGTGAGCTCGTTCCG-3′</td>
<td>180</td>
</tr>
</tbody>
</table>

Table 2: Growth of *C. parapsilosis* following contact with human oral epithelial cells, and viability of epithelial cells after contact with *Candida*.

<table>
<thead>
<tr>
<th>Contact time (h)</th>
<th>Nb. of <em>C. parapsilosis</em> (\times 10^5/\text{cm}^2)</th>
<th>Viability of epithelial cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1.6 ± 0.7</td>
<td>97.3 ± 0.01</td>
</tr>
<tr>
<td>6</td>
<td>0.9 ± 0.03**</td>
<td>97.0 ± 0.02</td>
</tr>
<tr>
<td>12</td>
<td>0.1 ± 0.09**</td>
<td>96.5 ± 0.32</td>
</tr>
<tr>
<td>24</td>
<td>0.03 ± 0.01**</td>
<td>96.9 ± 0.37</td>
</tr>
</tbody>
</table>

\* *C. parapsilosis* was seeded onto epithelial cell culture at \(10^5\) cells/cm². To assess the growth of *Candida* following contact with the epithelial cells, nonadherent (in the supernatant) and adherent (on the monolayer epithelial cell cultures) *C. parapsilosis* were collected and enumerated. To assess the viability of epithelial cells, cultures were treated with trypsin and cells were washed twice then viable epithelial cells were determined using trypan blue exclusion test.

\*\* \(P \leq .01\) is level of significance for growth inhibition of *C. parapsilosis* in infected oral epithelial cell cultures compared to initial seeding concentration of *C. parapsilosis*.

using a Microplate Reader Model 680 (Bio-Rad Laboratories Ltd, Mississauga, ON). The experiments were repeated three times and the means ± standard deviation (SD) were presented.

2.8. Statistical Analyses. Experimental values are given as means ± SD. The statistical significance of differences between the control values and the test values was determined using a one-way ANOVA. Posteriori comparisons were done using Tukey’s method. Normality and variance assumptions were verified using the Shapiro-Wilk test and the Brown and Forsythe test, respectively. All of the assumptions were fulfilled. \(P\)-values were declared significant at 0.05. Data were analyzed using the SAS version 8.2 statistical package (SAS Institute, Inc., Cary, NC).

3. Results

3.1. Susceptibility of *C. parapsilosis* to Amphoterin-B. *Candida* strains were isolated from Mediterranean Sea water and sediment samples. Api ID 32C analysis revealed that *C. parapsilosis* was the most isolated strain. Following the extraction and identification of *C. parapsilosis*, we were concerned about its possible side effects on humans; we therefore tested its susceptibility to an antifungal agent. Figure 1 shows that the growth rate of *C. parapsilosis* was very slow compared to that of *C. albicans* (ATCC 10231). In the presence of amphoterin B, the MIC was in the range of 60 ng/mL for both *C. albicans* and *C. parapsilosis*, which
suggests that *C. parapsilosis* was as sensitive to amphotericin B as *C. albicans*.

3.2. Isolated *C. parapsilosis* Was Able to Adopt the Filamentous Form. As the phenotype change (i.e., transition from yeast to filamentous form) of *Candida* is one of the major determinants of its virulence and is closely associated with its ability to cause disease, we investigated *C. parapsilosis* transition under various culture conditions. As shown in Figure 2, both *C. albicans* and *C. parapsilosis* were able to adopt the filamentous form under any favorable conditions. Indeed, at neutral pH with 10% serum, *C. parapsilosis* displayed filamentous forms after 3 and 6 hours of culture. The same observations were obtained at acidic pH in the presence of serum.

3.3. *C. parapsilosis* Adhered to Gingival Epithelial Cells. Adhesion of *C. parapsilosis* to epithelial cells was investigated at 6 and 24 hours postinfection. As shown in Figure 3, different clamps of *C. parapsilosis* were present on the epithelial cell monolayer 6 hours after infection and were distributed throughout the culture. Of great interest was that in the presence of serum, these clamps were bigger than were those without serum. Also, with serum, morphological changes (hyphae forms) were more visible as compared to culture without serum. The same results were obtained at 24 hours postinfection (data not shown). *C. parapsilosis* thus adhered to normal human gingival epithelial cells and adopt filamentous phenotype, which may explain its pathogenic capacity in humans.
3.4. Effect of Human Gingival Epithelial Cells on C. parapsilosis Growth. As epithelial cells downregulate *C. albicans* growth [9], and because *C. parapsilosis* adheres to gingival epithelial cells, we investigated the effect of epithelial cells on the growth of *C. parapsilosis*. Table 2 shows that following contact with epithelial cells, all of the collected *C. parapsilosis* were alive, yet their numbers were significantly reduced, varying from $1.5 \times 10^5$/cm$^2$ after a 3-h contact to approximately $3 \times 10^3$/cm$^2$ after a 24-h contact. These results suggest that gingival epithelial cells inhibited the growth of *C. parapsilosis*. It is interesting to note that to some extent, epithelial cells killed off *C. parapsilosis* because at the longer contact period (24 hours), there were far less ($3 \times 10^3$/cm$^2$) *C. parapsilosis* present than what was initially used ($10^5$/cm$^2$) to infect the epithelial cell cultures. On the other hand, epithelial cell viability following contact with *C. parapsilosis* was investigated showing that (Table 2) no significant differences were obtained between *C. parapsilosis*-infected and uninfected cultures, either in terms of cell viability or total numbers of viable cells.

3.5. *C. parapsilosis* Formed a Biofilm on the Engineered Human Oral Mucosa Tissue Which May Have Contributed to Tissue Disorganization. To direct our studies closer to the in vivo setting, we examined the ability of *C. parapsilosis* to form biofilm on the in vivo-like EHOM model, which mimics the physiologic conditions and complex structure of the oral mucosa in terms of three-dimensional structure, relationship between the various cell types, and secretion of soluble factors [28]. Our results reveal that *C. parapsilosis* adhered
to and formed biofilms on the EHOM layers (Figure 4(b), arrows). More importantly, _C. parapsilosis_ did penetrate the tissue to reach the lamina propria of the EHOM, therefore, showing an invasion of the tissue layers (Figure 4(c), arrows). This invasion by _C. parapsilosis_ may explain the disorganization observed in the infected tissue (Figure 4(b)) compared to the noninfected tissue (Figure 4(a)). Indeed, the tissue disorganization was characterized by differentiated cells (large cells, faint nuclei, large cytoplasm) in the upper layers of the epithelium, and the absence of layers including the stratum corneum. These studies show that _C. parapsilosis_ was able to form a biofilm on the tissue to deeply invade the connective tissue, which contributed to damaging the structure of the mucosal tissue.

3.6. _C. parapsilosis_ Modulated TLR Expression by Normal Human Gingival Epithelial Cells. Oral mucosa represents a major interaction site between the host and the environment. The host’s line of defense at this interface is therefore critical to controlling infection. Epithelial cells are involved in the control of _C. albicans_ pathogenesis through different pathways, including specific receptors such as TLRs [11, 17]. As epithelial cells downregulate _C. parapsilosis_ growth, we sought to investigate the involvement of TLR expression by epithelial cells in the recognition of _C. parapsilosis_. Using quantitative RT-PCR, we showed (Figure 5(a)) that _C. parapsilosis_ significantly increased TLR-2 mRNA expression, at 6 (P < .05) and 24 hours (P < .005) of contact. It should be noted that the epithelial cells in contact with _C. parapsilosis_ expressed more TLR-2 mRNA at 24 hours than at 6 hours (Figure 5(a)). Following TLR-2 activation, we then examined TLR-4 expression by the gingival epithelial cells. As demonstrated in Figure 5(b), the cells expressed high levels of TLR-4 mRNA later (24 hours), in response to _C. parapsilosis_. The TLR-6 mRNA expression (Figure 5(c)) showed that _C. parapsilosis_ induced a significant increase of TLR-6 mRNA expression by epithelial cells at 6 hours of culture but not at 24 hours. Finally, _C. parapsilosis_ modulated TLR-9 expression differently. As shown in Figure 5(d), _C. parapsilosis_ significantly (P < .05, P < .001) reduced TLR-9 gene expression. Overall results suggest that gingival epithelial cells involve TLR-2, -4, and -6, but not TLR-9 in the defense against _C. parapsilosis_ infections.

3.7. Human Gingival Epithelial Cells Prevented _C. parapsilosis_ Infection through β-Defensin Expression (HBD-1, HBD-2, and HBD-3). Antimicrobial peptides (AMP) are a component of mammalian innate immunity and are expressed by various cell types to prevent microbial infection. It is well known that epithelial cells involve defensins to control bacterial infection through TLRs. As we revealed that TLR expression was modulated by _C. parapsilosis_ (Figure 5), we also examined the expression of HBD-1, HBD-2, HBD-3, and HBD-4 in response to stimulation with _C. parapsilosis_. As reported in Figure 6(a), _C. parapsilosis_ significantly (P < .05) increased HBD-1 mRNA expression by epithelial cells at 24 hours. We also showed (Figure 6(b)) a significant increase of HBD-2 at 6 hours (P < .05) and 24 hours (P < .03) following cell contact with _C. parapsilosis_. Epithelial cells expressed elevated HBD-3 mRNA levels at 6 but not at 24 hours in response to _C. parapsilosis_ stimulation (Figure 6(c)). However, HBD-4 mRNA remained unchanged at the early

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**Figure 7:** Quantification of HBD2 and HBD3 levels secreted by gingival epithelial cells following infection with _C. parapsilosis_. Gingival epithelial cells were cultured with or without _C. parapsilosis_ for 6 and 24 hours. Supernatants were collected and used to quantify HBD2 (a) and HBD3 (b) using ELISA kits. Data are means ± SD of three separate experiments. The levels of significance were obtained by comparing the amount of AMP obtained with unstimulated samples to that obtained with samples stimulated _C. parapsilosis_.

![Graph (a)](image)

![Graph (b)](image)
contact period but decreased at the longer contact period (24 hours) (Figure 6(d)). These results suggest that gingival epithelial cells control *C. parapsilosis* pathogenesis through antimicrobial peptides, particularly HBD-1, HBD-2, and HBD-3. To confirm these results, we analysed the secretion of HBD2 and HBD3 following *C. parapsilosis* infection. As showed in Figure 7, the basal level of HBD2 and HBD3 increased following tissue infection with *C. parapsilosis*. This supports the increased mRNA gene expression of AMP by epithelial cells in response to *C. parapsilosis*.

3.8. Gingival Epithelial Cells Expressed High Levels of Proinflammatory Cytokines against *C. parapsilosis*. Cytokine proteins IL-1β, TNFα, and IFNγ are active in controlling oral infections and maintaining the symbiotic relationship between the oral microbial community and the host. We therefore examined the gene expression of these interleukins by gingival epithelial cells following contact with *C. parapsilosis*. As shown in Figure 8(a), epithelial cells expressed a high level of IL-1β mRNA in response to *C. parapsilosis*. This elevated expression was present at 6 and 24 hours.

**Figure 8:** Quantification of mRNA expression levels of IL-1β, TNFα, and IFNγ by gingival epithelial cells following infection with *C. parapsilosis*. Gingival epithelial cells were cultured with or without *C. parapsilosis* for 6 and 24 hours. Total RNA was extracted from the cells following each culture period and was used for quantitative RT-PCR of the IL-1β, TNFα, and IFNγ genes, as described in Section 2. Results are presented as a fold expression of the gene in the test sample compared to this gene expression in the control. Data are expressed as means ± SD from triplicate assays of three different experiments. (a) IL-1β expression, (b) TNFα expression, and (c) IFNγ expression. GEC: gingival epithelial cells.
postinfection but was not time-dependent. \textit{C. parapsilosis} also stimulated the epithelial cells to express a high level of TNFα mRNA basically after 24 hours of stimulation (Figure 8(b)). On the other hand, \textit{C. parapsilosis} significantly (\(P < .001\)) increased the IFNγ mRNA expression by the gingival epithelial cells at early (6 hours) contact period, but decreased IFNγ mRNA expression at 24 hours postinfection. Overall results suggest that gingival oral epithelial cells prevented \textit{C. parapsilosis}'s side effect through pro-inflammatory cytokines including IL-1β, IFNγ, and TNFα.

### 4. Discussion

It is well documented that \textit{nonalbicans Candida} (NAC) species cause high levels of human candidiasis [2] which is more prevalent in immunocompromised patients. The most involved NAC species in human disease are \textit{C. tropicalis}, \textit{C. krusei}, and \textit{C. glabrata} [30, 31]. To these we add another emerging species, \textit{C. parapsilosis}, responsible for multiple human candidiasis cases [32]. In comparison to other \textit{Candida} species, \textit{C. parapsilosis} has an extensive distribution in nature. Unlike \textit{C. albicans} and \textit{C. tropicalis}, \textit{C. parapsilosis} is not an obligate human pathogen, having been isolated from nonhuman sources [3] such as domestic animals, insects, soil, and marine environments [4]. This raises the question as to whether humans may be contaminated by \textit{C. parapsilosis}. Based on available data, the answer to this question may reside in the interaction of humans with various environmental sites such as rivers, recreational locations (beaches), or through marine products. Indeed, this study showed the presence of \textit{C. parapsilosis} in sea water and sediments. It also confirms previously reported data [33] showing the presence of different \textit{Candida} species (including \textit{C. parapsilosis}) in marine sites. As \textit{C. parapsilosis} may reach humans via various exposures, we investigated the susceptibility of the yeast to antifungal agent amphotericin-B. Interestingly, we found that \textit{C. parapsilosis} isolated from sea water and sediment was sensitive to this antifungal agent, thus confirming previous data [32]. Although isolated \textit{C. parapsilosis} was sensitive to the antifungal agents, we pursued further to investigate its interaction with host cells such as normal human gingival epithelial cells. Our results indicate that isolated \textit{C. parapsilosis} adhered to gingival epithelial cells, and adapted filamentous form. However, the hyphae from was not well expressed as the one observed with \textit{C. albicans}, which is in accordance with previously reported data [33, 34]. However, even if it did not translate from blastospore to clear hyphal form, \textit{C. parapsilosis} was able to reach the connective tissue of the infected EHAM. This invasion may be due to (1) the capacity of some \textit{C. parapsilosis} cells to overcome the epithelial cells' innate defenses or (2) the large number of \textit{C. parapsilosis} cells (10⁶/cm² of tissue) used in our experiments. This large number may have overwhelmed the capacity of the epithelial structure to prevent \textit{C. parapsilosis} invasion. Thus, further studies are required to shed light on the possible mechanisms used by \textit{C. parapsilosis} to invade gingival tissue.

The adhesion and interaction mechanisms between \textit{C. parapsilosis} and epithelial cells are not well known, and most of the available data corresponds to studies performed specifically with \textit{C. albicans} [9, 10]. According to these studies, \textit{C. parapsilosis} sensing by human epithelial cells may be through specific receptors such as TLRs. In light of this, our study confirms an increase of TLR-2, TLR-4, and TLR-6, but not TLR-9 mRNA expression following interaction between \textit{C. parapsilosis} and human epithelial cells. Although ours is the first study with \textit{C. parapsilosis}, it confirms the involvement of the TLR (-2, -4, and -6) pathway in the interaction between host and other \textit{Candida} species including \textit{C. albicans} [10, 17, 35]. The nonactivation of TLR-9 mRNA expression by \textit{C. parapsilosis} is surprising, as TLR-9 reportedly plays an active role in the recognition of \textit{C. albicans} [36]. The recognition of \textit{C. albicans} and \textit{C. parapsilosis} by epithelial cells appears to involve TLR-9 in a different manner. This may constitute an experimental clinical marker to discriminate between \textit{C. albicans} and \textit{C. parapsilosis} infections. Further studies will provide insight on this possible pathway. Additional research is also required to shed light on the role of each of the TLR2, 4, 6, and 9 in controlling \textit{C. parapsilosis} infection, and how the pro-inflammatory response is triggered and by which part of \textit{C. parapsilosis}.

It is well known that TLRs play a critical role as they recognize pathogens and activate the adaptive immune response [35]. In this study, epithelial cells, another key member of the innate immunity network, expressed various inflammatory mediators (IL-1β, TNFα, and IFNγ) following contact with \textit{C. parapsilosis}. These findings suggest that epithelial cells support the host’s defenses against \textit{C. parapsilosis} through proinflammatory cytokines, which is in agreement with previously reported data involving \textit{C. albicans} [19, 37, 38]. Proinflammatory cytokines IL-1β and TNFα were augmented by the \textit{C. parapsilosis}-infected oral epithelial cells at early and late infection periods, while IFNγ was involved only at early defense phase, as previously reported with \textit{C. albicans} [9]. This study therefore confirms the capacity of oral epithelial cells, through the expression of IL-β, TNFα, and IFNγ, to control \textit{C. parapsilosis} infection.

In vitro studies have shown the stimulatory effect of IFNγ on the phagocytosis and elimination of \textit{C. albicans} by neutrophils and macrophages [39, 40]. In accordance with these data, we found that \textit{C. parapsilosis} growth was reduced following culture in the presence of oral epithelial cell (Table 2). This situation may mimic the physiological and pathological environment in the oral cavity, and supports previously reported studies related to the growth inhibition of \textit{Candida} by epithelial cells [9]. This growth inhibition may be achieved through the contribution of antimicrobial peptides. Indeed, our study demonstrates that following infection with \textit{C. parapsilosis}, gingival epithelial cells expressed high levels of HBD-1, HBD-2, and HBD-3, which confirms the antifungal activity of β-defensins against \textit{C. albicans} [41], and suggests the involvement of these antimicrobial peptides in controlling \textit{C. parapsilosis} growth/pathogenesis by epithelial cells.

It is important to note that HBD-4 not only appeared to be unrequired but even decreased in this antifungal defense, suggesting the presence of different pathways involving
HBD-4 separate from the other HBDs. Our results on HBD-4 confirm those previously reported showing a different effect of HBD-4, depending on the microorganism used [42]. Indeed, HBD-4 has been shown to display weak antimicrobial activity against E. coli, S. cerevisiae, S. aureus, S. pneumoniae, and B. cepacea, and strong antimicrobial activity against S. carnosus and P. aeruginosa [43]. The effect of HBD-4 remains to be elucidated basically following infection with Candida.

5. Conclusion

This study demonstrated some key events related to the interaction between gingival epithelial cell/tissues and C. parapsilosis. Through this interaction, gingival epithelial cells involved TLRs and different mediators to control C. parapsilosis pathogenesis. Additional research will nevertheless be necessary to fully determine the key events involved in host/C. parapsilosis interactions and the mechanisms that prevent C. parapsilosis infection. Epithelial cells could be instrumental in helping us unravel the opportunistic process by which C. parapsilosis, found in proximity to humans, becomes a pathogen.

Acknowledgment

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References

Research Article

The p110α and p110β Isoforms of Class I Phosphatidylinositol 3-Kinase Are Involved in Toll-Like Receptor 5 Signaling in Epithelial Cells

Sabine M. Ivison, Mohammed A. S. Khan, Nicholas R. Graham, Leila A. Shobab, Yu Yao, Arnawaz Kifayet, Laura M. Sly, and Theodore S. Steiner

1 Division of Infectious Diseases, Department of Medicine, Vancouver Coastal Research Institute (VCRI), Faculty of Medicine, University of British Columbia, Rm D452 HP East, 2733 Heather St., Vancouver, BC, Canada V5Z 3J5
2 Child and Family Research Institute, B.C. Children’s Hospital, Vancouver, BC, Canada V6H 3V4

Correspondence should be addressed to Theodore S. Steiner, tsteiner@interchange.ubc.ca

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Background. Bacterial flagellin triggers inflammation in mammalian cells via Toll-like receptor (TLR) 5. Release of the chemokine IL-8 in response to flagellin involves NF-κB, p38 MAP kinase, and phosphatidylinositol 3-kinase (PI3K). However, PI3K has been reported to be either pro- or anti-inflammatory in different model systems. We hypothesized that this could be due to different activities of the p110α and p110β isoforms of PI3K.

Results. PI3K and Akt were rapidly activated in Caco-2 colon carcinoma cells by flagellin. Using a plasmid-based shRNA delivery system and novel p110 isoform-specific inhibitors, we found that flagellin-induced IL-8 production was dependent on both p110α and p110β. However, in the mouse, inhibition of p110β but not p110α reduced the increase of serum IL-6 levels induced by intraperitoneal injection of flagellin.

Conclusions. These data demonstrate that the p110α and p110β isoforms of class IA PI3K are both required for the proinflammatory response to flagellin.

1. Background

Flagellin is the major structural protein of bacterial flagella, the organelles that confer motility to a wide variety of bacterial species. Flagellin is also a potent trigger of innate immune responses in eukaryotic cells from plants [1] and vertebrates (reviewed in [2]). Most if not all of the responses to flagellin in vertebrate epithelial cells are mediated by Toll-like receptor (TLR) 5 [3–7]. In general, activation of TLRs by microbial products can lead to the induction of inflammatory responses via a MyD88-dependent or—independent pathways, leading to IκB degradation and ultimately NF-κB and/or IRF-3 activation [8]. However, divergence and modulation of TLR signalling with the receptor/ligand pair is an important component of the innate immune response. This divergence implicates the involvement of signalling components other than NF-κB. We and others previously reported that flagellin-induced production of the neutrophil chemokine interleukin (IL)-8 requires the activity of p38 MAP kinase, and that inhibition of NF-κB with the IκB degradation inhibitor Bay11-7082 results in only slight inhibition of this TLR5-mediated response [9–11]. Thus, IL-8 production in response to flagellin challenge depends significantly on NF-κB-independent pathways.

Phosphatidylinositol 3-kinase (PI3K) has been implicated in NF-κB-dependent and —independent inflammatory signaling following TLR ligation. Class IA PI3K consists of a p85 regulatory subunit and one of several p110 catalytic subunits (α, β, or δ), whose primary activity is the addition of a phosphate to the 3’ position of the phosphatidylinositol-4,5-diphosphate ring. The class IB PI3K, comprised of p101 regulatory and p110y catalytic subunits, has a similar activity. The resulting product of both enzymes, PI-3,4,5-P3, activates downstream signaling through several kinases, most notably Akt and 3-phosphoinositide-dependent kinase (PDK) 1, leading to cellular responses that vary with the cell...
type and stimulus studied [12]. A role for PI3K in TLR5 signaling was shown in two previous studies. The first of these, by Yu et al. [9], found that inhibition of PI3K with the broadly-reactive PI3K inhibitors wortmannin (WM) or LY294002 (LY29) increased IL-6 and IL-8 production in response to flagellin in T84 cells, suggesting that PI3K is inhibitory in the flagellin-mediated signaling of intestinal epithelial cells (IECs). Moreover, they found that systemic cytokine release from PI3K p85α−/− mice in response to intraperitoneal injections of flagellin was significantly higher than in heterozygous littersmates. WM increased MAPK activation but not I-κB degradation in response to flagellin. The second publication, by Rhee et al. [13], demonstrated Akt activation in murine colon and nontransformed human colonic cells (NCM460) treated with flagellin. In contrast to Yu et al, they found that inhibition of PI3K using dominant-negative p85 or Akt, or LY29, reduced IL-8 production in response to flagellin, indicating that PI3K augments flagellin-mediated inflammatory responses in IECs.

To help reconcile these discrepant results, we first confirmed that flagellin stimulates PI3K enzymatic activity in Caco-2 cells, and then assessed the impact of PI3K inhibition on flagellin-mediated inflammatory signaling by directly inhibiting the two class 1A PI3K isoforms expressed in IECs, p110α, and p110β [14], using both RNA interference and novel, isoform-specific pharmacological inhibitors. We found that inhibition of p110α or β significantly decreased flagellin-induced IL-8 release, although inhibition of these isoforms produced distinct effects on MAPK activation and IL-8 mRNA concentrations in human IECs as well as in an in vivo mouse model of flagellin inflammatory responses. The findings suggest that the PI3K pathway has a net proinflammatory effect in TLR5 signaling, and that the discrepant results reported in the literature could be due to differences in p110 isoform activities.

2. Methods

2.1. Reagents. Culture media and supplements were purchased from Sigma (St. Louis) except where otherwise indicated. Antibodies included mouse anti-PI3K (Serotec; Oxford, UK); mouse anti-PI3K p85, rabbit anti-pSer473Akt, and mouse 4G10 antiphosphotyrosine (Upstate; Charlotte, VA); rabbit antiphospho-p38 T180/Y182, total p38, phospho-p44/42 T202/Y204, total p44/42 (Cell Signaling; Beverly, MA); mouse anti-GAPDH (RDI; Flanders, NJ); rabbit anti-I-κBα C-15 and goat anti-actin (Santa Cruz Biotechnology; Santa Cruz, CA); HRP goat anti-mouse (Cedar Lane; Hornby, ON); HRP mouse anti-rabbit IgG (Sigma). LY294002, Bay 11-7085, and LY303511 were purchased from Calbiochem (San Diego). Isoform-specific PI3K inhibitors for in vitro studies were provided by Kevan Shokat (University of California, San Francisco) [7, 15]. TGX-221 and PI-103 used in mouse experiments were purchased from Chemdeca (Ridgewood, NJ) Recombinant LPS-free E. coli H18 flagellin was produced as previously described in [16]. The human IL-8 and mouse IL-6 ELISAs were from R&D Systems (Minneapolis, MN). Flagellin was used at saturating concentrations (500–1000 μg/ml) for proximal signaling events in Caco-2 cells, and at 100 ng/ml in experiments involving PI3K inhibitors or shRNA as this dose is the threshold dose for maximal IL-8 production.

2.2. Cell Culture. Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown in DMEM with 4.5 g/L D-glucose, 1x nonessential amino acids, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) with 10% fetal bovine serum (FBS; HyClone, Logan, UT). Caco-2 cells were seeded at a density of 104/ml and used for experiments 5–14 days after becoming confluent. HEK 293T cells were from ATCC and were grown in DMEM with 10% heat-inactivated FBS, penicillin, streptomycin, and nonessential amino acids. T84 cells from ATCC were grown in DMEM/F12 with 15 mM HEPES, pen/strep, and 5% FBS. They were seeded at 5 × 105 per well and used 3–4 days after seeding.

2.3. Western Blotting and Immunoprecipitation. Flagellin treatment of cells cultured in 6- or 12-well plates for various time points was followed by two washes with ice-cold phosphate-buffered saline (PBS). Treated cells were then lysed in 250–500 μl of lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM PMSF) and equal volumes of protein lysate from each sample analyzed by Western blotting. For immunoprecipitation of PI3K, 5 μg of anti-p85 was added to 500 μg of cell lysate and incubated at 4°C overnight. Immune complexes were bound to protein A-agarose for 1 hour, washed in lysis buffer, and analyzed by Western blot using 4G10 at 1:1000.

2.4. IL-8 Promoter/Reporter Assays. Caco-2 cells were electroporated with pEGFP and IL-8 promoter-luciferase reporter plasmids and seeded in 96-well plates as described in [16]. After 6 days, cells were stimulated and lysed in Bright-Glo reagent (Promega, Madison, WI). The ratio of luminescence to fluorescence in arbitrary units (to correct for cell number and transfection efficiency) was calculated for each sample, and the fold increase in this value compared to controls within the same experiment was defined as the fold increase in expression.

2.5. In Vitro Kinase Assay for PI3K. Caco-2 cells were grown at least 5 days postconfluence in 6-well plates. They were serum starved overnight, and then treated with flagellin for brief time points (1–30 minutes). Cells were rinsed with cold PBS, lysed, and immunoprecipitated with anti-p85 and protein A agarose. During the second wash step, 20% of the total volume of beads from each tube was removed and analyzed by Western blot for total amount of p85 (IP control). Remaining immunoprecipitates were subjected to in vitro kinase assay as described in [17]. Kinase autoradiographs and p85 Western blots were scanned and analyzed by densitometry, and the ratio of these two values calculated for each sample.
2.6. Electrophoretic Mobility Shift Assay for NF-κB. Caco-2 cells seeded in 6-well plates were used at least 7 days after plating. Cells were fed with 1 ml fresh growth medium and incubated with signaling inhibitors for 60 minutes followed by flagellin or IL-1β for 30–60 minutes. Nuclear extracts were incubated with 32P-labeled NF-κB-binding oligonucleotide and analyzed by EMSA as described in [18].

2.7. Quantitative RT-PCR. Caco-2 cells grown to differentiation in 24-well plates were pretreated with pharmacologic inhibitors for 30 minutes and then stimulated for 60 minutes. Cells were lysed and cytoplasmic RNA isolated (Rneasy mini, Qiagen; Mississauga, ON). Equal amounts of RNA from each sample were reversely transcribed using Superscript II (Invitrogen), and equal volumes from each reaction subjected to real-time PCR using Sybr Green (Applied Biosystems; Foster City, CA) for fluorescent detection. Fold changes in IL-8 mRNA were standardized to GAPDH standards for each sample and calculated as described in [18].

2.8. Construction and Analysis of shRNA Mediated Knockdown of p110 Subunits. Target sequences for shRNA-mediated knockdowns of p110α and p110β were as follows: PIK3CA (Acc. NM_006218), 224-244, and PIK3B (Acc. NM_006219) 1140-1161. Knockdown vectors were constructed by cloning the following oligonucleotides into each reaction subjected to real-time PCR using Sybr Green (Applied Biosystems; Foster City, CA) for fluorescent detection. Fold changes in IL-8 mRNA were standardized to GAPDH standards for each sample and calculated as described in [18].

2.9. Mouse Injections. 6-12-week-old female C57Bl/6 mice bred in our animal care facility were cared for in accordance with the guidelines of the UBC Animal Care and Use Committee in accordance with Canadian regulations. Mice were injected intraperitoneally with inhibitors or DMSO vehicle as described in Results, followed 30 minutes later by 10 μg of flagellin in 100 μl of sterile phosphate-buffered saline (PBS). Blood was taken via the saphenous vein prior to injection and 90 minutes and 3 hours after flagellin injection. At 6.5–7 hours after injection, mice were euthanized by CO2 asphyxiation and blood taken by cardiac puncture. Sera were stored at −80°C until testing for IL-6 by ELISA.

2.10. Statistical Analysis. Statistics were performed on original data using the VassarStats statistical computation website (http://vassun.vassar.edu/~lowry/VassarStats.htm). Except where noted, multiple groups were analyzed by ANOVA to verify the presence of significant differences; this was followed by testing of individual pairs by t-test or Tukey HSD.

3. Results

3.1. Flagellin Causes Enzymatic PI3K Activation in Caco-2 Cells. Activity of PI3K in cell lysates was measured by in vitro kinase assay. As shown in Figure 1(a), Caco-2 cells possessed measurable basal PI3K activity, consistent with their transformed nature and reduced intrinsic PTEN activity [19]. Within 5 minutes of stimulation with flagellin, a rapid and transient increase in PI3K activity was measured. This activity returned to baseline after 30 minutes (not shown). As an additional test for PI3K activation, Caco-2 lysates were immunoprecipitated with anti-PI3K p85 followed by Western blot using antiphosphotyrosine (4G10). As shown in Figure 1(b), flagellin-treated cells had a time-dependent increase in signal versus controls, indicating tyrosine phosphorylation of PI3K, which is associated with PI3K enzymatic activation [20].

3.2. Flagellin-Induced NF-κB Activity Is Not PI3K Dependent. We next analyzed I-κB degradation and NF-κB localization and DNA binding activity in response to flagellin. As shown in Figure 2(a), flagellin-induced degradation of I-κB in Caco-2 cells was not significantly affected by LY29, but was reduced by Bay11-7082. We next examined NF-κB nuclear localization and DNA binding activity by EMSA. As shown in Figures 2(b) and 2(c), flagellin caused a large increase in NF-κB activity in Caco-2 cells that was not reduced by LY29.

3.3. PI3K Inhibition Reduces IL-8 Promoter Activity, mRNA Production, and Secretion in Caco-2 Cells. Yu et al. [9] showed that LY29 increased IL-8 production in T84 transformed human colonocytes. This stands in contrast to Rhee et al. [13], who found that LY29 inhibited flagellin responses in NCM460 nontransformed human colonocytes. To reconcile this discrepancy, we measured IL-8 production in Caco-2 cells. As shown in Figure 3(a), Caco-2 cells transiently transfected with an IL-8 promoter/luciferase reporter construct that had approximately a 4-fold increase in luciferase expression after treatment with flagellin. Pretreatment of cells with 30 μM LY29 reduced flagellin-induced luciferase expression by about one-fourth (0.76 ± 0.29; P < .05). LY29 also inhibited IL-1β-induced luciferase expression (0.54 ± 0.023; P < .001, t-test; not shown).

The effect of PI3K inhibition on IL-8 mRNA production was measured by quantitative real-time RT-PCR. As shown in Figure 3(b), flagellin caused a marked increase in IL-8 mRNA at 1 hour compared to untreated cells. LY29 reduced
flagellin-stimulated IL-8 mRNA amounts by about one-third compared to DMSO vehicle (P < .05). As LY29 has been reported to affect kinases other than PI3K, such as the mammalian target of rapamycin (mTOR) and casein kinase 2 (CK2), we tested an analogue, LY303511 (LY30), which lacks PI3K inhibitory activity but does inhibit mTor and CK2 [21–23]. The inactive analog, LY30, did not significantly reduce IL-8 mRNA expression (not shown). In contrast, two isoform-specific PI3K inhibitors (described below) showed disparate effects, with TGX-221 (p110β) causing an increase in IL-8 mRNA, and PI-103 (p110α) producing no effect (Figure 3(b)).

We next measured the effects of PI3K inhibitors on IL-8 release from flagellin-stimulated Caco-2 cells. For these experiments we used LY29 as well as a newly-available, broadly-reactive PI3K inhibitor, GDC0941. Pretreatment of cells with LY29 or GDC0941 significantly inhibited flagellin-induced IL-8 release (Figure 4(a)). We also tested effects of LY30, rapamycin (the prototype mTOR inhibitor), and pp242 (a novel mTor inhibitor). None of these inhibitors significantly inhibited flagellin-induced IL-8 release from Caco-2 cells, suggesting that the effects seen with LY29 and GDC0941 were in fact due to PI3K inhibition rather than off-target effects on mTOR or CK2. (Figure 4(a)). Absolute values of released IL-8 in the DMSO + flagellin treated cells ranged from 400–800 pg/ml.

3.4. p110α and p110β Are Both Required for Flagellin-Induced Signaling in Caco-2 Cells. To determine whether flagellin-induced inflammation requires a particular isoform of class Ia PI3K, we employed a panel of novel chemical agents with specificity for different p110 catalytic subunits. A list of these and standard agents is found in Table 1. In addition to LY29, TGX-221, and PI-103 significantly inhibited IL-8 release, despite their disparate effects on IL-8 mRNA (Figure 4(b)). Both compounds also inhibited CCL20 release from Caco-2 cells, as did LY29 (see Figure 4(c) in Supplementary Material available online at doi: 10.1155/2010/652098). In contrast to Caco-2 cells, T84 cells showed no significant change in flagellin-induced IL-8 production with LY29, TGX-221, or PI-103, and had a significant increase in IL-8 with WM (4.8 ± 1.4-fold increase; P < .001, t-test, see Figure 4d in

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**Figure 1:** Activation of PI3K in Caco-2 cells by *E. coli* flagellin. (a) *In vitro* kinase assay. Caco-2 cells treated with flagellin (FliC) 1 μg/ml for the indicated times were lysed and immunoprecipitated with anti-p85. An aliquot of each immunoprecipitate was analyzed by Western blot for p85, and the remainder was reacted with phosphatidylinositol and γ-32P-ATP and separated by thin-layer chromatography. The PIP3 band is indicated by the arrow. Densitometry performed on autoradiograms from three independent experiments is shown below. *P < .05, FliC versus control (t-test). (b) Tyrosine phosphorylation of PI3K. Caco-2 cells treated with 500 ng/ml flagellin for the indicated times were lysed and immunoprecipitated with anti-p85. Precipitates were separated by SDS-PAGE and Western blots performed using 4G10 antiphosphotyrosine. Blots were stripped and reprobed with anti-p85 to confirm equal efficiency of immunoprecipitation. Results are representative of two separate experiments.
Figure 2: Flagellin-induced I-κB degradation and NF-κB activation in Caco-2 cells are PI3K independent. (a) I-κBα degradation in Caco-2 cells treated with IL-1β (10 ng/ml) or flagellin (FliC, 1000 ng/ml or 500 ng/ml) was not inhibited by LY29 (30 μM) but was inhibited by Bay 11-7082 (20 μM). Caco-2 cell lysates were analyzed by Western blot for anti-IκBα and anti–GAPDH and the band density measured. Density of I-κB bands was divided by GAPDH to normalize for protein loading and transfer, and the ratio was taken versus unstimulated cells in each experiment to calculate the amount of I-κBα degradation. Results were pooled from four separate experiments. *P<.01 versus DMSO by ANOVA, DMSO + FliC versus Bay11 + FliC. (b) NF-κB activation in Caco-2 cells measured by electrophoretic mobility shift assay, with the shifted NF-κB band indicated by the arrow. (c) Densitometry measurements of NF-κB-bound probe bands, pooled from 3 independent experiments.

Supplementary Material). These results support those of Yu et al. [9], and suggest that PI3K signaling in IECs varies between cell types.

As the specificity of pharmacological inhibitors is imperfect, we sought to confirm these results, using RNA interference. shRNA constructs were cloned into the pSuper vector, using sequences reported in the literature to be effective at knocking down the class IA PI3K catalytic isoforms p110α and p110β, both of which are known to be expressed in Caco-2 cells. Because Caco-2 cells have relatively low transfection rates, we looked at IL-8 secretion in HEK 293T cells, which are highly transfectable and produce IL-8 in response to flagellin when transfected to overexpress TLR5 (they are minimally responsive to flagellin otherwise). As with Caco-2 cells, LY29 decreased flagellin-mediated IL-8 release from HEK 293T cells (37.3 ± 4.1% inhibition with LY29; not shown). Multiple sequences were selected from each p110 isoform and cloned into pSuper to generate a panel of shRNA-expressing plasmids. Knockdown of specific p110 mRNA was confirmed by QPCR. The different knockdown constructs reduced IL-8 secretion in flagellin-stimulated, TLR5 transfected HEK cells in proportion with their degree of knockdown as determined by QPCR (Figure 5). All constructs shown in Figure 5 significantly reduced IL-8 secretion in comparison to pSuper transfected cells (P < .01 by ANOVA followed by t-test). As an additional control, we found that two knockdown constructs for p110γ failed to inhibit IL-8 production (not shown). The absolute concentration of IL-8 produced in TLR5-expressing cells carrying empty pSuper was 182.3 +/- 96 pg/ml (N = 4).
Figure 3: PI3K inhibition reduces flagellin-stimulated IL-8 promoter activation and mRNA production. (a) Caco-2 cells were electroporated with an IL-8 promoter/luciferase reporter plasmid and pEGFP as a transfection marker and grown as described in Methods. Cells were pretreated with inhibitors at the concentrations shown (or an equivalent volume of DMSO vehicle) followed 30 minutes later by flagellin 100 ng/ml. After 6 hours, cells were harvested for luciferase assay and fluorescence determination. The RLU/RFU value was calculated for each well, and these data normalized to the wells stimulated with DMSO + flagellin in each individual experiment. Data shown are compiled from at least 3 experiments for each inhibitor. * P < .01 (t-test). (b) IL-8 mRNA measurements by quantitative RT-PCR compiled from at least 3 experiments. Cytoplasmic RNA isolated from cells treated as shown was reverse transcribed. cDNA was amplified using actin and IL-8 primers with real-time fluorescent quantitation. Fold increases in mRNA were calculated as described in Methods. Results are expressed as the ratio compared to cells treated with flagellin plus DMSO vehicle. ** P < .05 versus flagellin plus DMSO (t-test).

Figure 4: PI3K inhibition reduces IL-8 production after flagellin stimulation in Caco-2 cells. Caco-2 cells seeded as described in Methods were treated with the inhibitors shown (all at 10 μM, except for 30 μM LY29 and LY30) or an equivalent dose of DMSO vehicle in complete culture media. Thirty minutes later, flagellin was added to a final concentration of 100 ng/ml, and cell supernatants harvested 3 hours later. IL-8 was measured by ELISA, and the values in each experiment were normalized to the flagellin + DMSO wells. (a) Effects of broadly reactive PI3K inhibitors. (b) Effects of isoform-specific inhibitors. * P < .001 versus DMSO; ** P < .01 versus DMSO (t-test). Results compiled from N of at least 10 for each inhibitor.
stimulation. In contrast, ERK phosphorylation followed an
less pronounced upregulation for as long as 4 hours after
substantial increase in p38 phosphorylation at 1 hour, with
(not shown). Interestingly, both compounds also caused a
induced Akt phosphorylation was also inhibited by LY29
mediated by both p110\(\alpha\) and p110\(\beta\) target two separate sites of p110\(\alpha\), while pSp110-\(\beta\) and
Figure 6: Effects of p110 inhibitors on flagellin-induced Akt, p38,
and ERK activation. Caco-2 cells were serum-starved overnight,
and then given serum-containing media with 10 \(\mu\)M PI-103 (P) or TGX-
221 (T) or an equivalent volume of DMSO vehicle (D). After 30
minutes, flagellin was added to a final concentration of 100 ng/ml,
and cells were harvested after 1, 2, or 4 hours. Total cell lysates
were separated by SDS-PAGE and tested for pSer473-Akt, phospho-p38,
and phospho-ERK by Western blot. Blots were stripped and probed
with total Akt and total p38 antibodies as loading controls. Results
shown are typical of three independent experiments.

Table 1: Activities of pharmacologic inhibitors used in this study (IC\(_{50}\) in nM).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>LY294002</th>
<th>LY303511</th>
<th>GDC0941</th>
<th>PI-103</th>
<th>TGX-221</th>
<th>PIK-93</th>
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<tr>
<td>p110(\alpha)</td>
<td>500</td>
<td>&gt;1000</td>
<td>3</td>
<td>8</td>
<td>&gt;1000</td>
<td>39</td>
</tr>
<tr>
<td>p110(\beta)</td>
<td>300–973</td>
<td>&gt;1000</td>
<td>33</td>
<td>88–340</td>
<td>7–8.5</td>
<td>590</td>
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<tr>
<td>p110(\gamma)</td>
<td>1000–7000</td>
<td>&gt;1000</td>
<td>75</td>
<td>15</td>
<td>&gt;1000</td>
<td>16</td>
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<tr>
<td>p110(\delta)</td>
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<td>&gt;1000</td>
<td>3</td>
<td>48–&gt;500</td>
<td>&gt;100</td>
<td>120</td>
</tr>
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<td>?</td>
<td>580</td>
<td>20</td>
<td>?</td>
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<tr>
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<td>CK2</td>
<td>?</td>
<td>Class II PI3K (0.67)</td>
<td>PI4KIIIb</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data compiled from [24–28].

Figure 5: Effect of p110\(\alpha\)- and p110\(\beta\)-targeted shRNA on IL-
8 production in TLR5-transfected, flagellin-stimulated HEK293T
cells. Cells were transfected with TLR5 and a pSuper derivative or
with pSuper alone and then stimulated with 500 ng/ml flagellin for
6 hours. IL-8 concentrations were determined by ELISA. pSp110-
\(\alpha\)-1 and -\(\alpha\)-2 target 2 separate sites of p110\(\alpha\), while pSp110-\(\beta\) and
-\(\beta\) target two separate sites of p110\(\beta\). Results show fold increase
versus baseline (IL-8 amounts measured in flagellin stimulated cells
which were not transfected with TLR5). The percentages above the
columns denote the amount of knockdown of either p110\(\alpha\)
or p110\(\beta\) mRNA as determined by quantitative RT-PCR; results
were compiled from at least 7 samples from a minimum of 3
separate experiments. All four knockdowns significantly reduced
IL-8 production (P < .001, ANOVA).

3.5. PI3K Inhibition Alters p38 and ERK Activation in Response
to Flagellin. To determine the mechanisms by which PI3K
inhibition reduced flagellin responses, we looked at the
effects of PI3K inhibition on Akt and MAPK activation. It
has been previously reported that flagellin causes phosphor-
ylation of Akt, p38, and Erk kinases; moreover, Yu et al
reported that PI3K inhibition led to prolonged activation
of these kinases in T84 cells [9]. Both TGX-221 and PI-103
inhibited flagellin-induced Akt phosphorylation at Ser473,
suggesting that the effects of flagellin on PI3K may be
mediated by both p110\(\beta\) and p110\(\alpha\) (Figure 6). Flagellin-
induced Akt phosphorylation was also inhibited by LY29
(not shown). Interestingly, both compounds also caused a
substantial increase in p38 phosphorylation at 1 hour, with
less pronounced upregulation for as long as 4 hours after
stimulation. In contrast, ERK phosphorylation followed an
unexpected kinetic course, with both inhibitors reducing
immediate and 2 hours phosphorylation, while TGX221, but
not PI-103, caused a significant boost in late phosphorylation
(4 hours after stimulation). Together, these results point to a
complex interaction between the PI3K and MAPK pathways
following TLR5 activation, and reveal different activities of
p110\(\alpha\) and p110\(\beta\) in the late regulatory pathways.

3.6. PI3K p110\(\beta\) Inhibition Reduces Inflammatory Responses to
Flagellin In Vivo. To measure the in vivo relevance of PI3K
isof orm activation, we examined the serum IL-6 response
to intraperitoneal injection of flagellin into mice. IL-6 is
commonly used as a serum marker for flagellin-induced
inflammatory responses in mice, which do not produce IL-
8. Yu et al performed similar experiments comparing wild-
type to p85\(\alpha\)/-- mice and found exaggerated responses in
the absence of functioning PI3K [9]. Female C57Bl/6 mice
were injected with 100–300 \(\mu\)l of TGX-221 or PI-103 in
DMSO, or an equivalent volume of DMSO alone, followed
30 minutes later by 10 \(\mu\)g of flagellin in PBS. Blood was
taken at 90 minutes, 3 hours, and 7 hours following the
flagellin injection, and serum IL-6 measured. Some mice also
had preinjection blood taken, to establish a baseline, which
in all cases was less than or equal to 125 pg/ml. All mice exhibited mildly reduced physical activity following these injections, which may be due to the known depressant effects of DMSO. No mice died or exhibited any other signs of distress such as stereotypic movements, hunched posture, ruffled fur, etc. As shown in Figure 7, mice treated with TGX-221 had a statistically significant reduction in serum IL-6 90 minutes after flagellin injection, compared to DMSO vehicle. Serum from PI-103-treated mice contained increased IL-6, although this was not statistically significant. At 3 hours, serum half-life of TGX-221 has not been reported, but antithrombotic activity in rats persists for at least 60–90 minutes following an intravenous bolus [16].

To verify the purity of our flagellin preparations, C57Bl/6 TLR5 −/− mice were injected with the same dose and preparation of flagellin and IL-6 measured. Three-hour IL-6 production was minimal in these mice compared to wild-type mice (345 ± 27.2 versus 7655 ± 378.5 pg/ml, P < .001, not shown). Serum IL-6 was below assay detection limits (50 pg/ml) in mice prior to flagellin treatment.

4. Discussion

Our results indicate that PI3K activated upon TLR5 activation by flagellin has a net proinflammatory effect in Caco-2 cells, and that this effect is not mediated through upregulation of NF-κB or p38 MAPK activity. Moreover, we have shown that both the p110α and p110β isoforms contribute to this effect, although likely through different mechanisms involving ERK phosphorylation and possibly IL-8 mRNA translation, which may lead to nonredundant effects in vivo.

These findings are particularly relevant because of the current controversy surrounding the role of PI3K in TLR signaling, with both pro- and anti-inflammatory effects seen in various epithelial and hematopoietic cells from mice and humans. Moreover, differences in TLR5 activity have been implicated in several human diseases, including Crohn’s disease [30], systemic lupus erythematosus [31], and Legionella pneumonia [32]. While TLR5 is expressed on both epithelial and hematopoietic cells, these cells differ tremendously in their exposure to the natural TLR5 ligand, flagellin. Hence, it is not surprising that signaling in response to flagellin would differ between these cell types, and the involvement of downstream kinase messengers such as PI3K is one way that this difference can be achieved.

Class IA PI3K is a proximal transducer of a wide variety of extracellular signals. Stimuli such as growth factors, G-protein coupled receptor ligands, cytokines, and vitamin D3 can recruit the p85 regulatory subunit to the plasma membrane, where the p110 catalytic subunit converts (preferentially) PI-4,5-P2 into PI-3,4,5-P3 [10]. PI-3,4,5-P3 binds to and regulates several protein kinases and other targets through their pleckstrin-homology domains. The most studied of these targets are phosphatidylinositol-dependent kinase 1 (PDK1), Akt (protein kinase B), tyrosine kinases, and guanosine nucleotide exchange factors [12]. Further downstream, other proteins such as I-κB kinase subtypes (IKK), mTOR, protein kinase C-ξ, and several antiapoptotic factors can then be activated, making PI3K an important proximal element in many signal transduction pathways [33].

There are two distinct p85 isoforms (α and β) and four p110 isoforms (α, β, γ, and δ). The tissue distributions of these are distinct, with all four p110 isoforms expressed on hematopoietic cells but only α, β, and γ expressed in normal epithelial cells. Of note, p110y expression is absent in colonic adenocarcinoma cells, including Caco-2 cells [15]. While all of the subunits appear to catalyze the same enzymatic reactions, there are different cellular responses associated with them, some of which may be due to unique localization, or even nonenzymatic activities. For example, p110β was shown to bind to Akt in the nucleus of 3T3 cells, facilitating DNA elongation during replication [34]. In contrast, p110α was required for cell survival in the same study.
In TLR and IL-1β signaling, PI3K is believed to be recruited to the membrane by binding of p85 to a specific YYXM motif on MyD88, leading to activation of PI3K [13]. However, in the case of TLR4, binding of PI3K may result in phosphoinositide sequestration and subsequent reduced activity of the PI3K axis [35]. Overall, the net effect of PI3K activity can be either pro- or anti-inflammatory depending on the system studied. For example, inhibition of PI3K increases COX-2 upregulation in response to TNF-α or IL-1 in colonic epithelial cells [36] but decreases COX-2 upregulation by lauric acid in RAW macrophages [37]. Inhibition of PI3K with LY29 or WM reduces IL-8 expression in some experimental models, but not others [38–41]. Even within the same cell type, PI3K can differentially affect inflammatory gene expression. For example, LY29 reduces expression of MIP-1α and MIP-1β mRNA, but increases IL-8 mRNA in dendritic cells incubated with LPS [42]. Effects of PI3K on NF-κB also vary with the system studied, and can involve either inhibition or activation of NF-κB in an IκB-dependent or –independent manner [43–47].

One problem with many published studies of PI3K effects is that the use of pharmacologic inhibitors is inconsistent, with wortmannin (WM), LY29, or both used in different reports. While WM at concentrations below 50 nM is quite specific for PI3K, it does have activity against smooth-muscle myosin light-chain kinase (IC50 of 260 nM) [23]. While more stable than WM, LY29 is less potent, and inhibits CK2 and mTOR at similar concentrations at which it inhibits PI3K [21, 23]. These activities could explain different or even opposite pharmacologic effects of LY29 and WM reported in various cell systems [48–50].

Because of these issues, several more recent studies have focused on the roles of specific p110 isoforms in TLR signaling. While there are no reports examining p110 isoforms in TLR5 signaling, they have been studied in TLR4 pathways. Utsugi et al. found that p110β, but not α, positively regulated LPS-induced IL-12 production in human DCs and macrophages [51]. In contrast, in murine macrophages, Tsukamoto et al. found that shRNA-mediated inhibition of p110α increased Akt phosphorylation and decreased iNOS activity and IL-12 production, while p110β inhibition had the opposite effect on these markers [52]. They concluded that p110β is involved in downregulation or negative feedback of TLR signaling. While these results are consistent with the late increase in pERK that we observed following flagellin stimulation in the presence of TGX-221, in other ways our findings were quite different. For example, we found that TGX-221 and PI-103 were equally effective at reducing flagellin-induced Akt phosphorylation, and that TGX-221, but not PI-103, reduced IL-6 production in response to flagellin injection in mice.

The isoform-specific pharmacologic inhibitors of PI3K used in this study are starting to prove very useful in determining the roles of individual p110 isoforms, particularly in models where shRNA is impractical (such as primary cells or in vivo). For example, Sly et al. found that inhibition of p110β with TGX-221 increased TLR4- or TLR9-mediated IL-6 production in murine macrophages whereas p110α, γ, and δ inhibitors had the opposite effect [53]. The highly specific nature of these inhibitors has generated interest in their use in diverse clinical settings, including platelet inhibition and cancer treatment. However, the effects of these agents on TLR5 signaling, or indeed on IECs in any model system, have not been examined previously.

The relevance of our findings is further increased by the existing controversy regarding the role of PI3K in TLR signaling in general, and particularly on TLR5. As mentioned above, Yu et al. and Rhee et al. found opposite effects of PI3K inhibition on TLR5 signaling (anti-inflammatory in the former and proinflammatory in the latter). An additional paper by Kato et al. reported inhibition of TLR5 signaling by WM in TLR5-transfected HEK cells, although this was not the major focus of their work [54]. We hypothesized that differential effects of p110α and β isoforms (which are the forms expressed in transformed IECs) would be a possible explanation for these discrepancies.

Our findings clearly tip the balance in favor of PI3K having a net proinflammatory activity on Caco-2 and HEK cells, in contrast to RAW cells and T84 cells. We found that inhibition of either p110α or p110β using shRNA or pharmacologic blockade led to reduced IL-8 production that was independent of the NF-κB and p38 pathways. This was despite either no effect or an activating effect on IL-8 mRNA detected by RT-PCR. In fact, we found, as did Yu et al., that PI3K inhibition led to increased p38 phosphorylation in Caco-2 cells, and a strong proinflammatory effect of WM (but not LY) in T84 cells. Given that blockade of p38 and NF-κB have both been shown to reduce flagellin-induced IL-8 production, this finding was somewhat surprising, and suggests that an alternative, PI3K-dependent pathway is involved in TLR5-induced IL-8 production in IECs (perhaps one involving posttranscriptional regulation). Future studies will be required to investigate proteins that may be involved in this pathway.

An additional unexpected observation was the late increase in ERK phosphorylation after blockade of p110β, but not p110α. This finding, along with our in vivo results in mice, confirm that there are in fact different proinflammatory pathways utilized by p110α and p110β. To the best of our knowledge, this is the first report of the use of TGX-221 in a live animal model, and its lack of overt toxicity suggests that it could be useful in further studies whereas specific inhibition of p110β may be useful. One interesting observation was that the inhibition of IL-6 production was evident only at the earliest time point after stimulation. Since the serum half-life of TGX-221 is not known, this could be strictly due to pharmacokinetic effects, but it is intriguing to speculate that the late increase of pERK seen in vitro would reflect a biphasic effect of p110β signaling.

In conclusion, it is apparent from our studies that the TLR5 inflammatory pathway involves activation of PI3K, and that inhibition of either p110α or p110β blocks production of IL-8 in Caco-2 IECs in vitro whereas only p110β appears to be required for IL-6 production from mice in vivo. Moreover, p110 inhibition reduced IL-8 production while increasing MAPK activity without altering NF-κB activity, suggesting the presence of other important molecular targets of PI3K that ultimately facilitate chemokine production in response
to flagellin. Future studies of these novel targets may lead to the development of novel treatments for flagellin-related diseases, including Crohn’s disease and bacterial gastroenteritis.

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References


Research Article

Expression of TLR4-MyD88 and NF-κB in the Iris during Endotoxin-Induced Uveitis

Shang Li,1 Hong Lu,1 Xiaofeng Hu,1 Wei Chen,2 Yingzhi Xu,1 and Jing Wang1

1 Department of Ophthalmology, Beijing Chaoyang Hospital, Capital Medical University, No. 8 Baijiazhuang Road, Chaoyang District, Beijing 100020, China
2 Department of Ophthalmology, Haidian Maternal and Child Health Hospital, Tongji Medical University, Beijing 100080, China

Correspondence should be addressed to Hong Lu, honglu111@gmail.com

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Purpose. To observe the expression of Toll-like receptor-4 (TLR4), myeloid differentiation factor 88 (MyD88), and nuclear factor kappa B p65 (NF-κB p65) in iris tissue during endotoxin-induced uveitis (EIU) and evaluate the significance of these factors in uveitis.

Methods. Wistar rats were randomly divided into 5 groups (0 h, 12 h, 24 h, 48 h, and 72 h, n = 10/group). Animal model of acute anterior uveitis was established by a hind footpad injection of 200 μg Cholera vibrio LPS. Expression of TLR4, MyD88, and NF-κB p65 in iris ciliary body tissue was detected through immunohistochemical staining.

Results. Expression of TLR4 was not detected in normal iris-ciliary body complex, TLR4 positive cells with round morphology appeared in the iris stroma 12 hours after injection, significantly increased (P < .001) 48 hours after injection, and decreased gradually 72 hours after injection. Expression of MyD88 and NF-κB p65 is consistent with the change of the TLR4.

Conclusions. The increased expression of TLR4 and its downstream signal transduction molecules MyD88, NF-κB indicate the potential role of pathway in the pathogenesis of acute anterior uveitis (AAU).

1. Introduction

The Toll-like receptors (TLRs) are a recently discovered family of innate immune recognition receptors (PRRs). Innate immune system is rapidly activated by recognition of pathogenic microorganisms and special structure of cell wall that is a highly conserved pathogen-associated molecular patterns (PAMPs) [1]. There are at least 13 human TLRs identified to date, in which Toll-like receptor 4 (TLR4) is the earliest discovered and the most studied. The main function of TLR4 is to identify the lipopolysaccharide (LPS) of Gram-negative bacterial cell wall and activates innate immune system. Chen et al. recently [2] reported that TLR4 was highly expressed in the surface of iris-ciliary macrophages in the rats with uveitis induced by footpad injection of Vibrio cholera lipopolysaccharide. It suggested that TLR4 may be involved in the pathogenesis of acute anterior uveitis (AAU). In our study, changes in the expression of TLR4, downstream transduction molecules MyD88, and NF-κB were investigated in iris-ciliary during EIU by the same research method. The role of TLR4 transduction pathway was further explored in in the pathogenesis of AAU.

2. Materials and Methods

2.1. Material

2.1.1. Animal. Adult male pathogen-free Wistar rats (8–10 weeks old, weighing 150–200 g) were obtained from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). Fifty animals were used in the study. Animals were randomly divided into five groups (n = 10 per group) for the following time points: before LPS injection (0 h; control group) and 6 h, 12 h, 24 h, 48 h, and 72 h after injection.

2.1.2. Reagents. Lipopolysaccharide (V. cholera, classical Biotype, serotype Ogawa) was kindly provided by Lanzhou Institute of Biologic Products (Lanzhou, China). Rabbit Polyclonal antibody TLR4 and MyD88, mouse monoclonal
antibody NF-κB p65 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Vectastain ABC-peroxidase kits were purchased from Vector Laboratory (U.S.A). Affinity purified normal rabbit IgG was purchased from Boster Biotechnology (Wuhan, China). Mouse IgG1 was purchased from Serotec (Oxford, UK), DAB kit was purchased from Zhongshan Goldbridge Biotechnology (Beijing, China).

2.1.3. Instruments and Equipment. Removal of the iris was under a stereo microscope (Leica-M165 C; Leica, Wetzlar, Germany). Slides were examined under an optical microscope (Leica-DM-4000B; Leica, Wetzlar, Germany). Images were captured by a slit lamp with an anterior segment camera system (Topcon SL-D7; Topcon, Japan), and analysis using image-management software (Adobe Photoshop CS3. 10.0; Adobe Systems, Mountain View, CA).

2.2. Methods

2.2.1. Animal Model. Endotoxin-induced uveitis was induced as previously described [3]. Mice received a single injection of 200 μg [4, 5] LPS dissolved in 100 μL sterile saline (NS) in one rear footpad. The eyes were examined by slit microscope before the injection and at different time after the injection, and the intensity of anterior segment inflammation was evaluated by slit lamp.

2.2.2. Inflammatory Scoring. The intensity of anterior segment inflammation was examined by slit microscope before the injection and at 2 h intervals after the injection. Inflammatory signs were recorded in detail, and photographs were taken. The severity of uveitis was graded from 0 to 4 by a investigator blinded to study protocol [6] as follows: 0 = no inflammation; 1 = discrete vasodilatation of the iris and the conjunctiva vessels; 2 = moderate dilatation of the iris and the conjunctival vessels with moderate flare in the anterior chamber; 3 = intense iridal hyperemia with intense flare in the anterior chamber; and 4 = the same clinical signs as 3 with fibrinous exudates in the pupillary area.

2.2.3. Animal Perfusion and Specimen Preparation. Intracardiac perfusion of rats was performed at different time points in order to eliminate the effect of blood to immunohistochemical staining. Animals were deeply anesthetized using 17.5% chloral hydrate (2 mL/kg) by intraperitoneal injection. Through an abdominal incision in the midsagittal plane to expose the chest wall, the rat was flushed through the left ventricle with 250–300 mL of phosphate-buffered saline (PBS), 1 IU heparin per mL of PBS until the outflow becomes colorless, then 4% paraformaldehyde (250 mL) was flushed through the heart. Movements in the limbs and tail (fixation has reached the extremities) indicated adequate perfusion [7]. After being fixed in 4% paraformaldehyde for additional 1-2 hs, the eyes were put into petri dishes filled with PBS. The iris-ciliary body complex was gently dissected into 3–4 segments under the stereomicroscope and stored in Eppendorf tube with PBS at −80°C.

2.2.4. Histopathology. Rats were killed by overdose of pentobarbital (100 mg/kg) 24 hours after being immunized with LPS. The eyes of rats were enucleated and placed in 10% neutral buffered formalin solution for 24 h. After stationary liquid was washed out, tissue sample was immersed in 50%, 75%, 80%, 90%, and 100% alcohol for 1 h, respectively, to dehydrate. Then, the tissue was put into paraffin for 1 h × 3 for embedding after being treated with xylene for 30 minutes. Sagittal sections (4 μm thick) were cut near the optic nerve head and stained with hematoxylin and eosin.

2.2.5. Immunohistochemistry. The prepared tissues were put into 24 well plate. After three rinses with PBS (5 min each), 0.3% triton-X 100 was used to perforate the cell membrane for 30 min. Then, endogenous peroxidase activity in the whole mounts was blocked by 0.3% H2O2 for 30 min at room temperature. After another three rinses with PBS, the tissues were blocked with 5% goat serum for 30 min, and incubated with primary antibody against TLR4, MyD88, and NF-κB p65 (dilution, 1 : 50) overnight at 4°C. After several washes in PBS, the tissues were incubated with biotinylated antirabbit and antimouse secondary antibody (1 : 200) for 2 h at room temperature. After three further washes in PBS, the tissues were incubated with mixture of A and B (1 : 100) for 30 min at 37°C. Staining was visualized by the biotin-avidin-peroxidase method using diaminobenzidine as chromogen. Negative controls were performed by replacing the primary antibody with species-matched and isotype-matched antibodies with the same concentration of the primary antibody.

2.3. Quantitative Analysis. The method employed in our study involved counting the total number of immunopositive cells per iris segment (a strip 0.29 mm or 1 graticule in width from the pupil margin to iris base chosen randomly around the circumference). The mean length of the irides (base-pupil margin) in the present study was approximately 1 mm (0.97 mm or 3.4 graticule lengths); therefore, the mean area of a segment was 0.281 mm2. A minimum of two and up to five segments were counted per iris from one eye, and an overall mean density/mm2 per animal was obtained [8]. Cells were counted by a blinded investigator (one of the authors, who was unaware of the treatment). Different layers of stained cells could be distinguished in separate focal planes of the whole mounts. All cells of different layers in one field were counted under a microscope with 20X or 40X objective lens.

2.4. Statistical Analysis. Quantitative data were expressed as means ± standard deviation (SD) and were analyzed with one-way analysis of variance (ANOVA) followed by Significant Difference Procedure (LSD) test for multiple comparisons among experimental groups with control groups. Statistical analysis was performed using the SPSS 11.5 (SPSS Inc., Chicago, IL) statistical software. P value less than .05 was considered statistically significant.
3. Result

3.1. Inflammatory Manifestation of EIU. In addition to the control group, ocular inflammatory signs were observed in the each rat of remaining four groups after LPS injection. Conjunctival edema, ciliary congestion, and blood vessels dilatation in the iris began to appearance within 4~6 h after the LPS injection. At 12~16 h, aqueous flaring and fibrinous pupillary membrane was seen, reaching a maximum at 22~24 h. Occlusion of pupil even was found by slit microscope (Figure 1(a)). Generally, inflammation subsided gradually after 24 h, and the exudation had decreased 48 h after LPS injection (Figure 1(b)). At 72~76 h, fibrinous pupillary membrane had been completely absorbed, but only mild ciliary congestion remained. The score of EIU in Wistar rat at different time was described in Figure 2(a) and Table 1.

3.2. Histologic Changes. H-E staining results were consistent with inflammatory manifestations in Wistar rats at 24 h after LPS immunization. A large number of infiltration of inflammatory cells and fibrin exudations could be seen in the anterior and posterior chamber (Figure 3(a)); massive neutrophil adhered behind corneal endothelial cell (Figure 3(b)); thickened iris stroma with vasodilatation and a majority of inflammatory cell infiltration in the vitreous were observed (Figure 3(c)).

3.3. Expression of TLR4. TLR4 positive cells were brown, which were located in the cellular membrane. TLR4 could not be detected in the iris-ciliary body complex in 0 h group (Figure 4(a)); at 12 h, much TLR4 positive cells were found around blood vessels (Figure 4(b)); the number of TLR4 positive cells significantly increased in the iris and ciliary body of all rats at 24 h (Figure 4(c)) and reached the peak at 48 h (Figure 4(d)). The number of positive cells had reduced at 72 h (Figure 4(e)). There was statistical significance to positive cells overall among these groups (F = 46.79, P < .05 ANOVA). A small amount of positive cells were also seen in ciliary body. However, no positive cells could be detected in negative control with a nuclear counterstain (Figure 4(f)).

3.4. Expression of MyD88. MyD88 positive cells were mainly located in the cytoplasm. The trend of changes in MyD88 expression was consistent with the TLR4 (Figure 2(b)). MyD88 could not be detected at 0 h, but positive cells were observed in iris at 12 h (Figure 5(a)) and reached the maximum during 24~48 h (Figures 5(b) and 5(c)). The number of positive cells had reduced at 72 h (Figure 5(d)). No positive cells could be detected in negative control. There was statistical significance to positive cells among these groups (F = 54.37, P < .05 ANOVA).

3.5. Expression of NF-κB p65. NF-κB p65 could not be found at 0 h group, but NF-κB p65 positive cells were located in the cytoplasm or nucleus at other groups and its expression was gradually increased from 12 h to 48 h. Immunopositive cells were predominantly round-ovoid cells mainly scattering in iris stroma. The number of positive cells had reduced at 72 h. There was statistical significance to positive cells among these groups (F = 85.32, P < .05, Figures 6(a)–6(d)).

4. Discussion

Uveitis is a common inflammatory disease that was a potential threat to visual loss, which mainly affects iris, ciliary body, and choroid [9]. At present, the pathogenic mechanisms of uveitis is not clear. The majority of uveitis may be caused by nonimmune factors, only a small part of the infectious uveitis is due to pathogen invasion. Acute anterior uveitis, especially HLA-B27-associated AAU is a common noninfectious uveitis, but clinical and laboratory research have proven gram negative bacteria such as Klebsiella, Salmonella, Yersinia, and Shigella species can trigger it [10]. TLR-4 is a main receptor that recognizes lipopolysaccharide of gram-negative bacterial cell wall. In our study, TLR4 was not expressed in the normal Wistar rat iris whereas Chang et al. [11] and Brito et al. [12] found that TLR4 positive cells expressed in the normal human iris-ciliary body. This may be due to the different subjects and methods applied since TLR4 expression is too low in normal Wistar rat to be detected by iris stretched preparation technology. In our study, we found that TLR4...
Table 1: Clinical scoring of endotoxin-induced uveitis and density of immunopositive cells in the rat iris.

<table>
<thead>
<tr>
<th>Time (after LPS injection)</th>
<th>Clinical grade (mean ± SD, n = 10)</th>
<th>TLR4⁺ (Cell/mm², n = 10)</th>
<th>MyD88⁺ (Cell/mm², n = 10)</th>
<th>NF-κB p65⁺ (Cell/mm², n = 10)</th>
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</thead>
<tbody>
<tr>
<td>0 h</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
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<tr>
<td>12 h</td>
<td>2.2 ± 0.4</td>
<td>167.9 ± 61.5</td>
<td>154.3 ± 41.6</td>
<td>220.0 ± 54.6</td>
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<tr>
<td>24 h</td>
<td>4.0 ± 0</td>
<td>780.0 ± 191.9</td>
<td>1040.7 ± 209.2</td>
<td>402.1 ± 55.4</td>
</tr>
<tr>
<td>48 h</td>
<td>3.1 ± 0.3</td>
<td>917.9 ± 194.6</td>
<td>1034.6 ± 267.3</td>
<td>807.9 ± 177.2</td>
</tr>
<tr>
<td>72 h</td>
<td>1.7 ± 0.5</td>
<td>573.6 ± 113.3</td>
<td>650.8 ± 97.8</td>
<td>150 ± 57.9</td>
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</table>

Figure 2: (a) The clinical score of EIU at different time (Data were expressed as means ± SD): the intensity of the anterior segment inflammation was evaluated at 0–12 h inflammation reached a maximum at 24 h inflammation subsided gradually after 24 h. (b) Expression of TLR4, MyD88 and NF-κB p65 during iris at different time (Data were expressed as means ± SD): positive cells gradually increased at 0–24 h. Expression of MyD88 at 48 h compared with 24 h was no significant difference (P = .940). Expression of TLR4 and NF-κB p65 at 48 h compared with 24 h was significant difference (P = .049, P = .000). Expression of TLR4 and NF-κB p65 declined at 72 h.

was expressed in iris-ciliary body after LPS administration. The inflammatory response reached the maximum at 24 h after LPS administration, then the degree of inflammatory response was gradually reduced, but TLR4 positive cells continued to increase until 48 h in the iris. TLR4 expression significantly decreased at 72 h comparing with it at 48 h (P < .001). In our study, we had observed that the changes of TLR4 were relative to the degree of anterior segment inflammation, which suggested that LPS-related gram negative bacteria could excessively activate TLR4-mediated innate immunity and adaptive immunity that may resulted in incidence of AAU.

Upon LPS recognition, TLR4 undergoes oligomerization and recruits its downstream adaptors through interactions with the TIR (Toll-interleukin-1 receptor) domains, resulting in inflammatory reaction finally [13]. TLR4 signaling has been divided into MyD88-dependent and MyD88-independent (TRIF-dependent) pathways. Our study found that many MyD88 positive cells were expressed in the iris at 24 h after LPS administration, peaked at 48 h, and then gradually decreased. The curve of MyD88 expression is consistent with TLR4. It showed that TLR4 activated its downstream signaling molecules through a MyD88-dependent pathway conduction in the pathogenesis of AAU. Su et al. [14] reported that MyD88-deficient mice were completely resistant to experimental autoimmune uveitis (EAU) in Th1 mediated autoimmune response. Taken together, these findings suggest MyD88-dependent pathway plays an essential role in LPS/TLR4 signaling.

After MyD88 activation, another adaptor protein TRAF6 (TNF receptor-associated factor 6) is critical for the MyD88-dependent pathway. It leads to the phosphorylation of IκB proteins which makes NF-κB/I-κB trimer complex degradation. Subsequently, NF-κB is activated and transferred into nucleus [13]. In our study, NF-κB p65 positive cells could not be observed in normal Wistar rat iris. At 12 h after LPS immunization, NF-κB p65 positive cells were detected in the cytoplasm and nucleus of iris, reaching the maximum at 48 h,
and then had decreased at 72 h. Compared to TLR4 and MyD88, NF-κB p65 reduction is more obvious from 48 h to 72 h, which can better reflect attenuation of inflammation directly. It is consistent with Chi et al. [15] report in that NF-κB p65 was activated in iris-ciliary body after a footpad injection of LPS in the rats. Under the normal circumstances, the p65 subunit of NF-κB binds with its inhibitor I-κB to form I-κB-NF-κBp50/p65 trimeric complex, which is in the nonactivation state. When the I-κB inhibitory protein degrades, NF-κB antibody recognizes and activates p65 [16]. In addition, Todaro et al. [17] found that NF-κB was highly expressed in Behcet’s peripheral blood T cells, which contributes to the regulation of the apoptosis-related factors and death receptors leading to apoptosis resistance in BD T cell subsets. In the research, the reduction of NF-κB p65 may be induced in the down regulation of the peripheral lymphocyte apoptosis by this transduction pathway, which results in regression of inflammation reaction. We found more positive TLR4 and MyD88 cells than NF-κB p65 cells in the same field at 72 h, which may be due to feedback in the pathway. Further studies, including functional studies, are required to identify the roles of nuclear factor in the network of uveitis.
Figure 5: Immunohistochemistry of MyD88 (DAB): (a) The MyD88 positive cells were shown at 12 h. (b) Most of MyD88 expressed in the iris at 24 h. (c) The immunopositive cells were predominantly round-ovoid cells at 48 h. (d) MyD88 positive cells had been decreased in the iris at 72 h (Bar = 20 μm).

Figure 6: Immunohistochemistry of NF-κB p65 (DAB): (a) The NF-κB p65 positive cells were shown at 12 h. (b) The NF-κB p65 positive cells increased at 24 h. (c) NF-κB p65 located in the cytoplasm or nucleus at 48 h. (d) NF-κB p65 positive cells had been decreased in the iris at 72 h (Bar = 10 μm).
5. Conclusions

Our study revealed that the expression of TLR4, MyD88, and NF-κB p65 in the iris changed during EIU. These findings suggest the important role of TLR4 and its associated factors in the pathogenesis of uveitis and will provide some insightful ideas of the mechanism of uveitis.

Acknowledgments

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References

Review Article
Pattern Recognition via the Toll-Like Receptor System in the Human Female Genital Tract

Kaei Nasu and Hisashi Narahara

Department of Obstetrics and Gynecology, Faculty of Medicine, Oita University, Oita 879-5593, Japan

Correspondence should be addressed to Kaei Nasu, nasu@med.oita-u.ac.jp

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The mucosal surface of the female genital tract is a complex biosystem, which provides a barrier against the outside world and participates in both innate and acquired immune defense systems. This mucosal compartment has adapted to a dynamic, non-sterile environment challenged by a variety of antigenic/inflammatory stimuli associated with sexual intercourse and endogenous vaginal microbiota. Rapid innate immune defenses against microbial infection usually involve the recognition of invading pathogens by specific pattern-recognition receptors recently attributed to the family of Toll-like receptors (TLRs). TLRs recognize conserved pathogen-associated molecular patterns (PAMPs) synthesized by microorganisms including bacteria, fungi, parasites, and viruses as well as endogenous ligands associated with cell damage. Members of the TLR family, which includes 10 human TLRs identified to date, recognize distinct PAMPs produced by various bacterial, fungal, and viral pathogens. The available literature regarding the innate immune system of the female genital tract during human reproductive processes was reviewed in order to identify studies specifically related to the expression and function of TLRs under normal as well as pathological conditions. Increased understanding of these molecules may provide insight into site-specific immunoregulatory mechanisms in the female reproductive tract.

1. Introduction

The mucosal surface of the respiratory, gastrointestinal, and urogenital tracts separates the external environment from the internal sterile environment and thus represents the first line of defense against microbes. These mucosal innate systems consist principally of mechanical, chemical, and cellular components. The first of these, the mechanical component, primarily carries out the physical barrier function of the mucosa, but also includes physiological functions such as ciliary action, motility, desquamation, and mucous secretion. The second component, the chemical component, can be further divided into three subcomponents: soluble or cell-associated pattern recognition molecules, proteins, and peptides, which are responsible for orchestration of the immune response. The third component of the innate immune system is the cellular component, which includes epithelial cells, stromal fibroblasts, and various inflammatory leukocytes.

Mucosal epithelial cells constitute a crucial part of the innate immune system and are actively engaged in the first line of defense against microbial infections. Defense at the epithelial barrier includes the mechanical aspect of preventing penetration of the structure by microorganisms. The mucosal epithelial cells are known to function as sentinels that recognize antigens, and they respond in a manner leading to bacterial and viral eradication, as well as send signals to underlying immune cells. When a pathogenic challenge exceeds the protective capacity of the mucosal epithelial cells, they trigger a series of alarm signals resulting in the secretion of chemokines ultimately important for the recruitment of other components of the innate defense network, which in turn leads to the development of an acute inflammatory reaction. Binding of a pathogen with the epithelium can lead to cell death by necrosis, apoptosis, or internalization of the organism; however, the invading organism may remain on the cell surface and induce disease from this location. At this stage, increased vascular permeability
leads to extravasation of acute-phase proteins and protein complement into the damaged tissue. Also affected are the endothelial adhesion molecules that reduce the activity of phagocytic granulocytes, allowing them to leave blood vessels and be transported along a chemotactic gradient towards the pathogen. These various steps, which run parallel rather than in a sequential manner, are controlled by cytokines secreted from the cellular components (e.g., epithelial cells and inflammatory leukocytes) of the innate system. While these immediate mechanisms are in progress, antigenic material is processed by dendritic cells and macrophages for presentation to T cells, a process which represents the initiation of the more slowly developing acquired responses. However, the mechanisms leading to these latter responses differ in the mucosal linings of different organs [1].

The major purpose of the innate immune system is to react rapidly to infectious agents with the initiation of an inflammatory response, and to form subsequent adaptive immune responses. After a pathogen makes contact with the epithelial surface, signals are generated that result in the production of chemokines, cytokines, prostaglandins, and leukotrienes by the epithelium, signaling cell injury [2]. However, the pathogen also interacts with other components of the innate immune system such as dendritic cells and macrophages. The basis of this activation of the innate immune system is pattern recognition [3]. Pathogens are characterized by specific arrangements of key molecules called pathogen-associated molecular patterns (PAMPs) and are recognized by pattern recognition receptors (PRRs). The PAMPs are vital structures of the microbial cell that have altered little over evolutionary time spans and include lipopolysaccharide (LPS), lipoproteins, peptidoglycan (PGN), lipoarabinomannan, and oligosaccharides. The PRRs are found on many cells of the innate immune system including epithelial cells, fibroblasts, and inflammatory leukocytes. There are several different families of PRRs such as scavenger receptors, Toll-like receptors (TLRs), nucleotide binding oligomerization domain- (NOD-) like receptors, retinoic-acid-inducible protein (RIG)-I-like receptors, formyl peptide receptors, mannose and glycan receptors, C-type lectin receptors, complement receptors, and CD14. Among the PRRs, TLRs are capable of sensing organisms ranging from bacteria to fungi, protozoa, and viruses, and they play a major role in innate immunity.

2. Structure and Characteristics of Human Female Genital Mucosa

The female genital tract is composed of a sequence of cavities. The external genital tract at the vulva leads into the vagina, which connects in succession to the uterine cervix, the endometrium, and then to the fallopian tubes (Figure 1). The lumen of the lower genital tract (vagina and ectocervix) is lined with squamous epithelium; whereas the upper genital tract (endocervix, endometrium, and fallopian tubes) is lined with columnar epithelium. The surface epithelium serves a critical function, that is, as the defensive front line of the mucosal innate immune system in the female genital tract. Under normal conditions, the mucosa of the female genital tract appears to be in a state of controlled inflammation.

The upper genital tract is virtually free of organisms, with little commensal microbial activity [4]. Recent evidences suggest that there is a site-specific mucosal immune system in the female upper genital tract, including the endometrium and fallopian tubes, which differs from that described for the intestinal, respiratory, and lower genital tracts. This putative immune system in the fallopian tubes and endometrium might contribute to the maintenance of an aseptic milieu, free of the microorganisms that sporadically colonize the upper genital tract. It is essential that the mucosal epithelium of the upper genital tract has the capacity to recognize and respond to ascending pathogens, while at the same time avoiding a state of chronic inflammation that might disrupt the epithelial barrier. The upper genital tract is vulnerable to the spread of microorganisms from the lower genital tract, resulting in the development of infectious diseases such as endometritis and salpingitis [5]. The sequelae of such chronic inflammation of the female genital tract would be highly detrimental to the host and would include increased transmission of sexually transmitted diseases [6].

The cervical and vaginal epithelium is constantly exposed to microorganisms including species of commensals as well as pathogenic organisms; as anaerobic bacterial flora is normally present in the vagina. Therefore, the mucosal surface of the lower genital tract represents a complex biosystem that provides a barrier against the outside world and participates in both innate and acquired immune defense systems. This mucosal component has adapted to a dynamic, nonsterile environment challenged by a variety of antigenic/inflammatory stimuli associated with sexual intercourse and endogenous vaginal microbiota. The cervicovaginal epithelial cells that line the mucosal surface are often the first cells to come into contact with microbial pathogens; normally there are very few immune cells that present in the cervicovaginal mucosa and lumen [6, 7]. The cervicovaginal epithelial cells initiate and coordinate the inflammatory response at this mucosal barrier.
response, altering the adjacent epithelium and the underlying stromal fibroblasts and immune cells to counter the potential danger posed by various microorganisms.

3. Pattern Recognition via the TLR System in Humans

Rapid innate immune defenses against microbial infection usually involve the recognition of invading pathogens by specific PRRs recently attributed to the family of TLRs. TLRs are present in plants, invertebrates, and vertebrates and they represent a primitive host defense mechanism against microorganisms [8–11]. As shown in Table 1, TLRs recognize conserved PAMPs synthesized by microorganisms including bacteria, fungi, parasites, and viruses as well as endogenous ligands associated with cell damage, such as heat-shock protein 60, heat-shock protein 70, polysaccharide fragments of heparin sulfate, hyaluronic acid, fibrinogen, fibronectin DA domain, and mRNA [12]. Members of the TLR family include 10 TLRs identified in humans thus far, which recognize distinct PAMPs produced by various bacterial, fungal, and viral pathogens. The recognition of bacterial PAMPs (e.g., LPS, PGN, flagellin) is mediated by TLR1, 2, 4, 5, and 6 [13–16]. Among these TLRs, four are designed to recognize nucleic acids: TLR3, TLR7, TLR8, and TLR9 [17–20]. TLR7 and TLR8 recognize nucleotide derivatives, such as self and viral single-stranded RNA [19, 20], and TLR9 binds unmethylated DNA found in bacteria [17]. In contrast, TLR3 recognizes double-stranded RNA (dsRNA) [18], a molecular signature of RNA viruses [21]. Therefore, it is likely that TLR3 plays a physiological role in antiviral innate immunity [22].

TLRs are transmembrane signaling proteins that are designed to recognize, with high specificity, various proteins, lipids, carbohydrates, and nucleic acids of invading microorganisms. In turn, TLRs activate signaling cascades in cells that can trigger immune and inflammatory responses to combat the infectious agent [3, 23, 24]. Although every member of the TLR family responds to a specific ligand, they all share strong similarities in terms of their structures and properties [25]. TLR proteins are located on either the plasma membrane or internal membranes. Their cytoplasmic signaling domain is separated by a single membrane-spanning domain from the ligand-recognizing extracellular or luminal domain, which contains multiple repeats of a leucine-rich repeats (LRRs) motif XXLXXLXX. The 19–25 tandem copies of LRRs are thought to provide a highly specific binding surface for the cognate ligand. The cytoplasmic domain of the TLR family shares extensive homology with that of the interleukin (IL)-1 receptor family and is referred to as the toll-IL receptor (TIR) domain, which extends to about 200 residues [26, 27]. Ligand binding to TLRs leads to a common signal transduction pathway involving TIR, which couples with adaptor molecules including MyD88 [28] that binds to TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR10 [29–31]; MAL/TIRAP, a MyD88 homologue that binds to TLR1, TLR2, TLR4, and TLR6 [32, 33]; TRAM that binds to TLR4; TRIF/TICAM1 that binds to TLR3 and TLR4 [34] (Figure 2) [28, 29]. There are two main pathways activated by the TLR family, the MAL/MyD88-dependent and the MyD88-independent TRAM/TRIF pathway. Signaling through MyD88 activates nuclear factor (NF)-κB and induces many cytokines including tumor necrosis factor (TNF)-α and IL-6. Stimulation of TRIF signaling pathway activates the interferon (IFN) regulatory factor (IRF) family to induce production of type I IFNs [35, 36]. Subsequently, a signaling complex is formed that includes the IL-1 receptor-associated kinases ( IRAKs), Tollip and TNF receptor-associated factor 6 (TRAF-6), transforming

<table>
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<tr>
<td>TLR1</td>
<td>triacyl lipopeptides, modulin (bacteria)</td>
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<tr>
<td></td>
<td>Pam3Cys-Ser-(Lys)4 (synthetic lipoprotein)</td>
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<tr>
<td>TLR5</td>
<td>flagellin (bacteria)</td>
</tr>
<tr>
<td>TLR6</td>
<td>diacyl lipopeptide, modulin, soluble tuberculosis factor (bacteria)</td>
</tr>
<tr>
<td>TLR7</td>
<td>ssRNA (virus)</td>
</tr>
<tr>
<td></td>
<td>ssRNA (host)</td>
</tr>
<tr>
<td>TLR8</td>
<td>imidazoquinoline (synthetic antiviral compound)</td>
</tr>
<tr>
<td></td>
<td>loxoribine (guanosine analog)</td>
</tr>
<tr>
<td>TLR9</td>
<td>unmethylated CpG DNA (bacteria, protozoa, virus)</td>
</tr>
<tr>
<td></td>
<td>hemozoin (protozoa)</td>
</tr>
<tr>
<td></td>
<td>CpG-ODN (synthetic CpG-rich oligonucleotide)</td>
</tr>
<tr>
<td></td>
<td>Chromatin-IgG complex (host)</td>
</tr>
<tr>
<td>TLR10</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
growth factor (TGF)-β-activated kinase (TAK1), and the TAK1 binding proteins TAB1 and TAB2. The formation of this complex ultimately results in the phosphorylation of the inhibitor of NF-κB (IκB) and the activation of the NF-κB pathway [30, 31, 37]. In this manner, TLRs regulate a number of consequences such as the production of proinflammatory cytokines, the upregulation of costimulatory molecules on antigen-presenting cells, and the maturation of naive dendritic cells. TLR binding to microbial ligands is thus a key step in the acute inflammatory response.

It should be noted that cell surface TLRs (TLR1, TLR2, TLR4, TLR5, and TLR6) appear to recognize microbial products such as LPS or lipopeptides; whereas intracellular TLRs (TLR3, TLR7, TLR8, and TLR9) recognize nucleic acids [28, 29]. The availability of endogenous ligands and the amount of cell-surface TLRs are both tightly restricted to maintain sufficient TLR responses for the containment of pathogens, without inducing detrimental responses in the host. All of the nucleic acid-recognizing TLRs are expressed on the endosomal membranes of cells, rather than on plasma membranes; hence, ligand binding by the LRR motifs of these TLRs occurs in the lumen of intracellular vesicles. It is generally accepted that the extracellular nucleic acids released from damaged tissues or cells, infected or uninfected, are endocytosed and presented to the internal TLRs. Alternatively, nucleic acids from bacteria or viruses, which multiply within a cell, can be captured in membranous vesicles and then transported to TLRs in the endosomes.

Activation of these TLRs leads to the induction of interferons, proinflammatory cytokines, and chemokines. Cell-surface TLRs also sense endogenous ligands, released in damaged tissue as a danger signal, resulting in the induction of inflammation under both infectious and noninfectious conditions. In a number of recent studies, TLRs have been found on a wide range of cells, including immune cells such as mast cells, macrophages, and dendritic cells. TLRs are also found on epithelial cells and mesenchymal fibroblasts, and these cells recognize microbial infections by sampling the exterior milieu using a group of receptors that are able to discriminate between potential pathogens and self-produced molecules.

4. Expression and Function of TLRs in the Human Female Genital Tract

4.1. TLR1, TLR2, and TLR6. TLR2 is structurally related to TLR1 and TLR6 [38]. TLR2 forms heterodimers with TLR1 and TLR6, which is involved in discriminating between the molecular structures of diacyl and triacyl lipopeptides [39–41]. Complexes of TLR1 and TLR2 recognize various microbial components, such as lipoproteins/lipopeptides, lipoarabinomannan, and PGNs from gram-positive and gram-negative bacteria and mycoplasma [14, 42, 43]. It also recognizes lipoteichoic acid from gram-positive bacteria, a phenol-soluble medulin from Staphylococcus aureus, glycolipids from Treponema pallidum [14, 44], GPI anchors
of protozoa [45], and zymosan and phospholipomannan from fungi [46, 47]. TLR2 is also reported to be involved in the recognition of atypical LPS from nonenterobacteria, the structures of which are different from the typical LPS of gram-negative bacteria [44, 48]. Experimentally, TLR2 has been shown to recognize synthetic lipopolysaccharides [Pam3Cys-Ser-(Lys)4] [40]. During microbial infection in the female genital tract, TLR2 is considered to recognize the PGN of C. trachomatis [42, 49, 50], LPS and fragments of PGN of Neisseria gonorrhoeae [51–53], and phospholipomannan of Candida albicans [54]. TLR2 is also involved in the recognition of viral components such as cytomegalovirus and herpes simplex virus type 1 [55–57]. CD36, a member of the class II scavenger family of proteins, was shown to serve as a facilitator or coreceptor for diacyl lipopeptide recognition through the TLR2/6 complex [58]. Both TLR2 and TLR6 are necessary for responding to mycoplasma-associated protein.

Constitutive TLR1 and TLR6 expression has been detected in the epithelial cells of the fallopian tubes, endometrium, endocervix, ectocervix, and vagina [59–67]. TLR1 expression has also been detected in uterine NK cells [68], vascular endothelial cells, and smooth muscle cells within the struma of the cervix and the myometrial smooth muscle cells of the uterus [69]. Whereas, TLR6 expression was detected in uterine NK cells [51] and in stromal fibroblasts within the vagina [63].

Constitutive expression of TLR2 has been reported in the epithelial cells of the fallopian tubes, endometrium, cervix, and vagina [59, 60, 62–66, 69, 70], smooth muscle cells of the cervix and vagina [63, 71], endometrial stromal cells [70], and uterine NK cells [68, 72]. The highest levels of TLR2 mRNA expression have been observed in the fallopian tubes and cervical tissues, followed by the endometrium and ectocervix [60]. The expression levels of TLR2 in endometrial stromal cells were comparable to those of endometrial epithelial cells. Significantly higher levels of expression of TLR2 and TLR6 in the endometrium have been observed during the secretory phase than in other phases of the menstrual cycle [56, 67, 73]. TNF-α upregulates the TLR2 expression in human cervical smooth muscle cells [71].

Pam3Cys-Ser-(Lys)4, a synthetic analog of bacterial lipopeptides that bind to TLR2/1 heterodimers, was found to induce the production of MIP-3α and TNF-α by endometrial epithelial cells [74]. Polyriboinosinic : polyriboycytidylic acid [poly (I : C)], a TLR3 agonist, induced the expression of TLR2 in human fallopian tube epithelial cells [65]. FLS-1, a TLR2/6 heterodimer agonist, induced the expression of proinflammatory cytokines and chemokines in the epithelial cells of cervix and vagina [66]. Lipoteichoic acid inhibits human cytomegalovirus infection in ectocervical tissue through induction of IFN-β production [75]. Mycoplasma genitalium and the C-terminal portion of the antigenic protein encoded by MG309 activate NF-κB via TLR2/6, resulting in cytokine secretion from the epithelial cells of the uterine cervix and vagina [37]. Whereas, polyionic microbicides, such as dextran sulfate and polystyrene sulfonate inhibits TLR1/2- and TLR2/6-mediated cytokine production by human cervical and vaginal epithelial cells [76]. TGF-β is reported to inhibit the TLR-2-mediated activation of uterine NK cells [72].

4.2. TLR3. TLR3 recognizes dsRNA and is considered to mediate various antiviral responses. dsRNA during viral infection can arise from several sources [25]. The genome of the infecting virion can itself be dsRNA, as in the case of the known natural dsRNA viruses. However, even ssRNA virus samples often contain defective particles contain primarily double-stranded defective genomes. Intracellular viral dsRNA can be generated in a number of ways. In the case of ssRNA viruses, the formation of dsRNA replication intermediates is an obligatory step in viral reproduction. In the case of DNA viruses, complementary mRNAs are often produced that are encoded by partially overlapping genes located on the opposite strands of the viral genome. Long viral polyencrionic mRNAs often contain abundant stable double-stranded stems. Such findings, taken together, have indicated that all viral infections induce dsRNA at some point during replication [77]. Recently, host-derived mRNA released by dying or dead cells was shown to activate TLR3, suggesting that activation via TLR3 can occur in a variety of situations [78]. It has been demonstrated that a secondary structure creating hairpin loops within the mRNA is responsible for TLR3 activation. In addition, TLR3 has been shown to recognize double-stranded nucleic acid from Schistosoma mansoni and to be involved in the antiparasite response [79]. At present, it is generally accepted that RNA from a number of different sources can activate TLR3, as long as the RNA displays a secondary structure containing double-stranded regions, provided that the RNA is present in the appropriate cellular vesicle.

In experimental models, poly (I : C), a synthetic analog of viral dsRNA, is utilized as a ligand for TLR3 to mimic viral infection [18, 22, 65, 80, 81]. The induction of TLR3 signaling via dsRNA activates transcription factors such as NF-κB and IRF3, resulting in the production of proinflammatory and antiviral cytokines and chemokines [18, 82–84]. In the clinical setting, a variety of viruses (e.g., herpes simplex virus, human papilloma virus, hepatitis B virus, hepatitis C virus, cytomegalovirus, human immunodeficiency virus, etc.) can be the causative of viral infection in the female genital tract.

Constitutive expression of TLR3 has been reported in female genital tissue samples from fallopian tubes, endometrium, cervix, and vagina [60, 65, 66]. The expression of TLR3 in the endometrium is significantly higher during the secretory phase than in other phases of the menstrual cycle [67, 83]. TLR3 expression has been detected in the epithelial cells of the fallopian tubes, endometrium, endocervix, ectocervix, and vagina [59, 62–65, 70, 84–87]. TLR3 expression was also detected in endometrial stromal cells, although expression levels were higher in endometrial epithelial cells than in endometrial stromal cells [70]. Jorgenson et al. [85] recently demonstrated the cycle-dependent expression of TLR3 in primary endometrial epithelial cells. TLR3 expression was also detected in the stromal fibroblasts of the vagina and endocervix [63], and in human uterine NK cells [68, 72].
Poly (I:C), a TLR3 agonist, induces the expression of proinflammatory cytokines, chemokines, and TLR3 in human fallopian tube epithelial cells [65, 87]. Cultured fallopian tube epithelial cells recognize viral dsRNA via TLR3 and secrete proinflammatory cytokines and chemokines via an NF-κB-mediated signal pathway [87]. Poly (I:C) alone does not stimulate IFN-γ production by IL-2-expanded uterine NK cells [88]. Whereas, the presence of autologous uterine macrophages led to a significant increase in IFN-γ production by IL-2-expanded uterine NK cells [88]. Whereas, polyanionic microbicides, such as dextran sulfate and polystyrene sulfonate, inhibit TLR3-mediated cytokine production by human cervical and vaginal epithelial cells [76].

It has been shown that human endometrial epithelial cells recognize dsRNA and produce proinflammatory cytokines and chemokines via a TLR3-mediated pathway [64, 85, 89]. Lesmeister et al. [90] showed that in vitro treatment of endometrial epithelial cell lines with 17β-estradiol had no effect on TLR3 expression, and treatment with 17β-estradiol suppressed the production of proinflammatory cytokines and chemokines resulting from TLR3 stimulation with poly (I:C); these findings suggest that 17β-estradiol modulates TLR3 function. Poly (I:C) also upregulated the production of IL-8 by the epithelial cells of the uterine cervix [66, 86] and the production of proinflammatory cytokines and chemokines by the epithelial cells of the vagina [66]. In addition, Poly (I:C) activated these cells and induced IFN-γ production [75]. TGF-β was shown to inhibit the TLR-3-mediated activation of uterine NK cells [91]. Poly (I:C) inhibits human cytomegalovirus infection in ectocervical tissue through induction of IFN-β production [75].

4.3. TLR4. LPS is a cell-wall component of gram-negative bacteria. LPS is composed of lipid A (endotoxin), core oligosaccharide, and O-antigen. TLR4 recognizes lipid A of LPS. In addition to bacterial LPS, TLR4 also recognizes heat-shock protein 60, glycoinositolphospholipids of protozoa [45], and viral envelope proteins [92–94]. The ligation of TLR4, in association with the accessory molecules MD-2 and CD14, leads to the recruitment of MyD88, the phosphorylation of IL-1 receptor-associated kinase, the oligomerization of TNF receptor-associated factor 6, and the subsequent degradation of IkB [30, 31]. These events lead to the activation of NF-κB, and to the resultant transcription of immune response genes, such as proinflammatory cytokines, chemokines, and costimulatory molecules, which are necessary for further immune responses [8, 95, 96]. The host response to a primary bacterial infection of a mucosal surface is acute inflammation and is characterized by the infiltration of neutrophils and monocytes. In the clinical setting, a variety of microorganism-derived substances including LPS derived from N. gonorrhoeae [51–53], LPS and heat shock protein derived from C. trachomatis [97, 98], and mannan derived from C. albicans [54] are putative ligands for TLR4 in the female genital tract.

Constitutive expression of TLR4 was reported in the following female genital tissues: the fallopian tubes, the endometrium, the cervix, and the vagina [60, 66]. TLR4 expression has been shown to decline progressively along the genital tract, with the highest levels of expression in the fallopian tubes and endometrium, followed by the cervix [60]. The expression of TLR4 in the endometrium is significantly higher during the secretory phase compared with that in other phases of the menstrual cycle [67, 83]. Conflicting findings regarding the expression of TLR4 in the epithelial cells of the female genital tract have been reported. Some authors’ groups have reported the presence of TLR4 in the epithelial cells of the fallopian tubes [63, 65], endometrium [62–64, 70, 99], endocervix [63, 66], and vagina [57, 66]. However, other authors have observed an absence of TLR4 in the epithelial cells of the fallopian tubes [100], endocervix [59], ectocervix [59, 63], and vagina [59, 63]. The expression of TLR4 has also been detected in endometrial stromal cells [70], myometrial cells [101], uterine NK cells [68, 72], and smooth muscle cells of uterine cervix [71]. Levels of expression of TLR4 were found to be higher in endometrial stromal cells than in endometrial epithelial cells [70].

CD14, a coreceptor of TLR4 for the recognition of LPS, is not expressed in human fallopian tube epithelial cells or stromal cells [100]. However, CD14 has been detected in endometrial stromal fibroblasts, although it was not found in endometrial epithelial cells [99]. Whereas, Herbst-Kralovetz et al. [66] demonstrated the expression of CD14 in the epithelial cells of cervix and vagina. CD14 is known to be expressed in human cervical smooth muscle cells [71]. MD2, an accessory molecule of TLR4-signaling, was found to be absent from cultured epithelial cells derived from samples of normal human vagina, endocervix, and ectocervix [59].

Binding to LPS, a TLR4 ligand, rapidly leads to NF-κB activation and cytokine expression via TLR4-mediated signaling in fallopian tube stromal fibroblasts [100]. However, fallopian tube epithelial cells that lack TLR4 do not respond to LPS. LPS was shown to stimulate the expression of IL-8 in endometrial epithelial cells and stromal fibroblasts via a TLR4-mediated pathway [99]. LPS also induced the production of MIP-3α in primary endometrial epithelial cells [74], but not in an endometrial epithelial cell line, HHUA [102]. It has been demonstrated that cultured endocervical epithelial cells were unresponsive to LPS from either N. gonorrhoeae or Escherichia coli [59]. LPS induced the translocation of the NF-κB p65 subunit in human myometrial cells via a TLR4-protein kinase ζ-mediated pathway [101]. IFN-γ was also found to enhance the expression of TLR4, CD14, MD2, and MyD88 in endometrial stromal fibroblasts [99]. LPS inhibits human cytomegalovirus infection in ectocervical tissue through induction of IFN-β production [75].

4.4. TLR5. TLR5 recognizes flagellin, a protein component of bacterial flagella [15]. It has been suggested that TLR5 serves as a sensor for pathogenic bacteria that is able to cross the epithelium [103].

TLR5 expression has been demonstrated in epithelial cells derived from the fallopian tubes, endometrium, vagina, endocervix, and ectocervix [59–65]. TLR5 expression was
also detected in smooth muscle cells and vascular endothelial cells within the stroma of the vagina and endocervix [63]. However, TLR5 expression was not detected in human uterine NK cells [68]. The expression of TLR5 in the endometrium is significantly higher during the secretory phase than during other phases of the menstrual cycle [67, 83].

Flagellin, a TLR5 agonist, induced the expression of proinflammatory cytokines and chemokines in the epithelial cells of cervix and vagina [66].

4.5. TLR7. TLR7 has been shown to recognize self- and guanosine- or uridine-rich viral ssRNA from viruses such as HIV, vesicular stomatitis virus, and influenza virus [19, 20]. TLR7 signaling is also induced by low molecular-weight antiviral compounds, that is, imidazoquinolines [104].

TLR7 expression has been detected in the epithelial cells of fallopian tubes, endometrium, cervix, and vagina [61, 65, 66, 84, 105]. TLR7 expression was also detected in uterine NK cells [68] and in the endometrial stroma [69].

Poly (I:C), a TLR3 agonist, was shown to induce the expression of TLR7 in human fallopian tube epithelial cells [65]. Imiquimod, a TLR7 agonist, was demonstrated to stimulate IL-8 production by the primary cultured cells isolated from fallopian tube, endometrium, and cervix [105].

4.6. TLR8. TLR8 has been shown to recognize both self- and guanosine- or uridine-rich viral ssRNA from viruses such as HIV, vesicular stomatitis virus, and influenza virus [19, 20, 106].

TLR8 is expressed in the epithelial cells of fallopian tubes, endometrium, cervix, and vagina [61, 63–65, 73, 86, 105]. TLR8 expression was also detected in the endometrial stroma, as determined by immunohistochemical analysis [73]. However, no TLR8 expression has been detected in human uterine NK cells [66]. CL075, a TLR8 agonist, was demonstrated to stimulate IL-8 production by the primary cultured cells isolated from fallopian tube, endometrium, and cervix [105].

4.7. TLR9. TLR9 recognizes DNA containing unmethylated deoxytidyl-phosphate-deoxyguanosine (CpG) motifs common to both bacterial and viral genomes [17, 104, 107]. CpG motifs are found in the genomes of DNA viruses such as herpes simplex virus [108–110], suggesting that TLR9 induces antiviral responses in herpes genitalis. TLR9 also recognizes nonDNA pathogenic components, such as hemozoin and genomic DNA derived from protozoa malarial parasites [43, 111]. Experimentally, TLR9 signaling has also been induced by synthetic CpG-rich oligonucleotides (CpG-ODN) [17].

TLR9 expression has been reported in fallopian tube, endometrium, and cervix [105]. TLR9 expression has been demonstrated in epithelial cells of the fallopian tube, endometrium, cervix, and vagina [61, 62, 64–66, 70, 73, 86]. TLR9 expression was also detected in endometrial stromal cells [70, 73]. The authors of the latter study noted observing comparable expression levels of TLR9 in endometrial epithelial cells and stromal cells. However, higher levels of expression of TLR9 in the endometrium have been reported during the secretory phase than during other phases of the menstrual cycle [67, 73]. Moreover, TLR9 expression was absent in human uterine NK cells [68].

CpG oligodinucleotides upregulated the production of IL-8 by the epithelial cells of the fallopian tube and uterine cervix [65, 86]. CpG oligodinucleotides, a TLR9 agonist, was demonstrated to stimulate IL-8 production by the primary cultured cells isolated from fallopian tube, endometrium, and cervix [105]. To date, there have been no studies of TLR9 expression in the vaginal mucosa. CpG oligodinucleotides inhibit human cytomegalovirus infection in ectocervical tissue through induction of IFN-β production [75].

4.8. TLR10. A specific ligand for TLR10 has yet to be identified [23, 112, 113]. TLR10 expression has been demonstrated in fallopian tube, but not in endometrium or cervix [105]. TLR10 expression is absent in human endometrial epithelial cells and endometrial epithelial cell lines [60, 64]. However, Aflatoonian et al. [73] demonstrated using immunohistochemistry that TLR10 is expressed in the endometrial epithelium and stroma. The same authors also reported that levels of expression of TLR10 in the endometrium are significantly higher during the secretory phase than during other phases of the menstrual cycle [73]. TLR10 expression has also been detected in human uterine NK cells [68]. TLR10 has not been detected in human fallopian tube epithelial cells [65]. Higher levels of expression of TLR9 in the endometrium have been reported during the secretory phase than during the proliferative phase of the menstrual cycle [67].

5. Conclusions

It has been suggested that there is a site-specific mucosal immune system in the female upper genital tract that differs from that described in the gastrointestinal and respiratory tracts [114]. Furthermore, the immune system in the upper genital tract differs from that of the lower genital tract. The putative immune system in the upper genital tract appears to contribute to the maintenance of an aseptic milieu; that is, this immune system inhibits the growth of microorganisms that sporadically colonize this region [4]. In contrast, the lower genital tract is constantly exposed to microorganisms, including species of commensals as well as pathogenic organisms; in general, abundant anaerobic bacterial flora is known to be present in the vagina. The mucosal components of the lower genital tracts have adapted to a dynamic, nonsterile environment challenged by a variety of antigenic/inflammatory stimuli associated with sexual intercourse and endogenous vaginal microbiota. Clearly, it is essential that these mucosal tissues develop mechanisms for selectively responding to pathogens, while simultaneously avoiding chronic inflammation due to immune responses to commensal microorganisms [5]. The sequela of a chronic inflammation in the female genital tract would be
highly detrimental to the host and would include increased transmission of sexually transmitted diseases [6].

As summarized in this paper, the innate immune system in the female genital tract is highly complex and multifactorial. Mucosal epithelial cells, fibroblasts, lymphocytes, macrophages, and dendritic cells associated with the female genital tract have evolved a unique mechanism for the recognition of pathogens. These cells express a variety of TLRs, allowing them to recognize the different repertoire of a wide range of PAMPs. It is likely that TLR distribution in the female genital tract reflects an immunological tolerance of commensal organisms in the lower portions of the tract (i.e., vagina, ectocervix, and to some extent, the endocervix), as well as an intolerance of commensal microbial flora in the upper portion of the tract (i.e., the endometrium and fallopian tubes). The mucosal surface of the upper portion of the female genital tract is generally considered a sterile site, in part due to the cervical mucus, which filters bacteria and other debris. However, this barrier can readily be crossed by a variety of infectious agents, typically leading to endometritis and salpingitis. Thus, it is essential that the upper genital tract epithelium has the capacity to recognize and respond to ascending pathogens while simultaneously avoiding a state of unnecessary inflammation that might disrupt the epithelial barrier. The sequelae of such inflammation in the upper genital tract would be highly detrimental to the defense and reproductive functions of the mucosal surface. If the luminal epithelial barrier is broken by acute inflammation, damaged epithelial cells initiate and coordinate the inflammatory response, alerting adjacent epithelium and underlying immune cells of the potential danger posed by various microorganisms.

STDs are a major worldwide health problem that compromise reproductive fecundity and cut short the lives of millions of men, women, and children [115, 116]. Despite extensive efforts, only limited success has been achieved in dealing with STDs such as N. gonorrhoeae, C. trachomatis, group B streptococcus, herpes simplex virus type 2, and HIV. These pathogenic organisms can be recognized by TLRs expressed in the female genital tract. Further investigation into TLR signaling in these tissues could provide new insights into the roles played by the immune system in maintaining health and combating STDs and other genital infectious diseases.

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References


Mediators of Inflammation


Review Article

Toll-Like Receptors Expression and Signaling in Glia Cells in Neuro-Amyloidogenic Diseases: Towards Future Therapeutic Application

Dorit Trudler, Dorit Farfara, and Dan Frenkel

Department of Neurobiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Sherman building, Room 424, Tel Aviv 69978, Israel

Correspondence should be addressed to Dan Frenkel, dfrenkel@post.tau.ac.il

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Toll-like receptors (TLRs) are known to be expressed by innate immune response cells and to play a critical role in their activation against foreign pathogens. It was recently suggested that TLRs have an important role in the crosstalk between neurons and glial cells in the central nervous system (CNS). TLR signaling was reported to be associated with a yin-yang effect in the CNS. While TLR signaling was linked to neurogenesis, it was also found to be involved in the pathogenesis of neurodegenerative diseases. This paper will focus on TLR signaling in glial cells in neurodegenerative diseases such as Alzheimer’s disease, prion diseases, amyotrophic lateral sclerosis, and Parkinson’s disease. Understanding the pattern of TLR signaling in the glial cells may lead to the identification of new targets for therapeutic application.

1. Introduction

Toll-like receptors (TLRs) recognize conserved pathogen-associated molecular patterns (PAMPs) of bacteria, viruses, yeast, fungi, and parasites [1]. At least 13 TLR genes exist in mammals, and functional ligands have been identified [2]. TLRs 1–9 are expressed in both mice and humans, whereas TLRs 10–13 are expressed only in mice [3].

A tight control of the TLR pathway is essential for maintaining homoeostasis, since overactivation of TLRs has been linked to various infectious and inflammatory diseases. TLR engagement leads to the activation of the transcription factor nuclear factor κB (NF-κB), which regulates the induction of proinflammatory cytokines such as tumor necrosis factor α (TNFα), Interleukin-1β (IL-1β), and Interleukin-6 (IL-6). It can also activate members of the mitogen-activated protein kinase (MAPK) family including p38 and c-Jun N-terminal kinase (JNK) [3]. These kinases are involved in the transcription of genes and regulate mRNA stability. A tight regulation of these pathways results from post-translational modification processes.

Activation of TLR-mediated signaling by various agonists does not always involve a straightforward lock-and-key mode of ligand-receptor binding. The extracellular domains of all TLRs share important structural features, yet mediate responses to widely different agonists, pointing out that more complex interactions are involved. Many additional proteins are required for the activation of TLR-mediated signaling by their agonists, including coreceptors and docking molecules on the cell surface and binding catalysts that promote certain interactions, such as heat shock proteins [4–6]. In addition to the essential contribution by coreceptors and accessory interaction partners, most TLRs also operate as homo- or heterodimers.

2. TLR Signaling Pathways

TLRs are type 1 transmembrane glycoproteins characterized by the presence of a leucine-rich repeat (LRR) domain and a Toll/IL-1 receptor (TIR) domain [7]. LRRs are found in a diverse number of proteins and are involved in ligand recognition and signal transduction [8]. The intracellular
TIR domain portion consists of approximately 200 amino acids and contains sequences that are highly conserved among family members. TLRs are proposed to dimerize following ligand binding, resulting in the recruitment of TIR domain-containing adaptor molecules to initiate downstream signaling through interactions within the TIR regions [9].

The family of TLRs share structural properties of not only their extracellular LRR structures but also their intracellular domains, which interact with intracellular adaptor proteins that relay the agonist engagement signal. Currently, five such adaptors are known. The dominant and founding member of this family of adaptors is myeloid differentiation factor 88 (MyD88), which relays the signal for most TLR family members and tends to predominantly induce an NF-kB-mediated activation of genes, including those encoding TNF-α, chemokine (C-C motif) ligand 5 (CCL5 or RANTES), IL-1β, and chemokine interleukin 8 (IL-8; CXCL8). The less dominant adaptors are MyD88-adaptor-like protein (Mal), TIR domain-containing adaptor inducing interferon-β (TRIF), and TRIF-related adaptor protein (TRAM). Recent evidence indicates that TRIF associates with TRAF6 and induces NF-kB signaling toward cytokine and chemokine production [10]. The fifth TIR adaptor SARM (sterile-α and HEAT/Armadillo motifs-containing protein) has been shown to inhibit TRIF [11].

TLR signaling may lead to different responses in distinctive cell types through an interaction with MyD88 unique variants. Not every cell expresses the same set of adaptors. For example, it is rapidly becoming clear that a selective expression of the less-frequently used type of MyD88 in neurons, renders these cells uniquely sensitive to TLR-expression of the less-frequently used type of MyD88 in neurons, renders these cells uniquely sensitive to TLRs [14–17]. All structures of TLRs bound to their ligands, provide an understanding of ligand-induced activation of the JNK pathway to apoptosis, instead mediated activation of genes, including those encoding TNF-α, chemokine (C-C motif) ligand 5 (CCL5 or RANTES), IL-1β, and chemokine interleukin 8 (IL-8; CXCL8). The less dominant adaptors are MyD88-adaptor-like protein (Mal), TIR domain-containing adaptor inducing interferon-β (TRIF), and TRIF-related adaptor protein (TRAM). Recent evidence indicates that TRIF associates with TRAF6 and induces NF-kB signaling toward cytokine and chemokine production [10]. The fifth TIR adaptor SARM (sterile-α and HEAT/Armadillo motifs-containing protein) has been shown to inhibit TRIF [11].

TLR signaling may lead to different responses in distinctive cell types through an interaction with MyD88 unique variants. Not every cell expresses the same set of adaptors. For example, it is rapidly becoming clear that a selective expression of the less-frequently used type of MyD88 in neurons, renders these cells uniquely sensitive to TLR-mediated activation of the JNK pathway to apoptosis, instead the NF-kB pathway towards inflammatory response as in glia cells. In this way, selective expression of adaptors strongly influences the quality of the response mounted by different types of cells to a given TLR agonist.

TLR4 was the first TLR to be identified as an orthologue of Drosophila Toll [12, 13]. Structures of TLR2, 3, and 4 with their ligands have been recently elucidated and provide an understanding of ligand-induced activation of TLRs [14–17]. All structures of TLRs bound to their ligands, reveal a common “M”-shaped architecture. The C-termini of the extracellular domains converge, therefore allowing the interaction between TIR domains to occur and initiate downstream signaling events [18].

TLR ligands encompass a broad spectrum of pathogens. Each pathogenic ligand binds to a specific receptor, for example: TLR2 plays an important role in the recognition of fungal, gram-positive, and mycobacterial components. TLR2 can form a complex with TLR1 or TLR6 and respond to lipopeptides from a wide variety of microbes. The TLR1–TLR2 dimer responds to triacylated lipopeptides, whereas TLR2–TLR6 responds to diacylated lipopeptides. TLR3 recognizes double-stranded RNA (dsRNA). TLR4 is responsible for the recognition of lipopolysaccharide (LPS) while TLR5 is responsible for the recognition of bacterial flagellin [19]. Single-stranded RNA is recognized by TLR7 and TLR8, and TLR9 recognizes DNA which can be either host or pathogen derived [3]. TLR9 is associated with the cellular response against bacterial cytosine phosphate guanosine (CpG) DNA [20]. Interestingly, several TLR family members, including TLR2 and TLR6, appear to cooperate in the recognition of different PAMPs in macrophages [21, 22]. For example, the TLR2-mediated response to phenol-soluble modulin is enhanced by TLR6 but inhibited by TLR1, indicating functional interactions between these receptors [23].

3. TLR Signaling Mediates Glial Cell Activation in the CNS

Innate immunity in the CNS depends primarily on the functions of glial cells, especially astrocytes and microglia, which are important for the early control of pathogen replication, direct recruitment, and activation of the adaptive immune system required for pathogen recognition and clearance [24].

Under resting conditions, in rodents in vivo, TLRs 1–9 have been detected in the CNS by quantitative real-time PCR, with particularly strong expression of TLR3 [25]. The levels of TLRs in the CNS can be upregulated by viral and bacterial infection, treatment with TLR stimuli, or CNS autoimmunity [25–28], providing a mechanism for amplification of inflammatory responses to pathogens infecting the CNS. Human glial cells do not necessarily display the same TLR profile as rodent glial cells. Immunostaining of cultured microglia and astrocytes for TLR3 and TLR4, revealed two opposite features. Both TLR3 and TLR4 were found exclusively localized in vesicular structures inside microglia and not on the surface of the cells. However, with cultured astrocytes, TLR3 and TLR4 were found only on the cellular surface [26]. This striking difference in subcellular localization of TLRs between microglia and astrocyte may relate to the difference in phagocytic and antigen processing properties of these cells [29–32].

3.1. TLR in Microglia. Microglial TLRs are crucial as a first line of defence against bacterial or viral infection. In response to the appearance of multiple bacterial or viral TLR agonists, TLR-mediated signaling promotes the production of a variety of inflammatory mediators (reviewed by [33, 34]). In addition, phagocytosis is stimulated by TLR activation, which may be particularly relevant to the clearance of bacteria as well as aggregated or abnormal proteins such as amyloid fibers from the CNS [35, 36] (Figure 1). Like other macrophage-like cells, microglia can express essentially all different TLR family members. While TLR expression is hardly detectable in resting microglia in a healthy CNS, multiple TLRs rapidly appear upon activation of the cells. Primary microglia in vitro constitutively express a wide array of TLRs (TLRs 1–9) at varying levels [26, 37]. Constitutive expression of TLRs is primarily in microglia and largely restricted to the circumventricular organs (CVOs) and meninges, areas with direct access to the circulation, although they may be expressed at lower levels in other regions, too [28, 38, 39]. This unique localization allows
the CNS to recognize pathogens which are present in the periphery as well as those that invade the CNS. Like in macrophage, TLRs are exclusively found within endosomal vesicles of microglia, illustrating their primary role in examining the phagocytosed debris. While microglia express all TLRs at readily detectable levels [26], TLRs 1–4 are the most dominant, with TLR2 being the most highly expressed TLR compared to other family members; this applies to microglia in rodents as well as in humans. Lehnard et al. [40] have indicated that microglial cells are the major cell type that expresses TLR4 in the mouse brain.

Exogenous and endogenous TLR ligands activate microglial cells. TLRs may mediate different pathways in microglia leading to either neuroprotective or neurotoxic phenotypes. The activation of microglia with peptidoglycan as TLR2 ligand [35], LPS as agonist for TLR4 [41], or TLR9 ligand CpG [42], markedly boosted the ingestion of apoptotic cells as TLR4 ligand [42], or LPS as agonist for TLR4 [35], markedly boosted the ingestion of apoptotic cells [42], or LPS injection may produce a variety of factors that are well-known mediators of both neuroprotection, such as ciliary neurotrophic factor, neurotrophin-4, and vascular endothelial growth factor, and anti-inflammatory cytokines, such as TGF-β, IL-10 and IL-11. Indeed, when poly I:C, an agonist for TLR3, is added to organotypic human brain slice cultures, survival of neurons significantly improves [48].

Astrocyte take much longer than microglia to either upregulate TLRs or produce cytokines and growth factors in response to TLR activation. Moreover, TLR3-mediated activation of astrocyte leads to a strong induction of indoleamine 2,3-dioxygenase [50]. This enzyme converts extracellular tryptophan into kynurenine, thereby reducing its concentrations in the microenvironment, which in turn markedly enhances the sensitivity of any nearby T-cell for Fas-ligand-induced apoptosis [51]. In this way, the TLR3-mediated induction of indoleamine 2,3-dioxygenase in astrocyte acts as a local immune-suppressive factor [50].

3.3. TLR in Neuronal Cells. Accumulating evidence indicates that TLRs play a role in tissue development, cellular migration, differentiation, and repair processes, especially in response to endogenous molecular ligands. Convincing evidence indicates that neurons can express different functional TLRs, including TLRs 2, 3, 4, and 8 [52–58]. As in other cells, expression levels are dynamic, and influenced by soluble mediators including interferon-γ, or by energy deprivation [56, 57, 59]. TLR3 was found to be expressed in cultured human neurons following viral infection [60], and on neurons in human brain tissue in cases of rabies or herpes simplex virus infection [61].

Most TLRs, except TLR3, that are expressed in different cells such as glial cells, signal via the founding family member of the MyD88 family, which predominantly activates an NF-κB-mediated response. The neuronal MyD88 variant, on the other hand, associates with mitochondria, microtubules, and JNK3, and regulates neuronal death during deprivation of oxygen and glucose. Preferred expression of MyD88 in neurons confers a different quality of TLR responsiveness to these cells as compared to cells such as glial cells that do not express this MyD88 variant, but use other adaptors to relay TLR-mediated signaling. As a consequence, TLR3, which is concentrated in the growth cones of neurons, triggers growth cone collapse [55]. TLR2 and 4 induce apoptotic death [54], and TLR8 inhibits neurite outgrowth and triggers apoptosis [53]. In all these mentioned phenomena, signaling pathways operate independently from NF-κB. Clearly, by introducing different MyD88 variants as the dominant adaptor for TLR-mediated intracellular signaling, neurons turn most TLR-mediated signals into negative signals for growth, development, and even survival.

Engagement of TLR4 on neurons induces the expression of nociceptin, an opioid-related neuropeptide [58]. However, this response differs from TLR4-mediated responses in many other cells, in that neurons distinctly use the co-receptor
Figure 1: Microglia TLR signaling in neurodegenerative diseases. Abnormal amyloid deposition in different neurodegenerative diseases may activate microglial cells through TLRs. Microglial activation may lead to further neuronal damage through secretion of proinflammatory cytokines (green), such as IL-6 and TNF-α, or to neuroprotection by secretion of anti-inflammatory cytokines (blue), such as IL-10, which may prevent further neuronal death. Furthermore, recent reports suggest the role of TLRs 2, 4, and 9 signaling in modulating the phagocytosis (red) and clearance of the neurotoxic amyloid deposition.

MD-1, instead of the routinely used MD-2, along with CD14 as interaction partners for binding the TLR4 agonist LPS. This illustrates that neurons actually modulate the TLR-signaling platform by not only introducing unusual intracellular adaptors for unique signaling pathways, but also by employing uncommon surface coreceptors, which modulate the response.

4. TLR Signaling Link to Neurotoxicity

Although the stimulation of TLRs on glial cells activate functions that are important for the elimination of pathogens, these same functions can be toxic to cells of the CNS that have limited regenerative capacity. LPS exposure causes profound microglial activation associated with oligodendrocyte death, demyelination, and increased vulnerability of neurons to injury, dependent on TLR4 signaling [40, 62]. Similarly, microglia exposed to group B streptococcus (GBS) or S. pneumoniae serotype 2 also display neurotoxic properties, dependent on TLR2 [63, 64]. Some reports have demonstrated that LPS-stimulated astrocytes are also neurotoxic, while others have shown that only microglia are required for toxicity [40, 65]. Toxicity appears to be mediated primarily via NO. Indeed, pharmacologic blockade of iNOS is able to prevent neuronal death in the presence of activated glia [64, 65] and to rescue substantia nigra neurons from death [66].

Stimulation of astrocytes with TLR ligands also inhibits their ability to uptake excess glutamate [65, 67], and therefore the role of astrocyte in neurotoxicity may be more critical in models in which glutamate excitotoxicity is a major mechanism of death.

Injection of poly I:C or Pam3CysSK4 into the CNS can cause neurodegeneration in a TLR3- or TLR2-dependent manner, respectively [63, 68]. Local injections of LPS directly into the CNS cause severe loss of dopamine neurons in the substantia nigra [69] and neurons in the hippocampus [70]. Newborn neurons in the hippocampus and dopaminergic neurons in the substantia nigra appear to be extremely sensitive to the effects of LPS, as peripheral injection of even low levels of LPS reduces the number of these cells [71, 72].
5. TLR Signaling Link to Neurogenesis

Neurogenesis is the process by which new neurons are created from neural progenitor cells in the adult brain. It occurs in two major brain regions—the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus [73]. The mechanisms for neurogenesis are emerging with time. Recent evidence suggest that neural progenitor cells also express TLRs [53, 74]. Rolls et al. [74] have proposed that since TLR2 is widely expressed in the brain, and in cells that express early neuronal markers, they may be involved in adult hippocampal neurogenesis. They have demonstrated that in TLR2-deficient mice, there is a reduction in the differentiation of neural progenitor cells into neurons, and an increase in the differentiation into astrocytic cells, and that TLR2 activators increased differentiation. The increase in differentiation was mediated through the NF-κB pathway. In addition, TLR4 has been found to be involved in proliferation via both MyD88-dependent and MyD88-independent pathways. A TLR4 deficiency caused an increase in proliferation and differentiation [74].

TLR8 is dynamically expressed during mouse brain development and localizes to neurons and axons where it may regulate neurite outgrowth and apoptosis [53]. TLR2 and TLR4 are expressed in adult neural progenitor cells and may influence the proliferation and differentiation [74]. Some TLRs are strongly expressed in the embryonic brain and TLR3 and TLR8 have been implicated in neurogenesis and neurite outgrowth in the developing brain whereas TLR2 and TLR4 have been shown to regulate adult neurogenesis [75, 76].

In embryonic development, TLR3 negatively regulates neural progenitor cell differentiation [77]. It has also been suggested recently that in the adult brain TLR3 mediates the production of anti-inflammatory and neuroprotective factors, and thus a TLR3 activation may promote cell survival [48]. TLR8 is a suppressor of murine neurite outgrowth and induces neuronal apoptosis, through an NF-κB independent mechanism. [59]

6. TLR Signaling in Neurodegenerative Diseases

A dysfunction of glial and neuronal receptors, which alter the cells sense of their environment, is of interest that a polymorphism in TLR4, which results in cell activation towards Aβ depositions, which results in a decrease in the amount of proinflammatory cytokines IL-6 and TNF-α and NO. TLR4 has also been found to contribute to Aβ-induced microglia neurotoxicity. A tri-molecular complex consisting of TLR4, MD-2, and CD14 has to be complete for full cellular stimulation by aggregated Aβ. There is also an elevated level of TLR4 in transgenic APP mice and in the brains of AD patients. [86].

Another line of evidence suggests that in AD there is a form of TLR dysfunction that appears in TLR4, which is localized on the surface of microglial cells (Figure 1). A loss-of-function mutation in TLR4 inhibits microglial cell activation towards Aβ depositions, which results in an increase in the amount of proinflammatory cytokines IL-6 and TNF-α. TLR4 has also been found to contribute to Aβ-induced microglia neurotoxicity. A tri-molecular complex consisting of TLR4, MD-2, and CD14 has to be complete for full cellular stimulation by aggregated Aβ. There is also an elevated level of TLR4 in transgenic APP mice and in the brains of AD patients. [86].

Tahara et al. [36] have shown that TLR4 loss-of-function mutation in APP transgenic mice increase diffuse and fibrillar Aβ deposition without an increased expression of APP, and that uptake of Aβ is reduced in TLR4 mutant microglia. They have also demonstrated that the activation of TLR 2, 4, and 9 increased clearance of Aβ [36, 42]. Balistreri et al. reported that a TLR4 polymorphism is involved in aging, and in some age-related diseases such as AD [91]. The phenotypes are associated with changes in cytokine expression. One such haplotype has reduced the production of proinflammatory cytokines [92]. AD patients
in the Italian population had an increased frequency of the proinflammatory haplotype [91].

Lotz et al. [93] showed that coadministration of Aβ 1–40 with TLR2 or TLR4 agonists, Pam3-cys and LPS, respectively, led to an additive release of NO and TNF-α. However, coadministration of Aβ 1–40 with TLR9 agonist CpG, led to a decrease in the release of NO and TNF-α. This suggests that not all TLR agonists enhance the stimulatory effect of Aβ on innate immunity [93]. In microglia (Figure 1), the TLRs functionally interact with other cell surface receptors, including CD36, αβ integrin, CD47, and scavenger receptor A, which bind to fibrillar Aβ, to initiate the activation of intracellular signaling pathways [94, 95].

6.1.1. Targeting TLR as Therapeutic Application in AD. APP mouse models with a TLR4 deficiency have an increase in insoluble Aβ in the cortex, as compared to TLR4 wild-type APP mouse models [36]. Thus, factors that increase the microglial cell clearance of Aβ, without producing inflammatory mediators, are candidates for the treatment of AD (Figure 1).

These results suggest that the TLR signaling pathways may be involved in the clearance of Aβ deposits in the brain and that TLRs can be a therapeutic target for application in AD [36]. Indeed, it was shown that an injection to the intrahippocampus of LPS derivatives (a TLR4 ligand) to the brains of AD mice reduced Aβ load, suggesting that the activation of microglia by TLR4 may be therapeutic in AD [96].

Bisdemethoxycurcumin is a natural curcumin, a minor constituent of turmeric (curry), that enhances phagocytosis and the clearance of Aβ in cells from most AD patients, and increases transcription of the MGAT and TLR genes [97]. Furthermore, administration of CpG, a TLR9 activator, in APP transgenic mice, resulted in clearance of Aβ from microglial cells [42].

In conclusion, TLR activation may modulate glial cell activity in AD. Recent research suggests the involvement of TLRs 2, 4, 5, 7, and 9 in the proinflammatory response of microglia toward Aβ, which may be linked to neurotoxicity (Figure 1). Nevertheless, the activation of TLRs 2, 4, and 9 were also linked to both phagocytosis of the neuropathological response towards TLR9, which may lead to neuroprotection (Figure 1). Therefore, elevated expression levels of TLRs 2, 4, and 9, through genetic modification or toward specific agonists, may be a therapeutic application in AD. Indeed, a recent publication [98] suggests the use of TLR2 and 4 agonist as a specific macrophage activator to increase the clearance of Aβ in an AD mouse model. An alternative therapeutic approach may be the reduction of TLR5 and 7, by using shRNA or specific antagonists (Figure 1).

6.2. Prion Diseases. Prions are infectious particles that are composed mainly of proteins. In prion diseases, prions create extracellular aggregates of beta-sheet-rich, misfolded form, in different tissues, such as the spleen, muscles, and brain. In the brain, aggregated prions are presented by neurons, followed by neurodegeneration. Prion diseases are characterized by their transmissibility and are therefore also termed transmissible spongiform encephalopathies (TSE). Prion diseases have occurred in humans and animals for many years. The human prion disease is Creutzfeldt-Jakob disease (CJD) [99]. All known prion disease affect the structure of the brain with neuropathological features such as neuronal loss, astrocytic activation (gliosis), and spongiform change, and all are currently untreatable and fatal [100].

Prions have a role in the activation of the innate immunity, which suggests functional and structural similarities with Drosophila Toll receptors [101]. The mice with mutated TLR4, wherein signaling is prevented, developed Prion disease in a shorter period of time than control mice, but did not exhibit different levels of prions. This indicates an involvement of TLR4 in the progression of the disease [102]. In addition, in human patients with CJD, there is an elevated level of IL-10, which has been suggested to have a protective role in the disease. TLR4 signaling induces IL-10 production, and this may be the pathway by which TLR4 dysfunction may mediate the rapid progression of the disease [103, 104]. Nevertheless, MyD88 knockout mice inoculated with prions have not shown different prion pathogenesis kinetics from the control mice, suggesting that TLRs 1, 2, 6, and 9 signal through the MyD88 pathway and are not involved in the progression of the disease [105, 106] (Figure 1).

6.2.1. Targeting TLR as Therapeutic Application in Prion Diseases. It has been suggested that TLR9 expression may be linked to the progression of prion diseases. Furthermore, treatment with synthetic oligodeoxynucleotides that contain cytosine phosphate guanosine (CpG-ODN) motifs, known to bind to TLR9, have been suggested as possible treatment for prion diseases in a mouse model, by delaying the disease onset [105, 107]. Another explanation may be the effect of CpG-ODN on microglia activation that may lead to prion degradation [108] (Figure 1). Furthermore, as the activation of TLRs in other amyloidogenic diseases, such as AD, has been linked to the clearance of neurotoxic amyloid, it may prove to be a potential therapeutic approach to the prion diseases.

6.3. Amyotrophic Lateral Sclerosis. Amyotrophic lateral sclerosis (ALS) is a devastating and chronic neurodegenerative disease, characterized by selective loss of lower and upper motor neurons [109]. Ten percent of ALS cases are familial (fALS) with 20–25% of these cases resulting from various mutations in the SOD1 gene [110]. The toxicity of the mutant SOD1 (mSOD1) is again of function, because mice that lack the gene do not develop the disease [111], and may be a noncell-autonomous progression [112]. One proposed mechanism for pathogenesis is the aberrant oligomerization of mSOD1 proteins in beta-sheet form, that can be stained by thioflavin S [113].

Several studies have demonstrated the involvement of microglia in ALS pathogenesis (Figure 1). For example, microglia with mSOD1 release more superoxide, nitrate and
nitrite and induce more neuronal death [114]. It has also been demonstrated that mSOD1 mice had an elevated level of TLR1, 2, 7 and 9 at 8 months of age, as compared to the matched age control group [83]. Treatment of mSOD1 mice with wild-type microglia improved the pathogenesis [114].

Kang and Rivest [115] have demonstrated that mSOD1 activates microglia through the MyD88-dependent pathway, and mice that were transplanted with bone marrow from MyD88 knockout mice exhibited earlier disease onset and a shorter life span. This suggests a crucial effect of MyD88 in an ALS mouse model. Nevertheless, there was no difference in the disease onset and life span between mSOD mice with MyD88 knockout and mice with normal MyD88. However, MyD88 knockout mice had a more activated microglia at the end stage of the disease, and they lost more motor neurons, which suggests that the context of MyD88 deficiency is linked to neurotoxicity.

Zhao et al. [116] have demonstrated that mSOD1 binds to CD14, which has a role in the activation and toxicity of microglia treated with extracellular mSOD1. CD14 is a co-receptor of TLRs 2 and 4, and blocking the signaling of both of these TLRs inhibited microglial activation following extracellular mSOD1 administration. However, they have found that CD14 knockout mice showed a similar disease progression profile as the control mice.

Nguyen et al. [117] activated the innate immune response in mSOD1 mice. They injected systemic LPS, which increased TLR2 expression across the brain and spinal cord in both wild-type and mSOD1 mice, without changing mSOD expression. Chronic systemic administration of LPS exacerbated disease progression and motor neuron degeneration, which shortened life span. The degree of TLR2 elevation showed a correlation to motor neuron degeneration.

6.3.1. Targeting TLR as a Therapeutic Application in Amyotrophic Lateral Sclerosis. While some research suggests TLR signaling has an important role in neurotoxicity in ALS, there is no clear evidence for a specific TLR that may mediate this effect. Further research should aim to distinguish between elevated expression of different TLRs in modulating an inflammatory response in ALS and their role in disease progression. A potential link between TLR signaling and an increase in neurotrophic factor secretion from glial cells may prove to be a therapeutic approach in ALS.

6.4. Parkinson’s Disease. Parkinson’s disease (PD) is a progressive neurodegenerative disorder characterized by resting tremor, muscular rigidity, and gait disturbances [118, 119]. PD is pathologically characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta and their terminus in the dorsal striatum [120]. The pathological hallmark of PD is the presence of deposits of aggregated α-synuclein in intracellular inclusions known as Lewy bodies [121, 122].

One of the Parkinson’s disease animal models is composed of an intranigral LPS injection, which stimulates dopaminergic cell death [123]. In this model, microglial cells are activated, and demonstrate an upregulation of proinflammatory cytokines and free radical production [124, 125]. LPS is a TLR4 activator, which suggests that there may be a TLR involvement in the pathogenesis of PD. In an MPTP mouse model of PD there was increased expression of TLR4 and CD14, suggesting an involvement of the TLR pathway in the pathogenesis of PD [126].

6.4.1. Targeting TLR as Therapeutic Application in Parkinson’s Disease. A recent paper showed that α-synuclein immunization in a PD animal model may ameliorate disease progression [127]. Targeting mechanisms in which α-synuclein activates TLR signaling, may open a new horizon for therapeutic application in PD.

7. Conclusion

TLRs play an essential role in modulating self-defense in different types of species: from fly to human. More recently, it has been suggested that TLRs are important to both cell development and cell-cell interaction. The complex of TLR cascade may trigger specific pathways, which affect the fate of cell activation. The CNS is monitored by the BBB from the peripheral immune response, and is dependent on glia surveying for innate immunity. Abnormal amyloid deposits in the CNS may mimic viral or bacterial infection, which may trigger glial cell activation through TLRs. Investigating the neurotoxic and neuroprotective mechanisms of TLR signaling in glial cells may be crucial for understanding their role in the pathogenesis of neurodegenerative diseases, and may pave the route for future therapeutic intervention. Currently, targeting TLRs is being used in different experimental settings, from animal model experiments to clinical trials, including several diseases, such as chronic lung disease and cancer [128]. However, there are very few tests regarding neurodegenerative diseases. We suggest that targeting TLRs and TLR pathways may also be applicable as a possible treatment for those diseases.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
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<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
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<td>Interleukin-6</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor</td>
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<tr>
<td>MyD88</td>
<td>Myeloid differentiation factor 88</td>
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<tr>
<td>CCL5</td>
<td>Chemokine (C-C motif) ligand 5</td>
</tr>
<tr>
<td>CXCL8</td>
<td>Chemokine interleukin 8</td>
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<tr>
<td>Mal</td>
<td>MyD88 adaptor-like protein</td>
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<tr>
<td>TRIF</td>
<td>TIR domain-containing adaptor inducing interferon-β</td>
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</table>
TRAM: TRIF-related adaptor protein
LPS: Lipopolysaccharide
dsRNA: Double-stranded RNA
CVO: Circumventricular organ
NO: Nitric oxide
ROS: Reactive oxygen species
GBS: Group B streptococcus
AD: Alzheimer’s disease
NFT: Neurofibrillary tangles
Aβ: Amyloid β
MGAT3: β-1,4-mannosyl-glycoprotein
TSE: Transmissible spongiform encephalopathies
CJD: Creutzfeldt-Jakob disease
CpG: Cytosine phosphate guanosine.

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Mediators of Inflammation


M. Fiala, P. T. Liu, A. Espinosa-Jeffrey et al., “Innate immunity and transcription of MGAT-III and Toll-like receptors in...


Review Article

Modulation of Toll-Like Receptor Activity by Leukocyte Ig-Like Receptors and Their Effects during Bacterial Infection

Louise E. Pilsbury,¹,² Rachel L. Allen,¹ and Martin Vordermeier²

¹ Centre for Infection, St George’s University of London, Cranmer Terrace, London SW17 0RE, UK
² Veterinary Laboratories Agency, Weybridge, New Haw, KT15 3NB, UK

Correspondence should be addressed to Louise E. Pilsbury, p0904768@sgul.ac.uk

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1. Toll-Like Receptor Activity and Antigen Presenting Cell Phenotype

Toll-like receptors (TLRs) are pattern recognition receptors with the ability to detect microbial products. They can provide the initial danger signal required to alert the body to bacterial or viral infection, playing a pivotal role in the activation of both innate and adaptive immune responses. TLRs recognise pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS), a membrane component of gram negative bacteria; lipoproteins, functional proteins anchored to the cell membrane; flagellin, a major component of flagellum; heat shock proteins which are highly expressed during cellular stress and microbial nucleic acids [1, 2]. Ligation of individual or complex TLRs can activate different signalling pathways. Most TLRs signal via MyD88 resulting in NF-κB or MAPK activation (Figure 1), ultimately leading to the transcription of genes associated with antimicrobial defence such as inflammatory cytokines, costimulation molecules, MHC, and nitric oxide (NO) [3, 4].

TLRs are widely expressed on immune cells and possess distinctive functions dependent on cell type and signalling pathway [5, 15]. We will focus on their effects on dendritic cells and macrophages, which can act as professional antigen presenting cells (APCs). The expression profile of TLRs on APCs varies between subsets (Table 1), which include plasmacytoid DCs (pDCs), myeloid DCs (mDCs), monocyte-derived DCs (moDCs), and macrophages. TLR signalling in these immune cell subsets triggers an activation programme that includes cytokine secretion; pDCs secrete type I interferons (IFN-α) which have a fundamental antiviral function through the recruitment of immune cells and their role in T cell differentiation [16]. In contrast, mDCs and moDCs primarily secrete IL-12, a potent proinflammatory cytokine involved in T cell differentiation and NK cell activation [17]. Macrophages, which provide an initial antimicrobial response, also secrete IL-12, NO, and TNF-α which play an important role in apoptotic cell death and bacterial lysis [18]. In addition to the production of soluble cytokines and chemokines, upregulation of various cell surface markers such as the costimulation molecules and MHC required for antigen presentation is also observed following TLR activity [19, 20]. Of these, the best defined costimulation molecules are CD80, CD86, and B7-H1. Both CD80 and CD86 bind to CD28 on T cells to provide an activating signal, whereas B7-H1 binds to PD1 to generate an inhibitory signal [21]. The
Table 1: Expression profile of TLR and LILR on different APC subsets: this table depicts the known expression levels of TLRs and LILR on subsets of APCs. + denotes high expression, −/+ denotes weak expression, and − is no expression. ? is used where expression levels are yet to be determined [5–14].

<table>
<thead>
<tr>
<th>Monocytes</th>
<th>Macrophages</th>
<th>pDC’s</th>
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The expression profile of TLR and LILR on different APC subsets shows that the expression levels of TLRs and LILR can vary depending on the type of APC. For example, TLR1 and TLR2 are expressed on all subsets, whereas TLR8 is expressed only on macrophages and pDCs. LILR expression is also variable, with some LILRs expressed on all subsets and others only on specific subsets.

type of costimulation molecules upregulated and cytokine profile secreted by an APC is thought to be determined by the activation signal given by individual TLRs, and can determine the nature of the downstream immune response [20, 22, 23].

Upon migration to the lymph nodes, activated DCs present microbial antigens to prime a specific T cell response. MHC class II-restricted antigens are recognised by T helper (Th) cells, which then secrete proinflammatory cytokines to recruit effector cells and aid in B cell maturation. Th cell responses are defined by their cytokine secretion profile. For example, the cytokines IL-12 and IFN-1 dominate Th1 responses, resulting in the recruitment of proinflammatory effector cells and the clearance of infection. Th17 cells are also proinflammatory and through the secretion of IL-17 and IL-22 stimulate, for example, the secretion of antimicrobial proteins from other effector cells. For Th2 responses the cytokines IL-4 and IL-25 dominate, resulting in inhibition of proinflammatory cytokine secretion and further proliferation. Th1 responses are required for pathogen clearance. However, during excessive immune activation, Th1 responses may damage host tissues, raising the possibility of pathology or chronic inflammatory diseases. In order to prevent this, populations of Th2 and regulatory T cells (Tregs) are required, the effects of which regulate the Th1 response [24].

2. LILR and Their Murine Equivalents (PIR and LILRB4)

LILRs are a family of innate immune receptors that are predominantly expressed on antigen-presenting cells and B cells. The eleven members of the human LILRs family are split into three distinct groups: activating, inhibitory and soluble. The LILR classed as inhibitory (LILRB1-5) have a cytoplasmic tail containing 2–4 immunoreceptor tyrosine-based inhibitory domains (ITIMs). LILRs classed as activating (LILRA1-2, 4–6) lack any signalling motif, but instead possess a charged arginine residue which enables association with the adaptor protein FceR1y [45]. To date, this is the only identified adaptor molecule shown to associate with LILR, although it is possible that other adapter molecules are capable of this association. Signalling is then directed through the FceR1y-associated immunoreceptors tyrosine-based activatory domains (ITAMs). Despite this classification, the so-called activating group contains receptors with the ability to exert inhibitory effects, a phenomenon that has been observed for several ITAM-bearing immune receptors and is thought to be related to strength of signalling [46].
LILRs are conserved throughout evolution and to date homologues have been identified in rodents [47], chickens [48], and cattle [49]. In rodents, LILR equivalents are known as paired immunoglobulin-like receptors, a family that contains multiple activating receptors (PIR-A) but only one inhibitory receptor, PIR-B [47, 50–52]. PIRs were classified as LILR homologues due to their similarities in genetic sequence and location, expression profile, structure, and function [53–55]. They have since proved to be an effective tool to examine the role of these receptors [51]. A further murine homologue, LILRB4 was previously known as gp49B [56].

Similar to TLRs, LILR expression varies between APC subsets (Table 1). MDC and moDCs express LILRA2, LILRB1, LILRB3, and LILRB4, whereas pDCs express LILRA4, LILRB1, and LILRB4 [6, 57]. LILRB1, LILRB4 and LILRA4 expression is decreased upon DC maturation [6, 58]. Individual receptors exert their regulatory function in a variety of ways. Upregulation and/or cross-linking of LILRB2 inhibits the upregulation of co-stimulatory molecules on APCs, resulting in T cell anergy [59]. In contrast, signalling through LILRA2 inhibits the upregulation of CD1b, HLA-DR, CD40, CD80, CD86, and CD206, and prevents effective T cell activation and proliferation [60]. Unlike LILRB2, which inhibits APC effector functions by downregulating costimulation molecules, LILRB4 has recently been shown to inhibit APC response to bacterial infection by upregulating IL-10 production and subsequently downregulating IL-8 secretion. Ligation of LILRB4 does not appear to affect the expression of costimulation molecules [61]. However,
Figure 2: Possible pathways for LILR regulation of Toll-Like Receptor Signalling Pathways: There are several possible mechanisms of inhibition LILR receptors may employ to regulate TLRs. SHP-1 has been shown to associate with IRAK1 and inhibit further downstream signalling. Similarly, SHP-2 and SHIP have been shown to inhibit downstream signalling of TBK-1 and PI3K, respectively, thereby dampening down the production of proinflammatory mediators [35–38].

Lu et al., demonstrated that LILRB4 was able to inhibit TNF-α release via inhibition of FcγRII signalling [62], in line with the ability of murine LILRB4 to inhibit inflammatory cytokine production [63]. LILRB4 has also been shown to be highly expressed in patients with malignancy, and this receptor is thought to play a critical role in the induction of tolerance. However, it is not known whether LILRB4 expression on malignant cells is induced by regulatory T cells, or if the LILRB4 expression binds to a T cell ligand rendering them anergic [63].

3. LILR Ligand Specificity and Signalling

Members of the LILR and PIR families have been shown to be involved in bacterial engagement [64]. Both PIR-A1 and PIR-B, and their corresponding human homologues LILRB1 and LILRB3 have been shown to bind bacteria including E. coli, H. pylori, and S. aureus [64]. The bacterial ligand(s) involved in this interaction are yet to be determined and individual receptors vary in their bacterial specificity. However, the most thoroughly characterised ligand for LILR is MHC class I (MHC-I). Unlike other MHC-I-specific receptors, LILRs show a broad specificity for classical and nonclassical forms of MHC-I; LILRB1 and LILRB2 bind all classical MHC-I as well as some nonclassical alleles [65]. LILRB2 has been shown to bind CD1d, which is an MHC-I-like molecule able to present nonprotein antigens to T cells [66]. CD1d is usually recognised by the TCR receptor of NKT cells, which results in the activation of proinflammatory effector cells and target cell lysis. LILR modulation of CD1d activity may therefore be of particular importance in bacterial infections,
which can result in an overaccumulation of lipids in the
infected cell [66, 67].

Inhibitory LILRs have been shown to exhibit their
functions both independently and in association with
activating receptors such as TLRs. Inhibitory LILR carry
their own signalling motifs, varying from 2 to 4 ITIMs in
their cytoplasmic tail. Variation in the number of ITIM
domains has been proposed to result in signal amplification
or the recruitment of alternative signalling molecules [7].
Upon activation phosphorylated tyrosines within an ITIM
become docking sites for either the Src homology 2 domain-
containing phosphatase 1 (SHP-1) or SHP-2, or the SH2
domain-containing 5’ inositol phosphatase (SHIP) [58].
These phosphatases then dephosphorylate key molecules
further downstream in the cascade or those involved in the
ITAM signalling of activating receptors, with consequent
inhibitory effects [7]. SHP-2 is particularly important in both
positive and negative regulation of cellular differentiation
[58]. Although yet to be fully defined, these signalling
patterns are likely to be found mimicked in the modulation
of TLR activation [68, 69].

As mentioned previously, activating LILR has a positively
charged arginine residue within the cytoplasmic domain,
which enables association with adapter proteins, such as
FcεRIγ. When activated, tyrosine molecules in the ITAM
domain of FcεRIγ are phosphorylated by protein tyrosine
kinases of the Src family kinases, creating binding sites
for further signalling molecules, such as zap70 or src [7].
The recruited signalling molecules may differ depending
on cell type, and therefore LILRs may be involved in
modulating a wide range of intracellular signalling pathways
[70].

3.1. LILR-Mediated Control of TLR Functions. LILR-
mediated control of TLR activity has been documented
for several different bacterial infections. In the case of S.
aureus, LILR and PIR-B receptors can bind the pathogen in
conjunction with TLR2 and trigger the release of inhibitory
cytokines such as IL-10 [64]. Inhibition of TLR2 signalling
by PIR-B was confirmed in a study of PIR-B−/− mice, where
an excessive Th2 response was observed, coupled with
impaired DC maturation. This inhibition of DC maturation
was thought to arise from the absence of PIR-B regulation
of a common signalling pathway used by IL-3, IL-5, and
GM-CSF [71].

Evidence of a role for LILR (and their murine homolo-
ugues) providing a counterbalance to TLR activity is most
strikingly illustrated by the high mortality rate of Salmonella
infection for mice lacking the inhibitory receptor PIR-B
[72, 73]. Interestingly, instead of the exacerbated immune
responses that might have been expected in the absence of
an inhibitory receptor, PIR-B-deficient mice were actually more
susceptible to Salmonella infection, caused by a decrease in
phagosomal oxidant production, necessary for bacterial lysis
within lysosomes [72].

Mycobacterium leprae infection can result in tuberculoid
(T-lep) or lepromatous leprosy (L-lep). Patients with T-lep
typically display a localised form of disease, with effective
bacterial clearance. In L-lep, patients suffer from disseminated
disease, with large numbers of bacilli. Although the
factors that influence disease course are currently unknown,
polymorphisms in TLR2/1 are thought to play a role [60]. In
a study by Bleharski et al., gene expression analysis identified
an up to 5.4-fold overexpression of LILRA2 in skin lesions
of L-lep, compared to T-lep [73]. Infected macrophages
stimulated with LILRA2 ligands showed a 40% reduction in
antimicrobial responses, indicating that LILRA2 also inhibits
antimicrobial functions in macrophages [73]. Furthermore,
ligation of LILRA2 considerably reduced IL-12 production
by TLRs, skewing cytokine activity towards a Th2-biased
response. This is important as the immune response in T-lep
lesions (where infection is generally contained then cleared)
is Th1-biased, whereas L-lep with its higher bacterial loads
and disseminated infection is Th2-biased. Therefore, it is
possible that the overexpression of LILRA2 in L-lep results
in an inadequate Th2-biased immune response and a more
severe form of disease [60]. LILRB5, LILRB3, and LILRA3
are also overexpressed in L-lep patient lesions, although their
relevance in infection has yet to be determined [70].

Signalling through different TLRs has been shown to
result in different LILR expression profiles [6, 61, 74]. In
human cells the inhibitory receptors LILRB2 and LILRB4
were upregulated following Salmonella infection, an effect
which appears to be mediated largely by LPS recognition, as
activation of LILRB4 also occurred by both heat-inactivated
Salmonella and Salmonella LPS [61]. In this respect, LILRB4
may play an important role in TLR4 regulation. Similar
relationships exist for other LILR and TLR: LILRA4 has been
shown to regulate TLR7/9 activity in pDC and LILRA2 has
been shown to inhibit TLR4-mediated activity.

In viral infections, the pDC subset plays an important
role in mediating antiviral immunity upon activation by
pathogenic ligands. TLR7/9, together with TLR3 and TLR8
are localised in endosomal/lysosomal compartments [75],
where their activation leads to the production of TNF-
α and IFN-I, a group of potent antiviral cytokines. The
newly-characterised receptor LILRA4 is expressed only on
pDCs, where it appears to play an important role in the
control of their activity in response to viral TLR stimulation.
Following recognition of its ligand, tetherin (also known as
BST2), LILRA4 downregulates TLR7 and TLR9-mediated
production of IFN-α and TNF-α, and decreases calcium
mobilisation [76]. However, LILRA4 activity does not affect
the maturation of the cell, as upregulation of CD80/CD86
is still observed. LILRA4 is also able to inhibit TLR7
and TLR9 signalling after prior antigen stimulation, but
is selectively downregulated upon pDC activation [76, 77].
Similarly in murine models, PIR-B has been shown to
inhibit TLR9-mediated signalling via Brutons tyrosine kinase
(Btk) phosphorylation, which subsequently inhibits NF-kB
activation [78].

4. Modifying TLR Activity through
LILR Signalling

Given the potent effects of LILR on TLR activity, there
is the potential in future to use these receptors as a
tool for therapeutic modulation of TLR signalling. For example, inhibitory LILR could be triggered by their highest affinity self-ligand, HLA-G [70]. This nonclassical MHC-I allele has a restricted distribution of expression, limited to placental trophoblast cells and thymus epithelial cells, but is overexpressed in certain pathologies including nonrejected allografts, HIV infection, and tumours [79, 80]. HLA-G expression is known to trigger the upregulation of LILR [80]. HLA-G also has a natural tendency to form disulphide-bonded dimers which in turn enhance binding to LILRB1 and LILRB2, resulting in enhanced immunosuppressive effects [81]. Thus, there is therapeutic potential for recombinant HLA-G to be used to downregulate TLR effects through enhancing LILR activity.

Enhancing LILR expression would be expected to exert a further dampening effect on TLR activity. Expression levels of LILR can also be enhanced by certain agents; Vitamin D3, Dexamethasone, and niflumic acid have been shown to up-regulate the expression of LILRB2 and LILRB4 on DCs, which is seen with an accompanying increase in IL-10 secretion and Treg differentiation. Although the exact mechanisms involved in tolerance induction in these studies are yet to be fully elucidated, high expression of LILRB4 is thought to be strongly associated with inhibition of NF-κB activation [82, 83]. Recently a study examining the effects of 1,25(OH)2D3 on DCs demonstrated that this agent is able to up-regulate LILRB4 on moDCs and mDCs but not pDCs. This may be due to the fact that the normal expression levels of LILRB4 are markedly higher on pDCs than mDCs [84]. Further to this, the IDO activity in tryptophan (trp) deficient cells was used in a study recently to define mechanisms of DC tolerance and induction of Tregs. Brenk et al. found that upon DC tolerisation, high levels of LILRB3 and LILRB4 were upregulated. However, replacement of trp was unable to reverse the tolerogenic conditions, and DCs continued to stimulate T cells to differentiate into a regulatory phenotype. Furthermore, only by using anti-LILRB4 antibodies were they able to restore any function to the DCs and subsequently the T cells. The authors predict that DC regulation induced in this manner may affect the epigenetics of foxp3 gene transcription and provide antigen-specific Treg cells, therefore providing a mechanism open to therapeutic manipulation [85].

Given the powerful inhibitory nature of LILR, it may be possible to modulate the expression of these receptors prior to or in conjunction with chemotherapies in order to enhance treatment efficacy. Blocking the inhibitory functions of LILR has been demonstrated recently in a study by Morel and Bellón, in which amoxicillin was shown to have the ability to interfere with LILRB1 recognition of MHC class I on NK cells, a finding which could potentially have a large impact on tumour immunology and therapeutics [86]. With further research, the bacterial interaction with LILR will most likely prove fundamental in defining regulatory pathways involved in TLR pathogen responses. As more ligands are discovered for these inhibitory receptors, the potential for development of novel therapies targeting specific LILR allows the possibility of shaping immune responses in disease settings.

References


Review Article

Eicosanoids in the Innate Immune Response: TLR and Non-TLR Routes

Yolanda Alvarez,1 Isela Valera,1 Cristina Municio,1 Etzel Hugo,1 Francisco Padrón,2 Lydia Blanco,2 Mario Rodríguez,1 Nieves Fernández,1 and Mariano Sánchez Crespo 1

1 Instituto de Biología y Genética Molecular, Consejo Superior de Investigaciones Científicas, 47003-Valladolid, Spain
2 Centro de Hemoterapia y Hemodonación de Castilla y León, 47007-Valladolid, Spain

Correspondence should be addressed to Mariano Sánchez Crespo, mscres@ibgm.uva.es

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The variable array of pattern receptor expression in different cells of the innate immune system explains the induction of distinct patterns of arachidonic acid (AA) metabolism. Peptidoglycan and mannan were strong stimuli in neutrophils, whereas the fungal extract zymosan was the most potent stimulus in monocyte-derived dendritic cells since it induced the production of PGE2, PGD2, and several cytokines including a robust IL-10 response. Zymosan activated κB-binding activity, but inhibition of NF-κB was associated with enhanced IL-10 production. In contrast, treatments acting on CREB (CRE binding protein), including PGE2, showed a direct correlation between CREB activation and IL-10 production. Therefore, in dendritic cells zymosan induces il10 transcription by a CRE-dependent mechanism that involves autocrine secretion of PGE2, thus unraveling a functional cooperation between eicosanoid production and cytokine production.

1. Polymorphonuclear Leukocytes and Eicosanoid Production

Myeloid cells are unique cell types as regards their content of high amounts of esterified arachidonic acid (AA) and the enzymes necessary to metabolize free AA into different products via cyclooxygenase and lipoxygenase pathways. However, unlike mast cell, which readily respond to cross-linking FcεRI receptors, and platelets, which release AA in response to classical aggregating agents, less information is available about the physiologically relevant stimuli in polymorphonuclear leukocytes (PMN) and macrophages, since most experiments have been carried out either with xenobiotics or combining chemoattractants with chemicals and priming agents. An example of the first setting has been the use of calcium ionophores. An example of the second situation has been the use of formylated peptides in combination with cytoschalasin B and thapsigargin, which extend the time span of calcium transients and allow the occurrence of Ca2+ dependent events such as translocation of the cytosolic phospholipase A2 (cPLA2) from the cytosol to lipid bilayers [1–4]. However, this scenario has suddenly changed with the emergence of new views on the function of the immune system based on the recognition of microbial patterns.

1.1. Polymorphonuclear Leukocytes Release Arachidonic Acid in Response to Ligands of Pattern Recognition Receptors. PMN are the first blood cell type able to migrate into tissues following microbial invasion. PMN respond to a large set of stimuli, including inflammatory mediators and microbial products. This group of stimuli is most relevant, since microorganisms have unique molecules, termed pathogen-associated molecular patterns (PAMPs), which are recognized through pattern recognition receptors (PRRs) by the host innate immune system. The Toll-like receptor family (for review, see [5, 6]) and nucleotide-binding oligomerization domain family proteins (NOD) (for review, see [7, 8]) are representative of what Janeway first called PRR [9]. C-lectin type receptors are also PRR that may interact with structural signatures expressed in microorganisms. Experiments in human PMN using as stimuli a set of PAMP signatures including the mannose
polymer mannan and peptidoglycan (PGN), a polymer of sugars and amino acids that forms a mesh-like layer outside the plasma membrane of bacteria, showed a robust release of $[^3H]$AA (Figures 1(a) and 1(b)) and the production of leukotriene (LT) B$_4$ and prostaglandin (PG) E$_2$ (Figures 1(c) and 1(d)). The release of $[^3H]$AA observed under these conditions was not blunted by calpeptin, an inhibitor of the formation of microvesicles, but was inhibited by treatment with the cPLA$_2$ inhibitor pyrrolidine-1. The released product was identified as genuine unesterified AA by thin layer chromatography analysis, since the radioactivity detected in the supernatants showed a $R_F$ distinct from that associated with $[^3H]$triglycerides and $[^3H]$phospholipids, which were only observed associated with the cell pellets. The release of AA obtained under these conditions was comparable to that elicited by the so far considered reference stimuli such as the formylated peptide combined with thapsigargin or cytochalasin B, and complement-coated zymosan particles. In sharp contrast, stimuli mimicking other bacterial PAMP, that is, lipoteichoic acid, bacterial lipopolysaccharide (LPS), muramylidipeptide (MDP), and the TLR2 agonist Pam$_3$CSK$_4$ did not induce AA release [10].

The effect of PGN was observed with PGN from both S. aureus and B. subtilis, thus indicating that PGN displaying the structural features of both Gram + and Gram – bacteria are equally active. Preincubation of PMN with anti-TLR2 mAb prior to the addition of PGN did not inhibit AA release, thus suggesting that TLR2 receptors are not involved in the response to PGN. Consistent with this result, Barrett et al. [11] reported TLR2-independent cysteinyll-LT release from mouse bone marrow dendritic cells stimulated with PGN, since the response was intact in TLR2$^{-/-}$ mice. The assignment of the biological effect of PGN to definite PRR has been a matter of debate [12, 13]. Both TLR and NOD receptors have been involved and the controversy stems from the assignment of the biological properties to either the PGN polymer or the block elements MDP and D-glutamic acid-meso diaminopimelic acid. Molecular weight fractionation of S. aureus PGN showed the association of AA-releasing activity with fractions of molecular weight >30 kDa, whereas no activity was detected in the <30 kDa ultrafiltrate, which is consistent with the $M_s$ of soluble PGN. The biological significance of the lipid mediators release by PMN in response to TLR ligands was recently underscored in an in vitro model of migration through endothelial cell monolayers. In this system, PMN migration was inhibited by LTB$_4$ receptor antagonist and platelet-activating factor (PAF) receptor antagonists and was associated with the production of these mediators [14].

1.2. Mechanism of Cyclooxygenase-2 Expression Induction in Human PMN. Current understanding of PMN biology has been modified by recent findings indicating that the life span of PMN can be prolonged by proinflammatory agonists [15], and also by the depiction of mechanisms of translational control of the expression of specific proteins that endow the PMN with the potential for rapid protein synthesis from constitutive mRNA without requiring new transcript generation [16–18]. The possibility that this mechanism could be operative in PAMP-dependent responses and might influence AA metabolism through the expression of COX-2, was a challenging hypothesis.

Since PGE$_2$ is a major product resulting from AA in the PMN (Figures 1(c) and 1(d)) that can be produced both by COX-1, the constitutive isoform of cyclooxygenase, and COX-2, the inducible isoform, the effect of a set of PAMP signatures on the expression of COX-2 was addressed. Unexpectedly, preformed mRNA encoding for COX-2 was detected in resting PMN, whereas COX-2 protein was only detectable after stimulation with either mannan or PGN [19]. COX-1 protein showed the same level of expression in the absence and presence of several stimuli, but well below the level detected in platelets, which are the archetypal source of COX-1. Pam$_3$CSK$_4$ showed a less robust effect and lipoteichoic acid, an agonist of TLR2/TLR6 heterodimers, did not elicit COX-2 protein induction. MDP, which is the archetypal ligand for NOD2, also failed to induce COX-2 expression. Since interaction between NOD2 and specific TLR pathways has been reported as a mechanism of cooperation in the innate immune response that lead to the synergistic activation of host cells [20–22], the effect of the combined addition of both S. aureus PGN and MDP was assessed. This combination of agonists did not modify the effect elicited by PGN alone. The induction of COX-2 protein by PGN was observed as soon as 30 minutes after addition of the stimulus and remained almost unchanged from 1 to 18 hours. A similar trend was observed for both C3bi-coated zymosan and mannan, although a decreasing tendency was observed around 18 hours in response to these ligands. These results indicate that PGN contains a structural signature not acting on NOD2 nor mimicked by lipoteichoic acid and Pam$_3$CSK$_4$, which could act via the TLR route in combination with an additional catch-up receptor(s) and/or by an as yet ill-defined TLR2-independent route.

Since PMN are terminally differentiated cells that contain regulators of transcriptional control and show signal-dependent activation of mRNA translation [17, 18], the hypothesis that COX-2 mRNA could be one of those mRNA controlled in the same manner was put forward. Contrary to this view, one could argue that the calculation of the predicted secondary structure energy of the 5′-untranslated region (UTR) of COX-2 mRNA is ~36.94 kcal/mol, as judged from the application of RNAfold software [23, 24] to sequences available in data banks (Figure 2(a)). This value is lower than that usually associated with transcriptional regulation (~50 kcal/mol); however, it fits well with those reported for many transcripts detected using cDNA library arrays which are regulated at the transcriptional level in human monocytes adherent to P-selectin [25]. Moreover, the presence of four tracts of 5–8 consecutive pyrimidine bases is an additional feature strongly suggesting the possibility of translational control by mammalian target of rapamycin (mTOR). The presence of the 5′-UTR in COX-2 transcripts in human PMN was confirmed by RT-PCR with a set of primers spanning the first 20 nucleotides of exon 1 and exon 2 of COX-2, which gave similar results to PCR reactions using the primers selected from exons 5 and 7 (Figure 2(b)). Preincubation of PMN with 100 nM rapamycin inhibited
the induction of COX-2 elicited by complement-coated zymosan, PGN, and mannan, thereby suggesting that the mTOR route is implicated in the translational regulation of COX-2 protein induction. Given that mTOR is integrated in a signalling cascade, the proximal component of which is phosphoinositide-3 kinase (PI3K), the effect of the PI3K inhibitor wortmannin was addressed. A significant inhibition of COX-2 induction was produced by wortmannin as well as by the translation inhibitor cycloheximide. PGN also induced a time-dependent threonine phosphorylation of eIF4E binding protein. This provides further evidence of the involvement of the mTOR route, since the phosphorylation of this translation inhibitor by mTOR disrupts its binding to eIF4E and activates cap-dependent translation [26].

Additional mechanisms of COX-2 mRNA regulation were explored using transcription inhibitors. Actinomycin D did not influence the induction of COX-2 protein elicited by mannan and PGN, whereas it fully inhibited the response to LPS. Since COX-2 mRNA stability in some cell types is regulated at the 3'-UTR, PMN were incubated in the presence and absence of 1 μg/ml actinomycin D for 30 minutes before the addition of PGN to address the half-life of COX-2 mRNA. In the absence of actinomycin D, 53 ± 7% of the starting COX-2 mRNA was detected in control cells versus 70 ± 9% (mean ± SD, n = 3) in
cells treated with PGN for 2 hours after addition of the stimulus. Actinomycin D treatment induced a further drop of the remaining mRNA in vehicle treated cells, whereas this additional drop was hardly observed in PGN-treated PMN. Further assessment of transcriptional regulation of COX-2 expression was carried out by looking at the effect of 2-hydroxy-4-trifluoromethylbenzoic acid, an inhibitor of both NF-κB and NF-AT [27, 28], which is a useful tool to address in a single step transcriptional regulation since both transcription factors have been involved in COX-2 regulation in different cell types [29, 30]. Hydroxy-4-trifluoromethylbenzoic acid lacked a significant effect on COX-2 protein expression in response to all of the stimuli tested. Taken collectively, these data suggest that transcriptional regulation is not the main mechanism whereby COX-2 expression is regulated in human PMN.

To ascertain whether the above described mechanisms are either a unique property of PMN or are also operative in other myeloid cells, COX-2 protein expression was assayed in monocytes; however, the time course was somewhat different from that observed in PMN, since COX-2 protein steadily increased up to 4–8 hours. Unlike PMN, rapamycin did not influence COX-2 protein expression in monocytes nor in macrophages, whereas actinomycin D significantly blocked COX-2 protein induction expression in response to zymosan, mannan, PGN, and the soluble β-glucan laminarin. These results strongly suggest that different mechanisms can be involved in COX-2 regulation in PMN and mononuclear phagocytes.

2. The Macrophage and Dendritic Cell System

Uptake of phagocytosable particles is strongly dependent on the expression of receptors involved in the recognition of serum proteins displaying opsonic functions such as complement factors and antibodies. This is relevant to the engulfment of fungi and bacteria since these microbes can be coated by the complement factor 3 derived protein C3b and by opsonic IgG class antibodies. The display of receptors on the different cell types including FcγR receptors, complement receptors, and PRR is a key factor to determine the inflammatory and phagocytic responses and it can widely vary among different cell types (Figure 3). In addition, signals elicited upon binding of receptors by their cognate ligands may be balanced by concomitant signals induced by associated PAMP or from the environment, or even by the expression of cell-specific adaptors [31]. This is particularly relevant to mononuclear phagocytes in view of the different patterns of differentiation they may undergo due to the presence of cytokines and growth factors in the inflammatory milieu.

2.1. The Opposing Effect of C3bi-Coating of Immune Complexes and Zymosan Particles on AA Release. AA metabolism was assessed in mononuclear phagocytes stimulated with antigen/antibody immune complexes and zymosan, a cell wall extract of the yeast Saccharomyces cerevisiae. Since formation of immune complexes (IC) in fluids containing complement is accompanied by the covalent linkage of
Figure 3: Cells and receptors involved in AA metabolism in the innate immune system. PMN respond to cooperative binding of dectin-1 and CR3, to the cross-linking of FcγR, to mannose-based PAMP, and to G-protein-coupled receptors (GPCR) in the presence of priming agents. The receptor involved in the response to PGN has not been characterized as yet. Macrophages express a similar array of receptors showing productive binding. In DC the most efficient response is obtained by triggering dectin-1 and DC-SIGN by PAMP containing β-glucan and mannose polymers, respectively.

C3bi onto the Ab and because C3bi coating of zymosan is known to increase inflammatory responses [32] and AA release in leukocytes [33], experiments were carried out with preformed IC treated with normal human serum to allow the formation of adducts between IgG γ-chain and C3b-α-chain, a process that has been related to the clearance of IC with a limited inflammatory response. Notably, the AA released by C3bi-IC was significantly lower than that induced by IC containing similar amounts of IgG, thus suggesting that the reaction of IC with C3bi gives rise to an IC lattice showing a distinct ability to interact with signaling receptors. The most likely interpretation of these findings is that the ability of C3bi-IC to interact with complement receptor 3 (CR3) blunts Fc/FcγR interactions and the attendant AA release associated with FcγR cross-linking. Treatment of C3bi-IC with anti-C3 IgG, but not with an irrelevant rabbit IgG, allowed the recovery of AA-releasing ability, thus indicating that masking the C3bi moieties with IgG in the C3bi-IC lattice, makes these complexes similar to those formed in the absence of complement [34]. Conversely, removal of the Fc portion of anti-OVA IgG, which preserves the ability of the F(ab′)2 fragment to bind covalently C3bi on the Ser-132 of the CH1 domain [35], abrogated AA-releasing activity, thus indicating that Fc-FcγR interaction is essential for IC-induced AA release and that stimulation through C3bi does not elicit productive binding in this system.

2.2. The Role of the Mannose Receptor in Human Monocytes. The mannose receptor (MR), first described by Stahl et al. [36] has been the object of detailed analysis regarding its ability to initiate the uptake of glycosylated molecules with terminal mannose, fucose, or N-acetylglucosamine moieties. Its capacity for ligand recognition makes this receptor suitable to phagocytose Candida albicans, Leishmania donovani, and Pneumocystis carinii, among other microorganisms [37–39]. The MR is the prototypic element of a homonymous
family of C-type lectin receptors, which includes the secreted phospholipase A2, M-type receptor, the dendritic cell receptor DEC-205, and Endo180/urokinase plasminogen-activated receptor-associated protein. These receptors contain carbohydrate recognition domains, although the chemical structure of the ligands interacting with those domains shows wide differences. The MR is mainly expressed in alveolar macrophages, peritoneal macrophages, and macrophages derived from blood monocytes [40] and seems to play a role in the early immune response against invading pathogens. Although the MR was shown to participate in intracellular signalling leading to target gene expression, the absence of signaling motifs in its intracellular tail makes it necessary the assistance from other receptors in order to trigger any signaling cascade. The MR has been found to exert some effect on the induction of effector T_{h}17 cells in mixed leukocytes populations [41] and binding of the mannose polymer mannan to the MR induced a mild expression of COX-2 protein above basal levels, whereas treatment with laminarin, zymosan particles, and preformed IC failed to do so. Notably, monocyte-derived macrophages obtained after two weeks of culture showed a prominent induction of COX-2 protein with concentrations of mannan as low as 0.1 mg/ml, thus suggesting that recognition of mannose-based molecular patterns by macrophages might play a central role in the induction of the innate immune response [42].

2.3. AA Metabolism in Monoctye-Derived Dendritic Cells. The main function of dendritic cells (DC) is the detection of pathogens and the initiation of the host response to microbial invasion. So far, few studies have been dedicated to the analysis of the production of AA metabolites [43], in spite of the relevant role of eicosanoids in DC function and the prominent changes in lipid metabolism elicited by M-CSF and IL-4 along the process of monocyte differentiation [44]. In addition, PGE2 is required for human DC migration in response to chemokines [45, 46], and consistent with this pivotal function, failure of DC to produce PGE2 has been considered a major obstacle for the successful application of DC in therapy [47, 48]. PG biosynthesis involves several steps catalyzed by different enzymes, but it depends primarily on the availability of free AA selectively released from phospholipids by cPLA2. COX-2 is involved in the sustained production of prostanoids, the activity of which is necessary for strong Ab response following vaccination [49]. In addition to the COX-2 route for AA metabolism, there are pathways dependent on constitutively expressed 5-lipoxygenase and COX-1, which are triggered shortly after cell activation. As regards 5-lipoxygenase products, deficient extracellular export of LTC4 is associated with a decreased migratory response of DC [50], whereas cysteinyl-LT increase IL-10 production by myeloid DC [51]. Recent studies have disclosed lipoxins as a unique class of lipoxygenase interaction metabolites with a strong ability to suppress the production of IL-12 and the function of DC [52].

In keeping with the changes in functional parameters observed upon DC differentiation, AA metabolism in DC showed different patterns in mature and immature DC. Whereas the release of AA elicited by zymosan and other ligands showed no difference between immature and TNFα-mature cells, increased expression of COX-2 was only observed in immature dendritic cells. Unlike PMN and monocytes, zymosan particles were the most potent stimulus for AA release, which was observed with concentrations as low as 0.1 mg/ml. In contrast, mannan induced AA release to a lower extent. Unlike the results observed in monocytes, neither C3bi coating nor opsonization with rabbit IgG modified the ability of these stimuli to release AA [53].

This raises important questions about the recognition of β-glucan particles and the coupled signaling mechanisms in different cell types. In fact, the main receptor involved in β-glucan recognition is dectin-1, which is expressed on the cell surface of PMN, monocytes, and DC; however, DC display a unique response to zymosan particles. At first glance, two mechanisms might explain the different responses: (i) expression in some myeloid cell types of an inhibitor, for example, tetraspanin CD37, that restricts dectin-1-CARD9 signaling [31], or (ii) gain of function of DC by differentiation-induced expression of a receptor cooperating with dectin-1.

Zymosan-induced AA release was inhibited by laminarin, mannan, and anti-dectin-1 and anti-DC-SIGN mAb, especially when the inhibitors were used in combination. These data would suggest cooperation of both dectin-1 and DC-SIGN in zymosan-induced AA release and would agree with the aforementioned hypothesis of the selective expression in DC of a receptor not expressed in other myeloid cell types. To obtain further insight into the type of receptors involved in the recognition of zymosan by DC, the binding of Alexa-Fluor 488 zymosan was studied in the presence of different inhibitors. Mannan, laminarin, anti-DC-SIGN mAb, and anti-dectin-1 mAb blocked zymosan binding, but combination of these inhibitors enhanced binding blockade. Taken together, these data show the existence of a cPLA2-dependent route for AA release in DC that is triggered by the binding of zymosan to dectin-1 and DC-SIGN.

2.4. Syk Activity Is Involved in AA Release. Protein tyrosine phosphorylation reactions play a central role in cell signaling through both FcγR and dectin-1 in murine DC [54, 55]. Since these receptors do not possess intrinsic enzymatic activity, their signal transduction pathways must rely on activation of nonreceptor tyrosine kinases. This explains why the Syk/Zap70 family member Syk has been found to be critical for linking receptor engagement to many early downstream events including calcium mobilization and activation of the Ras/mitogen-activated protein kinase pathway. The involvement of Syk in AA release and COX-2 induction in murine macrophages was first reported by Suram et al. [56], who also showed that AA release and LTC4 production stimulated by zymosan and Candida albicans were TLR2-independent. Studies in human DC were addressed by examining tyrosine phosphorylation of the kinase (a measure of Syk activation) and the effect of Syk inhibitors. Both IC and zymosan induced the phosphorylation of tyrosines in the activation loop of Syk and Syk inhibitors significantly
blunted AA release. However, Syk inhibitors only partially affected zymosan-induced cPLA₂ phosphorylation [53] and the Syk inhibitor piceatannol blunted the release of AA by 96% and 54% in response to IC and zymosan, respectively. R406, a very specific Syk inhibitor, also inhibited completely the response to IC and reduced zymosan-induced AA release by 30%. Zymosan-induced Syk phosphorylation was also inhibited with the addition of laminarin, but not by anti-DC-SIGN mAb. Taken collectively, these results are consistent with the notion that Syk activity is completely necessary for IC-induced AA release, but it is only partially involved in the signalling mechanism whereby zymosan elicits AA release in DC.

2.5. DC-SIGN Coimmunoprecipitates with Dectin-1. The inhibition of AA release by combinations of laminarin/anti-dectin-1 and anti-DC-SIGN mAb suggested cooperation between DC-SIGN and dectin-1. This was confirmed by showing that dectin-1 coimmunoprecipitated with DC-SIGN, particularly after the stimulation of DC with zymosan (Figure 4). Additional experiments in HEK293 cells transfected with vectors encoding DC-SIGN and Myc-dectin-1 showed a robust coimmunoprecipitation of both C-lectin receptors when immunoprecipitation was carried out with either anti-DC-SIGN mAb or anti-Myc mAb. These results are consistent with a system for zymosan recognition in DC involving the interaction of dectin-1 and DC-SIGN. Studies by confocal microscopy confirmed these findings by showing DC-SIGN clusters in areas of contact with zymosan particles, but not around engulfed particles as judged from the analysis of images taken after 10 minutes, where ingested particles were not surrounded by DC-SIGN staining (Figure 5). This finding agrees with recent reports indicating that DC-SIGN is a mannan-inhibitable zymosan receptor, but does not mediate phagocytosis [57, 58]. In contrast, engulfed zymosan particles were clearly surrounded by dectin-1. Taken collectively, these data would suggest that the differentiation of human monocytes into DC is accompanied by the induction of DC-SIGN, a receptor that cooperates with dectin-1 to elicit an active metabolism of AA. Further support of the role played by changes associated to the process of DC differentiation on AA metabolism is the enhancement of dectin-1-mediated AA release in alveolar macrophages by GM-CSF, a cytokine used to promote DC differentiation [59]. In sharp contrast, rat peritoneal macrophages respond to zymosan particles by promoting the mobilization of both type IIA phospholipase A₂ and cPLA₂ into the phagosomes in the absence of growth factors and cytokines [60, 61]. Taking collectively, these findings underscore the importance of environmental factors on the ability of mononuclear phagocytes to regulate the catalytic properties of phospholipases A₂. A diagram of the signaling routes involved in AA metabolism in DC stimulated with fungal stimuli is shown in Figure 6.

2.6. AA Metabolites and the Release of Cytokines from DC. Fungal PAMP acting through dectin-1 and DC-SIGN induce a cytokine response characterized by a high production of IL-10 and IL-23, and a low secretion of IL-12 p70 [55, 62, 63], as compared to the effect on IL-12 p70 production of archetypal TLR4 agonists. This fact may have pathophysiological consequences for the persistence of infection and raises the question of the signaling pathways involved in the predominant IL-10 response. The regulation of IL-10 production has been the subject of intense research in TLR4-dependent models and both transcriptional and posttranscriptional mechanisms have been reported. As regards transcriptional regulation, many transcription factors have been considered as master regulators, namely Stat3 [64–67], Sp1 and Sp3 [68, 69], c-Maf [70], NF-Y [71], NF-κB [72–74], Pbx1b (pre-B cell leukemia transcription factor-1b) [75], c/EBP [76], NFAT [77, 78], and CREB [79, 80]. In addition, posttranscriptional regulation of IL-10 message has also been proposed because of the high number of AU-rich elements in the 3′-UTR of IL-10 mRNA [81] and their binding by the RNA-binding protein tristetraprolin, which destabilizes the message [82]. After addressing the stability of IL-10 mRNA in the presence of actinomycin D, it was concluded that the regulation of IL-10 expression is best explained by transcriptional mechanisms [83]. Computer analysis of human and mouse il10 promoters was carried out using the MatInspector program and the TRANSFAC database to detect binding sites for transcription factors. In addition, both sequences were aligned with DNA Block Aligner software to define conserved areas, since these regions are more likely to represent functionally relevant elements. Several of the sites detected were previously associated with the transcriptional regulation of il10, but there have been some discrepancies regarding their functional relevance and studies with fungus-related stimuli have not been reported. The first approach was to search for the presence of binding activities to the consensus strings of the transcription factors found in the human il10 promoter. No binding activity to Stat and C/EBP consensus sequences was observed in the nuclear extracts of cells treated with zymosan, whereas binding activity to Stat1 and Stat3 was elicited by IFN-α. Constitutive binding activity to Sp sites compatible with both Sp1 and Sp3 was detected, as well as binding activity to CRE consensus sequences. NF-κB is activated by zymosan and has been associated with the regulation of il10 in mouse macrophages [72–74] and with the regulation of COX-2. Taking into account that the expression of COX-2 parallels IL-10 induction, experiments were conducted using probes containing the κB sites from the human cox2 promoter that have found to be of functional relevance. Zymosan and LPS were strong activators of NF-κB binding activity to cox2 sites. The response to zymosan was dose-dependent and binding was competed by the unlabeled sequence. However, as the sequence involved in NF-κB-dependent regulation of il10 expression in the mouse is not conserved in the human il10 promoter, the appearance of κB-binding activity in the nuclear extracts upon zymosan challenge is not a proof of the involvement of NF-κB in the regulation of IL-10 expression in human DC. Altogether, the above-mentioned results did not support the involvement of Stat1, Stat3, and c/EBP in the regulation of IL-10 induction and further experiments were
conducted focusing on the possible involvement of NF-κB and CREB.

2.7. Effect of the Pharmacological Modulation of CREB and NF-κB Activities on IL-10 Production. Since the activity of CREB and NF-κB can be modulated by pharmacological tools, experiments were conducted with 8-Br-cAMP, a cell permeable analogue of cyclic AMP, PGE2, and the protein kinase A inhibitor H-89. Elevation of the intracellular levels of cyclic AMP by both PGE2 and 8-Br-cAMP had on its own a limited effect on IL-10 production, whereas stimulation with zymosan in the presence of these compounds produced a synergistic increase of IL-10 production. Notably, the opposite effect was observed in the presence of the protein kinase A inhibitor H-89. In contrast with the effect of the chemicals producing inhibition of CREB activity, blockade of NF-κB activity by two structurally unrelated inhibitors was accompanied by an increase of IL-10 production. Since the regulation of CREB activity has been related to calcium/calmodulin kinases and CRE coactivators, the activity of which depends on a sensor of both Ca²⁺ and cyclic AMP levels [84], the Ca²⁺-dependence of IL-10 production was addressed. IL-10 production was blunted by Ca²⁺-chelation. Ionomycin induced a limited production of IL-10, thus suggesting that intracellular Ca²⁺ levels are not the only factor determining IL-10 production. Moreover, low micromolar concentrations of cyclosporin induced a significant decrease of IL-10 production, thus pointing to
the involvement of calcineurin in the regulation of IL-10 production. Since E prostanois receptors type 2 and 4 are involved in the regulation of the intracellular levels of cyclic AMP and zymosan is a strong inducer of COX-2 and PGE2 production, the effect of inhibiting COX-1 with the specific inhibitor SC560 and COX-2 with the specific inhibitor NS398 was addressed. The isolated addition of any of those compounds at concentrations of ∼1 μM to preserve their selectivity, did not show any significant effect on IL-10 production. Notably, combination thereof produced a significant inhibition, thus suggesting that both COX isoforms might be involved in an autocrine production of PGE2 that regulates intracellular cyclic AMP levels and zymosan-induced IL-10 production. Taken together, these results suggest that the polarization of DC cytokine response versus IL-10 production in response to the fungal surrogate zymosan depends on a fine-tuned balance between NF-xB and CREB activity, and that PGE2 plays a role in this balance.

2.8. The Role of Different Transcription Factors on IL-10 Induction. To address directly the involvement of the distinct transcription factors on IL-10 regulation, chromatin immunoprecipitation (ChIP) assays were conducted using antibodies reactive to P-CREB, CBP, c-Maf, NF-YA, Sp1, and Pbx1. Significant binding of P-CREB to the il10 promoter was observed in DC stimulated with zymosan, but not in control cells nor in samples treated with an irrelevant antibody. Notably, this was associated with a 64-fold increase of the amount of CBP associated to the il10 promoter, thus suggesting that zymosan induces both binding of P-CREB to CRE sites and recruitment of the coactivator CBP. ChIP was negative when the PCR reactions were carried out using primers from the IL12 p35 promoter, which does not contain CRE sites. P-CREB binding was also detected in the cox2 promoter upon zymosan stimulation, which agrees with the presence of two CRE sites in this promoter and with the functional relevance of these sites in cox2 transcriptional regulation [85, 86]. Binding of P-CREB and CBP to the promoters was coincidental with the detection of TORC2 (transducer of regulated CREB activity), a CREB coactivator also known as CREB-regulated transcription coactivator (CRTC), in the nuclear extracts. In
addition, TORC2 was found to coimmunoprecipitate with P-CREB.

Expression of the mRNA encoding both the long and the short form of c-Maf was detected in DC, thus agreeing with the reported induction of this factor by LPS and IL-4 in monocytes [70], but binding to the il10 promoter was not detected by ChIP assays. As regards Sp1 and Sp3, the detection of binding activity in resting cells was not accompanied by binding to the il10 promoter, which agrees with the notion that this family of transcription factors behaves as a constitutive activator of housekeeping genes and TATA-less genes. Stat3 has been associated with il10 transcriptional activation, especially in response to ligands of TLR4, which differ from zymosan because of their capacity to activate the Jak/Stat pathway by TRIF (TIR-domain-containing adapter-inducing interferon-β)-dependent mechanisms. Stat binding activity and tyrosine-phosphorylated Stat1 were not detected in nuclear extracts from zymosan-stimulated DC, whereas they were induced upon LPS and IFN-γ treatment [87]. These results show a major role for CREB in the transcriptional regulation of il10 in response to the fungal stimulus zymosan.

2.9. Langerhans Cells and the Th17 Response. The response of DC to fungal glucans is characterized by a high production of IL-23 and the development of a Th17 response. This is of interest because Th17 cells have been implicated in a number of inflammatory and autoimmune diseases, including multiple sclerosis, inflammatory bowel disease, asthma and psoriasis. So far, only preliminary data have suggested the involvement of lipid mediators in the expansion of Th17 cells. The phospholipid mediator PAF is released in response to zymosan in many cell types and is found in increased concentrations in inflammatory lesions. PAF has been shown to induce the production of IL-6 and the development of Th17 cells when added at picomolar concentrations to monocyte-derived Langerhans cells and to keratinocytes. Moreover, when Langerhans cells (LC) were pretreated with PAF and then cocultured with anti-CD3- and anti-CD28-activated T cells, the latter developed a Th17 phenotype, with a 3-fold increase in the expression of the transcriptional regulator RORγt and enhanced production of IL-17, IL-21 and IL-22. PAF-induced Th17 development was prevented by the PAF receptor antagonist WEB2086 and by neutralizing antibodies to IL-23 and IL-6. It was also dependent on LC-T cell contact as shown in Transwell experiments [88]. These data suggest that a lipid mediator, the biosynthesis of which is associated to the eicosanoid cascade, can stimulate LC to produce IL-6 and IL-23, which, in contact with TCR-activated T cells, can induce their differentiation into Th17 cells. This may constitute a previously unknown stimulus for the development and persistence of inflammatory processes that could be amenable to pharmacologic intervention.

3. Concluding Remarks

Release of AA and the sequential production of eicosanoid are a blatant outcome of PRR binding by their cognate ligands. The amounts of eicosanoids released under these conditions make PAMP the most potent and physiologically relevant stimuli of AA metabolism in myeloid cells. However, there are a number of significant differences regarding the effect of PRR ligands on the different cell types, even though the same types of receptors might be expressed. This raises...
relevant questions regarding the distinct signaling routes coupled to the receptors, the role of the concomitant expression of other receptors recognizing the same or other PAMP present on the same ligand, and the effect of positive and negative regulators. Of particular interest is the fact that PGN is the most relevant stimulus in PMN, thus underscoring the important role played by these cells in the pyogenic infections produced by Gram+ bacteria. Complement coating of PAMP seems essential for the activation of PMN and monocytes by particles mimicking the fungal cell wall, whereas monocyte-derived DC are a cell type specially endowed to respond to fungal patterns, even in the absence of opsonins. This property depends on the particular set of receptors expressed on their membranes, which cooperate to recognize β-glucans and mannose-containing patterns. The signaling network triggered by the combined binding of dectin-1 and DC-SIGN in DC is specially suited for the transcriptional regulation triggered by the combined binding of dectin-1 and DC-SIGN on their membranes, which cooperate to recognize fungal patterns, even in the absence of opsonins. This property depends on the particular set of receptors expressed on their membranes, which cooperate to recognize β-glucans and mannose-containing patterns. The signaling network triggered by the combined binding of dectin-1 and DC-SIGN in DC is specially suited for the transcriptional regulation of a pattern of cytokines characterized by a low production of IL-12 p70 and a high production of IL-10. This can be explained by the activation of the transcription factor CREB through a mechanism involving the coactivators CBP and TORC2/CRTC2, and autocrine production of prostaglandin E2. These findings emphasize the need of further work to transform these mechanistic data into valuable tools to treat infectious and autoimmune diseases.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
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<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
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<tr>
<td>IC</td>
<td>Immune complexes</td>
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<tr>
<td>MR</td>
<td>Mannose receptor</td>
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<tr>
<td>MDP</td>
<td>Muramylidipeptide</td>
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<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain family proteins</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans cells</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
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<tr>
<td>mTor</td>
<td>Mammalian target of rapamycin</td>
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<tr>
<td>PamCSK4</td>
<td>Palmitoyl-3-cysteine-serine-lysine-4</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
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<tr>
<td>PG</td>
<td>Prostaglandin</td>
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<tr>
<td>PAF</td>
<td>Platelet-activating factor</td>
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<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
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<tr>
<td>P13K</td>
<td>Phosphoinositide-3 kinase</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
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<tr>
<td>UTR</td>
<td>Untranslated region</td>
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Mediators of Inflammation


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Research Article

Interleukin-6 Contributes to Age-Related Alteration of Cytokine Production by Macrophages

Christian R. Gomez, 1 John Karavitis, 1, 2 Jessica L. Palmer, 1 Douglas E. Faunce, 1
Luis Ramirez, 1 Vanessa Nomellini, 1, 3 and Elizabeth J. Kovacs 1, 2, 3

1 Department of Surgery, and Immunology and Aging Program, The Burn and Shock Trauma Institute, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL 60153, USA
2 Department of Cell Biology, Neurobiology and Anatomy and Alcohol Research Program, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL 60153, USA
3 Stritch School of Medicine, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL 60153, USA

Correspondence should be addressed to Elizabeth J. Kovacs, ekovacs@lumc.edu

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1. Introduction

As a part of the age-associated deterioration of the immune system [1–3], there is a chronic proinflammatory state even in the absence of clinically-apparent disease. This state defined as “inflamm-aging” is characterized by elevated circulating levels of proinflammatory factors (IL-1β, IL-6, TNFα, and prostaglandin E2) and anti-inflammatory mediators, (IL-1 receptor antagonist, soluble TNF receptor, IL-10, transforming growth factor beta, acute phase proteins, C-reactive protein, and serum amyloid A) and contributes to the decreased ability of the elderly to mount an appropriate immune response following an infectious challenge [4–7].

One of the most prominent aspects of “inflamm-aging” is in presence of elevated circulating levels of the proinflammatory cytokine, interleukin (IL)-6 [8, 9]. In an attempt to better define the role of IL-6 in the systemic inflammatory response in the aged, we gave young and aged wild type (WT) and IL-6 knockout (KO) BALB/c female mice lipopolysaccharide (LPS). Following the inflammatory challenge, aged IL-6 KO mice had improved survival when compared to aged WT mice [10]. Moreover, we previously reported that aged IL-6 KO mice showed a decreased acute phase response when compared to that of aged WT mice [10]. In addition, hepatic injury was drastically reduced in aged IL-6 KO mice given LPS as compared with LPS-exposed aged WT mice [11]. Recently, Starr et al. reported similar findings in male C57BL/6 IL-6KO mice using a higher dose of LPS (5 mg/kg) and a slightly older group of mice (26 months old) [12] than our study. Taken together, these observations have suggested a crucial role for IL-6 as a component of the aberrant systemic innate immune responses of the aged after an inflammatory challenge or injury and that this role is not sex or strain specific.

Macrophages play critical roles in phagocytosis of antigens, microorganisms, and cellular debris; killing of invading
pathogens and tumors; and wound healing [13]. Many of the macrophage functions are carried out by secreted cytokines, which in turn, regulate multiple immune functions, especially inflammatory responses [14]. The effects of aging on cytokine production by macrophages have shown conflicting results with in vitro stimulation of purified macrophage populations with LPS and in vivo systemic administration of LPS. For example, our laboratory and others reported that macrophages from aged mice produce less TNF and IL-6 after in vitro exposure to LPS than comparably stimulated cells from young mice [15–19]. These findings have been observed despite the presence of “inflamm-aging” and the elevated baseline inflammatory state seen in healthy aged individuals [20, 21].

Since macrophages are considered to be an important source of proinflammatory cytokines in vivo, many possible explanations for this inconsistency can be proposed [22]. Among the multiple possibilities, the effect of the local environment in vitro versus the effect of the aged microenvironment in vivo has been considered [22–24]. We reported that serum from aged Fisher 344 rats increased the levels of IL-6 by macrophages obtained from young animals and cultured in vitro without stimulants [25]. These observations suggest that the elevated production of inflammatory mediators in the aged results, in part, from the interaction of macrophages with a variety of factors in their environment in situ.

To analyze the role that IL-6 might play in regulating the age-related defects in macrophages through alteration of proinflammatory cytokines, we exposed splenic macrophages from young and aged WT and IL-6 KO mice to LPS. Our findings suggest that IL-6 regulates the age-related defects in macrophages through alteration of proinflammatory cytokines and that these alterations are not due to changes in surface expression of TLR4 or the IL-6 receptor.

2. Materials and Methods

2.1. Animals. Young (2 months old) and aged (18 months old) WT female BALB/c mice were purchased from the National Institute of Aging colony at Harlan Laboratories (Indianapolis, IN). IL-6 KO mice, kindly provided by Dr. Manfred Kopf, Molecular Biomedicine, ETH Zurich, Switzerland, have a disruption of IL-6 in the second exon (first coding exon) by insertion of a neo cassette [26]. IL-6 KO mice were backcrossed onto the BALB/c background, bred, maintained, and aged at the Taconic Laboratories (Germantown, NY). Young (2 months old) and aged (18 months old) female BALB/c IL-6 KO mice were used in the experiments described here. Animals were housed under similar conditions at their respective facilities. They were free of potential endemic viral pathogens that could influence their inflammatory response. All animals where maintained in an environmentally controlled facility at Loyola University Medical Center for at least one week prior to experimentation. At the time of sacrifice, all mice were dissected and the organs were screened for visible tumors and/or gross abnormalities. If found, these animals were removed from the study. The experimental protocols described here followed the guidelines established by the publication, Principles of Laboratory Animal Care (NIH publication no. 86–23, revised 1985), and were approved by the Loyola University Chicago Institutional Animal Care and Use Committee.

2.2. Cell Isolation and Culture. Mice were sacrificed by CO₂ inhalation and subsequent cervical dislocation. In order to avoid confounding factors related to circadian rhythms, all animal protects were performed between 8 and 9 AM. Splenic macrophages were isolated by plastic adherence as previously described [17]. Briefly, spleens were aseptically removed and disrupted to yield a cell suspension in RPMI 1640 medium, supplemented with 5% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM glutamine (culture medium) (GIBCO-BRL, Grand Island, NY). Following red blood cell lysis with ACK Lysis Buffer (Invitrogen Corp., Carlsbad, CA), white blood cells were counted in a hemocytometer and viability was determined by Trypan Blue exclusion. Two × 10⁶ cells were seeded in 96-well plates in 200 μl culture medium. After incubation for 2 hours at 37°C and 5% CO₂, nonadherent cells were discarded by medium aspiration and washed twice with warm phosphate buffer saline (PBS). This method resulted in an adherent population characterized as ≥98% positive for Mac-3 and D1-1-acetylated low-density lipoprotein uptake, as we previously reported [27]. Adherent cells were treated in 200 μl culture medium alone or containing 100 ng/ml LPS from Escherichia coli 0111:B4 (Sigma Biosciences, St. Louis, MO). In the absence of stimulation, macrophage cytokine levels were undetectable (116) and data not shown. Supernatants were collected after 16 hours and stored at −80°C.

2.3. Measurement of Proinflammatory Cytokines. The concentrations of TNFα, IL-1β, IL-6, and IL-12 in macrophage supernatants were measured by commercially available OptEIA ELISA kits (BD Pharamingen, San Diego, CA) according to the manufacturer instructions. The lowest detectable limit of these kits is 15.6 pg/ml. Data are expressed as pg/ml.

2.4. Flow Cytometry. Total spleen cell suspensions were obtained as reported above. Flow cytometry was performed as previously described [16, 17]. Briefly, after blocking nonspecific staining with anti-CD16/CD32 (FcγIII/II; BD-PharMingen, San Diego, CA), total splenocyte suspensions were stained with APC-conjugated anti-CD3 (0.25 μg/1 × 10⁶ cells, clone 145-2C11, eBioscience, San Diego, CA), PE-conjugated anti-CD4 (0.125 μg/1 × 10⁶ cells, clone GK1.5, eBioscience, San Diego, CA), FITC-conjugated anti-CD8 (0.5 μg/1 × 10⁶ cells, clone 53–6.7, eBioscience, San Diego, CA), FITC-conjugated anti-Ly49c (0.5 μg/1 × 10⁶ cells, clone 5E6, eBioscience, San Diego, CA), Alexafafluor 750-conjugated anti-F4/80 (0.1 μg/1 × 10⁶ cells, clone BM8, Invitrogen Corp., Camarillo, CA), PE-conjugated anti-IL-68a (0.06 μg/1 × 10⁶ cells, clone D7715A7, BD-PharMingen, San Diego, CA), APC-conjugated anti-CD19 (0.5 μg/1 × 10⁶ cells,
clone MB 19–1, eBioscience, San Diego, CA), and biotin-conjugated anti-TLR4 (0.25 μg/1 × 10⁶ cells, clone MTS510, eBioscience, San Diego, CA), biotin-conjugated anti-gp130 (1 μg/1 × 10⁶ cells, clone 125623, R&D Systems Inc., Minneapolis, MN). Following biotin-conjugate incubation, cells were incubated with PerCP-conjugated streptavidin (0.125 μg/1 × 10⁶ cells, eBioscience, San Diego, CA). Flow cytometric determinations were made using Becton Dickinson FACSCanto flow cytometer (BDIS, San Jose, CA) and data was analyzed with FlowJo software (Tree Star Inc., Ashland, OR).

2.5. Statistical Analysis. Data are expressed as mean ± SEM. ANOVA and Tukey-Kramer Multiple Comparisons were used to determine statistical significance using GraphPad Prism Version 2.0 statistical package (San Diego, CA, USA). A P value less than .05 was considered significant.

3. Results

3.1. Cytokine Levels in Macrophages from Aged IL-6-Deficient Mice after LPS Stimulation. When stimulated in vitro with LPS, macrophages from aged mice produce less cytokines than comparably stimulated cells from young mice [15–19]. To determine if this phenotype may be determined in part by IL-6, which is elevated in the circulation of healthy aged individuals [9, 10, 28, 29], we evaluated proinflammatory cytokine production in splenic macrophages obtained from young aged WT and IL-6 KO mice. Macrophages from aged and young mice produced proinflammatory cytokines upon LPS stimulation (Figures 1(a)–1(d)). As previously reported [15–19], macrophages from aged WT mice had decreased production of TNFα and IL-6 (55%), as well as IL-1β (80%) and IL-12 (35%) relative to splenic macrophages obtained from young WT mice given LPS. Similar to macrophages from aged WT mice, the production of cytokines by macrophages from young IL-6 KO mice was reduced for TNFα (67%), IL-1β (31%), and IL-12 (71%) relative to that of young WT mice (Figure 1). In contrast, as compared to macrophages from young IL-6 KO mice, LPS exposure induced higher cytokine production by cells from aged IL-6 KO mice (1.3, 2.2 and 1.2-fold for TNFα, IL-1β and IL-12, resp.) (Figure 1). Cytokine production by macrophages from aged IL-6 KO mice was elevated when compared to cells from aged WT mice (1.8, 3.7 and 1.9-fold for TNFα, IL-1β and IL-12, resp.). Overall, these results show that IL-6 plays a role in regulating the age-related defects in macrophages through alteration of the production of proinflammatory cytokines.

3.2. Effects of Age and IL-6 on Splenocyte Cell Populations. Interleukin-6 is a pleiotropic cytokine, which has an important role in supporting the growth of T and B lymphocytes [30] in lymphoid tissues, mainly in the spleen [31]. Therefore, lack of expression of IL-6 could modify the phenotype of splenocytes and their production of cytokines when cultured in vitro. To test this hypothesis, total spleen cell suspensions were examined by flow cytometry for specific splenocyte cell populations. The percentage of T cells was determined by analyzing splenocytes bearing the cell surface markers CD3 (T cells), CD4 (CD3+/CD4+; helper/inducer cells), and CD8 (CD3+/CD8+; suppressor/cytotoxic cells). Relative to young WT mice, the percentage of CD3+ T cells in splenocytes from aged WT mice was reduced (13%, P < .05), Table 1. Interestingly, this difference was more pronounced in splenocytes from aged IL-6 KO mice relative to cells from young IL-6 KO mice (24% reduction in CD3+ T cells, P < .001). An analysis of splenic CD3+CD4+ helper/inducer T cells revealed no effects of aging or IL-6 on their percentage. However, CD3+/CD8+ suppressor/cytotoxic T cells were reduced in splenocytes from aged WT mice (25%, P < .05), relative to young WT mice. In addition, a marked (47%) decrease in CD3+/CD8+ suppressor/cytotoxic T cells was found in aged IL-6 KO animals, relative to splenocytes from young IL-6 KO mice. Analysis of B cell percentages in the spleen was also assessed by measuring B220+ splenocytes, Table 1. Spleens of young WT mice were composed of 40% B220+ cells. This percentage was slightly increased by aging and IL-6 deficiency, however, these differences failed to reach statistical significance. Similarly, analysis of the F4/80+ macrophage population showed no effects of aging or IL-6 deficiency on their percentage.

3.3. Effects of Age and IL-6 on TLR4 Expression in Macrophages. TLR4 involvement in age-related defects in macrophage activation following LPS activation has been documented extensively (reviewed in [1, 2]). However, TLR4 levels in the context of aging and IL-6 deficiency are unknown. To analyze the expression of TLR4 in macrophages from aged IL-6 KO mice, splenic macrophages were examined by flow cytometry. As we reported previously [16, 17], F4/80+ macrophages from aged WT mice did not exhibit age-associated differences in surface TLR4 expression (23.0% in young WT and 24.7% in aged WT), Table 2. In F4/80+ macrophages from IL-6 KO mice, a similar percentage of TLR4+ cells was found relative to those from WT mice, regardless of age or IL-6 deficiency (24.6% in young IL-6 KO versus 23.6% in aged IL-6 KO). These results suggest that changes in TLR4 expression in macrophages are not responsible for the increase in proinflammatory cytokine production observed in macrophages from aged IL-6 KO mice.

3.4. Effects of Age and IL-6 on IL-6 Receptor Expression in Macrophages. In vivo as well as in vitro studies suggest that stimulation with IL-6 affects the expression of the IL-6 receptor [32, 33]. To analyze the effects of aging and IL-6 on the initial steps of IL-6 signaling, we measured surface levels of the IL-6 receptor in total spleen cell suspensions by flow cytometry. IL-6 signaling is initiated by binding of the cytokine to an 80-kDa glycoprotein α chain (IL-6Ra/CD126) [34]. F4/80+ macrophages from aged WT mice did not differ in the percentage of IL-6Ra expressing cells (Table 2) or the level of the surface expression as measured by mean fluorescent intensity (MFI) (data not shown) relative to those from young WT mice. In F4/80+ macrophages from IL-6 KO mice, similar levels of IL-6Ra were found
Figure 1: Splenic macrophages obtained from young and aged WT and IL-6 KO mice were cultured for 18 hours with LPS (100 ng/ml). Supernatants collected were assayed for TNFα (a), IL-1β (b), IL-6 (c), and IL-12 (d), by ELISA. Data are shown as mean ± SEM of 4-5 mice per group. *P < .05 from young WT. #P < .05 from young WT. †P < .05 from young IL-6 KO. @P < .05 from aged WT.

Table 1: Effects of age and IL-6 on splenocyte cell populations.

<table>
<thead>
<tr>
<th>Cells (%)</th>
<th>WT</th>
<th>IL-6 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+ T Cells</td>
<td>47.1 ± 1.1</td>
<td>41.1 ± 1.1*</td>
</tr>
<tr>
<td>CD3+/CD4+ T Cells</td>
<td>34.1 ± 0.9</td>
<td>31.0 ± 0.8</td>
</tr>
<tr>
<td>CD3+/CD8+ T Cells</td>
<td>13.6 ± 0.9</td>
<td>10.2 ± 0.7*</td>
</tr>
<tr>
<td>Ly49c+ NK Cells</td>
<td>5.4 ± 0.1</td>
<td>4.2 ± 0.3*</td>
</tr>
<tr>
<td>F4/80+ Macrophages</td>
<td>15.9 ± 2.1</td>
<td>12.6 ± 1.2</td>
</tr>
<tr>
<td>CD19+ B cells</td>
<td>41.1 ± 1.5</td>
<td>43.4 ± 1.2</td>
</tr>
</tbody>
</table>

Splenocytes were incubated with anti-CD3, CD4, CD8, Ly49c, F4/80, or CD19 antibodies and analyzed by flow cytometry. Data are shown as mean ± SEM of 4-5 mice per group. *P < .05 from aged matched control. **P < .05 from young WT.

Table 2: Percentage of TLR4+, IL-6Ra+, and Gp130+ in F4/80+ splenocytes.

<table>
<thead>
<tr>
<th>F4/80+ cells (%)</th>
<th>WT</th>
<th>IL-6 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4+</td>
<td>23.0 ± 2.8</td>
<td>24.7 ± 1.7</td>
</tr>
<tr>
<td>IL-6Ra+</td>
<td>61.4 ± 1.3</td>
<td>60.1 ± 1.5</td>
</tr>
<tr>
<td>gp130+</td>
<td>35.6 ± 6.3</td>
<td>27.2 ± 2.0</td>
</tr>
</tbody>
</table>

Splenocytes were incubated with anti-F4/80 and anti-TLR4 antibodies or with anti-F4/80, anti IL-6Ra, and anti-Gp130 antibodies and analyzed by flow cytometry. Data are shown as mean ± SEM of 4-5 mice per group.
relative to those from WT mice, regardless of age or IL-6 deficiency. Following binding of IL-6 to IL-6Ra, dimerization of signal-transducing glycoprotein 130 (gp130/CD130) leads to activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) 3 signal transduction pathway. As shown in Table 2, gp130 levels on F4/80+ macrophages from young or aged WT mice were similar. No effects of aging or IL-6 were found in surface expression of gp130 in cells from IL-6 KO mice.

4. Discussion

In this report, we analyzed the effects of aging and IL-6 on cytokine production by macrophages in vitro. Our results add to published data [15–19] showing decreased synthesis and release of the proinflammatory cytokines, TNFα, IL-1β, IL-6, and IL-12, in macrophages from aged WT mice compared to those from young WT animals after LPS stimulation. In addition, we have found for the first time that knocking out IL-6 restores proinflammatory cytokine production by macrophages from aged mice to the levels of macrophages from young WT mice. When compared to aged WT mice, splenocytes from aged IL-6 KO mice had similar age-related decreases in the relative abundance of CD3+ T cells, CD3+/CD4+ T cells, and Ly49c+ NK cells, however, a more pronounced decrease was found in CD3+/CD8+ T cells. No effects of aging or IL-6 deficiency were found on the percentage of F4/80+ macrophages and CD19+ B cell populations, neither in the surface expression of TLR4, nor the components of the IL-6 receptor, IL-6Ra, and gp130. Overall, our results indicate that IL-6 plays a role in regulating the age-related defects in macrophages through alteration of proinflammatory cytokines. Additional investigation is needed to clarify the cellular mechanisms involved in such effects.

Macrophages show the impact of advanced aged in many of their biological properties including cytokine production (recently reviewed in [1, 2]). In general, when cultured in vitro with LPS, macrophages from aged mice produce lower levels of proinflammatory cytokines than comparably stimulated cells from young mice [15–19]. As previously reported, compared to macrophages from young WT mice, macrophages from aged WT mice had decreased production of TNFα and IL-6. IL-1β and IL-12 production after LPS stimulation also was reduced in macrophages from aged WT mice. These results are coincident with findings from Chelvarajan and collaborators and are independent of the source used to isolate the macrophages (plastic adherence utilized in current study versus positive selection from unfractionated splenocytes using magnetic sorting with CD11b microbeads used by Chelvarajan and collaborators [18]). Overall, our findings agree with published literature in demonstrating decreased cytokine production in macrophages from aged WT mice.

Interleukin-6 deficiency in aged mice restored the cytokine production profile of macrophages to that of young WT mice. Age-related alterations in the function of macrophages result from the combination of intrinsic and extrinsic defects and possibly the consequence of the complex interactions with other cell types or the aged milieu [22]. Our results, added to the previous findings [24, 25, 35–37], suggest that altered production of cytokines in macrophages from aged mice results in part from changes in the expression of extrinsic factors. Cells from young IL-6 KO mice had significantly lower levels of IL-1α, TNFα, and IL-12 when incubated with LPS relative to their WT counterparts. We still don’t have an explanation for these results, however, it can be speculated that a modification in the steady-state of components of cellular pathways shared between the IL-6 signaling cascade and proinflammatory cytokine expression (i.e., Erk and AP-1) may be a suitable common link. Additional studies will clarify this issue.

Interleukin-6 supports the growth of splenic T and B lymphocytes [30, 31]. It was therefore our interest to study if the effects of aging and the lack of IL-6 in cytokine production by macrophages could be attributed to changes in the numbers of these cells in the spleen. While some of the splenocyte populations we tested showed age-related decreases in cytokine release, regardless of the presence of IL-6, we failed to find changes for F4/80+ macrophages between young WT mice and young IL-6 KO mice. Unexpectedly, a more pronounced decrease was found in CD3+/CD8+ T cells (25% in aged WT versus 47% in aged IL-6 KO, P < .05). Our results agree with those from Kopf and collaborators indicating no effects of IL-6 deficiency on the B cell compartment in the bone marrow and spleen of young IL-6 KO mice, with normal expression of B220, IgM, IgD, and CD23 [38]. In this same study the expression of the T cell receptor α, β, γ, δ chains, CD4, CD8, CD44 (Pgpl), and CD24 (HSA) in splenocytes from young IL-6 KO mice was unchanged [38]. Kopf et al. also reported a 30% to 50% reduction in the total number of splenocytes in young IL-6 KO mice compared to young controls [38]. Similarly, we observed a 40% reduction in the number of splenocytes in young IL-6 KO mice as well as in their concanavalin-A induced proliferative response relative to young WT mice (data not shown). Overall, these results stress the involvement of IL-6 in the expansion and function of specific lymphoid cell subsets. Further investigation will help to clarify the effects of IL-6 on some of the age-related T cell defects, such as lymphocyte activation.

The impact of aging on cell signaling in macrophages has been analyzed by several groups (reviewed in [39]). Renshaw and collaborators linked defective production of cytokines in macrophages from aged mice following LPS stimulation to a decline in all subclasses of mRNA for TLRs as well as decreased surface expression of TLR4 on cells from the aged mice when compared with those from young mice [15]. However, two independent studies from separate groups failed to show age-dependent differences in cell-surface expression of TLR4 [16, 18]. In our study, we showed similar surface expression of TLR4 in macrophages from aged WT mice [16, 18]. TLR4 expression was not affected by either aging or lack of IL-6 in macrophages from senescent IL-6 KO mice. Previously, we reported that aged IL-6 KO mice had similar serum levels of lipopolysaccharide-binding protein (LPB) relative to aged WT mice [10]. Neither advanced age nor IL-6 deficiency modified the ability to induce production...
of this protein in vivo after an inflammatory challenge [10]. Therefore, age or IL-6 deficiency does not appear to affect surface expression of the initial molecules involved in LPS signal transduction. This conclusion also seems to be valid for the components of the IL-6 receptor whose levels remained unchanged in macrophages regardless of age or lack of IL-6. In humans, there was a significant increase of soluble IL-6 receptor until around age 70 and a gradual decline in its levels after age 70 [40]. In mice, administration of IL-6 triggered an upregulation of the components of the IL-6 receptor in several tissues in mice embryos early during their development [32]. However, aged MRL/lpr mice, genetically predisposed to the development of autoimmune diseases and reported to have elevated levels of IL-6 and sIL-6R levels, had a marked downregulation of gp130 in splenic T cells [33]. Our findings differ from the published literature and suggest that up or downregulation in expression of the IL-6 receptor seen in other models is unlikely to operate in macrophages when IL-6 is “chronically absent” and maybe alternative compensatory mechanisms are operating in cells from aged IL-6 KO mice. Most likely in our aged IL-6 KO mice, the mechanisms responsible for age-dependent effects of IL-6 in LPS-mediated cell activation observed in macrophages may originate at the intracellular level, such as that which has been observed in macrophages from aged WT mice. Examples of this may include defects in intracellular activation cascades [15–17, 41]. There is only a handful of publications on the effects of IL-6 and aging on specific cell types involved in inflammatory responses. Most of the work has been performed in mouse models of systemic inflammatory responses following burn injury, during sepsis, or after LPS administration. In one of these studies, we analyzed the effects of aging and IL-6 on the hepatic inflammatory response in two models of systemic injury: dorsal scald (burn) injury versus intraperitoneal LPS administration. Evidence obtained from histological observation showed comparable numbers of polymorphonuclear cells (PMNs) in the livers of burn-injured mice regardless of age or IL-6 deficiency. However, increased hepatic neutrophils were seen in aged wild type (WT) mice given LPS relative to young WT mice given LPS. Accumulation of hepatic PMNs was drastically reduced in aged IL-6 KO mice given LPS as compared with LPS-exposed aged WT mice. These results suggest a role of PMNs in the—insult specific—hepatic injury mediated by IL-6 in aged animals [11]. Starr and collaborators examined the expression of IL-6 in various tissues in aged WT mice during LPS-induced systemic inflammation. Among the different tissues tested, white adipose tissue from epididymal fat pad expressed the highest level of IL-6 mRNA in both young and aged mice with a 5.5-fold higher level in the aged. Immunohistochemistry revealed that LPS-induced IL-6 expression was associated to both the adipocytes and stromal cells. Aged IL-6 KO mice exhibited reduced mortality to LPS suggesting a deleterious effect of IL-6 overexpression in the aged [12]. These results suggest that increased vulnerability to systemic inflammation with age is due in part to augmented IL-6 production by the adipose tissue. Overall, a role for other cells, besides macrophages, in work documenting advanced age and IL-6 in vivo is suggested by the two publications showed above [11, 12]. This report is the first to analyze the effects of aging and IL-6 on cytokine production in macrophages in vitro. Additional research will clarify the cellular mechanisms involved in our findings as well as the effects of aging and IL-6 on other cell types.

**Abbreviations**

IL: Interleukin  
IL-6Ra: IL-6 receptor antagonist  
KO: Knockout  
LPS: Lipopolysaccharide  
MFI: Mean fluorescence intensity  
TLR4: Toll-like receptor 4  
TNFα: Tumor necrosis factor alpha  
WT: Wild type.

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**References**


Review Article

Modulation of Adult Mesenchymal Stem Cells Activity by Toll-Like Receptors: Implications on Therapeutic Potential

Olga DelaRosa and Eleuterio Lombardo

Cellerix S.A, Parque Tecnológico de Madrid, Calle Marconi 1, Tres Cantos, 28760 Madrid, Spain

Correspondence should be addressed to Eleuterio Lombardo, elombardo@cellerix.com

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Mesenchymal stem cells (MSCs) are of special interest as therapeutic agents in the settings of both chronic inflammatory and autoimmune diseases. Toll-like receptors (TLR) ligands have been linked with the perpetuation of inflammation in a number of chronic inflammatory diseases due to the permanent exposure of the immune system to TLR-specific stimuli. Therefore, MSCs employed in therapy can be potentially exposed to TLR ligands, which may modulate MSC therapeutic potential in vivo. Recent results demonstrate that MSCs are activated by TLR ligands leading to modulation of the differentiation, migration, proliferation, survival, and immunosuppression capacities. However inconsistent results among authors have been reported suggesting that the source of MSCs, TLR stimuli employed or culture conditions play a role. Notably, activation by TLR ligands has not been reported to modulate the “immunoprivileged” phenotype of MSCs which is of special relevance regarding the use of allogeneic MSC-based therapies. In this review, we discuss the available data on the modulation of MSCs activity through TLR signalling.

1. Introduction

Adult mesenchymal stem cells (MSCs) represent an innovative tool for cell-based therapy of degenerative disorders, chronic inflammatory, and autoimmune diseases or allograft rejection. The understanding of the mechanisms that mediate and/or modulate the therapeutic potency of MSCs is important from both a physiological and a clinical point of view. In this context, one key interest is to better understand the modulation of MSC biology by Toll-like receptors (TLRs) as they have been linked to the perpetuation of chronic inflammatory responses (Chron’s disease, Rheumatoid Arthritis) through the recognition of conserved pathogen-derived components or endogenous ligands (also known as “danger signals”) that MSCs will likely encounter in the sites of injury. Here we will review the most recent data on these topics.

2. Adult Mesenchymal Stem Cells and Cell Therapy

2.1. Differentiation Capacity of MSCs. MSCs have been isolated from multiple tissues of mesodermal origin, such as bone marrow (BM-MSCs), adipose tissue (AD-MSCs), umbilical cord blood and peripheral blood. MSCs can be easily isolated by adhesion to plastic and expanded in vitro in serum containing media with no additional requirements for growth factors or cytokines. In culture, they acquire a fibroblast-like morphology. Despite ample efforts, no exclusive surface markers have been identified for MSCs. Thus, to date they are defined according to the criteria of the International Society for Cellular Therapy by being negative for hematopoetic and endothelial markers such as CD11b, CD14, CD31, CD34, and CD45, and positive for a variety of many other markers, including HLA class I, CD105, CD73, CD29, and CD90 [1]. Therefore, MSCs can be identified in vitro by their ability to differentiate into mesenchymal-type cells (adipocytes, osteoblasts, and chondrocytes) but also neurons, endothelial cells, astrocytes, and epithelial cells when cultured in the appropriate conditions [2–6].

2.2. Immunogenicity of MSCs. The expression of human leukocyte antigen (HLA) molecules class I (also called Mayor Histo-Compatibility class I = MHC I) on all cells on the body allows the immune system to distinguish self from nonself. In the absence of immune suppression or
tolerogenic mechanisms, allogeneic cells are rejected by the immune system upon recognition of their foreign HLA. Cells expressing HLA molecules stimulate T cells directly only if they possess appropriate costimulatory molecules—CD80 (B7-1), CD86 (B7-2) or CD40. Allogeneic cells can also activate T cells through an indirect pathway where their HLA antigens are presented by professional antigen presenting cells (APCs). Aside from HLA class I, certain cells also express HLA class II constitutively or after induction. HLA II also plays an important role in antigen presentation and immune response. A remarkable unique feature of MSCs is that they are considered to be immunoprivileged as they express low levels of cell-surface HLA class I molecules whereas HLA class II, CD40, CD80, and CD86 are not detectable on the cell surface. Stimulation with interferon (IFN)-γ has been shown to increase both class I and class II molecules, however, MSCs do not express costimulatory molecules CD80 (B7-1), CD86 (B7-2) or CD40, even after IFN-γ stimulation. These features allow MSCs to escape to the immune surveillance [7–11]. MSCs not only fail to induce activation of CD4+ cells but also escape lysis by CD8+ cytotoxic lymphocytes [12]. Even whole lymphocytes stimulated in vitro to target Peripheral Blood Lymphocytes (PBLs) derived from a specific donor, will lyse lymphocytes from that individual but not MSCs derived from the same donor. Further analyses indicate that MSCs induce an abortive activation programme in fully differentiated CD8+ T cells so that major effector functions are not activated. MSCs also escape natural killer (NK) cell-specific lysis [12].

2.3. Immunosuppressive Capacity of MSCs. A third important feature of MSCs is that they are immunosuppressive and inhibit activation, proliferation, and function of immune cells, including T cells, B cells, NK cells and antigen-presenting cells (APCs) [6, 13–24]. Despite ample research in recent years, mostly in BM-MSCs, the specific molecular and cellular mechanisms involved in the immunoregulatory activity of MSCs remain controversial. There is evidence that the capability to modulate immune responses relies on both cell contact-dependent mechanisms and soluble factors secreted by MSCs in response to cytokines released by activated immune cells. MSCs may inhibit lymphocyte proliferation by a mechanism that requires, at least in part, the release of soluble factors such as hepatocyte growth factor (HGF), prostaglandin-E2 (PGE2), transforming growth factor (TGF)-β1, indoleamine 2,3-dioxygenase (IDO), nitric oxide, and interleukin (IL)-10 [14, 16, 18, 21, 22, 25–29]. On the other hand, other studies have shown that BM-MSCs may modulate T-cell phenotype resulting in the generation of cells with regulatory activity [14, 15, 19, 30–34].

2.4. MSCs Treatment as a New Therapeutic Tool. The biological characteristics mentioned above make MSCs an interesting tool for cellular therapy and regeneration. This is supported by a number of studies in animal models of inflammatory diseases demonstrating an efficient protection against allograft rejection, graft-versus-host disease, experimental autoimmune encephalomyelitis, collagen-induced arthritis, sepsis and autoimmune myocarditis [13, 28, 31–38]. In fact, MSCs are being used in several clinical trials with a focus on their immunomodulatory capacities (http://clinicaltrials.gov/search?term=stem+cells&term=stem+cells). In this regard, CelleriX is currently conducting a phase III clinical trial using human AD-MSCs to treat complex peri-anal fistula in Crohn patients after having successfully completed phase I and II trial with high efficacy rates [39, 40]. Importantly, it is believed that the therapeutic potency, safety, and efficacy of treatment of inflammatory diseases with MSCs reside to a large extent in their immunologically privileged phenotype and in their immunosuppressive capacity. Interestingly, TLR activation has been implicated in the pathology of a number of inflammatory diseases including rheumatoid arthritis or inflammatory bowel disease (IBD), since they can either initiate or perpetuate the chronic inflammation due to the continue exposure to TLR ligands [41, 42]. Therefore, the use of MSCs in cell therapy for the treatment of inflammatory diseases deserves further investigation regarding the potential effects of TLR signaling on MSCs biology and the potential implications in the immunogenicity and immunosuppressive capacity, which are of special relevance in terms of therapeutic potency.

3. Modulation of MSCs through TLRs

3.1. MSCs Express Active TLRs. Expression of TLRs at the RNA and protein levels have been studied by RT-PCR, flow cytometry, and immunofluorescence. It is well established that MSCs express a number of TLRs. So far, consistent results demonstrate high mRNA expression of TLR 1, 2, 3, 4, 5, and 6 in AD-MSCs and BM-MSCs from both human and mice, while inconsistent results have been reported on the expression of TLR 7 to 10 in the same studies. At the protein level, expression of TLR2, TLR3, TLR4, TLR7, and TLR9 has been reported by flow cytometry and immunofluorescence [23, 28–31, 43].

Expression of TLRs can be modulated in MSCs. Thus, Hwa Cho et al. have studied whether hypoxia can affect the expression of TLRs in human AD-MSCs, as these cells may be used as a therapeutic approach in ischemic tissues [44]. They demonstrated that exposure to hypoxic conditions significantly increase mRNA of TLR1, 2, 3, 5, 9, and 10. Moreover, lipopolysaccharide (LPS) challenge downregulates TLR2 and TLR4 expression in MSC-derived osteoprogenitors [45]. On the other hand, transduction of MSCs with baculoviruses (a DNA viral vector) upregulates expression of TLR3 and triggers TLR3 signaling pathway [46].

As reviewed extensively somewhere else in this issue, TLR activation trigger MyD88 dependent and independent downstream signalling cascades leading to the nuclear translocation of NF-κB and other transcription factors and the activation of a number of genes (see Figure 1) [47–52]. It has been demonstrated that when AD-MSCs or BM-MSCs were stimulated with ligands specific for different TLRs the nuclear factor-κappa B (NF-κB), mitogen-activated protein (MAP) kinases (MAPKs), PI3K signalling pathways were
activated with a subsequent induction of several genes and cytokines, mainly CXCL-10, IL-6 and IL-8. These results clearly demonstrate that MSC express active and functional TLRs. However, differences in the induction of genes in response to TLR activation have been reported. For instance, in contrast to our observations in AD-MSC, Hwa Cho et al., and Tomchuck et al. have reported the induction of tumor necrosis factor (TNF)-α and IL-1β by LPS and polyinosinic:polycytidylic acid (Poly IC) in BM-MSC and AD-MSC, respectively [44, 53].

3.2. Effect of TLR Activation on MSC Survival. In response to TLR stimulation human AD-MSCs induce the expression of manganese superoxide dismutase (MnSOD), a key protective protein against oxidative stress in the mitochondria [23]. It has been reported that induction of MnSOD protects cells from oxidative stress leading to increased survival [54]. In the generation of an oxidative milieu. Based on these data we speculated that increased expression of MnSOD by MSCs in response to TLR ligand exposure would provide them with improved engraftment or survival at injured or inflamed sites, leading to enhanced therapeutic effects [23]. This hypothesis is further supported by recent results showing that TLR4 activation protects MSCs from oxidative stress-induced apoptosis [55]. In fact, LPS preconditioning of mouse BM-MSCs can, when compared to unconditioned MSCs, improve their survival and engraftment of MSCs and increase the release of vascular endothelial growth factor (VEGF) in a model of rat acute myocardial infarction leading to an enhanced therapeutic effect (improved cardiac function, reduced apoptosis of myocardium, reduced fibrosis and elevated vascular density after myocardial infarction) [56] (see Table 1).

3.3. Effect of TLR Triggering in the Differentiation of MSCs. As indicated above, one of the main features of MSCs is the
potential to differentiate to several cell types of mesenchymal origin. Some groups have reported the effects of TLR activation on MSC differentiation with contradictory results. We found no effect on adipogenic differentiation but detected that Poly I:C and LPS increased osteogenic differentiation in human AD-MSCs [23]. However, Hwa Cho et al. [44] reported increased osteogenic differentiation of human AD-MSC by LPS and peptidoglycan (PGN) activation, whereas CpG oligodeoxynucleotides (CpG-ODN) impaired it. The increased osteogenic differentiation in the presence of LPS or PGN was accompanied by increased ERK activation. These authors reported reduced adipogenic differentiation when PGN was present. On the other hand, whereas Mo et al. [45] reported increased osteogenic differentiation of human BM-MSCs after prolonged LPS activation, Liotta et al. [57] found no effect of TLR activation on adipogenic, osteogenic or condrogenic differentiation of human BM-MSCs. However, Pevsner-Fischer et al. [58] reported that TLR2 activation by Pam3Cys reduced mouse BM-MSC differentiation into the three mesodermal lineages.

Interestingly, they also found that myeloid-differentiation primary-response protein 88 (MyD88)-deficient BM-MSCs, when cultured in the appropriate differentiation media without additional stimulation with TLR ligands effectively differentiated into adipocytes but failed to differentiate into osteocytes and chondrocytes, indicating that this pathway may be involved in MSC multipotency. These discrepancies could be due to differences in culture conditions, between bone marrow and adipose-derived MSCs, and between mouse and human cells (see Table 1).

3.4. Effect of TLR Activation on Proliferation and Migration of MSCs. The effect of TLR activation in the proliferation of MSCs has been studied by several groups. Stimulation with LPS or Pam3Cys promoted proliferation of mouse BM-MSCs [55, 58], but stimulation with LPS, Poly I:C, lipoteichoic acid (LTA) and PGN showed not significant effects on human AD-MSCs and BM-MSCs [23, 44, 45]. However, Hwa Cho et al. reported that stimulation of AD-MSCs with CpG-ODNs leads to a G1 arrest wich results in inhibition of proliferation [44] (see Table 1).

Migration to the appropriate site of injury is considered to play an important role in the therapeutic efficacy of MSCs. In this context, Tomchuck et al. demonstrated that TLR3 activation drives the migration of human BM-MSCs using transwell and Boyden chamber migration assays suggesting that this TLR signalling pathway may be manipulated to increase the biodistribution of infused MSCs at the injured sites [53]. Moreover, LPS, ODNs, LL-37 (an antimicrobial peptide), fibronectin fragment III 1C (Fn III1C) and flagellin resulted in moderate to limited induced migration. On the other hand, Pevsner-Fischer et al. found that TLR2 activation impaired mouse BM-MSC migration using “wound healing” assays [58] (see Table 1). Nevertheless, further investigation will be required to better understand the potential role of TLR signalling in migration and biodistribution of MSCs in vivo, which is of great clinical relevance.

3.5. TLRs and the Immunogenic Phenotype of MSCs. The potential use of allogeneic MSCs relies on the special capacity of these cells to escape to the immune recognition. It is well established that TLR activation can modulate expression of costimulatory molecules in immune cells. Therefore, it is of special interest from a therapeutic point of view to determine whether exposure of MSCs to TLR ligands may induce the expression of HLA-I, HLA-II, and costulatory molecules (CD40, CD80, CD86) leading to an augmented immunogenic phenotype. We analyzed the expression of HLA-I, HLA-II, CD80, and CD86 in human AD-MSCs by flow cytometry 72 hours after stimulation with LPS, Poly I:C, and PGN. We found that LPS, Poly I:C, and PGN did not alter the expression of HLA-II, CD80, and CD86. Poly I:C was the only TLR ligand capable to induce HLA-I to some extend. Furthermore, costimulation of human AD-MSCs with IFN-γ (a well-known inducer of HLA-I and HLA-II expression in MSCs) in combination with either LPS, Poly I:C or PGN did not alter the IFN-γ-mediated induction of HLA-I and HLA-II [23]. Similar results have also been reported in human BM-MSCs [43, 57]. These results indicate that TLR activation does not significantly affect the immunogenic properties of human MSCs (see Table 1). These results are of great relevance regarding the use of allogeneic MSC-based cell therapies.

3.6. Effect of TLR Activation on the Immunosuppressive Capacity of MSCs. BM-MSC and AD-MSCs have been shown to possess the capacity to inhibit proliferation of immune cells upon mitogenic or allogeneic activation. As mentioned above, this immunosuppressive capacity of all MSCs can become a key factor for their therapeutic use and potency. The mechanisms underlying the immunosuppression potential of MSCs are not fully understood, but seem to require both cell-to-cell contact-dependent mechanisms and the release of soluble immune modulators (IDO, PGE2, TGF-β1, nitric oxide, etc.) upon activation in response to immune cells. Interestingly, some of these immune modulators are downstream of signalling pathways triggered by TLRs in other cell types. Therefore, a feasible hypothesis is that TLR ligands may induce the production of such anti-inflammatory mediators in MSCs resulting in an enhanced immunosuppressive phenotype. Moreover, TLR signalling has been associated with the perpetuation of chronic inflammatory and autoimmune diseases such as Crohn’s disease and Rheumatoid Arthritis [41, 42]. Therefore, AD-MSCs and BM-MSCs employed in the treatment of such diseases will likely be exposed to TLR ligands, which may result in the modulation of MSCs activity and therapeutic potency. Therefore, it is very important to determine whether TLR signalling may modulate the immunosuppressive capacity of MSCs.

In recent years, several groups have reported inconsistent results regarding the role of TLR ligands on the modulation of MSCs capacity to suppress immune responses. In this context, we tested the role of TLRs in the immunosuppressive capacity of human AD-MSCs. To do so, we analyzed proliferation of activated CFSE-labeled PBLs, CD4+ T cells
and CD8+ T cells in the absence or presence of increasing amounts of human AD-MSCs precultured for up to 72 hours with medium alone or in the presence of LPS, Poly I:C or PGN. We found no significant effect of TLR activation on human AD-MSC-mediated suppression, indicating that activation through TLR2, TLR3, and TLR4 do not significantly interfere with the capacity of human AD-MSCs to modulate immune responses in vitro. Supporting these results, IDO, a key mediator of human AD-MSCs immunosuppression [24] was weakly induced by a high concentration of Poly I:C and was not induce upon TLR2 or TLR4 triggering [23]. Similar results were reported by Pevsner-Fischer et al., showing that TLR2 activation by Pam3Cys does not affect immunosuppression mediated by mouse BM-MSC [58]. However, other groups have reported that TLR activation may modulate the immunosuppressive properties of human BM-MSCs, although in very different ways. Thus, Liotta et al. found that TLR3 and TLR4 activation reduce the inhibitory activity of human BM-MSCs on T-cell proliferation without influencing IDO activity or PGE2 levels [57]. By using inhibitors of the Notch signalling pathway and anti-Jagged-1 neutralizing antibodies they found that TLR activation leads to the downregulation of the Notch ligand Jagged-1 in BM-MSCs. Based on these data, the authors suggested that Notch signalling pathway mediates the cell contact-mediated immunosuppression by MSCs. In contrast to these results, Opitz et al. have recently reported that TLR3 and TLR4 engagement enhances the immunosuppressive properties of human BM-MSCs through the indirect induction of IDO1 [43]. Induction of IDO1 involved an autocrine IFN-β signalling loop, which was dependent on protein kinase R (PKR) and independent on IFN-γ.

A common characteristic of all MSCs is that they constitutively express IL-6 and IL-8. The significance of a constitutive expression of IL-6 and IL-8, which can both be considered proinflammatory cytokines, in context to the immunosuppressive activity of MSCs is still unclear. Interestingly, it has been reported that MSCs inhibit the differentiation of dendritic cells, at least in part, through the release of IL-6 [59]. This observation links IL-6 production to the immunosuppression mediated by MSCs. Hence, it is tempting to speculate that induction of IL-6 secretion by TLR activation may enhance MSCs-mediated impairment of dendritic cells differentiation and maturation.

### Table 1: Summary of reported effects on MSC activity by TLR ligands.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Adipogenic</th>
<th>Condrogentic</th>
<th>Osteogenic</th>
<th>Immuno-genicity</th>
<th>Immuno-suppression</th>
<th>Migration</th>
<th>Proliferation</th>
<th>Survival</th>
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<td>[45]</td>
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</table>

= (no significant effect), ↑ (increase), ↓ (inhibition), AD (adipose derived MSCs), BM (Bone marrow MSCs). LPS: Lipopolysaccharide; PolyIC: Polynosinic:polycytidylic acid; PGN: Peptidoglycan; CpG-ODNs: CpG oligodeoxynucleotides; Fn III1C: Fibronectin fragment III 1C; LTA: Lipoteichoic acid.
These inconsistent and partly contradictory results demonstrate the complexity of the immune system, even under in vitro conditions. It is likely that differences in the experimental settings between laboratories might be behind these contradictory results (see Table 1). For instance, the use of PBMCs versus purified T cells or the method of activation of these cells may play a role. Liotta et al. employed purified CD4+ T cells stimulated with allogeneic T cell-depleted PBMCs and anti-CD3 mAbs, Opitz et al. used a mixed lymphocyte reaction (MLR) with total PBMC from two unrelated donors, one of which was irradiated, and we used either whole PBMC samples or purified CD4+ and CD8+ fractions stimulated with beads loaded with anti-CD3, anti-CD2 and anti-CD28 mAbs. Another important aspect could be the time of treatment with TLR ligands and the concentration of them (very variable among studies). While some left TLR ligands in the cocultures or pretreated the MSCs with TLR ligands for several days before initiating the coculture experiments [23, 57], others pretreated MSCs for 24 hours, washed and cocultured them with the MLR [43]. It has also been suggested that a long-term exposure to TLR ligands may lead to downregulation of factors induced shortly after activation of TLR [43].

As several studies have reported beneficial effects of MSCs treatment in animal models of LPS-induced sepsis or lung injury [32, 38, 60], an inhibition of a therapeutic capacity of MSC by TLR ligands does not appear to be the case. Thus, it is unclear whether in vivo potency of MSCs can be potentiated by TLR ligands, however it does not appear to be impaired.

4. Concluding Remarks

It is necessary to better define the role of TLR activation on MSC biology in the context of the development of new therapeutic strategies on inflammatory or autoimmune diseases, simply because MSCs are likely be exposed to activation through TLR ligands in the sites of injury or inflammation. The discrepancies shown by different authors need further investigation as it would be relevant to determine whether or not TLR activation interferes or enhances migration, biodistribution or immunosuppressive capacity of MSCs. Differences in the source of MSCs, type and concentration of the stimuli used, the experimental settings and method of detection or culture conditions may explain these discrepancies. On the other hand, the recognition of endogenous ligands by TLRs is now thought to have an important role in the regulation of inflammation, both in infectious and noninfectious diseases. A number of endogenous ligands have been identified, including heat shock protein (HSP) 60, HSP 70, heparan sulfate, hyaluronan, fibronectin extra domain A, uric acid, oxidized LDL, intracellular components of fragmented cells, myeloid-related proteins-8 and 14, eosinophil-derived neurotoxin, and human defensin-3 [61–72]. As these ligands are accessible to TLRs in the setting of injury or non-infectious threat, they have been called “danger signals”. A very important aspect that has not been studied in details so far is the activation and modulation of MSC activity by these danger signals. Given the capacity of MSCs to modulate immune responses it would be interesting to determine whether resident MSCs may be activated by danger signals in the settings of injury and if this activation results in a contribution of MSCs to the process of healing and cure in homeostasis.

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