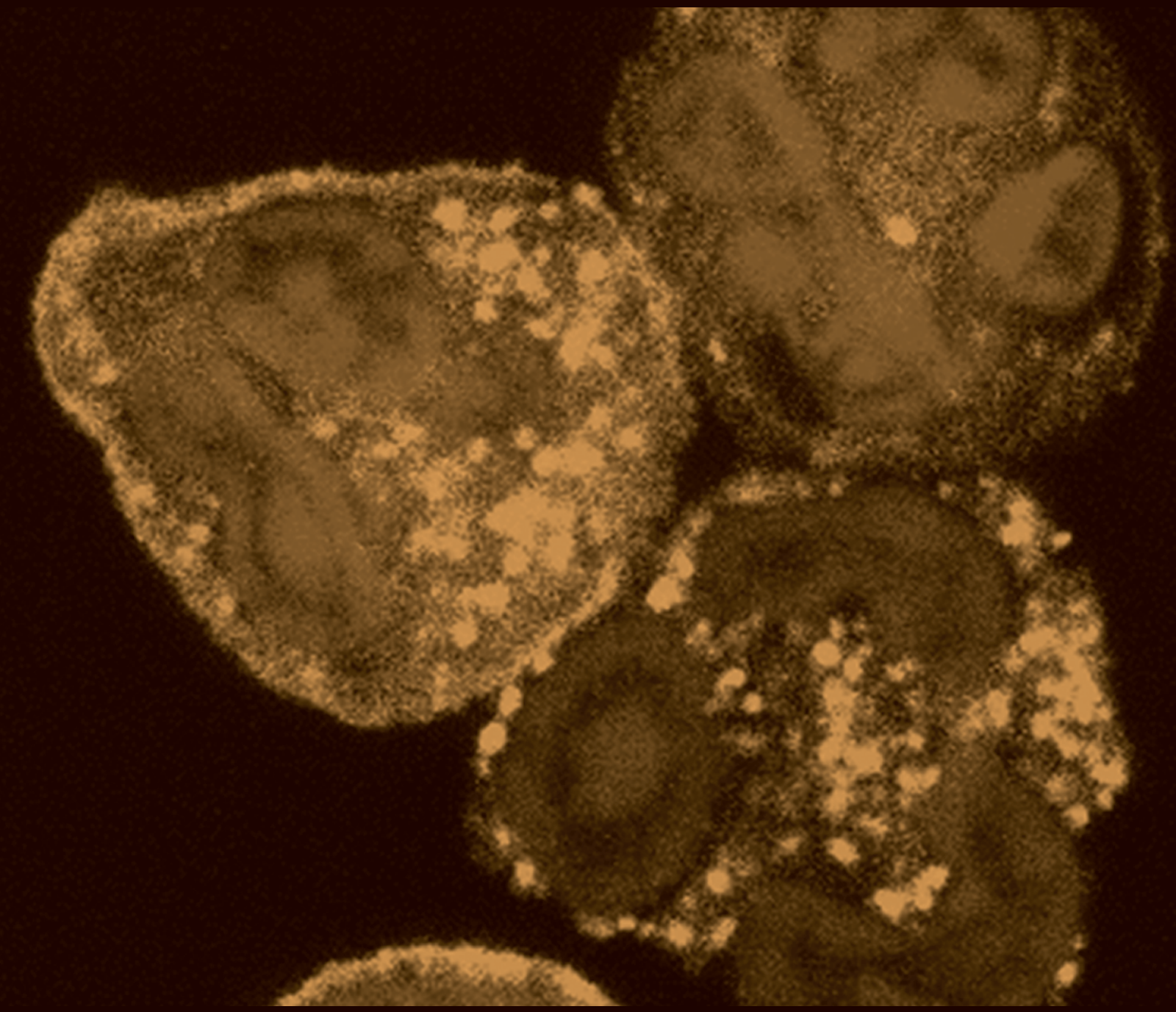
Mediators of Inflammation

Innate Immune Pathways in Host Defense

Guest Editors: Thirumala-Devi Kanneganti, Mohamed Lamkanfi, and Amal O. Amer



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Editorial Board

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Editorial

Innate Immune Pathways in Host Defense

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The innate immune system is a critical component of host defense against invading microbial pathogens. It is responsible for mounting proper inflammatory and repair responses that contribute to the elimination of the invading pathogen and for instructing the adaptive immune system to develop a prolonged immunity against microbial pathogens. This is accomplished through the regulation of transcriptional and posttranslational programs that culminate in the production of proinflammatory cytokines and chemokines, the induction of type I and II interferon responses and autophagy responses, and the induction of programmed cell death modes that eliminate infected host cells and expose intracellular pathogens to surveillance by the immune system. This issue includes eight published papers which are discussing the following issues.

In the article "*The EGF receptor and HER2 participate in TNF-α-dependent MAPK activation and IL-8 secretion in intestinal epithelial cells*," by H. B. Jijon et al., the authors provide evidence that TNF activates one or more metalloproteinases leading to the release of TGF-α in intestinal epithelial cells.

In the article "*Innate immune cells in liver inflammation*," by E. Liaskou et al., the authors discuss the innate immune cells that take part in human liver inflammation, and their roles in both resolution of inflammation and tissue repair.

In the article "*Optimizing dendritic cell-based immunotherapy*: *Tackling the complexity of different arms of the immune system*," by I. Brussel et al., the authors explore the molecular and cellular mechanisms underlying adequate immune responses and focus on most favourable DC culture regimens and activation stimuli in humans. Also, they envisage that by combining each of the features outlined in the current paper into a unified strategy, DC-based vaccines may advance to a higher level of effectiveness.

In the article "*Danger signals activating the immune response after trauma*," by S. Hirsiger et al., the authors focuse on the role of the dual function DAMPs in the initiation of the immune response after trauma. Moreover, they shed light on recently discovered mechanisms of activation of innate immunity by mitochondrial DAMPs released from disrupted cells which bear bacterial molecular motifs similar to PAMPs due to their endosymbiotic origin.

In the article "*Guilty molecules*, *guilty minds? The conflicting roles of the innate immune response to traumatic brain injury*" by S. Hellewell and M. Morganti-Kossmann, the authors discuss the positive, negative, and often conflicting roles of the innate immune response to TBI in both an experimental and clinical settings and highlights recent advances in the search for therapeutic candidates for the treatment of TBI.

In the article "*Interplay between human cytomegalovirus and intrinsic/innate host responses*: *A complex bidirectional relationship*" by G. Rossini., the authors review the viral and cellular partners that mediate early host responses to HCMV with regard to the interaction between structural components of virions (viral glycoproteins) and cellular receptors (attachment/entry receptors, toll-like receptors, and other nucleic acid sensors) or intrinsic factors (PML, hDaxx, Sp100, viperin, interferon inducible protein 16), the reactions of innate immune cells (antigen presenting cells

and natural killer cells), the numerous mechanisms of viral immunoevasion, and the potential exploitation of events that are associated with early phases of virus-host interplay as a therapeutic strategy.

In the article "*Lipopolysaccharides: From Erinyes to Charites*" by A. Focà et al., the authors focuse on recent data supporting a beneficial activity of both typical and atypical endotoxins. Such novel perspective looks promising for the development of new drugs for the prevention and therapy of several human diseases.

In the article "*Essential role of mast cells in the visceral hyperalgesia induced by T. spiralis infection and stress in rats*" by C.-Q. Yang et al., the authors show that the visceral hyperalgesia cannot be triggered by stress in MCs-deficient rats, although both stress and infection play an important role in visceral hyperalgesia in wild control rats.

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Research Article

The EGF Receptor and HER2 Participate in TNF-α-Dependent MAPK Activation and IL-8 Secretion in Intestinal Epithelial Cells

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TNF-α activates multiple mitogen-activated protein kinase (MAPK) cascades in intestinal epithelial cells (IECs) leading to the secretion of interleukin 8 (IL-8), a neutrophil chemoattractant and an angiogenic factor with tumor promoting properties. As the epidermal growth factor receptor (EGFR) is a known transducer of proliferative signals and a potent activator of MAPKs, we hypothesized that the EGFR participates in TNF-dependent MAPK activation and IL-8 secretion by intestinal epithelial cells (IECs). We show that the EGFR is tyrosine-phosphorylated following treatment of IECs (HT-29 and IEC-6) with TNF-α. This requires EGFR autophosphorylation as it was blocked by the EGFR kinase inhibitor AG1478. Autophosphorylation was also inhibited by both a Src-kinase inhibitor and the metalloproteinase inhibitor batimastat. TNF treatment of IECs resulted in the accumulation of soluble TGF-α; treatment of IECs with batimastat suppressed TGF-α release and immunoneutralization of TGF-α resulted in decreased EGFR and ERK phosphorylations. TNF-α treatment of IECs resulted in an association between EGFR and HER2 and inhibition of HER2 using a specific inhibitor AG879 in combination with AG1478-suppressed TNF-α-dependent ERK phosphorylation and IL-8 release. Downregulation of HER2 via siRNA resulted in a significant decrease in ERK phosphorylation and a 50% reduction in IL-8 secretion.

1. Introduction

Inflammatory bowel diseases (IBDs), comprised of ulcerative colitis and Crohn's disease, are chronic, relapsing-remitting inflammatory diseases of unknown etiology. Current understanding suggests a critical role for the innate immune system in the context of a permissive genetic background and the intestinal microflora [1]. Interestingly, like other chronic inflammatory disorders, IBD is associated with an increased risk of cancer. In patients with ulcerative colitis particularly, the presence of either extensive or prolonged colonic disease can lead to a 20–30-fold increased risk of developing colorectal cancer (CRC) [2, 3].

The mechanism(s) by which chronic inflammation contributes to carcinogenesis are poorly defined. Tumors, including CRC, are infiltrated by activated immune cells including T cells, neutrophils, macrophages, and dendritic cells which secrete various cytokines, chemokines, proteases, and growth factors. This results in the modification of the surrounding stroma creating an environment conducive to tumor growth, invasion, and eventual metastasis [2, 4–6].

Tumor necrosis factor-alpha (TNF-α) is a proinflammatory cytokine known to play a central role in the development of intestinal inflammation and IBD [7]. Elevated serum levels of TNF-α have been demonstrated in IBD patients [8, 9], and anti-TNF therapies can be effective in the treatment of patients with otherwise refractory CD and UC [10–12]. Interestingly, TNF-α was recently shown to play a critical role in the development of colorectal cancer in an animal model of chemically induced colitis-associated cancer [2, 3, 13].

TNF-α affects the growth, migration, differentiation, and function of intestinal epithelial cells (IECs) [14–18]. However, how TNF-α mediates these functional changes in IECs remains poorly understood. TNF-α is known to

act through two distinct cell-surface receptors, a 55-KDa receptor and a 75-KDa receptor referred to as TNFR-I and TNFR-II, respectively, although most biological activities are attributed to the type I receptor [19, 20]. Historically, the first defined molecular target of TNF-α receptor signaling was the EGF receptor (EGFR) [21], a 170 kDa protein prototypical of a family of growth factor receptors characterized by a conserved N-terminal extracellular ligand-binding domain, a single transmembrane domain, and an intracellular C-terminus which possesses tyrosine kinase activity [22]. However, until recently the significance of TNF-dependant EGFR phosphorylation has remained obscure. The EGF receptor is a well-characterized transmitter of proliferation and differentiation signals, and a potent activator of the ERK MAPK pathway. Engagement of the EGF receptor results in its dimerization and activation of its intrinsic tyrosine kinase activity leading to receptor autophosphorylation on tyrosine residues [22, 23]. These phosphotyrosine residues then serve as docking sites for molecules containing specific domains involved in protein-protein interactions (e.g., Src-homology-2 (SH2) domains). Thus tyrosine phosphorylation of the EGFR is necessary for the recruitment and subsequent activation of multiple signaling pathways including the ERK pathway [22, 23].

In contrast to EGFRs, TNF-α receptors (TNFRs) do not possess any known catalytic activity and instead rely exclusively on adaptor molecules for the recruitment and transmission of extracellular signals [24]. Work over the last two decades has unveiled a unique set of intracellular signaling cascades downstream of TNF receptors, which elicit TNF-α-dependent cellular changes in a cell- and tissue-specific manner. TNF is a potent activator of MAPK signaling; however, the mechanisms whereby TNF-α activates the ERK MAPK pathway, remain poorly understood [19, 20]. GRB2, an adaptor molecule which couples receptor tyrosine kinase receptors to the MAPK pathway has been reported to associate with the type I TNF-α receptor, suggesting a direct link between TNFR-I and ERK [25]. In addition, RIP2 and MADD, two TNFR-I-interacting proteins, have been proposed to activate the ERK pathway in response to TNF-α [26, 27]. Also, the kinase and adaptor molecule KSR has recently been suggested to couple TNF receptors to ERK signaling in intestinal epithelial cells, leading to protection from cytokine induced apoptosis [28, 29]. Other groups have reported evidence for TNF-α-dependent EGFR transactivation and suggest that this event is required for ERK pathway activation in both hepatocytes and mammary epithelial cells [30, 31].

We have previously described the activation of the ERK signaling pathway in response to TNF-α in the transformed intestinal epithelial cell line HT-29 leading to expression of the angiogenic and chemotactic cytokine interleukin 8 (IL-8) [32]. EGFR gene amplification and overexpression are deemed important mechanisms leading to colonic epithelial transformation while IL-8 is believed to not only stimulate new blood vessel growth but also participates in the epithelial-mesenchymal transition in the colon [5, 33]. Therefore, EGFR transactivation leading to IL-8 secretion may not only contribute to inflammatory cell recruitment and activation in the context of IBDs but could also constitute an important component of colonic epithelial transformation. In this study we examined whether the EGF receptor is required for TNF-α-mediated activation of the ERK pathway leading to the secretion of IL-8 in intestinal epithelial cells. We report that maximal ERK activation and IL-8 secretion in response to TNF-α requires the release of TGF-α and the activation of the EGFR family of receptors.

2. Materials and Methods

2.1. Materials. Unless otherwise stated all chemicals were purchased from Sigma (St. Louis, MO).

2.2. Cell Culture. HT-29 and IEC-6 cells were obtained from American Type Culture Collection (Rockwell, MA). HT-29 cells were cultured in RPMI 1640 media (Gibco, Burlington, Ontario) supplemented with 10% heat-inactivated fetal calf serum (Cansera, Rexdale, Ontario), 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 2% sodium bicarbonate, and 10 mmol/L HEPES. IEC-6 cells were cultured in DMEM supplemented with 5% fetal calf serum, 2 mmol/L glutamine, and 5 μg/mL insulin. For experimental treatments, cells were grown in either 6 or 12 well tissue culture plates (Falcon, NJ).

Confluent monolayers (passage 25–45) were incubated with human recombinant TNF-α (10 ng/mL, R&D systems, Minneapolis, MN) or epidermal growth factor (EGF, 50 ng/mL) in the presence or absence of the ERK pathway inhibitor PD98059 (Calbiochem, San Diego, CA), the platelet-derived growth factor (PDGF) receptor tyrosine kinase inhibitor AG1298, Src kinase inhibitor PP2 (Calbiochem, San Diego, CA), the tyrosine kinase inhibitor genistein (Calbiochem, San Diego, CA) the matrix metalloproteinase inhibitor batimastat (BB94) (Tocris, Ellisville, MO), the TNF-alpha converting enzyme (TACE) inhibitor TAPI-1 (Calbiochem, San Diego, CA), the EGF receptor tyrosine kinase inhibitor AG1478 (Calbiochem, San Diego, CA), and the HER2 receptor tyrosine kinase inhibitor AG879 or TGF-α neutralizing serum (R&D Systems, Minneapolis, MN). Cells were treated with the inhibitors for 30 mins prior to treatment with TNF-α or EGF. Control monolayers were treated with an equal volume of vehicle (DMSO for all inhibitors, PBS pH 7.4 for EGF and TNF-α). Prior to experiments designed to measure ERK activation or EGFR/HER2 transactivation, cells were incubated in serum-free media (OptiMEM, Invitrogen, Carlsbad, CA) overnight in order to reduce growth factor-mediated activation. All experiments were conducted in serum-free media.

2.3. Determination of IL-8 and TGF-α in Supernatants. For the purpose of measuring IL-8, HT-29 monolayers were stimulated with 10 ng/mL TNF-α or 50 ng/mL EGF for 3 hrs. IL-8 protein in supernatants was measured via ELISA as follows: 96 well Maxisorp ELISA plates (Nunclon, Rochester, NY) were coated with 4 μg/mL capture monoclonal anti-IL-8 antibody (R&D Systems, Minneapolis, MN) in PBS (pH 7.4) overnight. Plates were then blocked overnight (5% sucrose, 0.05% sodium azide, 1% BSA in PBS pH 7.4). Plates were

washed 4 times between all steps with 0.05% Tween-20 PBS pH 7.4. 100 μL samples and standards (0–4000 pg/mL human recombinant IL-8, R&D Systems, Minneapolis, MN) were incubated in the plates overnight. Biotinylated polyclonal anti-IL-8 antibody (R&D Systems, Minneapolis, MN) was added (20 ng/mL in PBS pH 7.4) and plates incubated for 2 hrs. 100 μL Streptavidin-HRP (Southern Biotechnology Associates, Birmingham, AL) was added for 1 hr, followed by development with 100 μL TMBS (Calbiochem, San Diego, CA). Reaction was stopped with acid (0.5 M H_2SO_4) and plates read immediately at 450 nm using an ELISA plate reader (UV max, Molecular Devices, Sunnyvale, CA). All steps were carried out at room temperature. ELISA was sensitive to <30 pg/mL. TGF-α in cell culture supernatants was measured using a commercial TGF-α ELISA following manufacturer's instructions (R&D Systems, Minneapolis, MN).

2.4. Immunoprecipitation and Neutralization Studies. Cells were grown in six well plates (100 mm dishes for IEC-6 cells) and treated in duplicate as described in figure legends (results). Cells were harvested in 200 μL/well (500 uL/dish for IEC-6 cells) ice-cold modified RIPA buffer (250 mM NaCl, 50 mM HEPES, 0.5% NP40, 10% glycerol, 2 mM EDTA pH 8.0, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin) and sonicated on ice for 30 secs. Lysates were centrifuged at 4000 RPM for 2 min and supernatants transferred to new tubes. Protein concentrations were determined using a commercial Lowry Assay, (Biorad DC, Biorad, Hercules, CA). Protein concentrations were adjusted to the same concentration (5 mg/mL) then, 5 μg of anti-EGFR, anti-her-2 antibody added (1 μg/μL, Santa Cruz Biotech, Santa Cruz, CA), or antiphosphotyrosine (4G10 monoclonal, kind gift from Dr. Stephen Robbins) and incubated on a rotator overnight at 4°C. Antibody was precipitated by the addition of 50 μL of a 50% protein A/G-sepharose bead suspension for 2 hr at 4°C. Beads were washed 4 times with ice-cold modified RIPA buffer, supernatant was aspirated, then 60 μL 2X protein sample buffer was added per sample. Samples were boiled for 5 min, centrifuged at 10000 rpm for 1 min, and proteins separated by SDS-PAGE as described above. For TGF-α immunoneutralization studies, HT-29 cells were treated with 1–10 μg/mL anti-TGF-α or Ig control sera (R&D Systems, Minneapolis, MN) for 30 min prior to stimulation with TNF-α for 15 min. Cells were then harvested and analyzed for phospho-ERK content as described in the following.

2.5. Western Blotting. Monolayers were stimulated with 10 ng/mL TNF-α or 50 ng/mL EGF and harvested in Mono Q buffer (1.08 g β-glycerophosphate, 38.04 mg EGTA, 0.5 mL Triton X-100, 200 μL $MgCl_2$ per 100 mL) at different times. Following sonication for 30 secs, samples were centrifuged at 12000 rpm for 1 min to remove insoluble material and protein concentrations were determined using a commercial Lowry Assay (Biorad DC, Hercules, CA) using BSA standards made in Mono Q buffer. Lysate concentrations were adjusted to ensure even protein loading, mixed with an equal volume of 2X protein sample buffer (130 mM Tris pH 6.8, 20% glycerol, 4% SDS, 5% β-mercaptoethanol, trace bromophenol blue, 4 mM Sodium orthovanadate (Calbiochem, San Diego, MN), 2 μM microcystin (Calbiochem, San Diego, MN)), boiled for 2 mins, and separated via electrophoresis (10% acrylamide gels). Proteins were transferred for 1.5 hrs (2 hrs for EGFR/HER2 immunoprecipitation experiments) at 400 mA in transfer buffer (25 mM Tris-base, 150 mM glycine, 10% methanol) onto a PVDF membrane (Millipore, MA). Membranes were blocked for 1 hr using 3% skim milk (5% BSA for antphosphotyrosine blots) and incubated overnight in primary antibody. The antibodies used were as follows: anti-ERK-1 (1 : 3000, rabbit, Upstate Biotech, Lake Placid, NY), anti-phospho-ERK 1/2 (1 : 1000, rabbit, New England Biolabs, Beverly, MA), anti-phosphotyrosine (1 : 1000, 4G10 monoclonal, kind gift from Dr. Stephen Robbins), anti-EGFR, and anti-HER2 (1 : 1000, Santa Cruz Biotech, Santa Cruz, CA). Secondary staining was conducted using HRP-conjugated goat sera specific for mouse or rabbit Ig as required (1 : 3000, Amersham, Baie d'Urfe, Quebec) followed by chemiluminescent detection using a commercial reagent following manufacturer's instructions (Lumilight, Roche, Laval, Quebec). Comparisons were made only among samples isolated and transferred together onto the same membrane. Multiple exposures were done to ensure that film was not overexposed. In order to confirm equal loading of protein, all western blots using phospho-specific antibodies were stripped and reprobed with antibody against the nonphosphorylated kinase.

2.6. TACE Activity. HT-29 cells were incubated in serum-free media overnight, washed once with serum-free media, and stimulated with 10 ng/mL TNF-α for 15 mins. Cells were washed 2X with ice-cold PBS and harvested on ice. TACE activity was measured using a commercially available fluorimetric TACE assay kit (Sensolyte 520, AnaSpec, San Jose, CA) as per manufacturer's instructions. Fluorescence was measured every 5 mins for 3 hrs and plotted over time. Data represents fluorescence following 1 hr incubation with fluorescent substrate which is within the linear portion for all curves.

2.7. HER2 siRNA Knockdown. Single-cell suspensions of HT-29 cells were prepared by trypsinizing 100 mm confluent monolayers. 5×10^5 cells were transfected with 80 pmols siRNA reagent (control siRNA-A and HER2, Santa Cruz Biotech, Santa Cruz, CA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Cells were cultured for a further 48 hrs in serum-free media prior to treatment with TNF-α as described in results and figure legends.

2.8. Statistical Analysis. Unless otherwise stated, data shown in figures are representative experiments. Comparable results were obtained in additional experiments. Bar graphs are expressed as mean ± SD from at least three separate experiments. Differences between mean values were analyzed

using the Student's t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. EGF Rapidly Stimulates the ERK Pathway in HT-29 Cells. We have previously shown that TNF-α rapidly stimulates the phosphorylation (activation) of multiple MAPK pathways in HT-29 cells, including the ERK pathway leading to IL-8 secretion [32]. Previous studies have suggested an interaction between the EGFR and TNF-α signaling, some studies suggesting that the EGFR acts downstream of TNF receptors [15, 21, 34–38]. In that the EGFR is a potent activator of the ERK pathway in IECs, we sought to determine whether the EGFR couples TNF to ERK/MAPK signaling leading to IL-8 secretion [14, 15]. As shown in Figure 1(a), the kinetics of EGF-dependent ERK activation in HT-29 cells are consistent with the possibility that the EGFR couples TNF to ERK activation. ERK was rapidly activated following EGF treatment with significant ERK phosphorylation evident by 5 mins after stimulation whereas TNF-dependant ERK activation was only evident by 15 mins.

3.2. TNF-α Stimulates EGFR Tyrosine Phosphorylation in HT-29 Cells. Previous studies have described changes in EGFR tyrosine phosphorylation in response to TNF-α stimulation in various cell types [15, 21, 34–38]. Kaiser and Polk have previously reported a reduction in EGF-dependent EGFR tyrosine phosphorylation in response to TNF-α in intestinal epithelial cells [15, 16]. Argast et al. and Chen et al. on the other hand have recently reported EGFR transactivation in response to TNF-α in hepatocytes and mammary epithelial cells, respectively [30, 31]. They propose a similar model to that recently described for GPCR-mediated transactivation of growth factor receptors. This involves the extracellular release of growth factors via what is referred to as the "triple membrane passing signal" model of EGFR transactivation. Under this model, GPCR activation results in the activation of a membrane-bound matrix metalloproteinase (MMP) which then cleaves membrane-tethered EGFR ligands resulting in autocrine EGFR activation and Ras/ERK signaling [39–41]. We sought to examine whether a similar mechanism mediates ERK activation by TNF-α in intestinal epithelial cells. HT-29 cells were cultured in serum-free media overnight, stimulated with 10 ng/mL TNF-α for various times, and the EGF receptor immunoprecipitated. EGFR tyrosine phosphorylation was then assessed by western blotting using antiphospho-tyrosine sera. As shown in Figure 1(b), there was a low level of constitutive EGFR tyrosine phosphorylation in control cells which increased significantly following 15 mins treatment with TNF-α.

To determine whether the increase in tyrosine phosphorylation of the EGFR observed following TNF-α treatment requires the intrinsic kinase activity of the EGFR (transactivation), HT-29 cells were treated as above, except cells were incubated with the EGF receptor tyrosine kinase inhibitor AG1478 for 15 mins prior to TNF-α stimulation. As shown in Figure 1(c), EGFR phosphotyrosine content

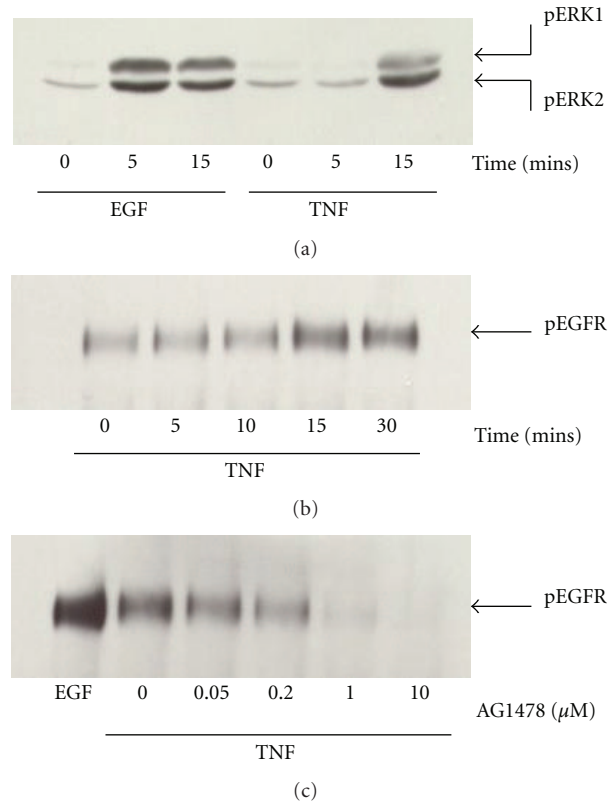

Figure 1: EGF rapidly stimulates the ERK pathway in HT-29 cells. HT-29 cells were cultured in serum-free media overnight and stimulated with 50 ng/mL EGF or 10 ng/mL TNF (a). ERK activation in response to TNF is relatively delayed (apparent by 15 mins) relative to EGF (apparent by 5 mins). (b) shows an antiphosphotyrosine blot of immunoprecipitated EGFR following stimulation of serum-starved HT-29 cells with TNF-α. TNF-α treatment results in the time-dependent tyrosine phosphorylation of the EGF receptor. (c) shows the effect of EGF receptor tyrosine kinase inhibition using the EGFR tyrosine kinase inhibitor AG1478. Cells were treated for 15 mins with AG1478 (0–10 μM) and stimulated with 10 ng/mL TNF-α for 15 mins. AG1478 dose-dependently inhibits EGFR phosphorylation on tyrosine. Data are representative of at least three separate experiments.

was dose-dependently reduced in the presence of AG1478. This effect was evident at 50 nM AG1478 with complete reduction apparent between 1 and 10 μM AG1478. AG1278 (5 μM), a PDGF-receptor tyrosine kinase inhibitor which is structurally similar to AG1478, did not affect EGF receptor tyrosine phosphorylation (Figure 2(a)). Interestingly, despite almost complete inhibition of EGFR phosphorylation, AG1478 had a modest effect on ERK phosphorylation (Figure 2(b)). TNF-α-dependant EGFR transactivation was also observed in the rat intestinal cell line IEC-6 (Figure 2(c)) suggesting that TNF-dependent EGFR transactivation is conserved across intestinal epithelial cell lines. On the other hand, there is a lack of correlation between the effects of AG1478 on EGFR phosphorylation and ERK activation.

3.3. TNF-Dependent EGFR Transactivation Is Matrix Metalloproteinase Dependent. We next examined whether MMP

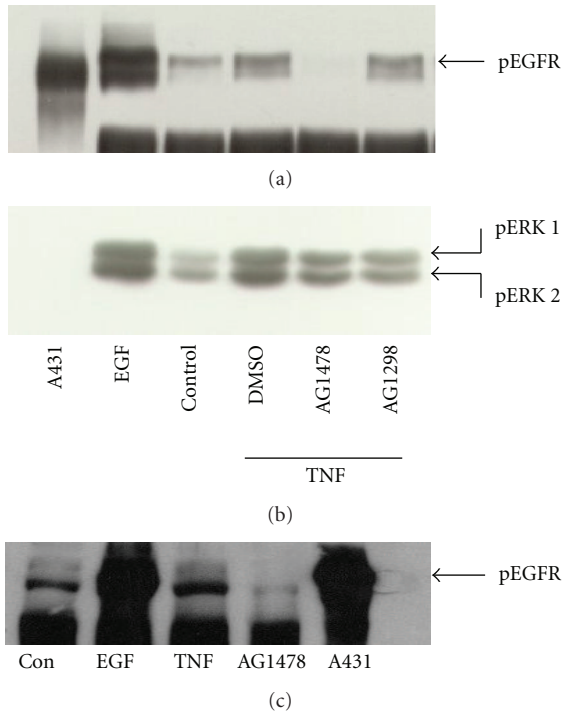

Figure 2: EGFR tyrosine phosphorylation is inhibited by the EGF receptor tyrosine kinase inhibitor AG1478 (10 μM) but not the PDGF receptor inhibitor AG1298 (5 μM) (a). In contrast, only a modest decrease in Erk1/2 phosphorylation was noted in response to pretreatment of HT-29 cells with either of these agents (b). Serum-starved cells were pretreated for 30 mins prior to stimulation with TNF-α for 15 mins, and EGFR tyrosine phosphorylation and ERK phosphorylation were assessed as described in *Materials and Methods*. Transactivation of the EGFR in response to TNF-α was also observed in the rat intestinal epithelial cell line IEC-6 (c). IEC-6 cells were treated with 10 ng/mL TNF-α for 15 mins in the presence or absence of 1 μM AG1478. Data are representative of at least three experiments.

activity is required for EGFR transactivation in response to TNF-α in HT-29 cells. Cells were serum-starved overnight and treated for 15 mins with 10 ng/mL TNF-α in the presence or absence of the pan-MMP inhibitor batimastat (BB94, 10 μM). As shown in Figure 3(a), treatment with BB94 resulted in almost complete inhibition of EGFR tyrosine phosphorylation in response to TNF-α, suggesting that EGFR tyrosine kinase activation in response to TNF-α requires MMP activity.

We next sought to identify the MMP responsible for TNF-dependent EGFR transactivation. TNF-α-converting enzyme (TACE) is a metalloproteinase which derives its name from its ability to cleave membrane-bound TNF-α leading to TNF-α release, but it also cleaves multiple EGFR ligands including amphiregulin, HB-EGF, epiregulin, and TGF-α [42]. TACE is expressed in HT-29 cells where it participates in TNF-α-stimulated TNF-α release [43]. We therefore examined whether TACE is required for TNF-dependent EGFR transactivation. As shown in figure 3B, pretreatment of HT-29 cells with the TACE-specific inhibitor TAPI-1 attenuated EGFR phosphorylation following TNF-α treatment.

TGF-α has previously been implicated in TNF-α-stimulated EGFR transactivation [31, 44]. We therefore stimulated HT-29 cells with TNF-α and measured TGF-α in the culture media. As shown in Figure 3(c), treatment with TNF-α resulted in a 60% increase in soluble TGF-α compared to unstimulated controls. Pretreatment of cells with BB94 completely blocked TNF-α-stimulated TGF-α release as well as basal TGF-α release in unstimulated cells. On the other hand, pretreatment of HT-29 cells with increasing concentrations of the TACE inhibitor TAPI-1 had a dose-dependant effect on TNF-stimulated TGF-α release but did not alter basal TGF-α production (Figure 3(c)). We next measured TACE activity in control and TNF-stimulated cells using a fluorescent peptide substrate harbouring a TACE cleavage site. Interestingly, TACE activity did not change in response to TNF-α treatment (Figure 3(d)).

3.4. Tyrosine Kinase Inhibitors Inhibit EGFR Transactivation in Response to TNF-α. The sensitivity of TNF-α-dependent EGFR phosphorylation to batimastat suggests that, similar to GPCRs, TNF-α utilizes a "triple membrane passing signal" mechanism in order to activate the EGFR. Unlike GPCRs, however, TNF-α does not trigger changes in intracellular calcium in HT-29 cells (data not shown); thus it is unlikely that TNF would act via Pyk, a calcium-dependent kinase suggested to play a role in other systems such as that of carbachol-stimulated EGFR transactivation in T84 intestinal epithelial cells [39]. Instead we asked whether tyrosine kinases such as Src family kinases are involved as has been suggested in other cell types [45]. HT-29 cells were treated with 10 μM AG1478, 2 μM PP2 (a Src-kinase inhibitor), or 100 μM genistein (tyrosine kinase inhibitor) for 15 mins prior to treatment with TNF-α for 15 mins. The results of this experiment are shown in Figure 4(a). As before, TNF-α treatment resulted in increased EGFR tyrosine phosphorylation and this was blocked by AG1478. Interestingly, PP2 also abrogated EGFR tyrosine phosphorylation as well as the phosphorylation on tyrosine residues on proteins that coprecipitate with the EGFR (data not shown). This was also true of genistein, a broad specificity tyrosine kinase inhibitor, although genistein had a smaller effect upon the phosphotyrosine content of coprecipitating proteins. These results suggest the participation of Src-like kinases in relaying the signal that links TNF-α to the EGFR. In parallel experiments, we looked at the effects of these inhibitors upon TNF-stimulated ERK phosphorylation (Figure 4(b)). Similarly, PP2 and genistein had almost no effect upon ERK activation despite having completely abrogated EGFR phosphorylation (Figure 4(b)).

3.5. Neutralization of TGF-α Blocks Both EGFR Transactivation and ERK Signaling. Having observed increased TGF-α release in response to TNF-α and considering the ability of a metalloproteinase inhibitor to attenuate both TGF-α release and EGFR phosphorylation, we next asked whether specific blockade of TGF-α using a neutralizing antibody

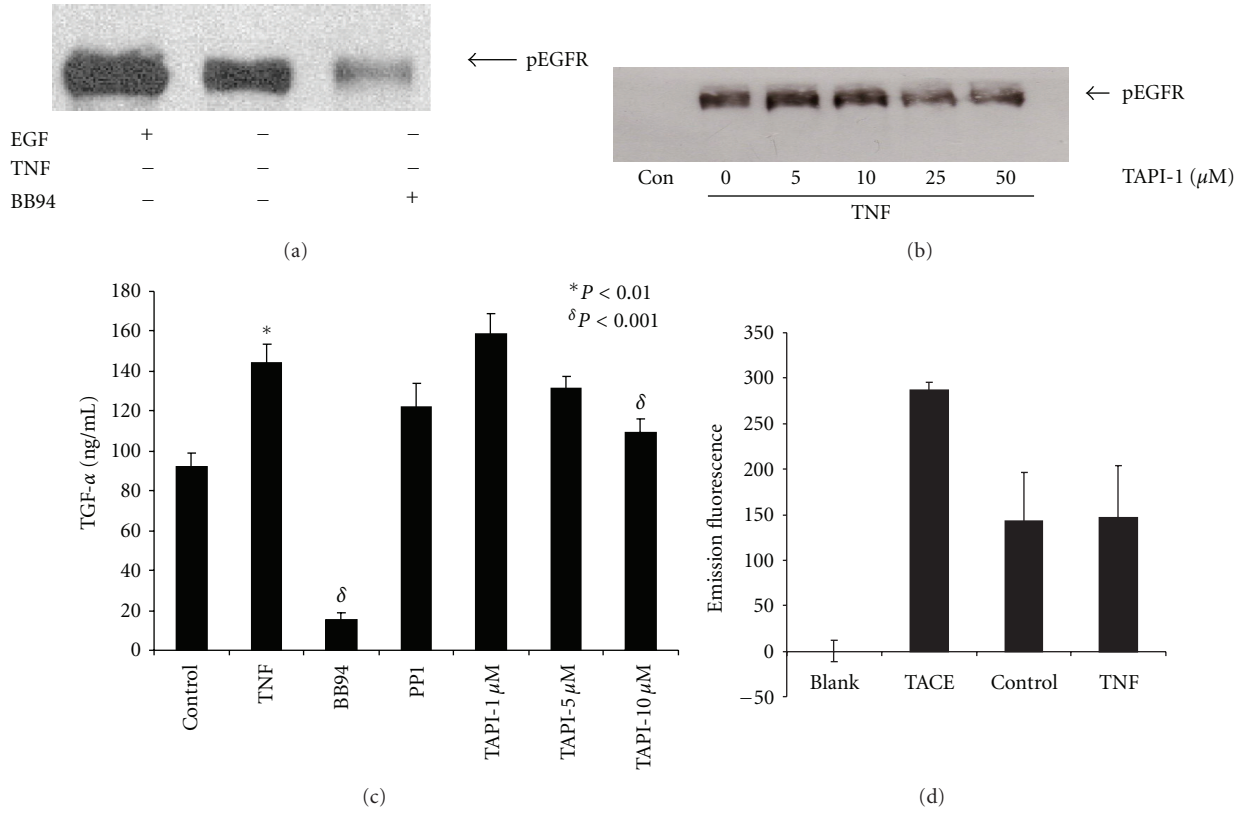

Figure 3: TNF-dependent EGFR transactivation requires metalloproteinase activity and results in TGF-α release. Serum-starved cells were treated for 30 min with the metalloproteinase inhibitor BB94 (batimastat, 10 μM) (a), or increasing concentrations of the TNF-converting enzyme (TACE) inhibitor TAPI-1 (b), and stimulated with 10 ng/mL TNF-α for 15 mins. EGFR tyrosine phosphorylation was assessed as described in Section 2. EGFR tyrosine phosphorylation is significantly reduced in the presence of BB94 and to a lesser extent by TAPI-1. (c) shows the effect of BB94 and TAPI-1 pretreatments on TNF-stimulated TGF-α release. Serum-starved cells were pretreated for 30 mins with BB94 or TAPI-1, stimulated with TNF-α for 3 hrs, and TGF-α measured via ELISA. (d) shows total TACE activity as measured using either recombinant TACE or membrane preparations from vehicle and TNF-treated HT-29 cells using a fluorescent substrate. Cells were pretreated with vehicle or TAPI-1 for 30 mins prior to stimulation with TNF-α (10 ng/mL) for 15 mins (see Section 2). Data are representative of at least three experiments.

could block both EGFR and ERK activations. HT-29 cells were incubated with increasing concentrations of TGF-α neutralizing sera or isotype control and both EGFR tyrosine phosphorylation and ERK activation examined. As shown in Figure 5(a), anti-TGF-α dose-dependently blocked EGFR tyrosine phosphorylation. This was paralleled by a significant reduction in ERK phosphorylation (Figure 5(b)).

3.6. HER2 Associates with EGFR and Participates in TNF-α-Dependent ERK Activation. EGFR is a member of the structurally related ErbB family of transmembrane receptor tyrosine kinases, which also includes HER2 (Neu/ErbB2), HER3 (ErbB3), and HER4 (ErbB4) [46]. Heterodimerization between ErbB family members is common and adds to the diversity of signals which can be elicited by multiple ligands with different binding affinities. HER2 is an orphan receptor and frequently partners with other ErbB family members. Zhou and Brattain demonstrated synergy between EGFR and HER2 tyrosine kinase inhibitors towards the induction of apoptosis in human colon cancer cell lines [47]. In this study, EGFR transactivation and ERK activation could both be blocked by neutralizing TGF-α, in contrast to AG1478 which does not block ERK activation to the same degree as it blocks EGFR phosphorylation, suggesting that TGF-α may activate another EGFR family receptor leading to the activation of ERK. We therefore asked whether HER2, in association with EGFR, participates in TNF-α stimulated ERK activation.

To answer this question, we first stimulated HT-29 cells with TNF-α for various times and immunoprecipitated the EGFR. These immunoprecipitates were then probed for the presence of HER2. As shown in Figure 6(a), TNF-α treatment resulted in the time-dependant recruitment of HER2 to EGFR with peak association at 15 mins. Interestingly, this association is transient as it is no longer evident by 30 mins. Next, we assessed whether HER2 becomes phosphorylated on tyrosine residues in response to TNF-α. For this purpose serum-starved HT-29 cells were stimulated as before and tyrosine-phosphorylated proteins immunoprecipitated using antiphosphotyrosine sera. Samples were then probed via western blotting using anti-HER2 sera. As shown in Figure 6(b), HER2 phosphotyrosine content was significantly

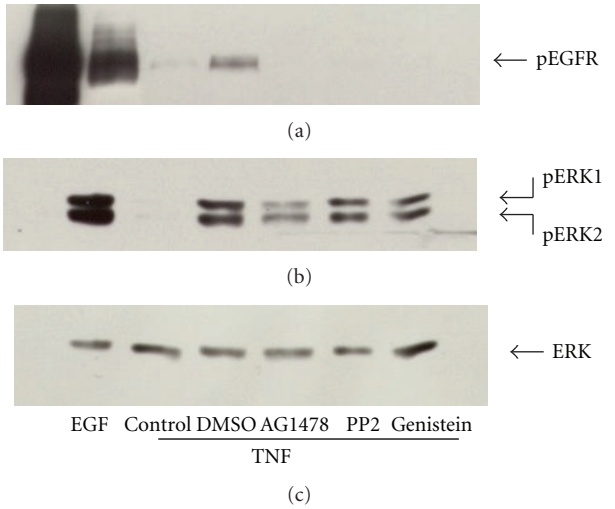

Figure 4: Tyrosine kinase inhibitors inhibit EGFR phosphorylation in response to TNF-α. HT-29 cells were treated with 2 μM PP2 (Src-kinase inhibitor), 100 μM genistein (tyrosine kinase inhibitor), or 10 μM AG1478 for 15 mins prior to 15 mins of TNF-α treatment. The EGF receptor was immunoprecipitated and tyrosine phosphorylation assessed (a). Both PP2 and genistein abrogated TNF-α-dependent EGFR tyrosine phosphorylation. In contrast, neither PP2 nor genistein had an appreciable effect upon TNF-stimulated ERK1/2 phosphorylation (b) and (c). Data are representative of at least three experiments.

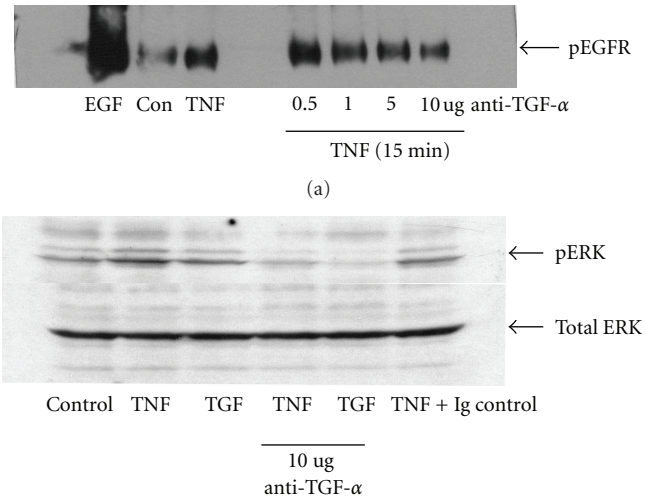

Figure 5: TGF-α release is required for TNF-α-stimulated EGFR transactivation and Erk1/2 phosphorylation. Serum-starved HT-29 cells were pretreated with increasing concentrations of TGF-α-neutralizing serum or Ig control and stimulated with TNF-α for 15 mins. EGFR was immunoprecipitated and phosphotyrosine content determined by western blotting (a). (b) shows the effect of TGF-α-neutralizing serum on TNF-stimulated Erk1/2 phosphorylation. Data are representative of at least 3 separate experiments.

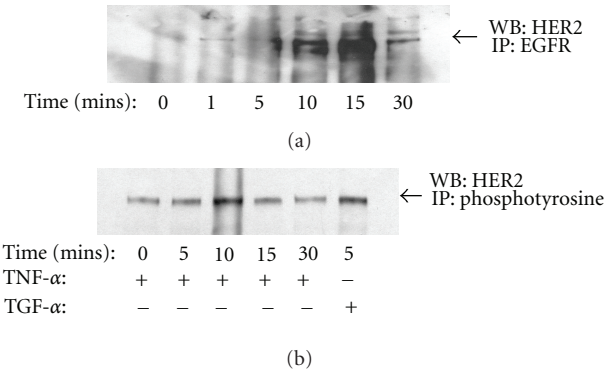

Figure 6: TNF treatment stimulates EGFR-HER2 heterodimerization, and HER2 tyrosine phosphorylation. HT-29 cells were serum-starved for 24 hrs prior to stimulation with 10 ng/mL TNF-α. EGFR was immunoprecipitated, and coprecipitating HER2 was measured via western blotting (a). HT-29 cells were serum-starved for 24 hrs, stimulated with 10 ng/mL TNF-α for 15 mins, and phosphotyrosine-containing proteins immunoprecipitated. Samples were separated by SDS-PAGE and HER2 content quantified by western blotting (b). Figures are representative of at least 3 separate experiments.

increased 10 mins after stimulation with TNF-α and after 5 mins of stimulation with TGF-α.

We next asked whether inhibition of HER2 in combination with inhibition of the EGFR would result in greater inhibition of ERK activation than inhibition of the EGFR alone. HT-29 cells were incubated in the presence or absence of 5 μM AG1478 and 2.5 μM of the HER2-specific inhibitor AG879 prior to stimulation with TNF-α. As shown in Figure 7, combined inhibition of HER2 and the EGFR resulted in greater inhibition of ERK signaling as compared to EGFR inhibition alone.

3.7. EGF Receptor and HER2 Tyrosine Kinase Inhibitors Block TNF-α-Stimulated IL-8 Secretion by HT-29 Cells. In a previous study we showed a requirement for ERK in TNF-α-stimulated IL-8 secretion by intestinal epithelial cells [32]. We therefore asked whether inhibition of EGFR tyrosine kinase activity would decrease TNF-stimulated IL-8 secretion. HT-29 cells were treated with increasing doses of AG1478 for 15 mins followed by treatment with 10 ng/mL TNF-α for 6 hrs. The amount of secreted IL-8 was then measured in the supernatants via ELISA. As shown in Figure 8(a), TNF-α-stimulated IL-8 release was inhibited only at 10 μM AG1478 ($\sim$50%, $P < 0.001$). On the other hand, while 1 μM AG1478 was sufficient to completely block EGFR phosphorylation (Figure 1(c)), it had no effect on IL-8 secretion. We next tested the effect of HER2 inhibition upon IL-8 secretion. As shown in Figure 8(b), the HER2 inhibitor AG879 dose-dependently inhibited TNF-induced IL-8 secretion. Further, combined AG879 and AG1478 at submaximal doses inhibited IL-8 secretion in an additive manner.

3.8. HER2 siRNA Blocks TNF-α-Stimulated ERK Activation and IL-8 Secretion in HT-29 Cells. Tyrosine kinase inhibitors selective for EGFR and HER2 suggested a role for these receptors in TNF-stimulated ERK activation and IL-8 secretion.

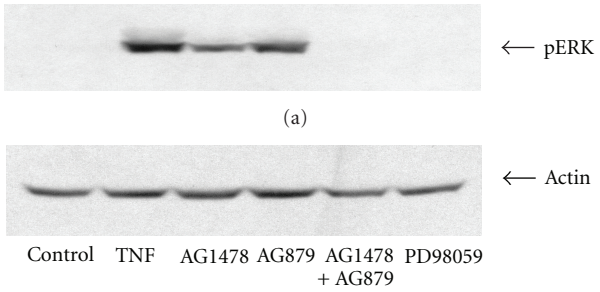

Figure 7: Inhibition of HER2 tyrosine kinase activity (2.5 μM AG879) in addition to that of EGFR results in greater inhibition of ERK1/2 phosphorylation as compared to EGFR inhibition alone (10 μM) (a) in HT-29 cells. (b) is a loading control, 25 μM PD98058. Figure is representative of three separate experiments.

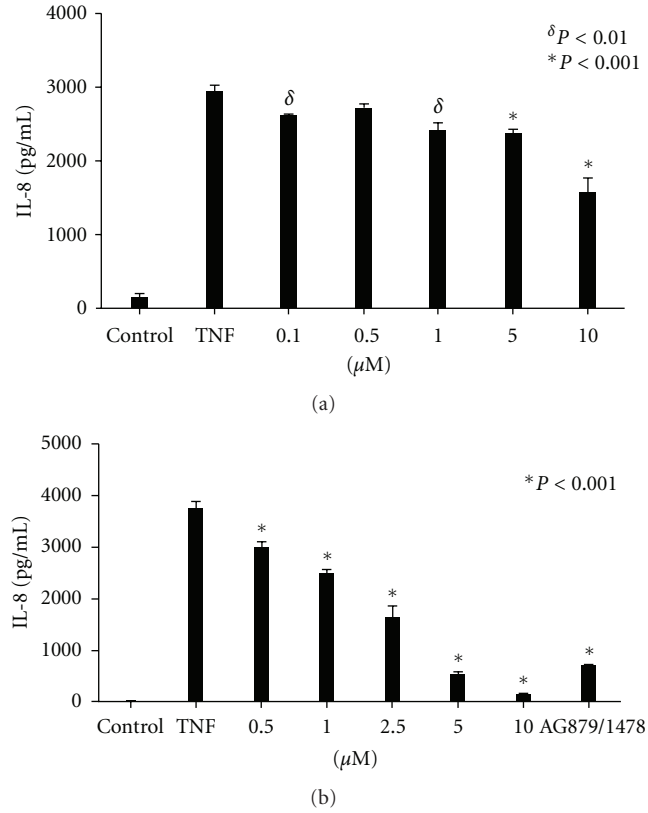

Figure 8: Effect of EGFR and HER2 inhibition on TNF-α-stimulated IL-8 secretion. HT-29 cells were treated with increasing doses of AG1478 for 30 mins prior to 6 hr stimulation with 10 ng/mL TNF-α (a). HT-29 cells were treated with increasing doses of the HER2 inhibitor AG879 or a combination of AG1478 and AG879 (b). Secreted IL-8 was measured via ELISA. Results are representative of three separate experiments. $^{*}P < 0.01$, $^{\delta}P < 0.001$.

To further demonstrate a role for ErbB2/Her2 in this process we made use of siRNA specific to HER2. HT-29 cells were transfected with HER2-specific siRNA for 48 hrs and both EGFR and HER2 protein levels determined by immunoblotting (Figure 9). As shown in Figure 9(a), HER2 protein expression levels were significantly decreased by treatment with HER2 siRNA. In contrast, the expression of EGFR was unaffected by treatment with HER2-specific siRNA (Figure 9(a) middle). We next took HER2 siRNA-treated HT-29 cells, stimulated them with TNF-α for 15 mins, and determined the levels of phospho-ERK. As shown in Figure 9(b), downregulation of HER2 via siRNA significantly reduced ERK activation in response to TNF-α. Lastly, HT-29 cells were transfected with HER2 siRNA for 48 hrs, stimulated for an additional 12 hrs with TNF-alpha, and IL-8 protein secretion measured via ELISA. As shown in Figure 9(c), inhibition of HER2 protein expression via siRNA led to a profound reduction in IL-8 secretion in response to TNF-α treatment.

4. Discussion

Various studies have described the phosphorylation of the EGF receptor in response to TNF-α. This has been shown to occur on tyrosine residues, threonine residues, or both and to result in different outcomes depending on the cell type studied. Donato et al. examined multiple fibroblast cell lines and suggested that phosphorylation of the EGF receptor occurs predominantly on threonine residues and results in a reduction in EGF receptor affinity in cell lines susceptible to TNF-α-mediated cytotoxicity [34]. On the other hand, Guazzoni et al. reported inhibition of EGFR tyrosine phosphorylation which was accompanied by a decrease in EGF receptor tyrosine kinase activity in a fibroblast cell line [35]. Further, Murthy et al. reported EGFR tyrosine phosphorylation in response to IL-1 and TNF-α in the intestinal epithelial cell line Caco-2, an event which mimics the effects of the EGFR ligand EGF [37]. In this last study, Murthy and coworkers identified 2 peaks in EGFR tyrosine phosphorylation in response to TNF, one at 30 mins and the other at 6.5 hrs. Interestingly, it was determined that the early peak was ligand independent whereas the later peak could be abolished using a receptor blocking antibody [37].

In this study we provide evidence that TNF activates one or more metalloproteinases leading to the release of TGF-α in intestinal epithelial cells. TNF-dependant EGFR phosphorylation was abrogated by the pan-MMP inhibitor BB94 (Figure 3(a)) and BB94 profoundly reduced TGF-α release both basally and in response to TNF-α (Figure 3(c)). Blocking TGF-α in turn led to reduced EGFR activation and ERK phosphorylation (Figures 5(a) and 5(b)). In a previous study, we demonstrated that ERK activation was necessary for maximal IL-8 secretion through a mechanism involving the stabilization of IL-8 mRNA. Thus, TNF activates multiple signaling cascades including the IκK/NFκB, p38 and ERK pathways which act at different points to stimulate maximal IL-8 release: stimulating NFκB nuclear translocation [48, 49], increasing NF-κB transcriptional activity [48, 49] and stabilizing IL-8 mRNA message [32].

Previously, Janes et al. showed that TNF-α stimulates EGFR transactivation and the ERK signaling pathway in HT-29 cells via an autocrine loop involving TGF-α. In this

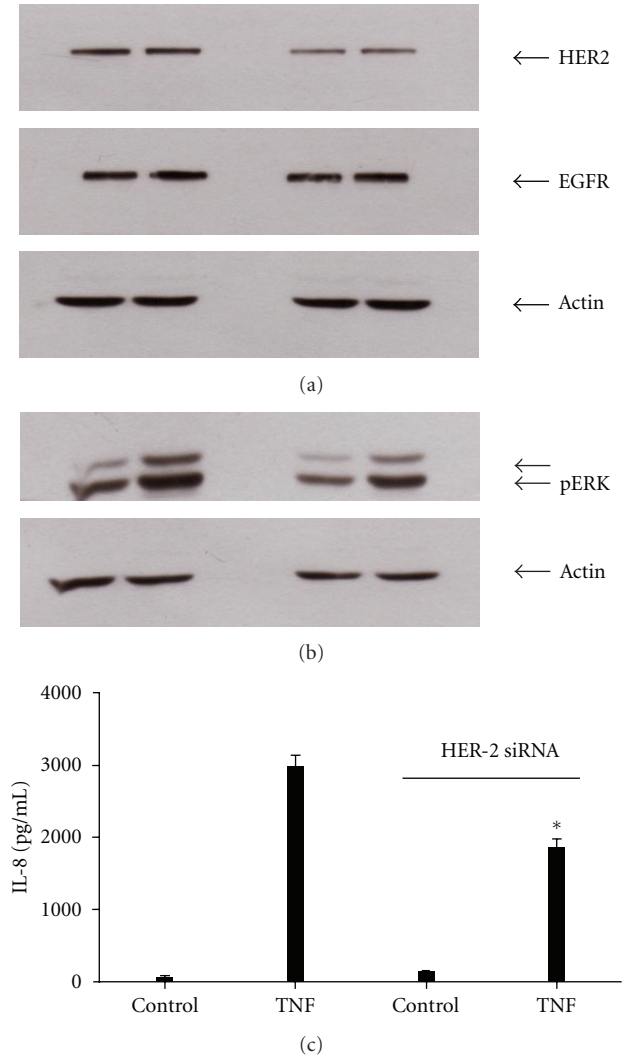

Figure 9: HER2 knockdown using siRNA attenuates TNF-dependant ERK activation and significantly inhibits IL-8 secretion. HT-29 cells were transfected with control or HER2-specific siRNA and incubated in serum-free media for 48 hrs. Cell lysates were prepared for western blotting as described in Section 2. HER2 specific siRNA reduced HER-2 protein expression but did not alter EGFR expression (a). siRNA-transfected cells were then stimulated with 10 ng/mL TNF-α for 15 mins, or 6 hrs and ERK phosphorylation, and IL-8 secretion measured as described previously ((b) and (c) resp.). Figures are representative of at least 3 separate experiments. $^*P < 0.001$.

study they showed that blocking TGF-α/EGFR signaling enhanced TNF-α/IFN-γ-induced apoptosis. They used an EGFR-neutralizing antibody (C225, Cetuximab/Erbitux) to completely block TNF-stimulated EGFR phosphorylation and downstream signaling. Our data with AG1478, the EGFR inhibitor, was initially very difficult to interpret. We observed a complete blockade of EGFR phosphorylation with AG1478; however, we could at best only partially block TNF-dependant ERK activation and had almost no effect upon IL-8 secretion with this drug alone. In the study by Janes et al, they pretreated cells with IFN-γ before all their experiments in order to enhance apoptosis in response to TNF-α. IFN-γ pretreatment is a key difference between their experimental design and ours; however, we were unable to completely block ERK activation or IL-8 secretion with AG1478 with or without IFN-γ pretreatment (unpublished data). However, using combined EGFR and HER2 inhibition, we can achieve greater ERK and IL-8 inhibition than either inhibitor alone. Interestingly, inhibition of HER2 using AG879 alone had a profound effect upon IL-8 secretion (~50% reduction at 2.5 μM), but combined inhibition using both AG1478 (1 μM) and AG879 (2.5 μM) resulted in greater than 80% inhibition. This may represent a nonspecific effect on the part of our inhibitors or a greater role for the EGFR/HER2 receptor complex upon IL-8 secretion, which may involve the activation of pathways other than the MEK/ERK pathway. Recently, Sethi et al. have suggested that the EGFR can stimulate NFκB activation independent of IKK through the phosphorylation of IκB on tyrosine 42 [50]. Although this pathway may contribute to NFκB activation and IL-8 secretion in IECs, there is significant evidence pointing to the importance of IKK-dependent IκB phosphorylation and degradation leading to NFκB activation and proinflammatory gene expression in these cells [49]. Future experiments will look at the effect of EGFR/HER2 inhibition upon NFκB activation and IκB degradation.

While this paper was in preparation, Hobbs and coworkers have shown TNF transactivation of the EGFR stimulates COX-2 expression in mouse intestinal cells. They provide evidence to suggest the participation of Src and p38, kinases in an MMP-independent manner. In our hands, a Src inhibitor (PP2) and a tyrosine kinase inhibitor (genistein) completely blocked EGFR phosphorylation and yet had no effect upon ERK activation (Figure 4). It would be expected that if Src or a related kinase lies upstream of MMP(s) and TGF-α release, Src inhibition should result in reduced EGFR and Her-2 activities leading to decreased ERK activation. Perhaps in the absence of Src, TNF-stimulates ERK activity through a yet undetermined mechanism. Alternatively, there could exist two parallel pathways leading to EGFR transactivation downstream from TNF-α, one Src-dependent, and one MMP-dependent. TNF-α has been shown to activate both in other systems [30, 31, 37, 44]. Janes and coworkers and now us have shown a requirement for TGF-α in TNF-dependent ERK activation using human HT-29 cells; perhaps there exist cell line/species-specific differences that underlie the noted discrepancies between these studies

TACE seemed like a good candidate to be the MMP activated by TNF leading to TGF-α release. The TACE inhibitor TAPI-1 inhibits TNF-stimulated TGF-α release but this is most evident at high concentrations. In addition, TNF failed to stimulate TACE activity. Interestingly, there is precedent for this as Myhre et al. have recently shown that TACE may be regulated by at the level of cellular localization as opposed to enzymatic activity [51].

In the present study we focused on the role of the EGFR/HER2 signaling pathway in TNF-stimulated IL-8 secretion. However, this pathway is likely to contribute to

many aspects of TNF signaling in IECs. Both the EGFR and TNF are known to profoundly affect intestinal epithelial cell function. Of particular interest in this regard, Janes et al. have shown that this pathway may modulate IEC apoptosis which may have implications towards the development of cancer in the context of inflammation [44]. Work by Yamaoka et al. has suggested that TNF-dependant transactivation of the EGFR/Her2 heterodimer activates Akt thus activating an antiapoptotic program which protects IECs from TNF-dependant apoptosis [52]. Likewise, in our study we provide evidence that the EGFR may contribute towards the production of the potent angiogenic chemokine IL-8. IL-8 not only acts as a potent neutrophil chemoattractant but also has been shown to be the most bioactive chemoattractant for microvascular endothelial cells in the context of human IBD, contributing to the development of an abnormal mucosal vascular bed in the context of intestinal inflammation [53]. Importantly, polymorphisms within the loci coding for IL-8 receptors A and B have recently been identified in genome-wide association studies supporting an important role for IL-8 in the pathogenesis of IBD [54].

IL-8 and other cytokines such as IL-6 have been shown to play a critical role in tumor growth in multiple cancer models independent of inflammation such as in Ras-driven models of cancer [6]. Il-8 has been shown to recruit regulatory T cells which via their immunosuppressive abilities may contribute to tumor escape from immune surveillance [55]. Interestingly, therapies targeting both EGFR and Her 2 have been shown to normalize tumor vascularization [56]. Thus, IL-8 secretion in the context of inflammation may act to stimulate angiogenesis in the absence of mutant Ras and therapies targeting EGFR signaling may act in part by blocking IL-8 production.

The fact that EGFR/HER2 participates in TNF signaling may have several important therapeutic implications. First, it suggests that therapies which target the EGFR/HER2 may potentially affect immune responses in the gut. Second, EGFR/HER2 activation by TNF may contribute to inflammation induced carcinogenesis. This possibility will have to await testing *in vivo* to see the effect of EGFR/Her2 signaling inhibition in the context of a colitis-induced cancer model. Third, attempts at abrogating EGFR signaling in the context of TNF-α signaling must keep in mind the participation of other EGFR binding partners such as HER2.

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Review Article

Innate Immune Cells in Liver Inflammation

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Innate immune system is the first line of defence against invading pathogens that is critical for the overall survival of the host. Human liver is characterised by a dual blood supply, with 80% of blood entering through the portal vein carrying nutrients and bacterial endotoxin from the gastrointestinal tract. The liver is thus constantly exposed to antigenic loads. Therefore, pathogenic microorganism must be efficiently eliminated whilst harmless antigens derived from the gastrointestinal tract need to be tolerized in the liver. In order to achieve this, the liver innate immune system is equipped with multiple cellular components; monocytes, macrophages, granulocytes, natural killer cells, and dendritic cells which coordinate to exert tolerogenic environment at the same time detect, respond, and eliminate invading pathogens, infected or transformed self to mount immunity. This paper will discuss the innate immune cells that take part in human liver inflammation, and their roles in both resolution of inflammation and tissue repair.

1. Introduction

The immune system is made up of a coordinated network of cells, tissues and organs, which are able to attack non-self-exogenous pathogens and self-endogenous danger with a complex set of defence mechanisms. It responds to pathogens in two fundamental pathways: the primal strategy of "identifying and destroying" (innate immunity) or the specific detection and targeted killing process with regulation and memory (adaptive immunity) [1].

The innate immune system is the first line of defence against initial invading organisms and environmental challenges during the initial critical hours and days of life [2]. The overall survival of the host depends on its ability to recognise and induce the appropriate defence signals for the elimination of infectious microbes. Through anatomical barriers (skin and mucosal epithelia of the gastrointestinal, respiratory and reproductive tracts), soluble antimicrobial factors (acute phase proteins, complement and cytokines), and cellular components, the innate immune system provides protective barriers between the inside of the body and the outside world.

Innate immune cells [monocytes, macrophages, mast cells, neutrophils and natural killer (NK) cells] are able to recognise pathogen associated molecular patterns (PAMPs) such as components of microorganisms [lipopolysaccharide (LPS), glycolipids, flagellin, lipoproteins, viral RNA and bacterial DNA] and endogenous ligands (such as heat shock proteins released by damaged or necrotic host cells) via their pattern-recognition receptors (PRRs), which include receptors for bacterial carbohydrates and toll-like receptors (TLRs). The TLRs and corresponding ligands, their impact on innate immune system are described in Table 1. Engagement of PAMPs with PRRs results in targeted and specific destruction of the activating organism, infected or tumour cells, by releasing cytotoxic agents or phagocytosis [3].

2. Liver as an Immunological Organ

Adult human liver is the largest internal organ in the body, weighing 1.2–1.5 kg. It has a dual blood supply with oxygenated blood entering through the hepatic artery (20%) and blood rich in nutrients and bacterial endotoxin

Table 1: Toll-like receptors and their ligands, target microbes, and effector molecules are described.

TLRs	Ligands	Target microbes	Effector molecules
TLR1	Triacyl lipopeptides	Mycobacteria	Inflammatory cytokines
TLR2	Peptidoglycans, Lipoprotein; Zymosan	G+ bacteria Mycobacteria Yeast/other fungi	Inflammatory cytokines
TLR3	Viral double stranded RNA	Viruses	IFNβ
TLR4	LPS	Gram-negative bacteria	IFNβ Inflammatory cytokines
TLR5	Flagellin	Bacteria	Inflammatory cytokines
TLR6	Yeast zymosan Diacyl lipopeptides	Mycobacteria Yeasts and Fungi	Inflammatory cytokines
TLR7/8	Viral Single-stranded RNA	Viruses	IFNα
TLR9	Bacterial and viral CpG DNA	Bacteria/virus	IFNα Inflammatory cytokines

entering the liver through the portal vein (80%). The arterial and portal-venous blood percolates through a network of liver sinusoids generating a mixed arterial-venous perfusion collected in the central vein and exit via three hepatic veins and drain back into the inferior vena cava [4, 5]. The liver is constantly exposed to antigenic loads of harmless dietary and commensal products from the gastrointestinal tract via portal vein and blood-borne antigens via hepatic artery. Thus, it is prerequisite for the liver immune system to be appropriately equipped in order to protect itself from pathogens and metastatic cells, whilst tolerating harmless self and foreign antigens. The liver innate cells (resident macrophages, named, Kupffer cells, dendritic cells, NK and NKT cells) and antimicrobial components (inflammatory cytokines, chemokines, acute phase proteins, complement) coordinate to achieve this critical task and eliminate invading pathogens and infected or transformed self [5].

In this paper, we will describe the innate immune cells phenotype, function in the context of human liver inflammation.

3. Innate Immunity in Liver Inflammation

3.1. Acute Phase Proteins (APPs) and Complement System

3.1.1. Acute Inflammation and Acute-Phase Proteins. During local liver injury or infection, resident Kupffer cells and monocyte/macrophages initiate an immune response. Upon phagocytosis of the pathogenic material, phagocytes release a variety of chemical messengers such as tumour necrosis factor alpha (TNFα), interleukin (IL)-1, and IL-6 that initiate the acute-phase response and inflammation. Such acute inflammation is characterised by the rise in concentration of numerous plasma proteins, collectively termed acute-phase proteins (APPs) [6]. APPs are a heterogeneous group of plasma proteins, which are exclusively synthesised in the liver and include pentraxins (C-reactive protein (CRP), serum amyloid P (SAP), and the long pentraxin 3 (PTX)), serum amyloid A (SAA), serum mannose-binding lectin, orosomucoid, inhibitors of proteases (α1-antitrypsin, α1-antichymotrypsin, α1-ACH, α2-macroglobulin), coagulation factors (fibrinogen, prothrombin, fVIII, plasminogen), transport proteins (haptoglobin, hemopexin, ferritin), and complement components [7]. The characteristic of these APPs is that their concentration can be increased (positive APPs) or decreased (negative APPs) by at least 50% in inflammatory disorders [8, 9].

APPs are critical components of the innate immune response restoring homeostasis after infection or inflammation. The important tasks they serve include haemostatic functions (e.g., fibrinogen), microbicidal and phagocytic functions (e.g., CRP and complement components), antithrombotic (e.g., α1-acid glycoprotein), and antiproteolytic properties which are required for maintaining protease activity at sites of inflammation (e.g., α2-macroglobulin, α1-antitrypsin and α1-antichymotrypsin) [10].

One of the major acute-phase proteins in humans is C-reactive protein. CRP belongs to the pentraxin superfamily of acute phase reactants that has originally been named for its ability to react with the C-polysaccharide of *Streptococcus pneumonia* [8, 11]. CRP production increases rapidly up to 1000-fold within 24–48 hours in response to infection, trauma, and tissue infection, and its concentration reduces the same rapidly after resolution of inflammation. Hence, the measurement of CRP is widely used to monitor various inflammatory conditions [8, 12]. CRP is produced mainly by hepatocytes, but it can also be produced by Kupffer cells, monocytes, and subsets of lymphocytes [11]. CRP binds to phosphocholine and phospholipid constituents of foreign pathogens and damaged cells and to chromatin in nuclear DNA-histone complexes, thus acts as an opsonin for various pathogens and activator of the complement system by binding to Fc receptors. Interaction of CRP with Fc receptors induces the production of proinflammatory cytokines that further enhance the inflammatory response. One characteristic of CRP is that it does not recognise specifically distinct antigenic epitopes, but recognises altered self and foreign molecules based on pattern recognition, thus provides early defence through production of proinflammatory signals and activation of the humoral and adaptive immune system

[13]. *In vivo* studies in transgenic mice overexpressing CRP have confirmed its anti-inflammatory effects. Increased CRP could prevent the adhesion of neutrophils to endothelial cells by decreasing the surface expression of L-selectin, inhibiting the generation of superoxide by neutrophils and stimulating the synthesis of IL-1rα by mononuclear cells [8].

3.1.2. Complement System. The complement system is a biochemical cascade of more than 35 proteins that plays an important role in innate immune defence against various pathogens through cytolysis, chemotaxis (e.g., C5a), opsonization (e.g., C3b), and activation of mast cells [14]. The complement system is activated through three different pathways: the classical, alternative, and mannose-binding lectin pathway. Its activation is initiated by the binding of one or more molecules of the above pathways on the surface of the target cells. The classical pathway destroys antibody-coated targets, apoptotic cells, Gram-negative bacteria, and some viruses. The alternative pathway destroys a variety of infectious agents including bacteria, viruses, and fungi in addition to playing a role in the immune surveillance of tumours, and the mannose-binding lectin pathway destroys mannose-bearing pathogens [15, 16]. All three complement activation pathways lead to the formation of C3 convertase, which in turn leads to the formation of membrane attack complex (MAC), a cytotoxic end-product of complement system made up of C5b, C6, C7, C8, and polymeric C9, that form a macromolecular pore capable of inserting itself into cell membranes and lysing heterologous cells, including bacteria and viruses, resulting in their death [16]. There is growing evidence suggesting that complement proteins not only serve as mediators of innate immune defence against foreign pathogens but can also modulate diverse developmental processes, such as cell survival, growth, and differentiation in various tissues [17]. The anaphylatoxins C3a and C5a, complement effector molecules released after complement activation, have been reported to be involved in the priming phase of liver regeneration, contributing to both the regulation of liver cell proliferation and hepatoprotection [17–19]. In complement deficient mice, lack of complement signalling results in impaired liver regeneration [19].

Depletion of serum complement before ischemia resulted in a significant attenuation of the KC-induced oxidant stress (enhanced oxidation of plasma glutathione) and also prevented the accumulation of PMNs in the liver during the reperfusion period suggesting that complement is involved in the induction of a KC-induced oxidant stress, the priming of KC and PMNs for enhanced reactive oxygen generation, and the continuous accumulation of PMNs in the liver during reperfusion [20]. Moreover, complement activation products can augment adhesion of leukocytes to endothelium, since C5b-9 and C5a can induce rapid translocation of P-selectin from Weibel-Palade bodies to the endothelial surface. The complement receptors CR3 and CR4 (CD18/CD11c) are members of the β-integrin family, which promote interactions between leukocytes and vascular endothelium [17].

3.2. Neutrophils. Neutrophils are polymorphonuclear cells that belong to the granulocyte family of leukocytes. They are the most abundant cells of the innate immune system and are indispensable for their defence against invading infectious pathogens. Neutrophils are generated in the bone marrow, where they remain for further 4–6 days, thus spending there the majority of their life [21, 22]. Their production is extensive in steady state with $1\text{-}2 \times 10^{11}$ cells being generated per day in normal human adult [23]. In systemic circulation neutrophils form the majority of circulating leukocytes, but they only consist <2% of total neutrophils. They have a very short half-life (~6–8 hours in humans and ~11 hours in mice) and are generally functionally quiescent [24]. During episodes of infection, their number can be increased by up to 10-fold. In steady-state conditions, circulating neutrophils can home either to the spleen, liver, or return to the bone marrow to be destroyed [25]. Alternatively, in the event of a pathogenic invasion, neutrophils from peripheral blood are rapidly recruited into peripheral tissues to fulfill their primary role to eliminate microbial organisms.

3.2.1. Neutrophil Recruitment in Human Liver. A unique feature of the liver is that it has several anatomical compartments for leukocyte recruitment, including the endothelial cells lining hepatic sinusoids, and the endothelial cells lining the portal and terminal hepatic veins [4, 26]. Leukocytes are able to adhere and migrate across such different regions of the hepatic microvasculature, but the majority of these cells seem to enter the parenchyma via the hepatic sinusoids. The endothelial cells lining the hepatic sinusoids have distinct characteristics as they lack underlying basement membrane and tight junctions but have fenestra [27]. They display differences in adhesion molecule expression compared with other endothelial cells of central and hepatic veins. Adhesion molecules such as E- and P-selectin, which are expressed on endothelial surfaces of hepatic arteries, portal and central veins are absent in sinusoidal endothelial cells [28, 29].

Leukocyte recruitment (Figure 3) is a highly regulated process dependent on sequential interactions with endothelial adhesion molecules and chemokines. The initial interactions between endothelium and leukocytes induce tethering and rolling of the leukocyte on the endothelial surface via transient bonds between selectins and their glycoprotein ligands. This initial contact allows leukocytes to sample the endothelial microenvironment for chemokines, which can be secreted by the activated endothelium and immune cells and are immobilized by glycosaminoglycans on the endothelial cell surface. The binding of chemokines to chemokine receptors on leukocytes leads to rapid G-protein coupled signalling that triggers cytoskeletal rearrangement and activation of leukocyte integrins. The activated integrins are then able to bind to their ligands, members of the immunoglobulin superfamily expressed on the endothelial surface, hence firmly arresting the leukocyte on the endothelium. In the final step, leukocytes pass through the endothelial monolayer in a process named transendothelial migration or diapedesis, following directional cues to the site of infection or tissue injury [30, 31].

In the case of neutrophils, the initial step includes the slowing of this leukocyte within the venule. The cell is loosely tethered to the vessel wall and rolls along the endothelial

surface at less than 50 μm/sec velocity. Neutrophil rolling along the endothelium is mediated by the three members of the selectin family (E-, P-, and L-selectin) and their ligands. After rolling, neutrophils are firmly arrested on the endothelium via CD18 integrin/intercellular adhesion molecules (ICAMs) interactions. The adherent neutrophils migrate through the endothelial junctions into the region between the endothelium and its basement membrane. After stopping briefly at this location, neutrophils migrate into the surrounding tissue via β2-integrins (LFA-1, Mac-1) and ICAM-1 [32, 33]. This neutrophil recruitment cascade occurs in mesentery, brain, and skin *in vivo* and *in vitro.* However, some of the adhesion mechanisms in sinusoids may not be the same as in postcapillary venules.

However, the recruitment of neutrophils in the liver displays a different pattern [34]. Neutrophil recruitment and accumulation in the hepatic sinusoids is independent of selectins and β2-integrins, which are though required for their recruitment to the postsinusoidal venules [35–37]. It has been suggested that accumulation of neutrophils into the sinusoids is mediated by mechanical trapping of these cells in the narrow sinusoidal vessels due to changes of the activated neutrophils themselves, sinusoidal endothelial cell swelling, and additional low stress in these capillaries [38]. McDonald et al. [39] have supported that CD44 and its hyaluronan ligand (HA), which is extensively expressed on the sinusoidal endothelial cells, are responsible for neutrophil recruitment in liver sinusoids, as proven by blocking antibodies directed against either CD44 and HA. Recent reports have also highlighted the CD44/HA interaction as the dominant mechanism for neutrophil adhesion in sinusoids during endotoxemia and ischemia reperfusion [39, 40]. Although the adhesion molecules are the important "tracks" for neutrophil movement, their driving forces however are the chemotactic factors that induce their migration from systemic circulation to the site of infection. Such factors are cytokines (TNFα, IL-1α, and IL-1β), activated complement proteins, and CXC chemokine IL-8 (CXCL8, specific neutrophil chemoattractant) [32, 41].

3.2.2. Neutrophil-Mediated Innate Immune Defence. Mature neutrophils are professional phagocytic granulocytes with numerous antimicrobial molecules (>300 proteins) stored in their cytoplasmic granules. These granules are unspecific molecules with high cytotoxicity and potential tissue-damaging activity that can be also involved in many neutrophilic processes including adhesion, migration, and antibacterial activities [42]. Thus, neutrophils are considered highly dangerous cells, whose action needs to be tightly controlled [43, 44]. This characteristic explains why neutrophils are mainly absent in tissues and body cavities in steady-state conditions and are predominantly kept in reserve pools as quiescent cells in the blood and bone marrow. This also explains the reason that they are the first cells to be recruited to the site of infection upon acute inflammation [45].

During an infectious insult in the liver, resident macrophages and dendritic cells detect the presence of invading pathogens (via PRRs/PAMPs mechanisms) and will release chemokines CXCL8 (IL-8), CXCL1, 2, 3, CCL2, 3, 4 to attract neutrophils and monocytes at the site of infection (Figure 1) [44, 46, 47]. Neutrophils are the first phagocytes to arrive at the foci of microbial invasion, where they change their phenotype, become activated, and release cytotoxic antimicrobial molecules (reactive oxygen species (ROS), oxidants, defensins, lactoferrin and cathelicidins) [42, 48–51] as well as chemokines to attract primarily more neutrophils as well as monocytes, which extend the lifespan of the former from 6–12 hrs (at resting state) to 24–48 hrs at the inflammatory sites [45] by factors such as IL-1β, TNFα, G-CSF and GM-CSF [52].

In order for the infection to be effectively controlled and resolved, the neutrophils that are present at the infectious foci need to undergo apoptosis, a mechanism that renders them functionally quiescent [53]. Apoptotic neutrophil itself represents an important anti-inflammatory stimulus to other cells by producing "eat me" signals recognised by the surrounding phagocytes to resolve the infection. Scannell et al. [54] have identified the release of annexin 1 by apoptotic cells as a soluble signal that promotes neutrophil phagocytosis by macrophages. Moreover, the exposure of phosphatidylserine (PS) residues on the apoptotic neutrophil membrane allows recognition of PS with its receptors on macrophages, which not only initiates phagocytosis but also modifies the transcriptional profile of the macrophage, increasing the production of IL-10 and TGFβ, two cytokines associated with resolving the inflammatory response and promoting tissue repair [43, 55].

3.2.3. Neutrophil-Mediated Liver Tissue Injury. Protective immunity is always beneficial when it is well contained and properly regulated. Excessive neutrophil accumulation at the site of liver tissue injury may contribute to pathology through relevant proinflammatory and tissue-damaging effects from these cytotoxic phagocytes [56]. Liver injury mediated by neutrophils has been reported in a number of experimental animal models such as Concanavalin (Con)A-induced hepatitis [57, 58], ischemia-reperfusion injury [59, 60], alcoholic hepatitis [61, 62], endotoxemia [63], and sepsis [64]. Although the neutrophils that are accumulated in sinusoids are partially activated and primed, they cannot cause liver injury. Prerequisite for their cytotoxicity is their extravasation and adherence to parenchymal cells via ICAM-1/Mac-1 interaction [38, 65]. Adherence to parenchymal cells triggers the formation of reactive oxygen species and release of proteases through degranulation [38]. Neutrophils generate superoxide through NADPH oxidase, and the resulting hydrogen peroxide can either directly diffuse into hepatocytes or generate an intracellular oxidant stress. Neutrophil myeloperoxidase also generates hypochlorous acid, a major oxidant that also diffuses into target cells leading eventually to hepatocyte death [66, 67]. The proteases cathepsin G and elastase can also cause parenchymal cell necrosis, as protease inhibitors have been shown to attenuate neutrophil-induced liver injury [32, 68]. Neutrophils are detected in acute liver injury such as alcoholic hepatitis. Recent study from Lemmers and colleague suggested that IL-17 secreted from Th17, a new lineage of T helper cells act on fibroblast which in turn secreted IL-8 to attract neutrophils to site of

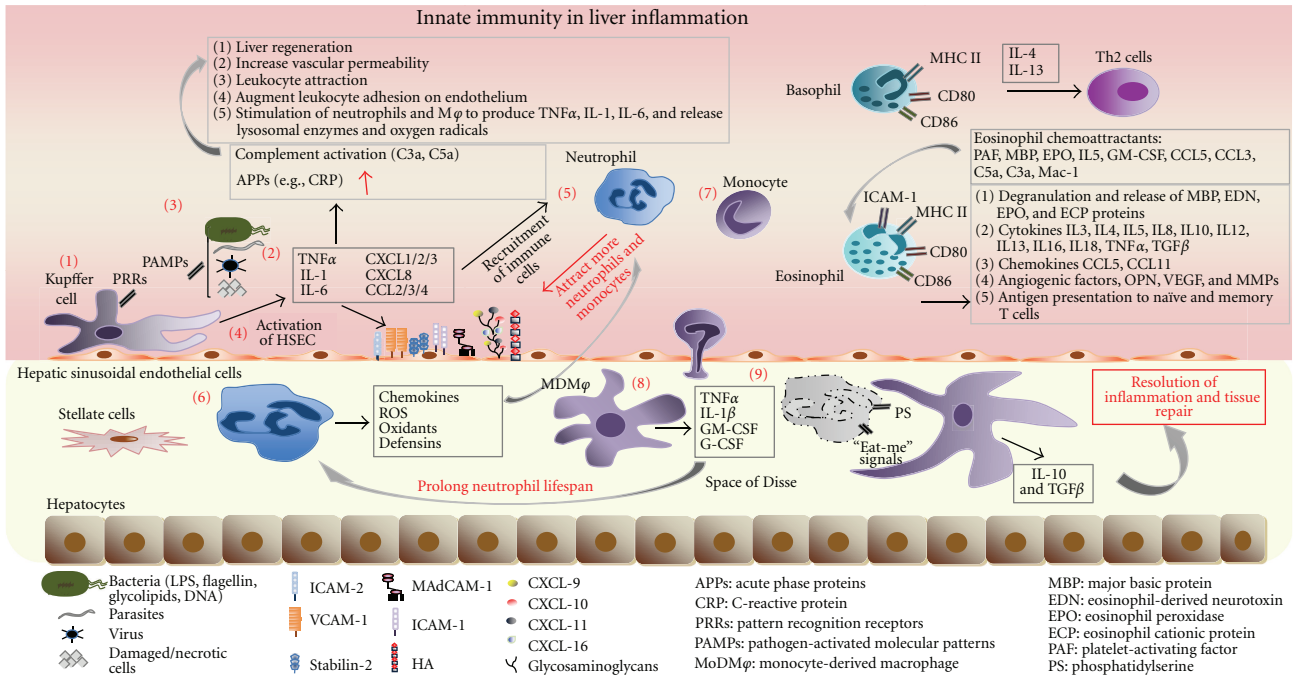

Figure 1: Innate immune cells in liver inflammation. During an infectious insult in the liver (1) resident macrophages, Kupffer cells, are the first immune cells to detect the presence of invading pathogens (bacteria, parasites, viruses, damaged, and/or necrotic cells) via PRRs/PAMPs. (2) Upon activation Kupffer cells release cytokines TNFα, IL-1, and IL-6 as well as chemokines CXCL 1–3, CXCL-8, CCL-2–4 that initiate (3) the acute-phase response and inflammation. Acute inflammation is characterized by the rise in plasma proteins, collectively named acute-phase proteins (APPs) that include C-reactive protein (CRP) and complement components. (4) Proinflammatory cytokines released from activated Kupffer cells can activate hepatic sinusoidal endothelial cells to upregulate adhesion molecules (ICAM1 and 2, VCAM-1, MAdCAM etc.) and in combination with the chemokines secreted from Kupffer cells can stimulate the recruitment of neutrophils and monocytes to the liver. (5) Neutrophils are the initial phagocytes to arrive at the site of microbial invasion, where (6) they change their phenotype, they become activated and release powerful and cytotoxic antimicrobial molecules such as reactive oxygen species (ROS), oxidants, defensins, as well as chemokines to attract more neutrophils and monocytes. (7) Following their recruitment to the tissue, monocytes undergo differentiation into (8) tissue macrophages (MDMφ), which release TNFα, IL-1β, G-CSF, and GM-CSF factors that can extend the lifespan of neutrophils thus sustaining their presence at the site of inflammation. (9) In order for inflammation to be resolved, the dangerous neutrophils at the inflammatory loci undergo apoptosis and terminate the inflammatory process quickly. Apoptotic neutrophils represent an important anti-inflammatory stimulus to other cells involved in the resolution of inflammation by producing "eat-me" signals recognised by the surrounding phagocytes. Phosphatidylserine (PS) residues on the apoptotic neutrophil membrane allow recognition by its receptor on macrophages, which not only initiates phagocytosis but also modifies the transcriptional profile of the Mφ, increasing the production of IL-10 and TGF-b, cytokines associated with resolution of inflammatory response and tissue repair. Basophils are short-lived cells that express MHC II and CD80/CD86 costimulatory molecules, thus are able to present antigens to CD4+ T cells promoting their differentiation into Th2 cells via release of IL-4 and IL-13. Eosinophils recruited to the liver release proinflammatory mediators including granule-stored cationic proteins, cytokines, and chemokines. They also express MHC II, CD80/CD86, CD40, and ICAM-1; thus they are able to present antigens to T cells initiating or amplifying antigenic-specific immune responses.

alcoholic hepatitis suggesting the link between adaptive and innate immune system via cytokine IL-17 [69].

3.3. Monocytes, Macrophages and Kupffer Cells

3.3.1. Monocytes: Origin, Heterogeneity, and Function. Monocytes originate from a common myeloid progenitor cell in the bone marrow that is shared with neutrophils. They are released in the bloodstream as nondifferentiated cells and circulate in the blood for 1–3 days [70]. Following recruitment to tissues, monocytes can differentiate into tissue macrophages (Mφs) or myeloid dendritic cells (DCs) [71–75], replenishing the existing populations and contributing to homeostasis maintenance, host defence, tissue remodeling, and repair [70, 76, 77] (Figure 2).

Circulating monocytes constitute ~5–10% of peripheral blood leukocytes that show morphological heterogeneity [78]. The heterogeneity among human monocytes has been described since 1989 [79]. The differential expression of CD14 (part of the receptor for LPS) and CD16 (also known as FcγRIII) was initially traced in order to define two major subsets in peripheral blood: the so-called "classical" CD14++CD16 monocytes, typically representing up to 80% of the monocytes in a healthy individual, and the "nonclassical" CD16+ monocytes comprising the remaining fraction of monocytes (Figure 3) (paper in submission). It is now

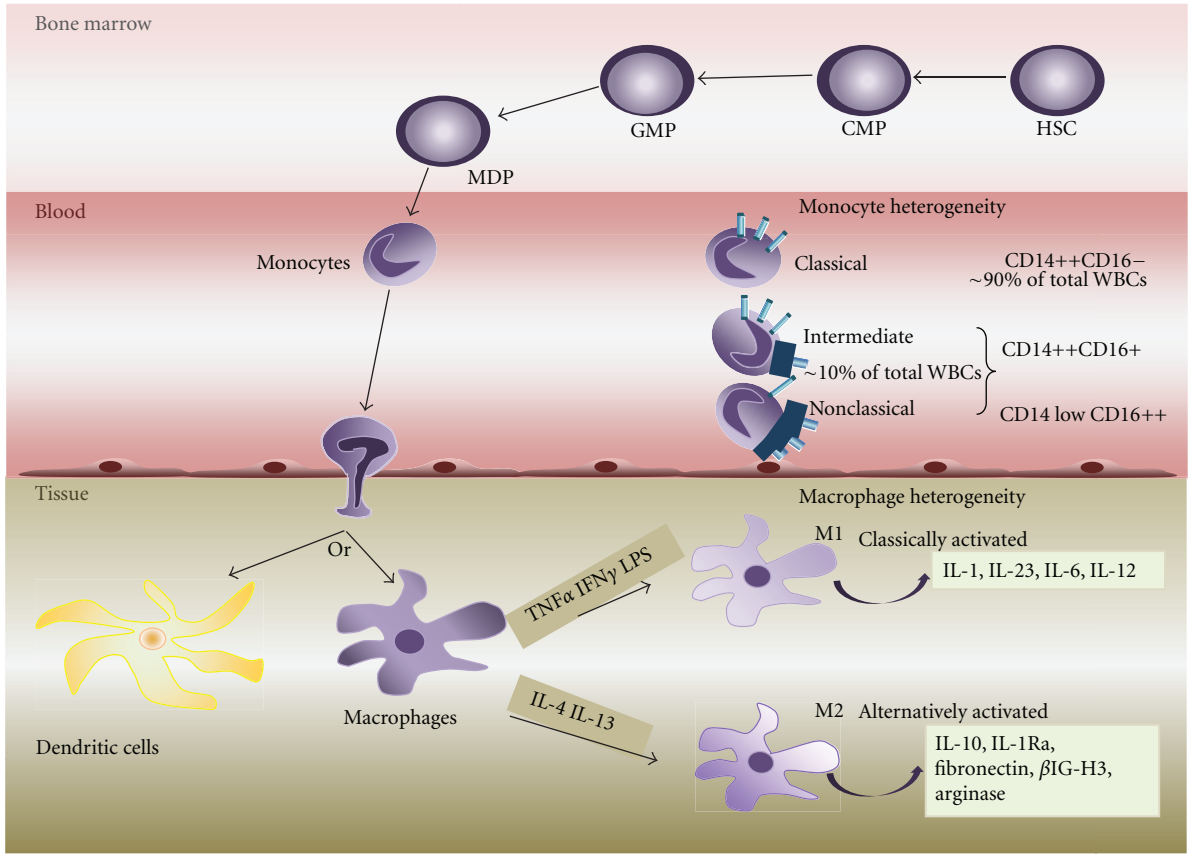

Figure 2: Monocyte and macrophage heterogeneity. Monocytes originate in the bone marrow where they develop from hematopoietic stem cells (HSCs) via several differentiation steps and intermediate progenitor stages that pass through the common myeloid progenitor (CMP), the granulocyte/macrophage progenitor (GMP), and the macrophage/DC progenitor (MDP) stages. The MDP gives rise to monocytes, which are released in blood circulation where they remain for 1–3 days. In peripheral blood, circulating monocytes represent ~5–10% of peripheral blood white blood cells (WBCs) and are a highly heterogenic population. Three main subtypes have been described based on the expression of CD14 and CD16 receptors: the classical CD14++CD16, intermediate CD14++CD16+, and nonclassical CD14 low CD16++ monocytes. In general, circulation monocytes are recruited to tissues where they can differentiate into dendritic cells or tissue macrophages (Kupffer cells in the liver; microglial cells in the brain, etc.), replenishing the existing populations. Additional heterogeneity also exists between the macrophages, with two major classes being identified: the classically activated (M1) and the alternatively activated (M2) macrophages. M1 macrophages are developed in response to TNFα and IFNγ as well as in response to microbial products such as LPS, and they produce in turn proinflammatory cytokines including IL-1, IL-23, IL-6, and IL-12. M2 macrophages can develop in response to IL-4 and IL-13 cytokines and play important roles in down-regulation of inflammation and tissue remodelling by releasing IL-10 and IL-1 receptor antagonist (IL-1Ra). They also produce high levels of arginase, fibronectin, and a matrix-associated protein, βIG-H3.

apparent that further heterogeneity exists and is that the nonclassical subset can be further divided into the intermediate CD14++CD16+ and the nonclassical CD14+CD16++ subpopulations. These subsets differ in many respects, including adhesion molecule and chemokine receptor expression [80, 81]. For mouse blood monocytes, a subdivision into three subsets similar to humans is also proposed that is classical, intermediate, and nonclassical. Specifically, in mouse the classical monocytes are Ly6Chi, CCR2hi, and CX_3CR1low, whereas the nonclassical monocytes are Ly6Clow, CCR2low, and CX_3CR1hi [81, 82].

Monocytes are members of the human mononuclear phagocyte system, which is important for the host nonspecific antimicrobial defence and tumour surveillance [82]. They are also a critical effector component of the innate immune system, equipped with chemokine receptors and adhesion molecules to recruit to site of infection. Monocytes secrete inflammatory cytokines, take up cells and toxic molecules, thus contributing to the immune defence against bacterial, protozoa, and fungal pathogens [83, 84]. Monocytes can kill bacteria by producing reactive nitrogen intermediates (RNIs), reactive oxygen intermediates (ROIs), and through the action of phagolysosomal enzymes [85].

3.3.2. Monocyte Recruitment to Human Liver. Monocyte recruitment to the site of infection follows the general paradigm of leukocyte trafficking cascades, which involves rolling, adhesion, and transmigration. Monocytes are heterogeneous group and human monocyte subpopulations are defined on the basis of the expression of cell-surface

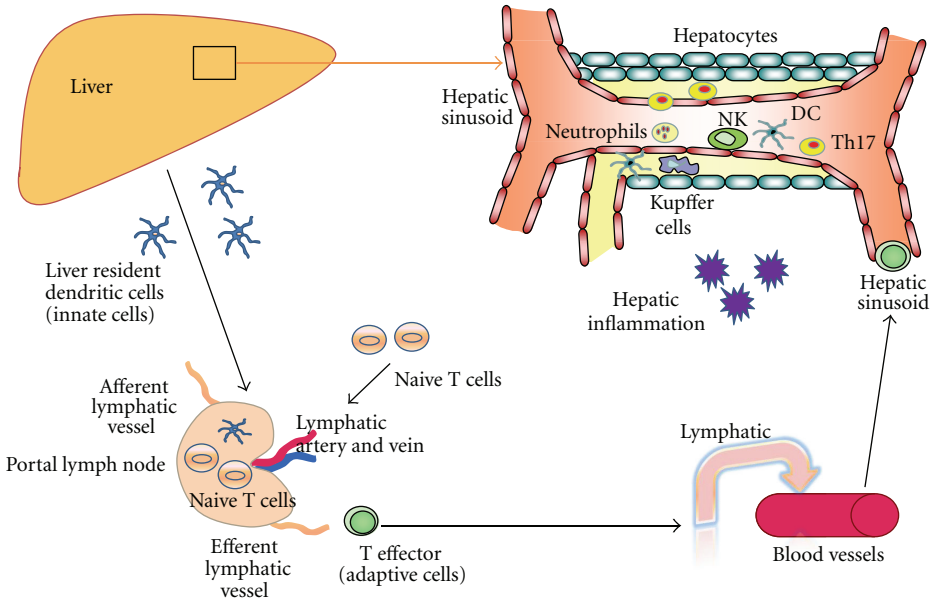

FIGURE 3: Innate immune cell (neutrophils, NK cells and monocytes) recruitment to hepatic inflammation. Human liver receives dual blood supply from both portal vein and hepatic artery. During the inital event of hepatic inflammation, innate immune cells such as neutrophils, monocytes and NK cells are recruited to the liver. Liver resident dendritic cells sample the foreign antigen and carry to local draining portal lymph nodes where antigens are presented to the adaptive naive T cells. Following the antigen presentation, different types of antigen-specific T effectors cells leave the nodes and drain back to systemic circulation. These T effector cells recruit via hepatic sinusoid towards the site of injury or inflammation. Th17 cells which secrete IL-17 attract neutrophils and also link innate and adaptive immunity.

markers. The classical CD14+ monocytes express high levels of CCR2 (the receptor for CCL2/MCP-1), low levels of CCR5 and low levels of CX_3CR1. Conversely, CD16+ monocytes express high levels of CX_3CR1 and CCR5 (receptors for CCL3/MIP1α). Therefore, both CX_3CL1 and CCL3 are able to induce the transendothelial migration of CD16+ cells, whereas the recruitment of classical CD14+ cells depends on CCL2 [76, 86]. Additional studies in human peripheral blood monocyte subsets have shown that classical CD14++CD16− monocytes express CCR1, CCR2, CCR4, CCR5, CCR6, CXCR1, CXCR3, and CXCR5 chemokine receptors, whereas the nonclassical CD16+ monocytes show a limited chemokine receptor repertoire compared to CD14+ cells [87]. In mice, inflammatory monocytes express CD62L (L-selectin), LFA-1 ($\alpha L\beta 2$ integrin), Mac-1 ($\alpha M\beta 2$ integrin), PECAM-1 (CD31), and VLA-4 ($\alpha 4\beta 1$). Therefore, initially, monocytes undergo CD62L selectin-dependent rolling along the vascular endothelium. Firm arrest is then mediated by integrins; interaction of $\beta 2$ integrins with ICAM-2 causes firm arrest of monocytes in the absence of inflammation, whereas interaction of $\beta 2$ integrins with their countereceptors ICAM-1 and ICAM-2 and of $\alpha 4\beta 1$ with VCAM-1 mediates firm arrest and transmigration to inflamed tissues. Monocyte transendothelial migration across endothelium involves PECAM-1, CD99, CD226, and the junctional adhesion molecules (JAMs), which are present at tight junctions [88, 89]. After migration to the peripheral tissue, monocytes uses $\alpha 4\beta 1$- and $\alpha 6\beta 1$ integrins to interact with the extracellular matrix [82]. Previous study by Aspinall et al. from our group has reported that the recruitment of CD16+ monocyte subset to the inflamed human liver is mediated by VAP-1 and CX_3CL1 [87].

3.3.3. Monocyte-Derived Macrophages and Kupffer Cells in Human Liver. Inflammatory monocytes recruited at the site of inflammation can differentiate into macrophages. Tissue macrophages have a broad role in the maintenance of tissue homeostasis, through the clearance of senescent cells and the remodelling and repair of tissues after inflammation [90]. They are considered to be important immune effector cells that can clear approximately 2×10^{11} erythrocytes each day. Macrophages are also involved in the removal of cellular debris generated during tissue remodelling and rapidly and efficiently can clear the cells that have undergone apoptosis. The receptors involved in these homeostatic processes include scavenger receptors, phosphatidyl serine receptors, the thrombospondin receptor, integrins and complement receptors [91]. Moreover, necrosis that results from trauma or stress generates cellular debris that need to be cleared by macrophages. Phagocytosis of necrotic debris leads to dramatic changes in their physiology, including alterations in the expression of surface proteins and the production of cytokines and proinflammatory mediators. Macrophages are able to detect endogenous danger signals that are present in the necrotic cell debris through TLRs, intracellular PRRs, and IL-1R, most of which signal through the adaptor molecule MyD88. This function makes macrophages one of the primary sensors of danger in the host [91].

Additional heterogeneity also exists between the macrophages, with two major classes of macrophages being identified: the classically activated macrophages (M1) and the alternatively activated macrophages (M2) (Figure 2). M1 Mφs whose prototypical activating stimuli are IFNγ and LPS (which induces TNF production) generate proinflammatory cytokines, bactericidal mediators, and promote strong IL-12-mediated Th1 responses. In contrast M2 Mφs whose stimuli are IL-4 or IL-13 play an important role in the downregulation of inflammation supporting Th2-associated effector functions, tissue remodelling, elimination of tissue debris, and apoptotic bodies, as well as induction of angiogenesis [75, 92–94]. In general, macrophages are equipped with a broad range of pathogen-recognition receptors that make them efficient at phagocytosis and induce the production of inflammatory cytokines [84].

Kupffer cells (KCs), named after the pathologist C. von Kupffer are the liver resident macrophages which account for 80–90% of total fixed tissue macrophages in the body [95]. The origin of Kupffer cells has been speculated to involve two mechanisms: replenishment by local self-renewal and proliferation [96] and from circulating bone-marrow-derived monocytes. Kupffer cells are present throughout the liver residing within the lumen of liver sinusoids. Large KCs are mainly located in the periportal region where they are optimally located for response to systemic bacteria and bacterial products that are transported from the gut to the liver via the portal vein. Accordingly, periportal KCs have higher lysosomal enzyme activities together with greater phagocytic capacity than smaller KCs in midzonal and perivenous regions. Furthermore, large KCs produce higher levels of TNFα, PGE2, and IL-1 in contrast to the higher levels of nitric oxide formation by small KC [97, 98].

Kupffer cells are active phagocytes, which uptake intravascular debris, dead bacterial cells, and other blood-borne particles, and are able to secrete various inflammatory cytokines including IL-1, IL-6, TNFα, GM-CSF, and chemokines such as MIP-1α (macrophage inflammatory protein 1 alpha) and RANTES (regulated on activation, normal T-cell expressed and secreted). However, overproduction of such inflammatory mediators by Kupffer cells can lead to liver injury [99, 100]. Kupffer cells express several cell-surface receptor complexes involved in immune stimulation. These include complement receptors, Fc receptors, receptors for lectin-containing opsonins such as plasma mannose-binding lectin, adhesion receptors including those that bind ICAM-1, TLRs, and receptors for polysaccharides of microbial and host origin [101]. They also express high-affinity Fcγ receptors, which facilitate phagocytosis of IgG-coated particles, as well as receptors for IgA, galactose, and mannose receptors, and scavenger receptors which are capable of directly binding microbial surface components [101].

3.3.4. Monocyte/Macrophage-Mediated Innate Immune Defence. Resident macrophages and dendritic cells are the first to detect the presence of invading pathogens by using invariant PRRs that recognise conserved PAMPs on extracellular and/or intracellular microbial components. Initially damaged cells spill cytoplasmic and nuclear components into the extracellular milieu, and these "alarm signals" activate tissue resident macrophages. CLEC4E is a transmembrane C type lectin, which has been reported to be involved in initiating the early inflammatory response after necrotic cell death [102]. The subsequent production of proinflammatory cytokines and chemokines including TNF, IL-6, CXCL1, CXCL2, CXCL3, CXCL8, CCL2, CCL3, and CCL4 can stimulate the recruitment of neutrophils and monocytes [103]. Granule proteins discharged from activated neutrophils anchor on endothelial proteoglycans and are recognised by monocytes that roll along the endothelium, thus promote their firm adhesion. Moreover, azurocidin, LL-37, and cathepsin G, proteases released from activated recruited neutrophils, activate formyl peptide receptors on classical inflammatory monocytes and promote their extravasation. Neutrophil granule proteins can promote *de novo* synthesis of monocyte-attracting chemokines by neighbouring endothelial cells and macrophages. In resolution of inflammation, apoptosis of neutrophils holds a central position as it brings to an end the sustained recruitment of neutrophils, while the phagocytic clearance of apoptotic neutrophils reprogrammes macrophages to an anti-inflammatory phenotype [104].

3.3.5. Monocyte/Macrophage-Mediated Liver Tissue Injury. Monocytes/macrophages have an essential role in antimicrobial immune defence and are able to promote tissue healing and repair. However, they can also contribute to tissue destruction during some infections and inflammatory diseases [82]. The cytotoxicity of infiltrating macrophages or Kupffer cells has been reported in ischemia-reperfusion injury [105], endotoxemia [106], galactosamine hepatotoxicity [107], and corynebacterium parvum/endotoxin-induced liver injury [108]. It has been suggested that infiltrating macrophages and Kupffer cells mediate their cytotoxic effects through the production of reactive free radicals and specifically reactive oxygen species and proinflammatory cytokines including TNFα, IL-1β and IL-6. In addition, activated Kupffer cells can induce the infiltration of neutrophils. Again, proinflammatory cytokines released by Kupffer cells are thought to be important in the development of neutrophil-mediated tissue injury [59]. Previous study of Duffield et al. [109] demonstrated that deletion of macrophages either during injury or during repair and resolution has dramatically different effects on the overall fibrotic response. Specifically, in progressive inflammatory injury, macrophage depletion results in amelioration of fibrosis, whereas depletion during recovery results in a failure of resolution with persistence of cellular and matrix components of the fibrotic response. Hepatic macrophages have been implicated in APAP-induced liver hepatotoxicity (acetaminophen overdose), through the production of proinflammatory cytokines and mediators such as TNFα, IL-1β, and NO [110]. On the other hand, however, there are studies which described protective role of kupffer cells in acetaminophen-induced hepatic injury [111, 112]. The current concept suggests the role of macrophages predominantly in tissue repairs especially the newly recruited tissue macrophages [113].

3.4. Mast Cells

3.4.1. Origin and Phenotype. The mast cell is originally derived from the pluripotent haemopoetic stem cell. An immature version of the mast cell, an undifferentiated $CD34^+$ and $CD117^+$ progenitor cell, is released from the bone marrow into the blood stream [114, 115]. Mast cells are sessile and predominantly inhabit perivascular dermal and submucosal (respiratory/gastrointestinal/genitourinary tracts) connective tissue and lymph nodes [116]. They mature only once they have reached their tissue destination. The stem cell factor, c-kit, plays a critical role in the maturation process of the mast cell. Mast cells can be broadly divided into two categories, connective tissue mast cells, which are known as mast cell tryptase and chymase (MC^{TC}) that release IL-4, and mucosal mast cells also known as mast cell tryptase (MC^{T}) and produce IL-5 and IL-6 [117]. Once resident in the tissue, the mast cell has a life span of several months. They can proliferate, have a plasticity potential [115], and are mainly involved in Th2 immune response at the infected sites.

3.4.2. Mast Cells in Innate Immune Response. Mast cells are among the first responders during infection that also provide immediate action by recruiting other immune cells to the scene of inflammation. Mast cells are large cells whose content is dominated by cytoplasmic granules. These cytoplasmic granules contain a variety of mediators including serotonin, histamine, cytokines, chemokines, and leukotriene. Histamine on its own composes 10% of the entire weight of the mast cell which illustrates the importance of the cytoplasmic granules to the function of the mast cell.

Degranulation of mast cells and release of the mediators occur primarily via an IgE-mediated pathway but also via surface receptor binding sites including TLRs and $\beta 2$ integrin. Mast cells have receptors, known as FcεRI, with high affinity for IgE on their surface. In fact the receptors have such high affinity for IgE that there is very little circulating IgE, as most is bound to mast cells already. The binding of IgE to FcεRI creates a sensitised mast cell ready to degranulate. The degranulation occurs when bi- or multi-valent antigen binds to the IgE causing cross-linking between the IgE. This leads to rapid exocytosis of the stored mediators, degranulation. This can also occur when substances such as neuropeptides and anaphylatoxins C3a and C5a bind to receptors on the mast cell surface. Toll-like receptor ligands can bind to toll-like receptors on the surface of mast cells and cause secretion, rather than degranulation of cytokines, chemokines, and lipid mediators [118].

Mast cells can amplify or suppress different areas of both innate and adaptive immunity depending on the concentration and type of the mediator released. The main mediators contained in the mast cell are histamine, heparin, cytokines, chemokines, and lipid mediators. Histamine and heparin are able to increase vascular permeability, cause smooth muscle contraction, and directly kill parasites. The major role of mast cells in innate immunity is to recruit neutrophils which can either enhance immune defence of host or can lead to immunopathology [118]. Lipid mediators are also involved in smooth muscle contraction, and can increase vascular permeability as well as neutrophil, eosinophil and platelet activation and mucus secretion.

3.4.3. Mast-Cell-Mediated Liver Tissue Injury. The number of mast cells within the liver is proportionately low in comparison to other tissues. The density of mast cells is calculated at between 1.2 and 3.9 cells per square millimetre of human liver. Hepatic mast cells are mostly situated within connective tissue adjacent to the hepatic artery, hepatic vein and bile ducts of the portal tract [119]. Recent studies have shown the role of intrahepatic mast cells in different chronic liver diseases [119]. Increased mast cell numbers have also been reported in liver fibrosis and hepatitis [120] and have been involved in acute hepatitis [121], primary biliary cirrhosis [122, 123], primary sclerosing cholangitis [123], hepatocellular carcinoma and cholangiocarcinoma [124, 125].

3.5. Basophils.

Basophils are granulocytes that develop from hematopoietic stem cells in the bone marrow. They leave bone marrow after maturation, enter systemic circulation, and finally migrate to the inflammatory sites, where they play essential effector functions in response to parasite infection and allergic inflammation [126, 127].

3.5.1. Origin and Phenotype. Basophils are short-lived cells (lifespan of 2-3 days) that account for less than 1% of circulating granulocytes in the blood [128]. However, their low baseline numbers can be expanded in response to growth factors such as IL-3, which has been reported to be important for basophil activation, population expansion, and survival [129]. Basophils express the high-affinity IgE receptor (FcεR1) present in a tetramer form ($\alpha\beta\gamma 2$) [130], and their activation can be induced in IgE-dependent (by IgE/FecεR1 interaction) and IgE-independent manner (by cytokines (IL-3, IL-6, IL-18, IL-33, TNFα, and GM-CSF), antibodies (IgG and IgD), allergens, parasite antigens, toll-like receptor (TLR) ligands and complement factors). Activation of basophils results in their degranulation and release of pro-formed (histamines) and newly synthesized lipid mediators, cytokines (IL-4, IL-13, IL-6, TNFα, and thymic stromal lymphopoietin (TSLP)) and chemokines, which are essential players in vascular reaction, exudation, leukocyte accumulation and wound healing [131, 132].

Basophils are mainly found in the blood and spleen and upon exposure to stimuli such as allergens or parasites they become activated. Activated basophils are then able to migrate to lymph nodes [133–135]. Basophils express a wide spectrum of chemoattractant receptors, such as cytokine receptors (e.g., IL-3R, IL-5R, GM-CSFR) [130, 136], chemokine receptors (CCR1, CCR2, CCR3, CXCR1, CXCR3 and CXCR4) [137–141], and receptors for more pleiotropic chemotactic factors such as receptors for complement components C3a and C5a, formyl-methionine-leucine-phebylalaning (fMLP), platelet-activating factor (PAF), leukotriene B4 (LTB4) [142–144]. Thus, basophils have the potential to respond to a wide variety of inflammatory stimuli, and some basophil populations migrate to

draining lymph nodes, while others accumulate in inflamed tissues during an ongoing inflammatory response.

3.5.2. Basophil Recruitment and Function in Lymph Nodes and Tissues. Basophil recruitment from the peripheral circulation to the sites of infection occurs through the multistep process of leukocyte recruitment that has been described above. *In vitro* studies have shown that TNFα and IL-1 enhance basophil adhesion on endothelial cells, possibly through induction of basophil adhesion molecule expression. Moreover, it has been reported that IL-3 increases basophil adhesiveness to endothelial cells, possibly by increasing CD11b, an integrin that interacts with ICAM-1, fibrinogen and C3bi. CD11b and CD11c are also induced on the surface of basophils after activation [145].

Although for many years it has been well accepted that basophils are late-phase effector cells that migrate to the site of inflammation after the establishment of a Th2 cytokine response, recent studies have provided evidence that basophils can also play a central role in the induction and propagation of a Th2 cytokine-mediated immunity and inflammation [146, 147]. In the lymph nodes, basophils are able to directly interact with naive CD4+ T cells and induce their differentiation into Th2 cells. They express MHC class II and costimulatory molecules CD80 and CD86, thus basophils can present antigen via MHC class II and can provide IL-4 that promotes the differentiation of naïve T cells [147]. Basophils can also produce IL-13 upon stimulation with Ag/IgE complexes and can strongly release IL-4 and IL-13 in response to IL-3 and IL-18 or IL-33, further supporting their role in the development of Th2 cells [147]. Interestingly, independent groups have demonstrated that basophils are the predominant antigen-presenting cell (APC) in inducing Th2 responses against helminth parasites and allergens [133, 146, 148].

3.5.3. Basophils in Liver Inflammation. Studies reporting the role of basophils in human liver inflammation are very limited. It has been described that infection with intestinal nematode *Nippostrongylus brasiliensis* induces robust Th2 immune responses and also enhances basophil generation in the bone marrow and subsequent accumulation in the peripheral tissues, including liver, lung, and spleen [149]. Further studies have also shown that basophils isolated from the spleen, liver or bone marrow are able to initiate Th2 cell development in the presence of antigens and DCs [150, 151].

3.6. Eosinophils

3.6.1. Origin, Phenotype, and Function. Eosinophils develop and mature in the bone marrow from multipotent hematopoietic progenitor cells of a myeloid lineage in IL-3, IL-5 and GM-CSF dependent manner. IL-5 has been described as the major lineage differentiation factor as well as the stimulus for eosinophils to leave the bone marrow and enter the circulation [152]. In the blood, mature eosinophils circulate for a short time (half-life of 8–18 hours), and then migrate out of the vessels into tissue. They consist approximately 1–3% of total circulating white blood cells, since a large pool remains in the bone marrow and the vast majority is located in the tissues, particularly at the mucosal surfaces of the gastrointestinal tract (lamina propria), mammary gland, respiratory and reproductive tracts [153–155].

3.6.2. Eosinophil Recruitment to Tissue. Eosinophils express an array of cell surface molecules including immunoglobulin receptors for IgG (FcγRII/CD32) and IgA (FCαRI/CD89), complement receptors (CR1, CR3, and CD88), leukotriene receptors (CysLT1R and CysLT2R, LTB4 receptor), prostaglandin receptors (PGD2 type 2 receptor), platelet activating factor receptor (PAF), and toll-like receptors (particularly TLR7/8), cytokine receptors (IL-3R, IL-5R, GM-CSF that promote eosinophil development, as well as receptors for IL-1α, IL-2, IL-4, IFNα, and TNFα), chemokine receptors (CCR1 and CCR3) and adhesion molecules (VLA/α4β1, α4β7, Siglec-8) [130].

The migration of eosinophils from the blood into tissues involves selective adhesion pathways and chemoattractants. Chemoattractants for eosinophils include platelet-activating factor (PAF), complement component C5a [156], IL-16 [157], RANTES [158], MCP-3 [138] and eotaxin [159, 160]. Eosinophils can pass through post-capillary venules into tissues following chemoattractants in several steps of recruitment cascades of rolling, firm adhesion, and transendothelial migration. At the initial steps of tethering and rolling on endothelium, eosinophils make use of the receptors L-selectin, PSGL-1 and VLA-4 (α4β1), that interact with their counter receptors GlyCAM-1, CD34 and MAdCAM-1 (all L-selectin ligands), P-selectin and VCAM-1, respectively on the surface of endothelial cells [161, 162]. Following rolling, eosinophil integrins LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), VLA-4 and α4β7 become activated and lead eosinophils to firmly arrest on ICAM-1, ICAM-2, VCAM-1 and MAdCAM-1, respectively on the endothelial surface [163]. In order to infiltrate into the tissue, eosinophils need to penetrate gaps between the endothelial cells. Utilizing Mac-1/ICAM-1 interactions and PECAM-1/PECAM-1 homotypical interactions between both cells at transendothelial junctions, eosinophils are able to translocate to the underlying basement membrane and through the extracellular matrix into the tissue [164, 165].

3.6.3. Eosinophils in Innate Immune Defence. Activated human eosinophils are able to defend host against parasites, fungi and invading bacteria, by using functionally important receptors such as TLRs (TLR1, TLR4, TLR7, TLR9, and TLR10), responsible for recognition of conserved motifs in those pathogens [166]. Proteolytic enzymes released by various microbes and allergens, cross-linking of IgG or IgA Fc receptors, IL-3, IL-5, GM-CSF, CC chemokines and PAF mediators can potentially induce activation of eosinophils [130].

Eosinophils are characterised by their cytoplasmic granules that contain cationic proteins: major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO).

These basic proteins play key roles in killing parasites, microorganisms, and tumour cells [156]. Degranulation of eosinophils can be induced by soluble stimuli, such as IL-5, GM-CSF, eosinophil-chemotactic cytokines CCL5 and CCL3, the lipid mediator PAF, the complement fragments C5a and C3a. The granule proteins, MBP and EPO acting in an autocrine manner, and the integrin Mac-1 which plays a role in eosinophil recruitment can also play a role in eosinophil degranulation [158, 167, 168].

At the sites of inflammation, recruited eosinophils release proinflammatory mediators including granule-stored cationic proteins, and newly synthesized eicosanoids, cytokines and chemokines including TGFα, TGFβ, IL 3–5, IL-8, IL-10, IL-12, IL-13, IL-16, IL-18, TNFα, CCL-5 and CCL11 and profibrotic and angiogenic factors such as osteopontin, VEGF and MMPs [169–172]. They also promote Th2 responses. Eosinophils also possess the ability to internalise, process and present antigenic peptides within the context of surface-expressed major MHC class II. They express CD80, CD86, CD40 and ICAM-1 thus they are capable to provide costimulatory signals to T cells, present antigens to naïve and memory T cells and initiate/amplify antigen-specific immune responses. In healthy individuals, circulating eosinophils are devoid of MHC class II, but they are induced to express MHC II and costimulatory molecules upon exposure to appropriate cytokine stimuli and transmigration through endothelial cell monolayer [173–175].

IL-5, IL-3 and GM-CSF besides being growth and maturation factors for eosinophils, can also enhance several eosinophil functions. Th2 cytokines, IL-4 and IL-13 can also activate eosinophils.

3.6.4. Eosinophils in Liver Injury. Activated eosinophils have been suggested to play important roles in the pathogenesis of various liver diseases including primary biliary cirrhosis (PBC) [122, 176, 177]; primary sclerosing cholangitis (PSC) [178, 179] idiopathic hypereosinophilic syndrome [180, 181], drug-induced liver injury [182, 183], graft-versus-host disease [184], and hepatic allograft rejection [185–189]. Experimental studies have shown that activated eosinophils could play a critical role in the pathogenesis of liver diseases through the release of highly cytotoxic granule proteins such as MBP, ECP, TNFα followed by cell damage. The first experimental model to prove *in vivo* eosinophil-induced hepatotoxicity was established by Tsuda et al. in 2001 [190] by using IL-5 transgenic mice with a consequent blood hypereosinophilia. These mice after injection of LPS developed an extensive hepatic lobular necrosis, associated with a transmigration of eosinophils through vascular endothelium and degranulation of their cytotoxic granules in inflamed areas. These eosinophilic injuries were transient but liver specific. A recent study by Takahashi et al. [191] has also demonstrated an increased expression of galectin-9 and eosinophilic chemoattractant in the liver biopsy of patients with drug-induced liver injuries. Tarantino et al. [192] have reported an association between liver fibrosis and eosinophilia infiltrate (EI), which could be explained by the eosinophils' ability to release TNF-α and other cytokines capable of increasing an inflammatory cascade and therefore stimulating the fibrogenic stellate cells.

3.7. Dendritic Cells (DCs)

3.7.1. Phenotype and Function. Dendritic cells (DCs), first discovered [193] by Steinman, are professional antigen-presenting cells which control immunity and tolerance. They initiate and regulate immune responses depending on signals received from the invading microbes and their cellular environment. They are a heterogeneous population which can be divided into two major population; myeloid CD11c+ DCs (mDCs) expressing DC-SIGN and plasmacytoid $CD123^+$ DCs (pDCs) which are also known as IFN producing cells [194, 195].

Myeloid DCs are HLA-DR^+CD11c^+ and express TLR 2, 3, 4, 5, 8. Myeloid DCs exist in three compartments; peripheral tissues, secondary lymphoid organs and in circulating blood. Peripheral tissue resident DCs consist of Langerhans cells (epidermis, gut) and dermal interstitial DCs [196]. Lymphoid organ resident DCs play a critical role in both induction of immunity to invading pathogens and maintenance of tolerance. They capture antigens and upon stimulation via pattern recognition receptors, they induce the proliferation of antigen-specific T cells. They are able to present antigens to $CD4^+$ and $CD8^+$ T cells as well as B cells.

Plasmacytoid DCs are HLA-DR^+CD123^+, express TLR 7, 9, 10 and are present in blood, secondary lymphoid organs and peripheral tissues (skin and lungs) [197]. Their main function is to secrete IFN-α in response to viral infections and to prime T cells against viral antigens [198]. Plasmacytoid DCs are also described as tolerogenic DCs because they could induce regulatory T cells [199].

3.7.2. Dendritic Cells in Innate Immunity. Both myeloid and plasmacytoid subsets are capable of initiating innate immune responses that lead to elimination of invading microbes. DCs express several receptors for recognising viruses including pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs) and C-type lectins [200]. pDCs secrete large amount of type I IFN in response to viral encounter [201]. Activated mDCs produce cytokines such as interleukin-12, IL-15, and IL-18. IL-12 is crucial for mDCs to induce T helper 1 (Th1) cell responses, which subsequently promote the potent cytotoxic T lymphocyte (CTL) responses that are necessary for clearing microbe-infected cells [202].

DCs detect microbes in peripheral tissue sites and, following activation and microbe uptake, migrate to draining lymph nodes, where they promote NK cell activation. DCs also activate NKT cells to secrete IFN-γ and IL-4 [203]. DCs trigger different types of adaptive T-cells immune responses based on antigen and cytokine environment; they can promote IL-10 secreting regulatory T-cell development [204]; induce Th1 response [205] through upregulation of IL-12 secretion and Th2 responses [206] via secreting Th2 cytokines.

3.7.3. Dendritic Cells in Hepatic Inflammation. Both plasmacytoid and myeloid DCs reside in the human liver. Hepatic

DCs play important roles in the induction and regulation of immune responses (Figures 3 and 4). Human liver is constantly exposed to gut pathogens thus liver resident DCs remain in an immature state expressing low levels of MHC and costimulatory molecules CD40, CD80, and CD86. Intrahepatic DCs tend to act as tolerogenic cells preferentially expressing IL-10 [207]. The constant exposure to bacterial LPS via portal blood down-regulates the expression of TLR4 on liver DCs thus limiting their response to danger signals and resulting in reduced or altered activation of the hepatic adaptive immune responses. DCs also have the capacity to expand functional $CD4^+CD25^+$ regulatory T cells [208, 209] and recent study has suggested that $CCR9^+$ plasmacytoid DCs (pDCs) are capable of inducing regulatory T cells and inhibiting antigen-specific immune responses both *in vitro* and *in vivo* [210]. The role of DCs has been widely described not only in viral and autoimmune diseases but also in hepatocellular carcinoma and liver transplantation [211, 212].

3.8. Natural Killer (NK) Cells

3.8.1. Phenotype and Function. NK cells, first described as "pit cells" [213] are a crucial component of innate immune system. They are abundant in the liver where they provide a first line of defence against viral infections and tumour immunity [214, 215]. Hepatic NK cells in mice consist of 5–10% of lymphocyte population and they are defined by $NK1.1^+$ (only for CD57BL/6 mice) $CD3^-$ or $DX5^+$ $CD3^-$. In the human liver, NK cells consist approximately 20-30% of liver resident lymphocytes [216] and they are $CD56^+CD3^-$.

Human NK cells can be divided into two major populations; $CD56^{dim}$ $CD16^{bright}$ $CD3^-$ and $CD56^{high}$ $CD16^{dim}$ $CD3^-$. The former comprise approximately 90% of peripheral circulating NK cell population. They constitutively produce high numbers of cytolytic granules and are capable of spontaneously lysing target cells in the absence of prior sensitization. The latter consist the remaining 10% of circulating NK cells that are poorly cytotoxic and express high levels of C-type lectins and natural cytotoxicity receptors (NCRs) and low levels of killer cell immunoglobulin-like receptors (KIRs) [217]. These two NK cell subsets represent different stages of NK cell maturation, with the $CD56^{dim}$ NK cells being the functionally and phenotypically mature cells [218]. A third population of NK cells consisting of $CD56^-$ cells has been demonstrated during chronic viral infections [219]. They express a similar receptor profile to $CD56^{low}$ NK cells but are poorly cytotoxic and do not secrete cytokines [220–222].

3.8.2. NK Cell Recruitment in Liver. NK cells arrive very early to the site of inflammation and generally reside in the hepatic sinusoids. They express chemokine receptors CCR2 (which responds to chemokine CCL2), CCR5 (ligands are CCL5, CCL7, CCL8), CXCR3 (CXCL9-11), CX_3CR1 (CX3CL1) and S1PR (SIP) thus responding to a variety of chemokines. Both $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets migrate to inflamed sites with more $CD56^{dim}$ being recruited to inflamed liver. Previous studies have suggested that Kupffer cell derived CCL2/MCP-1 recruits CCR2 expressing NK cells to the liver [223, 224]. During hepatic inflammation, activated liver sinusoidal endothelial cells express CXCL9-11 chemokines (CXCR3 ligands) [225] which subsequently recruit CXCR3 expressing NK cells to the liver. They also secrete chemokines CCL3/ MIP-1α and CCL4/MIP-1β which lead to subsequent T cells recruitment to the liver [226]. IFN-γ secreted from NK cells favours development of Th1 cells and upregulates CXCL9-11 chemokines (CXCR3 ligands) on human hepatic sinusoidal endothelium thus will recruit various inflammatory cells expressing CXCR3 chemokine receptors.

3.8.3. NK Cells in Hepatic Inflammation. NK cells play a significant role in antiviral and antitumour activity, liver fibrosis, liver repair and may also be involved in hepatic tolerance. NK cells main function in antiviral and antitumor immunity depends on their proinflammatory cytokine IFN-γ or their direct killing of infected or transformed target cells such as virus-infected hepatocytes or hepatocellular carcinoma. They have both inhibitory and stimulatory receptors which act on their corresponding ligands on target cells [227]. NK cells inhibitory receptors include killer cell immunoglobulin-like receptors (KIRs) and CD94/NKG2 which recognize MHC class I molecules on target cells and inactivate the function of NK cells. The activating receptors include NKG2D, NCRs, and CD266 [220]. Thus, following acute viral infection, chemokines from hepatic resident cells recruit NK cells to inflamed liver and keep them in an activated state to control the infection. However, in chronic hepatitis C, studies have shown that NKG2 expression is increased on NK cells which may contribute to persistence of viral infection [221].

NK cells have also been suggested to be involved in preventing hepatic fibrosis, via killing-activated stellate cells which are key player in fibrosis due to its matrix deposition. Depletion of NK cells in experimental murine models enhances liver fibrosis [222].

NK cells may also be involved in hepatic tolerance. It has been reported that LPS-stimulated Kupffer cells secrete higher levels of the immunosuppressive cytokine IL-10, which in turn leads to inactivation of NK cell function [222]. NK cells may also indirectly maintain hepatic tolerance via dendritic cells which can induce tolerogenic regulatory T cells in the presence of NK cells [228].

3.9. NKT Cells. NKT cells are part of the innate immune system. They express both T-cell receptor and natural killer cell surface markers. They are a heterogeneous group which recognises lipid antigen presented by CD1d [229]. They are classified based on MHC class I like molecule, CD1d restriction as invariant NKT and noninvariant NKT cells. CD1d-dependent NKT cells are again classified into Type I and Type II NKT cells. Human NKT cells express TCR $\alpha\beta$ or TCR $\gamma\delta$ and a variety of NK cell receptors, which include CD161, CD69 and CD56 [230, 231].

Human intrahepatic NKT cells are defined as $CD3^+$ $CD56^+$ and consist of 10–15% of lymphocyte population but

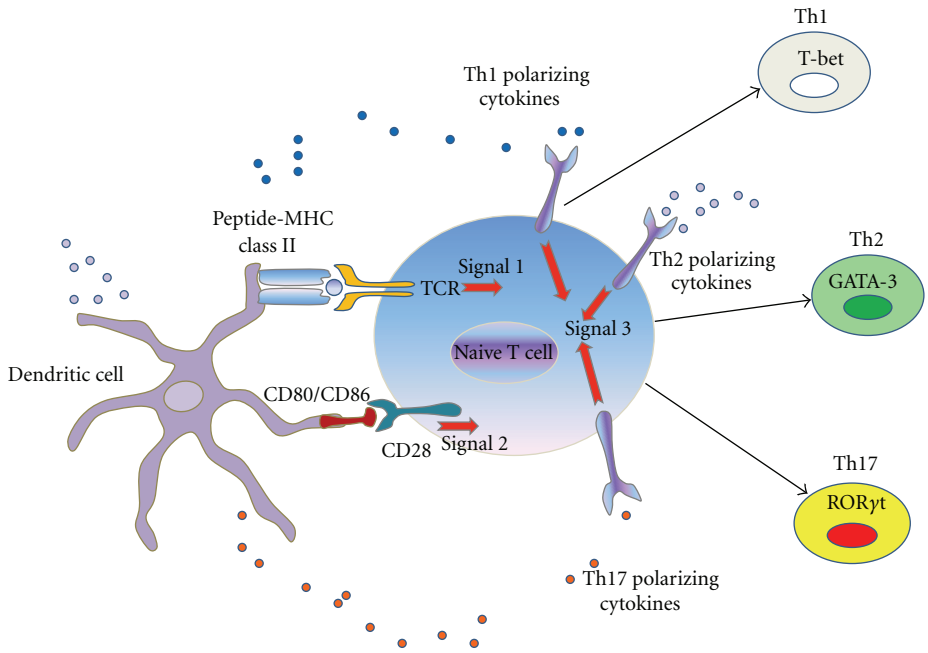

Figure 4: Linking innate and adaptive immune system. Dendritic cells from innate immune system present their antigen to naive T cells at local draining lymph nodes. T-cell receptor (TCR) ligation to MHC class II associated peptides processed from pathogens (signal 1) and binding of costimulatory molecule CD28 on lymphocyte to CD80 and CD86 expressed by dendritic cells (signal 2) leads to T-cell lineages differentiation. Signal 3 is the polarizing cytokines signals from the innate immune cells. Depending on type of antigen which is presented and nature of cytokines in the microenvironment, innate DC cells direct the development of Th1, Th2, Th17 lymphocytes lineages which plays crucial role in adaptive immune system.

of that <1% is CD1d restricted invariant NKT. Intrahepatic NKT cells play an important role in defence towards hepatic infection or inflammation. Host antigen presenting cells present microbial glycolipid antigens to CD1d and NKT cells release Th1 (IFN-γ, TNF-α), Th2 (IL-4, IL-5, IL-10) or Th17 (IL-17, IL-22) cytokines which in turn activate other innate immune cells and adaptive T cells [232].

3.9.1. NKT Cells and Hepatic Inflammation. NKT cells are enriched in liver and play a diverse role in acute liver injury, liver fibrosis and tolerance. It is due to different types of NKT cells and a variety of cytokines which they produce upon stimulation. In the acute injury setting, injection of α-GalCer, a specific ligand for invariant NKT will lead to acute hepatitis [233]. NKT cells also play a role in progressive fibrosis in nonalcoholic fatty liver disease both in human and murine models via activation of Hedgehog pathway [234]. NKT cells are implicated in hepatic tolerance. One elegant study suggested that IFN-γ secreted from NK cells upregulates CXCR3 ligands on hepatic sinusoid and subsequently recruits CXCR3 expressing regulatory T cells to control hepatic inflammation [235].

3.10. Innate Immune Cells Crosstalk Adaptive System in Hepatic Inflammation. Innate immune system provides signals to stimulate the adaptive immune system by proliferation and differentiation of antigen-specific T and B lymphocytes. Antigen peptide acts as a signal 1 which presents the antigen to [236] naive T cells via MHC-class II. Costimulatory molecules such as CD28, CD80, and CD86 are present on antigen presenting cells such as DCs to stimulate T lymphocytes thus acting as signal 2 to link the innate and adaptive immune response. Innate immune cells such as dendritic cells and macrophages produce polarizing cytokines in response to microbes that also promote the differentiation and growth of specific lymphocyte lineages. IL-12 stimulates naive T lymphocytes to develop into Th1 effector cells, IL-4 and IL-13 stimulate them into Th2 phenotype and IL-1, IL-6 and TGF-β into Th17 phenotype. Thus, polarizing cytokines in the microenvironment will shape the naive T cells into different T effectors lineages to counteract with different types of microbes (Figure 4).

3.11. Diagnostic and Therapeutic Clinical Application of Innate Immune Systems. Innate immune proteins and cells have been harnessed for many diagnostic and therapeutic applications in human diseases. Acute phase protein CRP, a mediator of inflammation and agent of innate immunity is now used as a key diagnostic marker of cardiovascular risk. Individuals with CRP levels <2 mg/L have significantly lower rate of coronary event. Thus, CRP levels are useful in evaluating the risk of myocardial infarction [237, 238]. Complement component levels are normally measured to assess the immune-mediated disorders and anaphylactic disorder such as hereditary angioedema. Tocilizumab, an anti-IL-6 therapy has been used in rheumatoid arthritis, cancer

therapy, and cancer-related anorexia [239]. Cell therapy utilizing innate immune cells such as NK cells and DC is always an attractive option for clinical immunologists. Human NK cells immunotherapy is currently a promising tool as an adjuvant therapy in acute myeloid leukemia patients along with standard therapy [240, 241]. Furthermore, administration of myeloid DCs that have been pretreated with inactivated HIV enhances immune control of HIV in patients [242] and myeloid DCs pulsed with tumour antigen lysate (APF) induce tumour specific immune responses along with transarterial chemoembolization (TACE) in hepatocellular carcinoma patients [243].

Many GMP grade clinical trials are now underway for development of DC-based vaccine strategies in viral (HIV) and carcinoma (such as hepatocellular carcinoma) to elicit strong cytotoxic immune responses to overcome the immune regulation. However, vaccine strategies and cell therapies that aim to promote DC and NK cell responses during viral infection and antitumour therapy would have to be carefully monitored to prevent any deleterious consequences of immune activation. Gradual understanding of how DCs and NK cells are involved during viral infection at molecular level may provide new targets for vaccine design or even therapeutic modulation of disease with autologous cell therapy in future.

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Review Article

Optimizing Dendritic Cell-Based Immunotherapy: Tackling the Complexity of Different Arms of the Immune System

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Earlier investigations have revealed a surprising complexity and variety in the range of interaction between cells of the innate and adaptive immune system. Our understanding of the specialized roles of dendritic cell (DC) subsets in innate and adaptive immune responses has been significantly advanced over the years. Because of their immunoregulatory capacities and because very small numbers of activated DC are highly efficient at generating immune responses against antigens, DCs have been vigorously used in clinical trials in order to elicit or amplify immune responses against cancer and chronic infectious diseases. A better insight in DC immunobiology and function has stimulated many new ideas regarding the potential ways forward to improve DC therapy in a more fundamental way. Here, we discuss the continuous search for optimal in vitro conditions in order to generate clinical-grade DC with a potent immunogenic potential. For this, we explore the molecular and cellular mechanisms underlying adequate immune responses and focus on most favourable DC culture regimens and activation stimuli in humans. We envisage that by combining each of the features outlined in the current paper into a unified strategy, DC-based vaccines may advance to a higher level of effectiveness.

1. Introduction

Dendritic cells (DCs), originally described by Steinman and Cohn [1], serve as a crucial link between innate and adaptive immunity [2]. Although they represent only a small population of leukocytes, they are the most powerful antigen presenting cells (APC) with the unique ability to activate naive T cells [3]. As sentinel members of the *innate immune system*, DCs respond to antigens and molecules containing pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs)—so-called danger signals—by the generation of protective cytokines [4]. As members of the *acquired immune system*, DCs respond to these harmful molecules by efficient antigen uptake, processing, and presentation, and hence DCs are crucial in the initiation of adaptive immune responses. Besides their potent capacity to stimulate naive T cells, effector T cells and memory T cells, as well as B cells, they are also involved in the maintenance of tolerance against harmless (auto)antigens [4, 5].

Because of their immunoregulatory capacities and because very small numbers of activated DCs are highly efficient at generating immune responses against antigens [6], DCs have been vigorously used in clinical trials in order to elicit or amplify immune responses against cancer and chronic infectious diseases [7]. Although an impressive amount of data has been obtained from these clinical trials completed thus far, the outcomes were not in line with initial expectations [8–10]. A critical issue in the development of DC-based vaccines is that their ability to stimulate immune responses depends largely on the activation state of DCs. In this paper, we discuss the continuous quest for the best in vitro conditions in order to generate clinical-grade DCs with a potent immunogenic potential. For this, we

explore the molecular and cellular mechanisms underlying adequate immune responses and focus on optimal DC culture regimens and activation stimuli in humans.

2. Origin and Subsets of DCs

DCs originate from $CD34^+$ haematopoietic stem cells in the bone marrow and circulate as precursors through the blood stream to target tissues. Additionally, it is well established that during physiological stress, monocytes are also a source of DC precursors and differentiate into immature DCs in the presence of GM-CSF and a variety of other cytokines. Immature DCs take residence at sites of potential antigen entry and are specialized in antigen capturing and processing. They recognize the so-called pathogen-associated molecular patterns (PAMPs) which are evolutionary conserved structures, including microbial lipids, carbohydrates, nucleic acids and intermediates of viral replication (double-stranded (ds)RNA), via pattern recognition receptors (PRRs) [11]. There are several types of PRRs that are involved in innate recognition of pathogens, including toll-like receptors (TLRs), nucleotide-binding-oligomerization-domain-(NOD-like) receptors, interferon (IFN-induced) dsRNA-activated protein kinase (PKR), and RIG-I-like helicases [12]. Once DCs have captured a foreign nonself-antigen, they undergo a highly regulated maturation process and remodel into fully activated antigen-presenting DCs [13] capable to elicit effective immune responses. Indeed, mature DCs express high levels of several costimulatory molecules as well as major histocompatibility complex (MHC) molecules on their surface [14]. Maturation of DCs also induces the production of chemokines that attract naive and memory T cells. During the maturation process, DCs exit the nonlymphoid tissues to migrate via afferent lymph to lymphoid tissues. Subsequently, mature DCs will activate (naive) T- and B-lymphocytes that recognize the presented antigen as peptide-MHC complexes on the surface of the DC. Yet, additionally, positive amplification of antigen presentation via costimulation and secretion of various cytokines is also crucial to induce proper immune responses [3, 15] (cfr. 3.1).

Besides the above-delineated classical view of the DC life cycle, it has gradually become clear that DCs do not represent a homogeneous population. Briefly, the first division is the distinction between plasmacytoid and myeloid or conventional DCs (cDCs). Plasmacytoid DCs (pDCs), also referred to as type I IFN-producing cells (IPCs), are the key effectors in the innate immune system because of their extraordinary capacity to produce type I IFN upon viral infection [16, 17]. The conventional DCs can be further subdivided according to their localization: (i) lymphoid organ-resident DCs, (ii) peripheral tissue-resident DCs (e.g., langerhans cells and interstitial DCs), and (iii) circulating DCs. In human blood, differences in DC subsets can be identified based on differential expression of specific markers: pDCs express CD303 (BDCA-2), CD304 (BDCA-4), and CD123 (IL-3Rα), whereas cDCs are characterized by their expression of CD1c (BDCA-1) and CD11c [18, 19]. In addition, pDCs and cDCs also express a different set of toll-like receptors TLRs [20]. In brief, pDCs express mainly TLR7 and TLR9, whereas cDCs exhibit strong expression of TLR1, TLR2, TLR3, TLR4, and TLR8. Accordingly, pDCs mainly recognize viral components and produce a large amount of IFN-α. In contrast, cDCs recognize bacterial components and produce proinflammatory cytokines such as TNF-α, IL-6, and IL-12p70 to activate proinflammatory T-cell subsets [T helper type 1 (Th1)/Th17] and consequently recruit cytotoxic T-lymphocytes (CTL). Because of the unique biological function of each DC subset, it was proposed that a specific DC lineage determines the outcome of T-cell contact, that is, tolerance or immunity. Indeed, it was initially thought that cDCs were inducers of immunity, while pDCs induced tolerance [21]. However, nowadays pDCs are believed to be the key effector cells in the early antiviral innate immune response by producing large amounts of type I interferons upon viral infection. Furthermore, it has been shown that pDCs augment immune responses by cross-talking with cDCs by the production of IFN-α, thereby playing a key role in effective stimulation of adaptive immunity as well. In addition to IFN-α production, it has been demonstrated that mouse pDCs also express CD40L, which activates cDCs to produce IL-12p70 [22] (Figure 1).

Recently, several groups identified a unique human DC subset ($CD11c^+BDCA\text{-}3^+$) as the homologue of mouse $CD8\alpha^+$ DC [23–26]. Of particular importance is their superior antigen cross-presentation capacity, expression of the XC chemokine receptor 1 (XCR1), and their capacity to produce high levels of bioactive IL-12p70. Initially, it was suggested that $BDCA\text{-}3^+$ DCs and $BDCA\text{-}1^+$ DCs may represent maturational stages of the same cell type. The fact that BDCA-3 expression is induced on a reasonable proportion of $BDCA\text{-}1^+$ DCs after culture-induced maturation may be considered an argument in favour of the former concept. However, since the same observation was also made for IL-3-stimulated pDC, such data could also be taken as an argument in favour of a similar relationship between $BDCA\text{-}3^+$ DCs and pDCs [27]. Nowadays, it is well accepted that $BDCA\text{-}3^+$ DCs represent a unique myeloid DC subset that effectively activates $CD8^+$ CTL, in analogy with mouse $CD8\alpha^+$ DCs. This supports a potential key role for the myeloid $BDCA\text{-}3^+$ DC subset in immunity to viruses, as well as other intracellular pathogens [28–30] and may have important implications in the design of human DC vaccines.

3. The Immune System against Cancer and Chronic Infectious Diseases

3.1. 3-Signal Theory for T-Cell Activation. Therapeutic vaccines to treat chronic infectious diseases (such as human immunodeficiency virus (HIV), cytomegalovirus (CMV), hepatitis B virus (HBV), and hepatitis C virus (HCV)), or numerous tumor types (including melanoma, leukaemia, breast, and prostate cancer) mainly aim to induce antigen-specific cell-mediated immunity to clear infected cells and eliminate tumor cells. Recent studies have shown that DCs play a critical role in directing effector T-cell responses towards a Th1, Th2, Th17, or regulatory T cell (Treg) response [31, 32].

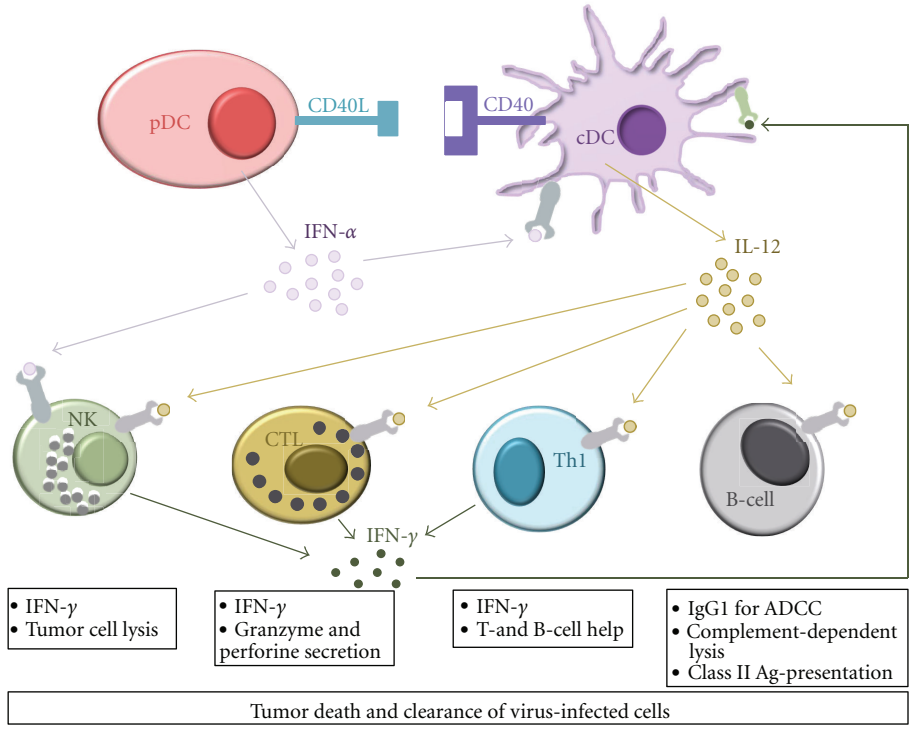

Figure 1: Cooperative action of different DC subsets to tackle both innate and adaptive immunity for clearance of tumors and viral infection.

Briefly, upon maturation, DCs upregulate the expression of certain products necessary to supply T lymphocytes with the 3 signals that will determine their activation status and general fate [33]: antigen-specific signalling via the T cell receptor (TCR), mediated by the binding of MHC-peptide complexes to the TCR drives the initial interaction between DCs and T cells (i.e., signal 1). Costimulation by surface molecules on APC, such as DCs, can either amplify or regulate the interaction with T cells (i.e., signal 2) [34]. Costimulatory molecules can be divided in two classes: Ig superfamily members, including CD28, that interact with several members of the B7 family (CD80/CD86) [35–37] on the one hand, and TNF receptor superfamily members, including CD27 and CD40, that bind to membrane-bound proteins of the TNF superfamily [35, 38, 39] on the other hand. CD28 is expressed on T cells, and is the receptor for CD80 and CD86 expressed on activated APC [37]. Ligation of CD28 provides costimulatory signals required for T cell activation: (i) altering the threshold level of TCR ligation required for activation, (ii) reducing the time needed to stimulate naive T cells and (iii) enhancing the magnitude of the T cell response. Without CD28 signalling, the T cell would either become apoptotic or anergic [40]. The B7 family of costimulatory molecules has been extensively reviewed elsewhere [41, 42]. CD27, a member of the TNF receptor superfamily, is constitutively expressed on the surface of naive T cells, in contrast to other members of the TNF receptor family [43] and can thus play a role during the initiation of T cell responses [35]. The contribution of CD27 to the immune response is dependent upon CD70 expression [44]. While during primary T cell activation there seems to be a certain redundancy in CD80/CD86 and CD70 costimulation, it is triggering of CD27 on T-lymphocytes by its ligand CD70 that enhances the magnitude of antigen-specific cytotoxic T cell reponses [38, 45], which is required for effective immunotherapy. CD27/CD70 interaction increases the initial expansion and survival of antigen-specific T cells [46] and improves their cytotoxic capacity [47]. Furthermore, a recent study has shown that CD70 expressed on mouse DEC205$^+$ cDCs represents an IL-12p70-independent Th1-inducing factor [48] (vide infra). Taken together, enhancing CD70 expression on DCs would lead to the development of a vaccine strategy capable of facilitating the CD27/CD70 interaction, and hence the induction of an adequate anti-tumor or antiviral immune response. Finally, mature DCs can secrete a variety of pro- and anti-inflammatory cytokines for differentiation from naive T cells to effector T cells (i.e., signal 3). One well-studied third signal agent is interleukin (IL)-12p70 for the induction of Th1 and CTL [49], which are essential for efficient tumor/pathogen rejection [50]. IL-12p70 is a multifunctional proinflammatory cytokine with pleiotropic effects and comprises two subunits: p35 and p40. Highly-coordinated p35 and p40 gene expression results in the formation of the biologically active form IL-12p70 and is essential for initiation of an effective immune response. Indeed, IL-12p70 activates natural killer (NK) and T cells to produce mainly IFN-γ, it favours the generation of CTL and it enhances the cytotoxic activation of activated NK cells [51]. Besides the activation of innate and antigen-specific adaptive immunity against the tumor cells, the antitumor effects of IL-12p70 are based on the ability to inhibit tumor angiogenesis through IFN-γ [52, 53]. In addition, IL-12p70

is crucial in the early phase of host defence against microbial infections [52, 54, 55], where it is produced within a few hours after bacterial, fungal or parasitic infection [52]. Thus, to develop an efficient vaccine against tumors or chronic infectious diseases, DCs producing the biologically active form IL-12p70 are desired [56].

3.2. Other Arms of Cellular Immunity Required to Fight Cancer and Chronic Infectious Diseases. Rather than simply recruiting Th1 cells and CTL, vaccines should be designed to recruit other cellular arms of the immune system as well, for example, NK cells and antibody-producing B cells. In this perspective, it has been shown that DCs also play a key role in the activation of NK cells that can have powerful effects against tumor cells, particularly those with attenuated MHC expression [57]. Indeed, in response to DC-derived cytokines, such as IL-12p70 and IL-18, NK cells are able to produce IFN-γ [58]. In turn, exposure to signals provided by activated NK cells subsequently induces DCs to mature into a highly stimulatory phenotype that produces sustained IL-12p70, thereby promoting adaptive immunity [59, 60]. Overall, these findings support the concept to include DC-NK interactions in order to improve DC-based immunotherapy. Furthermore, recent studies that have resulted in reappraisal of the potential of antibodies in the control of tumors and viruses support the strategy that DC-based vaccines should also be designed with antibody production in mind [61–63]. In addition to priming of T cells and NK cells, the group of Banchereau have recently demonstrated that DCs may also directly signal naive B-cell differentiation through the production IL-12p70 [64] and indirectly by promoting the differentiation of IL-21-producing T follicular helper cells (Tfh) in an IL-12p70-dependent manner [65, 66]. These observations suggest that IL-12p70 could constitute a potent vaccine adjuvant in situations when both the cellular and humoral arms of the immune system are required, such as cancer [62, 63] and HIV [61]. Indeed, studies with rhesus macaques have concluded that IL-12p70 enhances the induction of specific antibody responses in vivo when used as vaccine adjuvant [67–69]. Noteworthy, IL-12p70 also possesses a number of powerful nonimmunologically related anticancer activities. For example, IL-12p70 plays a role as an antiangiogenic agent that can strongly inhibit the formation of neovasculature [53].

Taken together, the goal of many DC-based vaccination protocols is to cultivate DCs that are capable of expressing immunostimulatory cytokines (IL-12p70) and costimulatory molecules (CD70) in parallel with antigen presentation. Since expression of costimulatory molecules and cytokine secretion can be influenced by environmental signals during DC maturation, it is necessary to find an optimal cytokine environment for DC maturation in order to create a powerful vaccine against several cancer types or chronic infectious diseases. Various attempts have subsequently been made in order to harness DC to achieve most powerful immunity, including strategies to enhance or stabilise antigen-specific stimulation, as well as essential costimulatory modulation of DCs.

4. Harnessing DCs for Clinical Use

4.1. Antigen Loading Strategy. To maximize the efficiency and stability of antigen presentation by DCs, several strategies have been developed. These include direct in vivo delivery of antigen to circulating DCs in patients [70], as well as a variety of ways for in vitro loading of DCs with antigen. Indeed, antigens coupled to antibodies specific for DC markers, such as 33D1 or DEC-205, have already been used in preclinical models to deliver antigens to DCs in vivo [71]. Additionally, DCs transduced ex vivo with tumor- or viral-derived mRNA or DNA [72–74], fused with tumor cells [75, 76], or directly loaded with tumor- or viral-derived peptides [77, 78] have been tested for the induction of antigen-specific immune responses in vitro and in vivo. While the use of peptides as a source of antigen has several limitations when implementing clinical trials with antigen-loaded DCs, including human leukocyte antigen (HLA) restriction as well as a limited number of identified immunodominant tumor- and virus-associated antigens, we [79] and others [80–84] have previously shown that DCs transfected with mRNA-encoding antigens are superior to other loading strategies to induce immune responses. In general, there are several advantages regarding the use of mRNA for antigen loading of DCs [72, 85] as compared to tumor-associated peptides. mRNA transfection will generate multiple antigenic epitopes, possibly more immunogenic than those already characterized, independent of the patient's HLA haplotype. In addition, mRNA can be isolated and amplified from autologous tumor or virally infected cells in order to obtain mRNA encoding patient-specific antigens [86–88]. Moreover, because mRNA only has a short half-life and does not integrate in the host genome, genetic modification of DCs by mRNA electroporation is considered to be highly safe and an easily applicable clinical tool.

4.2. Different Sources for Isolation or Generation of DCs. The earliest studies on DC vaccination were initiated in 1993 and utilized whole blood leukapheresis products with subsequent gradient centrifugation procedures to enrich for rare immature DC precursors of the peripheral blood before antigen loading and maturation [89]. However, because of low yield of circulating DCs and difficulty to obtain them, the clinical utility of DC vaccines was initially limited. In a second attempt to directly isolate DCs from peripheral blood, they were first mobilized by cytokines such as Flt3-ligand [90, 91]. Unfortunately, the in vivo expanded cells lacked efficient protein uptake properties [89]. Moreover, although blood DCs from patients with a malignant or chronic infectious disease may seem to have normal distributions, they might have some functional defects, such as a lower expression of costimulatory molecules or an impaired capacity to stimulate autologous antigen-specific T cells [92, 93]. Currently, DCs for vaccination studies are generally obtained in large numbers after in vitro generation. At first, human DCs were cultured from $CD34^+$ haematopoietic progenitors in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor alpha (TNF-α) [94, 95]. However, only few studies that used $CD34^+$-derived

DC preparations for vaccination protocols in clinical phase I studies have been reported [96, 97]. Nowadays, generating DCs from peripheral blood $CD14^+$ monocytes is a generally-accepted method and is extensively used in experimental and clinical vaccination studies. In doing so, large numbers of monocyte-derived (mo-)DCs are obtained without necessity for pretreatment of donors with any cytokines to mobilize DC progenitor cells [98]. Yet, the design of DC-based clinical trials varies greatly, including DC preparation, and therefore, standardization and further improvement for clinical use are needed [99].

While a combination of granulocyte-macrophage colony-stimulating factor (GM-CSF) with IL-4 is most commonly used to induce immature DCs from monocytes [100, 101], a variety of other cytokines, such as IFN-α [102–104], TNF-α [105, 106], and IL-15 [107] have been used in combination with GM-CSF for this purpose. In this perspective, Santini et al. [102] as well as Arimoto-Miyamoto et al. [34] reported independently that IFN-α induces rapid differentiation of freshly isolated GM-CSF-treated human monocytes into mo-DCs endowed with potent functional activities, both in vitro and in vivo [102, 103], possibly mediated by IFN-α-dependent induction of CD70 expression [34]. It must be noted however that IFN-α also induces activity of RNases [108], and can not therefore be used in in vitro culture regimens for DCs when mRNA-based in vitro modification of DCs is wanted [109]. In addition, others have demonstrated that $CD14^+$ monocytes respond to IL-15 by undergoing morphological transformation and acquiring characteristic DC features that facilitate antigen-specific responses of T cells [110]. In contrast to IFN-α-modulated DCs, mRNA electroporation appeared to serve as an efficient antigen-loading strategy for IL-15-treated DCs [111]. Furthermore, Chomarat et al. described that TNF-α facilitates the induction of adaptive immunity also by promoting DC differentiation from $CD14^+$ blood precursors in vitro [106]. However, it has been reported in contrast that TNF-α-treated semi mature DCs induce tolerance in experimental acute encephalitis (EAE), a mouse model for multiple sclerosis [112]. Moreover, due to strong plastic adherence before and to a lesser extent also after maturation, IL-15 and TNF-α treatment for DC generation results in a lower DC yield [34]. Consequently, the well-established and generally used combination of GM-CSF and IL-4 [100, 101] is the most efficient method to obtain mo-DCs that express acceptable levels of CD70 with minimal loss of cells by adherence [34] and with good compatibility with a mRNA approach [79].

4.3. Various Stimuli to Obtain Mature DC. Regardless of how they are generated, it is important that DCs are activated to a mature phenotype, since immature DCs are no longer considered as competent candidates for vaccination trials because of their low T-cell activation potential [113–115]. Most DC culture regimens that have been commonly employed in clinical trials have activated DCs through the use of individual cytokines associated with inflammation [101] or inflammatory cytokine cocktails [116].

Indeed, in an attempt to resemble a physiological environment for DC maturation, balanced cocktails of maturation agents that may be the most representative of various inflammatory states have often been used. In 1996, Romani was the first to describe a method to mature DCs from human blood by using a conditioned medium containing an unidentified cytokine mixture produced by adherent peripheral blood mononuclear cells (PBMC) stimulated by human immunoglobulins or fixed *Staphylococcus aureus* Cowan I strain [98]. Only one year later, Morse and colleagues described a way to mature mo-DCs by adding TNF-α to the culture medium. TNF-α appeared to enhance the number of cells expressing the maturation marker CD83, which seemed to be the most potent allostimulatory cells in mixed lymphocyte reactions [117]. Also, Jonuleit et al. reported for the first time a well-defined cytokine cocktail to induce DC maturation, consisting of IL-1β, IL-6, TNF-α, and PGE_2 [116]. This combination of proinflammatory mediators represents current "golden" standard for activation of DCs, although the concentration of the diverse components varies among studies. Fully-mature DCs induced by this combination of inflammatory cytokines have been consistently observed as superior to immature DCs in promoting a higher degree of specific T-cell priming in vitro and in vivo. While PGE_2 increases the expression of CCR7 and hence the capacity of DCs to migrate to the regional lymph nodes through chemotaxis by CCL-19 and/or -21 [118], PGE_2 also inhibits IL-12p70 secretion by DCs [56]. Although some details remain incompletely clarified, expression of IL-12p70 appears to be under unusually tight regulation and requires at least 2 signals activating both MyD88 (myeloid differentiation factor 88)- and TRIF (TIR domain-containing adapter-inducing IFN-β)-dependent pathways simultaneously for maximal expression [119, 120]. Of note, TLRs, commonly used for activation of DCs, are divided in those that are MyD88-dependent and those that are TRIF-dependent, hence explaining observed requirements of multiple TLR engagement for maximized IL-12p70 production. In this perspective, mature DCs with the potential to produce high amounts of biologically active IL-12p70 (10–15 ng/mL) were obtained by Mailliard et al. in 2004, who used a combination of IL-1β, TNF-α, IFN-α, IFN-γ, and poly I:C [121]. Although these mature mo-DCs displayed a slightly decreased migratory capacity [121], they induced significantly more antigen-specific cytotoxic T cells than did the "golden standard" counterparts, dependent on the high IL-12p70 secretion. In 2007, Zobywalski et al. proposed a cytokine cocktail consisting of TNF-α, IL-1β, IFN-γ, R848 and PGE_2 as the best cocktail to allow large-scale processing of clinical-grade mo-DCs with the capacity to secrete IL-12p70 [56]. Addition of poly I:C to this cocktail significantly increased IL-12p70 production even more, yet it disabled the mature DCs to express the transgene after exogenous RNA electroporation and it led to a decline in cell viability [56]. Dohnal et al. used a mixture of LPS and IFN-γ to mature DCs [122]. Although high IL-12p70 secretion by mature mo-DCs was previously attributed to the addition of IFN-γ [121], IFN-γ also appeared to be responsible for the low migratory ability of DCs cultivated in the presence of LPS and IFN-γ

[122, 123]. Nevertheless, this migratory problem could be fixed by including PGE_2 in the maturation-inducing cytokine cocktail [123, 124]. In addition, whereas DC maturation by TLR ligand alone (including LPS, CpG, and poly I:C) has been reported to increase expression of classical activation markers as well as many inflammatory cytokines [125], a TLR agonist alone does not result in a substantial $CD8^+$ T-cell response, which is probably due to no or very low levels of IL-12p70 secretion as well as insufficient induction of CD70 by TLR ligand stimulation alone [126]. According to Sanchez et al., expression of CD70 on mo-DCs requires combined TLR/CD40 stimulation [125]. In preliminary experiments, we experienced that addition of IFN-γ alone to a cocktail of proinflammatory cytokines is neither enough for optimal CD70 induction on mo-DC (unpublished data). In contrast, addition of IFN-γ in combination with the TLR7/8 agonist R848 to the standard maturation cocktail from which IL-6 was omitted resulted in a significant increase in CD70 expression (unpublished data).

From the above-mentioned observations, it may be evident that each compound added to a cytokine cocktail can influence DC phenotype and function in its own way and the "ideal" maturation mixture still needs to be well considered. Taken together, the "ideal" maturation cocktail to prime Th1-polarizing mo-DCs must contain PGE_2 [123, 124], for its migration-inducing potential, a TLR ligand (e.g., LPS [125] or R848 (own unpublished data), but not poly I:C [56]) in combination with CD40L [125] or IFN-γ [56], and some proinflammatory cytokines that have a positive impact on DC maturation (e.g., TNF-α [117] and IL-1β [116]). In addition, the cocktail must be free of IL-6 which has been described to inhibit IL-12p70 secretion [34, 56], while IL-4 [126] and IL-10 [126] need to be eliminated from the cocktail as well, since these cytokines prevent CD70 expression.

Alternatively, one can optimise DC immunogenicity through molecular modification of the cells [109], for example, by selective overexpression of genes encoding immune-stimulatory signals (e.g., IL-12p70 [127, 128], CD40 or CD40 ligands [129, 130], and CD80/CD86) or by selective downmodulation of negative regulatory molecules, such as IL-10 [131, 132], IDO [133], SOCS1 [134, 135], and TGF-beta [136].

4.4. Influence of Different Oxygen Levels and Culture Media on Mo-DC Physiology. Mo-DC generation as well as maturation does not solely depend on the cytokine environment, but can also be influenced by oxygen levels, culture media and medium supplements. Mo-DCs are generally differentiated ex vivo in incubators that maintain atmospheric oxygen levels of 21% O_2 in combination with 5% CO_2. In contrast, DCs do not come across such high oxygen levels in vivo. Indeed, the oxygen levels in tissues are usually 3–5% [137], whereas approximately 12% in arterial blood [138]. In many inflamed and tumor tissues, even extremely low oxygen levels (<1%) have been found [139]. Therefore it is evident that DCs experience rapid changes of oxygen supply during their migration in different tissues. Although it is well recognized that tissue microenvironments are involved in regulating the development and function of immune cells, including B- and T cells, only few studies have investigated the effect of hypoxia (<1% oxygen saturation) or physiological oxygen levels (±3% oxygen saturation) on the differentiation of human DCs from progenitors and their maturation. Yang et al. reported that monocytes remain able to differentiate into DCs under hypoxia. However, these hypoxia-conditioned DCs displayed poor T cell-stimulatory activity and shifted towards a Th2-stimulatory phenotype [140], presumably as a consequence of the marked reduction of MHC class II and costimulatory molecule expression, [141] as well as of reduced Th1-polarizing cytokine secretion [140, 141]. The observed inhibition of DC function by hypoxia could possibly explain why most tumors can efficiently escape from host immune surveillance. However, Wang et al. showed only one year later that reoxygenation of hypoxia-differentiated DCs results in complete recovery of their mature phenotype and function, including a strong ability of the reoxygenated DCs to drive immune responses towards a proinflammatory Th1/Th17 direction [141]. Besides hypoxic conditions, one study investigated the influence of physiological oxygen levels on DC physiology and antigen-presenting capacity. Surprisingly, no difference in expression of surface molecules (CD54, CD40, CD83, CD86, HLA-DR, CXCR4, CCR7) nor secretion of TNF-α, IL-6, and IL-10 was observed between DC cultures under physiological (3%) or atmospherical (21%) oxygen levels [138]. Albeit that DCs stimulated with LPS or CD40L under physiological O_2 conditions secreted higher amounts of IL-12p70, these DCs did not elicit increased $CD8^+$ T-cell responses in vitro, as measured by IFN-γ secretion [138]. Taken together, there is still some controversy on whether physiologically or atmospherically oxygen levels must be used for DC culture and not enough data exist to robustly support a conclusion.

For optimal production of clinical-grade DCs from peripheral blood monocytes, it is also important to choose the appropriate culture medium as well as potential serum supplements. Initially, most mo-DCs used for clinical trials were generated in medium supplemented with plasma or serum, such as fetal bovine serum (FBS) containing xenologous proteins. For this, FBS can be immunogenic and possibly transfer bovine-related infections, including bovine spongiform encephalopathy [98, 142]. However also the use of autologous or allogeneic (pooled) serum derived from patients or healthy controls, respectively, might lead to undesired immunomodulatory ingredients that can affect DC phenotype and function [143]. Therefore, it is clear that by eliminating the need for serum, an undesirable variable is removed making the medium more defined and consistent [142, 143]. For this reason, several clinical-grade serum-free media are now commercially available and have been tested, including XVivo15, XVivo20, and AIMV [98, 144, 145]. Although so far only a small amount of studies have compared mo-DCs differentiated in serum-free medium with cells cultured in medium containing serum, they all agree that serum-containing media were more able to generate mature mo-DCs as compared with serum-free media [143, 145, 146]. The latter resulted mainly in the generation of semimature mo-DCs that express CD83 (a mature DC

marker [147]) as well as CD1a (an immature DC marker [98, 101]), and were slightly but consistently less able to produce IL12p70 in response to maturation-inducing stimuli [142, 143]. Other characteristics, including yield, surface expression of maturation markers, in vitro survival, migratory capacities and induction of lymphocyte proliferation, were comparable between DCs differentiated in serum-free or serum-containing media [99, 143, 145, 146]. In vivo assays following transfer of such mo-DCs generated in serum-free medium into humans are needed to decide whether the limited difference in CD1a expression and cytokine production is of true biological relevance.

5. Taking DC into the Clinic

5.1. Completed and Ongoing Clinical Trials. Despite the use of mature DCs in vaccination trials, results from multiple clinical trials with DC-based vaccines have been contradictory and only fractions of enrolled patients show potent antitumor or antiviral immune responses with moderate clinical response rates (approximately 10–15%) (reviewed in [10, 148–151]) or partial control of viremia and immune reconstitution [77, 152–154], respectively. Several studies suggested that this is because of inefficient activation of Th1-polarized responses due to incomplete DC maturation [155–157]. For this, different strategies are currently being pursued in order to improve the efficacy and outcome of DC-based cancer vaccines. Considering the above-mentioned powerful immune-stimulatory properties possessed by IL-12p70, DC-based vaccination strategies may consistently benefit from incorporation or endogenous induction of this cytokine. In a first phase I clinical trial by the group of Czerniecki [158], 13 breast cancer subjects were injected intranodally with short-term DCs activated with a cytokine-cocktail consisting of IFN-γ and LPS in order to induce IL-12p70-secreting DCs. The authors reported induction of robust detectable immunity as evidenced by in vitro monitoring of circulating vaccine-induced antigen-specific $CD4^+$ and $CD8^+$ T cells, as well as both T-and B-cell infiltrates into tumor region as well as dramatic reductions in tumor volume. Additionally, Dohnal et al. [122] also showed the safety and feasibility of IFN-γ/LPS-activated DCs for the treatment of paediatric cancer patients. Besides that no adverse events were reported, they also demonstrated the potential of IL-12p70-secreting DCs to induce cellular immune responses. It should, however, be noted that Traxlmayr et al. [159] reported IL-12p70-dependent proliferation of immunosuppressive $\gamma\delta$ T cells in cancer patients vaccinated with IL-12p70-secreting DCs, pointing to a negative regulatory feedback mechanism for DC-controlled immune responses.

Furthermore, it has been demonstrated by others [160–162] that DCs electroporated with mRNA encoding CD40 ligand, CD70, and constitutively active toll-like receptor 4, so-called TriMix DCs, display increased potential for the induction and amplification of tumor-specific responses in patients with advanced melanoma. Noteworthy, a positive delayed-type hypersensitivity assay (DTH) postvaccination correlated with Il-12p70 secreting capacity of vaccinated DCs.

5.2. Overcoming Tumor and Virus Immune Escape. One of the major obstacles against successful DC vaccination, is certain immunosuppressive mechanisms triggered by the tumor cells or viruses. Indeed, under the influence of the tumorigenic microenvironment, the host DCs may acquire a tolerogenic phenotype. These tumor-conditioned DCs could, in return, produce a variety of immunosuppressive molecules and thus further supporting tumor immune escape [163]. For example, many tumors produce IL-10 [164], a potent immunosuppressive cytokine. We (unpublished data) and others have previously shown that DC differentiation and functional activities are tightly regulated by this cytokine [165, 166]. In return, DCs can secrete IL-10 and effectively inhibit T-cell activation. Additionally, numerous viruses, including human CMV, HIV, herpes simplex virus type 1, and measles virus, target DCs [167, 168], and have evolved strategies to specifically modulate DC phenotype and/or function, thereby promoting virus-mediated immune escape. For example, DCs infected by human CMV are characterized by reduced expression of MHC class I and II molecules, costimulatory molecules, and proinflammatory cytokines, which consequently results in reduced T-cell activation [169]. Nowadays, emerging evidence indicates that one of the most effective ways to enhance the efficacy of DC-based immunotherapy is by targeting the negative arm of immune regulation. For future clinical trials, this may be achieved by the use of small interfering RNA (siRNA) for knocking down IL-10 expression by DCs [131, 132], or other negative regulatory molecules, such as indoleamine 2,3-dioxygenase (IDO) [133], suppressors of cytokine signalling 1 (SOCS1) [134, 135], and transforming growth factor (TGF)-beta [136]. Indeed, inhibition of expression of these regulatory molecules has been demonstrated to significantly enhance the abilities of DCs to present tumor antigens, to produce IL-12p70, and to induce effectively antitumor responses.

5.3. Future Perspectives. With respect to tackle different arms of the immune system, many different approaches are currently being pursued. In particular, considering the distinct ability of different DC subsets in inducing both innate and adaptive immunity, the exploitation of specific subsets of DCs to elicit the desired immune response is foreseen. Although pDCs primarily contribute to innate antiviral immune responses by producing IFN-α/β [16], this ability also has been reported to activate other DCs, including those involved in cross-priming [170], and consequently greater activation of adaptive immune responses. In doing so, pDCs may play a critical role in provoking cancer immunity. Hence, combination therapies aiming at interaction of pDCs and cDCs to stimulate T-cell priming, and hence effective anti-tumor or antiviral immunity are needed in cancer patients and chronically infected patients (Figure 1).

Additionally, differentiation of monocytes into DCs with cocktails including GM-CSF and IL-15 will generate cells with the phenotype and characteristics of Langerhans cells (LC), which are far more efficient in vitro in priming antigen-specific $CD8^+$ T cells than DCs derived with GM-CSF and IL-4 [111, 171]. As described by others, LCs are

very efficient in cross-presenting peptides to $CD8^+$ T cells, which acquire potent cytotoxicity and are able to efficiently kill target cells, including tumor cell lines that express peptide-HLA complex, only at low amounts [172] in an IL-15-dependent manner. The pivotal role of LC to allow maximal stimulation of both humoral and cellular immune responses, supports the important concept for targeting LC in the design of vaccines that aim at eliciting strong cellular immune responses [66, 173, 174].

The recent identification of human $CD141^+$ DCs that can effectively cross-present antigens has clear implications for the design of new therapies to treat cancers and infectious diseases with improved efficacy. It has been reported that the limiting cytokine for the development of the murine counterpart is the Fms-related tyrosine kinase 3 ligand (Flt3L), rather than GM-CSF or M-CSF, which has major influence on the development of inflammatory and migratory DCs [175–177]. However, although Poulin et al. [24] made a first attempt to delineate in vitro culturing conditions for the generation of $CD141^+$ DCs from human progenitor cells, further optimization of such protocols is necessary to allow for their use in adoptive transfer immunotherapy approaches.

6. Conclusion

Current efforts for DC-based modalities have been compromised by a failure to utilize the full potential of DCs. However, even though only limited success rates have been achieved to date, DC vaccination remains a promising immunological approach against tumors and/or viruses and deserves further exploration. Alternative strategies to enhance DC immunogenicity by functional conditioning and molecular modifications have been investigated in vitro. The different findings discussed here, indicate that DCs can indeed be functionally conditioned and genetically modified to acquire an enhanced immunogenic phenotype. For this, time has come to bring DC-based immunotherapy to the next level and implement above-mentioned observations in a standardized regimen for alternatively conditioned DCs. Results from first clinical trials will subsequently reveal their potential in order to improve treatment of cancer and chronic infections.

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Review Article

Danger Signals Activating the Immune Response after Trauma

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Sterile injury can cause a systemic inflammatory response syndrome (SIRS) that resembles the host response during sepsis. The inflammatory response following trauma comprises various systems of the human body which are cross-linked with each other within a highly complex network of inflammation. Endogenous danger signals (danger-associated molecular patterns; DAMPs; alarmins) as well as exogenous pathogen-associated molecular patterns (PAMPs) play a crucial role in the initiation of the immune response. With popularization of the "danger theory," numerous DAMPs and PAMPs and their corresponding pathogen-recognition receptors have been identified. In this paper, we highlight the role of the DAMPs high-mobility group box protein 1 (HMGB1), interleukin-1α (IL-1α), and interleukin-33 (IL-33) as unique dual-function mediators as well as mitochondrial danger signals released upon cellular trauma and necrosis.

1. Introduction

Trauma and tissue damage trigger an inflammatory response, which is required for postinjury regeneration and tissue repair. In the case of severe trauma, an overwhelming, systemic inflammatory response can result in additional multiorgan damage to the host cells and the development of multiorgan failure (MOF) [1]. The inflammatory response after severe trauma correlates with the severity of injury and is associated with mortality and the development of complications, such as MOF or sepsis [2, 3]. Inflammation following tissue damage is a dynamic process, which is driven by numerous inflammatory mediators. The innate and the adaptive immune system can be activated by endogenous signals that originate from stressed, injured, or necrotic cells, signifying "danger" to the host [4]. The notion that endogenous and exogenous molecular patterns can cause a similar host response through the same receptors challenged the model of the immune system discriminating between "self" and "nonself", but could be sufficiently explained by the "danger theory." In 1994, the "danger theory" of the inflammatory response following trauma or infection has been introduced and has meanwhile significantly broadened our understanding of the immune response [4–6]. Endogenous danger signals released from necrotic or stressed cells which trigger the inflammatory response after trauma have been termed alarmins or danger-associated molecular patterns (DAMPs) [6, 7]. Initially, it was believed that apoptotic cells are not a source for DAMPs, but it became meanwhile evident that DAMPs can also be released during a specific modality of programmed cell death, referred to as immunogenic apoptosis [8–10]. DAMPs share structural and functional similarities with exogenous, conserved microbial surface structures released from invading microorganisms, so-called pathogen-associated molecular patterns (PAMPs), which, like DAMPs, are recognized by a set of receptors, termed pathogen-recognition receptors (PRRs) [4, 11–15]. However, this definition of DAMPs is not always used consistently, and sometimes endogenous alarmins and exogenous PAMPs are collectively classified as danger-associated molecular patterns (DAMPs) [6].

Well-known alarmins include but are not limited to heat shock proteins (Hsp), hyaluronan, uric acid (UA, monosodium urate), galectins, thioredoxin, adenosine, high-mobility group box protein 1 (HMGB1), interleukin-1α (IL-1α), and interleukin-33 (IL-33) [6]. As unique features,

HMGB1, IL-1α, and IL-33 exert dual functions as intracellular transcription factors and as extracellular inflammatory mediators.

In this paper, we focus on the role of the dual function DAMPs in the initiation of the immune response after trauma. Moreover, we shed light on recently discovered mechanisms of activation of innate immunity by mitochondrial DAMPs released from disrupted cells which bear bacterial molecular motifs similar to PAMPs due to their endosymbiotic origin.

2. High-Mobility Group Box Protein 1

HMGB1 was originally described as a DNA-binding nuclear protein that acts as a transcription factor [16]. A decade ago, HMGB1 has been rediscovered as a proinflammatory cytokine in sepsis and endotoxemia which, under these conditions, is released downstream of the early cytokine production [17]. Meanwhile, HMGB1 has emerged as a prototypical DAMP and has been shown to play an important role in the response to sterile injury, such as hemorrhagic shock and ischemia/reperfusion injury [18]. As a ubiquitous protein, HMGB1 is virtually expressed by all cell types with a nucleus.

HMGB1 can be released by active secretion predominantly from macrophages and monocytic cells but also from other cell types that are exposed to proinflammatory cytokines or bacterial products [19]. Besides PAMPs and cytokines, active HMGB1 secretion from monocytes can be triggered by the complement activation product C5a [20]. This mechanism not only seems to play a role in sepsis but also in sterile injury since in patients with severe trauma levels of HMGB1 correlate with the extent of complement activation [21]. Interestingly, active HMGB1 secretion is controlled by the autonomic nervous system as activation of the cholinergic anti-inflammatory pathway suppresses HMGB1 secretion from macrophages [22]. In this context, it has been postulated that the spleen is an abundant source for HMGB1 and that mediator secretion by splenic macrophages in the red pulp is under influence of the vagus nerve [23, 24].

In its role as an endogenous danger signal, passive release of HMGB1 from necrotic or disrupted cells stimulates innate immunity, while it was initially believed that HMGB1 is not released from apoptotic cells [16]. However, recent research revealed that after initial nuclear retention in apoptotic cells HMGB1 may be released during late apoptosis (secondary necrosis) due to increased cellular permeability and nucleosomal degradation [25].

When actively released by macrophages, HMGB1 undergoes posttranslational modifications, including acetylation, phosphorylation, and methylation [25–27]. Moreover, posttranslational redox modifications of certain cystein residues regulate the activity of (actively and passively released) HMGB1, including receptor interaction and subsequent signaling events [25, 28]. The redox sensitivity of HMGB1 has particular ramifications for systemic inflammation and sepsis with increased oxidative stress and release of reactive oxygen species (ROS). Oxidation of HMGB1 not only occurs in necrotic cells but also during apoptosis, which is associated with generation of ROS by mitochondria [29]. On the other hand, HMGB1 can promote the production of ROS in neutrophils [30]. It has been postulated that oxidation might temporarily inactivate HMGB1, and in turn, the activity of HMGB1 is prolonged and maintained in a reduced environment [29, 31].

With respect to its functional roles in inflammation, HMGB1 exerts pleiotropic proinflammatory effects on various organ systems. These effects include activation of phagocytic and endothelial cells and the loss of epithelial barrier functions, resulting in typical signs of inflammation and other symptoms, collectively referred to as "sickness syndrome" [32, 33]. In addition, HMGB1 promotes processes required for host defense, tissue repair, and regeneration, including chemotaxis, angiogenesis, maturation of dendritic cells, and recruitment and proliferation of stem cells [34]. Other reports suggest that HMGB1 amplifies the inflammatory response by binding endogenous and exogenous inflammatory mediators, such as cytokines or endotoxins [35, 36]. In fact, the intrinsic capacity of HMGB1 to trigger immune responses has been questioned lately since recombinant HMGB1 failed to induce cytokine production *in vitro*. A possible explanation for these conflicting results might be the redox state of cysteine residues since commercially available preparations of HMGB1 may contain reducing agents [25]. In the case of formation of HMGB1-containing immunostimulatory complexes, it has been suggested that inflammation is primarily promoted through the receptor of the partner molecule in independence of the redox state of HMGB1 [25, 37]. However, to date only limited information is available about the mechanisms, kinetics, and conformational changes involved in HMGB1-complex formation.

The activities of HMGB1 are mediated through interaction with pathogen-recognition receptors, which also recognize products from invading microorganisms. HMGB1 has been shown to be a ligand of various toll-like receptors and to signal through TLR2 and TLR4, the latter being required for HMGB1-mediated activation of macrophages and the development of secondary damage in ischemia/reperfusion injury [18, 38–40]. In addition to TLRs, HMGB1 interacts with the receptor for advanced glycation endproducts (RAGE) on a variety of cell types which is involved in the initiation of a rapid and sustained inflammatory response as well as in mediating the chemotactic and mitogenic activities of HMGB1 [6, 41, 42].

However, RAGE not only senses and transmits danger upon HMGB1 release but also contributes to inactivation or neutralization of HMGB1 in form of soluble RAGE, which is released simultaneously to HMGB1 in severe trauma [43, 44]. But it is also conceivable that soluble RAGE functions as a carrier to convey HMGB1 activities to remote tissues/cells, as it has been suggested for various cytokines, although this putative mechanism of action currently lacks direct evidence. Binding of HMGB1 to thrombomodulin on endothelial cells represents another mechanism by which HMGB1-triggered inflammation can be attenuated [45]. On the other hand, interference of HMGB1 with the thrombin-thrombomodulin complex may inhibit the anti-inflammatory protein-C pathway. Furthermore, HMGB1 can drive coagulation towards a procoagulatory state by

stimulating tissue factor expression and inhibiting tissue plasminogen activator on endothelial cells, putatively paving the way for the development of manifest coagulopathy and disseminated intravascular coagulation [46].

The knowledge about HMGB1 as an inflammatory mediator gained during the last decade after its rediscovery is mainly based on results from experimental studies, with models of sepsis and endotoxemia in particular. In the setting of experimental sepsis, HMGB1 has been defined as a late mediator, as compared to other cytokines, such as IL-6 and TNF-α [17]. Antibody-induced neutralization or use of recombinant A box domain of HMGB1 (antagonist of B box proinflammatory activity) in sepsis could improve the outcome, even when applied after the onset of disease [17, 47, 48]. Other preclinical models, in which blockade of HMGB1 led to promising results, include arthritis, ischemic brain injury, liver injury, and organ transplantation [25]. In severe trauma in humans, HMGB1 acts as an early proinflammatory mediator, which is systemically released within 30 to 60 minutes, peaking from 2 to 6 hours after injury [21, 49]. The concentration of HMGB1 correlates with the severity of injury and the systemic inflammatory response. Moreover, patients who develop organ dysfunction and nonsurvivors of severe trauma show higher levels of HMGB1 [21]. In a conflicting report, no correlation between increased HMGB1 levels after trauma and injury severity or parameters of patient outcome was found, which might be due to a rather small sample size in the latter study [49]. However, there are striking differences in the absolute values of HMGB1 concentrations measured in the patient populations of both studies, which may reflect the difficulty in HMGB detection. As a matter of fact, current standard detection assays do not distinguish between different forms of HMGB1 as a result of posttranslational changes, redox reactions, or complex formation [25]. In contrast to sterile injury, the peak of HMGB1 release during sepsis occurs during later stages of the disease, and the levels of HMGB1 do not always decrease in patients who have recovered from sepsis [17, 50]. Although neutralization of HMGB1 has been protective against tissue injury in numerous preclinical models of inflammatory diseases, the complexity of its mechanisms of action currently precludes the clinical use of HMGB1-neutralizing agents, and clinical studies targeting HMGB1 have not been performed at present.

Owing to its pleiotropic proinflammatory activities, HMGB1 still represents a promising therapeutic target in various inflammatory conditions. However, targeting HMGB1 for protection in sterile injury and infection-associated inflammation in the clinical setting requires thorough understanding of the underlying molecular mechanisms.

3. Interleukin-1α

The interleukin 1 family (IL-1F) currently consists of 11 known members [51]. Their effects in inflammation are complex as they have anti-inflammatory as well as proinflammatory properties. In general, IL-1 activates lymphocytes, enhances the defensive activity of monocytes and macrophages by inducing the production of inflammatory mediators, and acts as costimulant on natural killer cells [52]. IL-1 is divided into 2 subtypes, IL-1α and IL-1β. IL-1β is the most thoroughly investigated member of the IL-1F due to its role in autoimmune diseases [53]. Interleukin-1 receptor antagonist (IL-1Ra) is a specific inhibitor of IL-1α and IL-1β on their membrane-bound receptor IL-1R, and generic IL-1Ra is routinely used in the treatment of patients with rheumatoid arthritis [54].

IL-1α belongs to the group of dual function alarmins (formerly also known as endokines), describing the ability to induce an inflammatory response upon release by necrotic cells besides intracellular functions in intact cells [7]. In contrast to IL-1β, IL-1α is constitutively expressed in epithelial cells, keratinocytes, and fibroblasts. Its precursor molecule (pIL-1α) is also biologically active.

Cells constitutively expressing IL-1α rarely secret it in an active manner. From these cells, IL-1α is only released after loss of cellular integrity. In contrast, monocytes and macrophages do not constitutively express IL-1α but are capable of de novo synthesizing IL-1α. Upon activation of monocytic cells, membrane calpain is activated to cleave pIL-1α, followed by secretion of IL-1α. In contrast to cellular necrosis, IL-1α remains attached to chromatin during apoptosis, which reduces its intracellular mobility and possibly limits its passive release [55]. Thus, IL-1α is predominantly released by necrotic cell disintegration, but stays intracellular under physiological conditions, during apoptosis, and even in the presence of inflammatory diseases [56–59]. As mentioned above, the precursor can be cleaved by membrane-bound calpain, a calcium-activated cysteine protease, which is not found in all cell types expressing pIL-1α [60]. Both the uncleaved (pIL-1α) and the mature form of IL-1α can bind to the IL-1R, but membrane-bound IL-1α can also exert juxtacrine functions in a receptor-independent manner [61]. Like IL-33 (see below) but unlike other members of the IL-1F, IL-1α not only acts on intra- or extracellular membrane receptors but also as a nuclear transcription factor [62]. While pIL-1α contains the sequence for the nuclear localization site, the mature form (IL-1α) has no ability to function as a transcription factor [63]. In cultured resting cells, pIL-1α is distributed evenly in the cytoplasm. After exposition to inflammatory stimuli, such as lipopolysaccharides (LPS) or TNFα, pIL-1α locates to the nucleus without further processing and acts as a transcription factor. This leads to the IL-1R-independent production of NF-κB and proinflammatory cytokines, such as IL-6 and IL-8 [63]. However, the biological role of constitutively expressed IL-1α is not entirely clear. In unstimulated cells expressing IL-1α, large amounts of intracellular IL-1Ra are present at the same time, competing for binding sites [64]. IL-1α has a costimulatory effect on T-cell function and is expressed consistently by thymic epithelial cells, but mice deficient of IL-1α show normal antibody production and proliferation [51, 65]. The expression of interferon-γ largely depends on IL-1α and antibodies to IL-1α but not IL-1β block its activity [66]. Antibodies to IL-1α inhibited the immune response in sterile inflammation in mice, corroborating its role as an alarmin [67]. Interestingly, mesothelial cells have been proposed to play a key role in sensing cell death. Exposed to recombinant IL-1α, they

produce CXCL-1, a cytokine with neutrophil attractant properties [68, 69]. The intraperitoneal injection of lysed cells *in vivo* leads to neutrophil infiltration that is markedly reduced in CXCR2- (receptor to CXCL-1-) deficient mice. The CXCL-1 production following exposure to cytosolic extracts of necrotic tissues can be abolished by IL-1Ra or in IL-1α-deficient in mice. Conversely, injection of lysed CXCR2$^{-/-}$ cells results in reduced neutrophil recruitment as compared to cell lysates from wild-type mice [69]. However, mesothelial bone marrow-derived cells cannot only sense IL-1α but also secrete it upon exposure to lysed cells *in vitro* [70].

Although IL-1α is known for a fairly long time, the information about its distinct roles in the *in vivo* setting in SIRS is limited since most clinical studies focused on the role of IL-1β rather than the other subtype IL-1α. Moreover, only few studies have investigated the isolated effects of either pIL-α or mature IL-α [63]. With respect to trauma, there is only a single report available investigating the release of IL-1α in patients with systemic inflammation after accidental injury. However, in this study IL-1α was not detectable in any patient at any time point investigated over a 5-day period [71]. While this does not necessarily mean that IL-1α is not systemically released during trauma, the findings might be based on limitations of the detection assays used two decades ago. Other possible explanations include unknown internal clearance mechanisms of systemically released IL-1α, complex formation with endogenous antagonists or other inflammatory mediators, or tissue sequestration. In humans, injection of IL-1 leads to symptoms of systemic inflammation, including fever, myalgia, arthralgia, and a lowered pain threshold. Although preclinical studies treating SIRS and sepsis in mice with IL-1Ra revealed promising results, it failed to significantly reduce the overall mortality of sepsis in humans in large double-blind, placebo-controlled clinical trials [72]. To our knowledge, no clinical studies have been conducted yet to test the effect of specific blockade of IL-1α in systemic inflammation after trauma in humans.

In summary, IL-1α fulfills the definition criteria for a dual function alarmin since it not only functions as a powerful inductor of systemic inflammation when released into the extracellular space but also acts as a nuclear transcription factor. However, although IL-1α represents the first dual function protein described, its particular role in sterile inflammation and trauma is less thoroughly investigated than HMGB1, its archetypical partner in crime.

4. Interleukin-33

Due to similarities to IL-1α and HMGB1 with respect to constitutive, nuclear tissue expression and passive release after loss of cellular integrity, it has been suggested that IL-33 represents the third dual function protein of the alarmin family [73]. IL-33 was previously known as nuclear factor from high endothelial venules (NF-HEV) and was identified by computational data base analysis as the ligand for ST2 (also known as IL-1RL1), which, until then, was designated as an orphan receptor [74]. IL-33, the latest member of the IL-1 cytokine family, is mainly expressed in structural and lining cells, including endothelial cells, fibroblastic reticular cells of lymphoid tissues, and epithelial cells of tissues exposed to the environment [73]. In the absence of inflammatory stimuli, IL-33 localizes to the nucleus, which is mediated by the amino terminus of full-length IL-33 [75].

IL-33 was originally considered to be actively released to the extracellular space after proteolytic cleavage of its precursor pIL-33 [74]. The active release of IL-33 from macrophages can be triggered by PAMPs, such as LPS, while dendritic cells or mast cells have not been found to be source of active IL-33 secretion [76]. In contrast to other members of the IL-1 family, active IL-33 secretion is independent of caspase-1 and caspase-8 (required for cleavage of pIL-1β and/or pIL-18) or calpain (required for cleavage of pIL-1α) [76]. Although recombinant pIL-33 is cleaved by recombinant caspase-1 *in vitro* [74], the *in vivo* role of caspase-1 in the cleavage of pIL-33 (full-length IL-33) remains controversial [76].

With respect to its role as a DAMP, it has been demonstrated that nuclear, full-length IL-33 is biologically active and can be released following cellular damage [77–79]. It has been suggested that different biologically active forms of IL-33 exist [80, 81]. However, to date the distinct roles of various forms of IL-33 as well as the corresponding mechanisms of release are enigmatic, as are potential posttranslational modifications or environment-dependent functional/conformational alterations of IL-33. Although our knowledge about IL-33 secretion is limited due to its recent discovery, passive release of IL-33 from necrotic tissues seems to be the major pathway since pIL-33 does not exhibit typical peptide sequences for active secretion, and full-length IL-33 is thought to be the biologically most active form [78]. In line with this, inactivation of IL-33 through proteolytic cleavage during apoptosis may limit the release of biologically active full-length IL-33.

With respect to the mechanisms of release and activation/inactivation of IL-33, various studies reported conflicting results. Initially, it was believed that IL-33 is activated through caspase-1-dependent cleavage of pIL-33 into an active form [74]. However, more recently, it has been demonstrated that the functional activity of IL-33 is independent of caspase-1-cleavage and that IL-33 may even be inactivated by caspase-1 [77, 80]. According to another study, cleavage of IL-33 into less active forms is presumably mediated by the proapoptotic caspases-3 and -7, while caspase-1 cleavage only seems to play a minor role under physiological conditions [78]. Moreover, an alternative splice variant of IL-33 has recently been identified, the functional role of which has yet to be defined [81].

As a dual function protein, IL-33 is active as a nuclear transcription factor and as a cytokine. But unlike IL-1α and HMGB1, IL-33 exerts repressive transcriptional activity and features some anti-inflammatory properties [82]. IL-33 can activate cells of the innate as well as the adaptive immune system via interaction with membrane ST2, which, in particular, is abundantly expressed on the surface of T helper 2 (Th2) cells and mast cells [83]. Through interaction with membrane-bound ST2, IL-33 promotes Th2-type immune responses, with enhanced production of the anti-inflammatory cytokines IL-5 and IL-13, drives the

differentiation of naïve T cells towards a Th2 phenotype, and functions as chemotactic factor in Th2 cell mobilization [84, 85]. On mast cells, IL-33 triggers the production and release of proinflammatory cytokines and chemokines, such as IL-1β, IL-6, or TNFα, promotes maturation, and induces degranulation [79, 86, 87]. Therefore, IL-33 has been attributed to mediate anaphylactic shock [88]. Furthermore, IL-33 amplifies the polarization of alternatively activated macrophages, upregulates TLR4, and enhances TLR4-mediated cytokine production by macrophages [79, 89, 90]. The receptor for IL-33, ST2, exists in different splice variants, resulting in a localized form bound to the cellular membrane and a soluble form [91]. The soluble variant, termed sST2, is generated by alternative splicing and is not thought to induce signaling, therefore acting as a decoy receptor for IL-33 [79]. Similar to IL-33, sST2 has been linked to the pathogenesis of various inflammatory conditions, including sepsis, asthma, autoimmune diseases, and cardiovascular diseases [92–95]. In general, sST2 is considered as a marker of poor prognosis.

Since IL-33 represents the most recent member of dual-function DAMPs, to date there are no specific clinical data available on the role and kinetics in patients with severe trauma. However, there is indirect evidence of its involvement in trauma since it has been demonstrated that patients with SIRS after major trauma or sepsis exhibit elevated levels of the soluble receptor sST2, possibly associated with a poor outcome [10]. In contrast to sterile injury and trauma, the role of IL-33 in sepsis is better defined. In experimental sepsis, IL-33 has beneficial effects by enhancing the accumulation of neutrophils at the site of infection and reducing systemic but not local proinflammatory responses, resulting in an improved outcome [96]. However, it remains to be determined in future studies if administration of IL-33 in fact represents a therapeutic strategy in the clinical treatment of patients with sepsis or SIRS.

Taken together, the novel cytokine and alarmin IL-33 functions as an important activator of the innate and the adaptive immune system. However, its particular roles remain enigmatic to date since, depending on the environment, IL-33 can either play a beneficial role and lead to the resolution of inflammatory processes, or, on the other hand, IL-33 can contribute to aggravation of inflammation.

5. Mitochondrial DAMPs

Bacterial infection and major trauma both can elicit responses that are summarized as systemic inflammatory response syndrome (SIRS) or sepsis, respectively [97–99]. The clinically similar phenotype led to the hypothesis that the molecular pathways may have resemblances as well. According to the "danger theory," traumatic cell destruction causes release of substances that are usually hidden intracellularly, but signify "danger" to the host once they appear in the extracellular environment [5]. Searching for endogenous agents that provoke activation of the immune response, mitochondria could be recently identified as potent effectors. Based on striking similarities between bacteria and mitochondria, the endosymbiotic theory was established already more than a century ago, according to which bacteria with the ability to conduct respiration were incorporated by eukaryotic cells by endocytosis [100]. However, a direct link between the development of SIRS and the release of mitochondrial constituents following cellular damage in trauma could not be established until recently when it has been shown that mitochondrial DAMPs (MTDs) are markedly elevated in severely injured patients [101]. These MTDs mainly comprise circular DNA strands containing CpG DNA repeats and N-formylated peptides [102].

Mitochondrial DNA (mtDNA) is released by shock and can directly activate neutrophils after binding to TLR9 [103, 104]. Moreover, proinflammatory cytokine secretion by monocytes/macrophages was found to be augmented after exposure to mtDNA [105]. Mitochondrial DNA released after trauma can directly activate neutrophils after binding to TLR9 and activation of the intracellular p38 MAPK signaling pathway [103, 104]. In accord, systemic administration of mtDNA in mice resulted in systemic inflammation and the development of lung injury [101]. Therefore, it has been hypothesized that the release of soluble mitochondrial degradation products may be the missing link between tissue injury and sterile SIRS [106]. As an example for the relevance in the clinical setting, it has been shown that femoral reaming during fracture fixation causes release of MTDs into the wound and circulation, which may be associated with the development of acute lung injury [107].

N-formyl peptides synthesized by mitochondria are strong chemoattractants as they closely resemble those derived from bacteria [108]. They bind to formyl-peptide receptors (FPRs) and its functional variant FPR-like 1 receptor (FPRL1) [109]. It has been demonstrated that isolated mitochondrial peptide fragments bind to FPRL1 and trigger proinflammatory responses through chemotactic effects [110]. Especially phagocytic cells, which are specialized in defending the host against invading microorganisms, express FPR and FPRL1 on their surface [111]. Signal transduction through G-proteins following engagement of FPR and FPRL1 results in chemotaxis, Ca^{2+} mobilization, activation of MAP-kinase signaling pathways, cytokine production and release, desensitization of other chemoattractant receptors, and respiratory burst [112–115]. Interestingly, isolated N-formyl peptides do not trigger an inflammatory response in monocytes unless coupled with mitochondrial transcription factor A (TFAM), a homologue to the nuclear HMGB1 [116]. These effects of mitochondrial N-formyl peptides can be attenuated by receptor antagonists or silencing of FPR [116].

With respect to the role in ATP-generation, mitochondrial function was found to be reduced in sepsis and trauma patients, resulting in ATP-depletion and eventually cellular necrosis [117]. Furthermore, the mitochondrial production of ROS is enhanced during the inflammatory response in trauma and sepsis. It has been suggested that mitochondrial ROS can regulate NF-κB levels in immune cells, thereby inducing an inflammatory response [117].

Moreover, mitochondria have emerged as crucial mediators in the induction of apoptosis during SIRS in trauma and shock [117, 118]. Besides this well-known form of programmed cell death, there is now evidence that necrosis can also occur in an organized manner, called necroptosis. It can

be induced by so-called death receptors, such as TNF-R, during SIRS and involves activity of the receptor-interacting protein kinase pathway (RIPK). Activation of RIPK1 and RIPK3, a signaling complex also referred to as necrosome, is followed by active disintegration of mitochondrial and plasma membranes [119, 120]. In a recent report, RIPK3-deficient mice were protected from lethality in experimental models of sterile SIRS and polymicrobial sepsis [119]. Interestingly, levels of mitochondrial DNA in plasma were lower in RIPK3-deficient mice, suggesting reduced tissue damage in absence of RIPK3 [119].

In summary, besides their known functions in ATP-generation, apoptosis, biosynthesis, and calcium homeostasis, mitochondria play an important role in activating innate immunity since they contain constituents of their bacterial ancestors which are potentially immunogenic [121].

6. Conclusions

The dual-function alarmins HMGB1, IL-1α, and IL-33 represent crucial mediators in the initiation and perpetuation of the inflammatory response following loss of cellular integrity. While HMGB1, IL-1α, and IL-33 share the unique features of acting as transcription factors and extracellular mediators of inflammation, each dual-function protein exerts distinct functions, which we are just beginning to understand. Further, the dual-function mediators substantially differ in their mechanism of action and release. Based on the information available to date, the role of the dual-function mediators in systemic inflammation provides a possible explanation for the enigmatic question of why patients with severe (sterile) injury present with a syndrome that is indistinguishable from sepsis. The discovery of mitochondrial DAMPs, which activate the immune response after cellular disruption by mimicking bacterial infection, has opened up a new avenue for the investigation of danger sensing and transmission. However, future basic science research as well as clinical studies in this fascinating field are necessary to further unravel the complexity of the host response after trauma and tissue damage. In the setting of sterile injury and trauma, the roles of IL-1α and IL-33 and their various forms as a result of posttranslational modifications and corresponding environments need to be defined in detail. In this context, novel preclinical models of trauma may help characterize the role of DAMPs and investigate mechanisms/kinetics of release after tissue injury in single-organ injury and multisystem trauma, followed by rapid transfer of findings to the setting of human disease. Further possible future research directions may include posttranslational modifications of DAMPs and their dynamics after tissue injury, which may be associated with alterations of the functional roles, ranging from activation of inflammation to tissue repair, or even anti-inflammatory activity. In addition, only little is known about mutual interactions of DAMPs prior and after passive or active release and direct crosstalk with other mediators of inflammation and signaling systems. Moreover, mechanisms of intracellular retain of DAMPs during different modalities of cell death appear to be an interesting field for future research. With respect to potential therapeutic strategies, besides agents that neutralize or block DAMPs, the development of compounds that cause intracellular DAMP retention and limit DAMP release upon tissue damage might represent a promising approach. The versatility of DAMPs and associated signaling systems is an impressive example for the plasticity of innate immunity, and with increasing understanding of the underlying mechanisms and interactions, routine clinical application of DAMP-targeting strategies for the treatment of patients with SIRS may be in reach.

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Review Article

Guilty Molecules, Guilty Minds? The Conflicting Roles of the Innate Immune Response to Traumatic Brain Injury

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Traumatic brain injury (TBI) is a complex disease in the most complex organ of the body, whose victims endure lifelong debilitating physical, emotional, and psychosocial consequences. Despite advances in clinical care, there is no effective neuroprotective therapy for TBI, with almost every compound showing promise experimentally having disappointing results in the clinic. The complex and highly interrelated innate immune responses govern both the beneficial and deleterious molecular consequences of TBI and are present as an attractive therapeutic target. This paper discusses the positive, negative, and often conflicting roles of the innate immune response to TBI in both an experimental and clinical settings and highlights recent advances in the search for therapeutic candidates for the treatment of TBI.

1. Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability, particularly in young adults who fall victim to motor vehicle accidents, falls, sporting injuries, and increasingly common assaults. Despite advances in prehospital and clinical care, a vast majority of severe TBI survivors will not be able to live independently or return to work [1]. Aside from the enormous personal burden of TBI, a substantial economic cost exists, estimated at $8.6 billion dollars each year in Australia alone [2] whilst in the United States this cost exceeds $55 billion dollars per year [3].

TBI has been described as the most complex disease in the most complex organ of the body; a sentiment which highlights both the multifactorial nature of brain injury in terms of type and spatial distribution of damage, and the intricacies of the brain's responses to insult. The pathology caused by a TBI can be classified in two broad temporal phases: the primary or initial injury to the head, which cannot be treated or prevented; the secondary injury, which is instigated by the primary injury, results in a complex cascade of pathophysiological and neurochemical events [4–6]. This ongoing secondary injury process is potentially amenable to intervention and, thus, has been the focus of research in the past two decades, with a view to halting or limiting these factors to avoid the progression of initial injury.

Alas, many compounds showing promise in experimental models have shown largely disappointing results in the clinical setting [7, 8], and to date, no effective therapies exist to treat TBI [4]. This failure is likely due to the aforementioned complexities of the brain, and the propensity for use of rodents in preclinical trials of compounds, which overlooks the fundamental differences between human and rodent brains. Another key aspect has been the use of pharmacological agents that target a single factor of the complex interconnected pathways leading to secondary brain damage [9].

The immune system consists of two important components: the "innate" system, which is responsible for immediate, nonspecific action against pathogens or insults, and the "adaptive" system, a response tailored to the specific

threat or insult at hand [10]. It is increasingly clear that far from being distinct, these systems are highly interrelated, with the innate system shaping and modifying the responses of the adaptive system [11]. Recently, the role of the innate immune system has been under the spotlight, as these early inflammatory responses implicitly designed to minimise the deleterious outcomes of injury have a somewhat paradoxical role in that they are increasingly implicated in the mediation of secondary pathogenic cascades.

The central nervous system (CNS) was traditionally thought to be a site of immune privilege due to the impermeable shield of the blood brain barrier (BBB). However, over the past two decades, it has been well established that under injury and inflammatory conditions, immune cells are able to cross the BBB and enter the brain parenchyma. The brain is also equipped with its own resident immune cells, the microglia, which undergo marked recruitment, proliferation, and activation in response to virtually any neuropathological insult [12].

This paper aims to provide an insight into the innate immune responses elicited by TBI, and the beneficial or detrimental roles these pivotal responses may exert in the pathogenesis of brain injury. We will also discuss therapies and strategies currently under investigation to minimise the inflammatory response to TBI or modulate it to a more beneficial phenotype.

2. Pathophysiological Responses to Traumatic Brain Injury

Initial or primary brain injury results in mechanical damage to the brain as a result of motor vehicle accidents, falls, sporting injuries, and violence [13]. The complex pathology caused by the primary TBI is further complicated by the intrinsic nature of the damage involved: focal or diffuse [5, 14]. Patients with focal injuries often present with skull fractures and subdural, epidural, or intraparenchymal haematomas [15], with the damage that occurs being largely dependent on the site of impact to the head. In contrast, diffuse brain injury is characterised by widespread damage to the white matter as well as the vasculature caused by acceleration/deceleration forces to the head [16]. Diffuse injury leads to axonal perturbation and impaired axonal transport, with gradual axonal disconnection from the soma [17]. Whilst patients with focal injuries are readily diagnosed using conventional CT scans, diffuse injuries often show no overt pathology and thus can potentially be missed during early imaging-assisted diagnosis [1, 7]. In addition, focal and diffuse injuries often coexist, particularly in motor vehicle accidents, falls, and assaults [5].

Both focal and diffuse TBIs can cooccur with insults such as hypoxia, hypotension and ischemia, or cerebral hypoperfusion [18–20]. These insults are commonly reported, occurring in approximately one-third of severe TBI patients [21] and are known to exacerbate pathology, with prolonged cognitive deficits and poorer long-term outcome when compared to patients experiencing an isolated TBI [22–25]. Animal studies have further elucidated this observation, with posttraumatic insults such as hypoxia and hypotension found to worsen behavioural outcomes and heighten pathology in models of both focal and diffuse injury [26–33].

At the time of the primary TBI, mechanical damage to the brain results in the activation of a multitude of pathways, including (but not limited to) excitotoxicity and oxidative stress, influx of Ca^{2+} and Na^{+}, and efflux of K^{+} [34–36]. Subsequently, disruption of cell membranes, mitochondrial disturbance leading to energy failure, and a lack of ATP availability hamper reparative mechanisms the brain may attempt [37, 38]. High intracellular Ca^{2+} levels also trigger the activation of Ca^{2+}-dependent proteases including calpains, caspases, and phospholipases, resulting in damage to the axonal cytoskeleton [39, 40]. Secondary injury cascades triggered by these primary injurious events include breakdown of the blood brain barrier (BBB) and extravasation of vascular fluid into the parenchyma, ultimately culminating in vasogenic oedema [41–43]. Increased BBB permeability facilitates the infiltration of peripheral immune cells and activation of resident immune cells, which release chemokines and cytokines and thus perpetuate the inflammatory response in the injured brain, with the end result of cellular dysfunction and death [44–46].

3. The Blood Brain Barrier Allows Transient Passage of Immune Cells into the Injured Brain

The brain and the CNS have traditionally been considered to be sites of immunological privilege due to the BBB, however during certain inflammatory states, the BBB allows the transient passage of immune cells from the vasculature [47]. The BBB is composed of tight junctions at three sites: endothelial cells in the cerebral capillaries, the arachnoid barrier, and the blood-CSF barrier formed by the choroid plexus [48, 49] and is further defined by the associated cells—pericytes and astrocytes [49]. Under normal circumstances, the BBB tightly controls the exchange between plasma and the interstitial fluid, however the dysfunction caused by TBI allows for excess permeability, with disruption of tight junctions and transcytosis allowing passive diffusion. BBB disruption is typically transient, with an immediate period of hyperpermeability, in which immune cells and other products in the plasma may freely cross into the parenchyma [41, 50] (Figure 1).

4. Extravasation of Immune Cells into the Traumatically Injured Brain

Though peripheral immune cells may enter the CNS via the dysregulated BBB, the BBB is open for only limited periods of time, and thus cells must also cross the vasculature into the CNS via a process of extravasation. In focal TBI, neutrophils are the first immune cells to enter the injured brain, appearing first on the vascular endothelium within the first 24 hours of injury [51]. The passage of immune cells through the BBB to the parenchyma is mediated by adhesion molecules (Figure 1). These molecules, expressed on both the vascular endothelium and the immune cells themselves, are

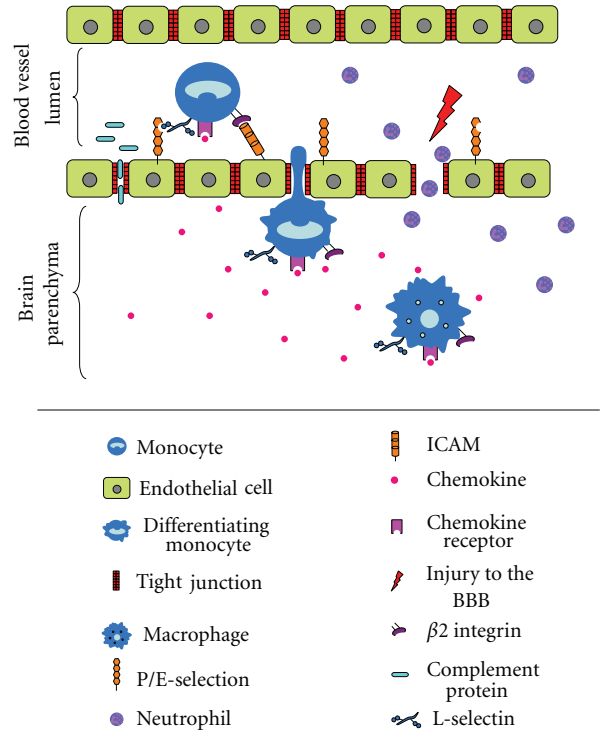

Figure 1: Passage of innate immune components through the blood brain barrier (BBB) after TBI. Injury to the brain results in transient opening of the BBB, in which complement proteins and neutrophils are able to directly enter the parenchyma. Peripheral monocytes enter the brain through a process of extravasation, in which several adhesion molecules are upregulated in turn on both the monocyte and endothelial cell to first tether, then provide passage for the cell through the BBB. First, constitutively expressed L-selectin binds to upregulated P/E-selectin on the endothelial cell surface. Once tethered to the endothelium, monocytes are exposed to chemokines that bind to their cognate receptors on the cell, inducing conformational change and upregulation of $\beta 2$ integrins, which bind to ICAMs expressed on endothelial cells. This final interaction between adhesion molecules signals the cell to migrate across the endothelium into the parenchyma, where it begins to differentiate and take on the morphology of an activated macrophage. Under the influence of chemokines, the cell continues the transition to an activated macrophage state migrates to the site of injury. Figure adapted from [58].

important mediators of brain injury as their expression and binding largely regulates the extent of peripheral immune cell entrance to the injured brain [52]. These adhesion molecules are sequentially upregulated to first tether, tightly adhere, and then provide passage for the cell through the vessel wall, beginning with P- and E-selectin expressed on the endothelium, whilst L-selectin is constitutively expressed on leucocytes [53]. Binding of these molecules tethers the cell to the endothelium, and, once secured, the cell is exposed to chemokines also present on the endothelium, which are highly upregulated in response to injury [54]. The binding of chemokines to their receptors on migratory cells induces conformational change and subsequent activation of the next family of adhesion molecules in the sequence, $\beta 2$ integrins. These proteins, namely, CD11a (LFA-1), CD11b (Mac-1), and CD11c (p50.195) are expressed on the leucocyte cell surface and bind to endothelial cells expressing intercellular adhesion molecules (ICAMs) [52]. ICAMs belong to the immunoglobulin "superfamily" consisting of ICAM-1, ICAM-2, and vascular adhesion molecule (VCAM)-1, as well as ICAM-3, which is expressed on the leucocyte cell surface [53]. It is this binding which gives the final signal for extravasation of the cell through the endothelium into the parenchyma.

In rats, upregulation of E-selectin has been demonstrated on endothelial cells as early as 4 h after weight-drop injury and remained elevated until 48 h [55]. ICAM-1 has also been shown to be increased on the endothelium after weight-drop injury 4 h post-TBI [55]. In diffuse TBI, the number of ICAM-1 positive vessels was also increased by 4-fold compared to sham at 24 h [46]. This expression pattern showed late stage amplification, with an 8-fold maximal value observed at 4 days after injury, and only returning to sham levels 1 week after TBI [46].

Children suffering from TBI have also been found to have increased CSF levels of soluble ICAM-1, which correlated with poor outcome [56]. In our adult TBI study, we have reported that patients with large focal contusions had elevated levels of soluble ICAM-1 in their CSF, whilst interestingly, patients with small or absent lesions after TBI showed no such elevations [57]. These differences likely reflect the inconsistencies seen between distinct forms of TBI and may be indicative of the reported contrasts in inflammatory cell infiltrates in animal studies of focal and

diffuse brain injuries, which will be discussed in more detail in the following sections.

5. Innate Immune Cells in the Pathogenesis of Brain Injury

The innate cellular response to TBI involves both infiltrating and resident immune cells, which share many functions in resolving, and at times prolonging the pathological response to injury [11]. Each cell type involved is briefly discussed below.

5.1. Infiltrating Immune Cells. Neutrophils (often referred to as polymorphonuclear cells or leukocytes) are bone-marrow-derived cells which function to phagocytose cellular debris and bacteria [59]. They produce a number of factors designed to be harmful to bacteria and other pathogens, however these substances also have neurotoxic effects on mammalian cells and their release overtly contributes to tissue damage [47]. These molecules include reactive oxygen/nitrogen species (ROS/RNS), matrix metalloproteinases, and proinflammatory cytokines that perpetuate damage in the CNS [37]. After focal TBI, neutrophils are the first immune cell to cross the BBB and enter to sites of injury, though this response is short-lived, with a peak at 24–48 h after injury and a resolution in neutrophil numbers by 7 days [60–62]. Interestingly, diffuse TBI causes no such infiltration of neutrophils, with only sham-level numbers observed after injury in both immature and adult rats [46, 63].

Monocytes/macrophages are also bone marrow derived and contribute to neuroprotection and recovery after CNS injury by phagocytosing cellular debris and preserving healthy tissue. These cells have an important function in antigen presentation to T cells, and as such are also essential for activation of the adaptive immune response. Acutely after injury, infiltrating macrophages are able to produce growth factors and neurotrophins such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), insulin-like growth factor 1 (IGF1), and anti-inflammatory cytokines such as interleukin (IL)-10 and transforming growth factor-β (TGF β) [64]. However, these cells may also be neurotoxic to the injured brain, mediating glutamate release, generating ROS/RNS, and producing chemokines such as CXCL2, CXCL1, CXCL3, and CXCL8 to induce migration of neutrophils [65], and CCL-2 and RANTES to induce migration of monocytes [66]. Activated monocytes/macrophages are also key producers of proinflammatory cytokines such as tumor necrosis factor (TNF), IL-1β, and IL-6 [67]. As with neutrophils, the recruitment of monocytes is variable between focal and diffuse injury types, with substantially less monocyte recruitment after diffuse injury [51, 68].

5.2. The CNS Resident Innate Immune Cells. Microglia are the dynamic surveillance cells of the immune system, constantly exploring their environment for noxious agents and injurious processes [69, 70]. Microglia play a predominant role in the phagocytosis of cellular debris and respond to extracellular signals by functional transformation from a "resting" to an "activated" phenotype, in which their processes retract, making these cells morphologically and functionally indistinguishable from macrophages [71, 72]. Activated microglia are highly motile and able to rapidly move through the brain to sites of injury [70]. Several neuroinflammatory factors are able to stimulate microglial migration, including the chemokines CCL-2 and fractalkine [73–75] and complement anaphylatoxin C5a [76]. Microglia have long been scrutinized for their role in neuronal damage and particularly in synaptic stripping after TBI [77], however it has now been suggested that, rather than being the perpetrators of neuronal and axonal injury, it is more a case of "guilt by association," since microglia may not be active participants in neuronal damage (for excellent review see [78]). This hypothesis has been corroborated by in vitro experiments of rat neuronal and microglial coculture, in which even when exposed to inflammatory factors, microglia did not cause direct neuronal damage [74].

Astrocytes are the most numerous cell type in the brain and become rapidly activated in response to injury in a process of "reactive astrogliosis," in which cells undergo hypertrophy and proliferation proportional to injury severity [79, 80]. The role of astrocytes after TBI is controversial, as they are known to produce many proinflammatory cytokines including TNF, IL-1, and IL-6 and are also major producers of chemokines [81]. Astrocytes have also been shown to inhibit axonal spouting in lesioned tissue by formation of a dense fibrous glial scar [80, 82]. However, this glial scar also restricts tissue damage by forming a protective barrier, confining injury to a defined space and preventing further spread of damage [79, 83]. After TBI, astrocytes decrease the expression of glutamate transporters, with reduced glutamate uptake thus intensifying the excitotoxic response [84]. Conversely, reactive astrocytes upregulate the expression of matrix metalloproteinase (MMP) after TBI, and in particular release of MMP-3, which has been shown to be released from these cells in the vicinity of neurons undergoing synaptogenesis [85], suggesting that astrocytes may play a role in the clearance of damaged tissue in order to make a more permissive environment for neuronal plasticity.

6. Complement Proteins Are Pivotal in the Pathogenesis of Traumatic Brain Injury

Best known for its role in the recognition and elimination of pathogens, the complement system has recently emerged as a key innate mediator of the inflammatory response after brain injury. Complement is a complex network of soluble and cell-associated factors [48] and can be activated through three different pathways depending on the stimulus: the classical pathway, the alternative pathway, and the lectin pathway [86]. Within the CNS, complement has been shown to be upregulated both clinically in TBI patients and in various models of experimental TBI [87]. Under normal physiological conditions, complement proteins are detected at very low levels in the brain due to the precise compartmentalization of the vasculature and the parenchyma by the BBB [49], and thus peripheral complement proteins

are unlikely to enter the brain without disruption of the BBB. After TBI, the disruption of the BBB allows an influx of serum complement proteins into the injured CNS [48, 87] (Figure 1). However, complement proteins can also be produced endogenously in the brain by astrocytes, microglia, and neurons in response to infection or injury [48].

Whilst the role of complement is intrinsically one of elimination and resolution of infection, the infiltration and/or activation of complement proteins after TBI may lead to inflammatory-induced damage by way of C3b deposition and subsequent opsonisation and phagocytosis, and C5a-induced recruitment and activation of immune cells from the periphery, with neutrophils being the "early responders" [88]. Overt tissue destruction may also occur with the final formation of the membrane attack complex (MAC), the primary role of which is mechanoporation [86]. Clinically, elevated levels of two crucial components of the alternative pathway, C3 and factor B have been demonstrated in the CSF of severe TBI patients, with concomitant BBB dysfunction in more than 50% of patients, suggesting that the elevated levels of C3 and factor B, were due to serum leakage across the dysfunctional BBB rather than *de novo* synthesis [89]. Similarly, C5b-9 (MAC), the cytolytic end product of the complement system has been shown to be increased in the CSF of TBI patients and was accompanied by a loss of integrity of the BBB. Interestingly, several patients in this study experiencing secondary insults such as hypoxemia or hypoperfusion had more pronounced levels of C5b-9 in their CSF [90].

Complement protein synthesis has also been demonstrated in the brain after TBI both experimentally and clinically, with postmortem analysis of human brain tissue revealing the upregulation of C1q, C3b, C3d, and C5b-9 in close association with neurons in patients with focal brain contusions [91]. Experimentally, TBI-induced C3 deposition has been demonstrated by immunohistochemistry after lateral fluid percussion TBI [92].

The deleterious role of C5b-9 after TBI has also been demonstrated experimentally in mice null for the C5b-9 regulator, CD59. CD59 is able to prevent the formation of C5b-9 and thus acts as an essential inhibitor of complement activation and protector from cell death [93]. Consistent with its role, deletion of CD59 led to worsened neurological outcomes and heightened neuronal cell death, demonstrating the key role of the complement pathway is the pathophysiology of TBI [94]. This detrimental property was corroborated in transgenic mice overexpressing the soluble complement inhibitor Crry (complement receptor, related protein y), which had reduced neurological impairment and improved BBB dysfunction following TBI compared to wild type controls [95]. Furthermore, the pathogenesis of complement activation after TBI has been demonstrated by dual inhibition of both the classical and alternative pathways by pretreatment of rats with a soluble complement receptor type 1 (sCR1) prior to experimental weight-drop TBI. This dual pharmacological inhibition resulted in a significant decrease in posttraumatic neutrophil infiltration, suggesting that complement activation is an essential mediator of the early neutrophil inflammatory response after TBI [96]. Similarly, experimental TBI using mice deficient for C3 or the downstream C5, or treatment of wild type mice with the C5a receptor agonist lessened neutrophil extravasation and resulted in smaller lesions [88]. When C3 was injected intracerebrally into C3 deficient mice, the extravasation of neutrophils to the lesion site was amplified, suggesting that that locally produced C3 is important in brain inflammation [88].

7. Chemokines Mediate Posttraumatic Neuroinflammation and Tissue Damage

With the ability to dictate directional migration of neutrophils and leukocytes, chemokines are considered essential mediators of posttraumatic neuroinflammation as they control immune cell trafficking from circulation to extravasation [54, 97]. Two main families of chemokines have been described: CXC and CC. The CXC cytokines, including CXCL2, CXCL1, CXCL3, and CXCL8, are predominantly chemoattractant for neutrophils [65], whilst the CC chemokines CCL-2 (MCP-1) and RANTES attract monocytes and lymphocytes [66]. Additionally, a third class of chemokines has been implicated in the pathogenesis of brain injury, the CX3C subfamily, with the only characterised member being fractalkine (CX_3CL1). Fractalkine has the unique ability to attract both neutrophils and monocytes, as well as T cells [98].

Clinically, CXCL8 has been found to be acutely elevated in the CSF and extracellular fluid of patients with severe TBI and correlated with BBB dysfunction and NGF production [99, 100]. In paediatric TBI, elevation of CXCL8 strongly correlated with mortality [101]. Severe TBI patients also experienced a sustained elevation in levels of CCL-2 for 10 days after injury, though this was highest on days 1 and 2 [97]. Using cerebral microdialysis, several groups have recently demonstrated acutely elevated levels of CCL-3, CCL-4, and RANTES after severe TBI [100, 102]. A prolonged elevation of fractalkine in the CSF has also been observed in patients after TBI, with a strong correlation to BBB dysfunction and corresponding low fractalkine levels in the serum [103].

Evidence suggests that CXCL1, and particularly CXCL2, are the key mediators of neutrophil migration early after focal brain injury, with both CXCL1 and CXCL2 found to be acutely upregulated within 5 h of experimental cortical impact injury in both mice and rats [61, 104], while after lateral fluid percussion injury CXCL2 expression has been shown to peak at 4 h in the injured hemisphere [105]. Using mice null for the CXCL2 receptor (CXCR2) in a cortical impact model, our group demonstrated a significant attenuation in the numbers of neutrophils migrating to the site of injury as early as 12 h after injury, and found that this correlated with reduced amounts of cell death and tissue damage [62].

Ample experimental evidence also exists to demonstrate the presence of monocyte-attracting chemokines acutely after injury, with elevated mRNA for CCL-2, CCL-4 and RANTES all observed after experimental cortical injury [97, 106]. By 4 h, production of CCL-2 and CCL-4 is significantly

upregulated both *in vivo* and *in vitro* [61, 104, 107], with levels of CCL-2 peaking between 8 and 12 h after injury [97, 105]. Elevation of these chemokines after both focal and diffuse TBI is strongly correlated with poor functional outcome [46, 97], with more evidence of this provided using a CCL-2 knockout mouse for cortical injury, in which improved neurological function and reduced lesion volume were attributed to a reduction in macrophage accumulation [97].

This experimental evidence certainly suggests that chemokines play a deleterious role in the pathogenesis of focal brain injury, however their effects in diffuse brain injury are rather different, particularly with respect to CXC (neutrophil-attracting) chemokines. Without the presence of a gross pathological lesion, very low levels of CXCL2 have been observed in diffuse TBI, correlating with absent neutrophil migration into the brain [46]. However, diffuse TBI is associated with abundant accumulation of monocytes/activated microglia in the white matter tracts, colocalising with axonal pathology [28, 108, 109]. This cellular infiltration/activation also correlates with elevated CCL-2 levels acutely after diffuse injury [46]. So, it appears that CC chemokines play a more significant role in diffuse injury, whilst focal injuries involve both CXC and CC chemokines. These distinct molecular profiles very much reflect individual modes of cellular infiltration in these injury subsets.

8. Proinflammatory Cytokines Have Dual Roles in Traumatic Brain Injury

Proinflammatory cytokines are produced by several types of resident CNS cells such as microglia, astrocytes, and neurons in response to pathological challenge. Cytokines are usually preformed peptides that are activated by cleavage, and swiftly released in response to various stimuli. Once released, cytokines upregulate the expression of cell adhesion molecules and signal the secretion of chemokines in the early postinjury period [47], thus stimulating the infiltration of inflammatory cells to the injured regions. The activation of proinflammatory cytokines in human and rodent TBI has been reported since the early 1990s [99, 101, 102, 110–115]. Their role within the injured brain is, however, one of duality, in that they inherently promote repair, but often bring about additional tissue degeneration by activating a number of cytotoxic pathways leading to cell death [67]. It appears that both the timing of proinflammatory cytokine release and their concentrations are critical to ongoing secondary damage after TBI. The cytokines interleukin IL-1β, TNF, IL-6 and granulocyte-colony macrophage stimulating factor (GM-CSF) have been intensely investigated in a multitude of human and experimental paradigms to elucidate their role within the injured brain (see Table 1). Each of these cytokines is discussed in more detail below.

9. IL-1

IL-1 is known to induce many signaling pathways stimulating the production of other proinflammatory cytokines and thus is thought to be a key player in initiating the "cytokine cycle" [136]. IL-1 exists in both membrane-bound (IL-1α) and -secreted (IL-1β) forms, however it is IL-1β that has earned a reputation as the perpetrator of the acute inflammatory response to TBI. An important distinction is to be made, however, between IL-1β and other cytokines, in that IL-1β itself is not directly toxic when produced; rather it is the propensity to incite other cytokines that lends to its cytotoxic reputation. In noninjured tissue, IL-1β administration alone has been demonstrated to have no ill effects [137], however after TBI IL-1β mRNA is upregulated within minutes, and increased protein levels are detectable within an hour [110, 138–141]. Clinically, acutely elevated levels of IL-1β have been detected after injury by microdialysis [100, 102, 117], in patient CSF [116, 128], and directly in perioperative and postmortem brain tissue after TBI at both protein and mRNA levels [131, 142]. IL-1β levels have also been demonstrated to decrease rapidly; in rat models of focal cortical impact and lateral fluid percussion, IL-1β peaks at 6 h post-injury and returns to baseline by 72 h [143, 144]. This early and transient rise in IL-1β was also consistent with our recent findings in diffuse TBI, with a peak in IL-1β levels at 2 h in the cortex of rats subjected to diffuse TBI [27]. When combined with posttraumatic hypoxia, production of IL-1β was prolonged to 24 h, suggesting that this combinatory insult significantly amplified and sustained this early inflammatory response.

Evidence for the detrimental role of IL-1β is found in experiments in which its expression is modified, with neutralisation of IL-1β in a model of focal TBI in mice resulting in reduced tissue loss and improved visuospatial learning [145]. Furthermore, mice null for the IL-1 receptor (IL-1R1) had decreased VCAM-1 mRNA and a subsequently reduced extravasation of peripheral macrophages after stab wound injury. An overall reduction of inflammation resulted in fewer activated microglia and delayed and depressed expression of cerebral IL-1 and IL-6 [146]. Similarly, blockage of IL-1β signaling by use of an IL-1 receptor agonist (IL-1ra) has also been shown to delay the production of other proinflammatory cytokines, reduce cell death, and improve neurological recovery after experimental focal TBI and ischaemia [147, 148]. Clinically, endogenous IL-1ra microdialysate levels in have also been correlated with improved outcomes in TBI patients [117]. This largely negative role of IL-1β after injury has also been corroborated by peripheral administration of IL-1β after TBI, leading to larger lesions and impaired behavioural outcomes in rats subjected to fluid percussion injury [118].

10. TNF

Along with IL-1β, TNF has long been thought of as a cytokine of detriment following injury and still remains a subject of controversy, particularly as both cytokines have many signaling cascades in common and share the same physiologic effects, with the neurotoxic effects of IL-1β synergistically enhanced in the presence of TNF [149]. TNF is produced by microglia and astrocytes and its expression is regulated in an autocrine manner [150]. In TBI patients,

Table 1: Key studies highlighting the dual roles of proinflammatory cytokines after traumatic brain injury.

(a)

IL-1β			
Finding	Clinical/experimental	Experimental setting	Reference
Acutely upregulated after TBI	Clinical	Cerebral microdialysis; adult and pediatric patient CSF	[100, 102, 116, 117]
Peripheral administration after TBI results in larger lesions and impaired behavioural outcomes	Experimental (rat)	Fluid percussion injury	[118]
Expression exacerbated and prolonged by secondary insult	Experimental (rat)	Diffuse axonal injury with posttraumatic hypoxia	[27]
Causes BBB dysfunction *in vivo*	Experimental (rat; *in vitro*)	Cerebral endothelial cells	[119]

(b)

TNF			
Finding	Clinical/experimental	Study methodology	Reference
High levels observed acutely after injury	Clinical	Cerebral microdialysis, adult patient CSF	N [102, 120, 121]
Acutely upregulated in rats after focal TBI	Experimental (rat)	Controlled cortical injury; lateral fluid percussion	[115, 122]
Administration causes BBB dysfunction and increased recruitment of peripheral leukocytes	Experimental (rat, newborn piglet, rat; *in vitro*)	Healthy animals/cerebral endothelial cells	N [119, 123, 124]
Inhibition of TNF ameliorates BBB dysfunction	Experimental (rat)	Controlled cortical injury	[125]
Deficiency of TNF/TNF-R causes exacerbated BBB damage and impairs long-term recovery	Experimental (mouse)	Controlled cortical injury	N [126, 127]
Expression exacerbated and prolonged by secondary insult	Experimental (rat)	Diffuse axonal injury with posttraumatic hypoxia	[27]

(c)

IL-6			
Finding	Clinical/experimental	Study methodology	Reference
CSF levels correlate with improved outcome	Clinical	Adult and pediatric patient CSF	[128, 129]
Production within 24 h localised to neurons	Experimental (rat)	Diffuse axonal injury	[130]
IL-6 deficient mice have heightened neurodegeneration, increased oxidative stress, poor behavioural recovery	Experimental (mouse)	Controlled cortical injury; aseptic cerebral injury	[158–160]

(d)

GM-CSF			
Finding	Clinical/experimental	Study methodology	Reference
Significantly upregulated in brain tissue within minutes of TBI	Clinical	Postmortem brain tissue	[131]

(d) Continued.

GM-CSF			
Finding	Clinical/experimental	Study methodology	Reference
Promotes neuronal stem cell differentiation *in vitro*	Experimental (rat; *in vitro*)	Neural stem cell culture	[132]
Promotes tissue sparing when administered in conjunction with IL-3	Experimental (rat)	Stab-wound injury	[133]
Minimises tissue damage and promotes behavioural recovery	Experimental (rat)	Spinal cord contusion	[134, 135]

high levels of TNF in the CSF have been observed acutely after injury [102, 120, 121], although the concentrations of TNF have been detected at considerably lower levels compared to other cytokines such as IL-6, TGF-β, and IL-8. TNF is also upregulated acutely in various experimental rat models of focal injury [115, 151] and has been fingered as a key mediator of the inflammatory response, with exogenous TNF administration in healthy brains causing breakdown down of the BBB and increasing recruitment of peripheral leukocytes [119, 123, 124]. Consistent with the hypothesised early detrimental role of TNF in the setting of TBI, its inhibition resulted in ameliorated BBB dysfunction [125] and decreased neuronal damage [152]. Whilst most of the evidence to date has documented the deleterious role of TNF in brain injury, this is increasingly becoming an issue of contention, particularly with longer-term studies of TNF-deficient mice, which showed a robust improvement in neurological function initially after TBI, but which then failed to progress in the long term compared to wild type mice [126]. In addition, TNF-deficient mice have also been shown to have exacerbated tissue and BBB damage after TBI [127]. These findings suggest a key detrimental role for TNF in the acute phase, but demonstrate that it may also have a crucial reparative role essential for long-term recovery. The intrigue of TNF action is not only of its temporal benefit or detriment, but also in its differential expression in focal and diffuse brain injuries and species-specific expression. Interestingly, the majority of studies examining TNF expression have used rat focal TBI models, and we and other groups have not observed any changes in TNF levels in rats subjected to diffuse TBI [27, 140], despite the fact that, like focal injuries, diffuse TBI evokes a substantial microglial and astrocytic response. However, when rats were subjected to diffuse TBI with posttraumatic hypoxia, our group showed a significant increase in TNF levels at 2 h, which was maintained until 72 h after injury [27]. In contrast to rat models of focal TBI, in the mouse closed head injury model we have not observed significant upregulation of TNF at any time examined [61, 97], and it is becoming increasingly apparent that there may be a species-specific production of TNF in CNS pathologies, in that rats produce more and mice less when subjected to similar levels of brain damage [153].

11. IL-6

IL-6 is a true pleiotropic cytokine, with roles in both pro- and anti-inflammation, and deleterious and beneficial effects after TBI [154–156]. However, it is known most often for its role as an immune stimulator, able to regulate chemokine production, cell adhesion molecule expression, and enhance leukocyte recruitment [157]. Clinical studies have indicated that IL-6 is, for the most part, neuroprotective, with maximal expression observed two days after injury [102, 112, 116] and CSF levels correlating with improved outcome in both children and adults [128, 129]. Previously, we have demonstrated an increase in IL-6 in the CSF over the first 24 h after mild experimental diffuse TBI, with production of both IL-6 mRNA and protein localised to neurons [130]. The most telling evidence of the beneficial role for IL-6 has come from studies of IL-6 gene deficient mice, which have been shown to have poor behavioural recovery, as well as increased oxidative stress, a more compromised immune response, and heightened neurodegeneration [158–160].

12. GM-CSF

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hematopoietic cytokine produced by monocytes, macrophages, and endothelial cells [161], with its receptor expressed on most cell types in the CNS [162]. GM-CSF has been shown to have a positive role in promoting neuronal differentiation of adult stem cells *in vitro* [132], though as one of the least-examined cytokines after TBI, the role of GM-CSF is still largely to be elucidated. However, GM-CSF concentrations have been found to be significantly upregulated in human postmortem brain tissue within minutes of injury [131], indicating that GM-CSF plays an important role in the acute inflammatory response. This role appears to be one of neuroprotection, with a recent study employing stab-wound injury in rats observing that tissue loss was reduced by 40% when rats were administered a combination of exogenous GM-CSF and IL-3 [133]. Similarly, in models of rat spinal cord injury, rats treated with GM-CSF had reduced numbers of apoptotic cells and significantly improved neurological function [134] as well as reduced glial scar formation, preserved axonal cytoskeleton integrity, and higher numbers of regenerating axons [135]. In addition, rats exposed to focal cerebral ischemia had smaller infarct volumes and altered expression of apoptosis-related genes, with significantly increased levels of the antiapoptotic Bcl-2 and decreased levels of the pro-apoptotic genes Bax and p53 after treatment with GM-CSF [163]. In a mouse model of cerebral ischemia, GM-CSF administration also

reduced the infarct size and increased the numbers of circulating blood monocytes/macrophages [164]. Taken together, these studies indicate that GM-CSF may play a beneficial role in neuroprotection, however more studies are required to clarify its full potential after TBI.

13. Toll-Like Receptors Mediate Innate Immune Responses to CNS Trauma

The toll-like receptors (TLRs) are a family of pattern recognition receptors which mediate innate immune responses to diverse pathogen-associated molecular patterns (PAMPs) [165]. Following injury or neurodegenerative disease without an infectious etiology, the engagement of danger-associated molecular patterns (DAMPs) by TLRs leads to exacerbated immune activation and enhanced neuropathology [166, 167]. Like all innate immune responses discussed here, TLR signaling is typically beneficial, yet it has become increasingly clear that following injury signaling through TLRs has particularly pathological consequences, contributing to the activation of microglia and subsequent induction of NFκB leading to the transcription of proinflammatory mediators [168, 169]. Microglia are known to express all recognised TLRs [169], however the expression of TLRs on astrocytes is a contentious topic, with some researchers observing the presence of TLR-2 and TLR-4 mRNA in astrocyte culture [170], whilst others were unable to identify the expression of any TLR in 99% pure human astrocytic culture [171].

Many molecules may act as endogenous ligands for TLR signaling, with evidence suggesting that the TLRs involved most in TBI are TLR-2 and TLR-4, and that signaling through these TLRs triggers NFκB activation and gene transcription [12]. Whilst research on the role of TLRs after TBI is scant, levels of TLR2 has been noted to be significantly upregulated after mouse bilateral cortical contusion [172], and significant infiltration of TLR-2 positive macrophages/microglia has been observed in the lesioned area and subcortical white matter after weight-drop injury in rats [173]. It appears though that the most compelling evidence of the roles of TLRs in TBI comes from experiments in which they are suppressed or deleted, with TLR-2 knockout mice showing an 18-fold reduction in GFAP mRNA, and 4-fold reduction in CD11b mRNA after stab-wound injury when compared to wild type. The authors also found less infiltrating astrocytes in the lesioned area, with those present possessing a less-activated morphology [174], suggesting that activation of TLR-2 was a substantial contributing factor to glial activation. In another study, suppression of TLR-4 using the monosomic alkaloid oxymatrine after focal TBI led to reduced gene expression of NFκB and lower concentrations of TNF-α, IL-1β, and IL-6, with fewer apoptotic neurons as a consequence, suggesting a negative role for TLR-4 in neuroinflammation [175]. A double-knockout of TLR-2 and TLR-4 also resulted in decreased IL-1β and MCP-1 signaling after sciatic nerve damage, as well as significantly decreased macrophage recruitment/microglial activation, however these rats were noted to have poor locomotor recovery, impaired Wallerian degeneration, and inhibited axonal regeneration [176]. Interestingly, a single microinjection of the TLR-2 and TLR-4 ligands at the lesion site resulted in faster clearance of degenerating myelin, and significant and sustained improvement in motor function, indicating that while TLR signaling may be detrimental in terms of the acute neuroinflammatory response, it may in fact be important for long term recovery in terms of myelin clearance and nerve regeneration [176].

14. Immunotherapies for TBI

Despite more than 30 years of research, not a single effective therapy has been developed for the treatment of TBI. A multitude of compounds showing promise in animal studies have failed to exhibit beneficial effects in clinical trials, with more than 20 compounds reaching phase II/III trials but showing no long-term benefit [7]. In one of the largest clinical trials for TBI to date, the corticosteroid randomisation after significant head injury (CRASH) trial investigators found that despite encouraging results in animal studies in which corticosteroid treatment was found to be efficacious, in a clinical setting the administration of corticosteroids after TBI was strongly correlated with excess mortality [177].

The lack of success of clinical trials has been attributed to several factors, including superficial examination in animal models with premature translation to the clinic, variations in therapeutic windows in animals and humans and variable dosing schedules, and failure of experimental models to include secondary insults which are commonplace in clinical TBI. Finally, animal models of TBI are by design well-controlled and reproducible, whilst clinical TBIs are far more complex and inherently heterogeneous [178, 179]. In order to address these problems, experimental studies are increasingly employing more clinically relevant species with secondary insults, and many compounds are trialed in larger animal models in order to establish efficacy in more clinically relevant brains before moving to clinical trial. Compounds that are currently under investigation for the treatment of TBI fall broadly into two categories: those with multiple targets and modes of action in CNS pathologies, and those with a single target of action. Examples of each with relevance to innate immunity are presented below.

14.1. Compounds of Multifunctional Modality

14.1.1. Erythropoietin. Erythropoietin (Epo) is a haematopoietic cytokine produced mainly by the kidney which is rapidly upregulated in response to hypoxia [180]. Epo has been used extensively in the treatment of chronic renal and anaemic patients and has been shown to reduce mortality in trauma patients [181]. In recent years, Epo has been highlighted as a promising neuroprotective candidate due to its current clinical use with few side effects and feasible therapeutic window of ~6 hours [182]. Epo and its receptor EpoR are rapidly upregulated in the brain after various insult models [183], and its administration after experimental injury was shown to be efficacious in a number of experimental TBI paradigms. Importantly for the treatment of TBI, Epo has numerous targets in the brain, with robust

benefits including anti-inflammation, with a reduction in immune mediators' levels and subsequent reduction in inflammatory cell infiltrates, diminished cell death, reduction of oedema, rectification of BBB dysfunction, resolution of cerebral vasospasm, as well as enhanced neurogenesis, and angiogenesis and improvement in sensorimotor function [183–187]. Currently, Epo is being investigated in a phase III clinical trial within multiple sites in Australia, with an estimated completion date of 2014.

14.1.2. Minocycline. The tetracycline derivative minocycline has been posited as a neuroprotective candidate in several experimental models of CNS injury due to its potent anti-inflammatory actions [61]. After focal TBI in mice, minocycline has been shown to attenuate microglial activation and reduce the expression of IL-1β [61, 188, 189], as well as acutely reduce the size of focal brain lesions [61, 188, 190] and decrease cerebral oedema [189]. Minocycline may also improve neurological function, however several studies report this effect may be transient, with beneficial outcomes only observed acutely [61, 190]. Minocycline is currently being investigated in a phase I trial in Detroit, Michigan, with imminent completion.

14.1.3. Progesterone. The hormone progesterone has been shown to have multiple functions in the treatment of brain injury, and is able to exert its effects through steroidal, neuroactive and neurosteroidal mechanisms [191]. Experimentally, progesterone acts as a potent anti-inflammatory agent by dampening the cytokine response and limiting immune cell activation and extravasation [192], as well as decreasing NFκB-mediated inflammatory gene transcription [193]. Progesterone has proved to be particularly effective in the treatment of focal brain injuries, in which it has been demonstrated to reduce neuronal damage, minimise oedema and improve neurological outcomes in a variety of focal contusion models [194–198]. Progesterone can also affect the complement system, with significant reductions in C3 cleaved fragments observed after bilateral frontal contusion in rats [193]. Although limited in number, studies of progesterone's effects on diffuse TBI have also demonstrated benefit with a reported reduction in BBB permeability and subsequent oedema [199] as well as a decreased number of apoptotic cells and the apoptotic precursor caspase 3, and a substantial decline in axonal pathology [200]. Importantly for translation to the clinic, delaying the administration of progesterone for 24 h still resulted in benefit, with a diminished oedema observed after cortical contusion injury [201]. Due to these benefits in rodent models, progesterone has been applied clinically, with evidence from the ProTECT clinical trial and other pilot studies suggesting that progesterone may reduce mortality and improve neurological outcomes after TBI [202, 203], warranting further investigation in a large multicentre trial. The ProTECT trial has now entered phase III, with an estimated completion date of 2015.

14.2. Single-Target Compounds

14.2.1. Complement Inhibition. The complement system presents as an attractive target for immune modulation after TBI due to its prominent role in inflammatory cell extravasation. Several aspects of the complement system are amenable to interventions such as selective antagonists, making them viable candidates for clinical translation. Experimentally, administration of the soluble complement receptor 1 after weight drop injury in the rat significantly attenuates neutrophil infiltration into the injured brain [96]. A similar effect on neutrophil extravasation was also observed after cryoinjury in mice with deletion of either the C3 or C5 gene, or administration of the C5a receptor antagonist, with corresponding reductions in the chemokines CCL5 and CCL2, and smaller lesions as a consequence [88]. Inhibition of the alternative pathway has also shown promising results, with targeted deletion of the factor B gene or delivery of antifactor B neutralising antibody resulting in significantly decreased C5a serum levels and a reduction in cell apoptosis [204, 205]. Although it appears that targeting the complement system in the acute phase may be beneficial, it may also have deleterious consequences for long-term recovery. For example, treatment with the C5a receptor antagonist in rats after spinal cord injury resulted in a significantly less macrophages/microglia in the injury site at 7 days, however these rats also had poor locomotor recovery and reduced myelination, suggesting that while early inhibition of C5a may be beneficial, the long-term outcome of reducing this aspect of inflammation is detrimental [206].

14.2.2. Anticytokine Antibodies. Whilst cytokines appear a natural target for neutralisation as the perpetuators of the inflammatory response, they must be considered in the context of the whole organism, in that the beneficial effects of abolishing such a targeted response may have more broad adverse consequences in recovery. Studies suggest, however, that there may be some benefit to inhibiting the actions of several cytokines, with neutralisation of IL-1β after focal TBI in mice attenuating neutrophil infiltration and microglial activation, minimising the number of ICAM-1 positive cells, and reducing oedema and improving cognitive outcome [145, 207, 208]. Treatment of mice with the IL-1ra has also resulted in benefit in various models and species, with better behavioural scores and attenuation of oxidative stress, as well as smaller lesion volumes [209, 210]. Importantly for the clinic, IL-1ra is able to penetrate the BBB in concentrations considered to be experimentally therapeutic [211], and even when administration is delayed by 4 h under experimental conditions, smaller lesion volumes are still observed in an animal model of TBI [210].

Therapeutic inhibition of TNF has also been demonstrated with good result after closed head injury in rats, with a reduction in oedema and recovery of motor function reported [125, 212]. However, this effect may vary depending on the model of TBI employed, with other researchers finding no benefit when employing neutralising antibodies to TNF in a lateral fluid percussion injury in the rat with respect to oedema, motor, or cognitive outcomes up to one week

after injury [213]. Results of TNF neutralisation may also vary between species, with no effect observed on behavioural outcomes, lesion volumes or cell death in mice subjected to closed head injury [214].

14.2.3. Antibodies to Cell Adhesion Molecules. Targeting the passage of immune cells through the BBB via inhibition of cell adhesion molecules presents an interesting avenue to dampen the neuroinflammatory response to TBI. Experimentally, administration of antibodies to ICAM-1 resulted in a substantial decrease in neutrophil recruitment [55, 122], however neutrophil accumulation was not completely abolished, thus suggesting a prominent role for other cell adhesion molecules in the absence of ICAM-1 [55]. Neutralisation of ICAM-1 also significantly improved motor performance after lateral fluid percussion injury in the rat, however a significant effect was also seen with IgG injection, indicating that there may be a nonspecific antibody effect [122]. In mice deficient in ICAM-1, however, no beneficial effect was observed with regard to neutrophil accumulation, lesion volume, or motor or cognitive function [215]. In mice double knockout mice for both ICAM-1 and P-selectin, whilst a significant reduction in oedema was observed, no differences to wild type were found with regard to histopathology, motor or cognitive function [216], providing more supporting evidence for a compensatory role of other cell adhesion molecules.

15. Summary

The innate immune response plays an intrinsic role in the governance of TBI, with both beneficial and deleterious consequences. This response is largely mediated by resident innate immune cells (microglia and astrocytes), while passage of peripheral immune cells into the brain is facilitated by opening of the BBB, or by upregulation of adhesion molecules and chemokines to aid their movement into the injured tissue. Chemokines such as CXCL2 and CCL-2, and cytokines such as IL-1β, TNF, and IL-6 also play essential roles in dictating migration and recruitment of immune cells to sites of injury, with reparative or destructive consequences depending on the timing of their release and their concentrations. Whilst the intention of the innate immune response is to promote repair, restorative efforts are often hampered by the presence of additional inflammatory factors such as complement proteins and increased signaling through microglial TLRs, which results in a disproportionate and self-perpetuating immune response. This dysregulation has become a key target for therapeutic intervention, with both single-target and multifunctional drugs evaluated in efforts to curb the innate immune response. Therapeutic targets are wide ranging, with a focus ranging from adhesion molecules to cytokines in an effort to minimise cell entry, activation and expansion. As yet, no one compound has proven efficacious when applied in multiple models or translated to the clinic, highlighting the need for more rigorous investigation in multiple pathological scenarios prior to clinical application.

16. Conclusion

It has become increasingly clear over the last two decades that the innate immune system plays a crucial role in the pathogenesis of TBI. The innate immune system is, by nature, complex and interrelated, with each crucial aspect shaping the structure for the next, and ultimately determining the outcome following TBI. It is this intricate nature, however, which heightens the challenge faced by researchers and clinicians alike in both understanding and combating the secondary consequences of brain trauma. While research into the pathogenesis of TBI is rapidly advancing, many of the complex interactions between compartments of the innate immune response are still unknown. However, with further understanding and more thorough preclinical screening of neuroprotective candidates, the development of an effective therapy for the treatment of TBI could be achieved.

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Review Article

Interplay between Human Cytomegalovirus and Intrinsic/Innate Host Responses: A Complex Bidirectional Relationship

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The interaction between human cytomegalovirus (HCMV) and its host is a complex process that begins with viral attachment and entry into host cells, culminating in the development of a specific adaptive response that clears the acute infection but fails to eradicate HCMV. We review the viral and cellular partners that mediate early host responses to HCMV with regard to the interaction between structural components of virions (viral glycoproteins) and cellular receptors (attachment/entry receptors, toll-like receptors, and other nucleic acid sensors) or intrinsic factors (PML, hDaxx, Sp100, viperin, interferon inducible protein 16), the reactions of innate immune cells (antigen presenting cells and natural killer cells), the numerous mechanisms of viral immunoevasion, and the potential exploitation of events that are associated with early phases of virus-host interplay as a therapeutic strategy.

1. Introduction

Human cytomegalovirus (HCMV) is a ubiquitous, highly specific herpesvirus. As the other herpesviruses, after an initial primary infection HCMV establishes latency for the life of the host with periodic and spontaneous reactivation. In immunocompetent subjects, primary HCMV infection is usually asymptomatic but occasionally gives rise to a self-limited mononucleosis-like syndrome. In immunocompromised patients, HCMV is one of the most common opportunistic pathogens and causes different clinical syndromes, whose severity parallels the degree of the immunosuppression [1]; in these patients HCMV infection causes both direct effects, reflecting cell destruction by the virus, and indirect effects, such as acute and chronic rejection, cardiovascular disease, and HCMV-associated opportunistic infections [2]. During the acute phase of infection, HCMV can infect a remarkably broad cell range within its host, including endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, neuronal cells, hepatocytes, trophoblasts, monocytes/macrophages (Mφs), and dendritic cells (DCs) [3].

HCMV induces many hallmarks of innate immune responses, such as the production of inflammatory cytokines and activation of the interferon (IFN) pathway in both immunocompetent and immunocompromised patients. This induction is rapid and does not require transcriptionally active viral particles [4]. The ability of the soluble forms of envelope glycoproteins B (gB) and H (gH) to effect a similar pattern of cellular responses suggests that their interactions with host cell components, such as integrin heterodimers, toll-like receptors, and entry receptors, are sensed by host cells, leading to early signaling and transcriptional events in infected cells and activating innate immune responses before the outset of viral replication [4–6].

Proper activation of innate immunity appears to be crucial to efficiently combat infections; in addition to the release of primary IFNs, professional antigen-presenting cells (APCs) are activated and natural killer (NK) cells are recruited and stimulated, triggering APCs and T cells. Further, unlike the innate and adaptive components of the immune system that require pathogen-induced signaling cascades for activation, intrinsic immune mechanisms are

significant, forming an antiviral frontline defense that is mediated by cellular proteins, called restriction factors, that are constitutively expressed and active, even before a pathogen enters a cell [7–9]. Notably, interplay exists between innate and intrinsic immune mechanisms, wherein several restriction factors are upregulated by IFN, enhancing their antiviral activity [10, 11].

This paper describes the viral and cellular partners that mediate early host responses to HCMV with regard to the interaction between structural components of virions and cellular receptors and intrinsic factors, the reactions of innate immune cells, the mechanisms of viral immunoevasion, and the potential exploitation of events that are associated with these early phases of virus-host interplay as a therapeutic strategy.

2. Binding and Activation: Function of Receptors in Early Stages of HCMV Infection

Several receptors, including epidermal growth factor receptor (EGFR) [12, 13], platelet-derived growth factor receptor (PDGFR)-α [14], and integrins [15, 16], mediate HCMV attachment and entry. Virus-receptor interactions appear to be cell-type specific. For example, in the interaction between HCMV and monocyte-derived dendritic cells (Mo-DCs), viral envelope glycoprotein gB binds to the DC membrane protein DC-SIGN [17]. Polymorphisms in the promoter of *DC-SIGN* that enhance its expression on the surface of Mo-DCs are linked to higher levels of HCMV infection *in vitro* and *in vivo* [18], implicating DC-SIGN in viral entry into DC-SIGN-positive immune cells.

In addition to its binding to receptors, facilitating its entry, the virus is sensed by pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), which initiate immune responses by recognizing pathogen-associated molecular patterns (PAMPs). TLR activation is followed by inflammatory cytokine secretion, upregulation of costimulatory molecules on APCs, and, in most cases, type I IFN production [19].

The initial evidence that HCMV activates innate immunity in a TLR-dependent manner was obtained with TLR2; stimulation of TLR2 by HCMV is replication independent and results in the activation of NF-κB and the release of inflammatory cytokines [20] without affecting the IFN pathway [21]. The envelope glycoproteins gB and gH also interact with TLR2, and neutralizing antibodies against TLR2, gB, and gH inhibit inflammatory cytokine responses to HCMV infection in permissive human fibroblasts [22]. Further, HCMV fusion inhibitors block virus-induced IFN signaling but not inflammatory cytokine secretion, suggesting that the latter is effected by surface sensing by TLR2 and does not require viral entry [21]. These findings indicate that HCMV-induced activation of cell surface TLR2 occurs at the earliest stages of infection; that is, the recognition and binding of envelope glycoproteins.

In addition to the *in vitro* findings, there is clinical evidence that implicates TLR2 in the pathogenesis of HCMV infection; liver transplant recipients who carry the homozygous Arg753Gln mutation of TLR2 have a higher incidence of HCMV-related disease that is associated with increased levels of HCMV DNA in the peripheral blood [23]. This clinical finding is explained by *in vitro* data that cells with the Arg753Gln mutation in TLR2 fail to recognize HCMV gB. Thus, impaired innate viral recognition might impede the development of a robust antiviral immune response, resulting in symptomatic disease in immunocompromised transplant recipients [24]. Chan and Guilbert have also demonstrated the significance of TRL2 in the immunopathogenesis of HCMV, reporting that UV-inactivated virions stimulate apoptosis in syncytiotrophoblast-like cells in a TLR2-dependent manner, likely contributing to chronic villitis and disruption of syncytiotrophoblasts, which often develop in placentas on delivery of newborns with congenital HCMV [25].

Intracellular TLRs, including TLR3, TLR7, TLR8, and TLR9, detect nucleic acids and are primarily involved in viral detection; TLR3, 7, and 9 recognize microbial nucleic acids in endolysosomes and trigger innate and downstream adaptive immune responses [26]. Endosomal TLR3 and TLR9 are essential components in the innate response to murine CMV (MCMV) in DCs and Mφs, and TLR9 is critical for NK cell activation and control of MCMV infection [27–29]. TLR9 also functions in the early responses to HCMV in humans; HCMV induces IFN-α secretion from human plasmacytoid DCs (PDCs) by engaging the TLR7 and/or TLR9 pathways *in vitro* [30] and upregulates TLR9 expression in human PDCs [30] and fibroblasts [31].

Notably, the stimulation of TLR9 by its ligand, CpG-B, when added after viral entry, enhances HCMV infection in fibroblasts by an unknown mechanism, suggesting that the virus exploits TLR9 signaling to further its replication during infection of stromal cells. Moreover, the presence of T-1237C polymorphism that alters TLR9 promoter activity [32] correlates with symptomatic HCMV infection in stem cell transplants [33], implicating the TLR9 pathway in the recognition of and response to HCMV.

HCMV infection in fibroblasts is also influenced by the TLR3 and TLR4 pathways; stimulation of fibroblasts with TLR3 and TLR4 ligands inhibits viral replication through an IFN-β-dependent mechanism [31, 34]. Nevertheless, TLR3 has no function in the innate/early phases of the cellular response to HCMV in human Mo-DCs, as recently demonstrated by experiments in which TLR3 was silenced before HCMV infection [35]. HCMV also triggers TLR-independent DNA sensing mechanisms [36], as evidenced by findings that the DNA sensor ZBPI/DNA-dependent activator of IFN-regulatory factors (DAI) activates IFN regulatory factor (IRF) 3 and upregulates type I IFN on HCMV infection [37]. Further, HCMV modulates the activity of other innate immunity receptors that induce type I IFN secretion, such as retinoic acid-inducible gene I (RIG-I-) like helicases (RLHs); RIG-I is upregulated quickly in the early phase of HCMV infection in fibroblasts [38].

Other HCMV attachment/entry receptors might mediate the development of innate responses. Because they associate with TLRs [39] and HCMV glycoproteins [15, 40, 41],

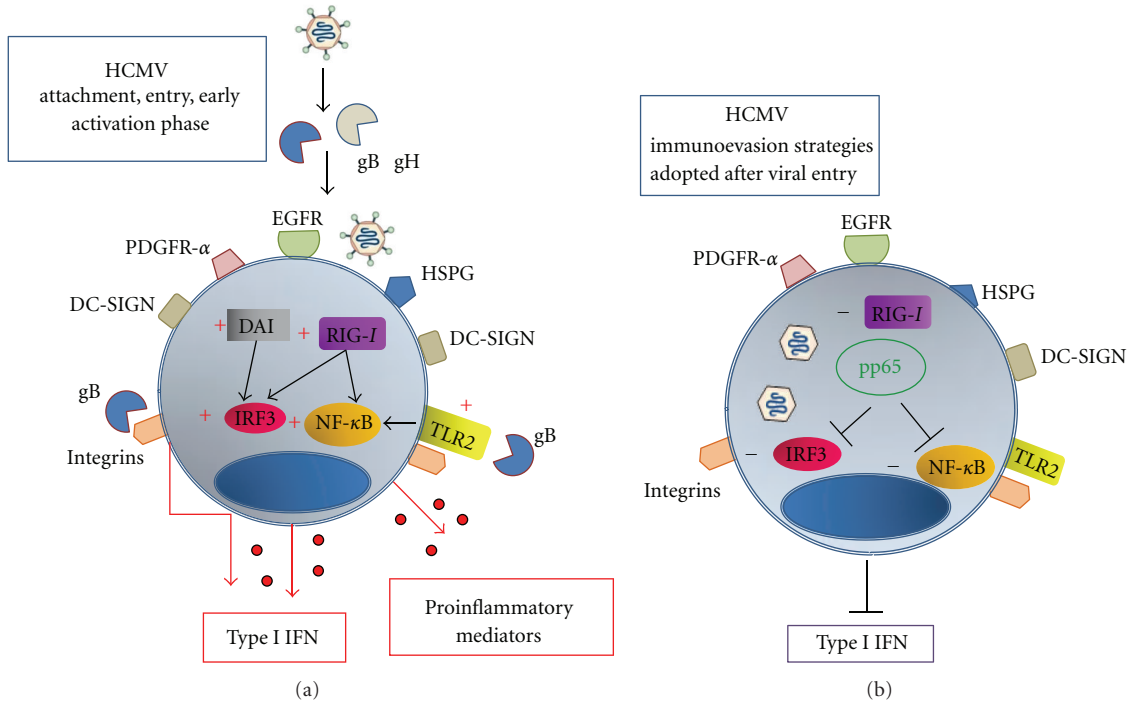

Figure 1: Activation and viral-induced modulation of early phases, HCMV attachment, entry, and intracellular phases of the viral cycle. (a) The binding of viral glycoprotein B (gB) induces the release of type I interferons (IFN) *via* IFN regulatory factor (IRF) 3, whereas contact between viral glycoproteins gB and gH and toll-like receptor (TLR)2 induces the activation of NF-κB and the release of proinflammatory cytokines. Expression of the intracellular receptor retinoic acid-inducible gene I (RIG-I) is also upregulated in the early phases, the DNA sensor DNA-dependent activator of IFN-regulatory factors (DAI) is activated, triggering IRF-3 activation and type I IFN production. (b) After viral entry, HCMV immunoevasion strategies are activated. Virion-associated and newly produced pp65 prevents IRF3 activation and subsequently impairs the production of type I IFN. Viral pp65 also inhibits NF-κB activation. RIG-I is downmodulated by an unknown mechanism, likely contributing to reduced IFN production. +; upregulation or activation, −; downmodulation or inhibition.

surface integrins have been proposed to facilitate the interactions of gB and gH with TLR2 [22, 42]. However, the ligation of gB to β_1 integrin stimulates IFN signaling but not NF-κB-mediated inflammatory signalling [21], suggesting that this interaction induces a TLR-independent antiviral state before viral entry. The activation of innate mechanisms following HCMV attachment and entry and virus-induced modulation of host responses is depicted in Figure 1.

HCMV infects a variety of nonimmune cells *in vivo*, including fibroblasts, endothelial cells, epithelial cells, smooth muscle cells, and stromal cells; each of which expresses a unique subset of TLRs and other innate receptors, allowing them to respond specifically to HCMV infection and contribute to early antiviral defense. The activation of immune receptors on HCMV infection has significant function in fibroblasts [21, 22, 31]. HCMV-induced activation of innate receptors in other nonimmune cells might also be critical, an area that merits further study.

2.1. Viral Escape Starts at Very Early Phases. After viral entry, HCMV immunoevasion strategies are activated. The expression of HCMV pp65/UL83 blocks IRF3 signaling, which lies downstream of the RIG-I, DAI, and TLR3 pathways; pp65-mediated impairment of IRF3 signalling occurs by reducing IRF phosphorylation status and by inhibiting its nuclear accumulation [43]. pp65 also blocks IRF1 and NF-κB activation by an unknown mechanism [44], suggesting that HCMV counteracts the activation of the IFN and proinflammatory pathways at several steps. Further, RIG-I is downmodulated by an unknown mechanism starting at 48-hour postinfection [38], likely contributing to reduced IFN production.

3. Function of IFN Inducible Restriction Factors in Antiviral Defense

Intrinsic immune mechanisms were discovered as being active against retroviruses and involving the APOBEC3 class of cytidine deaminases, a large family of proteins that are collectively termed the TRIM family, and tetherin, an IFN-inducible protein whose expression blocks the release of HIV-1. Increasing evidence, however, suggests that such mechanisms also counter other viruses [45, 46]. Moreover, four proteins, promyelocytic leukemia protein (PML) [47], hDaxx [48], Sp100 [49], and viperin [50], have been identified as restriction factors that mediate intrinsic immunity against HCMV infection.

PML and hDaxx are components of subnuclear structures called nuclear domain 10 (ND10) or nuclear bodies

(NBs). Direct evidence for their antiviral function comes from studies of cells that lack ND10. Primary human fibroblasts from which PML was depleted by small interfering RNA (siRNA) significantly increased the plaque-forming efficiency of HCMV due to enhanced immediate early (IE) expression. hDaxx represses HCMV IE expression and replication through histone deacetylases (HDACs), inducing transcriptionally inactive chromatin around the major IE promoter (MIEP) [51]. These findings demonstrate that the ND10 proteins PML and hDaxx are restriction factors that silence HCMV IE expression, thus controlling viral replication.

Viperin is an IFN-inducible iron-sulfur (Fe-S) cluster-binding antiviral protein that is induced in various cell types by type I, II, and III IFNs and on infection by many viruses, including HCMV. Ectopic expression of viperin in fibroblasts has no effect on the expression of HCMV IE1 or IE2, whereas the synthesis of early late (pp65), late (gB), and true late (pp28) genes is reduced significantly in viperin-expressing cells compared with control [52]. Because it interferes with the secretion of soluble proteins by disrupting lipid rafts of the plasma membrane, viperin likely exerts its antiviral effects by preventing virion assembly at a late stage of the viral life cycle.

An IFN-inducible family of proteins, previously known as the p200 family, has recently been demonstrated to suppress HCMV replication. This family, now designated PYHIN, comprises homologous human and mouse proteins that have an N-terminal Pyrin domain (PYD) and 1 or 2 partially conserved 200-residue C-terminal domains (HIN domain) [53]. These proteins are pleiotropic, based on their ability to bind to various target proteins (e.g., transcription factors, signaling proteins, and tumor suppressors) and modulate various cell functions. Increasing evidence implicates them as regulators of many processes, including proliferation, differentiation, apoptosis, senescence, inflammasome assembly, and the control of organ transplants.

Two members of the PYHIN family, AIM2, and IFN inducible protein 16 (IFI16), bind to and function as PRRs of virus-derived intracellular DNA [8]. In particular, IFI16 interacts with the adaptor molecule ASC and procaspase-1, forming a functional inflammasome during Kaposi sarcoma-associated herpesvirus (KSHV) infection [54]. Moreover, the induction of IRF3 and NF-κB-dependent genes by herpes simplex virus (HSV)-1 infection in RAW264.7 cells is impaired by siRNA that targets p204, the murine ortholog of IFI16 [55].

Using two approaches, we recently determined IFI16 to be an antiviral factor against HCMV [56]; IFI16 expression was knocked down by specific siRNA, enhancing HCMV replication, and transduction with dominant-negative IFI16 (lacking the PYD) increased HCMV replication, whereas overexpression of wild-type IFI16 impaired HCMV viral yield. In the latter set of experiments, early (E) and late (L), but not IE, mRNA and protein were downregulated, indicating that IFI16 exerts its antiviral effects by hindering viral DNA synthesis. The HCMV UL54 (also called *pol*) is the catalytic subunit of HCMV DNA polymerase and represents a prototypical early gene required for viral DNA replication. We have shown that IFI16 overexpression induces a significant inhibition of UL44, UL54, and UL83 mRNAs. These data were also confirmed at protein level. Moreover, transfection and electrophoretic mobility shift assay experiments performed with nuclear extracts of HCMV infected cells demonstrated that the UL54 promoter is the target of IFI16-induced viral suppression. In fact, using luciferase constructs that were driven by a site specifically mutated HCMV DNA polymerase (UL54) promoter, we noted that IFI16 suppresses UL54 transcription [56]. These data indicate that IFI16 has antiviral activity against HCMV and provide novel insights into the functions of IFI16 as a viral restriction factor.

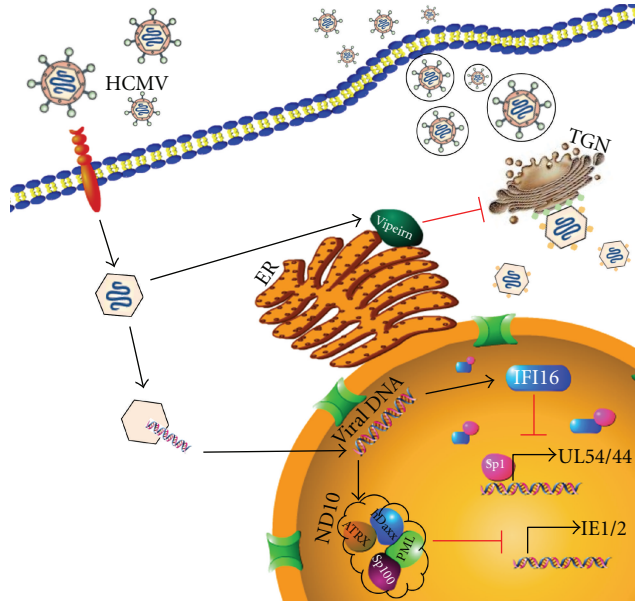

Figure 2: Type I IFN restriction factors that target HCMV. Type I interferons (IFN) are effector molecules of the immune response to virus. This antiviral action is mediated by IFN-stimulated genes. ND10 proteins are induced by IFN and function as part of an intrinsic antiviral defense mechanism of the cell by suppressing viral immediate early (IE) gene expression. The IFN-inducible protein IFI16 interacts with and displaces the transcription factor Sp1 from its DNA cognate element, the IR-1 element, in the viral UL54 promoter. This interaction inhibits the UL54 promoter and decreases HCMV DNA synthesis. The IFN-inducible protein viperin exerts its antiviral effects at a late stage of the HCMV life cycle. During infection, viperin is redistributed from the endoplasmic reticulum (ER) to the Golgi apparatus (TGN, *trans* Golgi network) and then to cytoplasmic vacuoles that contain gB and pp28.

Type I IFN-induced restriction factors, briefly described and summarized in Figure 2, constitute a potent antiviral defense mechanism against HCMV infection, rendering viral replication a true hurdle race.

3.1. Strategies Adopted by HCMV to Escape Activity of IFN Restriction Factors. In response to the antiviral action of type I IFN factors, HCMV has evolved regulatory proteins and counteracting mechanisms that subvert and inactivate such

factors. For example, IE1 disrupts ND10 by inducing the deSUMOylation of PML [47]. Recent evidence has demonstrated that HCMV relocates viperin from the endoplasmic reticulum to the mitochondria, where it reduces the generation of ATP, disrupting the actin cytoskeleton and enhancing viral infection [57]. Nuclear IFI16 appears to become inactivated, following its egress from the nucleus, during early gene expression by molecular mechanisms that appear to rely on protein ubiquitination (Landolfo et al. unpublished results).

4. Function of Innate Immunity Cells during HCMV Infection

HCMV infects host cells of the myeloid lineage, such as monocytes, Mφs, and myeloid DCs. Despite their resistance to HCMV infection, lymphoid lineage cells, such as NK cells and PDCs [58], are also activated rapidly by viral components, confirming the importance of early virus-host interactions in the induction of prompt host defense mechanisms. However, HCMV has developed myriad immunoevasion strategies, allowing it to subvert host cell functions for its own advantage.

4.1. HCMV Efficiently Infects APCs and Employs These Cells as Vehicle of Viral Dissemination. APCs, including monocytes and various DC and Mφ subsets, are critical in initiating specific naive and memory T-cell responses and coordinating and modulating host responses. Nevertheless, it is evident that HCMV hijacks these cells, transforming them into vehicles for viral dissemination in the first phase of infection and sheltered reservoirs in which the virus can persist, reactivate, and replicate under favorable conditions [59].

HCMV infects myeloid APCs, based on the detection of viral genome and antigens [60–63]. Monocytes do not support productive viral replication, and viral gene expression is restricted to early events [64, 65], whereas infected fully differentiated Mφs and myeloid DCs undergo lytic viral cycles, express late HCMV genes, release infectious virus, and stimulate T-cell responses *in vitro* [62, 63, 66, 67]. Thus, the ability of HCMV to replicate in myeloid cells depends on their stage of differentiation, as shown in an experimental model of HCMV latency, which was established by infecting human monocytes with a clinical isolate *in vitro*, in which monocytic differentiation to Mφs or DCs induced viral reactivation [68].

During the differentiation of DC progenitors to mature DCs *ex vivo*, chromatin structure is altered, permitting robust IE expression and, consequently, reactivation of latent HCMV [69]. Consistent with these observations, the inhibition of viral lytic genes that occurs during latency in undifferentiated myeloid precursors, including monocytes, is attributed to their inability to sustain high IE levels; the histone modifications present on the MIEP impart on it a repressive chromatin structure preventing transcriptional activity [70]. Recent evidence implicates IL-6 signaling and activation of the ERK/MAPK pathway in HCMV reactivation from potentially permissive cells, such as interstitial DCs [71]. Thus, myeloid cell differentiation, which is driven by inflammation and proinflammatory factors, such as IL-6, contribute to reactivation of latent HCMV infection (Figure 3(a)).

Conversely, the virus can enhance inflammation by acting on APCs; HCMV infection of peripheral monocytes induces a proinflammatory state, resulting in their adhesion to endothelial cells and transendothelial migration [72] and the secretion of proinflammatory cytokines and chemotactic factors [73]. Further, Mo-DCs [74, 75] and monocyte-derived Mφs [76] release proinflammatory factors on productive HCMV infection *in vitro*.

4.2. Immunoevasion Mechanisms Adopted by HCMV against APC Responses. In addition to enhancing inflammation for its own sake, HCMV hampers APCs in taking up and presenting the proper antigen to T lymphocytes. Several counteracting mechanisms have been evolved by HCMV to circumvent APC activity (Figure 3(a)). Immunoevasive viral transcripts, such as gpUS3 and gpUS8, that block human leukocyte antigen (HLA-) mediated antigen presentation pathways predominate during the early phases of HCMV infection of myeloid DCs [77]. HCMV inhibits the differentiation of Mφs and DCs from monocytic precursors, blocking their phagocytic, migratory, and allostimulatory activities [78, 79].

HCMV also impairs the immunophenotype and function of differentiated APCs. For example, it downmodulates integrin-like receptors, such as CD11b/CD18 (CR3) and CD11c/CD18 (CR4), on the surface of monocyte-derived Mφs, reduces their phagocytic activity [80], and impairs migration by downregulating CCR1 and CCR5, reorganizing the cytoskeleton, and inducing the secretion of soluble inhibitors [76]. Further, HCMV-infected, immature Mo-DCs have fewer surface HLA class I and class II molecules and impaired migratory and immunostimulatory capacity [74, 81, 82]. The virus also inhibits Mo-DC maturation and impedes the migration of mature DCs in response to lymphoid stimuli and induction of T-cell proliferation [75, 82, 83]. Similarly, on infection with HCMV, activation markers are downregulated in mature Langerhans DCs, decreasing their ability to stimulate T-cell proliferation [84, 85].

Many events have been implicated in the HCMV-induced impairments to immunostimulation by DCs, such as the release of soluble CD83 [86], upregulation of apoptosis-stimulating molecules [87], expression of the HCMV-encoded HLA class I-like homolog pUL18 [88], and secretion of the viral homolog of IL-10, which is expressed during the productive phase of infection (cmvIL-10) [89]. cmvIL-10 also impairs CD1-mediated antigen presentation (by reducing CD1 transcription) [90], monocyte function [91, 92], and TLR-induced transcriptional activation of IFN α/β genes in PDCs [93]. cmvIL-10 enhances HCMV infectivity by upregulating the viral entry receptor DC-SIGN [89]. Thus, secretion of cmvIL-10 during HCMV infection has many effects in hindering APC function.

4.3. APCs and HCMV: A Double-Edged Sword. Despite the subversion of APC function by the virus, specific effector

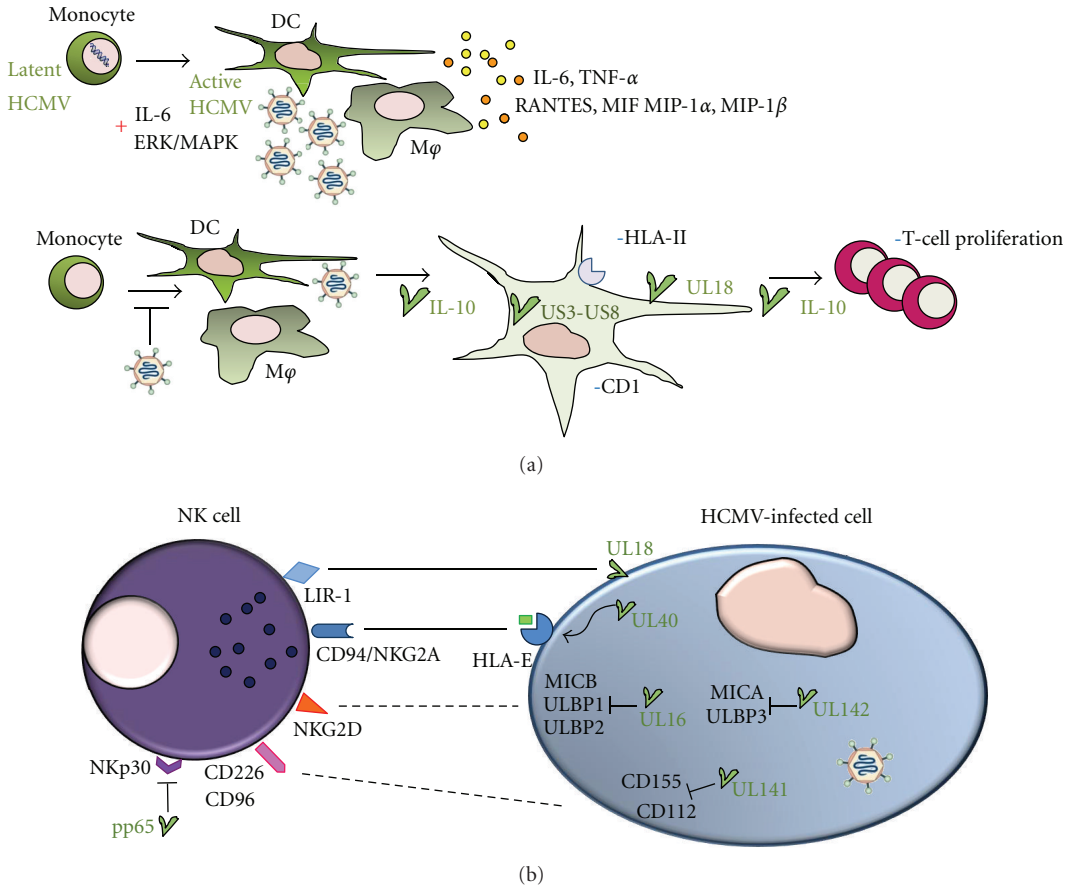

Figure 3: Cells of innate immunity, activation and virus counterattack. (a) HCMV reactivates from latency in infected monocytes by inflammation or cellular differentiation, in which IL-6 and ERK/MAPK signaling are involved. Differentiated macrophages (Mφ) and dendritic cells (DC) are permissive for viral replication and, once infected, release proinflammatory factors. HCMV hampers the ability of Mφ and DC to properly differentiate from monocytes and present antigens to T lymphocytes by downregulating surface expression of CD1 and HLA class II molecules. DC-induced T-cell proliferation also decreases through mechanisms that involve virally encoded IL-10 and pUL18. IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; MIF, macrophage migration inhibitory factor; MIP-1α, macrophage inflammatory protein-1α; MIP-1β, macrophage inflammatory protein-1β. +; upregulation or activation, −; downmodulation or inhibition. (b) HCMV-encoded proteins modulate NK-cell recognition of infected cells. pUL40 binds to HLA-E and upregulates its surface expression, potentiating its interaction with the inhibitory receptor CD94/NKG2A. pUL18, an HLA-I viral homolog, binds to the inhibitory receptor LIR-1. Expression of the ligands of the activating receptor NKG2D is inhibited by pUL16 (which targets MICB, ULBP1, and ULBP2) and pUL142 (targeting MICA and ULBP3). pUL141 prevents the expression of CD112 and CD155, ligands of the activating receptors CD226 and CD96, whereas pp65 interferes with the signal transduction of the activating receptor NKp30. Solid lines: possible interactions resulting in NK-cell inhibition. Dotted lines: impairment of interactions between activating receptors and their ligands.

and memory T cells develop during acute HCMV infection [94, 95] and robust adaptive immune responses develop to many HCMV antigens, of which IE1 is a significant target of $CD4^+$ and $CD8^+$ T-cell responses [94]. Whereas immunostimulation by DCs is profoundly impaired by the virus, HCMV-infected Mφs induce efficient T-cell activation through presentation of endogenous IE antigen [62]. Further, mechanisms of crosspresentation, the exogenous acquisition of antigen that is presented directly to $CD8^+$ T cells without endogenous processing, are also initiated during HCMV infection of APCs [96]. However, the effective role of cross-presentation in inducing an efficient cellular imunity to HCMV has not yet been addressed.

4.4. NK Cell Activation during HCMV Infection. NK cells are a critical component of early innate immune responses against certain viruses, including HCMV. Individuals with NK-cell defects have increased susceptibility to herpesviruses and, in particular, HCMV [97, 98]. Moreover, the extensive mechanisms that HCMV implements to prevent NK-cell activation are indirect evidence of their importance in the innate response to HCMV.

NK cells accumulate rapidly in several organs during viral infections, taking active part in the direct elimination of injured target cells by cytotoxicity and in the activation and recruitment of other cells of the immune system by secreting cytokines and chemokines, including IFN-γ and TNF-α [99].

In secondary lymphoid organs and damaged tissues, NK cells establish a dialog with APCs, thus regulating innate and adaptive immune responses [100].

NK cells recognize virus-infected cells, using a repertoire of stimulatory and inhibitory cell surface receptors [101] that control NK-cell activation, proliferation, and effector functions; their cytotoxic function depends primarily on stimulatory receptors. Different receptors are expressed to respond to different ligands on target cells: (i) HLA class I molecules (HLA-I), frequently downmodulated in virus-infected cells are recognized by specific inhibitory receptors, including killer cell-Ig-like receptors (KIRs), leukocyte Ig-like receptor 1 LILRB1 (LIR-1), and C-type lectin receptor CD94/NKG2A; (ii) pathogen-derived molecules are recognized by activating receptors, and (iii) self-proteins that are upregulated on "stressed" or damaged cells bind to a major activating receptor, NKG2D [102].

4.5. Mechanisms of Viral Immunoevasion Employed against NK Cells. Many inhibitory receptors on NK cells, including KIRs and LIR-1, recognize HLA-I, and under normal conditions, the engagement of inhibitory receptors by self-molecules suppresses NK-cell attack. However, HCMV is able to reduce cell surface expression of HLA-I by several mechanisms (reviewed in [103]). Consequently, it was predicted that according to the *missing self* hypothesis, low levels of HLA-I on HCMV-infected cells render them vulnerable to NK-cell lysis [104]. Yet, NK cells fail to discriminate between normal and infected cells on the basis of virus-induced HLA-I downmodulation [105, 106]. HCMV circumvents other aspects of the NK cell-target cell interaction [107], and HCMV-infected cells become resistant to be attacked by NK cells, due to a vast array of virally encoded immunomodulatory molecules [108].

Two mechanisms describing HCMV-mediated inhibitory signalling have been proposed. In the first, HCMV encodes for pUL18, an HLA-I homolog [109] that, like HLA-I, binds β2-microglobulin [110] and peptides [111] and engages the inhibitory receptor LIR-1 with 1000-fold higher affinity compared with HLA-I [112–114]. pUL18 inhibits LIR-1$^+$ NK cells but has additional effects, because LIR-1 is expressed on other cells of the immune system, including APCs [115]. For example, the binding of pUL18 to DCs impairs cell migration and CD40 ligand-induced maturation, reducing T-cell proliferation [88]. Thus, pUL18 can be exploited by HCMV to avoid host immune responses [116]. Clinical isolates of HCMV retain *UL18*, underscoring its importance for viral survival in the host [117, 118].

In the second mechanism, HCMV uses the host HLA-E pathway to suppress NK cells through the inhibitory receptor complex CD94/NKG2A. A nonameric peptide that is derived from the leader sequence of the viral protein pUL40 is a canonical ligand for the nonclassical HLA-I molecule HLA-E and promotes HLA-E expression on the cell surface [119–121], facilitating the interaction between HLA-E and CD94/NKG2A receptor and conferring resistance to NK-cell lysis [122–125].

Because the decision by NK cells to attack relies on the sum of signals from inhibitory and activating receptors, it is important for the virus to prevent the engagement of activating receptors. HCMV encodes five genes that impede signaling by activating receptors on NK cells: UL16, UL141, UL142, UL83, and microRNA-UL112-1 (miRNA-UL112) [108]. pUL16, pUL142, and miRNA-UL112 inhibit the expression of ligands of a major activating receptor, NKG2D. In humans, the ligands for NKG2D are the human major histocompatibility complex (MHC) class I chain-related genes (MIC)A, MICB, and ULBP1-6 molecules, which are particularly expressed under stress and on stimulation by innate cytokines that are produced during viral infections (reviewed in [126]).

Because NKG2D has an important role in controlling both NK- and T-cell-mediated immunity, it is reasonable that this receptor and its ligands forced the virus to evolve specific strategies of evasion. pUL16 prevents cell surface expression of MICB, ULBP1, and ULBP2 by binding and sequestering them in the endoplasmic reticulum or Golgi [127–129]. The selective pressure that is exerted by pUL16 likely contributes to drive the diversification of NKG2D ligands, eventually leading to the emergence of proteins that do not interact with UL16, such as MICA and ULBP3; the expression of which, however, is countered by the HCMV protein pUL142, which retains them in the *cis*-Golgi [130–132]. In addition, MICB is under the control of the virally encoded miRNA-UL112 which specifically reduces its cell surface expression [133].

Another tactic that was evolved by HCMV to interfere with activating receptors relies on pUL141, which sequesters the adhesion molecules CD155 (PVR/necl-5) [134] and CD112 (nectin-2) intracellularly [135]; these proteins are ligands for the NK-cell activating receptors CD226 (DNAM-1) and CD96 (TACTILE) [136]. Notably, pUL141 is the most robust modulator of NK cells that has been tested *in vitro*, inhibiting a wide range of human NK-cell populations [134]. This important function explains in part the increased resistance to NK-cell lysis of low-passaged HCMV clinical isolates compared with the laboratory strain AD169 [105], from which 13–15 kbp of DNA has been deleted due to extensive passaging *in vitro* [137], a segment that contains UL141 [108, 134].

The pp65 tegument protein also affects NK-cell functions, dissociating the ζ-chain from the natural cytotoxicity receptor NKp30 and preventing it from transducing signals through an unknown mechanism [138]. The outcome of these disparate strategies is impaired NK-cell-mediated recognition and elimination of HCMV-infected cells, as depicted in Figure 3(b).

4.6. NK Cells and HCMV: Windows of Opportunity for Host Counterattack. Despite the many viral strategies that modulate the antiviral functions of NK cells, there is a *window of opportunity* during which host responses can prevail, potentially rendering infected cells detectable by the immune system. Such a circumstance could be achieved through several mechanisms, depending on genetic variations in

the virus and host. For example, some allelic variants of NKG2D ligands are unaffected by known viral strategies. The MICA*008 allele, the most frequent allele in several populations, does not bind to viral pUL142. This variant has a truncated cytoplasmic tail, making it resistant to pUL142 and allowing it to persist on the surface of infected cells, where it can induce NK cells to lyse [132, 139]. This finding suggests that UL142 may be driving the selection of certain MICA alleles in humans [140, 141].

Genetic variations have also been detected in *UL142* from different clinical isolates of HCMV, some of which are more efficient in downregulating MICA expression [132]. Variations have also been identified in pUL40 and pUL18 [117, 118, 124].

Despite of the wide range of strategies that are used by HCMV to modulate NK-cell function, there is still the possibility of a time interval during which host responses prevail. MICA and MICB expression appears to be regulated by IE1 and IE2 proteins, indicating that viral *trans* activation is largely mediated by these HCMV gene products [142]. Notably, this effect might allow NK-activating ligands to be expressed before late immunoevasion genes are expressed and exert their effects. Collectively, this evidence suggests that the cellular response to infection could be sufficiently robust in some individuals against certain viral strains and/or at a specific time after infection, allowing to achieve elevated, functionally relevant levels of activating signals.

4.7. Interplay between NK Cells and APCs during HCMV Infection. NK-DC crosstalk is bidirectional, NK cells can kill immature DCs or promote their maturation, and in turn, mature DCs can stimulate NK-cell cytotoxicity and proliferation. These processes depend primarily on the activating receptors NKp30 and DNAM-1 and on the production of cytokines, such as IL-12, IL-15, IL-18, and IFN-α/β [100, 143–149].

Recent evidence has demonstrated that NK cells regulate HCMV infection through interactions with autologous APCs, such as Mo-DCs and polarized Mφs; NK cells respond vigorously against infected Mo-DCs by producing IFN-γ and becoming cytotoxic, where NKp46 and DNAM-1 have a dominant role [150]. Such a response is evident early after infection, whereas later, the virus-mediated downregulation of the DNAM-1 ligands CD155 and CD112 prevails, illustrating the significance of the course of infection with regard to the efficacy of the host response. Further, the production of IFN-γ by NK cells is influenced by the polarization of Mφs, wherein proinflammatory Mφs induce more efficient IFN-γ responses than anti-inflammatory Mφs on HCMV infection [151].

5. Early Events of HCMV Replication as Potential Targets for Therapeutic Intervention

The identification of cellular and viral components that regulate early HCMV-host cell interactions has increased our understanding of the pathogenesis of HCMV diseases and formed the rationale for the design of novel antiviral interventions that target these initial events.

The need for anti-HCMV drugs with novel mechanisms of action is underscored by the findings that conventional standard therapy is often associated with considerable adverse events and that prolonged treatment can lead to the emergence of drug-resistant strains [152]. Further, agents that target viral DNA polymerase are unable to prevent viral attachment or entry or the expression of IE proteins, which mediate proinflammatory responses and immunomodulation. Thus, blocking pre-IE events and IE expression and function may represent an alternative strategy of combating HCMV-induced immunopathological phenomena [153]. Several molecules that effect such outcomes have been identified (reviewed in [154]). However, with the sole exception of hyperimmune globulin preparations, compounds that target HCMV attachment and entry remain at the preclinical stage of development. We briefly review the properties of those experimental agents that have been shown to inhibit HCMV attachment and entry *in vitro*.

The adsorption of HCMV virions to cell surface heparan sulfate proteoglycans (HSPGs) is mediated by positively charged regions of the viral gM/gN complex and is essential for stabilizing virions at the cell surface prior to the engagement of entry receptors [4]. Several experimental inhibitors of HCMV attachment have been characterized, including sulfated polysaccharides, lactoferrin, and peptide-derivatized dendrimers. Negatively charged polyanions, such as sulfated polysaccharides from bacteria, algae, and animals and semisynthetic compounds, such as dextran sulfate and pentosan polysulfate, disrupt the electrostatic interactions between the positively charged region of HCMV envelope glycoproteins and the negatively charged sulfate/carboxyl groups of heparan sulfate (HS) chains in HSPGs; these compounds show potent anti-HCMV activity against laboratory strains and clinical isolates [155]. HSPGs can also be bound by the N-terminal region of lactoferrin, an iron-binding glycoprotein that exists in most mucosal secretions and body fluids, suggesting that it acts by preventing virions from tethering to the cell surface [156].

Dendrimers are synthetic hyperbranched molecules that may have potential applications as antivirals, based on their small size (nanomolar), ease of preparation, and ability to display multiple copies of surface groups (multivalency) that are required for recognition, including the initial interactions that occur between an infecting virus and the target cell [157]. Recently, two peptide-derivatized dendrimers, SB105 and SB105_A10, were shown to inhibit HCMV replication directly by preventing viral adsorption to HSPGs onto cells [158, 159].

The use of compounds that target viral attachment could be curbed by the cell-to-cell spread of clinical HCMV isolates. In a normal host, however, the release of cell-free virus depends on the site of infection; whereas cell-free viral transmission during hematogenous dissemination is believed to be unlikely (because HCMV replication is highly cell associated), cell-free virus is commonly found in body fluids, such as urine, saliva, and breast milk, often at high

titers [160]. Thus, molecules that block viral adsorption may be used to prevent HCMV transmission *via* such excretions.

HCMV-exploits its coding capacity for glycoproteins to form different envelope complexes [3]. The gH/gL heterodimer can participate in two distinct glycoprotein complexes; it can associate with gO to form a heterotrimeric complex that regulates pH-independent fusion at the cell surface in fibroblasts or it associates with pUL128, pUL130, and pUL131 to form a pentameric complex, required for entry by endocytosis, followed by low pH-dependent fusion in endothelial and epithelial cells, DCs, and monocytes [67, 161–163]. gB is also required for viral entry and cell-to-cell spread [164]. Thus, compounds that bind to virion components that mediate entry or interfere with the protein-protein interactions required to induce membrane fusion can be termed HCMV entry inhibitors.

Experimental agents that have been shown to interfere with HCMV entry include CFI02, β-peptides, and CpG ODNs. gB is the target of a small-molecule thiourea derivative, CFI02, which suppresses HCMV replication. Mechanism-of-action studies indicate that CFI02 acts at an early stage in HCMV replication by inhibiting gB-mediated fusion of the virion envelope to the cell membrane [165]. Further, heptad repeat motifs, characteristic of α-helical coiled-coil interactions, have been identified within gB and gH. Peptides that correspond to these regions have been shown to inhibit the entry of clinical and laboratory HCMV strains, thus providing the proof of concept that blocking the coiled-coil interactions required for viral entry is a feasible strategy of preventing HCMV infection [166]. These potential new targets for therapeutic intervention have been exploited, based on the development of oligomers of β-aminoacids (β-peptides) that mimic the heptad repeat domain of gB and block viral infection during virus-cell membrane fusion [167]. β-peptides showed to be more potent than gB-derived α-peptides and blocked the activation of the type I IFN pathway in HCMV-infected fibroblasts [21], suggesting that β-peptides can impede both HCMV replication and viral-induced immunopathogenesis.

Short synthetic oligodeoxynucleotides that contain deoxycytidyl-deoxyguanosine motifs (CpG ODNs) can mimic bacterial and viral DNA to stimulate TLR9 and activate innate responses [168, 169]. Their antiviral activity has been proposed to be secondary to CpG-induced IFN responses that are triggered through TLR9 activation. Luganini et al. [170] recently reported, however, that *in vitro* replication of HCMV was suppressed by several CpG ODNs in a TLR9-independent mechanism. The B-class prototype CpG ODN 2006 was shown to prevent the nuclear localization of pp65 and input viral DNA, thus suggesting that it inhibits HCMV entry [170]. Notably, when added after the onset of HCMV replication, CpG ODN 2006 stimulates viral replication [31], as discussed, indicating that once the virus establishes its transcriptional programs, it takes advantage of the TLR9 stimulation pathway to propagate. These findings also suggest that CpG ODNs should be considered for antiviral intervention solely to prevent HCMV infection.

Yet, the window of opportunity for the mentioned experimental compounds that target the attachment and entry phases of HCMV infection is narrow. Their development as candidate drugs for future intervention should be considered in combination with conventional anti-HCMV therapeutics, such as ganciclovir and foscarnet that inhibit viral replication.

Conversely, intravenous immunoglobulins that are enriched for antibodies against HCMV (HCMV-IVIG) have been approved for use in preventing HCMV diseases in transplant recipients. The rationale for their clinical application lies in their ability to neutralize the virus and prevent entry into several cell types. Therefore, HCMV-IVIG represents the first example of a drug capable of blocking a pre-IE event that has been extensively used in patients at risk of HCMV disease. Further, the immunomodulatory activity of IVIG [171] might help reduce HCMV-induced immunopathology. However, in spite of their widespread clinical application, the role of HCMV-IVIG in the prevention of HCMV infection and disease remain to be fully elucidated. In fact, prophylactic administration of HCMV-IVIG has been associated with improved total survival, reduced HCMV disease, and lower HCMV-associated deaths in solid organ transplant recipients [172], whereas in patients who are undergoing hematopoietic stem cell transplantation, routine prophylaxis with HCMV-IVIG remains controversial [173]. Moreover, observational clinical studies indicate that administration of HCMV-IVIG to pregnant woman with primary HCMV infection may be effective in treating and preventing fetal infection [174].

The low neutralization potency of these preparations, however, may limit their clinical use. Thus, human monoclonal antibodies (mAbs) that neutralize HCMV infection have recently garnered interest as more effective and safer passive immunotherapeutic agents. Panels of human mAbs against gB and gH [175] or those that recognize conformational epitopes that require two or more proteins of the gH/gL/pUL128-131 pentameric complex [176] were developed from immortalized memory B cells of HCMV-immune donors. Notably, the human mAbs against the UL128-131 locus gene products [161] showed a neutralizing activity 2-3 logs more potent than neutralizing mAbs directed to gB or gH [176]. Although their protective activity *in vivo* remains to be investigated, these new human mAbs are promising next-generation immunotherapeutic compounds for the therapy/prophylaxis of HCMV infection and disease.

6. Concluding Remarks

The complex interaction between HCMV and the host begins immediately on viral contact with many cell types, including innate immune cells. Virion recognition and binding and entry-related events induce inflammation and IFN responses, the latter upregulating restriction factors that, in turn, contribute to the creation of an intracellular antiviral state. However, the induction of the IFN response is modulated by many counteracting viral mechanisms, as well as the inactivation of IFN restriction factors and modulation

of innate cell functions that facilitate evasion of host intrinsic and innate immunity.

The identification of the mechanisms of host-HCMV interactions during attachment and entry has provided the rationale for the design of novel experimental compounds that target these events. Blocking the early phases of infection may provide a window of opportunity that allows such interventions to inhibit HCMV gene expression and replication and modulate inflammatory and IFN host responses, thus hindering viral-induced immunopathogenesis.

HCMV uses several immunoevasion strategies to evade host NK cells and APCs, most of which involve protein products of L viral genes that are used to complete the viral cycle. Novel therapeutics that block the viral cycle before the late stages of replication might also prevent HCMV from exploiting such strategies, thus increasing the immunocompetence of the host.

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Review Article

Lipopolysaccharides: From Erinyes to Charites

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Following the discovery of endotoxins by Richard Pfeiffer, such bacterial product was associated to many severe disorders produced by an overwhelming inflammatory response and often resulting in endotoxic shock and multiple organ failure. However, recent clinical and basic sciences investigations claimed some beneficial roles of typical as well as atypical endotoxins. The aim of this paper is to focus on recent data supporting a beneficial activity of both typical and atypical endotoxins. Such novel perspective looks promising for development of new drugs for prevention and therapy of several human diseases.

1. Introduction

The Erinyes were three netherworld goddesses depicted as ugly, winged women with hair, arms, and waists entwined with poisonous serpents and personified the tormenting madness inflicted upon a patricide or matricide. They could make people suffer, and a nation harbouring such people could experience dearth, and with it hunger and disease. On the contrary, the Charites, also commonly known as the Graces, were three goddesses daughters of Zeus and named Aglaia, Thalia, and Euphrosyne. They were often associated with grace, beauty, adornment, mirth, festivity, dance, and songs of revel [1].

The endotoxins of Gram-negative bacteria are lipopolysaccharides (LPSs), which are vital to both the structural and functional integrity of the bacterial outer membrane [2, 3].

In the first reports on endotoxin by Pfeiffer (1892) and Centanni (1894), only one side of the coin (the toxic activity) has been considered [4]. Lewis Thomas reported the reaction of higher animals (including humans) to endotoxins as "… a uncontrolled and auto-destructive behaviour of the host, leading to the consideration of endotoxin as a venom. All this seems unnecessary, panic-driven. There is nothing intrinsically poisonous about endotoxin, but it must look awful, or feel awful, when sensed by cells …" (Lewis Thomas, Germs, 1974). However, endotoxins may behave not only as Erinyes but also as Charites. Indeed many LPS activities are being increasingly revealed to be beneficial to the host. Some of such beneficial activities have been published a few years after endotoxin discovery, including the inhibitory effects on human sarcoma studied (since late 1890s) by William Bradley Coley, who used killed *Serratia marcescens*, and the successful therapy of lethal tertiary syphilis reported by the 1927 Nobel laureate Julius Wagner von Jauregg, who used different types of microbial suspensions [4].

The purpose of this paper is to focus on recent data supporting beneficial activities of both typical and atypical endotoxins.

2. Chemistry of Typical and Atypical Lipopolysaccharides

The lipid A, the core oligosaccharide, and the O-antigen polysaccharide chain are the three domains usually found in an LPS molecule.

The innermost, hydrophobic region, lipid A, is responsible for the major toxic and beneficial properties of bacterial endotoxins [2]. Lipid A is the least variable part of the molecule among the different species of a genus, and its structure generally consists of a diglucosamine backbone substituted with varying numbers (usually four to seven) of ester- or amide-linked fatty acids.

Phosphate and/or other substituents are linked to carbons at the C-1 and C-4′ positions of the glucosamine disaccharide [3] (Figure 1). A 2-keto-3-deoxyoctonate (Kdo)

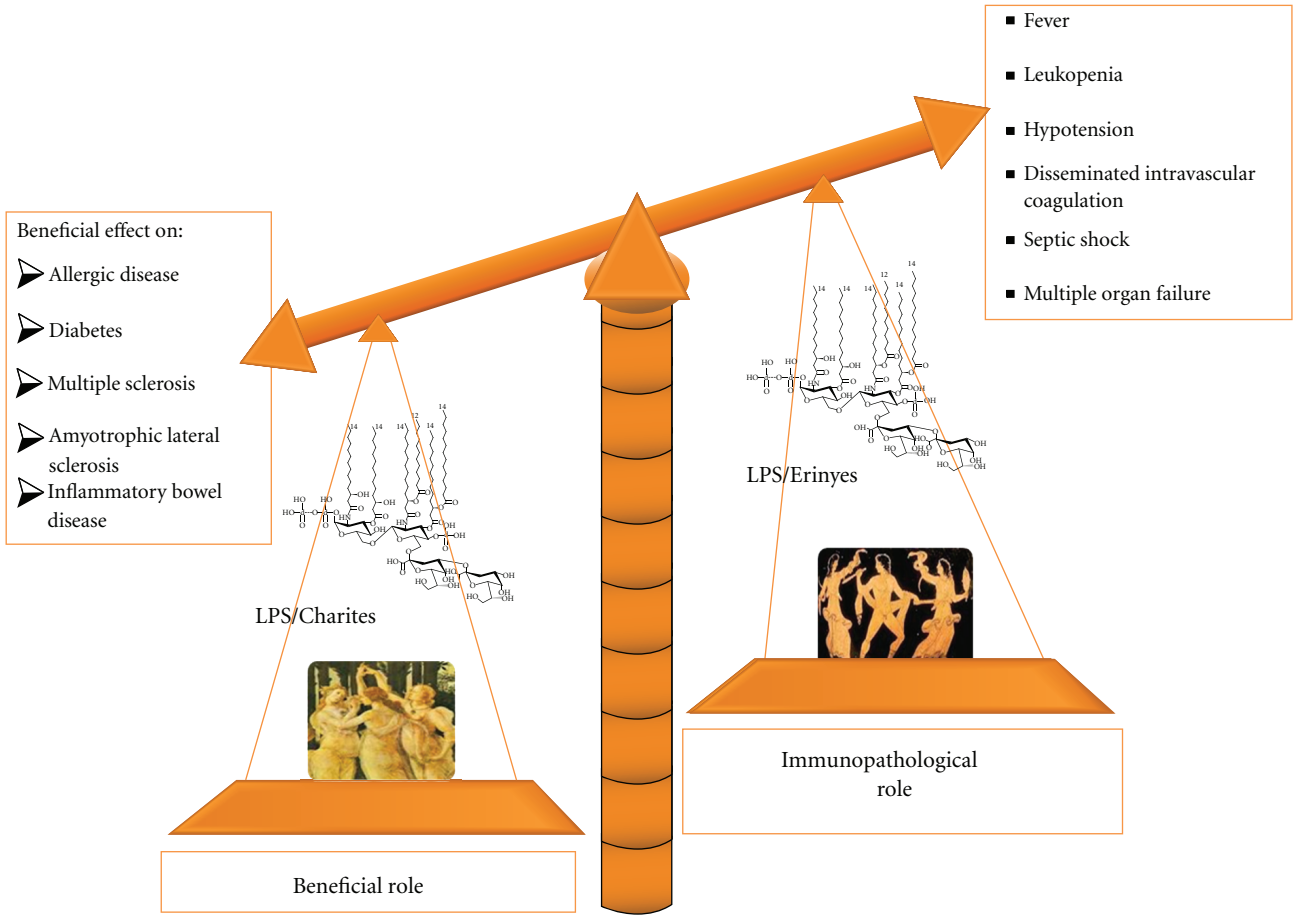

Figure 1: Balance between beneficial and immunopathological roles of LPSs.

unit links the lipid A to a core oligosaccharide (OS) composed of about 10 sugar residues. The core is linked to a third outermost region of a highly immunogenic and variable O-chain polysaccharide (PS) or O-antigen made up of repeating OS units. The latter region of the LPS molecule is responsible for bacterial serological strain specificity and is present only in smooth-type bacteria. The core region of enterobacterial LPS includes an outer portion, distal from lipid A (proximal to the O-polysaccharide chain), and an inner portion directly linked to the lipid A. The complete outer core region (Ra-structure) mainly consists of hexoses and hexosamines, whereas inner core region is composed of KDO and heptose. The so-called rough-type bacteria produce LPSs lacking O-antigens [2, 3]. A successive truncation of Ra-structure LPSs associated with specific alterations of core oligosaccharide biosynthesis in different *Salmonella* strains (R-mutants) results, respectively, in the Rb, Rc, Rd, and Re core structures. The last structure, which contains only lipid A and KDO residues, is a minimal LPS structure. The lipooligosaccharides (LOSs) consist of lipid A and an oligosaccharide core. The structural organization of LOS allows us to assign them to the group of intermediate molecules between typical R- and S-LPS structures.

The lipooligosaccharides (LOSs) contain a recognizable, well-conserved inner core (including KDO and heptose residues) from which extend one or two/three mono- or oligosaccharide branches (such as α-, β-, and γ-chains in Neisseria LOSs), that exhibits serological specificity [3]. In classical LPSs, the core provides an acceptor for O-polysaccharide, on the contrary in LOSs (distinct from R-LPS) the core is destined to terminate without O polysaccharide addition [2, 3].

LOSs are identified in such Gram-negative bacteria as *Bordetella pertussis*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Haemophilus ducreyi, Burkholderia (Pseudomonas) multivorans, Burkholderia (Pseudomonas) cenocepacia, Alteromonas addita* KMM 3600T, and *Campylobacter jejuni* [2, 3].

Atypical LPSs reportedly exhibit a lipid A chemistry which is different from archetypal structure found in *Escherichia coli* and *Salmonella*. Namely, atypical lipids A from different bacteria have the same general structure, but differ in the head-group substituents (e.g., phosphate groups) and in the number, distribution, and composition of fatty acids [2, 3].

In addition to the presence of fatty acids with hydrocarbon chain longer than 14 carbon atoms, the charge of lipids A from *Helicobacter pylori, Porphyromonas gingivalis, Francisella tularensis* was lower than the charge of lipids A from *E. coli* and compound 506, which could also affect the

binding. The low affinity of LPS binding with LBP and/or sCD14 is likely to influence the rate of endotoxin delivery to membranes of target cells and as a result to decrease the effectiveness of LPS signalization [2].

Matera et al. [5] reported that *Bartonella quintana* LPS exhibited a migration pattern of the deep rough chemotype.

Bartonella henselae has been found to exhibit a deeply atypical LPS with an approximate molecular weight of 5000 and with a Lipid A containing an acyloxyacyl residue 16:0[3-O(28:0(27-OH))] [6].

Therefore, LPS of *Bartonella henselae* has a deep-rough structure without an O-chain polysaccharide and contains an unusual penta-acylated lipid A with a long-chain fatty acid. The absence of O-side chain could conceivably decrease complement fixation and provide a degree of serum resistance on *Bartonella*, but this possibility has not been explored. The unusual fatty acid composition renders *Bartonella henselae* endotoxin at least 1000-fold less potent at Toll-like receptor (TLR)4 activation (as measured by IL-8 production), as compared with LPSs from *Salmonella* [3, 6].

LPS also serves as one of the primary targets of the innate arm of the mammalian immune system, whose Toll-like receptors (TLRs) are the primary Pathogen Recognition Receptors (PRR). A wealth of publications indicated TLR4 and TLR2 as the receptors involved in the recognition of most of the LPS studied [2, 3, 6, 7].

LPSs are known as endotoxins, which cause the prominent pathophysiological symptoms associated with sepsis and septic shock, that is, fever, leukopenia, hypotension, disseminated intravascular coagulation, and multiple organ failure [2, 3]. The well-known typical LPS from enteric bacteria, such as *Escherichia coli* and *Salmonella enterica*, are highly potent molecules with regard to their biological, that is, endotoxic activities [6].

3. Beneficial Activities of Typical LPSs

Naturally occurring (often typical) LPSs modulate the immune system of higher vertebrates in order to keep pathogens away and to avoid the possibility of saprophytes/commensals to become invaders (translocation); moreover, it has been demonstrated that the immune system is dependent on certain microbial products including LPSs for normal development [7].

Epidemiology studies in young children have found that LPS exposure at home is inversely correlated with the development of atopic diseases, following the "hygiene hypothesis" for allergic disorders [8].

The growing prevalence of broadly diffused chronic, inflammatory, and degenerative diseases in the industrialized world (allergic illnesses, diabetes and other metabolic disorders, inflammatory bowel diseases and, within the central nervous system (CNS), demyelinizing inflammatory pathologies, as well as stroke) might ask for a broadening of such "hygiene hypothesis" [9], which should also include the above reported chronic/inflammatory diseases [10].

In an asthma model, nonobese diabetic (NOD) mice were immunized intraperitoneally on day 0 with ovalbumin (OVA) in presence of alum, challenged one week later with 3 consecutive OVA aerosol administrations and analyzed 24 hrs after the last challenge. Following this protocol, mice presented allergic inflammation and abnormal lung function. Allergic inflammation resulted in an increase of cell recruitment including eosinophils in the BALF, and of cytokine and chemokine production, IL-4, and eotaxin, respectively, in the lung. Mice treated with TLR agonists, particularly LPS, showed a decreased eosinophilia and IL-4 and eotaxin production as compared to control mice [11].

In a NOD mice experimental model, the effect of TLR ligands, including LPSs, on development of spontaneous diabetes was evaluated. In NOD protected (LPS-treated) animals, the histological analysis of the pancreas showed a reduction in destructive islet infiltration (i.e., invasive insulitis). This form of insulitis is associated with active destruction of insulin-secreting β-cells; this is the point in time, where the first mice showing overt hyperglycemia can be seen. It appears that in the case of LPS treatment a control of insulitis progression and hyperglycemia can be observed [11, 12].

To address the intricate relationship between gut microbiota and host cells, colitis was induced in C57BL/6J mice with dextran sodium sulfate (DSS) or by transferring CD45Rb(hi) T cells into RAG1$^{-/-}$ mice. Colitis severity was assessed by disease activity index (DAI) and histology. The effect of anti-TLR4 antibodies (Ab) on the inflammatory infiltrate was determined by cell isolation and immunohistochemistry. Mucosal expression of inflammatory mediators was analyzed by real-time PCR and ELISA. Blocking TLR4 at the beginning of DSS administration delayed the development of colitis with significantly lower DAI scores. Anti-TLR4 Ab treatment decreased macrophage and dendritic cell infiltrate and reduced mucosal expression of CCL2, CCL20, TNF-alpha, and IL-6. Anti-TLR4 Ab treatment during recovery from DSS colitis resulted in defective mucosal healing with lower expression of COX-2, PGE(2), and amphiregulin. In contrast, TLR4 blockade had minimal efficacy in ameliorating inflammation in the adoptive transfer model of chronic colitis. Therefore, anti-TLR4 therapy may decrease inflammation in IBD but may also interfere with colonic mucosal healing [12].

Deficient TLR signaling may cause an imbalance in commensal-dependent homeostasis, facilitating injury and leading to inflammatory bowel disease. Accordingly, systemic administration of a TLR4-blocking antibody impairs restoration of tissue integrity during DSS-colitis, despite limiting exaggeration of acute inflammatory responses induced by recruited cells. Several recent studies suggest that TLR signaling exerts many important cytoprotective functions in the intestinal epithelium (and adjacent cell subsets), which are required for barrier preservation, cell survival and stability, and restitution, including, for example, inhibition of apoptosis, migration, and proliferation [13, 14].

Thus TLR4 agonists such LPS could be beneficial in colonic mucosal healing during IBD.

Animals exposed to LPS as neonates displayed induction of IL-10 within the CNS, and there was a robust inverse correlation between experimental autoimmune

encephalomyelitis severity and the frequency of CNS-infiltrating FoxP3$^+$ T lymphocytes. These observations were supported by reduced FoxP3 expression in brain tissue from multiple sclerosis (MS) patients compared with non-MS patients [15].

A small dose of LPS given systemically confers ischemic protection in the brain, a process that appears to involve activation of an inflammatory response before ischemia. LPS preconditioning in the brain shares some hallmarks that are characteristics of ischemic preconditioning in other organs. Interestingly, it has been reported that pretreatment of animals with LPS increases myocardial functional recovery in ischemia/reperfusion heart injury model. Such LPS-induced beneficial effect has been shown to be mediated through inhibition of NF-κB via increase of HSP70. These include delayed induction of tolerance after preconditioning and dependence on "de novo" protein synthesis. The systemic route of LPS administration and the induction of some systemic changes are unique aspects of LPS preconditioning that might offer some clinical advantages [14–16]. Also, the very recent paper by Mouihate et al. [17], underlined that early postnatal LPS exposure remodulates neuroimmune axis allowing enhanced activation of a novel prostaglandin-mediated activation of the hypothalamic-pituitary-adrenal (HPA) axis brought about by increased constitutive expression of TLR4 and COX2 [17]. Reprogramming the neuroimmune axis during infancy might be beneficial in the rest of animal and human life. Such LPS-driven tight regulation of overwhelming or inappropriate immune system activation would pay off during acute systemic inflammatory reaction (e.g., sepsis/septic shock) or severe allergic disorders (e.g., asthma attack) in adult life.

4. Beneficial Effects of Atypical LPSs

Some bacteria (e.g., *Bartonella, Yersinia, Rhodobacter, Chromobacterium*) contain an atypical LPS with low endotoxic activity and/or prominent antagonistic effect on LPSs from enteric bacteria [2, 17–19].

Coevolution of organisms bearing a deeply modified/atypical LPS with a vertebrate host would be beneficial to both of them. Indeed the microorganisms factors including LPS may reduce/inhibit the inflammatory potential of same tissue/district (respiratory and digestive mucosal, CNS).

Rough mutants of *Yersinia enterocolitica* exhibited atypical LPS and attenuated virulence and lack of ability to colonize organs as spleen and liver. Even more interestingly such mutants showed a substantial impairment of several other virulence factors, which depend on a full structure of LPS for proper function and/or expression [20]. Therefore, these strains might be exploited for preparation of vaccines or adjuvants.

Similarly to other atypical LPS-bearing bacteria also *Bartonella spp.* are endowed with anti-inflammatory activities which might be exploited for medical purposes [18, 19].

Bartonella spp.LPS have been found to behave in a manner that is substantially different from other LPSs from saprophytic, commensal, and pathogenic microorganisms.

Matera et al. [5] reported that *B. quintana* LPSs exhibited a migration pattern of the deep rough chemotype, a strong reactivity following the chromogenic Limulus amoebocyte lysate test and a very low cytokine release from human whole blood samples. In human leucocytes or in endothelial cells [21], as well as in a rat model [22], *B. quintana* LPS was not able to induce significant levels of blood TNFα. Moreover, *B. quintana* LPS induced an increase in the white blood cell count without a substantial change in heart rate, hematocrit, platelet count, or blood pressure [22]. Remarkably, *Bartonella quintana* LPS possesses antagonistic properties for TLR4 and does not activate TLR2 [18, 19]. However, the physical-chemical features of *B. quintana* LPS warrant further investigations for a more in-depth knowledge of the structure-activity relationship.

The atypical LPS attributes undoubtedly contribute to the establishment and maintenance of mild although persistent infection, since the bacterium's major surface component is subinflammatory and antagonistic to the host's innate immune response. Interestingly, long-chain fatty acids are a conserved feature in the LPS of intracellular bacteria that establish long-term symbioses with their host, including *Legionella, Chlamydia*, and closely related rhizobia [2].

The control of inflammatory illnesses and the decrease of allergic/atopic disorders might be obtained by the administration of such antagonistic LPS species. Also the control of experimental rheumatic disease has been obtained by administration of TLR4 antagonist LPS from *B. quintana* [23]. However, impaired NF-κB translocation by LPS pretreatment was also observed in TLR4-transfected overexpressing cells, suggesting that downregulation of TLR4 or TLR4 antagonism are not necessary events in impaired signal transduction in LPS-tolerant cells/tissues [24] and pointing to downstream site(s) of regulation and control of TLRs-dependent cascades carried out by LPSs and other bacterial products [7].

The complex population of microbes that we harbor within our mucosal cavities is not just passive bystanders, rather these organisms seem to actively shape our immune system responses both along the mucosal surface and in very remote tissues/organs [11].

Therefore, we suggested that some pivotal virulence factors, such as LPSs, control broad and increasingly diffused chronic, inflammatory, and degenerative diseases during the human evolution [25].

More interestingly some atypical LPSs could be plausible candidates to be developed into useful drugs for many diseases such as allergic illness, inflammatory bowel disease, and demyelinizing pathology of CNS [25].

5. LPS Derivatives as Adjuvants and Vaccines

Furthermore, enzymes involved in Lipid A biosynthesis/modification [3] not only provide access to new lipid A derivatives that may be useful as adjuvants or endotoxin antagonists, but also can be exploited for generating novel live bacterial vaccines. Heterologous expression of lipid A modification enzymes like LpxE, LpxF, LpxR, or PagL in

pathogens such as *Salmonella* might attenuate these bacteria by altering lipid A structural elements recognized by the TLR-4/MD2 complex [3].

Monophosphoryl lipid A (MPL) has been obtained from *Salmonella minnesota* R595 by removal of core KDO, one phosphate and one acyl chain from disaccharide backbone. MPL is among the recently licensed adjuvants and is used in combination with alum in recently approved vaccines for human papillomavirus and hepatitis B virus. Adjuvants can modify the delivery of the antigen or act as immunopotentiators, influencing both the amount and the quality of the adaptive immune response. Delivery can be modified through the slow release of antigen and enhancement of uptake by APCs in emulsions and liposomes, for example, whereas immunopotentiators act through the activation of the innate immune system [26].

6. Conclusions

While it seems clear that the microbiota influences progression and/or prevention of disease, the mechanism by which it can accomplish this task remains to be assessed. We have presented evidence that there is an intimate relationship between host and microbe that involves bacterial LPSs and host intricate mechanisms.

As many other molecules in biology, LPSs appeared as a "double-edged sword" [19]. Beneficial activity of both typical and atypical endotoxins look promising for the development of new drugs for prevention and the therapy of several human diseases.

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Conflict of Interests

The authors declare that they have no competing interests.

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Research Article

Essential Role of Mast Cells in the Visceral Hyperalgesia Induced by *T. spiralis* Infection and Stress in Rats

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Mast cells (MCs) deficient rats (Ws/Ws) were used to investigate the roles of MCs in visceral hyperalgesia. Ws/Ws and wild control (+/+) rats were exposed to *T. spiralis* or submitted to acute cold restraint stress (ACRS). Levels of proteinase-activated receptor 2 (PAR2) and nerve growth factor (NGF) were determined by immunoblots and RT-PCR analysis, and the putative signal pathways including phosphorylated extracellular-regulated kinase (pERK1/2) and transient receptor potential vanilloid receptor 1 (TRPV1) were further identified. Visceral hyperalgesia triggered by ACRS was observed only in +/+ rats. The increased expression of PAR2 and NGF was observed only in +/+ rats induced by *T. spiralis* and ACRS. The activation of pERK1/2 induced by ACRS occurred only in +/+ rats. However, a significant increase of TRPV1 induced by *T. spiralis* and ACRS was observed only in +/+ rats. The activation of PAR2 and NGF via both TRPV1 and pERK1/2 signal pathway is dependent on MCs in ACRS-induced visceral hyperalgesia rats.

1. Introduction

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder seen by gastroenterologists. Patients classically present with chronic abdominal pain, associated with an alteration in bowel habits, and visceral hyperalgesia which is generally considered to be a hallmark of IBS by their lowered thresholds for pain, increased intensity of sensations, and/or exaggerated visceromotor response to colorectal distension (CRD) [1, 2]; however, the underlying pathogeneses of visceral hyperalgesia are still unknown.

At present, growing evidences have indicated that mast cells (MCs) play an important role in visceral hyperalgesia [3, 4]. MCs take part of host defense against parasitic and bacterial infections. A subset of patients with IBS have an increased number of MCs in the colonic mucosa [5]. It has also been shown that MCs infiltration and release of mediators in the close proximity of mucosal innervation may contribute to abdominal pain perception in IBS patients [6]. Accumulated evidences have indicated that MCs tryptase is known to be involved in promoting pain and visceral hyperalgesia by activating the proteinase-activated receptor 2 (PAR2) which is expressed on primary afferent nociceptive neurons [7]. Nerve growth factor (NGF) can be released from MCs due to stimulus-induced degranulation, which also plays a pivotal role in colonic hyperalgesia [8].

On the processing of signal transduction of pain sensation, the transient receptor potential vanilloid receptor 1 (TRPV1) is also expressed and colocalized with PAR2 on C-fiber primary sensory afferent neurons [9]. Previous studies have shown that upregulation of PAR2 and NGF enhance the activation of TRPV1 channel [10]. Moreover, as a sensor for thermal and acidic nociception, TRPV1 plays critical roles in the processing of visceral inflammatory pain [11]. It has been demonstrated that pancreatic pronociceptive stimuli with PAR2 agonists cause extracellular-regulated kinase (ERK 1 and ERK2) phosphorylation in the spinal dorsal horn through activation of TRPV1 channels [12]. Noxious stimuli cause phosphorylation of ERK (pERK) in the afferent neuron that contributes to facilitation of pain sensation and is often used as an immediate marker for excitation of afferent neuron following colonic nociception [12]. It has been revealed that NGF activates ERK1/2 and pERK1/2 inhibition decreases excitability in DRG neurons

in culture [13]. Although the relationship between visceral hyperalgesia and MCs in IBS animal models and patients has been reported from several laboratories, the essential role of MCs in the progress of various stimulate is not well understood. In the present study, on the basis of using MCs deficient rats, we attempted to identify colonic (PAR2 and NGF) and peripheral sensory neuronic alterations (pERK1/2 and TRPV1) that can be involved in the visceral hyperalgesia triggered by both intestinal infection and stress.

2. Materials and Methods

2.1. Animals. Male MCs deficient rats (Ws/Ws) and their normal wild-type littermates (+/+) were obtained from TGC Inc. (Kanagawa, Japan). Rats were housed in standard polypropylene cages containing 2.5 cm of wood chip bedding material, which was maintained at 22°C with an automatic 12 hour light/dark cycle. Rats received a standard laboratory diet and tap water ad libitum. The experiments were conducted when the rats reached approximately 12 weeks of age. All procedures were aimed to minimize both animal number and suffering of the animals and were approved by the Animal Care Committee of Peking University.

2.2. T. spiralis Induced Colitis. Rats were induced by administering 1.0 mL of 0.9% saline solution containing 1500 *T. spiralis* larvae by gavage. An equivalent volume of vehicle (saline) was administered into control rats. The postinfection (PI) rats were allowed to have a recovery during 100 days period following administration.

2.3. Acute Cold Restraint Stress Procedure (ACRS). Briefly [14], 100 days after recovery, one half of the control and one half of the PI rats were restrained in individual polymethyl methacrylate restraint cages, and these animals were designated as ACRS and PI + ACRS groups. The animals were then placed in their cold home cages at 4°C for 2 hour. ACRS was routinely performed between 10:00 AM and 12:00 AM.

2.4. Visceromotor Response to CRD. Sensitivity to CRD was determined using the abdominal withdrawal reflex (AWR) as previously widely described. The rats received a standard CRD procedure, and the first balloon dilation used was 1 mL, and then increasing phases of distension (0.2 mL ascending increments) were applied for 20 second every 5 minutes until the AWR score reached 3. This evaluation was performed by three independent observers, and the AWR score was assigned as follows: 0: no behavioral response to distension; 1: brief head movements followed by cessation of movement; 2: contraction of abdominal muscle without lifting of abdomen; 3: lifting of abdomen; 4: body arching and lifting of pelvic structure.

2.5. Immunofluorescence. L6S1 DRGs segments were removed and fixed for overnight in 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer saline (PBS) at 4°C and then cryoprotected overnight in 30% sucrose in PBS. The tissue was embedded in Tissue-Tek OCT compound medium (Sakura Finetek, USA) and frozen in isopentane at −45°C. Cryostat sections (10 μm) were postfixed with acetone (10 minute, −20°C), and permeabilised with 0.3% Triton X-100 for 2 hours, and then blocked with 10% normal goat serum in PBS with 0.3% Triton X-100 for 30 minutes at room temperature. Sections were incubated overnight at 4°C with rat anti-TRPV1 (1 : 500; Chemicon) and rabbit anti-phospho (p) ERK1/2 (pERK; 1 : 1000; Cell Signaling Technology). Sections were then washed with PBS and incubated for 30 minutes at 37°C with FITC-conjugated goat anti-rat (1 : 100) and FITC-conjugated goat anti-rabbit (1 : 100) antibodies (Sigma). Sections were used to counterstain with DAPI-fluoromount (SouthernBiotech, USA). Digital images of five slices per individual DRG per animal were captured under the same parameters in the fluorescence microscope (Leica DM3000, Leica Microsystems, Germany) at lower magnification (×200 objective). The mean gray level intensity for a region of interest of the images was determined by using Image Pro Plus 6.0 image analysis software system (Media cybernetics, Silver Spring, MD, USA).

2.6. Western Blot Analysis. L6S1 DRGs and distal colon were dissected, and the samples were homogenized in ice-cold RIPA lysis buffer containing 50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% v/v Triton X-100, 1% sodium deoxycholate, 1% SDS, and "Complete," mini, EDTA-free protease inhibitor cocktail (Roche Diagnostics, Germany). Proteins were separated in 10% SDS-polyacrylamide gel electrophoresis and then transferred to PVDF membranes (BioRad). Nonspecific binding sites were blocked for 1 hour with 5% nonfat milk in tris-buffered saline Tween-20 (TBST). The blots were then incubated overnight at 4°C with the following primary antibodies in 2% nonfat milk in TBST : PAR2 (1 : 200; Chemicon); rabbit anti-p44/42 MAPK (1 : 1000; Cell Signaling Technology, MA, USA); phospho-p44/42 (Thr202/Tyr204) MAPK (1 : 1000; Cell Signaling Technology, MA, USA); rat anti-TRPV1 (1 : 1,000, Chemicon); and rabbit anti-β-actin (1 : 2,000; CWBiotech; China). The membranes were then incubated in appropriate secondary antibodies (IRDye 800CW conjugated goat-anti-rat IgG, 1 : 10,000, or goat-anti-rabbit IgG, 1 : 10,000, li-cor, USA) for 1 hour at room temperature in darkness. Images of the bands in the membranes were captured and analyzed with a Licor odyssey scanner (Licor Biotechnology, USA). The relative expression of each protein was calculated as the ratio of signal density to β-actin density. The mean value of the two bands (pERK1 and pERK2) was calculated and normalized with the loading control (total ERK).

2.7. RT-PCR. The RNA extraction from colonic tissues was carried out using the RNeasy Micro Kit (QIAGEN) according to manufacturer's instructions. RNA concentration was determined by absorbance at 260 nm, and its integrity was verified by electrophoresis. The first-strand cDNA was synthesized from 2 μL of total RNA by using SuperScript II RNase H reverse transcription (Invitrogen) and oligo-(dT) 12–18 primers according to its protocol. Quantitative PCR was carried out using following primer while using

18S ribosomal RNA as an endogenous control. Samples were amplified in duplicate using the following thermal cycling conditions: 95°C for 3 minutes followed by 40 cycles of amplification at 95°C for 15 seconds and then 60°C for 1 minute to allow for denaturation and annealing-extension. After amplification, a dissociation curve was plotted against melting temperature to ensure amplification of a single product. Comparative cycle threshold values were recorded, and the relative expression of mRNA species was then quantified in duplicate using the $2^{-\Delta\Delta CT}$ method using iCycler optical system interface software (v2.0, Bio-Rad).

Primers for target genes included the followings:

NGF	Sense: 5′ AGCGTAATGTCCATGTTGTTCTACA 3′ Antisense: 5′ TGTCAAGGGAATGCTGAAGTTTAGT 3′
PAR2	Sense: 5′ CCGAACGAAGAAGAAGCACCCT 3′ Antisense: 5′ GGAGCAGTACATATTGCCGTAGAAA 3′
TRPV1	Sense: 5′ TGGTACTGTACTTCAGCCAACGC 3′ Antisense: 5′ GAACACGAGGTAGACGAACATAAA 3′
18S rRNA	Sense: 5′ GTAACCCGTTGAACCCCATT 3′ Antisense: 5′ CCATCCAATCGGTAGTAGCG 3′

2.8. Statistical Analysis. Data were presented as mean ± standard error of the mean (SEM). The statistical significance of data was determined using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) or the Student-Newman-Keuls (S-N-K) tests. Statistical calculations were performed using SPSS for windows (version 13.0; SPSS Inc., IL, USA). A P value of <0.05 was considered significant in all instances.

3. Results

3.1. Visceral Hyperalgesia Induced by ACRS Dependent on MCs. In response to CRD, the distension volume to reach AWR score = 3 was significantly lower in +/+ rats triggered by transient *T. spiralis* intestinal infection ($P < 0.01$) and ACRS ($P < 0.01$). Although *T. spiralis* intestinal infection decreases visceral threshold of pain sensitivity to CRD in Ws/Ws rats ($P < 0.01$), it seems to be inoperative for Ws/Ws rats triggered by ACRS (Figure 1).

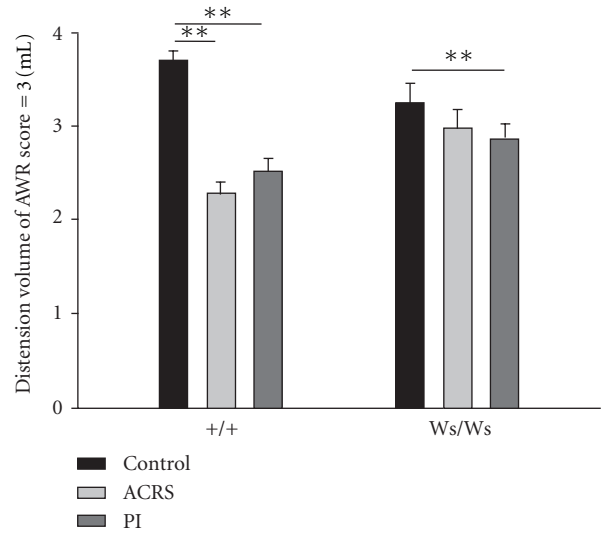

Figure 1: The distension volumes needed to reach an abdominal withdrawal reflex (AWR) score of 3 were significantly lower in postinfection (PI) rats, rats induced by acute cold restraint stress (ACRS), and PI rats received ACRS procedures (PI + ACRS). Each group represents the mean ± SEM of 6 rats. $^{**}P < 0.01$.

3.2. The Increased Transcription and Expression of PAR2 Induced by T. spiralis Infection and ACRS in Distal Colon Dependent on MCs. The changes in PAR2 expression in rats were confirmed using western blot analysis. As shown in Figures 2(a) and 2(b), the protein level of PAR2 increased to 1.38 ± 0.03 ($P = 0.001$) and 1.32 ± 0.04 ($P = 0.004$) in distal colon in +/+ rats induced by ACRS, and *T. spiralis* intestinal infection, respectively, compares with controls (1.07 ± 0.03) by quantitative densitometry of the immunoblots. Although we only see a significant difference ($P = 0.036$) between the PI group (1.82 ± 0.15) and control group (1.00 ± 0.09) in +/+ rats (Figure 2(c)) in PAR2 mRNA levels, neither ACRS nor *T. spiralis* intestinal infection had an effect on both PAR2 protein and mRNA levels in Ws/Ws rat.

3.3. The Increased NGF mRNA Levels Induced by T. spiralis Infection and ACRS in Distal Colon Dependent on MCs. Compared with control (1.000 ± 0.079), a significant upregulation of NGF mRNA induced by transient *T. spiralis* intestinal infection (2.274 ± 0.338, $P = 0.010$) and ACRS (2.213 ± 0.397, $P = 0.014$) in the distal colon in +/+ rats. However, the NGF levels induced by transient *T. spiralis* intestinal infection and ACRS have not been changed in Ws/Ws rats (Figure 3).

3.4. ACRS Evokes Phosphorylation of ERK in L6S1 DRGs Dependent on MCs. In this study, we determined pERK1/2 expression in L6S1 DRGs using immunofluorescence. It was found that ACRS mediates a 0.6-fold increase in pERK1/2 immunoreactivity- (IR-) positive neurons in L6S1 DRGs in +/+ ($P = 0.001$) but not Ws/Ws rats (Figures 4(a)–4(g)). ERK phosphorylation was further confirmed by western blot with a phospho-ERK1/2 antibody. The mean immunoblot band density for pERK1/2 was greatly increased not only in +/+ rats induced by ACRS ($P < 0.01$) and *T. spiralis* intestinal infection ($P < 0.05$) but also in Ws/Ws rats induced by

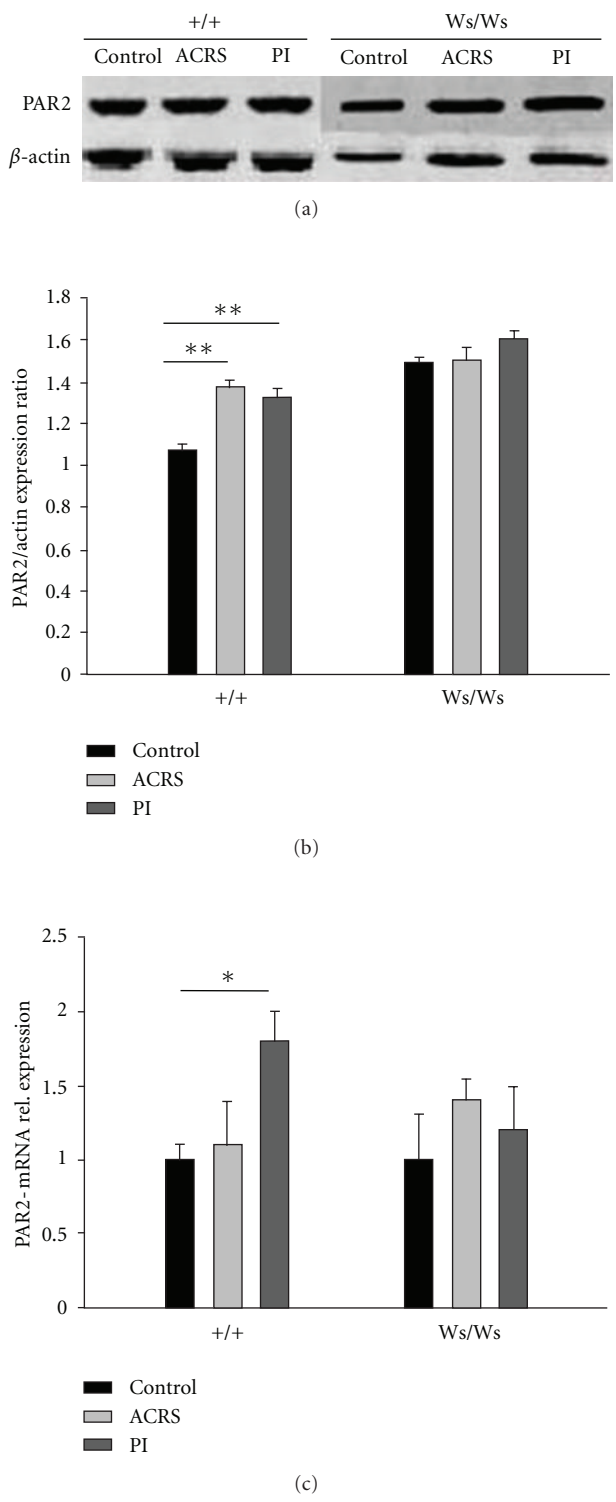

Figure 2: The increased PAR2 expression induced by intestinal infection (PI) and acute cold restraint stress (ACRS) in distal colon of +/+, but not Ws/Ws, rats. (a) Representative western blotting for PAR2 in extracts from colon tissue. (b) Quantitative analysis of PAR2 protein. Data was expressed as normalized density to β-actin. (c) Relative levels of PAR2 mRNA in colon tissue. Data was normalized to 18S ribosomal RNA and expressed using the $2^{-\Delta\Delta Ct}$ method. $^{*}P < 0.05$; $^{**}P < 0.01$.

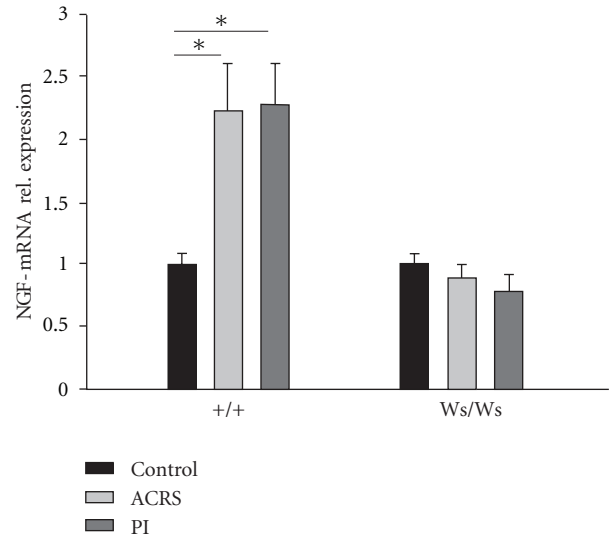

Figure 3: Relative levels of NGF mRNA were increased in the distal colon in +/+ but not Ws/Ws rats induced by intestinal infection (PI) and acute cold restraint stress (ACRS). Data was normalized to 18S ribosomal RNA and expressed using the $2^{-\Delta\Delta Ct}$ method. $^{*}P < 0.05$.

T. spiralis intestinal infection ($P < 0.01$). However, ACRS seems to be inoperative in visceral hyperalgesia in Ws/Ws rats (Figures 4(h) and 4(i)).

3.5. The Increased TRPV1 Expression Induced by T. spiralis Intestinal Infection and ACRS in L6S1 DRGs in +/+, but Not Ws/Ws, Rats. Immunofluorescence was used to assess the expression of TRPV1 in L6S1 DRG neurons. As shown in Figures 5(a)–5(g), in +/+ rats, the cytoplasmic TRPV1 IR signal was (0.047 ± 0.002) in L6S1 DRGs from control animals, whereas it was significantly increased to 0.09 ± 0.007 ($P = 0.004$) in ACRS group and 0.100 ± 0.016 ($P = 0.001$) in PI group. No changes were observed in TRPV1 IR signal in Ws/Ws rats. The TRPV1 expression was further confirmed by western blot analysis. Compared with control group, the mean immunoblot band density for TRPV1 was also greatly increased not only in the ACRS group ($P < 0.05$) but also in the PI group ($P < 0.05$) in +/+ rats. In contrast, there is no significant difference observed in TRPV1 protein levels in Ws/Ws rats (Figures 5(h) and 5(i)).

4. Discussion

In this study the proposed MCs deficient rats (Ws/Ws), triggered by transient *T. spiralis* intestinal infection and ACRS, have been used to investigate the effects of MCs on the visceral hyperalgesia. In our study, we found that *T. spiralis* intestinal infection and ACRS can cause visceral hyperalgesia in wild control rats. However, its lose effect on visceral hyperalgesia induced by ACRS in Ws/Ws rats. In our previous study we have demonstrated that the number of MCs was enhanced in rats induced by infection. However, stress could not stimulate the hyperplasia of MCs. Furthermore, when we observed the MCs with transmission electron microscopy, we

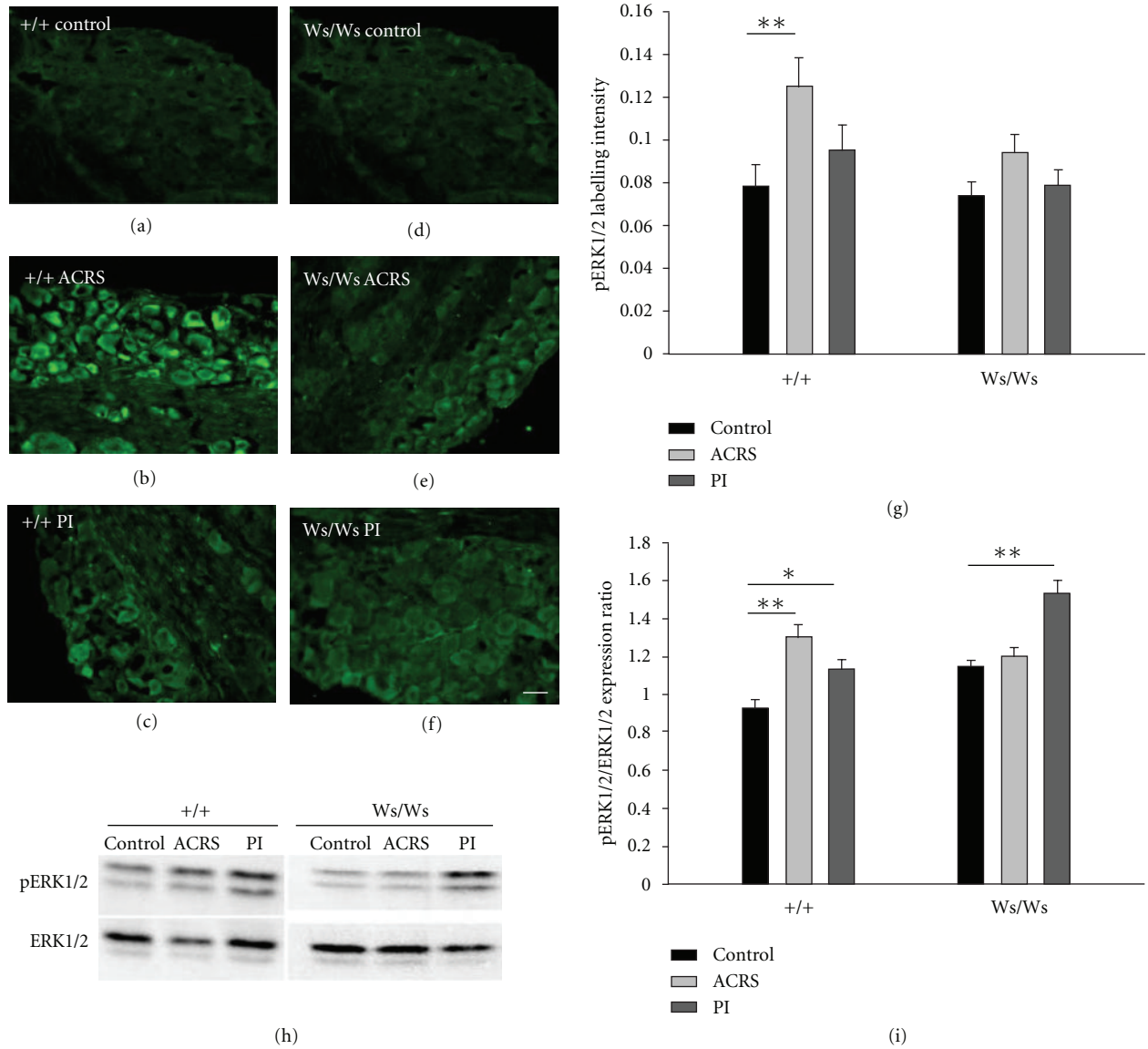

Figure 4: Role of ERK phosphorylation in +/+ and Ws/Ws rats induced by intestinal infection (PI) and acute cold restraint stress (ACRS). (a–f) Representative immunofluorescence images of pERK1/2 immunoreactivity- (IR-) positive neurons in L6S1 DRGs in +/+ rats (a–c) and Ws/Ws rats (d–f). Scale bar: 100 μm. (g) Quantification of pERK1/2 IR labelling intensity in L6S1 DRGs. (h) Representative western blot for phosphorylated ERK1/2 in L6S1 DRG extracts using a phospho-ERK specific Ab (pERK1/2, upper panel). Protein loading was confirmed by reprobing the membrane with ERK1/2 Ab (lower panel). (i) Quantitative analysis of phosphorylated ERK1/2 protein. Data was expressed as normalized density to ERK1/2. $^*P < 0.05$; $^{**}P < 0.01$.

noticed that, in the wild control rats, the MCs of the control group and infection group retained a full complement of electron-dense secretory granules, while those of the stress groups underwent piecemeal degranulation [15]. Thus we deduced that visceral hyperalgesia induced by ACRS is dependent on MCs, although the accurate pathogenesis is still unknown.

We investigated the role of PAR2 and NGF in rats due to their close relationship with MCs. MCs are some important proinflammatory cells, which not only participate in host-defense immune responses but also regulate the functions of peripheral nerves and smooth muscles in the gastrointestinal tract [16]. Upon activation, mucosal MCs released act on PAR2 to sensitize sensory afferents in the proximity [17]. PAR2, a G-protein-coupled receptor for trypsin and MCs tryptase, has been identified in colonic myocytes, enterocytes, enteric neurons, terminals of mesenteric afferent nerves, and immune cells [18]. It has been demonstrated that activation of PAR2 on the plasma membrane of nociceptive DRG neurons innervating the mouse colon leads to sustained hyperexcitability, and ERK1/2 mediates the PAR2-induced hyperexcitability [19]. Intracolonic administration of the synthetic selective PAR2 agonist in rats increases paracellular permeability and produces visceral hyperalgesia [17]. PAR2-mediated dysfunction of colonic epithelial barrier and subsequent allodynia or hyperalgesia may play an important role

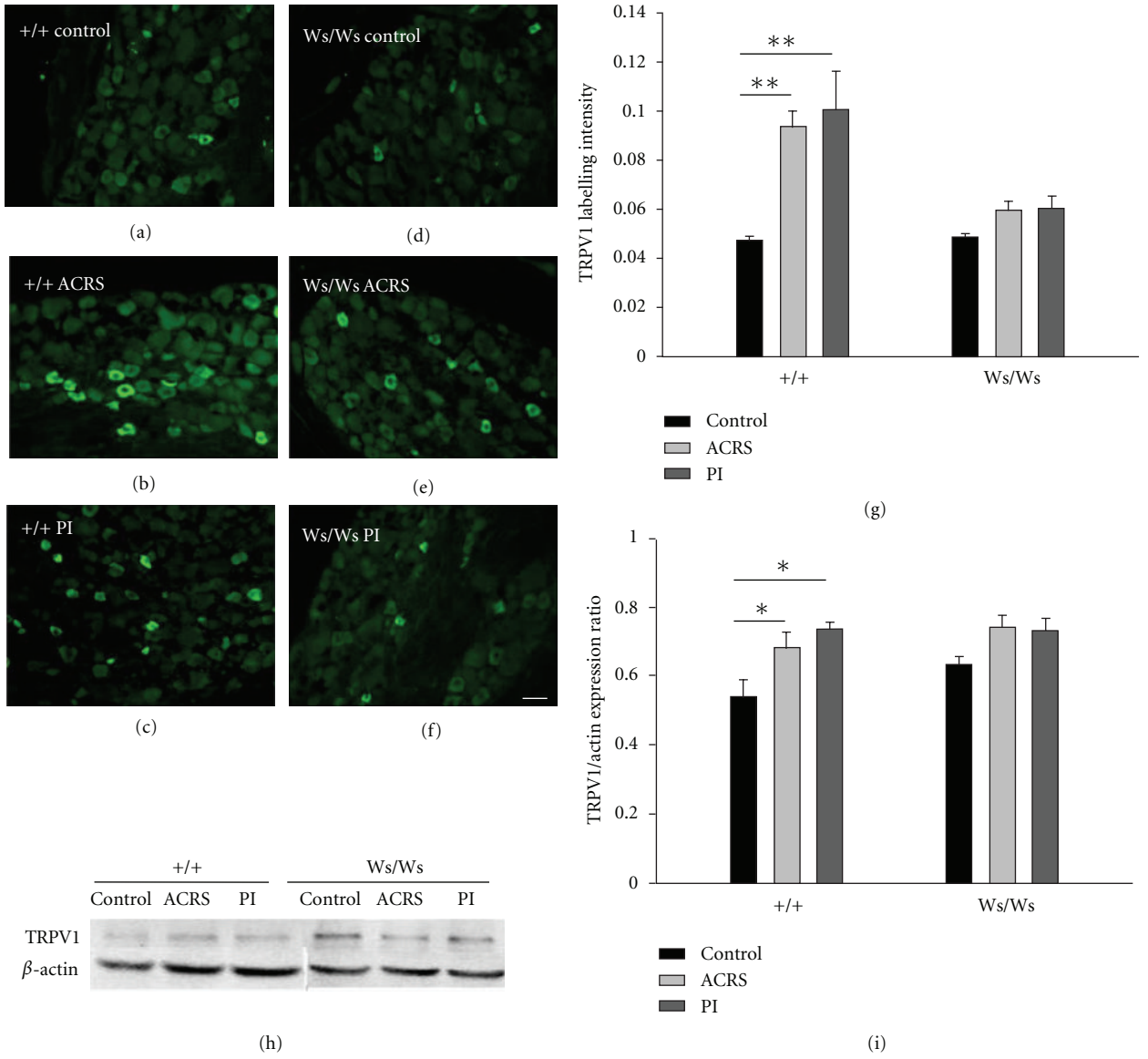

Figure 5: The expression of TRPV1 in L6S1 DRG neurons in +/+ and Ws/Ws rats induced by intestinal infection (PI) and acute cold restraint stress (ACRS). (a–f) Representative immunofluorescence images of TRPV1 immunoreactivity (IR)-positive neurons in L6S1 DRGs in +/+ rats (a–c) and Ws/Ws rats (d–f). Scare bar: 100 μm. (g) Quantification of TRPV1 IR labelling intensity in L6S1 DRGs. (h) Representative western blotting for TRPV1 in extracts from L6S1 DRG extracts in +/+ and Ws/Ws rats. (i) Quantitative analysis of TRPV1 protein. Data was expressed as normalized density to β-actin. $^{*}P < 0.05$; $^{**}P < 0.01$.

in the pathogenesis of IBS [20]. Our results demonstrated that the increased expression of PAR2 was induced by both *T. spiralis* infection and ACRS in distal colon in +/+ but not Ws/Ws rats. If the results of visceral pain threshold detected by AWR score are taken into account as shown in Figure 1, we concluded the upregulation of PAR2 induced by ACRS in visceral hyperalgesia dependent on the activation of MCs. Moreover, the activation of MCs plays an important role in the upregulation of PAR2 induced by *T. spiralis* infection in visceral hyperalgesia.

NGF may be produced in part by MCs and recognized as a potent immunomodulator, behaving like a bridge between neuronal and immune cells [21]. The peripheral stress mediator norepinephrine induces visceral hypersensitivity to CRD in response to heterotypic chronic stress by increasing the expression of NGF in the colon wall [22]. NGF can further sensitize afferent nociceptors directly by binding to the high-affinity receptor tyrosine kinase A (trkA) expressed on primary afferent neurons and indirectly by triggering MCs degranulation [23]. Moreover, there are interactions among them; that is, the increased NGF production can be induced by activation of MCs, whereas NGF induces histamine release from MCs [24, 25]. In addition, anti-NGF treatments were effective in preventing the motor alterations induced by the *T. spiralis* infection, that is, inhibited increased spontaneous motor activity and reversed

altered response to cholecystokinin (CCK) [26]. In this study we observed that the upregulation of NGF mRNA in intestine of +/+ rats, but not Ws/Ws rats, was induced by *T. spiralis* infection and stress. If the results of visceral pain threshold detected by AWR score are taken into account as shown in Figure 1, we concluded the upregulation of NGF induced by ACRS in visceral hyperalgesia dependent on the activation of MCs. At least we can infer that the interaction between MCs and NGF plays an important role in visceral hyperalgesia. Moreover, the activation of MCs may also be involved in the upregulation of NGF induced by *T. spiralis* infection in visceral hyperalgesia. As the NGF protein level is undermeasured with western blot method, we only detected the NGF mRNA by RT-PCR. This is the deficiency in our studies.

DRG is the primary afferent neuron in the information transmission of visceral sensation. Nociceptive processing in the visceral afferent neuronal pathways is thought to be mediated primarily via ERK and TRPV1 signal pathway. Thus, in our studies the changes of ERK and TRPV1 were investigated in rats. Visceral stimuli cause prompt phosphorylation of ERK in the spinal dorsal horn that contributes to facilitation of pain sensation and is often used as an immediate marker for excitation of spinal neurons following colonic nociception [27]. ERK1/2 is involved in the transduction of NGF neurotrophic signals by interactions with TrkA [28]. ERKs are phosphorylated in the nervous system after visceral stimulation or inflammation and play roles in central sensitization and pain hypersensitivity [29]. Our results demonstrated that phosphorylation of ERK1/2 can be induced by *T. spiralis* infections; however, the ACRS evokes phosphorylation of ERK1/2 in L6S1 DRGs dependent on MCs.

The TRPV1 pathway may also plays an important role in the process of information transfer from the peripheric receptor to central nervous system. Several studies indicated that TRPV1 plays a critical effect in visceral hyperalgesia and pain in IBS which may be associated with the increased MCs [30]. And evidences indicated that stress-induced visceral hyperalgesia in rats occurs in the absence of overt inflammation and dependents on MCs degranulation and subsequent TRPV1 activation [5]. PAR2 activation has been proven to sensitize several downstream TRPV1 channels via its G-protein-coupled receptor on sensory afferent to induce hyperalgesia [31]. If the results of visceral pain threshold detected by AWR score are taken into account as shown in Figure 1, we concluded the increased TRPV1 expression in DRGs induced by ACRS in visceral hyperalgesia dependent on the activation of MCs. Moreover, the activation of MCs also plays an important role in the increased TRPV1 expression in DRGs induced by *T. spiralis* infection in visceral hyperalgesia.

5. Conclusions

Our results have shown that the visceral hyperalgesia can not been triggered by stress in MCs deficient rats, although both stress and infection play an important role in visceral hyperalgesia in wild control rats. We also found that the upregulation of mediators (PAR2 and NGF) and signal proteins (pERK1/2 and TRPV1) has a close relationship with the presence of MCs. Our studies provide new evidence that the activation of PAR2 and NGF via both TRPV1 and pERK1/2 signal pathway is dependent on MCs in stress-induced visceral hyperalgesia rats. And MCs also play an important role in infection-induced visceral hyperalgesia rats.

Authors' Contribution

C.-Q. Yang and Y.-Y. Wei contributed equally to this work.

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