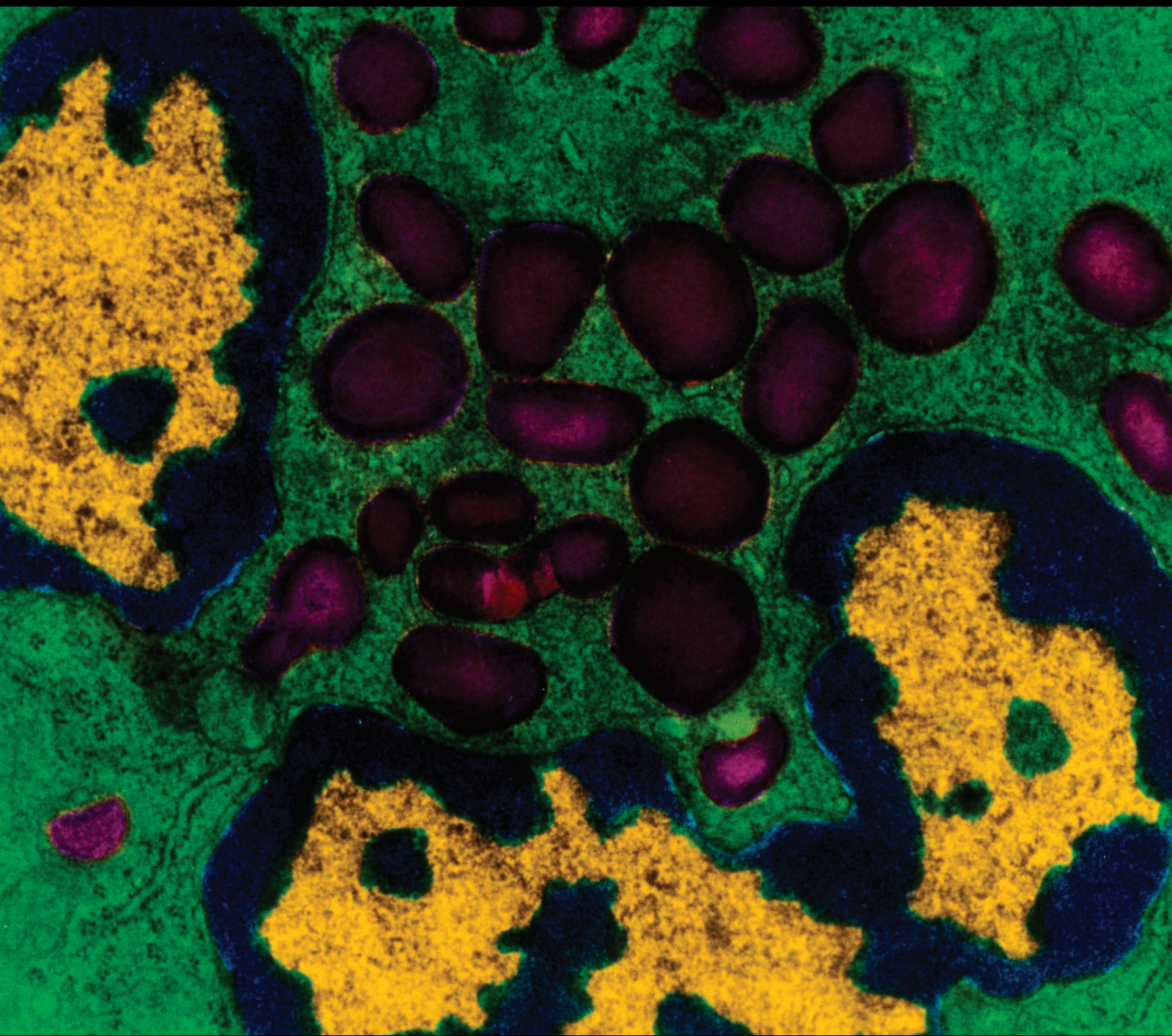
Mediators of Inflammation

Holding the Inflammatory System in Check: TLRs and NLRs

Guest Editors: Eda K. Holl, Irving C. Allen, and Jennifer Martinez



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Editorial Board

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 8156816, 2 pages
http://dx.doi.org/10.1155/2016/8156816

Editorial

Holding the Inflammatory System in Check: TLRs and NLRs

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Received 1 August 2016; Accepted 1 August 2016

Aberrant inflammation has been linked to the development of a diverse spectrum of human diseases. We now appreciate that Toll-like receptor (TLR) and Nod-like receptor (NLR) signaling pathways play an important role in the onset and/or severity of these pathologies, and targeting of these receptors has shown promise in preclinical studies of infectious diseases, autoimmune disorders, tissue injury, and cancer. Recently, clinical trials have been initiated to establish the efficacy of compounds targeting TLR and NLR pathways to improve disease outcomes. Despite advancements in the field, major challenges remain in potential ways to target these receptors at various stages of disease pathogenesis and develop effective therapeutics.

The articles contained in this special issue include reviews, translational studies, and basic science findings that are focused on characterizing the contribution and molecular mechanisms associated with TLR and NLR signaling in diverse disease processes. Together, these studies serve to illustrate the essential role that these pattern recognition receptors play in maintaining immune system homeostasis in both human disease pathobiology and preclinical animal models.

In particular, this special issue emphasizes the role of TLRs and NLRs in modulating signaling pathways associated with inflammatory disease processes and cytokine production. For example, in the article entitled "Effect of Toll-Like Receptor 4 on Synovial Injury of Temporomandibular Joint in Rats Caused by Occlusal Interference" by J. Kong et al., the authors show that activation of TLR4 participates in the initiation and development of synovial membrane inflammation by regulating the expression of inflammatory mediators like IL-1β. This inflammation reaction and increased IL-1β could be restrained by treatment with a TLR4 signaling inhibitor. In addition to TLR signaling and synovial injury, the importance of NLR family members is also explored in a series of articles evaluating their contribution either following traumatic brain injury (TBI) or in the context of parasite infection. There are currently several therapeutics targeting NLR family members under development to treat or attenuate brain injury following trauma. In this special issue, T. Brickler and colleagues examine the role of the NLRP1 inflammasome in TBI. In their article entitled "Nonessential Role for the NLRP1 Inflammasome Complex in a Murine Model of Traumatic Brain Injury" the authors conclude that the NLRP1 inflammasome has only a minor role in TBI using a moderate controlled cortical impact injury model. While some cytokine expression differences were observed in *Nlrp1*$^{-/-}$ mice, these animals showed no significant difference in motor recovery, cell death, or contusion volume, as compared to wild-type animals. Beyond the canonical NLRP1 inflammasome, the contribution of the recently described noncanonical NLR inflammasome is also explored in the context of parasite infection and toxoplasmosis. In the article entitled "Caspase-11 Modulates Inflammation and Attenuates *Toxoplasma gondii* Pathogenesis" by S. L. Coutermarsh-Ott et al., the authors explore the contribution of this unique inflammasome signaling pathway. Here, the authors show that caspase-11 functions to protect the host by enhancing

systemic inflammation during the early phase of *Toxoplasma gondii* infection and subsequently minimizes disease pathogenesis and brain cyst development during later stages of toxoplasmosis.

In addition to the preclinical animal studies described above, a trio of articles are also included in this special issue evaluating TLR and NLR regulation of inflammation as potential therapeutic targets in human disease. In the research article entitled "Maternal Vitamin D Level Is Associated with Viral Toll-Like Receptor Triggered IL-10 Response but Not the Risk of Infectious Diseases in Infancy" by S.-L. Liao et al., the authors investigate the role of maternal and cord blood vitamin D levels in TLR-mediated innate immune responses and its effect on infectious disease outcomes. The study concludes that maternal vitamin D, but not cord vitamin D, is inversely correlated with viral TLR-triggered IL-10 responses, yet it does not impact the risk of infectious disease in infancy. In addition to this research article, the review article by Z. Dong and colleagues, entitled "Holding the Inflammatory System in Check: TLRs and Their Targeted Therapy in Asthma," focuses on describing the role of TLRs and NLRs in asthma. While not often considered major components of allergic airway inflammation, the authors present a compelling overview of ways in which these receptors contribute to disease exacerbation and potential therapeutic avenues for targeting asthma in human patients. This article suggests that combination therapies of well-timed corticosteroids and TLR agonists may represent a more effective way to control inflammation in asthmatic patients. Finally, G. Lopez-Castejon and M. J. Edelmann present a highly mechanistic perspective on the role of deubiquitinases in TLR and NLR signaling. In the review article entitled "Deubiquitinases: Novel Therapeutic Targets in Immune Surveillance?" the authors review the role of deubiquitinases in the NF-κB pathway and inflammasome activation, two intrinsically related events triggered by the activation of the membrane TLRs as well as the cytosolic NLRs. The article also discusses the advances and challenges of using deubiquitinases as therapeutic targets during pathological inflammation.

With the ever-expanding realization of the role that inflammation plays in disease pathogenesis, we hope that this special issue will be of interest to the scientific community involved in studying TLRs and NLRs in the context of aberrant inflammation. We further hope that these articles emphasize the diverse function of these pattern recognition receptors in maintaining immune system homeostasis in a variety of human diseases.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 3481371, 13 pages
http://dx.doi.org/10.1155/2016/3481371

Review Article

Deubiquitinases: Novel Therapeutic Targets in Immune Surveillance?

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Received 24 March 2016; Revised 1 June 2016; Accepted 4 July 2016

Academic Editor: Luca Cantarini

Inflammation is a protective response of the organism to tissue injury or infection. It occurs when the immune system recognizes Pathogen-Associated Molecular Patterns (PAMPs) or Damage-Associated Molecular Pattern (DAMPs) through the activation of Pattern Recognition Receptors. This initiates a variety of signalling events that conclude in the upregulation of proinflammatory molecules, which initiate an appropriate immune response. This response is tightly regulated since any aberrant activation of immune responses would have severe pathological consequences such as sepsis or chronic inflammatory and autoimmune diseases. Accumulative evidence shows that the ubiquitin system, and in particular ubiquitin-specific isopeptidases also known as deubiquitinases (DUBs), plays crucial roles in the control of these immune pathways. In this review we will give an up-to-date overview on the role of DUBs in the NF-κB pathway and inflammasome activation, two intrinsically related events triggered by activation of the membrane TLRs as well as the cytosolic NOD and NLR receptors. Modulation of DUB activity by small molecules has been proposed as a way to control dysregulation or overactivation of these key players of the inflammatory response. We will also discuss the advances and challenges of a potential use of DUBs as therapeutic targets in inflammatory pathologies.

1. Introduction

Ubiquitination is a posttranslational modification (PTM) that involves the attachment of a ubiquitin molecule (~9 kDa) to a target protein. It is now well accepted that most of the cellular processes required for the maintenance of the cell homeostasis are regulated by the ubiquitin-proteasome system (UPS), including the regulation of innate immune signalling. Ubiquitination is mediated by a set of three enzymes, a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). Ubiquitin (Ub) is attached as a monomer or as polyubiquitin (poly-Ub) chains. This attachment occurs between a lysine group in the target protein and the carboxy-terminal glycine of Ub. The formation of Ub chains, however, occurs by formation of a bond between the carboxy-terminal glycine of Ub and one of the seven lysines (K6, K11, K27, K29, K33, K48, and K63) or the methionine (M1) present in the acceptor Ub molecule [1] allowing the generation of a wide variety of poly-Ub chains. Each poly-Ub chain type will influence the fate of the target protein differently. For instance, K48-conjugated Ub chains are considered a signal for protein degradation at the proteasome while K63 and M1 chains play important roles in signalling pathways [1]. Ubiquitination is a reversible process, and its reversibility is mediated by a family of proteases called deubiquitinases (deubiquitinating enzymes, DUBs). Keeping the balance between the addition and removal of ubiquitin moieties is crucial in maintaining cellular homeostasis and any disturbances in this balance can have adverse consequences for the cell.

2. Mechanisms of Regulation of DUBs

The human genome encodes ~100 DUBs that fall into five different families. There are four thiol protease families,

the ubiquitin-specific proteases (USP), ubiquitin C-terminal hydrolases (UCH), ovarian tumour domain containing proteases (OTU), and Machado Joseph disease (MJD)/Josephin domain DUBs, and one zinc-metalloprotease group, the JAB1/MPN/Mov34 metalloenzyme family [2]. The main functions of DUBs are (i) generation/release of free ubiquitin from Ub precursors (*de novo* Ub synthesis), (ii) subtle editing of poly-Ub chains, and (iii) removal of the poly-Ub chains from substrates prior to degradation by proteasome-bound DUBs. DUBs, similarly to other proteases, are tightly regulated to avoid aberrant function that could be therefore detrimental to the cell. This is achieved by a combination of different layers of regulation at transcriptional and nontranscriptional levels.

As many other proteins DUBs are regulated at the transcriptional level. One of the best examples of transcriptional regulation is A20 (TNFAIP3), which is a member of the OTU family of DUBs. A20 expression levels are highly upregulated in a proinflammatory environment (i.e., in response to TLR4 activation) [3], reflecting its important role as a negative regulator in the inflammatory response, as we will discuss below. There are other DUBs, which are regulated in response to cytokines, including DUB1, DUB2, USP17 (DUB3), and OTUD-6B. DUB1 is specifically induced by IL-3, IL-5, and GM-CSF, while DUB2 is stimulated by IL-2. USP17 (DUB3) is involved in the regulation of cell growth and survival and it is regulated by the cytokines IL-4 and IL-6 [4]. Ovarian tumour domain containing 6B (OTUD-6B) is a DUB, whose expression in B lymphocytes is induced by secretion of IL-3, IL-4, IL-13, or GM-CSF. With prolonged stimulation, these cytokines have an opposite effect and instead lead to a decrease in OTUD-6B expression. A higher expression of OTUD-6B was associated with inhibition of cell growth, an increase in apoptosis, and arrest of cells in G1 phase [5].

DUBs are also heavily regulated at the activity level by different mechanisms. DUBs can acquire specificity due to recruitment factors that guide them towards a specific substrate. One example is USP10 that requires the protein MCPIP-1 (monocyte chemotactic protein induced protein 1) to interact with and deubiquitinate its substrate NEMO inhibiting the NF-κB signalling cascade [6]. In other cases binding of the substrate actively contributes to DUB catalysis. For instance, USP7, whose catalytic triad exists in an inactive configuration, changes towards an active one upon ubiquitin binding suggesting that USP7 catalytic domain is only fully active when a ubiquitin molecule is correctly bound [7]. The presence of DUBs in molecular complexes is a common way to modulate their activity. This mechanism is essential for USP1, an inefficient enzyme alone, but its activity highly increases when bound to the WD40-repeat protein UAF-1 due to conformational changes that increase its catalytic activity [8]. USP1 is involved in DNA damage response, mainly in the Fanconi anemia (FA) pathway where it mediates the deubiquitination of FANCD2 and FANCI, a crucial step for the correct function of the FA pathway [9, 10].

Additionally DUB activity can be further adjusted by posttranslational modifications such as phosphorylation or ubiquitination [11]. For instance, phosphorylation of CYLD at Ser418 or USP7 at Ser18 led to an increase in the activities of these two DUBs. In the case of CYLD, this modification can be induced by LPS (lipopolysaccharide) or TNF-α (tumour necrosis factor) treatment and it can suppress its deubiquitinating activity on TNF receptor-associated factor 2 (TRAF2) [12]. Furthermore, this phosphorylation also occurs in dendritic cells (DCs) treated with LPS/Lex, which leads to a diminished activity of CYLD but not to its complete loss. This effect can be reversed by an inhibition of DC-SIGN signalling and also by depletion of IKKε [13].

Changes in the cellular microenvironment can also have an effect on DUB activity. One example is the production of reactive oxygen species (ROS) generated during mitochondrial oxidative metabolism as well as in cellular responses to cytokines or bacterial invasion, which can inhibit cellular DUB activity by oxidation of the catalytic cysteine residue [14, 15].

To summarize, more than one regulatory mechanism can apply to certain DUBs, which highlights the importance of a fine and multifaceted control of DUB expression and activity.

3. Deubiquitination in TLR- and NLR-Mediated Immune Signalling

Innate immunity is triggered in response to either PAMPs, which are derived from microbial pathogens, or DAMPs such as ATP, cholesterol, or monosodium urate crystals. These danger signals are recognized by Pattern Recognition Receptors either at the cell membranes by Toll-Like Receptors (TLRs) or at the cytosol by receptors such as the NOD-like receptors (NLRs) [16]. Activation of these PRR receptors results in a variety of immune signalling cascades which lead to the induction of immune mediators and proinflammatory cytokines, such as TNFα or IL-1β, capable of triggering appropriate immune responses. These cytokines lead to the recruitment of immune cells to the site of infection or tissue damage, which initiates an inflammatory response. TLR- and NLR-mediated signalling is heavily controlled by the ubiquitin system, which plays an essential role in maintaining the appropriate regulation of these cellular pathways [1]. Although DUBs can be involved in many other inflammatory aspects, here we will discuss how DUBs contribute to TLR and NLR-induced pathways, focusing on the activation of two very important and related processes, NF-κB pathway and inflammasome activation.

3.1. TLR Signalling. TLRs are transmembrane glycoproteins, which play a key role in the immune response against microbes. Ten human TLRs have been identified to date and they either localize to the cell surface (TLRs 1, 2, 4, 5, 6, and 10) or have endosomal localization (TLRs 3, 7, 8, and 9 [17]).

There are two distinguishable pathways of TLR signalling, one via the MyD88 (myeloid differentiation primary-response protein 88) and the second one via TRIF (TIR domain containing adaptor protein inducing interferon α/β) and apart from TLR3, most other TLRs are associated with the MyD88 pathway [18]. Ubiquitination is critically

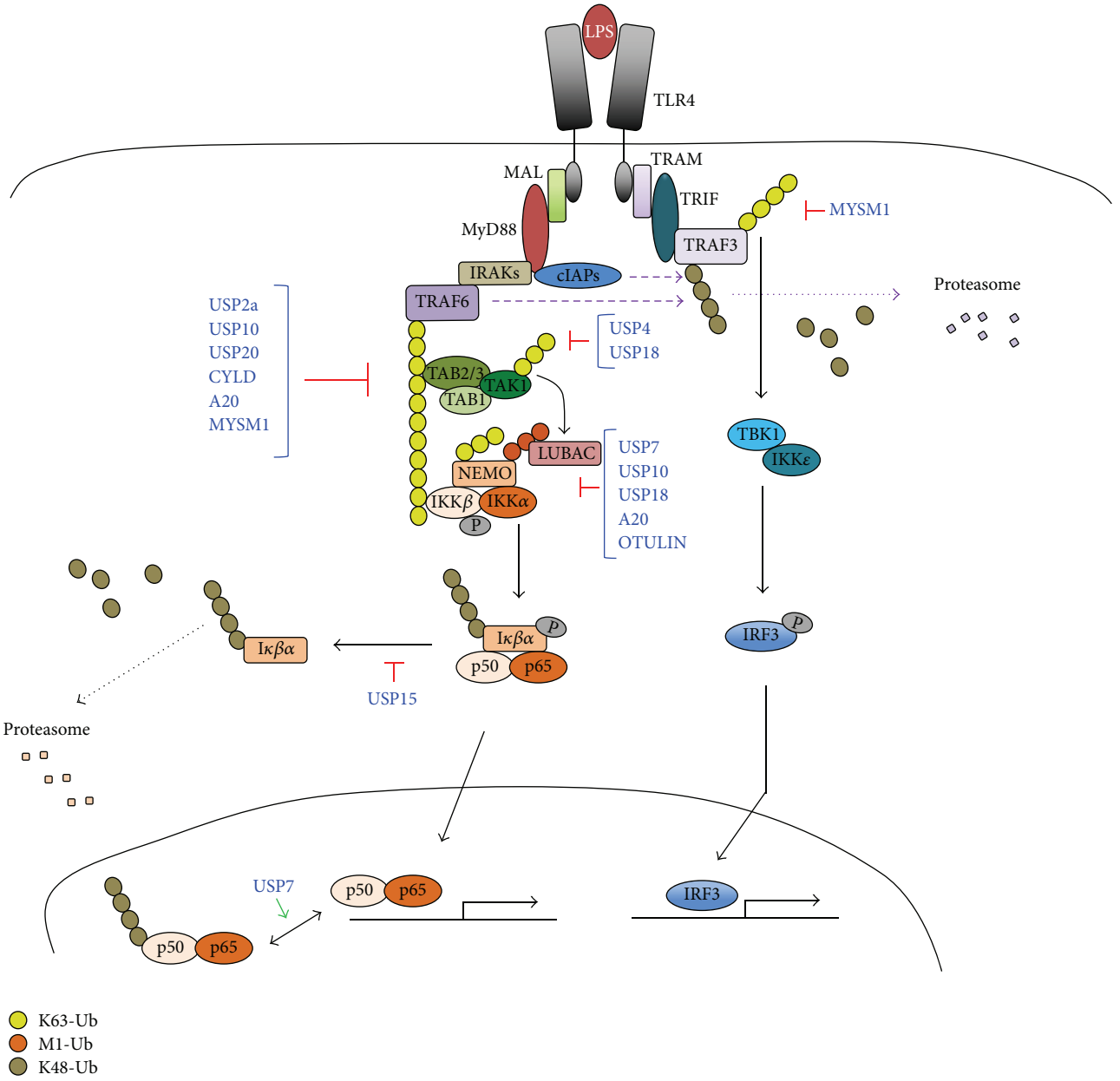

FIGURE 1: Regulation of TLR4 signalling by the ubiquitin-proteasome system. In MyD88-dependent signalling, TRAF6 and cIAP1/2s mediate K48 polyubiquitination and consequent degradation of TRAF3 by the proteasome. TRAF6 synthesizes K63 poly-Ub chains, which act as a scaffold for TAK1 and IKK complexes, TAB2/3 and NEMO. This occurs with the help of LUBAC, which leads to the linear ubiquitination of NEMO required for the recruitment of the IKK complex (IKKα and IKKβ). As a result, TAK1 phosphorylates IKKβ, which in turn phosphorylates IκB and subsequently undergoes ubiquitination and proteasomal degradation. This event frees NF-κB (p50/p65) to translocate to the nucleus and initiate transcription. Several DUBs (in blue) remove ubiquitin chains from TRAF6, NEMO, or NF-κB, negatively regulating this signalling pathway. USP7 can also prevent NF-κB degradation hence positively regulating transcription. MyD88-independent signalling occurs through TRAM/TRIF. In this case K63 poly-Ub chains are added to TRAF3, which consequently recruits the TBK1/IKKε kinase complex. This phosphorylates IRF3 allowing nuclear translocation and initiation of transcription. The DUB MYSM1 can deubiquitinate TRAF3, controlling the extent of this signalling.

involved in optimal TLR-triggered MyD88 and TRIF signalling (Figure 1). TLR3 engagement induces the recruitment of TRIF and modification of TRAF3 with K63 poly-Ub, which consequently recruits the TBK1 (TRAF family member-associated NF-κB activator-binding kinase)/IKKε kinase complex. Finally, this cascade of events causes IRF3 activation and INFγ production. In contrast, TLR4 or TLR2 activation leads to the assembly of the MyD88 signalling complex, recruiting TRAF6, cIAP1, and cIAP2. These ubiquitin ligases mediate K48-linked poly-Ub of TRAF3, and TRAF3

is consequently degraded by the proteasome [19]. TRAF6 ubiquitin ligase activity is essential for the synthesis of K63-linked poly-Ub chains, which act as a scaffold to recruit other proteins required for signalling. TRAF6 K63-linked poly-Ub chains recruit both the TAK1 and IKK complexes through their respective ubiquitin-binding subunits, TAB2/3 and NEMO. This occurs with the help of the LUBAC ubiquitin ligase complex, which leads to the linear ubiquitination of NEMO required for the recruitment of the IKK complex (IKKα and IKKβ). As a result, TAK1 phosphorylates IKKβ, which in turn phosphorylates IκB and subsequently undergoes ubiquitination and proteasomal degradation [20]. This allows NF-κB to translocate to the nucleus from cytosol and regulate the transcription of a variety of target genes (Figure 1).

Deubiquitination also plays a key role in TLR signalling pathways by reversing the effect of ubiquitination and controlling the intensity of the immune response (Figure 1). Several DUBs have been identified to participate in the TLR signalling, the most studied and best characterized being A20 (TNFAIP3) and CYLD. A20 plays an essential role in restricting TLR signalling and maintaining immune homeostasis. A20 contains an OTU domain, which has DUB activity specific towards several NF-κB signalling factors, such as TRAF6, RIPK1, or NEMO, which consequently leads to suppressed NF-κB activation [21]. A20 is an unusual DUB because it encodes seven zinc-finger (ZnF) motifs, which confer E3 ubiquitin ligase activity on A20. This allows A20 to perform an editing function: in addition to removing K63-linked polyubiquitin chains from substrates such as RIPK1, A20 can introduce K48-polyubiquitin chains in the same substrate tagging it for a proteasomal degradation [21]. In addition to this, A20 can also regulate NF-κB independently of its enzymatic activity. A20 can bind polyubiquitin chains through its ZnF domain allowing the interaction of ubiquitinated NEMO with A20. This ubiquitin-induced recruitment of A20 to NEMO is sufficient to block IKK phosphorylation by its upstream kinase TAK1 preventing NF-κB activation [22]. In contrast CYLD is a tumour suppressor, whose loss leads to familial cylindromatosis, a skin tumour hereditary disorder, but that also controls NF-κB activation. CYLD achieves this by specifically cleaving K63-linked poly-Ub chains and linear poly-Ub chains from RIPK1, TRAF2, and NEMO and similarly to A20 negatively regulates NF-κB signalling [23].

USP7 was first identified as a herpesvirus associated protein, hence its alternative name HAUSP (herpesvirus associated USP). USP7 presents dual roles in the regulation of NF-κB. It can regulate NF-κB transcriptional activity in the nucleus, by deubiquitinating NF-κB and preventing its degradation, hence increasing its transcriptional activity [24]. But USP7 can also act as a negative cytosolic regulator by deubiquitinating NEMO and consequently decreasing proteasomal degradation of IκBα. This in turn retains NF-κB in the cytoplasm and further suppresses NF-κB activity [25]. These two reported and opposing roles suggest that USP7 can perform different functions roles, depending on substrate recognition or cellular localization, highlighting the tight activity control of this protease.

As previously mentioned, USP10 is required for mediated inhibition of NF-κB activation. By mediating USP10-dependent deubiquitination of NEMO, MCPIP1 serves in a negative feedback mechanism for attenuation of NF-κB activation [6]. TRAF family member-associated NF-κB activator (TANK) interacts with both MCPIP1 and USP10, which leads to decrease in TRAF6 ubiquitination and the termination of the NF-κB activation in response to TLR activation [26]. In accordance with this, depletion of USP10 is associated with TLR-triggered increase in NF-κB activation [26].

USP18 is responsible for counteracting ISG15 conjugation and it is an important negative regulator of the IFN responses, thereby playing important roles in viral responses [27]. However, we now know that USP18 also mediates and regulates TLR-induced NF-κB activation by cleavage of K63-polyubiquitin chains, but not K48 chains, of TAK1 and NEMO [28].

In addition to the DUBs described here there are several others implicated in the downregulation of the NF-κB pathway upon TLR activation, although these are not well characterized. These include the USP family members USP2a, USP4, USP15, USP21, and USP31 and the member of the JAMM family MYSM1 and their substrates have been summarized in Table 1.

3.2. NLR Signalling. The NLR family presents a characteristic tripartite domain architecture with a variable C-terminus, a middle NACHT domain, and a Leucine Rich Repeat (LRR) N-terminus. The C-terminal LRR domain is involved in the ligand binding or activator sensing while the N-terminal domain performs effector functions by interacting with other proteins. NLRs are classified into four subfamilies according to their N-terminal domains: the acidic transactivation domain (NLRA), the baculoviral inhibitory repeat-like domain (NLRB) that includes NOD1 and NOD2, the caspase activation and recruitment domain (CARD; NLRC), and the pyrin domain (NLRP). NLRs can recognize a wide variety of ligands including pathogens, endogenous molecules, or environmental factors [29]. Their functions can vary and they are divided into four steps: inflammasome formation, signalling transduction, transcription activation, and autophagy [29]. Similarly to TLRs, NLR activation is also tightly regulated and PTMs play an important role here. Although ubiquitination in NLR signalling is well accepted, the role of DUBs in these pathways is just emerging.

3.2.1. NOD1 and NOD2. NOD1 and NOD2 receptors are important bacterial sensors, which recognize peptidoglycan (PGN). NOD1 senses the iE-DAP dipeptide, which is found in PGN of all Gram-negative and certain Gram-positive bacteria, while NOD2 recognizes MDP (muramyl dipeptide), the minimal bioactive peptidoglycan motif common to all bacteria (Figure 2(a)). Upon encountering with these ligands, NOD1 and NOD2 form oligomeric complexes, leading to the activation of NF-κB and MAPK. IAPs (cIAP1, cIAP2, and XIAP) are central regulators of NOD1 and NOD2 signalling. Upon oligomerization RIPK2 is recruited to this complex. cIAP1, cIAP2, and XIAP contribute to K63-linked

Table 1: DUBs involved in TLR, NOD1/2, or inflammasome activation. Knock-out mouse available for these DUBs has been indicated. Mouse model validation of target in which these mice have been used to demonstrate their function on that substrate. This table does not include studies where these mice have been used in other models of inflammation.

DUB	PRR	Target	KO mouse available	Mouse model validation of target	Ref.
USP2a	TLR	TRAF6	Yes	No	[89]
USP4	TLR	TAK1	Yes	No	[90]
USP7	TLR	NF-κB, NEMO	No, lethal	No	[24, 25, 82]
USP10	TLR	NEMO, TRAF6	No, lethal	No	[6, 26, 91]
USP15	TLR	IκBα	Yes	No	[92]
USP18	TLR	TAK1, NEMO	Yes	Yes	[28, 77]
USP20	TLR	TRAF6	No	No	[93]
USP21	TLR	RIPK1	Yes	No	[94, 95]
USP25	TLR	TRAF3	Yes	Yes	[96, 97]
USP31	TLR		Yes	No	[98]
A20	TLR	TRAF6, RIPK1, NEMO	Yes	Yes	[3, 76, 99, 100]
	NOD1/2	RIPK2		Yes	[34]
	NLRP3 inflammasome			Yes	[57, 58]
Cezanne	TLR	TRAF6	Yes	Yes	[101, 102]
OTULIN	TLR	NEMO	No, lethal	No	[103, 104]
	NOD2	RIPK2			[103, 105]
CYLD	TLR	RIPK1, TRAF2, NEMO	Yes	Yes	[12, 72]
MYSM1	TLR	TRAF3, TRAF6	Yes	Yes	[105, 106]
BRCC3	NLRP3 inflammasome	NLRP3	No	No	[51]

ubiquitination of RIPK2. This allows the recruitment of TAK1/TAB2/TAB3 complex and LUBAC, which can also mediate the linear ubiquitination of RIPK2, and further contributes to the NF-κB and MAPK pathway activation by ubiquitination of NEMO [30, 31]. Ubiquitin can directly bind to the CARD domain of NOD1 or NOD2 and compete with RIPK2 for its association with these receptors, suggesting that ubiquitin might play a negative regulatory role [32, 33] (Figure 2(b)). A20 also plays a regulatory role in NOD2 signalling by deubiquitinating RIPK2 to control the extent of the inflammatory signals. A20-deficient cells present an amplified response to MDP, including increased RIPK2 ubiquitination and NF-κB signalling [34].

One of the DUBs, which is relatively poorly characterized but which has been shown to play key functions in NOD2 signalling, is OTULIN. This protein specifically deconjugates linear (M1) poly-Ub chains assembled by LUBAC and in this way it modulates linear ubiquitination of LUBAC's substrates and provides fine-tuning of the initial activation of NF-κB. By deubiquitinating RIPK2, OTULIN prevents NEMO binding and hence decreases its downstream signalling. Because LUBAC continuously ubiquitinates itself and other substrates, OTULIN plays an important role to avoid accumulation of Met1-Ub chains and overactivation of this pathway [35] (Table 1, Figure 2(b)).

3.2.2. The Inflammasome. Another crucial function of NLR receptors is their contribution to the inflammasome. The inflammasome is a molecular complex, which consists of a sensor molecule (NLR, e.g., NLRP1, NLRP3, NLRC4, or NLRP6), an adaptor protein (ASC, apoptosis-associated speck-like protein containing a CARD domain), and an effector molecule (caspase-1) [36]. The main function of the effector molecule is to induce the cleavage and activation of the proinflammatory cytokines, IL-1β and IL-18. These proinflammatory proteins are synthesized as precursor molecules and require caspase-1 activation within the inflammasome in order to be released and cleaved and perform their biological activity. Activation of inflammasomes occurs in two steps. First, an NF-κB mediated initial step leads to increased expression of NLRP3 and pro-IL-1β. Then an activating signal triggers rapid activation of caspase-1. Caspase-1 activation can be achieved by several K^+-releasing molecules, including nigericin, crystals, or extracellular ATP through the activation of the ATP-gated P2X7 receptor (P2X7R) [37]. After the inflammasome is fully activated, it can lead to pyroptotic cell death, which can be distinguished from other cell death types by pore formation in the plasma membrane followed by osmotic cell lysis and finally the release of IL-1β and IL-18 [36].

Given the important role of ubiquitin in signalling cascades derived from TLR and NLR activation, it is not surprising to find that assembly and activation of an inflammasome is also regulated by the ubiquitin system. Ubiquitination can regulate canonical inflammasome activation by modulation of three major components: NLR, ASC, and caspase-1.

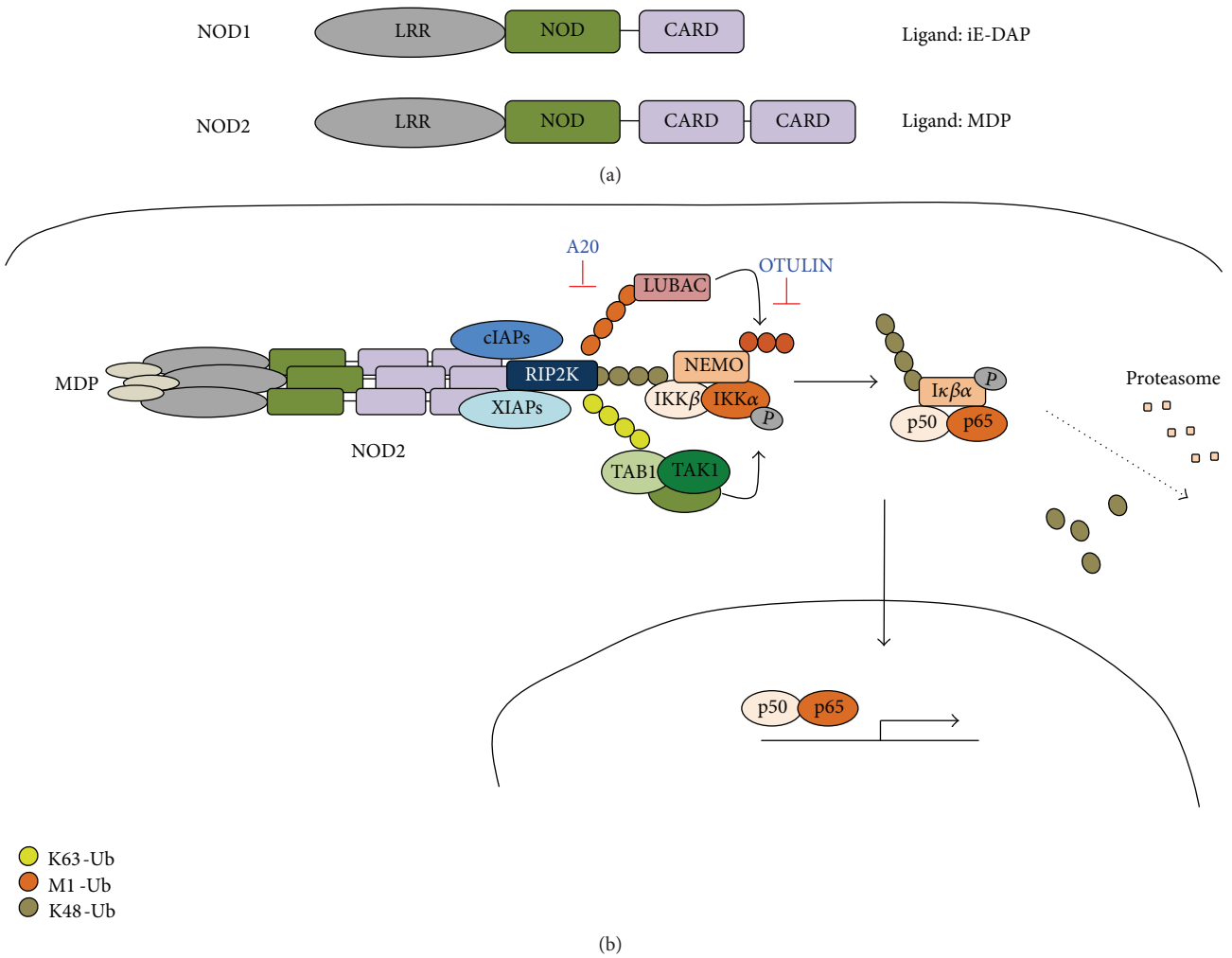

Figure 2: Regulation of NOD signalling by the ubiquitin-proteasome system. (a) NOD1 receptors recognize iE-DAP while NOD2 main ligand is muramyl dipeptide (MDP). (b) Similarly to NOD1, NOD2 receptors oligomerize upon ligand binding. This triggers the recruitment of RIPK2 to this complex and cIAP- and XIAP-mediated K63-ubiquitination of RIPK2. This allows the recruitment of TAK1/TAB2/TAB3 complex and LUBAC, which can also mediate the linear ubiquitination of RIPK2. TAK1 then phosphorylates IKKβ, which in turn phosphorylates IκB and subsequently undergoes ubiquitination and proteasomal degradation. This frees NF-κB (p50/p65) to translocate to the nucleus and initiate transcription. Deubiquitinases A20 and OTULIN are negative regulators of these events by deubiquitinating K63 and M1 poly-Ub chains, respectively.

Ubiquitin ligases can also directly influence NLRP3 inflammasome activation. This can be exemplified by MARCH7, which promotes ubiquitination of NLRP3, and this causes its degradation upon dopamine stimulation as a mean to control inflammasome activation [38]. Another example is SCFFBXL2, whose activity is impaired upon LPS priming preventing NLRP3 ubiquitination and its consequent degradation [39] (Figure 3). Other ubiquitin ligases have also been involved in control of NLRP3 ubiquitination. For instance, TRIM30 can negatively regulate NLRP3 inflammasome by modulating the levels of ROS species in the cell. TRIM30$^{-/-}$ macrophages produce higher levels of ROS and potentiate NLRP3 inflammasome activation; however the mechanisms by which TRIM30 controls this remain unknown [40]. However, TRIM33 is essential for cytosolic RNA-induced NLRP3 inflammasome activation. TRIM33 ubiquitinates DHX33, a cytosolic dsDNA sensor for NLRP3, allowing DHX33-NLRP3 interactions and consequent inflammasome activation [41]. Similarly to the NOD2 receptor activation, cIAP E3s are also involved in the inflammasome activation. Attenuation of cIAP activities, either by their deletion or by inhibition, triggers NLRP3 and caspase-1 activation as well as RIP3 kinase-dependent IL-1β processing and secretion [42].

On the other hand, cIAP1 and cIAP2 can attach K63-linked poly-Ub chains to caspase-1, thereby facilitating caspase-1 activation and IL-1β release [43]. Caspase-1 ubiquitination also occurs in response to the NLRP1 activator anthrax lethal toxin [43, 44] although the type of ubiquitin chains and whether this is a requirement for caspase-1 activation still remain unclear.

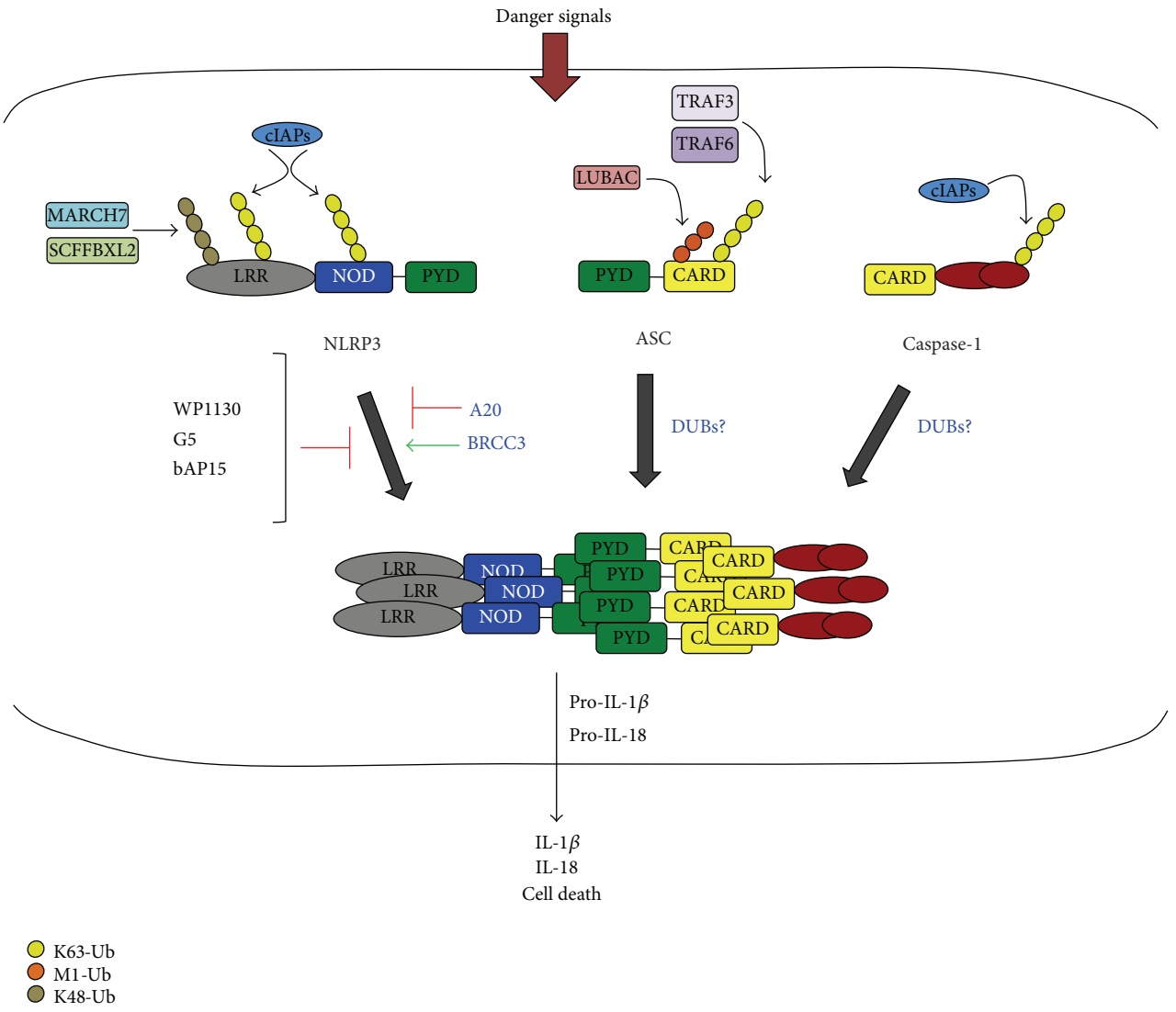

Figure 3: Regulation of the NLRP3 inflammasome activation by the ubiquitin-proteasome system. Assembly of the NLRP3 inflammasome complex occurs in response to a wide variety of danger signals including ATP, bacterial toxins, or particulate matter such as monosodium urate crystals. The ubiquitin ligases MARCH7 and SCFBXL2 add K48-linked poly-Ub chains to NLRP3 as a mean to control its levels by proteasomal degradation. cIAPs on the contrary add K63 poly-Ub chains to NLRP3 and caspase-1, contributing to the assembly of the complex. A20 also acts as a negative regulator of this complex. However, BRCC3 can deubiquitinate NLRP3, allowing it to form the complex and acting as a positive regulator of this pathway. TRAF3, TRAF6, and LUBAC also ubiquitinate ASC by K63 or M1 poly-Ub chains and this contributes to complex assembly. How other DUBs contribute to the assembly of the NLRP3 complex still remains unknown.

In addition to NLR and caspase-1, ubiquitin-mediated inflammasome activation can be also promoted by modification of the adaptor protein ASC. Activation of the inflammasome can induce autophagy as a mean to control inflammasome activation. In this situation, K63 poly-Ub modification of ASC allows for its interaction with the autophagic adaptor p62 and delivery of ASC to the autophagosome [45]. TRAF3 ubiquitin ligase ubiquitinates ASC, and abolishment of the target lysine (K174) prevents inflammasome activation and IL-1β release in response to viral infection [46]. Also, TRAF6-mediated ASC ubiquitination has been recently reported in response to far-infrared and proposed to constitute a mechanism, which dampens inflammasome activation in repair processes [47]. Interestingly ASC has been identified as a substrate of HOIL-1L, a member of linear ubiquitination complex LUBAC, and HOIL deficient macrophages present an impaired inflammasome response [48]. In line with this, macrophages deficient in SHARPIN, which is a different member of the LUBAC complex, are not able to mount an optimal inflammasome response [49].

All this evidence reveals that ubiquitination is an essential modification for the control of the inflammasome activation. It is then logical to assume that DUBs are important players of these regulatory mechanisms. This was first suggested by Juliana et al., who showed that NLRP3 is ubiquitinated in resting macrophages and that, upon cell activation with

priming (LPS) and activating signals (ATP, nigericin, and MSU crystals), these ubiquitin chains are removed by DUBs, allowing activation of the complex [50]. This report was quickly followed by two other studies supporting these results [51, 52], and it was Py et al. who identified BRCC3 as the first DUB to be directly involved in inflammasome activation. These reports showed that inhibition of DUB activity with the DUB inhibitors bAP-15, WP1130, PR-619, and G5 blocks NLRP3 but not NLRC4 or AIM2 mediated IL-1β release and pyroptosis (Figure 3; Table 1). Moreover, a recent report has demonstrated that histone deacetylase 6 (HDAC6) negatively regulates NLRP3 inflammasome activation. HDAC6 interacts with NLRP3's ubiquitin-binding domain and treatment with the DUB inhibitor PR-619 results in an increased interaction of NLRP3 with HDAC6. The authors suggest this is due to an increased ubiquitination of NLRP3 and the consequent inhibition of NLRP3-dependent caspase-1 activation [53]. The ability of these DUB inhibitors to block inflammasome activation could explain the inhibitory effect of the compound Bay 11-7082 on NLRP3 inflammasome independently of its NF-κB inhibitory activity [54] since this compound can inhibit components of the ubiquitin system, including DUBs [55, 56]. The other DUB, which has been directly implicated in the inflammasome activation, is A20. In contrast to BRCC3, A20 acts as a negative regulator of NLRP3 and suppresses inflammasome activation by restricting ubiquitination of IL-1β and NLRP3 activation [57, 58].

Given the fine-tuning and the layers of regulation required for both the inflammasome and DUB activation, it is quite likely to think that different DUBs might perform opposing functions pertaining to the inflammasome activation. Whether DUBs regulate the ubiquitination state of ASC or caspase-1 involved in the inflammasome assembly still remains unknown.

4. Pathogen Manipulation of DUBs to Control PRR Signalling

During pathogenesis, deubiquitinating enzymes are regulated both by microorganisms and by a host cell. Pathogens can exploit the host ubiquitin system by expressing their own ubiquitin-specific enzymes, and the host cell can up- or downregulate expression and/or activity of host DUBs [59].

First, an example of a pathogen-encoded deubiquitinase disturbing the host innate immune pathways is *Salmonella*'s AvrA, which is a DUB that facilitates inhibition of the NF-κB pathway. AvrA leads to stabilization of IκBα and prevents nuclear translocation of NF-κB p65. Also, depletion of AvrA in *Salmonella* leads to significantly increased secretion of cytokine IL-6 in the host cell, which is dependent on NF-κB pathway [60–63]. As a second example, *Chlamydia trachomatis* encodes two DUBs, ChlaDub1 and ChlaDub2, which are specific for ubiquitin but they also harbour deneddylating activity [64]. ChlaDub1 binds and stabilizes IκBα, most likely via its deubiquitination, and finally this can lead to an inhibition of NF-κB activation [65]. Since several known bacterial DUBs directly target important functions in the host immune system, development of selective inhibitors for pathogenic DUBs could be exploited as a therapeutic approach in the treatment of infections.

Bacterial infection can induce inflammasome activation in the host cell [36] and deubiquitination has been implicated in this process. *Salmonella* Typhimurium infection leads to changes in the activity of several host DUBs, such as USP4, USP5, UCHL3, and UCHL5, and increased activity of UCHL5 was found to contribute to the inflammasome activation during this infection [66]. Additionally, enteropathogenic *Escherichia coli* protein NleA associates with and interrupts deubiquitination of NLRP3, thereby repressing inflammasome activation [67].

5. Deubiquitinases and Inflammatory Disease

Accumulating evidence indicates that somatic mutations in DUBs are correlated with human disease. DUBs are genetically altered in many human cancers (i.e., CYLD, A20, or USP6) or contribute to the stability of oncogenes or tumour suppressors (i.e., USP7, USP8, or BRCC3) [68]. Here we will highlight DUBs with potential implications in immune disease although the scope for other DUBs contributing to disease is very high. Although many of the studies mentioned in this review have been performed *in vitro* in cell culture models, the involvement of DUBs in inflammatory responses has been also studied by using animal models, highlighting the relevance of these proteases in a relevant tissue and immune context (Table 1).

Mutations in the CYLD gene lead to a subtype of the benign cancer predisposition syndrome of skin appendages also known as Brooke-Spiegler syndrome, although inactivation or downregulation of CYLD is also observed in a variety of other cancers, including melanoma, and breast, colon, lung, breast, cervical, and, recently, prostate cancer. As previously mentioned CYLD can bind to NEMO and NF-κB that have been identified as its substrates. It is possible that the negative regulation of NF-κB mediated by CYLD contributes to its tumour suppression function given the increasingly recognized role for NF-κB in cancer advancement. CYLD deactivation could provide specific advantage to tumour cells by enhanced NF-κB signalling [69–71]. CYLD-deficient mice present abnormalities in their immune system. They show increased basal and induced NF-κB activation and can develop autoimmune symptoms and colonic inflammation with features of human inflammatory bowel disease [72], and their inflammatory responses in response to pathogenic infection are potentiated [73].

A20 is an important negative regulator of immune response as we have mentioned before. Multiple mutations in the A20 gene have been identified; however no inheritable syndrome has so far been linked with A20 abnormalities. This could be explained if these mutations were developmentally critical. A20 mutations are strongly linked to autoimmunity, lymphomas, and asthma [74, 75], highlighting important differences to CYLD despite both targeting NF-κB. This might be explained by different chain preference, K48 and K11 for A20 compared to the K63 and M1 chain preference showed by CYLD [68]. A20$^{-/-}$ mice fail to regulate NF-κB

responses, develop severe inflammation and are hypersensitive to LPS or TNFα leading to premature death [76]. Cell specific ablation of A20 has revealed important knowledge about the contribution of A20 to disease pathogenesis and generated very useful mouse models for several conditions like rheumatoid arthritis, lupus erythematosus, or inflammatory bowel disease [75].

USP18 has been thoroughly studied in the context of viral responses, since it regulates protein ISGylation in response to viral infection. However Liu et al. also demonstrated that USP18 deficient mice are resistant to experimental autoimmune encephalomyelitis (EAE) [77]. This study proposes that USP18 regulates TAK1-TAB interaction and is hence necessary for Th17 differentiation and autoimmune response.

DUBs can contribute to disease not only by mutations, but also by an altered expression or activity. An example of this is USP7, whose increased activity mediates the deubiquitination and destabilization of a number of critical tumour suppressors, including p53 or PTEN, and is by inference an oncogenic prosurvival protein. The interrelationship between p53, USP7, and MDM2 ubiquitin ligase is quite unique and complex. USP7 can deubiquitinate and stabilize p53, but interestingly it can also deubiquitinate and stabilize MDM2 indirectly leading to p53 destabilization and its degradation by the proteasome [78]. USP7 also interacts and stabilizes the ICP0 ubiquitin E3 ligase of herpes simplex virus (HSV), which is required for the effective initiation of the lytic cycle, facilitating lytic viral growth [79]. USP7 can also interact with other viral proteins, such as the EBNA1 protein of the Epstein-Barr virus (EBV) [80] and the Viral Interferon Regulatory Factor 1 (vIRF1) of a Kaposi sarcoma herpesvirus protein [81]. In addition, and as mentioned before, USP7 plays a role by regulating NF-κB signalling [24, 25]. Unfortunately USP7$^{-/-}$ mice are embryonically lethal explaining the lack of *in vivo* studies to further characterize the role of USP7 in immune responses and associated pathologies [82].

6. Modulating DUB Activity as a Novel Inflammatory Therapeutic Approach

Given the importance of DUBs in inflammatory and other pathological responses, it is certainly easy to think of DUBs as potential therapeutic targets, whose modulation could be beneficial for inflammatory conditions. However, up to date there are no DUB targeting compounds that have been approved for clinical use, either in the inflammatory or in cancer context. The identification and success of inhibitors that target other elements of the ubiquitin system suggest that altering inflammation by targeting the ubiquitin system, including DUBs, could be a viable approach to develop novel anti-inflammatory treatments. An example of successful development of UPS inhibitors has been achieved with the proteasomal inhibitors Bortezomib or Carfilzomib, which have been effected in multiple myeloma treatment [83]. Another compound, MLN4924 (Nedd8-E1 enzyme inhibitor), has reached phase I clinical trials [84] and SMAC mimetics, which promote proteasomal degradation of cIAPs, have recently proved to work in cancer patients through phase I clinical trials [85].

DUB targeting drugs present a great potential as novel therapeutic agents. DUBs present the advantage of being druggable targets since they have a catalytic domain, and unlike other UPS members, such as the E3 ubiquitin ligase family with approx. 600 members, targeting the DUB family seems an achievable target. Given the clear evidence of the contribution of DUBs to disease there is a considerable effort put into the development of compounds that modulate DUB activity. Intensive research is being channelled to develop selective DUB inhibitors, which could be applied to such diseases like cancer, neurological and inflammatory disorders, or infectious disease.

Despite these intensive efforts and great advances in the DUB field, selective compounds have not reached clinical trials yet. Although no DUB-selective compound has yet reached clinical trials, the field is moving fast and in the right direction. MISSION Therapeutics is developing new DUB inhibitors that present good oral bioavailability and low EC_{50}s in cell viability assays. Proteostasis Therapeutics in collaboration with Biogen is developing very promising USP14 inhibitor series, while Genentech and Almac might be developing a new therapeutic generation of USP7 inhibitors [86, 87].

This is due to two main challenges: first not all DUBs work in the same manner hence different strategies need to be followed to develop these compounds and second we do not completely understand how these enzymes function and/or are regulated. In addition, many of the studies, which address DUB functions, have been developed in *in vitro* systems using either isolated proteins or cell lines that are not relevant to function or disease. This might not reflect the reality of DUB behaviour in a tissue-specific context and more work has to be developed using *in vivo* mice models and primary human cells. To achieve this, new and more powerful tools are required, including in-cell based assays to discriminate selective DUB function and cytotoxicity and the development of inducible mouse models, which would allow for the study of tissue-specific DUB functions. It is fundamental that basic research and drug development teams work in close collaboration to allow the success of these compounds [86, 87].

Based on our actual knowledge on DUBs thinking that not all DUBs will be good therapeutic targets is likely, since some of them might share more than one substrate, which play opposing roles in different tissues or be essential to maintain homeostasis and health. For instance, targeting USP7 in the oncology context would be a good therapeutic strategy [88]; however we need to very carefully consider the possible effects of inhibiting USP7 on the inflammatory response to the tumour. Whether this would be detrimental or beneficial still remains unknown. Similarly, we could argue that potentiating A20 function in an inflammatory context would be a plausible treatment; however more detailed studies in the consequences of this approach are required. The presence of DUBs in pathogens causative of disease, such as virus, bacteria, or parasites, has also highlighted the possibility of developing DUB inhibitors, which specifically

target the pathogen and not the host. In the following years new knowledge emerging from ongoing research will allow scientists to discern those that constitute good targets and offer promising new alternatives to existing therapeutics.

7. Concluding Remarks

Immune responses are strongly regulated by the addition and removal of ubiquitin molecules, and although the roles of E3 ubiquitin ligases in these signalling pathways are well established, it is still unclear how DUBs contribute to PRR signalling. The advances in this field due to novel tools and approaches including advanced mass spectroscopic techniques, ubiquitin linkage-specific antibodies, and structural and biochemical studies will provide new insights into the regulatory mechanism of immune signalling molecules by DUBs and vice versa.

Since the involvement of DUBs in several inflammatory conditions is clear, development of potent and selective DUB-specific inhibitors or agonists could provide new therapeutics to treat these conditions. For instance, given the high regulation of NOD1/2 by ubiquitin and the contribution of NOD mutations to inflammatory diseases such as inflammatory bowel disease (IBD) or Crohn's disease, it is possible that DUBs could be used as a target in NOD-associated inflammatory conditions.

Similarly to the kinase research area 20 years ago the DUB field is in its infancy. There are many challenges that remain to be solved to further advance our understanding of DUB function, specificity, and activity and to develop compounds that inhibit this activity. However, the field is advancing quickly, and hopefully new highly selective DUB inhibitors will be developed very soon.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

Gloria Lopez-Castejon is supported by a Sir Henry Dale Fellowship from the Wellcome Trust/Royal Society (UK); Mariola J. Edelmann's work was supported by USDA National Institute of Food and Agriculture, Hatch Multistate Project FLA-MCS-005482.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 7694921, 7 pages
http://dx.doi.org/10.1155/2016/7694921

Research Article

Effect of Toll-Like Receptor 4 on Synovial Injury of Temporomandibular Joint in Rats Caused by Occlusal Interference

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Received 25 February 2016; Revised 9 May 2016; Accepted 25 May 2016

Academic Editor: Eda Holl

Synovitis is an important disease that causes intractable pain in TMJ. Some investigations suggested that the increasing expression of IL-1β secreted by synovial lining cells plays an important role in synovial inflammation and cartilage destruction in TMJ. In our previous research, the results demonstrated that TLR4 is involved in the expression of IL-1β in SFs from TMJ with lipopolysaccharide stimulation. However, the inflammatory response that occurred in synovial membrane is not caused by bacterial infection. In the current study, we investigated whether or not TLR4 participates in the inflammatory responses and the expression of IL-1β in synovial membrane of rats induced by occlusal interference. The results showed that obvious inflammation changes were observed in the synovial membranes and the expression of TLR4 and IL-1β was increased at both mRNA and protein levels in the occlusal interference rats. In addition, the inflammation reactions and the increased expression of IL-1β could be restrained by treatment with TAK-242, a blocker of TLR4 signaling. The results prompted us that the activation of TLR4 may be involved in the inflammatory reactions and increased expression of IL-1β in patients with synovitis and participate in the mechanisms of the initiation and development of synovial injury by regulating the expression of inflammatory mediators like IL-1β in synovial membranes.

1. Introduction

Temporomandibular disorder (TMD) is one of common and frequently occurring diseases in department of stomatology. A survey found that 9.7% of the population suffers from conditions covered by TMD group I diagnosis (myofascial pain) and 11.4% from conditions covered by TMD group II a diagnosis (disk displacement with reduction) [1]. The patients can experience some symptoms that seriously affect human normal life and work, for example, pain in Temporomandibular Joint (TMJ) and masticatory muscle and limited mouth opening. The mechanisms of the initiation and development of this disease are complicated and not completely clear, and a lot of etiologic factors may be attributed to the onset of disorder, as biomedical and psychological as well as psychosocial impact factors and occlusal interferences [2–4].

Synovitis is an inflammation mainly occurs in synovial membrane and joint capsule of TMJ. A series of investigations [5, 6] on TMD has revealed the occurrence of inflammation in the synovial membrane. Various inflammatory mediators are thought to be involved in joint pathology, including interleukin- (IL-) 1β, tumor necrosis factor- (TNF-) α, and matrix metalloproteinases (MMPs) [7–9]. IL-1β was reported to be expressed by synovial lining cells and endothelial cells of blood vessels [10], and it is suggested that the increasing expression of IL-1β plays an important role in synovial inflammation and cartilage destruction [11, 12].

In our previous research, we found that treatment with lipopolysaccharide (LPS) could increase Toll-like receptor (TLR) 4 (a transmembrane protein) and IL-1β expression at both mRNA and protein levels in synovial fibroblasts (SFs) separated from TMJ of rat, and the increased expression

of IL-1β could be blocked by treatment with TAK-242, a blocker of TLR4 signaling, and the cell surface receptor TLR4 is involved in the expression of IL-1β in SFs from TMJ with LPS stimulation [13]. However, the inflammatory response that occurs in synovial membrane is not caused by bacterial infection as we all know. In the current study, we created an occlusal interference animal model [14] to induce synovial injury by bonding crowns with a thickness of 0.6 mm to the right mandibular first molars of rats and describe inflammatory response in the synovial membranes and expression of TLR4 and IL-1β. Besides, we injected TAK-242 into upper compartment of TMJ to describe the change of TLR4 and IL-1β expression in synovial lining cells, and we wanted to investigate whether or not TLR4 participates in the inflammatory responses and the expression of IL-1β in rats induced by occlusal interference.

2. Materials and Methods

2.1. Subjects. Thirty-six male wistar rats (6-week old, obtained from the Shandong University Center of Laboratory Animals, China) were housed under a 12-h light/dark cycle with food and water available ad libitum. This study was approved by the Animal Care and Use Committee at the Shandong University.

2.2. Animal Model of Occlusal Interference. Rats were anesthetized with intraperitoneal injection of pentobarbital sodium (0.5%, 40 mg/kg). A metallic crown (0.6 mm, uniform thickness) was bonded to the right mandibular first molar using resin cement (Super-Bond C&B, Osaka, Japan). Crowns were fabricated using cobalt chromium casting alloys and designed to cover the occlusal, buccal, lingual, and medial surfaces of the molars. Sham-treated rats in the control group were anesthetized and their mouths were forced opened for approximately five minutes using a protocol similar to the occlusal interference groups; however, no crowns were cemented.

Thirty-six rats were randomly divided into three groups (twelve rats in each group) and treated as follows: (1) control group, these rats were anesthetized and mouths were forced open for about 5 min and received saline injections (10 μL, twice a week) into upper joint cavities of both sides of TMJs, (2) occlusal interference group, these rats were treated to create an occlusal interference animal model according to methods above and received saline injections (10 μL, twice a week) into upper joint cavities of both sides of TMJs, (3) TAK-242 group, these rats were treated to create an occlusal interference animal model according to methods above and received TAK-242 injections (3 mg/kg [15], diluted in 10 μL DMSO, twice a week; Invitrogen, San Diego, CA, USA) into upper joint cavities of both sides of TMJs.

2.3. Tissue Preparation. After two weeks, six rats in each group were randomly selected and were euthanized by overdose pentobarbital sodium. Then, rats were perfused with heparinized saline followed by a cold fixative containing 4% paraformaldehyde in 0.01 M phosphate buffer saline (PBS, pH 7.2). The right TMJs were removed, fixed in 4% paraformaldehyde, and then demineralized in 15% EDTA. After decalcification in 10% EDTA, the TMJs were dehydrated, embedded in paraffin, and sectioned on the sagittal plane at a thickness of 4 μm.

The other six rats in each group were also anesthetized with overdose pentobarbital sodium. The synovial tissues were harvested from the right TMJs, rinsed with cold sterile saline solution, and stored at −80°C for real-time quantitative polymerase chain reaction (PCR) assay.

2.4. Histopathologic Examination. The sagittal sections of the central portion of the rat TMJ were selected from each TMJ in all rats and stained with hematoxylin and eosin. The histopathological findings were evaluated using measure that is described as follows [16, 17]:

(1) Synovial lining hyperplasia was graded on a scale from 0 to 2: grade 0, staining of 1–3 layers; grade 1, staining of 4–6 layers; and grade 2, staining of 7 or more layers.

(2) Dilated vasculature was graded on a scale from 0 to 3: grade 0, not present; grade 1, involving less than one-third of the synovial membrane length; grade 2, involving one-third to two-thirds of the synovial membrane length; grade 3, involving more than two-thirds of the synovial membrane length.

(3) Fibrin deposits were graded on a scale from 0 to 3 (as described for the vasculature).

(4) Vascularity was graded on a scale from 0 to 2: grade 0, a limited number (less than 5) of blood vessel profiles/mm^2; grade 1, focal occurrence of 5–10 small blood vessel profiles/mm^2; grade 2, focal occurrence of a large number (more than 10) of small blood vessel profiles/mm^2.

2.5. Immunohistochemistry. After routine deparaffinization and rehydration, the sections underwent antigen retrieval in 0.125% trypsin-EDTA (Solarbio, Beijing, China) for 20 min at 37°C. Histostain™-Plus kits (ZSGB-Bio, Beijing, China) were used according to the manufacturer's recommendations. After incubation in goat serum, sections were incubated with the primary antibodies against TLR4 and IL-1β (1 : 1000, Cell Signaling, Beverly, MA, USA), respectively, overnight at 4°C. After rinsing with 0.01 M PBS, the sections were exposed to goat anti-rabbit secondary antibody (ZSGB-Bio, Beijing, China) for 30 min at 37°C, then were exposed to a solution of horseradish peroxidase-conjugated avidin-biotin complex (ZSGB-Bio, Beijing, China) for 20 min at 37°C. Then, sections were visualized with 0.1% 3, 30-diaminobenzidine dihydrochloride (DAB) (ZSGB-Bio, Beijing, China), and the sections were counterstained with hematoxylin. The digital images were captured using a microscopy digital camera system (Olympus, Tokyo, Japan). The results were evaluated semiquantitatively using the Image-Pro Plus 6.0 software. Five sections per rat were assayed in high power, and the mean optical density (MOD) was measured, respectively. The

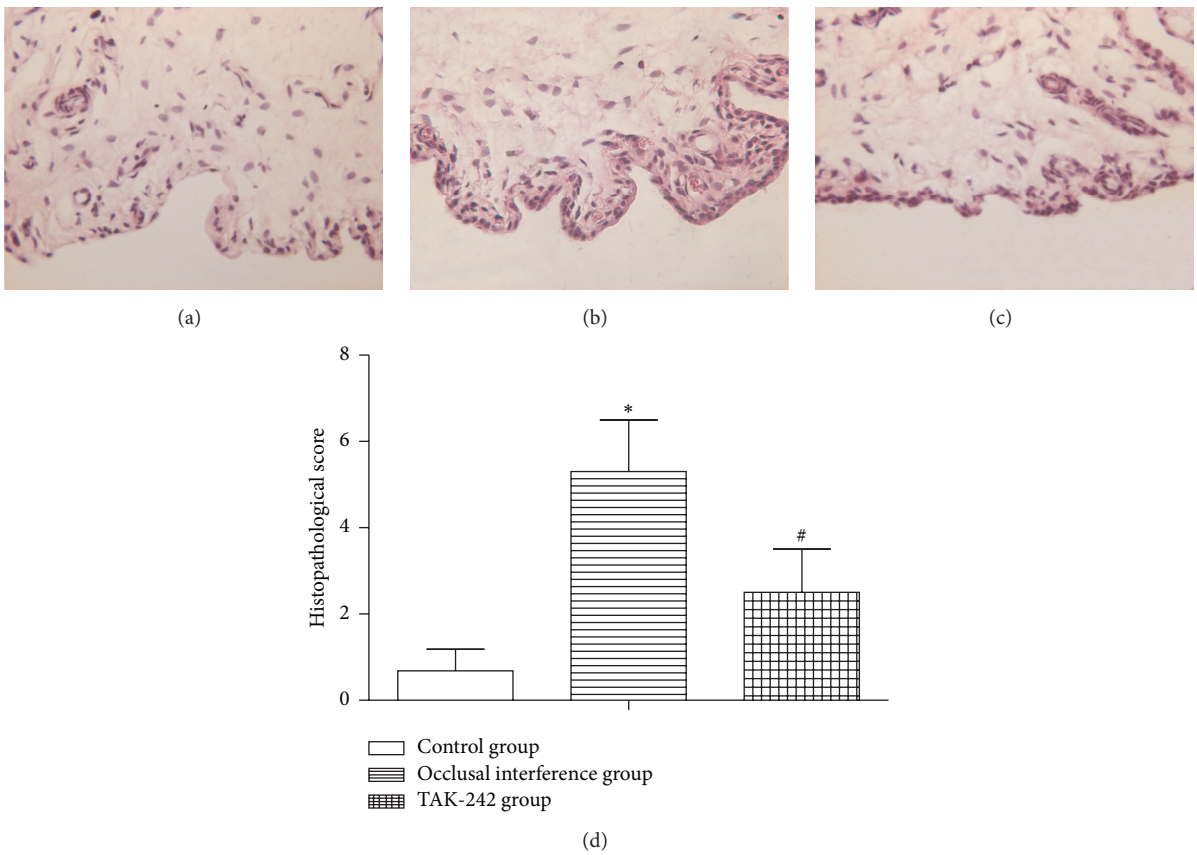

Figure 1: Histological examination of synovial membranes. (a) Control group. (b) Occlusal interference group. (c) TAK-242 group. (d) The histopathological score of each group. As the results shown, in comparison with the control group, the histopathological score was significantly increased in the occlusal interference group. However, this effect could be inhibited significantly after treatment with the TAK-242. Data shows all the values from independent samples of $n = 6$, $^{*}P < 0.05$ versus control group and $^{\#}P < 0.05$ versus occlusal interference group.

mean of MOD of five sections was seen as relative protein expression of this rat.

2.6. Real-Time Quantitative PCR. Total RNA was extracted from synovial tissues using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The first strand complementary DNA (cDNA) was synthesized by reverse transcription using SYBR Prime Script TM RT reagent Kit (Takara, Dalian, China). The levels of target mRNA in synovial tissues were analyzed by quantitative real-time PCR using SYBR Green I dye (Takara, Dalian, China). The primer pairs used for PCR were as follows: forward 5′-CCTGTGCAATTTGACCATTG-3′ and reverse 5′-AAGCATTCCCACCTTTGTTG-3′ for TLR4, forward 5′-ACAAGGAGAGACAAGCAACGA-3′ and reverse 5′-TCTGCTTGAGAGGTGCTGATG-3′ for IL-1β, and forward 5′-GAAGGTGAAGGTCGGAGTCG-3′ and reverse 5′-GAAGATGGTGATGGGATTTC-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The amplification was performed in triplicate on a LightCycler 480 QPCR System (Roche Diagnostics Ltd., Bern, Switzerland). Each gene was normalized against the corresponding GAPDH levels and relative gene expression of each sample was fold change ($2^{-\Delta\Delta Ct}$) using the control group as calibrator.

2.7. Statistical Analysis. Normally distributed variables were expressed as means ± SD. Unpaired Student's t-test was used to compare differences between groups. Differences in data values were defined significant at a $P < 0.05$ using SPSS statistical software package Version 17.0.

3. Results

3.1. Histological Examination. In the control group (Figure 1(a)), the synovial membranes of the TMJs did not show inflammatory changes. In the occlusal interference group (Figure 1(b)), obvious inflammation changes were observed in the synovial membranes, such as apparent hyperplasia of synovial lining cells, dilated blood vessels, proliferation of blood vessels, and fibrin deposition. As shown in Figure 1(d), in comparison with that in the controls, the histopathological score was significantly increased in the occlusal interference group. In the TAK-242 group (Figure 1(c)), the treatment of TAK-242 markedly inhibited the inflammatory reactions, although slight hyperplasia of synovial lining cells and dilated blood vessels were still present. As shown in Figure 1(d), the histopathological score became significantly lower after treatment with the TAK-242 when compared with the occlusal interference group.

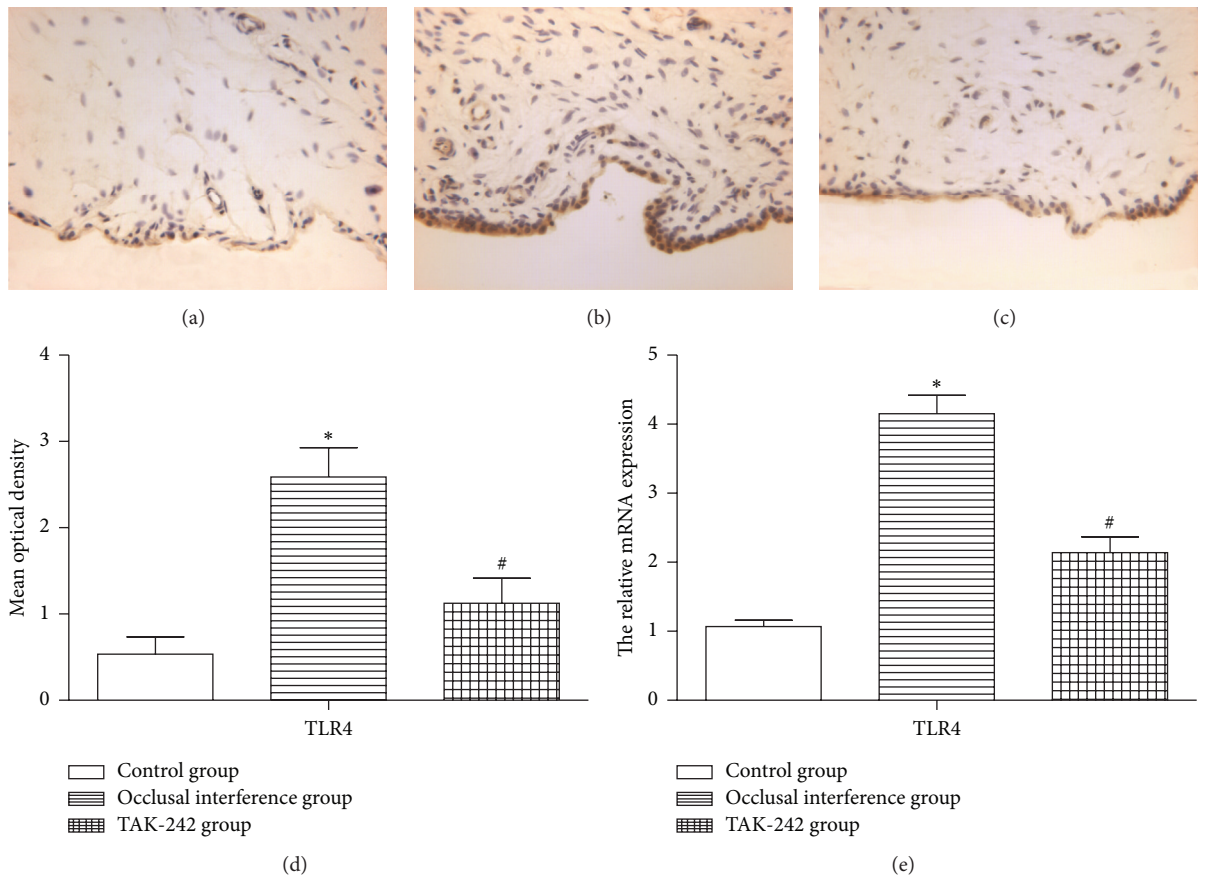

Figure 2: The expression of TLR4 in the synovial membranes. (a) Immunohistochemical staining for TLR4 in the membranes of the control group. (b) Immunohistochemical staining for TLR4 in the membranes of occlusal interference group. (c) Immunohistochemical staining for TLR4 in the membranes of TAK-242 group. (d) The mean optical density of each group. (e) The relative mRNA expression of TLR4 of each group. As the results shown, in comparison with the control group, the expression of TLR4 was significantly increased in the occlusal interference group at both protein and mRNA levels. However, this effect could be inhibited significantly after treatment with the TAK-242. Data shows all the values from independent samples of $n = 6$, $^{*}P < 0.05$ versus control group and $^{\#}P < 0.05$ versus occlusal interference group.

3.2. The Expression of TLR4 in the Synovial Membranes. As shown in Figure 2(a), the immunohistochemistry revealed that there are few synovial membranes could be stained, and the synovial membranes in the control group hardly expressed TLR4. Compared with the control group, the area of synovial membranes stained was increased by the experiment of occlusal interference (Figure 2(b)), and the expression of TLR4 (Figure 2(d)) in the occlusal interference group was improved. The same result was also found in the mRNA expression (Figure 2(e)). In the TAK-242 group, treatment with TAK-242 could reduce the area of synovial membranes stained (Figure 2(c)). As shown in Figure 2(d), the occlusal interference induced increased expression of TLR4 was significantly reduced by the injection of TAK-242 compared with the occlusal interference group. Consistent with the protein change, the mRNA expression of TLR4 was also reduced (Figure 2(e)).

3.3. Effect of TLR4 on the Expression of IL-1β in the Synovial Membranes. The results of immunohistochemistry staining for IL-1β in the synovial membranes of the TMJ in each groups were shown in Figures 3(a), 3(b), and 3(c). Compared with the control group, the expression of IL-1β of synovial membranes in the occlusal interference group was improved at both protein (Figure 3(d)) and mRNA (Figure 3(e)) levels. In the occlusal interference group, the treatment with TAK-242 significantly reduced occlusal interference-enhanced IL-1β expression at both protein (Figure 3(d)) and mRNA (Figure 3(e)) levels compared with the occlusal interference group.

4. Discussion

The patients of synovitis often suffered from pain in TMJ, and pain was the main reason that prompts patients to seek treatment at the hospital. The disease will continue to progress if patients do not receive effective treatment. Occlusion was defined as the balanced relationship between the incising or masticating surfaces of the maxillary and mandibular teeth. Experimental occlusal interference in animals could result in mandibular condyle bone remodeling [18], and another study [19] showed changes in blood flow in TMJ

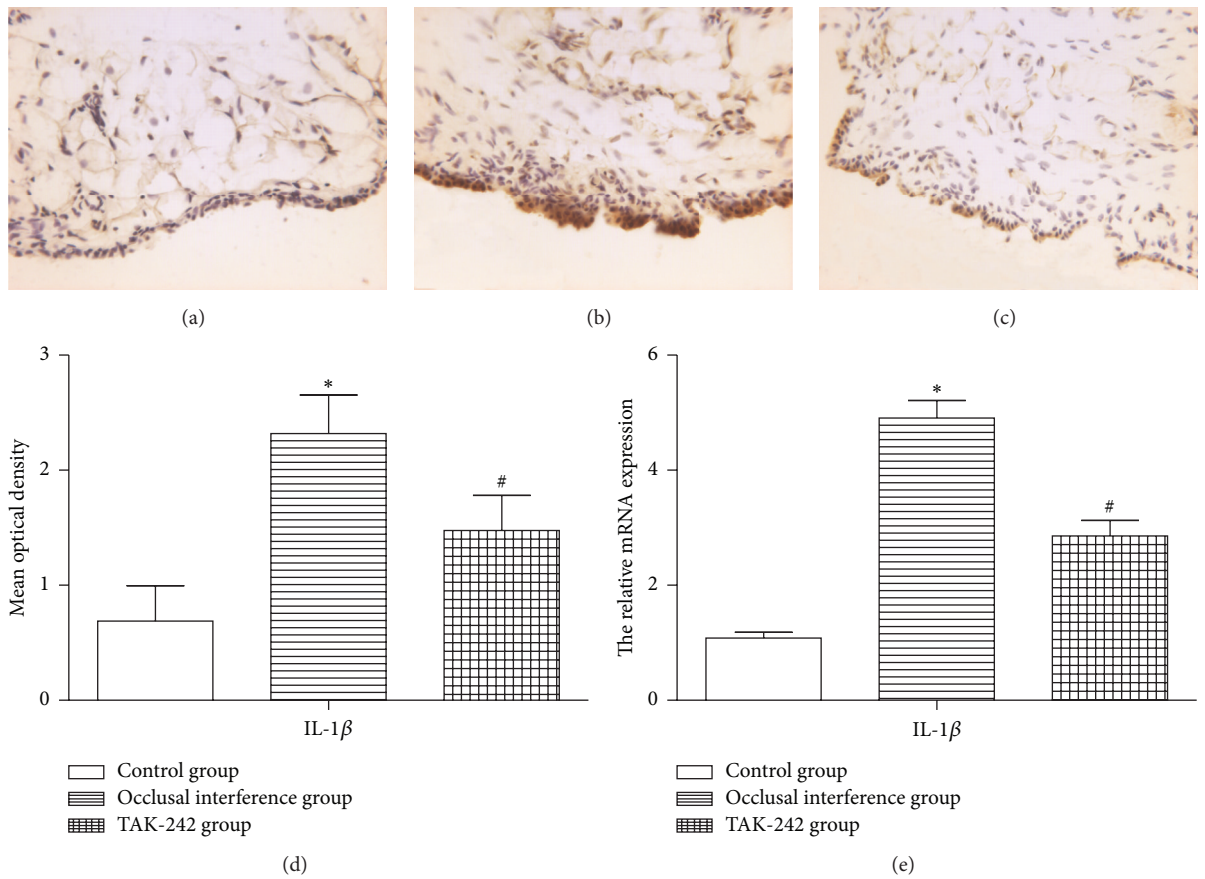

Figure 3: Effect of TLR4 on the expression of IL-1β in the synovial membranes. (a) Immunohistochemical staining for IL-1β in the membranes of the control group. (b) Immunohistochemical staining for IL-1β in the membranes of occlusal interference group. (c) Immunohistochemical staining for IL-1β in the membranes of TAK-242 group. (d) The mean optical density of each group. (e) The relative mRNA expression of IL-1β of each group. As the results shown, in comparison with the control group, the expression of IL-1β was significantly increased in the occlusal interference group at both protein and mRNA levels. However, this effect could be inhibited significantly after treatment with the TAK-242. Data shows all the values from independent samples of $n = 6$, $^{*}P < 0.05$ versus control group and $^{\#}P < 0.05$ versus occlusal interference group.

induced by experimental occlusal interference, and these changes possibly related to tissue damage and inflammation in TMJ. Occlusal interference was a tooth contact that inhibits the remaining occluding surfaces from achieving stable and harmonious contacts and changed the stress in articular cavity. The synovial membrane was sensitive tissue that feels stress in the articular cavity, and may occurred pathologic changes. In this study, we created an occlusal interference animal model by bonding crowns with a thickness of 0.6 mm to right mandibular first molar and observed obvious inflammation changes in the synovial membranes, such as apparent hyperplasia of synovial lining cells, dilated blood vessels, proliferation of blood vessels, and fibrin deposition. We induced synovial injury successfully by this method and provided experimental basis for the following research.

TLR4 is a member of the TLR (Toll-like receptor) family of transmembrane proteins, recognizes conserved pathogen associated molecular patterns like lipopolysaccharide (LPS), viral double-stranded RNA, bacterial flagella, and viral and bacterial CpG DNA, and generates innate immune responses to pathogens by activating a cascade of proinflammatory events [20]. Recent studies have found that endogenous ligands such as saturated free fatty acids [21] and high mobility group box-1 protein [22] can also activate TLR4. When a ligand binds to TLR4 and its coreceptors CD14 and MD-2, the adaptor molecules are recruited to the Toll/IL-1 receptor (TIR) domain of TLR4. This interaction cascade enables downstream signaling and mediates activation of a transcriptional factor and nuclear factor- (NF-) κB, resulting in induction of proinflammatory genes, such as those encoding TNF-α, IL-6, and IL-1β [23, 24]. A serious of studies has demonstrated that the TLR4 signaling pathways play an important role in the progression of many diseases by mediating the expression of proinflammatory cytokines. Edfeldt et al. suggested that hyporesponsive TLR4 polymorphisms affect the susceptibility to myocardial infarction in men and that TLR4-mediated innate immunity plays a role in the pathogenesis of myocardial infarction [25]. A report identified that the interaction TLR4 signaling pathway is involved in the development of lung ischemia reperfusion

injury (LIRI) [26]. Kim et al. cultivated the cartilage cells isolated from patients with osteoarthritis and detected increased expression of TLR4 mRNA [27]. In our previous study, we found that the expression of TLR4 and IL-1β was significantly increased in SFs separated from rat TMJ with LPS stimulation at both mRNA and protein levels, and LPS activated the TLR4 signaling pathway in SFs. However, the inflammatory response that occurred in synovial membrane is not caused by bacterial infection as we all know. In the current study, we wanted to investigate whether or not TLR4 participate in the inflammatory responses and the expression of IL-1β in rats induced by occlusal interference. As the results showed, the expression of TLR4 and IL-1β in the synovial membranes of the occlusal interference group was significantly increased at both protein and mRNA levels. This finding prompted us that maybe TLR4 participates in inflammatory response of synovial membranes in rat. So, which endogenous ligands are involved in the activation of TLR4 signaling in synovial membranes of rats in the occlusal interference group? This question remains an issue waiting for us to explore and research.

TAK-242 is a specific inhibitor of TLR4, which could selectively suppress TLR4-mediated myeloid differentiation factor 88- (MyD88-) dependent pathway as well as TIR domain-containing adapter-inducing IFN-β (TRIF) dependent pathway by binding to Cys747 in the intracellular domain of TLR4 and its inhibitory effect, is largely unaffected by LPS concentration and types of TLR4 ligands, and finally inhibits the expression of NO, TNF-α, IL-6, and IL-1β [28, 29]. In previous researches, TAK-242 played a protective effect in LPS-induced lung injury [30], and treatment with TAK-242 showed benefits for sepsis [31]. In the current study, we created an occlusal interference animal model by bonding crowns with a thickness of 0.6 mm to right mandibular first molars and observed inflammation changes as well as increased expression of IL-1β at both mRNA and protein levels. However, the effect of occlusal interference was significantly decreased by the use of TAK-242. In the TAK-242 group, the histologic severity score of synovial membranes became significantly lower after treatment with the TAK-242 when compared with the occlusal interference group. Consistent with the inflammatory reactions, the increased expression of IL-1β was obviously reduced at both mRNA and protein levels. These may represent an important link between activation of TLR4 and the increased expression of proinflammatory cytokines like IL-1β and the inflammatory reactions of synovial membranes in rats treated with occlusal interference. However, the adaptor molecules participate in intracellular signaling and the pathways of intracellular signaling transduction triggered by TLR4 and induce production of inflammatory mediators like IL-1β, which were not studied.

In the current study, we demonstrated that TLR4 involved in the inflammatory reactions of synovial membranes and the expression of IL-1β at both mRNA and protein levels caused by occlusal interference in rats. Additionally, the injection of TAK-242 could inhibit the development of this disease. The results prompted us that the activation of TLR4 may be involved in the inflammatory reactions and increased expression of IL-1β and participate in the mechanisms of the initiation and development of synovial injury by regulating the expression of inflammatory mediators like IL-1β in synovial membranes. Our research results provided new theoretical evidences for study about pathogenesis of synovitis in TMJ.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

This research was supported by science and technology development plans of Shandong province (Grant no. 2014GSF118027) and the Specialized Research Fund for the Doctoral Program of Higher Education of China (Grant no. 20120131110074).

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 9848263, 14 pages
http://dx.doi.org/10.1155/2016/9848263

Research Article

Caspase-11 Modulates Inflammation and Attenuates *Toxoplasma gondii* Pathogenesis

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Received 22 January 2016; Revised 20 April 2016; Accepted 12 May 2016

Academic Editor: Denis Girard

Toxoplasma gondii is an obligate intracellular parasite that is the etiologic agent responsible for toxoplasmosis. Infection with *T. gondii* results in activation of nucleotide binding domain and leucine rich repeat containing receptors (NLRs). NLR activation leads to inflammasome formation, the activation of caspase-1, and the subsequent cleavage of IL-1β and IL-18. Recently, a noncanonical inflammasome has been characterized which functions through caspase-11 and appears to augment many biological functions previously considered to be dependent upon the canonical inflammasome. To better elucidate the function of this noncanonical inflammasome in toxoplasmosis, we utilized $Asc^{-/-}$ and $Casp11^{-/-}$ mice and infected these animals with *T. gondii*. Our data indicates that caspase-11 modulates the innate immune response to *T. gondii* through a mechanism which is distinct from that currently described for the canonical inflammasome. $Asc^{-/-}$ mice demonstrated increased disease pathogenesis during the acute phase of *T. gondii* infection, whereas $Casp11^{-/-}$ mice demonstrated significantly attenuated disease pathogenesis and reduced inflammation. This attenuated host response was associated with reduced local and systemic cytokine production, including diminished IL-1β. During the chronic phase of infection, caspase-11 deficiency resulted in increased neuroinflammation and tissue cyst burden in the brain. Together, our data suggest that caspase-11 functions to protect the host by enhancing inflammation during the early phase of infection in an effort to minimize disease pathogenesis during later stages of toxoplasmosis.

1. Introduction

Toxoplasma gondii is an intracellular apicomplexan parasite that can infect a variety of vertebrate hosts including humans and domestic animals. Transmission can occur through food or drinking water contaminated with infective oocysts, ingestion of meat infected with tissue cysts, or transplacentally from the mother to the fetus. Infection rates in humans are high but clinical disease is most problematic in immunocompromised individuals or when the infection is congenital. It is well established that the Toll-like receptor (TLR) family of pattern recognition receptors (PRRs) plays a critical role in host defense against *T. gondii*. The TLR associated adaptor protein myeloid differentiation primary response gene 88 (MyD88) has been found to be essential in the production of proinflammatory cytokines including IL-12 and IFN-γ [1–3]. Likewise, roles have been described for TLR-2, TLR-4, TLR-5, TLR-9, TLR-11, and TLR-12 [4–11].

In addition to the TLRs, other families of PRRs have been shown to play a role in the innate immune response against *T. gondii*. Recent studies have focused on members of the nucleotide binding domain and leucine rich repeat containing family of receptors (also referred to as NLRs). NLRs are cytosolic PRRs which are important modulators of inflammation through their regulation of the proinflammatory cytokines IL-1β and IL-18, as well as their role in the proinflammatory form of cell death termed pyroptosis [12]. Once a ligand binds the protein receptor, there is oligomerization with procaspase-1 and the adaptor molecule apoptosis-associated speck-like protein containing carboxy-terminal caspase activation and recruitment domain (ASC) to form a multimeric protein complex termed the inflammasome. This

process cleaves cytosolic procaspase-1 into its active form, which then cleaves cytosolic pro-IL-1β and pro-IL-18 into their active forms. A diverse subgroup of NLRs have been identified as forming inflammasomes following the sensing of specific signals associated with intracellular pathogens [13]. The inflammasome forming NLR NLRP1 (nucleotide binding domain and leucine rich repeat containing receptor P1) has previously been identified as an essential sensor of *T. gondii* in rodents and mutations in *NLRP1* have been shown to confer susceptibility for human congenital toxoplasmosis [14–18]. Likewise, the inflammasome forming NLR NLRP3 has also been shown to confer host protection following *T. gondii* infection [17].

Recently, a noncanonical inflammasome has been identified and characterized [19]. This noncanonical inflammasome is responsible for recognizing intracellular lipopolysaccharide (LPS), as well as recognizing and regulating the host immune response to *Escherichia coli*, *Citrobacter rodentium*, and *Vibrio cholera* [19]. Activation of the noncanonical inflammasome appears to occur during acute inflammatory conditions, such as sepsis, and activation results in IL-1β and IL-18 cleavage through a canonical inflammasome-dependent mechanism [19]. Activation of the noncanonical inflammasome can also lead to pyroptosis; however, this occurs through a mechanism that is independent of the canonical inflammasome [19]. Unlike the canonical inflammasome, there is currently a paucity of data pertaining to noncanonical inflammasome activation and function. For example, it has recently been shown that LPS can directly bind caspase-11, which is a critical caspase, to activate the noncanonical inflammasome [20]. However, it is not clear if other pathogen associated molecular patterns (PAMPs) can stimulate this pathway or if noncanonical signaling plays any role in host-pathogen responses that are not associated with bacteria.

In the present study, we investigated the role of caspase-11, which is an essential component of the noncanonical inflammasome, in the pathogenesis of *T. gondii*. We hypothesized that caspase-11 would significantly contribute to the host innate immune response following *T. gondii* infection, particularly during the acute phase, which is characterized by robust inflammation. Our data show that IL-1β levels are partly dependent on caspase-11 *ex vivo* in macrophages and *in vivo* in mice. *Casp11*$^{-/-}$ animals appear to be relatively resistant to *T. gondii* as they show significantly attenuated changes in morbidity and mortality and reduced inflammation during the early phase of disease. This is in stark contrast to the increased sensitivity observed in the *Asc*$^{-/-}$ mice also evaluated in this study. Unfortunately, these protective effects do not extend into later phases of the disease as *Casp11*$^{-/-}$ mice develop significantly increased neuroinflammation and brain tissue cysts, likely due to the attenuated local inflammatory response during the acute phase. Thus, our findings establish a role for caspase-11 beyond the host immune response to bacteria and demonstrate a critical role in the host-pathogen response following *T. gondii* infection.

2. Materials and Methods

2.1. Experimental Animals. All studies were conducted in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines and according to the institutionally approved animal protocol. The generation and characterization of *Casp11*$^{-/-}$ and *Asc*$^{-/-}$ mice have previously been described [19, 21]. All experiments were conducted with 6–8-week-old, female mice backcrossed for at least 12 generations onto the C57Bl/6 background. Mice were injected intraperitoneally with 1,000 *T. gondii* tachyzoites (strain ME49) diluted in 400 μL phosphate-buffered saline (PBS). Morbidity and mortality were monitored daily for 25 days after inoculation (d.p.i.). Weight loss and clinical scores were assessed daily following *T. gondii* inoculation. The clinical score is derived from individual assessments of weight loss, body condition, behavior, and gait, each individually scored on a scale of 0–5. Individual scores are combined and averaged to generate the composite clinical score. Mice were euthanized at day 15 after inoculation to evaluate acute (or early) *T. gondii* infection or at day 25 after inoculation to evaluate chronic (or late) *T. gondii* infection. At euthanasia, whole blood was collected via cardiac puncture and tissues were collected for further processing.

2.2. Assessments of Inflammation. At harvest, samples of brain, lung, heart, liver, spleen, and intestine were collected and either stored at −80°C for protein analysis or placed into 10% buffered formalin for histopathology. Peritoneal lavage fluid (PLF) was taken prior to organ collection. Briefly, the skin overlying the abdominal cavity was incised and reflected to reveal an intact peritoneum. A 27-gauge needle was inserted and the abdomen was flushed with 5 mL of sterile PBS. The samples were spun down and cell supernatants utilized for cytokine analysis. Cell pellets were resuspended and evaluated on a hemacytometer for total nucleated cell counts. Additionally, cytospin preparations were made and evaluated with Dif-Quik for differential cell counts.

2.3. Histopathologic Examination. Formalin-fixed tissues were routinely processed for histopathology. The paraffin-embedded tissues were sectioned at 5 μm and prepared for hematoxylin and eosin (H&E) staining. H&E stained sections were evaluated by a board-certified veterinary pathologist (S.C.O.). Composite scores for brain inflammation were determined by the numbers of inflammatory cells within the leptomeninges (0–2) as well as within the parenchyma itself (0–3). For the leptomeninges, scores were given as follows: 0, no inflammatory cells identified, 1, less than 3 layers of inflammatory cells identified, or 2, greater than or equal to 3 layers of inflammatory cells identified. For the parenchyma, scores were given as follows: 0, no inflammatory cells identified, 1, less than 3 layers of inflammatory cells identified in perivascular spaces only, 2, greater than 3 layers of inflammatory cells identified in perivascular spaces only, or 3, inflammatory cells identified in perivascular spaces as well as within the neuroparenchyma. *T. gondii* cysts were identified as present or absent.

2.4. Bone Marrow Derived Macrophage Isolation and Evaluation. Bone marrow derived macrophages (BMDMs) were isolated from the femurs of C57Bl/6, *Casp11*$^{-/-}$, and *Asc*$^{-/-}$ mice using standard procedures. The cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 20% L929-conditioned cell culture supernatant, 1x L-glutamine, and 1x nonessential amino acids for 7 days. BMDMs with or without LPS priming (100 ng/mL for 30 minutes) were infected with Me49 tachyzoites (Moiety of Infection = 1) for 24 hours. Supernatants were removed for cytokine measurements.

2.5. Cytokine Assessments. Cytokine analysis on cell-free supernatants from PLF, BMDM supernatants, and tissue homogenates as well as serum was performed using standard enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences). All results were normalized per weight of tissue or volume of original sample.

2.6. Statistical Analysis. We utilized GraphPad Prism 5 statistical software to conduct Analysis Of Variance (ANOVA) followed by either Tukey-Kramer honest significant difference or Newman-Keuls posttest to evaluate statistical significance for multiple comparisons. Single data point comparisons were evaluated by Student's two-tailed t-test. Group survival was assessed utilizing the Kaplan-Meier test. All data are presented as the mean ± the standard error of the mean (SEM) and in all cases a p value less than 0.05 was considered statistically significant.

3. Results

3.1. The Canonical NLR Inflammasome and Caspase-11 Are Associated with IL-1β Production in Murine Macrophages following Toxoplasma gondii Infection. While the role of the canonical NLR inflammasome and caspase-1 in *T. gondii* infection has been characterized, the contribution of the noncanonical inflammasome and caspase-11 has yet to be evaluated [14–18]. To determine if caspase-11 functions in the host-parasite response, primary BMDMs were isolated from wild type, *Asc*$^{-/-}$, and *Casp11*$^{-/-}$ mice, as previously described [22]. Previous studies have shown that canonical inflammasome activation and secretion of IL-1β by macrophages following exposure to *T. gondii* Me49 are dependent upon LPS priming and parasite internalization and occur independent of cell death [17]. To evaluate these findings in the *Casp11*$^{-/-}$ macrophages, cells were primed with 100 ng/mL of LPS for 30 minutes followed by exposure to *T. gondii* Me49 tachyzoites (MOI = 1) or vehicle. *T. gondii* infection resulted in a significant increase in IL-1β in wild type macrophages compared to LPS primed mock infected macrophages (Figure 1). Consistent with the previously demonstrated essential nature of the canonical NLR inflammasome in sensing *T. gondii*, IL-1β levels were markedly reduced in macrophages isolated from *Asc*$^{-/-}$ mice (Figure 1). *Casp11*$^{-/-}$ macrophages infected with *T. gondii*, interestingly, displayed intermediate levels of IL-1β when compared to infected wild type and infected *Asc*$^{-/-}$ macrophages (Figure 1). The attenuation in IL-1β levels, rather than full ablation, observed in the *Casp11*$^{-/-}$ macrophages suggests that caspase-11 and the noncanonical inflammasome likely function to augment the activity of the canonical inflammasome. This is consistent with prior observations that suggest a synergistic model for caspase-11 function in bacteria sensing, where the noncanonical inflammasome complements canonical inflammasome signaling and IL-1β maturation during acute inflammation [19, 23–25].

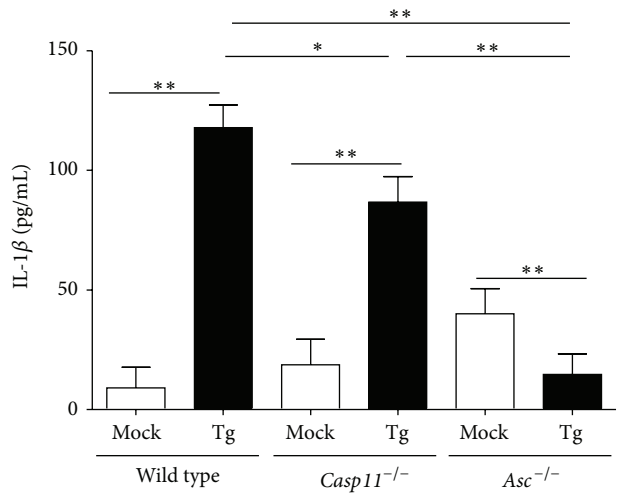

Figure 1: IL-1β levels are ablated in macrophages harvested from *Casp11*$^{-/-}$ and *Asc*$^{-/-}$ mice following *ex vivo* infection with *Toxoplasma gondii*. Bone marrow derived macrophages were primed with 100 ng/mL of LPS for 30 mins prior to stimulation with either *Toxoplasma gondii* Me49 (MOI = 1) or vehicle for 24 hrs. IL-1β levels in cell-free supernatants were quantified using ELISA. $^{*}p < 0.05$; $^{**}p < 0.01$. Data shown are representative of 3 independent experiments utilizing 2 mice per genotype.

3.2. In Vivo Caspase-11 Increases Toxoplasma gondii Pathogenesis in the Early Stages of Disease. Activation of the canonical NLRP1 and NLRP3 inflammasomes controls *T. gondii* proliferation and minimizes disease pathogenesis [16, 17]. This conclusion is based on prior studies, which have revealed that *Nlrp1*$^{-/-}$, *Nlrp3*$^{-/-}$, *Casp1/11*$^{-/-}$, and *Asc*$^{-/-}$ mice are highly susceptible to acute toxoplasmosis [16, 17]. To evaluate the contribution of caspase-11 in disease pathogenesis, wild type, *Casp11*$^{-/-}$, and *Asc*$^{-/-}$ mice were infected with 1,000 *T. gondii* Me49 tachyzoites via intraperitoneal (i.p.) injection. Survival, weight change, and clinical parameters associated with toxoplasmosis were assessed daily (Figure 2). During early stages of the disease, typically characterized by tachyzoite proliferation and acute inflammation, we observed a significant decrease in survival of both wild type and *Asc*$^{-/-}$ mice compared to mock injected animals (Figure 2(a)). Fifty percent of the wild type mice required euthanasia by 14 d.p.i. In the *Asc*$^{-/-}$ mice, we observed a significant decrease in survival beginning at 9 d.p.i. and extending through 14 d.p.i. Ultimately, 70% of the *Asc*$^{-/-}$ mice required euthanasia due to acute toxoplasmosis (Figure 2(a)). Conversely, no *Casp11*$^{-/-}$ mice required euthanasia and all of the animals survived

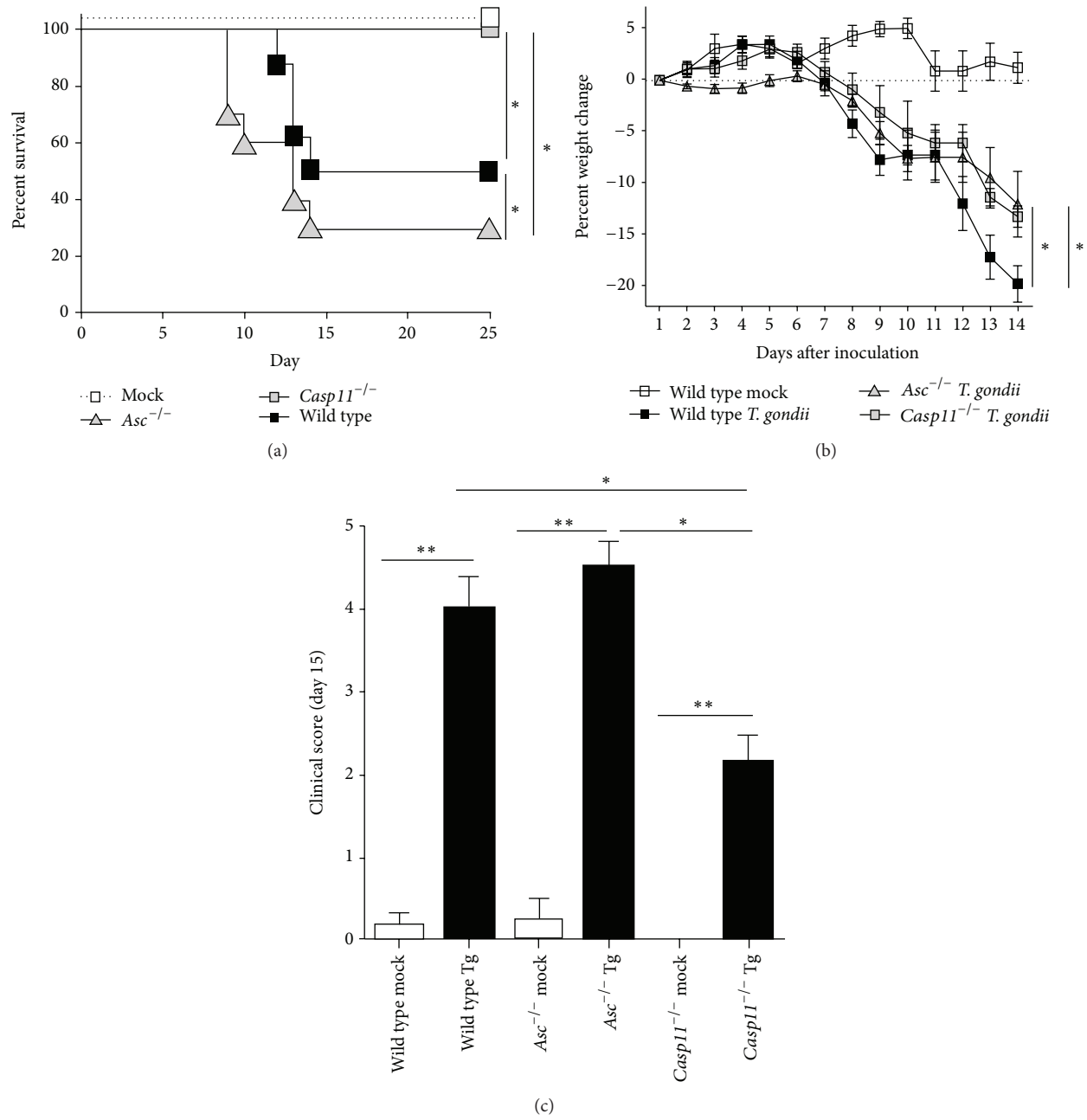

FIGURE 2: *Casp11*$^{-/-}$ mice exhibit reduced susceptibility to *Toxoplasma gondii* infection. Wild type, *Casp11*$^{-/-}$, and *Asc*$^{-/-}$ mice were infected with 1,000 *Toxoplasma gondii* Me49 tachyzoites via i.p. administration. Morbidity and mortality were monitored daily. (a) Kaplan-Meier plot of WT, *Asc*$^{-/-}$, and *Casp11*$^{-/-}$ mice survival. Mice were considered moribund when weight loss was sustained at or below –15% from baseline and/or clinical parameters necessitated euthanasia. (b) Weight loss was evaluated daily and the percent change from baseline for each animal was calculated. Data shown reflect 1–14 days after inoculation due to the significantly reduced survival of the wild type and *Asc*$^{-/-}$ mice at day 15. (c) *Casp11*$^{-/-}$ mice demonstrate significantly attenuated clinical parameters associated with disease progression. The clinical score is a composite of scores associated with weight loss, clinical condition, and behavior. $^{*}p < 0.05$. Wild type mock, $n = 9$; *Asc*$^{-/-}$ mock, $n = 9$; *Casp11*$^{-/-}$ mock, $n = 6$; wild type Tg, $n = 18$; *Asc*$^{-/-}$ Tg, $n = 18$; *Casp11*$^{-/-}$ Tg, $n = 13$. Data shown are representative of 3 independent studies.

(Figure 2(a)). These findings were also reflected in the weight loss and clinical score data. All animals inoculated with *T. gondii* demonstrated significantly increased weight loss (Figure 2(b)). Wild type animals demonstrated a consistent decrease in body weight between 8 and 14 d.p.i., which was significantly greater than the weight loss observed for either the *Asc*$^{-/-}$ or *Casp11*$^{-/-}$ mice (Figure 2(b)). While weight loss was more rapid in the wild type animals, the *Asc*$^{-/-}$ mice demonstrated higher clinical scores reflecting more severe disease presentation (Figure 2(c)). Assessments of clinical parameters associated with toxoplasmosis revealed that disease progression was indeed severe in both wild

type and *Asc*$^{-/-}$ mice while it was significantly attenuated in the *Casp11*$^{-/-}$ animals (Figure 2(c)). Weight loss and clinical parameters associated with disease were monitored through day 25 in the surviving animals (data not shown). While these parameters remained significantly reduced (5–10% decrease) in all genotypes compared to the mock treated animals, all of the animals that survived the acute phase of *T. gondii* infection demonstrated a significant improvement in morbidity and mortality throughout the remainder of the study (data not shown). Together, these data confirm previous findings by demonstrating the essential role of the canonical inflammasome in maintaining host resistance to *T. gondii*. In addition, these findings also reveal a unique contribution for caspase-11 which appears to be distinct from ASC and suggests that caspase-11 actually contributes to disease severity during the acute phase of *T. gondii* infection.

3.3. Severe Toxoplasmosis Was Observed during the Acute Phase of Toxoplasma gondii Infection That Resolved during the Chronic Phase in All Surviving Animals. Previous studies have shown that *Casp1/11*$^{-/-}$ mice are highly susceptible to *T. gondii* infection in part due to failure to control parasite load in the peritoneal cavity [16, 17]. These prior studies utilized green fluorescent protein-luciferase (GFP-LUC) expressing tachyzoites and whole body imaging to evaluate parasite load. To investigate these findings in our studies utilizing *Asc*$^{-/-}$ and *Casp11*$^{-/-}$ mice, we evaluated histopathologic sections of peritoneal organs to directly assess disease pathogenesis (Figure 3). All histopathology was evaluated by a board certified veterinary pathologist (S.C.O). At 15 days after infection, we detected robust signs of infection and inflammation. Spleens from mice harvested at Day 15 were characterized by expansion of the lymphoid follicles by large numbers of macrophages occasionally containing intracellular tachyzoites as well as moderately increased numbers of lymphocytes and plasma cells. Lymphocytolysis was a prominent feature in the spleen and numerous animals exhibited pronounced accumulations of fibrin, cellular debris, and intrahistiocytic and extracellular tachyzoites on the serosal surface (Figures 3(a) and 3(b)). No significant difference was observed between wild type, *Asc*$^{-/-}$, or *Casp11*$^{-/-}$ animals. Spleens from mice harvested at day 25 exhibited milder evidence of disease characterized by significantly less inflammation and no evidence of intracellular or extracellular tachyzoites (Figure 3(a)). Additionally, by day 25, all infected animals had developed mild evidence of inflammation in the liver as well (Figure 3(a)). Additionally, we did detect a significant tachyzoite burden and moderate-to-severe inflammation in the lungs in infected animals harvested at day 25 (data not shown). However, our analysis revealed that there were no significant differences in histopathology or parasite burden between the wild type, *Asc*$^{-/-}$, and *Casp11*$^{-/-}$ mice in any of the abdominal or thoracic organs evaluated. This is in contrast to the prior study that suggested increased parasite replication in the absence of the canonical inflammasome [17]. The differences observed between the prior studies and the current data can be reconciled by considering methodological differences between the models as well as temporal and spatial differences in disease pathogenesis. Both prior studies utilized bioluminescence as an indirect assessment of disease pathogenesis [16, 17], whereas the current study utilized histopathological evaluation. Histopathological assessments are a more direct method of assessment and provide higher resolution associated with parasite localization. However, this approach is less quantitative compared to the bioluminescence approach. Likewise, the prior study evaluating *Asc*$^{-/-}$ mice focused on earlier time points (days 5 and 7), which may reflect the peak in tachyzoite proliferation [17]. Meanwhile, the current study evaluated day 15 which represented the peak in morbidity and mortality and may better reflect the peak in the host immune response.

3.4. Inflammation during the Acute Phase of Toxoplasma gondii Infection Is Increased by Caspase-11. *T. gondii* infection results in the rapid and acute induction of the innate immune response, which results in significant leukocyte migration. To evaluate this response in the absence of ASC and caspase-11, we collected PLF from animals 15 d.p.i. The total cellularity of each animal was evaluated using trypan blue staining and counted on a hemacytometer. *T. gondii* infection resulted in a significant increase in leukocytes in the peritoneal cavity in wild type mice (Figure 4(a)). We observed a significant increase in PLF cellularity in the *Asc*$^{-/-}$ mice compared to the wild type animals (Figure 4(a)). These findings are consistent with a loss of immune system homeostasis and increased overall inflammation in these animals as described in other models of acute disease [25–27]. Unlike *Asc*$^{-/-}$ mice, we observed a significant attenuation in PLF cellularity in *Casp11*$^{-/-}$ animals compared to wild type mice (Figure 4(a)). Overzealous inflammation is a major contributing factor to increased morbidity and reduced survival during acute disease. Thus, the significantly attenuated leukocyte infiltration observed in *Casp11*$^{-/-}$ mice in the peritoneal cavity is consistent with the improved survival and reduced clinical features observed in these animals. In order to better characterize the infiltrating leukocytes, cytospins were generated from each PLF sample and differential staining was utilized to determine the specific cell populations associated with the immune response for each genotype. The cellular composition of the PLF was significantly altered 15 d.p.i. with *T. gondii* (Figures 4(b)–4(e)). Monocyte derived cells represented the dominant cellular population in all mock treated animals (Figure 4(b)). However, following *T. gondii* infection, we observed a significant decrease in the percentage of monocytes and a significant increase in polymorphonuclear (PMN) cells in the PLF (Figures 4(b) and 4(d)). In both *Asc*$^{-/-}$ and *Casp11*$^{-/-}$ mice, we observed significant decreases in the PMN cell population and significant increases in the lymphocyte population compared to the wild type mice (Figures 4(d) and 4(e)). We also observed a significant increase in the percentage of monocytes in *Casp11*$^{-/-}$ mice compared to wild type mice (Figure 4(b)). Interestingly, we observed a significant decrease in the mast cell populations in both mock and *T. gondii* infected *Asc*$^{-/-}$ mice (Figure 4(c)). Decreased mast cell populations have not been reported in

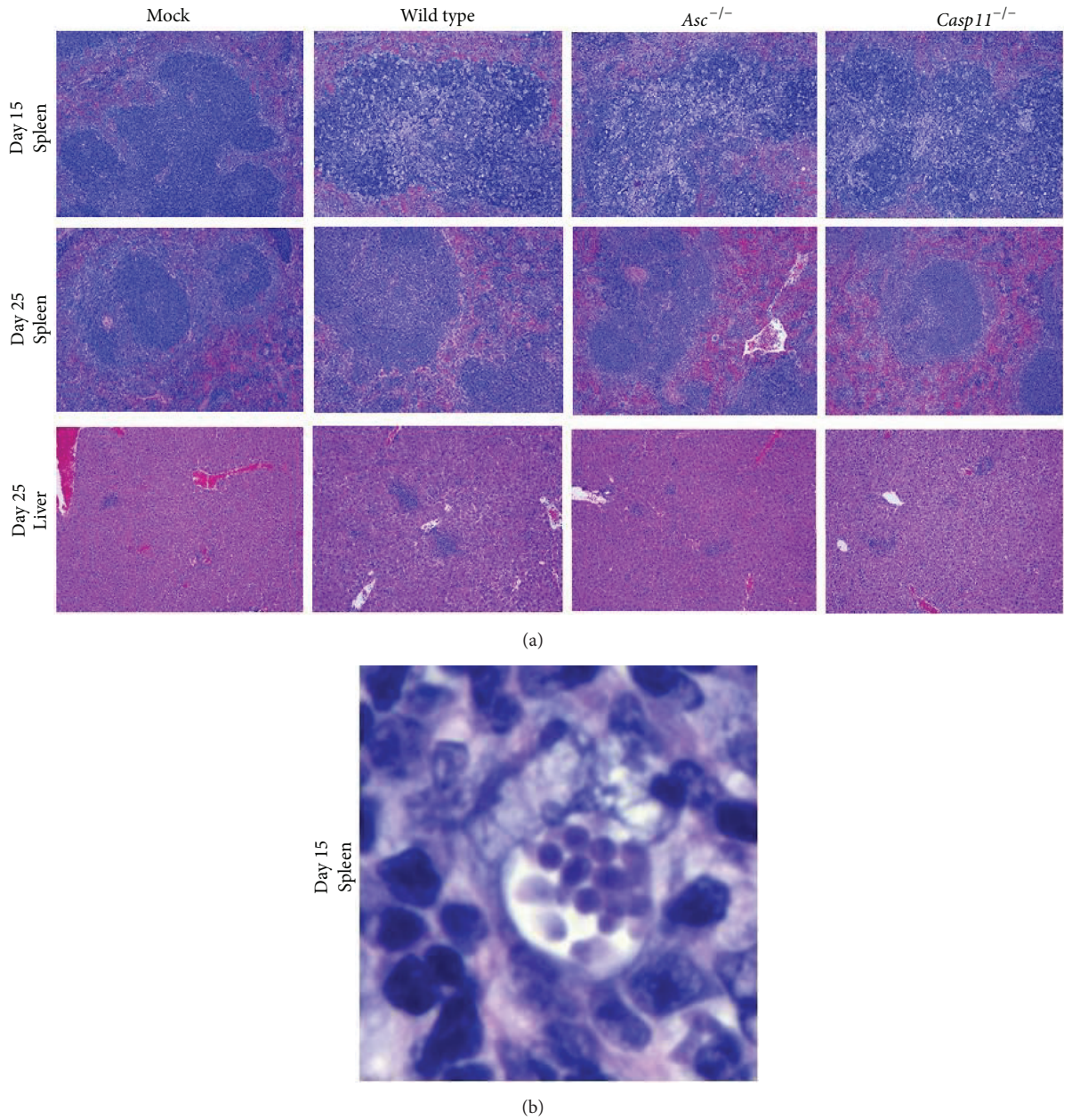

FIGURE 3: *Toxoplasma gondii* tachyzoites induced severe toxoplasmosis which resolved during the chronic phase of disease in all surviving animals. Wild type, *Casp11*$^{-/-}$, and *Asc*$^{-/-}$ mice were infected with 1,000 *Toxoplasma gondii* Me49 tachyzoites via i.p. administration and necropsied at 15 and 25 days after inoculation (d.p.i.). (a) Tachyzoite injection resulted in significantly increased spleen histopathology at 15 d.p.i. in all animals regardless of genotype. By 25 d.p.i., spleen histopathology was markedly improved with only minimal evidence of prior disease for all inoculated genotypes. Mild-to-moderate inflammation was observed in the livers of all infected mice. 20x magnification. (b) *T. gondii* tachyzoites were found in high concentrations in the spleen of all inoculated animals at 15 d.p.i. with no significant difference noted between genotypes. 100x magnification. Wild type mock, $n = 9$; *Asc*$^{-/-}$ mock, $n = 9$; *Casp11*$^{-/-}$ mock, $n = 6$; wild type Tg, $n = 18$; *Asc*$^{-/-}$ Tg, $n = 18$; *Casp11*$^{-/-}$ Tg, $n = 13$. Data shown are representative of histopathology evaluated over the course of 3 independent studies.

Asc$^{-/-}$ mice and, if confirmed, may represent an interesting direction for future studies. In the context of *T. gondii* infection, reduced numbers of mast cells have been correlated to increased disease pathogenesis [28] and may underlie the increased disease severity in *Asc*$^{-/-}$ mice reported here and in prior studies [16, 17]. Together, these data reflect significant differences in the overall immune response between *Asc*$^{-/-}$ mice and *Casp11*$^{-/-}$ animals, whereas the composition of the cells being recruited to the peritoneal cavity is similar between the two mouse strains during acute toxoplasmosis.

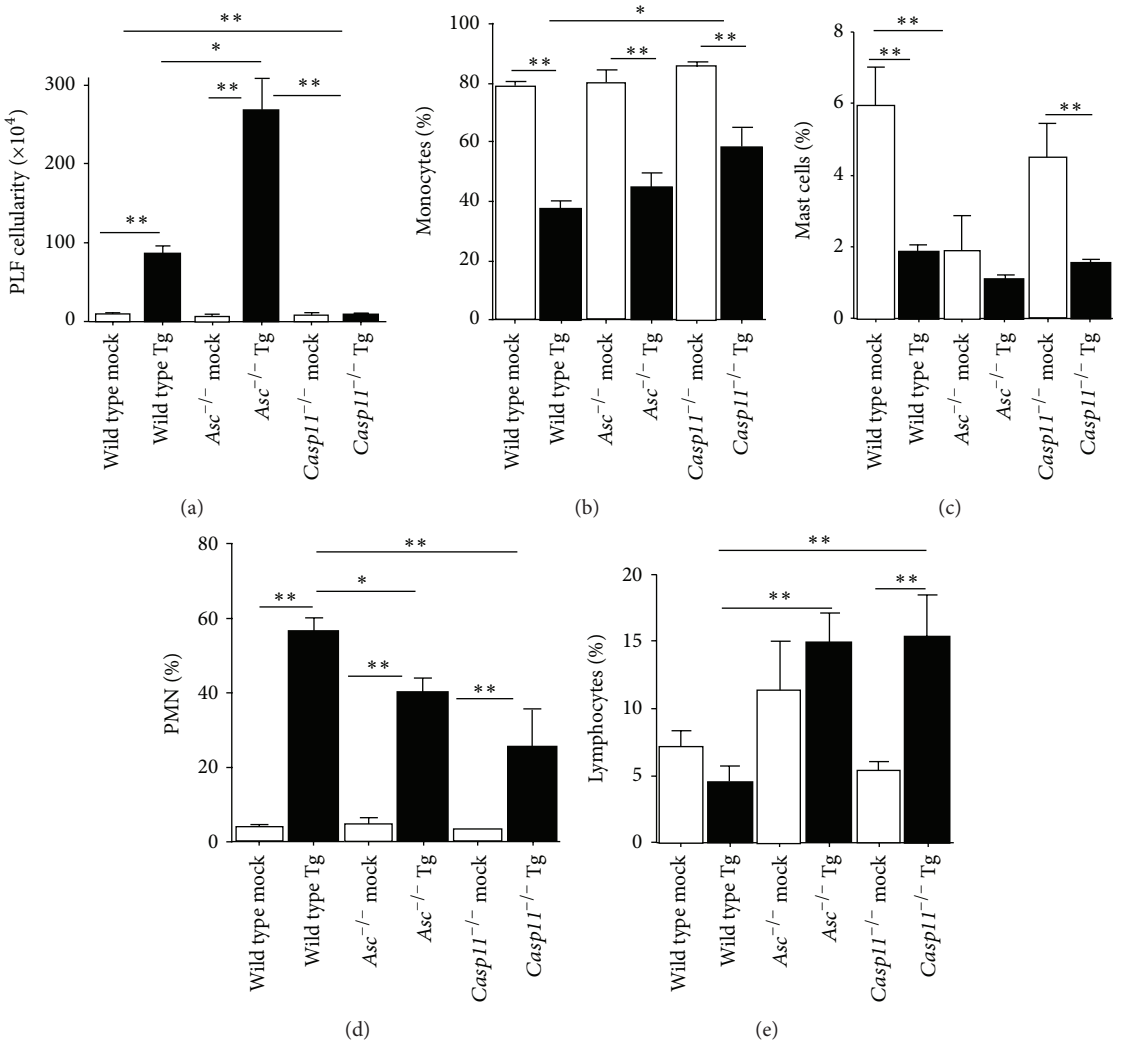

Figure 4: Peritoneal inflammation was significantly attenuated in *Casp11*$^{-/-}$ mice. Wild type, *Casp11*$^{-/-}$, and *Asc*$^{-/-}$ mice were infected with 1,000 *Toxoplasma gondii* Me49 tachyzoites via i.p. administration and peritoneal lavage fluid (PLF) was collected 15 days after inoculation. (a) Total PLF cellularity was determined for each treatment group using trypan blue staining and a hemacytometer. (b–e) PLF leukocyte populations were determined following differential staining of cytospun samples. The percentages of monocytes (b), mast cells (c), polymorphonuclear cells (neutrophils and eosinophils) (d), and lymphocytes (e) were determined for each animal. $^{*}p < 0.05$; $^{**}p < 0.01$. Data shown are representative of histopathology evaluated over the course of 3 independent studies. Wild type mock, $n = 9$; *Asc*$^{-/-}$ mock, $n = 9$; *Casp11*$^{-/-}$ mock, $n = 6$; wild type Tg, $n = 18$; *Asc*$^{-/-}$ Tg, $n = 18$; *Casp11*$^{-/-}$ Tg, $n = 13$. Data shown are representative of 3 independent studies.

3.5. Local and Systemic Levels of Proinflammatory Cytokines Are Significantly Attenuated during the Acute Phase of Toxoplasma gondii Infection in the Absence of Caspase-11. IL-1β production following *T. gondii* infection has been shown to be dependent upon canonical NLRP1 and NLRP3 inflammasome activation [16, 17]. To evaluate the contribution of caspase-11 in this process, we evaluated systemic and local cytokine levels in the serum and PLF, respectively, from our wild type, *Asc*$^{-/-}$, and *Casp11*$^{-/-}$ mice. *T. gondii* infection resulted in a significant increase in both systemic and local levels of both IL-1β and IL-6 in the wild type animals 15 d.p.i. (Figures 5(a)–5(d)). Following infection, IL-1β was significantly attenuated in both serum and PLF from *Asc*$^{-/-}$ mice compared to the wild type animals (Figures 5(a) and 5(c)). This appears to be specific to IL-1β as IL-6 levels were significantly higher in *Asc*$^{-/-}$ mice compared to the wild type animals (Figures 5(b) and 5(d)). In addition to our findings in *Asc*$^{-/-}$ mice, we also observed a significant attenuation in both local and systemic IL-1β levels during acute *T. gondii* infection in the *Casp11*$^{-/-}$ animals (Figures 5(a) and 5(c)). However, unlike the data reported for mice lacking ASC, we also observed a significant attenuation in systemic IL-6 levels in the serum from the *Casp11*$^{-/-}$ mice compared to the wild type mice (Figure 5(b)). Local levels of IL-6 were significantly increased in the PLF from the *Casp11*$^{-/-}$ mice, similar to wild type levels, following *T. gondii* infection (Figure 5(d)). The reduction in systemic IL-6 likely reflects the overall improvement in morbidity and mortality

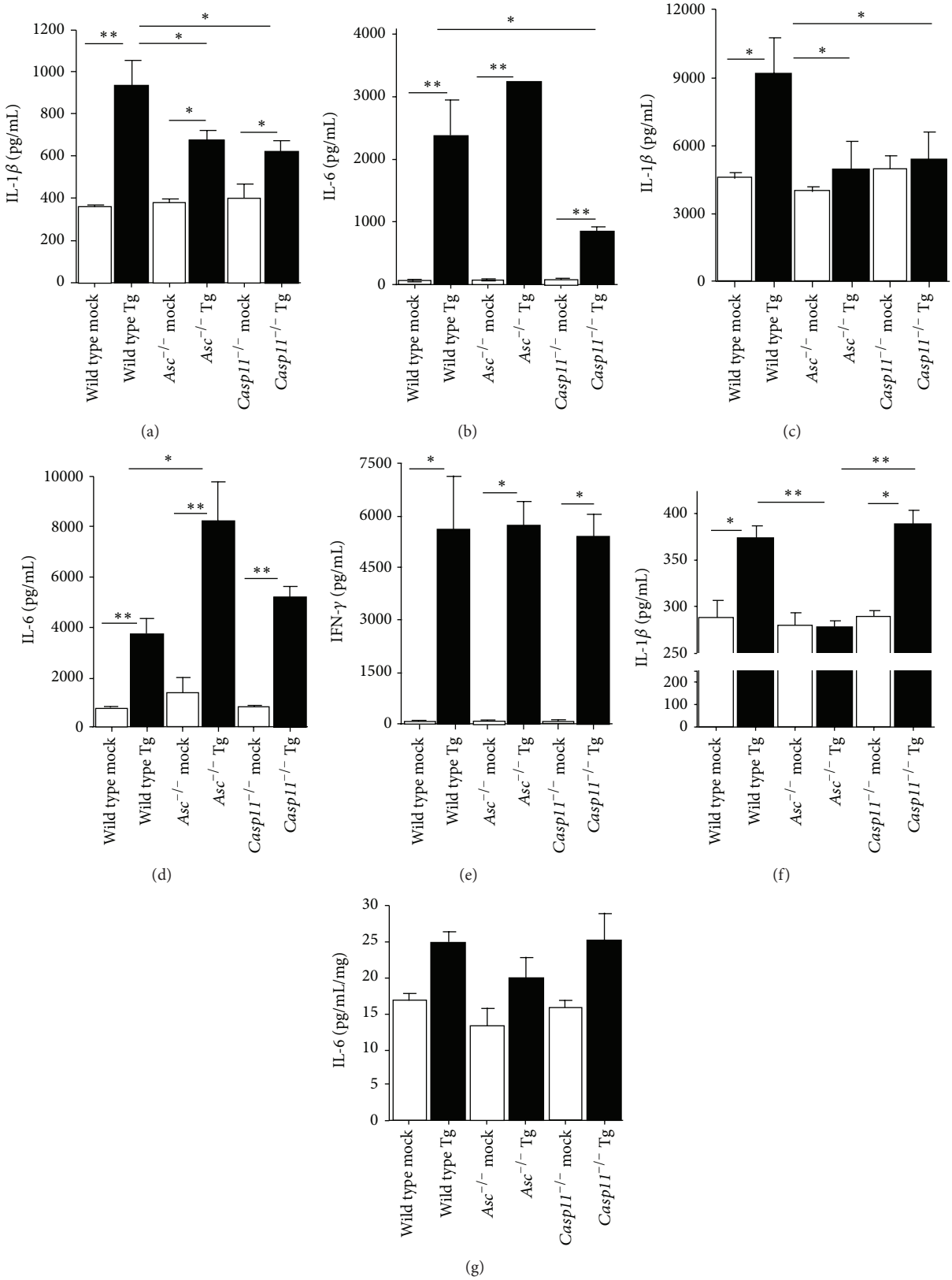

Figure 5: Local and systemic levels of IL-1β and IL-6 were significantly attenuated in *Casp1l*$^{-/-}$ mice following infection with *Toxoplasma gondii*. Whole blood, peritoneal lavage fluid (PLF), and brains were collected from wild type, *Casp1l*$^{-/-}$, and *Asc*$^{-/-}$ mice 15 days after inoculation with *Toxoplasma gondii* Me49 (a–d). IL-1β and IL-6 levels were determined in (a, b) serum and (c, d) cell-free PLF supernatant by ELISA. (e) IFN-γ levels were also evaluated in the cell-free PLF supernatant. (f, g) Brain samples were weighed and homogenized, and IL-1β and IL-6 levels were determined using the resultant cell-free supernatants. $^{*}p < 0.05$; $^{**}p < 0.01$. Wild type mock, $n = 9$; *Asc*$^{-/-}$ mock, $n = 9$; *Casp1l*$^{-/-}$ mock, $n = 6$; wild type Tg, $n = 18$; *Asc*$^{-/-}$ Tg, $n = 18$; *Casp1l*$^{-/-}$ Tg, $n = 13$. Data shown are representative of 3 independent studies.

and attenuated immune response observed in the *Casp11*$^{-/-}$ animals compared to the wild type and *Asc*$^{-/-}$ mice. In addition to IL-1β and IL-6, we also evaluated IFN-γ levels in the PLF (Figure 5(e)). IFN-γ is a critical cytokine that plays an essential role in controlling *T. gondii* proliferation and host protection [3, 29]. IFN-γ levels were significantly increased 15 d.p.i. in all of the infected mice compared to the mock treated animals. However, no significant difference in IFN-γ was detected between the different genotypes (Figure 5(e)). In addition to local and systemic cytokine responses, we also evaluated IL-1β and IL-6 levels in the brain at 15 d.p.i. The brain is one of the primary target organs of *T. gondii*. During the acute phase of infection, we observed a significant increase in IL-1β in brains collected from both wild type and *Casp11*$^{-/-}$ mice compared to the mock treated animals, with no differences between genotypes (Figure 5(f)). However, IL-1β was significantly attenuated in *Asc*$^{-/-}$ mice compared to either wild type or *Casp11*$^{-/-}$ animals (Figure 5(f)). It should be noted that the background levels of IL-1β were significantly increased in the brains from all animals regardless of genotype or treatment (Figure 5(f)). This is consistent with previous data that reported increased pro-IL-1β transcription levels in the brain at baseline [30]. In general, we have found this to be typical in tissue homogenates, which often include high levels of pro-IL-1β which is recognized by the ELISA based assays used in the current study. IL-6 was also evaluated in these samples. While we observed a slight increase in IL-6 in all infected mice, the overall levels of this cytokine were very low in the brain at this stage of *T. gondii* infection (Figure 5(g)). Together, these data are consistent with the prior studies evaluating ASC function in the acute stages of *T. gondii* infection and confirm the previous observations that IL-1β processing is deficient in the absence of canonical inflammasome signaling [16, 17]. Prior studies have shown that the noncanonical inflammasome augments canonical inflammasome function and the maturation of IL-1β during acute inflammation [31]. The data shown here are consistent with this previously described mechanism and further suggest that caspase-11, and more broadly the noncanonical inflammasome, likely functions through similar mechanisms as those previously reported for sepsis and acute bacteremia to modulate the host-parasite response to *T. gondii* [19].

3.6. Caspase-11 Mediated Inflammation during Acute Toxoplasma gondii Infection Results in Decreased Neuroinflammation during the Chronic Phase. The chronic phase of *T. gondii* infection is typically characterized by attenuated inflammation and the transition of the parasite from the tachyzoite stage to the cyst stage [32]. During this stage of the disease, the brain becomes an important refuge for *T. gondii* and is usually associated with benign clinical features of disease [33]. Mice surviving early infection were monitored for additional 10 days and necropsied 25 d.p.i. to evaluate disease pathogenesis in later stages. The majority of tissues showed only minor signs of prior disease (Figure 3). However, we did observe a significant amount of neuroinflammation in the brains of all infected mice (Figure 6). Brain histopathology was evaluated by a board certified veterinary pathologist (S.C.O.) following H&E staining. Brain inflammation scores were generated based on assessments of the presence and amount of inflammatory cells within perivascular spaces and whether or not they extended into the adjacent parenchyma as well as the amounts of inflammatory cells present in the leptomeninges. The presence of *T. gondii* cysts within the tissue was also evaluated. *T. gondii* infection resulted in a significant increase in neuroinflammation in all of the infected mice at 25 d.p.i. compared to the mock treated animals (Figures 6(a) and 6(b)). However, the neuroinflammation observed in the *Casp11*$^{-/-}$ mice was significantly increased at this time point compared to the wild type and *Asc*$^{-/-}$ mice (Figures 6(a) and 6(b)). In the *Casp11*$^{-/-}$ mice, leukocytes were observed within perivascular spaces and often extended into the brain tissue (Figure 6(a)). We also observed significant differences in leptomeningitis following *T. gondii* infection (Figures 6(c) and 6(d)). Leptomeningitis refers to inflammation associated with the subarachnoid space in the brain and has been previously associated with *T. gondii* infection in humans [34, 35]. Leptomeningitis developed in all of the *T. gondii* infected mice. However, the condition was significantly increased in the *Casp11*$^{-/-}$ animals compared to the wild type and *Asc*$^{-/-}$ mice (Figures 6(c)-6(d)). The underlying cause of this highly serious presentation of toxoplasmosis in humans is unknown. However, the condition appears to occur more often in the context of immunosuppression or attenuated innate immune responses [34, 35].

3.7. Caspase-11 Attenuates Toxoplasma gondii Brain Cyst Burden. The formation of tissue cysts is the basis of *T. gondii* persistence in infected humans and animals. Indeed, the most frequent mechanism of primary infection is the ingestion of these tissue cysts [36]. In the brain, *T. gondii* generates latent cysts, which have been suggested to be associated with tachyzoite invasion of microglia, astrocytes, and neurons [37–41]. While these cysts tend to be considered asymptomatic in healthy individuals, reactivation in immunocompromised patients may result in fatal toxoplasmic encephalitis [42]. Cyst formation in the brain was evaluated by histopathology 15 and 25 d.p.i. (Figures 7(a)–7(c)). No brain cysts were identified in samples from mice harvested at 15 d.p.i. but they were present in mice harvested at 25 d.p.i. Brain cysts were detected less frequently in histopathology sections from wild type (37.5%) and *Asc*$^{-/-}$ (12.5%) mice compared with the *Casp11*$^{-/-}$ (85.7%) animals (Figure 7(b)). Increased cyst formation is a response of the pathogen to the stresses of the tissue environment including the host immune response (reviewed in [43]). Thus, this finding is consistent with our neuroinflammation observations in the *Casp11*$^{-/-}$ mice (Figure 6). Interestingly, these data are also consistent with prior studies that have noted a significant increase in brain cyst formation in caspase-1/caspase-11 double knockout mice and in *MyD88*$^{-/-}$ animals [16, 44]. The mechanisms underlying these observations are still undetermined. However, similar to the findings from the caspase-1/caspase-11 and MyD88 studies, our data reveals that caspase-11 also plays a critical role, either directly or indirectly, in attenuating brain cyst burden.

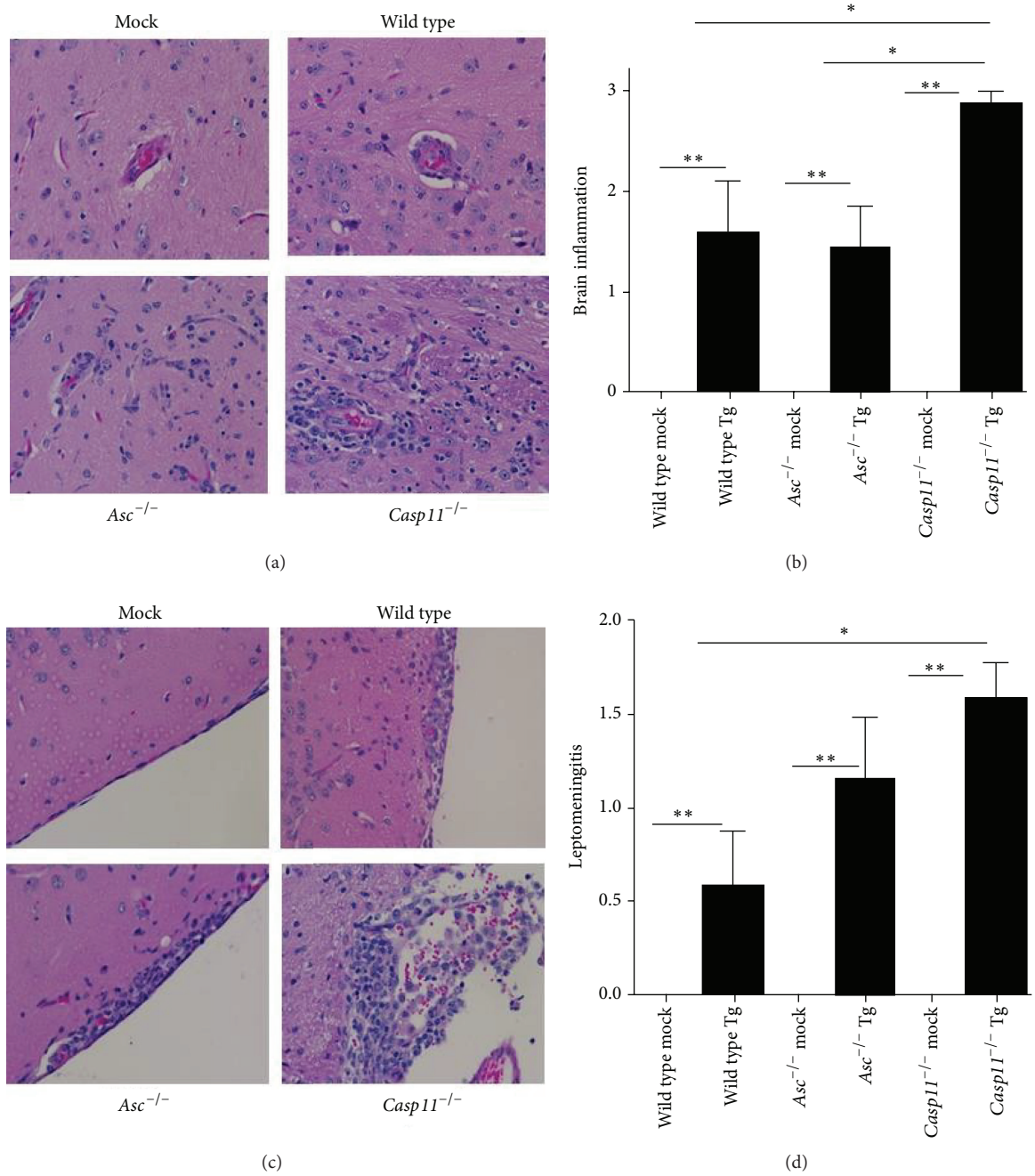

FIGURE 6: *Toxoplasma gondii* infection resulted in increased brain inflammation in *Casp11*$^{-/-}$ mice during late stages of disease. Wild type, *Casp11*$^{-/-}$, and *Asc*$^{-/-}$ mice were infected with 1,000 *Toxoplasma gondii* Me49 tachyzoites via i.p. administration and brains were evaluated 25 days after inoculation in surviving mice. (a) Histopathologic assessments of H&E stained sections revealed increased inflammation in the brains from all animals infected with *T. gondii*. 20x magnification. (b) Parameters associated with neuroinflammation were assessed and scored for each animal. Composite scores for each animal were averaged to generate a semiquantitative brain inflammation score. (c) Leptomeningitis was a predominate feature in all *T. gondii* infected animals. 20x magnification. (d) Histopathology scoring revealed significant increases in leptomeningitis in *Casp11*$^{-/-}$ mice. $^{*}p < 0.05$; $^{**}p < 0.01$. For all mock treated animals, $n = 3$. For all *T. gondii* inoculated animals, $n = 7$. Data shown are representative of 2 independent studies.

4. Discussion

Host resistance against intracellular pathogens, such as *T. gondii*, relies on a complex network of PRRs. The majority of studies to date have focused on TLR signaling pathways and have shown that resistance to *T. gondii* is driven by a diverse range of TLRs and is dependent upon the adaptor protein MyD88 [5–7, 44]. In addition to TLR signaling, members

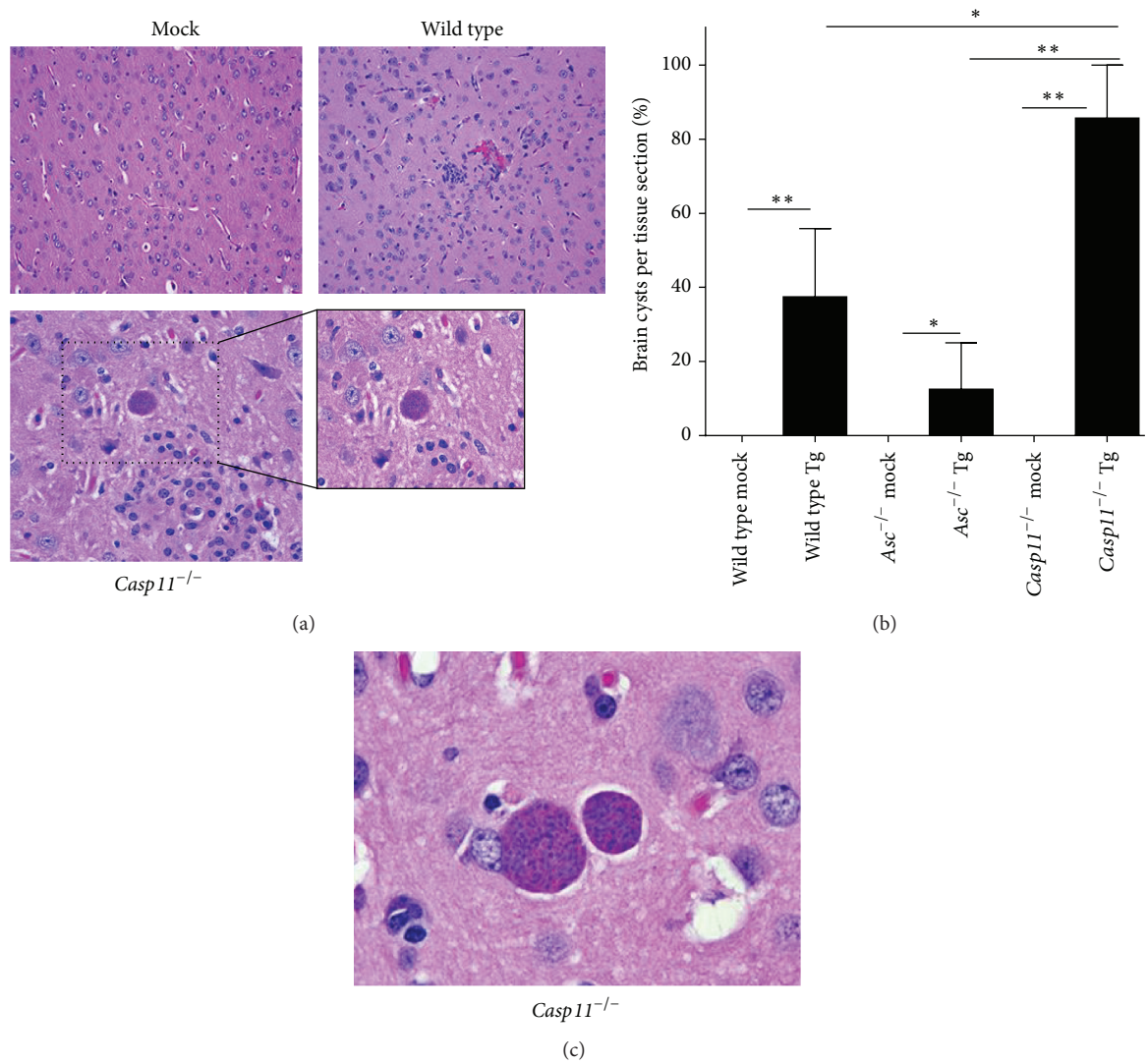

Figure 7: *Toxoplasma gondii* infection resulted in a greater brain tissue cyst burden in *Casp11*$^{-/-}$ mice during the chronic phase of disease. Wild type, *Casp11*$^{-/-}$, and *Asc*$^{-/-}$ mice were infected with 1,000 *Toxoplasma gondii* Me49 tachyzoites via i.p. administration and brain tissue cyst formation was evaluated by histopathology 25 days after inoculation in surviving mice. (a) Histopathology assessments of H&E stained sections revealed tissue cyst formation in the brains from all genotypes infected with *T. gondii*. 20x magnification, 40x insert. (b) The presence of tissue cysts with the brain was assessed for each animal and the percent of animals with tissue cysts was determined. (c) High magnification image of a brain tissue cyst from a *Casp11*$^{-/-}$ mouse. 100x magnification. $^{*}p < 0.05$; $^{**}p < 0.01$. For all mock treated animals, $n = 3$. For all *T. gondii* inoculated animals, $n = 7$. Data shown are representative of 2 independent studies.

of the inflammasome forming NLR family have also been identified as critical mediators of host resistance to *T. gondii* [17]. Initial studies using human monocytes and genetic ablation of ASC revealed that IL-1β release was dependent upon canonical inflammasome components in this *in vitro* system [45]. Additional mechanistic studies revealed that canonical NLR inflammasome activation and IL-1β production in human monocytes were associated with the recognition of *T. gondii* protein GRA15 [45]. These studies supported previous observations, also in the human monocyte system, which revealed NLRP1 as the NLR responsible for inflammasome formation following *T. gondii* infection. Though we did not test individual NLRs as a part of our current study, our findings using BMDMs from *Asc*$^{-/-}$ mice largely support these prior observations [16, 17]. However, the direct sensing of GRA15, or any other known PAMP associated with *T. gondii*, by an NLR family member is yet to be established and the mechanism of inflammasome activation by this parasite is still quite unclear.

Beyond macrophage studies, the NLRP1 and NLRP3 inflammasomes have also been shown to significantly modulate *T. gondii* pathogenesis *in vivo* [17]. Mice lacking ASC, NLRP1, and NLRP3 are highly susceptible to parasite infection [17]. In these studies, the sensitivity to *T. gondii* appears to be associated with reduced systemic IL-18, rather than IL-1β, which allows increased parasite replication and reduced animal survival [17]. Mice deficient in IL-18 and IL-18 signaling were subsequently utilized to confirm this

mechanism and the resultant findings revealed that these animals were also sensitive to *T. gondii* infection [17]. Our *in vivo* findings utilizing the $Asc^{-/-}$ mice are highly consistent with these prior observations and strongly imply that canonical inflammasome activation plays a vital role in promoting host resistance to *T. gondii*. While our overall conclusions support the prior studies, our current data does differ in the potential mechanism described. The apparent discrepancy between studies can be reconciled based on a variety of factors that can influence local and systemic cytokine levels following *T. gondii* infection in mice, including the infection dose/severity of disease, the timing of sample collection, the parasite strain, genetic modifications of the parental parasite strain, and the life cycle stage of the parasite at the time of sample collection [46].

It has recently come to light that studies of caspase-1 function have utilized $Casp1/11^{-/-}$ double knockout mice [17, 19]. Consistent with the loss of both canonical and noncanonical inflammasome signaling, macrophages from these animals also show ablated IL-1β [16, 17]. However, because both caspases are deficient in these animals, it has been difficult to discern a specific role for caspase-1 or caspase-11 in *T. gondii* pathogenesis. To better address this issue, we utilized $Casp11^{-/-}$ single knockout mice [19]. These animals have proven to be highly useful in characterizing canonical versus noncanonical inflammasome function following bacterial infection [19]. Here, we show that IL-1β production in $Casp11^{-/-}$ macrophages is significantly attenuated to levels which are comparable to those observed in $Asc^{-/-}$ macrophages (Figure 1). This is consistent with prior findings associated with caspase-11 function following LPS stimulation [19]. The current model of caspase-11 function focuses on its sensing of bacterial LPS, which has been shown to be a potent trigger of both canonical and noncanonical inflammasome signaling [47]. Extracellular LPS is recognized by TLR4 and serves as a priming signal for the canonical NLR inflammasome [48]. However, intracellular LPS appears to directly bind to and trigger caspase-11, resulting in canonical and noncanonical inflammasome activation in the cytoplasm [20]. Since *T. gondii* does not have LPS, this suggests that caspase-11 is associated with the recognition of some other aspect of this intracellular parasite to modulate IL-1β production. One possible mechanism could be associated withpotassium efflux, which is important to *T. gondii* egress from infected cells [49]. Recent studies using acute LPS exposure have suggested that potassium efflux can directly modulate caspase-11 activation of the NLRP3 inflammasome [50]. While this has not been directly evaluated in the context of *T. gondii* infection, this may be a possible mechanism underlying caspase-11 function.

Our data suggest that the loss of caspase-11 plays a protective role shortly after *T. gondii* exposure as $Casp11^{-/-}$ mice have improved survival and reduced local and systemic inflammation during earlier time points. These observations are consistent with the reduced levels of IL-1β and other proinflammatory cytokines, such as IL-6 (Figure 5). The pathogenesis is significantly different between the $Casp11^{-/-}$ and $Asc^{-/-}$ mice. Combined with the prior data related to ASC and caspase-1/11, these data suggest that caspase-1 and caspase-11 have distinct, nonredundant functions following *T. gondii* infection. It is possible that the canonical inflammasome modulates IL-18 and controls parasite replication, as previously reported [17], whereas caspase-11 and the noncanonical inflammasome modulate IL-1β and/or cell death, which act to increase local inflammation and minimize parasite migration to the brain. Supporting this hypothesis, in the $Casp11^{-/-}$ mice, we show attenuated local inflammation during acute infection yet with limited effects on tachyzoite burden in the local tissues (spleen and liver) (Figure 3). However, during the later stages of infection, the $Casp11^{-/-}$ mice present with greater neuroinflammation and cyst burden compared to the surviving wild type and $Asc^{-/-}$ animals (Figures 6 and 7). Similar data has been reported for $MyD88^{-/-}$ mice [44]. However, the mechanisms associated with the increased parasite migration and neuropathological effects were not fully discovered. Together, these data suggest that reduced local inflammation can result in improved morbidity and survival during early stages of disease, while creating an environment favorable for systemic expansion and ultimately increased brain localization.

The findings presented here suggest that caspase-11 function extends well beyond the currently characterized mechanisms associated with LPS recognition and acute bacterial infections. Our data show that caspase-11 is associated with increased inflammation during acute *T. gondii* infection, which results in attenuated neuroinflammation and reduced brain cyst burden during chronic phases of the disease. These data may suggest a role for caspase-11 in response to a currently undefined molecular pattern associated with *T. gondii* or may suggest that the noncanonical inflammasome is being activated by intracellular changes driven by the parasite, such as changes in potassium efflux. It is clear that additional mechanistic insight is needed to better define the interaction between these inflammasome pathways and parasite associated factors. Likewise, additional insight pertaining to the relationship between members of the TLR and NLR families in initiating the host immune response following *T. gondii* infection is necessary to better define the host-pathogen interactions. We anticipate that better defining the contribution of specific elements associated with the host innate immune response to this parasite will result in new therapeutic options and strategies to protect against this pervasive human pathogen.

Disclosure

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or any other funding agency.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Sheryl L. Coutermarsh-Ott and John T. Doran contributed equally to this paper.

Acknowledgments

The authors would like to acknowledge Dr. Vishva Dixit (Genentech) for the generous supply of *Casp11*$^{-/-}$ mice utilized in this work. This work was supported by the Virginia-Maryland College of Veterinary Medicine, Virginia Tech, through internal funding and research support to David S. Lindsay and Irving C. Allen. The authors would also like to acknowledge the financial support of the Virginia Tech Open Access Subvention Fund. Student work on this publication was supported by the National Institute of Allergy and Infectious Diseases' Animal Model Research for Veterinarians (AMRV) training grant (T32-OD010430). The authors would also like to recognize the Virginia Tech Postbaccalaureate Research and Education Program (VT-PREP) and Undergraduate Research Programs for student support.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 8175898, 8 pages
http://dx.doi.org/10.1155/2016/8175898

Research Article

Maternal Vitamin D Level Is Associated with Viral Toll-Like Receptor Triggered IL-10 Response but Not the Risk of Infectious Diseases in Infancy

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Received 21 January 2016; Revised 14 April 2016; Accepted 27 April 2016

Academic Editor: Irving Allen

Reports on the effect of prenatal vitamin D status on fetal immune development and infectious diseases in childhood are limited. The aim of this study was to investigate the role of maternal and cord blood vitamin D level in TLR-related innate immunity and its effect on infectious outcome. Maternal and cord blood 25 (OH)D level were examined from 372 maternal-neonatal pairs and their correlation with TLR-triggered TNF-α, IL-6, and IL-10 response at birth was assessed. Clinical outcomes related to infection at 12 months of age were also evaluated. The result showed that 75% of the pregnant mothers and 75.8% of the neonates were vitamin deficient. There was a high correlation between maternal and cord 25(OH)D levels ($r = 0.67$, $p < 0.001$). Maternal vitamin D level was inversely correlated with IL-10 response to TLR3 ($p = 0.004$) and TLR7-8 stimulation ($p = 0.006$). However, none of the TLR-triggered cytokine productions were associated with cord 25(OH)D concentration. There was no relationship between maternal and cord blood vitamin D status with infectious diseases during infancy. In conclusion, our study had shown that maternal vitamin D, but not cord vitamin D level, was associated with viral TLR-triggered IL-10 response.

1. Introduction

Vitamin D has been shown to play an important role in both the innate and adaptive immune system. In vitro studies have demonstrated vitamin D to correlate with alterations of several cytokines such as IL-4, IL-5, IL-6, IL-10, IL-13, and interferon γ [1–3]. Hence, as a potent immune modulator, vitamin D was shown to be associated with childhood asthma and allergic diseases [4–6]. In addition to allergic diseases, vitamin D also partakes a potential role in airway inflammation, thus, strongly linked with acute infectious illness such as upper or lower respiratory tract infections, sepsis, and hospitalization [7–10]. Given the important immune modulatory role of vitamin D, the link between Toll-like receptors- (TLRs-) mediated innate immunity and vitamin D deserves in-depth investigation. Studies have shown vitamin D to downregulate TLR expression in order to dampen immune cytokine response in multiple basic science models [11–13]. In contrast, some reports have shown treatment with vitamin D to result in increased TLR activation. In a cohort study of 225 infants, higher 25(OH)D_3 level at 6 months was associated with greater cytokine responses to TLR ligands [14–16]. Thus, despite a wide variety of studies that acknowledged the immunomodulatory role of vitamin D,

the results have been conflicting. Furthermore, very few studies address the impact of prenatal vitamin D status on TLR-related innate immune response in neonatal infants. Because of the apparent importance of vitamin D in immune development, we aimed to investigate the effect of maternal and/or cord vitamin D level on TLR-triggered cytokine response in neonates at time of birth and disease outcome in early childhood. We focused attention on neonatal innate immunity since early life events appear to have a critical influence on the ultimate pattern of immune maturation. In addition, we sought to investigate whether maternal blood and cord blood vitamin D correspond in their association with TLR-related innate immunity.

2. Methods

2.1. Study Population. Data for this analysis came from an ongoing prospective birth cohort study called the PATCH (The Prediction of Allergy in Taiwanese Children). The Chang Gung Ethics Committee approved the study, and informed consent was obtained from the parents/legal guardians of the neonates. Pregnant women undergoing routine prenatal exam were approached randomly by a study nurse and invited to join our research program. All mothers and their offspring were enrolled upon agreement, but those born under the gestational age of 37 weeks, had major congenital anomaly, or were suspicious of congenital infections were subsequently excluded from this analysis. The result from this study comprised the first 372 eligible mother-neonatal pairs. A baseline questionnaire survey was conducted at birth to obtain parental information such as demographic characteristics, medical and obstetric history, and smoking exposure history. Standardized questionnaires on atopic heredity, environmental factors, infection, and allergic diseases were answered at 2, 4, 6, and 12 months and every year thereafter. Infants were defined as ever having lower respiratory tract infection (bronchiolitis, pneumonia, and/or croup) if there was a diagnosis from a health care professional, and the infant either had been hospitalized or received medical treatment. Other infections such as infectious enteritis and urinary tract infection were also obtained from medical records with physicians' diagnosis. By the time of analysis, 321 children included in this study were at least 1 year of age and had adequate follow-up data.

2.1.1. Sample Collection, Cell Culture, and TLR Ligands Stimulation. The details of our experimental procedures have been published previously [17]. Briefly, maternal blood was obtained during third gestation and umbilical cord blood collected at the time of delivery. Mononuclear cells were isolated and stimulated with TLR ligands. These included synthetic bacterial lipoprotein (PAM3csk4) that is recognized by TLR1-2; a synthetic analog of double stranded RNA for TLR3; ultrapure LPS for TLR4; and R848, which is activated via the TLR7/TLR8 signaling pathway. As a positive control, cells were treated with the NF-κB activator phytohemagglutinin (Murex Pharmaceuticals) at 4 μg/mL in R10-FBS. To determine TLR responses, 3×10^5 PBMCs in 100 μL R10-FBS were added to each of the media or ligands (in duplicate), containing wells and incubated at 37°C for 20 h with 5% CO_2. All assay preparations were performed using sterile technique in a laminar flow hood. The concentrations of the ligands used for this experiment are as follows: 10 μg/mL of PAM3csk4, 10 μg/mL of poly(I:C) directly administered, 20 ng/mL of LPS, and 10 μg/mL of R848 (InvivoGen, San Diego, CA).

2.1.2. Measurement of Cytokines. TNF-α, IL-10, and IL-6 levels in culture supernatants were determined by enzyme-linked immunosorbent assays according to the manufacturer's instructions (ELISA; R&D systems, MN). The detection limits were 15.6 pg/mL for TNF-α, 3.12 pg/mL for IL-6, and 7.8 pg/mL for IL-10.

2.1.3. Serum 25(OH)D Measurement. Serum samples obtained from the pregnant mother and cord blood were stored frozen in aliquotsat −80°C until analysis. Serum 25(OH)D levels were measured by Elecsys Vitamin D total assay (Roche Diagnostics, Mannheim, Germany). This method is a new automated electrochemiluminescence-based assay that measures both the $25(OH)D_2$ and $25(OH)D_3$ as total 25(OH)D level. Results from this assay have shown close agreement to other well-established methods such as liquid-chromatography tandem mass spectrometry (LC-MS/MS) [18].

2.2. Statistical Methods. Spearman's rank correlation test was performed to analyze the correlation between maternal and cord blood 25(OH)D level. Since the concentrations of 25(OH)D and cytokines were not normally distributed, values were logarithmically transformed as continuous variables in the statistical models. Regression analysis was used to determine the relation between maternal/cord blood 25(OH)D concentration and TLR-induced cytokine response as continuous variables. Association between serum vitamin D level and binary outcomes (bronchiolitis, pneumonia, croup, infectious enteritis, and urinary tract infection) was analyzed by using logistic regression. Models were adjusted for gestational age, gender, birth body weight, mode of delivery, maternal allergy, and season of birth. All statistical analysis was carried out using IBM SPSS Statistics Version 20 (Armonk, NY).

3. Result

3.1. Subject and Demographic Data. Characteristics of the mother-neonatal pairs are summarized in Table 1. Mean maternal age was 29.4 years. The incidence of maternal allergy was 34.8%, compatible with that of the general population. All neonates included in this analysis were above gestational age of 37 weeks with adequate birth body weight. Slightly more babies were delivered during the season of spring (28,8%). Of the original 372 maternal-neonatal pairs, fifty-one participants either were lost to follow-up, refused to further participate, or had yet to return. By the age of 12 months, 321 infants with complete questionnaires and

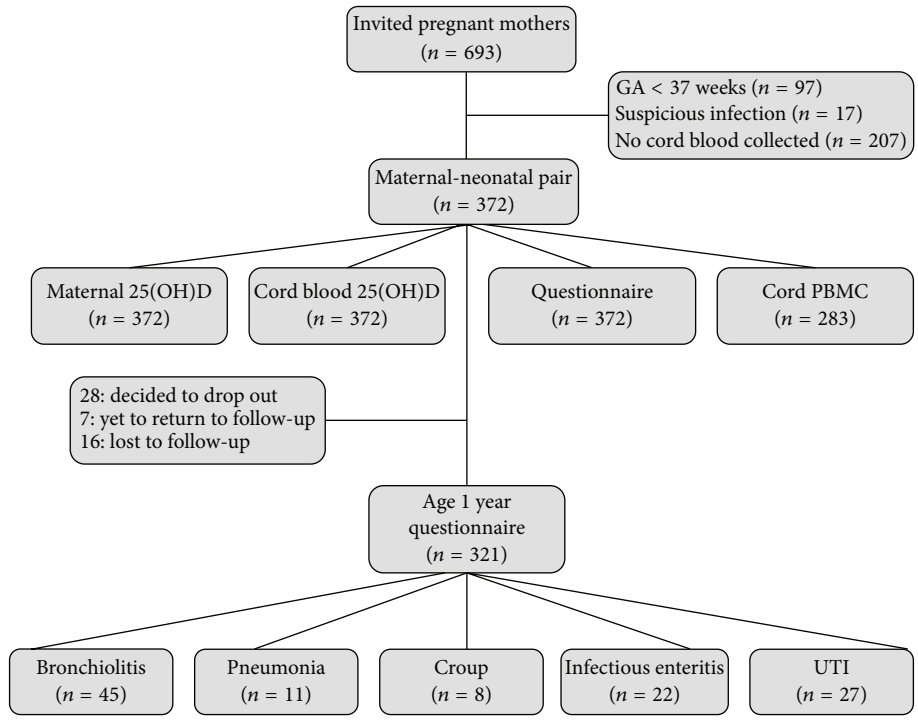

Figure 1: Flowchart of the analyzed maternal-neonatal pairs: demonstrating the number of study participants after consideration of available maternal and cord 25(OH)D measurements, cell culture data, and questionnaire information.

Table 1: Characteristics of mother-neonatal pairs at delivery.

Characteristics	Data
Mothers (*n*)	372
Age at enrollment (y)	29.4 (28.6–30.1)
History of allergy	129 (34.8)
Smoking during pregnancy	35 (9.5)
Education	
Primary or secondary	11 (3.0)
High school	99 (26.6)
College or above	262 (70.4)
Mode of delivery (NSD)	234 (62.9)
25(OH)D (ng/mL); median (IQR)	15.18 (10.85–19.10)
Neonates (*n*)	372
Sex (male)	179 (48.6)
BBW (g)	3087 ± 481
Gestational age (weeks)	38.3 ± 1
Season of birth	
Spring	107 (28.8)
Summer	105 (28.2)
Autumn	88 (23.7)
Winter	68 (18.3)
25(OH)D (ng/mL); median (IQR)	14.80 (10.02–18.86)

Values are listed as *n* (%) or mean ± SD, as appropriate.
NSD: natural spontaneous delivery.
IQR: interquartile.

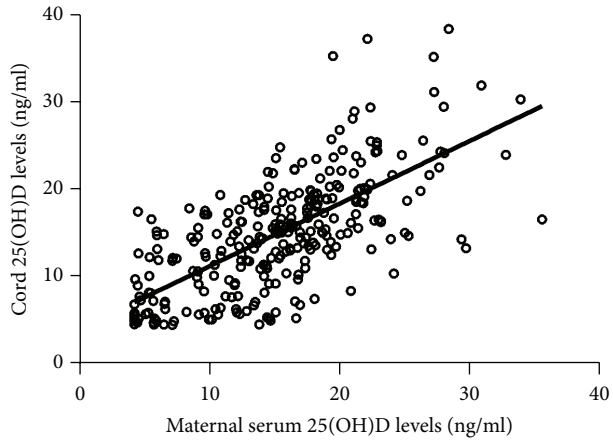

Figure 2: Correlation between maternal and cord blood 25(OH)D levels (ng/mL).

medical records were available for analysis. Detailed number of participants and test samples are listed in Figure 1.

3.2. Maternal and Cord Vitamin D Levels. The median maternal blood 25(OH)D concentration was 15.18 ng/mL (interquartile range (IQ): 10.85–19.10 ng/mL), and the median cord blood 25(OH)D was 14.80 ng/mL (IQ: 10.02–18.86 ng/mL). Of the 372 maternal participants, 280 (75%) had 25(OH)D levels less than 20 ng/mL (considered deficient), and 71 (19%) had levels between 20 and 30 ng/mL (considered insufficient). An even higher percentage of vitamin D deficiency was found in the neonatal cord blood, with 279 (75.8%) having levels less than 20 ng/mL, and 55 (15%) between 20 and 30 ng/mL. There was a high correlation between maternal and cord 25(OH)D levels ($r = 0.67$, $p < 0.001$; Figure 2).

3.3. Association of Maternal and Cord Vitamin D Level with TLR-Stimulated Cytokine Response. Because the distribution of most cytokine levels and vitamin D concentrations was highly skewed (data not shown), we used natural log-transformed 25(OH)D and cytokine levels for correlation analysis. The result showed significant inverse correlation between maternal 25(OH)D level and IL-10 response to TLR3 and TLR7-8 stimulation ($p = 0.007$ and $p = 0.008$, resp.) in cord blood mononuclear cells. The result still remained significant after adjusting for potential confounding factors ($p = 0.004$ for TLR3 and $p = 0.006$ for TLR7-8) (Table 2(a)). However, neonatal cord 25(OH)D concentration was not associated with any of the TLR-triggered cytokine productions (Table 2(b)). Preliminary analysis was also performed on maternal innate immune function; the result showed no correlation between maternal vitamin D status and cytokine response to TLR ligands in maternal mononuclear cells (Supplement 1) (see Supplementary Material available online at http://dx.doi.org/10.1155/2016/8175898).

3.4. Vitamin D Level and Clinical Outcome. By the age of one year, 321 infants had their medical records reviewed and completed the questionnaires administered at 6 and 12 months of age. Analysis was made to investigate whether maternal or cord blood vitamin D status was associated with lower respiratory tract infection (bronchiolitis, pneumonia, and croup), infectious enteritis, and urinary tract infection at 1 year of age. The results, summarized in Table 3, showed no significant association between maternal vitamin D status and the incidence of infection during the first year of life. Cord blood vitamin D level was also not correlated with any of the infectious disorders by 12 months of age.

4. Discussion

The result from our study showed that maternal, but not cord blood vitamin D level, was associated with TLR-3 and TLR7-8 triggered IL-10 response. To our knowledge, this is the first cohort study to simultaneously assess the effect of both maternal and cord blood vitamin D status on TLR-related immune response and various infectious diseases during infancy. Despite extensive investigations on vitamin D, its role in neonatal immune development and health outcome remains inconsistent. The reasons for various conflicting results might be due to differences in the study designs, age of the study population, definition of clinical outcomes, and also disparities in the assessment of vitamin D concentration (use of maternal blood or cord blood). Our result suggested that the latter might be an important issue to consider, because despite a strong correlation between maternal and cord vitamin D level, our study had observed a distinction between maternal and cord vitamin D status in their association with TLR-triggered cytokine response. Thus, when addressing the impact of prenatal vitamin D status on various outcomes, it might be important to consider that vitamin D levels in the pregnant mothers and cord blood might not always correspond uniformly. Similar observations were noted in few other studies that concurrently assessed maternal and cord vitamin D concentrations. In the study of Weisse et al., although both maternal and cord blood vitamin D levels were associated with clinical food allergy, only maternal $25(OH)D_3$ was associated with an increase in allergen sensitization [19]. Another recent publication had demonstrated that although low levels of vitamin D in the cord blood were associated with higher airway resistance in childhood, maternal vitamin D level was not related to the children's airway resistance [20].

The relationship between vitamin D and IL-10 has been established considerably. Our result had observed a negative correlation between maternal vitamin D concentration and TLR-induced IL-10 response. In contrast to our study, Vijayendra Chary et al. had observed lower cord blood IL-10 level in vitamin D deficient or insufficient subjects. In vitro studies have also shown direct vitamin D supplementation in culture human cells to upregulate IL-10 secretion [3, 21–24]. The difference between our findings and those of published reports might be explained by distinctive study design, as ours assessed cytokine response to TLR ligands under different vitamin D concentrations, and not cytokine response to direct vitamin D stimulation. Because direct vitamin D supplementation deemed to increase IL-10 production, our result suggested a reduced IL-10 response to TLR stimulation with higher vitamin D concentration. Similar result was observed in the study of Belderbos, in which they found high concentration of 1,25-OHD to suppress IL-10 response to LPS stimulation in adult PBMC [25]. As a potent anti-inflammatory immune modulator, studies have shown vitamin D to downregulate TLR expression in monocytes resulting in reduced downstream cytokine production [11, 13]. Thus, we speculated that, with increasing vitamin D level, the ability of TLR to trigger IL-10 production is diminished when compared to lower concentration, thus displaying a negative correlation between vitamin D level and cytokine response to TLR stimulation. In addition, studies have demonstrated higher vitamin D levels at birth to be associated with lower number of T regulatory (Treg) cells in the cord blood [2, 16]. Since one potential source of IL-10 is Treg, it was speculated that higher vitamin D level would be associated with less Treg cells to respond to TLR stimulation, thus resulting in lower IL-10 response as seen in our study. Further research is warranted to investigate the mechanism in which maternal vitamin D status affects neonatal TLR-related IL-10 response.

Evidences have shown low production of IL-10 at birth to be strongly associated with susceptibility to acute respiratory tract infections in children aged 5 years [26]. However, in present analyses, albeit an association between maternal vitamin D level and viral TLR-triggered IL-10 response, there was no effect on the prevalence of infectious diseases during the first 12 months of life. Our observations are at odds with several studies that showed lower vitamin D status to be associated with increased incidence or severity of infection during early childhood. However, most studies that showed protective effect of vitamin D against respiratory diseases were of interventional studies that used supplementary vitamin D and did not measure serum vitamin D level. Although the study of Belderbos et al. demonstrated an association between low cord blood vitamin D level and increased

Table 2: (a) Association between maternal vitamin D level and Toll-like receptor triggered cytokine response. (b) Association between cord blood 25(OH)D level and Toll-like receptor triggered cytokine response.

(a)

	Univariate analysis β (95% CI)	p	Multivariate analysis β (95% CI)	p
TLR1-2				
TNF-α	0.42 (0.44, 1.47)	0.36	0.55 (−0.53, 1.59)	0.30
IL-6	0.35 (−0.85, 0.79)	0.92	0.13 (−0.79, 1.05)	0.79
IL-10	0.04 (−0.79, 0.86)	0.93	0.12 (−0.82, 1.06)	0.76
TLR3				
TNF-α	0.15 (−0.64, 0.96)	0.73	0.15 (−0.68, 0.94)	0.93
IL-6	−0.45 (−1.15, 0.33)	0.26	−0.42 (−1.48, 0.54)	0.40
IL-10	−0.85 (−1.45, −0.24)	0.007	−1.05 (−1.70, −0.34)	0.004
TLR4				
TNF-α	0.12 (−0.19, 0.50)	0.43	0.08 (−0.28, 0.44)	0.44
IL-6	−0.17 (−0.46, 0.12)	0.27	−0.24 (−0.54, 0.08)	0.14
IL-10	−0.03 (−0.16, 0.11)	0.69	0.19 (−0.55, 0.15)	0.28
TLR7-8				
TNF-α	−0.35 (−0.84, 0.12)	0.64	−0.31 (−0.83, 0.20)	0.19
IL-6	−0.05 (−0.43, 0.28)	0.79	−0.18 (−0.59, 0.21)	0.89
IL-10	−0.85 (−1.47, −0.22)	0.008	−0.99 (−1.70, −0.30)	0.006
PHA				
TNF-α	0.52 (−0.59, 1.73)	0.39	0.30 (−1.07, 1.55)	0.39
IL-6	0.52 (−0.55, 1.62)	0.33	0.30 (−1.02, 1.59)	0.67
IL-10	−0.36 (−0.34, 1.14)	0.35	0.08 (−0.80, 0.96)	0.87

Adjusted for gestational age, gender, birth body weight, mode of delivery, maternal allergy, and season of birth.

(b)

	Univariate analysis β (95% CI)	p	Multivariate analysis β (95% CI)	p
TLR1-2				
TNF-α	0.63 (−0.06, 1.50)	0.09	0.73 (−0.17, 1.64)	0.12
IL-6	0.21 (−0.63, 1.20)	0.67	0.51 (−0.77, 1.51)	0.51
IL-10	0.21 (−0.74, 1.11)	0.62	0.51 (−0.68, 1.50)	0.41
TLR3				
TNF-α	0.77 (−0.01, 1.55)	0.06	0.63 (−0.27, 1.50)	0.16
IL-6	−0.13 (−0.83, 0.63)	0.73	0.18 (−0.86, 1.18)	0.71
IL-10	−0.17 (−0.67, 0.41)	0.54	−0.18 (−0.83, 0.50)	0.62
TLR4				
TNF-α	0.20 (−0.22, 0.72)	0.41	0.21 (−0.41, 0.69)	0.70
IL-6	−0.05 (−0.38, 0.26)	0.77	0.01 (−0.39, 0.39)	0.96
IL-10	−0.03 (−0.16, 0.11)	0.69	0.19 (−0.55, 0.15)	0.28
TLR7-8				
TNF-α	0.14 (−0.29, 0.58)	0.55	0.39 (−0.05, 0.85)	0.95
IL-6	0.14 (−0.36, 0.60)	0.60	−0.03 (−0.48, 0.47)	0.89
IL-10	−0.51 (−1.33, 0.23)	0.16	−0.49 (−1.64, 0.52)	0.40
PHA				
TNF-α	0.94 (−0.06, 2.02)	0.78	0.61 (−0.45, 1.79)	0.29
IL-6	0.94 (−0.10, 2.00)	0.08	0.61 (−0.47, 1.74)	0.30
IL-10	0.56 (−0.27, 1.34)	14	0.47 (−0.56, 1.34)	0.30

Adjusted for gestational age, gender, birth body weight, mode of delivery, maternal allergy, and season of birth.

Table 3: Association of maternal and cord serum 25(OH)D levels with clinical outcome at 1 year of age.

	Univariate OR (95% CI)	p	Multivariate OR (95% CI)	p
Maternal serum 25(OH)D				
Bronchiolitis	1.26 (0.47, 3.37)	0.65	1.25 (0.42, 3.71)	0.69
Pneumonia	2.57 (0.41, 16.07)	0.31	4.58 (0.52, 40.51)	0.17
Croup	7.10 (0.85, 60.81)	0.07	13.29 (0.88, 199.8)	0.06
Enteritis	1.20 (0.36, 4.02)	0.77	1.47 (0.38, 5.62)	0.58
UTI	0.85 (0.25, 2.94)	0.80	0.92 (0.26, 3.24)	0.92
Cord serum 25(OH)D				
Bronchiolitis	2.22 (0.90, 5.47)	0.08	1.13 (0.42, 3.04)	0.81
Pneumonia	1.70 (0.36, 7.94)	0.51	2.38 (0.22, 25.46)	0.47
Croup	3.82 (0.67, 21.81)	0.13	4.64 (0.75, 28.80)	0.10
Enteritis	0.98 (0.36, 2.69)	0.97	1.42 (0.43, 4.69)	0.56
UTI	0.75 (0.25, 2.29)	0.62	0.78 (0.25, 2.45)	0.67

Total number of children aged 1 year: 321.
Number of bronchiolitis: 45 (14%), pneumonia: 11 (3.4%), croup: 8 (2.5%), infectious enteritis: 22 (6.9%), and UTI (urinary tract infection): 27 (8.4%).
Adjusted for gestational age, sex, birth body weight, mode of delivery, season of birth, and maternal allergy.

respiratory syncytial virus infections in infancy, however, unlike our participants, around 50% of their neonates were vitamin D sufficient [27–29]. Since the majority of our participants were vitamin D deficient, it is possible that higher serum levels might be required to reach optimal protective effect to result in significant clinical differences. Nonetheless, in support of our results, several studies also failed to show a difference in serum vitamin D level between children with and without respiratory infections [30–32]. These inconsistent observations point to the complicated role of vitamin D in the immune modulation and disease process. The null results from our observation suggested that since the immune system is composed of multiple cells and variable pathways, having effect on only certain cytokines, such as IL-10, might not have an overall effect on disease outcome.

Our study had several limitations. First, the predominantly low serum level of 25(OH)D in this study has limited our ability to determine whether there is an association between higher concentration of vitamin D and infection. It has also limited the applicability of our result in representing the general population, although inadequate vitamin D concentration seems particularly common among pregnant woman [3, 19]. In addition, having a majority of population with suboptimal vitamin D level, we were unable to perform analysis with commonly used clinical cut-offs of vitamin D (deficient < 20 ng/mL, insufficient 20–29.9 ng/mL, sufficient ≥ 30 ng/mL). Thus, future studies related to the effect of prenatal vitamin D level on neonatal innate immunity and subsequent health outcomes will need to focus on populations with more vitamin D sufficient pregnant mothers. In addition, because only cultured supernatants were harvested in this study, we did not perform tests on T regulatory cells or maturation of the monocytes. Thus, our data could not provide detailed mechanism on how maternal vitamin D level affected neonatal TLR-triggered IL-10 response. Finally, although present study had observed an association between prenatal vitamin D status and viral TLRs-triggered response (TLR3 and TLR7/8, both of which recognize viral RNA), the effect of vitamin D on CpG double stranded DNA motif (TLR9) of the viral genome also demands further exploration. However, due to technical issues, analysis was only completed in very few participants. The result showed no correlation between maternal vitamin D status and neonatal TLR9 triggered cytokine response (Supplement 2), though such conclusion needed to be interpreted with caution for the null correlation might be due to lack of power owing to small number of test samples.

In conclusion, our study had shown that maternal vitamin D level, but not cord vitamin D, was associated with TLR3 and TLR7-8 triggered IL-10 response. This study emphasized that even though cord vitamin D level was strongly correlated with maternal vitamin D status, the extent of impact on fetal immune cytokine response might be distinctive. Although our study did not show maternal vitamin D concentration to have a significant impact on the magnitude or functional capacity of the young infant to defend against infection, we believe our study may contribute to a better understanding of the effect of prenatal vitamin D status on neonatal innate immunity and infectious disease during early life.

Competing Interests

The authors declare no competing interests.

Acknowledgments

This work was financially supported by Chang Gung Memorial Hospital (Grants CMRPG2B0021-3 and 2E0111). The authors thank all the families for their participation and Mr. Hao-Chun Cheng for the technical assistance. The PATCH (The Prediction of Allergy in Taiwanese Children) Cohort Study.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 2180417, 8 pages
http://dx.doi.org/10.1155/2016/2180417

Review Article

Holding the Inflammatory System in Check: TLRs and Their Targeted Therapy in Asthma

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Received 24 February 2016; Accepted 13 April 2016

Academic Editor: Eda Holl

Inflammation is a complex biological response to detrimental stimuli and can be a double-edged sword. Inflammation plays a protective role in removing pathogenic factors, but dysregulated inflammation is associated with several major fatal diseases such as asthma, cancer, and cardiovascular diseases. Asthma is a complex heterogenous disease caused by genetic and environmental factors. TLRs are the primary proteins associated with the innate and adaptive immune responses to these fatal factors and play an important role in recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), which initiates the downstream immune response. Due to the complex TLRs cascade and nowadays unsuccessful control in asthma, new studies are focused on TLRs and other potential targets in TLR cascade to minimize airway inflammation.

1. Introduction

Inflammation is a complex host response to detrimental stimuli including tissue injury, microbial infection, and irritant exposure. It is classically characterized by redness, swelling, heat, pain, and tissue dysfunction [1]. When inflammation involves mucosal surfaces, there are accompanying mucus hypersecretions and epithelial shedding. Inflammation plays a protective role in the body in negating pathogenic factors such as microbial infections and oxidative stress and is a healing process enabling repair of damaged tissue [2]. On the contrary, persistence of inflammation with overproduction of cytokines by immune cells including macrophages, neutrophils, eosinophils, dendritic cells, mast cells, natural killer cells, and structural cells such as endothelial cells, mucosal epithelial cells, and fibroblasts can be harmful. Dysregulated inflammation is associated with several diseases including asthma, cancer, cardiovascular disease, autoimmune diseases, and metabolic disease.

Asthma is a complex heterogeneous disease associated with local tissue chronic inflammation of the airway and is characterized by variable and recurring symptoms (including wheezing, coughing, chest tightness, and shortness of breath), reversible airflow obstruction, airway remodeling, and airway hyperresponsiveness. According to Chung [3], asthma is ranked as the 14th most important chronic disease worldwide regarding the prevalence, extent, and duration of disability and affects 334 million individuals of all ages, resulting in 90 and 170 deaths per million in female and male individuals, respectively. In addition, asthma causes a heavy economic burden for the government and individuals. For example, in Europe, total cost per patient ranges from £509 for controlled asthma to £2281 for uncontrolled asthma [4]. Asthma is caused by a complex and incompletely understood combination of genetic (polymorphisms of multiple genes) and environmental (such as respiratory infections and particulates PM2.5) factors, which induce an immune response via the infiltration of inflammatory cells into the airway and consequent cytokine release. Emerging evidence shows that Toll-like receptors (TLRs) are associated with the inflammatory response and chronic airway inflammation in asthma [5]. TLRs are a subgroup of pattern recognition

receptors (PRRs) that are expressed by cells of the innate immune system and that sense two classes of molecules such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), which then initiates the downstream immune cascades.

Many previous studies have focused on the discovery, structure, and roles of TLR family members and related signaling pathways in airway diseases, but few studies emphasize TLR expression in asthma, especially in the different phenotypes. This review will highlight the roles of TLR members in airway inflammation and their association with the pathogenesis of distinct asthma phenotypes and in addition will discuss the potential for TLR-targeted therapies in the treatment of asthma.

2. TLRs Family and Related Signal Pathways

Toll-like receptors (TLRs) are a class of single, transmembrane, and noncatalytic proteins named PRR and are expressed on specific immune cells (i.e., macrophages and dendritic cells) as well as nonimmune cells (e.g., epithelial, fibroblast, and endothelial cells) [6]. TLRs bind to and recognize endogenous molecules named DAMPs (e.g., structurally conserved components of microbes) and exogenous molecules that are named PAMPs (e.g., viral and bacterial products). Additionally, after the recognition by TLRs, downstream cascades are initiated. TLRs are involved in the initiation of innate immune responses and play a protective role against microbial infections. Once microbes invade physical barriers such as the skin or intestinal tract mucosa, TLRs on the cellular surface respond to microbial membrane materials (e.g., lipids and lipoproteins) and intracellular TLRs recognize microbial nucleic acids to initiate a host response [7].

So far, a total of 10 TLR genes in humans (TLR1–TLR10) and 12 (TLR1–TLR9 and TLR11–TLR13) in mice have been discovered. The 10 TLRs family members in humans are categorized into two subgroups. The first subgroup that recognizes the components of microbial membranes includes TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 of humans and TLR11 and TLR12 of mice and is primarily expressed on the cell surface [8]. The second subgroup that responds to microbial nucleic acids includes TLR3, TLR7, TLR8, and TLR9 and is expressed intracellularly in vesicles (e.g., lysosomes, endosomes, and the endoplasmic reticulum). TLR signaling is divided into two distinct signaling pathways, that is, the myeloid differentiation factor 88- (MyD88-) dependent and Toll/IL-1 receptor-domain containing adapter-inducing interferon-β- (TRIF-) dependent pathway. Both pathways are involved in innate immunity. MyD88 and TRIF bind independently to TLRs, resulting in the production of cytokines such as TNF-α, IL-1β, IL-6, and type I IFNs [9].

2.1. MyD88 Pathway. MyD88 possesses an amino- (N-) terminal death domain (DD), a shorter linker sequence, and a carboxy- (C-) terminal Toll/interleukin-1 receptor (TIR) domain. MyD88 also has an intermediate domain (ID) that interacts with IL-1R-associated kinases 4 (IRAK4) in TLR signaling [10, 11]. MyD88-dependent signaling is used by all TLRs except TLR3. The knockout of MyD88 in mice showed no responses to the ligands of TLR family members including TLR2, TLR4, TLR5, TLR7, and TLR9, indicative of the key role of MyD88 in TLRs-mediated inflammatory responses [12–16].

2.2. TRIF Pathway. TRIF is a large protein containing 712 amino acids in humans and directly binds to TLR3 and indirectly binds to TLR4 via connection with another adaptor protein, TRIF-related adaptor molecule (TRAM) [10]. The knockout of TRIF in mice triggers defective expression of IFN-β production and IFN-related genes that are mediated by TLR3 and TLR4, although early-phase activation of NF-κB and TLR4-mediated activation of MyD88 pathway were observed [17]. Similarly, TRIF was confirmed to have a key role in the induction of inflammatory mediators contributing to antiviral innate immune responses via MyD88-independent signaling that is mediated by both TLR3 and TLR4 [18].

3. What Is Asthma?

Asthma is a common heterogeneous disease characterized by chronic airway inflammation and is defined by recurring respiratory symptoms (such as wheezing, cough, shortness of breath, and chest tightness) that vary over time and in intensity, as well as by airflow obstruction according to GINA report [19]. Asthma causes a serious global health threat to patients of all age groups and is increasing in many countries in its prevalence, especially among children. Some countries have experienced a significant decline in hospitalizations and mortality from asthma; however, asthma still imposes a heavy burden on public health systems and on society through productivity decreases.

Due to both exposures (such as allergen and microbial infection) and treatment, there is heterogeneity in the inflammatory response in asthmatic airway. Wang et al. [20] previously categorized asthma into four phenotypes such as neutrophilic, eosinophilic, mixed granulocytic, and paucigranulocytic asthma according to inflammatory cell counts in induced sputum. Individualized precise diagnosis and treatment based on inflammatory phenotypes are now advocated because of limitations on the premise of current management of asthma. Individualized therapy is the customization of health care tailored to the individual and uses previously infeasible technologies based primarily on recent cluster analyses, molecular phenotyping, biomarkers, and differential responses to therapies, distinguishing a given patient from other patients with similar clinical presentations [21, 22]. Nowadays, the mainstay of asthma treatment is daily long acting β_2 agonists and inhaled corticosteroids (LABA/ICS) [3]. Maintenance treatment with LABA/ICS relieves asthma symptoms and reduces the frequency of exacerbations; however, there are limits in treatment options for people who do not gain control on combination LABA/ICS [23]. Targeted therapies at IgE, interleukin-4 (IL-4), IL-4 receptor, IL-5, IL-13, tumor necrosis factor-α, and CRTh2 are new treatment paradigms for asthma [24]. Emerging studies demonstrate that TLRs-targeted therapies potentially play a

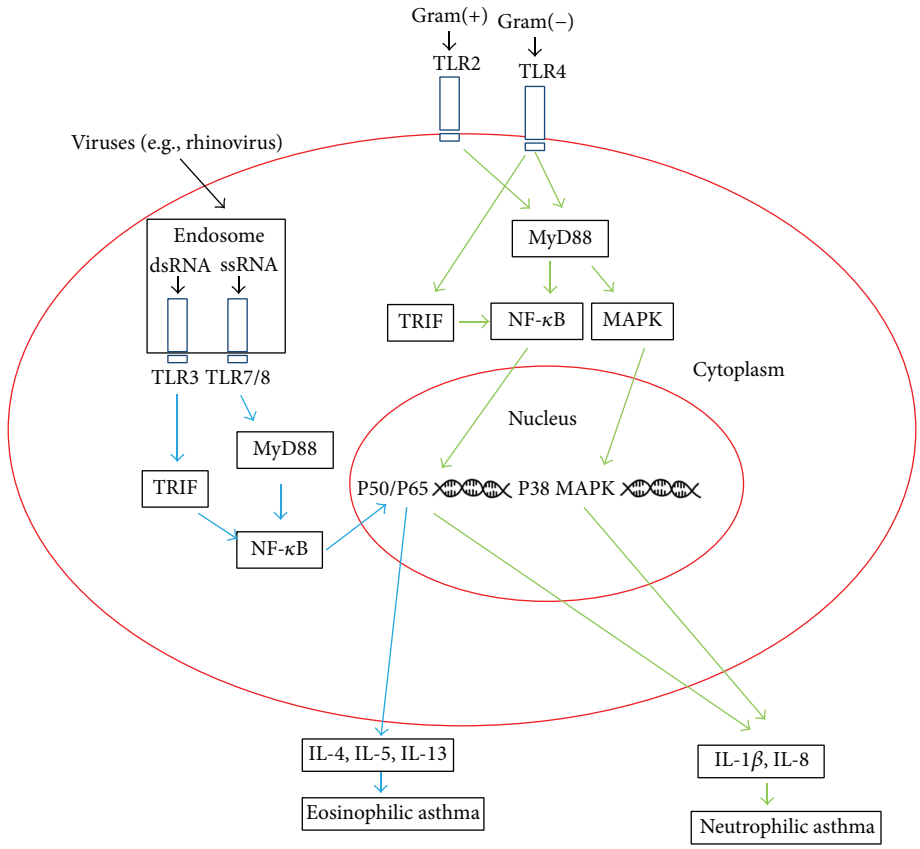

FIGURE 1: Schematic overview of TLR signaling pathway in neutrophilic and eosinophilic asthma. Gram-negative and Gram-positive bacteria as well as respiratory viruses (e.g., rhinovirus) are detected by TLRs. Subsequently, TLR3 and TLR7/8 trigger TRIF and MyD88 pathways, respectively, followed by the transcription of NF-κB in nucleus and the production of IL-4, IL-5, and IL-13, inducing eosinophilic asthma. TLR2 and TLR4 induce MyD88 and MyD88 as well as TRIF cascades, respectively, followed by the transcriptions of MAPK and NF-κB into nucleus, triggering the release of IL-1β and IL-8 and the onset of neutrophilic asthma. MAPK: mitogen-activated protein kinase; MyD88: myeloid differentiation primary-response gene 88; NF-κB: nuclear factor-κB; TLR: Toll-like receptor; TRIF: Toll/IL-1R (TIR) domain containing adaptor protein inducing IFN-β.

key role in effectively controlling airway inflammation in asthma.

4. TLRs in Asthma

4.1. TLR2 and TLR4 in Neutrophilic Asthma. The role of adaptive immune responses in asthma is well studied and involves T helper type 2 lymphocyte activation by allergen, accompanied by eosinophilic airway inflammation. The innate immune system is also associated with the pathogenesis of asthma and the onset of inflammation in the airway. Simpson first discovered that an upregulation of the innate immune receptors TLR2 and TLR4 as well as proinflammatory cytokines IL-8 and IL-1β was involved in neutrophilic asthma [25]. TLR2 plays an important role in recognizing Gram-positive bacteria and TLR4 is responsible for the detection of Gram-negative bacteria through their microbial components such as lipopolysaccharides (LPS) (Figure 1) [8].

4.2. TLR7 and Eosinophilic Asthma. TLR7 is intracellularly expressed on the surface of airway epithelia and airway smooth muscle as well as innate immune cells (such as macrophages, natural killer cells, and dendritic cells) [26, 27] and plays a significant role in the pathogenesis of autoimmune disorders such as Systemic Lupus Erythematosus (SLE) and in the regulation of antiviral immune responses [28]. TLR7 recognizes single-stranded RNA, a common molecular component to respiratory viruses, resulting in regulating downstream interferon production and the activation of Th1 antiviral responses [28]. TLR7 exhibits its antiviral activity in combination with TLR8, the homologue of TLR7 that also recognizes single-stranded viral RNA (Figure 1).

TLR7 plays an important role in reduction of airway inflammation, promoting Th1 responses in immune cells, reversing airway hyperresponsiveness, and preventing airway remodeling. Airway inflammation is essential to the pathogenesis of asthma and is triggered by respiratory viral infections and inhaled allergen, leading to the activation of T helper 2 (Th2) cell differentiation and the secretion of Th2 cytokines such as IL-4, IL-5, and IL-13 [29]. IL-5 matures eosinophils in the bone marrow and, together with chemokines such as eotaxins, promotes recruitment of eosinophils into the airways, resulting in local eosinophilic

inflammation [30]. TLR7 stimulation suppresses eosinophilic airway inflammation in a variety of animal models of asthma through reducing Th2 cytokines such as IL-4 and IL-5 as well as eotaxin in the lung [31] and IgE [32]. On the other hand, IL-5 induced airway eosinophilia can act as negative regulator of TLR7 expression and antiviral responses [30]. The role of TLR7 is not limited to Th2 responses; besides, it is involved in Th1 responses in immune cells. TLR7 activation promotes the reduction of Th2 cells and the enhancement of Th1 cells, which results in increases in Th1-cytokine release and decrease in IgE production [33–35], exhibiting the immunomodulatory activity of TLR7 in maintaining Th2/Th1 balance. IL-13 is responsible for inducing airway hyperreactivity (AHR) and mucus production in eosinophilic asthma [36]. TLR7 stimulation ameliorates ovalbumin-induced AHR when animals are treated with TLR7 agonists. A number of emerging studies suggest that the suppression of AHR involves NF-κB and p38 MAP intracellular signaling and is dependent on iNKT cells and IFN-γ production [26, 37]; however, *in vivo* investigation on the mechanism of AHR amelioration remains incomplete. Additionally, TLR7 ligand prevents chronic irreversible asthmatic airway remodeling including smooth muscle proliferation and goblet cell hyperplasia [38, 39].

4.3. TLR Genetic Polymorphisms and Asthma. Genetic polymorphisms in TLRs may be responsible for individual susceptibility and severity of asthma. Genetic diversity in specific alleles determines the differences in susceptibility to a specific disease to some extent [40]. Polymorphisms in the TLR4 gene affect sensitivity to allergens [41, 42]. Zhang et al. [42] discovered a harmful effect of the TT homozygote allele in the TLR4 gene rs1927914 on the forced expiratory volume in 1s (FEV_1), implicating impaired lung function. Additionally, the AA homozygote genotype and A allele in Asp299 Gly of the TLR4 gene may correlate with a reduced asthma risk, as indicated by the association between TLR4 polymorphisms and the development of asthma in the study by Tizaoui et al. [43]. In addition to TLR4, variants of the TLR2 gene were reported to have some association with childhood asthma in Caucasians [44], and TLR2 polymorphism affects the asthma risk and lung function [45]. It has been shown that variants in the TLR7/8 genes as well as the TLR10 gene showed no significant association in some alleles despite the relevance between other polymorphisms in the TLR10 gene and asthma [42, 45–48]. In terms of TLR1 and TLR5, studies on the association between genetic polymorphisms and the development of asthma have not been reported. Future investigations should emphasize TLR genetic variants such as haplotype analysis and gene-environmental interaction [43].

4.4. TLR and Viral Infection. Viral infection is a common acute trigger of asthma and exacerbation of asthma. Approximately 80% of asthma exacerbations are caused by respiratory viral infection [49, 50]. The PRRs in the detection of viral infection include TLR7 and TLR8 which detect single-stranded RNA and TLR3, retinoic acid-inducible gene I (RIG I), and melanoma differentiation associated gene 5 (MDA5) that are activated by double-stranded RNA. TLR7 expression is associated with the severity of the disease [51]. Airway cells from asthmatic patients are vulnerable to viral infection due to impaired innate antiviral responses compared to healthy subjects. This vulnerability is triggered by aberrant production of type I IFN, an antiviral cytokine [51]. TLR7 deficiency was discovered in alveolar macrophages from severe asthmatic and affected the interferon responses to rhinovirus infection. In the same study, the abnormal expression of the three microRNAs such as miR-150, miR-152, and miR-375 was the trigger of TLR7 deficiency. When these miRs were blocked, this resulted in restored TLR7 expression and augmented interferon responses to rhinovirus infection, indicating that TLR7 is associated with the vulnerability of asthmatic subjects [51]. In addition to this finding, *in vivo* research shows that a lack of TLR7 signaling in a rhinovirus-induced asthma exacerbation leads to reduced IFN production and exaggerated Th2-driven inflammation, suggesting the role of TLR7 signaling in rhinovirus-induced asthma exacerbation [30]. Other investigations support this finding. Bronchoalveolar lavage (BAL) cells from nonsevere asthma possess a deficient IFN response to rhinovirus infection [52, 53]; additionally, TLR7 dysfunction was shown in asthmatic peripheral blood mononuclear cells [54]. TLR3 also detects double-stranded RNA genome of respiratory virus which represents the replication of RNA viruses and protects the host by the induction of inflammatory responses including type I IFN production and activation of NK cells and cytotoxic T lymphocytes [55]. In an investigation by Parsons et al. [56], although no difference in the expression of TLR3 was observed, primary bronchial epithelial cells (pBECs) from asthmatics demonstrated an ineffective innate immune response following RV infection, with impaired type I and type III interferon responses to the infection. In addition, RV infection of healthy pBECs triggered a robust upregulation of TLR3, while inhibition of TLR3 signaling leads to a marked inhibition of both type I and type III interferon responses.

4.5. TLR9 and Asthma. TLR9 is intracellularly expressed in the immune cells such as B lymphocytes, monocytes, and plasmacytoid dendritic cells and detects unmethylated CpG motifs in microbial DNA molecules [57]. In allergic asthma subjects, TLR9 expression on plasmacytoid dendritic cells and TLR9-induced responses are upregulated by IL-25 that originates from airway epithelial cells [58]. In an *in vivo* investigation in severe asthma, Duechs et al. [59] discovered that TLR9 activation significantly reduced some features of the asthmatic phenotype such as a reduction in eosinophil influx and IgE levels in serum. The same study also observed a decreased release of cytokines such as IL-4, IL-5, IFN-γ, IL-1β, and IL-12, indicative of enhanced Th1 response, suggesting that TLR9 activation may suppress the Th2 response via promoting a Th1 response. Similarly, a Th1 response induced by the exposure to CpG DNA opposes a Th2 response in a murine model of asthma [60]. TLR9 is also involved in the inhibition of airway remodeling [61–64], suggesting a potential protective role of TLR9 in asthma. This was evaluated in *in vivo* models where TLR9 activation was found to be associated with a reduction in antigen-induced respiratory allergic responses [65, 66], suggesting that TLR9

ligands could be used as prophylactic and therapeutic agents in asthma [67]. However, TLR9 targeted treatment was not found to be efficacious in preexisting severe allergic inflammation in the airway, in either animal experiments or clinical trials [68, 69]. The role of TLR9 agonists in asthma requires further evaluation.

5. TLRs Targeted Therapeutics

5.1. Effect of TLR Agonists in Asthma. The typical treatment for asthma and asthma exacerbations includes inhaled corticosteroids for their ability of enhancing β-adrenergic responses and repressing inflammation in airways [70]. Nevertheless, in the treatment of severe asthma, corticosteroids are ineffective in alleviating symptoms, probably because oxidative stress as well as subsequent DNA damage leads to decreased activity of transcriptional corepressors such as histone deacetylase-2 (HDAC-2) [8]. Recently, TLRs agonists have been considered as agents in controlling asthma. TLRs agonists can be categorized into cellular surface TLRs agonists and intracellular TLRs agonists based on the distribution of TLRs. Cell surface TLRs sense structural components of microbia ranging from Gram-positive bacteria to Gram-negative bacteria and some respiratory viruses in the onset and development of asthma and asthma exacerbation [67]. Targeting TLR4 to treat asthma is based on the activation of TLR4 as an adjuvant in allergy vaccines to induce tolerance and inhibition of TLR4 expression. TLR4 agonists such as MPL® (monophosphoryl lipid A) seem to work effectively as allergy vaccines due to overexpression of TLR4 in asthmatic patients [8]. Another cell surface TLRs agonist is Pam3CSK4 that acts as a synthetic TLR2 agonist and exhibits antiasthmatic effects by reducing Th2 cytokine release, AHR, IgE levels, airway inflammation, and asthmatic symptoms in animal models of asthma [67]. Intracellular TLRs agonists such as TLR7/TLR8 agonists have also been evaluated in asthma. Resiquimod is a typical TLR7/TLR8 agonist and *in vivo* suppresses AHR as well as airway remodeling in asthma [31, 39, 71–73]. In addition, this drug was also found to suppress both Th1 and Th2 cytokine production in the lungs of experimental animals and decrease lung eosinophilia, goblet cell hyperplasia, and IgE levels [39, 67, 71]. Many other agents that target TLRs have been found to control airway inflammation, eosinophilia, and AHR in distinct animal models of allergic inflammatory diseases [67]. It is obvious that in the future a wide variety of TLR agonists are likely to be evaluated as effective asthma controllers. On the contrary, future emphasis should be on the side effects of TLR agonists, especially on asthmatic children due to a lack of investigation on allergic children. Nowadays clinical trials are mainly conducted in adults, and besides uncertain targeting of the immature immunity in children as well as timing, dosage, and patient selection regarding the formulation to best employ TLRs agonists still needs further studies, which may hinder wider application of TLRs agonists.

5.2. Effect of Corticosteroid on TLR Expression. Corticosteroids are the most effective agents in inflammation management in asthma, and classical corticosteroids such as budesonide are recommended by guidelines for asthma treatment [74]. When inhaled corticosteroids (ICS) were introduced into asthma management, symptom control of asthma and lung function were improved, and exacerbations and asthma-related mortality decreased [19]. Corticosteroids influence TLRs and can upregulate TLR4 expression *in vivo* in peripheral blood mononuclear cells from asthmatic patients [75]. In addition to this finding, after *in vitro* stimulation with LPS, the production of both TNF-α and IFN-γ in PBMC supernatant was significantly increased by oral corticosteroids [75]. Similarly, Pace et al. [76] reported that TLR4 and TLR2 expression were increased in Treg lymphocytes from allergic asthmatic subjects after budesonide treatment compared to healthy controls, providing further understanding of the action mechanism of budesonide on the control of inflammation in asthma. Furthermore, an increased level of IL-10 and decreased level of IL-6 and TNF-α were observed after budesonide administration, confirming the modulatory potential of budesonide in immune responses to allergic subjects.

6. Conclusions

The invasion of antigens into airways causes the activation of PRRs such as TLRs in response to PAMPs. TLRs play an important role in the detection of invading pathogens by the innate immune system, and a total of 10 TLRs family members have been discovered in humans (TLR1–TLR10). TLRs induce the activation of MyD88 and TRIF signaling pathways, resulting in the production of inflammatory mediators via the NF-κB pathway. Different pathogens trigger distinct immune activation of TLRs. TLR2 plays an important role in recognizing Gram-positive bacteria and TLR4 is responsible for the detection of Gram-negative bacteria, leading to the production of cytokines such as IL-1β and IL-8 and to the infiltration of neutrophils in asthmatic airways. In addition, TLR7 senses single-stranded viral RNA which inhibits Th2 immune responses and eosinophilic asthma, and TLR9 detects unmethylated CpG motifs in microbial DNA molecules in the development of asthma and asthma exacerbation. Furthermore, genetic polymorphisms affect the susceptibility and severity of asthma, making effective control of airway inflammation in asthma more complex. Nowadays, corticosteroid therapy is commonly used for asthma treatment, and some findings confirmed the modulatory role of corticosteroid in the mediation of TLR expression in asthmatic subjects. Combination therapy of corticosteroid and TLRs agonists may be potentially more effective in controlling inflammation in asthmatics compared to the traditional treatment by corticosteroid. However, the timing, dosage, patient selection, and many other questions regarding the formulation to best employ TLRs agonists remain unclear, and future work needs to address these difficulties in order to hold airway inflammation in check in asthma.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by Training Program of Scientific and Technical Innovative Talent (Grant no. 201205014), Science and Technology Development Plan of Jilin Province of China (Grant no. 20150519015JH), and Bethune B Plan of Jilin University (Grant no. 2012218).

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2016, Article ID 6373506, 11 pages
http://dx.doi.org/10.1155/2016/6373506

Research Article

Nonessential Role for the NLRP1 Inflammasome Complex in a Murine Model of Traumatic Brain Injury

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Received 8 January 2016; Revised 8 March 2016; Accepted 24 March 2016

Academic Editor: Luc Vallières

Traumatic brain injury (TBI) elicits the immediate production of proinflammatory cytokines which participate in regulating the immune response. While the mechanisms of adaptive immunity in secondary injury are well characterized, the role of the innate response is unclear. Recently, the NLR inflammasome has been shown to become activated following TBI, causing processing and release of interleukin-1β (IL-1β). The inflammasome is a multiprotein complex consisting of nucleotide-binding domain and leucine-rich repeat containing proteins (NLR), caspase-1, and apoptosis-associated speck-like protein (ASC). ASC is upregulated after TBI and is critical in coupling the proteins during complex formation resulting in IL-1β cleavage. To directly test whether inflammasome activation contributes to acute TBI-induced damage, we assessed IL-1β, IL-18, and IL-6 expression, contusion volume, hippocampal cell death, and motor behavior recovery in *Nlrp1*$^{-/-}$, *Asc*$^{-/-}$, and wild type mice after moderate controlled cortical impact (CCI) injury. Although IL-1β expression is significantly attenuated in the cortex of *Nlrp1*$^{-/-}$ and *Asc*$^{-/-}$ mice following CCI injury, no difference in motor recovery, cell death, or contusion volume is observed compared to wild type. These findings indicate that inflammasome activation does not significantly contribute to acute neural injury in the murine model of moderate CCI injury.

1. Introduction

Mechanical trauma to the CNS results in the disruption of the cellular microenvironment leading to massive necrotic and apoptotic loss of neuronal and glia populations. The progressive cascade of secondary events, including ischemia, inflammation, excitotoxicity, and free radial release, contributes to neural tissue damage. Activation of the innate immune response, including microglia, peripheral-derived macrophages, and astrocytes [1–3], can lead to the expression of proinflammatory cytokines, chemokines, and reactive oxygen species, thereby triggering the inflammatory responses in the central nervous system (CNS). Recently, the initiation of such a response following tissue injury was shown to involve a multiprotein complex called the inflammasome [4]. This cytosolic complex enables the activation of proinflammatory caspases, mainly caspase-1 [5, 6], resulting in a potent inflammatory response. Inflammasome complexes generally have three main components: an NLR protein; the enzyme caspase-1; and an adaptor protein that facilitates the interaction between the two. The NOD-like receptors (NLRs) are critical in this process and members of the inflammasome forming NLR subfamily recruit the adapter apoptosis-associated speck-like protein (ASC) to activate caspase-1. Currently, at least 8 different NLR proteins are well characterized as being capable of inflammasome formation under a diverse range of stimuli. In the brain, the inflammasomes forming NLRs, NLRP1, NLRP2, and NLRP3 have each been shown to modulate caspase-1 activation and the subsequent processing of IL-1β and IL-18, primarily from glia cells [7–10]. The CNS is particularly sensitive to IL-1β and IL-18 signaling, since multiple neural cell types express receptors for these cytokines [11–13]. In addition, activated caspase-1 can mediate a form of necrotic cell death known as pyroptosis

[14–16], making it a potential candidate for cell death signaling within neurons following injury. Therefore, induction of the NLR-mediated inflammasome complex could contribute to the proinflammatory milieu as well as neuronal pyroptosis following immunopathogenic conditions such as traumatic brain injury (TBI). Interestingly, recent studies have demonstrated assembly of the NLRP1- and NLRP3-inflammasome complex including increased expression of ASC, activation of caspase-1, and processing of IL-1β in a rat model of TBI [8, 9]. Furthermore, therapeutic administration of anti-ASC neutralizing antibodies was shown to reduce the innate immune response and significantly decrease contusion volume in the same model [9]. These studies suggest that inflammasome activation plays a critical role in acute neural injury and that pyropotosis may be a key element of neuronal cell death following brain trauma.

To better understand the role of the NLRP1 inflammasome complex in TBI-induced damage, we sought to evaluate the effects of NLRP1 and ASC gene deletion on cortical tissue loss in a murine model of controlled cortical impact (CCI) injury. This model produces a well-demarcated cortical lesion that mimics the contusions commonly observed in TBI patients. Because the pathophysiological sequela of TBI is dependent on impact severity and location, we investigated whether the absence of inflammasome activation impacts the histopathological outcome using this distinct model. The overall goal of this study was to assess the role of the NLR inflammasome following CCI injury by quantifying IL1-β and IL-18 expression and determine whether inflammasome disruption impacts cortical contusion volume and motor recovery in wild type, *Nlrp1*$^{-/-}$, and *Asc*$^{-/-}$ mice.

2. Materials and Methods

2.1. Animals/Ethics Statement. The *Nlrp1*$^{-/-}$ mice were provided by Dr. Beverly H. Koller (UNC Chapel Hill) and the *Asc*$^{-/-}$ (*Pycard*$^{-/-}$) mice were acquired from Genetech. The generation and characterization of *Nlrp1*$^{-/-}$ and *Asc*$^{-/-}$ mice have been previously described [17, 18]. All mice were maintained on the C57Bl/6 background and all animals were genotyped using standard PCR analysis prior to each study. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were conducted under the approval of the Virginia Tech Institutional Animal Care and Use Committee (IACUC; #12-081) and the Virginia-Maryland College of Veterinary Medicine.

2.2. CCI Injury. Male mice, 2–4 months old, were anesthetized with ketamine and xylazine by intraperitoneal (i.p.) injection and positioned in a stereotaxic frame [19, 20]. Body temperature was monitored with a rectal probe and maintained at 37°C with a controlled heating pad set. A 5 mm craniotomy was made using a portable drill over the right parietal-temporal cortex (−2.5 mm A/P and 2.0 mm lateral from bregma). Injury was induced by moderate CCI using the eCCI-6.3 device (Custom Design & Fabrication, Richmond, VA; 4 mm impounder) at a velocity of 3.5 m/s, depth of 0.5 mm, and 150 ms impact duration [19, 20]. Moderate CCI injury results in a well-demarcated cortical lesion or cavity that mimics the contusions commonly observed in TBI patients. Sham controls received anesthesia, skin incisions, and sutures only. Following injury, the incision was closed using Vetbond tissue adhesive (3M, St. Paul, MN, USA) and the animals were placed into a heated cage to maintain body temperature for 1 h after injury. At 1, 3, or 14 days after CCI injury, mice were anesthetized and brain tissue removal was performed following decapitation. Fresh frozen tissue was embedded in OCT and sectioned at 30 μm thick. Five serial coronal sections were (300 μm apart) stained for Nissl substance [21].

2.3. Rotarod Assessment. Motor function was evaluated using Rotarod testing, as previously described [21, 22]. Assessment was performed on the Economex (Columbus Instruments, Columbus, OH) at 3, 7, and 14 days after CCI by an observer unaware of experimental groups. The starting velocity was set at 10 rpm and accelerated to 0.1 rpm/sec. Animals were pretrained for 4 consecutive days prior to CCI injury with 3 trials (2 minutes resting in between) each day. Each trial ended when the animal fell off the Rotarod or gripped the rod and passively spun more than once. Time to fall was recorded for each trial per animal. A baseline (seconds) was collected on the fourth day of training. Evaluation of motor function after injury was based on individual scores relative to each animal's baseline latencies and represented as percentage of baseline.

2.4. Evaluation of Contusion Volume. Lesion or contusion volume was assessed by a blinded investigator using Cavalieri estimator from StereoInvestigator (MicroBrightField, Williston, VT, USA) and an Olympus BX51TRF motorized microscope (Olympus America, Center Valley, PA, USA). Contusion volume (mm^3) was determined as previously described [21]. Briefly, volume analysis was performed by estimating the area of tissue loss in the ipsilateral cortical hemisphere for five coronal serial sections at or around the epicenter (−1.1 to −2.6 mm posterior from bregma) of injury. Nissl stained serial sections were viewed under brightfield illumination at a magnification of 4x. A random sampling scheme was used that estimates every 10th section from rostral to caudal, yielding five total sections to be analyzed. A randomly placed grid with 100 μm spaced points was placed over the ipsilateral hemisphere and the area of contusion was marked within each grid. Contusion boundaries were identified by loss of Nissl staining, pyknotic neurons, and tissue hemorrhage. The contoured areas, using grid spacing, were then used to estimate total tissue volume based on section thickness, section interval, and total number of sections within the Cavalieri program, StereoInvestigator. Data is represented as volume of tissue loss or contusion volume (mm^3) for wild type, *Nlrp1*$^{-/-}$, and *Asc*$^{-/-}$ mice.

2.5. Evaluation of Protein Cytokine Levels in Cortical Tissue Samples. Protein was isolated from cortical tissue samples of wild type, *Nlrp1*$^{-/-}$, and *Asc*$^{-/-}$ mice 1 day after CCI injury

as previously described [21]. Briefly, fresh brain tissue was dissected in L15 (Gibco) media on ice and homogenized in RIPA buffer (pH 7.5, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 2 mM EDTA, and 0.01 M sodium phosphate) in the presence of complete protease inhibitor cocktail (Roche, Florence, SC, USA) and phosphatase inhibitor cocktail 2 (Sigma-Aldrich, St. Louis, MO, USA). Supernatant was collected by centrifuging at 14 000 ×g for 30 min at 4°C and the Lowry assay was used for the determination of protein concentration (Pierce, Rockford, IL, USA). Protein samples were then tested for IL-1β, IL-18, and IL-6 expression levels using ELISA (BD Biosciences), as previously described [23]. Final concentrations of each cytokine were calculated based on internal standard controls and represented as pg/mL, then normalized to the amount of protein (mg) loaded per well of the ELISA and represented at (pg/mL)/mg for each sample. This is a standard procedure to account for differences in starting protein levels that could significantly influence the final concentration of each cytokine [24–26].

2.6. Metadata Analysis. The microarray data was generated following a metadata analysis or data mining of publically available datasets using a publically accessible microarray meta-analysis NextBio search engine, available at http://www.nextbio.com/b/search/ba.nb. The data analysis was performed from the following datasets: human: GSE2392, 1432, 10612, 12837, 12305 12679; mouse: GSE17256, 10246, 9566, 11288; TBI study: GSE2392.

2.7. Statistical Analysis. Data was graphed using GraphPad Prism, version 4 (GraphPad Software, Inc., San Diego, CA). Student's two-tailed t-test was used for comparison of two experimental groups. Multiple comparisons were done using one-way and two-way ANOVA, where appropriate, followed by Tukey test for multiple pairwise examinations. Changes were identified as significant if P was less than 0.05. Mean values were reported together with the standard deviation (SD).

3. Results

3.1. NLRP1 Inflammasome Activation Does Not Contribute to Acute Cortical Damage after CCI Injury. Inflammasome complex formation has been shown to play a critical role in initiating inflammation in a variety of settings [27], though our understanding of its role in neuroinflammation is limited. Here, we sought to analyze the effects of inflammasome disruption on acute neural tissue damage and cytokine production following TBI. Specifically, we evaluated injury outcome in *Nlrp1*$^{-/-}$ and *Asc*$^{-/-}$ mice, using the controlled cortical impact (CCI) model [19–21], at 3 days after injury. Serial sections were subjected to Nissl staining and contusion boundaries were demarcated by loss of Nissl stain, pyknotic neurons, and tissue hemorrhage. Using the Cavalieri estimator, we found no significant difference in contusion volume ($F = 1.37$, $P = 0.3$) among wild type ($4.22 \pm 0.97\,\text{mm}^3$; $n = 8$), *Nlrp1*$^{-/-}$ ($3.70 \pm 1.12\,\text{mm}^3$ $n = 7$), and *Asc*$^{-/-}$ ($4.57 \pm 1.43\,\text{mm}^3$; $n = 5$) mice at 3 days after CCI (Figures 1(a)–1(c) and 1(g)) or 14 days ($F = 1.07$; $P = 0.49$); ($3.113 \pm 0.85\,\text{mm}^3$ $n = 8$; $3.0 \pm 1.2\,\text{mm}^3$ $n = 5$; $3.76 \pm 0.66\,\text{mm}^3$ $n = 5$, resp.) (Figures 1(d)–1(f)) after CCI injury. These results indicate that genetic ablation of specific genes known to be involved in the formation of the NLR inflammasome complex has no effect on neural tissue loss in the cortex following acute TBI. We also performed Rotarod behavioral analysis to test whether motor deficit and recovery were affected by inflammasome disruption following CCI injury. Mice were pretrained on the Rotarod 4 days prior to CCI injury then subjected to motor assessment at 3, 7, and 14 days after sham or CCI injury. Time to fall was recorded then normalized to the average baseline time for each mouse. No differences between groups were seen following sham injury for each time point tested (Figure 1(h)). Although motor deficits were observed following CCI injury, no difference between groups was observed in motor ability at 3 days (wild type 60.07% ± 18.4 $n = 9$; *Nlrp1*$^{-/-}$ 55.7% ± 9.1 $n = 5$; *Asc*$^{-/-}$ 45.44% ± 10.5 $n = 5$) compared to baseline, or at any other time point tested (Figure 1(i)). These data correlate with contusion volume estimates and confirm that inflammasome disruption has no effect on neural tissue loss or motor function after CCI injury.

Next, we investigated the protein expression of the proinflammatory cytokines IL-1β, IL-18, and IL-6 in the cortex of *Nlrp1*$^{-/-}$, *Asc*$^{-/-}$, and wild type mice following CCI injury. Following injury, we collected cortical tissue samples from the ipsilateral and contralateral hemispheres of sham- and CCI-injured mice at 24 hours. Total IL-1β, IL-18, and IL-6 levels were quantified using ELISA. Our findings demonstrate that CCI injury results in a significant increase in IL-1β (1.3-fold; 78.2 ± 13.6 pg/mL per mg protein) and IL-6 (5-fold; 229.3 ± 30.9) levels in the wild type CCI-injured ipsilateral cortex compared to uninjured contralateral (IL-1β 56.8 ± 2.9; IL-6 43.3 ± 29.2) or sham-injured ipsilateral tissue (IL-1β 45.3 ± 5.7; IL-6 56.6 ± 12.9) (Figures 2(a) and 2(c)). However, ipsilateral IL-1β levels are significantly attenuated in CCI-injured *Nlrp1*$^{-/-}$ (51.9 ± 6.5) and *Asc*$^{-/-}$ (56.8 ± 3.7) mice, where levels reach that of uninjured wild type samples (Figure 2(a)). On the other hand, we observed a similar increase in IL-6 in the injured cortex of wild type, *Nlrp1*$^{-/-}$, and *Asc*$^{-/-}$ mice (Figure 2(c)). Ipsilateral IL-6 levels are slightly reduced in *Nlrp1*$^{-/-}$ (172.6 ± 34.9) but not in *Asc*$^{-/-}$ (197.4 ± 29.7) compared to wild type. Interestingly, no significant difference in IL-18 levels was found following CCI injury, although a reduced trend in all ipsilateral samples is observed (Figure 2(b)). We find that disruption of the NLRP1 inflammasome complex leads to an attenuation of IL-1β production, an end-product of the inflammasome complex, while having minimal effects on IL-6 following CCI injury. These data indicate that IL-1β does not significantly contribute to the neural tissue injury in this model. We further characterized the histopathological changes induced by CCI injury in wild type (Figure 2(d)), *Nlrp1*$^{-/-}$ (Figure 2(e)), and *Asc*$^{-/-}$ (Figure 2(f)) using H&E staining. For each strain of mice, we found a well-demarcated area of oncosis,

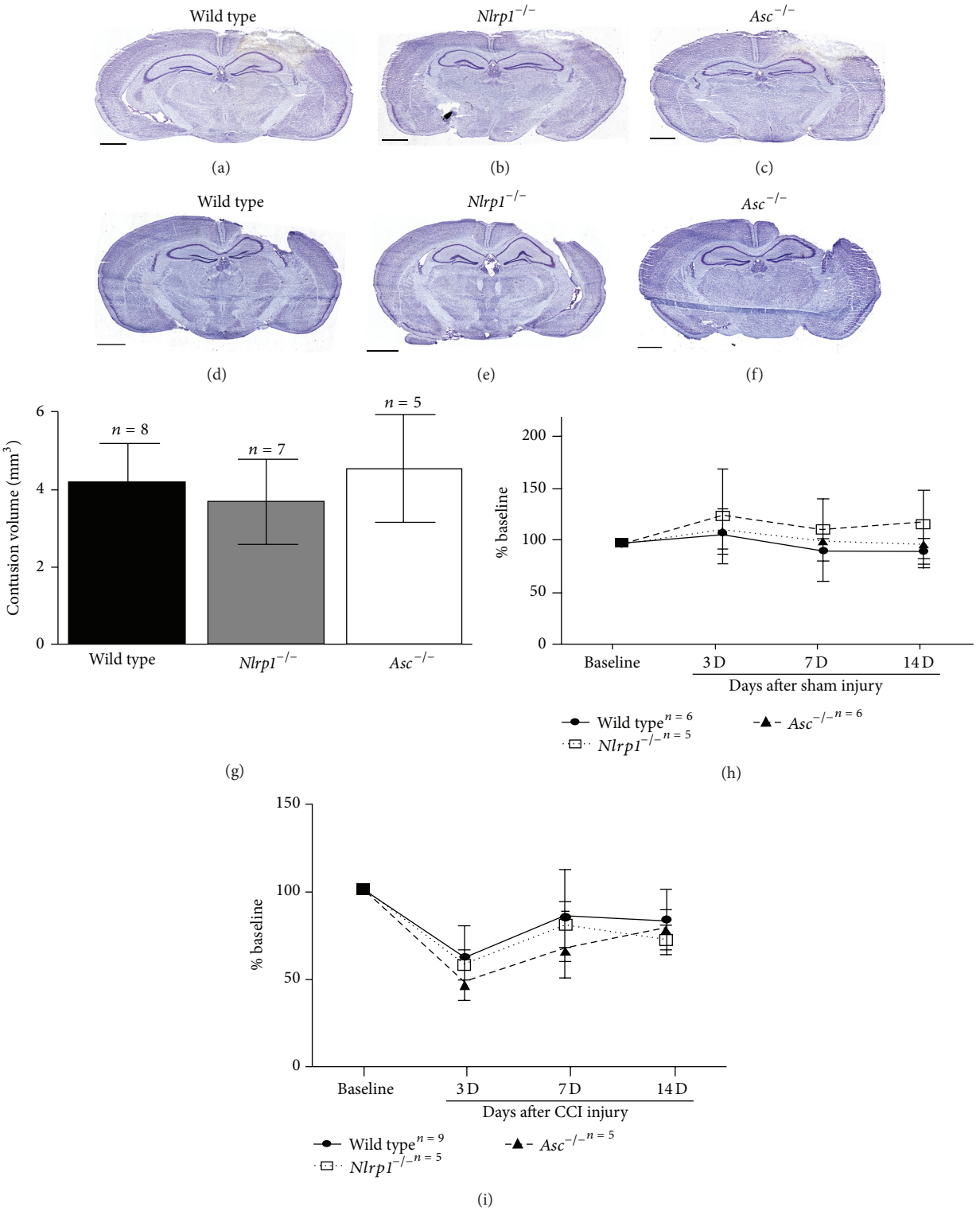

Figure 1: Genetic disruption of the NLRP1 inflammasome complex has no effect on contusion volume and motor deficits following CCI injury. Using Cavalieri estimator on Nissl stained sections collected from (a, d) wild type, (b, e) *Nlrp1*$^{-/-}$, and (c, f) *Asc*$^{-/-}$ brains at 3 and 14 days after injury, respectively, shows no significant change in contusion volume (mm^3). (d) Bar graph represents mean contusion volume ± SD in wild type, *Nlrp1*$^{-/-}$, and *Asc*$^{-/-}$ mice (n = 5–8 per group). Rotarod assessment or motor function was performed in each group and demonstrates no significant difference between sham-injured mice (h) or CCI-injured mice (i). n = 5–9 per group, represented as mean ± SD.

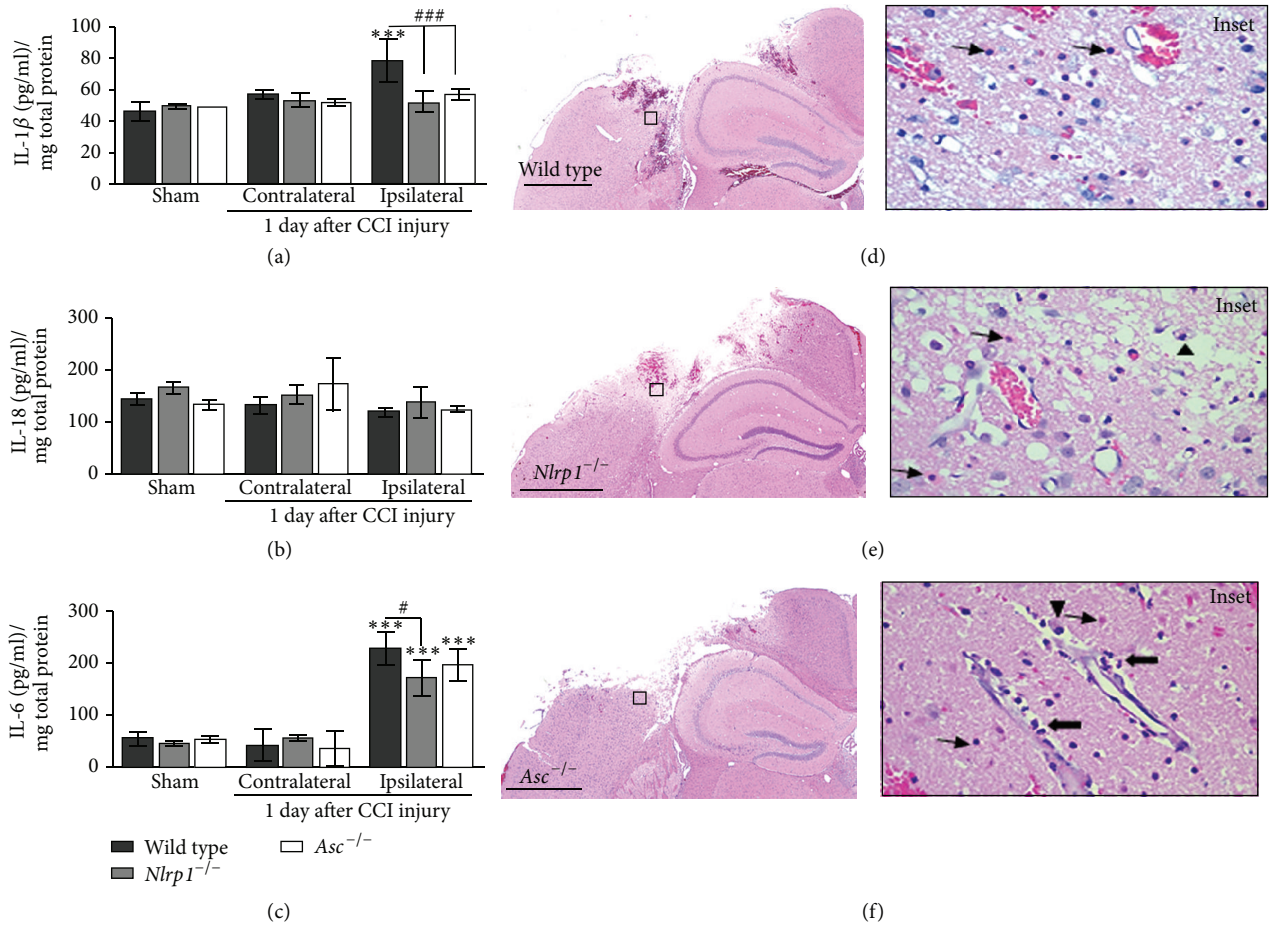

FIGURE 2: Cytokine protein expression and histopathology in wild type, *Nlrp1*$^{-/-}$, and *Asc*$^{-/-}$ mice. Quantification of IL-1β, IL-18, and IL-6 in wild type, *Nlrp1*$^{-/-}$, and *Asc*$^{-/-}$ cortical tissue samples analyzed by ELISA 1 day after CCI injury. (a) IL-1β, a direct release product of the inflammasome signaling cascade, is significantly reduced in the ipsilateral cortex of *Nlrp1*$^{-/-}$ and *Asc*$^{-/-}$ compared to wild type cortex. (b) No significant differences were observed in IL-18 levels 1 day after injury. (c) IL-6 is increased in wild type, *Nlrp1*$^{-/-}$, and *Asc*$^{-/-}$ mice following trauma with a slight attenuation in *Nlrp1*$^{-/-}$ ipsilateral cortex compared to wild type. $^{***}P < 0.001$ compared to the contralateral and sham-injured cortical control samples. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ compared to wild type ipsilateral cortex. (d) H&E staining on coronal brain sections from wild type, (e) *Nlrp1*$^{-/-}$, and (f) *Asc*$^{-/-}$ mice shows no difference in histopathological outcome. Prominent cellular features present in all sections include large numbers of necrotic neurons (thin arrows), few macrophages (arrowheads), and neutrophils (thick arrows); insets. Scale bar = 1 mm. n = 4-5 per group, represented as mean ± SD.

hemorrhage, and loss of neuropil at 3 days after CCI injury. In all sections tested, the surrounding devitalized brain tissue was vacuolated and contained numerous oncotic neurons (Figure 2 insets; thin arrows), low numbers of macrophages containing phagocytized erythrocytes and cellular debris (Figure 2 insets; arrowheads), and numerous neutrophils present in the perilesion site and perivascular spaces (Figure 2 insets; thick arrows). Histopathological assessments revealed no significant differences in inflammatory cell composition or quantity between the wild type, *Nlrp1*$^{-/-}$, and *Asc*$^{-/-}$ mice in our assessments 3 days after CCI injury. Likewise, no overt differences in the pathological phenotype were observed between the strains of mice following CCI injury.

In order to gain more robust insight regarding gene expression patterns related to the NLRP1 inflammasome following CCI injury, we compared our ELISA data with previous microarray data obtained from murine cortical tissue samples following sham and CCI injury at 4–72 hrs after trauma using meta-analysis [28]. At each time point tested from the datasets GSE2392, no change was observed in gene expression for *Nlrp1* or *Asc* between sham and injury levels (Figure 3). However, in agreement with our ELISA results, the microarray metadata shows an increase in *Il1b* (1.8-fold) and *Il6* expression (2.54-fold) at 24 hr after CCI injury (Figure 3). Likewise, *Il1a* (data not shown; 8 hr after injury) and *Il1r1* (Figure 3) are also increased during acute trauma. Interestingly, at this time, there was also an observed decrease (−1.28-fold) in Il18 expression. We also observed that *Ccl2* expression consistently showed the highest fold increase at each time point tested (5.78-, 17.2-, 8.86-, and 5.59-fold, resp.). These results emphasize that trauma-induced changes occur at the transcriptional level in genes related to inflammasome associated pathways.

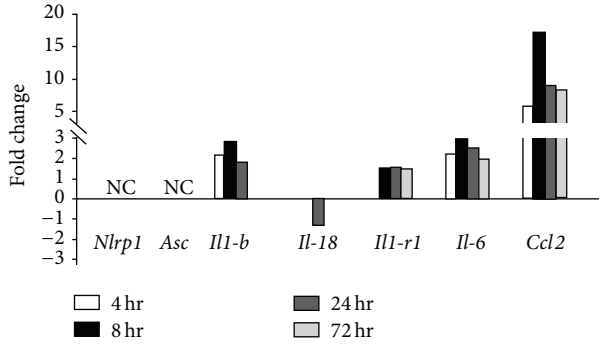

Figure 3: Time course of gene expression in the murine parietal cortex after CCI injury. Expression of several inflammasome related genes was compiled using the metadata-analysis system. Cortical tissue samples from sham- and CCI-injured mice were subjected to microarray analysis [28, 29]. Changes in expression are represented as fold change from uninjured levels. No change in *Nlrp1* or *Asc* was detected, while greater expression is noted for *Il-1b*, *Il-1r1*, *Il-6,* and *Ccl2* at 4, 8, 24, or 72 hrs after trauma. Conversely, there was a reduced fold change (−1.28) in the expression of *Il-18* at 24 hours after CCI injury.

Hippocampal dysfunction and cellular loss are the hallmark of TBI [21, 30–32]. In addition to cortical lesion volume, we evaluated cell death using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) in the dentate gyrus (DG) and cortex of wild type, $Nlrp1^{-/-}$, and $Asc^{-/-}$ mice (Figure 4). Serial coronal sections were stained with TUNEL and optical fractionator, and StereoInvestigator was used to quantify the total number of TUNEL-positive cells in the contralateral and ipsilateral DG at 3 days after CCI. In all three strains, our analysis revealed a similar increase in cell death in the ipsilateral DG compared to contralateral; however, no significant difference was observed (Figure 4(a)) between wild type (1507.7 ± 1221.2), $Nlrp1^{-/-}$ (1840.6 ± 862.3), and $Asc^{-/-}$ (1635.8 ± 315.8) mice in the ipsilateral DG (Figures 4(b), 4(c), and 4(d), resp.). Similarly, no differences in TUNEL were seen in the cortex between the experimental groups (Figures 4(e)–4(g)). These findings indicate that the extent of cell death induced by cortical impact injury was not affected by the loss of Nlrp1 or Asc expression.

3.2. Meta-Analysis of NLRP1, ASC, and IL-1β Gene Expression. Prior studies have shown that therapeutic targeting of the NLRP1 inflammasome attenuates the immune response and significantly improves histopathologic features associated with traumatic brain injury in rats (PMID: 19401709; 22781337). Likewise, the inflammasome has been suggested to be a promising target for therapeutic development in humans to treat a variety of conditions, including following CNS injury (reviewed in 26024799). Due to the therapeutic interest in targeting the NLR inflammasome, we sought to evaluate cell-specific expression in murine and human samples using a data mining bioinformatics approach. *NLRP1*, *ASC,* and *IL-1β* expression were analyzed in immune and neural cell types from both humans and mice using a publically accessible microarray meta-analysis search engine (NextBio website, available at http://www.nextbio.com/b/search/ba.nb), as previously described [29]. This analysis revealed highly variable levels between select cell populations relevant to the immune response compared to neural tissue. Overall, human *NLRP1, ASC,* and *IL-1β* show greater median cell expression in the naïve peripheral blood (PB) cells known to respond to trauma. These include neutrophils, macrophages, and monocytes (Figures 5(a)–5(c)), with the exception of *IL-1β*, which has the highest level of expression in human microglial cells (Figure 5(c)). Brain-derived cell types show lower median expression levels of these genes suggesting that the greatest activity occurs in response to immune activation. *Nlrp1* (*Nlrp1a; Nlrp1b; Nlrp1c*) (Figure 5(d)) expression data has not been added to the datasets evaluated using this method. However, similar results to the human findings were obtained for *Asc* and *Il1-b* in naïve C57Bl/6 mice (Figures 5(e) and 5(f), resp.).

4. Discussion

New insights into the role of the NLR inflammasome complex during acute inflammation have prompted its investigation in the pathogenesis of numerous neurological diseases, including TBI [33, 34]. The current study shows that genetic deletion of NLRP1 (*Nlrp1a, Nlrp1b*, and *Nlrp1c*) or ASC, essential proteins for the assembly of the NLRP1 inflammasome complex, has no direct effect on cortical tissue loss, hippocampal cell death, or motor behavior deficits. In the present study, we utilized the Rotarod as a measure of functional recovery following CCI injury in the motor cortex. This technique measures aspects of motor impairment that are not evident by either the beam-balance or beam-walking tasks in our model. For this reason our current experiments focused on the Rotarod, which is the most robust, sensitive, and efficient index for assessing motor impairment produced by CCI injury [22]. Although we find a significant attenuation of IL-1β in $Nlrp1^{-/-}$ or $Asc^{-/-}$ mice, histopathological changes seen following cortical trauma were similar to that found in wild type mice. These results are the first to identify a nonessential role for the NLR inflammasome in injury outcome following controlled cortical impact using a genetic approach. Our data is not consistent with previous findings that demonstrate significant protection following administration of ASC neutralizing antibodies in the rat lateral fluid percussion injury (FPI) model of TBI [9]. This discrepancy may be due to species differences between rats and mice or possible variations in the cytokine profile induced between the CCI and fluid percussion TBI models. Likewise, recent studies indicate that mouse and rat genetic factors may also mediate some of this variability [35]. It is also possible that other compensatory mechanisms may be associated with TBI progression in the $Nlrp1^{-/-}$ and $Asc^{-/-}$ mice, which are completely devoid of NLRP1 inflammasome function from birth. The discrepancies observed in our model versus the prior studies in rats underscore the need for further investigation to develop additional mechanistic insight into the role of the NLRP1 inflammasome following traumatic brain injury.

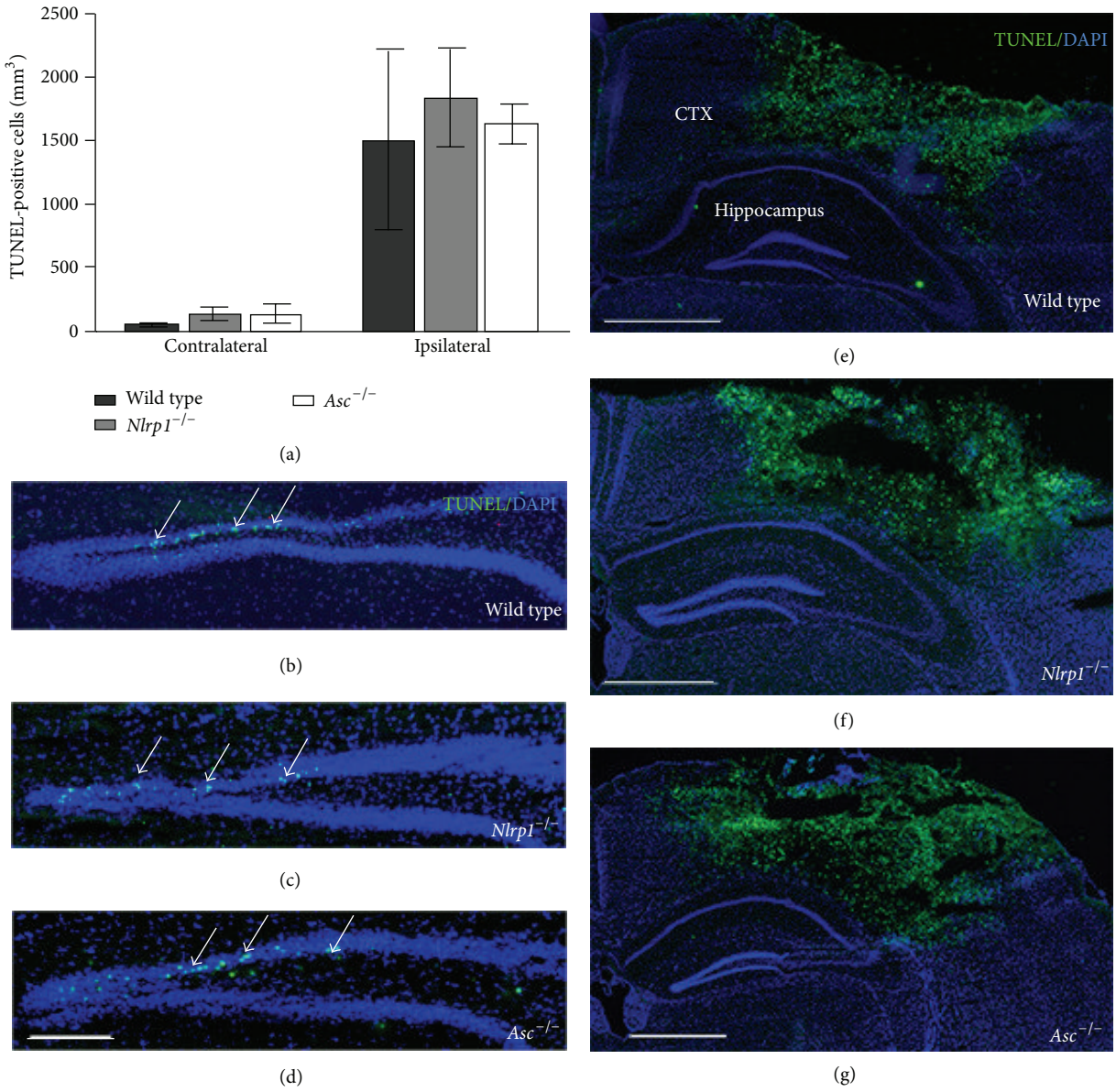

FIGURE 4: TUNEL analysis in the dentate gyrus and cortex of wild type, $Nlrp1^{-/-}$, and $Asc^{-/-}$ mice. (a) Quantification of TUNEL-positive cells in the ipsilateral and contralateral dentate gyrus (DG) 3 days following CCI injury. Increased cell death is observed in the ipsilateral compared to contralateral DG in all groups; however, no significant difference in TUNEL is observed between wild type, $Nlrp1^{-/-}$, and $Asc^{-/-}$ mice following trauma. Representative immunofluorescence images of TUNEL (green) DAPI (blue) colabeled cells in the ipsilateral DG and cortex of (b and e) wild type, (c and f) $Nlrp1^{-/-}$, and (d and g) $Asc^{-/-}$ mice, respectively. Images from 4x magnification; scale bar = 0.5 mm (b–d) and 1 mm (e–g). n = 4-5 per group, represented as mean ± SD.

In the present study, we utilized NLRP1 and ASC gene targeted knockout mice, which have been previously shown to prevent NLRP1-mediated inflammasome complex formation during acute inflammation [17, 18, 36]. We demonstrate attenuated inflammasome function in the $Nlrp1^{-/-}$ and $Asc^{-/-}$ mice, as evidenced by reduction of IL-1β following acute CCI injury. Interestingly, IL-18 levels were unaffected in the cortex after trauma (Figure 2(b)). In fact, we observed a trend towards reduced IL-18 levels in wild type, $Nlrp1^{-/-}$, and $Asc^{-/-}$ mice indicating that noninflammasome regulated pathways may be acting to suppress IL-18 induction in response to cortical impact. This is further supported by our metadata analysis that also shows reduced expression of *Il-18* at the transcription level (Figure 3). Overall, we find that abolishing IL-1β expression in the absence of ASC and NLRP1 does not correlate with changes in lesion volume or behavioral outcome after controlled cortical impact. Therefore, in order to identify key cytokines that may play a more central role, we further analyzed IL-6, as this cytokine has been largely implicated in tissue damage and progression of cavity formation. IL-6 generation has also been shown to be

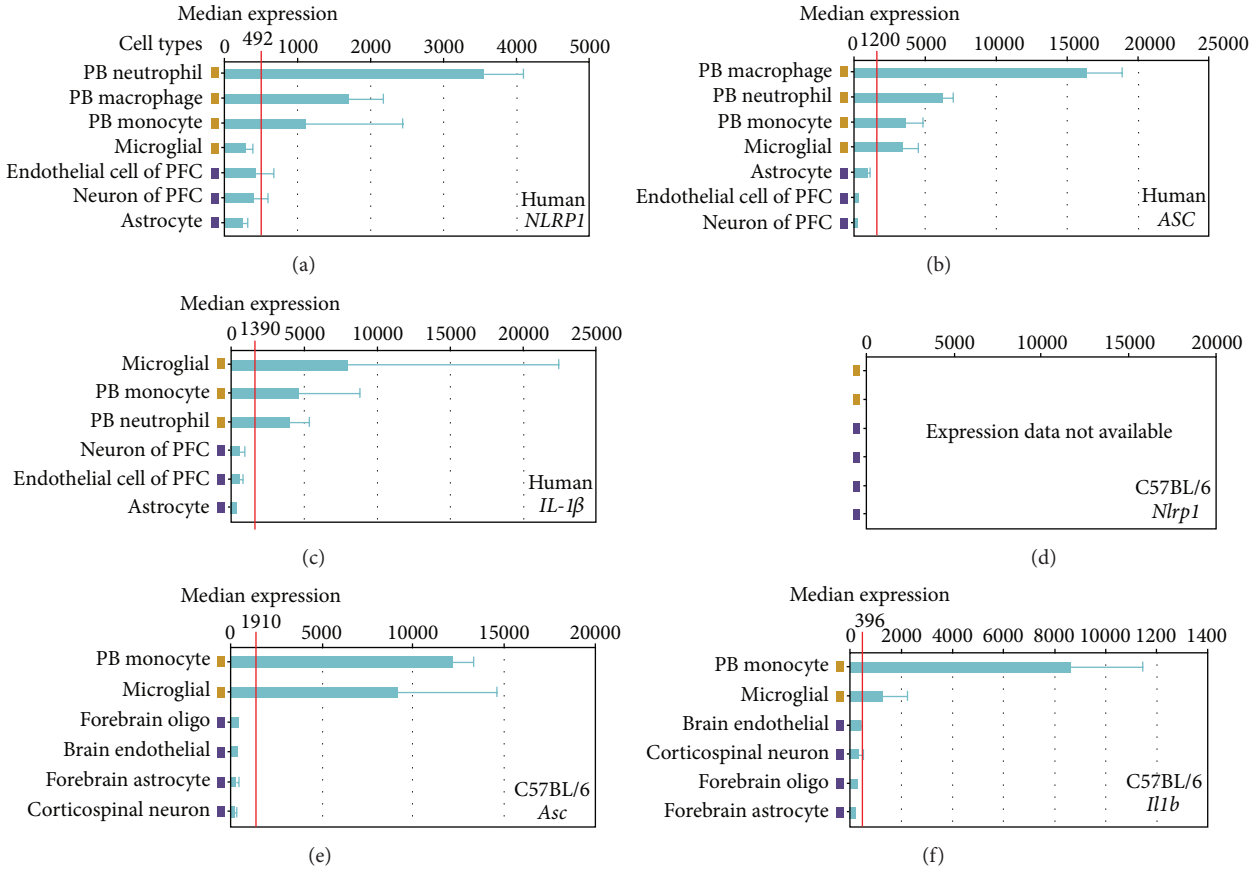

Figure 5: Cell-specific gene expression of *NLRP1*, *ASC*, and *IL-1β* in human and murine tissue. Expression of human (a) *NLRP1*, (b) *ASC*, and (c) *IL-1β* in relevant immune and neural system cell types was compiled using a publically accessible microarray meta-analysis search engine (NextBio website: http://www.nextbio.com/b/search/ba.nb. Accessed on September 8, 2014). Expression of mouse (d) *Nlrp1*, (e) *Asc*, and (f) *Il1b* from the C57Bl/6 strain can be directly compared to the human expression profile in specific immune and neural cells. No gene expression data was available for *Nlrp1* (*Nlrp1a, Nlrp1b, and Nlrp1c*) at the time the search engine was accessed. PB: peripheral blood; PFC: prefrontal cortex.

significantly modulated by several NLRs and is often dysregulated in NLR deficient mice in other models beyond the nervous system [36–38]. Compared to the ~2-fold increase in IL-1β, there was a ~6-fold increase in IL-6 expression after TBI implying that this protein, among others, is more central to the inflammatory response in brain trauma. NLRP1 has not previously been shown to regulate IL-6 and the small reduction seen in the cortical tissue samples of TBI-injured *Nlrp1*$^{-/-}$ mice (Figure 2(c)) is somewhat surprising. We do not believe such minimal changes would impact the overall downstream effects on IL-6 production. However, it is possible that earlier induction (6–12 hrs) of IL-6 is unaffected, which is also critical for stimulating the inflammatory milieu and progression of injury.

ASC, NLRP1, and IL-1β are expressed in neurons and glial, vascular endothelial and peripheral-derived immune cells [39, 40] and have been shown to be upregulated after TBI [8, 9]. These immune mediators play a vital role in activating the inflammatory response, which is a necessary component of repair and healing [41, 42]. However, acute inflammation also exacerbates tissue damage in the brain for which IL-1 has been implicated as a major player [43, 44]. Previous studies in ischemic stroke have demonstrated that exogenous IL-1 administration can exacerbate neuronal injury [45], while inhibition of caspase-1 or IL-1 receptor provides protection [46, 47]. Deletion of IL-1α and IL-1β also can significantly reduce ischemic injury in mice [48]. Furthermore, current clinical trials of IL-1 receptor antagonist are underway in patients who suffer acute stroke [49]. However, our studies indicate that attenuation of IL-1β does not correlate with neuroprotection in CCI-injured *Nlrp1*$^{-/-}$ and *Asc*$^{-/-}$ mice suggesting that NLR inflammasome activation and IL-1 production may not play a significant role in neuronal damage after TBI. Injury-induced expression of IL-1β in the current model may be too weak to participate in eliciting a majority of the immune response. Our data shows that IL-1β is minimally upregulated at 24 hr (1.3-fold increase) as compared to IL-6 (5-fold increase) suggesting that other proinflammatory pathways may play a more prominent role [50]. For example, TNF expression is consistently upregulated across several TBI models in rodents including CCI, FPI, and stab wound injury, with detectable levels at 1 h after injury,

maximal concentration at 3–8 h, and a decline at 24 h after injury [51, 52]. Similarly, IL-6 is also an important mediator of neuroinflammation in the brain [53, 54] suggesting that these and other cytokine pathways may predominate following CCI injury. Indeed, human clinical studies have demonstrated that levels of TNF, IL-6, IL-8, and IL-10 correlate with TBI severity and rates of complication [55–58].

We conclude that disruption of the NLRP1 inflammasome has no effect on injury outcome in the murine moderate CCI model. Inflammasome activation and subsequent IL-1β expression are not a limiting factor in the behavioral deficits, neuronal loss in the cortex, or hippocampus associated with acute CCI injury. NLR inflammasomes have been shown to be involved in a diverse range of conditions associated with aberrant inflammation, including many neurological and neurodegenerative conditions. While our current negative findings using a genetic approach were unexpected, they emphasize the need to further explore the clinical relevance and mechanistic details underlying the NLRP1 inflammasome in brain injury and other related central nervous system disorders.

Disclosure

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or any other funding agency.

Competing Interests

The authors would like to disclose that Dr. Irving C. Allen currently serves as a Guest Editor of this Special Issue of Mediators of Inflammation.

Acknowledgments

The authors would like to thank Dr. Beverly H. Koller (UNC Chapel Hill) for kindly providing the *Nlrp1*$^{-/-}$ mice used in this study. The authors would also like to recognize Genetech for providing the *Asc*$^{-/-}$ (*Pycard*$^{-/-}$) mice. This work was supported by R01 NS096281, R15 NS081623, and VT's OASF. The authors recognize the VT-PREP and VT-IMSD Programs for student support (Tere M. Williams; Armand Meza) and the Virginia-Maryland College of Veterinary Medicine (Irving C. Allen; Daniel E. Rothschild). Student work (Sheryl Coutermarsh-Ott) on this paper was supported by the National Institute of Allergy and Infectious Diseases Animal Model Research for Veterinarians (AMRV) training grant (T32-OD010430).

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