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Immune-Regulatory Mechanisms in Systemic Autoimmune and Rheumatic Diseases

Guest Editors: Britt Nakken, Philip Alex, Ludvig Munthe, Zoltan Szekanecz, and Peter Szodoray

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Editorial Board

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Editorial

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Britt Nakken,[1] Philip Alex,[2] Ludvig Munthe,[1] Zoltan Szekanecz,[3] and Peter Szodoray[1]

[1] *Institute of Immunology, Rikshospitalet, Oslo University Hospital, University of Oslo, Oslo 0027, Norway*
[2] *Department of Medicine, Johns Hopkins University, Baltimore, MD 21205, USA*
[3] *Department of Rheumatology, University of Debrecen Medical and Health Sciences Center, Debrecen 4012, Hungary*

Correspondence should be addressed to Peter Szodoray, szodoray@gmail.com

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Autoimmune diseases encompass a wide range of organ-specific and systemic disorders with a complex etiology. An intricate interplay of genetic, environmental, as well as immunological factors leads to the development of these debilitating diseases. In the absence of infections, regulatory processes inhibit immune responses towards antigen. Moreover, the immune system has multiple levels of negative feedback mechanisms that dampen immune responses and counteract establishment of chronic and destructive immunity. These immune-regulatory functions include a broad spectrum of cellular and molecular mechanisms, which control autoimmune responses. In autoimmune animal models and in patients with autoimmune conditions, various disorders of such regulatory mechanisms have been described. Knowledge and understanding of the immunomodulatory and pathogenic mechanisms that contribute to these conditions can lead to the development of novel diagnostic strategies, and future therapies, providing better life expectancies to patients with autoimmune diseases.

In this special issue, we present original research articles, as well as review papers on the role of derailed regulatory mechanisms underlying autoimmune diseases.

The paper by Y. Takakubo and Y. T. Konttinen gives an overview of the most important immune-regulatory mechanisms in systemic autoimmune and rheumatic diseases, encompassing the failure of crucial tolerogenic mechanisms, with a special emphasis on tolerogenic dendritic cells, regulatory T and B cells, Th17 cells, inflammatory and tolerogenic cytokines, and intracellular signaling pathways. The paper also introduces the next-generation therapies, beyond the currently used biologic therapies, targeting derailed immune-regulatory processes.

The paper by C. López−Pedrera et al. addresses epigenetic mechanisms of immune-regulatory functions in conjunction with cardiovascular risk in systemic autoimmune diseases. Epigenetic regulatory mechanisms comprise DNA methylation, histone modifications, and microRNA activity, which influence the development of autoimmune diseases. Other two review articles describe novel immunopathologic roles of diverse cytokines, chemokines, signaling molecules and pattern-recognition receptors in systemic lupus erythematosus, as well as addressing the interaction of CD154 with its various receptors, outlining the role of CD54 in the pathogenesis of lupus and rheumatoid arthritis (RA).

Three papers present various immune-regulatory mechanisms in connection with RA.

The paper by J. Furuzawa-Carballeda et al. evaluates the effect of intramuscular administration of polymerized collagen in early and established collagen-induced arthritis (CIA) in mice and analyzes changes in Th subsets following therapy. Polymerized-type I collagen induces upregulation of Foxp3-expressing CD4+ regulatory T-cells and downregulates IL-17-producing CD4+ T-cells (Th17) cells in CIA. Based on these findings, polymerized-collagen may be an effective therapeutic agent in early and established RA by exerting down-regulation of autoimmune inflammation.

The paper by Y. Shi et al. shows that enhanced high mobility group box chromosomal protein 1 (HMGB1) expression can contribute to Th17 cell activation, and thereby to the perpetuation of autoimmune processes in RA. Another research article in the RA-section of the special issue suggests the Notch pathway may be involved in the pathophysiology of RA, by mediating TNF-α-induced IL-6 production in cultured fibroblast-like synoviocytes.

The paper by B. Szalay et al. assesses the phenotype of T-cell subsets and describes early T-cell activation characteristics in patients with Ankylosing Spondylitis (AS) in conjunction to intravenous therapy with the anti-TNF agent, infliximab. The paper describes that the frequency of Th2 and Th17 cells is higher in AS compared to healthy individuals. This abnormal immune phenotype together with functional disturbances of CD4+ and CD8+ cells in AS can partially be restored by infliximab administration.

The paper by D. Mieliauskaite et al. describes the expression of IL-17, IL-23 and their receptors in minor salivary glands of patients with primary Sjögren's syndrome.

The paper by E. D. Abston et al. investigates the role of virus-activated Toll-like receptor (TLR)3 and its adaptor TRIF on the development of autoimmune coxsackievirus B3 (CVB3) myocarditis in mice and shows that TLR3 versus TRIF deficiency results in altered Th2 responses that uniquely influence the progression to chronic myocarditis.

The paper by B. De Paepe et al. gives an overview on the TNF superfamily of cytokines in idiopathic inflammatory myopathy. For each TNF family member, the possibilities for treating inflammatory diseases in general and the idiopathic inflammatory myopathies in particular are explored.

The paper by S.-J. Chen et al. introduces the current status of immune-regulatory processes and immunomediated therapeutic strategies for multiple sclerosis and highlights the growing evidence that Th17 cells play a pivotal role in the complex adaptive autoimmunity of the disease and discusses the roles of the associated immune cells and cytokines.

The paper by N. Rieber et al. presents current concepts of hyperinflammation in the pathogenesis of chronic granulomatous disease (CGD). The paper summarizes the role of reduced neutrophil apoptosis and efferocytosis, dysbalanced innate immune receptors, altered T-cell surface redox levels, induction of Th17 cells, the enzyme indolamine-2,3-dioxygenase (IDO), impaired Nrf2 activity and inflammasome activation, as well as their potential therapeutic implications in CGD.

This special issue encompasses basic, molecular mechanisms of immune-regulation in connection with autoimmune processes, cellular and molecular immune-regulatory functions, which can aid as biomarkers for diagnostics, as well as potential targeting of the immune-regulatory machinery as part of future therapeutic interventions in patients with autoimmune diseases.

Britt Nakken
Philip Alex
Ludvig Munthe
Zoltan Szekanecz
Peter Szodoray

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Review Article

Immunopathological Roles of Cytokines, Chemokines, Signaling Molecules, and Pattern-Recognition Receptors in Systemic Lupus Erythematosus

Shui-Lian Yu,[1] Woon-Pang Kuan,[2] Chun-Kwok Wong,[3] Edmund K. Li,[1] and Lai-Shan Tam[1]

[1] *Department of Medicine and Therapeutics, Prince of Wales Hospital, The Chinese University of Hong Kong, 30-32 Ngan Shing Street, Shatin, Hong Kong*
[2] *Department of Rheumatology, Hospital Selayang, Lebuhraya Selayang-Kepong, 68100 Batu Caves, Malaysia*
[3] *Department of Chemical Pathology, Prince of Wales Hospital, The Chinese University of Hong Kong, 30-32 Ngan Shing Street, Shatin, Hong Kong*

Correspondence should be addressed to Lai-Shan Tam, lstam@cuhk.edu.hk

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Systemic lupus erythematosus (SLE) is an autoimmune disease with unknown etiology affecting more than one million individuals each year. It is characterized by B- and T-cell hyperactivity and by defects in the clearance of apoptotic cells and immune complexes. Understanding the complex process involved and the interaction between various cytokines, chemokines, signaling molecules, and pattern-recognition receptors (PRRs) in the immune pathways will provide valuable information on the development of novel therapeutic targets for treating SLE. In this paper, we review the immunopathological roles of novel cytokines, chemokines, signaling molecules, PRRs, and their interactions in immunoregulatory networks and suggest how their disturbances may implicate pathological conditions in SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease which is characterized by a loss of tolerance to nuclear antigens and various immunological abnormalities, including dysregulated activation of both T and B lymphocytes and subsequent polyclonal activation of circulating B lymphocytes which produces a large quantity of autoreactive antibodies and the formation of immune complexes causing tissue and organ damage [1]. This is a complex process involved interaction between various cytokines, chemokines, signaling molecules, and pattern-recognition receptors (PRRs) in the immune pathways. With the advent of new and advanced technique which include intracellular cytokine analysis by flow cytometry combined with multiplex quantization of cytokine levels in recent years, it had provided us a reasonable understanding of the activation profile of cytokine production and new insight in the immune and cellular mechanism in the pathogenesis of SLE, which further clarify the significance of the current body of literatures. This had provided valuable information on the development of novel therapeutic targets for treating SLE. This article will focus on the recent advances of cytokines, chemokines, signaling molecules, and the role of PRRs in immunopathogenesis in SLE.

2. Imbalance of Th1/Th2 Cytokines in SLE

Cytokines are a group of small peptides or glycoprotein produced by a wide variety of cells with molecular weights between 8 and 30 kDa. They had been shown to play an essential role in modulating the immune response against foreign or self-antigens. These mediators have been classified according to their cellular source and effector functions, with the paradigmatic T helper (Th)1 and Th2 cytokine families best illustrating this division of function. Th1 cells arise in response to dendritic cells- (DCs-) derived interleukin- (IL-) 12, produce tumor necrosis factor- (TNF-) α, interferon- (IFN-) γ, and are involved in mediating strong inflammatory responses to intracellular pathogens. IL-4-mediated Th2 cell

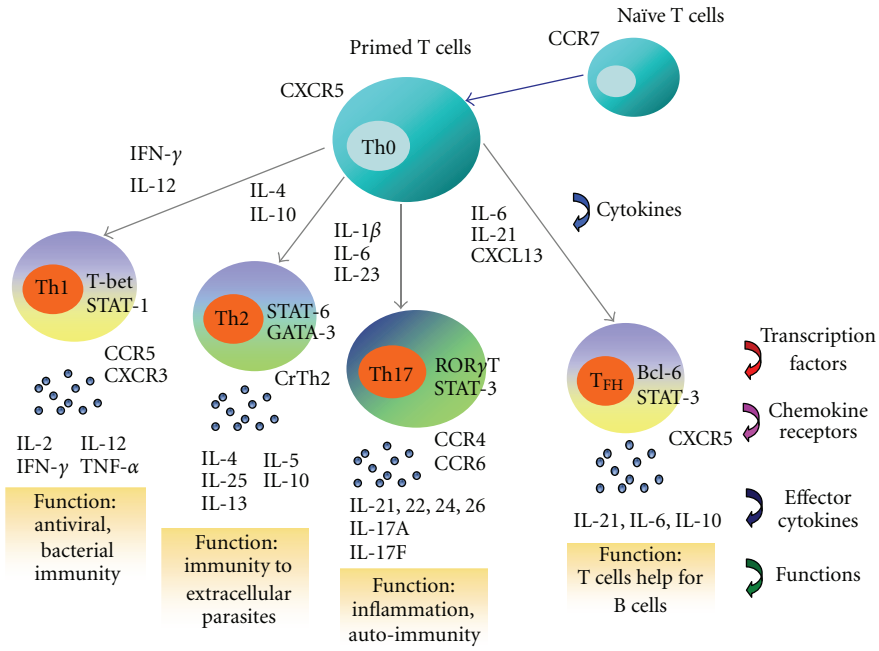

Figure 1: Effector T-cell differentiation (Th1, Th2, Th17 and T_{FH}), the expression of transcription factors, effector cytokines, chemokine receptors, and T-cell functions.

differentiation results in cells that produce cytokines, including IL-4, IL-5, and IL-13, which mediate antibody responses to extracellular pathogens (Figure 1).

The ratios of Th1 and Th2 cytokines have been investigated to determine the cytokine homeostasis in order to determine whether Th1 or Th2 predominance during the development of SLE [2, 3]. SLE was thought to be a Th2-polarized disease because of the production of auto-antibodies specific for self-antigens [4]. However, significantly elevated cytokines for Th1 response including IL-12, TNF-α, and IFN-γ were also found in the plasma of SLE patients [5–8]. Th1 dominant immune responses have been generally considered to be pathological in autoimmune disease via the induction of inflammatory reaction. Recently, few cytokines which had been shown to be of great importance in pathogenesis of SLE had surfaced with advent of new technology in detection, which enhances our understanding of their role in SLE-related immune pathway. These cytokines, including IL-12, IL-23, IL-18, IL-21, and IL-33, will be discussed below.

2.1. IL-12. IL-12 is a heterodimeric cytokine of 70 kDa comprising covalently linked p40 and p35 subunit which has been shown to be a central stimulator of Th1-related proinflammatory cytokine that induces IFN-γ in both innate and adaptive immunity [9, 10]. IL-12 had been suggested to be associated with progression of severe glomerulonephritis [11]. Moreover, mRNA levels of p19, p40, and p35 of IL-12 were found to be significantly higher in active SLE patients compared with those patients with inactive disease [12]. Accordingly, serum level of IL-12 was also found to be significantly elevated in SLE patients, and it is associated with the increased level of Th1 cytokine IFN-γ but decreased level of Th2 cytokine IL-13 [5, 13, 14]. Conversely, another study reported the decreased *ex vivo* production of IL-12 from peripheral blood polymorphonuclear leukocytes (PMN) stimulated by lipopolysaccharide (LPS) in patients with active SLE [15] using a different ELISA kit. Recently, the elevated plasma IL-12 concentration has been shown to exhibit positive correlation with systemic lupus erythematosus disease activity index (SLEDAI) in SLE patients with renal impairment, supporting IL-12 could play a pathological role in the development of autoinflammatory response in SLE patients with severe disease, probably through the recruitment of the effector leukocytes to the inflamed tissue for orchestrating the immunoresponse at the site of inflammation [16].

2.2. IL-23. IL-23 is a novel heterodimeric cytokine composed of a unique p19 subunit, and a common p40 subunit shared with IL-12. IL-23 shares similar intracellular signal transduction molecules with IL-12, therefore both cytokines exhibit some overlapping function in promoting cellular immunity [17]. Different from IL-12, IL-23 does not promote the development of IFN-γ-producing Th1 cells, but is crucial for the expansion of a pathogenic $CD4^+$ T-cell population characterized by the production of IL-17 and IL-22 [18, 19]. Recent studies had shown that the mRNA levels of IL-23p19 were significantly higher in active SLE patients when patients were stratified into different disease activity groups, thereby suggesting that IL-23 should play a role in SLE disease exacerbation [12]. Moreover, the likely significance of IL-23 in autoinflammatory responses was further supported by a more recent report indicated that Th1 transcription factor T-bet could upregulate IL-23 receptor expression and the differentiation of Th1 and Th17 cells in autoimmunity [20] (Figure 1). IL-23 has been reported to enhance the IL-17

secretion by peripheral blood mononuclear cells (PBMC) from healthy subjects [20]. Moreover, the pathogenic Th17 subgroup expresses elevated level of IL-23 receptor via the activation by T-bet, thereby representing a distinct inflammatory Th cell lineage for the development of organ-specific autoimmune inflammation [18, 20–22]. In order to better elucidate the involvement of IL-23 in the IL-23/IL-17 autoinflammatory axis and the immunopathological mechanisms of the activation of Th17 cells in SLE, Wong et al. have used IL-23 as an activating agent to demonstrate the direct involvement of IL-23 in the IL-23/IL17 inflammatory axis. It acts to induce a distinct T-cell activation state that produces IL-17 as the effector cytokine that promotes the autoinflammatory responses in SLE [16].

2.3. IL-18. IL-18 was originally identified as a factor that enhances IFN-γ production in macrophages, T lymphocytes, and DCs [23]. Previous studies also reported that the involvement of this Th1-related cytokine in initiating both innate and acquired immune responses [24, 25]. It has been elucidated that IL-18 along with IL-12 is a potent inducer of the inflammatory mediators by T lymphocytes, causing severe inflammatory disorders in autoimmune diseases such as rheumatoid arthritis (RA) [26]. In SLE, previous studies by our group and others have demonstrated the increased levels of IL-18 in serum/plasma of affected persons, which positively correlated with disease severity [13, 27–29]. Of interest is the elevated urinary IL-18 levels that were found significantly increased in patients with established acute tubular necrosis [30] and the increases within 24 hours after kidney transplantation in patients with delayed allograft dysfunction [31], suggesting that IL-18 may serve as an prognostic marker of renal involvement useful to identify patients at risk of renal failure. Possible pathogenic role of IL-18 in lupus has been studied in a mouse model of progressive disease, demonstrating that IL-18 has a multifaceted role in autoimmune lupus, being apparently involved both in the effector phases of the late organ damage and, in some organs, in the initial pathogenic events [32, 33].

2.4. IL-21. IL-21 is a pleiotropic cytokine, produced by $CD4^+$ Th cells, that modulates the differentiation and function of T cells, B cells, natural killer (NK) cells, and DCs by binding to the receptor composing of the IL-21 receptor-α (IL-21Rα) and the common γ chain [34, 35]. Recent study has intimated that IL-21 can mediate the differentiation and generation of follicular helper T cells (T_{FH}) [34, 36] (Figure 1). Nevertheless, autocrine production of IL-21 from T_{FH} cells can potently stimulate the differentiation of B cells into antibody-forming cells through IL-21R [37]. As a result, dysregulation of T_{FH} cell function may relate to the pathogenesis of SLE. IL-21 has been shown to contribute to the development of autoimmune diseases in different animal models of SLE, experimental autoimmune encephalomyelitis, and RA [35]. The genetic association of IL-21 polymorphisms has also been demonstrated in SLE [38]. Recent animal study has revealed that elevated production of IL-21, T_{FH} dysfunction within germinal centers, and aberrant positive selection of germinal center B cells are required for the production of autoantibodies and systemic autoimmunity [39, 40].

2.5. IL-33. IL-33, a novel member of the IL-1 cytokine family [41], has recently been shown to be involved in the pathogenesis of chronic inflammatory disease [42–44] similar to other family members IL-1 and IL-18 [45]. IL-33 is responsible for the protection against helminth infections and prevention of atherosclerosis by promoting Th2 immune responses [46]. The IL-33 receptor, consisting of ST2 and IL-1 receptor accessory protein, is also widely expressed, particularly on Th2 cells and mast cells [47], to mediate important effector Th2 functions [48]. Although the elevated ST2 protein in the sera of SLE and other patients with autoimmune diseases has been reported [49], its causal relationship with disease activity is still unclear. Recently, significantly elevated serum soluble ST2 (sST2) but not IL-33 has been detected in SLE patients, and the levels of sST2 were found to correlate with the disease activity and severity of these medical conditions. It therefore suggested that the sST2 level may have a potential role as a surrogate marker of disease activity [44]. In this study, no correlation was found between serum IL-33 level and sST2 level, lupus disease activity, or specific organ involvement. In contrast, others reported that serum IL-33 level was significantly increased in SLE compared with healthy controls (HCs). Increased IL-33 level was significantly associated with thrombocytopenia, erythrocytopenia, and anti-SSB antibody, suggesting IL-33 may exert biologic effects on erythrocytes and platelets or their precursors in SLE [43]. In summary, the role of the IL-33/ST2 system in the pathogenesis of SLE remained unclear.

3. Imbalance of Th1/Th2 Transcription Factors in SLE

Although the control of the Th1/Th2 imbalance has been unclear, there is growing evidence to suggest that two major transcription factors, T-box expressed in T cells (T-bet) and GATA binding protein 3 (GATA-3), are the determining factors of T-helper cell differentiation [50]. T-bet, a Th1-specific transcription factor, has been postulated to initiate Th1 development while inhibiting Th2 cell differentiation [51]. GATA-3 is a member of the GATA zinc finger protein family, and enhances the development of the Th2 phenotype while inhibiting Th1 cells [52–54]. Recent study had demonstrated that the mRNA levels of T-bet and IFN-γ and the relative expression levels of T-bet/GATA-3 and IFN-γ/IL-4 were significantly higher, in contrast to the lower expressions of GATA-3 and IL-4, in SLE patients [55]. There were also significant correlations in mRNA expression of T-bet with IFN-γ and of GATA-3 with IL-4. Additionally, the relative expressions of T-bet/GATA-3 and IFN-γ/IL-4 were found to correlate with lupus disease activity. Moreover, the elevated plasma Th1/Th2 cytokine ratio of IL-18/IL-4 was also shown to correlate positively with disease activity in all SLE patients, suggesting the functional activation of peripheral blood Th1 cells in SLE patients. Thus, previous study provided us with new insight that ratio of T-bet/GATA-3 expression is more informative than the level of either transcription

factor alone, which may be disproportionately affected by the changes in their coexpression in cell populations. The T-bet/GATA-3 expression ratio not only enhances our understanding of Th1/Th2 polarization, it may also serve as a supplementary tool for further assessment of Th1/Th2 status and development of SLE disease activity (Figure 1).

4. Th17-Mediated Inflammation of SLE

Apart from Th1 and Th2 cells, there is a novel subset of IL-17 producing effector T helper cells, called Th17 cells, whose dysregulation is thought to participate in the pathogenesis of SLE [56, 57]. Transforming growth factor (TGF)-β, IL-6, IL-21, and IL-23 have been implicated for Th17 formation [58, 59]. Other proteins involved in their differentiation are signal transducer and activator of transcription 3 (STAT3) and the retinoic-acid-receptor-related orphan receptors alpha (RORα) and gamma (RORγ) [58]. Moreover, effector cytokines associated with this cell type are IL-17, IL-21, and IL-22 [60] (Figure 1). We herein highlighted some of the biological effects of IL-17 implication for Th17-mediated inflammation of SLE.

IL-17 is a type I 17-kDa transmembrane protein that comprises six members and five receptors mostly produced by activated T cells [61]. It is a pleiotropic proinflammatory cytokine that enhances T-cell priming and stimulates epithelial, endothelial, and fibroblastic cells to produce multiple proinflammatory mediators, including IL-1, IL-6, TNF-α, and chemokines [62]. Additionally, IL-17 also exerts its effects through the recruitment of monocytes and neutrophils by increasing the local production of chemokines (IL-8, monocyte chemoattractant protein-1, growth-related oncogene protein-α), the facilitation of T-cell infiltration and activation by stimulating the expression of intercellular adhesion molecule-1 by T cells as well as the amplification of the immune response by inducing the production of IL-6, prostaglandin E_2, granulocyte-macrophage colony-stimulating factor, and granulocyte colony-stimulating factor [63]. Lastly, this cytokine synergizes with other cytokines, in particular with IL-1β, TNF-α, and IFN-γ [63].

Wong et al. have demonstrated that SLE patients have higher plasma/serum levels of IL-17 than HCs [13, 16, 56], which positively associated with SLE disease activity [16]. Accordingly, the frequency of IL-17-producing T cells is increased in peripheral blood of SLE patients [16, 64]. Significant levels of IL-17 and IFN-γ were detected in T cells from SLE patients [64]. Additionally, overproduction of total immunoglobulin G (IgG), antidouble stranded DNA, and IL-6 by PBMC of patients with lupus nephritis was observed upon the stimulation with IL-17 [65], suggesting a potential role of IL-17 in human lupus progression. On the other hand, no elevation of IL-17 was found in serum of cohort of Japanese lupus patients [66]. Most recent evidence suggested that the ability of regulatory T cells (T_{regs}) to express IFN-γ and IL-17 was impaired in SLE patients, whereas the proportion of T_{regs} was similar between SLE patients and HCs [67]. Additionally, studies in mice support the concept that IL-17 and Th17 cells may be involved in the development of lupus nephritis [56, 68]. For instance, IL-17 was recently found to be critical for the formation of autoreactive germinal centres in autoimmune BXD2 mice, a strain that develops a lupus-like syndrome [69]. In a spontaneous mouse model of lupus, the New Zealand Black (NZB) mice, stimulation of splenocytes with nucleosomes as an autoantigen results in the activation of large numbers of IL-17-secreting T cells [70]. Upon adoptive transfer to naïve recipient mice, IL-23-dependent IL-17 producing $CD4^+$ effector T-cell subset Th17 can invade the target organ and promote the development of organ-specific autoimmune inflammation. Consistently, Wong et al. also found that the proinflammatory cytokine IL-23 and IL-12 can promote the disease severity by activating pathogenic Th1 and Th17 cells via the induction of downstream Th1 chemokine CXCL10 and inflammatory cytokine IL-17 in SLE, demonstrating that the IL-23/IL-17 axis of inflammation and related molecules may arise as a therapeutic target for treating autoimmune disease.

5. Chemokines in SLE

Chemokines in itself refer to a group of smaller cytokines (mass between 8 to 12 kDa) with chemotactic properties, which are classified into four families according to the location of cysteine residues. The four chemokine groups are CC, C, CXC, and CX_3C, where C is a cysteine and X is any amino acid residue [71]. These small molecules have had well-defined roles in directing cell migration necessary for the initiation of T cell immune response, attraction of appropriate effector cells to sites of inflammation, and regulation of differential recruitment of T helper (Th1 and Th2) lymphocytes [72–74]. There has been growing evidence suggesting that infiltration of T lymphocytes and other leucocytes into the sites of inflammation plays a critical role in organ involvement in SLE [75]. Recent studies have also shown that chemokines and their receptors are intimately involved in regulating organ-specific leucocyte trafficking and inflammation, suggesting their important roles in the pathophysiology of autoimmune diseases such as RA, multiple sclerosis, and SLE [76–78]. Chemokine CXCL13 in emerging studies had consolidated the important role of these chemokines in pathogenesis of SLE. Other chemokines that will be briefly discussed in this article mainly include CC and CXC chemokines which had been shown to play some roles in SLE disease.

5.1. CXCL13. CXCL13/B lymphocyte chemoattractant (BLC) is a small cytokine belonging to the CXC chemokine family that is produced by cells in the omentum, peritoneal macrophages, and DCs [79, 80], which is selectively chemotactic for B cells including both the B1 and B2 subsets by interacting with specific chemokine receptor CXCR5 [79, 81]. The accumulation of B1 cells in the peritoneal cavity and spleen are responsible for the body cavity immunity and the production of autoantibody for the development of autoimmune disease in the murine model [79, 82, 83]. Elevated levels of B1 cells have been documented in patients with autoimmune disorders such as Sjogren's syndrome and RA [84, 85]. Previous studies using murine model of SLE

showed that CXCL13 is highly produced by $CD11b^+$ $CD11c^+$ DCs in the target organs including thymus and kidney for the chemoattraction of B1 cells into target organ [83, 86–88]. Therefore, the elevated expression of CXCL13 by myeloid dendritic cells (mDCs) in the target organs may play a crucial role in breaking the immune tolerance in the thymus leading to the activation of self-reactive $CD4^+$ Th cells and the recruitment of autoantibody producing B cells in the development of murine lupus [83, 87, 88]. In addition to that, studies have revealed that CXCL13 can induce the trafficking of distinct $CXCR5^+$ T cells designated as T_{FH} which are specifically involved in high-affinity IgG production in germinal centers developed within B-cell follicles of secondary lymphoid tissues including lymph nodes, spleen, and tonsils [36, 89–91]. $CD4^+$ T_{FH} cells, located at B-cell follicles, provide a T helper function to B cells and represents one of the most numerous and important subsets of effector T cells in lymphoid tissue [37, 92]. Several studies demonstrated that B-cell chemokine CXCL13 is ectopically and highly expressed in thymus and kidney in murine model for SLE. Studies on humans also demonstrated that serum CXCL13 level was significantly elevated in SLE patients and the elevation correlated significantly with SLE disease activity [93, 94]. As anti-TNF-α treatment was found to be able to reduce the plasma level of CXCL13 in RA patients [95], it had been postulated that serum level of CXCL 13 can act as a disease activity marker for both RA and SLE patients.

5.2. CC Chemokines. Monocyte chemoattractant protein-1 (MCP-1/CCL2) is a prototype CC chemokine, which can attract monocytes, T cells, NK cells, and basophils [96, 97]. An increase of serum MCP-1/CCL2 was observed with the progression of disease activity in SLE patients compared to HCs [98]. Further investigation reported that cerebral spinal fluids (CSF) MCP-1/CCL2 levels were significantly higher in neuropsychiatric syndromes of systemic lupus erythematosus (NPSLE) patients than those non-NPSLE patients [99]. Regulated upon activation, normal T-cell-expressed and secreted (RANTES)/CCL5 is another CC chemokine which attracts monocytes, memory T cells, and NK cells [100]. Increased plasma RANTES/CCL5 concentrations were found in SLE patients more than in controls, and correlated significantly with SLEDAI score [101]. Moreover, the expression of miR-125a was found to contribute to the elevated expression of RANTES/CCL5 in SLE [102]. In addition to that, studies from animal models and patients with lupus nephritis demonstrated that inflammatory chemokines, especially CCL2 and CCL5, are detectable in kidney tissues and urine before other signs of inflammation [103–106]. With this finding, urine chemokines had been proposed as a possibility to serve as biomarkers for renal SLE flare [107], suggesting that the reduced plasma concentration of these circulating chemokines in lupus patients with renal involvement may result from a protein leakage in the urine.

5.3. CXC Chemokines. Interferon-gamma inducible protein-10 (IP-10)/CXCL10 and monokine induced by gamma-interferon (MIG)/CXCL9, the prototype of the CXC family, have chemotactic activity mainly for activated Th1 cells and are involved in the pathogenesis of various Th1-dominant autoimmune diseases [71, 108]. Their synthesis and expression from neutrophils, macrophages, and other immune cells are induced by IFN-γ, and this response is suppressed by IL-10 and IL-4 [71, 109]. Th1 cells and IFN-γ had been shown to be important for cell-mediated inflammation in developing autoimmune disease such as SLE [5], thus implicated that these chemokines might have an important role in pathogenesis of SLE. Furthermore, several studies have shown that levels of IP-10/CXCL10 and MIG/CXCL9 were significantly elevated in active SLE [98, 110, 111]. Moreover, Okamoto et al. reported that IP-10/CXCL10 was upregulated in the central nervous system (CNS) fluid of NPSLE [112, 113], suggesting that IP-10/MCP-1 ratio in CSF is a useful diagnostic marker of NPSLE [112]. On the other hand, CXC chemokines CXCL8 and CXCL1 are potent chemoattractants and activators of T cells, neutrophils, thereby enhancing their proinflammatory and proangiogenic activities [114]. They also stimulate neutrophil degranulation to release reactive oxygen radicals, thereby inducing an acute inflammatory reaction [115, 116]. They had also been shown to be significantly elevated in serum of patient with active lupus, and the elevation was associated with disease activity [111].

6. Intracellular Signaling Pathways in SLE

Signal transduction refers to an ordered biochemical process by which a signal or stimulus is transferred within a single cell. This cascade begins with binding of extracellular signaling molecules to cell surface receptors, triggering an initial stimulus that propagated into the cytoplasm. Nowadays, the most well-known and established signal transduction pathway that has been identified is mitogen-activated protein kinase (MAPK) pathway. MAPKs are serine and threonine protein kinases that can be activated by phosphorylation in response to extracellular stimuli, such as mitogens, growth factors, cytokines, and osmotic stress [117, 118]. Nuclear translocation of activated MAPKs can induce and transactivate transcription factors including nuclear factor- (NF-) κB and activator protein 1, which facilitate the modulation of gene transcription in cellular activation, proliferation, apoptosis, and the expression of cytokines, chemokines, adhesion molecules, and metalloproteinases [117, 118]. Three main distinct MAPKs, p42/p44 extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal protein kinase (JNK), and p38 MAPK, have been identified in mammalian cells. The activation of NF-κB, JNK, and p38 MAPK plays crucial roles in cytokine-mediated signaling pathways regulating the release of chemokines and the expression of adhesion molecules of eosinophils and Th cells [119–121]. Activation of p38 MAPK has been shown to be crucial for B-cell activation leading to Ig production, and p38 MAPK regulates the production of a number of cytokines, including IL-6 that promotes the differentiation and survival of plasma cells [122]. Moreover, B-cell-activating factor of the TNF family, an essential factor for B-cell activation and differentiation, was regulated through JNK and p38 MAPK [123]. Furthermore, nuclear factor of activated T cells (NFAT), a downstream

transcription factor of the ERK and JNK pathways, is essential for T and B lymphocyte activation and differentiation [124], and specific anti-NFAT drug therapy has been shown to be pharmacologic armamentarium against RA, inflammatory arthropathies, and related autoimmune disorders [125].

7. Interaction between Cytokines, Chemokines, and Signaling Molecules in SLE

As mentioned before, immunopathogenesis of SLE is a complex process that involved the interaction and synergistic effect of various cytokines, chemokines, and signaling molecules which perpetuate the disease activity in SLE. This section below will highlight the recent update on the interaction between all these agents in promoting the disease activity in SLE.

7.1. Role of IL-18 and Chemokines. The potential role of IL-18 and chemokines in the exacerbation of SLE disease had been highlighted in a study, which provided valuable information on the development of SLE disease markers [111]. In this study, plasma concentration of CXCL10, CCL5, CXCL9, CXCL8, CXCL1, and CCL2 was significantly elevated in SLE patients and the elevation was correlated significantly with disease activity. Furthermore, plasma concentration of IL-18 was found to be correlated positively with production of CXCL10, CXCL9, CXCL1, and CXCL8 in SLE patients, it was also shown to be a potent costimulus for the induction of these chemokine release from activated PBMC as there was a significant increase in *ex vivo* production of these inflammatory chemokines when their PBMC were cultured in the presence of IL-18.

This enhances our knowledge that successful delivery of the appropriate population of leucocytes to sites of acute inflammation will depend on the repertoire of inducible chemokines synthesized locally, and the temporal expression of chemokine receptors on the leucocytes. Meanwhile, the chemokine expressions are influenced by proinflammatory cytokines, mainly IL-18, to present in the local environment of the cells at the time of stimulation. Furthermore, inflammatory activities of IL-18, together with the induction of Th1 cytokine IFN-γ and the activation of Th cells, natural killer cells (NK), and cytotoxic T lymphocytes-inflammatory chemokines, may even enhance the Th1-mediated inflammatory process, the activation of NK and T cells, and the migration of macrophages for initiating and perpetuating the Th1 immune response in SLE. In summary, the correlation of raised plasma concentration and *ex vivo* production of inflammatory chemokines with disease activity, and their association with IL-18, supports that the chemotaxis of Th1/Th2 lymphocytes and neutrophils is important in SLE pathogenesis.

7.2. Role of CXCL13 and IL-21. A recent study [93] had shown that CXCL13 and IL-21 may relate with the immunopathogenesis mediated by the function of T_{FH} cells in SLE as serum level of all these cytokines were found to be significantly elevated in lupus patient with the increase in CXCL13 concentration correlated positively and significantly with SLEDAI score. Furthermore, cell surface expression of CXCR5 on Th and B cells and IL-21R on B cells was found to be significantly lower in SLE patients, which indicated that most differentiated T_{FH} cells migrate out from circulation into lymphoid organ upon activation during the disease development of SLE. This piece of information suggests that the elevated production of CXCL13, BAFF, and IL-21 may be associated with the function of T_{FH} for the immunopathogenesis in SLE, and CXCL13 may serve as a potential disease marker of SLE.

7.3. Role of IL-23, IL-17, IL-18, Th17, and CXCL10. The pathogenic role of IL-23/IL-17 autoinflammatory axis in SLE had been elucidated in a recent study [16]. First, parallelly elevated plasma IL-12, IL-17, and CXCL10 concentrations exhibited positive correlation with the SLEDAI in their lupus patients with renal impairment, which supported that these cytokines cascade could play a pathological role in the development of autoinflammatory response in SLE patients with severe disease, through the recruitment of the effector leukocytes into the inflamed tissue for orchestrating the immunoresponse at the site of inflammation. Second, when using IL-23 as activator, the CD3 and CD28 costimulated PBMC responded with an aberrant *ex vivo* production of IL-17, which provided robust evidence on the direct involvement of IL-23 in the IL-23/IL-17 inflammatory axis, which acts to induce a distinct T-cell activation state that produces IL-17 as the effector cytokine that promotes the autoinflammatory responses in SLE. Third, *ex vivo* production of IL-12, IL-23, and IL-17 from PMBC was significantly enhanced by the presence of IL-18 which indicated that the expressions of inflammatory cytokines IL-12, IL-23, and IL-17 and activation of Th17 cells are in part influenced by proinflammatory cytokine IL-18 present in the local environment of the cells during stimulation. IL-23-mediated activation of IL-17-producing Th cells in SLE patients may closely be influenced by IL-18 activation, which orchestrates the inflammation of SLE. In conclusion, proinflammatory cytokine IL-18 and IL-12 family cytokines IL-12 and IL-23 can promote the disease severity by activating pathogenic Th1 and Th17 cells via the induction of downstream Th1 chemokine CXCL10 and inflammatory cytokine IL-17 in SLE.

7.4. Role of MAPK, IL-18, and CXCL10. As for the roles of MAPK transduction pathway in pathogenesis of SLE, highly abnormal ERK and NF-κB activities in T lymphocytes of lupus patients had been reported [126, 127]. The lyn kinase deficiency in B lymphocytes and decreased ras-MAPK in T lymphocytes had also been demonstrated in SLE patients [128–130]. A recent study had further consolidated the facts that p38 MAPK and JNK are the key signaling molecules in regulating the inflammation-mediated hyperactivity of T and B lymphocytes in SLE [131]. In this study, the basal expressions of p38 MAPK in $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, and B lymphocytes had been shown to be significantly higher in SLE patients, and the expression of phospho-p38 MAPK in $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, and B lymphocytes, and phospho-JNK in $CD8^+$ T

lymphocytes and B lymphocytes was also significantly elevated in SLE patients upon the activation by IL-18, exhibiting significant correlation with the plasma concentrations of Th1 chemokine CXCL10. Furthermore, the expression of phospho-JNK in IL-18-activated $CD8^+$ T lymphocytes and the relative percentage (%) fold increase of the expression of phospho-JNK upon IL-18 activation in B lymphocytes were significantly correlated with SLE disease activity index. Therefore, the inflammation-mediated activation of JNK and p38 MAPK signaling pathways in T and B lymphocytes can be the underlying intracellular mechanism causing lymphocyte hyperactivity in SLE.

8. Pattern-Recognition Receptors in SLE: Friend or Foe

An infectious etiology of SLE has been a longstanding hypothesis [132–134] and with the discovery of PRRs in SLE, the role of bacteria and viruses in the pathogenesis of SLE has been invigorated. PRRs can alert and activate the innate immune system through recognizing the conserved molecular patterns to distinguish extrinsic pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs). Several PRRs participated in the recognition of viral components, such as genomic DNA and RNA, in a replication-independent way. Additionally, cells express intracellular RNA helicases that function as PRRs of actively replicating viruses [135]. These PRRs are also essential in establishing antiviral immunity by triggering type I interferon responses.

8.1. Toll-Like Receptor in SLE. Being the most studied PRRs, Toll-like receptors- (TLRs-) mediated intracellular signaling is a crucial link between innate and adaptive immunity [136], which principally sense structurally conserved molecular motifs called PAMPs for triggering NF-κB, p38 MAPK, JNK, and the IFN pathways, which results in the translocation of transcription factors, cytokine modulation, and IFN-stimulated gene regulation leading to inflammatory responses [137]. The stimulation of TLR by PAMPs is an important prerequisite for the induction of various autoimmune diseases [138]. To date, at least 10 human TLRs have been identified, and the functions of human TLR1-9 have been characterized [138, 139]. Cell surface TLRs (TLR-1, 2, 4, 5, and 6) are designed for the engagement of extracellular pathogens, whereas the intracellular TLRs (TLR-3, 7, 8, and 9) are against intracellular pathogen-derived products [140].

Animal studies of SLE have indicated that TLRs are important in the pathogenesis of lupus mouse. For instance, in myeloid differentiation primary response gene (MyD) 88-deficient MRL/MpJ-Fas(lpr) (MRL/lpr) mice, both MyD88-dependent and -independent innate signals were found to play a crucial role in the development of autoimmune nephritis [141]. Treatment of lupus-prone mice with a dual inhibitor of TLR-7 and TLR-9 was found to lead to the reduction of autoantibody production and amelioration of disease symptoms [142]. Lupus-prone mice deficient in TLR-7 also failed to generate antibodies against RNA-containing antigens such as Smith, which decreased lymphocyte activation and serum IgG [143]. Conversely, the absence of TLR-9 can exacerbate the disease activity by the activation of lymphocytes and plasmacytoid dendritic cells (pDCs), inducing the subsequent increase of serum IgG and IFN-γ [143]. Emerging evidence revealed that TLR-9 was involved in class-switching to pathogenic autoantibody production in SLE [144, 145]. Accordingly, patients with active SLE had been shown to have upregulated expression of TLR-9 in peripheral blood memory and plasma B lymphocytes, suggesting that endogenous nucleic acids released during apoptosis may stimulate B lymphocytes via TLR-9 and contribute to SLE pathogenesis [146]. Upregulated expression of TLR-7 and TLR-9 mRNA, together with IFN-γ mRNA in PBMC, may also contribute to the pathogenesis of human lupus [147]. Consistently, other study also revealed that PBMCs of SLE patients with a higher expression of TLRs are more prone to be activated by diverse TLR ligands when compared to HCs [147, 148], suggesting that the innate immune response for extracellular pathogens and self-originated DNA plays immunopathological roles via TLR activation in SLE.

Recent study by our group found that antagonist-mediated diminished intracellular TLRs might act as potent activators of innate immune responses involved in the higher prevalence of human papillomavirus infection (HPV) in SLE [149]. TLR antagonist, such as hydroxychloroquine, might decrease the expression of intracellular TLRs in SLE patients, thereby increasing the risk of acquiring HPV infection. Moreover, high-risk HPV infections may play a predominant role in further downregulating the expression of intracellular TLR in SLE patients with HPV infection resulting in a higher prevalence of persistent infection, suggesting that the avoidance of stimulation and downregulation of the innate immune system, which might permit persistence of HPV in SLE, is evidently part of an immune evasion strategy used by oncogenic HPV establishing of persistence infection [149].

8.2. Nucleotide-Binding Oligomerization Domain Containing 2 in SLE. In contrast to the well-elucidated membrane-bound TLRs, cytoplasmic nucleotide binding oligomerisation domain (NOD) receptors are a new family of PRRs for the recognition of extracellular PAMPs [150, 151]. Two NOD-like receptor (NLR) proteins, namely, NOD1 and NOD2, can participate in the signaling events triggered by host recognition of specific motifs of bacterial peptidoglycans (PGNs) and, upon activation, induce the production of proinflammatory mediators [150]. NOD1 recognizes products from gram-negative bacteria (diaminopimelic acids), whereas NOD2 senses muramyl dipeptide (MDP), a peptidoglycan derived peptide from gram-negative as well as gram-positive bacteria [152]. It has been shown that NLRs complement and synergize with TLRs in innate immune responses [153–156]. NLRs are associated with Crohn's disease and inflammatory arthritis [155–157]. However, the precise mechanisms by which NOD-mediated recognition of PGNs in the pathogenesis of inflammatory diseases are still unclear. Apart from the putative link between the genetic variants of NOD2 and SLE [158–163], little is known about the expression and function of NOD2 in SLE [164]. Our recent study demonstrated an overexpression of NOD2 in

monocyte of immunosuppressant naïve SLE patients with longer process might lead to activation of PBMCs to produce proinflammatory cytokines, implicating the innate immune response for extracellular pathogens in immunopathological mechanisms in SLE [165]. Conversely, immunosuppressive therapy may downregulate the expression of NOD2 in $CD8^+$ T, monocytes, mDCs, and pDCs in SLE which subsequently reduce regulatory cytokine IL-10, allowing for an aberrant inflammatory response contributing towards the regulation of immunopathological mechanisms of SLE, at the expense of increasing risk of bacterial infection [165]. NOD1 expression in PBMC subsets of SLE patients and HCs could not be detected using flow cytometry [165].

Recently, increased prevalence of HPV and tuberculosis (TB) in SLE has been reported by our group and others [166–169]. Whether the immune evasion strategy, specific bacteria, or virus could escape PRRs recognition, establishment of persistent infection in SLE playing a significant part in host-pathogenic interaction need further considerations. Further elucidation of the infectious process and immune response against infections and exploration of the efficacy of agonists as therapeutic tools for eliminating infected cells in SLE will be worth investigation.

9. Conclusion

The understanding of the immunopathologic mechanisms of SLE has been gradually evolving with budding studies on assessing the activation of monocytes, T, and B lymphocytes upon stimulation of various stimuli and also underlying intracellular signaling mechanisms. This further enhanced our current and limited knowledge regarding the cellular mechanism and pathway in the immunopathogenesis of SLE, which had shed light on developing potential and novel therapies in treating this chronic immunological disorder. Therapeutic inhibitors of the pathways of JNK or p38MAPK [170, 171] and antibodies against IL-21, CXCL13 [172, 173], and TLR [174, 175] have been shown to exhibit some promising beneficial effects. Hopefully, with the advent of more advanced technology and emergence of more studies, our understanding for this elusive disease can be further strengthened in the future.

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Research Article

Expression of IL-17, IL-23 and Their Receptors in Minor Salivary Glands of Patients with Primary Sjögren's Syndrome

Diana Mieliauskaite, Irena Dumalakiene, Rita Rugiene, and Zygmunt Mackiewicz

Department of Experimental and Clinical Medicine, State Research Institute Center for Innovative Medicine, Zygimantu Street 9, 01102 Vilnius, Lithuania

Correspondence should be addressed to Diana Mieliauskaite, diana.mieliauskaite@ekmi.vu.lt

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The main purpose of this study was to determine the expression of interleukins-17/-23 (ILs-17/-23) and receptors of interleukins-17/-23 (IL-17R, IL-23R) in minor salivary glands (MSGs) of patients with primary Sjögren's syndrome (pSS). Expression of IL-17, IL-23 and receptors of IL-17/-23 was analyzed in MSGs from 25 patients with pSS, 25 patients with probable preclinical pSS, and 25 patients with nonautoimmune sicca syndrome by immunohistochemistry. Comparison of the expression of IL-17, IL-23 and receptors of IL-17, IL-23 in MSG of patients with pSS with probable preclinical pSS, and with nonautoimmune sicca syndrome showed significant differences between three groups. However, the expression of IL-17, IL-23 and receptors of IL-17/-23 in MSG was comparable in pSS and probable preclinical pSS patients. We did not find correlation between the expression of IL-17 and IL-23 and of IL-17R and IL-23R in patients with pSS. These results demonstrate an involvement of IL-17/-23 system in the early pSS pathogenesis.

1. Introduction

Primary Sjögren's syndrome (pSS) is a systemic chronic inflammatory autoimmune disorder that affects secretory organs. pSS is not only characterized by sicca syndrome but also by extraglandular manifestations that reveal the severity of this disorder. Patients with pSS also present broad spectrum analytical features (cytopenias, hypergammaglobulinemia, and cryoglobulins) and autoantibodies (antinuclear antibodies, anti-Ro/SSA, and anti-La/SSB antibodies). Thus, the spectrum of the disease ranges widely from minimal local symptoms of the eyes and oral mucosa to systemic involvement and the development of malignant lymphoma, the latter being the most worrisome complication of primary Sjögren's syndrome [1–8]. Pathophysiology of Sjögren's syndrome is not yet fully understood. Cytokines play a central role in the regulation of immunity and are often found to be deregulated in autoimmune diseases [9]. Recently, much attention has been focused on the relationship between innate responses and subsequent activation of specific adaptive immunity in an attempt to understand subsequent immune dysregulation. With the identification of CD4+ Th17 cell population, which initially challenged the long-standing Th1/Th2 paradigm, several of these important relationships between innate and adaptive immunity are now being uncovered. A subset of CD4+ memory T cells are characterized by their unique ability to secrete interleukin-17 (IL-17) family cytokines. Importantly, Th17 cells appear to be intimately involved in autoimmunity. As mature memory cells, Th17 cell survival and maintenance appear to be dependent on IL-23 [9–16].

The main purpose of this study was to determine the expression of interleukins-17/-23 and receptors of interleukins-17/-23 (IL-17R, IL-23R) focusing on autoimmune epithelitis in patients with primary Sjögren's syndrome. We set tasks to investigate a relationship between expression of IL-17 and IL-23 in patients with primary Sjögren's syndrome.

2. Material and Methods

2.1. Patients. Participants of the study were selected from patients seen at the State Research Institute Center for Innovative Medicine: 25 patients with pSS who meet the American-European criteria for primary SS, 25 patients with

Table 1: Clinical and serologic characteristics of the patients.

Variable	pSS ($n = 25$)	Probable preclinical pSS ($n = 25$)	Nonautoimmune sicca syndrome ($n = 25$)
Age, mean (s.d)	59.6 (11.9)	58.4 (13.4)	63.1 (10.1)
Ocular symptoms, *n* (%)	25 (100)	0 (0)	25 (100)
Oral symptoms, *n* (%)	25 (100)	0 (0)	25 (100)
Schirmer *I* test, ≤5 mm/5 min., *n* (%)	25 (100)	0 (0)	25 (100)
Unstimulated salivary flow rate, ≤1.5 mL/15 min., *n* (%)	25 (100)	0 (0)	25 (100)
Salivary secretion, mean (s.d)	1.0 (0.22)	2.1 (0.4)	1.4 (0.17)
Histopathology, focus score ≥1, *n* (%)	25 (100)	20 (80)	0 (0)
Salivary gland enlargement, *n* (%)	5 (20)	0 (0)	0 (0)
Arthralgias, *n* (%)	18 (72)	15 (60)	14 (56)
Raynaud phenomenon, *n* (%)	3 (12)	1 (4)	0 (0)
Cutaneous vasculitis, *n* (%)	1 (4)	0 (0)	0 (0)
Pulmonary involvement, *n* (%)	3 (12)	0 (0)	0 (0)
Renal involvement, *n* (%)	0 (0)	0 (0)	0 (0)
Fatigue, *n* (%)	17 (68)	19 (76)	0 (0)
ESR, ↑, *n* (%)	7 (28)	12 (48)	0 (0)
Leucopenia, *n* (%)	6 (24)	8 (32)	0 (0)
Anemia, *n* (%)	8 (32)	6 (24)	0 (0)
RF, >15 kU/I, *n* (%)	25 (100)	25 (100)	25 (100)
ANA, >1 : 40, *n* (%)	25 (100)	25 (100)	25 (100)
ACA, negative, *n* (%)	25 (100)	25 (100)	25 (100)
Anti-dsDNA, negative, *n* (%)	25 (100)	25 (100)	25 (100)
Anti-Scl-70, negative, *n* (%)	25 (100)	25 (100)	25 (100)
Anti-Sm, negative, *n* (%)	25 (100)	25 (100)	25 (100)
Anti-Ro/SSA, *n* (%)	21(84)	23 (92)	0 (0)
Anti-La/SSB, *n* (%)	0 (0)	0 (0)	0 (0)
Both anti-Ro/SSA and anti-La/SSB, *n* (%)	4 (16)	2 (8)	0 (0)

probable preclinical pSS who did not met fully the American-European criteria for primary SS, and 25 patients with nonautoimmune sicca syndrome. A new international consensus for Sjögren's syndrome diagnosis requires objective signs and symptoms of dryness including a characteristic appearance of a biopsy sample from a minor salivary gland or autoantibody such as anti-SSA/Ro or/and anti-SSB/La [3].

Symptoms and signs of Sjögren'syndrome are subtle and can be intermittent or nonspecific [4, 6, 17–20]. Our experience has revealed that about one-third of patients with pSS had extraglandular manifestations before the onset of sicca ocular and oral symptoms and signs including mild asymptomatic hematologic manifestations (anemia, leucopenia, and increased erythrocyte sedimentation rate). It has been estimated that these signs are present for a mean of seven years before primary Sjögren's is properly diagnosed. Patients who had mild asymptomatic hematologic abnormalities (anemia, and leucopenia, increased erythrocyte sedimentation rate), positive anti-SSA/Ro or/and anti-SSB/La, and negative serology for hepatitis C, EBV, cytomegalovirus, and human immunodeficiency virus were included in the probable preclinical pSS group. All patients underwent an extensive medical examination. Following the initial evaluation by a rheumatologist, each patient was referred to Sjögren's syndrome specialists for a review of his/her medical history, an oral and an ocular examination, Schirmer's *I* test, unstimulated salivary flow rate, and a labial gland biopsy. Also, all patients underwent extensive serologic evaluations which included test for the presence of rheumatoid factor (RF), antinuclear antibodies (ANA), anticentromere antibodies, anti-double-stranded DNA, anti-Scl-70, anti-Sm, anti-SSA/Ro, and anti-SSB/La (Table 1). Patients of all groups had negative serology for hepatitis C, EBV, cytomegalovirus, and human immunodeficiency virus.

Informed and written consent was obtained from all patients who participated in this study. The study has been approved by the Lithuanian Bioethics Committee (2009-06-03, No.: 158200-6-061-15).

2.2. Labial Salivary Gland Biopsy. Labial salivary gland biopsies were performed on patients by the oral medicine specialist. A local anesthetic was injected into the lower lip followed by a small incision to the right or left of the lip midline. Five or six minor salivary gland lobules were harvested and placed into formalin fixative. Standard paraffin preparations were prepared, sectioned, and stained with hematoxylin and eosin. The slides were examined for the presence of lymphocytic infiltrates by 2 board-certified pathologists.

2.3. Immunohistochemical Staining. Biopsy specimens from the lips of patients were placed in 10% phosphate-buffered formalin for 24 hours. Fixed tissues were embedded in paraffin and sectioned at 4 μm thick sections with *Microm* microtome. 4 μm tissue sections were deparaffinized by immersion in xylene followed by dehydratation in ethanol. Antigen retrieval was performed in 0.01 M sodium citrate buffer, pH 6.0, at +98°C for half an hour in *Milestone* histoprocessor or with 0.05% trypsin solution (pH 7.8) for 20 minutes at 37°C., followed by cooling down at +22°C for 30 min. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 minutes at +22°C. Following a 5-minute wash with PBS, sections were incubated at +22°C for 30 minutes with primary antibody at the room temperature.

Primary antibodies were used: rabbit anti-human IL-17 (H-132) polyclonal antibody, 2 μg/mL (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA); goat anti-human IL-17 RD/SEF receptor polyclonal IgG, 4 μg/mL (R&D Systems, Minneapolis, MN, USA); mouse anti-human IL-23 (p19) monoclonal IgG1, κ, 4 μg/mL (BioLegend, San Diego, CA, USA); goat anti-human IL-23 receptor polyclonal antibody, 4 μg/mL (Capralogics, Inc. Hardwick, MA, USA).

Incubation with appropriate biotinylated secondary antibodies: rabbit anti-goat, horse anti-mouse, or goat anti-rabbit, all from the VECTASTAIN ABC kit, 1 : 200 (Vector Laboratories, Burlingame, CA, USA), was performed at +22°C for half an hour and was used following manufacturer's instructions. All incubations were performed in humid atmosphere. The staining was developed by using diaminobenzidine substrate (Vector Laboratories, Burlingame, CA, USA), and counterstaining was performed with hematoxylin (Merck, Darmstadt, Germany), followed by mounting in HistoGel (Lab. Storage Systems Inc., St. Peters, MO, USA). Slides were rinsed in PBS 2 × 5 minutes after each step.

2.4. Evaluation of the Results. Semiquantitative microscopic assessment of immunohistochemical staining was performed in light microscope *Olympus BX51* under ×400 (high-power fields) scoring 5 random fields in 10 random similarly stained slides using four grades: "0" = no immunoreactivity; "1" = low immunoreactivity; "2" = moderate immunoreactivity; "3" = high immunoreactivity. In every slide were separately scored: (1) acini; (2) glandular ducts; (3) intraglandular interstitium; (4) blood vessels; (5) infiltrating inflammatory cells. The immunoreactivity grades of IL-17, IL-23, IL-17R, and IL-23R in the separate localizations sum is a total expression of mentioned interleukins and their receptors. Theoretically, maximum score could reach the value of 15. Practically, such a value never occurred in this study. Two histologists evaluated the results of histological and immunohistochemical staining independently using this predefined and a very simple scoring system leading to very similar readouts and followed by a consensus session.

2.5. Statistical Analysis. All values presented are the mean ± SD. Statistical differences were analyzed with the Mann-Whitney, Kruskal-Wallis tests using standard program SPSS 19.0. P values less than 0.05 were considered significant.

3. Results

IL-17, IL-23, IL-17R, and IL-23R were detected in glandular ducts (d), acini (a), blood vessels (bl v), intraglandular interstitium (i), infiltrating inflammatory cells (ly) of minor salivary glands (MSGs) of patients with pSS ($n = 25$), probable preclinical pSS ($n = 25$), and nonautoimmune sicca syndrome ($n = 25$). Representative photomicrographs of the expression patterns in patients with pSS, probable preclinical pSS, and nonautoimmune sicca syndrome are shown in figures (Figures 1 and 2).

Assessment of the total expression of IL-17, IL-23 and receptors of IL-17, IL-23 in MSGs of patients with pSS, probable preclinical pSS, and nonautoimmune sicca syndrome and comparison of the total expression of IL-17, IL-23 and receptors of IL-17, IL-23 between three groups showed significant differences ($P < 0.05$) (Tables 2 and 3). The mean total expression of IL-17, IL-23 and receptors of IL-17, IL-23 was increased in MSGs of pSS patients compared with nonautoimmune sicca syndrome patients ($P < 0.05$). However, the total expression of IL-17, IL-23 and receptors of IL-17, IL-23 in MSGs of pSS was comparable to probable preclinical pSS patients. Comparison of the total expression of IL-17, IL-23, and IL-17R between probable preclinical pSS and nonautoimmune sicca syndrome patients showed significant differences. Furthermore, the highest expression of IL-17, IL-23 was revealed in glandular ducts and inflammatory cells of patients with pSS and probable preclinical pSS. Expression of IL-17R in glandular ducts was increased in MSGs of pSS ($P < 0.05$) and was comparable in the glandular ducts of patients with probable preclinical pSS and nonautoimmune sicca syndrome.

The total expression of IL-23 was increased in MGS of pSS patients compared with nonautoimmune sicca syndrome patients. We found the highest expression of IL-23 in glandular ducts and acini of patients with pSS and probable preclinical pSS. The IL-23 expression in blood vessels, intraglandular interstitium, and infiltrating inflammatory cells of minor salivary glands was comparable in three groups. Our study revealed that the total expression of IL-23R was significantly higher compared with the total expression of IL-23 in MSGs of patients with probable preclinical pSS. The total expression of IL-23R and of IL-23R in glandular ducts, acini, intraglandular interstitium, and infiltrating inflammatory cells was comparable in pSS and

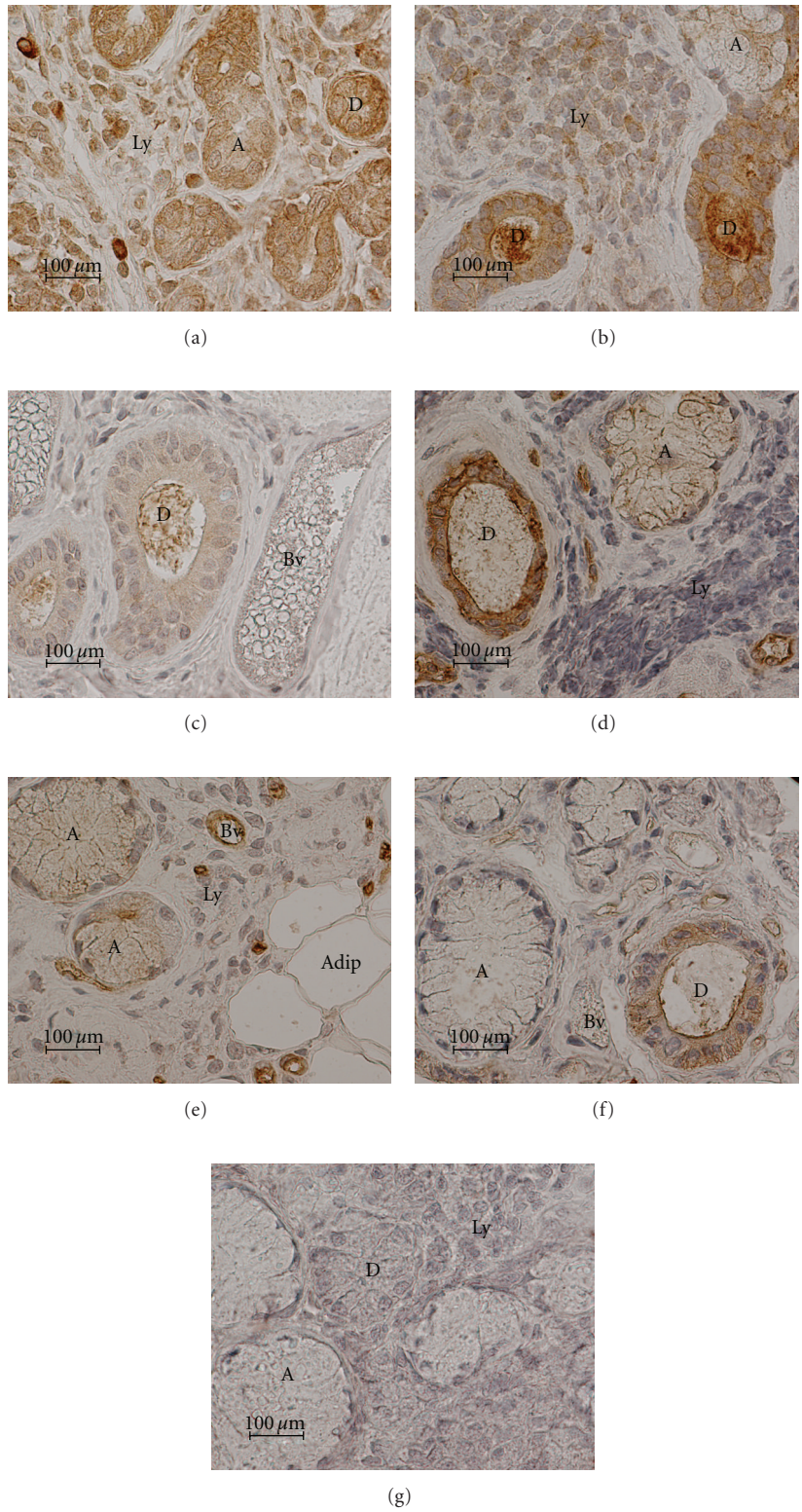

Figure 1: Expression of IL-17 and IL-17R in minor salivary glands of patients with pSS, probable preclinical pSS, and nonautoimmune sicca syndrome. IL-17: (a) pSS, (b) probable preclinical pSS, (c) nonautoimmune sicca syndrome. IL-17R: (d) pSS, (e) probable preclinical pSS, (f) nonautoimmune sicca syndrome, (g) negative staining control. Counterstained with hematoxylin. Original magnification ×400. Abbreviations: A: acinus, Adip-adipocytes, Bv: blood vessel, D: ductus, and Ly: lymphocyte infiltration.

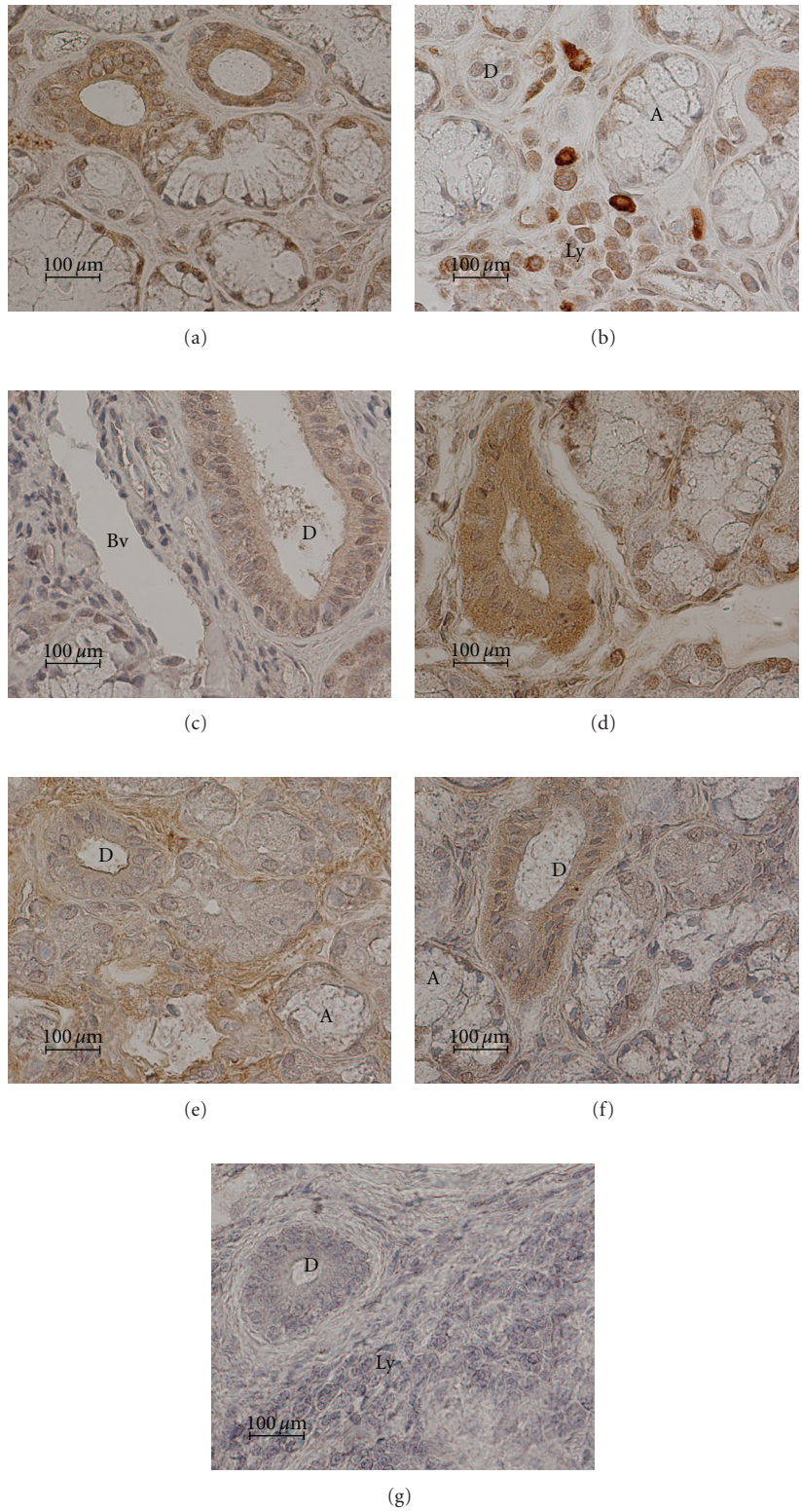

Figure 2: Expression of IL-23 and IL-23R in minor salivary glands of patients with pSS, probable preclinical pSS and nonautoimmune sicca syndrome. IL-23: (a) pSS, (b) probable preclinical pSS, (c) nonautoimmune sicca syndrome. IL-23R: (d) pSS, (e) probable preclinical pSS, (f) nonautoimmune sicca syndrome, (g) negative staining control. Counterstained with hematoxylin. Original magnification ×400. Abbreviations: A: acinus, Bv: blood vessel, D: ductus, and Ly: lymphocyte infiltration.

Table 2: Expression (grades) of IL-17 and IL-17R in minor salivary glands of patients with primary Sjögren's syndrome (pSS), with probable preclinical primary Sjögren's syndrome (probable preclinical pSS), and with nonautoimmune sicca syndrome: ducti (d), acini (a), blood vessels (bl v), interstitium (i), lymphocytes (ly)] [mean ± SD, median (*M*)].

Variable	pSS		Probable preclinical pSS		Nonautoimmune sicca syndrome	
	Mean ± SD	*M*	Mean ± SD	*M*	Mean ± SD	*M*
IL-17	4.6 ± 1.38*,2,3	5.0	4.32 ± 1.31	4.0	3.8 ± 1.15	3.0
IL-17 a	0.88 ± 0.33*,2,3	1.0	0.96 ± 0.54	1.0	0.56 ± 0.51	1.0
IL-17 d	1.68 ± 0.48*,2	2.0	1.44 ± 0.51	1.0	1.20 ± 0.41	1.0
IL-17 i	0.60 ± 0.50*,1,2	1.0	0.16 ± 0.37	0.0	0.12 ± 0.33	0.0
IL-17 bl v	0.32 ± 0.56*,1,3	0.0	0.60 ± 0.50	1.0	0.20 ± 0.41	0.0
IL-17 ly	1.16 ± 0.372,3	1.0	1.20 ± 0.41	1.0	1.00 ± 0.00	1.0
IL-17R	4.68 ± 1.84*,2,3	5.0	4.44 ± 1.89	4.0	3.08 ± 1.68	3.0
IL-17R a	0.88 ± 0.33*,2,3	1.0	0.96 ± 0.45	1.0	0.56 ± 0.51	1.0
IL-17R d	1.68 ± 0.48*,1,2	2.0	1.32 ± 0.48	1.0	1.20 ± 0.41	1.0
IL-17R i	0.24 ± 0.44	0.0	0.44 ± 0.51	0.0	0.20 ± 0.41	0.0
IL-17R bl v	1.04 ± 0.79*,2,3	1.0	0.84 ± 0.55	1.0	0.48 ± 0.51	0.0
IL-17R ly	0.84 ± 0.37	1.0	0.88 ± 0.44	1.0	0.64 ± 0.49	1.0

*$P < 0.05$, comparison between all groups (Kruskal-Wallis test);
[1]$P < 0.05$, comparison between patients with pSS and probable preclinical pSS (Mann-Whitney *U* test);
[2]$P < 0.05$, comparison between patients with pSS and nonautoimmune sicca syndrome (Mann-Whitney *U* test);
[3]$P < 0.05$, comparison between patients with probable preclinical pSS and nonautoimmune sicca syndrome (Mann-Whitney *U* test).

Table 3: Expression (grades) IL-23 and IL-23R in minor salivary glands of patients with primary Sjögren's syndrome (pSS), with probable preclinical primary Sjögren's syndrome (probable preclinical pSS), and with nonautoimmune sicca syndrome: ducti (d), acini (a), blood vessels (bl v), interstitium (i), lymphocytes (ly)], [mean ± SD, median (*M*)].

Variable	pSS		Probable preclinical pSS		Nonautoimmune sicca syndrome	
	Mean ± SD	*M*	Mean ± SD	*M*	Mean ± SD	*M*
IL-23	4.08 ± 1.75*,2	4.0	3.32 ± 1.35	4.0	2.68 ± 1.68	3.0
IL-23 a	0.96 ± 0.54*,2,3	1.0	0.84 ± 0.47	1.0	0.52 ± 0.51	1.0
IL-23 d	1.64 ± 0.49*,2,3	2.0	1.44 ± 0.51	1.0	1.08 ± 0.49	1.0
IL-23 i	0.28 ± 0.46	0.0	0.2 ± 0.41	0.0	0.32 ± 0.48	0.0
IL-23 bl v	0.24 ± 0.44	0.0	0.16 ± 0.37	0.0	0.16 ± 0.37	0.0
IL-23 ly	0.88 ± 0.60	1.0	0.68 ± 0.48	1.0	0.64 ± 0.57	1.0
IL-23R	4.28 ± 1.97*,2,3	4.0	4.52 ± 1.96	5.0	2.76 ± 1.79	3.0
IL-23R a	0.96 ± 0.54*,2,3	1.0	0.80 ± 0.41	1.0	0.52 ± 0.51	1.0
IL-23R d	1.52 ± 0.51*,2,3	2.0	1.36 ± 0.49	1.0	1.0 ± 0.41	1.0
IL-23R i	0.48 ± 0.51*,3	0.0	0.76 ± 0.52	1.0	0.48 ± 0.51	0.0
IL-23R bl v	0.44 ± 0.51	0.0	0.72 ± 0.54	1.0	0.20 ± 0.41	0.0
IL-23R ly	0.88 ± 0.44*,2,3	1.0	0.88 ± 0.44	1.0	0.56 ± 0.51	1.0

*$P < 0,05$, comparison between all groups (Kruskal-Wallis test);
[1]$P < 0,05$, comparison between patients with pSS and probable preclinical pSS (Mann-Whitney *U* test);
[2]$P < 0,05$, comparison between patients with pSS and nonautoimmune sicca syndrome (Mann-Whitney *U* test);
[3]$P < 0,05$, comparison between patients with probable preclinical pSS and nonautoimmune sicca syndrome (Mann-Whitney *U* test).

probable preclinical pSS patients. Furthermore, we found that the total expression of IL-23R and of IL-23R in glandular ducts, acini, intraglandular interstitium, and infiltrating inflammatory cells was significantly higher in pSS and probable preclinical pSS patients compared with nonautoimmune sicca syndrome patients.

Expression of IL-17/IL-23 and IL-17R/IL-23R significantly correlated in minor salivary glands of patients with probable preclinical pSS (accordingly, $r = 0.683$, $P < 0.001$ and $r = 0.406$, $P = 0.044$) and nonautoimmune sicca syndrome patients (accordingly, $r = 0.740$, $P < 0.001$ and $r = 0.506$, $P = 0.010$). We did not find correlation between expression of IL-17R and IL-23R in patients with pSS ($r = 0.181$; $P = 0.386$).

4. Discussion

In this study, we sought to determine the expression of interleukins-17/-23 (IL-17/-23) and receptors of

interleukins-17/-23 (IL-17R, IL-23R) in minor salivary glands (MSGs) of patients with primary Sjögren's syndrome (pSS), with probable preclinical pSS, and with nonautoimmune sicca syndrome. Patients who had mild asymptomatic hematologic abnormalities (anemia, leucopenia, and increased erythrocyte sedimentation rate), positive anti-SSA/Ro or/and anti-SSB/La, and negative serology for hepatitis C, EBV, cytomegalovirus, and human immunodeficiency virus were included in the probable preclinical pSS group. Among the antinuclear autoantigens targets, the ribonucleoprotein particles (Ro/SSA and La/SSB) have a prominent role in pSS diagnosis and systemic activity [6, 17].

Cytokine-mediated immunity plays a substantial role in the pathogenesis of various autoimmune diseases including primary Sjögren's syndrome. Dysregulation of the cytokine network contributes to both systemic and exocrine manifestations of SS [9]. Recently some studies have added primary Sjögren's syndrome to the rapidly expanding list of autoimmune diseases (e.g., multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, and psoriasis) in which cytokines IL-17 and IL-23 are now implicated [12, 21–27].

Besides being produced by Th17 cells, IL-17A is also produced by a variety of cell types, including NK cells, neutrophils, and eosinophils. Thus, IL-17 is an effector cytokine that is produced by cells of both the innate and the adaptive immune systems, suggesting a bridging function of this type of immunity between innate and adaptive immune responses. IL-17A has proinflammatory properties and act on broad range of cell types to induce expression of cytokines, chemokines, and metalloproteinases. IL-17 was reported to be a factor that contributes to the formation of germinal centers (GCs) of lymph follicles retaining B cells within GCs an enhancing through modulation of chemokine activity. The IL-17 receptors constitute a distinct family of cytokine receptors. IL-17RA and IL-17RC are the receptors for IL-17A and IL-17F. IL-17RA not only conveys proinflammatory IL-17 effects, but also contributes in IL-25 signaling. IL-17RA binds IL-17A with higher affinity compared with IL-17F. IL-17RA is not only highly expressed on hematopoietic cells, but also at lower levels on endothelial cells, epithelial cells, osteoblasts, and fibroblasts [10, 15].

Recent studies revealed that IL-23, not IL-12, is regarded as a crucial cytokine for the pathogenesis of autoimmune diseases. IL-23 plays a key role in the development of pathogenetic Th17 cells that produce the cytokine IL-17 and finally contribute to hyperproduction of IL-17 and other cytokines. IL-23 is expressed predominantly by activated dendritic and phagocytic cells. IL-12 and IL-23 share a common p40 subunit binding to common IL-12 receptor (IL-23R). IL-23R is expressed on activated/memory T cells, T-cell clones, and natural killer cell lines in human. IL-23R has now been proposed as a common genetic marker for a variety of autoimmune diseases [28].

Comparison of the total expression of IL-17 and IL-17R in minor salivary glands between three groups showed significant differences. However, the total expression of IL-17 and IL-17R in MSGs of pSS was comparable to probable preclinical pSS patients. The highest expression of IL-17 was revealed in glandular ducts and inflammatory cells of patients with pSS and probable preclinical pSS. Resent study also revealed that the staining of the IL-17 and IL-23 appeared localized to lymphocytic infiltrates and ductal cells, with less staining occurring in acini. Another resent study showed that ageing induces specific changes in lacrimal-keratoconjunctivitis in CD25KO mice, with a mix of Th1 and Th17 cytokines, and that the peak severity of corneal epithelial disease corresponded to the peak of IL-17 expression [29].

The expression of IL-17R in glandular ducts was significantly higher in pSS patients compared with probable preclinical pSS patients, but the expression of IL-17R in acini of pSS patients was comparable to probable preclinical pSS patients. These results suggest that autoimmune inflammation may appear in salivary glands before sicca symptoms and signs in patients with probable pSS.

The total expression IL-23 and IL-23R in minor salivary glands of pSS was comparable to probable preclinical pSS patients. In contrast to our results, before-mentioned study revealed that IL-17 expression appeared stronger and more widely distributed than IL-23 expression in pSS patients [12]. We suppose that this contrast might be explained by different sizes of study groups. We found the highest expression of IL-23 in glandular ducts and acini of patients with pSS and probable preclinical pSS. Our study revealed that the total expression of IL-23R was significantly higher compared with the total expression of IL-23 in MSGs of patients with probable preclinical pSS. The total expression of IL-23R and expression of IL-23R in glandular ducts, acini, intraglandular interstitium, and infiltrating inflammatory cells was comparable in pSS and probable preclinical pSS patients. These results may demonstrate an influential role of IL-23R in the onset of autoimmune epithelitis. Future investigations need to take these data into consideration in designing studies to examine effects of IL-23R. Furthermore, IL-23R has now been proposed as a common genetic marker for a variety of autoimmune diseases [28].

The expression of IL-17/IL-23 and IL-17R/IL-23R significantly correlated in minor salivary glands of patients with probable preclinical pSS and nonautoimmune sicca syndrome patients. We did not find correlation between expression of IL-17R and IL-23R in patients with pSS. These data reflect that cytokines networks may contribute to the pathogenesis of SS in various and separate ways.

We speculate that the particular expression of interleukins-17/-23 and their receptors may reflect ongoing autoimmune inflammation in various target organs and suggest a role for these interleukins and their receptors in the early SS stage.

5. Conclusion

These results demonstrate an involvement of interleukin-17/-23 in the early Sjögren's syndrome pathogenesis.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Notch Signaling Mediates TNF-α-Induced IL-6 Production in Cultured Fibroblast-Like Synoviocytes from Rheumatoid Arthritis

Zhijun Jiao,[1] Wenhong Wang,[2] Jie Ma,[3] Shengjun Wang,[3] Zhaoliang Su,[3] and Huaxi Xu[3]

[1] *Key Laboratory of Medical Immunology and Department of Laboratory Medicine, Affiliated Hospital of Jiangsu University, 438 Jiefang Road, Zhenjiang 212001, China*
[2] *Department of Pathogenic Biology, School of Medical Science and Laboratory Medicine, Jiangsu University, Zhenjiang 212013, China*
[3] *Department of Immunology, School of Medical Science and Laboratory Medicine, Jiangsu University, Zhenjiang 212013, China*

Correspondence should be addressed to Zhijun Jiao, jiaozhijun@yahoo.com.cn

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It has been reported that Notch family proteins are expressed in synovium tissue and involved in the proliferation of synoviocyte from rheumatoid arthritis (RA). The aim of this paper was to investigate whether Notch signaling mediated TNF-α-induced cytokine production of cultured fibroblast-like synoviocytes (FLSs) from RA. Exposure of RA FLSs to TNF-α (10 ng/ml) led to increase of Hes-1, a target gene of Notch signaling, and a marked upregulation of Notch 2, Delta-like 1, and Delta-like 3 mRNA levels. Blockage of Notch signaling by a γ-secretase inhibitor (DAPT) inhibited IL-6 secretion of RA FLSs in response to TNF-α while treatment with recombinant fusion protein of Notch ligand Delta-like 1 promoted such response. TNF-α stimulation also induced IL-6 secretion in OA FLSs; however, the Hes-1 level remained unaffected. Our data confirm the functional involvement of Notch pathway in the pathophysiology of RA FLSs which may provide a new target for RA therapy.

1. Introduction

Rheumatoid arthritis (RA) is characterized by chronic and progressive inflammation of multiple joints, resulting in leukocyte invasion, formation of pannus, progressive degradation of the cartilage, and erosion of the bones [1]. Although the exact mechanism of RA pathogenesis is not well defined, it has been suggested that activated synoviocytes may play an important role, primarily through proliferation in inflamed synovia, and production of proinflammatory cytokines, matrix metalloproteinases (MMPs), and chemokines [2]. Cytokines secreted by RA synoviocytes include tumor necrosis factor-α (TNF-α), interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and granulocyte macrophage-colony stimulating factor (GM-CSF) [3]. Among these cytokines, TNF-α appears to be the major proinflammatory cytokine because it is known to strongly induce the production of IL-6, IL-8, GM-CSF, and even itself in synoviocytes; however, the precise molecular mechanism of cytokines production in response to TNF-α stimulation is not clarified [4].

The Notch signaling pathway is highly conserved beyond species and plays a critical role in a variety of cellular functions, including cell proliferation, differentiation, and apoptosis [5]. To date, four Notch receptors (Notch 1–4) and five of their ligands (Delta-like 1, 3, 4; Jagged-1, 2) have been identified in mammals. Upon ligand binding, the intracellular domain (ICD) of the receptor is proteolytically cleaved and translocated into the nucleus, where it associates with the RBP-Jκ transcription factor and regulates expression of several target genes, such as the basic helix-loop-helix (bHLH) proteins hairy-enhancer of split-like 1- (Hes-1) and Hes-5 [6].

Several early reports have shown the functional involvement of Notch pathway in the pathophysiology of RA synovitis. Ishii et al. and Yabe et al. showed that the expression pattern of Notch homologues among synovium from OA and RA patients differed from that of normal subjects [7, 8].

Another report demonstrated the expression of Notch-1 in synoviocytes and the presence of Notch-1 fragment in the nuclei of RA synoviocytes and suggested the involvement of Notch-1 signaling in the TNF-α-induced proliferation of RA synoviocytes [9]. TNF-α induced the expression of IL-6, MMP11, Notch-1, Notch-4, and Jagged-2 in RA FLSs [10, 11]. As yet, however, the expression pattern of Notch molecules on cultured synoviocyte is controversial and the relationship between increased expression of IL-6 or MMP11 and Notch signaling molecules response to TNF-α-stimulation remains unclear. In this study, we demonstrate that Notch signaling mediates TNF-α-induced secretion of IL-6 in RA FLSs while the expression pattern of Notch receptors and ligands upon TNF-α-stimulation differs from the previously reported.

2. Materials and Methods

2.1. Patients. Synovial tissue samples were obtained from patients with RA ($n = 4$, one male, three females, the mean age 54.7 ± 20.1) and OA ($n = 3$, one male, two females, the mean age 51.3 ± 10.4) at the time of total knee joint replacement. All RA patients fulfilled the respective American Rheumatism Association criteria [12]. All OA patients were evaluated by a certified rheumatologist and diagnosed based on the criteria developed by the ACR Diagnostic Subcommittee for OA [13]. Written consent was obtained from all patients after a full explanation of the procedure in conformity with requirements of the Committee on Ethics of Biomedicine Research of Affiliated Hospital of Jiangsu University.

2.2. Preparation of Fibroblast-Like Synoviocytes. RA FLSs were isolated from synovial tissues according to the method previously described [14]. Briefly, the collected synovial tissues were minced and cultured as explant pieces in a flask. Within 14 days, fibroblast-like cells migrated out from the tissue explants and formed confluent monolayers. The cells were collected by trypsinization and reseeded into flasks for expansion at 37°C in Dulbecco's modified Eagle medium (Gibco, BRL, USA) supplemented with 10% heat inactivated fetal calf serum (Gibco, BRL, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin. Fibroblasts between passages 3 and 5 were used for each experiment after being identified by morphology and purity analysis. Osteoarthritis (OA) FLSs were similarly prepared.

2.3. Treatment of FLSs with TNF-α and Notch Inhibitor and Ligand Fusion Protein. For kinetic analysis of the TNF-α-induced expression of Notch target gene Hes-1 and cytokines production, 1×10^5 FLSs per well were seeded into 12-well culture plates and subsequently stimulated with TNF-α (10 ng/mL, R&D Systems, Abingdon, UK) for various times. After stimulation, culture supernatants were collected and kept at −80°C for the measurement of IL-6 and IL-8 by ELISA, while the cells were lysed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for RNA isolation. To observe the effect of Notch signaling in the TNF-α-induced cytokine production, a Notch inhibitor, DAPT (Sigma, St. Louis, MO, USA) and Notch ligand fusion protein, Delta-like 1 (R&D Systems, Abingdon, UK), were also added into the culture system together with TNF-α.

2.4. Measurement of IL-6 and IL-8 by ELISA. The concentrations of IL-6 and IL-8 in culture supernatants were determined by using commercial ELISA kits (eBioscience, San Diego, USA) according to manufacturer's recommendations.

2.5. RNA Isolation and Real-Time RT-PCR. For the determination of mRNA expression of Notch receptors, ligands and target gene Hes1, total RNA was extracted from FLSs with or without stimulation by using TRIzol reagent (In vitrogen, Carlsbad, CA, USA). cDNA was prepared by reverse transcription with oligo (dT) from total RNA extraction. Real-time PCR for Notch signaling molecules and a reference gene (β-actin) was performed in a LightCycler instrument (Roche Molecular Diagnostics, Mannheim, Germany) with the SYBRgreen mastermix kit (Takara, Shiga, Japan). The target gene expression was then normalized relative to β-actin. Primers used were forward (5′-TCAGCGGGATCCACTGTGAG-3′) and reverse (5′-ACACAGGCAGGTGAACGAGTTG-3′) for Notch 1; forward (5′-TGCCAAGCTCAGTGGTGTTGTA-3′) and reverse (5′-TGCTAGGCTTTGTGGGATTCAG-3′) for Notch 2; forward (5′-GGTTCCCAGTGAGCACCCTTAC-3′) and reverse (5′-GTGGATTCGGACCAGTCTGAGAG-3′) for Notch 3; forward (5′-ACCTGCTCAACGGCTTCCA-3′) and reverse (5′-AGCTTCTGCACTCATCGATATCCTC-3′) for Notch 4; forward (5′-ACCTGCTCAACGGCTTCCA-3′) and reverse (5′-AGCTTCTGCACTCATCGATATCCTC-3′) for Jagged-1; forward (5′-ACCAGGTGGACGGCTTTGAG-3′) and reverse (5′-CCCGGGATGCAATCACAGTAATA-3′) for Jagged 2; forward (5′-TGGGCTACTCCGGCTTCAAC-3′) and reverse (5′-ACAGGTAGGCATCACCGAGGTC-3′) for Delta-like 1; forward (5′-TCAACAACCTAAGGACGCAGGAG-3′) and reverse (5′-CTACATCTTCAGGGCGATTCCAA-3′) for Delta-like 3; forward (5′-GTCCAACTGTGGCAAACAGCA-3′) and reverse (5′-AGCATATCGCTGATATCCGACACTC-3′) for Delta-like 4; forward (5′-GACTGTGAAGCACCTCCG-3′) and reverse (5′-GTCATGGCGTTGATCTGG-3′) for Hes1; forward (5′-GAAGTCCCTCACCCTCCCAA-3′) and reverse (5′-GGCATGGACGCGACCA-3′) for β-actin.

2.6. Statistical Analysis. Two-tailed Student's t-test was used for determining significant differences ($P \leq 0.05$) between groups.

3. Results

3.1. TNF-α Stimulation Induces the Activation of Notch Signaling in Fibroblast-Like Synoviocytes (FLS) from RA. We first determined the effect of TNF-α on the expression of Notch signaling molecules in RA FLSs by real-time PCR. Exposure of RA FLSs to TNF-α led to a gradual, time-dependent increase of Hes-1 mRNA levels, a target gene

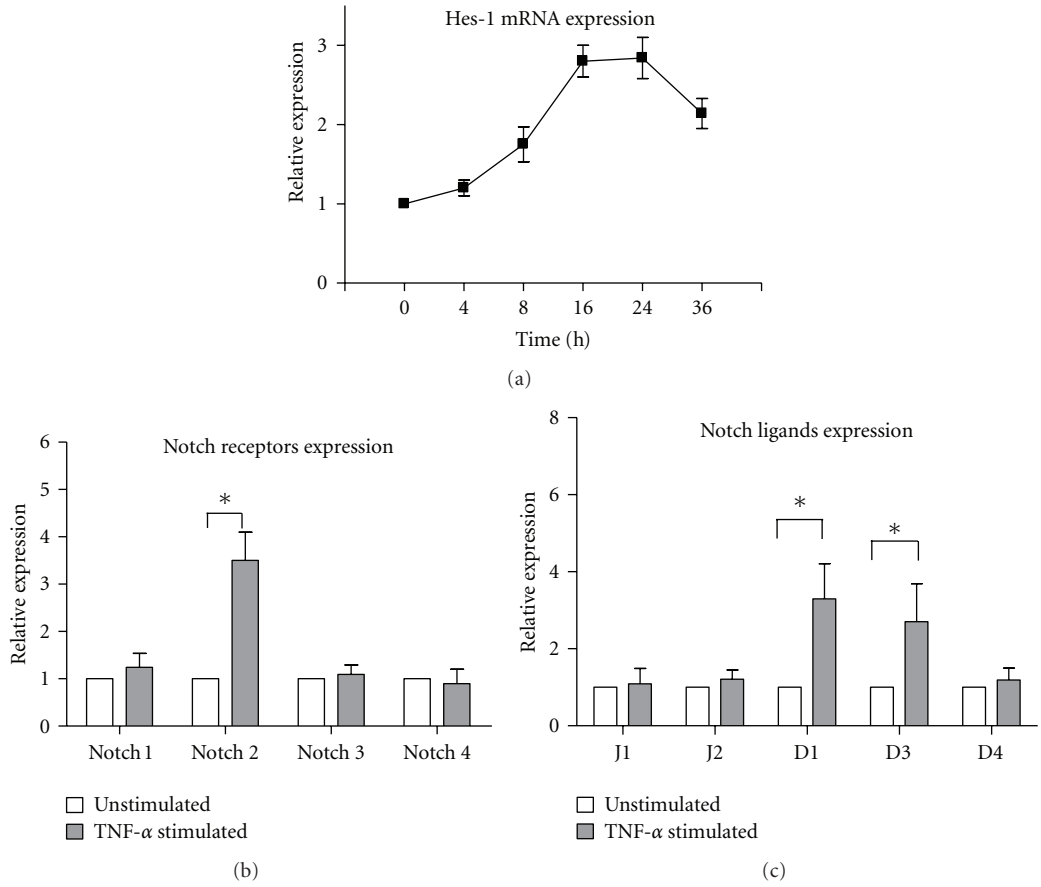

Figure 1: TNF-α stimulation induces the mRNA expression of Notch signaling molecules in fibroblast-like synoviocytes (FLS) from RA. (a) FLSs from 4 RA patients were cultured in the presence of TNF-α (10 ng/mL) for 0, 4, 8, 16, 24, or 36 h, and then analyzed for Hes-1 expression by PCR; (b) FLSs from 4 RA patients were cultured in the presence of TNF-α (10 ng/mL) for 24 h, followed by analysis of Notch receptors expression by PCR; (c) FLSs from 4 RA patients were cultured in the presence of TNF-α (10 ng/mL) for 24 h, and then analyzed for Notch ligands expression by PCR; J1: Jagged 1; J2: Jagged 2; D1: Delta-like 1; D3: Delta-like 3; D4: Delta-like 4. Results were normalized to unstimulated condition (basal value = 1). Each column is the mean ± SD. $^*P < 0.05$ for unstimulated group versus TNF-α-stimulated group.

of Notch signaling, which reached a maximum after 16–24 h of stimulation (Figure 1(a)). mRNAs of four receptors and five ligands of Notch signaling were also detected in RA FLSs stimulated with TNF-α (10 ng/mL) for 24 h. As shown in Figure 1(b), TNF-α stimulation induced a marked upregulation of Notch 2, in contrast, the levels of other three receptors were unchanged. TNF-α also significantly increased the mRNA expression of two ligands, Delta-like 1 and Delta-like 3, while the expression of the other three ligands was not affected (Figure 1(c)). These data suggest that Notch signaling is activated upon TNF-α stimulation in RA FLSs.

3.2. Notch Signaling Mediates TNF-α-Induced IL-6 Production in FLSs from RA. TNF-α stimulation induced a marked secretion of IL-6 and IL-8 from RA FLSs, which reached a maximum at 24 h of stimulation (Figure 2(a)). To test whether the activated Notch signaling mentioned above mediates this process, we first determined the effect of DAPT, a Notch signaling inhibitor, on TNF-α-stimulated cytokines secretion. Coincubation with such blocking reagent inhibited IL-6 secretion in response to TNF-α in a dose-dependent manner; however, DAPT did not significantly reduce the IL-8 secretion (Figure 2(b)). To confirm a role of Notch signaling as a mediator of TNF-α-induced IL-6 secretion, we examined the effect of human recombinant fusion protein of Notch ligand Delta-like 1 on TNF-α-induced IL-6 secretion. As demonstrated in Figure 2(c), when Delta-like 1 protein was added together with TNF-α, augmentation of IL-6 production was observed in a time-dependent manner. The facilitation of Delta-like 1 protein on IL-6 secretion was also dose-dependent with maximal response at 10 μg/mL (Figure 2(d)).

3.3. Effect of TNF-α Stimulation on IL-6 and Hes-1 Production in OA FLSs. To test whether Notch signaling could also mediate IL-6 production in OA FLSs, we determined the ability of TNF-α to induce IL-6 secretion and Hes-1 mRNA expression in primary human OA FLSs. The spontaneous secretion of IL-6 in cultured OA FLSs was significantly lower

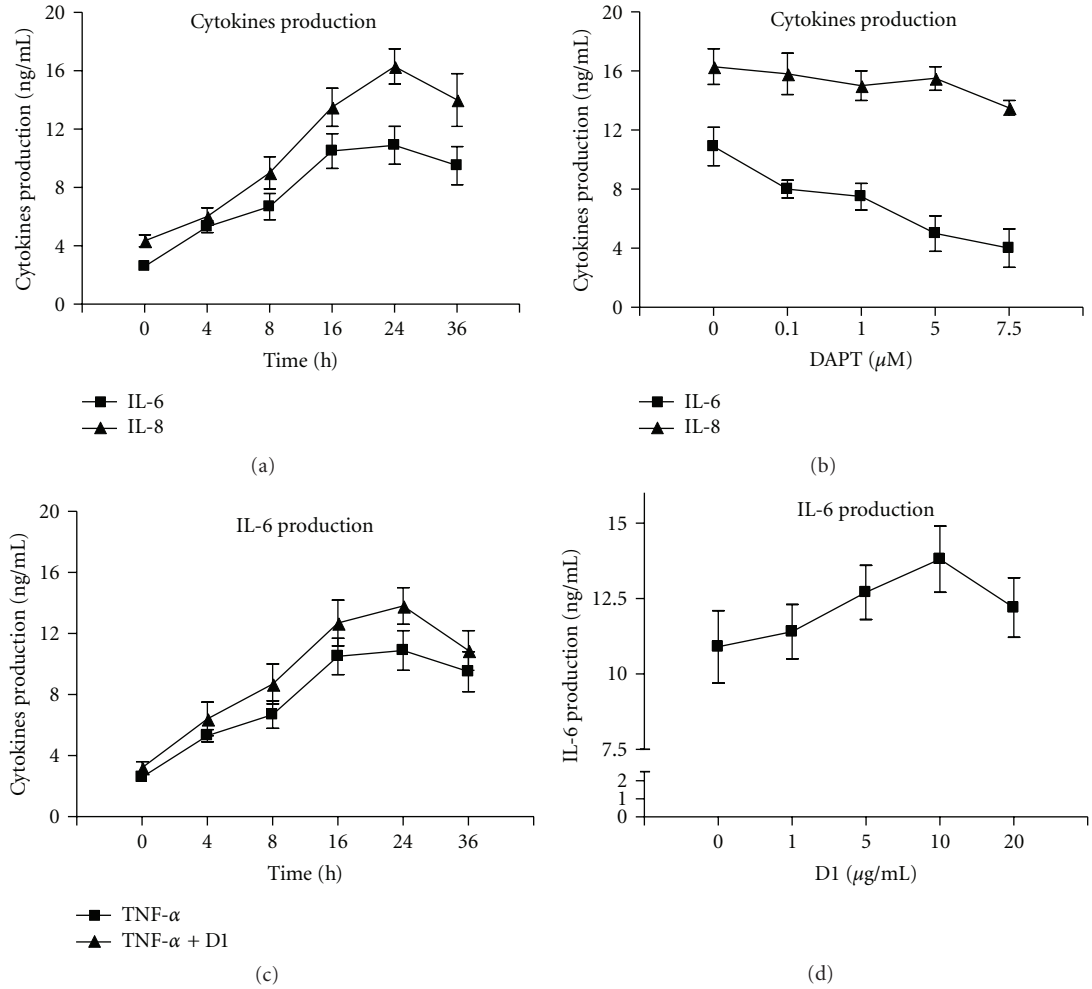

Figure 2: Notch activity contributes to TNF-α-induced IL-6 production of FLSs from RA. (a) FLSs from 4 RA patients were cultured in the presence of TNF-α (10 ng/mL) for 0, 4, 8, 16, 24, or 36 h, and then measured the levels of supernatant IL-6 and IL-8 by ELISA; (b) RA FLSs were cultured in the presence of TNF-α (10 ng/mL) in addition to the indicated concentration of DAPT (Notch signaling blocking reagent) for 24 h. Supernatant IL-6 and IL-8 were determined by ELISA; (c) RA FLSs were cultured in the presence of TNF-α (10 ng/mL) in addition to Delta-like 1 fusion protein (10 μg/mL) for indicated times. Supernatant IL-6 was determined by ELISA; (d) RA FLSs were cultured in the presence of TNF-α (10 ng/mL) in addition to indicated concentrations of Delta-like 1 fusion protein for 24 h. Supernatant IL-6 was determined by ELISA. Values are the mean ± SD.

than that of RA FLSs, while TNF-α stimulation induced marked IL-6 secretion in both OA FLSs and RA FLSs (Figure 3(a)). The basal Hes-1 mRNA expression in OA FLSs was also significantly lower than that of RA FLSs; however, unlike in RA FLSs, TNF-α stimulation did not increase the Hes-1 mRNA expression in OA FLSs (Figure 3(b)). This result indicates that involvement of Notch signaling in TNF-α-induced IL-6 production should be unique to RA FLSs.

4. Discussion

Fibroblast-like synoviocytes, (FLSs) also called synovial fibroblasts (SF), are resident mesenchymal cells of synovial joints [15]. Activation of FLSs in the setting of RA leads to the production of a variety of cytokines, small molecule mediators of inflammation, and proteolytic enzymes which are responsible for the progressive destruction of articular cartilage and bone [4, 10]. Activation of FLSs could be initiated by cytokines, among which TNF-α is paramount. Activated FLSs by TNF-α in turn produce IL-6, IL-1β, and even itself to sustain regulatory feedback loops that perpetuate local joint inflammation. Such mechanism has been confirmed by the clinical efficacy of TNF-α blocking reagent in the treatment of RA synovitis. However, it remains less clear how many signal pathways are activated within FLSs upon TNF-α stimulation, and therefore which signal pathway may be the alternative target for clinical intervention.

Several early studies, which reported the expression of Notch molecules in RA synovium and the involvement of Notch signaling in the activation of cultured FLSs [9, 11, 16], led us to test the role of Notch signaling in the cytokine

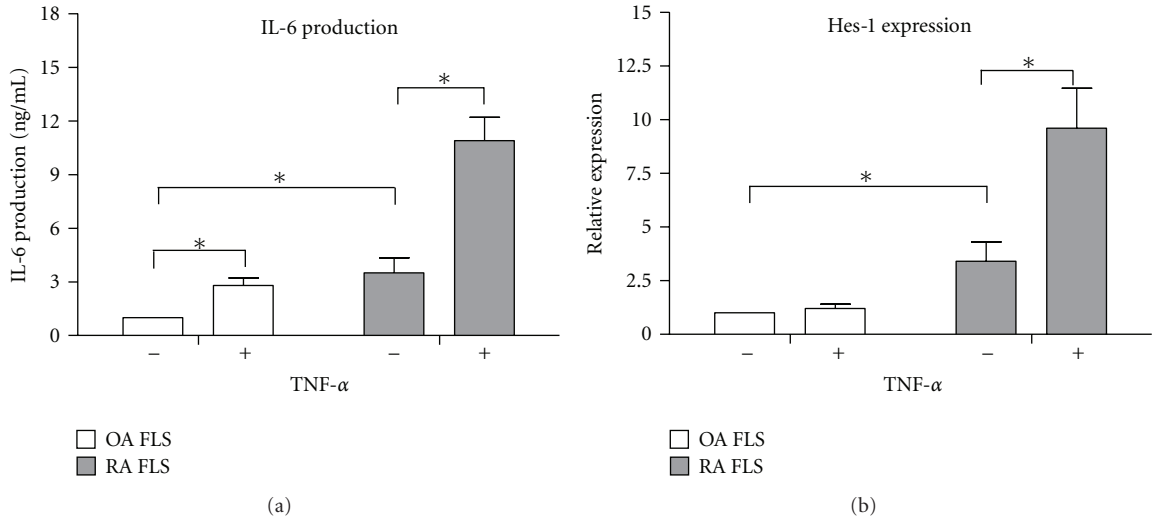

Figure 3: Effect of TNF-α stimulation on IL-6 and Hes-1 production in OA FLSs. (a) OA and RA FLSs were incubated with TNF-α (10 ng/mL) for 24 h, and then the supernatants were collected and analyzed for the IL-6 production by ELISA; (b) OA and RA FLSs were incubated with TNF-α (10 ng/mL) for 24 h, and then the cells were collected and analyzed for the Hes-1 mRNA expression by PCR. Each column is the mean ± SD. $^*P < 0.05$.

secretion of RA FLSs in response to TNF-α stimulation. The present study first observed that TNF-α stimulation led to a gradual, time-dependent increase of Hes-1 mRNA levels in RA FLSs, which reached a maximum after 16–24 h of stimulation. Hes-1 is the most well characterized, γ-secretase-dependent transcriptional target gene of Notch, and upregulated expression of Hes-1 represents the activated Notch signaling. A previous study has also demonstrated that TNF-α induced the elicitation of the Notch signaling by the observation of nuclear translocation of Notch intracellular domain (NICD) in cultured RA FLSs [9]. However, the same study reported that TNF-α treatment (200 pg/mL) upregulated the expression of Notch 1, Notch 4, and Jagged 2, which is quite different from our results that TNF-α stimulation (10 ng/mL) induced a marked upregulation of Notch 2 and two ligands, Delta-like 1 and Delta-like 3. This diversity may be due to the different stimulation concentration of TNF-α used in experimental system or the different culture times. Indeed, the expression profile of Notch receptors or ligands in RA local synovium tissue also varied among previous reports [7, 8, 11]. Nevertheless, here we confirmed the result that TNF-α could induce the activation of Notch signaling in cultured RA FLSs.

TNF-α has been shown to induce cytokine production in RA FLSs, such as IL-6 and IL-8 [3, 10, 11]. We also observed the increased production of IL-6 and IL-8 upon TNF-α stimulation, which was consistent with the previous reports. FLSs in the intimal lining have been shown to be the primary source of IL-6 by in situ hybridization and immunohistochemistry studies. Cultured RA FLSs spontaneously produce IL-6, and their production is markedly increased by TNF-α, thus, targeting the function of FLSs to produce IL-6 might improve clinical outcomes in inflammatory arthritis without suppressing systemic immunity [3, 10].

To test whether the activated Notch signaling mediate the cytokine production in response to TNF-α stimulation, DAPT, a γ-secretase inhibitor, was added into the culture system to block the activation of Notch signaling. We found that DAPT inhibited IL-6 secretion in response to TNF-α in a dose-dependent manner, however, it did not significantly reduce the IL-8 secretion. To confirm a role of Notch signaling as a mediator of TNF-α-induced IL-6 secretion, we also demonstrated that Delta-like 1 fusion protein added together with TNF-α, augmented IL-6 production in a time and dose-dependent manner. In our study, TNF-α stimulation could also induce marked IL-6 secretion in OA FLSs other than RA FLSs. However, unlike in RA FLSs, TNF-α stimulation did not increase the Hes-1 mRNA expression in OA FLSs. Such inconsistent result indicated that the targets of Notch-mediated transcriptional activation should be explored other than Hes-1. Alternatively, there may be target sequences of CSL in the IL-6 promoter. Similar research strategies had been reported in a newly published paper [17]. By ChIP analysis, Keerthivasan et al. report NICD directly binds to both ROR-γt and IL-17 promoters and regulates Th17 differentiation (IL-17 induction). Further experimentation is required to test whether Notch can bind directly to the IL-6 promoters.

The function of FLSs to produce IL-6 has been reported to be regulated in an NF-κB-dependent pathway. In fact, some effective anti-RA drugs are now known to inhibit NF-κB and its activation cascade. Here we reported that Notch signaling also mediated TNF-α-induced IL-6 production in cultured RA FLSs which can also potentially serve as therapeutic target signaling. We have also demonstrated the activation of Notch signaling in helper T cells from RA patients [18]. Based on these considerations, using inhibitors of γ-secretase (as is already in use for the treatment of

Alzheimer's disease) to block the activated Notch signaling might be a feasible approach to RA therapy [19].

Conflict of Interests

The authors declare that they have no conflict of interest.

Acknowledgments

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Review Article

Current Status of the Immunomodulation and Immunomediated Therapeutic Strategies for Multiple Sclerosis

Shyi-Jou Chen,[1,2] Yen-Ling Wang,[3] Hueng-Chuen Fan,[1] Wen-Tsung Lo,[1] Chih-Chien Wang,[1] and Huey-Kang Sytwu[2,4,5]

[1] *Department of Pediatrics, Tri-Service General Hospital, National Defense Medical Center, Taipei 114, Taiwan*
[2] *Department of Microbiology and Immunology, National Defense Medical Center, Taipei 114, Taiwan*
[3] *Center for Composite Tissue Allotransplantation, Chang Gung Memorial Hospital, Linkou, New Taipei City 333, Taiwan*
[4] *Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 114, Taiwan*
[5] *Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei 114, Taiwan*

Correspondence should be addressed to Huey-Kang Sytwu, sytwu@ndmctsgh.edu.tw

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Multiple sclerosis (MS) is an autoimmune disease of the central nervous system, and $CD4^+$ T cells form the core immunopathogenic cascade leading to chronic inflammation. Traditionally, Th1 cells (interferon-γ-producing $CD4^+$ T cells) driven by interleukin 12 (IL12) were considered to be the encephalitogenic T cells in MS and experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Currently, Th17 cells (Il17-producing $CD4^+$ T cells) are considered to play a fundamental role in the immunopathogenesis of EAE. This paper highlights the growing evidence that Th17 cells play the core role in the complex adaptive immunity of EAE/MS and discusses the roles of the associated immune cells and cytokines. These constitute the modern immunological basis for the development of novel clinical and preclinical immunomodulatory therapies for MS discussed in this paper.

1. Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

Multiple sclerosis (MS) was initially identified in 1868 by Charcot. This disease often begins in young adulthood with intermittent episodes of neurological dysfunction, including visual impairment, ataxia, motor and sensory deficits, and bowel and bladder incontinence. These are attributable to recurrent inflammatory attacks on the white matter of the brain and spinal cord, which lead to the accumulation of perivascularly distributed inflammatory cells within the brain and spinal cord white matter [1].

Beeton et al. first established an animal model of MS in the 1930s, when they immunized monkeys with a central nervous system (CNS) homogenate to induce what is now known as experimental autoimmune encephalomyelitis (EAE) [2]. Since this pilot animal study, EAE has become the most accepted animal model of MS. In recent decades, pathogenic hypotheses have been investigated and novel therapeutic agents tested in this model in the fields of CNS inflammation and demyelination. Therefore, EAE provides a valuable tool for the investigation of the T-cell-dependent pathogenesis of autoimmune inflammation in the CNS and the orchestration of the autoimmune demyelinating inflammation in the CNS of MS patients. Mice and/or genetically modified mice have also been of fundamental value in the exploration of the complex pathogenesis of MS [3, 4]. EAE is undoubtedly the best animal model in which to study autoimmune diseases and particularly the demyelinating diseases of the CNS, such as MS [5].

2. Basic Immunopathogenic Mechanism and the Role of T Cells in EAE and MS

Myelin basic protein-(MBP)-specific T cells isolated from the peripheral lymphocytes of human individuals with MS and encephalitogenic T cells recovered from circulating

autoreactive T cells of either immunized or naïve animals have shown that autoreactive T-cell lines that recognize the encephalitogenic part of MBP in vitro can be distinguished from an unprimed rat T-cell population. This confirms that autoreactive T cells play a central role in the pathology of MS [6–8]. EAE can also be induced by adoptively transferring an expanded population of myelin-reactive encephalitogenic $CD4^+$ (T helper [Th]) cells, which allows the further dissection of the immunopathogenic potency of different encephalitogenic $CD4^+$ cell populations [9].

In the 1990s, Mosmann and Coffman postulated that Th cells can be classified into two distinct subsets, Th1 and Th2. Th1 cells produce large quantities of interferon γ (IFNγ), driven by interleukin 12 (IL12), which promotes cellular immunity directed against intracellular pathogens. Alternatively, Th2 cells, which secrete IL4, IL5, IL13, and IL25, are essential in the destruction of extracellular parasites and the mediation of humoral immunity [10, 11]. Self-reactive Th1 clones derived in vitro are capable of adoptively transferring EAE to naïve recipients [12]. Increased levels of Th1 cytokines are particularly evident during EAE/MS relapse, whereas increased Th2 cytokines are found during remission in MS patients when compared with control levels [13]. Clinical and hematological symptoms are exacerbated in relapsing/remitting MS patients following the administration of IFNγ, and this is also observed in other Th1-type diseases, whereas it is less apparent in Th2 diseases [14, 15]. Th1 cells were earlier thought to be pathogenic T cells, whereas Th2 cells were thought to confer an anti-inflammatory potential, constituting protective T cells in both MS and EAE [16–19].

However, this clear-cut immunodysregulation of the Th1/Th2 balance in EAE and MS may be part of a hidden complex of interactions underlying EAE and MS [20]. The Th1-driven nature of the EAE/MS disease was challenged by the finding that IFNγ- and IFNγ-receptor-deficient mice, as well as mice that lack other molecules involved in Th1 differentiation, such as IL12p35, IL12 receptor β2 (IL12Rβ2), and IL18, were not protected from EAE, but instead were more susceptible to the disease [21–25]. Unexpectedly, mice deficient in IL12α (IL12p35), a component of the Th1 paradigm, are vulnerable to EAE. Similarly, IL12Rβ2-deficient mice develop more severe clinical manifestations of EAE, whereas IL12p40-deficient mice are resistant to EAE [23, 24, 26]. These discrepancies and conflicting data indicate that an imbalance in the Th1/Th2 milieu cannot explain the overall immunopathogenic mechanisms underlying EAE and MS.

3. Immunopathogenic Role of Th17 Cells and Cytokines in EAE/MS

p19, a novel cytokine heavy-chain homologue of the IL6 subfamily, was discovered as a computational sequence [27]. When the p19 chain is linked to the p40 chain, a subunit of IL12 (another subunit of the IL12 heterodimers is the p35 chain), it forms a novel cytokine designated IL23. Therefore, the deletion of IL12p40 will affect the functions of both IL12 and IL23. Cua and colleagues verified that Il23 but not Il12 is essential for the induction of EAE by generating Il23p19 knockout (KO) mice and comparing them with IL12p35 KO mice [28]. Furthermore, an IL17-producing T-cell subset, driven and expanded by IL23, can pathogenically induce EAE when adoptively transferred into naïve wild-type mice [29, 30]. These IL17-producing T cells were dramatically reduced in the CNS of IL23p19-deficient mice. Based on these studies, researchers confidently suggested that IL17-producing $CD4^+$ T cells are a distinct and novel Th subset that exacerbates autoimmunity, and designated them Th17 cells [31, 32]. Th17 cells are a Th-cell subset distinct from Th1 and Th2 cells in terms of their differentiation, expansion, and effector functions [33, 34]. The discovery of Th17 cells further clarifies the cytokine profile of MS [35]. Recently, the levels of IL17 produced by MBP-stimulated peripheral blood cells obtained from MS patients or controls were shown to correlate with the active lesions in MS patients observed with magnetic resonance imaging (MRI) [36].

Like other Th subsets, the Th17 lineage is activated by a specific cytokine milieu. However, IL23 cannot produce Th 17 cells de novo from naïve T cells, and the IL23 receptor (IL23R) is not expressed on naïve T cells [37]. Transforming growth factor β (TGFβ) upregulates IL23R expression, thereby conferring responsiveness to IL23, which confirms that TGFβ is a critical cytokine in the commitment to Th17 expansion in vitro and in vivo [38]. In mice, TGFβ together with IL6 can activate antigen-responsive naïve $CD4^+$ T cells to develop into Th17 cells [39]. In humans, naïve $CD4^+$ cells exposed to IL6, TGFβ, and IL21 can develop into Th17 cells, and the production of IL23 plays a role in maintaining these Th17 cells [40, 41]. Altogether, Th17 cells require IL23, TGFβ, IL6, and IL1 for their generation. Th17 cells produce IL17A and IL17F, which are upregulated in chronic lesions [42], and IL22, which is also involved in the pathogenesis of MS. Thus, Th17 cells are a recently discovered, unique Th lineage that produces a repertoire of signature cytokines, including IL17A, IL17F, IL21, and IL22, that are essential for the development of autoimmune diseases such as MS [43].

The discovery of transcription factors that are key regulators of the cytokine expression required to launch lineage-specific transcriptional programs has greatly extended our understanding of Th-cell lineage commitment [44]. It has been shown that T-bet and STAT4 program the commitment of the Th1 lineage and Th1 cytokine production [45], whereas GATA-binding protein 3 (GATA3) and STAT6 drive Th2 population expansion and Th2 cytokine production [46, 47]. The T-bet and STAT4 (necessary for Th1 differentiation) transcription factors are important in the differentiation of autoimmune T cells in the EAE model [48], and T-bet- and STAT4-deficient mice are resistant to EAE. However, these transcription factors do not mediate the induction of Th17 cells. Instead, in a unique inductive milieu, Th17 differentiation is driven by distinct transcription factors: retinoic acid receptor-related orphan receptor-γt (Rorγt) and Rorα [33, 34]. Stat3 deletion in T cells also prevents autoimmune uveitis and EAE and increases the expression of IL10 and forkhead box P3 (FoxP3) [49], and the expression of FoxP3 programs the development and functions of Treg

cells [50]. In humans, IL23 and IL1β also induce the development of Th17 cells expressing IL17A, IL17F, IL22, IL26, IFNγ, the chemokine CCL20, and the transcription factor RORγ [51–53], as illustrated in Figure 1 (adapted from Hirota et al. [54]).

Recent microarray studies of lesions in MS patients demonstrated an increased expression of IL17, confirming that Th17 cells play an important role in the development of inflammation and demyelination and in the eventual damage of the CNS. IL17 is a recently described cytokine produced in humans almost exclusively by activated memory T cells and can induce the production of proinflammatory cytokines and chemokines from parenchymal cells and macrophages. Patients with MS have greater numbers of IL17-mRNA-expressing mononuclear cells in the cerebrospinal fluid (CSF) than in the blood. Previously, no increase in the numbers and expression of IL17 mRNA by mononuclear cells isolated from the CSF was observed in patients with MS, but higher levels of IL17 mRNA were observed in the CSF than in the blood, with the highest levels in the blood detected during clinical exacerbations [56]. These data confirm the pivotal role of IL17 in MS both peripherally and centrally.

4. Recruited and Residential Innate Immune Cells in EAE and MS

Myelin is expressed in the circulation, and other CNS antigens are thought to be expressed in the cervical lymph nodes, which can trigger the conversion of autoaggressive myelin-reactive T cells to pathogenic T cells. Adhesion molecules, the integrins, allow these myelin-reactive T cells to penetrate the blood-brain barrier (BBB) under inflammatory conditions, and in this way, activated and memory T cells can enter the CNS [57]. Autoaggressive myelin-reactive T cells migrate into the CNS, where they recognize their cognate target antigens, and the movement of antigen-presenting cells (APCs) into the CNS is essential for lymphocyte reactivation within the CNS compartment and the initiation of the inflammatory cascade in the development of EAE [58]. Subsequently, inflammatory and immune cells, such as granulocytes and macrophages, are attracted into the CNS parenchyma, where they mediate tissue inflammation, leading to demyelination and tissue damage [59].

The brain was formerly considered an immunoprivileged organ, but this perspective has been revised in the last two decades [60]. Today, we understand that any damage to the CNS can activate immune cells in situ in the CNS, particularly microglial cells. Deshpande et al. demonstrated the transient inactivation of microglial cells via a cell-specific deficiency of CD40 expression, indicating that microglial cells are crucial for maintaining the autoimmune responses in the CNS [61]. The major histocompatibility complex (MHC, also known as "human leukocyte antigens" in humans) class II molecules are only displayed on specialized APCs (e.g., dendritic cells [DCs], B cells, and macrophages), whereas MHC class I molecules are expressed by all cells in the inflammatory milieu of the CNS [62]. Microglial cells upregulate the expression of MHC and costimulatory molecules to initiate the generation and maintenance of the inflammatory milieu. DCs seem to play a critical role in antigen presentation to invading T cells and in the release of cytokines and chemokines, thereby guiding the entry of monocytes, lymphocytes, and cells with a phenotype similar to that of DCs into the lesion [63].

Th cells recruit macrophages, which release proinflammatory cytokines and destructive molecules (such as nitric oxide [NO], IL1, IL6, tumor necrosis factor α [TNFα], and matrix metalloproteinases (MMPs]), and $CD8^+$ T cells also directly attack MHC class I-expressing cells, such as oligodendrocytes and neurons [64, 65]. The secretion of destructive molecules, such as NO and TNFα, and the degradation of myelin are consequences of this cascade. TNF receptor 1 (TNFR1) but not TNFR2 signaling is critical for demyelination and the limitation of T-cell responses during immune-mediated CNS disease [66]. This complicated process triggers the recruitment of innate immune cells, generally consisting of T cells, macrophages, and microglia, which in turn mediate demyelination, axonal damage, and lesions.

5. Th17 and Immune Cells In Situ

In autopsy samples from MS patients, the expression of IL17 is evident in perivascular lymphocytes and in astrocytes and oligodendrocytes located in the active areas of CNS lesions. IL17R is also identifiable in acute and chronic MS plaques of patients with MS, suggesting the enrichment of Th17 and $CD8^+$ T cells in active MS lesions, and confirming an important role for IL17 in the pathogenesis of MS [67]. Th17 cells are identified by their expression of IL23R and the memory T-cell marker CD45RO in situ. Other markers that have been investigated including the chemokine receptor, CCR6, and RORC variant 2, which is a central transcription factor for Th17-cell development [42, 68]. Microarray analysis of MS lesions has also demonstrated increased transcripts of genes encoding inflammatory cytokines, particularly IL6, IL17, and IFNγ and associated downstream pathways [56]. A significant increase in IL23 mRNA and protein expression is found in lesion tissues compared with nonlesion tissues. Activated macrophages/microglia have been shown to be important sources of IL23p19 in active and chronically active MS lesions. IL23p19-expressing mature DCs are preferentially located in the perivascular cuffs of active lesions. This data on the expression of IL23p19 in MS lesions improves our understanding of the pathogenesis of MS [69].

There is also evidence that MS endothelial cells express high levels of IL17R and are more permeable to IL17 than are non-MS endothelial cells. Perivascular DCs also express high levels of granzyme B in inflammatory lesions, polarizing naïve $CD4^+$ T cells into Th17 cells. These Th17 cells transmigrate efficiently across BBB endothelial cells (BBB-ECs), leading to the destruction of human neurons and initiating CNS inflammation through Th-cell recruitment [70]. Similarly, the expression of IL17R and IL22R on BBB-ECs has been examined in MS lesions, and IL17 and IL22 have been shown to disrupt BBB tight junctions in vitro and

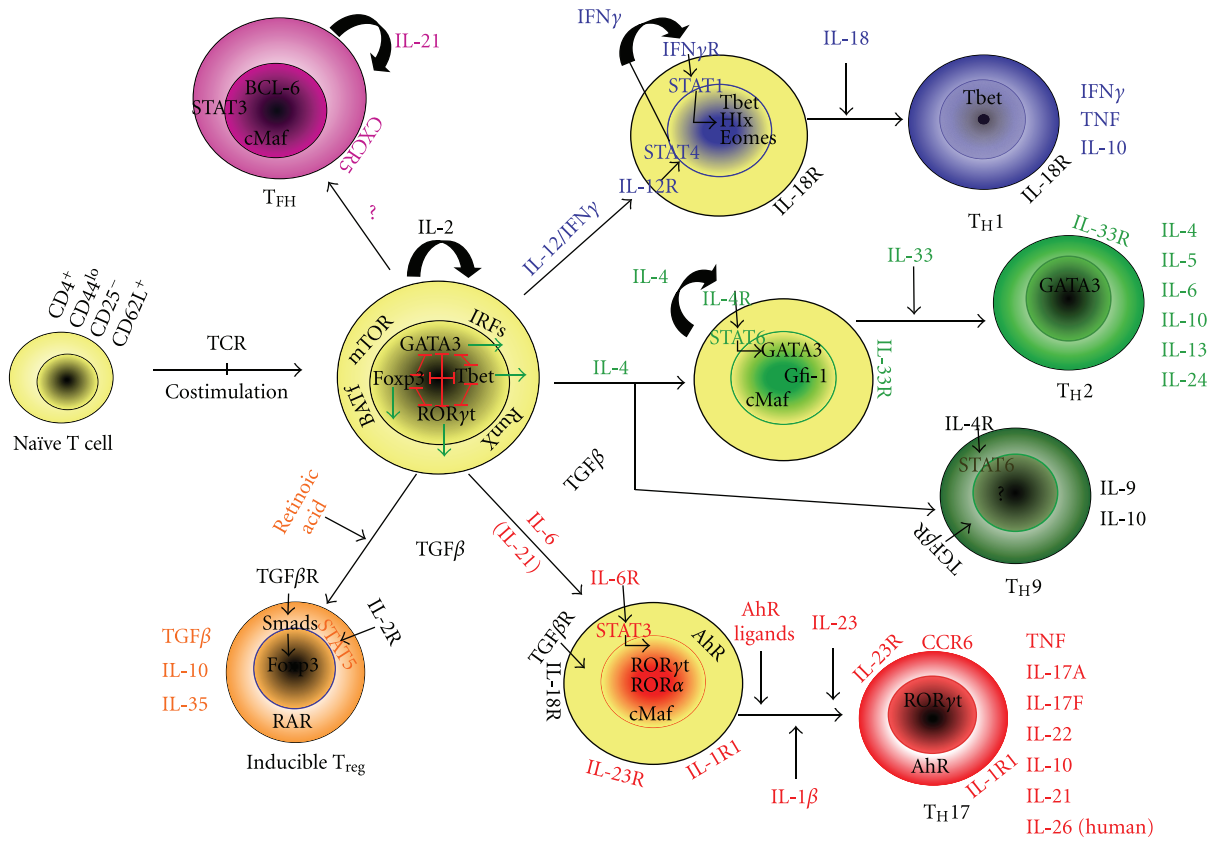

Figure 1: Current schedule of T-helper-cell differentiation. When naïve $CD4^+TCR\alpha\beta^+$ T lymphocytes, classified by their low expression of CD44, absence of CD25, and high levels of CD62L, encounter their cognate antigens, they can differentiate into several previously identified effector subsets. It is likely that several "master" transcription factors, individually required for T-cell differentiation towards one of the end effector stages, are initially expressed upon engagement of the TCR with costimulatory receptors. Each transcription factor drives a specific set of genes required for lineage commitment and the expression of signature cytokines and negatively affects alternative pathways. However, the local microenvironment is the driving force that determines the outcome of the differentiation course. Th1 cells are established in the presence of IFNγ and IL12 and signaling via STAT1 and STAT4, resulting in the expression of the master transcription factor T bet. Th2 cells depend on IL4 and STAT6 for the increased expression of GATA3, whereas the simultaneous presence of TGFβ results in the development of Th9 cells, utilizing an undefined master transcription factor. The presence of TGFβ, with IL2 signaling via STAT5, is known to generate, at least in vitro, inducible Treg, which utilize FOXP3 like those Treg generated in the thymus. Again, it is TGFβ in combination with IL6 signaling via STAT3 that drives the expression of RORγt, resulting in the differentiation of Th17 cells. However, the initiation of the developmental program of these T helper subsets may not be completed in the presence of only these driving cytokines. Several additional factors may be required for their subsequent functional maturation or may be responsible for the fine tuning of their effector phases. Several of these factors are indicated, together with the characteristic cytokine profiles of each subset (adapted from [54]).

in vivo. IL6 transsignaling may also play a role in the autoimmune inflammation of the CNS, mainly by regulating the early expression of adhesion molecules, possibly via cellular networks at the BBB [71]. Ifergan et al. demonstrated that a subset of $CD14^+$ monocytes migrate across the inflamed human BBB and differentiate into $CD83^+CD209^+$ DCs under the influence of BBB-secreted TGFβ and granulocyte-macrophage colony-stimulating factor (GM-CSF). These DCs can produce IL12p70, TGFβ, and IL6 and promote the proliferation and expansion of distinct populations of Th1 and Th17 cells. The abundance of such DCs in situ is strongly associated with microvascular BBB-ECs within acute MS lesions and with a significant number of Th17 cells in the perivascular infiltrate [72].

Astrocytes play significant physiological roles in CNS homeostasis and act as a bridge between the CNS and the immune system. Astrocytes also contribute to the complex interactions during CNS inflammation. IL17 functions in a synergistic manner with IL6 to induce IL6 expression in astrocytes. Astrocytes upregulate the expression of IL17 and IFNγ genes and proteins in T cells, which is consistent with the astrocytes' capacity to express IL23 subunit p19 and the common IL12/IL23 subunit p40, but not IL12 subunit p35 when these two cell types are cocultured [73]. Das Sarma et al. demonstrated increased IL17RA expression in the CNS of mice with EAE and the constitutive expression of functional IL17RA in mouse CNS tissues. They also identified the expression of IL17RA in both astrocytes and microglia in

vitro. In that study, the secretion of the chemokines Mcp1, Mcp5, Mip2, and CxcL1 was upregulated in these cells, suggesting that the upregulation of chemokines by glial cells is the result of IL17A signaling through constitutively expressed IL17RA [74].

Ma et al. demonstrated that the suppressor of cytokine signaling 3 (Socs3) participates in IL17 functions in the CNS as a negative feedback regulator, using mouse models of Socs3 small interfering RNA (siRNA) knockdown and Socs3 deletion. These mice with loss of Socs3 function showed enhanced IL17 and IL6 signaling in astrocytes via the activation of the NF-κB and Mapk pathways, indicating that astrocytes can act as a target of Th17 cells and IL17 in the CNS [75]. Similarly, Kang et al. constructed specific deletion mutants of Act1, a critical component required for IL17 signaling, in mice with EAE to examine CNS inflammation in endothelial cells, macrophages, microglia, and the neuroectoderm (neurons, astrocytes, and oligodendrocytes). In these Act1-deficient mice, Th17 cells showed normal infiltration into the CNS but failed to recruit lymphocytes, neutrophils, and macrophages. Therefore, astrocytes are critical in IL17–Act1-mediated leukocyte recruitment during EAE [76].

Interestingly, Merkler et al. demonstrated that macrophages respond to the Th1 milieu and neutrophils respond to Th17 cytokines in a marmoset monkey model of EAE. They also showed dense accumulations of T and B lymphocytes, MHC-II-expressing macrophages/microglia, and early activated macrophages at the sites of perivascular and parenchymal lesions in the neocortex and subcortical white matter, indicating that the inflammatory response, especially macrophage and microglia activation, may be regulated differently in the gray matter areas of the primate brain [77].

In summary, DCs in the peripheral tissues and microglia in the CNS are responsible for cytokine polarization and the expansion of Th17 cells. The complex interactions of Th17 cells with different DCs, such as microglia, astrocytes, and peripheral DCs (including neutrophils and macrophages), all contribute to the immunopathogenesis of EAE and MS.

6. Reciprocal Interactions of Cytokines on Th Subsets in EAE/MS

IL1R KO mice have impaired Th17 cells and are protected from EAE [78], and IL1β increases the susceptibility to and progression of relapse onset in MS [79], implying a role for IL1β in the development of EAE and MS. EAE was abolished by a virus-expressing IL4 but not by a virus-expressing IL10 in chronic relapsing EAE. Therefore, the cytokine environment was converted from a disease-promoting IL23-producing condition to a disease-limiting IL4-producing condition by the local expression of IL4 from a Herpes simplex virus vector delivered to the brain [80]. Moreover, the increased expression of IL4 in glial cells was associated with the reduced severity of EAE [81], suggesting that the upregulation of Th2 cytokines inhibits the propagation of the inflammation of EAE/MS by encephalitogenic Th17 cells. $CD4^+CD25^+Foxp3^+$ T cells, well-known regulatory T cells (Tregs), retain the potential to inhibit the autoimmune response, and protect against inflammatory injury. TGFβ is a key cytokine in the generation of Tregs. Tregs are not only primarily involved in the regulation of Th17 cells but can also regulate the functions of Th1/Th2 cells [82]. A distinction has been drawn between the generation of pathogenic Th17 cells that induce autoimmunity and the generation of Tregs that inhibit autoimmune tissue injury [39].

Although EAE was once considered a classical Th1 disease, it has been proposed that it is predominantly Th17 driven. Recently, Singh et al. demonstrated that the overexpression of IL17 in T cells did not exacerbate EAE. Moreover, genetic and antibody studies have indicated that the absence of IL17A or IL17F does not reduce the incidence or severity of EAE. The collective findings of IL17 and IFNγ studies indicate that their roles may depend on the nature of the immune response and that the IL17 that occurs in the brain may overcome the inhibitory effect of IFNγ, which generally prevents inflammation at that site [83]. When pure Th17 cells from myelin oligodendrocyte glycoprotein-(MOG-) immunized mice, polarized with TGFβ to deplete any IFNγ production, are adoptively transferred to mice, they do not induce EAE, suggesting that the reciprocal interactions among Th17-related cytokines enrol and activate the involvement of associated immune cells. Interestingly, when Th17 cells are combined with Th1 cells, they can fully induce EAE disease [84]. Liu et al. also demonstrated that the loss of STAT3 by Th cells results in an intrinsic developmental defect that renders $STAT3^{-/-}$ mice resistant to CNS inflammatory diseases. STAT3 is required for the production of IL17 by Th17 cells, the generation of double positive T cells expressing IL17 and IFNγ, and T cell trafficking into CNS tissues. This suggests that STAT3 may be a therapeutic target for modulating CNS autoimmune diseases, and that Th1 cells can facilitate the entrance of Th17 cells into the CNS during EAE [85].

An encephalitogenic Th1 cell line that induces the recruitment of host Th17 cells to the CNS during the initiation of EAE has been reported [49]. Stromnes et al. showed significant differences in the regulation of inflammation in the brain and spinal cord, depending on different Th17/Th1 ratios, by demonstrating that specific T-cell populations targeting different myelin epitopes are characterized by different Th17/Th1 ratios in EAE [86]. Therefore, Th1 cells have the potential to reciprocally regulate Th17 cells during EAE.

IL21 is a type I four-α-helix bundle cytokine that belongs to the IL2 family and functions as a "growth hormone"-like cytokine. After the antigen-responsive differentiation phase, Th17 cells enter the amplification stage, and IL21 plays a pivotal role in the expansion and differentiation of the Th17 lineage, providing an autocrine and paracrine stimulus for Th17 cells [41, 87]. During clonal expansion, IL21 also promotes IL23R expression in differentiated Th17 cells, which plays an important role in the stabilization of the Th17 lineage in the presence of IL23 [88]. Although no effects were observed when Il21 was administered after EAE progression, the administration of IL21 boosted natural killer (NK)

cell functions before the induction of EAE, including the secretion of Ifnγ. Therefore, IL21, by affecting NK cells, has various effects during the initiation and progression of EAE [89].

Alternatively, IL27, an IL12/IL23 family member, is a negative regulator of Th17 cell differentiation and can prevent inflammatory demyelination in the EAE model [44]. IL27 drives the expansion and differentiation of IL10-producing Tr1 cells by inducing the expression of three key molecules: the transcription factor c-MAF, the cytokine IL21, and ICOS. Moreover, IL27-driven c-MAF expression transactivates the production of IL21, which acts as an autocrine growth factor for the expansion and/or maintenance of IL27-induced Tr1 cells. ICOS also promotes IL27-driven Tr1 cells. Each of these elements is essential, because the loss of c-MAF, IL21 signaling, or ICOS reduces the frequency of IL27-induced differentiation of Tr1 cells (Figure 1) [90]. Exacerbation of EAE was demonstrated in IL27-deficient mice, and interestingly, Il27-treated mice had markedly reduced CNS inflammatory infiltration, indicating the downregulation of Th17 phenomena [91].

Recently, a novel effector T-cell subset, Th9 cells, has been identified, and the ability of this T-cell subset to induce EAE is currently being investigated. Jäger et al. generated Mog-specific Th17, Th1, Th2, and Th9 cells in vitro to directly characterize their encephalitogenic potency after their adoptive transfer. They found that Mog-specific Th1, Th17, and Th9 cells, but not Th2 cells, induce EAE. Interestingly, each T-cell subset induced disease in a distinct pathological manner, suggesting that the different effector Th subsets that induce EAE do so differently and implying that the pathological heterogeneity in MS lesions might be partly attributable to various characteristics of myelin-reactive effector T cells [92]. The authors also suggested that MS might be a disease caused by multiple distinct myelin-reactive effector cells. The disease induced by Th17 cells in some animals exhibited symptoms atypical of EAE, including ataxia, severe imbalance, and weight loss associated with high mortality. Some animals had a mixture of atypical and typical EAE symptoms. When cells were recovered from the CNS, it appeared that the transferred Th9 cells produced IFNγ. The identities of the other cell populations did not seem to drift after their in vivo transfer [93].

Nowak et al. recently demonstrated that like other T cells cultured in the presence of TGFβ, Th17 cells produce IL9. Th17 cells generated in vitro with IL6 and TGFβ and ex vivo-purified Th17 cells both produced IL9. Data show that IL9 neutralization and IL9R deficiency attenuate the disease, and this correlated with reductions in Th17 cells and IL6-producing macrophages in the CNS. These authors also confirmed the role of IL9 in the development and progression of EAE and implicated Il9 as a Th17-derived cytokine that contributes to inflammatory disease [94].

Together, Th2 cells, Tr1 cells, and Tregs exert repressive effects on Th17 cells, and Th9 cells have a stimulatory effect on Th17 cells, suppressing EAE and MS. However, Th1 cells play dual roles in EAE.

7. Clinical Applications, Limitations, and the Future of Immunomediated Therapies for MS

Our understanding of the pathophysiology and neurodegenerative processes of MS has led to the development of novel therapeutic strategies. Since the early 1990s, disease-modifying drugs have been introduced for the selective management of MS, including IFNβ and glatiramer acetate (GA), which have become the standard treatment for relapsing/remitting MS [95]. Most recommendations previously made by the Multiple Sclerosis Therapy Consensus Group (MSTCG) on the use of disease-modifying drug therapies remain valid [96, 97]. Hermmer and Hartung have published an apparent review of the development of rational therapies in MS [98]. Therefore, we will discuss four domains of novel immunomediated therapeutics used for MS and their current status.

The first domain includes immunosuppressive agents, such as mitoxantrone, laquinimod (ABR-215062), cladribine (Mylinax), and teriflunomide (probably via the suppression of TNFα and IL2 production). The second domain includes immunomodulatory agents: (1) cytokine inhibitors such as IFNβ; (2) agents that deplete specific immune cell subsets, such as alemtuzumab (a human monoclonal antibody [mAb] that targets CD52 expressed by T and B cells, producing long-term T-cell depletion) [99, 100] and rituximab (which targets CD20 to deplete human B cells) [99, 101]; (3) agents that selectively block coreceptors and costimulators, such as daclizumab (an anti-CD25 mAb that inhibits activated T cells and induces regulatory immune cells) [102]. The third domain involves the development of migration-modifying therapies: (1) agents that affect adhesion molecules, such as natalizumab (an mAb that blocks very late antigen 4 [VLA-4]) and (2) sphingosine 1-phosphate receptor (S1PR) agonists: fingolimod (FTY720). The fourth domain includes neuroprotective agents associated with immunomodulation, including broad-spectrum immunomodulators such as statins, PPAR agonists (e.g., pioglitazone, gemfibrozil), the sex hormone estriol (E3), fumarate, minocycline, and erythropoietin (EPO), all of which have been effective in the treatment of both EAE, and MS. IFNβ has been clinically introduced to treat patients with MS based on its ability to shift a Th1-mediated response to a Th2-mediated response [92]. However, microarray studies have indicated that a number of genes in patients with MS are upregulated by the cytokines associated with the differentiation of cells into Th1 lymphocytes rather than into Th2 lymphocytes, suggesting that this shift may not be the only therapeutic mechanism of IFNβ in MS [103]. IFNβ therapy also reduces IL23 mRNA levels [104]. IFNβ inhibits human Th17 cell differentiation, so the Th17 axis could be another target of IFNβ therapy [105]. IFNβ-mediated IL27 production by innate immune cells has been shown to play a critical role in the immunoregulatory role of IFNβ in EAE by inhibiting Th17 cells in EAE mice and MS patients [91, 106, 107]. Besides, Galligan et al. evidence further that IFN$\beta(-/-)$ mice exhibited an earlier disease onset and a more rapid progression of EAE compared to IFN$\beta(+/+)$ mice of EAE and IFN$\beta(-/-)$ mice of EAE had increased

numbers of CD11b(+) leukocytes infiltrating affected brains and an increased percentage of Th17 cells in the CNS with augmentation of autoreactive T cells,suggesting that IFN-β acts to suppress the production of autoimmune-inducing Th17 cells during the development of disease as well as modulating proinflammatory [108]. In addition, the therapeutic effect of IFNβ is probably attributable to the induction of the regulatory cytokine IL10 [104]. Furthermore, Axtell et al. design a delicate study to further clarify the role of IFNβ in MS/EAE [109]. Likewise, They demonstrate that IFNβ was effective in reducing EAE symptoms transferred by Th1 cells transfer but exacerbated disease by Th17 cells transfer and effective treatment of IFNβ in Th1-induced EAE correlated with augmented IL10 production; differently, in Th17-induced EAE, the amount of IL10 was unaffected by treatment of IFNβ. Likewise, a high IL17F level in the serum of people with RRMS is associated with fail of IFNβ therapy. This characteristic of IFNβ might contribute to explore some logical biomarkers for predictive assessment of the response to a popular therapy for MS [109, 110]. Although, B cells may have a dual role in the pathogenesis of MS that they contribute to the induction of the autoimmune response but also mediate the resolution of the CNS inflammatory infiltrate [111, 112]. However, Ramgolam et al. demonstrate further that supernatants transferred from IFNβ-1b-treated B cells inhibited Th17 cell differentiation, as they suppressed gene expression of the RORC and IL-17A and secretion of IL-17A. Likewise, IFNβ-1b also induces B cells' IL-10 secretion which may mediate their regulatory potent [113]. Thus, IFNβ-1b exerts its therapeutic effects at least in part by targeting B cells' functions that contribute to the autoimmune pathogenesis of RR MS, which may uncover extra mechanisms of the B-cell contribution to the autoimmune effects and provide novel targets for future selective treatment of MS [113].

Glatiramer acetate (GA; Copaxone; copolymer 1) exerts a clinical response in MS patients via its modulation of IFNγ and IL4 by reducing the expression of IFNγ and ensuring the stable expression of IL4 in anti-CD3/CD28-stimulated peripheral blood mononuclear cells (PBMCs) [114]. Moreover, GA enhances the suppressive effects of Tregs in both EAE and MS [115, 116]. Studies of human DCs have shown that GA modulates the production of inflammatory mediators without affecting DC maturation or immunostimulatory potential. DCs exposed to GA secrete low levels of the Th1-polarizing factor IL12p70 in response to lipopolysaccharide and triggering of the CD40 ligand [117]. Human DCs exposed to GA also induce IL4-secreting effector Th2 cells and increase their expression of IL10 [118]. These results show that APCs, including DCs, are essential for the GA-mediated shift in Th-cell phenotypes and indicate that DCs are an important target of the immunomodulatory effects of GA.

Patients with MS show a threefold to fourfold increase in the expression of the α4 subunit of the integrin VLA-4, which is normally expressed on activated lymphocytes, monocytes, and other cell types in the CSF and circulation [119]. Elovaara et al. confirmed that methylprednisolone reduces the adhesion molecules in the blood and CSF in patients with MS [120], implying that targeting leukocyte trafficking may be a possible therapeutic strategy for MS [121]. Therefore, natalizumab, a humanized mAb directed against the VLA-4 adhesion complex, has been introduced into the treatment of MS and reduces the risk of sustained progression of disability and the rate of clinical relapse in patients with relapsing MS [122]. However, during clinical trials, two natalizumab-treated MS patients developed progressive multifocal leukoencephalopathy (PML), which resulted in the voluntary removal of the drug from the market in February 2005 [123, 124]. A retrospective safety evaluation was subsequently conducted, and natalizumab was consequently returned to the market as a monotherapy in July 2006 for the treatment of relapsing MS; however, there were 111 cases of PML reported subsequently in natalizumab-treated MS patients as of April 2011 [125]. More evidently, the risk of developing PML for a MS patient on natalizumab (Tysabri) is almost 100 times higher if the patient (1) has been taking the drug for more than two years, (2) has a prior history of immunosuppressant use, and (3) tests positive for antibodies to the JC virus [126], compared to a patient with none of these three risk factors [127]. Instead, there is currently no convincing evidence that natalizumab-associated PML is restricted to combination therapy with other disease-modifying or immunosuppressive agents [128]. Nevertheless, natalizumab use must be restricted to the indicated patients.

Mitoxantrone, a cytotoxic drug with immunomodulatory properties, is used to treat progressive forms of MS [129]. Mitoxantrone increases the ex vivo production of the Th2 cytokines IL4 and IL5, but with no significant changes in IFNγ, TNFα, IL10, or IL17 expression by PBMCs or $CD4^+$ T cells, indicating that the immunomodulation afforded by mitoxantrone treatment in MS acts through the enhancement of Th2-type cytokines [130].

Currently, a head-to-head race for approval had initially developed between two under spotlight oral immunomodulatory agents—fingolimod and cladribine (Figure 2) [131]. Fingolimod (FTY720/Gilenya, Novartis), an S1PR modulator [132], is under the spotlight because it has completed phase III trials [133] and has been approved by the US Food and Drug Administration as the first oral, first-line treatment for relapsing MS [134, 135]. S1PR is mainly expressed by immune cells, neuronal cells, endothelial cells, and smooth muscle cells [136–139]. The key roles of S1PR in angiogenesis, neurogenesis, and the regulation of immune cell trafficking, endothelial barrier function, and vascular tone were demonstrated with the genetic deletion of S1pr in a murine model [140–142]. The immunomodulatory effect of fingolimod acts in two pathways. In one pathway, it inhibits the function of S1PR, which facilitates the CC-chemokine receptor 7-(CCR7-) mediated retention of lymphocytes in the lymph nodes, including naïve T cells and central memory T cells, but not effective memory T cells. This significantly reduces the infiltration of inflammatory cells into the CNS [143, 144] and reduces the numbers of autoreactive Th17 cells that are recirculating via the lymph and blood to the CNS [145–147]. The second pathway prohibits neuroinflammation via the modulation of the

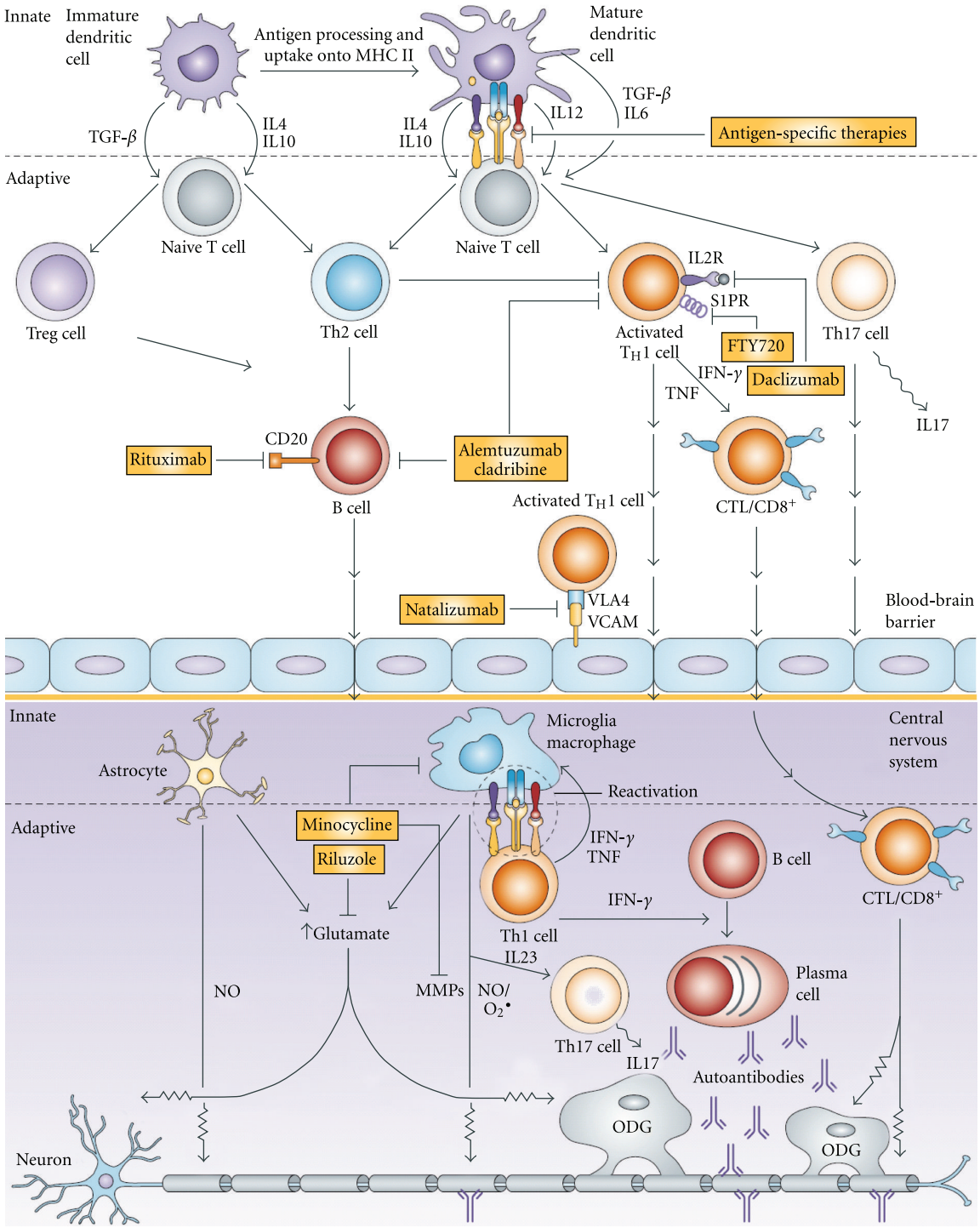

Figure 2: Multiple sclerosis immunopathogenesis and therapeutic targets. Immature dendritic cells (DCs) are central players in the innate immune response and are involved in the maintenance of peripheral tolerance by promoting the suppressor Treg and anti-inflammatory Th2-cell responses. Abnormally activated (mature) antigen-presenting DCs can be found in patients with multiple sclerosis (MS). This activation results in the increased production of proinflammatory cytokines, which lead to the aberrant activation of Th1 and Th17 proinflammatory responses. Activated encephalitogenic adaptive immune effectors (such as Th1 cells, Th17 cells, $CD8^+$ cells, and B cells) express surface molecules that allow them to penetrate the blood-brain barrier and to enter the central nervous system (CNS). The presence of autoreactive immune effectors, together with abnormally activated CNS astrocytes and microglia, leads to the increased production of reactive oxygen species, excitotoxicity, autoantibody production, and direct cytotoxicity, which are all involved in the demyelination and axonal and neuronal damage that is present in patients with MS. Potential therapeutic interventions at different levels of the immunopathological cascade are shown in the filled yellow boxes (cytotoxic T lymphocytes [CTL]; interferon γ [IFNγ]; IL2 receptor [IL2R]; major histocompatibility complex class II [MHC II]; matrix metalloproteinases [MMPs]; nitric oxide [NO]; oligodendrocyte [ODG]; sphingosine 1-phosphate receptor [S1PR]; transforming growth factor β [TGFβ]; tumor necrosis factor [TNF]; regulatory T cells [Treg]; vascular cellular adhesion molecule 1 [VCAM1]; very late antigen 4 [VLA-4] (This figure was adapted and partly revised from [55].).

S1PR1 expressed on oligodendrocytes, neurons, astrocytes, and microglia [76, 148, 149]. Another oral immunomodulatory drug Cladribine (2-chlorodeoxyadenosine) is a synthetic chlorinated deoxyadenosine analog [150] that is activated by intracellular phosphorylation in specific cell types, resulting in preferential and sustained reduction of peripheral T and B lymphocytes, mimicking the immune-deficient status of hereditary adenosine deaminase deficiency [151]. Orally administered cladribine shows significantly efficacy in patients with RR-MS [152]. Relative to placebo, oral cladribine reduces relapses by 55–58% and has an impact on disability progression and all MRI outcome markers in patients with RR-MS [152–154]. Nevertheless, to exactly weight the benefits of both novel immunomodultory agents against the potential risks is necessary and must be monitored continually.

These advances in identifying unique therapeutic targets for MS have instigated numerous phase II and phase III clinical trials, for example, trials of various mAbs, including those directed against CD52 (alemtuzumab), CD25 (daclizumab), and CD20 (rituximab), and trials of disease-modifying therapies, such as teriflunomide, laquinimod, and fumarate [135, 155]. For example, alemtuzumab, a humanized mAb, targets the surface molecule CD52 on all T-cell populations and other cellular components of the immune system, such as thymocytes, B cells, and monocytes [156].

Offner reported that estrogen and its derivatives exert neuroimmunoprotective effects against EAE and that E2 upregulates the expression of Foxp3 and Ctla4, which contribute to the activity of Tregs, suggesting the therapeutic application of estrogen to MS [157]. Papenfuss et al. also demonstrated that estriol (E3), a pregnancy-specific estrogen, has therapeutic efficacy in MS and EAE and they confirmed that E3 protects mice against EAE by inducing DCs to increase their expression of inhibitory costimulatory markers (PD-L1, PD-L2, B7-H3) and deviate towards a Th2 phenotype [158].

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily, which includes receptors for steroids, retinoids, and thyroid hormones, all of which are involved in the immune response [159]. Natarajan et al. demonstrated that PPARγ agonists inhibit EAE by blocking IL12 production, IL12 signaling, and Th1 cell differentiation [160]. Kanakasabai et al. further demonstrated that the PPARδ agonists ameliorate EAE by blocking IFNγ and IL17 production by Th1 and Th17 cells. The inhibition of EAE by PPARδ agonists is also associated with reductions in IL12 and IL23 and increases in IL4 and IL10 expression in the CNS and lymphoid organs. This indicates that PPARδ agonists modulate the Th1 and Th17 responses in EAE, and suggests their use in the treatment of MS and other autoimmune diseases [161].

Minocycline, an oral semisynthetic tetracycline antibiotic, can penetrate the CNS and has interesting pleiotropic biological functions and neuroprotective effects, including in demyelinating diseases such as MS [55]. Nikodemova et al. have shown that minocycline attenuates EAE in rats by reducing T-cell infiltration into the spinal cord and downregulating LFA-1 on T cells, but without modifying the production of dominant cytokines [162]. Zabad et al. demonstrated in a cohort study the impact of oral minocycline on clinical and MRI outcomes and serum immune molecules during the 24 months of open-label minocycline treatment. No relapses occurred between months 6 and 24, and the levels of the p40 subunit of IL12 were elevated during the 18 months of treatment, which might have counteracted the proinflammatory effects of IL12R. The downregulation of MMP9 activity was reduced by minocycline treatment [163].

Brines et al. have demonstrated that EPO mediates neuroprotection against experimental ischemic brain injury [164]. Agnello et al. have shown that EPO exerts an anti-inflammatory effect that ameliorates EAE [165]. Yuan et al. also demonstrated that EPO retains its immunomodulatory capacity in both the periphery and the inflamed spinal cord by promoting a massive expansion of Treg cells, inhibiting Th17 polarization and abrogating the proliferation of antigen-presenting DCs [166]. We observed significantly reduced levels of both Th1 and Th17 cells in the CNS and a significantly increased proportion of splenic Tregs in EPO-treated Mog-EAE mice. We also demonstrated that MOG-specific T-cell proliferation was suppressed in the EPO-treated group [167].

The immunomodulatory mechanisms of immunomediated therapeutic agents are not fully understood. Here, we report our current understanding of the immunomodulatory effects of clinically proven and clinically tried agents, and of potential candidate agents, such as decoy receptor 3 (DcR3). We have selectively reviewed their immunomodulation in EAE and MS. Demjen et al. showed that the neutralization of CD95L (FasL) promoted axonal regeneration and functional improvement in an injured animal model, suggesting that this therapeutic strategy may constitute a potent future treatment for human spinal injury [168]. DcR3 is a recognized member of the TNFR superfamily and is predominantly expressed in tumor cells, allowing them to evade immune attack [169]. DcR3 is a soluble receptor that binds to members of the TNF family and can competitively inhibit the binding of TNF to TNFRs [170]. FasL, LIGHT, and TNF-like molecule 1A (TL1A) are all confirmed ligands of DcR3 [171, 172]. When DcR3 binds to FasL, it inhibits FasL-induced apoptosis [169]. It has also recently been shown that DcR3 counteracts the effects of Th17 cells by interfering with FasL-Fas interactions [173]. We have demonstrated that DcR3 ameliorates EAE by directly counteracting inflammation and downregulating Th17 cells in situ [174], implying that DcR3 downregulates the Th17 response and inhibits the inflammation of the CNS in situ during EAE by blocking ligand-receptor interactions, such as Fas-FasL, DR2–LIGHT, and/or DR3–TL1A. Therefore, we introduce DcR3, another immunomodulatory molecule, as a potential candidate for consideration in the clinical treatment of MS.

In summary (Figure 2), these immunomodulatory agents and neuroprotective therapies for MS have great value as clinical agents, to be tested in clinical trials or preclinical studies, and in the development of novel therapeutic strategies for MS [55].

8. Concluding Remarks

MS is the most common disabling CNS disease in young adults. It is characterized by recurrent relapses and/or progression, which are attributable to multifocal inflammation, demyelination, and axonal pathology within the brain and/or spinal cord [175]. The effector Th cells play a well-recognized role in the initiation of autoimmune tissue inflammation, and these autoreactive effector $CD4^+$ T cells have an established association with the pathogenesis of this disorder [17]. However, in models thought to be driven by Th1 cells, mice lacking the hallmark Th1 cytokine IFNγ were not protected from EAE but tended to display enhanced susceptibility to this disease [26]. The identification of Th17 cells has shed light on this apparent discrepancy. Like Th1 cells, polarized Th17 cells have the capacity to cause inflammation and autoimmune disease. A deficiency of the Th17-related cytokine IL23, but not of the Th1-related cytokine IL12, induces resistance to EAE, implying that Th17 cells are the chief contributors to EAE/MS [28], whereas Th1 cells can consistently transfer EAE disease [16, 17]. Komiyama et al. demonstrated that EAE was significantly suppressed in $Il17^{-/-}$ mice, manifested as delayed onset, reduced maximum severity, ameliorated histological changes, and early recovery [176]. However, the outcomes have varied when the differentiation and/or functions of Th17 cells have been blocked in clinical trials of human autoimmune diseases, with notable success only in psoriasis and Crohn's disease, but negative results in relapsing/remitting MS. The strategy of inhibiting the Th17 response has had even less support in preclinical studies in animal models [177].

These data raise the questions of whether MS is mediated solely by Th1 cells or solely by Th17 cells, whether it is mediated by both pathways, or whether perhaps it is mediated by neither pathway [175]. There is growing evidence that autoreactive T cells (particularly Th1 and Th17 cells) participate in the pathophysiology of MS. Although the exact roles of Th1 and Th17 cells in the development of MS lesions are not well understood, it appears that both these effector T-cell populations can cause CNS inflammation and demyelinating lesions in MS and EAE [50, 178].

Our increasing understanding of the immunopathogenic roles of Th1, Th2, and Th17 cells and Tregs in MS/EAE should facilitate the development of novel immunomodulatory therapeutic approaches to MS [179, 180]. The treatment of MS has always been hampered by the untoward adverse effects caused by immunosuppression with agents such as natalizumab [128]. Currently approved disease-modifying treatments achieve their effects primarily by blocking the proinflammatory response in a nonspecific manner. Their limited clinical efficacy calls for a more differentiated and specific therapeutic approach. We can confidently say that IFNβ, GA, and mitoxantrone are fairly clinically effective for MS patients. The addition of estrogen(s) or minocycline has also shown benefits in the treatment of MS. We have established the protective effects of DcR3 and EPO against EAE [174, 181], but further evidence is required before they can be used clinically for the treatment of MS. More immunomodulatory therapeutic agents are currently in clinical trials, including fingolimod (FTY720), alemtuzumab, and rituximab add-on therapies [182]. The extensive clinical application of these potential novel immunomodulatory therapeutic agents will be under close scrutiny in the near future.

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Review Article

Immune-Regulatory Mechanisms in Systemic Autoimmune and Rheumatic Diseases

Yuya Takakubo[1,2] and Yrjö T. Konttinen[1,3,4,5]

[1] *Department of Medicine, Biomedicum Helsinki, University of Helsinki, P.O. Box 700 (Haartmaninkatu 8), 00029 HUS, Finland*
[2] *Department of Orthopedic Surgery, Yamagata University School of Medicine, Yamagata 990-9585, Japan*
[3] *Department of Medicine/Invärtes Medicin, Helsinki University Central Hospital, 00029 Helsinki, Finland*
[4] *ORTON Orthopedic Hospital of the ORTON Foundation, 00281 Helsinki, Finland*
[5] *COXA Hospital for Joint Replacement, 33101 Tampere, Finland*

Correspondence should be addressed to Yuya Takakubo, takakubo-y@med.id.yamagata-u.ac.jp

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Systemic autoimmune and rheumatic diseases (SAIRDs) are thought to develop due to the failure of autoimmune regulation and tolerance. Current therapies, such as biologics, have improved the clinical results of SAIRDs; however, they are not curative treatments. Recently, new discoveries have been made in immune tolerance and inflammation, such as tolerogenic dendritic cells, regulatory T and B cells, Th 17 cells, inflammatory and tolerogenic cytokines, and intracellular signaling pathways. They lay the foundation for the next generation of the therapies beyond the currently used biologic therapies. New drugs should target the core processes involved in disease mechanisms with the aim to attain complete cure combined with safety and low costs compared to the biologic agents. Re-establishment of autoimmune regulation and tolerance in SAIRDs by the end of the current decade should be the final and realistic target.

1. Introduction

Recently, several important new discoveries have been made on both immunogenicity and autoimmune regulation and tolerance. Immune tolerance is necessary for the homeostatic and balanced host defense. It is important to recognize the role of the self-antigens which should be protected (non-dangerous) or self- and nonself-antigens which should be eliminated (dangerous) for the induction and maintenance of autoimmune regulation and tolerance. Immune system is constantly in contact with numerous self-antigens including autologous necrotic or apoptotic tissues, and it uses multiple strategies to prevent autoimmunity [1]. The antigen-specific or nonspecific immune tolerance can be generated primarily in thymus or secondarily in peripheral lymphatic and extralymphatic tissues. Various elimination mechanisms of autoreactive T cells and B cells such as presentation of autoantigen by antigen-presenting cells (APCs) and regulation by regulatory T cells (Tregs) play a role, not only for the central tolerance in the thymus, but also for the peripheral tolerance in extrathymic tissues, which exert an ongoing control to avoid systemic autoimmune and rheumatic diseases (SAIRDs). Within the thymus, T cells go through positive and negative selection processes in anatomically different locations to shape the entire peripheral T-cell repertoire when the central tolerance is established. Peripheral tolerance is then produced by engagement of dendritic cells (DCs) via several mechanisms, including generation and expansion of Treg cells and regulatory cytokines [2–5].

The onset and progression of SAIRDs depends on multiple factors, and many types of cells are involved in the multiple pathways of the immune reaction [5–7]. In all SAIRDs, the ultimate goal should be the re-establishment of self-tolerance [8]. In this paper, we describe new insight and

topics related to immune regulation and tolerance in SAIRDs and their potential in the management of these diseases.

2. The Pathogenic Role of Dendritic Cells, Regulatory T and B Cells, and Regulatory Cytokines in SAIRDs

2.1. Dendritic Cells (DCs). The functional abilities of the DCs vary depending on their subset and state of maturation. The two major categories of DCs are the conventional DCs (cDCs) and the plasmacytoid DCs (pDCs). They can be further functionally classified to mature and immature DCs. DCs for a heterogeneous group of cells, which play an important role in immunogenicity but also in the maintenance of immunotolerance, including their effects on the induction of antigen-specific T cell responses resulting in anergy, apoptotic deletion, or formation of Tregs [9]. Tolerogenic DCs populations have been generated as experimental therapeutic tools, which have been used with some success in murine disease models [10]. However, direct evidence implicating a particular DC subset in the breach of self-tolerance leading to SAIRDs is lacking although some novel murine experimental arthritis models allow delineation of early, preclinical events leading to the loss of self-tolerance [11, 12].

2.2. Conventional Dendritic Cells (cDCs). CDCs are detected in blood, skin, secondary lymph nodes, spleen, and inflammatory synovitis [1, 4, 7, 13]. They are subdivided into two categories, migratory DCs and resident DCs, for example, Langerhans cells in the skin and mucosal membranes. In addition, there are other types of cDC that are derived from monocytes during inflammation [14–16].

In immune reactions mature cDCs play a more important role as an APC to activate naïve T cells than pDCs [12, 17]. In addition, these cells play a central role in the tumor necrosis factor (TNF) α-dependent experimental autoimmune arthritis initiated by an irrelevant nonarticular antigen and no other APC is by itself sufficient for breach self-tolerance mediated by endogenous pathways [8]. External (pathogen-associated molecular patterns) and internal (alarmins) danger signals or a combination of both leads to the maturation of cDCs in various inflammatory and infection diseases through membrane receptors, such as Toll-like receptors (TLR) 1-6, TLR8, and intracytoplasmic inflammasome [8, 12, 17].

On the other hand, engagement of immature cDCs with naïve T cells is thought to result in immunological tolerance. Immature cDCs are characterized by low surface expression of major histocompatibility complex- (MHC-) II and costimulatory molecules [9, 17]. Immature cDCs can drive naïve T cells to assume Tregs phenotype and/or promote the function of already existing Tregs as has been shown in experiments in which antigen was administered to mice without a concomitant maturation signal. Under these conditions, antigen accumulated on cDCs in secondary lymphoid organs and triggered the differentiation and/or proliferation of Tregs, resulting in antigen-specific tolerance that could prevent or reverse autoimmune processes [18–20].

2.3. Plasmacytoid Dendritic Cells (pDCs). PDCs can function to limit self-reactivity and consequent pathology [11]. These cells are also known as interferon- (IFN-) $\alpha\beta$ (type I IFN-) producing cells [11, 21]. Several systemic autoimmune diseases cause a prominent IFN signature (interferon-regulated genes) in the affected target tissue which pDCs produce, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), psoriatic arthritis (PsA), systemic sclerosis (SSc), and Sjögren's syndrome (SjS). PDCs are activated to produce type I IFN by recognition and internalization of immune complexes consisting of autoantibodies and self-nucleic-acids, which after endocytosis are recognized through TLR7 and TLR9. On the other hand, pDCs are reported to be key players in the establishment of oral and transplant tolerance. Human pDCs activated by the TLR9 DNA ligand CpG-ODN can induce Tregs [22, 23]. PDCs has the potential to express indoleamine 2,3-dioxygenase (IDO), an enzyme that via its products inhibits effector T cell proliferation in SAIRDs [13, 24]. In the presence of extracellular IDO, T cell proliferation is compromised, and adaptive differentiation Tregs is enhanced although the precise molecular basis for this effect is still unclear [20, 25, 26].

2.4. Regulatory T Cells (Tregs). Adaptive effector T cell responses to self and nonself-antigens can be efficiently controlled by regulatory T cells belonging to the CD4+CD25+ Treg subset. Tregs are phenotypically heterogeneous and include both CD4+ and CD8+ T cells, most of which express forkedhead box p3 (Foxp3) [20, 27]. Tregs can express anti-inflammatory molecules, such as interleukin (IL)-10, transforming growth factor (TGF)-β and IL-35, and/or inhibitory receptors, such as cytotoxic T-lymphocyte antigen 4 (CTLA4), lymphocyte-activation gene 3 (LAG-3), glucocorticoid-induced tumor necrosis factor receptor (GITR), CD39, and CD73 [19, 28].

DCs constantly present innocuous self- and nonself-antigens in a fashion that promotes tolerance, at least in part, through the control of Tregs [29]. Failure of Tregs function has been implicated in the development of many autoimmune processes and, *vice versa*, cellular therapies by adoptive transfer of Tregs have shown efficacy in these disorders [20, 30]. In addition, DCs and Tregs regulate homeostasis of each other [18–20].

Natural Tregs (nTregs) in thymic tissue develop and maintain central tolerance during the fetal period. Conventional naïve T cells can develop to so-called adaptive Tregs (aTregs) in extrathymic tissues such as secondary lymphoid organs. This is important, because the autoimmune T cell have not been completely deleted by the central tolerance mechanisms. Therefore, aTregs play a complementary role in the maintenance of the peripheral tolerance by regulating peripheral autoimmune T cells. The role of these inflammation-induced aTregs is not fully understood, but they seem to limit immunohistopathologic changes by suppressing autoaggressive responses and/or by promoting

restitution of tissue homeostasis (via TGF-β) or T and B cell memory (via IL-10). Antigen-specific Tregs can spread their tolerance-promoting capacity to local DCs and naïve T cells through a mechanism called "infectious tolerance", which means that tolerant T cells, which are incompletely stimulated by APCs, induce new naïve T cells toward a similar state of tolerance [20, 27, 31].

2.5. Regulatory-B Cells (Bregs). The efficacy of B cell suppression with anti-CD20 therapy such as Rituximab in the treatment of RA indicates an important role for B cells in the pathogenesis of RA. In RA, transient B cell depletion with Rituximab can ameliorate disease for a prolonged period but typically not indefinitely. Autoreactive B cells have been found in bone marrow and peripheral tissues [32]. Anti-TNF agents decreased the peripheral blood memory and germinal center B cells in RA patients restoring the early B cell tolerance in RA [33]. In addition, B cells are the main producers of LTα and an important producer of TNFα in RA synovitis. B cells can also secrete multiple other cytokines, including IL-1, IL-4, IL-6, IL-7, IL-8, and IL-12, granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) [34, 35].

On the other hand, B cells are able to suppress autoimmunity in various animal models through production of IL-10 and/or TGF-β and by cytokine-independent mechanisms. Murine models have also revealed a suppressive role for B lymphocytes. Following stimulation of CD40, B cells inhibit the induction of arthritis through secretion of IL-10 [36]. In Palmerston North SLE mouse model, regulatory B cells (Bregs) produce high levels of IL-10 that inhibit production of IL-12, which leads to diminished inflammatory response to bacterial DNA [37]. A role for Bregs has been reported in a mouse model of inflammatory bowel disease and experimental autoimmune encephalomyelitis [38, 39]. Depending on the model studies, antigen-specific autoreactive T and B cells, DCs, macrophages and/or Tregs are regulated by the Bregs populations [40]. These observations contribute to the new and important concept of Bregs [32, 41].

3. Advances in Molecular Genetics and Molecular Diagnostics

3.1. Genetics in Systemic Autoimmune and Rheumatic Diseases (SAIRDs). Advances in the field of human genetics have led to a rapid increase in the number of new disease risk alleles and loci identified in SAIRDs patients. In particular, genes involved in the nuclear factor (NF)-κB pathway and T cell-DC interactions seem to be involved in SAIRDs [42–44].

New candidate genes have been reported in RA, and they include single-nucleotide polymorphisms (SNPs) of IL-6, signal transducer and activator of transcription (STAT) 4, IL-2RA, CC chemokine ligand (CCL) 21, CD40, CD244, TNF alpha-induced protein (TNFAIP) 3, sprouty-related protein with enabled/vasodilator stimulated phosphoprotein homology 1 domain (SPRED) 2, recombination signal binding protein for immunoglobulin kappa J region (RBPJ), CC chemokine receptor (CCR) 6, interferon-regulatory factor (IRF) 5, PX domain containing serine/threonine kinase (PXK), cyclin-dependent kinase (CDK) 6, and vitamin D (Vit.D) Fokl found in genome-wide association studies (GWAS) [42, 43, 45, 46]. The heritability of RA has previously been estimated to be about 60%. Recently, it was reported that the heritabilities of anticitrullinated protein antibody- (ACPA-) positive and ACPA-negative RA are rather similar, 68% and 66%, respectively [47–50]. In particular, human leukocyte antigen- (HLA-) DRB1 and shared epitope (SE) alleles of it were reported the strong association with ACPA-positive RA. Apart from perhaps gender, the main known genetic factor predisposing to RA is HLA and its contribution to the genetic variation has previously been estimated to be 37% [51].

In SLE, the IRF5 haplotype has been reported to relate to an increased production of type I IFN with a concomitantly increased risk for SLE [52], and a variant of STAT4 increases the sensitivity to type I IFN in patients with SLE [53]. A variant of TNFAIP 3, which encodes an ubiquitin-editing enzyme inhibiting NF-κB-dependent signaling and prevents inflammation, and polymorphic haplotypes of the human T-lymphotropic virus-1-related endogenous sequence (HRES) 1 long terminal repeat (LTR) have been associated with SLE [54, 55]. New candidate changes include SNPs in CD40 region, B-lymphoid tyrosine kinase (BLK) gene, vascular endothelial growth factor (VEGF) receptor 2 gene, and Vit.D Bsml gene [44, 45].

Polymorphisms in IRF5 are not only associated with RA and SLE, but also with SSc, inflammatory bowel diseases and multiple sclerosis. An association with STAT4 was first identified in RA and SLE, but later also found in SSc, inflammatory bowel diseases, type 1 diabetes, psoriasis, and primary antiphospholipid antibody syndrome [44].

GWASs have disclosed over twenty candidate genes in PsA, including IL-4, IL-12B, IL-13, IL-23A, IL-23R, TNFAIP3, TNFAIP3 interacting protein (TNIP) 1, TNF receptor-associated factor (TRAF) 3 interacting protein (IP) 2, nitric oxide synthase (NOS) 2, v-rel reticuloendotheliosis viral oncogene homolog (REL), endoplasmic reticulum aminopeptidase (ERAP) 1, F-box and leucine-rich repeat protein (FBXL) 19, NF-κ light polypeptide gene enhancer in B cells inhibitor, alpha (NFKBIA), interferon induced with helicase C domain (IFIH) 1, IL-28RA, and tyrosine kinase (TYK) 2 [56, 57].

In SSc, the newly reported candidate genes and risk loci include CD247, MHC, IRF5, and STAT4 gene regions, psoriasis susceptibility 1 candidate (PSORS1C) 1, TNIP1, and putative one close to *ras* homolog gene family, member B (RHOB) gene [58].

In SjS, the newly reported candidate genes and risk loci include early B-cell factor 1 (EBF1) gene, family with sequence similarity 167 member A (FAM167A)-BLK locus, TNF superfamily4 gene, HLA-DRB1$*$0301, -DQA1$*$0501, and -DQB1$*$0201, with SNPs in IL-10 promoter, Fas/FasL, TGF-β1, and TNFα genes, IRF5 and STAT4 [44].

In addition to the genetic factors also environmental risk factors and their interactions with the genetic factors contribute to SAIRDs, in particular to RA [42]. One important

environmental risk factor for RA is smoking, which increases the odds ratio to about 1.8 [59]. SE alleles, lymphoid-specific tyrosine phosphatase (LYP)/protein tyrosine phosphatase, nonreceptor type (PTPN) 22, and cigarette smoking are risk factors for the development of ACPA positive RA patients [47, 60]. Further, it has been reported that also the noninherited maternal HLA antigens (NIMA), originally discovered in transplantation immunology [61], containing the RA-protective DERAA sequence, protect DERAA-negative children against RA [49, 62].

It has been recently reported that the gene polymorphism may not only be associated with the pathogenesis of SAIRDs, but also with the outcome of the treatment and the progression of the disease. GWAS and new generation sequencing disclosed that the response of RA patients to anti-TNF therapy was associated with ATP-binding cassette, subfamily A (ABCA) 1, solute carrier family 44 (SLC44A) 1 gene and granzime B SNP were associated with joint destruction, and protein tyrosine phosphatase, receptor type, C (PTPRC) locus (rs10919563) and TRAF 6 [63–67].

3.2. MicroRNA. MicroRNAs have attracted attention as potential new biomarkers useful for the diagnosis of early disease and for the prediction of drug response in SAIRDs. MicroRNAs form an abundant class of endogenous, short, noncoding single stranded RNA molecules (19–23 nucleotides) that function as posttranslational regulators of the expression of genes, such as inflammatory cytokines. MicroRNAs were first reported in cancer [68]. Recently, many microRNAs, such as microRNA-16, -146a, -155, and -223, have been detected in SAIRDs samples, and it is thought that their expression regulates disease onset/activity and drug responses [68, 69].

4. New Insights into Defective Regulatory Function in SAIRDs

4.1. Toll-Like Receptor (TLR) and Interferon (IFN) Signature. In the last decade, a relationship has been described between TLR and IFN signature. IFN signature is refers to a pattern of genes regulated by IFN in inflamed target tissues. IFN signature is typical for many SAIRDs, such as RA, SLE, and SSc. DNA- or RNA-containing immune complexes, formed as a result of autoantibodies binding antigenic material derived from apoptotic or necrotic cells, can act as endogenous type I IFN inducers [70–72]. Prolonged exposure of the immune system to type I IFN increases the risk for the loss of self-tolerance and for subsequent autoimmune reactions. Indeed, high-serum type I IFN serum levels have been associated with the development of SAIRDs like SLE [73–75].

PDCs are activated to produce type I IFN when they recognize immune complexes consisting of autoantibodies and self-nucleic acids. Following FcγRIIa- (CD32-) mediated internalization, they trigger endosomal TLR7 and TLR9 [76, 77]. PDCs are low in numbers in the blood of SLE patients, but large amounts of type I IFN are in lupus produced to serum by the activated pDCs located in the lymph nodes, skin, and other target organs [74, 78]. Proper clearance of apoptotic cell rests is normally thought to prevent harmful exposure of the immune cells to self-antigens and alarmins and to prevent their autoaggressive activation [16, 79]. Type I IFN plays powerful roles in shaping immune responses in SAIRDs by activating lymphocytes, macrophages, DCs, and natural killer (NK) cells with the expression of IFN signature [80–82]. If apoptotic cells are not adequately cleared, condensed, and fragmented cell rests enter late stage apoptosis or undergo secondary necrosis, which causes release of nucleosomes, small nuclear ribonucleoproteins (snRNPs), and DNA, but also high-mobility group box 1- (HMGB1-) nucleosome complexes and other signals able to trigger inflammation through TLRs [83–85]. Only extracellular free HMGB1 or nucleosomes alone were not able to activate DCs or induce cytokine production. HMGB1 was thought to inhibit and diminish the phagocytosis of apoptotic neutrophils by macrophage through binding to phosphatidylserine on the neutrophil membrane and the remains of them might activate to produce proinflammatory mediators through TLR2 or TLR4 [86]. From studies of lupus-prone mice, it appears that there are multiple defects in the process that regulates autoreactive T and B cells, allowing the maturation of autoreactive B cells. In SLE patients, several abnormalities interfere with the clearance of apoptotic cell rests, microparticles and chromatin, such as complement defects [87] and reduced levels and activity of DNase I in serum [88]. In lupus-prone mice model, a deficiency in a negative regulator (Tir8/Sigirr) of TLR signaling accelerates disease progression [89]. Some TLR7-deleted lupus-prone mice strains have decreased lymphocyte and pDC activation, decreased serum IgG, and ameliorated autoimmune disease [90]. TLR7-dependent production of autoantibodies is decreased in such mouse strains. In contrast, although CpG-containing DNA, which is a ligand of TLR9, is induced lupus-like disease, lack of TLR9 in the MRL/lpr mouse background exacerbated autoantibody production and disease activity, suggesting that TLR9 protects against lupus in this model [90, 91]. TLR7 and TLR9 appear to have different roles in the development of murine lupus. Taken together, these studies suggest TLR9 may either protect or exacerbate autoimmune conditions depending on the genetic background of the lupus-prone mice. Jin et al. demonstrated that peripheral circulating pDCs in patients with SLE were functionally abnormal and that they lacked TLR9 [23]. The role of TLR9 in SLE development is still unclear.

4.2. Th17 and Regulatory T Cells (Tregs). Th 17 cells produce IL-17 (or actually IL-17A), IL-21 and IL-23 and have been reported to play a crucial role in inflammation in SAIRDs [92, 93]. A combination of IL-6 and TGF-β is required for the development of Th 17 cells [92]. Apoptotic bodies and nucleosomes induce maturation of mouse cDCs to produce high amounts of IL-6 [79]. On the other hand, the development and maturation of Tregs is inhibited by IL-6 [15, 94, 95]. IL-6 produced from LPS-treated DC activated effector T cells and blocked the suppressive effects of Tregs [94]. In fact, SLE patients have significantly lower numbers

of CD4+CD25+ Tregs than healthy controls [23, 96]. Anti-TNF therapy increased Foxp3 mRNA and protein expression by Tregs and restored their suppressive function [97].

Recently, it was shown that retinoic acid, which binds the nuclear retinoic acid receptor α (RARα), increases the expression of Foxp3 and Smad3 in T cells and inhibits the generation of Th 17 cells [98]. Retinoic acid may be a candidate drug for RA therapy, because alltrans-retinoic acid (ATRA) improves clinical symptom in collagen-induced arthritis mice [99].

4.3. Interleukine (IL)-27 and IL-35. IL-27 and IL-35 are the dominant anti-inflammatory cytokines. IL-27, a member of the IL-12 family, is a heterodimeric cytokine consisting of Epstein-Barr virus-induced gene 3 protein (EBI3) and a unique IL-27-p28 [100]. In collagen-induced mice, IL-27 reduced disease development and was associated with downregulation of *ex vivo* synthesis of IL-17 and IFN-γ [101].

IL-35, also a member of the IL-12 family, is composed of EBI3 and p35. IL-35 is thought to be specifically produced by Tregs as a novel inhibitory cytokine and is required for maximal suppressive activity [102].

4.4. Transforming Growth Factor- (TGF-) β. TGF-β has variant roles due to the influence of the environments on its effects. DCs derived from tolerized mice, especially pDCs, produced increased levels of TGF-β and decreased levels of IL-6 after stimulation with nucleosomes, which favors the development of Tregs [79, 103]. TGF-β is unique among cytokines, because it can induce Foxp3 expression and aTregs differentiation in the absence of DCs [104].

4.5. Follicular Helper CD4 T (T_{FH}) Cells. T_{FH} cells are important in the regulation of CD4 T cell lineage to Th1, Th2, Tregs, or Th17 cells [105]. T_{FH} cells expressed transcription factor Bcl6, which is a master regulator of T_{FH} differentiation. Bcl6 can antagonize transcription factors important for the differentiation of CD4 T cells to Th1, Th2, or Th17 cells. Bcl6 inhibits Th1 differentiation by binding to the *T-bet* gene, Th2 differentiation by inhibiting GATA3 protein and Th17 differentiation by inhibiting RORγt activity and the human RORγt promoter. Antagonism of Tregs by Bcl6 has not been reported, but gut Tregs lose Foxp3 and differentiate into Bcl6+ T_{FH} cells under inflammatory conditions [105]. T_{FH} cells seem to play important roles in common autoimmune diseases such as RA and SLE [106, 107]. T_{FH} cell pathway and effector molecules are potent therapeutic target candidates in SAIRDs.

4.6. B Cells. Natural autoreactive B cells specific for unmodified parts of self-antigens are normally present. In SLE patients, this autoreactive B cell fraction is probably larger than in healthy individuals [108] and a large proportion of B cells express TLR9, which correlates with high titers of anti-DNA autoantibodies [109]. In murine B cells, TLR9-MyD88-dependent signaling is critical for the class switch to pathogenic IgG antibodies [110]. Thus, at least in B cells, TLR9 with the BCR signaling appear to activate B cells and promote autoimmunity.

4.7. Skin Dendritic Cells (Skin DCs). Psoriasis is a common chronic inflammatory disease of the skin in which the local activation of autoimmune DCs and T cells induces an abnormal differentiation of epidermal keratinocytes [111, 112]. TNF-α and inducible nitric oxide synthase (iNOS) producing dermal CD11c+ DCs, so called TIP-DCs, are thought to be the human equivalent of a similar DC subset necessary for the clearance of some pathogens in mice [113]. In addition, also pDCs are increased in psoriatic skin compared with normal skin [114]. Infiltration of pDCs into psoriatic skin and their activation to produce type I IFN represent key upstream events that initiate the activation of autoimmune T cells, leading to the formation of the typical skin lesions [115].

LL37, an endogenous antimicrobial peptide, which is overexpressed in psoriatic skin, seems to be the key mediator of pDCs activation in psoriasis. LL37 breaks innate self-tolerance by forming a complex with self-DNA that is delivered to and retained within early endocytic compartments of pDCs to trigger TLR9 to induce type I IFN production, which then activates IFN signature [116]. LL 37 also binds self-RNA forming a complex which stimulates pDCs through TLR7 [117]. Self-RNA-LL37 complexes also interact with TLR8 on cDCs and promote their differentiation into mature DCs, which secrete TNF-α and IL-6 [117]. LL37 is released during skin injury, breaks innate tolerance to self-DNA and self-RNA so that they can act as "danger signals" that potently activate innate antiviral-like immune responses through activation of endosomal TLRs in DCs [116, 117].

Recently, Vit. D3-activated epidermal Langerhans cell have been shown to induce the development of either TGF-β-dependent Foxp3+ Treg or IL-10-dependent IL-10+ Treg. This may be the mechanism via which Vit. D3 exerts its immunosuppressive function in inflammatory skin diseases [118]. In addition, Vit. D3 is thought to exert immunoregulation of DCs and T cells via the upregulation of CTLA-4.

5. Potential Treatment Options Based on the Modulation of Immune-Regulatory Processes (Table 1)

5.1. Glucocorticosteroids-Induced Immune Regulation. Glucocorticosteroids were the first immunosuppressants, which were used in the clinic. The inhibitory effect of glucocorticosteroids on the canonical NF-κB pathway probably plays a key role in the generation of mature DCs and cytotoxic T cells [119].

Nonspecific immunosuppressive drugs such as glucocorticosteroids have numerous adverse effects and their use is sometimes limited by a lack of efficacy [120]. Specific interference in the production of cytokines, intracellular signaling pathway, autologous stem-cell transplantation, gene therapy, immune regulation-induced antigen therapy

Table 1: Potential treatment options for SAIRDs therapy.

	Immunogenicity	Immunoregultion and tolerance
Cytokine and receptor	TNF, TNFR	
	IL-1	IL-10?
	IL-2	IL-27
	IL-6, IL-6R	IL-35
	IL-12	
	IL-15	
	IL-17	
	IL-18	
	IL-21	
	IL-23	
	Type I IFN	TGF-β
	IFN-γ	
	BAFF	
	CD20	
	CD22	
	TCR (vaccination)	CTLA4-Ig
Intracellular signaling pathway	JAK-1	
	JAK-2	
	JAK-3	
	SyK	
Stem cells and immune regulation		Autologous stem cell transplantation
Gene therapy		TNFR: Fc
Immune regulation-induced antigen		HSPs?
DC therapy		Tolerogenic DC (DC vaccination)

TNF: tumor necrosis factor, TNFR: tumor necrosis factor receptor, IL: interleukine, R: receptor, IFN: interferon, TGF: transforming growth factor, BAFF: B-cell activating factor, CTLA: cytotoxic T-lymphocyte antigen, TCR: T cell receptor, JAK: Janus kinase, SyK: spleen tyrosine kinase, TNFR: Fc: soluble form of the tumor necrosis factor receptor, HSPs: heat shock proteins, and DC: dendritic cell.

and DC vaccination are promising treatment targets for induction of SAIRDs at present.

5.2. Anticytokine Therapy. Therapies with biological agents to attain anti-inflammatory and immunosuppressive actions, using drugs such as anticytokines and cytokine receptors, have remarkably improved the results of the treatments for many SAIRDs patients [5, 12]. Successful identification of biological targets and their therapeutic translation (anti-TNF, anti-IL-6 receptor, anti-CD20, CTLA4-Ig, and anti-IL-1) will help many patients refractory to conventional intervention for RA, PsA, Crohn's disease, SLE, and so on [121]. Concentrations of proinflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-12, IL-15, IL-18, type I IFN, IFN-γ, and B cell stimulating agents, are increased in SAIRDs. They play important roles in the inflammatory processes that lead to tissue and organ damage [122].

5.3. Tumor Necrosis Factor (TNF). TNF-α stimulates migration of mature cDCs to the draining lymph nodes. TNF-α blockade can prevent this influx of antigen-presenting DCs to secondary lymphatic tissue, which is an important step for the activation of T cell responses [123]. The TNF blockers have been successfully used in the management of RA, PsA, and Crohn's disease; however, they can in some cases induce autoantibodies and lupus-like syndromes. Thus, their use in SLE is controversial [122, 124–126]. In SLE, the use of anti-TNF blockers has been associated with polyarthritis, cutaneous manifestations, disease activity, proteinuria, and nephritis but also severe infusion reactions [127, 128]. In SjS, anti-TNF blockers have not shown any clinical efficacy [129].

5.4. Interleukine- (IL-)1 and IL-18. Blockade of IL-1 and IL-18 has raised interest in human SLE. The results of two

small, open-label trials of the recombinant human IL-1 receptor antagonist anakinra in SLE have been published, both of which reported beneficial effects [7, 130]. In RA, the effectiveness of IL-1 receptor antagonist therapy is clearly lower than that of the TNF blockers [131]. IL-18 acts as a chemoattractant stimulating the migration of pDCs to the glomeruli in the kidney [78] so its blockade might be a good therapeutic target.

5.5. Interleukine (IL)-6. IL-6 is a pleiotropic cytokine that is overexpressed in patients with several SAIRDs. In contrast to other cytokines, IL-6 can bind to a soluble IL-6 receptor (sIL-6R) without being inhibited. On the contrary, this IL-6/sIL-6R complex can bind to IL-6R-negative cells via a nonligand binding but signal transducing pg130 component of the receptor complex. Therefore, antibodies against IL-6R inhibit such IL-6 actions. Accordingly, good clinical results were reported on the use of a humanized antibody against IL-6R, tocilizumab, in RA and juvenile idiopathic arthritis (JIA) [132]. An open-label study conducted using tocilizumab in SLE reported moderate effectiveness [133]. Some phase II trials of IL-6 blocking monoclonal antibodies are ongoing in SLE [134].

5.6. Interleukine (IL)-10. Disease activity in many SAIRDs has been considered to be driven by an imbalance between proinflammatory and anti-inflammatory cytokines. IL-10 is a powerful anti-inflammatory cytokine, which can be produced by both leukocytes and structural cells within tissues, being produced in particular by Tregs *in vivo* [20, 135]. However, high serum levels of IL-10 have been reported in patients with SLE and they correlated positively with the disease activity [136]. Therefore, somewhat paradoxically, in an open-label pilot study, a single injection of a mouse anti-IL-10 monoclonal antibody, given to a small group of active SLE patients, seemed to have beneficial clinical effects [137]. It was concluded that it was beneficial to boost the cell-mediated immune responses in SLE by inhibiting IL-10, because IL-10 impairs antigen presentation and Th1 lymphocyte activation.

5.7. Interleukine- (IL-) 17 and Others. IL-17 (or actually IL-17A) has been discovered to be a powerful proinflammatory cytokine and the recent detection of a Th17 T-helper cell subset that secretes it has focused attention on the role of IL-17 and Th17 cells in SAIRDs, in particular RA, PsA, multiple sclerosis and Crohn's disease, which are considered to represent Th17-related diseases. Several therapeutically interesting compounds have been reported, including anti-IL-17A agent (AIN 457 and LU2439821), anti-IL-17 receptor (AMG827) or anti-IL-17A/F receptor [92, 93, 138].

IL-12 and IL-23, which belongs to the IL-12 family, are important regulators of Th17 lymphocytes and dominant candidates for the treatment of SAIRDs with IL-17 involvement [139–141]. Anti-IL-12/23p40 agents (CNTO1275: Ustekinumab and ABT-874) are directed against both IL-12 and IL-23. An ustekinumab study is ongoing in phase III in PsA [142]. In addition, IL-21 and IL-22 are attractive therapeutic targets as cytokines related to Th17 cells [7, 122, 143]. IL-27 and IL-35 also belong to the IL-12 family and may be candidate tools to inhibit Th17 cells in the future [101, 102].

5.8. B Cell Related Cytokine. B-cell activating factor (BAFF, also known as B-lymphocyte stimulator (BLyS) and TNF ligand superfamily member 13B) promotes B-cell survival and autoantibody production. BAFF blocker (Belimumab) seems to be close to the public authority approval as they have been completed in large, double-blind, placebo-controlled studies of patients with active SLE in a phase III study [7, 144], completed in phase II study in RA [145]. Anti-CD22 monoclonal antibody (Epratuzumab) has finished a phase III trial in SLE [146].

Recent experimental and clinical evidence obtained in SSc-like mouse models and SSc patients suggests a role for B cells in the development of inflammation and fibrosis characteristic for this disease [147]. Antihuman CD20 antibody, that has been reported to be effective in the treatment of RA (Rituximab and Ofatumumab in phase III), had some beneficial effects also on skin fibrosis and lung involvement in SSc patients [148, 149]. In SjS patients, a phase II trial of Rituximab has been finished and Belimumab prepared [150, 151]. BAFF blocker as well as anti-CD20 blocker is one of potential drugs in the treatment of SAIRDs.

5.9. Intracellular Signaling Pathway. Among various signaling molecules activated by cytokine-receptor interaction, the small molecules targeting in particular Janus kinase-(JAK-) STAT pathway form attractive candidate drugs in the treatment of SAIRDs. Inhibitors of various JAK molecules (JAK-1/2, JAK-2, JAK-3) might be useful in the treatment of SAIRDs [5, 152, 153]. Some trials of JAK-3 inhibitor in patients with RA (CP690,550 in phase III), PsA and inflammatory bowel diseases have already been published [153–156]. JAK-3 is critical for signal transduction for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, and a selective inhibition of JAK-3 has immunomodulatory effects, which affect T cells, B cells, macrophages, and NK cells, without significantly affecting other organ systems. JAK inhibitors may find clinical applications in SAIRDs in the near future.

Spleen tyrosine kinase (SyK), which is another nonreceptor tyrosine kinase, could to be one of treatment targets in SAIRDs. A phase III trial of SyK inhibitor (R788) in RA (OSKIRA trial) is ongoing [157].

In addition to monoclonal antibodies and receptor fusion proteins, other alternative approaches to neutralize inflammatory cytokines are being developed in experimental models. These include vaccinations against cytokines, which have been found to be important in the disease mechanisms [158] and administration of small interfering RNAs (siRNAs) that target the messenger RNAs encoding key cytokines [3]. Another approach is to use small molecules to block certain cytokine pathways. For example, T-cell receptor (TCR) vaccinations and epitope-mimetic peptides (e.g., A9 in collagen-induced arthritis) are also in experimental use [159, 160].

5.10. Stem Cells and Immune Regulation. At first, most of the experience with autologous stem-cell transplantation in arthritis derived from studies with SSc, multiple sclerosis, and systemic JIA that were refractory to conventional therapies. Recently, also patients of RA and SLE have been treated with autologous hematopoietic stem cells, but their effect was limited in RA. Phase II trials of autologous stem-cell transplantation are ongoing in SLE and SSc [161]. In this procedure, autologous hematopoietic stem cells are collected and stored but transferred back to the donor after the remaining bone marrow and blood cells have been destroyed. The autotransplant then reconstitutes the hematopoietic and immune system and is hoped to restore the self-tolerance. Indeed, a small study reported that Tregs were restored by transplantation of autologous hematopoietic stem cells [162]. Autologous stem-cell transplantation therapy has potential in the treatment of also other SAIRDs in the future.

5.11. Gene Therapy. Some small clinical trials of the gene therapy have been done in RA *in vivo* and *ex vivo* [163]. Local viral gene therapies by tgACC 94, which is a recombinant adeno-associated virus serotype 2 vector genetically engineered to contain the cDNA for a human TNFR-immunoglobulin (IgG1) Fc fusion gene, are going on phase I in RA and phase II in PsA [164]. However, several reasons such as the limitation of efficacy and logistic and financial issues have deterred to step up the next trial phases. Technically insertions, alterations, or removals of genes can be done, but gene therapy is difficult to apply in polygenic diseases. Generation of an appropriate gene construct is already challenging but gene transfer and host immune responses against the gene therapy vectors form additional barriers to clinical applications. Due to the haphazard and random location of the gene in the host genome, insertional mutagenesis, and cancer development pose a potential threat. Further studies are needed for the gene therapy with new techniques such as siRNA [163, 165].

5.12. Immune Regulation Induced by Antigens. Secondary antigens (T-cell epitopes) that are involved in the amplification and maintenance of immune-inflammation, but independent of the initial and evasive "factor X" triggering the SAIRDs, might have therapeutic potential. Heat shock proteins (HSPs) are often targeted by proinflammatory T-cell responses in arthritis and induction of mucosal tolerance induced against their proinflammatory, immunodominant epitopes could provide a means to alleviate autoimmune inflammation in RA [166]. The dnaJP protein, which encodes a 15mer peptide (dnaJP1) derived from the bacterial HSP, was administered to induce immune tolerance in RA patients in a small trial study [167].

5.13. DC Therapy. Maintenance of the immature, immunoincompetent state of DCs, especially after *in vivo* delivery, remains a challenging but also promising task. For progress in this field, the methods for generation and delivery of tolerogenic or vaccinated DCs have first to be standardized.

Recently, chronic stimulation of pDCs with self-nucleosomes through TLR7 and TLR9 to produce type I IFN was reported to reduce therapeutic effects of glucocorticosteroids in SLE [90, 168]. *Vice versa*, inhibition of TLR7 improved disease manifestations in a lupus mouse model but not TLR9 [90, 91, 95].

The endogenous proapoptotic agents and the death receptors involved in the maturation of DCs and activation of specific T cell subsets, such as Th 17 cells, may become novel targets for the individualized, disease process-specific treatments of SAIRDs. Human monocyte-derived tolerogenic DCs, generated with dexamethasone and Vit. D3, maintained their tolerogenic function upon activation with lipopolysaccharide (LPS-tolerogenic DCs), while acquiring the ability to present human type II collagen (autoantigen) and migrate in response to CCR 7 ligand, CCL 19 [169, 170]. Vaccination with autologous tolerogenic DCs (RHEUMAVAX) is in preliminary phase I human trial [171].

6. Conclusion

The pathogenesis and mechanism of SAIRDs are not fully understood. However, various molecules, signal transduction, and immune effector pathways able to regulate immune regulation and tolerance are being increasingly revealed. For the generation of new drugs and therapies, it is important to know molecules and understand mechanisms responsible for the maintenance, failure, and restoration of tolerance in SAIRDs. Molecularly targeted and highly specific biologic agents have caused a paradigm shift of the treatment. However, in spite of top-of-the-line drugs applied according to current management strategies, some 20%–30% the patients do not respond adequately. Further, there are still several issues to be resolved, also with the currently used drugs, such as severe adverse events and the high cost although small chemical molecules, such as JAK inhibitors, might offer low cost options possible to administer easily per os. It is expected that the development leads to new drugs which are able to re-establish autoimmune regulation and tolerance in SAIRDs, drugs, which are more effective and tailored based on genetic polymorphism and at the same time safer, low cost, and easy to administer.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Enhanced HMGB1 Expression May Contribute to Th17 Cells Activation in Rheumatoid Arthritis

Yan Shi,[1,2] Siamak Sandoghchian Shotorbani,[1] Zhaoliang Su,[1] Yanfang Liu,[1] Jia Tong,[1] Dong Zheng,[1] Jianguo Chen,[3] Yingzhao Liu,[1,3] Yan Xu,[1] Zhijun Jiao,[1] Shengjun Wang,[1] Liwei Lu,[3] Xinxiang Huang,[1] and Huaxi Xu[1]

[1] *Department of Immunology, Institute of Laboratory Medicine, Jiangsu University, Xuefu Road 301, Zhenjiang 212013, China*
[2] *Suzhou Municipal Hospital, Suzhou 215002, China*
[3] *The Affiliated People's Hospital of Jiangsu University, Zhenjiang 212002, China*

Correspondence should be addressed to Zhaoliang Su, szl30@yeah.net and Huaxi Xu, xuhx@ujs.edu.cn

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Rheumatoid arthritis(RA) is a common autoimmune disease associated with Th17 cells, but what about the effect of high-mobility group box chromosomal protein 1 (HMGB1) and the relationship between Th17-associated factors and HMGB1 in RA remains unknown. In the present study, we investigated the mRNA levels of HMGB1, RORγt, and IL-17 in peripheral blood mononuclear cells (PBMCs) from patients with rheumatoid arthritis by quantitative real-time PCR (RT-qPCR), and the concentrations of HMGB1, IL-17, and IL-23 in plasma were detected by ELISA. And then, the effect of HMGB1 on Th17 cells differentiation was analyzed *in vitro.* Our clinical studies showed that the mRNAs of HMGB1, RORγt, and IL-17 in patients were higher than that in health control ($P < 0.05$), especially in active RA patients ($P < 0.05$). The plasma HMGB1, IL-17, and IL-23 in RA patients were also higher than that in health control ($P < 0.05$); there was a positive correlation between the expression levels of HMGB1 and the amount of CRP, ERS, and RF in plasma. *In vitro*, the IL-17-produced $CD4^+$T cells were increased with 100 ng/mL rHMGB1 for 12h, which indicated that the increased HMGB1 might contribute to Th17 cells activation in RA patients.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation in the small joints leading to the destruction of articular cartilage and bone. TNF-α, IL-1, and IL-17 as well as T, B lymphocytes and macrophages are implicated in the pathogenesis of RA [1–3]. Recently, high-mobility group box chromosomal protein 1 (HMGB1), a nonhistone nuclear DNA-binding protein, is proved to be a potent proinflammatory mediator in rheumatoid arthritis [4–7]. Increased HMGB1 was found in the joints of RA patients [8–10], and the HMGB1 transferred into health mouse joint could induce the arthritis [11]. HMGB1 is secreted or released from lymphocytes, dead and/or apoptosis cells [12–15]. Previous studies have showed that HMGB1 in milieu could contribute proinflammatory such as IL-6, IL-1β, and IL-10 secretion by macrophages, sustain inflammation [15]. It is clear that IL-6 and IL-1β can prime the naïve $CD4^+$T cells differentiation into Th17 cells [16].

Whether HMGB1 involved in the pathogenesis of RA by promoting the Th17 cells activation was unclear. In the present study, we examined the expression levels of HMGB1 and Th17-associated factors in RA patients, analyzed the relationship between them, and explored the potentiality of HMGB1 in Th17 differentiation *in vitro.*

2. Patients and Methods

2.1. Patients. 80 patients with RA enrolled in the affiliated hospital of Jangsu University were included in this study from January 2008 to September 2009. Among 80 patients, 59 females and 21 males, ranged from 36 to 80 years old. 48 patients were in active phase, and 32 patients were in inactive phase. Diagnoses were established according to the American College of Rheumatology (ACR) criteria [17] and

the disease activity score calculated for 28 joints (DAS28). 48 patients in active phase untreament during the past 2 years which did not accompany other chronic diseases; all the 48 patients included 8 males and 40 females, the age was 43 ± 10 years, the disease duration was 10.3 ± 4.5 months, and the range of DAS28 was 4.21–6.32 (the mean was 5.60 ± 0.78); the RA patients in inactive phase were 32 cases, 6 males and 26 females, the age was 49 ± 13 years, the course of disease was 40.1 ± 25.7 months, and the DAS28 range was 1.92 to 0.67 (the average was 1.75 ± 0.23). 50 healthy volunteers, 38 females and 12 males ranged from 28 to 41 years old, acted as control. This study was approved by the ethical committee of the Affiliated Hospital of Jiangsu University. All individuals were informed consensus.

2.2. Reagent. rHMGB1 was expressed in *Escherichia coli* (*E.coli*) and purified by Ni-column. The control protein eGFP was produced from *E.coli* and purified by the same method.

2.3. Blood Samples. Peripheral blood samples were collected from healthy volunteers and patients. The collection tubes contained 0.2 mL sodium heparin. The blood samples were centrifugalized at 1000 r/min 4°C for 5 min, then the supernatant was collected and stored at −70°C for use, and sediment were separated from PBMCs by standard Ficoll-Hypaque density centrifugation. TRIzol was added to the PBMCs for total RNA.

2.4. Primers Design. According to Genbank sequences, the primers were designed by Premier 5.0 software and synthesized by Shanghai Sangon Biological Engineering Technology and Service Company. All sequences of primers were shown in Table 1.

2.5. RNA Extraction and cDNA Synthesis. Following the manufacturer's instructions, total RNA from PBMCs was extracted with Trizol (Invitrogen, USA). cDNA was synthesised with reverse transcription reagent kits (TOYOBO, Japan). All RNA samples were heated at 65°C for 10 min to denature the secondary structure with the template then put in ice for 5 min. Total RNA (1 μg) was reversely transcribed in a total volume of 20 μL, containing Oligo (dT) 1 μL, dNTP (10 mM) 2 μL, 5 × RT buffer 4 μL, ReverTraAce (100 U/μL) 1 μL, RNase Inhibitor 1 μL, DEPC free H_2O add up to 20 μL, response conditions: 42°C for 20 min; 99°C for 5 min; 4°C for 5 min. The cDNA was stored at −20°C.

2.6. Construction of Recombinant Plasmid Calibrator. PCR amplification was performed in the Thermon Hybaid System (Eppendorf, USA). The program consisted of an initial denaturation step for 5 min at 94°C followed by 30 cycles, with each cycle consisting of a 30 s denaturing step at 94°C, a 30 s annealing at 56°C and a 30 s extension at 72°C. The reaction was completed by a final 5 min extension at 72°C. Purified HMGB1, RORγt, IL-17, and β-actin PCR fragments were transformed to PMD18-T vector (Invitrogen, USA) to establish recombinant plasmids PMD18-HMGB1, RORγt, IL-17, and β-actin. All these recombinant plasmids were transformed into competent *E. coli* DH5α, transferred on a 1.5% agar Amp-resistant plate, and then cultured at 37°C for 12 ∼ 14 h. Positive clones were initially identified by sequencing. Part of positive clones were further amplified and extracted and accurately quantified with a nucleic acid-protein ultraviolet instrument. 10-fold serial dilution of the recombinant plasmid DNAs were used as calibrator and stored at −20°C until use.

2.7. RT-qPCR-Detected Objective Genes Expression. The objective genes expression (HMGB1, RORγt, and IL-17) were detected by quantitative real-time polymerase chain reaction (RT-qPCR), and all samples were calibrated by β-actin. All PCR reactions were performed using the Rotor-Gene 6000 System (Corbett Research, Australia) in a total volume of 20 μL, containing 1 μL cDNA, 10 μL 2 × sybr1 premix (Takara, China), 0.3 μL 10 μM each primer, and 8.4 μL water. The specificity of the amplification products was controlled using a melting curve analysis. The copy number of RORγt, IL-17, and β-actin transcripts in samples was calculated with the Corbett software according to corresponding standard curves. The copy number of gene/%β-actin represented the ratio of the gene. A no-template negative control was also included in each experiment, and all samples were measured in triplicate.

2.8. The Function of rHMGB1 in Th17 Differentiation In Vitro. $CD4^+$T cells from C57BL/6 mice spleen were prepared by magnetic column; 1×10^6/well cells were put into precoating 24-well plates by anti-CD3, anti-CD28, and cultured in PRIM-1640 including 10% FCS at 5% CO_2, 37°C, the cells were stimulated with different dose of rHMGB1. The eGFP and LPS from *E. coli* was used as controls. Collected cells after 0, 3, 6, 9, 12, 24, and 48 hours and supernatants were used to detect the related cytokines as previously described.

2.9. Enzyme-Linked Immunosorbent Assays (ELISAs) for IL-17 and IL-23. The levels of HMGB1, IL-17, and IL-23 in plasma or cell culture supernatants were measured by ELISAs, following the manufacturer's protocols (eBioscience, USA). All samples were measured in triplicate.

2.10. Flow Cytometry Analysis. The procedures of flow cytometry analysis were performed as described elsewhere [18]. Briefly, 1×10^6 PBMCs were stained with anti-CD3-PE-cy5 (eBioscience), anti-CD8-FITC (eBioscience), and anti-IL-17-PE (eBioscience). 1×10^6 $CD4^+$T cells from the spleen of mice were stained with anti-IL-17-FITC (eBioscience). The stained cells were applied for data acquisition on Coulter EPICS XL Cytometer (Beckman Coulter) and analyzed by software WinMDI (version 2.9).

2.11. Statistical Analysis. All statistical analysis were performed using SPSS17.0 statistical analysis software. Data are expressed as the mean ± standard deviation (SD) in text and figures. Comparisons between paired or unpaired groups were performed using the appropriate Student's t-test. For

Table 1: The primers used in this study.

Gene	Sequence(5′–3′)	Length (bp)
HMGB1	5′-GATGGGCAAAGGAGATCCTA-3′ 5′-CTTGGTCTCCCCTTTGGGGG-3′	233
RORγt	5′-CCTGGGCTCCTCGCCTGACC-3′ 5′-TCTCTCTGCCCTCAGCCTTGCC-3′	171
IL-17	5′-CAAGACTGAACACCGACTAAG-3′ 5′-TCTCCAAAGGAAGCCTGA-3′	231
β-actin	5′-CACGAAACTACCTTCAACTCC-3′ 5′-CATACTCCTGCTTGCTGATC-3′	265

nonparametric data, differences between two groups were analyzed by the Mann-Whitney test. Spearman's correlation was used to test correlation between two continuous variables. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Electrophoresis Identification the Amplicons. The amplicon length of HMGB1, RORγt, IL-17, and β-actin was 233, 171, 231, and 265 bp, respectively, and it was consistent with the expected data. The positive clone recombinant plasmid was identified by sequencing. These objective gene sequences were in accordance with Genbank seqence (detailed data not shown).

3.2. The Linear Range and Reproducibility. The detection range of recombinant plasmid DNAs was from 10 to 10^8 copies, and the coefficients of variation values ranged from 2.20% to 8.32%. Amplification efficiency ranged from 0.88 to 0.92, and $r^2 > 0.99$.

3.3. Levels of HMGB1, RORγt, and IL-17 mRNA in RA Patients. The expression levels of HMGB1, RORγt, and IL-17 mRNA from RA patients and healthy controls were measured by RT-qPCR. As shown in Figure 1, the mRNAs of Th17-associated cytokines and transcription factor were significantly increased in PBMCs from RA patients, especially in active phase of RA patients, and it was quite different from that in inactive phase of patients and healthy controls ($P < 0.05$).

3.4. The Correlations between the mRNA Levels of HMGB1 and Th-17 Cells-Related Factors. To assess the relationships between the mRNA levels of HMGB1 and Th-17 cells-related factors in RA patients. We examined the correlation between the mRNA levels of HMGB1 and Th17 cells-related factors in PBMCs of RA patients. There was a significantly positive correlation among them (Figure 2).

3.5. Increased Cytokine Concentrations in Plasma from Patients with RA. Concentrations of plasma HMGB1, IL-23, and IL-17 measured by ELISA in each group are shown in Table 2. The Th17 cell-associated cytokines were significantly increased in plasma from active phase of RA patients, but no obvious difference between inactive RA and healthy controls.

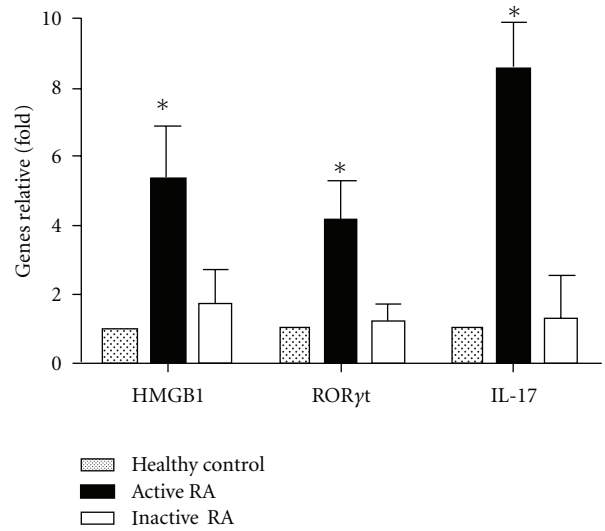

Figure 1: Expression of gene ratio by RT-qPCR. The mRNA expression level were determined by RT-qPCR, the values were expressed as the fold of the healthy control. the ratio of target genes used the healthy control as 1. $^*P < 0.05$ compared with the healthy control and inactive RA group.

The correlations analysis among HMGB1, IL-23, IL-17, and other clinical targets in the serum of active RA patients showed that there was a significantly positive correlation (Table 3).

3.6. Increased Frequencies of $CD3^+CD8^-IL\text{-}17^+$T Cells in PBMCs from RA Patients. Flow cytometry was used to assess frequencies of $CD3^+CD8^-IL17^+$T cells in PBMCs from patients and controls, and the results showed that $CD3^+CD8^-IL17^+$T cells in active phase of patients (1.36 ± 0.98%) were higher than those in controls (0.39 ± 0.16%), and the difference was statistical significance ($P < 0.05$), whereas there was no significant difference was found between inactive phase of patients (0.45 ± 0.23%) and controls (Figure 3).

3.7. The mRNA Expression Levels of Th17 Cell-Related Factors in rHMGB1-Stimulated Mice $CD4^+$T In Vitro. To further confirm the relationship between HMGB1 and Th17 cells, rHMGB1 was used to stimulate $CD4^+$T cells *in vitro* and then

Table 2: The plasma concentration of HMGB1, IL-23, and IL-17 in RA patients.

	Active RA	Inactive RA	Health control
sample	48	32	50
HMGB1 (ng/mL)	8.420 ± 1.780*	6.315 ± 0.725	5.892 ± 0.901
IL-23 (pg/mL)	203.825 ± 99.321*	148.332 ± 91.278	103.825 ± 73.427
IL-17 (pg/mL)	409.239 ± 152.324*	188.325 ± 76.143	165.672 ± 46.238

* $P < 0.05$ compared with healthy control.

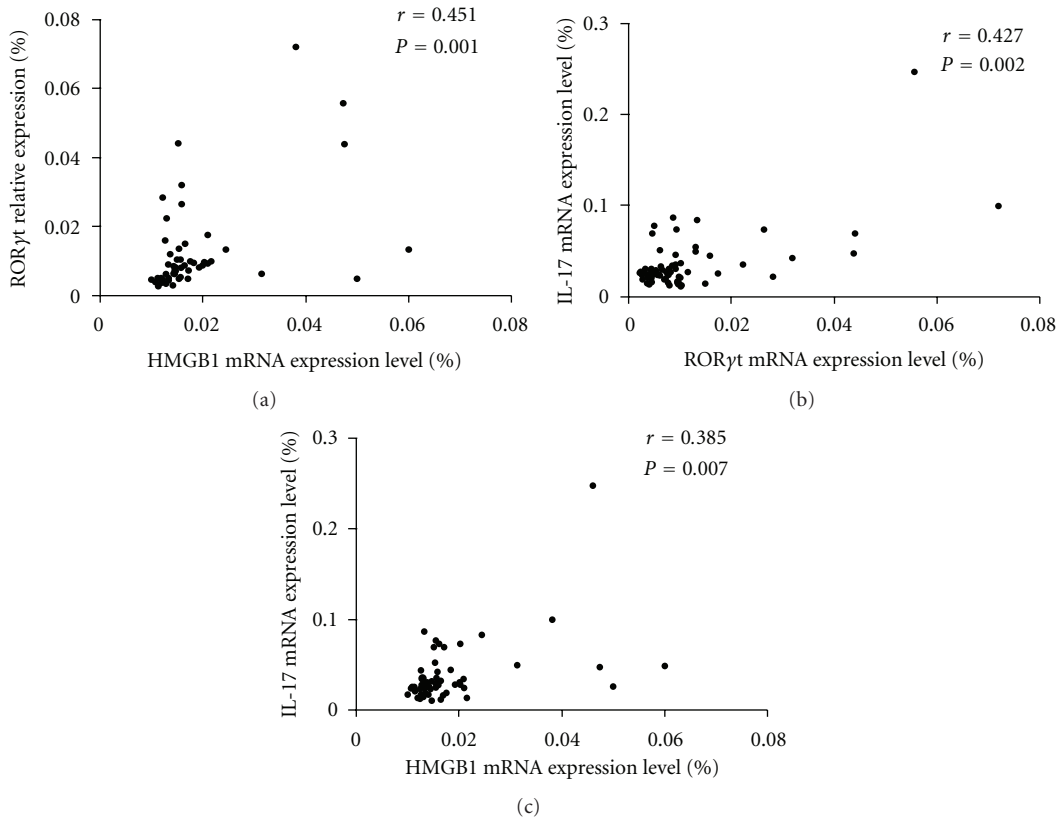

Figure 2: Correlations of IL-17 and RORγt and HMGB 1 mRNA level in RA active patients. The mRNA expression levels as determined by RT-qPCR, the values were expressed as the target genes versus β-actin mRNA expression.

to detect the mRNA levels of Th17 cell-associated factors by RT-qPCR. Our data indicated that rHMGB1 could enhance the expression levels of Th17 cell-related factors, and the levels changed with the dose and time stimulated by rHMGB1. In the 0.1–100 ng/mL rHMGB1 stimulus dose range, Th17-related factors expression was a dose dependence, 100 ng/mL was the best concentration. Cells were collected at the different points after rHMGB1 stimulation, and Th17 cell-related factors was up to the peak at 12 h (Figure 4).

3.8. $CD4^+IL\text{-}17^+T$ Cells Ratio Was Increased under the rHMGB1 Stimulation. Flow cytometry analysis showed that the ratio of IL-17-producing cells (Th17) was up to the maximum (1.50 ± 0.43%), while 100 ng/mL of HMGB1 was used for 12 h, it shown significant difference compared with other groups ($P < 0.05$) (Figure 5).

4. Discussion

Th17 cells and their specific transcription factor or related cytokines are being recognized as important mediators in inflammatory and autoimmune diseases including RA, but relatively little is known about HMGB1 roles and the relationship between Th17 and HMGB1 in RA. In the present study, we found that in RA patients, the mRNAs of HMGB1, RORγt, and IL-17 in PBMCs and the levels of HMGB1, IL-17, and IL-23 in plasma were increased, and there was a positive correlation between HMGB1- and Th17-cell, especially in active phase of RA. Furthering analysis showed that HMGB1 and Th17 related factors also had the positive correlation with other RA clinical related detections. To study the relationship of HMGB1 and Th17 cell, we explored the function of HMGB1 on $CD4^+$T cells *in vitro* and observed that rHMGB1 could enhance the ratio of Th17 cell.

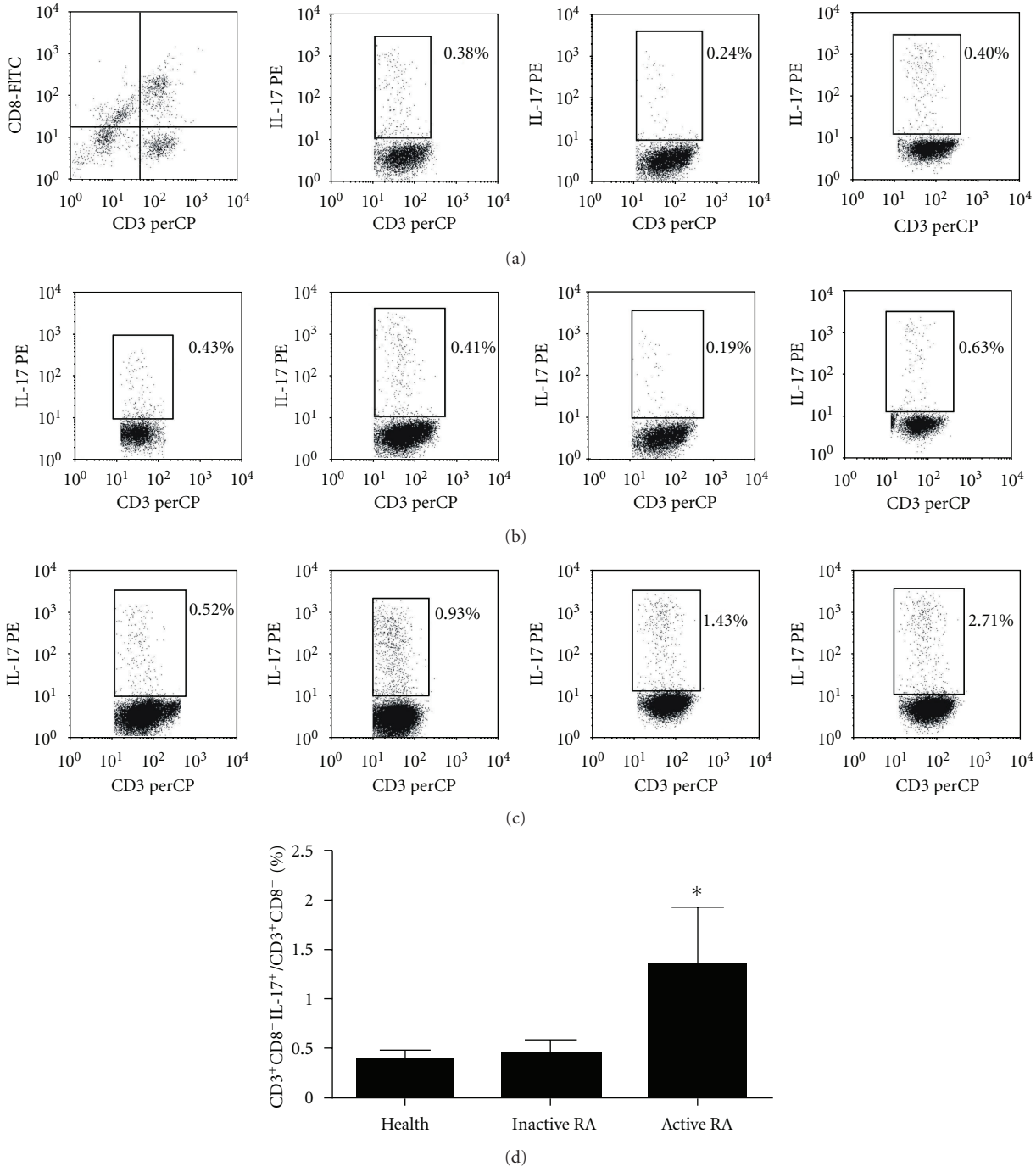

Figure 3: FACS analyzed the $CD3^+CD8^-IL\text{-}17^+$ cell ratio in RA patient The PBMCs were isolated by standard Ficoll-Hypaque density centrifugation. The cells were stained by anti-CD3-PE-cy5, anti-CD8-FITC, and anti-IL-17-PE. (a) First figure presented $CD3^+CD8^-$T cells were considered $CD4^+$T cells in region RL, and the other three were presented healthy controls. (b) Representative IL-17 expression in $CD3^+CD8^-$T subsets from RA patients in inactive phase. (c) Representative IL-17 expression in $CD3^+CD8^-$T subsets from RA patients in active phase. (d) The results were shown as means ± SD. $^*P < 0.05$ compared with the control group.

Neither the same source protein nor the endotoxin LPS had the function. We revealed that HMGB1-triggered the RA may via the Th17 pathway in RA pathogenesis.

There are two pathway of HMGB1 transit from intracellular to extracellular, one is secreted by activated innate immune cells, the other is released by the death or apoptosis cells [13–16]. HMGB1 in milieu was involved in the innate and adaptive immune system [7]. Previous data showed that HMGB1 could prime the naïve $CD4^+$ T lymphocytes toward T helper 1 phenotype. Increasing evidence indicated

Table 3: Correlations of HMGB1, IL-23, IL-17, and clinical index in the serum of active RA patients.

	HMGB1 (pg/mL)		IL-23 (pg/mL)		IL-17 (pg/mL)	
	r	*P*	*r*	*P*	*r*	*P*
Age	0.122	0.410	0.065	0.660	0.007	0.965
CRP (mg/dL)	0.894	0.000#	0.405	0.004#	0.454	0.001#
ESC (mm/h)	0.817	0.000#	0.328	0.02*	0.371	0.009#
RF (IU/mL)	0.707	0.000#	0.325	0.024*	0.370	0.010#

*$P < 0.05$, #$P < 0.01$ compared with healthy control.

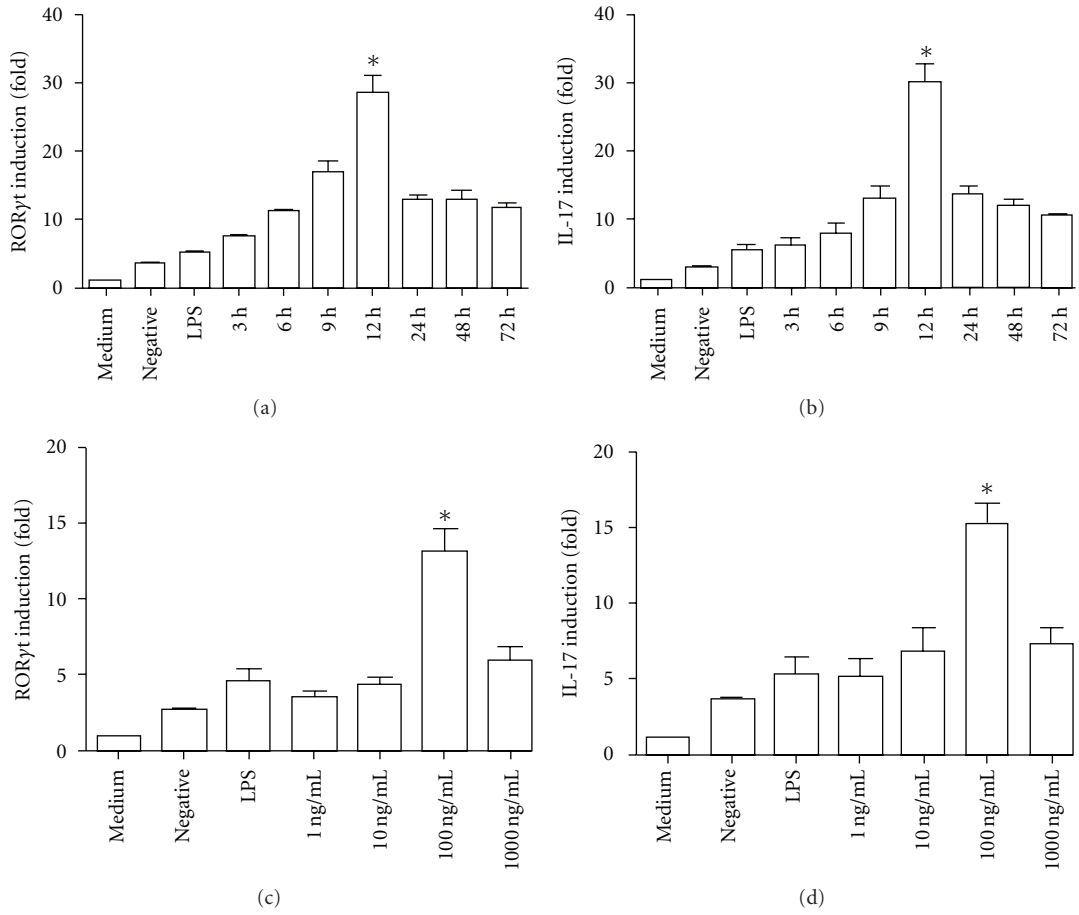

Figure 4: Th17 cell-related factors expression stimulated by HMGB 1 *in vitro*. Th17 cell-related factors expression by HMGB 1 stimulus *in vitro* were detected by RT-qPCR. The $CD4^+$T cells were isolated from mouse spleen, preactivated by anti-CD3 and anti-CD28, and then added to rHMGB 1. (a) RORγt mRNA expression levels after rHMGB1 stimulating in different time; (b) IL-17 mRNA expression leves after rHMGB 1 stimulus; (c) RORγt mRNA expression levels after rHMGB 1 stimulus; (d) IL-17 mRNA expression levels after $CD4^+$T cells stimulated by rHMGB 1 stimulus. RT-qPCR analysis for target genes versus β-actin mRNA expression, the ratios of target genes used the control as 1. Data from 3 independent experiments were presented as means ± s.d. *$P < 0.05$ versus control.

that HMGB1 acts as an early inflammatory mediator in the pathogenesis of arthritis [11, 19].

Th17 cells and their effector cytokines are being recognized as important factors in organ-specific autoimmune diseases, especially which were thought mediating by Th1 cells before [20–25]. Th17 cells have emerged as critical effector cells in EAE pathogenesis [20, 21]. HMGB1 is a potent inducer of several proinflammatory cytokines, such as IL-1β and IL-6, which were considered as crucial mediators in inducing of Th17 cells [26]. Recently, Liu reported that HMGB1 can induce IL-23 through TLR4 pathway and IL-23 can enhance the IL-17 levels [27]. Philippa indicates that IL-23/IL-17 axis exist in the pathogenesis of RA [28]. HMGB1 also played important roles in other autoimmune diseases as well as in acute allograft rejection [29–32].

In the present study, we not only confirmed the previous results, but also indicated that the HMGB1 involved in pathogenesis of RA. There is a positive correlations

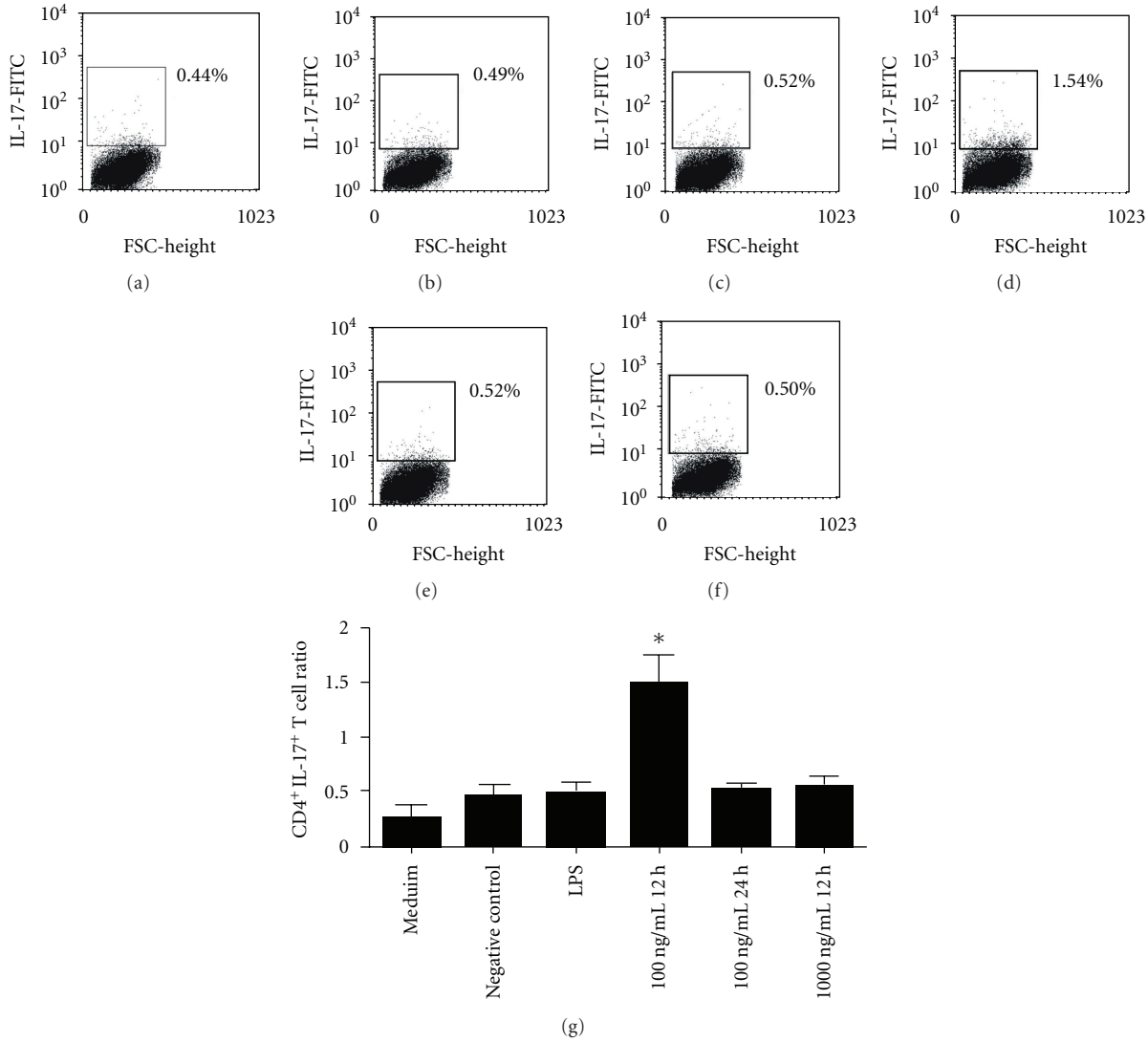

Figure 5: The number of IL-17-expressed $CD4^+$T cell stimulated by HMGB 1. The conditions were designed as before. The cells were collected; before 6h, 1 μL monosion, 5 μL 10 ng/mL PMA, and 1 μL 1 Mm/mL inon were added. (a) Presents medium control, stimulated for 12 h; (b) Presents independence protein, stimulated for 12 h; (c) Presents LPS, stimulated for 12 h; (d) Presents 100 ng/mL HMGB 1 stimulated for 12 h; (e) Presents 100 ng/mL HMGB 1 stimulated for 24 h; (f) Presents 1000 ng/mL HMGB 1 stimulated for 12 h.

between HMGB1 and Th17 or other clinical index. Our data from FACS also showed that HMGB1 might upregulate $CD3^+CD8^-$IL-17^+T cells in RA patients, and also in our *in vitro* study, we observed that HMGB1 directly acted on $CD4^+$ T cells to enhance IL-17 production following activation by CD3 and CD28 mAbs, which was consistent with our recent report [33]. In brief, our data provide a strong association between increased Th17 activity and HMGB1 in RA, and HMGB1 may upregulate Th17 cells *in vivo* or *in vitro*, which opens a new avenue in the studies of RA immunotherapy and pathogenesis.

Acknowledgments

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Review Article

CD154: An Immunoinflammatory Mediator in Systemic Lupus Erythematosus and Rheumatoid Arthritis

Nada Alaaeddine,[1] Ghada S. Hassan,[2] Daniel Yacoub,[2] and Walid Mourad[2]

[1] *Department of Pathology, Faculty of Medicine, P.O. Box 11-5076, St Joseph University, Beirut, Lebanon*
[2] *Laboratoire d'Immunologie Cellulaire et Moléculaire, Centre Hospitalier de l'Université de Montréal, Hôpital St-Luc, Montréal, QC, Canada H2X 1P1*

Correspondence should be addressed to Walid Mourad, mw.mourad@umontreal.ca

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Systemic lupus erythematosus and rheumatoid arthritis are two major chronic inflammatory autoimmune diseases with significant prevalence rates among the population. Although the etiology of these diseases remains unresolved, several evidences support the key role of CD154/CD40 interactions in initiating and/or propagating these diseases. The discovery of new receptors (αIIbβ3, α5β1, and αMβ2) for CD154 has expanded our understanding about the precise role of this critical immune mediator in the physiopathology of chronic inflammatory autoimmune diseases in general, and in systemic lupus erythematosus and rheumatoid arthritis in particular. This paper presents an overview of the interaction of CD154 with its various receptors and outlines its role in the pathogenesis of systemic lupus erythematosus and rheumatoid arthritis. Moreover, the potential usefulness of various CD154-interfering agents in the treatment and prevention of these diseases is also discussed.

1. Introduction

Over the last decade, considerable new insights into the pathogenesis of systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) have been provided. SLE involves the production of autoantibodies against many self antigens, and the formation of immune complexes affecting many tissues and organs. RA, on the other hand, mainly implicates joints and articulations, where chronic inflammation of the synovial tissues leads to destructive tissue damage. Mechanistically, interactions between the extracellular matrix, cell surface receptors, and soluble mediators are at the center of the inflammatory response that takes place in these autoimmune diseases. Indeed, most of the new biological therapies currently in use or in clinical development are directed at membrane associated targets or their ligands. In this matter, CD154, in its membrane-bound or soluble form, was shown to be an important modulator of immunoinflammatory events in autoimmune diseases [1]. Here we review the major and most recent findings on the role of CD154 in various inflammatory autoimmune diseases, while focusing in particular on SLE and RA. Accumulating knowledge will allow us to better understand the CD154 axis in disease states, thereby, facilitating the design of new preclinical approaches and more efficient therapies for the treatment of CD154-associated pathologies.

2. CD154

CD154, also known as CD40 ligand (CD40L), previously referred to as gp39, TRAP, or TBAM [2–4], is a 39 kDa type II membrane glycoprotein of the TNF family [5]. Located on the long arm of the X chromosome, region q26.3–q27.1, the human CD154 gene is composed of five exons and four introns. It encodes a polypeptide of 261 amino acids (aa), consisting of a 215 aa extracellular domain, a 24 aa transmembrane region, and a 22 aa cytoplasmic tail [6–9]. Like other members of the TNF-family, CD154 forms a trimeric structure and promotes as such trimerization of the receptor, namely, CD40 [10]. The CD154/CD40 interaction is stabilized by charged residues, namely, the basic chains on CD154 and the acidic ones on CD40 [11, 12].

It has been reported that the CD154/CD40 interaction is required for the proteolysis of membrane-bound CD154 and the subsequent release of soluble CD154 (sCD154) by activated platelets [13]. Soluble CD154 is an 18 kDa fragment comprised of residues 113–261 of the membrane-bound CD154 molecule and remains a functional trimer retaining its ability to bind receptors. Indeed, sCD154 shares similar activities with the membrane bound form and has been shown to be associated with many autoimmune diseases [14]. The CD154 homotrimer is nonconstitutively expressed on different cell types, including activated T lymphocytes, basophils, eosinophils, monocytes, macrophages, natural killer cells, B lymphocytes, platelets, dendritic cells, as well as endothelial, smooth muscle, and epithelial cells [14].

Accumulating evidence now indicates that CD154 can bind to receptors other than CD40, namely, the integrins αIIbβ3, α5β1, and αMβ2 [15–19].

3. CD154 Receptors

3.1. CD40. CD40 is a 48 kDa type I membrane glycoprotein and member of the tumor necrosis factor receptor (TNFR) super family [5]. The human CD40 gene was localized to the long arm of chromosome 20 along 2q12–q13.2. [20], and Tone et al. have showed that it contains 9 exons [21]. Transcription of the gene yields a phosphoprotein of 277 amino acids composed of a signal peptide, an extracellular domain including 22 cysteine residues implicated in its ligand binding, two potential N-linked glycosylation sites, a transmembrane region central for its translocation and clustering into lipid rafts microdomains, and a cytoplasmic region [11, 22–25].

The precise structure by which CD40 is found on the cell surface is still a matter of controversy. Despite the initial suggestion that CD40 exists as a dimer that is being trimerized upon CD154 ligation, more recent studies describe CD40 as a constitutively preassembled trimer [26]. Moreover, we have shown that upon CD154 ligation, CD40 can oligomerize into dimers between cysteine residues 238 located in the intracellular region of the molecule. Interestingly, this dimerization appears essential for phosphoinositide-3 kinase (PI-3K) activation and the subsequent activation of B7.2, as well as the production of IL-8 in B cells. Recently, the CD154/CD40 complex has been described as a trimeric CD154 molecule interacting with a CD40 dimer, as the third CD40 molecule is pushed out of the complex by charged residues [12]. Hence, additional studies are required for further clarification of the exact structure of CD40 on the cell surface.

CD40 is constitutively expressed on a wide variety of cells including B cells, dendritic cells, macrophages, endothelial cells, fibroblasts, platelets, osteoblasts, smooth muscle cells, neurons, pancreatic beta cells, and ductal cells. CD40 was also shown to be induced by various cytokines such as TNF-α and interleukin-1β (IL-1β) in a variety of cell types [9, 27]. Upon its engagement, CD40 induces a pattern of gene expression depending on the particular cell type involved. On B cells, it is critical for survival and proliferation, isotype switching, germinal center formation, memory generation, and production of numerous cytokines and chemokines such as IL-1, IL-6, IL-8, IL-10, IL-12, TNF-α, and cytotoxic radicals. On T cells, CD40 can influence cell priming and cell-mediated effector functions including macrophage and natural killer cell activation [14]. On vascular endothelial cells, CD40 stimulates cytokine production, upregulation of adhesion molecules, release of superoxide anions, and expression of cyclooxygenase-2 [1, 28, 29]. On dendritic cells, monocytes and macrophages, CD40 stimulates the production of cytokines such as TNF and contributes to the rescue of circulating monocytes from apoptosis [14] (Figure 1). The extensive distribution and various inflammatory functions of the CD154/CD40 axis explain its involvement in the pathogenesis of many autoimmune diseases.

3.2. αIIbβ3. The αIIbβ3 integrin, also known as GPIIb/IIIa, is a major platelet integrin of critical importance for platelet adhesion, aggregation, and thrombus formation. When activated by "inside out" signaling, in response to various platelet agonists (thrombin, collagen, ADP, or epinephrine), αIIbβ3 will change its conformation allowing binding of its major ligands, including fibrinogen, fibronectin, and von Willebrand factor [30]. Binding is mediated through the RGD sequence found on many αIIbβ3 ligands. Interestingly, CD154 was also found to interact with αIIbβ3, primarily through the RGD sequence contained within the extracellular portion of the CD154 molecule. Binding of CD154 to αIIbβ3 induces phosphorylation of tyrosine residues within the cytoplasmic domain of the β3 chain and appears necessary for stability of arterial thrombi [31]. These findings demonstrate that CD154, through its interaction with αIIbβ3, is a platelet agonist that functions in an autocrine manner to regulate platelet biology (Figure 2).

3.3. α5β1. The α5β1 integrin has recently been identified as a novel functional receptor for CD154 [32]. Like αIIbβ3, active α5β1 binds to its classical ligands fibrinogen and fibronectin through their RGD sequence [30]. Interestingly, we have previously shown that CD154 binds to inactive α5β1. In addition, we demonstrated that simultaneous binding of CD154 to CD40 and α5β1 is possible, indicating that CD154 interacts with α5β1 outside the CD40-binding site. The binding of CD154 to a monocytic cell line expressing α5β1 leads to the phosphorylation of the extracellular signal regulated kinase 1/2 (ERK1/2) and the expression of IL-8 mRNA in these cells, which is indicative of a functional consequence of the CD154/α5β1 interaction [32] (Figure 2). Even though α5β1 is expressed on endothelial cells, smooth muscle cells, and platelets, its role upon interacting with CD154 in these cells has not been elucidated yet.

3.4. αMβ2 (Mac-1). AlphaMβ2 or Mac-1 (CD11b/CD18), a member of the integrin family mainly expressed on monocytes/macrophages and neutrophils, has also recently been identified as a CD154 receptor [19]. As with its classical ligands, such as C3bi [33], intracellular adhesion molecule-1 [34], fibrinogen [35], vitronectin [36], factor Xa [37, 38], heparin [39, 40], glycoprotein Ibα [41, 42],

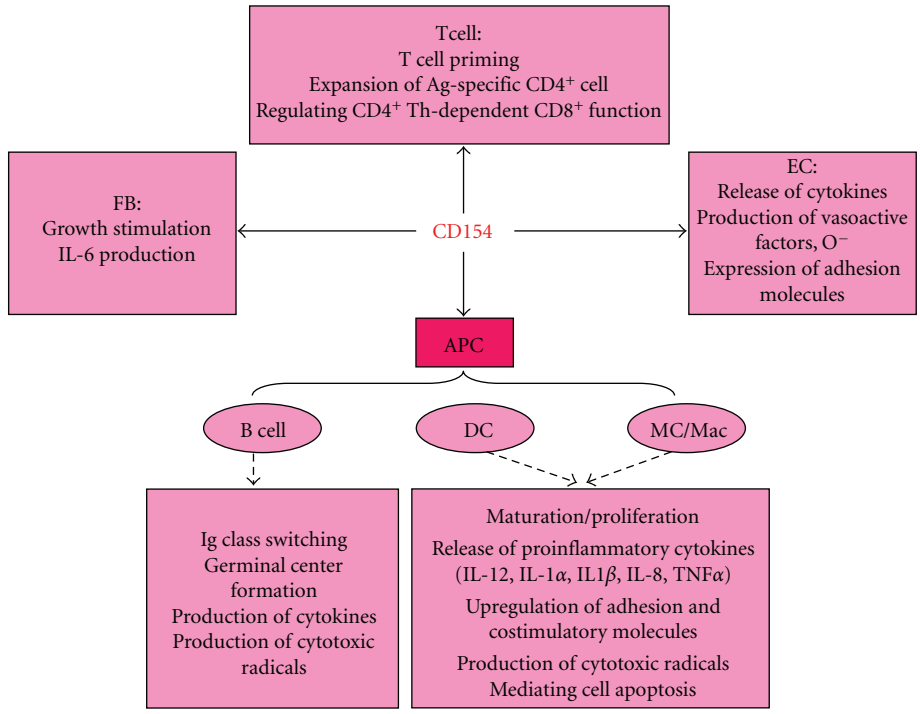

Figure 1: Biological function of the CD154/CD40 interaction. CD154 mediates numerous inflammatory functions on a wide variety of cell types by interacting with its classical CD40 receptor. These include T cell priming, B cell-dependent Ig class switching and germinal center formation, cell proliferation, regulation of apoptosis, release of proinflammatory cytokines, upregulation of adhesion molecules and costimulatory molecules, and production of cytotoxic radicals. FB: fibroblast, EC: endothelial cell, APC: antigen presenting cells, DC: dendritic cell, MC/Mac: monocyte/macrophage.

junctional adhesion molecule-3 [43], and lipoproteins [44], only the active conformation of Mac-1 can interact with CD154. This pleiotropic binding ensues Mac-1 a role in immune responses, coagulation, and inflammation [45]. Indeed, Zirlik et al. demonstrated that CD154 functionally enhances monocyte adhesion and migration via its binding with Mac-1 on monocytes. Interestingly, CD154 stimulation was shown to trigger Mac-1-dependent nuclear factor-kB (NFkB) activation and enhance myeloperoxidase release from monocytes [19] (Figure 2). In addition, the CD154/Mac-1 interaction may play a significant role in atherogenesis, since Mac-1 inhibition in the $LDLR^{-/-}$ atherosclerosis mouse model attenuates arterial plaque development and lesional macrophage accumulation [19]. Furthermore, Li et al. showed that CD154 upregulates Mac-1 expression on neutrophils and enhances leukocyte recruitment and neointima formation after arterial injury in $ApoE^{-/-}$ mice, another atherosclerosis-prone mouse model [46]. These findings unravel a novel mechanism by which CD154 contributes to inflammatory events at the level of vascular cells.

4. CD154 in Autoimmune Diseases

CD154 contributes to the potentiation of autoimmune diseases in which B and T cell activation plays a major role, such as SLE, RA, lupus nephritis, multiple sclerosis, and autoimmune diabetes [47]. Indeed, a role for CD154/CD40 interactions have been identified in the development of Type I diabetes [48]. Moreover, signaling through CD40 was shown to induce the production of inflammatory cytokines in human and nonhuman primate islet cells [49]. High expression of $CD154^{+}$ cells was also detected in the brains of patients with multiple sclerosis [47]. In addition, patients with psoriatic arthritis were found to exhibit CD40 expression on keratinocytes and endothelial cells within psoriatic plaques and increased expression of CD154 on peripheral blood T cells [50–52]. Increased levels of soluble CD154 were also reported in patients with SLE, RA, and Sjorgren's disease, in association with disease activity [53]. In fact, the CD154/CD40 interaction triggers a series of immune responses contributing to T-cell-dependent immunity at different levels in these diseases [54]. First, CD154 signaling could disrupt negative selection in the thymus allowing escape of self-reactive T cells and thus failure of central tolerance. Second, aberrant CD154-dependent production of proinflammatory cytokines could direct the differentiation of T cells to Th17 cells, a process that is augmented by activation of antigen-presenting cell. Third, CD154 interactions could stimulate inflammatory chemokines and cytokines within the target tissue, which contribute to tissue damage and propagation of the inflammatory assault [49, 55–58].

This review will focus primarily on recent findings concerning the role of CD154 in SLE and RA.

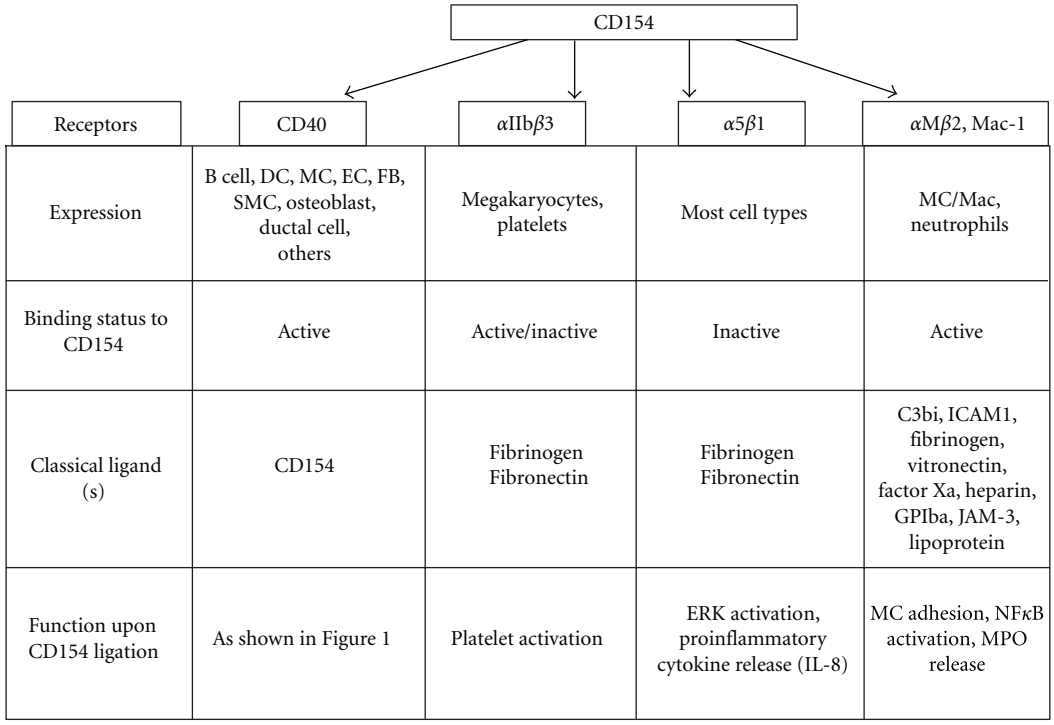

Figure 2: Structural and functional characteristics of CD154 receptors. Four receptors for CD154 have been identified, namely, CD40, αIIbβ3, α5β1, and αMβ2. These receptors are found on various cell types and induce different biological functions upon CD154 binding. APC: antigen presenting cells, DC: dendritic cells, MC: monocyte, EC: endothelial cell, FB: fibroblast, SMC: smooth muscle cell, Mac: macrophage, ICAM: intercellular adhesion molecule, GPIbα: glycoprotein Ibα, JAM-3: junctional adhesion molecule-3, MPO: myeloperoxidase.

4.1. Systemic Lupus Erythematosus (SLE). SLE is a multiorgan target autoimmune disease characterized by a defect in the innate and adaptive immune systems, in which B cells are at the center of the pathogenesis. Indeed, B cells, with the aid of $CD4^+$ T cells, secrete autoantibodies, activate the complement system, and favor the production of cytokines and other mediators potentially involved in inflammation, tissue damage, and progression of the disease [59]. Central to all these processes is signaling events mediated by CD154 [60, 61].

4.1.1. Role of CD154 in Pathogenesis of SLE. CD154 is overexpressed on T cells and atypically expressed on B cells and monocytes in patients with active SLE [62–64]. Ectopic expression of CD154 on B cells is also observed in lupus-prone BXSB mice [65]. Higushi et al. demonstrated that CD154-transgenic mice spontaneously produce autoantibodies such as anti-DNA Abs and develop lupus like glomerulonephritis with age [66]. Immunohistochemical analysis of CD154 expression in the biopsies of lupus kidney specimens showed an upregulation of CD154 expression on renal endothelial and tubular cells, and on interstitial infiltrating T cells [60]. Moreover, CD154 was shown to contribute to SLE pathogenesis by inducing the production of various chemokines in renal endothelial and tubular cells, thereby, increasing local inflammatory responses [67, 68]. The abnormally prolonged expression of CD154 on T cells and high levels of circulating sCD154 can activate bystander autoimmune B cells and initiate autoantibody secretion in SLE (Figure 3). Moreover, the enhanced CD154 expression on activated T cells is implicated in the overexpression of costimulatory molecules such as CD86 on B cells isolated from SLE patients. CD154-induced overexpression of CD86 is essential for anti-DNA antibody production in these patients [69]. In addition, it has been suggested that the atherosclerotic complications seen in patients with SLE are mediated by CD154 and its receptors [70, 71]. In fact, CD154 on activated platelets derived from patients with SLE can upregulate the expression of CD40 on mesangial cells and induce the release of soluble CD40. Such CD154-mediated responses activate mesangial cells, thus, stimulating their proliferation and production of TGF-β1 [72]. Interestingly, these responses are associated with glomerular injury and glomerulosclerosis in SLE, respectively.

4.1.2. Serum Levels of CD154 in SLE Patients. Circulating levels of sCD154 in patients with SLE correlate with the titers of anti- double-stranded DNA (dsDNA) autoantibodies and with disease activity [73, 74]. Soluble CD154, at concentrations reported in some SLE sera, is capable of inducing the upregulation of several accessory molecules on B cells, which favors B cell survival and differentiation, thereby, exacerbating the immune response [74]. Moreover, SLE patients exhibiting antiphospholipid antibodies in their sera

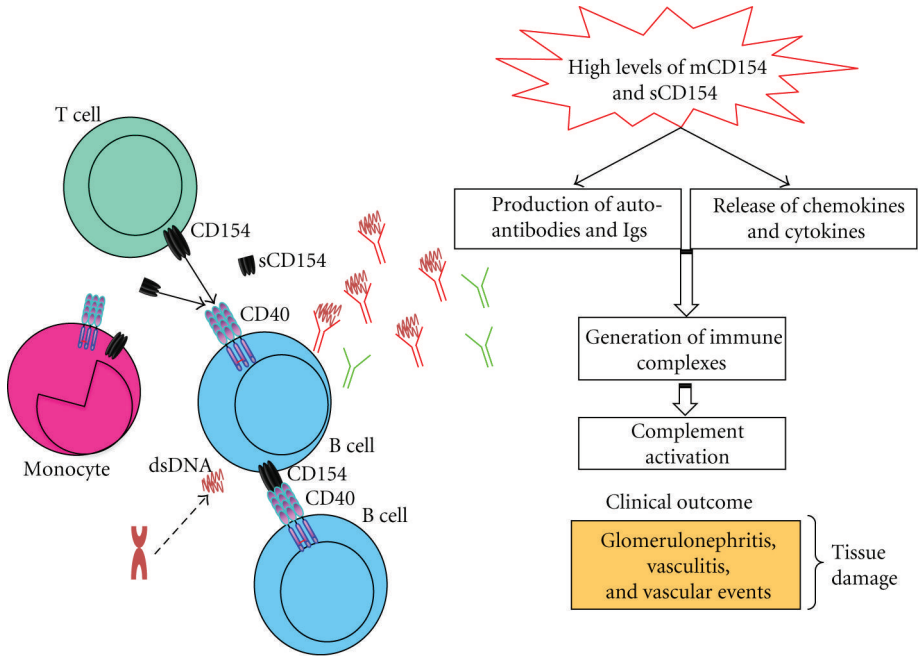

Figure 3: CD154-dependent mechanistic events in SLE patients. High levels of membrane bound and sCD154 activate various immune cells in SLE, including T cells, B cells, and monocytes. These CD154 interactions induce the release of inflammatory mediators and the production of autoantibody and Igs, leading to the generation of immune complexes and the activation of the complement system at the forefront of many clinical manifestations in SLE patients.

and presenting with a history of thrombosis have higher circulating sCD154 levels than SLE-antiphospholipid antibody-positive patients with no history of thrombosis [75]. These data further support a possible role for the CD154 axis in the increased vascular events seen in SLE. Moreover, Aleksandrova et al. showed that enhanced levels of sCD154 in patients with SLE and secondary antiphospholipid syndrome are associated with pronounced intima-media thickness of the carotid arteries, hypercholesterinemia, and diastolic dysfunction [76]. These findings also support the association of CD154 with cardiovascular abnormalities in SLE.

4.1.3. Anti-CD154 Treatment in SLE. In view of all the suggested roles of CD154 in SLE, many approaches using CD154 blocking monoclonal antibodies (mAbs) have been tested in murine models of the disease and resulted in positive outcomes. Treatment with anti-CD154 mAbs prior to disease onset prevents proteinuria, prolongs survival, ameliorates or even prevents kidney disease, and decreases anti-DNA autoantibody titers in the New Zealand Black/New Zealand White F_1 systemic lupus erythematosus (NZBxNZW) F1 and (SWRxNZB) F1 mice models. Moreover, anti-CD154 treatment when nephritis has already developed still slows disease progression, reverses proteinuria, and induces remissions in mice, despite ongoing renal immune complex deposition. Responding mice show rapid downregulation of TNF-α, IL-10, and TGF-β mRNA levels [77, 78]. In addition, a short-term combination therapy with the costimulatory antagonists CTLA4-Ig and anti-CD154 in NZB/NZW F_1 mice delays the onset of renal dysfunction, significantly decreases the frequency of B cells producing anti-DNA IgGs, partially suppresses class switching, and inhibits T cell activation and switching to memory phenotypes [79].

To date, two clinical studies investigating the use of anti-CD154 mAbs for the treatment of SLE have been conducted. In clinical trials, ruplizumab (BG9588) showed good clinical and laboratory responses in some SLE patients. However, the study was stopped earlier than expected because of thromboembolic events [80]. Toralizumab (IDEC-131) was tested in a double-blind, placebo-controlled study in patients with mild-to-moderately active SLE over 16 weeks. although the systemic lupus erythematosus disease activity index (SLEDAI) scores improved from the baseline levels of disease activity in all groups, these scores were not statistically significant among the IDEC-131 treated and placebo groups [61]. New reagents inhibiting CD154-mediated events without increasing the risk of thromboembolic complications are in development [81]. It is important to note at this level that developing new CD154 interfering agents should take into account the new receptors identified for CD154 [1].

4.2. Rheumatoid Arthritis (RA). RA is a chronic, progressive, and debilitating autoimmune disease that occurs in approximately 1% of adults. A vicious cycle of inflammation and cartilage destruction is at the hallmark of the ongoing autoimmune reactions in the synovial tissue. Synovial inflammation, pannus formation, neoangiogenesis, and destruction of joint cartilage are mediated by the constant synthesis of matrix metalloproteinases (MMPs) and proinflammatory cytokines, such as TNF-α and IL-1. The

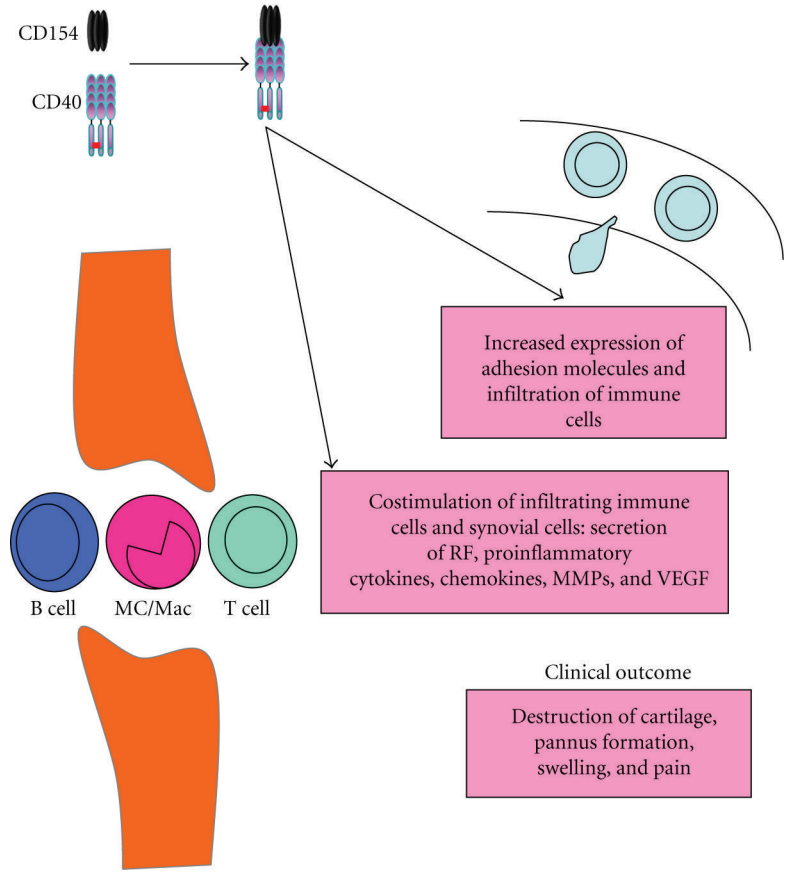

Figure 4: Biological role of CD154 in RA. CD154 contributes to the pathogenesis of RA by stimulating expression of adhesion molecules, production of RF, release of inflammatory mediators including cytokines, chemokines, MMPs, and others.

infiltrating leukocytes and synovial cells destroy the cartilage tissue and erode bones, thereby, resulting in the loss of articular surfaces and joint motion [82].

4.2.1. Role of CD154 in the Pathogenesis of RA. Many reports have demonstrated the implication of CD154 and CD40 in the pathogenesis of RA. In fact, CD154 is implicated in all pathogenic events of RA that ultimately lead to cartilage destruction and bone erosion. These include the T cell-mediated response, the presence of rheumatoid factors (RFs), the expression of adhesion molecules, synovial hyperplasia, and pannus formation, as well as the secretion of proinflammatory cytokines and MMPs (Figure 4). The enhanced expression of CD154 on T cells supports the theory of a T-cell-driven disease. CD154 mRNA and protein were shown to be upregulated on peripheral blood and synovial fluid T cells from RA patients, in comparison to control patients [83]. In addition, CD154 as well as CD40 are overexpressed by $CD4^+$ and $CD8^+$ T lymphocytes and macrophages of the synovial fluid of rheumatoid patients. This aberrant expression was postulated to contribute to the development of synovial hyperplasia [84, 85]. It was also suggested that the increased and prolonged expression of CD154 on T cells from RA patients might be contributing to enhanced cell function and articular inflammation [85, 86].

The inflammatory process in RA is dependent on both humoral- and cell-mediated immunity. CD154 on activated T cells is linked to RF synthesis [87], B cell-dependent IgG overproduction, and secretion of IL-12 by synovial dentritic cells and macrophages [85, 88, 89]. On the other hand, CD154 expressed on T lymphocytes induces other critical costimulatory molecules, namely, CD80 and CD86 on B cells, macrophages, and dendritic cells, which in turn can favor T cell activation. Activated T cells can then initiate specific cellular immune responses, through the secretion of proinflammatory cytokines. For instance, the CD154/CD40 interaction was shown to increase the production of TNF-α, which plays a major role in the pathogenesis of RA. Indeed, Harigai et al. demonstrated that ligation of CD40 on freshly isolated synovial cells, using a recombinant sCD154 protein, induced TNF-α and IL-1β production, a response further amplified by IFNγ [88].

Other investigators have also demonstrated that ligation of CD40 on $CD68^+$ synovial macrophage cells as well as fibroblast-like synoviocytes enhances the expression of CD154, CD106, IL-6, stromal cell-derived factor 1, vascular endothelial cell growth factor (VEGF), IL-8, and regulated upon activation normal T-cell expressed and secreted (RANTES) [84, 90–95]. In addition, ligation of CD40 on synovial fibroblasts from RA patients by T cell CD154

was shown to stimulate neovascularization at the site of synovitis by enhancing VEGF protein and mRNA levels [96]. Moreover, the CD154/CD40 axis is directly linked to the inflammatory process by increasing the expression of important adhesion molecules on fibroblastic cells, such as E selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1). These responses thereby exacerbate the recruitment and infiltration of immune cells at the sites of inflammation [91]. CD154 further contributes to a deleterious degradative cycle in RA by inducing the expression and activation of MMPs (MMP-1, MMP-9, and MMP-3) [97, 98], which are well known to drive the degradation of extracellular matrix proteins in RA.

Hence, it is now believed that CD154, through its involvement in the production of chemokines, cytokines, MMPs, adhesion molecules, and growth factors, contributes to pannus formation and perpetuation of inflammation in RA (Figure 4). It is also worth noting, that the CD154/CD40-dependent induction of inflammatory mediators by RA synovial cells is mediated via the activation of MAPKs, especially ERK-1/2, p38 and NFκB [99].

4.2.2. Serum Levels of CD154 in RA Patients. The importance of the CD154/CD40 axis in the pathophysiology of RA is further consolidated by the levels of circulating sCD154 found in patients. Indeed, high levels of sCD154 have been reported in most patients with juvenile idiopathic arthritis [100]. Moreover, serum levels of sCD154 are higher in patients with RA than in healthy subjects and significantly correlate with both IgM-RF and IgG-RF titers [101].

4.2.3. Anti-CD154 Treatment in RA. CD154/CD40 signaling was demonstrated to be critical in the initiation and progression of the mouse collagen-induced arthritis (CIA) model. Treatment of mice with agonistic anti-CD40 Abs at the time of CIA induction exacerbates the disease [102]. Conversely, the administration of antagonistic anti-CD154 mAbs prior to induction of CIA significantly ameliorates the disease [103], as manifested by the inhibition of symptoms such as development of joint inflammation, serum antibody titers to collagen, infiltration of inflammatory cells into the subsynovial tissue, and erosion of cartilage and bone in treated mice [104]. Anti-CD154 treatment in the K/BxN arthritis mouse model was shown to induce prophylactic effects, as antibody administration inhibits development in mice when given before the onset of the clinically apparent disease [105]. However, anti-CD154 therapy has no effect when administered after clinical onset. These results support the CD154 axis as a therapeutic target in the treatment of RA.

5. Conclusions and Future Studies

The current review supports the role of CD154 as a major participant in the pathogenesis of autoimmune diseases, particularly RA and SLE. As previously detailed, the CD154 axis, through its diverse distribution on many cell types, has acquired pleiotropic functions and contributes to inflammatory diseases by triggering the secretion of critical inflammatory mediators potentially involved in tissue degradation and damage. It is now well established that CD154 interacts with many receptors, namely, CD40, αIIbβ3, α5β1, and αMβ2. For instance, it can constitutively bind to CD40 and α5β1, as well as the active forms of αIIbβ3 and αMβ2. Interestingly, CD154 can simultaneously bind to two receptors expressed on the same cell surface. This phenomenon may grant CD154 distinct biological activities, depending on the receptor in question and the signaling pathway it might trigger. Even though, most of the biological functions of CD154 are believed to involve its interaction with CD40, the discovery of other receptors for CD154 unravels new roles for this molecule in mediating immune and inflammatory events at the forefront of the pathogenesis of many diseases including autoimmune disorders. However, little is known about the signaling pathways and cellular responses triggered by the interaction of CD154 with its other receptors. Indeed, the elucidation of the precise molecular pathways induced by the CD154/αIIbβ3, CD154/αMβ2, and CD154/α5β1 interactions will allow a better understanding of the biological roles of CD154 in associated diseases and allow the design of better therapeutic strategies for the management of these disease conditions.

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Review Article

The Tumor Necrosis Factor Superfamily of Cytokines in the Inflammatory Myopathies: Potential Targets for Therapy

Boel De Paepe, Kim K. Creus, and Jan L. De Bleecker

Laboratory for Myopathology, Department of Neurology and Neuromuscular Reference Center, Ghent University Hospital, 9000 Ghent, Belgium

Correspondence should be addressed to Boel De Paepe, boel.depaepe@ugent.be

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The idiopathic inflammatory myopathies (IM) represent a heterogeneous group of autoimmune diseases, of which dermatomyositis (DM), polymyositis (PM), and sporadic inclusion body myositis (IBM) are the most common. The crucial role played by tumor necrosis factor alpha (TNFα) in the IM has long been recognized. However, so far, 18 other members of the TNF superfamily have been characterized, and many of these have not yet received the attention they deserve. In this paper, we summarize current findings for all TNF cytokines in IM, pinpointing what we know already and where current knowledge fails. For each TNF family member, possibilities for treating inflammatory diseases in general and the IM in particular are explored.

1. Introduction

The idiopathic inflammatory myopathies (IM) are characterized by distinct immune effector mechanisms. Dermatomyositis (DM) is a complement-mediated endotheliopathy associated with perimysial inflammation and perifascicular muscle fiber atrophy. In polymyositis (PM) and sporadic inclusion body myositis (IBM), muscle fibers are injured by autoaggressive immune cells which predominantly infiltrate the endomysium [1]. Additional degenerative phenomena occur in IBM muscle, such as muscle fiber vacuolation and deposition of β-amyloid and other ectopically localized proteins [2]. Other less well-delineated IM include myositis-associated cancer or connective tissue diseases and immune-mediated necrotizing myopathy. Macrophages, dendritic cells (DCs), and T-cells are prominently present in muscle tissues of the different IM. In DM, large numbers of helper T-cells are found within the perimysial, often perivascular, inflammatory infiltrates. In PM and IBM, activated cytotoxic T-cells surround and invade nonnecrotic muscle fibers, while helper T-cells are found at more distant parts of the infiltrates. B-cell-mediated immunity is an important component of DM/PM pathogenesis, and autoantibodies can be detected in up to 70% of patients [3]. The most common is Jo-1, an antihistidyl-tRNA-synthetase, but more autoantibodies directed against aminoacyl-tRNA-synthetases or other muscle antigens are continuously being described. Autoantibody profiles are associated with clinical subsets of patients [4]. In IBM on the other hand, humoral autoimmunity is a more controversial subject. However, it has been established that IBM muscle contains large numbers of plasma cells and an environment permissive of ectopic lymphoneogenesis, which suggests the possibility of local maturation of B-cells and autoantibody production. Indeed, a recent report describes IBM-specific autoantibodies directed against a yet unidentified muscle antigen [5].

The important role played by cytokines in the IM has long been recognized [6]. In this respect, a key role for type I interferon (IFN)-mediated innate immunity has been shown in DM and PM [7, 8]. In this paper, the tumor necrosis factor (TNF) family will be systematically overviewed, discussing current knowledge on their involvement in the IM and exploring whether they could represent appropriate targets for future therapeutic intervention. TNF cytokines affect immune cell proliferation, differentiation, and survival. Up till now, 19 members have been identified in man, and they have been assigned systematic names starting from TNFSF1 to TNFSF18 based on the encoding genes. Ectodysplasin A (EDA) 1 and 2 have not been assigned systematic names and will not be discussed in this paper. Most TNF members

are type II transmembrane proteins whose extracellular C-terminal TNF homology domain can be cleaved off by specific metalloproteinases, generating soluble cytokines. The TNF-like receptors (TNFR) are type I transmembrane proteins of which cystein-rich domains are the hallmark structural motif [9].

2. TNFSF2—TNFα

TNFα or cachectin, the prototypic member of the TNF family, is produced mainly by activated macrophages and T-cells. TNFα activates T-cells, B-cells, and macrophages and induces the expression of other cytokines and cell adhesion molecules through interaction with its receptor TNF-R55 (TNFR1). The alternative receptor TNF-R75 (TNFR2) has been shown to chiefly function as a concentrator at cellular surfaces, transferring the cytokine to TNF-R55 [10]. TNFα augments the activity of nuclear factor-κB (NF-κB) signaling pathways [11].

In the IM, TNFα is by far the most studied cytokine of its family. The endomysial and perimysial inflammatory cells express varying levels, with macrophages being the primary source of the cytokine. TNFα is also prominently expressed in blood vessel endothelial cells of DM tissues [12–14]. The soluble forms of the receptors TNF-R55 and TNF-R75 are increased in DM/PM sera [15]. TNF-R75 expression is notably increased near inflammatory infiltrates in all IM and on the perimysial and perifascicular blood vessel endothelium in DM even remote from inflammation [12]. Polymorphisms in the gene encoding TNFα have been linked to either an increased risk of, or protection against, the development of juvenile DM [16, 17].

Neutralization of TNFα is efficacious for treating several autoimmune diseases. The important catabolic role of TNFα as a regulator of the chronic inflammation associated with the IM has made it a therapeutic target for these diseases as well. Fortunately, knocking out TNFα appears relatively safe and does not seem to hamper skeletal muscle regeneration [18]. Four agents, that generate excellent results in rheumatoid arthritis (RA) and Crohn's disease, can be considered for IM patients: (1) a mouse/human chimeric anti-TNFα monoclonal antibody termed infliximab (Remicade), (2) a TNFα-neutralizing receptor fusion protein termed etanercept (Enbrel), (3) a humanized anti-TNFα monoclonal antibody termed adalimumab (Humira), and (4) the humanized polyethyleneglycol conjugated Fab′ anti-TNFα fragment certolizumab pegol (Cimzia). For the first two compounds, reports so far have revealed variable outcomes in IM patients. Trial results are summarized in Table 1 [19–26]. Several phase II clinical trials have been started up, but, in general, studies suffer from low inclusion rate and notably high drop-out rates mostly due to disease deterioration and adverse events. However, it appears that anti-TNFα treatment could be of benefit to a subset of IM patients. The identification of responsive patients remains difficult, as no specific marker has been identified yet that may predict the therapeutic outcome.

3. Other TNF Members Already Investigated to Some Extent in the IM

3.1. TNFSF1/3—LTα/β. Lymphotoxins (LTs) are versatile cytokines. They are crucial for robust immune responses and are key elements required for lymphoid organogenesis and organization. LTα, also termed TNFβ, is secreted as the homotrimer LTα3, or complexed on the cell surface with LTβ, predominantly as LTα1β2 heterotrimers. LTα can bind to the receptor LTβR as well as to the receptors TNFR1 and TFNR2. LTβ signals through LTβR ligation only.

It appears that LTs are important factors orchestrating sustained inflammation in the IM. LTα has been implicated in the cytotoxic response of $CD8^+$ T-cells towards nonnecrotic muscle fibers in PM [27]. LTβ is increased in muscle tissues of DM patients, where it localizes to blood vessels and intramuscular follicle-like structures. The latter contain large numbers of T-cells, B-cells, and DCs organized in functional compartments [28]. Recent data also show that LTβ may well be an early marker for muscle disease [29].

LTs have been pinpointed as important targets for suppressing inflammation in autoimmune diseases. Studies showed that depletory monoclonal anti-LTα and the receptor antagonist LTβR:Ig inhibit disease in murine collagen-induced arthritis [30, 31]. In addition, administering LTβR:Ig inhibited T-cell-driven intestinal inflammation in murine inflammatory bowel disease [32]. In human RA, synovial LTα and LTβ expression is elevated [33], but targeting the expression by administering LTβR:Ig failed to meet clinical end points in a phase IIb clinical trial. As TNFα and LTα share the receptors TNFR1 and TNFR2, strategies targeting these receptors influence the activities of both cytokines. Therefore, the therapeutic effects of competitive antagonists of TNFR1 and TNFR2, namely, etanercept and lenercept, are presumed to result from combined inhibition of TNFα and LTα.

3.2. TNFSF4—OX40L. The primary source of the transmembrane glycoprotein OX40L, also termed CD252 or gp34, are antigen presenting cells, and expression is further induced when B-cells, DCs, T-cells, and macrophages become activated. The cytokine promotes clonal expansion of T-cells, leading to long-term T-cell survival and enhanced memory T-cell development. The receptor OX40, also termed CD134, is expressed on activated T-cells, B-cells, and vascular endothelial cells. Proinflammatory cytokines, including TNFα, can further augment the expression of the receptor. The OX40/OX40L interaction provides a costimulatory signal for T-cells and enhances ongoing immune responses driven by either helper T-cell type 1 (Th1), Th2, or Th17 cells [34].

OX40 is present on mononuclear cells in the endomysium and at perivascular sites in PM muscle. OX40 positive cells are mostly $CD4^+$ cells, few are $CD8^+$ cytotoxic T-cells. Autoaggressive immune cells invading nonnecrotic muscle fibers are invariably OX40 negative [35]. OX40L expression has not yet been described in the IM.

Blocking OX40L has produced strong therapeutic effects in multiple animal models of autoimmune and inflammatory

Table 1: Tumor necrosis factor inhibitors for treating inflammatory myopathies: published trial results for infliximab and etanercept.

Compound and treatment regimen	Diagnosis/patients continued to end point	Follow-up time	Clinical outcome at end point	Reference
infliximab 6 mg/kg 4-weekly or more frequent	R-JDM/5	32 to 130 weeks	I (5/5)	[19]
infliximab 10 mg/kg (week 0, 2, 6, 14)	R-DM/1 R-PM/4 R-IBM/4	16 weeks	NC (1/1) I (2/4) W (2/4) I (1/4) NC (3/4)	[20]
infliximab 10 mg/kg (week 0, 2, 4)	R-DM/1 R-PM/1	12 weeks	I (1/1) I (1/1)	[21]
infliximab 10 mg/kg (week 20) infliximab 10 mg/kg (week 14, 18, 22)	R-DM/1 R-PM/1	66 weeks	I (1/1) I (1/1)	[22]
infliximab 10 mg/kg (week 0, 2, 6, 14, 22)	PM/2	26 weeks	I (2/2)	[23]
infliximab 8 mg/kg (week 0, 2, 6)	R-DM/1	6 weeks	I (1/1)	
infliximab 10 mg/kg (week 0, 2, 4, 6, 9)	R-PM/1	69 weeks	I (1/1)	[24]
infliximab 3 mg/kg (week 0, 2, 6, every 8) and etanercept 25 mg twice weekly	R-DM/1 R-PM/2	36 to 96 weeks	PR (1/1) I (2/2)	[25]
etanercept 25 mg twice weekly	R-DM/1	56 weeks	I (1/1)	[26]

Abbreviations: dermatomyositis (DM), improved (I), inclusion body myositis (IBM), juvenile DM (JDM), no change (NC), partial response (PR), polymyositis (PM), refractory DM/PM/IBM (R-DM/PM/IBM), worsened (W).

disease, which include inflammatory bowel disease [36] and arthritis [37]. Neutralizing antibodies to OX40L are currently being tested in phase II clinical trials for treating asthma.

3.3. TNFSF5—CD40L. CD40L, also termed CD154 or gp39, is expressed by activated T-cells, mainly on the $CD4^+$ subsets. Its receptor CD40 is present on antigen presenting cells and on endothelial cells. CD40L positive T-cells activate monocytes and upregulate adhesion molecules and monocyte chemoattractant protein 1 (CCL2) production by blood vessel endothelial cells [38].

The CD40/CD40L system seems to be involved in the immunopathogenesis of the IM. A subset of inflammatory cells in IM tissues express CD40L, of which the majority are $CD4^+$ cells. Also, part of the muscle fibers in PM/DM tissues express CD40. In vitro IFNγ-stimulation of myoblasts induces CD40 expression, leading to increased levels of IL-6, IL-8, IL-15, and CCL2 [39]. The induction of proinflammatory factors through the CD40/CD40L system could contribute to T-cell recruitment and activation found within IM muscle tissues.

CD40L/CD40 interaction engages antigen presenting cells, provokes B-cell responses and enhances the production of proinflammatory cytokines, pinpointing the interaction as an important regulatory mechanism in inflammatory disease. In murine collagen-induced arthritis, for example, an agonistic anti-CD40 antibody exacerbates disease [40], while a blocking anti-CD40L antibody protects against disease [41]. Phase I and II trials in humans have already been initiated, testing the effects of a humanized anti-CD40L antibody in inflammatory bowel disease [42]. However, the development of IDEC-131, another humanized monoclonal anti-CD40L, is no longer pursued after a placebo-controlled trial demonstrated no clinical activity in systemic lupus erythematosus (SLE) [43].

3.4. TNFSF6—FASL. FasL is expressed on activated T-cells and NK-cells. The cytokine comes in a 40 kDa membrane-bound and a 29 kDa-soluble variant. Its receptor Fas, also termed CD95 or apoptosis 1 (Apo1), is constitutively expressed on many cell types. An alternative soluble receptor termed decoy receptor 3 (DCR3) has been described, possibly serving to counteract Fas function. Fas ligation leads to a conformational change, which causes binding of death domain-containing adaptor proteins, subsequently activating caspases and nucleases.

A role for the Fas/FasL system in muscle damage is suspected. Proinflammatory in vitro conditions have been shown to induce apoptosis in muscle cells, a process that can be partially inhibited by an anti-FasL antibody [44]. However, apoptosis is not a prominent feature of IM, and data concerning Fas/FasL expression appear somewhat confusing. FasL was found absent from IM muscle tissue [45] or expressed only by some T-cells [27, 46]. Fas expression has been reported with very different frequencies, but the sarcolemma of regenerating muscle fibers seems to represent the main site of immunoreactivity. Also, some nonnecrotic invaded muscle fibers in PM/IBM and some atrophic perifascicular muscle fibers of DM are Fas positive [45, 47, 48]. Serological studies report unchanged FasL levels, while Fas levels were significantly higher in PM/DM patients compared to normal controls [49]. The peripheral blood of DM patients contains significantly lower percentages of regulatory T-cells, but the

fraction of Fas positive cells is similar, indicating no increased susceptibility of regulatory T-cells to FasL-mediated apoptosis [50].

The involvement of Fas/FasL interactions in human inflammatory disease is complex. It has been shown that Fas/FasL deficiencies are associated with the accumulation of lymphocytes and establishment of autoimmune disease. Indeed, a number of inflammatory diseases seem to be associated with decreased serum levels of soluble FasL. Administering DCs overexpressing FasL resulted in protection against murine collagen-induced arthritis [51] possibly through elimination of autoreactive T-cells. Nonetheless, in RA synovial fluid, increased levels of soluble FasL have been found [52]. More research is needed to unravel the precise involvement of the cytokine in human diseases.

3.5. TNFSF7—CD27L. CD27L, also termed CD70, is expressed on T-cells, B-cells, and NK-cells. It is a T-cell costimulatory molecule whose expression is upregulated upon activation. CD27L regulates the formation of effector and memory T-cells and induces their secretion of cytokines. In the B-cell compartment, CD27L promotes differentiation into plasma cells and subsequent antibody production, commitment to memory B-cell responses, and the formation of germinal centers. The receptor CD27 is constitutively expressed on resting T-cells and is upregulated upon T-cell activation. In B-cells, CD27 expression is induced by antigen-receptor activation.

Very limited data is currently available on CD27L expression in the IM. We do know that unlike in the series of SLE patients, CD27L is not increased on peripheral $CD4^+$ cells of a single DM patient, who was included as a disease control in the study [53].

Under physiological conditions, expression of CD27L is restricted and transient in nature. Thus, CD27L offers a potential mechanism to selectively target only the activated cells of the immune system, B-cells in particular, potentially avoiding generalized immunosuppression and overt toxicity. Anti-CD27L was found to improve disease and reduce autoantibodies in murine collagen-induced arthritis [54].

3.6. TNFSF10—TRAIL. TNF-related apoptosis-inducing ligand (TRAIL), also termed Apo2L, can be expressed by various cell types. It binds TRAIL receptors 1 to 4 and osteoprotegrin (OPG), inducing target cell apoptosis.

Only one report is available that describes TRAIL in PM, stating that many inflammatory cells are TRAIL positive [55]. TRAIL is expressed in the endomysial capillaries of healthy skeletal muscle and patients alike.

TRAIL potentially dampens autoimmune responses by silencing autoreactive T-cell populations and, therefore, could be beneficial to patients suffering from inflammatory diseases. A study showed that a blocking anti-TRAIL monoclonal antibody, on the one hand, exacerbates murine experimental autoimmune encephalomyelitis, while recombinant TRAIL, on the other hand, suppressed disease [56]. Thus, strategies delivering a soluble TRAIL equivalent may be effective in suppressing disease episodes. In addition, a human study identified TRAIL as a prognostic marker for IFNβ-response in multiple sclerosis (MS). Upregulated concentrations of TRAIL in response to IFNβ distinguished drug responders from nonresponders [57].

3.7. TNFSF11—RANKL. Receptor activator of NF-κB ligand (RANKL), expressed on the membranes of T-cells, is also called TNF-related activation-induced cytokine (TRANCE), OPG ligand (OPGL), or osteoclast differentiation factor (ODF). Two putative receptors for RANKL have been proposed, being RANK and OPG. RANK is a transmembrane receptor present on DCs and T-cells. OPG is a soluble secreted decoy receptor for RANKL. RANKL acts in synergy with TNFα, activating a cascade of intracellular signaling events which lead to osteoclast activation.

In mice, RANKL mRNA is expressed in healthy skeletal muscle [58], which points to a role in normal muscle physiology. A study reported serum RANKL concentrations to be significantly higher and RANK levels to be significantly lower in juvenile DM than in healthy age-matched controls [59].

RANKL/RANK is of major pathophysiological importance in the bone and joint destruction associated with RA [59], where RANK expression appears to be limited to the sites of immune reaction. The development of compounds that mimic OPG action may prevent osteoclast-mediated bone loss in patients [60]. Denosumab (AMG-162), a monoclonal anti-RANKL antibody, is currently being tested for treating RA.

3.8. TNFSF13—BAFF and APRIL. B-cell activating factor (BAFF), also termed TNFSF13b or B-lymphocyte stimulator (BLyS), is expressed on the surface of monocytes, DCs, and activated T-cells. BAFF binds three receptors: transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI), BAFF receptor (BAFFR), and B-cell maturation antigen (BCMA). BAFF is crucial for B-cell maturation and survival and antibody production by plasma cells. In addition, BAFF regulates T-cell activation and differentiation. A proliferation-inducing ligand (APRIL) or TNFSF13 is homologous to BAFF and exists only as a soluble homotrimer. APRIL binds TACI and BCMA and is important for B-cell development and function [61].

Serological studies have shown that BAFF levels are significantly increased in DM patients [62] but not in PM/IBM [63]. BAFF levels in serum correlated with IL-7, IL-12, and CXCL10 [64] and with Jo-1 expression, supporting a role for BAFF in autoantibody production. In addition, BAFF transcripts were found markedly upregulated in muscle extracts from DM (12-fold), PM (14-fold), and IBM (21-fold) patients [65]. In DM muscle, BAFF localizes to the muscle fibers in perifascicular areas [66]. Interestingly, mononuclear cells infiltrating IM muscle express IFNα [67], a potent BAFF inducer. Serum APRIL levels were found unaltered in IM patients [64].

Blocking BAFF and APRIL potentially diminishes autoreactive B-cells, which would interrupt B-cell differentiation and prevent autoantibody production. Thus, BAFF and APRIL represent appropriate targets for intervention

in autoimmune diseases with an important humoral pathogenic component. B-cells are especially associated with DM infiltrates, where IFNα expression could well be the trigger to activate autoantibody production. In addition, differentiated plasma cells can also be encountered in PM/IBM muscle samples [68].

The anti-BAFF monoclonal antibody belimumab has been tested in two phase III trials for the treatment of SLE. In both trials, belimumab met the primary endpoints, showing significant clinical improvement compared to standard of care alone. LY2127399, another BAFF neutralizing antibody, has entered phase II trials for RA. Atacicept, an Ig fusion protein of the extracellular domain of the TACI receptor that binds BAFF and APRIL, has currently reached phase II/III for treating SLE [69].

4. TNF Members Not Yet Explored in IM

4.1. TNFSF8—CD30L. CD30L is expressed on the membranes of activated T-cells, resting B-cells, and monocytes. Interaction with its receptor CD30, expressed on T-cells, and B-cells, leads to their proliferation and activation. In inflammatory diseases, CD30L/CD30 interactions seem to represent both deleterious and beneficial effects. A blocking monoclonal anti-CD30L antibody aggravates allograft rejection in mice by suppressing regulatory T-cell function [70], while soluble CD30-Ig fusion protein ameliorates murine experimental colitis through inhibition of Th17 responses [71]. Elevated levels of soluble CD30 have been observed in autoimmune diseases such as RA [72] and SLE [73].

4.2. TNFSF9—4-1BBL. 4-1BBL is predominantly expressed on activated antigen presenting cells and interacts with the 4-1BB receptor expressed early and transiently on activated T-cells and on DCs. The 4-1BBL/4-1BB interaction is relevant to the pathogenesis of inflammatory disease. In sera of RA patients, soluble 4-1BB and 4-1BBL levels are increased and correlate with disease severity [74]. Treatment with an antagonistic anti-4-1BB antibody reduces severity of arthritis in animal models, ameliorating inflammation, and the associated B-cell responses [75].

4.3. TNFSF12—TWEAK. The multifunctional cytokine TNF-like weak inducer of apoptosis (TWEAK) triggers multiple and often seemingly conflicting cellular responses, which range from cell proliferation to cell death. Moreover, TWEAK signaling through the FN14 receptor [76] has an impact on normal muscle functioning. In vitro, TWEAK inhibits the differentiation of myoblasts to myotubes [77] and induces the expression of proinflammatory CCL2 [78]. TWEAK knockout mice exhibit augmented muscle tissue regeneration [79], while overexpression results in inhibited myofiber regeneration and increased expression of proinflammatory cytokines including TNFα, IL-1β, IL-6, and CCL2 [80–83]. The proinflammatory cytokines inducible by TWEAK are important regulators of IM [84], which warrants further exploration. Also, circulating TWEAK levels are significantly increased in other human autoimmune diseases such as MS and SLE [85]. A TWEAK-neutralizing monoclonal antibody ameliorates collagen-induced arthritis in mice, reducing serum and joint CCL2 levels significantly [86].

4.4. TNFSF14—LIGHT. Lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpes simplex virus entry mediator (HVEM) on T-cells (LIGHT), also termed LTγ, binds the receptors LTβR, HVEM, and the decoy receptor DCR3. LIGHT is expressed by activated T-cells and immature DCs, and is a potent T-cell costimulatory molecule [87] with profound effects on T-cell-mediated disease [88]. LIGHT enhances Th1-mediated immune responses and in vitro strongly induces CXCR3 ligands [89]. The predominance of Th1-mediated immunity and CXCL10 expression have been demonstrated in the IM [90]. In synovial tissues from RA patients, both LIGHT and its receptor HVEM are expressed by CD68+ macrophages, and their interaction induces the proinflammatory cytokines TNFα, IL-6, and IL-8 [91]. Blocking LIGHT activity significantly reduces graft versus host disease [92, 93].

4.5. TNFSF15—TL1. TNF-like 1 (TL1), also termed vascular endothelial growth inhibitor (VEGI), is expressed by macrophages, lymphocytes, and plasma cells. TL1 binding enhances the expression of IFNγ by T-cells [94] and induces apoptosis in endothelial cells. TL1 has been implicated in human inflammatory bowel disease, where it is found to be increased in macrophages, plasma cells and lymphocytes [95], and TL1 gene variants have been linked to disease susceptibility [96, 97].

4.6. TNFSF18—GITRL. Glucocorticoid-induced TNF receptor ligand (GITRL), also termed TNF-like 6 (TL6), is expressed by endothelial cells, DCs, macrophages, and B-cells. Its receptor GITR is present on naive, activated, and regulatory T-cells that, upon ligation, proliferate and produce cytokines. In RA, both GITR and GITRL are expressed in synovial macrophages that, in response to in vitro stimulation with an anti-GITR monoclonal antibody, produce TNFα, IL-6, IL-8, and CCL2 [98]. Agonistic anti-GITR monoclonal antibodies exacerbate joint inflammation and cytokine production [99].

5. Conclusions and Future Prospects

Oral corticosteroids are still standard treatment for DM and PM, but they come with serious side effect and incomplete treatment responses. Patients anxiously await more selective treatment options. Moreover, IBM patients do not respond to the immunosuppressive and immunomodulatory drugs currently available. A better understanding of the deleterious and beneficial effects of the different players that make up the IM muscle microenvironment is necessary to aid the development of novel routes for therapy. In addition, such knowledge could provide markers to distinguish potentially responsive from nonresponsive patients, better predicting the outcome of costly immune interventions.

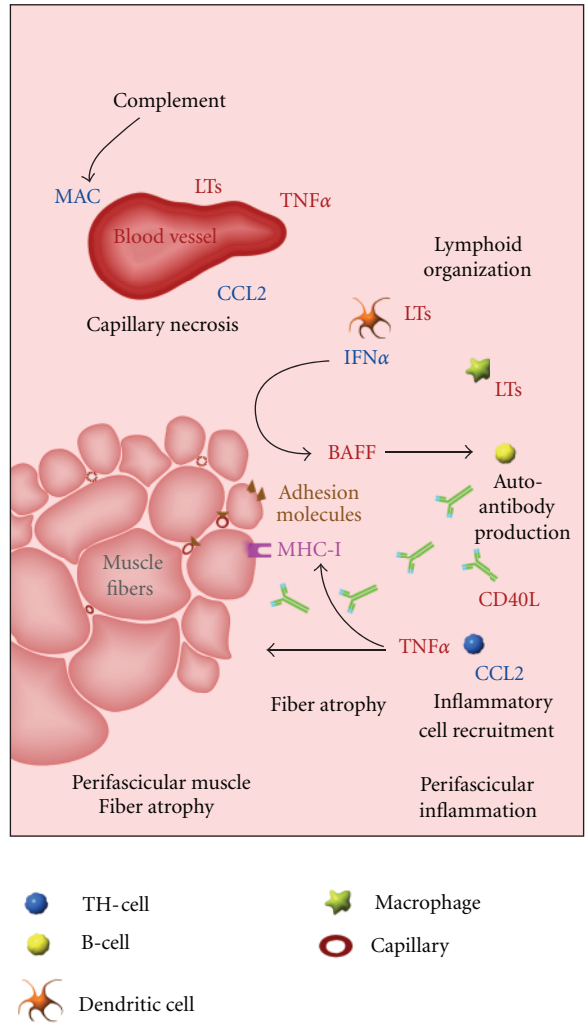

Figure 1: Potential roles for tumor necrosis factor cytokines in the muscle damage associated with dermatomyositis. Based on current knowledge, a model is developed describing the possible involvement of tumor necrosis factor (TNF) cytokines in the sustained inflammation in perifascicular regions of muscle. Activation of the complement system leads to membrane attack complex (MAC) deposition on blood vessels and subsequent necrosis. Endothelial monocyte chemoattractant protein-1 (CCL2) recruits inflammatory cells that accumulate and organize in perimysial areas. Lymphocytes organize into functional compartments and produce lymphotoxins (LTs), TNFα and CD40L, which further recruit responsive immune cells from the circulation, leading to the buildup of perimysial inflammation. Dendritic cells (DCs) produce IFNα, which stimulates muscle fibers to secrete B-cell activating factor (BAFF). The latter activates B-cells that, in response, begin to produce autoantibodies. TNFα, mostly produced by Th-cells, provokes muscle fiber atrophy and stimulates major histocompatibility complex I (MHC-I) and expression of adhesion molecules.

In this paper, we summarized current knowledge on the involvement of the TNF superfamily of cytokines in IM, finding ourselves humbled by the lack of data regarding some of them. The TNF cytokines represent plausible therapeutic targets for the IM, as they are regulators of the complex inflammatory cascade that leads to sustained inflammation. For PM and IBM, limited information is available for TNF cytokines other than TNFα. However, for DM, a picture is slowly emerging in which TNF cytokines no doubt play a crucial role. Based on current knowledge, we developed a model describing the TNF-mediated sequence of events that lead to the characteristic muscle damage, being blood vessel loss, perifascicular muscle fiber atrophy, and inflammation (Figure 1).

Selectively targeting individual TNF members provides new promises and opportunities to develop more efficacious therapies for IM while avoiding the toxicity seen in existing systemic anti-inflammatory therapeutics.

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Research Article

Polymerized-Type I Collagen Induces Upregulation of Foxp3-Expressing CD4 Regulatory T Cells and Downregulation of IL-17-Producing CD4$^+$ T Cells (Th17) Cells in Collagen-Induced Arthritis

Janette Furuzawa-Carballeda,[1] Perla Macip-Rodríguez,[1] Angeles S. Galindo-Feria,[1] David Cruz-Robles,[2] Virgina Soto-Abraham,[2] Sergio Escobar-Hernández,[2] Diana Aguilar,[3] Deshiré Alpizar-Rodríguez,[1] Karen Férez-Blando,[1] and Luis Llorente[1]

[1] *Department of Immunology and Rheumatology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, Colonia Sección XVI, Tlalpan, 14000 Mexico City, DF, Mexico*
[2] *Department of Pathology, Instituto Nacional de Cardiología Ignacio Chávez, Juan Badiano No. 1, Colonia Sección XVI, Tlalpan, 14080 Mexico City, DF, Mexico*
[3] *Department of Experimental Pathology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, Colonia Sección XVI, Tlalpan, 14000 Mexico City, DF, Mexico*

Correspondence should be addressed to Janette Furuzawa-Carballeda, jfuruzawa@gmail.com

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Previous studies showed that polymerized-type I collagen (polymerized collagen) exhibits potent immunoregulatory properties. This work evaluated the effect of intramuscular administration of polymerized collagen in early and established collagen-induced arthritis (CIA) in mice and analyzed changes in Th subsets following therapy. Incidence of CIA was of 100% in mice challenged with type II collagen. Clinimorphometric analysis showed a downregulation of inflammation after administration of all treatments ($P < 0.05$). Histological analysis showed that the CIA-mice group had extensive bone erosion, pannus and severe focal inflammatory infiltrates. In contrast, there was a remarkable reduction in the severity of arthritis in mice under polymerized collagen, methotrexate or methotrexate/polymerized collagen treatment. Polymerized Collagen but not methotrexate induced tissue joint regeneration. Polymerized Collagen and methotrexate/polymerized collagen but not methotrexate alone induces downregulation of CD4$^+$/IL17A$^+$ T cells and upregulation of Tregs and CD4$^+$/IFN-γ^+ T cells. Thus, Polymerized Collagen could be an effective therapeutic agent in early and established rheumatoid arthritis by exerting downregulation of autoimmune inflammation.

1. Introduction

Rheumatoid arthritis (RA) is a common systemic disorder characterized by autoimmunity and chronic inflammation of multiple joints. Collagen-induced arthritis (CIA) is a well-established animal model of RA [1]. CIA is induced in genetically susceptible strains of mice by immunization with type II bovine collagen (CII). Although the effector mechanisms of inflammation ultimately result in pathogenic lesions of joints (inflammatory component of CIA), there is considerable evidence implicating CII-specific CD4$^+$ T cells as primary mediators of disease induction (T-cell immunity component of CIA) [2, 3]. After antigenic stimulation naive CD4$^+$ T cells develop into different types of helper T cells, each produces its own set of cytokines that mediate different responses in CIA. It has been well documented that Th1 cells produce interferon (IFN)-γ and interleukin (IL)-2 and have been considered to be the major mediator of the disease [4, 5]. However, the notion that CIA is a Th1-mediated disorder has been challenged by studies using

Th1-defective mice [6]. Mice lacking IFN-γ, IFN-γ receptor, or IL-12p35 develop accelerated arthritis after induction of CIA [7, 8]. Furthermore, recent studies have suggested that highly proinflammatory IL-17-producing Th17 cells, rather than Th1 cells, are central to the pathology of autoimmune arthritis [9, 10]. IL-17-producing $CD4^+$ T cells contribute to severe synovitis, pannus formation, joint destruction in arthritis joints and autoimmune inflammation [6]. On the other hand, there is ample evidence that $CD4^+/CD25^+/Foxp3^+$ regulatory T (Treg) cells also play a critical role on the inhibition of autoimmune reaction. Thus, a reciprocal relationship between the differentiation of Th17 and Treg cells has been reported [11]. Hence, therapeutic strategies of RA should consider the regulation of Th17 and Treg differentiation as well as the inhibition of proinflammatory cells and Th1 cytokines.

In order to shift the cytokine balance to anti-inflammatory cytokines in RA and to delete hyperactive proinflammatory Th17 and Th1 cells that closely associate with etiology of RA, it is of utmost importance to discover novel biological substances that can selectively suppress the function or downregulate activated Th1 and Th17 cells, whilst at the same time enhance Treg cell function.

This study focuses on the effect of polymerized-type I collagen on Th subsets and its primary mechanism of action in early and established CIA in mice. Polymerized Collagen is a γ-irradiated mixture of atelopeptidic porcine type I collagen and polyvinylpyrrolidone, which has immunomodulatory properties [12–14]. One percent Polymerized Collagen addition to synovial tissue cultures from non-RA and RA cultures does not induce any change in DNA concentration or metabolism. However, the addition of the biodrug to RA synovial tissue cultures modifies the histological and biochemical pattern of fibrosis, without changing the total collagen content. Polymerized Collagen induces the recovery of type III collagen at similar levels to those detected in normal synovial tissue. The biodrug diminishes the accumulation of dense and tightly packed type I collagen fibers and contributes to establish similar tissue architecture to that observed in normal synovium. Polymerized Collagen induces a decrease of collagenolytic activity, mainly calcium-independent collagenase activity (cathepsins) and the increase of TIMP-1, as well as type III collagen production. The chronic inflammatory process is altered by Polymerized Collagen action, presumably due to the downregulation of IL-1β and TNF-α, ELAM-1, VCAM-1, ICAM-1, and Cox-1 [15, 16].

Subcutaneous or intramuscular administration of Polymerized Collagen in combination with methotrexate to RA patients was safe and well tolerated in the treatment of this pathology [17]. The biodrug induced a statistically significant clinical improvement in basal versus 3- or 6-month treatment [18]. Besides, intramuscular administration diminished C-reactive protein (CRP) and rheumatoid factor (RF) levels, and patients required lower doses of methotrexate versus placebo. Thirty percent achieved remission. No differences in serological or hematological variables were found. Adverse events were not detected, except pain lasting <5 min at the injection site [18].

In this study, we show that Polymerized Collagen suppresses the development of CIA by enhancing a proportion of Treg cells while simultaneously diminishing Th17 cells suggesting that Polymerized Collagen ameliorates autoimmune arthritis through the regulation of both Treg/Th17 differentiation and Th1/Th2 balance.

2. Materials and Methods

2.1. Mice. Male DBA1/J mice (7-8 weeks old, 20–22 g) were obtained from Harlan S.A. de C.V. (Mexico City, Mexico). Mice were kept under specific pathogen-free conditions. All animals were set aside under standard conditions in a 12 h day/night rhythm with access to food and water *ad libitum*. All animal procedures and experiments were carried according to international guidelines (ICLAS-WHO) and national law (NOM 062-ZOO-1999). The protocol of this study was approved by the Animal Care and Research Committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán.

2.2. Induction of CIA. Arthritis was induced according to methods as described previously [1]. Chicken CII (SIGMA-Aldrich Co. St. Louis, MO, USA) was dissolved in 0.05 M acetic acid to a concentration of 2 mg/mL at 4°C, and emulsified with an equal volume of complete Freund's adjuvant (SIGMA-Aldrich Co.) Male mice were injected intradermally at the base of the tail with 0.1 mL of emulsion containing 100 μg of chicken CII. Twenty-one days after the primary immunization, mice were boosted with 0.1 mL of the mixture of 2 mg/mL chicken CII and incomplete Freund's adjuvant (SIGMA-Aldrich Co.) via the same route. In each experiment, a control group mice were injected with citric/citrate buffer alone.

2.3. Evaluation of Arthritis Severity in Mice with CIA. The severity of arthritis was measured by scoring each limb from 0 to 4 grades and by summing up the scores of four limbs: 0 = normal; 1 = erythema or swelling of one or several digits; 2 = erythema and moderate swelling extending from the ankle to the mid-foot (tarsals); 3 = erythema and severe swelling extending from the ankle to the metatarsal joints; 4 = complete erythema and swelling encompassing the ankle, foot, and digits, resulting in deformity and/or ankylosis. The maximum score for each animal is thus 16 [3]. Paw edema in each animal was measured with a vernier (Mitutoyo America Co.). All the evaluations were made by the same observer.

2.4. Disease Models

2.4.1. Toxicity. Sixteen mice (n = 4 per group) without arthritis were treated by intramuscular administration once weekly during six weeks with 100 μL of (a) placebo (citric/citrate buffer), (b) polymerized-type I collagen (0.17 mg of collagen), (c) Methotrexate (2.5 mg/kg) [19], or (d) methotrexate (2.5 mg/kg)/Polymerized-type I collagen

(0.17 mg of collagen). Two mice of each group were sacrificed at the 6th and 13th weeks.

2.4.2. Early Arthritis. Forty-eight male mice ($n = 12$ per group) were injected with emulsion of chicken CII. Twenty one days after the primary immunization, mice were boosted. At the same time, mice were treated with 100 μL of (a) placebo (citric/citrate buffer), (b) Polymerized-Type I Collagen, (c) methotrexate (2.5 mg/kg) or (d) methotrexate/Polymerized-Type I Collagen. Six mice of each group were sacrificed at the 6th and 13th weeks.

2.4.3. Established Arthritis. Forty eight male mice ($n = 12$ per group) were injected with emulsion of chicken CII. Twenty one days after the primary immunization, mice were boosted. One week later, mice were treated with 100 μL of (a) placebo (citric/citrate buffer), (b) Polymerized-Type I Collagen, (c) methotrexate, or (d) Methotrexate/Polymerized-Type I Collagen. Six mice of each group were sacrificed at the 8th and 13th weeks.

2.5. Drug Treatment. One hundred μL of (a) placebo (citric/citrate buffer), (b) Polymerized-Type I Collagen, (c) methotrexate (2.5 mg/kg), or (d) methotrexate/polymerized-type I collagen was intramuscularly administered once weekly during six weeks.

2.6. Histological Analysis. On day 35 and day 98, the mice were killed in a carbon dioxide chamber, and the hind paws were collected, fixed with 10% buffered formalin, and then decalcified in 5% formic acid and embedded in paraffin. Sections (5 μm) of whole hind paws were stained with hematoxylin and eosin and PAS technique. Histopathological changes were scored by a blinded observer using the previously reported parameters: 0 = normal joint structure; 1 = mild changes, synovitis and pannus front with view discrete cartilage focal erosions; 2 = moderate changes, accompanying loss of large areas of cartilage, eroding pannus front and synovial hyperplasia with infiltrating mononuclear cells and polymorphonuclear cells; 3 = severe synovitis, cartilage, and bone erosion; 4 = total destruction of joint architecture [20].

2.7. Cell Preparations and Flow Cytometric Analysis. Spleens were isolated from mice. Splenocytes were stained with 5 μL of anti-CD4-FITC-labelled monoclonal antibody (BD Biosciences, San Jose, Calif, USA) at room temperature in the dark for 20 min. After two washes, cells were permeabilized with 200 μL of cytofix/cytoperm solution (BD Biosciences) at 4°C for 20 min. After two washes with permwash solution (BD Biosciences), cells were stained for intracellular cytokines and transcription factors with 5 μL of anti-IL-17A-PE-labelled, anti-IFN-γ-PE-labeled anti-IL-4-PE-labelled, and PE-labelled anti-Foxp3 for Tregs (BD Biosciences), for 30 min at 4°C in the dark. Finally, after washing with permwash solution, splenocytes were analyzed by flow cytometry with a FACScan (BD Biosciences). A total of 10000 events were recorded for each sample and analyzed with the CellQuest software (BD Biosciences). Results are expressed as the relative percentage of IL-17A, IFN-γ, IL-4, or Foxp3-expressing cells in each gate. In order to avoid false positive PE results and also for setting compensation for multicolor flow cytometric analysis, we performed instrument calibration/standardization procedures each day according to established protocols of our laboratory. Briefly, we run an unstained (autofluorescence control) and permeabilized PBMCs sample. Autofluorescence control (unstained cells) was compared with single-stained cell-positive controls to confirm that the stained cells were on scale for each parameter. Besides, BD Calibrite 3 beads were used to adjust instrument settings, set fluorescence compensation, and check instrument sensitivity (BD CaliBRITE, BD Biosciences).

2.8. NFκB/IκBα Flow Cytometric Analysis. Splenocytes were permeabilized with 200 μL of cytofix/cytoperm solution (BD Biosciences) at 4°C for 20 min. After two washes with permwash solution (BD Biosciences), cells were stained for intracellular transcription factors with 5 μL of anti-NFκBp65-FITC-labelled monoclonal antibody (Santa Cruz, Calif, USA) and 5 μL of anti-IκBα PE-labeled monoclonal antibody (IκBα binds to the p65 subunit of p50–p65 heterocomplex through ankyrin repeats) (Santa Cruz), for 30 min at 4°C in the dark. After washing with permwash solution, splenocytes were analyzed by flow cytometry with a FACScan (BD Biosciences). A total of 10000 events were recorded for each sample and analyzed with the CellQuest software (BD Biosciences). Results are expressed as the relative percentage of NFκBp65$^+$/IκBα^+ cells in each gate.

2.9. Statistical Analysis. Statistical analysis was performed using the SigmaStat11 program by one way analysis of variance on Ranks and by Holm-Sidak method for all pairwise multiple comparison procedures. Data were expressed as the mean ± SEM. The P-values smaller than or equal to 0.05 were considered as significant.

3. Results

3.1. Effect of Polymerized-Type I Collagen on Toxicity Model. Treatments evaluated in this study have no *in vivo* toxicity for mice. It was evaluated by macroscopical and histopathological analysis of kidneys, heart, lungs, spleen, lymph nodes, and hind paws. All tissues resembled normal architecture. No inflammatory infiltrates or other abnormalities were observed (*data not shown*).

3.2. Effect of Polymerized-Type I Collagen on Attenuation of Early and Established Arthritis Model. To investigate the effect of polymerized-type I collagen in the progression of CIA, intramuscular administration of biodrug was initiated one week before booster of CII. The dose selected was based on our previous *in vitro* and *in vivo* results [16, 17]. Typically, DBA1/J mice developed signs of arthritis after the second immunization given at 21 day after the first CII immunization and showed maximum arthritis

around day 35–42 (Figure 1). Mice of early arthritis model treated with polymerized-type I collagen showed significant reductions in the severity of CIA compared with placebo (Figures 1(a) and 1(b)). On day 8–16, arthritis score and paw thickness reached their peaks. Polymerized Collagen and methotrexate/Polymerized Collagen treatments more than methotrexate alone significantly suppressed arthritis severity scores. A similar pattern of response was observed on established arthritis model (Figures 1(c) and 1(d)). Paw thickness was normal in early and established CIA models in groups treated with Polymerized Collagen and methotrexate/Polymerized Collagen (Figures 1(a) and 1(c)). However, methotrexate treatment does not diminish edema and induces fever ($\triangle \approx 1.5^{\circ}$C).

3.3. Histopathological Analysis of the Polymerized-Type I Collagen Effect on Early and Established Arthritis Model. Histopathological analysis was performed on hind paws of CIA mice harvested on day 35 and 98 after booster immunization by two blinded investigators. Representative images of hematoxylin and eosin-stained and PAS-stained joint tissue sections from the groups treated with placebo, Polymerized-Type I Collagen, methotrexate, and methotrexate/polymerized-type I collagen are presented in Figure 2. Mice showed typical arthritis, which is characterized by extensive infiltration of inflammatory cells, synovial hyperplasia, and bone erosion. Treatment with polymerized-type I collagen showed no signs of inflammation. Whereas methotrexate/polymerized-type I collagen resulted in significant reduction in cellular infiltration, pannus formation, and destruction of cartilage and bone in arthritic joints with a low pathogenic score of CIA mice in both models. Methotrexate induced other tissue abnormalities such as the presence of nodules (amorphous fibrin tissue like) and a low quality wound repair tissue as well as inflammatory infiltrates. The effect of polymerized-type I collagen and methotrexate/polymerized-type I collagen was sustained until the second sacrifice. Thus, histological evaluations confirmed the characteristic arthritic lesions and showed an excellent correlation with clinical grading (Figure 2).

3.4. Polymerized-Type I Collagen Modulates Th Spleen Cell Population on Early and Established CIA Model. To investigate Th cytokine patterns, mice were sacrificed by a blinded researcher at two different periods after booster immunization, on day 35 and 98. One control mice group without CIA was included. Cell suspensions from spleens were obtained and analyzed for *ex vivo* cytokine production by intracellular staining using flow cytometry (Figure 3).

Proportions of Th17 were consistently ~0.7% in splenocytes from mice without CIA. Higher percentage of IL-17-producing $CD4^+$ T cells were determined in CIA mice in early arthritis (2.5% and 4.3%, for the first and second sacrifice, resp. Figures 4(a) and 4(b)) and established arthritis (2.4% and 3.6%, for the first and second sacrifice, resp. Figures 4(c) and 4(d)). A reduction on percentage of IL-17A-producing $CD4^+$ T cells similar to normal levels was determined in mice with early and established arthritis treated with polymerized-type I collagen (1.2% and 1.3% for the first and second sacrifice in early arthritis, Figures 4(a) and 4(b) and 0.5% and 1.3% for the first and second sacrifice in established arthritis Figures 4(c) and 4(d)) and methotrexate/Polymerized Type I Collagen (0.8% and 1.4% for the first and second sacrifice in early arthritis, Figures 4(a) and 4(b) and 0.7% and 1.3% for the first and second sacrifice in established arthritis Figures 4(c) and 4(d)). Methotrexate treatment was not so effective compared to the biodrug and the mixture of it with methotrexate (1.4% and 2.2% for the first and second sacrifice in early arthritis, Figures 4(a) and 4(b) and 1.2% and 1.1% for the first and second sacrifice in established arthritis Figures 4(c) and 4(d)). Polymerized-Type I Collagen and methotrexate/Polymerized Collagen treatments had a sustained effect in early and established arthritis model until the second sacrifice (follow up) on the percentage of IL-17A-producing $CD4^+$ T (Figures 4(b) and 4(d)). IFN-γ-producing Th1 cells were also induced by CII *in vivo* immunization, and their frequency was similar than that of Th17 in early and established arthritis. However, IFN-γ producing Th1 cells were lower after treatment in established arthritis model (Figures 4(c) and 4(d)).

Proportions of Treg cells were consistently ~3.0% in splenocytes from mice without CIA. Lower percentage of Treg cells were determined in CIA mice in early arthritis (2.2% and 1.6%, for the first and second sacrifice, resp. Figures 4(a) and 4(b)) and established arthritis (1.5% and 1.2%, for the first and second sacrifice, respectively, Figures 4(c) and 4(d)). A statistically significant increase on percentage of Treg cells was determined in mice with early and established arthritis treated with polymerized-type I collagen (4.5% and 3.5% for the first and second sacrifice in early arthritis, Figures 4(a) and 4(b) and 2.4% and 2.5% for the first and second sacrifice in established arthritis Figures 4(c) and 4(d)) and methotrexate/Polymerized Collagen (4.4% and 3.0% for the first and second sacrifice in early arthritis, Figures 4(a) and 4(b) and 2.4% and 1.7% for the first and second sacrifice in established arthritis Figures 4(c) and 4(d)). Methotrexate-treated mice had a slightly increased response on percentage of Treg cells (2.0% and 1.6% for the first and second sacrifice in early arthritis, Figures 4(a) and 4(b) and 1.2% and 1.8% for the first and second sacrifice in established arthritis Figures 4(c) and 4(d)). However, this subpopulation was reduced in 50% compared to mice without CIA.

Taken together, these results indicate that polymerized-type I collagen is a biological compound that downregulated Th17 and upregulated Treg cell differentiation *in vivo* on early and established arthritis.

3.5. Effect of Polymerized-Type I Collagen on NF-κB on Early and Established CIA Model. We infer that polymerized-type I collagen mechanism of action might be mediated through the regulation of certain transcription factors such as NF-κB and AP-1. In particular, NF-κB regulates the expression of proinflammatory enzymes, cytokines, chemokines, immunoreceptors, and cell adhesion molecules as well as apoptosis. In the light of this knowledge, NF-κBp65 and IκBα were analyzed in splenocytes *ex vivo* (Figure 5). Percentage

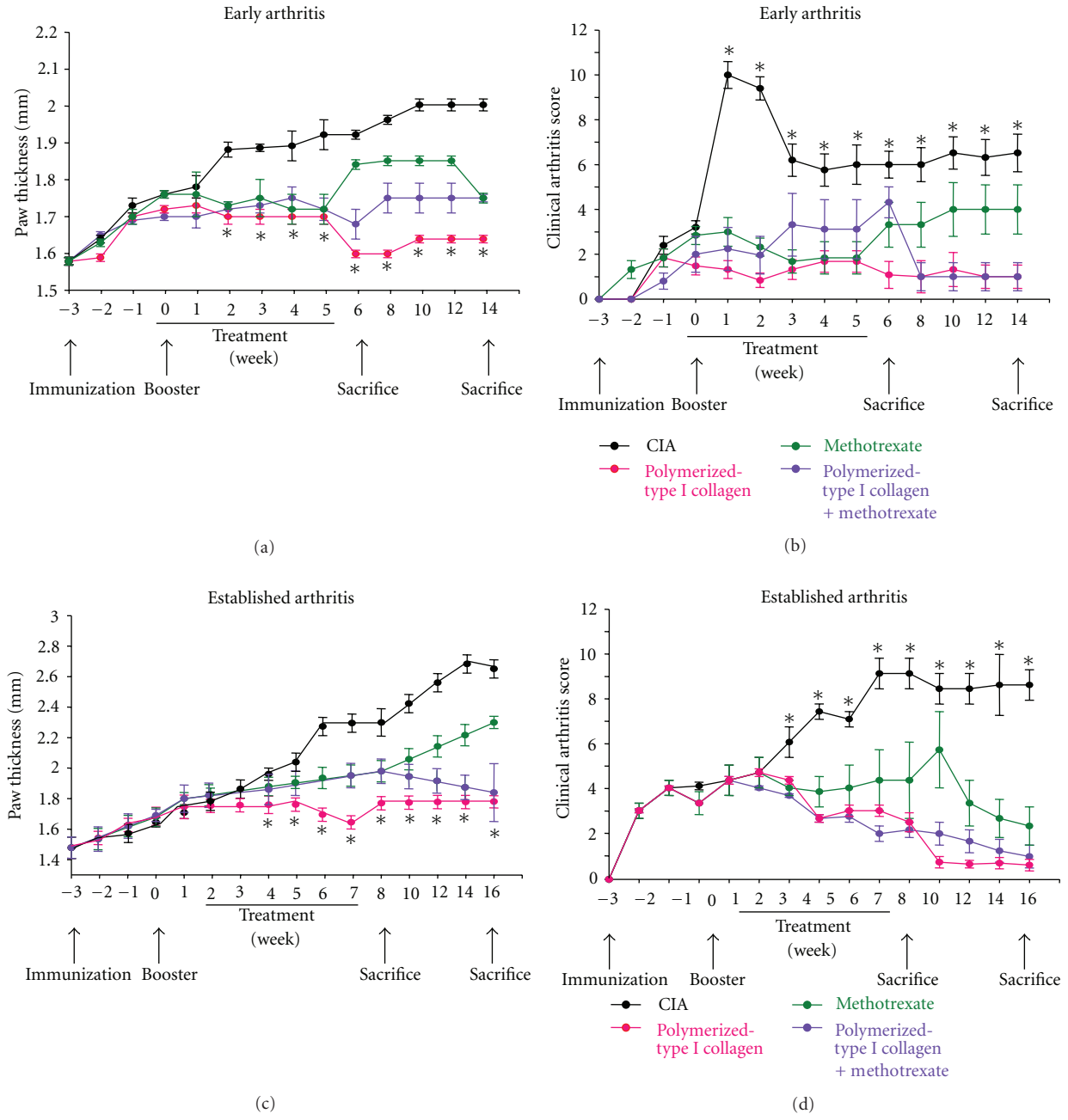

Figure 1: Preventive and therapeutic effects of polymerized-type I collagen on mouse CIA. (a) Paw thickness in early arthritis model. One hundred microliters of Polymerized Collagen, 100 μL of Polymerized Collagen, and 100 μL of methotrexate (2.5 mg/kg) or 100 μL of methotrexate (2.5 mg/kg)/Polymerized Collagen were administered intramuscular once a week during 6 weeks at the same time of that of the booster. Citric/citrate buffer was injected as a vehicle control. Data represent mean ± SEM (each group, $n = 6$). (b) Clinical arthritis score in early arthritis model ($n = 6$). (c) Paw thickness in established arthritis model. One hundred microliters of Polymerized Collagen, 100 μL of Polymerized Collagen, and 100 μL of methotrexate (2.5 mg/kg) or 100 μL of methotrexate (2.5 mg/kg)/Polymerized Collagen were administered intramuscularly once a week during 6 weeks. Treatments were started on 14 days after the booster. Citric/citrate buffer was injected as a vehicle control. Data represent mean ± SEM (each group, $n = 6$). (d) Clinical arthritis score in established arthritis model ($n = 6$). $^*P < 0.05$.

of NF-κBp65 (~1.5%), IκBα (~1.1%), and NF-κB/IκBα cells (~2.2%) were consistently found in splenocytes from mice without CIA.

Higher percentage of IκBα^+ and NF-κB$^+$/IκBα^+ cells were found in CIA mice under polymerized-type I collagen and methotrexate/polymerized-type I collagen treatment in early arthritis in the first and second sacrifice compared with CIA mice without treatment (Figures 5(a) and 5(b)). Interestingly, normal levels of NF-κBp65 were observed in CIA mice under Polymerized Collagen and methotrexate/Polymerized

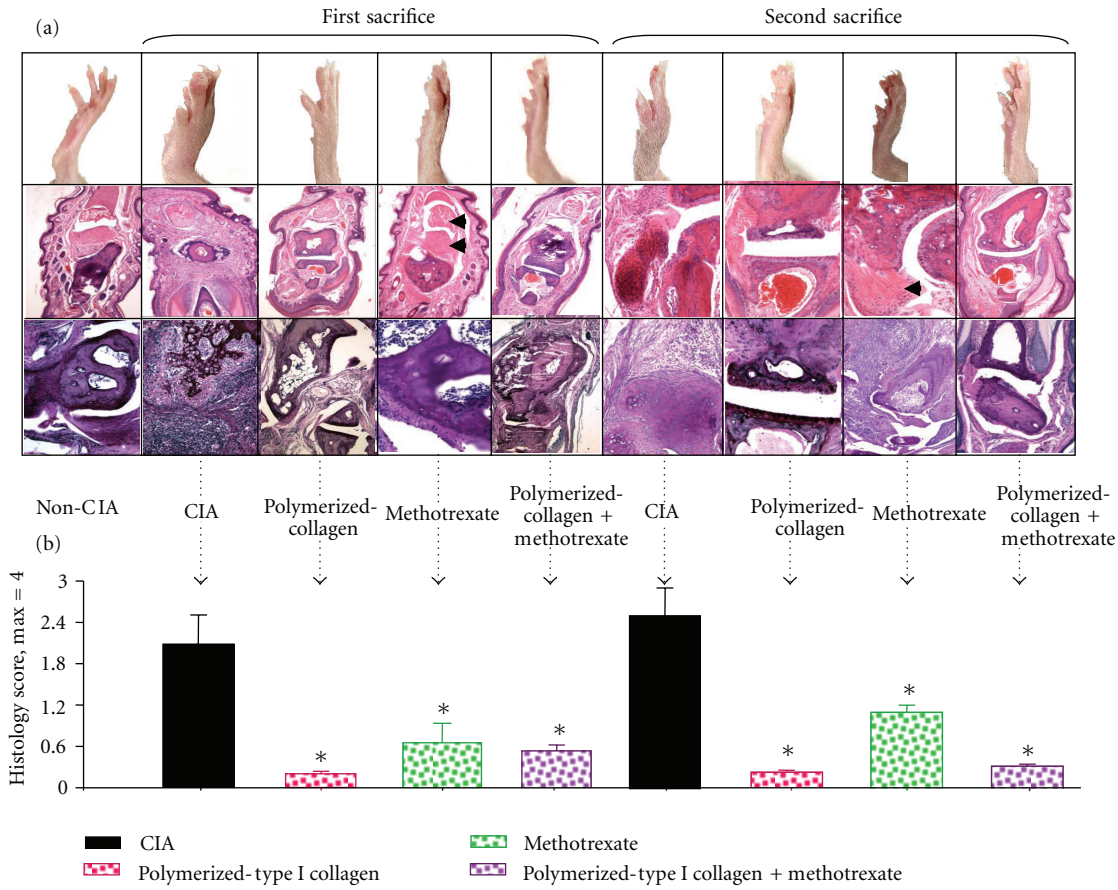

FIGURE 2: Preventive effect of polymerized-type I collagen on the histological damage in CIA mice. (a) Representative section of joint histopathology is shown. Upper panel: hematoxylin and eosin stained and lower panel: PAS stained; magnification: 10x. Arrow heads point out nodules. (b) Pathology scores of each group were calculated and expressed as mean ± SEM ($n = 6$). $^*P < 0.05$.

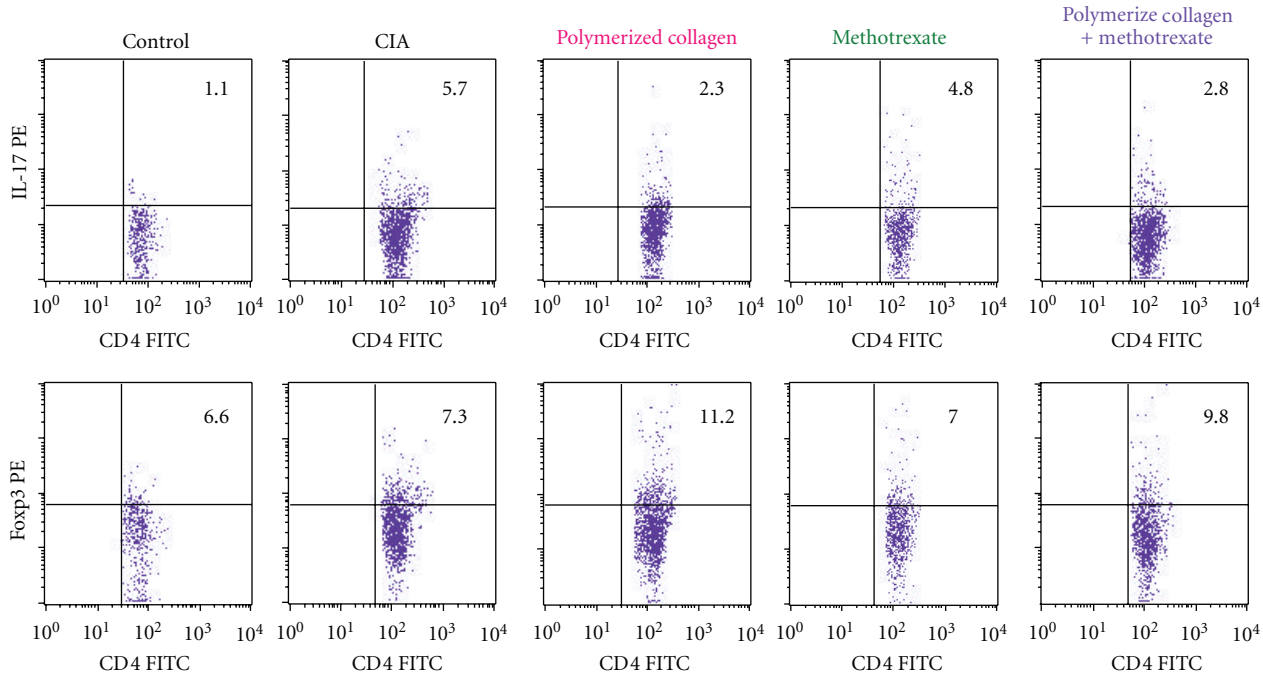

FIGURE 3: Representative flow plots of IL-17A- and Foxp3-expressing $CD4^+$ T cells.

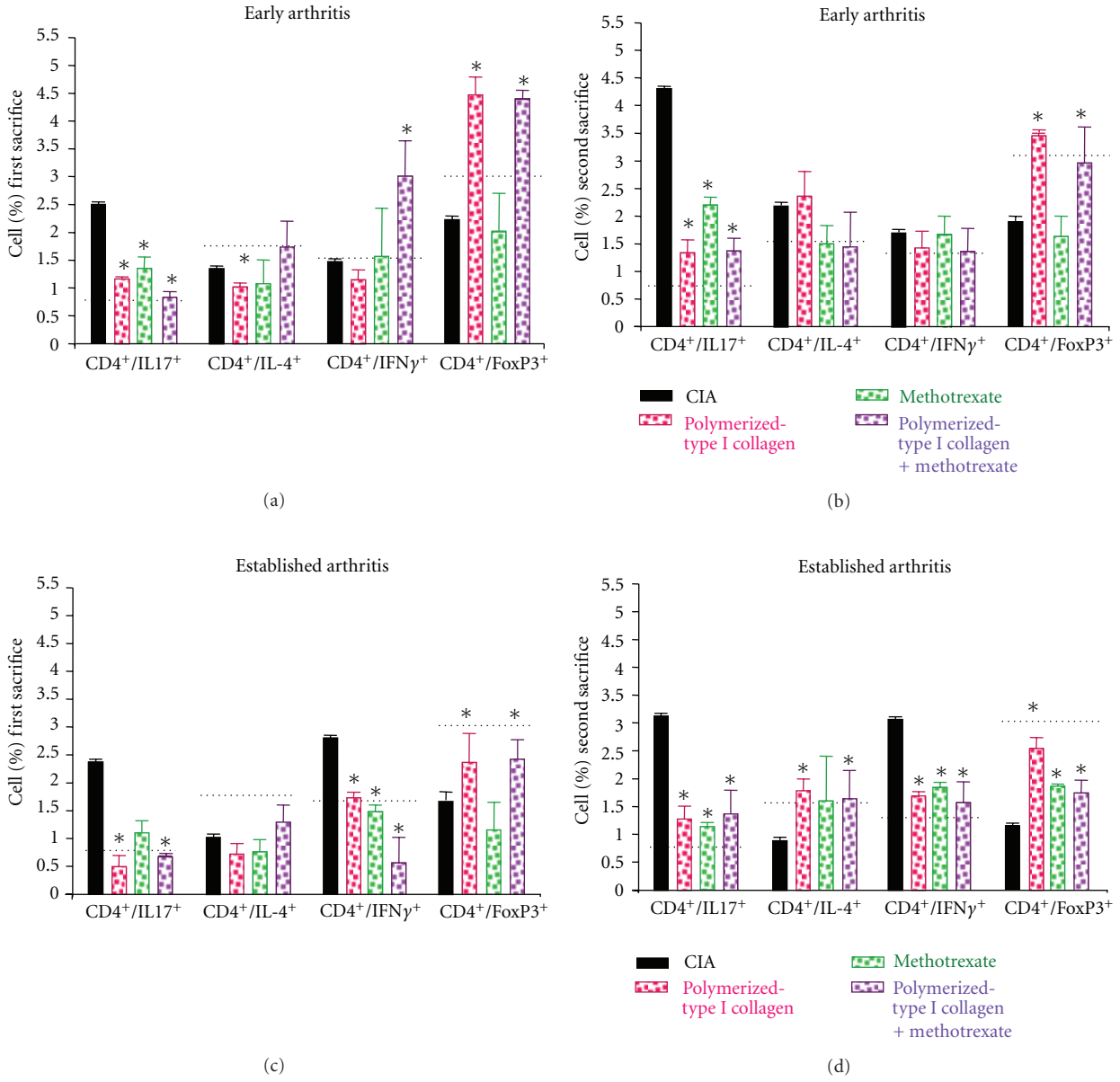

Figure 4: Effect of polymerized-type I collagen on the *ex vivo* intracellular cytokine production and on $CD4^+$ T cell subsets regulation in splenocytes. (a) Spleen cells obtained immediately *ex vivo* in early arthritis model on day 35 after booster immunization. (b) Splenocytes obtained immediately *ex vivo* in early arthritis model on day 98 after booster immunization. (c) Spleen cells obtained immediately *ex vivo* in established arthritis model during first sacrifice. (d) Splenocytes obtained from established arthritis model during second sacrifice. Intracellular production of IL-17A, IL-4, IFN-γ, and Foxp3 by $CD4^+$ T cells was detected by flow cytometry. Results are representative of 6 mice analyzed in each group. Horizontal dotted line represents mean normal values, obtained from mice ($n = 3$) without CIA. Data represent mean ± SEM. $^*P < 0.05$.

Collagen treatment in early arthritis during second sacrifice (Figure 5(b)). In established arthritis model, normal percentages of NF-κBp65 were found in mice under Polymerized Collagen, methotrexate, or methotrexate/Polymerized Collagen treatment in the first and second sacrifice (Figures 5(c) and 5(d)), as well as, IκBα levels during second sacrifice (Figure 5(d)). Finally, NF-κB^+/I$\kappa B\alpha^+$ cells were increased in treated mice compared with CIA mice in the first and second sacrifice (Figures 5(c) and 5(d)).

4. Discussion

In the present work, we demonstrated that polymerized-type I collagen as monotherapy as well as in combination with methotrexate exhibits both preventive and therapeutic effects on mouse CIA, through downregulation of Th17 subset and upregulation of Treg cells.

Th17 cells represent a new subset of T helper cells, which mainly produce IL-17A and IL-17F, and, to a lesser extent,

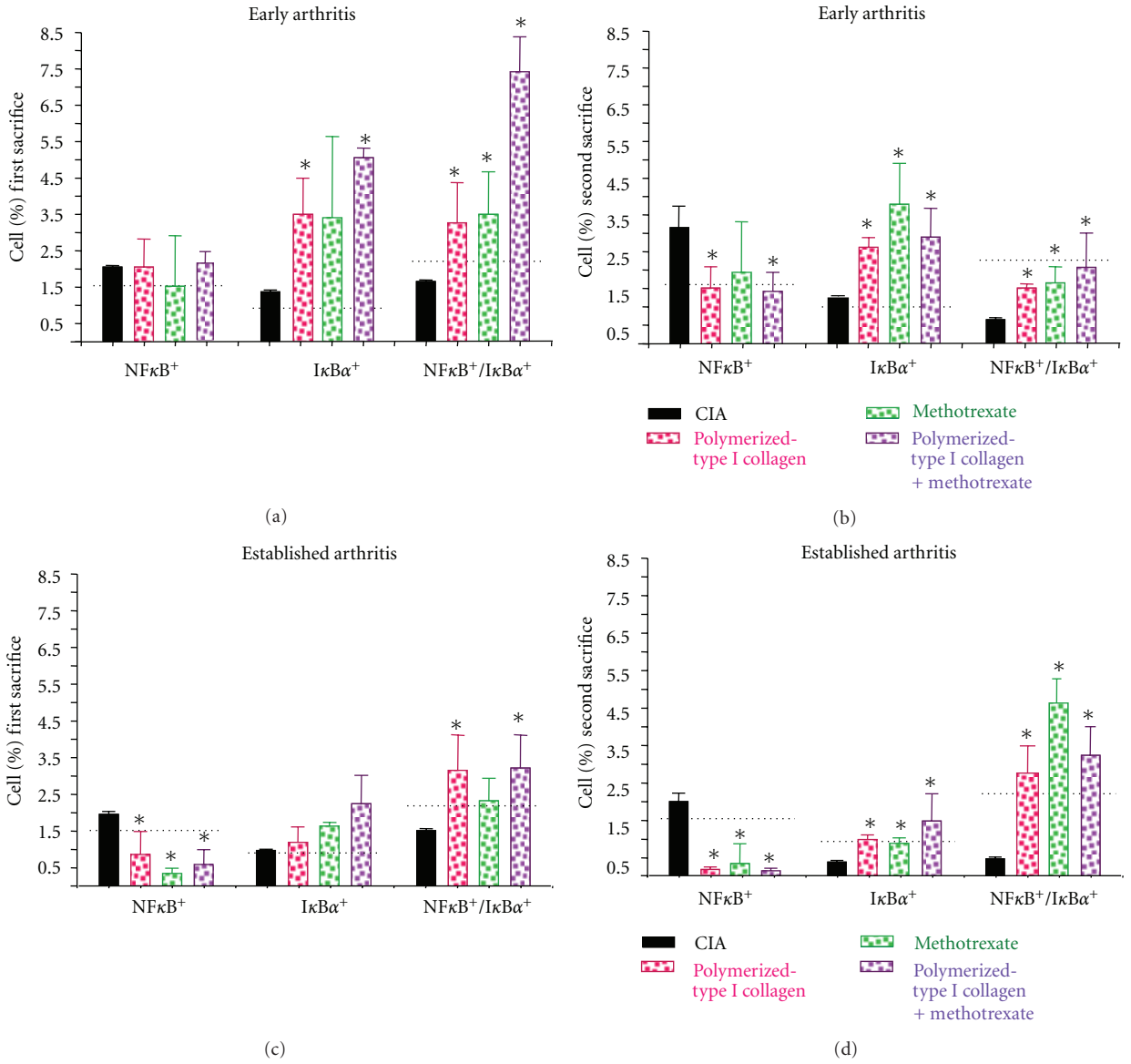

Figure 5: Effect of polymerized-type I collagen on the *ex vivo* NF-κB and IκB-α in splenocytes. (a) Spleen cells obtained immediately *ex vivo* in early arthritis model on day 35 after booster immunization. (b) Splenocytes obtained immediately *ex vivo* in early arthritis model on day 98 after booster immunization. (c) Spleen cells obtained immediately *ex vivo* in established arthritis model during first sacrifice. (d) Splenocytes obtained from established arthritis model during second sacrifice. Intracellular levels of NF-κBp65 and IκBα cells were detected by flow cytometry. Results are representative of 6 mice analyzed in each group. Horizontal dotted line represent mean normal values, obtained from mice ($n = 3$) without CIA. Data represent mean ± SEM. $^*P < 0.05$.

TNF-α and chemokines. The first report on IL-17-producing CD4$^+$ T cells came from a study of *in vitro*-primed TCR transgenic T cells where the addition of *Borrelia burgdorferi* lysate induced IL-17 production [21].

However, in the last years, the outstanding importance of Th17 cells has most convincingly been demonstrated in the pathogenesis of organ-specific autoimmune diseases. This was in fact, a broken paradigm, as previously Th1 cells had been regarded as the preponderant cell subpopulation driving autoimmune tissue damage. This concept was challenged when it became clear that IFN-γ and IFN-γ-receptor deficient mice were not protected from CIA but developed more severe disease [22–26]. Moreover, IL-17-deficient mice or mice treated with anti-IL-17 antibody reduce joint inflammation, cartilage destruction, and bone erosion [27–29]. Collectively, these data corroborate the importance of Th17 cells for the induction of autoimmune tissue inflammation.

Our results are in agreement with the subcutaneous, intramuscular or mucosal administration of type II collagen or altered CII263–272 peptide that suppress Th17 cells and expand regulatory T cells in the early stage of the disease [30–33]. However, the mechanism of action could be quite different meanwhile altered CII263–272 peptides

could bind to RA-associated HLA-DR4/1 with no T cell stimulating effects and might inhibit T cell activation in RA, polymerized-type I collagen could be acting likely as a tolerogenic molecule.

Methotrexate therapy is associated with depletion of all $CD4^+$ T cell subsets, including Tregs, and also with the development of rheumatoid nodules following improvement of arthritis, as has been reported in previous studies [34–36].

Methotrexate/polymerized-type I collagen induced effective downregulation of inflammatory T cell subsets although, unexpectedly, there was no synergistic effect between both treatments.

On the other hand, it is well known that impaired Treg function is also associated with pathogenesis of autoimmune disease, and that $CD4^+/CD25^+/Foxp3^+$ cells represent one of the major Treg cells involved in susceptibility/resistance to autoimmunity [37]. In the present experiments, we found that the percentage of Treg cells in the spleen of CIA mouse was significantly upregulated by Polymerized Collagen and methotrexate/polymerized-type I collagen treatments but not by methotrexate alone. These results support the hypothesis that the reciprocal downregulation of IL-17 expression at the time of upregulation of Foxp3 induced by Polymerized Collagen is involved in the balance of Treg/Th17, and this can lead to prevention or full blown autoimmune arthritis.

It is important to note that, 28 days after the last polymerized-type I collagen or methotrexate/polymerized-type I collagen administration, mice were without any sign of disease. Besides, side effects were not determined in mice under Polymerized-Type I Collagen, albeit methotrexate induced other tissue abnormalities such as the presence of nodules (amorphous fibrin tissue like) and hyperthermia ($\triangle \approx 1.5^\circ C$) determined during all the study, which was also previously described by Lange et al. [19].

Furthermore, animals treated with Polymerized Collagen in the early and established CIA model not only remained without clinical manifestations of the disease and without hyperthermia, but their joints were free from inflammatory cell infiltrates. Indeed, histological sections of ankle from polymerized-type I collagen but not methotrexate treatment showed normal joint tissues, without inflammatory infiltration of bone erosions and preservation of proteoglycans content (PAS staining), compared to CIA. Thus, histological evaluations confirmed the characteristic arthritic lesions and showed an excellent correlation with clinical grading.

NF-κB has been often termed a "central mediator of the immune response," because of its critical role in the control of key physiological and pathological states, from immune to autoimmune response. Regulation of the so-called "canonical" NF-κB transcription factor by the IKK complex involves its cytosolic-to-nuclear translocation mediated by the phosphorylation of the inhibitory molecule IκB-α [38]. Imbalance of NF-κB and IκBα has been associated with development of common inflammatory diseases including ulcerative colitis, Crohn's disease, rheumatoid arthritis, systemic lupus erythematosus, psoriatic arthritis, giant cell arthritis, type 1 diabetes, multiple sclerosis, celiac disease, and Parkinson's disease, as well as susceptibility of several cancers, such as oral squamous cell carcinoma, colorectal cancer, hepatocellular carcinoma, breast cancer, and myeloma. In the present work, we found that Polymerized Collagen alone or in combination with methotrexate is capable to increase IκB-α inhibitor in early and established arthritis models. Thus, it is not preposterous to speculate that, this biodrug could contribute considerably to the downmodulation of inflammation and tissue regeneration effects observed.

Summing up, polymerized-type I collagen may be one of the novel therapeutic candidates that can suppress autoimmune inflammation by regulating the T cell differentiation and the balance of pathogenic and regulatory T cells, in such a way that proinflammatory Th17 cells are downregulated and Treg cells are expanded. Besides, Polymerized Collagen induces downregulation of proinflammatory cytokine expression and tissue regeneration that could be regulated probably through NF-κB modulation. Our results shed further light into the preponderant role of polymerized-type I collagen in downregulation of inflammation and tissue regeneration and certainly deserve to be studied in depth in order to determine the precise mechanism(s) of action.

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Research Article

Th2 Regulation of Viral Myocarditis in Mice: Different Roles for TLR3 versus TRIF in Progression to Chronic Disease

Eric D. Abston,[1] Michael J. Coronado,[1] Adriana Bucek,[1] Djahida Bedja,[2] Jaewook Shin,[1] Joseph B. Kim,[1] Eunyong Kim,[1] Kathleen L. Gabrielson,[2] Dimitrios Georgakopoulos,[3] Wayne Mitzner,[1] and DeLisa Fairweather[1,2]

[1] *Department of Environmental Health Sciences, Johns Hopkins University Bloomberg School of Public Health, 615 N. Wolfe Street, Baltimore, MD 21205, USA*
[2] *Department of Pathology, Johns Hopkins University School of Medicine, 615 N. Wolfe Street, Baltimore, MD 21205, USA*
[3] *CVRx Inc., 9201 West Broadway Avenue, Minneapolis, MN 55445, USA*

Correspondence should be addressed to DeLisa Fairweather, dfairwea@jhsph.edu

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Viral infections are able to induce autoimmune inflammation in the heart. Here, we investigated the role of virus-activated Toll-like receptor (TLR)3 and its adaptor TRIF on the development of autoimmune coxsackievirus B3 (CVB3) myocarditis in mice. Although TLR3- or TRIF-deficient mice developed similarly worse acute CVB3 myocarditis and viral replication compared to control mice, disease was significantly worse in TRIF compared to TLR3-deficient mice. Interestingly, TLR3-deficient mice developed an interleukin (IL)-4-dominant T helper (Th)2 response during acute CVB3 myocarditis with elevated markers of alternative activation, while TRIF-deficient mice elevated the Th2-associated cytokine IL-33. Treatment of TLR3-deficient mice with recombinant IL-33 improved heart function indicating that elevated IL-33 in the context of a classic Th2-driven response protects against autoimmune heart disease. We show for the first time that TLR3 versus TRIF deficiency results in different Th2 responses that uniquely influence the progression to chronic myocarditis.

1. Introduction

Heart failure (HF) is the end consequence of a number of cardiovascular diseases including myocarditis and dilated cardiomyopathy (DCM). In spite of advances in diagnosis and treatment, HF remains a growing medical problem associated with major hospitalization, mortality, and poor prognosis [1]. Myocarditis is an autoimmune disease that is responsible for around half of all DCM cases in the United States [2–4]. A recent long-term study of myocarditis patients revealed that inflammation was the best predictor for the progression to HF following acute myocarditis [5]. Viruses like coxsackievirus B3 (CVB3) are often detected in patient myocardial biopsies [3, 6], and antiviral treatments such as interferon (IFN)-β reduce inflammation and HF in animal models and patients [7, 8], implying that viral infections are an important cause of myocarditis cases that lead to HF. Viral infections including CVB3 are able to induce autoimmune myocarditis that progresses to DCM in susceptible strains of mice [9, 10].

Toll-like receptor (TLR)3 binds to double-stranded RNA and inhibits viral replication by upregulating IFNs [11, 12]. TLR3 and TLR4 uniquely signal through TIR domain-containing adaptor protein-inducing IFN-β (TRIF) at the endosomal surface [12]. Recently, TLR3 polymorphisms in a patient population were associated with an increased occurrence of viral myocarditis and DCM [13]. Previously, TLR3- or TRIF-deficient mice were found to develop increased viral replication and acute myocarditis [11, 14]. Because TRIF participates in TLR3 and TLR4 signaling and TLR3 is known to protect against CVB3 myocarditis [14] while TLR4 increases disease [15], we were interested in determining whether TLR3- and TRIF-deficient mice developed similar disease.

To examine the effect of TLR3 versus TRIF deficiency on myocarditis, DCM, and HF, we used an autoimmune model

of CVB3 myocarditis where mice receive infectious virus and heart proteins [9, 16]. We found that TLR3-deficient mice developed a classic T helper (Th)2 response with increased interleukin (IL)-4 and markers of alternative activation during acute CVB3 myocarditis, while TRIF-deficient mice did not. Although both knockout strains developed similarly worse acute CVB3 myocarditis and viral replication compared to wild-type (WT) mice, disease was significantly worse in the absence of TRIF compared to TLR3. Our results indicate that TLR3 protects against CVB3 myocarditis by increasing IFN-γ and decreasing a classic IL-4-driven Th2 response, while TRIF protects by increasing IFN-β and decreasing the Th2-associated cytokine IL-33. Treatment of WT mice with recombinant (r)IL-33 increased CVB3 myocarditis and impaired cardiac function. However, in the context of an IL-4-skewed Th2 response (i.e., TLR3−/−), rIL-33 treatment prevented cardiac dysfunction. We show for the first time that TLR3 versus TRIF deficiency results in different Th2 responses that uniquely influence the progression to chronic myocarditis.

2. Materials and Methods

2.1. Experimental Model. Wild-type C57BL/6 (BL/6), B6.129, TLR3-(B6; 129S1-**Tlr3^{tm1Flv}/J**)-deficient (TLR3−/−) and TRIF-(C57BL/6-**Ticam1^{Lps2}/J**)-deficient (TRIF−/−) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained under pathogen-free conditions in the animal facility at Johns Hopkins School of Medicine, and approval was obtained from the Animal Care and Use Committee of the Johns Hopkins University for all procedures. CVB3 (Nancy strain) was obtained from the American Type Culture Collection (ATCC, VA), grown in Vero cells (ATCC) and passaged through the heart as described previously [16]. Mice inoculated ip with uninfected cardiac tissue supernatant diluted in PBS, or PBS alone, do not develop myocarditis (data not shown). Eight-to-ten-week-old male mice were inoculated with **10^3** PFU of heart-passaged CVB3 containing infectious virus and heart tissue diluted in sterile PBS or PBS alone ip and tissues collected at day 10 (acute myocarditis) or day 35 (chronic myocarditis) pi as described previously [16]. Recombinant rIL-33 (1 μg/0.1 mL, Cat#3626-ML) from R&D Systems (Minneapolis, MN) was diluted in sterile PBS, or PBS only was injected ip on days 1, 3, 5, 7, and 9 pi following CVB3 injection on day 0. All experiments were conducted three or more times with 7 to 12 mice per group except for rIL-33 treatment of TLR3-deficient mice.

2.2. Histology. Hearts were fixed in 10% buffered formalin and stained with haematoxylin and eosin (H&E) to assess inflammation. Myocarditis was assessed as the percentage of the heart section with inflammation compared to the overall size of the heart section using a microscope eyepiece grid according to [15]. The development of DCM was assessed by gross observation of histology sections at low magnification and by pressure-volume relationships, as previously described [17, 18].

2.3. Cardiac Function. Cardiac function was assessed by pressure-volume catheter (1.2F Scisense Inc., London, ON) placed in the left ventricle via the apex in open-chest mice anesthetized with 3% isoflurane (Baxter, Deerfield, IL), as previously described [19, 20].

2.4. Plaque Assay. Hearts from individual mice were homogenized at 10% weight/volume in 2% minimal essential medium (MEM) (MediaTech, Manassas, VA) and individual supernatants used in plaque assays to determine the level of infectious virus, as previously described [15]. Virus levels are expressed as the mean plaque-forming unit (PFU)/g tissue ± standard error of mean (SEM), and the limit of detection is 10 PFU/g of tissue.

2.5. ELISA. Hearts were homogenized at 10% weight/volume in 2% MEM and individual supernatants used in ELISA [15, 17]. Cytokines were determined in homogenized supernatants using R&D Systems ELISA kits (Minneapolis, MN), according to the manufacturer's instructions. Levels were expressed as pg/g of heart tissue ± SEM.

2.6. RNA Extraction and qRT-PCR. Hearts and spleens were harvested and flash frozen in liquid nitrogen and stored at −80°C. Tissues were homogenized in 2 mL TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The PureLink Micro-to-Midi Total RNA Purification System (Invitrogen) was used for extraction and purification of RNA. RNA was quantified using a NanoDrop spectrophotometer and quality assessed by RNA Nano LabChip analysis on an Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA). Total RNA from hearts or spleens was assessed by quantitative real-time (qRT) PCR using Assay-on-Demand primers and probe sets and the ABI 7000 Taqman System from Applied Biosystems (Carlsbad, CA). Data were normalized to hypoxanthine phosphoribosyltransferase 1 (HPRT) according to [21]. There was no significant difference in HPRT expression in the heart or spleen before or after infection. The mRNA data are presented as a relative gene expression (RGE). RGE is calculated as the ratio of target gene expression (fold change of mRNA of interest) to the normalization control gene expression (fold change of normalization control mRNA).

2.7. Statistical Analysis. Two-group analysis of normally distributed data was performed by Student's t-test. The Mann-Whitney U-test was used to evaluate nonparametric data comparing two groups. Multiple comparisons were analyzed by ANOVA with a Bonferroni correction. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Inflammation and Viral Replication Increase in a Similar Manner in TLR3- or TRIF-Deficient Mice during Acute CVB3 Myocarditis. Because TRIF participates in TLR3 and TLR4 signaling and TLR3 is known to protect against CVB3 myocarditis [14] while TLR4 increases disease [15], we were

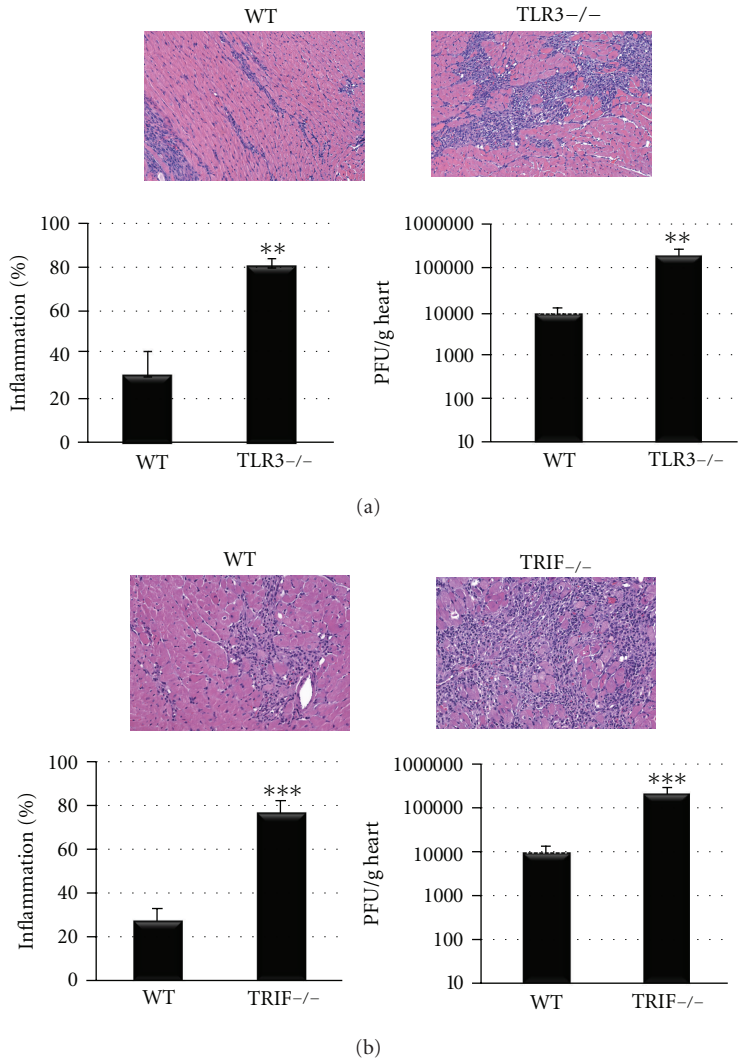

Figure 1: Inflammation and viral replication increase in a similar manner in TLR3-deficient (TLR3−/−) or TRIF−/− mice during acute CVB3 myocarditis. (a) TLR3−/− mice developed increased inflammation and viral replication at day 10 pi compared to WT B6.129 controls. (a) Representative histology sections of inflammation in WT and TLR3−/− hearts stained with H&E (top), magnification ×64. (b) TRIF−/− mice develop increased inflammation and viral replication at day 10 pi compared to WT BL/6 controls. (b) Representative histology sections of inflammation in WT and TRIF−/− hearts stained with H&E (top), magnification ×64. Data show the mean ± SEM of at least three separate experiments using 7 to 12 mice/group. **: $P < 0.01$, ***: $P < 0.001$.

interested in determining whether TLR3- and TRIF-deficient mice developed similar disease. As expected, there was a significant increase in viral replication and acute myocarditis at day 10 pi in TLR3- or TRIF-deficient mice compared to WT controls (Figure 1), confirming previous reports [11, 14]. When we began these studies, only TLR3-deficient mice on a B6.129 background were available, and it was only very recently that Jackson Laboratories began to offer TLR3-deficient mice on a BL/6 background. Our preliminary studies with TLR3-deficient mice on a BL/6 background provide similar results as we found with TLR3-deficient B6.129 mice (data not shown), but we have not performed a complete analysis of BL/6 TLR3-deficient mice. Importantly, both BL/6 and B6.129 WT strains had a very similar level of inflammation and viral replication in the heart at day 10 pi (Figure 1). Based on these results, it appears that activation of TLR3 and TRIF inhibits CVB3 myocarditis in a similar manner.

3.2. Deficiency in TLR3 or TRIF Has a Different Effect on Survival. Although the role of TLR3- or TRIF-deficiency appeared similar when examining the severity of viral replication and acute inflammation in the heart at day 10 pi (Figure 1), their effect on survival was distinctly different (Figure 2). Note that with this autoimmune CVB3 model of myocarditis nearly 100% of WT BL/6, B6.129, or BALB/c mice survive to day 35 pi [16, 17]. Most WT, and TLR3-deficient mice survived to day 35 pi (Figure 2). In contrast, 40% of TRIF-deficient mice died by day 25 pi ($P < 0.001$). These findings suggest that there are distinct differences in

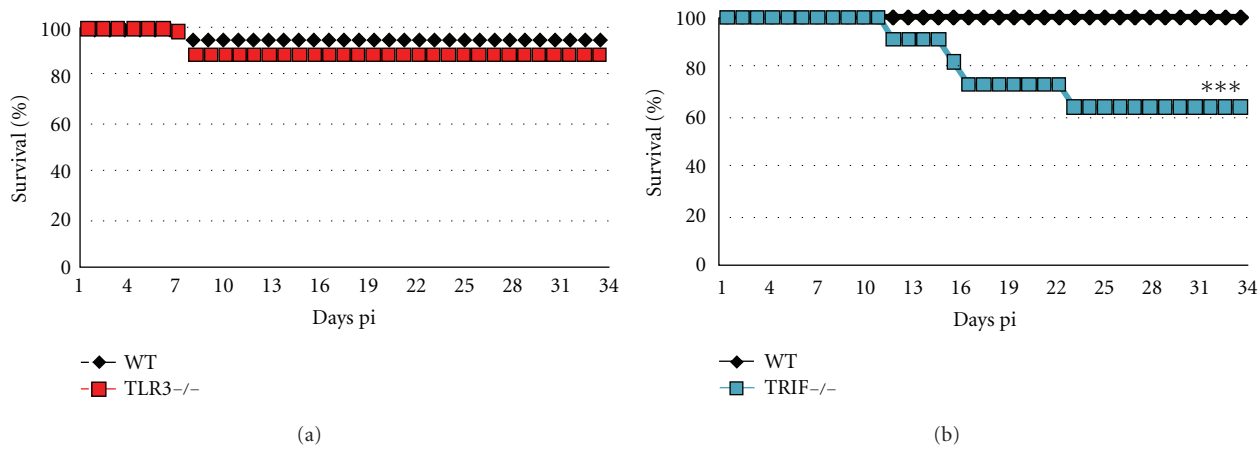

Figure 2: Survival differs between TLR3-deficient (TLR3−/−) and TRIF−/− mice with CVB3 myocarditis/DCM. (a) Survival of TLR3−/− mice compared to WT B6.129 controls (TLR3−/− versus WT, $n = 77$/group). (b) Survival of TRIF−/− mice compared to WT BL/6 controls (TRIF−/− versus WT, $n = 45$/group), ***: $P < 0.001$.

TLR3- versus TRIF-deficient mice that affect progression to chronic disease and HF.

3.3. TRIF-Deficient Mice Develop Significantly Worse Heart Function at Day 10 pi. Next, we compared heart function in TLR3- versus TRIF-deficient mice during acute CVB3 myocarditis by echocardiography. We found that although TLR3- or TRIF-deficient mice developed significantly worse heart function compared to their WT controls (Figures 3(a) and 3(b), resp.), that TRIF-deficient mice were significantly worse compared to TLR3-deficient mice (Figure 3(c)). Importantly, there were no differences in heart function as assessed by echocardiography during acute CVB3 myocarditis between WT B6.129 or BL/6 strains (Figure 3). Echocardiography in-dicated that TRIF-deficient mice were already dilated by day 10 pi (see left ventricular end diastolic dimension/LVEDD) (Figure 3). Dilation in TRIF-deficient mice during acute CVB3 myocarditis was also observed by histology and is indicated by a thinning of the left ventricular wall in TRIF-deficient mice that was not observed in TLR3-deficient mice at day 10 pi (Figure 4). In susceptible mouse strains like BALB/c or A/J, dilation does not usually develop until day 35 pi, while B6.129 and BL/6 strains are resistant to the development of DCM in this model [16]. These findings in-dicate that TRIF is critically important in protecting against progression to DCM in resistant strains of mice like BL/6 and B6.129.

3.4. TLR3 versus TRIF Deficiency Affects Heart Function Differently during Acute and Chronic CVB3 Myocarditis. Further evidence that TLR3 and TRIF have separate roles in regulating the progression from myocarditis to DCM was obtained by comparing acute and chronic heart function using pressure-volume relationships in WT and knockout mice. Recall that B6.129 and BL/6 WT mice are resistant strains that do not develop the chronic phase of autoimmune CVB3 myocarditis and DCM [16]. We confirmed that these WT strains did not develop DCM in this study (Table 1 and Figure 5, see EDV day 35 pi versus day 0). No significant difference was observed in the severity of acute myocarditis between B6.129 and BL/6 WT mice at day 10 pi (Figure 1), or in their cardiac function prior to infection (day 0) or at day 10 and 35 pi for most parameters (Table 1 and Figure 5). Thus, cardiac function in WT B6.129 and BL/6 mice during acute and chronic CVB3 myocarditis is nearly identical (Table 1).

In contrast, a comparison of TLR3 to TRIF-deficient mice using pressure-volume relations demonstrated significantly diminished left ventricular (LV) function and increased dilation in TRIF-deficient mice at day 10 and 35 pi (Figure 5). End diastolic volume (EDV) is a measure of LV dilation and was significantly increased in TRIF-deficient mice by day 10 and 35 pi compared to TLR3-deficient mice, which were not dilated similar to WT B6.129 and BL/6 mice (Figure 5(b)). Thus, once TRIF-deficient mice became dilated at day 10 pi, they remained dilated to day 35 pi (Figure 5(b), see EDV). During acute myocarditis, ejection fraction (EF) was significantly lower in TRIF- than TLR3-deficient mice (26% ± 2.8 versus 48% ± 4.7, $P < 0.05$). An EF less than 40% indicates the risk for heart failure [22]. The peak rate of pressure rise (dP/dT Max) was significantly lower in TRIF-deficient mice compared to TLR3-deficient mice (5535 ± 688 versus 8803 ± 760 mmHg/s, $P < 0.05$). End systolic pressure (ESP) was significantly lower in TRIF- compared to TLR3-deficient mice during acute myocarditis (65 ± 4.5 versus 96 ± 4.7 mmHg, $P < 0.05$). Thus, many important functional parameters were significantly worse in TRIF- compared to TLR3-deficient mice by day 10 pi that persisted to day 35 pi, but these changes were not observed in WT mice (Figure 5 and Table 1). In fact, most functional parameters were not significantly different between BL/6 and B6.129 mice at baseline (day 0), day 10, or day 35 pi (Table 1). Thus, TRIF-deficient mice rapidly progressed to DCM and HF (e.g., low EF and reduced survival), while TLR3-deficient mice did not.

3.5. TLR3- and TRIF-Deficient Mice Differ in the Type of Th2 Response Induced during Acute Myocarditis. Wild-type BL/6

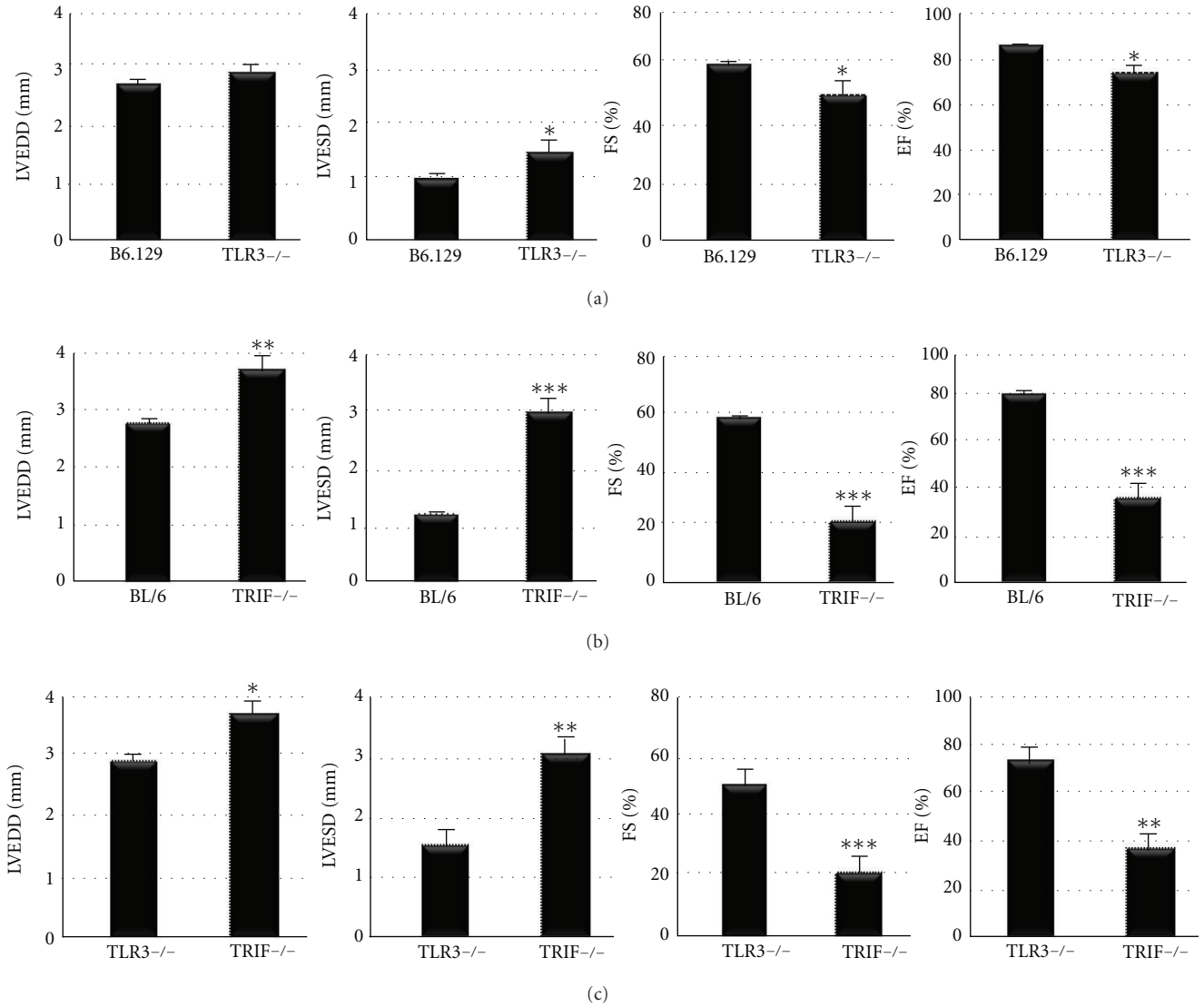

Figure 3: TRIF-deficient mice develop significantly worse heart function at day 10 pi. Echocardiography was used to assess heart function in (a) TLR3-deficient (TLR3−/−) versus WT B6.129 mice, (b) TRIF-deficient (TRIF−/−) versus WT BL/6 mice, or (c) TLR3- versus TRIF-deficient mice. LVEDD, left ventricular end diastolic dimension; LVESD, left ventricular end systolic dimension; FS, fractional shortening; EF, ejection fraction. Data show the mean ± SEM of at least three separate experiments using 7 to 12 mice/group. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

and B6.129 mice characteristically produce Th1-type cytokines in response to infections or other stimuli. Because TLR3- and TRIF-deficient mice are known to have a defective Th1 response (i.e., reduced IFNs) following CVB3 infection [11, 14], we examined whether the Th1/Th2 balance had shifted in TLR3- and TRIF-deficient mice during acute myocarditis at day 10 pi by examining cytokine levels in the heart by ELISA. Previous reports had not described the type of Th2 response TLR3- or TRIF-deficient mice develop during CVB3 myocarditis [11, 14], and since susceptibility to chronic autoimmune myocarditis is dependent on a Th2-type immune response [16, 17], we were interested in determining whether a shift had occurred. We found that TLR3-deficient mice had significantly increased IL-4 ($P = 0.03$) and decreased IFN-γ ($P = 0.03$) levels in the heart at day 10 pi (Figure 6), suggesting a shift to a Th2 response. In contrast, TRIF-deficient mice developed a different type of Th2 response characterized by significantly reduced IFN-β (a Th1 cytokine) ($P = 0.009$) and increased IL-33 (a Th2 cytokine) ($P = 0.007$) (Figure 6). To our knowledge, there are no reports describing that TLR3- or TRIF-deficient mice develop different types of Th2 responses.

Although the level of myocarditis, viral replication, and cardiac function can be compared between different experiments, a direct comparison of cytokines in WT mice should not be made because cytokine levels vary considerably between experiments (even in the same mouse strain) due to differences in processing the samples. So even though it appears as if BL/6 mice have higher levels of IL-4 and IFN-β during acute myocarditis (Figure 6, WT for TRIF−/−)

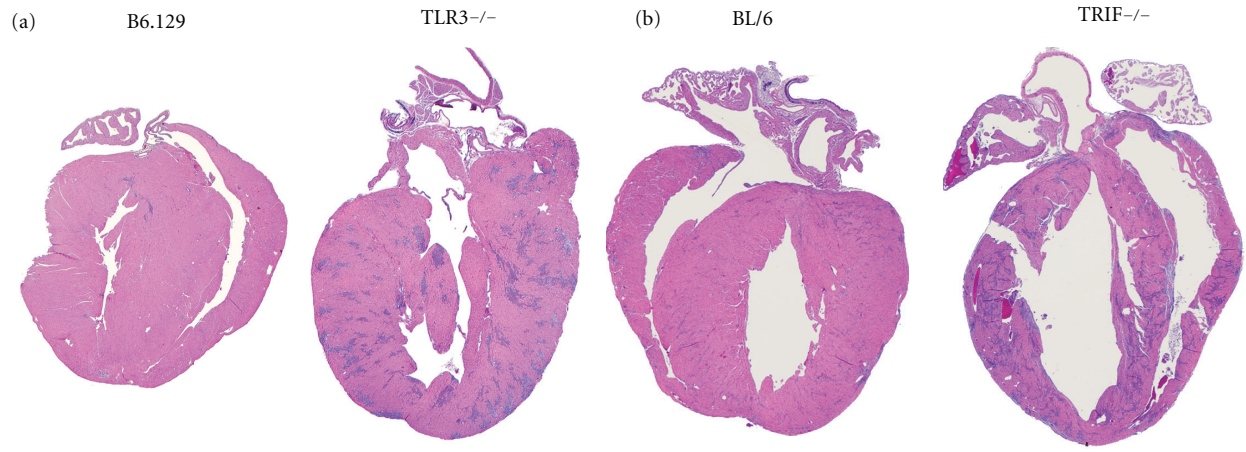

Figure 4: TRIF-deficient (TRIF−/−) mice develop DCM by day 10 pi, but TLR3−/− mice do not. Representative histology sections of (a) TLR3−/− compared to WT B6.129 controls at day 10 pi, magnification ×5. (b) Representative histology sections displaying dilation in TRIF−/− compared to WT BL/6 mice at day 10 pi, magnification ×5.

compared to B6.129 mice (Figure 6, WT for TLR3−/−), this may only be an artifact of processing. We did not conduct an experiment to directly compare Th1 versus Th2 cytokine levels in WT BL/6 versus B6.129 mice. Thus, the only valid comparison for cytokine levels is the WT to the knockout. We consistently observed that TLR3-deficient mice developed significantly increased IL-4 and reduced IFN-γ, while TRIF-deficient mice developed significantly increased IL-33 and reduced IFN-β.

3.6. TLR3-Deficient Mice Have Increased Markers of Alternative Macrophage Activation during Acute Myocarditis. To confirm that the increased IL-4 and decreased IFN-γ levels that we had observed in the heart of TLR3-deficient mice during acute myocarditis were due to a shift to a classic Th2 response, we examined markers of IL-4-driven alternative activation in the heart by qRT-PCR (Figure 7) [23, 24]. We found that arginase-1 (Arg-1), chitinase (Ym1), the IL-4 receptor (IL-4R), and the macrophage mannose receptor (Mrc1) were significantly increased in TLR3-deficient hearts compared to WT controls (Figure 7), indicating a switch to a Th2 response. Note the high-fold increase in Arg1 and Ym1 in TLR3-deficient hearts compared to WT controls or TRIF-deficient mice (Figure 7). In contrast, these classical markers of alternative activation were not increased in the heart of TRIF-deficient mice except for the macrophage mannose receptor (Figure 7). These data show that TLR3-deficient mice switch to a Th2 response during CVB3 myocarditis, while TRIF-deficient mice develop a distinctly different cytokine response involving the Th2-associated cytokine IL-33.

3.7. IL-33 Decreases Cardiac Function in WT BL/6 Mice. Our data showed that although TRIF-deficient mice developed similar myocarditis compared to TLR3-deficient mice, they had significantly worse cardiac function. The elevation of IL-33 in TRIF-deficient hearts suggested that this cytokine could be responsible for increasing cardiac dysfunction in TRIF-deficient mice. We treated WT BL/6 mice with recombinant rIL-33 or PBS on day 1 through 9 pi following CVB3 infection on day 0 and examined heart function at day 10 pi. We found that rIL-33 treatment increased acute CVB3 myocarditis (Figures 8(a) and 8(c)), increased cardiac IL-33 levels (Figure 8(b)), and decreased cardiac function at day 10 pi (Figure 8(d)), indicating that elevated IL-33 in TRIF-deficient mice could lead to poor heart function in this strain.

3.8. IL-33 Improves Cardiac Dysfunction in TLR3-Deficient Mice during Acute Myocarditis. Because IL-33-treatment was found to decrease cardiac function during acute CVB3 myocarditis in WT mice, we tested whether rIL-33 treatment of TLR3-deficient mice (that have a classic Th2 response) could worsen myocarditis. rIL-33 was administered ip to TLR3-deficient mice every other day from day 1 to 9 pi, as above, and heart function examined at day 10 pi. Surprisingly, pressure-volume analysis of heart function showed that rIL-33-treated TLR3-deficient mice had significantly improved heart function during acute CVB3 myocarditis (Figure 9, Table 2). Thus, in the context of a Th2-driven IL-4 response elevated IL-33 protects the heart from cardiac dysfunction during acute CVB3 myocarditis.

4. Discussion

Proinflammatory Th1 and Th17 immune responses are known to be important in the pathogenesis of experimental autoimmune myocarditis (EAM) and autoimmune CVB3 myocarditis mouse models [25–27]. Interestingly, Th2 responses have also been implicated in the pathogenesis of autoimmune myocarditis promoting DCM and HF [2, 17, 28, 29]. Only Th2-type responding susceptible mouse strains like BALB/c and A/J progress to chronic myocarditis and DCM in EAM and CVB3 myocarditis models [9, 16]. Additionally, IFN-γ-deficient mice, which have an elevated IL-4/Th2 response, develop severe DCM and HF following CVB3 myocarditis or EAM [17, 18]. Furthermore, IL-4 was found to increase EAM using rIL-4 treatment or anti-IL-4 antibodies [2, 28]. Evidence so far suggests that IL-4 increases

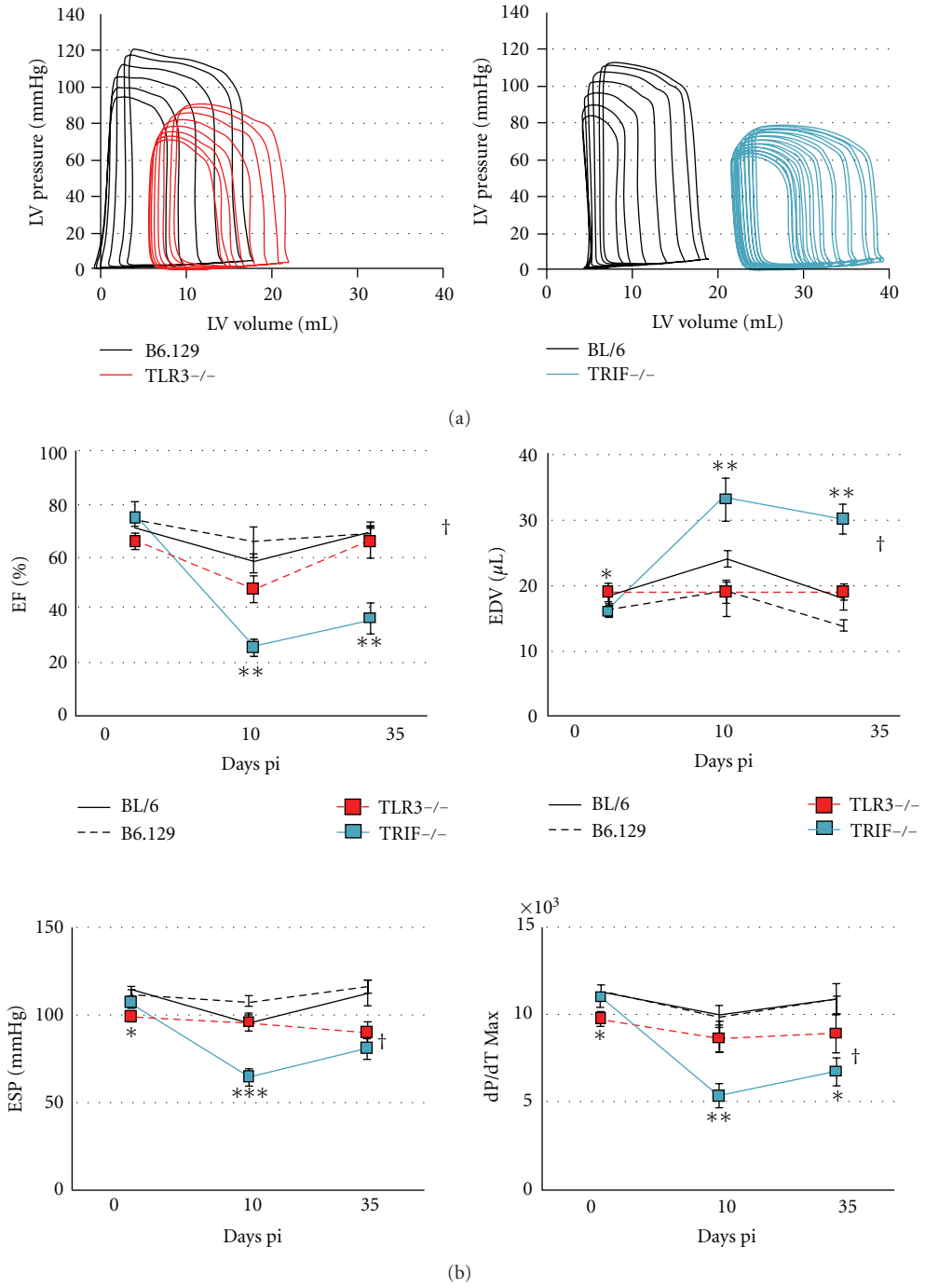

Figure 5: Cardiac function differs between TLR3 deficient (TLR3−/−) and TRIF−/− mice during acute and chronic CVB3 myocarditis. (a) Representative pressure-volume loops for TLR3−/− (left) and TRIF−/− (right) hearts at day 10 pi compared to their WT controls. (b) Comparison of pressure-volume relationships of TLR3−/−, TRIF−/− and WT mice at day 10 pi, $n = 10$ to 12 mice/group. Student's t-test compares TLR3−/− to TRIF−/− at day 0, 10 or 35 pi; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. ANOVA compared TLR3−/− to TRIF−/− mice over time; †, $P < 0.05$. EF: ejection fraction; dP/dT Max (mm Hg/s) measures the peak rate of pressure rise; ESP: end systolic pressure; EDV: end diastolic volume.

autoimmune myocarditis by elevating autoantibody production and activating mast cells and alternatively activated macrophages that produce cytokines and enzymes needed for remodeling and fibrosis [2, 9, 17, 30–32].

In this study, TLR3-deficient mice had significantly increased markers of alternative activation and switched from IFN-γ to increased IL-4 production during acute CVB3 myocarditis, yet they did not develop severe DCM and HF. This suggests that an IL-4-driven Th2 response is not capable on its own of inducing HF, at least not in resistant strains of mice. Additionally, administration of rIL-33 to Th2-skewed TLR3-deficient mice reduced cardiac dysfunction during

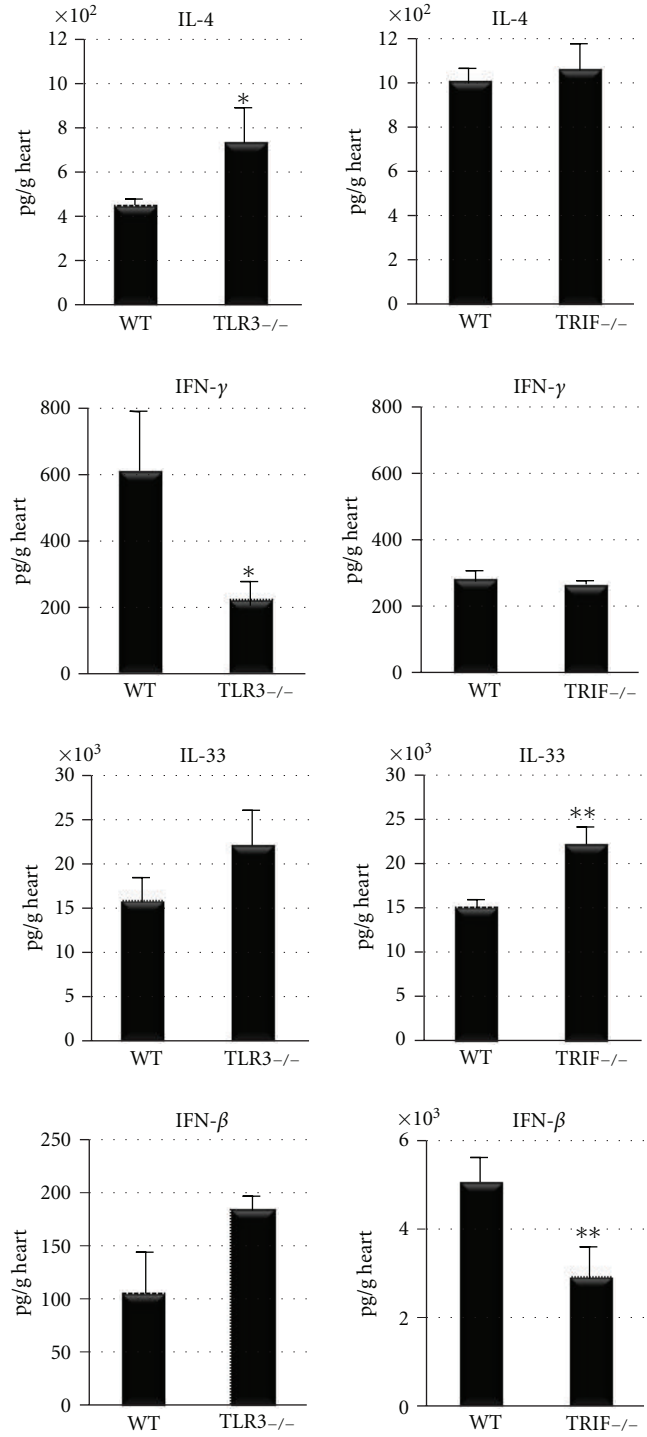

Figure 6: TLR3-deficient (TLR3−/−) mice have reduced IFN-γ and elevated IL-4 levels in the heart compared to WT B6.129 controls, while TRIF−/− mice have reduced IFN-β and increased IL-33 com-pared to WT BL/6 mice by ELISA. Similar results were obtained in at least three separate experiments and show the mean ± SEM of 7 to 12 mice/group. *: $P < 0.05$; **: $P < 0.01$.

acute myocarditis. IL-33 is known to be able to induce Th2 responses on its own or with IL-4, but IL-33 is unique in that it can also increase proinflammatory Th1-type immune responses [33]. Our data show that if IL-33 is elevated when IL-4 is not also high, it has deleterious consequences on heart function. We are currently investigating the mechanism of action of IL-33, which is outside the scope of this paper. Although the mechanism has not yet been elucidated, the difference in cardiac function and survival/HF between TLR3- and TRIF-deficient mice suggests that TLR4-mediated TRIF

Table 1: Similarity in cardiac function of C57BL/6 versus B6.129 mice prior to infection (day 0) and during acute (day 10) or chronic (day 35) CVB3 myocarditis based on pressure-volume analysis.

	Day 0		Day 10 pi		Day 35 pi	
Parameter	BL/6	B6.129	BL/6	B6.129	BL/6	B6.129
HR	591 ± 3.9	596 ± 3.7	535 ± 6.0	567 ± 11.5*	569 ± 9.0	601 ± 5.7**
ESP	114 ± 2.1	111 ± 3.8	98 ± 2.1	108 ± 2.6**	109 ± 3.9	116 ± 3.4
EDP	6.3 ± 0.6	6.9 ± 2.5	4.1 ± 0.5	5.5 ± 0.5	4.8 ± 0.4	4.6 ± 0.7
dP/dT Max	11336 ± 463	10766 ± 250	10019 ± 538	10241 ± 395	11042 ± 852	10658 ± 555
dT/dT Min	−10628 ± 290	−10022 ± 309	−8484 ± 306	−9549 ± 454	−10017 ± 528	10852 ± 432
EF	71 ± 4.3	74 ± 2.2	58 ± 3.5	66 ± 5.7	69 ± 4.4	69 ± 2.4
ESV	5.7 ± 1.1	4.0 ± 0.4	10 ± 1.1	7.4 ± 1.8	6 ± 1.0	4 ± 0.2
EDV	18 ± 1.2	16 ± 1.0	24 ± 1.2	18 ± 2.5	18 ± 1.7	14 ± 0.7
CO	7499 ± 408	6940 ± 318	7276 ± 495	6083 ± 485	6885 ± 672	5960 ± 417
Ees	9.2 ± 0.8	8.3 ± 0.9	7.5 ± 0.5	8.4 ± 1.0	11.5 ± 1.2	15 ± 1.4
Ea/Ees	1.05 ± 0.1	1.3 ± 0.2	1.0 ± 0.0	1.1 ± 0.2	0.9 ± 0	0.8 ± 0.06
V_0	−7.7 ± 1.3	−11.3 ± 1.6	−5.4 ± 1.2	−6.4 ± 2.1	−4.2 ± 1.9	−5.6 ± 1.0
Tau	5.2 ± 0.2	5.1 ± 0.3	5.6 ± 0.2	5.6 ± 0.2	5.6 ± 0.2	5.1 ± 0.2

CO (μL/min), cardiac output; dP/dT Max, peak rate of pressure rise (mmHg/s); dP/dT Min, peak rate of pressure decline (mmHg/s); Ea/Ees, arterial elastance normalized to Ees; EDP (mmHg), end diastolic pressure; EDV (μL), end diastolic volume; Ees (mmHg/μL), LV end systolic elastance (stiffness); EF (%), ejection fraction; ESP (mmHg), end systolic pressure; ESV (μL), end systolic volume; HR (bmp), heart rate; PFR/EDV (s^{-1}), peak flow rate normalized to EDV; PMX/EDV^2, maximum ventricular power normalized to EDV^2 ($mW/\mu L^2$) × 100; PRSW (mmHg), preload recruitable stroke work; SV (μL), stroke volume; SW, stroke work; Tau, Weiss (ms), time constant of diastolic relaxation; V_0 (μL), X-intercept of the ESP-volume relationship. * : $P < 0.05$, ** : $P < 0.01$, and *** : $P < 0.001$ compare BL/6 to B6.129 by Student's t-test at each timepoint. Data shown as mean ± SEM for 10 mice/group per timepoint.

Table 2: *In vivo* hemodynamics of recombinant (r)IL-33-treated TLR3-deficient mice during acute CVB3 myocarditis (day 10 pi) based on pressure-volume analysis.

Parameter	PBS	rIL-33	P value
Heart rate	560 ± 12.0	555 ± 7.6	0.75
Developed pressure	66 ± 5.6	82 ± 3.9	0.04
EDP	11 ± 1.6	6 ± 0.8	0.04
dP/dT Max	4891 ± 537	7177 ± 523	0.01
dP/dT Min	−3597 ± 446	−6168 ± 850	0.02
EF	54 ± 4.0	60 ± 2.8	0.24
ESV	8 ± 1.6	5 ± 1.7	0.27
EDV	17 ± 1.9	14 ± 3.2	0.34
CO	5.0 ± 0.43	4.5 ± 0.96	0.58
PMX	4 ± 0.7	6 ± 0.7	0.03
PRSW	35 ± 6.0	62 ± 11.2	0.04
Ees	5 ± 0.4	14 ± 1.0	0.00001
E Max	13 ± 2.7	32 ± 3.9	0.002
Ea/Ees	1.7 ± 0.18	0.89 ± 0.19	0.01

CO, cardiac output (μL/min); dP/dT max, peak rate of pressure rise (mmHg/s); dP/dT min, peak rate of pressure decline (mmHg/s); EDV, end diastolic volume (μL); Ees, LV end systolic elastance; EF, ejection fraction (%); developed pressure, (ESP-EDP) (mmHg); ESV, end systolic volume (μL); PRSW, preload recruitable stroke work; PMX, maximum ventricular power (mW); Ea/Ees, arterial elastance normalized to Ees; E Max, slope of line from end systole to end diastole. P values compare PBS-treated TLR3−/− to rIL-33-treated TLR3−/− by Student's t-test at day 10 pi. Data are shown as mean ± SEM for 11 to 12 mice/group. All mice were infected with CVB3 10 days prior to assessment.

may be responsible for reducing the negative cardiac effects of IL-33 in the heart during acute CVB3 myocarditis. We showed previously that more severe acute CVB3 myocarditis in male BALB/c mice is associated with elevated $TLR4^+$ IL-$1\beta^+$ alternatively activated M2 macrophages in the heart [34]. Perhaps elevated IL-33 negatively impacts heart disease by increasing a proinflammatory M2 macrophage population in the heart leading to remodeling and HF. We are currently investigating this possibility.

Previous reports have found that increased myocarditis and HF in TLR3- or TRIF-deficient mice is due primarily to increased viral replication [11, 14]. However, this study suggests that high viral replication does not necessarily result in HF because TLR3-deficient mice had high levels of viral

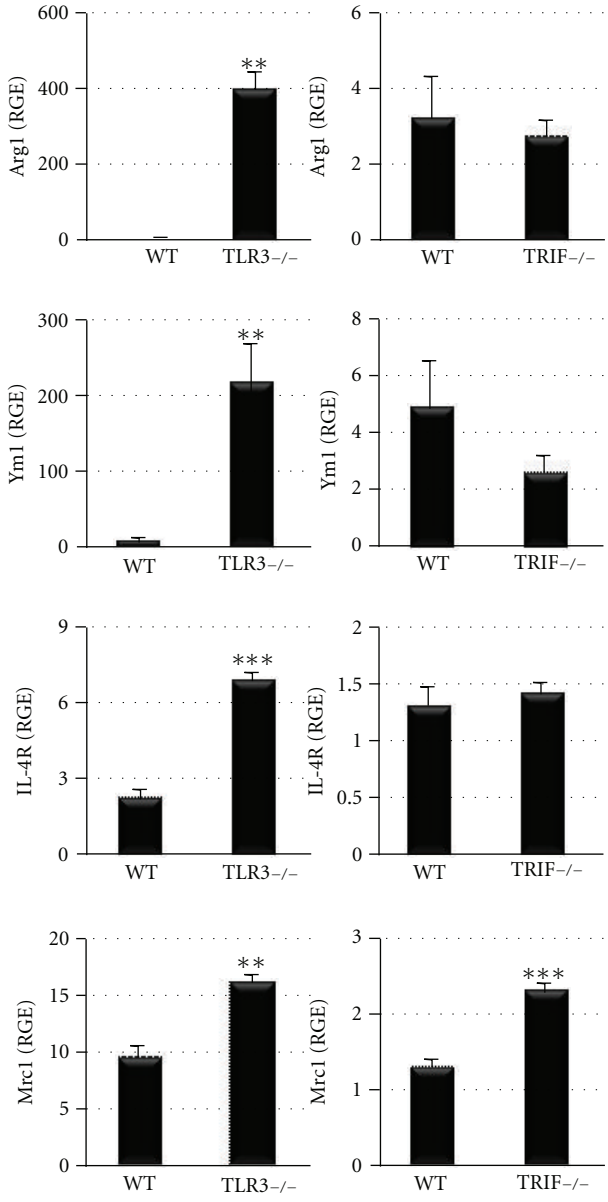

Figure 7: TLR3-deficient (TLR3−/−) mice have increased markers of IL-4-driven alternative activation compared to TRIF−/− mice. Markers of alternative activation included arginase-1 (Arg1), chitinase (Ym1), IL-4R, and macrophage mannose receptor (Mrc1) by qRT-PCR. Relative gene expression (RGE) was normalized to hypoxanthine phosphoribosyltransferase 1 (HPRT). Data show the mean ± SEM of 10 mice/group. **: $P < 0.01$; ***: $P < 0.001$.

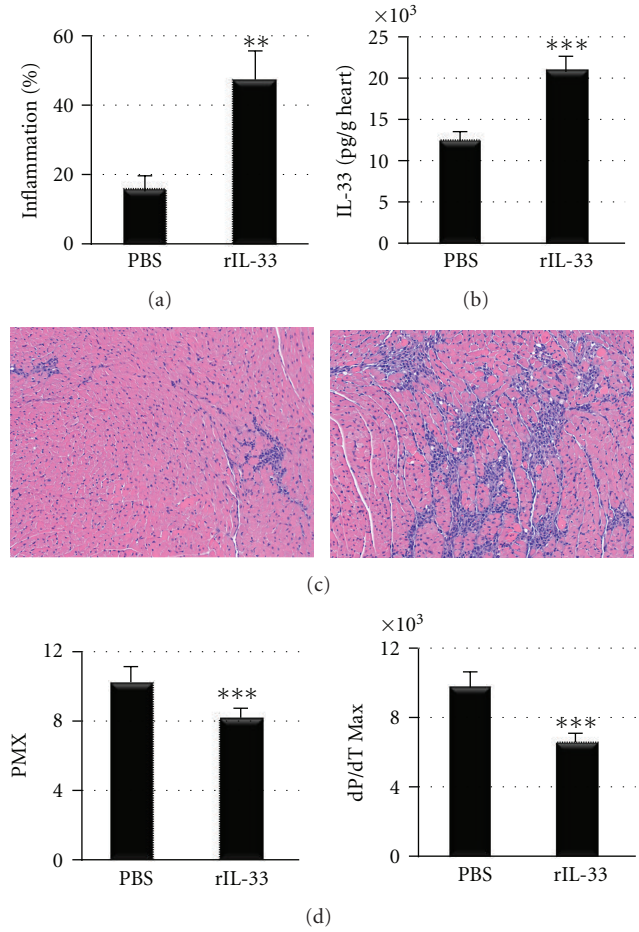

Figure 8: Recombinant IL-33 treatment increases myocarditis and cardiac dysfunction in BL/6 mice. Male BL/6 mice were treated with recombinant IL-33 (rIL-33) or PBS every other day from day 1 to 9 pi and (a,c) myocarditis, (b) cardiac IL-33 levels by ELISA and (d) heart function assessed using pressure-volume relationships at day 10 pi. (c) Representative histology sections of inflammation in PBS-treated (left) and rIL-33-treated (right) BL/6 mice stained with H&E, magnification ×64. Data show the mean ± SEM of 10 mice/group. **: $P < 0.01$; ***: $P < 0.001$.

replication in the heart but were protected from severe chronic disease and HF. These findings suggest that additional factors beside direct viral damage are required for progression to DCM and HF such as elevated IL-33. IL-33 has been called an "alarmin" because it acts as a nuclear transcription factor until it is released from damaged cells when it acts as a cytokine via its receptor [35]. We show in this study that high viral replication does not itself account for increased IL-33 levels in the heart during CVB3 myocarditis because similar amounts of viral replication occurred in TLR3- and TRIF-deficient hearts, but IL-33 levels were only increased in TRIF-deficient mice. Because IL-33 is missing the signal peptide sequence required for secretion as a traditional cytokine [33], perhaps TRIF regulates the level of IL-33 within cells and then IL-33 is released by cellular damage due to viral replication thereby accounting for the differences in IL-33 levels between knockout strains.

The major limitation to this study is that TLR3- and TRIF-deficient mice are on a different background strain (TLR3−/− BL/6 mice were not available when we started these studies). Yet we provide evidence in this study that for all major parameters WT BL/6 and B6.129 strains responded in a similar manner during acute and chronic CVB3 myocarditis and are thus comparable backgrounds. This evidence includes (1) that both strains are "resistant" to the development of the chronic phase of CVB3 myocarditis and DCM

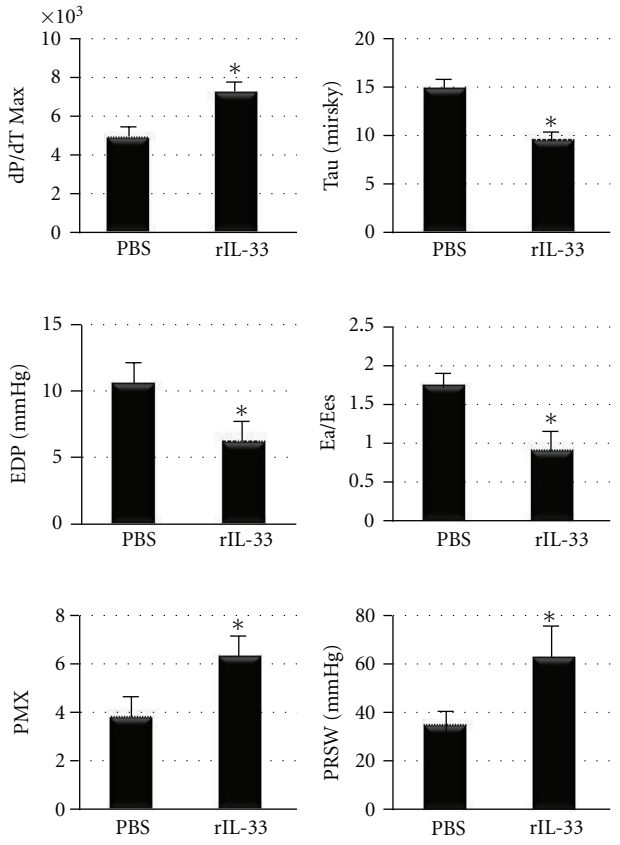

Figure 9: Summary of pressure-volume relationships at day 10 pi in TLR3−/− mice treated with recombinant IL-33 (rIL-33) or PBS every other day from day 1 to 9 pi. dP/dT Max measures the peak rate of pressure rise (mmHg/s); Tau, time constant of diastolic relaxation; EDP, end diastolic pressure; Ea/Ees, arterial elastance normalized to Ees which is left ventricular end systolic elastance (stiffness); PMX, maximum ventricular power; PRSW, preload recruitable stroke work. $n = 10$ to 12 mice/group. *: $P < 0.05$.

(Figures 2 and 5, Table 1), (2) that both strains have almost-identical heart function prior to infection (Table 1, day 0), (3) that both strains have nearly identical heart function as assessed by echocardiography and pressure-volume relationships during acute (day 10 pi) and chronic (day 35 pi) CVB3 myocarditis (Figures 3 and 5, Table 1), (4) that the severity and histologic appearance of acute myocarditis is the same for both strains (Figure 1), (5) that the level of viral replication in the heart during acute myocarditis is the same for both strains (Figure 1), and (6) that there is a similar level of M2 markers in the heart by RT-PCR for both WT strains (Figure 7, note difference in scale). However, it is possible that important differences exist between these backgrounds. We are currently examining this possibility by assessing TLR3-deficient mice on a BL/6 background.

In summary, we have shown in this study that elevated IL-4 or IL-4 plus IL-33 in the context of a Th2-driven response does not cause severe chronic disease resulting in HF. Yet outside of a classic IL-4-driven Th2-response, IL-33 is capable of causing cardiac dysfunction and HF as occurs in TRIF-deficient mice. Our findings suggest that the combination of high viral replication and elevated IL-33 levels in the heart is likely to be important in the progression from acute CVB3 myocarditis to severe DCM and HF. Our data also suggests that activation of TLR3 and TRIF in response to CVB3 infection protects Th1-responding mouse strains like BL/6 and B6.129 from progressing to chronic myocarditis, DCM, and HF. These findings provide insight into why most individuals that acquire CVB3 infection do not develop DCM and HF, and why polymorphisms in TLR3 signaling may predispose certain individuals to develop viral myocarditis, DCM, and HF.

Acknowledgments

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Research Article

Adaptive Immunity in Ankylosing Spondylitis: Phenotype and Functional Alterations of T-Cells before and during Infliximab Therapy

Balázs Szalay,[1] Gergő Mészáros,[1] Áron Cseh,[1] Lilla Ács,[2] Magdolna Deák,[2] László Kovács,[2] Barna Vásárhelyi,[3,4] and Attila Balog[2]

[1] *First Department of Pediatrics, Semmelweis University, Bókay János Utca 53-54, 1083 Budapest, Hungary*
[2] *Albert Szent-Györgyi Health Center, Department of Rheumatology, University of Szeged, Kálvária sgt. 57, 6725, Szeged, Hungary*
[3] *Research Group of Pediatrics and Nephrology, Hungarian Academy of Sciences, Bókay János Utca 53-54, 1083 Budapest, Hungary*
[4] *Department of Laboratory Medicine, Semmelweis University, Bókay János Utca 53-54, 1083 Budapest, Hungary*

Correspondence should be addressed to Balázs Szalay, szalay.b@gmail.com

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Our aim was to assess the phenotype of T-cell subsets in patients with ankylosing spondylitis (AS), a chronic inflammatory rheumatic disease. In addition, we also tested short-term T-cell activation characteristics. Measurements were done in 13 AS patients before and during the intravenous therapy with anti-TNF agent infliximab (IFX). Flow cytometry was used to determine T-cell subsets in peripheral blood and their intracellular signaling during activation. The prevalence of Th2 and Th17 cells responsible for the regulation of adaptive immunity was higher in AS than in 9 healthy controls. Although IFX therapy improved patients' condition, immune phenotype did not normalize. Cytoplasmic and mitochondrial calcium responses of CD4+ and CD8+ cells to a specific activation were delayed, while NO generation was increased in AS. NO generation normalized sooner upon IFX than calcium response. These results suggest an abnormal immune phenotype with functional disturbances of CD4+ and CD8+ cells in AS.

1. Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disease, the best characterized of the diseases belonging to the concept of spondylarthritides. It affects mainly the axial skeleton and the sacroiliac joints [1]. In time, the chronic inflammation of the spine (spondylitis) causes extra bone formation and eventually leads to the fusion of the vertebrae (ankylosis) [2]. The pathogenesis of AS is still unclear, but it is considered to be an autoimmune disease with a strong association with the MHC class I genetic marker HLA-B27 [3]. In any stages of the disease, autoimmune reactions can be associated with peripheral-arthritis, enthesitis, and extra-articular manifestations such as inflammations in the eye, the gastrointestinal tract, and the heart [4], indicating that AS is a systemic immune-mediated disease. This is supported by the number of alterations found in lymphocyte subgroups in peripheral blood.

Specifically, increased numbers of circulating Th2 helper lymphocytes [5] as well as increased numbers of Th17 cells [6] were reported in AS. Regulatory T-cells as major suppressors of the immune system show decreased prevalence, in the blood of AS patients indicating that their lack may contribute to the pathogenesis of the disease [7]. Along the alterations observed in cell prevalence one can assume that T-cell activation properties may also be altered in AS. In rheumatoid arthritis (RA), a further common example of chronic inflammatory arthritides, T-lymphocytes present an increase in intracellular nitric oxide (NO) production along with increased cytoplasmic Ca^{2+} concentrations [8]. This finding raised the notion that some functional alterations of T-lymphocytes were indeed present in autoimmune rheumatic disorders and would contribute to ongoing inflammation risk and progression of the disease. However, to date, no studies have been performed to

characterize functional characteristics of short-term T-cell activation in AS.

Effective therapy in AS includes the administration of nonsteroidal antiinflammatory drugs (NSAIDs) and, in unresponsive patients, the use of anti-tumor necrosis factor (TNF)-α agents such as infliximab (IFX). IFX is a chimeric anti-TNF antibody that has been shown to be highly effective for the treatment of AS. Although NSAID treatment has only a symptomatic effect and probably does not alter the disease course, IFX targets the specific inflammatory processes of the disease, and thus may potentially influence disease progression [9]. In addition to its action on soluble TNF-α, an increasing body of evidence supports the effect of IFX on immune cell prevalence [10–12]. However, no data regarding the impact of IFX on adaptive immune phenotype were reported in AS.

The aim of our comprehensive study was to assess the prevalence of major regulatory cells of adaptive immunity, and also to investigate the short-term T-cell activation characteristics in AS before and during IFX therapy.

2. Materials and Methods

2.1. Patients. Eleven male and two female patients with active AS meeting the modified New York criteria [13] were enrolled to the study. The age of the patients was (mean ± SD) 43.7 ± 9.2 years, and disease duration was 10.0 ± 5.4 years. Inclusion criteria were (1) Bath ankylosing spondylitis disease activity index (BASDAI) [14] higher than 4, (2) no response to 2 types of NSAIDs and (3) HLA-B27 positivity. Each of the patients was candidate for IFX therapy. The patients are given infliximab at a dose of 5 mg/kg bw intravenously on week 0, 2, and 6 then on every 8th week. Exclusion criteria were any significant comorbidity and the use of any drug excluding NSAID and a proton pump inhibitor. Nine male age-matched healthy volunteers (39.6 ± 6.5 years) served as controls. Written informed consent was obtained from each participant. The work was approved by a local ethical committee, and it was conducted in accordance with the Declaration of Helsinki (1964).

2.2. Cell Preparation. 24 mL of lithium-heparin anticoagulated blood was taken from all participants. In AS patients, blood samples were taken at 3 distinct time points: just before starting IFX when each of them was on NSAID therapy alone then on Week 2 and 6 after initiation of IFX therapy. Peripheral blood mononuclear cells (PBMCs) were separated with gradient centrifugation using Ficoll-Paque (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and washed twice with Phosphate Buffered Saline pH 7.4 (PBS, Central Pharmacy of Semmelweis University, Budapest, Hungary). 20% of the PBMCs (approximately 5×10^6 cells) was resuspended in PBS and used for cell surface staining, while 80% (2×10^7 cells) was resuspended in modified RPMI (Sigma-Aldrich, St. Louis, Mo, USA) medium (the Ca^{2+} concentration was set to 2 mM) for intracellular measurements.

2.3. Surface Staining. To characterize T-cell subset prevalence values, PBMCs were stained with fluorescent antibodies (Becton Dickinson, San Diego, Calif, USA) against cell surface markers according to the manufacturer's instructions. Samples were measured within 1 hour after staining; at least, 300,000 events were recorded for each acquisition.

Cell types were defined as: helper T-cells (CD4+), cytotoxic T-cells (CD8+), Th1 cells (CD4+CXCR3+), Th2 cells (CD4+CCR4+), Th17 cells (CD4+CCR4+CCR6+), regulatory T-cells (Tregs; CD4+CD25+CD127−), naive T-cells (CD4+CD45RA+ and CD8+CD45RA+), and memory/effector T-cells (CD4+CD45RO+ and CD8+CD45RO+). The prevalence of CD4+ and CD8+ cells expressing early and late activation markers (i.e., CD25, CD69, and HLA-DR, resp.) was also determined.

2.4. Intracellular Staining. In order to characterize intracellular processes associated with a specific T-cell stimulation, each specimen marked with CD4+ and CD8+ surface markers was loaded with Fluo3AM, Rhod2-AM, Dihydroethidium and DAF-FM diacetate (Molecular Probes, Carlsbad, Calif, USA) sensitive to cytoplasmic Ca^{2+} levels, mitochondrial Ca^{2+} levels, superoxide generation and nitric oxide production, respectively. Staining conditions were identical to those recently reported [15]. The changes in fluorescent signals were monitored up to 10 minutes after the addition of 20 μg/mL in final concentration of phytohaemagglutinin (PHA) (Sigma-Aldrich, St. Louis, Mo, USA), a specific activator of T-cells.

2.5. Equipment and Statistical Analysis. All measurements were performed on a BD FACSAria flow cytometer (Becton Dickinson, San Jose, Calif, USA). Cell prevalence values were determined with conventional gating using FACSDiVa software (Becton Dickinson, San Jose, Calif, USA).

The kinetic parameters of intracellular processes were determined using R (R Foundation for Statistical Computing, Vienna, Austria) as follows. Measurement timeframe was divided into 100 time intervals of equal length, and the medians of fluorescent values were calculated in each interval. Lowess smoothing method was applied to the median values, and each value was related to that measured at the beginning of the experiment (the resulting values became relative parameter values, rpv). The following parameters were calculated from the rpv values: area under the curve (AUC), maximum value (Max) and time to reach maximum (t_{max}) (Figure 1). One unit (u) of the AUC value is defined as one rpv in one second. Detailed explanation of these parameters can be found in our previous work [16].

Further statistical analysis was based on the values of these parameters and was performed using Statistica 7 software package (Statsoft, Tulsa, Okla, USA). For the comparison of controls and patients before IFX, a Mann-Whitney test was used, while data obtained on Week 2 and 6 were compared to those measured before IFX using paired Wilcoxon test. All data are given as median and [interquartile range]. $P < 0.05$ was considered significant.

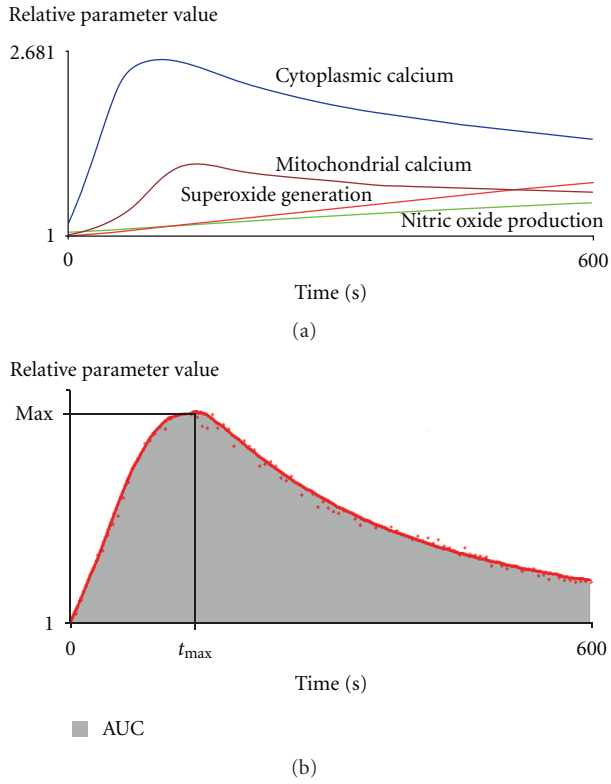

FIGURE 1: Schematic demonstration of characteristics changes of intracellular processes following PHA activation of lymphocytes. AUC: area under the curve, Max: maximum value, t_{max}: time to reach maximum.

3. Results and Discussion

Originally, 13 patients were enrolled; on week 2 and 6, 11, and 8 patients provided blood samples, respectively. 2 patients did not return after the initial IFX administration because of technical reasons (moving to a different region), while the others did not attend appointments at the required time.

At the beginning, BASDAI was ≥5 in each AS patient (median [interquartile range]: 6.88 [6.07–7.6]. After 6 weeks of IFX therapy, it decreased significantly: 1.79 [0.60–3.83], $P < 0.0001$.

3.1. Cell Prevalence Values. We found several important differences in cell prevalence values between AS with or without IFX and healthy controls (Table 1). The overall prevalence of CD4+ cells within lymphocytes increased in AS, while that of expressing CD25 decreased. In AS, Th1 prevalence values increased by approximately 30 per cent, while Th2 prevalence was double, resulting in a skewness of Th1/Th2 ratio to a Th2 direction. Th17 prevalence increased by 70 per cent, while Treg numbers were comparable to that in controls.

During IFX-treatment, these abnormalities did not disappear. Instead, the prevalence of naive cells on Week 2 and 6 decreased, while the prevalence of memory/effector cells increased on Week 6.

In general, CD8+ prevalence was comparable in patients and controls irrespectively of IFX therapy.

3.2. Functional Characteristics. CD4+ and CD8+ cells presented with a delayed increase in cytoplasmic Ca^{2+} levels after activation in AS compared to controls (Table 2, Figure 2). Mitochondrial Ca^{2+} kinetics also changed in a similar manner in CD8+ cells (Figure 2). The amount of NO generation, max and time to reach max NO levels were significantly higher in CD4+ and CD8+ cells of AS patients (Figure 3). Superoxide generation of T-cells did not differ between AS and controls. (For data on AUC levels see Table 2; additional information on comparable t_{max} and Max levels is available upon request.)

With IFX, therapy the delay in CD4+ cytoplasmic Ca^{2+} levels did not normalize in AS. For CD8+ cells, cytoplasmic and mitochondrial Ca^{2+} kinetics during activation normalized by week 6 on IFX (but not on week 2). NO kinetics became comparable in AS patients to that in controls even after 2 weeks of IFX therapy.

3.3. Discussion. AS is a chronic, systemic disorder exhibiting autoimmune reactions particularly against large joints and the axial skeleton [9]. The pathogenesis is still unclear, but both innate and adaptive immune responses could have a role in disease development [1].

Different T-lymphocyte subpopulations are implicated in AS [17]. Previous studies extensively investigated the prevalence of different T-cell subtypes in AS in different body fluids. A number of papers consistently reported that peripheral CD4+ [18] and CD8+ [19] cell numbers in blood are increased. In line with these data, we also observed higher than normal CD4+ prevalence in AS patients' blood.

Simultaneously with increased CD4+, Th1 and Th2 prevalence also increased. This finding supports earlier observations that indicated higher than normal Th2 [5] activity in AS. In addition to CCR4+, the prevalence of CXCR3+ cells were also found to be increased, but to a less extent, leading to a skewness to Th2 direction. Therefore our results relying on cell surface markers reinforce previous data based on the cytokine producing capability of T-cells suggesting a Th2 dominance in AS [20]. (The CXCR3 and CCR4 markers are increasingly accepted as surrogate markers of Th1 and Th2 cells, resp. [12].)

We also tested the prevalence of Th17 and Treg cells due to their central role in the regulation of T-cell subset functions. The clinical importance of Treg cells in autoimmune disorders are underlined by their reduced prevalence in a number of conditions [21], particularly when the prevalence of proinflammatory Th17 cells is increased [22, 23]. For AS, Wu et al. demonstrated that Th17/Treg cell ratios are increased and speculated that this abnormality in immune phenotype may be a contributing factor to disease development [7]. In our patients, we also found a skewness of Th17/Treg ratios to Th17 supporting this theory.

The discrepancy between the normal Treg prevalence found in our study and the lower Treg reported earlier may be partly due to different techniques used for the assessment

Table 1: Prevalence and ratios of T-cell subsets in ankylosing spondylitis patients before and during infliximab (IFX) therapy.

Members of adaptive immunity	Healthy controls ($n = 9$)	Before IFX therapy ($n = 13$)	Week 2 after IFX therapy ($n = 11$)	Week 6 after IFX therapy ($n = 8$)
CD4+	35.80 [29.43-40.95]	42.7 [38.35–48.65]*	47.10 [43.00–49.10]*	44.30 [42.15–48.85]*
CD4+CD45RA+	49.50 [40.68–61.68]	50.80 [45.20–65.95]	50.90 [36.20–55.40]#	42.55 [39.55–60.78]#
CD4+CD25+	7.55 [6.20–7.88]	4.82 [4.22–5.42]*	3.92 [3.60–4.85]*	4.35 [4.04–4.98]*
CD4+CD69+	3.23 [2.83–4.48]	3.05 [2.72–3.28]	3.04 [2.69–3.43]	2.88 [2.21–3.36]
CD4+HLA-DR+	2.8 [2.53–3.81]	3.03 [2.09–3.46]	1.95 [1.56–2.33]	2.89 [2.21–3.51]
CD4+CD45RO+	42.95 [33.33–49.65]	41.50 [29.40–49.55]	45.50 [37.80–54.60]	47.45 [33.10–54.78]#
Th1	9.81 [8.95–12.53]	12.90 [11.75–13.80]*	14.00 [11.50–15.70]*	13.80 [12.43–18.83]*
Th2	4.54 [4.19–4.84]	9.18 [7.32–11.45]*	8.70 [7.98–11.30]*	10.90 [8.32–12.50]*
Th1/Th2 ratio	2.38 [1.95–2.64]	1.31 [1.06–1.85]*	1.40 [1.16–1.83]*	1.38 [1.11–1.72]*
Th17	0.69 [0.60–0.77]	1.18 [1.02–1.69]*	1.2 [0.84–1.55]*	1.26 [0.91–1.51]*
Treg	4.42 [3.76–5.58]	4.45 [3.57–5.51]	4.29 [3.66–5.72]	4.42 [4.05–5.24]
Th17/Treg ratio	0.14 [0.13–0.21]	0.27 [0.19–0.45]*	0.25 [0.17–0.39]*	0.29 [0.18–0.37]*
CD8+	18.00 [14.35–27.98]	17.70 [14.65–21.45]	18.30 [14.20–21.90]	16.65 [14.43–21.60]
CD8+CD45RA+	62.85 [53.38–75.78]	70.30 [61.70–78.75]	69.10 [55.20–72.50]	60.20 [54.43–70.33]
CD8+CD25+	2.84 [1.87–4.06]	2.81 [2.23–3.63]	2.85 [2.07–3.23]	3.29 [1.84–3.54]
CD8+CD69+	4.0 [3.53–12.78]	4.88 [3.37–5.89]	3.71 [3.26–6.77]	5.06 [3.55–6.27]
CD8+HLA-DR+	3.58 [3.10–5.31]	3.43 [2.95–4.33]	4.37 [2.93–5.15]	3.57 [2.91–5.15]
CD8+CD45RO+	23.65 [15.90–28.15]	19.40 [14.25–28.20]	26.30 [19.10–34.90]	28.45 [18.53–40.20]
CD4/CD8 ratio	2.07 [1.32–2.83]	2.26 [2.02–3.15]	2.61 [1.99–3.21]	2.82 [1.90–3.36]

Data are expressed as median [interquartile range] *versus control $P < 0.05$; #versus before IFX $P < 0.05$.

of Tregs. While Wu et al. identified Tregs according to FoxP3 expression, we defined Tregs as CD4+CD25+CD127−. Although it is in general accepted that CD127 inversely correlates with FoxP3 in CD4+ cells [24] and that the absence of CD127 expression can be used as an alternative to the transcription factor FoxP3 [25], a recent study suggests that these markers do not necessarily represent the same population of Tregs and, therefore, likely cannot be used for the comparison of results of studies with different methodology [26]. (However, when we analysed the prevalence of cells with CD4+CD25+ positivity—that was used earlier for the detection of Tregs—we did find decreased levels, confirming a decrease in Tregs in AS.)

While CD45RA+ and CD45RO+ (naive and memory/effector) T-cells have an important role in some autoimmune disorders [27], we found no difference in the prevalence values of these cell types between AS and healthy controls confirming the findings of other studies [28]. In addition, the prevalence of T-cells expressing early- and late-onset activation markers (CD69, HLA-DR) was also comparable in AS and controls as in other studies [29].

Overall, these results suggest that the prevalence of some CD4+ subsets including Th1, Th2, and Th17 cells is increased suggesting a general alteration in immune phenotype in AS. Of note, no major alterations in CD8+ subsets were found in AS patients. It is worth emphasizing, however, that these results relate to peripheral blood and, therefore, do not necessarily reflect the local conditions in affected joints, where the local immune cell numbers and ratios may be different.

In this study, we also followed up the cell prevalence values during IFX therapy of AS patients. While during IFX we observed a significant decrease in disease activity, this change was not directly accompanied by the normalization of CD4+ subset prevalence. Instead, the prevalence of naive and memory/effector CD4+ cells significantly altered in an opposite manner leading to an increase in the ratio of memory/naive cells. As the prevalence of cell types investigated did not normalize during IFX, it is reasonable to postulate that the prevalence of these T-cell subtypes are not linked to clinical efficacy of IFX in AS.

These results are in contrast with the effects of IFX on immune cells in other diseases. An increase in the prevalence of CD4+ and CD8+ cells in Crohn's disease [10], that of Tregs [11] and Th1 committed T-cells in RA [12], was observed during IFX treatments. The discrepancy between our results and other studies may suggest that the impact of IFX on cell prevalence may differ between disorders.

Increased chronic TNF-α exposure is the hallmark for many inflammatory rheumatological disorders. In AS, high TNF-α levels have consistently been reported [30, 31]. High TNF-α induces systemic and local inflammation leading to the clinical signs and symptoms of AS. More recently, an increasing amount of evidence has suggested the impact of TNF-α on intracellular T-cell signaling [32, 33].

TCR stimulation induces Ca^{2+} influx and, through inositol-1,4,5-triphosphate (IP3), the release of Ca^{2+} from intracellular stores. (Almost immediately, the reuptake of Ca^{2+} to endoplasmic reticulum and mitochondria is also initiated.) This process was clearly detected in CD4+ and

Table 2: Functional characteristics of intracellular processes in CD4+ and CD8+ cells following PHA activation in ankylosing spondylitis patients before and during infliximab (IFX) therapy.

	Parameter	Healthy controls ($n = 9$)	Before IFX therapy ($n = 13$)	Week 2 after IFX therapy ($n = 11$)	Week 6 after IFX therapy ($n = 8$)
Cytoplasmic Ca^{2+}					
CD4+	AUC (U)	63.24 [58.00–107.9]	82.43 [64.22–255.8]	93.57 [56.89–201.0]	96.21 [87.13–158.10]
	Max (rpv)	1.156 [1.132–1.256]	1.268 [1.174–1.623]	1.321 [1.149–1.477]	1.254 [1.216–1.420]
	t_{max} (s)	258.4 [192.1–283.5]	527.5 [327.9 595.3]*	471.1 [288.2–594.3]*	594.7 [290.5–595.5]*
CD8+	AUC (U)	48.76 [38.53–93.22]	88.52 [54.65–327.8]	104.8 [42.14–180.8]	81.55 [58.51–407.1]
	Max (rpv)	1.139 [1.089–1.262]	1.247 [1.124–1.852]	1.318 [1.120–1.434]	1.220 [1.125–1.819]
	t_{max} (s)	228.6 [162.1–593.2]	594.3 [294.6–595.6]*	594.4 [422.9–596.1]*	282.7 [232.9–531.0]
Mitochondrial Ca^{2+}					
CD4+	AUC (U)	61.64 [37.97–94.43]	66.00 [41.55–95.52]	64.87 [42.90–105.4]	67.60 [49.29–104.2]
	Max (rpv)	1.194 [1.121–1.272]	1.207 [1.103–1.241]	1.149 [1.114–1.281]	1.172 [1.159–1.266]
	t_{max} (s)	594.0 [211.9–594.9]	593.3 [384.7–595.3]	595.2 [593.0–596.2]	595.1 [386.6–596.4]
CD8+	AUC (U)	106.9 [71.64–144.5]	87.79 [62.47–101.4]	71.31 [53.46–175.0]	118.8 [67.55–249.9]
	Max (rpv)	1.250 [1.152–1.404]	1.187 [1.130–1.217]	1.154 [1.131–1.400]	1.259 [1.143–1.539]
	t_{max} (s)	144.2 [121.6–160.6]	577.1 [167.8–594.2]*	468.8 [165.3–594.7]*	198.7 [169.8–248.4]
Nitric oxide production					
CD4+	AUC (U)	3.318 [−8.315–18.84]	47.76 [1.727–97.18]*	22.20 [−8.903–37.95]	−3.055 [−13.24–9.005]#
	Max (rpv)	1.013 [1.000–1.043]	1.113 [1.011–1.282]*	1.046 [1.000–1.081]	1.001 [1.000–1.034]#
	t_{max} (s)	198.7 [34.55–321.8]	594.3 [228.2–594.8]*	331.2 [0.000–556.5]	107.9 [0.000–252.5]#
CD8+	AUC (U)	−9.481 [−19.97–24.30]	41.31 [6.068–109.8]*	9.641 [−11.20–29.92]	−11.80 [−29.93–4.094]#
	Max (rpv)	1.003 [1.000–1.051]	1.099 [1.011–1.330]*	1.025 [1.001–1.078]	1.000 [1.000–1.001]#
	t_{max} (s)	51.08 [0.000–185.2]	594.2 [185.2–594.8]*	289.1 [54.03–553.5]	0.001 [0.000–130.5]#
Superoxide generation					
CD4+	AUC (U)	79.27 [64.68–88.90]	75.65 [70.70–92.61]	82.64 [58.40–96.68]	70.99 [56.09–83.99]
CD8+	AUC (U)	73.15 [62.21–80.33]	74.97 [61.28–98.75]	83.87 [58.13–90.74]	60.34 [51.38–85.42]

Data are expressed as median [interquartile range] *versus control $P < 0.05$; #versus before IFX therapy $P < 0.05$; AUC: area under the curve, Max: maximum value, t_{max}: time to reach maximum.

CD8+ lymphocytes in our experiments: after a specific stimulation of TCR with PHA cytoplasmic Ca^{2+} levels and, in a parallel manner, mitochondrial Ca^{2+} levels increased in CD4+ and CD8+ cells in each sample. Of note, the increase of cytoplasmic Ca^{2+} levels in CD4+ and CD8+ cells from AS is delayed compared to controls. As a consequence, mitochondria (at least in CD8+ cells) also presented a delayed calcium response during short-term activation. (For CD4+ cells, the time when max calcium values in mitochondria reached was beyond the measurement in a large number of control and AS samples.) The delayed calcium response of AS fits well into the observations done in *in vitro* tests with

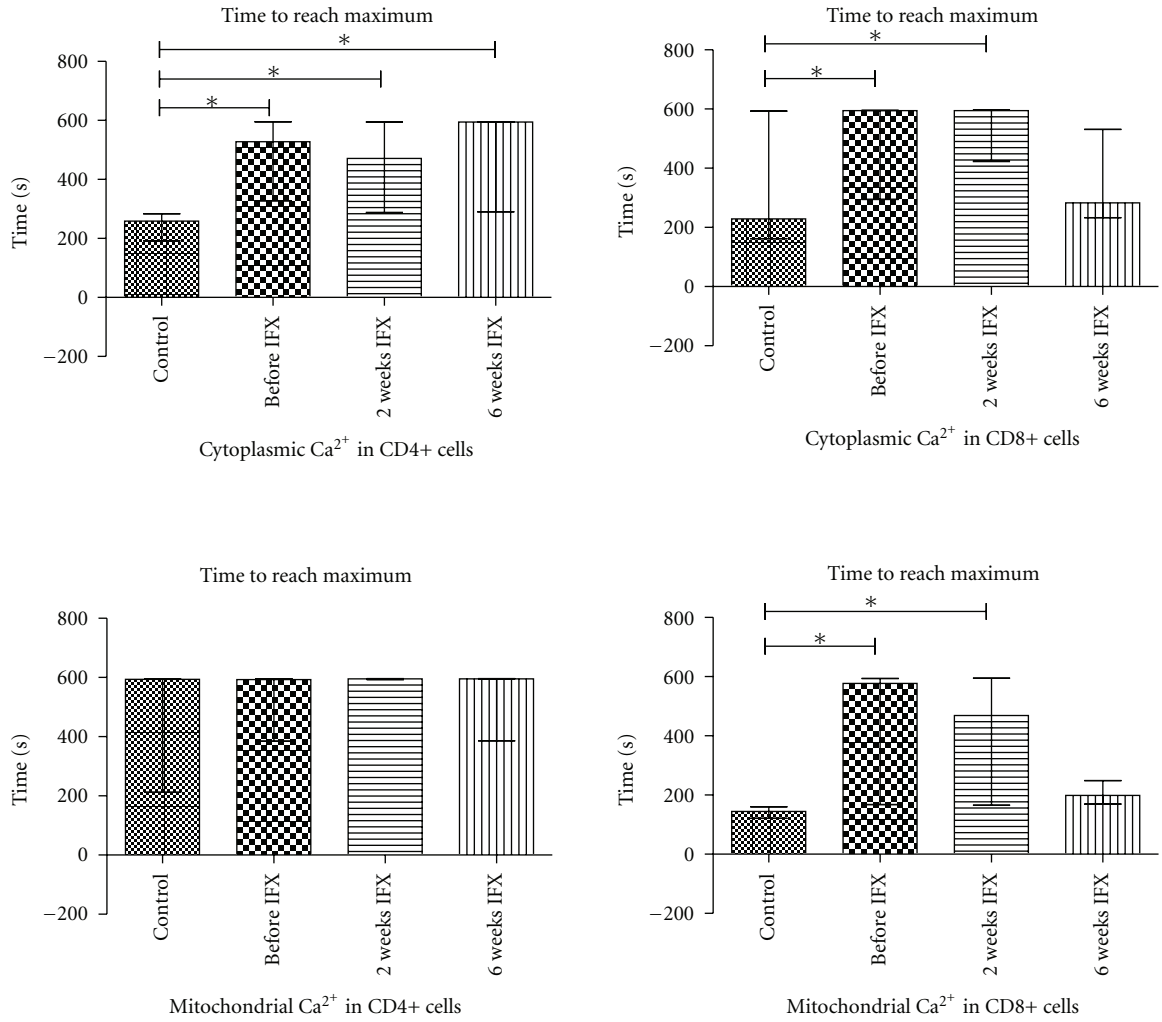

Figure 2: Time to reach maximum in cytoplasmic and mitochondrial Ca^{2+} levels in CD4+ and CD8+ cells following activation in healthy controls ($n = 9$) and ankylosing spondylitis patients before infliximab (IFX) therapy ($n = 13$) then 2 and 6 weeks after initiation of IFX ($n = 11$ and 8, resp.). $^{*}P < 0.05$.

T-cells exposed to TNF-α. Church et al. reported that TNF-α suppressed the Ca^{2+} peak after PHA stimulation; they have suggested that either signalling pathways upstream of Ca^{2+} mobilisation or the Ca^{2+} signalling itself were impaired by prolonged TNF-α exposure [33]. In AS, only one study has been performed with a more robust analytical approach: Lee et al. did not observe a significant difference in intracellular Ca^{2+} between AS patients and normal controls in activated peripheral blood mononuclear cells [34]. The inconsistency between their and our results is probably due to different methodology and cell types investigated.

The increase in Ca^{2+} levels following TCR stimulation triggers a large number of intracellular events. These include elevated NO production [35]. There is also a negative feedback, as high NO levels inhibit TCR signaling by decreasing the zeta-chain expression [36]. The effect of TNF-α on intracellular NO production, however, is rather independent on TCR as it induces iNOS activity directly in macrophages [37]. Therefore, in the presence of high TNF-α levels, in addition to the decrease of calcium response in T-cells, one can assume an increase in NO levels upon stimulation. This theory is clearly supported by our measurements indicating high NO levels in AS patients.

IFX is an effective anti-TNF agent that suspends TNF-mediated effects. In the prospective phase of our study, cell calcium handling (i.e, cytoplasmic and mitochondrial calcium response upon activation) of CD8+ cells normalized on week 6 (but not on week 2) of IFX therapy. For CD4+ cells, calcium handling remained abnormal even in the presence of significant improvement of clinical status of AS patients on IFX therapy. On the other hand, intracellular NO metabolism became normal already on week 2 and remained comparable to controls on week 6 of IFX therapy both in CD4+ and CD8+ cells. Therefore, one can conclude that the impact of IFX therapy on calcium handling and NO metabolism of T-cells differs. As NO kinetics in activated T-cells normalizes much earlier than calcium-kinetics, the major factors leading to NO abnormalities in IFX untreated patients seem independent of those leading to abnormalities in calcium kinetics. In addition, as the clinical condition of

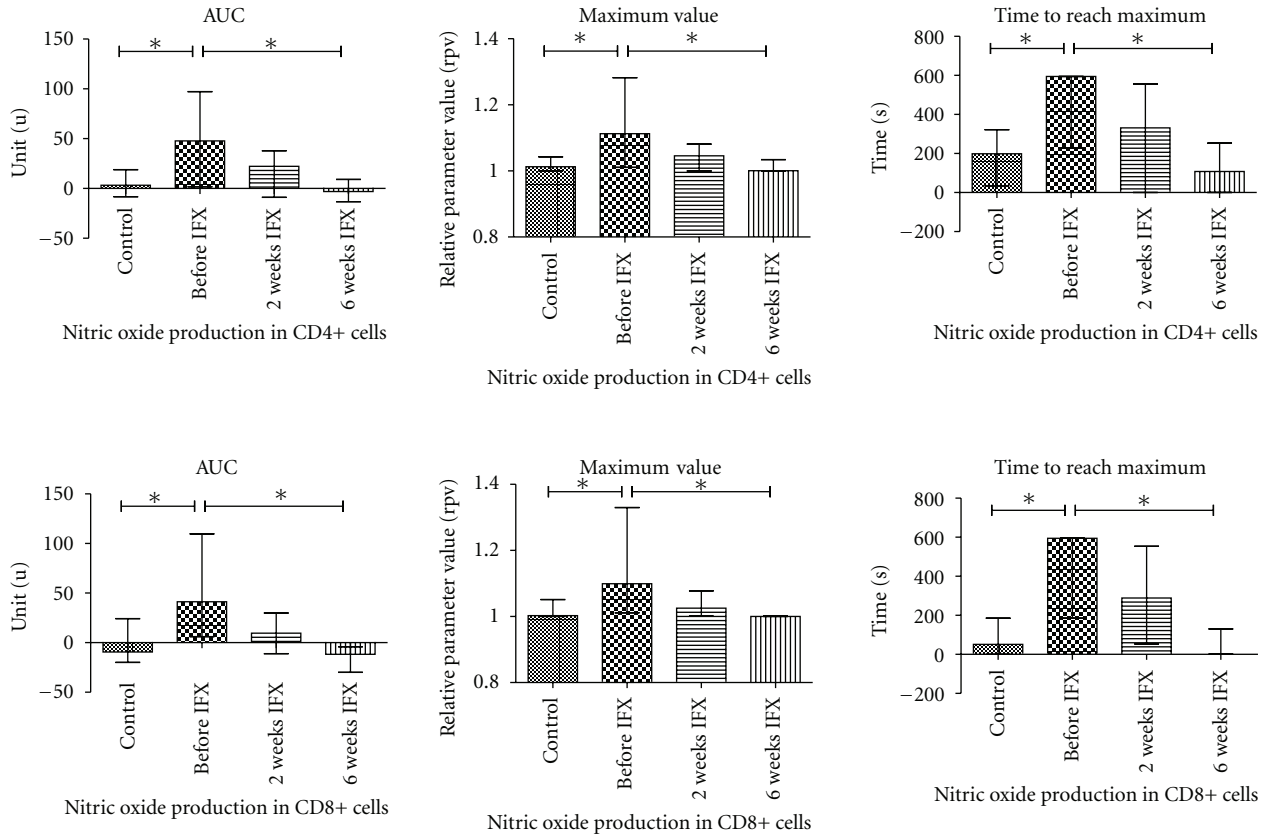

Figure 3: Nitric oxide generation kinetics in CD4+ and CD8+ cells following activation in healthy controls ($n = 9$) and ankylosing spondylitis patients before infliximab (IFX) therapy ($n = 13$) then 2 and 6 weeks after initiation of IFX ($n = 11$ and 8, resp.). $^*P < 0.05$ AUC: area under the curve. For details of IFX therapy, see text.

AS patients improved irrespectively of calcium kinetics, it is also reasonable to postulate that Ca^{2+} handling abnormalities of stimulated T-cells do not have a dominant role in the development of clinical signs and symptoms in AS.

In addition to Ca^{2+} and NO, we also tested superoxide generation of activated T-cells. No difference was observed between control and AS patients with or without IFX.

4. Conclusions

In this study, we found that the prevalence of Th2 and Th17 cells responsible for the regulation of adaptive immunity is different between AS and healthy controls. While IFX therapy improved the overall condition of patients, cell prevalence abnormalities did not disappear.

Some intracellular processes that are integral parts of lymphocyte activation are also altered in AS. These include intracellular nitric oxide and calcium handling. There are marked differences in the impact of anti-TNF therapy on these processes.

Acknowledgments

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Review Article

Cardiovascular Risk in Systemic Autoimmune Diseases: Epigenetic Mechanisms of Immune Regulatory Functions

Chary López-Pedrera,[1] Carlos Pérez-Sánchez,[1] Manuel Ramos-Casals,[2] Monica Santos-Gonzalez,[2] Antonio Rodriguez-Ariza,[1] and Ma José Cuadrado[3]

[1] *Unidad de Investigación e Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Hospital Universitario Reina Sofía, Avda. Menéndez Pidal s/n, 14004 Córdoba, Spain*
[2] *Laboratory of Autoimmune Diseases Josep Font, IDIBAPS, Department of Autoimmune Diseases, Hospital Clínic, C/ Villarroel 170, 08036 Barcelona, Spain*
[3] *Lupus Research Unit, St Thomas Hospital, Lambeth Palace Road, London SE1 7EH, UK*

Correspondence should be addressed to Chary López-Pedrera, rosario.lopez.exts@juntadeandalucia.es

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Autoimmune diseases (AIDs) have been associated with accelerated atherosclerosis (AT) leading to increased cardio- and cerebrovascular disease risk. Traditional risk factors, as well as systemic inflammation mediators, including cytokines, chemokines, proteases, autoantibodies, adhesion receptors, and others, have been implicated in the development of these vascular pathologies. Yet, the characteristics of vasculopathies may significantly differ depending on the underlying disease. In recent years, many new genes and signalling pathways involved in autoimmunity with often overlapping patterns between different disease entities have been further detected. Epigenetics, the control of gene packaging and expression independent of alterations in the DNA sequence, is providing new directions linking genetics and environmental factors. Epigenetic regulatory mechanisms comprise DNA methylation, histone modifications, and microRNA activity, all of which act upon gene and protein expression levels. Recent findings have contributed to our understanding of how epigenetic modifications could influence AID development, not only showing differences between AID patients and healthy controls, but also showing how one disease differs from another and even how the expression of key proteins involved in the development of each disease is regulated.

1. Introduction

Autoimmune diseases are a heterogeneous group of disorders characterised by humoral, cell-mediated immune responses against various self-constituents. It is widely known that AIDs are the result of interaction between predisposing genetic factors, deregulation of the immune system, and environmental triggering factors [1]. Several systemic autoimmune conditions, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS), and primary Sjögren Syndrome (pSS) are linked to enhanced atherosclerosis, and consequently higher cardiovascular morbidity and mortality rates. The development of cardiovascular disease involves genetic factors as well as other acquired and modifiable risk factors (e.g., hypercholesterolemia, diabetes mellitus, and hypertension). Inflammatory components of the immune response, as well as autoimmune elements (e.g., autoantibodies, autoantigens, and autoreactive lymphocytes), seem to be also involved in these processes [2–6].

From the genetic standpoint, it is known that in the case of AID there is a complex interaction between the product of various genes, and genomic high-throughput analyses can tell us which genes are turned on or off in different tissues from patients with autoimmune diseases. Recent genomic and transcriptomic profiling studies have implicated certain cytokines, surface receptors, signalling pathways, and cell types in the pathogenesis of inflammatory diseases [7–17]. This paper is focused in epigenomic approaches used to deep into the origin

of the mechanisms associated with both disease development and vascular involvement in systemic autoimmune diseases.

2. Epigenetic Mechanisms in Autoimmune Diseases

Systemic autoimmune diseases (AIDs) are of complex aetiology, characterised by an intricate interplay of various factors. A myriad of genes lies behind the heterogeneous manifestations of these diseases, and the overexpression and repression of particular genes form a specific gene expression profile (GEP) (genetic fingerprints) that is characteristic to the given disease phenotype. Pathophysiological mechanisms that might connect atherosclerosis and cardiovascular disease with SLE and RA have been greatly broadened with the application of genomic technologies, which have allowed explaining how these alterations might be associated to each AID [7–10, 12, 13]. One important and emerging mechanism controlling gene expression is epigenetics.

Epigenetics, the control of gene packaging and expression independent of alterations in the DNA sequence, is providing new directions linking genomics and environmental factors. The epigenetic process is important for controlling patterns of gene expression during the cell cycle, development, and in response to environmental or biological modifications. Moreover, epigenetic changes may be reversed [18].

A remarkable example of disease in which epigenetic abnormalities and patterns of inheritance are extremely complex is SLE. The high incidence of twin pairs in which SLE develops in only one of the siblings supports the notion that environmental factors and their involvement in epigenetic modifications could affect the onset of disease.

In the last year, several new findings about epigenetic modifications of gene expression were reported in different AIDs. These modifications describe changes in the expression of DNA that result from methylation, post-translational modifications of the histone proteins, including acetylation/deacetylation, methylation, and microRNAs (Figures 1 and 2). Most interestingly, these modifications seem to act in concert [19].

2.1. Histone Modifications. Histone modifications are regulated during the cell cycle, cellular development, and differentiation [20]. The two main histone modifications, histone acetylation and histone methylation, are tightly controlled. Indeed, histone acetylation is counterbalanced by histone deacetylation: histone acetyl transferase (HAT) adds the acetyl group, and the histone deacetylases (HDAC) remove it. HATs and HDACs reciprocally regulate the acetylation status of cellular proteins. Acetylation of histones promotes unwinding of compacted chromatin and allows access of transcription factors to gene promoter regions, while deacetylation of terminal lysine residues contributes to the silencing of transcription. Changes in relative HAT/HDAC activity would influence the sensitivity of cellular gene transcription in response to extracellular stimuli.

Similarly, the impact of lysine or arginine methylation by histone methyltransferases (HMTs) is reversed by demethylating enzymes, such as lysine-specific demethylase (LSD)1 and JmjC domain-containing histone demethylase (JMJC).

Global H3 and H4 hypoacetylation and hypermethylation characterize CD4+ T cells from SLE patients [21]. Moreover, a very recent study has demonstrated that there are significant clusters of aberrantly expressed genes in SLE (including those codifying for a set of chemokines) which are strongly associated with altered H4 acetylation [22].

2.2. DNA Methylation Alterations. DNA methylation occurs by covalent addition of a methyl group from the methyl donor S-adenosylmethionine (SAM) to the 5′ carbon of the cytosine ring in CpG pairs. Throughout the genome CpG is often found clustered in particular regions called CpG islands. CpG islands are typically methylated in silenced genes and hypomethylated in the regulatory areas of transcriptionally active genes. DNA methyl addition is carried out by at least five DNA methyl transferases (DNMT1, DNMT3a, DNMT3b, DNMT3L, and DNMT2). DNMT1 contributes to the maintenance of DNA methylation patterns, while DNMT3a and -b methylate unmethylated DNA and thus contribute to the novo methylation. DNMT2 displays weak DNA methyl transferase activity [18].

It has been shown that hydralazine and procainamide remove the methyl group from cytosines present in CpG islands through their interaction with DNA [23].

Procainamide and 5-azacythidine are competitive inhibitors of DNMT1 [24, 25], while hydralazine prevents DNMT1 upregulation during mitosis by blocking ERK pathway signaling at PKCdelta [26].

Patients with idiopathic lupus have changes in T cell signaling identical to those caused by hydralazine. In fact it has been demonstrated that T cells from patients with active lupus have hypomethylated DNA, due to decreased DNMT1 levels and activity [27, 28]. Interestingly, the decrease in DNMT1 levels is due to impaired ERK pathway signaling caused by a block at PKCdelta, also inhibited by hydralazine [26].

Candidate gene studies have revealed several pathways in which aberrant gene expression due to DNA demethylation is linked with the development of SLE. These genes include the ITGAL (also known as CD11A) [29], which is important for cell-cell adhesion, CD70 (encoding CD70), (also known as tumor necrosis factor ligand super family member 7) [30], which is required for T cell proliferation, clonal expansion, and the promotion of effector T cell formation, and CD40LG (encoding for CD40 ligand) [31], which stimulates B cell IgG overproduction. Other factors, such as the gene encoding perforin 1 (PRF1), [32] which contributes to autoreactive killing of macrophages and release of apoptotic material, are also hypomethylated in CD4+ T cells from individuals with SLE.

In 2009, Garaud and colleagues [33] reported that the E1B promoter of CD5 is hypomethylated in resting SLE B cells. This study also showed that high levels of interleukin 6 in SLE B cells, which is known to be positively associated with SLE disease activity, reduce the expression of DNMT1.

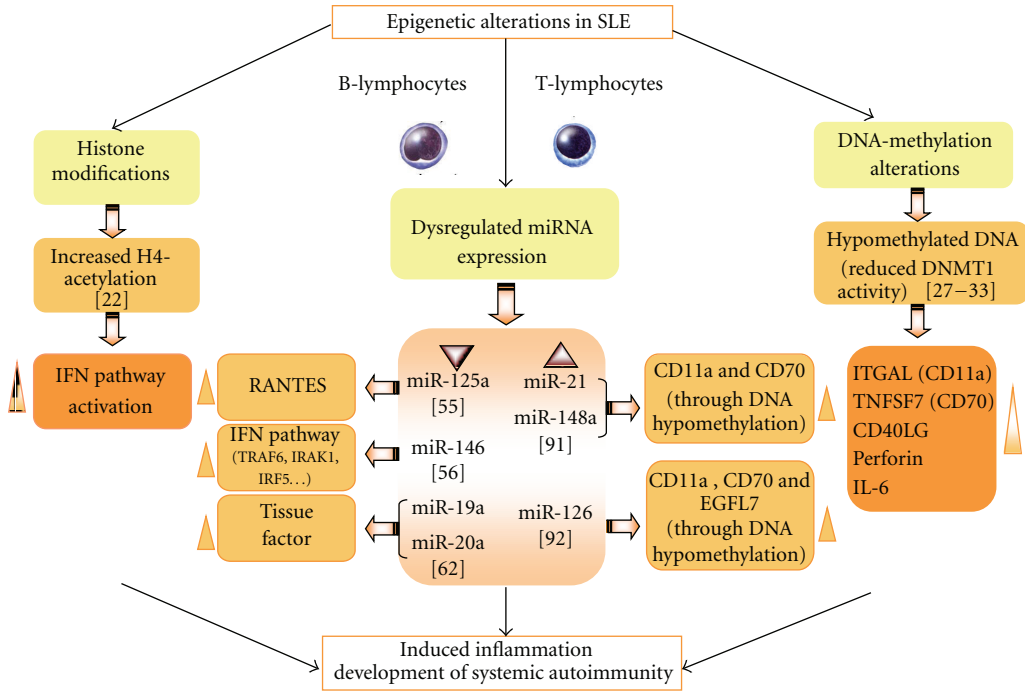

Figure 1: Epigenetics alterations in SLE and potential pathogenic contributions to inflammation, CVD, and autoimmunity. See the text for further details.

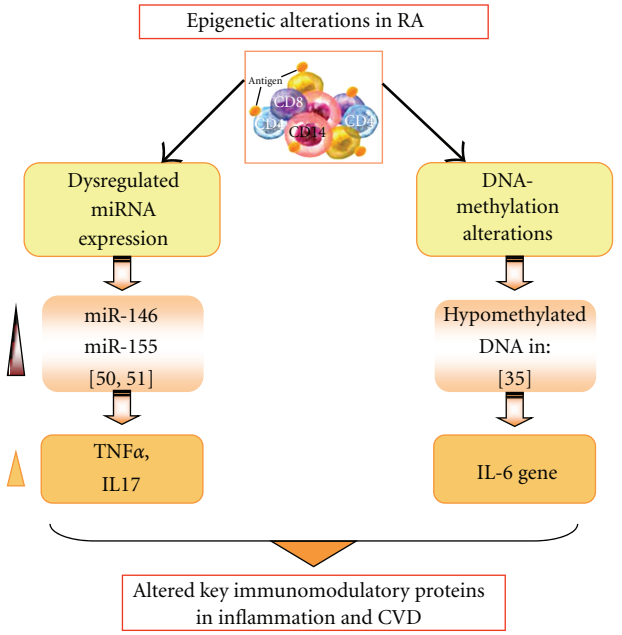

Figure 2: Epigenetic alterations in RA and potential pathogenic contributions to inflammation and CVD. See the text for further details.

Defective DNA methylation was also described in RA. T cell DNA is demethylated in RA [34] and may result in the generation of auto reactive T and/or B cell clones in RA as it does in lupus. The CD21 promoter is also demethylated in RA PBMC and synovial fluid cells. IL-6 has been further shown to be hypomethylated in PBMCs from individuals with RA [35]. However, further analysis of isolated B cells is necessary to confirm whether deregulated IL-6 expression occurs in these cells as a consequence of epigenetic changes in RA. Nevertheless, altered methylation of IL6 in RA reinforces the notion of the importance of applying epigenetic studies to the investigation of pathways that are affected in AIDs.

In pSS, recent studies have focused on analyzing DNA methylation alterations in mechanotransduction and hemidesmosome (HD) organization-mediated mechanisms. Changes in cell behaviour depend on mechanical signals received by the cell from the environment (mechanotransduction) [36, 37]. Experimental evidence suggests that a physical continuum directly connects the extracellular matrix (ECM) to the cellular nucleus [37]. Recent studies have investigated the role of these new mechanisms in the pathogenesis of SS and, specifically, the role of epigenetic processes in the development of glandular damage. An example of a mechanotransduction-mediated mechanism is the production of lactotransferrin by glandular cells: high levels of mRNA for lactotransferrin have been detected in a cell fraction enriched in epithelial cells from salivary glands of patients with SS, together with an altered distribution of $\alpha 6\beta 4$ integrin and an acinar cell shape [38]. Increased transcription of the lactotransferrin gene suggests a role of mechanotransduction-signalling pathways in the etiopathogenesis of SS.

A recent study by González et al. [39] found alterations in type I HD components in the salivary glands of SS

patients suggestive of epigenetic control. HDs are protein complexes that mediate epithelial cell adhesion to the ECM and are composed of an $\alpha 6\beta 4$ integrin dimer that binds to laminin, plectin, and other proteins (BP230 and BP180) [40, 41]. The study found reduced levels of BP230 mRNA in epithelial cells of patients with SS in comparison with controls and, in contrast, an accumulation of BP230 on the basal surface of acini [39]. An increased methylation index of CpG islands might explain the reduced levels of BP230 mRNA, and the authors suggest that differential changes in methylation of the BP230 gene promoter may explain the up- and downregulation detected in patients with SS. More recently, Yin and coworkers [42] evaluated whether the epigenetic regulation of CD70 expression is abnormal in pSS. They found that CD70 expression was significantly elevated and correlated with a decrease in TNFSF7 promoter methylation in pSS CD4(+) T cells compared to controls.

This study indicated that, as for SLE, demethylation of the CD70 promoter regulatory elements contributes to CD70 over expression in pSS CD4(+) T cells and may further contribute to auto reactivity.

2.3. MicroRNAs in Autoimmune Diseases. miRNAs are short (approximately 22 nucleotides in length) molecules of RNA that are transcribed from noncoding regions of the genome and exhibit significant secondary structure [43].

The biosynthesis of miRNAs is mediated by the nucleases Drosha, Pasha, and Dicer, involved in processing the miRNA from a hairpin configuration into a short RNA duplex and finally into a single-stranded miRNA, which is then loaded into the mRNA-induced silencing complex (RISC). The RISC and associated miRNA then bind complementary sequences in 3′ untranslated regions (base pairing of microRNA nucleotides 2–8, termed seed sequence) of mRNA species and inhibit their translation by two distinct mechanisms: degradation of the message by the RISC protein argonaute and prevention of ribosomal binding and translation initiation [43].

miRNAs play a key role in biological processes, such as embryogenesis, differentiation and proliferation of cells, production of cytokines, and apoptosis. More than 700 miRNAs have been identified in mammalian cells, and up to one-third of all protein-encoding genes are estimated to be regulated by these small molecules [44].

miRNA expression is tightly regulated during hematopoiesis and lymphoid cell differentiation, and disruption of the entire miRNA network of selected miRNAs may lead to dysregulated immune responses. In fact, abnormalities in miRNA expression related to inflammatory cytokines, Th-17, and regulatory T cells as well as B cells have been described in several AIDs [45].

In addition, it has been shown that miRNAs are present in human plasma in a stable form. Moreover, other body fluids such as synovial fluids contain measurable miRNAs [46]. Thus, synovial fluid and plasma miRNAs have additional potential as diagnostic biomarkers for some AIDs as well as a tool for the analysis of their pathogenesis.

2.3.1. MicroRNAs in Rheumatoid Arthritis. The expression levels of several miRNAs in PBMCs have been found associated with inflammation and cytokine production, and some of them correlated with RA disease activity [47].

In reports from different research groups, miR-146a and miR155 have been consistently found to be up regulated in synovial fibroblasts (RASFs), PBMCs, synovial fluid, PBMC-derived CD4+ T cells, and Th-17 cells from patients with RA when compared with healthy controls or patients with osteoarthritis (OA) [40, 48–51].

The study of the by Li and colleagues [50] of the expression profile of miRNAs in CD4+ T cells from synovial fluid and peripheral blood of 33 RA patients showed that miR-146a expression was significantly up regulated, while miR-363 and miR-498 were down regulated in RA patients. Moreover, the level or miR-146a expression was positively correlated with levels of tumor necrosis factor-alpha (TNFα). In addition, miR-146a overexpression was found to suppress T cell apoptosis, thus indicating a role for miR-46a in RA pathogenesis.

As a proof of the additional involvement of miR-146a in inflammation and cytokine production, Niimoto and coworkers [51] recently found that the expression of miR-146a was associated with IL-17 expression in the PBMC and synovium in RA patients and that the increased expression of both molecules correlated with disease activity.

A parallel survey further demonstrated that a polymorphism in the 3′-UTR of interleukin-1 receptor-associated kinase (IRAK1), a target gene of miR-146a, is associated with RA susceptibility [52].

Simultaneous investigations in PBMCs from acute coronary syndrome patients further showed significantly increased expression of miR-146a [53]. Moreover, the over expression of miR-146a was found to significantly unregulate the function of Th1 cells. This study also provided evidence that miR-146a treatment in vitro could induce the protein expression of TNFα, MCP-1 and NFκB p65, which are, respectively, key proinflammatory cytokines and critical transcription factor in atherosclerosis. Although further detailed studies are required, these results support the hypothesis that miR-146a may be directly involved in the pathogenesis of CVD associated to AIDs such as RA.

2.3.2. miRNAs in Systemic Lupus Erythematosus and Antiphospholipid Syndrome. The first study reported about miRNA expression in PBMCs from SLE was the study by Dai and coworkers [54], performed in 23 SLE patients and 10 healthy controls. In those SLE patients, 7 miRNAs (miR-196a, miR-17-5p, miR-409-3p, miR-141, miR-383, miR-112, and miR-184) were down regulated and 9 miRNAs (miR-189, miR-61, miR-78, miR-21, miR-142-3p, miR-342, miR299-3p, miR-198, and miR-298) were up-regulated as compared with healthy controls. However, that study did not provide further data to show how these changes in miRNA may play a role in SLE disease pathogenesis.

A recent study has shown the involvement of miR-125a in the inflammatory chemokine pathway in SLE [55]. In

SLE patients, the expression of miR-125a was found to be reduced, and the expression of its predicted target gene, KLF13, was increased. This study also showed that miR-125a negatively regulated RANTES expression by targeting KLF13 in activated T cells.

It has also been recently demonstrated the down regulation of miR146 in PBMCs from SLE patients, as well as its involvement in IFN over expression [56].

APS and SLE are two conditions known to provoke increased tissue factor (TF) expression in monocytes and endothelial cells [57–61]. In a recent study by our group, we checked in patients with APS or SLE the hypothesis that miRNA levels may influence TF levels in those patients. Thus we measured by RT-PCR the levels of miR-19b and miR-20a (reported to targeting TF expression in several web databases and algorithms of miRNA target prediction) in monocytes from APS and SLE patients. In APS we found that the levels of these two miRNAs had an approximate 3-4-fold decrease in comparison with monocytes from healthy controls. In monocytes from SLE, miR-20a levels were also lower than those from healthy subjects (3-fold decrease). In addition, the reduced expression of miR-19b and miR-20a was inversely correlated with TF cell surface expression [62]. These results shed light on new mechanisms that may regulate the expression of TF and thus the occurrence of thrombotic events in these AIDs.

2.3.3. miRNAs in Primary Sjögren Syndrome. Studies on the role of microRNAs (miRNAs) in the pathogenesis of pSS have centred on analysing miRNAs from salivary exosomes. Exosomes are small cellular vesicles (30–100 nm) that contain a wide range of surface and internal proteins specific to their cellular origin [63], and recent studies have shown that exosomes can also transport mRNA and miRNA [64, 65]. Alevizos et al [66] tested the potential of salivary gland miRNAs as a biomarker of SS, using Agilent microRNA microarrays to profile miRNAs isolated from the salivary glands of healthy controls ($n = 8$) and patients with SS, who were classified according to a high focus ($n = 8$) or low focus score ($n = 8$). MicroRNA expression patterns distinguished salivary glands from control subjects and the two groups of patients with SS. The authors identified two miRNAs (768-3p and 574) which were inversely correlated with the focus score. In addition, they found down-regulation of the mir-17-92 cluster in half the SS patients with a high focus score. Previous studies have associated down-regulation of the mir-17-92 cluster with an accumulation in pro-B cells and a marked reduction of pre-B cells, which has been associated with lymphoproliferative and autoimmune diseases [67, 68]. Larger studies are currently underway to validate miRNAs from salivary glands as diagnostic markers in SS [69]. Moreover, parallel analyses on PBMCs from both mice and human are currently underway so that Lu and co-workers [70] have presented preliminary data describing two miRNAs (150 and 146) that are up regulated in both, target tissues and in PBMCs of the B6DC mice, and in PBMCs and salivary glands of SS patients.

3. Epigenetic Alterations, Inflammation, and Cardiovascular Involvement in Autoimmune Diseases

Significant evidence has shown that there is heterogeneity in the characteristics of vasculopathies underlying different autoimmune diseases such as APS, SLE, RA, and pSS. It has been also shown a relevant heterogeneity with respect to inflammatory risk factors. The data presented in this revision further indicated that epigenetic mechanisms also seem to influence inflammation and cardiovascular disease in those autoimmune conditions.

In SLE, relevant factors directly influencing the development of CVD and AT comprise immune complex generation, complement activation, and changes in the production and activity of a complex network of cytokines, including type I and II interferons, B lymphocyte stimulator (BLyS), TNFα, IL-6, IL-17, and migration macrophage inhibitor (MIF) [71–82]. Epigenetic analyses have demonstrated aberrant gene expression due to DNA methylation linked to both, the development of the disease and to inflammation and AT (such as ITGAL, CD70, and CD40L) [28–30]. There have been also found significant clusters of aberrantly expressed genes in SLE (codifying for a set of chemokines) strongly associated with altered H4 acetylation [22]. Concerning altered miRNA expression, various studies have demonstrated in PBMCs altered expression of some miRNAs (miR-125a and miR-146) involved in the regulation of inflammatory chemokine pathways as well as of IFN over expression [54, 55].

In Antiphospholipid Syndrome (APS), an autoimmune disease in which thrombosis development constitutes a major pathological feature, procoagulant cell activation, accompanied with TF expression and TF pathway up regulation, is one of the key events considered explaining the prothrombotic tendency [57–61]. Although this pathology has been also associated with inflammatory condition and early AT development, no epigenetic studies have been developed to date to prove any relationship with those pathogenic processes. Nevertheless, along with SLE, the over expression of TF has been demonstrated in APS to be accompanied with an epigenetic change: the altered expression of miR-19b and miR-20a [62]. Although more deep studies are required, this association reveals a new mechanism involved in the pathophysiology of thrombosis in both autoimmune diseases.

In RA, pathogenic mechanisms involved in CVD and AT development include prooxidative dyslipidemia, insulin resistance, prothrombotic state, and immune mechanisms such as T cell activation that subsequently leads to endothelial dysfunction and arterial stiffness [83, 84]. Anticyclic citrullinated peptide antibodies (anti-CCP), IgM rheumatoid factor, circulating immune complexes, proinflammatory cytokines including TNFα and IL-6, Th0/Th1 cells, decreased folate and vitamin B12 productions, and impaired paraoxonase activity, among others, may all be further involved in the development of vascular disease in RA [85–87]. Epigenetic data indicate that defective DNA methylation

might also be relevant to CVD and AT development in RA, so that it has been shown hypomethylation of IL-6 promoter [35]. Furthermore, the expression levels of some miRNAs in PBMCs have been found associated with inflammation and cytokine production, including the over expression of miR-146a (which positively correlated with levels of TNFα and IL-17) [50, 51].

In primary SS, various studies concluded that there is evidence that those patients have both early subclinical AT and altered lipid profile with potential AT risk. In that pathology, the key role in determining the acceleration of AT seems to be played by immune-mediated mechanisms. Numerous interferon regulatory genes have been also found to be highly expressed in T cells and in salivary gland tissue [14–17].

Concerning epigenetic studies suggesting inflammation and CVD involvement in SS, to date only one group have reached concluding results. The study group of Cha and coworkers has developed non obese diabetic mice (B6DC) that develop a disease similar to human SS. They have described two miRNAS (150 and 146) that are up regulated in target tissues and in PBMCs of the B6DC mice compared to control mice. That group further reported that miR-146 expression is increased in PBMCs and salivary glands of SS patients [70], which might be related to IFN production. Hypomethylation and over expression of CD70 (TNFSF7) in CD4+ T cells of patients with primary Sjögren's syndrome have been also demonstrated [42]. Although the studies delineating the precise role of epigenetic alterations in inflammation and/or CVD in SS are just beginning, preliminary data suggest that pSS may represent an interesting model to study the factors involved in early development of AT.

4. miRNA Common to Different Autoimmune Diseases But Controlling Distinct Inflammatory Profiles: The Master Role of miR-146a

miR-146 and miR-155 have been shown to be induced by proinflammatory stimuli such as IL-1, TNFα, and Toll-like receptors (TLRs) [88]. They have also been detected in synovial fibroblasts, and rheumatoid PBMCs and synovial tissue, as well as in regulatory T cells serum, and urine cell free samples from SLE patients [89]. Both miRNAs have multiple targets, with miR-146 inhibiting TLR signalling and miR-155 regulating Th1 cells and also, interestingly, positively regulating mRNA for TNFα.

Concomitantly, Tang et al. [56] have shown that miR-146 regulates the level of at least TRAF6, IRAK1, STAT-1, and IFN regulatory factor 5 (IRF-5), all of which are important for the IFN pathway. The reported reduction of miR146 in PBMCs from SLE patients will likely affect the levels of these factors significantly and contribute to over expression of type I IFN and, in turn to disease activity.

Independent studies have demonstrated an increased level of miR146 in RA patients, but a decreased level in SLE patients, as compared with healthy controls. Given that RA and SLE are both systemic rheumatic diseases, one may be surprised by the finding that miR-146 levels are opposite in these diseases. Yet, as suggested by Chan et al. [90], this may simply reflect a difference in the overall cytokine profiles between the two diseases, with type I IFN playing a dominant role in SLE, whereas TNFα, interleukin-1, and IL-6 are the main cytokines in RA. That data reinforce the idea of the existence of some miRNAs as master gene regulators in different autoimmune diseases.

5. Control of DNA Methylation via miRNAs

Only recent studies have suggested that miRNAs can regulate DNA methylation by targeting the DNA methylation machinery in SLE. Pan et al. [91] identified 2 miRNAs, miR-21 and miR-148a, as being up-regulated in CD4+ T cells in both patients with lupus and MRL/lpr mice, an animal model of lupus. Moreover, both miRNAs down regulate the protein levels of DNMT1, thus resulting in hypomethylation status in CD4+ T cells. In particular, miR-21 indirectly down-regulates DNMT1 by targeting its upstream regulator, Ras guanyl-releasing protein 1, while miR-148a directly down regulates DNMT1 by targeting the protein-coding region of its transcript. The final result is the derepression of autoimmune-associated methylation-sensitive genes in CD4+ T cells, such as CD70 and lymphocyte function-associated antigen 1 (LFA-1; CD11a). These investigators were also able to induce the potential alleviation of hypomethylation in CD4+ T cells from patients with lupus by transfection with miR-21 and miR-148a inhibitors.

The study by Zhao et al. [92] further expanded the role of miRNAs and epigenetic changes in SLE. The novel finding was that, among the 11 microRNA that were observed to have increased or decreased expression in CD4+ T cells from patients with SLE, miR-126 was significantly over expressed, and its up-regulation was inversely correlated with DNMT1 protein levels. Zhao and colleagues were then able to demonstrate that miR-126 can directly inhibit DNMT1 translation by interacting with its 3′-UTR, leading to a significant reduction in DNMT1 protein levels. Through this mechanism, over expression of miR-126 causes demethylation and up-regulation of genes encoding for LFA-1 (CD11a) and CD70, two autoimmune-related proteins, which are directly proportional to disease activity. The miR-126 host gene EGFL7 was also over expressed in SLE CD4+ T cells, in a hypomethylation-dependent manner.

They were also able to show that knocking down miR-126 in SLE CD4+ T cells reduced their autoimmune activity and their stimulatory effect on IgG production in the cocultured B cells.

6. Therapeutic Potential of Epigenetic Modifications in Autoimmune Diseases

Unlike genetic alterations, which are permanent, epigenetic alterations are reversible. This opens up the possibility of using epigenetic drugs to reverse the pattern of epigenetic alterations to relieve the phenotype. To date, HDAC inhibitors such as suberoylanilide hydroxamic acid and trichostatin A (TSA) have proved to be useful for relieving

lupus disease in mice [93]. The effects of TSA on human T cells are predominantly immunosuppressive and reminiscent of the signaling aberrations that have been described in patients with SLE.

Inhibition of HDACs has also been shown to alleviate renal disease in a mouse model of SLE [94, 95]. In line with this, it has been recently reported that HDAC inhibition is efficient in the treatment of juvenile idiopathic arthritis [96].

In autoimmune diseases, inhibiting DNA methylation would not be appropriate to revert DNA methylation changes, as the changes identified to date are hypomethylation, not hypermethylation; thus, agents should be designed to specifically increase methylation, and no such specific methylating agent exists. Nevertheless gene-gene specific hypermethylation cannot be ruled out [93].

Furthermore, DNA demethylating agents such as hydralazine have been shown to subvert B lymphocyte tolerance and to contribute to the generation of pathogenic auto reactivity [97].

It is not clear whether the increased expression of specific miRNAs is an indirect effect rather than the cause of SLE, and this point also needs further investigation in future studies. The hypothesis that many miRNAs are master regulators of gene expression remains attractive to select interesting target miRNAs for the development of new therapeutics.

7. Conclusions

There is a wealth of emerging evidence showing that epigenetics processes are involved in promoting autoimmunity. Yet, it remains unclear whether epigenetic changes in autoimmune diseases are causally related to the pathogenetic features, such as immune responses or inflammatory status, or whether they merely represent a consequence of the ongoing pathological process. However, epigenetic changes could at least partly explain poorly understood environmental effects on disease development and the enhanced cardiovascular risk observed in AIDs.

Epigenetic alterations can be used as clinical markers of disease progression or response to therapy. As stated above, hypomethylation, although very relevant to the pathology of the autoimmune disease, cannot be considered as a biomarker for development of alternative therapies. However, the current studies have indicated a huge potential of using miRNAs as gene therapy targets in vivo to treat cancer. In the same way, it can be anticipated that in the near future novel effective miRNA-based gene therapies will be developed to replace the traditional immune-suppressive therapies to treat autoimmune diseases.

Identification of novel epigenetic targets, a better understanding of the epigenetic mechanisms involved in cardiovascular disease, and development of novel compounds directed against them will surely open up novel therapeutic approaches in systemic autoimmune diseases.

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Review Article

Current Concepts of Hyperinflammation in Chronic Granulomatous Disease

Nikolaus Rieber,[1,2] Andreas Hector,[1,2] Taco Kuijpers,[3,4] Dirk Roos,[3] and Dominik Hartl[1,2]

[1] *Children's Hospital, University of Tübingen, 72076 Tübingen, Germany*
[2] *DFG Emmy-Noether Group, Research Center, Children's Hospital, Ludwig-Maximilians-University, 80337 Munich, Germany*
[3] *Sanquin Research Institute, Landsteiner Laboratory, 1006 AD Amsterdam, The Netherlands*
[4] *Emma Children's Hospital, Academic Medical Centre, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands*

Correspondence should be addressed to Dominik Hartl, dominik.hartl@med.uni-tuebingen.de

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Chronic granulomatous disease (CGD) is the most common inherited disorder of phagocytic functions, caused by genetic defects in the leukocyte nicotinamide dinucleotide phosphate (NADPH) oxidase. Consequently, CGD phagocytes are impaired in destroying phagocytosed microorganisms, rendering the patients susceptible to bacterial and fungal infections. Besides this immunodeficiency, CGD patients suffer from various autoinflammatory symptoms, such as granuloma formation in the skin or urinary tract and Crohn-like colitis. Owing to improved antimicrobial treatment strategies, the majority of CGD patients reaches adulthood, yet the autoinflammatory manifestations become more prominent by lack of causative treatment options. The underlying pathomechanisms driving hyperinflammatory reactions in CGD are poorly understood, but recent studies implicate reduced neutrophil apoptosis and efferocytosis, dysbalanced innate immune receptors, altered T-cell surface redox levels, induction of Th17 cells, the enzyme indolamine-2,3-dioxygenase (IDO), impaired Nrf2 activity, and inflammasome activation. Here we discuss immunological mechanisms of hyperinflammation and their potential therapeutic implications in CGD.

1. Chronic Granulomatous Disease

Chronic granulomatous disease (CGD) is the most common inherited disorder of phagocytic functions, caused by genetic defects in the leukocyte nicotinamide dinucleotide phosphate (NADPH) oxidase. Phagocytes of CGD patients are unable to kill ingested microorganisms through reactive oxygen species (ROS), resulting in an augmented susceptibility of the patients to bacterial and fungal infections [1, 2]. Most CGD patients are known to suffer from recurrent infections from the first year of life. To prevent severe life-threatening infections, these patients require prophylactic antibiotics and antimycotics [2, 3]. Bone marrow transplantation is considered curative, and gene therapy approaches have also been implemented to overcome severe infections in CGD [4–8]. In addition to their impaired antibacterial and antifungal innate host defense, CGD patients frequently present with autoimmune phenomena, such as granuloma formation, Crohn-like disease, pulmonary fibrosis, or others, suggesting a dysbalanced uncontrolled inflammatory response in CGD [3]. Today, most of the CGD patients reach adulthood due to improved treatment options for the bacterial and fungal infections, yet the autoinflammatory manifestations become more prominent. The severe recurrent infections can be explained by the lack of ROS required for the killing of pathogens, whereas the underlying reasons for the hyperinflammatory reactions in CGD remain poorly understood, and causative therapy for CGD hyperinflammation is lacking.

2. Inflammation in Chronic Granulomatous Disease

Several lines of evidence support the concept that CGD features hyperinflammation [9]. Notably, microarray analysis in neutrophils from CGD patients has revealed upregulation

of several proinflammatory genes [10]. Upon stimulation with TLR2 or TLR4 ligands, leukocytes from CGD patients yield an increased production of proinflammatory cytokines, which is, surprisingly, independent of NADPH oxidase activity [11].

2.1. Neutrophil Apoptosis and Efferocytosis. Apoptosis (programmed cell death) of inflammatory cells represents a physiological mechanism to prevent secondary uncontrolled necrosis and hyperinflammation followed by tissue damage. Apoptotic cells externalize phosphatidylserine (PS), which is recognized through PS-receptors. This interaction enables the uptake of apoptotic cells by phagocytes (for instance, macrophages), a process termed "efferocytosis" [12]. This controlled removal of apoptotic cells is of pivotal relevance for short-lived inflammatory cells, prototypically neutrophils. Efferocytosis leads to the secretion of the anti-inflammatory cytokine TGF-β by macrophages and thereby facilitates resolution of acute inflammation [13]. Both apoptosis and efferocytosis of apoptotic cells by macrophages have been reported to be impaired in CGD patients and/or mice (see Figure 1 for illustration). Constitutive apoptosis has been shown to be delayed in both human and murine CGD neutrophils due to impaired PS exposure. This may lead to unbalanced neutrophil necrosis with release of intracellular proteases/oxidants and an increased risk of developing lupus disease in CGD patients [14]. However, other groups could not determine any significant impairment of apoptosis in CGD monocytes [15] or neutrophils [16]. The latter group also observed higher rates of necrosis induced by specific bacteria in CGD neutrophils [16].

Besides apoptosis, also uptake/efferocytosis of apoptotic neutrophils has been shown to be impaired by murine CGD macrophages and has recently been shown to contribute to hyperinflammation in mice [17–19]. Two distinct mechanisms have been reported that play a role in the impaired removal of apoptotic cells by macrophages (Figure 1). Fernandez-Boyanapalli et al. demonstrated that the impaired phagocytosis of apoptotic cells by CGD macrophages could be reversed by IFN-γ treatment [18]. Further studies showed that the IFN-γ priming rescue effect was mediated through NO production, endogenous TNF-α production, and Rac activation.

Following up the consequences of the deficient PS exposure in CGD neutrophils, studies demonstrated that the impaired PS/PSR-dependent production of IL-4 resulted in reduced generation of 12/15-lipoxygenase and reduced activation of the transcription factor PPARgamma (peroxisome proliferator-activated receptor gamma) [17]. This leads to altered macrophage programming (M2 macrophage phenotype) and decreased efferocytosis in CGD macrophages. The authors showed that it was possible to overcome this IL-4-dependent defect in efferocytosis by injecting PS in CGD mouse models *in vivo*. However, the authors of these studies also discuss that the impaired clearance of apoptotic cells, observed in X-CGD, may not be contributable to the NADPH oxidase deficiency, but could be favoured by the cytokine microenvironment.

2.2. Innate Immune Receptors. Neutrophils are the key effector cells in antibacterial host defense and are in the focus of CGD research [20–23]. Effector functionality of neutrophils is orchestrated by innate immune receptors such as Toll-like receptors (TLRs) and complement receptors [21–23]. Neutrophils from CGD patients show lower expression levels of TLR5, TLR9, CD11b, CD18, CD35, and CXCR1 compared with those from healthy control subjects, whereas similar or increased receptor expressions are found in patients with bacterial pneumonia [24]. Reduced TLR5 expression resulted in impaired neutrophil activation by bacterial flagella, and reduced CD11b/CD18 expression is associated with impaired phagocytosis of *Staphylococcus aureus*.

TLR5 and CD18 expression levels correlate with disease severity in CGD patients. TLR5 and TLR9 expressions are higher in patients with residual NADPH oxidase activity. *In vitro* inhibition of the NADPH oxidase in control neutrophils decreases TLR5 and TLR9 expression and impairs TLR5 function. TLR5 expression correlates with the frequency of lymphadenitis in CGD patients, suggesting a clinically relevant role for TLR5 dysregulation in the course of CGD. Previous studies of CGD neutrophils have found a reduced expression of CD35 on neutrophils from patients with CGD compared to healthy controls and patients with recurrent infections [25]. When viewed in combination, these studies indicate that CGD neutrophils are not only restricted in terms of NADPH-mediated intracellular oxidative killing, but display distinct phenotypical and functional abnormalities. In particular, TLR5 and TLR9 are impaired in CGD through a mechanism linked to the deficient ROS production in these cells. Whether and how this dysbalance of innate immune receptors on CGD neutrophils contributes to inflammatory manifestations awaits further investigation.

2.3. ROS-Dependent Oxidization of T-Cell Membrane Proteins. Rats and mice with a lower capacity to produce ROS because of polymorphisms or mutations in the gene encoding the p47phox protein of the NADPH oxidase complex are more susceptible to develop severe arthritis [26, 27]. The lower capacity to produce ROS is associated with an increased number of reduced thiol groups on T-cell membrane surfaces. This influences activation and proliferation of T cells and the susceptibility to arthritis development [28]. In a recent study the Holmdahl group further showed that ROS-deficient macrophages can actually prime autoreactive T cells and initiate autoimmune arthritis in a murine model of collagen-induced arthritis [29].

2.4. Th17 Cells. Recently, IL-17-producing effector cells (Th17 cells and $\gamma\delta$ T cells) have been found to be involved in chronic inflammatory processes and several autoimmune diseases, for example, multiple sclerosis or rheumatoid arthritis. These highly proinflammatory cells are essential for the defence against pathogens [30, 31]. They are considered to be counterbalanced by regulatory T cells (Treg) [32]. A fine-tuned activity of both of these T-cell subsets is crucial for controlling infections, inflammation, autoimmunity, and malignancies [33, 34]. Evidence supporting a contribution

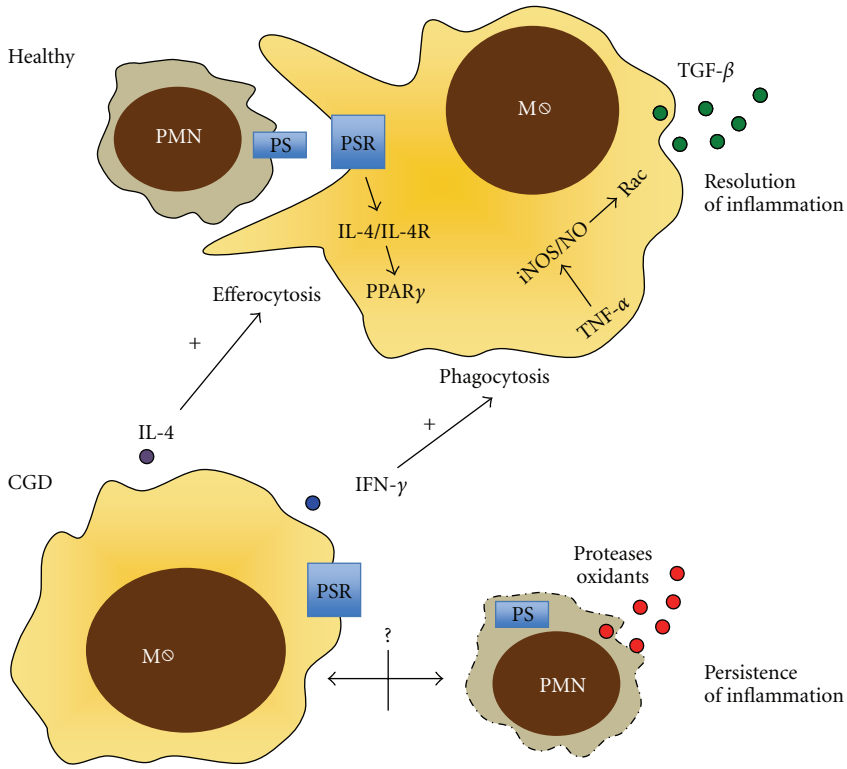

Figure 1: Impaired apoptosis/efferocytosis underlying hyperinflammation in CGD. Apoptotic cells (here shown for neutrophils, PMN) externalize phosphatidylserine (PS), which is recognized through PS-receptors on macrophages. This interaction enables the uptake of apoptotic cells by macrophages, a process termed "efferocytosis". Successful efferocytosis leads to the secretion of the anti-inflammatory cytokine TGF-β by macrophages and thereby facilitates resolution of acute inflammation. Due to an impaired externalization of PS by CGD neutrophils and/or other neutrophil-macrophage interaction mechanisms, both apoptosis and efferocytosis have been described to be dysfunctional in CGD. This leads to unbalanced neutrophil necrosis with release of intracellular proteases/oxidants and persistence of (sterile) inflammation. Two pathways have recently been proposed to be involved in this impairment: PS/PSR interactions were found to trigger downstream pathways comprising IL-4 and PPARγ, which are critically involved in the regulation of efferocytosis. Beyond that, impaired phagocytosis of apoptotic cells by CGD macrophages could be reversed by IFN-γ treatment, an effect that was mediated through NO production, endogenous TNF-α production, and Rac activation.

of IL-17 in CGD hyperinflammation was recently derived from animal model data [35, 36]. When challenged with either intratracheal zymosan or LPS, NADPH oxidase-deficient p47phox$^{-/-}$ mice and gp91phox$^{-/-}$ mice develop exaggerated and progressive lung inflammation, augmented NF-kappaB activation, and elevated downstream proinflammatory cytokines (TNF-alpha, IL-17, and G-CSF) compared to wild-type mice [36]. Replacement of functional NADPH oxidase in bone marrow-derived cells restores the normal lung inflammatory response. Studies *in vivo* and in isolated macrophages have demonstrated that in the absence of functional NADPH oxidase, zymosan fails to activate Nrf2, a key redox-sensitive anti-inflammatory regulator. Consistent with these findings, zymosan-treated peripheral blood mononuclear cells from X-linked CGD patients show impaired Nrf2 activity and increased NF-kappaB activation [36] (Figure 2).

2.5. Indolamine-2,3-Dioxygenase. In addition to regulatory cells and their specific cytokines, immune responses are controlled by the enzyme indolamine-2,3-dioxygenase (IDO). IDO is required for the catabolism of the aromatic amino acid L-tryptophan and the generation of L-tryptophan metabolites. Whereas IDO-dependent metabolites of L-tryptophan like L-kynurenine are well-known immunosuppressive agents [37, 38], L-tryptophan itself or IDO-independent metabolites of tryptophan have been shown to activate proinflammatory Th17 cells [39]. IDO is primarily expressed in monocytes and dendritic cells [40]. The impact of IDO on immune regulation has become evident in the maternal tolerance to the allogeneic fetal tissue [41], in several autoimmune disorders like type-1 diabetes, multiple sclerosis, chronic inflammatory bowel disease, rheumatoid arthritis and systemic lupus erythematodes [37], as well as in the tolerance against malignant tumors [42]. Presently, a phase-III clinical trial with synthetic immunomodulatory tryptophan metabolites is being conducted in multiple sclerosis patients [43]. IDO function has been reported to be dependent on superoxides [35, 44], which are not produced in suitable amounts when the NADPH oxidase system is deficient as in CGD. IDO deficiency could thus be an important factor for the excessive inflammatory reactions in this disease (Figure 2). Recently, in p47phox$^{-/-}$ mice a

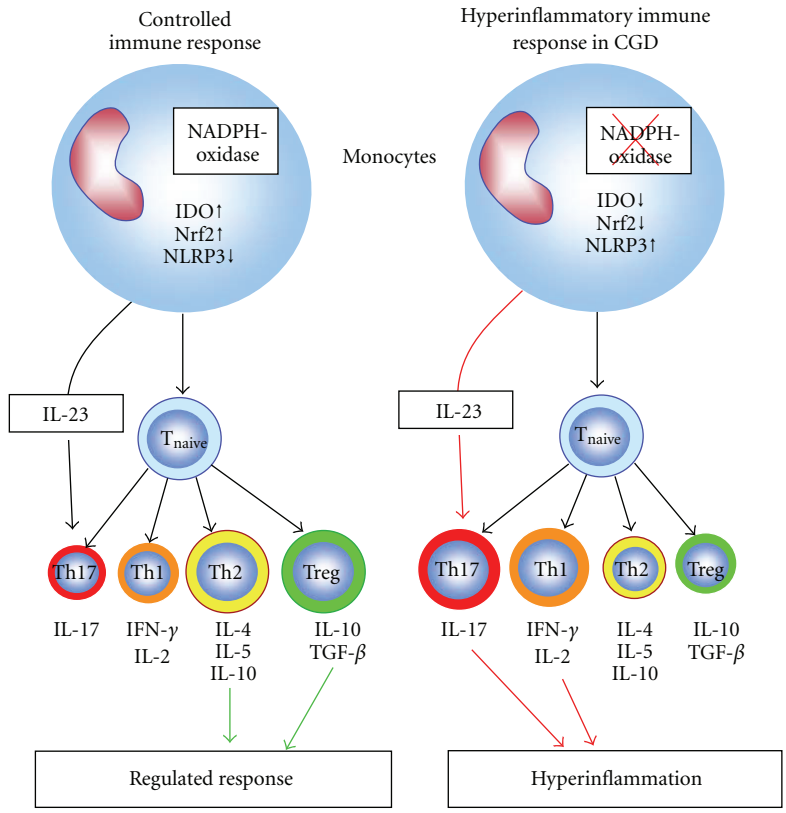

Figure 2: Other recently proposed mechanisms of hyperinflammation in CGD. Three independent mechanisms leading to hyperinflammation with increased numbers of Th17 cells have been recently proposed in CGD: Reduced IDO and Nrf2 activity and increased NLRP3 activation.

pathogenic link between defective NADPH oxidase, reduced tryptophan catabolism, and IL-17-mediated inflammation [35] has been shown. After infection with *Aspergillus fumigatus*, these mice develop an enormous inflammatory response with a shift in the $\gamma\delta$ T cell system and overproduction of IL-17 in comparison to wild-type mice. Furthermore, the hyperinflammatory phenotype of the CGD mice can be mimicked in wild-type mice by IDO blockade and abolished by replacement therapy with a natural kynurenine [35]. In humans, however, two independent studies recently showed, that monocyte-derived dendritic cells generated from patients harbouring X-linked and autosomal recessive forms of CGD, and from healthy controls, produced similar amounts of the tryptophan metabolite kynurenine upon activation with lipopolysaccharide and interferon-gamma [45, 46]. Thus, in humans, ROS apparently are dispensable for IDO activity and hyperinflammation in human CGD cannot be attributed to disabled IDO activation.

2.6. Inflammasome Activation. Some *in vitro* studies suggest that ROS are crucial for secretion of IL-1beta via inflammasome activation [47], whereas mice defective for ROS and patients with CGD have a proinflammatory phenotype. In three current studies, the activation of the IL-1beta inflammasome in cells from CGD patients was evaluated [48–50]. In contrast to previous studies using the small molecule diphenylene iodonium (DPI) as an ROS inhibitor, these studies did not find a decrease in either caspase-1 activation or secretion of IL-1beta and IL-18 in primary CGD monocytes. Moreover, activation of CGD monocytes by uric acid crystals induced a 4-fold higher level of IL-1beta secretion compared with that seen in control monocytes. This increase was not due to increased synthesis of the IL-1beta precursor. In addition, Western blot analysis of CGD cells revealed that caspase-1 activation was not decreased, but rather was increased compared with control cells [48] (Figure 2). Caspase-1 activation was especially strong in CGD patients with noninfectious inflammatory conditions. Treatment with IL-1 receptor antagonist reduced IL-1 production in monocytes ex vivo and during medical therapy [49]. These recent findings support the concept that ROS likely dampen inflammasome activation and identify phagocyte oxidase defective monocytes as a source of elevated IL-1. This provides new potential therapeutic options for inflammatory conditions associated with CGD.

3. Summary

CGD patients suffer from various autoinflammatory symptoms, such as granuloma formation, Crohn-like colitis, and lung fibrosis. As the majority of CGD patients reaches adulthood, autoinflammatory manifestations become more prominent and determine morbidity of many CGD patients. Here we summarize evidence on mechanisms underlying

hyperinflammation in CGD. These include reduced neutrophil apoptosis, dysbalanced innate immune receptors, induction of Th17 cells, impaired Nrf2 activity, and increased inflammasome activation. Further studies are required to determine the clinical relevance of and, in particular, the therapeutic options for manipulation of these mechanisms in CGD patients.

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