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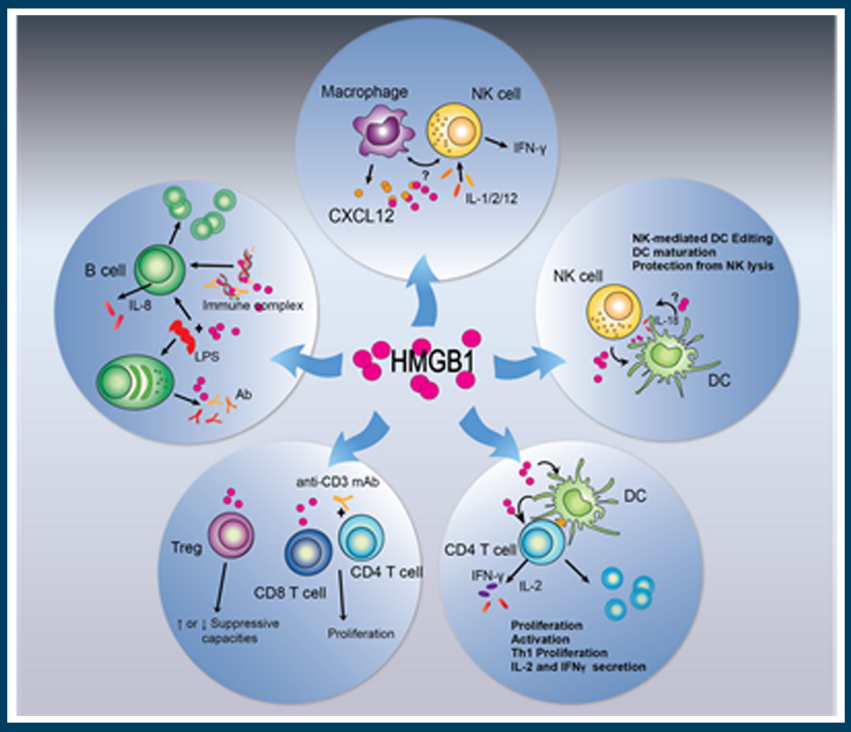

MECHANISMS OF STERILE INFLAMMATION

Topic Editors
Anna Rubartelli, Michael T. Lotze,
Eicke Latz and Angelo A. Manfredi

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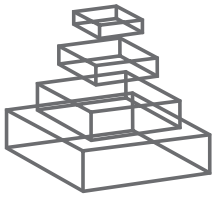

frontiers

ISSN 1664-8714
ISBN 978-2-88919-218-2
DOI 10.3389/978-2-88919-218-2

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MECHANISMS OF STERILE INFLAMMATION

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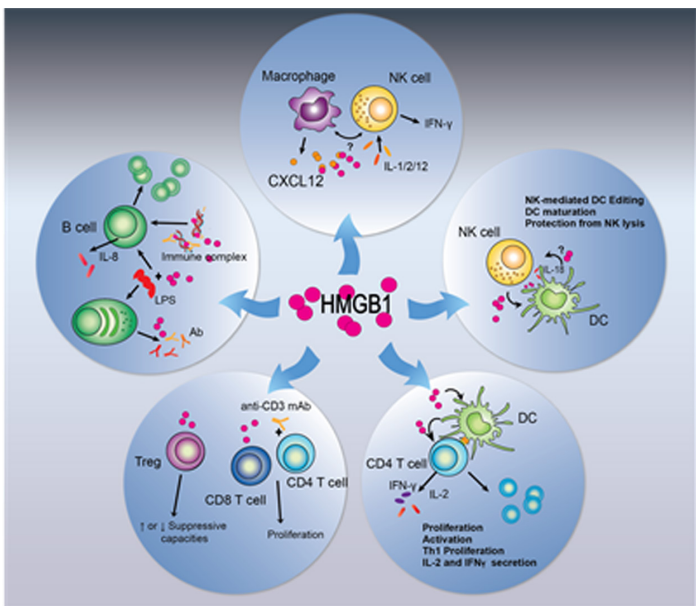

HMGB1. A central mediator of sterile inflammation.
A damage associated molecular pattern molecule (DAMP), HMGB1 stimulates recruitment and effector function in immune cells. It thus acts as a central mediator of sterile inflammation, promoting neutrophil, macrophage, and lymphocyte recruitment and activation. In many instances, HMGB1's function has been to promote interaction between lymphoid and myeloid cells. Its clearest definition has been with NK/macrophage or NK/DC interactions although suspected roles in myeloid interaction with B and T-cells are supported. What is unclear is whether these roles are cell type specific and whether HMGB1 might also act in trans between a myeloid and T-cell. The role of HMGB1 in binding other factors/substances including IL-1, LPS, DNA, CXCL12, and lipoteichoic acid places it as a central mediator for both sterile and pathogen (PAMPs, pathogen associated molecular pattern molecules) associated inflammation. Figure by Guanqiao Li.

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frontiers in
IMMUNOLOGY

EDITORIAL
published: 22 November 2013
doi: 10.3389/fimmu.2013.00398

Mechanisms of sterile inflammation

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Keywords: stress, inflammasome activation, HMGB1, IL-1, DAMPs, PRR, acute inflammation, chronic inflammation

Inflammation is a coordinated response of the immune system which is aimed at maintaining or restoring tissue integrity. Sterile inflammation can be triggered by physical, chemical, or metabolic noxious stimuli. The individual stimuli (genomic stress, ER stress, hypoxic stress, nutrient stress, etc.) promote a carefully choreographed set of cell responses to stress. Many types of stress responses exist (e.g., the unfolded protein response, integrated stress response, oxidative stress response, autophagy, etc.), and these can influence each other. Stress responses induce recruitment of inflammatory cells and result in inflammation. When the noxious stimuli persist over and cannot be eliminated or cleared inflammation fails to resolve, resulting in the development of a vicious circle which is part of the pathophysiology of many human diseases, including cancer, autoimmunity, chronic viral infections, chronic graft versus host disease, metabolic syndromes, and several acquired and inborn genetic disorders. Several proximal factors have been identified and proposed to play a role in the individual types of sterile inflammation, including redox responses, the occurrence of damage-associated molecular patterns molecules (DAMPs) and immune stimulatory heat shock proteins, and vascular remodeling. However, the detailed mechanism(s) linking stressful events and the development of inflammation have thus far remained elusive. The identification of the major molecular species in induction, development, and outcome of sterile inflammation, and the illumination of their mechanisms of action are therefore of paramount relevance for the design of effective therapeutic strategies for the treatment of the most common diseases of the Western world. Thus, this Special Topic focuses on articles that can shed new light on the molecular mechanisms of sterile inflammation. This collection of papers, written by experts in this field, addresses the most important current challenges in the topic of sterile inflammation.

A significant focus is placed on the factors that mediate sterile inflammation: DAMPs, released during tissue injury, and cytokines of the IL-1 family. In this family, some members such as IL-1β and IL-18 are true cytokines, in that they undergo active secretion by inflammatory cells, highly regulated at the post-translational level by inflammasomes (1) and regulatory receptors (2). Others, such as IL-1α (3), are molecules that are both DAMPs and cytokines, in that they initiate and perpetuate inflammation either after active secretion or when released by stressed cells undergoing necrosis (4). Interestingly, a similar behavior features the prototypic DAMP high mobility group box 1 (HMGB1) (5). In addition, this series describes the most recent observations on the cells involved in the process of sterile inflammation, not only professional inflammatory cells such as myelomonocytic cells but also innate lymphoid cells (6), granulocytes (7), and glial cells (8). Finally, sterile inflammation as a mechanism of disease is illustrated in important in pathologies such as type 2 diabetes (9) and endometriosis (10), in fungal infection, where DAMPs cooperate with pathogen associated molecular pattern molecules (PAMPs) in switching protective versus pathogenic inflammation (11), and in the regulation of physiologic processes such as parturition (12).

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Received: 25 October 2013; accepted: 07 November 2013; published online: 22 November 2013.
*Citation: Rubartelli A, Lotze MT, Latz E and Manfredi A (2013) Mechanisms of sterile inflammation. Front. Immunol. **4**:398. doi: 10.3389/fimmu.2013.00398*

This article was submitted to Inflammation, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 20 March 2013
doi: 10.3389/fimmu.2013.00068

HMGB1: the central cytokine for all lymphoid cells

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High-mobility group box 1 (HMGB1) is a leaderless cytokine, like the IL-1 and FGF family members, that has primary roles within the nucleus and the cytosol. Within the nucleus, it serves as another guardian of the genome, protecting it from oxidant injury and promoting access to transcriptional complexes such as nuclear hormone/nuclear hormone receptors and p53/p73 complexes. Within the cytosol it promotes autophagy and recruitment of the myddosome to Toll-like receptor (TLR) 9 vesicular compartments. Outside of the cell, it can either bind to specific receptors itself, or with high affinity to DNA, nucleosomes, IL-1β, lipopolysaccharide, and lipoteichoic acid to mediate responses in specific physiological or pathological conditions. Currently identified receptors include TLR2, TLR4, the receptor for advanced glycation end products, CD24-Siglec G/10, chemokine CXC receptor 4, and TIM-3. In terms of its effects or functions within lymphoid cells, HMGB1 is principally secreted from mature dendritic cells (DCs) to promote T-cell and B-cell reactivity and expansion and from activated natural killer cells to promote DC maturation during the afferent immune response. Some studies suggest that its primary role in the setting of chronic inflammation is to promote immunosuppression. As such, HMGB1 is a central cytokine for all lymphoid cells playing a role complementary to its better studied role in myeloid cells.

Keywords: lymphocytes, HMGB1, TLR2, TLR4, RAGE, NK cells, T cells, B cells

INTRODUCTION

Damage-associated molecular pattern (DAMPs) molecules, are endogenous danger signals that elicit inflammation and subsequent immune responses once released from dead or stressed cells following injury or infection (Rubartelli and Lotze, 2007; Sims et al., 2010; Tang et al., 2012). Although various DAMPs have been identified, the best characterized is the prototypic nuclear protein high-mobility group box 1 (HMGB1). HMGB1 is an evolutionarily ancient protein that was first discovered as a chromatin-associated protein more than three decades ago (Goodwin et al., 1977). We now know that it displays many other functions depending on its location and its synergizing partners. Present within the nuclei of almost all eukaryotic cells, HMGB1 functions as a DNA chaperone that stabilizes nucleosome formation and promotes access to transcriptional factors that target specific genes (Müller et al., 2001; Thomas, 2001), although HMGB1 itself is not sequence specific. Our group demonstrated that cytosolic HMGB1 also promotes autophagy, a conserved programed survival pathway evoked following environmental and intracellular stress (Tang et al., 2010a, 2012). Apart from its nuclear and cytosolic roles, HMGB1 possesses a previously unexpected multifaceted role in immunity when released or secreted into the extracellular milieu. This occurs in two principal ways: either (1) passively released from necrotic cells (Scaffidi et al., 2002) or (2) actively secreted by inflammatory cells, such as monocytes or macrophages (Gardella et al., 2002; Bonaldi et al., 2003; Tang et al., 2007) and natural killer (NK) cells (Semino et al., 2005; Gougeon and Bras, 2011). It this way, HMGB1 evokes innate immune response via its interaction with cell surface receptors.

Previous studies highlight the importance of HMGB1 at the core of inflammation-associated events, acting as an irreplaceable modulator of immune responses and the "universal" biosensor for nucleic acids (Yanai et al., 2012). In spite of its well-established divergent functions in myeloid cells which predominantly participate in innate immune response, its roles in adaptive immunity involving T-cells and B-cells is so far not fully understood and surprisingly, one which needs substantially more study. Here, we describe the cytokine-like biology of HMGB1 protein, with a focus on lymphoid cells, including NK cells, T-cells, and B-cells.

LESSONS FROM HMGB1 KNOCKOUTS

High-mobility group box 1 is vital for *ex utero* growth, as shown by inborn defects and rapid death (within 24 h following birth) in *hmgb1*$^{-/-}$ mice, as early as E15 in inbred species, because of hypoglycemia. This was initially postulated to be the result from deficient glucocorticoids receptor function (Calogero et al., 1999), but we would now attribute this to reduced autophagy, critically important for survival in the neonatal period (Kuma et al., 2004). Necrotic HMGB1$^{-/-}$ cells only weakly activate dendritic cells (DCs) (Rovere-Querini et al., 2004), and HMGB1-deficient DCs display sharply impaired capacity to trigger inflammation (Scaffidi et al., 2002). We now know that floxed HMGB1 deleted in a tissue- or cell type-specific fashion within the pancreas, liver, small bowel, DCs, and NK cells, is associated with prolonged viability of animals compared with complete knockout of HMGB1

(unpublished observations) in the whole animal, suggesting that these are not the target tissues associated with lethality.

HMGB1 AS THE CYTOKINE FOR LYMPHOID CELLS

High-mobility group box 1 was identified as a delayed mediator of inflammation released from macrophages (Wang et al., 1999), found in the serum 24–48 h later than secretion IL-1β and tumor necrosis factor (TNF)-α, the classical early pro-inflammatory cytokines which are dissipated by 24 h. Afterwards, it was demonstrated to be liberated from cells undergoing necrosis, followed by production of TNF-α from monocytes (Scaffidi et al., 2002). Subsequent investigations uncovered an amazingly profligate role in mediating local or systemic immune responses through its interaction with several receptors. As a cytokine, it transduces signals and coordinates cellular activities through several pattern-recognition receptors including the receptor for advanced glycation end products (RAGE), Toll-like receptor (TLR)2, TLR4, TIM-3, chemokine CXC receptor (CXCR)4, CD24-Siglec G/10 (Park et al., 2004, 2006; Dumitriu et al., 2005; Lotze and Tracey, 2005; Bianchi, 2009; Chen et al., 2009; Tang and Lotze, 2012; Tang et al., 2012; Yanai et al., 2012), and TLR-9 when combined with DNA (Tian et al., 2007). Extracellular HMGB1 thus functions as a modulator, modifying the immunogenic potentials of DNA and potentially other PAMPs and DAMPs and cytokines. Indeed, given the differences in the all thiol form of HMGB1, promoting primarily chemokine activity and the dithiol form which promotes TNF/IL-6 production (cytokine activity), it is quite likely that the molecule secreted by activated cells, endowed with autocrine and paracrine actions, differs biochemically and functionally from the molecule released as a consequence of cell and tissue necrosis (Venereau et al., 2012). Given this difference with the all thiol form promoting release of the chemokine CXCL12, and the dithiol not, environmental conditions likely dictate the eventual outcome of HMGB1 interactions with lymphoid cells in the tissues. For example, well perfused and non-hypoxic environments may promote different T-cell responses that hypoxic, reducing conditions (Venereau et al., 2012). TLRs, the best-studied pattern-recognition receptors (PRRs), are highly conserved proteins initiating immune responses following recognition of various molecules derived from pathogens (PAMPs) as well as endogenous danger signals (DAMPs) sharing similar structures (Medzhitov and Janeway, 1997; Aderem and Ulevitch, 2000; Medzhitov, 2001). The intracellular signaling cascades after recognition principally involve two specific adaptors, the Toll/IL-1R domain-containing adaptor TRIF and myeloid differentiation primary response protein (MyD88), which is primarily involved in HMGB1-mediated signaling pathway and acts as a component of myddosome with IRAK2 and IRAK4 assembled in response to primary stimulation (Motshwene et al., 2009; Lin et al., 2010; George et al., 2011). RAGE is a PRR with a wide variety of ligands including advanced glycation end products (AGEs) and DAMPs (Sparvero et al., 2009; Sims et al., 2010). The list of receptors that interact with HMGB1 continues to grow, as does interest in understanding the signaling pathways and their cooperative functions in specific cell types. Current insights on these receptors, based on experimental observations, is that TLRs principally are involved in the activation of myeloid cells, whereas RAGE is primarily activated in endothelial and somatic cells (Yanai et al., 2012).

High-mobility group box 1 signaling has been studied in many cell types following interaction with individual receptors, with most studies centering on myeloid cells – the maturation of conventional DCs, their role in plasmacytoid DCs, activation of monocytes or macrophages, and the production of pro-inflammatory cytokines (Lotze and Tracey, 2005; Yang et al., 2007; Bianchi, 2009; Yanai et al., 2012). Their effects on lymphoid cells, however, are surprisingly not well characterized. We have extraordinarily limited information about the expression of receptors RAGE and, TLR2/4 and TIM-3 on both helper and regulatory T-cells (Wild et al., 2012), RAGE and TLR2/4/9 on B-cells (Tian et al., 2007; Avalos et al., 2010), and TLR2/4 and TIM-3 on NK cells (Tang and Lotze, 2012), shown in **Figure 1**. Beyond that there is quite little information. In this review, we summarize the critical roles of HMGB1 in lymphoid cells (**Table 1**), with a focus on its extracellular role acting as a cytokine.

Most cytokines function distinctly in synergy or antagonism with other cytokines acting collectively. This is also true for HMGB1. Moreover, HMGB1 shares pleiotropic and redundant characteristics with other cytokines (Lotze and Tracey, 2005), sometimes binding them to enhance immunologic function, thereby endowing them with a more potent capacity to elicit biological and immunological responses, consequences depending on the local microenvironmental factors and presence of other circumstances. Here, we list out different cellular responses of lymphoid cells to HMGB1 in different conditions or settings (**Table 2**) which could act as a reference for readers to make comparisons or conduct experiments. Also, we summarize the common consequences in response to HMGB1 (**Figure 2**).

HMGB1 AND NK CELLS

There is little information about the direct effects of HMGB1 on NK cells, with the exception of elevated secretion of IFN-γ by macrophage-stimulated NK cells in concert with other pro-inflammatory cytokines like IL-2 and IL-12 (DeMarco et al., 2005), work done by our group almost a decade ago. Nevertheless, whether it occurs depending on the activation of monocytes or in a direct NK-cell specific manner is still unclear. Further clarification as to which cell type is responsive to HMGB1 under these specific circumstance is needed and of significant interest, since little is known about the interaction between monocytes (macrophages) and NK cells, both of which are key sentinels and instigators of immune responses.

Natural killer cells do, however, secrete HMGB1. HMGB1 undergoes abundant, regulated secretion from activated NK cells into the immunological synapse during NK/iDC (immature DC) crosstalk, thus inducing maturation of DCs and limiting NK cell-mediated cytotoxicity of the DCs (Semino et al., 2005). The secretion of HMGB1 is markedly elevated following engagement of NKp30 (one type of natural cytotoxicity receptor or NCR) expressed on human NK cells, thereby triggering maturation of autologous DC (Semino et al., 2007). Whether or not DC can in turn secrete HMGB1 for further activation of NK cells and promote the quality of the crosstalk remains

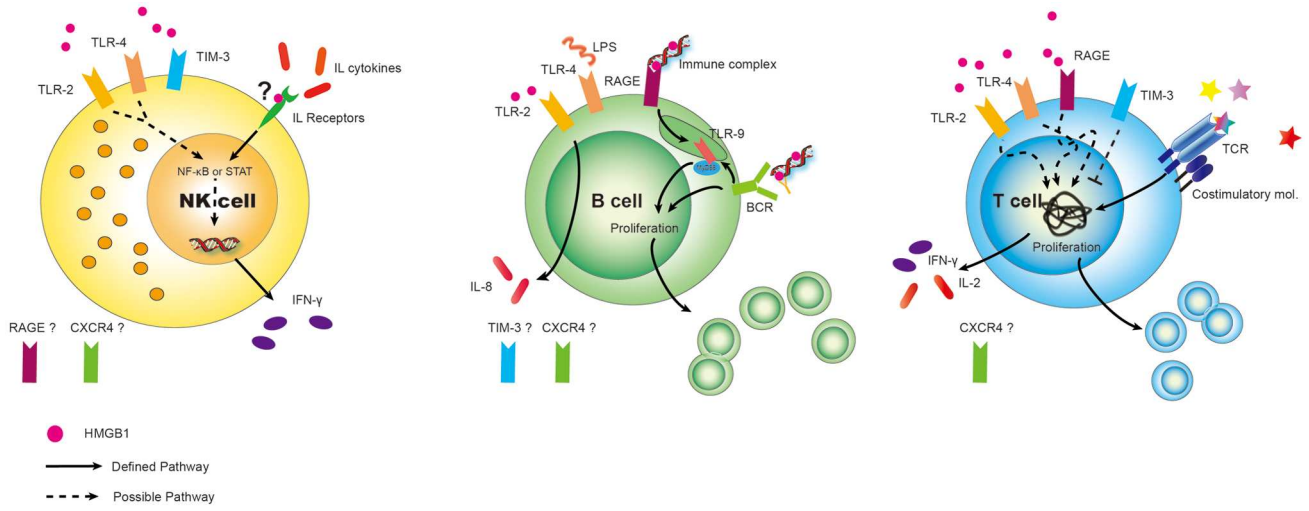

FIGURE 1 | Differential expression of DAMP receptors for HMGB1 on lymphoid cells. Surprisingly, given the surfeit of receptors now shown to be expressed on various cell types, relatively little has been done to fully characterize the panoply of receptors on lymphoid cells. Best defined are the expression of RAGE on Ba and T-cells (but not to our knowledge on NK cells). Similarly TLR2, TLR4, CXCR4, and possible TIM-3 could play critical roles on subserving cell functions in response to HMGB1 on immune cells and this needs to be more carefully studied. Possible and defined roles are shown. "?" represents we hypothesize that HMGB1 binds to IL receptors, assisting in interleukin (cytokine) interaction. Also, expression of other HMGB1 receptors are unknown at present.

Table 1 | Lymphoid cells respond to HMGB1.

	NK cells	B-cells	T-cells
Nuclear	Modulation of transcriptional activity of various genes, including steroid hormone receptors, NF-κB, p53/p73 transcriptional complexes, and some homeobox-containing protein (Erlandsson Harris and Andersson, 2004; Lotze and Tracey, 2005)		
		Nuclear assistance in assembly of recombination activating gene 1/2(RAG1/2)-DNA complex for V(D)J recombination of B-cell receptors (BCR) and T-cell receptors (TCR) (Agrawal and Schatz, 1997; Dai et al., 2005)	
Cytosolic	Regulation of autophagy (Tang et al., 2010a) (other cell types, not discovered in lymphoid cells yet)		
		Recruitment of MyD88 to TLR-9 (Ivanov et al., 2007)	
	Universal biosensor of nucleic acid (Yanai et al., 2012)		
Extracellular	Synergy with other cytokines to modulate cell functions via binding cytokine receptors (CXCR4 for example)		
	Increased IFN-γ secretion in macrophage-stimulated NK cells (DeMarco et al., 2005)	Activation and proliferation in the form of immune complex (HMGB1 + DNA) (Tian et al., 2007; Avalos et al., 2010)	Expansion, activation, and polarization of Th1 cells (Messmer et al., 2004; Dumitriu and Baruah, 2005; Sundberg et al., 2009)
		Spontaneous IL-8 production (McDonnell et al., 2011)	Infiltration of T-cells expressing lymphotoxin and tumor progression (He et al., 2012a)

undefined. Specific cell types without HMGB1 expression would be needed to uncover the critical role of HMGB1 in intracellular communication.

High-mobility group box 1-mediated NK/DC crosstalk is important in the setting of HIV infection (Saïdi et al., 2008; Melki et al., 2010; Gougeon and Bras, 2011). DCHIV (infected by HIV virus) are resistant to the NK cell-induced editing process. Interestingly, HMGB1, essential for DC maturation presumably within secondary lymphoid tissues, also contributes to viral replication and DC persistence via up-regulation of apoptosis inhibitors against TRAIL (TNF-related apoptosis-inducing ligand)-mediated apoptosis.

Given that the cooperative dialog between NK cells and DCs is pivotal for sustaining innate immunity and initiating the subsequent adaptive immune response, it is worth investigating the detailed mechanism by which NK/DC crosstalk and its altered processes link to clinical manifestations of diseases, including cancer, autoimmune, and infectious diseases.

Table 2 | Cellular responses of lymphoid cells to HMGB1 located differently.

Cell types	Species	Disease	HMGB1 effect	Stimulation	Secreted from	Receptors	Summary	Reference
T-cell expressing lymphotoxin	Mouse	Prostate cancer		Specific antigens	Cancer cells or inflammatory cells (?)		HMGB1 is required for infiltration and activation of antigen-experienced T-cells expressing lymphotoxin α1β2(LT), but not helper or regulatory T-cells, followed by recruitment of macrophages to the tumor site in an LTβR-dependent manner, thus prompting tumor malignant progression	He et al. (2012a)
CD4 T-cell	Human	–	Direct	Activated or α-CD3/α-CD28 Abs	Endotoxin-stimulated DC	RAGE on DC	HMGB1 is translocated and secreted by human DC upon stimulation, maintaining itself maturation, and improving CD4+ T-cell expansion, survival, and Th1 polarization. Blockade with anti-HMGB1 Abs or Box A, the effect is drastically impaired. However, T-cell activation cannot be stimulated by HMGB1 alone, but also requires Ag receptor and co-stimulatory signals (CD3 and CD8 crosslinking mimics the event *in vitro*)	Dumitriu and Baruah (2005)
CD4 T-cell	Human	–	Indirect	HMGB1-stimulated DC		RAGE on DC	HMGB1 as well as B box trigger phenotypic maturation and pro-inflammatory cytokine secretion via both RAGE-mediated NF-κB and p38 MAPK pathway. And activated DC will further drive Th1 polarization, as evidenced by secretion of IL-2 and IFN-γ	Messmer et al. (2004)
CD4 CD8 T-cell	Human	–	Direct	α-CD3 mAb			HMGB1 behaves as a proliferative signal for both human CD4 and CD8 T-cells in response to suboptimal anti-CD3 mAb stimulation	Sundberg et al. (2009)
CD4 Treg Tcon	Human	–	Direct	TCR/co-stimulation (CD2/CD3/CD28 beads)		RAGE TLR4	HMGB1 prompts survival and suppressive capacities of Treg in a RAGE-mediated fashion, whereas suppresses IFNγ release of Tcon (conventional) and inhibits their proliferation via TLR4, indicating that TCR/co-stimulatory signal is abrogated by HMGB1	Wild et al. (2012)
CD4 T-cell↓	Mouse		Indirect	$CD11C^{low}$ $CD45RB^{high}$ DC			IL-10 producing $CD11C^{low}$ $CD45RB^{high}$ mouse DCs display mature phenotype and secrete IL-10 upon HMGB1 stimulation in a dose-dependent manner, therefore potentially diminish T-cell response and driving Th2 polarization	Liu et al. (2011)
CD4 T-cell	Rat	Burn	Direct				HMGB1 markedly limits the proliferation of rat T-cells during post-burn, consistent with decreased expression of IL-2 and IL-2Rα. T-cells polarized to Th2 after HMGB1 stimulation *in vivo*	Zhang et al. (2008)
CTL (CD8 T-cell)	Human mouse		Indirect	DC + dying tumor cells	Dying tumor cells	TLR4/MyD88 on DC	In the context of chemo- or radio-therapy, functional binding between HMGB1 released by dying cells and its receptor TLR4 on DC is prerequisite for efficient antigen presentation of tumor antigens and induction of CTL immunity	Apetoh et al. (2007)

(Continued)

Table 2 | Continued

Cell types	Species	Disease	HMGB1 effect	Stimulation	Secreted from	Receptors	Summary	Reference
Treg	Mouse		Direct			TLR4	HMGB1 modulates the suppressive capacity of Treg through TLR4-dependent pathway. The expression level of CTLA4 and Foxp3 in Treg cells as well as IL-10 secretion were significantly diminished after HMGB1-treatment, which was restored by administration of anti-TLR4 antibody	Zhu et al. (2011)
Autoreactive B-cell	Mouse	SLE	Direct	Immune complex (+CpG)		TLR-9 and RAGE	HMGB1 acts to activate pDCs and IgG2a-reactive B-cell receptor (BCR) transgenic B-cells in form of DNA-containing immune complex via TLR-9-dependent pathway. The response is considerately elicited with the help of surface RAGE	Tian et al. (2007)
Autoreactive B-cell	Mouse	SLE	Direct	Immune complex (+DNA)		TLR-9 and BCR Not RAGE	B-cells can undergo activation and proliferation in response to chromatin immune complexes (ICs) containing HMGB1-DNA in a TLR-9-mediated manner by specific antibody engagement of BCR but not RAGE	Avalos et al. (2010)
B-cell	Human	Inflammatory bowel disease	Direct	±LPS		TLR2 and CD36	Endogenous HMGB1 induces B-cell activation through TLR2 and CD36, whereas exogenous endotoxin may exhibit disease-specific effects on B-cells, unexpectedly evoking pro- or anti-inflammatory responses. Moreover, serum levels of HMGB1 are linked with spontaneous IL-8 production	McDonnell et al. (2011)
B-cells	Mouse			LPS	LPS-stimulated splenic plasma cell		Non-canonical inflammatory cytokine HMG-1 is released from plasma cells into the extracellular milieu following B-cell maturation, demonstrating its pro-inflammatory role	Vettermann et al. (2011)
NK cells	Human		Direct/ indirect	+IL-2/1/12 + monocyte			HMGB1 in concert with IL-2 and IL-1 or IL-12 facilitates interferon gamma release from macrophage-stimulated NK cells	DeMarco et al. (2005)

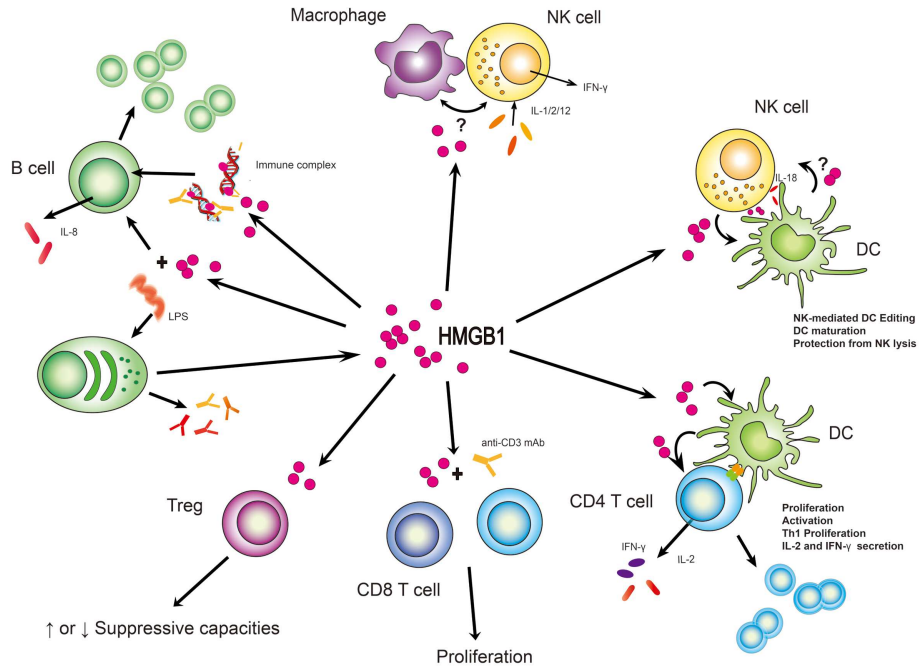

FIGURE 2 | HMGB1 stimulates effector function from immune cells. In many instances, HMGB1's function has been to subserve cell:cell interactions between lymphoid and myeloid cells. Its clearest definition has been with NK/macrophage or NK/DC interactions although suspected roles in B and T-cell activities are supported. What is unclear is whether these roles are cell type specific and whether HMGB1 might act in trans between a myeloid and lymphoid cell. "?" represents the state of uncertainty if HMGB1 plays a role in the setting of macrophage and NK cell interaction or HMGB1 is secreted from DCs to affect NK cell function.

HMGB1 AND T-CELLS

Our knowledge of HMGB1 effector functions to T-cells are principally based on observations and inferences from the evaluation of T-cell subsets with the treatment of HMGB1 co-cultured with DCs. In the presence of other cytokines, HMGB1 can modify the fate of the overall immune response, promoting immunity or tolerance as demonstrated by targeting effector T-cells and regulatory T-cells (Tregs) reciprocally in response to individual stimuli. Differences that may have been found in terms of immunity or tolerance when comparing various observations might be, at least in part, due to differences in the experimental systems utilized (dosage, duration, and the presence of other factors) and the pharmacological inhibitors utilized to block these complex biological systems *in vitro* and *in vivo*. We know little about the effects of HMGB1 on naïve and memory T-cells as regards alteration in phenotype or cytokine proficiency among the defined T-cell subsets. In addition, the ability of HMGB1 to either recruit T-cells to sites of tissue damage or injury, thus allowing effector T-cell function, or to induce Treg infiltration and expansion is largely unknown.

DIRECT EFFECTS ON T-CELLS

Acting as a pro-inflammatory cytokine, HMGB1, is not only released by stressed or necrotic tissues but also translocated and secreted by human DC following PAMP [endotoxin/lipopolysaccharide (LPS)] stimulation. It plays a critical role in promoting expansion, survival, and helper T (Th) 1 polarization of CD4+ T-cells (Dumitriu and Baruah, 2005; Jube et al., 2012). Similarly, HMGB1, is also a proliferative signal for both human CD4+ and CD8+ T-cells in response to suboptimal anti-CD3 mAb stimulation (Sundberg et al., 2009). The expression level of CTLA4 and Foxp3 in Treg cells as well as IL-10 secretion are significantly diminished following HMGB1 treatment. This is restored by administration of an anti-TLR4 antibody (Zhu et al., 2011). Altogether, HMGB1 is seemingly necessary for enhancing immunity through activation of effector T-cells and suppression of Treg's. In contrast, HMGB1 can also promote migration and survival of Treg, whereas it suppresses IFNγ release of conventional T-cells and inhibits their proliferation via TLR4, indicating that the TCR/co-stimulatory signal is abrogated by HMGB1. Furthermore, HMGB1 elicits increased suppressive capacity of Treg when co-cultured with effector T-cells in a RAGE-dependent fashion. Additionally, several reports provide evidence suggesting that HMGB1 may contribute to Th17 cells proliferation and activation in the context of autoimmune disease, including rheumatoid arthritis, myocarditis, as well as acute allograft rejection (Duan et al., 2011; Su et al., 2011; He et al., 2012b; Shi et al., 2012).

When we examine immune responses *in vivo*, the findings are totally different. HMGB1 is essential for infiltration and activation of T-cells expressing lymphotoxin α1β2(LT) in mice with prostate cancer, therefore recruiting macrophages to promote tumor malignant progression (He et al., 2012a). This work further confirms the notion that HMGB1 can prompt progression of many types of cancers (Tang et al., 2010b). Surprisingly, neither T effectors nor Tregs are detected differentially between normal and cancerous tissues. The source of extracellular HMGB1 needs to be

further characterized, whether arising from stressed tumor cells or recruited inflammatory cells, including NK cells or DCs, or all. HMGB1 markedly limits the proliferation of murine (rat) T-cells and induces Th2 polarization following burn injury, consistent with decreased expression of IL-2 and IL-2Rα (Zhang et al., 2008).

DC-MEDIATED INDIRECT EFFECTS ON T-CELLS

High-mobility group box 1 is an inducer of DC maturation (Messmer et al., 2004; Rovere-Querini et al., 2004; Semino et al., 2005, 2007). Mature and activated DC will further drive Th1 polarization, as evidenced by secretion of IL-2 and IFN-γ (Messmer et al., 2004). It is worth noting that one of the promising mechanisms underlying the chemo- or radio-therapy-based anti-tumor responses is due to the functional binding between HMGB1 released by dying cells and one of its receptors, TLR4 expressed on DC, which allows for antigen presentation and subsequent cytotoxic CD8+ T-cell (CTL) effector function (Apetoh et al., 2007).

On the other hand, IL-10 producing $CD11C^{low}$ $CD45RB^{high}$ mouse DCs also display a mature phenotype and secrete IL-10 following HMGB1 stimulation in a dose-dependent manner, thereby potentially diminishing T-cell responses with down-regulation of IL-2 and IL-2Rα and driving Th2 polarization, just the opposite in the case of $CD11C^{high}$ $CD45RB^{low}$ DCs. This finding is in concordance with the potential of HMGB1 to polarize Th2 cells in rats following thermal injury (Zhang et al., 2008).

HMGB1 AND B-CELLS

Compared with T-cells, the role of HMGB1 in B-cells has not been fully delineated. Some studies have supported a role for HMGB1 in B-cell activation. In the form of immune complexes (ICs), HMGB1 promotes proliferation of autoreactive B-cells in response to endogenous TLR-9 ligands (e.g., DNA) (Tian et al., 2007; Avalos et al., 2010). This suggests are markedly immune-regulatory function in the pathogenesis of autoimmune diseases. TLR-9 is responsive to immune complex in intracellular endosomes, while the internalization of DNA may be mediated by RAGE which bound with HMGB1 (Tian et al., 2007) or by specific IgG and B-cell receptor interaction, followed by BCR engagement (Avalos et al., 2010). However, given that B-cell proliferation and Ig gene recombination share the same pathway but contrary states of molecules involved (e.g., FOXO degradation or dephosphorylation) and autoreactive antigen, the capacity of antibody production could be further investigated in terms of individual receptors of IC interaction, thus providing a comprehensive role for HMGB1 in B-cell activation. Furthermore, in the context of inflammatory bowel disease (IBD), enhanced serum levels of HMGB1 is accompanied by spontaneous IL-8 production by B-cells via interaction with TLR2 and CD36 (McDonnell et al., 2011). On the other hand, plasma cells release HMGB1 into the extracellular milieu following LPS-stimulated maturation (Vettermann et al., 2011), demonstrating its pro-inflammatory effects in promoting autoimmune disease and chronic inflammation.

CONCLUDING REMARKS

High-mobility group box 1, like other cytokines, is able to function as an agonist, an antagonist, to synergize with other factors and to have multiple pleiotropic functions on multiple cell types, including lymphoid cells. Unlike typical cytokines however, it interacts with a panoply of receptors, many of which are notably promiscuous with functions quite disparate from each other, depending upon the local microenvironment, location, and coordination with individual stimuli. In addition, unlike cytokines which interact with picogram or nanogram quantities to promote full receptor activation, HMGB1 requires, in many instances, microgram quantities in order to elicit a meaningful response *in vitro*. Increasing advances in understanding the role of HMGB1 in immunity have extended the knowledge and led to widespread acceptance of the notion that HMGB1 acts as a centrally important, potent, ubiquitous cytokine which exerts effect on both myeloid and lymphoid cells. It thus plays a multifaceted modulatory role in both innate and adaptive immune responses. Although there is much information about the diverse, sometimes even opposite effects of HMGB1 on various kinds of immune cells in culture, it is of great importance to understand the precise mechanism by which HMGB1 functions *in vivo*, in particular during altered pathology or physiology. In a complicated balance of guiding and choreographing disparate biologies, HMGB1, interspersed with DAMPs and PAMPs, develops the plot line and provides impetus to the emergent immune response. Improved understanding of when, where, which cell types produce/respond to HMGB1 and what levels at intimate cell:cell contact or released into tissues or systemically would provide a basis for suitable therapeutic implementation or interventions in the clinic.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 January 2013; accepted: 01 March 2013; published online: 20 March 2013.

Citation: Li G, Liang X and Lotze MT (2013) HMGB1: the central cytokine for all lymphoid cells. Front. Immunol. ***4****:68. doi: 10.3389/fimmu.2013.00068*

This article was submitted to Frontiers in Inflammation, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 14 September 2012
doi: 10.3389/fimmu.2012.00290

The transcription of the alarmin cytokine interleukin-1 alpha is controlled by hypoxia inducible factors 1 and 2 alpha in hypoxic cells

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During hypoxia, cells undergo transcriptional changes to adjust to metabolic stress, to promote cell survival, and to induce pro-angiogenic factors. Hypoxia-induced factors (HIFs) regulate these transcriptional alterations. Failure to restore oxygen levels results in cell death by necrosis. IL-1α is one of the most important mediators of sterile inflammation following hypoxia-mediated necrosis. During hypoxia, IL-1α is up-regulated and released from necrotic cells, promoting the initiation of sterile inflammation. This study examined the role of IL-1α transcription in initiation of hypoxic stress and the correlation between IL-1α transcription and HIFα factors. In an epithelial cell line cultured under hypoxic conditions, IL-1α transcription was up-regulated in a process mediated and promoted by HIFα factors. IL-1α transcription was also up-regulated in hypoxia in a fibroblast cell line, however, in these cells, HIFα factors inhibited the elevation of transcription. These data suggest that HIFα factors play a significant role in initiating sterile inflammation by controlling IL-1α transcription during hypoxia in a differential manner, depending on the cell type.

Keywords: alarmin, IL-1, sterile inflammation, HIF-1α, HIF-2, DAMPs, cytokines and inflammation

INTRODUCTION

During infections, injuries, infarcts, or other ischemic events, tissue cells experience hypoxic stress, which can result in cell necrosis that induces inflammation. In infectious diseases, in addition to molecules originating in necrotic tissue, bacterial products alert the immune system following pattern recognition. However, dying cells in a sterile environment use self-molecules alone to signal the surrounding cells and the immune system of the danger which confronts the tissue (Matzinger, 1994). Among the cell molecules released from necrotic cells, some are inducers of sterile inflammation, and were termed "alarmins" or "danger-associated molecular patterns" (DAMPs; Oppenheim and Yang, 2005; Bianchi, 2007; Rubartelli and Lotze, 2007; Chen and Nunez, 2010). The immune response to dead cells includes myeloid cell recruitment. These cells, mostly granulocytes and macrophages, migrate to the hypoxic area, counter to the oxygen gradient (Lotfi et al., 2009; Eltzschig and Carmeliet, 2011), where they can promote debris clearance and tissue repair or a pro-angiogenic response (Nizet and Johnson, 2009). Alternatively, myeloid cells can expand the inflammatory response which can lead to additional tissue damage. Several alarmin molecules have been described. Among these are HMGB1, S100 proteins, heat-shock proteins, and IL-33 (Hofmann et al., 1999; Basu et al., 2000; Raucci et al., 2007; Moussion et al., 2008; Chen and Nunez, 2010; Andersson and Tracey, 2011). IL-1α is a major alarmin molecule that was shown to be a key inducer of sterile inflammation (Chen et al., 2007; Eigenbrod et al., 2008; Cohen et al., 2010; Dinarello et al., 2012). In addition to its alarmin property in response to dying cells, IL-1α can also differentiate between apoptosis and necrosis, by its restricted release from necrotizing but not apoptotic cells (Luheshi et al., 2009; Cohen et al., 2010).

During hypoxia, cells alter their transcriptome in order to adjust to changes in the availability of oxygen and to metabolic stress. The alteration in transcription is mediated by hypoxia-induced factors (HIFs), which are heterodimer transcription factors, composed of a stable beta subunit and an alpha subunit that has a half-life of several minutes. The alpha subunit is targeted for the ubiquitin proteasome degradation pathway, as long as its specific proline residues are hydroxylated by prolyl hydroxylases (PHDs; Bruick and McKnight, 2001), which allows recognition by von Hippel–Lindau (VHL) ubiquitin E3-ligase (Jaakkola et al., 2001). HIFα proteins include HIF-1 to -3, while HIF-1α and HIF-2α are considered to be the major HIF transcription factors that control vast gene transcription and can have differential or even opposing effects (Hu et al., 2003; Wang et al., 2005; Keith et al., 2012). HIF-1α and HIF-2α correlated not only with cell metabolism and angiogenesis accompanied by hypoxia, but also with the inflammatory response during infection or tissue damage (Nizet and Johnson, 2009).

It was shown that during hypoxia, macrophage secretion of IL-1α increased in conjunction with pro-angiogenic factors such as VEGF (Carmi et al., 2009). While bone marrow-derived cells can respond to tissue stress or infection by secreting many pro-inflammatory cytokines, chemokines, proteases, reactive oxygen species (ROS), etc., non-hematopoietic tissue resident cells can also induce inflammation, but in a more restricted manner, by secreting IL-1α, one of the most potent cytokines found in these

cells. Among such cells are fibroblasts (Kawaguchi et al., 2006), keratinocytes (Kong et al., 2006; Lee et al., 2009; Rider et al., 2011), endothelial cells (Berda-Haddad et al., 2011), and hepatocytes (Sakurai et al., 2008; Kamari et al., 2011). Recently, we showed that hypoxic cell-derived IL-1α induces inflammation in Matrigel plugs. IL-1α was up-regulated in hypoxic cells which eventually died by necrosis. The up-regulation was observed both on the mRNA and protein levels in keratinocytes cells. However, whether HIFα proteins where involved in this up-regulation was not yet studied. The up-regulation and release of IL-1α mediated an influx of neutrophils in early stage, followed by macrophage infiltration, which was an IL-1β-dependent phase of the inflammatory process (Rider et al., 2011). Thus, in the present study, we examined the role of major hypoxic transcription factors, the HIFα proteins, on the initiation of the transcription and regulation of IL-1α, the alarm cytokine, which characterizes sterile inflammation. A link between HIFα regulation and the elevation of IL-1α can add a new functional role for the HIFα proteins, as regulators of sterile inflammation, which when chronic local angiogenesis is switched on.

MATERIALS AND METHODS

CELL CULTURE

WI-38, A549, HeLa, and HEK-T293 cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Biological Industries, Beit Haemek, Israel). For hypoxic stress, cells were cultured in a sealed anaerobic workstation (Concept 400; Ruskinn Technology/Jouan) providing conditions of $O_2 < 0.3\%$, 5% CO_2, 95% N_2, and 37°C.

siRNA SILENCING

Cells were transfected using the jetPRIME transfection reagent (Polyplus transfection), according to the manufacturer's instructions, with 100 nM of non-targeting pool control siRNA, on-target plus SMARTpool human HIF1A (3091) or EPAS1 (2034), all from Thermo Scientific.

VECTORS AND TRANSFECTIONS

A549, WI-38, and HeLa cells were transfected using the Jet-PEI reagent (Polyplus transfection), while HEK-T293 were transfected using the calcium-phosphate method as described before (Rider et al., 2011), with plasmids encoding HA-HIF-1α-P402A/P564A and HA-HIF-2α-P405A/P531A mutated sequences which were previously described (Kondo et al., 2003), and were a gift from Professor William Kaelin (addgene plasmid #18955 and #18956). In order to obtain a control plasmid, the insert of HA-HIF-2α-P402A/P564A vector, was liberated with *Bam*HI and *Not*I restriction enzymes, overhang ends were filled with DNA polymerase I large (Klenow) fragment enzyme and ligated to obtain control circular plasmid encoding HA with no HIFα insert. All enzymes in this procedure were from New England Biolabs.

WESTERN BLOT

Nuclear and cytosol fractions of HEK-T293 transfected cells were prepared with NE-PER Nuclear and cytoplasmic extraction reagents (Thermo Scientific). Nuclear fractions were separated over 8% PAGE and transferred to PVDF membranes (Millipore). Detection of HIFα proteins was performed using mouse anti-HIF-1α (Novus Biological) and rabbit anti-HIF-2α (Abcam) antibodies. To detect IL-1α, cells were centrifuged and pellets were re-suspended in 0.5% Triton-X100 in PBS supplemented with protease inhibitor cocktail (Calbiochem). Lysates were centrifuged and protein concentrations were calculated using the Bradford reagent (Bio-Rad). Lysates were separated over 15% PAGE, and IL-1α was detected on PVDF membranes using mouse anti-IL-1α antibodies (R&D).

QUANTITATIVE RT-PCR

Total RNA was extracted from the cells using RNeasy kit (Qiagen, Valencia, CA, USA), and quantified using a NanoDrop spectrophotometer (ND-1000 spectrophotometer, NanoDrop Technologies, USA). cDNA reverse-transcription was performed with 1 μg of total RNA as a template, using the qScript cDNA Synthesis Kit (Quanta Biosciences). The quantitative RT-PCR was performed with PerfeCta SYBR Green FastMix, Low ROX (Quanta Biosciences) on ABI Prism 7500 sequence detection system (Applied Biosystems). In house SYBR Green based assays were used to quantify human β-actin: AGCCTCGCCTTTGCCGATCC, TTGCACATGCCGGAGCCGTT; IL-1α: GCCCAAGATGAAGACCAACCAGTGC, GCCGTGAGTTTCCCAGAAGAAGAGG; VEGF: CTACCTCCACCATGCCAAGTGGTCC, ATGTCCACCAGGGTCTCGATTGGA; HIF-1α: AGACTTTCCTCAGTCGACACAGCCT, GCGGCCTAAAAGTTCTTCTGGCTCA; and EPAS1: TGCTCCACGCCCAATAGCCC, GGGTGCCAGTGTCTCCAAGTCC.

Relative quantification was calculated by the 2^ΔΔCq method. Averages of ΔCq from biological replicates or from different experiments were analyzed by two-tailed Student's *t*-test for statistical significance using GraphPad Prism 4 (GraphPad Software).

RESULTS

IL-1α TRANSCRIPTION IS UP-REGULATED DURING HYPOXIA IN THE HUMAN EPITHELIAL CELL LINE A549

We recently reported that IL-1α is up-regulated in mouse keratinocytes during hypoxia (Rider et al., 2011). This up-regulation of IL-1α together with the accompanied necrosis following extended periods of hypoxia (24 h) resulted in increased levels of IL-1α in the cell supernatants. Therefore, we sought to elucidate the initial steps of up-regulation of IL-1α transcription before cells are damaged due to acidosis and necrosis. We cultured the lung epithelial A549 cell line in either normal or hypoxic conditions, and detected up-regulation of the 31 kDa precursor protein and to some extent the 17 kDa mature cytokine (**Figure 1A**), similar to data with mouse keratinocytes reported in our recent paper (Rider et al., 2011). Since our goal was to study the initial phase of IL-1α up-regulation, we evaluated mRNA levels during hypoxia. Cells were cultured in a hypoxic chamber for 6 h and then analyzed for IL-1α transcription by real-time PCR. Indeed, IL-1α transcription during the initial phase of hypoxia was up-regulated compared to cells cultured in normoxia (**Figure 1B**).

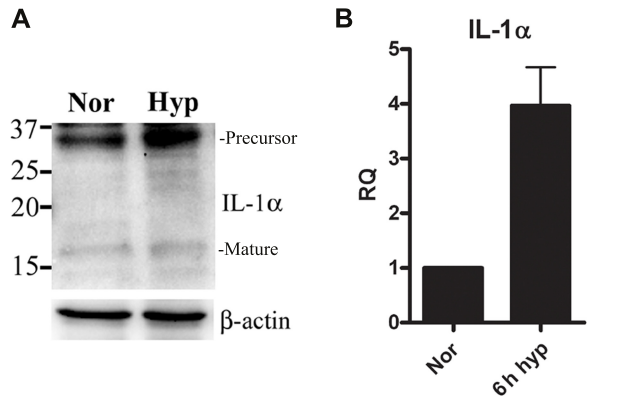

FIGURE 1 | IL-1α transcription is up-regulated during hypoxia in A549 epithelial cells. (A) A549 cells were cultured in either normoxic or hypoxic conditions for 12 h. Cells lysates were analyzed by Western blot for IL-1α detection. Bands are marked as precursor or mature. **(B)** A549 cells were cultured in either normoxic or hypoxic conditions for 6 h, and were analyzed for relative quantification (RQ) of IL-1α transcription. Graph represents mean ± SD of three biological repeats.

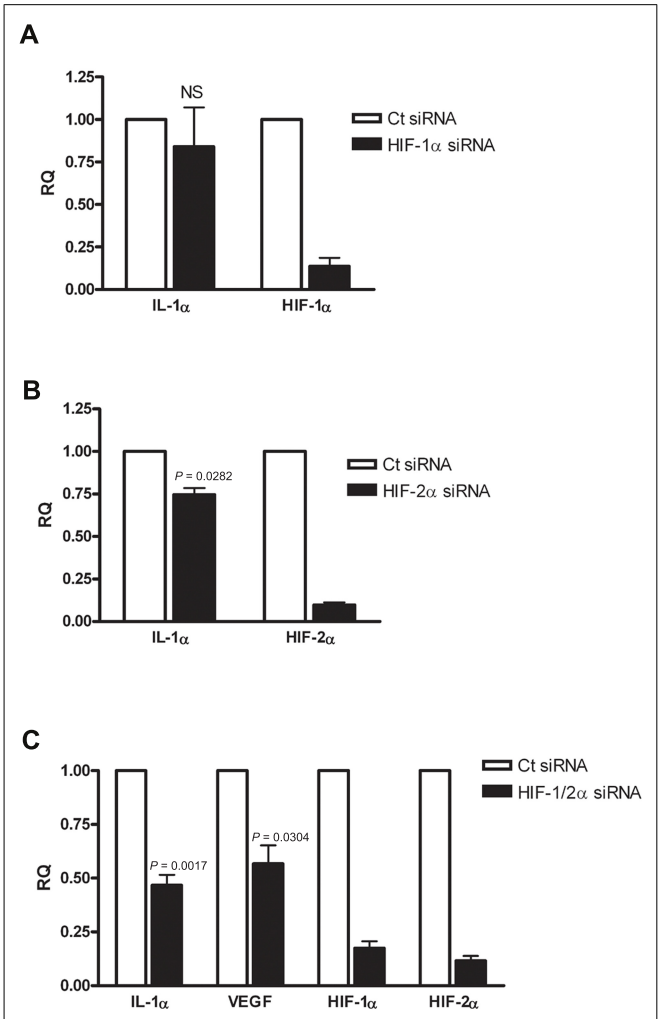

FIGURE 2 | Silencing HIFα proteins down-regulates IL-1α transcription in hypoxic A549 epithelial cells. A549 cells were transfected with HIF-1α siRNA **(A)** or HIF-2α siRNA **(B)** 24 h prior to hypoxia (6 h). Each graph represents mean ± SEM of three independent experiments. **(C)** Cells were transfected with both HIF-1α and HIF-2α mixed siRNA 24 h prior to hypoxia (6 h). Graph represents mean ± SEM of five independent experiments. *P* values of HIF-2α or HIF-1/2α siRNA-transfected cells vs. control (Ct) siRNA-transfected cells are annotated in the graphs.

SILENCING HIFα PROTEINS DURING HYPOXIA RESULTS IN ATTENUATED IL-1α TRANSCRIPTION

Since IL-1α transcription was altered during hypoxia, we examined whether HIFα proteins are involved in IL-1α up-regulation. First, we assessed HIF-1α siRNA silencing, and no significant change in IL-1α transcription was observed (**Figure 2A**). Levels of HIF-1α were also measured in order to assure that silencing was successful. Next, we examined whether silencing of the other major HIFα transcription factor, HIF-2α, altered the transcription of IL-1α. We observed that HIF-2α silencing resulted in a minor reduction of IL-1α mRNA levels (**Figure 2B**). However, the use of both HIF-1α and HIF-2α as targets for siRNA silencing resulted in significantly attenuated levels of IL-1α mRNA in A549 cells, similar to that of VEGF (**Figure 2C**). These data indicates that HIFα factors promote the up-regulation of IL-1α during hypoxia and increase the inflammatory potential in cases of hypoxia-mediated necrosis. In addition, silencing HIFα factors in order to decrease angiogenesis, for example, by inhibiting VEGF transcription, can result in decreased levels of IL-1α as well.

SILENCING HIFα PROTEINS DURING HYPOXIA RESULTS IN INCREASED IL-1α TRANSCRIPTION IN WI-38 LUNG FIBROBLASTS

Following the observation of decreased IL-1α transcription in A549 cells following HIFα silencing, we sought to confirm or decline whether the up-regulatory effect of HIFα factors on IL-1α expression is general observation or it is unique to this cell type, by testing different type of cells. Since fibroblasts and epithelial cells play different roles in inflammation and are known to interact and promote structural changes during inflammatory disease, such as asthma (Knight, 2001), we decided to test WI-38 fibroblasts cell line. These cells are capable of expressing IL-1α in similar way to the A549 cells (**Figure S1** in Supplementary Material). The cells were incubated in hypoxic conditions for 2–6 h, time periods in which initial transcription alterations can be observed. Indeed, real-time PCR analyses showed that elevated levels of IL-1α mRNA correlated with the prolongation of hypoxia (**Figure 3A**). WI-38 fibroblast cells responded differently to HIFα silencing than A549 cells. While silencing HIF-1α or HIF-2α alone resulted in no significant change in levels of IL-1α mRNA (**Figures 3B,C**), silencing both HIF-1α and HIF-2α resulted in elevated levels of IL-1α transcription (**Figure 3D**). These data indicate that although IL-1α transcription is inhibited following HIFα silencing in A549 cells, fibroblasts respond in an opposite manner. To assure that this elevation was genuine and unaffected by the treatment itself, we ruled out differences in levels of cell death between A549 and WI-38 cells following the hypoxic culture (**Figure S2** in Supplementary Material), and also examined VEGF levels, as VEGF is the most well-known pro-angiogenic factor controlled by HIFα

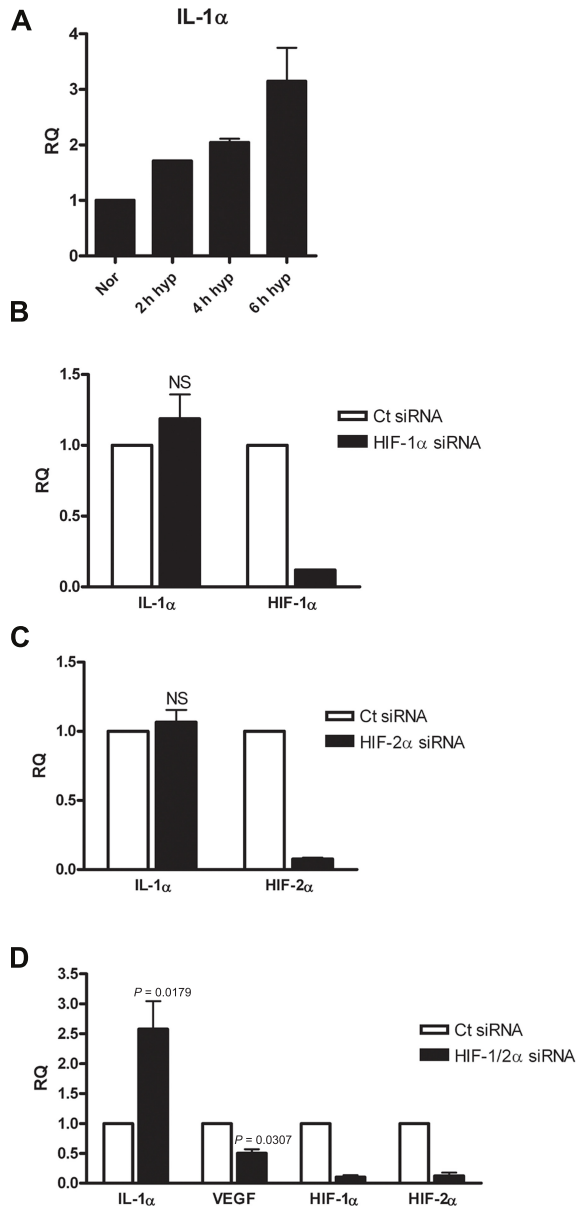

FIGURE 3 | Silencing HIFα proteins up-regulates IL-1α transcription in hypoxic WI-38 fibroblasts. (A) WI-38 fibroblasts were cultured in either normoxic or hypoxic conditions for 2, 4, or 6 h, and were analyzed by qRT-PCR for RQ of IL-1α transcription. Graph represents the average ± SEM of two different experiments. RQ of IL-1α transcription in WI-38 cells transfected with HIF-1α **(B)**, HIF-2α **(C)** siRNA 24 h prior to 6 h culture in hypoxic conditions. Each graph represents mean ± SEM of three independent experiments. **(D)** Cells were transfected with both HIF-1α and HIF-2α mixed siRNA 24 h prior to hypoxia (6 h). Graph represents mean ± SEM of four independent experiments. *P* values of HIF-1/2α siRNA transfected cells vs. control (Ct) siRNA-transfected cells are annotated in the graphs.

factors. Indeed, while IL-1α mRNA levels increased following HIFα silencing, VEGF mRNA levels decreased as expected. These results indicate that in hypoxic fibroblasts, IL-1α transcription up-regulation is restrained by HIFα factors, and silencing these factors, for example for therapeutic intervention, can result in an increased inflammatory response, due to increased levels of IL-1α.

OVEREXPRESSION OF HIFα FACTORS INCREASE IL-1α TRANSCRIPTION IN EPITHELIAL CELLS BUT NOT IN WI-38 FIBROBLASTS

We next decided to use a different approach to verify the results we obtained by siRNA silencing during hypoxia. We transfected WI-38 and A549 cells with plasmids encoding a proline to alanine muted form of HIF-1α and HIF-2α. These specific proline residues are hydroxylated under normal oxygen levels, and therefore mediate the degradation of the proteins by allowing the recognition of pVHL ubiquitin E3-ligase. In order to verify the stability of the muted proteins during normoxia, we obtained transfectant nuclear fractions and analyzed them by Western blot with anti-HIF-1α and anti-HIF-2α specific antibodies (**Figure 4A**). Indeed, increased levels of the proteins were obtained, as described before (Kondo et al., 2003). Next, we evaluated the effects of HIFα transfection on IL-1α levels in WI-38 and A549 cells. While higher mRNA levels of IL-1α were seen in A549 cells, IL-1α levels were not up-regulated in WI-38 cells, where, in fact, we noted a non-significant reduction in IL-1α levels (**Figures 4B,C**). Another epithelial cell line, the HeLa cell line, was transfected and evaluated for IL-1α by real-time PCR, and showed similar patterns to A549 cells, i.e., up-regulation of IL-1α transcription following an increase of HIFα factors by transfection (**Figure 4D**).

DISCUSSION

Sterile inflammation is a process in which the immune system recognizes danger rather than stranger (Matzinger, 1994). IL-1RI was found to be the major innate receptor mediating the sterile inflammation response to dying cells (Chen et al., 2007). Moreover, a study made by our group shows that IL-1α, and not IL-1β, is the major mediator of the inflammatory response to necrotizing cells. In addition, IL-1α is retained together with the chromatin of apoptotic cells, preventing its release and the subsequent induction of inflammation. However, necrotic cells release IL-1α and recruit myeloid cells in an IL-1α-dependent manner (Cohen et al., 2010). Recently, we demonstrated that supernatants of hypoxic cells contain IL-1α, thus inducing sterile inflammation by recruiting neutrophils to the site of injury (Rider et al., 2011). The initial infiltration of neutrophils was dependent on IL-1α originating from necrotic cells. The cells were necrotic due to prolonged hypoxia, in which IL-1α was up-regulated, and finally released. As in the inflammatory process the first few hours are critical (Serhan and Savill, 2005) and include an influx of neutrophils, it was of interest to study the initial transcriptional response of IL-1α in cells undergoing hypoxic stress. Here, we show that IL-1α transcription increases during hypoxia in human cell lines. This allows the cells to adjust their transcriptome in order to alarm the surroundings of an approaching danger. When normoxic conditions are not restored, the cell will eventually die by necrosis. Some cells, such as keratinocytes, express large amounts of IL-1α under homeostatic conditions; nonetheless, IL-1α increases during hypoxia (Rider et al., 2011). Other cells should also exhibit increased expression of IL-1α immediately upon stress conditions,

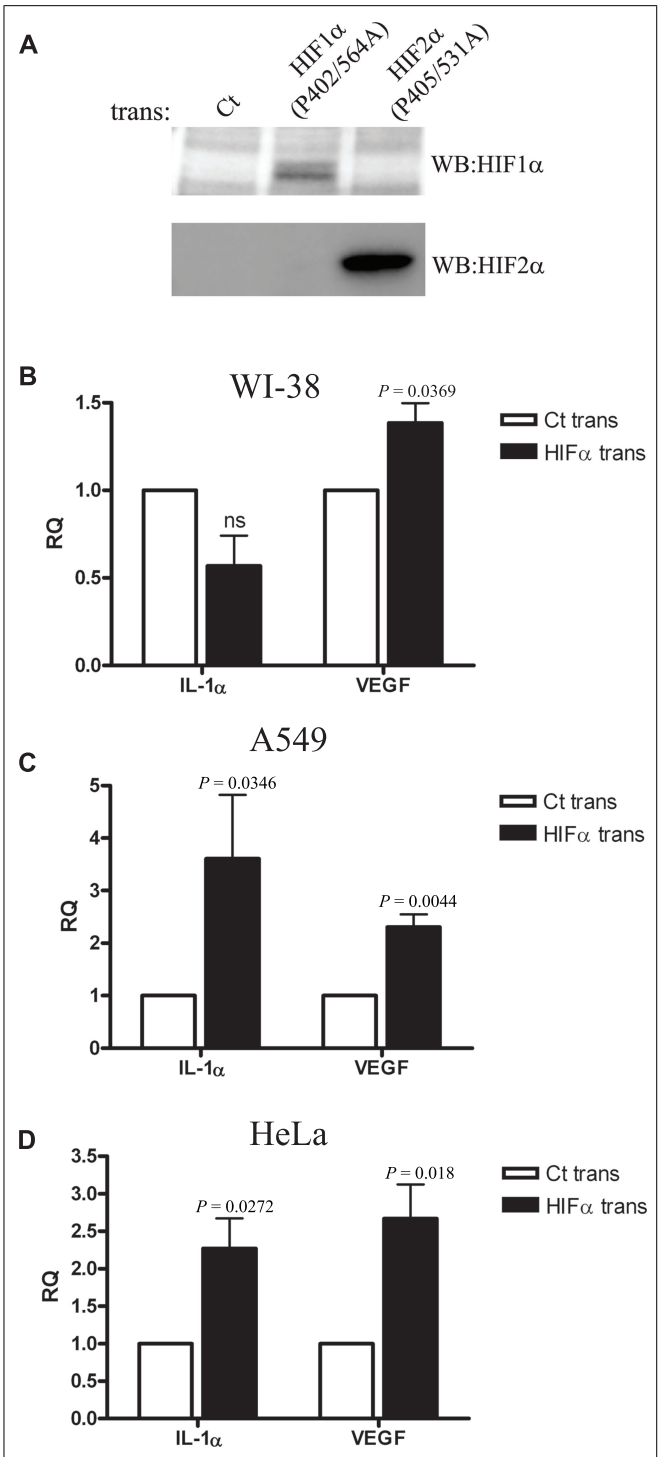

FIGURE 4 | Overexpression of HIFα proteins increases IL-1α transcription in A549 and HeLa cells but not in WI-38 fibroblasts. (A) Western blot using anti-HIF-1α or anti-HIF-2α antibodies of nuclear fractions obtained from HEK-T293 cells transfected with the annotated vectors. WI-38 **(B)**, A549 **(C)**, or HeLa **(D)** cells were transfected with either control vector or with HIF-1α (P402A/P564A) and HIF-2α (P405A/P531A), and 24 h later were analyzed for IL-1α and VEGF RQ of mRNA levels by real-time PCR. Graphs represent mean ± SEM of four independent experiments for each cell type. *P* values of HIFα-transfected cells vs. control (Ct) transfected are annotated in the graphs.

such as hypoxia. Indeed, both human epithelial cells and fibroblasts exhibit up-regulation of IL-1α during culture under hypoxic conditions. These data raised the question of whether HIFα factors were involved in IL-1α transcription. HIF-1α and HIF-2α were shown to up-regulate IL-1β in hypoxic macrophages (Fang et al., 2009). However, it was not evaluated whether these factors regulate also the expression of the alarmin cytokine IL-1α and whether it happens also in non-hematopoietic cells. Culturing the cells in a hypoxic chamber following siRNA silencing of HIFα factors, enabled us to determine that HIFα factors do regulate IL-1α transcription. In addition, we were able to distinguish between two different effects of HIFα regulation. In hypoxic A549 epithelial cells IL-1α is up-regulated by HIFα factors. However, in WI-38 fibroblasts, which originate in the lungs, as do A549 cells, HIFα factors regulate and restrain the transcription of IL-1α. This was verified using HIFα encoding vector transfections in normoxic cultures. The differential regulation of IL-1α transcription by HIFα proteins is still not clear. Further study is required in order to elucidate the opposing effect of these transcription factors on IL-1α in different types of cells. Indeed, in spite of numerous studies published concerning HIFα and inflammation, there have been no clear conclusions about the role of HIFα in inflammation. Injection of HIF-1α encoding vectors into mice showed a reduction in the IL-1α cytokine in splenocytes obtained one week after injection (Ben-Shoshan et al., 2009). Several other models of inflammation showed attenuating effects of HIFα (Kojima et al., 2007; Cummins et al., 2008; Kobayashi et al., 2012). However, HIFα factors in hypoxia were shown to cause an increase in cytokine levels, myeloid cell infiltration, and in the innate response (Nizet and Johnson, 2009). During inflammation, HIFα factors can play a significant role even without hypoxia, as HIFα proteins can be stabilized by NF-κB (Cummins et al., 2006; Rius et al., 2008). IL-1 signaling itself can increase the stability of HIFα and increase transcription of its target genes (Hellwig-Burgel et al., 1999). In addition, A549 epithelial cells, stimulated with IL-1β, were shown to increase the stability of the HIF-1α protein (Jung et al., 2003). Furthermore, viral infections of the lungs can stabilize HIFα proteins (Haeberle et al., 2008). However, when sterile inflammation occurs, hypoxia and not pathogens drives the inflammatory response (Nizet and Johnson, 2009). A growing number of studies show that IL-1α is a major mediator of sterile inflammation (Eigenbrod et al., 2008; Luheshi et al., 2009; Berda-Haddad et al., 2011; Lee et al., 2011; Rider et al., 2011; Gross et al., 2012; Norton et al., 2012). As such, its transcription during hypoxia has a special significance. Linking IL-1α up-regulation during hypoxia, a process which results in increased myeloid cell recruitment and HIFα transcription regulation raises the issue of the physiological relevance of these transcription factors in cases of sterile inflammation. Hypoxic factors are targeted in cancer therapy to use hypoxia-mediated cell death to kill cancerous cells; therefore, it is important to take into consideration that while pro-angiogenic factors, such as VEGF will be down-regulated, this treatment may either up- or down-regulate IL-1α, depending on the cell type. Elevated IL-1 levels can induce massive inflammation in the tissue; however, IL-1 itself can induce angiogenesis and compensate for the anti-angiogenic effects of HIFα inhibition, since IL-1 is an

important mediator in angiogenesis (Carmi et al., 2009). Overall, our data suggest that HIFα factors can control the transcription of IL-1α during hypoxia. While transcription of IL-1α increased with HIFα in the lung epithelial cell line, A549, and in the HeLa cell line, IL-1α mRNA levels were attenuated by HIFα factors in lung fibroblasts. This is novel data concerning the induction of IL-1α-mediated sterile inflammation at the transcriptional level, in cells which are sensitive to hypoxic stress and are prone to necrosis.

ACKNOWLEDGMENTS

We are grateful to Mrs. Rosalyn M. White for editing the manuscript. Ron N. Apte was supported by the Israel Ministry of Science (MOST) jointly with the Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany, the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities, the Israel Cancer Association and the Israel Ministry of Health Chief Scientist's, FP7: Cancer and Inflammation (INFLA-CARE), Deutsch-Israelische Projektkooperation (DIP) and the National Institute of Biotechnology (NIBN). Professor Ron N. Apte is an incumbent of the Irving Isaac Sklar Chair in Endocrinology and Cancer. Elena Voronov was supported by the Israel Cancer Association, the Israel Ministry of Health Chief Scientist's Office and the Concern Foundation, Deutsch-Israelische Projektkooperation (DIP), FP7: Cancer and Inflammation and the Israel Science Foundation.

SUPPLEMENTARY MATERIAL

Supplementary Material for this article can be found online at: http://www.frontiersin.org/Inflammation/10.3389/fimmu.2012.00290/abstract

Figure S1 | A549 and WI-38 cells express IL-1α. Cells were stained by immunofluorescence for IL-1α and were analyzed by confocal microscopy (×600 magnification).

Figure S2 | Cell viability following 6 h hypoxia. A549 and WI-38 cells were cultured with either normoxic or hypoxic conditions for 6 h. Cells were analyzed for annexin–PI by flow cytometer.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 July 2012; accepted: 29 August 2012; published online: 14 September 2012.

*Citation: Rider P, Kaplanov I, Romzova M, Bernardis L, Braiman A, Voronov E and Apte RN (2012) The transcription of the alarmin cytokine interleukin-1 alpha is controlled by hypoxia inducible factors 1 and 2 alpha in hypoxic cells. Front. Immun. **3**:290. doi: 10.3389/fimmu.2012.00290*

This article was submitted to Frontiers in Inflammation, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 09 October 2012
doi: 10.3389/fimmu.2012.00315

IL-1 family cytokines trigger sterile inflammatory disease

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Inflammation plays vital roles in protective responses against pathogens and tissue repair, however, improper resolution of inflammatory networks is centrally involved in the pathogenesis of many acute and chronic diseases. Extensive advances have been made in recent years to define the inflammatory processes that are required for pathogen clearance, however, in comparison, less is known about the regulation of inflammation in sterile settings. Over the past decade non-communicable chronic diseases that are potentiated by sterile inflammation have replaced infectious diseases as the major threat to global human health. Thus, improved understanding of the sterile inflammatory process has emerged as one of the most important areas of biomedical investigation during our time. In this review we highlight the central role that interleukin-1 family cytokines play in sterile inflammatory diseases.

Keywords: Sterile inflammation, IL-1, IL-18, IL-33, NLRP3, inflammasome, caspase-1, autoinflammatory disease

INTRODUCTION

Infection and cellular injury are the two principal stimuli that provoke inflammation. Detection of infectious agents and danger signals by pattern recognition receptors orchestrates a coordinated series of events that ensure removal of the insult and promote tissue repair. Following recognition of the foreign agents a cascade of inflammatory cytokines are induced that instruct the recruitment of neutrophils and macrophages to the site of infection or injury. Once mobilized to the tissue sites neutrophils and macrophages engulf and contain the insult, and also recruit additional immune cells to the site of inflammation through the production of cytokines and chemokines. Many of the pathogen-derived factors that trigger inflammation have been formally characterized, and include conserved molecules that are required for microbial survival. In comparison, sterile activators of inflammation are structurally diverse and can originate from both endogenous and exogenous sources. For instance; mechanical trauma, hypoxia, metabolic distress, chemical and environmental insults, and ischemia can all provoke sterile inflammation. Emerging data suggests that man-made and environmental irritants (silica, asbestos, alum, alloy particles, and car exhaust), metabolic factors (cholesterol, amyloids, saturated fatty acids, and glucose) and endogenous danger signals that are released as a result of aberrant cell death [ATP, reactive oxygen species (ROSs), uric acid, and interleukin-1α (IL-1α)] can all trigger sterile inflammation (Rock et al., 2010).

Although inflammatory responses play critical roles in the eradication of pathogens and sterile insults, excessive and unremitting inflammation causes damage to healthy tissue and centrally contributes to disease pathology. Dysregulated production of cytokines, ROSs, proteases, and growth factors by both innate and adaptive immune cells can lead to collateral damage and disrupt tissue homeostasis. Sterile inflammation has been implicated in a spectrum of acute and chronic disorders that include obesity, atherosclerosis, type 2 diabetes, gout, and multiple neurodegenerative diseases (**Figure 1**). In this review we focus on the pivotal role of IL-1 family cytokines in various sterile inflammatory diseases.

IL-1 FAMILY CYTOKINES

The IL-1 family of cytokines consists of 11 members that are centrally involved in regulating inflammatory responses to both infections and sterile insults. IL-1 family cytokines include IL-1α, IL-1β, IL-1Ra, IL-18, IL-33, IL-36Ra, IL-36α, IL-37, IL-36β, IL-36γ, and IL-38 (Dinarello, 2011). In this review we specifically focus on the roles of IL-1α, IL-1β, IL-18, and IL-33 in sterile inflammatory disease as these cytokines have been described to significantly influence disease pathogenesis. The emerging roles of the other IL-1 family cytokines in biology are beyond the scope of this review and are described in detail elsewhere (Dinarello, 2010).

Interleukin-1 has been shown to promote sterile inflammatory disease pathogenesis at multiple levels. For example, IL-1 can directly cause tissue destruction, altered fibroblast proliferation, and collagen deposition (Schmidt et al., 1984; Zucali et al., 1986; Ishida et al., 2006; Steer et al., 2006). Moreover, IL-1 receptor (IL-1R) signaling potently induces the production of secondary inflammatory cytokines and chemokines such as IL-6, TNFα, KC, and G-CSF (Di Paolo et al., 2009; Orjalo et al., 2009). IL-1 also contributes to the perpetuation of inflammatory disease by promoting the induction of pathogenic cytokines (IFN-γ, IL-17, and GM-CSF) by T cells and innate effector cells (Sutton et al., 2009; Lukens et al., 2012). Much like IL-1, IL-18 has also been shown to stimulate proinflammatory signaling and has historically been classified as a potent inducer of IFN-γ production. Likewise, IL-18 also promotes the activation and recruitment of inflammatory immune cells including macrophages, neutrophils, natural killer (NK) cells, and T cells (Nakanishi et al., 2001; Dinarello, 2007).

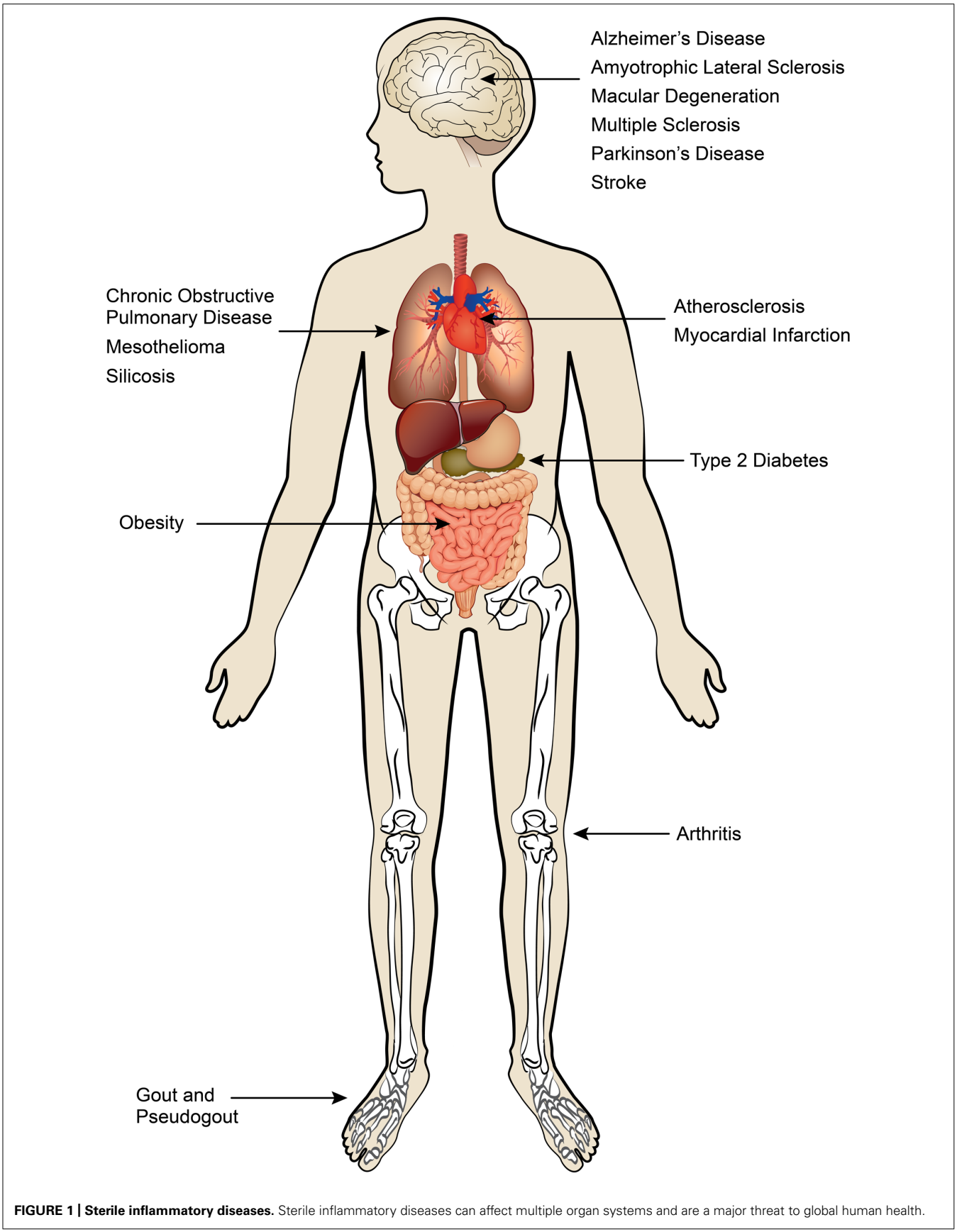

FIGURE 1 | Sterile inflammatory diseases. Sterile inflammatory diseases can affect multiple organ systems and are a major threat to global human health.

IL-33 is one of the newest members of the IL-1 cytokine superfamily and its roles in inflammatory disease are just now being uncovered (Liew et al., 2010). Engagement of the ST2 receptor by IL-33 provokes the induction of T helper 2 (Th2) cytokines including IL-5 and IL-13, and thus IL-33 uniquely contributes to the pathogenesis of Th2-mediated inflammatory diseases. Below we describe the molecular mechanisms that control the release of these potent cytokines and highlight emerging data that suggest central roles for IL-1 family cytokines in sterile inflammatory diseases.

INFLAMMASOME-DERIVED IL-1β AND IL-18

Over the past decade extensive research from various groups has shed light on the ability of inflammasome complexes to regulate the processing and activation of IL-1β and IL-18. Inflammasomes are comprised of a Nod-like receptor (NLR) or a pyrin- and HIN-200 domain-containing protein (PYHIN), an adaptor protein, and caspase-1 (**Figure 2**). NLRs are sensor molecules that detect both pathogen- and danger-associated molecular patterns (PAMPs and DAMPs, respectively). Activation of NLR proteins promotes the recruitment of the inflammasome-adaptor protein, ASC (also known as PYCARD), and pro-caspase-1 into a molecular platform known as the inflammasome. These multi-protein complexes mediate the proximity-induced autoactivation of caspase-1. Active caspase-1 subsequently cleaves pro-IL-1β and pro-IL-18, which is required for their secretion and to elicit their inflammatory properties. The activation of inflammasomes can occur in response to an array of different stimuli. For instance, inflammasome-mediated recognition of pathogen-derived molecules is a critical first line of defense during infection (Kanneganti et al., 2006; Kanneganti, 2010; Lupfer and Kanneganti, 2012). On the other hand, inflammasomes are also involved in the detection of danger- and stress-associated signals that are generated during sterile inflammation. Sterile stimuli that trigger inflammasome activation include endogenous danger signals that are released during aberrant cell death (ATP and uric acid), metabolic factors (saturated fatty acids and cholesterol crystals), and exogenous irritants (asbestos and silica; **Figure 2**). Mutations in NLR-inflammasome proteins are associated with both monogenic and polygenic human inflammatory disorders. Most of these rare genetic disorders are associated with mutations that result in exacerbated secretion of inflammasome-derived cytokines (Aksentijevich et al., 2007). Importantly, therapeutics that block IL-1 signaling have proven successful in the treatment of these disorders (Hoffman et al., 2004; Church and McDermott, 2009).

INFLAMMASOME-INDEPENDENT SOURCES OF IL-1β

Bioactive IL-1β can also be generated by inflammasome-independent mechanisms, however, the contributions of these non-canonical sources of IL-1β to the inflammatory environment and disease pathology are poorly understood. Proteases that are expressed by neutrophils are primarily responsible for the majority of caspase-1 independent IL-1β. Examples of neutrophilic proteases that have been shown to cleave pro-IL-1β independently of caspase-1 activity include elastase, proteinase-3, cathepsin G, granzyme A, and chymase (Dinarello, 2011). Roles for inflammasome-independent IL-1β in disease pathogenesis have only been described in a few settings to date. For instance,

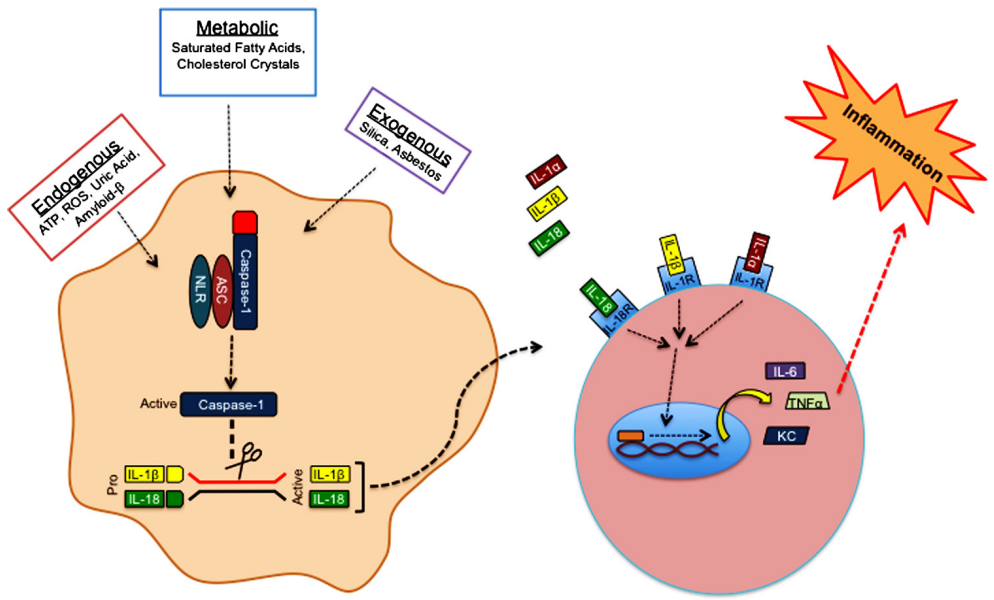

FIGURE 2 | Sterile inflammasome activation. Endogenous, metabolic, and exogenous danger-associated signals activate NLRP3 in the cytosol. Detection of danger signals by NLRP3 promotes the recruitment of ASC and caspase-1 into a multi-protein complex known as the inflammasome. Inflammasome formation provides the necessary molecular platform to promote self-cleavage and activation of caspase-1. Activated caspase-1 cleaves the pro-forms of IL-1β and IL-18, which is required for their secretion and biological activity. Inflammasome-derived IL-1β, and IL-1α that is released from dying cells bind IL-1R, whereas IL-18 has its own receptor. Engagement of IL-1R or IL-18R promotes the robust production of secondary proinflammatory molecules (TNFα, IL-6, KC, etc.) that can then trigger additional immune cell recruitment and activation.

capase-1 autonomous IL-1β was reported to provoke sterile inflammation in models of urate crystal-induced peritonitis and joint damage (Guma et al., 2009; Joosten et al., 2009). Cell death that ensues in sterile inflammatory environments results in the passive release of inactive pro-IL-1β. Extracellular pro-IL-1β can then be cleaved and activated by activated neutrophils that express surface-bound proteases (Netea et al., 2010). As most sterile inflammatory diseases are associated with enhanced neutrophil recruitment and tissue damage, it is likely that protease-dependent activation of IL-1β is involved in pathogenesis at some level. One possible scenario is that inflammasomes are involved in the initial sensing of the sterile threat and that caspase-1 independent sources of IL-1β are important in perpetuating the inflammatory environment later in the response. Regardless, it is clear that additional studies are needed to elucidate the unique contributions of inflammasome-independent sources of IL-1β in sterile disease.

IL-1α RELEASE

Historically, IL-1α and IL-1β have been believed to possess overlapping biological functions. Indeed, it is true that recombinant IL-1α and IL-1β both bind to IL-1R to induce a proinflammatory signaling cascade. However, several lines of evidence point to distinct biological roles for IL-1α. For one, IL-1α belongs to a family of "dual function cytokines", which can exert distinct biological functions in the nucleus and also when released into the extracellular compartment. The nuclear location sequence in the N-terminus of IL-1α allows it to translocate to the nucleus where it affects transcription (Buryskova et al., 2004; Werman et al., 2004). Both IL-1α and IL-1β are first synthesized as precursor proteins that can be enzymatically cleaved. The precursor form of IL-1β is not biologically active and requires cleavage to elicit its inflammatory activity. In comparison, the precursor form of IL-1α is biologically active. However, under certain poorly defined situations, precursor IL-1α can also be cleaved by the calcium-activated protease calpain to release the N-terminal propiece and produce the mature form of IL-1α. The secreted mature form of IL-1α can then bind IL-1R to induce inflammation. Why IL-1 exists in three different biologically active forms is an important question in the field. Furthermore, the discrete roles of each form of IL-1α (precursor, propiece, and mature) in disease progression remains to be formally addressed.

Under homeostatic conditions cells typically undergo apoptotic cell death, which does not provoke inflammation. However, cellular stress that occurs in response to trauma, hypoxia, chemical and environmental insults, and complement-mediated lysis can promote necrotic cell death. During programmed apoptosis IL-1α is trafficked to the nucleus to prevent its release into the extracellular compartment (Cohen et al., 2010). In contrast, necrosis is associated with the release of IL-1α, which then acts on macrophages, neutrophils, and parenchymal cells to trigger production of IL-6, TNFα, KC, G-CSF, and other inflammatory mediators (**Figure 3**). The passive secretion of IL-1α by necrotic cells following trauma or sterile insults orchestrates the recruitment of neutrophils and macrophages to the site of injury where they are needed to sequester the injurious agent, remove the dead cells, and initiate the healing process (Chen et al., 2007; Eigenbrod et al., 2008). However, exacerbated IL-1α release leads to pathogenic neutrophilic responses and collateral tissue damage, and thus is centrally involved in disease progression.

A recent report also suggested that IL-1α is secreted in a caspase-1 dependent fashion (Gross et al., 2012). They showed that processed IL-1α is secreted in high amounts following stimulation with various inflammasome activators. Intriguingly, caspase-1 catalytic activity was not required for IL-1α secretion in these scenarios. In the future it will be important to define the physiological relevance of catalytic independent caspase-1-mediated IL-1α release *in vivo*. Regardless, it is clear that a more complete understanding of the cellular and molecular pathways that regulate IL-1α processing and secretion is critically needed. These findings will aid in the design of novel autoinflammatory therapeutics and also provide insight into the etiology of sterile inflammatory diseases.

IL-33

Interleukin-33 is a newly described member of the IL-1 family that is predominantly expressed by non-hematopoietic cells including endothelial and epithelial cells (Moussion et al., 2008). Similar to IL-1α, IL-33 has also been characterized as a dual function cytokine that can exert transcriptional activity in the nucleus (Carriere et al., 2007). Originally it was thought that caspase-1 is required for the processing and secretion of IL-33, however, it was later found that active IL-33 release occurs independently of capsase-1 (Talabot-Ayer et al., 2009). Paradoxically, it has been formally shown that full-length IL-33 is fully bioactive and that caspase-mediated cleavage of IL-33 actually results in its inactivation (Cayrol and Girard, 2009; Luthi et al., 2009). Release of IL-33 is believed to occur passively during cell death (Luthi et al., 2009; Talabot-Ayer et al., 2009).

Interleukin-33 signals through the ST2 receptor, which is abundantly expressed on Th2 cells, mast cells, and most other immune cell populations (Ali et al., 2007; Chackerian et al., 2007). Stimulation of Th2 cells with IL-33 induces secretion of the Th2 cytokines IL-4, IL-5, and IL-13 (Schmitz et al., 2005). In a similar fashion, IL-33 has also been shown to drive potent production of Th2 cytokines by type 2 innate lymphoid cells (ILCs; Spits and Cupedo, 2012). Furthermore, engagement of ST2 by IL-33 on mast cells has been suggested to centrally contribute to anaphylactic shock by triggering the production of proinflammatory cytokines and degranulation (Pushparaj et al., 2009).

ROLE FOR IL-1 FAMILY CYTOKINES IN STERILE INFLAMMATORY DISEASES

NEURODEGENERATIVE DISEASE

Interleukin-1 is a potent neurotropic cytokine that has been implicated in numerous neurodegenerative diseases including Alzheimer's disease (AD), stroke, Parkinson's disease, and amyotrophic lateral sclerosis (ALS; Allan et al., 2005). These diseases are typically associated with elevated local and systemic levels of IL-1. Furthermore, IL-1 can cause neuronal cell injury, breakdown of the blood–brain barrier, and astrogliosis (Shaftel et al., 2008). In the case of AD, fibrillar peptide amyloid-β (Aβ) accumulation that accompanies dementia and neuronal cell death was found to incite NLRP3 inflammasome-dependent IL-1β production (Halle

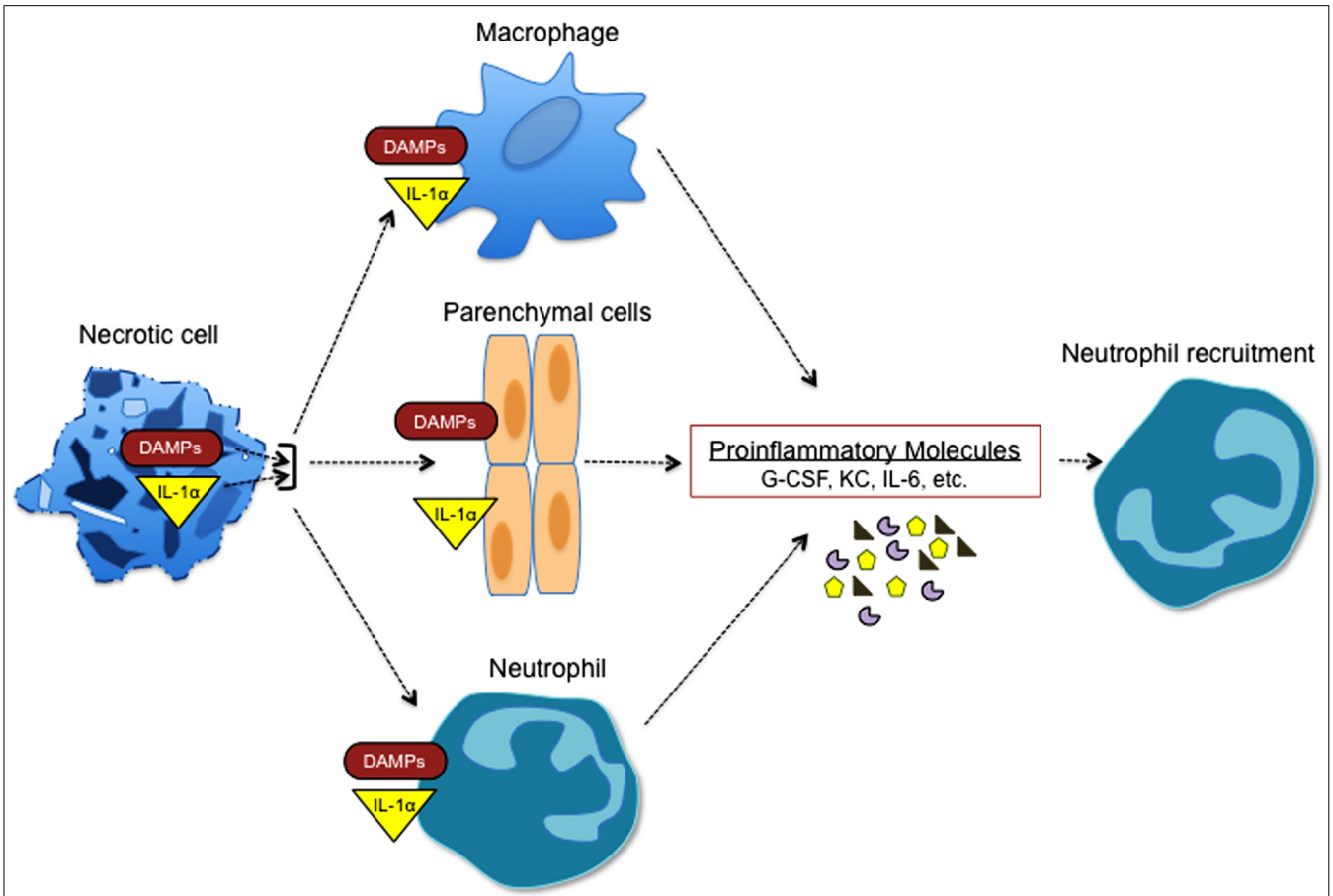

FIGURE 3 | Necrotic cells trigger sterile inflammation. Necrotic cells potently induce sterile inflammation through multiple mechanisms. IL-1α that is released from necrotic cells induces the generation of cytokines and chemokines that are associated with granulopoiesis and neutrophil recruitment (G-CSF, KC, IL-6, etc.). In addition, multiple danger-associated molecular pattern molecules (DAMPs) including uric acid, reactive oxygen species (ROS), and ATP are released during necrosis and can promote the expression of factors that are involved in inflammation and neutrophil recruitment.

et al., 2008). Genetic deletion of NLRP3/caspase-1/IL-1β axis molecules markedly attenuated the production of proinflammatory cytokines and neurotoxic factors in responses to Aβ, and also abrogated microglia activation in the brain. Genetic studies have also recently identified the *Il33* gene as a potential genetic determinant of AD (Chapuis et al., 2009). In this study they show that *Il33* expression is markedly reduced in AD patients. Intriguingly, they demonstrate that IL-33 overexpression hinders the production of Aβ. Additional studies are needed to follow-up on this exciting link and also to define how IL-33 mechanistically limits Aβ secretion.

Recent advancements in IL-1 cytokine family biology have also uncovered important roles for these proinflammatory mediators in both ALS and Parkinson's disease. The most common genetic cause of ALS is dominant gain of function mutations in superoxide dismutase 1 (SOD1). Recently it was described that mutant SOD1 accelerates ALS pathogenesis through the induction of inflammasome-derived IL-1β (Meissner et al., 2010). In the case of Parkinson's disease, polymorphisms in *Il1β* are associated with exacerbated neuropathology, and IL-1β was found to increase the rates of dopamine neuron degeneration in a Parkinson's disease mouse model (McGeer et al., 2002; Koprich et al., 2008).

Ischemic or hemorrhagic conditions that are responsible for strokes result in the rapid induction of proinflammatory cytokines and inflammatory cells. The initiation of this inflammatory cascade ultimately causes neuronal cell death and functional impairment. IL-1 expression is one of the most highly upregulated factors in stroke patients, thus randomized clinical trials using IL-1R antagonist (anakinra) to treat acute stroke were conducted (Rothwell et al., 1997; Wang et al., 1997; Emsley et al., 2005). Strikingly, stroke patients treated with anakinra exhibited reduced proinflammatory cytokine levels and improved cognitive functions.

Collectively, these findings highlight a critical role for inflammasome-induced inflammation and tissue damage in various neurodegenerative diseases. Moreover, they have helped to improve our understanding of the etiology of neurodegenerative disorders and have uncovered novel pathways to target in the treatment of these debilitating diseases.

PULMONARY DISEASE

The lung is constantly exposed to a multitude of airborne pollutants. Deposition of environmental or man-made irritants in the

lung is known to cause fibrosis and extensive cellular infiltration. Prolonged damage can ultimately result in devastating pulmonary disease. IL-1R signaling has emerged as a crucial regulator of many irritant-induced lung diseases. For instance, IL-1R deficient mice are highly resistant to lung damage in a myriad of chemically induced pulmonary disease models (Gasse et al., 2007). In these studies, disruption of IL-1 signaling was shown to attenuate proinflammatory cytokine production, immune cell recruitment, and fibrosis in response to a variety of irritants including bleomycin, cigarette smoke, diesel fuel, silica, and asbestos (Cassel et al., 2008; Hornung et al., 2008; Wilson et al., 2010; Yazdi et al., 2010). Moreover, it was clearly demonstrated that NLRP3 is a primary sensor of airborne pollutants in the lung. Indeed, asbestos and silica potently induce inflammasome activation and IL-1β secretion following NLRP3 recognition. Aerosolized asbestos-exposure in NLRP3 deficient mice resulted in diminished recruitment of inflammatory cells to the lung and concomitant reductions in proinflammatory cytokines (Dostert et al., 2008). Collectively, work in this field suggest that IL-1 blocking therapeutics may prove beneficial in the treatment of asbestos-induced mesothelioma, silicosis, and potentially other chronic obstructive pulmonary diseases (COPD).

Important roles for IL-33 in airway inflammation have also been recently described. In particular, IL-33 has been found to pivotally contribute to asthma-induced pathology. Expression of IL-33 is highly up-regulated in the lungs of asthmatic patients and in mouse models of asthma (Kurowska-Stolarska et al., 2008; Prefontaine et al., 2009). Damage to epithelial cells lining the airways is believed to be the major source of IL-33 that triggers asthmatic flares (Schmitz et al., 2005). IL-33 release promotes the recruitment of dendritic cells, Th2 cells, eosinophils, and mast cells into the airways and ultimately results in Th2-mediated inflammation (Liew et al., 2010; Borish and Steinke, 2011). Direct injection of IL-33 into the lungs of mice has been shown to rapidly induce eosinophilic inflammation and airway-hyperresponsiveness (AHR; Kondo et al., 2008; Kurowska-Stolarska et al., 2008). Furthermore, in the ovalbumin (OVA)-induced mouse model of airway inflammation, IL-33 causes exacerbated lung damage and inflammatory cell infiltration (Kurowska-Stolarska et al., 2008). Treatment of OVA-induced AHR mice with ST2 and IL-33 blocking antibodies was also found to ameliorate airway inflammation, which highlights the great promise that IL-33 neutralizing therapies hold in the treatment of asthma (Lohning et al., 1998; Coyle et al., 1999; Liu et al., 2009).

ATHEROSCLEROSIS

Atherosclerosis is an inflammatory disorder that occurs when fats and cholesterol accumulate around the arterial walls and cause disruptions in blood flow and heart failure. Numerous lines of clinical data point to instrumental roles for IL-1 family molecules in the induction and progression of atherosclerosis. For instance, the expression of IL-1β and IL-1R are markedly up-regulated in arterial plaques, and expression levels are linked to disease severity (Moyer et al., 1991; Galea et al., 1996). Moreover, circulating IL-18 levels can be utilized to predict the risk of atherosclerosis-related death in patients (Mallat et al., 2001; Blankenberg et al., 2002). Recently, the NLRP3 inflammasome has been identified as a central regulator of atherosclerosis pathogenesis. It was shown that cholesterol crystals incite NLRP3 inflammasome activation and subsequent IL-1β and IL-18 secretion (Duewell et al., 2010). Important roles for inflammasomes have also been established in other mouse models of atherosclerosis. For example, LDL-receptor-deficient mice (genetically prone to atherosclerosis) that are reconstituted with bone marrow cells from mice that lack NLRP3, ASC, or IL-1β are remarkably resistant to plaque formation (Duewell et al., 2010). The apolipoprotein E (APOE) mouse model is also routinely utilized to study the events that are responsible for atherosclerosis pathogenesis. APOE deficient mice on a high fat diet (HFD) develop severe hypercholesterolemia and arterial plaques around the heart. Interestingly, blockade of IL-1 signaling by treatment with IL-1R antagonists or genetic ablation of IL-1R results in marked resistance to the development of atherosclerosis in the APOE model (Chi et al., 2004). These findings suggest that IL-1 contributes to HFD-induced arterial plaque formation. However, a recent study also found that genetically crossing APOE mice to mice that lack NLRP3 does not diminish the severity or incidence of atherosclerosis (Menu et al., 2011). It remains unclear why these two different mouse models of atherosclerosis have produced opposite findings in regards to the role of the NLRP3-inflammasome in cardiovascular inflammation. Therefore, additional studies are needed to formally elucidate the role of IL-1 in atherosclerosis models.

In contrast to the pathogenic role of IL-1 and IL-18 in atherosclerosis pathogenesis, IL-33 has been described to attenuate cardiovascular inflammation and plaque formation (Miller et al., 2008). Administration of IL-33 to APOE deficient mice promotes the induction of Th2-associated cytokines (IL-4, IL-5, and IL-13) that play a protective role in atherosclerotic plaque formation. Furthermore, IL-33 treatment also limits plaque development and cardiovascular disease by promoting the production of atheroprotective anti-oxidized low-density lipoprotein (oxLDL) antibodies and limiting macrophage foam cell maturation (Miller et al., 2008; McLaren et al., 2010).

ISCHEMIA-INDUCED INFLAMMATION

Disruption in blood flow to organs causes aberrant cell death and is responsible for triggering ischemia-associated disease. Inflammation arises due to aberrant cell death and hypoxia in the organ. Myocardial infarction, hypoglycemia, hypotension, and surgery can trigger ischemia-induced inflammation and tissue damage. Altered IL-1 signaling has been identified to be a major culprit in the pathology of many ischemia-related diseases (Boutin et al., 2001; Thornton et al., 2010). Indeed, massive production of both IL-1α and IL-1β occurs during ischemia, and blockade of IL-1 signaling can significantly attenuate tissue damage and cytokine production (Shito et al., 1997; Pomerantz et al., 2001; Abbate et al., 2008; Luheshi et al., 2011). The release of the major alarmin molecule, IL-1α, following necrosis and hypoxia-induced cell death is a crucial initiating step of the ischemia-induced inflammatory cascade (Chen et al., 2007). In contrast, inflammasome-mediated IL-1β production by macrophages is believed to play important roles in sustaining local inflammation later in the response (Rider et al., 2011).

Recently emerging data suggests that IL-33 plays a protective role during ischemia-induced inflammation. IL-33 is highly expressed in both mice and humans following myocardial infarction, and studies utilizing mice that are deficient in ST2 suggest that IL-33 limits tissue destruction that results from myocardial infarction (Weinberg et al., 2002; Sanada et al., 2007; Seki et al., 2009). Soluble ST2 levels also directly correlate with impaired left ventricular function post myocardial infarction and thus circulating ST2 levels has been proposed as a biomarker for heart failure (Weinberg et al., 2003).

JOINT AND BONE DISEASE

Chronic inflammatory bone and joint diseases can cause debilitating pain, physical impairments, and significant morbidity. Importantly, the rates of these diseases are projected to rise substantially in coming years due to increased life expectancies, sedentary lifestyles, and the ongoing obesity epidemic. IL-1 can affect various aspects of bone and joint integrity, and as a result IL-1 is critically involved in the pathogenesis of rheumatoid arthritis (RA), periodontal disease, osteoarthritis, and gout (Walsh et al., 2006; Takayanagi, 2007; Jones et al., 2011). For instance, IL-1 stimulates bone resorption bone by directly impinging on osteoclast and osteoblast functions (Nguyen et al., 1991; Kwan Tat et al., 2004). On the other hand, IL-1 signaling contributes to cartilage deterioration by impairing chondrocyte proteoglycan synthesis and stimulating the production of joint damaging mediators such as matrix metalloproteinases and nitric oxide (Joosten et al., 2004; Zwerina et al., 2007). In the case of RA, insufficient levels of the naturally occurring IL-1R antagonist in the synovium are associated with joint pathology (Firestein et al., 1994). Treatment of RA with IL-1 pathway inhibitors has achieved moderate clinical success and is currently prescribed to limit the progression of joint damage in individuals with moderate to severe RA (Arend et al., 1998; Bresnihan et al., 1998). Similar to RA, dysregulated IL-1-mediated events also impinge on the development of osteoarthritis; however, therapeutic targeting of IL-1 has only provided modest improvements in clinical trials (Chevalier et al., 2009).

Gout and pseudogout are sterile inflammatory disorders that occur when monosodium urate (MSU) or calcium pyrophosphate dihydrate (CPPD) crystals, respectively, deposit in the joints and periarticular tissue. These crystalline agents cause aggravated inflammation and neutrophil recruitment that can result in bone and joint damage. This sterile inflammatory response is dependent on NLRP3 inflammasome activation both *in vitro* and *in vivo* (Martinon et al., 2006). Moreover, colchicine, which is commonly used to treat gout and pseudogout, was found to dampen inflammasome-triggered IL-1β production. Currently, inhibition of crystal uptake is targeted to treat gout, however, this recent data suggests that addition of IL-1R blockade could substantially improve current therapeutics.

Excessive Th1- and Th17-mediated inflammatory responses have traditionally been associated with joint destruction, especially in the case of arthritis. IL-33 has been extensively shown to potently induce Th2-driven inflammation, and thus it came as a great surprise that the absence of IL-33-mediated signaling results in attenuated joint disease (Xu et al., 2008). Appreciable levels of IL-33 and ST2 can be detected in the synovium of RA patients (Carriere et al., 2007; Palmer et al., 2009). Furthermore, the disruption of ST2 activation, either by genetic abrogation or antibody-induced blockade, resulted in decreased joint disease in the mouse model of collagen-induced arthritis (CIA; Xu et al., 2008; Palmer et al., 2009). The lack of functional ST2 contributed to protection in this model through the dampening of inflammatory cytokine and anti-collagen antibody production. In contrast, treatment of CIA-induced mice with exogenous IL-33 at the onset of disease resulted in exacerbated joint destruction. Expression of ST2 on mast cells was shown to be critical for disease pathology in the CIA model, thus suggesting a critical role for IL-33-mediated activation of mast cells in joint disease (Xu et al., 2008).

MACULAR DEGENERATION

Age-related macular degeneration (AMD) is the leading cause of vision loss and blindness in older adults. It is characterized by the accumulation of protein aggregates – known as drusen deposits – between the retina and choroid of the eye. Recently, the importance of inflammasome-derived IL-18 in this debilitating eye disease was described (Doyle et al., 2012; Tarallo et al., 2012). In one study it was shown that the accumulation of *Alu* RNA transcripts that are associated with disease progression in AMD patients can activate the NLRP3 inflammasome (Tarallo et al., 2012). Using both genetic and pharmacological inhibition of inflammasome components they found that the NLRP3/ASC/caspase-1 axis and IL-18, in particular, was required for ocular damage in response to *Alu* RNA. Intriguingly, they showed that regulation of inflammasome activation in ocular-specific epithelial cells and not immune cells was involved in disease pathology. A separate study also reported critical roles for inflammasome-derived IL-18 in AMD. Their findings revealed that drusen droplets isolated from AMD patients incite inflammasome activation and the subsequent release of IL-1β and IL-18 (Doyle et al., 2012). However, in contrast to the other study, NLRP3 inflammasome-induced IL-18 was found to have a protective role in macular degeneration. It is currently unclear why these two recent studies have yielded disparate findings, however, differences in mouse models may account for the different outcomes. Regardless, it is clear from these studies that inflammasome-induced IL-18 critically regulates ocular damage.

MULTIPLE SCLEROSIS

Interleukin-1 signaling has also been found to play instrumental roles in the pathogenesis of multiple sclerosis (MS) in animal models and patients. Mice that are deficient in IL-1R are protected from the development of neuroinflammation and demyelinating disease in the experimental autoimmune encephalomyelitis (EAE) mouse model of MS. Disruption in IL-1R was shown to confer protection by abrogating the induction of pathogenic $CD4^+$ T cells and γδ T cells (Matsuki et al., 2006; Sutton et al., 2006; Chung et al., 2009). Moreover, $IL\text{-}1R^{-/-}$ mice exhibit marked reductions in IL-17 and GM-CSF production (Sutton et al., 2009; Lukens et al., 2012). Genetic ablation of caspase-1 provided significant protection during the early phase of the response, however, inflammasome deficiency did not lead to the same levels of protection as seen in mice that lack IL-1R (Gris et al., 2010; Shaw

et al., 2010; Inoue et al., 2012a). This suggests that inflammasome-independent sources of IL-1 are also important in the pathogenesis of EAE. Future studies are needed to further characterize these sources of IL-1 in neuroinflammatory disease.

IFN-β is routinely prescribed to treat MS and the mode of action of this therapy was recently described to involve inhibition of inflammasome-mediated IL-1β production (Guarda et al., 2011a). Interestingly, one-third of MS patients do not respond to this regimen (Inoue et al., 2012b). It is possible that inflammasome-independent sources of IL-1β drive neuroinflammation in MS patients that are non-responsive to IFN-β. Strategies that inhibit inflammasome-independent IL-1 generation may hold great promise for the treatment of MS in this group of patients.

Both protective and pathogenic roles have been recently assigned to IL-33 in EAE. In one study, it was found that genetic abrogation of ST2 promotes exacerbated neuroinflammation (Jiang et al., 2012). They suggest that IL-33 provides neuroprotection by dampening IL-17 and IFN-γ production, and also by promoting the induction of alternatively activated macrophages that possess suppressive functions. Another study, on the other hand, showed that blockade of IL-33 during the induction phase can limit the development of demyelinating disease by curtailing the expression of pathogenic cytokines (Li et al., 2012). Both studies agree that IL-33 and its receptor, ST2, are highly expressed in the central nervous system (CNS) of EAE mice. Additional studies on the role of IL-33 in EAE are thus needed to clarify these disparate results.

OBESITY AND METABOLIC SYNDROME

The incidence of obesity worldwide has increased at a staggering rate in recent decades and as a result the obesity epidemic has become a major threat to global human health. Obesity is associated with increased rates of type 2 diabetes, atherosclerosis, and joint disease. Current strategies to combat obesity that include lifestyle and dietary changes have been unsuccessful in curtailing the obesity epidemic. Thus, therapies that target the specific molecular pathways that promote obesity-related diseases are desperately needed and are at forefront of biomedical investigation. Immune cell-mediated inflammation is now recognized to contribute to the development and progression of obesity (Gregor and Hotamisligil, 2011; Osborn and Olefsky, 2012).

Extensive research has shown that IL-1 family cytokines drive the development of obesity and related diseases. Of note, obesity progression in both diet-induced and genetically prone obese mice coincided with enhanced caspase-1 and IL-1β activity (Stienstra et al., 2010; Vandanmagsar et al., 2011). Importantly, genetic and pharmacological abrogation of the inflammasome was demonstrated to lower obesity-associated inflammation and improve insulin-sensitivity in HFD fed mice (Masters et al., 2010; Stienstra et al., 2010, 2011; Vandanmagsar et al., 2011; Wen et al., 2011). During obesity the adipose tissue undergoes considerable expansion and remodeling to store excess energy in the form of fat (Shoelson et al., 2006). Differentiation of adipocytes during adipose tissue expansion is associated with the activation of caspase-1, and IL-1β has been reported to convert adipocytes into a more insulin-resistant phenotype (Stienstra et al., 2010). Furthermore, hypertrophic and hyperplastic changes that occur during weight gain are typically associated with increased cell death and the release of cell death-related stimuli (ATP, uric acid, ROS, and damage mitochondria) in metabolic tissue can trigger inflammasome activation and enhanced secretion of IL-1β and IL-18 (Strissel et al., 2007; Khan et al., 2009).

Saturated fatty acids and other obesity-related metabolites that are markedly elevated in obese individuals have long been suspected to contribute to inflammation and disease pathology. However, the mechanistic link connecting metabolic factors derived from HFDs to chronic inflammation and insulin resistance remained elusive for many years. A collection of recent studies have identified the NLRP3 inflammasome as a central sensor that detects obesity-related stress signals and that triggers IL-1β production and subsequent disease pathogenesis (Masters et al., 2010; Stienstra et al., 2010, 2011; Vandanmagsar et al., 2011; Wen et al., 2011; Henao-Mejia et al., 2012). Specifically, it was discovered that saturated fatty acids (i.e., palmitate and ceramide) elicit NLRP3 inflammasome activation and subsequent release of IL-1β and IL-18 (Vandanmagsar et al., 2011; Wen et al., 2011). It is believed that saturated fatty acids disrupt AMPK signaling, which results in defective autophagy and the accumulation of dysfunctional mitochondria and ROSs. ROS production is a well-established activator of the NLRP3 inflammasome and thus the accumulation of ROS in response to fatty acid-induced impairment of autophagy is believed to be responsible for inflammasome activation in this setting (Zhou et al., 2009; Wen et al., 2011). Collectively these findings have provided us with important insight into the immunological underpinnings of obesity and define pivotal roles for the NLRP3 inflammasome in obesity-induced sterile inflammation.

TYPE 2 DIABETES

Type 2 diabetes mellitus (T2DM) is an obesity-related inflammatory disorder characterized by insulin resistance and uncontrolled glucose levels. It has become increasing clear that inflammasome-derived cytokines centrally regulate many of the inflammatory processes that are responsible for the impairment of metabolic function and the insulin resistance that underlies T2DM. For example, IL-1β can cause apoptosis of insulin-producing β-cells in the pancreas and also promotes insulin resistance in adipocytes (Bendtzen et al., 1986; Lagathu et al., 2006). Moreover, IL-18 is up-regulated in T2DM patients and has been linked to increased secondary renal failure and atherosclerosis (Blankenberg et al., 2002; Thorand et al., 2005). Perhaps the best example of the contribution of IL-1 family cytokines in the pathogenesis of T2DM comes from the recent clinical success of IL-1R antagonists in the treatment of this chronic disease (Larsen et al., 2007). In these clinical trials, IL-1 signaling inhibition was found to stabilize blood glucose levels and improve β-cell function.

Intriguingly, islet amyloid polypeptide (IAPP), was recently discovered to stimulate inflammasome activation and exacerbate T2DM (Masters et al., 2010). IAPP is a metabolite secreted at the same time as insulin by β-cells in the pancreas. IAPP forms amyloid deposits in the pancreas during T2DM progression (Wei et al., 2011). This formation of amyloid plaques is a salient hallmark of T2DM and has been speculated to influence disease severity. Recent findings now identify that IAPP amyloid plaques impinge

on T2DM pathogenesis by activating the NLRP3 inflammasome and triggering the secretion of IL-1β.

FUTURE PERSPECTIVES AND CONCLUSIONS

Recent discoveries have identified IL-1 family cytokines as pivotal regulators of a spectrum of sterile inflammatory diseases. The effectiveness of IL-1R blockade in the treatment of T2DM and other diseases suggest that additional therapeutics that target IL-1 family cytokines may provide effective strategies to treat other devastating inflammatory disorders. The identification of the central role of IL-1-associated cytokines in inflammatory disease progression and the discovery of the specific danger signals that trigger inflammation has provided an important foundation in our understanding of the etiology of many human diseases. Despite these recent advancements, numerous important questions remain to be addressed in order to gain a more complete understanding of the role of IL-1 family cytokines in non-communicable chronic diseases.

For example the ability of inflammasomes to influence autoinflammatory pathogenesis in cell types other than macrophages and dendritic cells has not been studied in detail. Multiple immune and organ-specific cell types express NLRs and inflammasome-associated proteins (Guarda et al., 2011b; Kufer and Sansonetti, 2011). Investigation of their contribution to disease should provide novel insight. Furthermore, up to now our understanding of the role of inflammasomes in sterile inflammatory disease has been limited to findings generated from studying the NLRP3 inflammasome. Much of this can probably be attributed to the unique role that the NLRP3 inflammasome plays in sensing multiple different stress and danger signals. However, multiple new NLR–inflammasome complexes have been discovered in recent years. Evaluation of the involvement of the AIM2, caspase-11, and caspase-8 inflammasomes in sterile inflammatory disorders is a very exciting area of future investigation (Maelfait et al., 2008; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Kayagaki et al., 2011; Gringhuis et al., 2012; Vince et al., 2012). We also still lack a complete understanding of the regulatory pathways and molecules that help to dampen excessive IL-1-mediated events. Identification of key molecules and regulatory networks that facilitate in suppressing IL-1-induced inflammation is paramount to the identification of improved therapeutics to treat sterile inflammatory diseases.

Investigation of the role of commensal bacteria in sterile inflammatory disease is another burgeoning frontier in this field. Although by definition sterile inflammation diseases are non-communicable in the infectious sense, it has become apparent recently that gut microflora can shape sterile inflammatory responses. Gut commensal bacteria are involved in calibrating the activation threshold of both adaptive and innate immune cells (Abt et al., 2012; Hooper et al., 2012). Moreover, lipopolysaccharide that translocates from the gut has been reported to directly influence both obesity and cardiovascular disease (Michelsen et al., 2004; Henao-Mejia et al., 2012).

Recent advancements in the field of IL-1 biology have greatly enhanced our understanding of the etiology of numerous autoinflammatory disorders. These studies have positioned IL-1 family cytokines as central regulators that link cellular stress that results from danger/stress signals to the induction and progression of sterile inflammatory diseases. These findings and future discoveries in the IL-1 field should provide novel strategies in the treatment of autoinflammatory diseases.

ACKNOWLEDGMENTS

We apologize to authors whose work could not be referenced in this review due to space limitations. Thirumala-Devi Kanneganti is supported by grants from the National Institute of Arthritis and Musculoskeletal and Skin Diseases under Award Number AR056290, the National Institute of Allergy and Infectious Diseases under Award Number AI101935 of the National Institutes of Health and the American Lebanese Syrian Associated Charities.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 July 2012; paper pending published: 23 August 2012; accepted: 22 September 2012; published online: 09 October 2012.

*Citation: Lukens JR, Gross JM and Kanneganti T-D (2012) IL-1 family cytokines trigger sterile inflammatory disease. Front. Immun. **3**:315. doi: 10.3389/fimmu.2012.00315*

This article was submitted to Frontiers in Inflammation, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 08 January 2013
doi: 10.3389/fimmu.2012.00414

ATP release and purinergic signaling in NLRP3 inflammasome activation

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The NLRP3 inflammasome is a protein complex involved in IL-1β and IL-18 processing that senses pathogen- and danger-associated molecular patterns (PAMPs and DAMPs). One step- or two step-models have been proposed to explain the tight regulation of IL-1β production during inflammation. Moreover, cellular stimulation triggers adenosine triphosphate (ATP) release and subsequent activation of purinergic receptors at the cell surface. Importantly some studies have reported roles for extracellular ATP, in NLRP3 inflammasome activation in response to PAMPs and DAMPs. In this mini review, we will discuss the link between active ATP release, purinergic signaling and NLRP3 inflammasome activation. We will focus on the role of autocrine or paracrine ATP export in particle-induced NLRP3 inflammasome activation and discuss how particle activators are competent to induce maturation and secretion of IL-1β through a process that involves, as a first event, extracellular release of endogenous ATP through hemichannel opening, and as a second event, signaling through purinergic receptors that trigger NLRP3 inflammasome activation. Finally, we will review the evidence for ATP as a key pro-inflammatory mediator released by dying cells. In particular we will discuss how cancer cells dying via autophagy trigger ATP-dependent NLRP3 inflammasome activation in the macrophages engulfing them, eliciting an immunogenic response against tumors.

Keywords: ATP, danger signal, inflammasome, P2R, NLRP3, purinergic signaling, autophagic cell death

THE NLRP3 INFLAMMASOME

Innate immunity is triggered by endogenous or environmental danger events through assembly of the NLRP3 inflammasome. The NLRP3 inflammasome is a cytosolic multiprotein platform which is activated in response to a variety of signals including infection, tissue damage, and metabolic dysregulation. Activation of the NLRP3 inflammasome results in the assembly of scaffold components: the cytoplasmic receptor NLRP3, the adaptor protein ASC and the effector protein caspase-1 (Agostini et al., 2004; Martinon et al., 2004, 2009; Martinon and Tschopp, 2004; Kanneganti et al., 2006; Mariathasan et al., 2006). This association leads to the activation of caspase-1, allowing the processing of pro-IL-1β and pro-IL-18 to their mature and secreted forms which are biologically active. IL-1β production is a tightly controlled process playing a pivotal role in inflammation and in recruitment of neutrophils into tissues. A two-signal model has been proposed to explain the regulation of IL-1β production. First the synthesis of pro-IL-1β and NLRP3 is triggered by transcriptional induction via ligands for Toll-like receptors (TLRs), whereas a second stimulus leads to inflammasome oligomerization, caspase-1 autoactivation, caspase-1-dependent cleavage of pro-IL-1β and then release of the biologically active, mature IL-1β. This second signal may be induced by a broad variety of chemically and biologically unrelated molecules classified as either pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). DAMPs originate from environmental pollutants including silica and asbestos (Dostert et al., 2008; Halle et al., 2008), from vaccines such as aluminum salt (alum) adjuvant (Eisenbarth et al., 2008) or from endogenous metabolic stresses such as high concentration of glucose (Schroder et al., 2010), cholesterol (Duewell et al., 2010), amyloid-β protein (Halle et al., 2008), biglycan (Babelova et al., 2009), adenosine triphosphate (ATP; Mariathasan et al., 2006), or monosodium urate (MSU) crystals (Aganna et al., 2002; Eisenbarth et al., 2008). Two major upstream mechanisms are currently proposed for NLRP3 inflammasome activation: plasma membrane disruption (for bacterial toxins and ATP) or internalization of particulate activators by phagocytosis (Cassel et al., 2009). Extracellular ATP (eATP) or bacterial toxins lead to K^+ efflux and pore formation (Pelegrin and Surprenant, 2007). Phagocytosis of particles including silica, alum, fibrillar amyloid-β protein, or MSU was shown to result in lysosomal destabilization/permeabilization with release of the endosomal–lysosomal protease cathepsin B into the cytoplasm (Halle et al., 2008) and/or to mediate K^+ efflux and reactive oxygen species (ROS) driven activation. Recently, we and others have described mechanistic links between ATP- and particle-mediated inflammasome activation pathways (Riteau et al., 2012). Here we review the role of active ATP release and purinergic signaling in NLRP3 inflammasome activation.

ATP AND PURINERGIC SIGNALING

Adenosine triphosphate signaling is emerging as an important mechanism to control various cell functions (Burnstock, 2006; Praetorius and Leipziger, 2009). Cellular stimulation triggers ATP release and subsequently activation of purinergic receptors at the cell surface (autocrine activation) and/or on adjacent cells

(paracrine activation), thereby regulating or modulating cellular functions in immunity. Although essentially all cells are able to release nucleotides, the mechanisms underlying nucleotides release by epithelial, endothelial, or other non-excitable cells are poorly understood (Lazarowski et al., 2003; Praetorius and Leipziger, 2009). After release, eATP interacts with specific purinergic receptors or is degraded via different ecto-ATPases to ADP and AMP and then to adenosine. ATP or its metabolites are able to signal through different purinergic receptors (P2X, P2Y, or adenosine P1 receptors; Yegutkin, 2008). In pathological conditions, high levels of ATP are passively released from necrotic cells and act as a pro-inflammatory danger signal, activating the NLRP3 inflammasome through binding to the ionotropic P2X7 receptor (P2X7R; Iyer et al., 2009).

NLRP3 INFLAMMASOME ACTIVATION: ONE OR TWO STEP SIGNAL?

During NLRP3 inflammasome activation, a two-signal model has been proposed to explain the regulation of IL-1β production by macrophages, dendritic cells, or microglial cells. After a first signal induced by LPS triggers accumulation of pro-IL-1β, exposure to high concentrations of eATP (5 mM) acts as a powerful second signal to elicit the processing of pro-IL-1β into mature IL-1β in murine (Perregaux and Gabel, 1994) and human macrophages (Ataman-Onal et al., 2006) via P2X7R signaling (Di Virgilio, 2007). In contrast, primary human monocytes were shown to require only one signal because LPS alone was sufficient to induce secretion of mature IL-1β, with exogenous ATP acting to further accelerate the LPS-triggered IL-1β processing and secretion (Ferrari et al., 2006; Netea et al., 2009). In addition, primary stimulation of human monocytes with several other PAMPs and one DAMP was sufficient to provide both the first and the second signals via a mechanism involving active release of endogenous ATP to the extracellular environment with consequent activation of the P2X7R in an autocrine loop; this allows the triggering of mature IL-1β secretion in a one step model of inflammasome activation (Piccini et al., 2008). In these studies, eATP and mature IL-1β were measured in the absence/presence of either, P2X7R pharmacologic inhibitors, or apyrase, an ATP/ADP degrading enzyme, to demonstrate the roles of ATP release and purinergic signaling in secretion of mature IL-1β. Moreover, an inhibitor of ATP degradation ARL67156 greatly increased both eATP and IL-1β contents, further supporting the role of endogenously released ATP in secretion of mature IL-1β. These studies demonstrated that LPS or MSU can trigger ATP release from stimulated cells, pointing to fundamental roles for ATP and/or its metabolites as important molecules that mediate the NLRP3 inflammasome activation responses to PAMPs or DAMPS.

Surprisingly, two other recent studies have implicated purinergic signaling but not ATP release in NLRP3 inflammasome activation by suggesting that certain non-nucleotide inflammasome activators may interact directly with purinergic receptors. First, it was shown that soluble biglycan, an ubiquitous leucine-rich repeat proteoglycan of the extracellular matrix, acts as an endogenous danger signal that activates the NLRP3 inflammasome and the release of IL-1β without additional co-stimulatory factors (e.g., exogenous ATP) in murine primary peritoneal macrophages (Babelova et al., 2009). By signaling through TLR2/4, biglycan stimulated the expression of NLRP3 and pro-IL-1β mRNA. Biglycan-induced inflammasome activation was completely inhibited by oxidized-ATP (oATP), which broadly inhibits several P2X and P2Y receptors, and partially inhibited by KN-62, a selective inhibitor of P2X7R, demonstrating the involvement of the P2 purinergic receptors. Moreover, co-stimulation of the biglycan-treated cells with ATP further increased IL-1β secretion. Nevertheless, the authors claimed direct activation of the NLRP3 inflammasome by biglycan and excluded an autocrine role for ATP because biglycan did not affect the secretion of ATP. They proposed that the interaction of biglycan with both TLR2/4 and purinergic P2X4/P2X7Rs induces receptor cooperativity and NLRP3 inflammasome activation (Babelova et al., 2009).

Another study characterized inflammasome responses to serum amyloid A (SAA), an acute-phase protein which undergoes up to a 1000-fold increase in serum levels during inflammation, and which has a pathogenic role in amyloid A-type amyloidosis. SAA provides a signal for both the induction of pro-IL-1β expression and NLRP3 inflammasome activation, resulting in secretion of IL-1β without fibril formation and lysosomal destabilization in human and mouse macrophages (Niemi et al., 2011). Blocking TLR2 and TLR4 attenuated SAA-induced expression of IL-1β, whereas inhibition of caspase-1 or P2X7R by oATP or KN-62 abrogated the release of mature IL-1β. However apyrase treatment did not diminish SAA-mediated IL-1β release and no increase in ATP levels was observed in response to SAA. Thus, the authors proposed that SAA-induced inflammasome activation is mediated by a direct interaction between SAA and P2X7R and that it is not associated with the release of ATP or ADP (Niemi et al., 2011). In conclusion, whether a one- or two-step model of inflammasome activation is involved, ATP and/or purinergic signaling seem to play key roles in NLRP3 inflammasome activation by PAMPs or DAMPs.

A ROLE FOR ATP IN PARTICLE-INDUCED NLRP3 INFLAMMASOME ACTIVATION

In a recent study, we showed that uric acid, silica, or alum particles induce the active release of intracellular ATP from human macrophage to extracellular compartments via mechanisms that depend on purinergic signaling and connexin/pannexin channels (Riteau et al., 2012). We observed a strong correlation between ATP release and secretion of mature IL-1β after stimulation of phorbol myristate acetate (PMA)-primed THP1 human macrophages. In presence of the first signal (PMA), MSU, silica, or alum salt crystals acted as second signal triggers leading to mature IL-1β production by these human myeloid cells via ATP release and subsequent purinergic signaling. Importantly, allopurinol crystals, which do not elicit NLRP3 inflammasome activation (Ataman-Onal et al., 2006) were also unable to trigger ATP release. In addition, exposure of LPS-primed murine macrophages to the different crystalline stimuli also lead to maturation of IL-1β via pathways dependent on autocrine purinergic signaling loops involving multiple purinergic receptor subtypes (Riteau et al., 2012). Moreover, crystal-induced secretion of IL-1β was abrogated by apyrase to

further confirm the role of ATP and autocrine purinergic signaling in inflammasome activation. Nevertheless, P2X7R deficiency in murine macrophages did not change the ability of these cells to secrete IL-1β even though high and non-specific concentrations of the P2X7R inhibitor A740003 severely impaired cytokine production. This suggested the involvement of P2 receptor subtypes in addition to P2X7R. Indeed, several other studies have reported that blocking or deleting P2X7R did not affect MSU- (Ataman-Onal et al., 2006), silica- (Iyer et al., 2009), or alum- (Eisenbarth et al., 2008) induced IL-1β production, thereby implicating roles for other purinergic receptors in non-pathologic conditions. Previous reports have indicated that not only ATP but other nucleotides such as ADP, UTP, or UDP are released into the extracellular space of mechanically stressed cells, in particular endothelial and epithelial cells (Di Virgilio et al., 2001; Lazarowski et al., 2003). These compounds may act on different purinergic receptors to generate a finely tuned response. Our data strongly suggest that members of the two major purinergic receptor families – P2X and P2Y – are involved because ADP and UTP act only on P2Y-family receptors. Thus, our study provides a novel link between the particle internalization- and membrane permeabilization-models of NLRP3 inflammasome activation. Indeed, prior to this analysis, particulate molecules and eATP were considered as two completely independent stimuli for NLRP3 inflammasome activation. The model presented in **Figure 1** summarizes these results and our model.

In another recent report, the P2Y6 receptor was identified as an essential mediator for MSU-induced inflammation in the human THP1 monocyte/macrophage cell line because the specific P2Y6 antagonist MRS2578 completely inhibited MSU-induced IL-1β production (Uratsuji et al., 2012). Because P2Y6 receptors are known to be coupled to the activation of phospholipase C (PLC; Abbracchio et al., 2006), a PLC inhibitor, U-73122, was also tested and shown to suppress the MSU-induced IL-1β production by THP1 cells. These results demonstrate that the P2Y6–PLC signaling pathway mediates MSU-induced inflammatory responses in human monocytes (Uratsuji et al., 2012) and thus point to the involvement of P2Y receptors in particle-induced NLRP3 inflammasome activation.

Interestingly, ATP is a potential mediator of neuroinflammation and an extracellular signaling molecule between neurons and glial cells (Abbracchio et al., 2006). Multiple P2X and P2Y receptor subtypes are expressed by astrocytes, oligodendrocytes, and microglia (James and Butt, 2002). Thus, microglial cells are a good model to investigate physiological functions of purinergic receptors in the immune system. First, eATP was shown to cause a large release of IL-1β from microglial cell lines and from freshly isolated microglial cells by activating the P2X7R (Di Virgilio et al.,

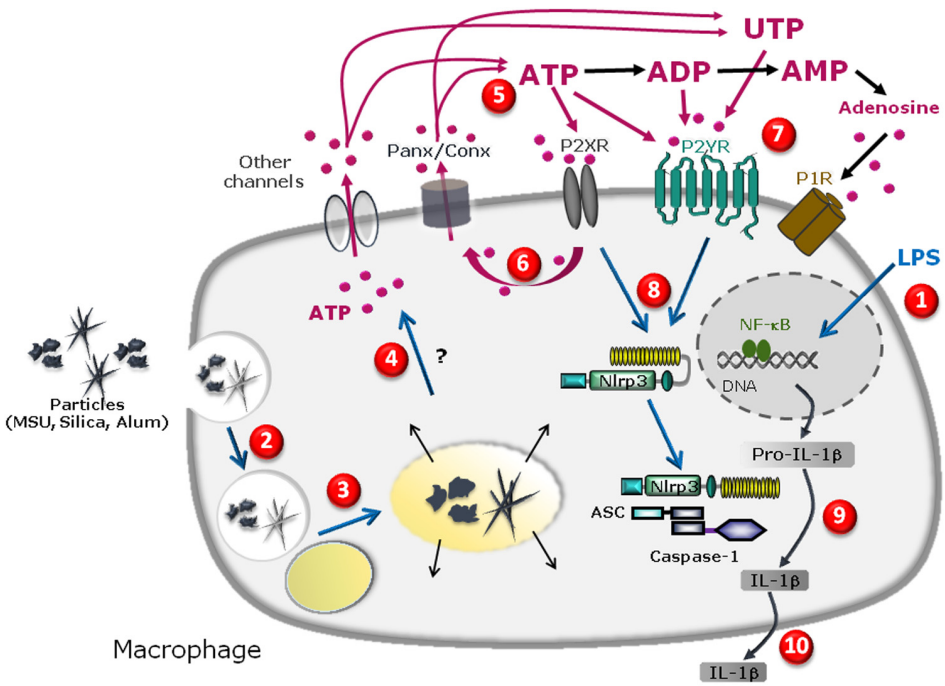

FIGURE 1 | Schematic diagram illustrating the specific cascade and signaling pathway in LPS-primed macrophages stimulated with MSU, silica, or alum salt crystals. LPS priming induces transcription of pro-IL-1β gene and other genes in the nucleus upon activation of the transcription factor NFkB and subsequent production of pro-IL-1β protein in the cytosol **(1)**. Particle internalization **(2)**, fusion to lysosome **(3)** and further phagolysosome destabilization may lead to cathepsin leakage **(4)** which precedes pannexin/connexin (Panx/Conx) hemichannel and purinergic signaling-dependent intracellular ATP release **(5)**. Extracellular ATP may act through P2X7 receptor to amplify ATP release in a P2X7 receptor-dependent way **(6)**. ATP, UTP, or their derived degradation products such as ADP, UDP, and adenosine, generated by ecto-endonucleases, may act through an autocrine loop on other purinergic receptor P2X, P2Y, and/or P1 receptors **(7)** leading to NLRP3 receptor activation **(8)**. This allows inflammasome complex formation and maturation of pro-IL-1β to IL-1β production **(9)** and IL-1β secretion **(10)**.

1996). Second, stimulation of microglia cells through TLRs has been proposed to induce the release of endogenous ATP acting in an autocrine manner to activate the ion channel P2X7 (Ferrari et al., 1997). Release of ATP and other nucleotides seems to modulate microglial responses via P2Y and P2X receptors, with the P2X7 subtype standing out for its known pro-inflammatory activity and for its up-regulation in both a transgenic mouse model of Alzheimer's disease and in the brains from Alzheimer's disease patients (Parvathenani et al., 2003; McLarnon et al., 2006). Moreover, another particulate NLRP3 activator, amyloid-β protein aggregates was shown to promote IL-1β release through P2X7R-mediated ATP release and therefore to activate the NLRP3 inflammasome in microglia (Sanz et al., 2009).

In summary, four different particulate activators, MSU, silica, alum crystals, and amyloid-β protein aggregates have been shown to activate the NLRP3 inflammasome through mechanisms involving ATP release and autocrine purinergic signaling. Based on the use of pharmacological inhibitors, a role for P2X7R has often been reported. However, the uncertain selectivity of P2X7R antagonists (which can vary with concentration and cell type) may yield questionable or equivocal conclusions. Our recent study used pharmacological agonists, antagonists, and P2R subtype-deficient mice to suggest that multiple purinergic signaling pathways are involved in NLRP3 inflammasome regulation through activation by ATP, ADP, UTP, UDP, and/or adenosine.

A ROLE FOR ATP RELEASE IN CELL DEATH-INDUCED INFLAMMASOME ACTIVATION

Cells which die in response to non-developmentally programmed cues, such as the necrosis produced by pressure disruption, hypoxic injury, or complement-mediated damage, are potent activators of the innate immune system and can promote sterile inflammation through sensing by the NLRP3 inflammasome that results in the subsequent release of the pro-inflammatory cytokine IL-1β. This activation may be triggered in part by ATP produced by mitochondria and released from damaged cells (Iyer et al., 2009). Cells which die as part of physiological responses, such as apoptotic or autophagic cells, are removed from tissues to prevent immune reactions and maintain tissue homeostasis. Although apoptotic cells have anti-inflammatory properties due to their surface exposure of anti-inflammatory molecules (Fadok et al., 1998; Cvetanovic et al., 2006), cells dying via autophagy can trigger pro-inflammatory responses through the release of danger signals that drive NLRP3 inflammasome activation (Petrovski et al., 2007b; Ghiringhelli et al., 2009; Michaud et al., 2011). Since the first description of autophagy in 1966 (De Duve and Wattiaux, 1966), numerous studies have described autophagy as a survival mechanism response to poor nutritional conditions (Gozuacik and Kimchi, 2007; Maiuri et al., 2007). However, it is now clear that autophagy has a dual role (Codogno and Meijer, 2005): under certain circumstances, autophagy constitutes a stress adaptation that avoids cell death (and suppresses apoptosis) by degradation of long-lived proteins and damaged organelles through the autophago-lysosomal pathway, whereas in other cellular settings, it constitutes an alternative cell-death pathway (Petrovski et al., 2007a). Cell dying through autophagy were shown recently to induce a pro-inflammatory response in human macrophages (Petrovski et al., 2007b). Moreover, phagocytosis of human cancer cells dying through autophagy was shown to trigger NLRP3 inflammasome activation and maturation of IL-1β in human macrophages via an ATP-dependent mechanism (Petrovski et al., 2011). Indeed, multiple manipulations including blockade of K^+ efflux during phagocytosis, incubation in the presence of apyrase, addition of P2X7R antagonist, or silencing NLRP3 protein expression, all acted to inhibit this IL-1β secretion response (Petrovski et al., 2011). Moreover, phagocytosis of murine cells, dying via autophagy by mouse macrophages was found to activate the NLRP3 inflammasome in the engulfing macrophages (Ayna et al., 2012). Studying the mechanism of inflammation illuminated roles for ATP release via pannexin-1 channels in the autophagic dying cells, phagocytosis of autophagic dying cells, P2X7R activation and subsequent K^+ efflux in macrophages as obligatory steps for NLRP3 inflammasome activation in this model (Ayna et al., 2012). Together, these studies have demonstrated that ATP release is required for NLRP3 inflammasome activation in both human and murine macrophages. Some mechanistic details may differ in these models; during phagocytosis of human autophagic dying cells, ATP is released by macrophages and acts on macrophage purinergic receptors in an autocrine loop (Petrovski et al., 2007b), whereas during engulfment of murine autophagic dying cells, ATP is released by autophagic dying cells and acts on macrophages in an paracrine loop (Ayna et al., 2012). Importantly, autophagic death was reported to contribute to making apoptotic cancer cells immunogenic (Michaud et al., 2011; Petrovski et al., 2011) and thereby capable of activating the inflammasome in dendritic cells (Ghiringhelli et al., 2009). These authors first showed in a murine model that treatment of cancer cells with anticancer chemicals (such as oxaliplatin and mitoxantrone) causes immunogenic cancer cell death (Ghiringhelli et al., 2009). ATP released from dying tumor cells was shown to activate P2X7R signaling in dendritic cells, leading to inflammasome activation and further IL-1β secretion (Ghiringhelli et al., 2009). These authors also demonstrated that oxaliplatin- or mitoxantrone-treated tumor cells die via autophagy which induces an immunogenic response *in vivo* by recruiting dendritic cells and T cells into the tumor through the release of ATP into the extracellular fluid (Michaud et al., 2011). They subsequently reported that autophagy is essential for the immunogenic release of ATP from dying cells (Michaud et al., 2011). Furthermore, such an immunogenic anti-tumor response could also be elicited when autophagy and cell death was induced by cytokine depletion. The mechanisms for the NLRP3 activation triggered through murine cells dying via autophagy are summarized in **Figure 2**.

In conclusion, an increasing body of evidence suggests that ATP and/or purinergic signaling are cornerstone regulators of NLRP3 inflammasome activation in many, but not all, biological contexts wherein the purinergic pathways do not exclude the existence of other mechanisms. First, a high amount of passive ATP release from necrotic cells activates the inflammasome through the P2X7R. Second, PAMP recognition and signaling through their receptors trigger active ATP release in some cell types such as human monocytes. Third, phagocytosis of several inflammasome activators following by ATP release appears to be common

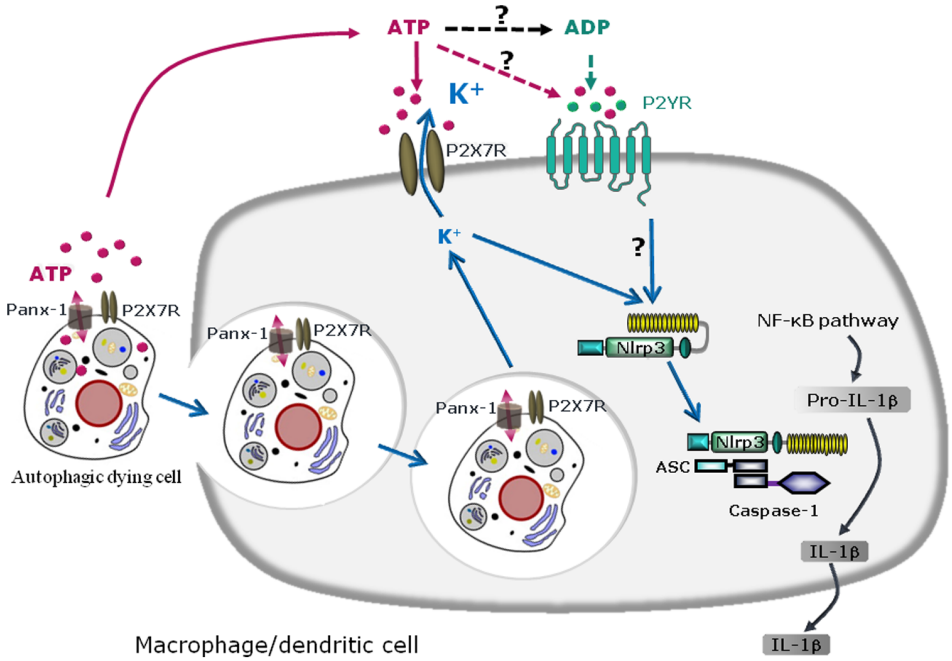

FIGURE 2 | Schematic diagram illustrating the mechanisms of NLRP3 inflammasome activation by autophagic dying cells through ATP leakage and purinergic signaling in murine macrophages or dendritic cells. Autophagic dying cells release ATP through pannexin-1 (Panx-1) hemichannel resulting in activation of the purinergic receptor P2X7R on macrophages or dendritic cells. Phagocytosis of autophagic dying cells and K^+ efflux are required for NLRP3 inflammasome activation in macrophages or dendritic cells. Since extracellular ATP is rapidly degraded in ADP, AMP, and adenosine, ATP metabolites could also act through other purinergic receptors and in particular, ATP and ADP could signal through P2YR. Purinergic signaling and K^+ efflux result in inflammasome assembly and caspase-1 activation leading to maturation of pro-IL-1β in IL-1β and IL-1β secretion.

pathway for activating the NLRP3 inflammasome. Fourth, ATP leakage from autophagic dying cells and the engulfment of these cells by macrophages trigger immunity through NLRP3 inflammasome activation. It will be important to identify the events linking, on the one hand, phagocytosis of particles or autophagic dying cells to ATP release by macrophages, and on the other hand, ATP leakage and engulfment of autophagic dying cells to NLRP3 activation. Finally, the events triggering NLRP3 inflammasome assembly and activation downstream of purinergic signaling are unknown. Another important issue is that ATP and P2X7R are not the only purinergic "players" in this response because different nucleotide metabolites such as ADP, UTP, UDP, and adenosine, and other members of the purinergic receptor family, i.e., the P2X, P2Y, and P1 receptors may contribute through complex purinergic signaling networks. Better understanding of these mechanisms will facilitate identification of new targets for inflammatory diseases and improve our understanding of the immune response to cancer.

ACKNOWLEDGMENTS

The authors are grateful to the support of the "Agence National de Recherche" (France), the "Conseil Général du Loiret" (France), the "Fonds de Dotation pour la Recherche en Santé Respiratoire" (France), and the "Région Centre" (France). The authors also thank George Dubyak (Cleveland, Ohio) for excellent review of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 October 2012; accepted: 19 December 2012; published online: 08 January 2013.

*Citation: Gombault A, Baron L and Couillin I (2013) ATP release and purinergic signaling in NLRP3 inflammasome activation. Front. Immun. **3**:414. doi: 10.3389/fimmu.2012.00414*

This article was submitted to Frontiers in Inflammation, a specialty of Frontiers in Immunology.

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IMMUNOLOGY

REVIEW ARTICLE
published: 29 October 2012
doi: 10.3389/fimmu.2012.00322

TIR8/SIGIRR is an interleukin-1 receptor/toll like receptor family member with regulatory functions in inflammation and immunity

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Interleukin-1R like receptors (ILRs) and Toll Like Receptors (TLRs) are key receptors of innate immunity, inflammation, and orientation of the adaptive response. They belong to a superfamily characterized by the presence of a conserved intracellular domain, the Toll/IL-1R (TIR) domain, which is involved in the activation of a signaling cascade leading to activation of transcription factors associated to inflammation. The activation of inflammatory responses and immunity by ILRs or TLRs signaling is potentially detrimental for the host in acute and chronic conditions and is tightly regulated at different levels by receptor antagonists, decoy receptors or signaling molecules, and miRNAs. Recent evidence suggests that the ILRs family member TIR8 (also known as SIGIRR) is a regulatory protein acting intracellularly to inhibit ILRs and TLRs signaling. In particular, current evidence suggests that TIR8/SIGIRR dampens TLRs-mediated activation and inhibits signaling receptor complexes of IL-1 family members associated with Th1 (IL-18), Th2 (IL-33), and Th17 (IL-1) differentiation. Studies with Tir8/Sigirr-deficient mice showed that the ability to dampen signaling from ILRs and TLRs family members makes TIR8/SIGIRR a key regulator of inflammation. Here, we summarize our current understanding of the structure and function of TIR8/SIGIRR, focusing on its role in different pathological conditions, ranging from infectious and sterile inflammation, to autoimmunity and cancer-related inflammation.

Keywords: cytokine, interleukin-1, toll like receptors, inflammation, infection, inflammation-associated cancer

INTRODUCTION

Interleukin-1R like receptors (ILRs) and Toll Like Receptors (TLRs) are key receptors of innate immunity and inflammation. They are members of a superfamily of phylogenetically conserved proteins characterized by the presence of a conserved intracellular domain, the Toll/IL-1R (TIR) domain (**Figure 1**).

Upon ligand binding, dimerization of receptor TIR domains, recruitment of TIR domain containing adapter proteins and activation of a signaling pathway occur. This pathway involves Myeloid differentiation factor 88 (MyD88), IL-1R associated kinases (IRAKs), and tumor necrosis factor receptor-associated factor 6 (TRAF6) and leads to activation of nuclear factor kappa B (NF-κB), activator protein-1 (AP-1), c-Jun N-terminal kinase (JNK), p38 mitogen-associated protein kinase, and members of the interferon regulatory factor family (O'Neill, 2006, 2008; Dinarello, 2009).

The family is subdivided in TLRs bearing leucine-rich repeats in the extracellular domain, and ILRs bearing Ig-like domains. Nowadays, 10 TLRs have been reported in humans and 12 in the mouse (Kang and Lee, 2011), whereas ILRs family includes 10 proteins. TLRs are receptors for specific pathogen associated molecular patterns (PAMPs) and of necrotic cell-derived danger signals (DAMPs) and act as sensors for microorganisms and tissue damage (Cavassani et al., 2008). The ILRs family includes components of signaling receptor complexes as well as molecules with regulatory function (IL-1RII and TIR8/SIGIRR). In particular TIR8, so called because at the time of cloning it was the eighth molecule with a TIR domain and also known as single immunoglobulin interleukin-1 receptor related molecule (SIGIRR), is a fringe member of the family with structural features incompatible with conventional signaling (Li and Qin, 2005; Garlanda et al., 2009; **Figure 1**).

Interleukin-1R like receptors ligand family includes pro-inflammatory molecules such as IL-1α and IL-1β (IL-1F1), IL-18/IL-1F4, IL-36α/IL-1F6, IL-36β/IL-1F8, and IL-36γ/IL-1F9 (Dinarello et al., 2010; Boraschi et al., 2011). Other members of IL-1 family show anti-inflammatory activity. IL-1 receptor antagonist (IL-1Ra)/IL-1F3 binds to IL-1RI inhibiting the recruitment of IL-1RAcP and compete with IL-1α and IL-1β for receptor binding (Dinarello, 2000); IL-36Ra binds IL-1Rrp2 antagonizing IL-36α, IL-36β, and IL-36γ; IL-37 produces anti-inflammatory effects; and finally IL-33 binds to T1/ST2, recruits IL-1RAcP and induces the expression of anti-inflammatory cytokines (Schmitz et al., 2005).

Uncontrolled or deregulated activation of ILRs- or TLRs-dependent inflammatory and immune responses can be

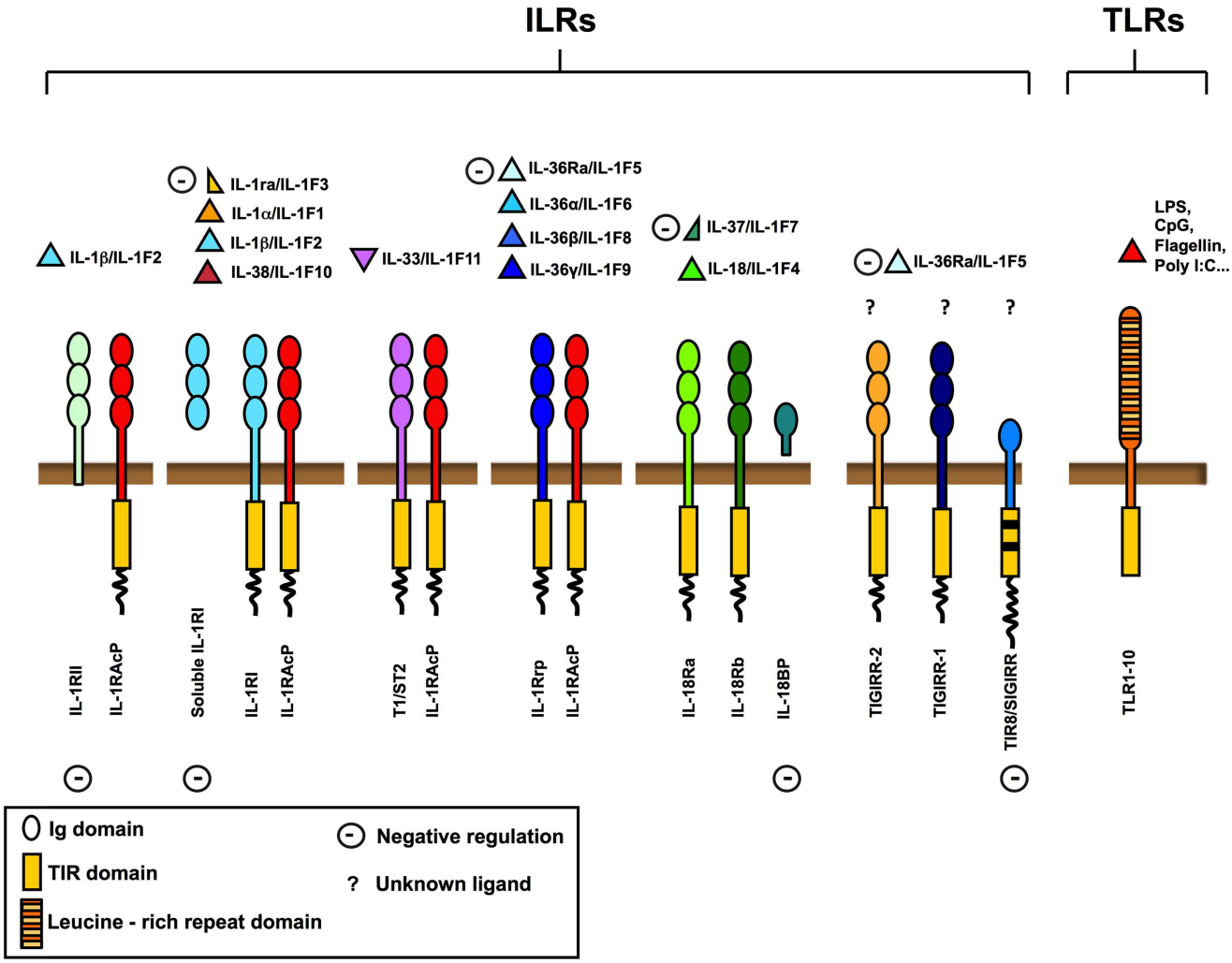

FIGURE 1 | The IL-1 receptor (ILRs) and Toll like receptor (TLRs) superfamily. Ligands, receptors, adaptors, and extracellular and intracellular negative regulators are shown. The ILRs family includes the receptors (IL-1RI, IL-18R, T1/ST2, IL-1Rrp2) and the accessory proteins (IL-1RAcP) for IL-1, IL-18, IL-33, and other IL-1 family members (IL-36α, IL-36β, and IL-36γ). IL-1Ra, soluble IL-1RI, IL-36Ra, IL-37, IL-18 binding protein (IL-18BP), IL-1RII, and TIR8/SIGIRR are negative regulators acting at different levels, as receptor antagonists, decoy receptors, scavengers, or dominant negative. TIR8/SIGIRR, TIGIRR-1, and TIGIRR-2 are still orphan receptors. Microbial moieties (LPS, Poly I:C, CpG, Flagellin, and others) and necrotic cell-derived danger signals are the ligands for TLRs.

detrimental for the host and potentially cause tissue damage and acute or chronic inflammatory disorders. The activation of inflammatory responses and immunity by ILRs or TLRs signaling is tightly regulated at different levels. For instance, IL-1Ra and IL-36Ra are polypeptide antagonists for IL-1RI and IL-1Rrp2, respectively (Towne et al., 2004; Costelloe et al., 2008; Aksentijevich et al., 2009; Dinarello, 2009; Reddy et al., 2009). Decoy receptors, such as IL-1RII, bind ligands that are no longer available for the transducing receptors or form dominant negative non-signaling complexes with AcPs (Mantovani et al., 2001). IRAK-M and MyD88s are negative regulators of ILRs and TLRs signaling, acting intracellularly (Kobayashi et al., 2002; Janssens et al., 2003). TIR8/SIGIRR, which will be discussed in detail below, inhibits the activation of the signaling pathway by TLRs and IL-1R by interfering with the association of adaptor molecules to the receptor complex. Finally, a novel level of feedback system has been identified, consisting of targeting of ILRs or TLRs signaling proteins or transcription factors by miRNAs. In particular, miR-155, miR-21, miR-146a, miR-132, miR-9, and miR-147 have all been significantly implicated in the immune response initiated by IL-1R or TLRs (Bazzoni et al., 2009; Nahid et al., 2011; Quinn and O'Neill, 2011). For instance, the induction of transcription of miR-146a or miR-9 by LPS, tumor necrosis factor alpha (TNFα) and IL-1α is dependent on NF-κB, and in turn, miR-146a potentially targets TRAF6 and IRAK-1, whereas miR-9 targets the NFkB1 transcript, dampening the immune response (Bazzoni et al., 2009; Quinn and O'Neill, 2011).

Here, we summarize our current understanding of the structure and function of TIR8/SIGIRR, focusing on its regulatory role in different pathological disorders dependent on ILRs and TLRs activity, ranging from inflammation in infectious and sterile conditions, to autoimmunity and cancer-related inflammation.

TIR8/SIGIRR GENE AND PROTEIN

TIR8/SIGIRRis an orphan receptor. The human gene is localized on chromosome 11, band p15.5, and encompasses 10 exons spanning about 11700 bp, whereas the murine gene is localized on chromosome 7, band F4, and includes nine exons spanning about 4000 bp (Thomassen et al., 1999). The human protein is 410 amino acid-long and is organized in an extracellular domain comprising of a single Ig domain, in contrast with others ILRs family members which have three, a transmembrane domain, an intracellular conserved TIR domain, and a peculiar terminal 95 aa long tail, which is not present in other mammal TIR family members. Two residues of the TIR domain (Ser447 and Tyr536) considered necessary for signal transduction of IL-1R1 are replaced by Cys222 and Leu305 in TIR8/SIGIRR, but the functional relevance of this replacement has not been addressed. TIR8/SIGIRR is extensively *N*- and *O*-glycosylated. Indeed five potential *N*-glycosylated sites in human and four in mouse are present in the extracellular portion of the molecule (Thomassen et al., 1999; Lech et al., 2007).

TIR8/SIGIRR shows a conserved sequence and pattern of expression among vertebrates, from chicken to humans (Riva et al., 2009). In particular, protein sequences of human and mouse TIR8/SIGIRR are 82% identical.

Little is known about *TIR8/SIGIRR* phylogenetic evolution, but the recent discovery in teleost fish of DIGIRR is helpful in defining the evolutionary history of TIR family members. DIGIRR presents two Ig-like domains in its extracellular region, a Arg-Tyr-mutated TIR domain and a unique expression within the Golgi apparatus. *In vitro* experiments demonstrate that DIGIRR is a negative regulator of LPS and IL-1β-mediated NF-κB activation (Gu et al., 2011). The authors suggest that DIGIRR could represent a "transitional molecule" between a potent receptor such as IL-1R1 and a negative regulator such as TIR8/SIGIRR.

TIR8/SIGIRR is ubiquitously expresses in tissues, in particular in kidney, digestive tract, liver, lung, and lymphoid organs (Thomassen et al., 1999; Polentarutti et al., 2003). Particularly, in kidney it is expressed on the luminal border and basolateral membrane of proximal tubular cells (Polentarutti et al., 2003; Lech et al., 2007), in the intestinal tract by epithelial cells and in lymphatic organs by NK cells, B lymphocytes, monocytes, and immature dendritic cells (DCs)(Polentarutti et al., 2003; Garlanda et al., 2004; Lech et al., 2007; Xiao et al., 2007), in the lung by bronchial epithelium, blood endothelial cells, and leukocytes (Veliz Rodriguez et al., 2012).

Mechanisms of regulation of TIR8/SIGIRR expression are still poorly defined (**Table 1**).

Kodota et al. recently showed that *TIR8/SIGIRR* proximal promoter presents a binding site for the transcription factor SP1, a zinc finger proteins, which binds directly to DNA and enhances *TIR8/SIGIRR* gene transcription. In the presence of LPS, SP1 binding to *TIR8/SIGIRR* promoter consensus sites was reduced, and consequently *TIR8/SIGIRR* expression was transiently inhibited in epithelial cells (Kadota et al., 2010). These data potentially explain previous findings showing that human and murine *TIR8/SIGIRR* expression was usually found downregulated by LPS stimulation or in other inflammatory conditions compared to homeostatic conditions (Polentarutti et al., 2003; Wald et al., 2003; Huang et al., 2006). For instance, ulcerative colitis in human and colitis in mouse were associated with reduced TIR8/SIGIRR expression by epithelial cells (Kadota et al., 2010). Bacterial infection of intestinal epithelial cells and exposure to flagellin transiently decreased TIR8/SIGIRR protein expression. Conversely, stable overexpression of TIR8/SIGIRR diminished NF-κB–mediated IL-8 responses to TLRs ligands (Khan et al., 2010). In psoriatic patients peripheral blood cells expressed decreased levels of *TIR8/SIGIRR* and other anti-inflammatory molecules

Table 1 | Regulation of TIR8/SIGIRR expression.

Stimuli	Organism	Cell type	mRNA/protein	Reference
UPREGULATION				
Th2-polarization	Mouse	T lymphocytes	Protein	Bulek et al. (2009)
Vasoactive intestinal peptide	Mouse	Macrophages and langerhans cells	mRNA	Jiang et al. (2012)
LPS	Mouse	Payer's patch DCs	mRNA	Davies et al. (2010)
DOWNREGULATION				
LPS	Mouse and human	Epithelial cells, DC, monocytes, polymorphonuclear granulocytes	mRNA, protein	Polentarutti et al. (2003), Wald et al. (2003), Huang et al. (2006), Garlanda et al. (2004), Kadota et al. (2010)
Flagellin, bacterial infection	Mouse and human	Intestinal epithelial cells	Protein	Khan et al. (2010)
Psoriatic arthritis	Human	Peripheral blood leukocytes	mRNA	Batliwalla et al. (2005)
Necrotizing enterocolitis	Human	Fetal enterocytes	mRNA	Nanthakumar et al. (2011)
Asymptomatic bacteriuria	Human	Neutrophil polymorphonuclear granulocytes	Protein	Ragnarsdottir et al. (2007)
P. aeruginosa acute lung infection	Mouse	Respiratory epithelium, polymorphonuclear granulocytes	mRNA	Veliz Rodriguez et al. (2012)
Toxoplasma gondii	Mouse	Intestinal epithelial cells	mRNA	Gopal et al. (2008)

(Batliwalla et al., 2005). Nanthakumar et al. (2011) demonstrated that *TIR8/SIGIRR*, together with other anti-inflammatory genes, was expressed at low levels in fetal human enterocytes and at lower levels in necrotizing enterocolitis intestinal cells, whereas pro-inflammatory genes were present at high levels, potentially explaining the excessive inflammatory response of the immature intestine. In asymptomatic bacteriuria patients, *TIR8/SIGIRR* and *TLR4* expression were significantly lower than in the age-matched control subjects (Ragnarsdottir et al., 2007). In mouse, *Tir8/Sigirr* mRNA was down-regulated upon acute lung infection by *Pseudomonas aeruginosa* in the lung and in neutrophils (Veliz Rodriguez et al., 2012) or in intestinal epithelial cells upon *Toxoplasma gondii* (Gopal et al., 2008). However, Adib-Conquy et al. (2006) reported that human monocytes up-regulated the *TIR8/SIGIRR* transcript during sepsis and sterile systemic inflammation, which was associated to reduced TNFα and enhanced IL-10 production in response to LPS and Pam3CysSK4.

Stimuli inducing *TIR8/SIGIRR* expression are not well defined yet and mechanisms involved are not known. In T lymphocytes, Th2-polarization induced higher levels of *TIR8/SIGIRR* expression than Th1-polarization or non-differentiating conditions (Bulek et al., 2009). The neuropeptide vasoactive intestinal peptide (VIP) was found to up-regulate *Tir8/Sigirr* in a cAMP-independent manner in the cornea of *P. aeruginosa* infected mice as well as in macrophages and Langerhans cells (Jiang et al., 2012). Recently, the probiotic microorganism *Lactobacillus jensenii* was found to up-regulate *TIR8/SIGIRR*, *A20*, and *IRAK-M* in porcine Payer's patch antigen presenting cells through TLR2, and to increase the expression of IL-10 and TGF-β thus inducing tolerogenic properties (Villena et al., 2012).

Finally, *in vitro* and *in vivo* experiments showed that LPS-induced up-regulation of *Tir8/Sigirr*, *tollip*, and *T1/ST2* messenger RNA in murine Payer's patch DCs, but not in spleen DCs, suggesting that Payer's patch DCs use these molecules in their arsenal to prevent the initiation of TLRs signaling (Davies et al., 2010).

REGULATION OF ILRs AND TLRs SIGNALING BY TIR8/SIGIRR

TIR8/SIGIRR lacks a specific ligand, even if it was demonstrated that glial cell TIR8/SIGIRR interacts with IL-36Ra regulating inflammatory response in the brain (Costelloe et al., 2008). TIR8/SIGIRR inhibits NF-κB and JNK activation dependent on ILRs or TLRs family member activation, but not TNFα-dependent NF-κB activation or IFNγ-dependent STAT1 activation (Wald et al., 2003; Garlanda et al., 2004). The inhibitory activity is exerted on IL-1RI, IL-18R, T1/ST2, TLR4, TLR7, and TLR9 (Wald et al., 2003; Garlanda et al., 2004; Qin et al., 2005; Lech et al., 2007, 2008; Bulek et al., 2009). TIR8/SIGIRR regulates also TLR3 and TLR1/2-dependent signaling in kidney monocytes (Lech et al., 2007; **Figure 2**).

Little is known about the interaction between TIR8/SIGIRR and other members of the superfamily and the data are sometimes contradictory. This could be explained by specific expression of TIR8/SIGIRR or other receptor complex components in different cell types or by posttranscriptional modifications, such as glycosylation, that modify biological function of TIR8/SIGIRR (Garlanda et al., 2004).

It has been proposed that the extracellular Ig-like domain of TIR8/SIGIRR interferes with the dimerization of IL-1RI and IL-1RAcP. In addition, the TIR8/SIGIRR cytoplasmic TIR domain sequesters proximal TIR-containing adaptor molecules, such as IRAK and TRAF6 after IL-1 stimulation. Mutagenesis studies showed that only the TIR domain (excluding the C-tail part) was necessary for TIR8/SIGIRR to inhibit TLR4 signaling (Wald et al., 2003; Qin et al., 2005). In contrast, T1/ST2 interacts with both TIR8/SIGIRR extracellular and intracellular domains, even if the TIR domain is crucial for TIR8/SIGIRR activity (Bulek et al., 2009).

Gong et al. (2010) addressed by a computational approach the molecular mechanism for the interaction of TIR8/SIGIRR and TLR4 or TLR7 and constructed a three-dimensional model for the TLR4, TLR7, MyD88, and TIR8/SIGIRR TIR domains. Receptor activation would trigger the formation of TLR4 and TLR7 TIR dimers recruiting MyD88 TIR dimers resulting in a signaling tetramer, in line with previous data (Loiarro et al., 2005; Nunez Miguel et al., 2007). The model proposed revealed that TIR8/SIGIRR binds through its BB-loop region to TLR4 and TLR7 by occupying their self-interacting sites and that the proper shape and electric environment of the MyD88-binding pocket are completely disturbed. In addition, TIR8/SIGIRR replaces a MyD88 monomer, interrupting the MyD88 homodimer formation (Gong et al., 2010). The BB-loop region is highly conserved in TIR-containing molecules and plays a crucial role in dimer formation (Gay et al., 2011). Thus, the interference exerted by TIR8/SIGIRRBB-loop region prevents the dimerization of the components of the receptor complex (receptor, accessory protein and adapter molecule), abolishing the signal transduction. In contrast, TIR8/SIGIRR unique C-tail is distant from the active BB-loop, consistent with the observation that this tail is not required for TIR8/SIGIRR inhibitory effect on TLRs signaling (Qin et al., 2005).

TIR8/SIGIRR also modulates IL-1-induced phosphorylation of JNK and mTOR kinase in Th17 lymphocytes, playing a non-redundant role in controlling mTOR-dependent Th17 differentiation, proliferation, and cytokine production (Gulen et al., 2010). On the same line, TIR8/SIGIRR was found to regulate IL-1- or commensal-TLRs-dependent activation of the Akt-mTOR axis in intestinal epithelial cells. The Akt-mTOR axis promotes cycle progression through its impact on posttranscriptional control of key cell cycle regulators and consequent genetic instability (Xiao et al., 2010; **Figure 2**). These findings open a new scenario for TIR8/SIGIRR acting as a modulator of autoimmune diseases and as tumorigenesis suppressor (see below).

ROLE OF TIR8/SIGIRR IN INFECTION-DEPENDENT INFLAMMATION

In different infectious conditions, TIR8/SIGIRR emerged as a non-redundant molecule involved in dampening inflammation and tissue damage by controlling TLRs-, but in particular ILRs-induced inflammatory response to pathogens (**Figure 3**; **Table 2**).

In *Mycobacterium tuberculosis* lung infection, Tir8/Sigirr-deficient mice showed exaggerated susceptibility to mortality, associated to overwhelming systemic inflammatory response, despite there was no difference in tissue bacterial load compared to control mice (Garlanda et al., 2007a). Indeed, treatment of

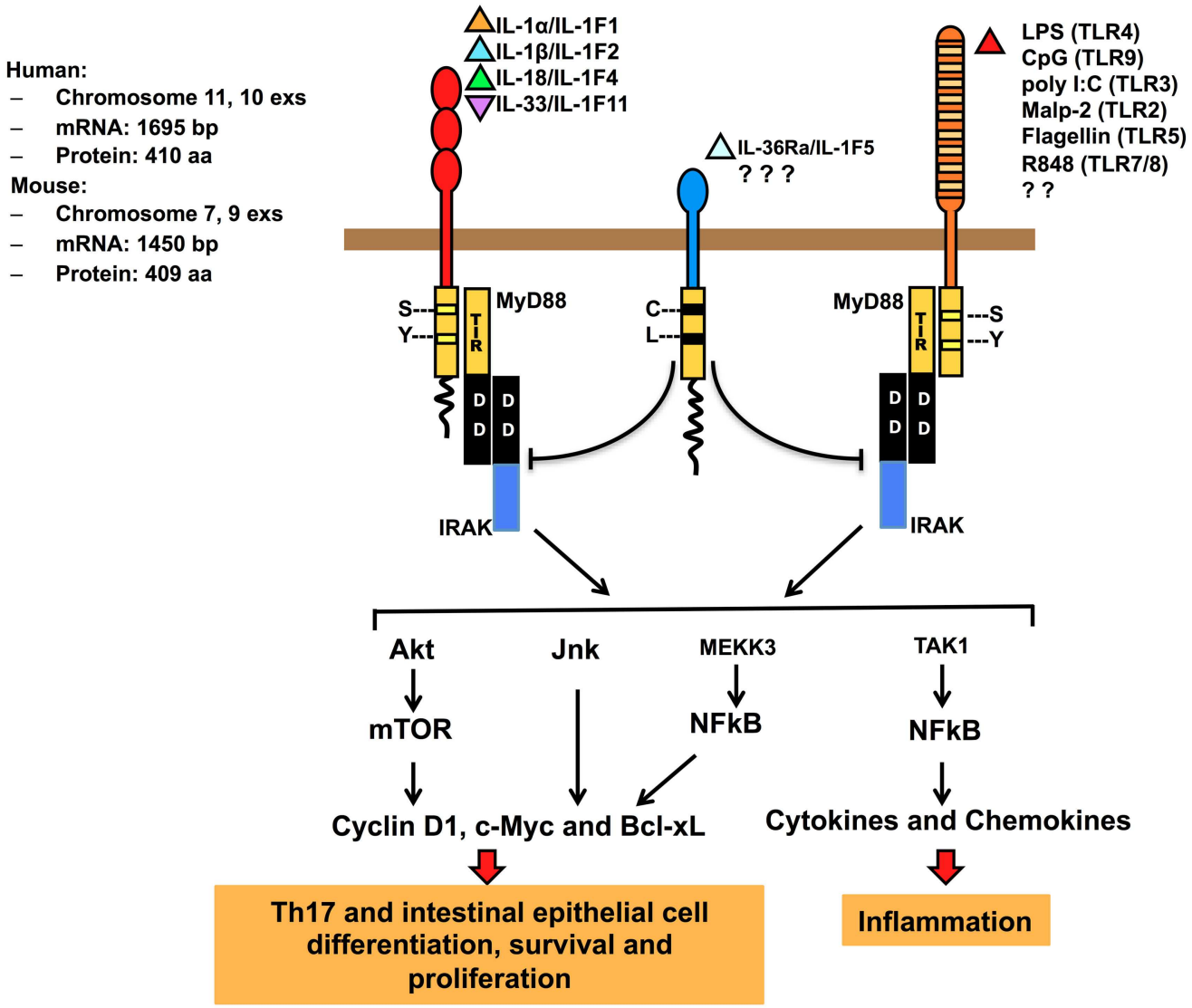

FIGURE 2 | Regulatory function of TIR8/SIGIRR in signaling. TIR8/SIGIRR is a receptor composed by a single extracellular Ig domain (amino acids 17–112), a transmembrane domain (amino acids 117–139); an intracellular conserved TIR domain (amino acids 166–305), and a 95 amino acid-long intracellular tail. Two conserved amino acid (Ser, Tyr in TIR domain) necessary for IL-1R-signaling are replaced in TIR8/SIGIRR (Cys 222, Leu 305) potentially leading to a non-conventional activation. TIR8/SIGIRR is an orphan receptor, but IL-36Ra has been proposed as a TIR8/SIGIRR ligand in glial cells. TIR8/SIGIRR inhibits ILR (IL-1RI, IL-18R, T1/ST2) and TLR (TLR2, TLR3, TLR4, TLR5, TLR7/8, TLR9, and possibly others) signaling and NF-κB activation. In T cells and epithelial cells, TIR8/SIGIRR inhibits IL-1-dependent activation of the Akt-mTOR pathway and of JNK.

Tir8/Sigirr-deficient mice with blocking anti-IL-1 and anti-TNFα antibodies during *M. tuberculosis* infection significantly prolonged survival (Garlanda et al., 2007a).

In *Candida albicans* or *Aspergillus fumigatus* infections, Tir8/Sigirr-deficient mice showed increased susceptibility to mucosal and disseminated or lung infections, respectively, in terms of mortality and fungal burden, which were associated to increased IL-1 signaling and heightened Th17 cell response (Bozza et al., 2008). The IL-17 pathway plays an important role in the protective antifungal host defense (Van De Veerdonk et al., 2011), however, rigorous control of the inflammatory Th17 response is necessary to prevent immunopathology (Park et al., 2005; Zelante et al., 2007). Thus, Tir8/Sigirr-deficient mice phenotype was possibly dependent on IL-1-mediated deregulated Th17 responses.

In a model of keratitis induced by *P. aeruginosa*, TIR8/SIGIRR was involved in down-regulating Th1 immunity and associated IL-1RI and TLR4-activation, thus preventing tissue damage and promoting resistance to infection (Huang et al., 2006). On the same line, in acute lung infections caused by *P. aeruginosa*, a Gram-negative pathogen responsible of life-threatening infections

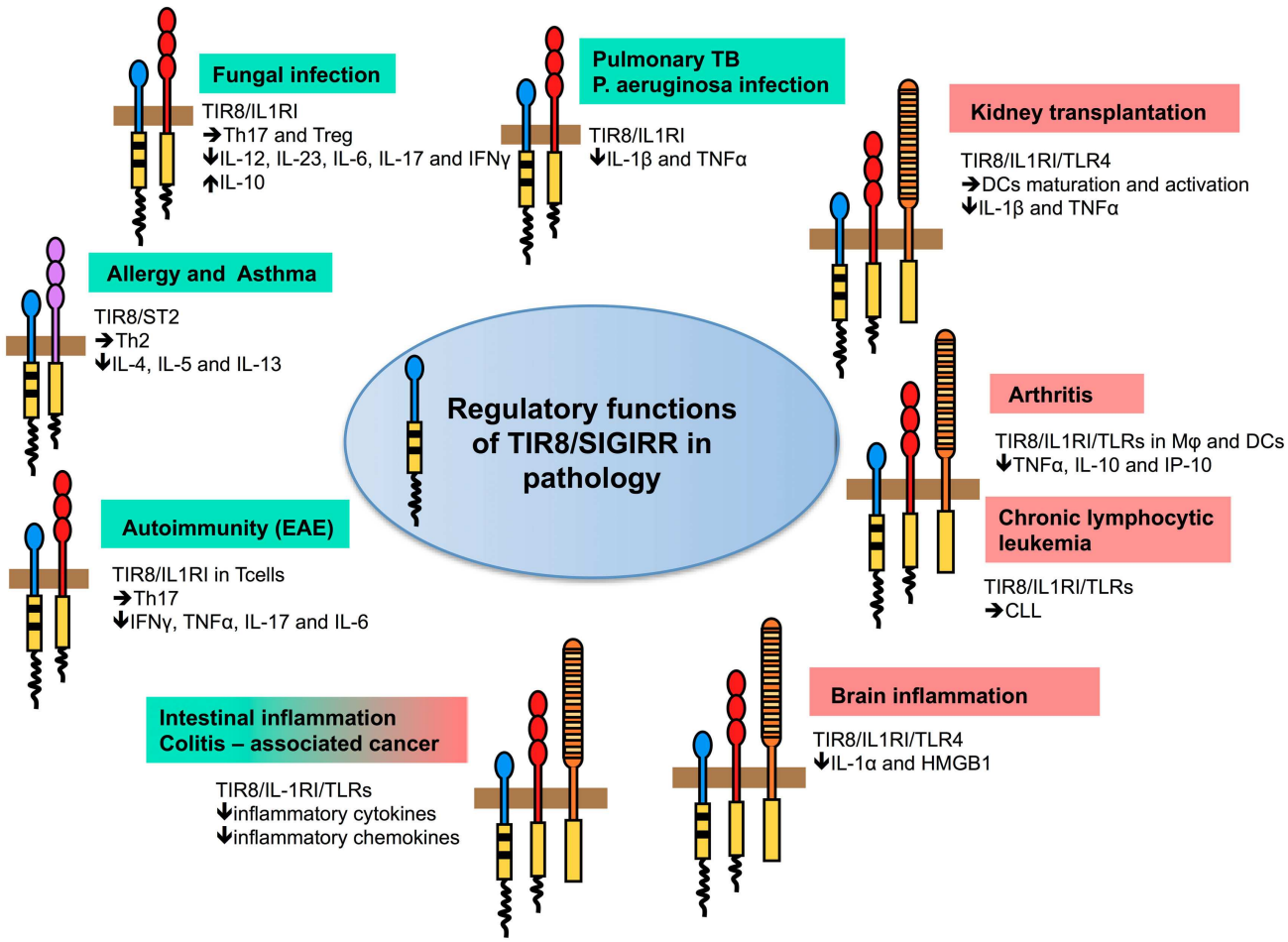

FIGURE 3 | Regulatory function of TIR8/SIGIRR in pathology. TIR8/SIGIRR inhibits ILR (IL-1RI, IL-18R, T1/ST2) and TLR (TLR4, TLR7, TLR9, and possibly others) signaling and NF-κB activation. Gene-targeted mice demonstrate that Tir8/Sigirr acts as a non-redundant negative regulator *in vivo* in different inflammatory conditions dependent on ILR and TLR activation. For some of these, the pathway prominently regulated by TIR8/SIGIRR and playing a relevant role in the pathogenesis of the disease has been defined. For instance, in autoimmunity (EAE) TIR8/SIGIRR regulates IL-1-dependent Th17 cell differentiation, survival, and proliferation; in asthma TIR8/SIGIRR regulates the IL-33/ST2 pathway and Th2 responses; in models of infections (*Candida albicans*, *Aspergillus fumigatus*, *Mycobacterium tuberculosis*, and *Pseudomonas aeruginosa*) TIR8/SIGIRR regulates IL-1RI signaling controlling Th17 and Treg responses (fungal infections) and pro-inflammatory cytokine production (bacterial infections). In colitis-associated colon cancer, TIR8/SIGIRR plays a major role in controlling microbial flora-dependent activation of TLRs, but the control on ILRs-dependent inflammation can not be excluded. In other contexts (chronic lymphocytic leukemia, arthritis, kidney transplantation, and brain inflammation), the molecular pathway regulated by TIR8/SIGIRR remains to be addressed.

in immunocompromised individuals and cystic fibrosis patients, Tir8/Sigirr-deficiency was associated to increased susceptibility in terms of mortality and bacterial load, and to exacerbated local and systemic production of pro-inflammatory cytokines and chemokines. IL-1RI-deficiency rescued the phenotype observed in Tir8/Sigirr-deficient mice. This suggests the non-redundant role of TIR8/SIGIRR in negatively regulating IL-1RI signaling, which plays a major role in the pathogenesis of *P. aeruginosa* infection (Veliz Rodriguez et al., 2012).

Thus, in both tuberculosis, *Candida*, and *P. aeruginosa* infections, the major protective role identified for TIR8/SIGIRR was the suppression of excessive IL-1-dependent inflammatory pathology, since inhibition of IL-1-signaling was sufficient to rescue the phenotype of Tir8/Sigirr-deficient mice in these infections.

In dextran sodium sulfate (DSS) colitis model, Tir8/Sigirr-deficient mice exhibited a dramatic intestinal inflammation compared to wild type mice in terms of weight loss, intestinal bleeding, local tissue damage, and mortality, which was associated to increased leukocyte infiltration and pro-inflammatory cytokine, chemokine, and prostaglandin production (Garlanda et al., 2004; Xiao et al., 2007). In homeostatic conditions, TIR8/SIGIRR was described to be involved in controlling gut homeostasis. Indeed, TLRs signaling activated by commensal gut microflora provides the survival signal for intestinal epithelial cells (Rakoff-Nahoum et al., 2004; Karin et al., 2006), and Tir8/Sigirr-deficiency was associated to increased proliferation in colon crypts and decreased apoptosis (Xiao et al., 2007). Tir8/Sigirr-deficient colon epithelial cells revealed constitutive NF-κB and JNK activation and

Table 2 | Regulatory functions of TIR8/SIGIRR in disease.

	Reference
INFECTION-DEPENDENT INFLAMMATION	
Mycobacterium tuberculosis lung infection	Garlanda et al. (2007a)
Mucosal and disseminated *Candida albicans* infections	Bozza et al. (2008)
Aspergillus fumigatus lung infections	Bozza et al. (2008)
Pseudomonas aeruginosa keratitis	Huang et al. (2006)
Pseudomonas aeruginosa lung infection	Veliz Rodriguez et al. (2012)
Dextran sodium sulfate (DSS) colitis model	Garlanda et al. (2004), Xiao et al. (2007)
Survival to endotoxin	Wald et al. (2003)
LPS-dependent acute lung injury	Chen et al. (2011)
AUTOIMMUNE DISEASES AND ALLERGY	
Experimental autoimmune encephalomyelitis (EAE)	Gulen et al. (2010)
Systemic lupus erythematosus(lpr and pristane models)	Lech et al. (2008, 2010)
Rheumatoid arthritis	Drexler et al. (2010)
OVA-induced allergic pulmonary inflammation	Bulek et al. (2009)
KIDNEY STERILE INFLAMMATION	
Postischemic acute renal failure	Lech et al. (2009)
Kidney transplantation	Noris et al. (2009)
BRAIN INFLAMMATION	
LPS-dependent sickness behavior and age-related neuroinflammation	Watson et al. (2010)
Cognitive and synaptic functions	Costelloe et al. (2008), Costello et al. (2011)
CANCER	
Colitis-associated cancer (AOM/DSS model)	Garlanda et al. (2007b), Xiao et al. (2007)
Spontaneous intestinal cancer ($Apc^{min/+}$ model)	Xiao et al. (2010)
Chronic lymphocytic leukemia (TCL1 transgenic mouse model)	Bertilaccio et al. (2011)

up-regulated expression of Cyclin D1 and Bcl-xL, which were further increased by treatment with IL-1 or LPS and returned to the control level after depletion of the commensal bacteria (Xiao et al., 2007). This spontaneous phenotype was not confirmed in other studies (Garlanda et al., 2004, 2007b), possibly a reflection of animal house-dependent variation in microbiota.

Finally, after endotoxin challenge, survival was reduced in Tir8/Sigirr-deficient mice on a BALB/c background (Wald et al., 2003), and overexpression of Tir8/Sigirr in lung epithelial cells suppressed the inflammatory reaction and improved the survival of BALB/c mice in a model of LPS-dependent Acute Lung Injury (Chen et al., 2011). However, Tir8/Sigirr-deficient mice on a C57BL/6 × 129/Sv background showed normal systemic or local inflammatory reactions to LPS (Garlanda et al., 2004).

The relevance in human of data obtained in mice is supported by a recent genetic analysis suggesting for the first time associations of TIR8/SIGIRR gene region SNPs with susceptibility to an infectious disease, in particular with the development of both pulmonary tuberculosis and tuberculous meningitis in independent cohorts of Vietnamese subjects (Horne et al., 2012).

ROLE OF TIR8/SIGIRR IN AUTOIMMUNE DISEASES AND ALLERGY

Members of the ILRs family have emerged as key players in T cell polarization (Schmitz et al., 2005; Acosta-Rodriguez et al., 2007). In particular, IL-1 has been identified as a critical factor in the differentiation and activation of Th17 cells, which mediate the development of autoimmune and inflammatory diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus, multiple sclerosis, psoriasis, and inflammatory bowel disease (IBD; Mills, 2011); whereas, IL-33 has been implicated in the initiation and propagation of Th2 immune responses, involved in allergy and asthma (Lloyd, 2010).

Gulen et al. (2010) recently showed that TIR8/SIGIRR was induced during Th17 cell differentiation and that it suppressed Th17 cell differentiation, proliferation, and cytokine production. TIR8/SIGIRR was shown to act through direct inhibition of multiple IL-1-dependent signaling pathways in T cells, in particular mTOR, a key transcription factor in IL-1-dependent Th17 cell proliferation. TIR8/SIGIRR suppressed IL-1 signaling during initial differentiation of Th17 cells as well as in differentiated effector Th17 cells, acting as key regulator to prevent overactivation of Th17 cell-mediated pathogenic effects. Indeed, regulation of IL-1R-mTOR pathway of Th17 development and activation by TIR8/SIGIRR was critical for the control of Th17 cell-dependent development of central nervous system (CNS) autoimmune inflammation (**Figure 3**; **Table 2**). In the absence of this IL-1 regulatory mechanism, Tir8/Sigirr-deficient mice were more susceptible to experimental autoimmune encephalomyelitis (EAE) resulting from hyperactivation of Th17 cells upon immunization with myelin oligodendrocyte glycoprotein (MOG) peptide, which infiltrated the CNS in greater numbers and showed enhanced pathogenic functions compared to wild type Th17 cells (Gulen et al., 2010).

In addition to IL-1 signaling in Th17 cells, TLRs signaling in innate immune cells, antigen presenting cells, and T

cells plays a pathogenetic mechanism in different autoimmune diseases (Mills, 2011). In particular, immune complexes containing the lupus autoantigen U1snRNP or nucleosomes activate DCs and autoreactive B-cells via TLR7 and TLR9, respectively, contributing in this way to pathogenesis of systemic lupus erythematosus (Leadbetter et al., 2002; Marshak-Rothstein and Rifkin, 2007). Genetic approaches showed that Tir8/Sigirr-deficiency alone did not induce autoimmunity against DNA, however, the deficiency of Tir8/Sigirr in C57BL/6lpr/lpr mice, which develop delayed autoimmunity due to impaired Fas-induced apoptosis of autoreactive B and T cells, caused massive lymphoproliferation, peribronchial inflammation and mesangioproliferative glomerulonephritis (Lech et al., 2008). This autoimmune tissue damage was associated with increased production of autoantibodies (anti-dsDNAIgG, anti-nucleosome, anti-Sm antigen, anti-snRNP, and rheumatoid factor) and early development of hypergammaglobulinemia. Tir8-deficient lpr/lpr mice showed enhanced activation of DCs to complexed lupus autoantigens, increased production of pro-inflammatory cytokines (e.g., CCL2, IL-6, and IL-12p40) and B-cell survival factors (e.g., Baff/BlyS and Bcl-2), increased B cell proliferation upon exposure to RNA and DNA immune complexes and other TLRs agonists, and production of lupus autoantibodies. Thus, the pathogenetic mechanisms underlying the susceptibility of Tir8/Sigirr-deficient mice in lupus include increased activation of antigen presenting cells that handle autoantigens, proliferation of autoreactive B lymphocytes, and production of immunoregulatory factors. On the same line, Tir8/Sigirr-deficiency caused increased susceptibility to lupus nephritis in a model, which mimics environmentally induced autoimmunity: intraperitoneal injection of hydrocarbon oil (pristane) causes persistent abundance of apoptotic peritoneal cells, chronic granulomatous peritonitis, ectopic lymphoid tissue formation, and evolution of antinuclear antibodies, immune complex disease, and lupus nephritis. In this model, a major pathogenetic role is played by TLR7 signaling and type I interferon (Savarese et al., 2008). Tir8/Sigirr protected from hydrocarbon oil-induced lupus by suppressing the TLR7-mediated activation of DCs and expansion of IgG and RNA autoreactive lymphocyte clones (Lech et al., 2010). Structure model prediction identified the BB-loop of TIR8/SIGIRR intracellular TIR domain to interact with TLR7 and MyD88 (Gong et al., 2010) and indeed, BB-loop deletion was sufficient to completely abrogate TIR8/SIGIRR inhibitory effect on TLR7 signaling in DCs (Lech et al., 2010). The relevance of genetic variants of the TIR8/SIGIRR gene in susceptibility to SLE was recently investigated in a large European-descent population, but the analysis was restricted to a single missense polymorphism (rs3210908), which results in the replacement of a glutamine (Gln) for arginine (Arg) at amino acid 312 (Q312R) and it did not reveal any association between *TIR8/SIGIRR* gene variants and SLE (Sanchez et al., 2012).

Increasing evidence implicate TLRs signaling also in RA (Sacre et al., 2007; Abdollahi-Roodsaz et al., 2009; Drexler and Foxwell, 2010). Overexpression of TIR8/SIGIRR in RA synovial cells led to a significant inhibition of spontaneous release of pro-inflammatory mediators, suggesting that either ILRs and/or TLRs signaling was at least partly responsible for the chronic production of those mediators in RA (Drexler et al., 2010). The role of TIR8/SIGIRR as an inhibitor of inflammation was confirmed *in vivo*, since Tir8/Sigirr-deficient mice developed a more severe disease in both the zymosan-induced arthritis and collagen antibody-induced arthritis models, which was due to increased cellular infiltration into the affected joints (Drexler et al., 2010). IL-1 plays a significant role in zymosan-induced arthritis model, and IL-1Ra reduced the disease severity in Tir8/Sigirr-deficient mice, but did not completely rescued the phenotype, suggesting that additional factors, including TLRs ligands, are driving pathology and are under Tir8/Sigirr control (**Figure 3**; **Table 2**). In agreement with this study, a gene expression study showed that *TIR8/SIGIRR* was one among the genes with the most significantly reduced expression in peripheral blood cells of patients with psoriatic arthritis compared to control subjects. *TIR8/SIGIRR* clustered with other genes involved in downregulation or suppression of innate and acquired immune responses, suggesting inappropriate control that favors pro-inflammatory responses (Batliwalla et al., 2005).

T1/ST2, the receptor of IL-33 preferentially expressed in Th2 cells, is a further ILRs controlled by TIR8/SIGIRR. IL-33 plays a major role in Th2 responses by inducing Th2 cytokines IL-4, IL-5, IL-13, splenomegaly, eosinophilia, and allergy (Schmitz et al., 2005). TIR8/SIGIRR was shown tobe expressed during Th2-polarization and to inhibit IL-33-and T1/ST2-mediated signaling and Th2 cytokine (IL-4, IL-5, and IL-13) production *in vitro* and *in vivo* (Bulek et al., 2009). Indeed, Tir8/Sigirr-deficient mice showed hyper responsiveness to IL-33 with increased serum levels of IL-5 and IL-13, splenomegaly, lung inflammation, and exacerbated Th2 responses in OVA-induced allergic pulmonary inflammation. These results indicate that Tir8/Sigirr serves as a negative feedback control in Th2-polarization and restimulation, thus controlling allergic inflammatory responses (Bulek et al., 2009; **Figure 3**; **Table 2**). However, a genetic analysis performed on about 850 asthma patients and 640 healthy subjects failed to identify any association between 12 *TIR8/SIGIRR* polymorphisms or haplotypes identified in this Japanese population with asthma susceptibility or asthma-related phenotype (Nakashima et al., 2006).

ROLE OF TIR8/SIGIRR IN KIDNEY STERILE INFLAMMATION

Among solid organs and tissues, TIR8/SIGIRR is highly expressed in the kidney, in particular by tubular epithelial cells, DCs, and macrophages, but functional activity of TIR8/SIGIRR in suppressing TLRs-induced expression of pro-inflammatory cytokines has been demonstrated in renal immune cells but not in renal parenchymal cells (Lech et al., 2007). The relevance in pathology of TIR8/SIGIRR expression in kidney has been demonstrated in different conditions, such as lupus nephritis (Lech et al., 2008, 2010; discussed above) and postischemic acute renal failure or kidney transplantation (Lech et al., 2009; Noris et al., 2009), conditions associated to TLRs activation by nucleosomes and DAMPs released during ischemic cell necrosis, respectively (**Figure 3**; **Table 2**).

Postischemic acute renal failure represents a state of sterile inflammation, in which DAMPs activate innate immune elements, mostly neutrophils and macrophages, which rather enhances the subsequent tissue injury than promoting the healing phase, in particular through TLR4 and TLR2 (Leemans et al., 2005). In a postischemic renal failure model, intrarenal DCs or macrophages

were excessively activated in Tir8/Sigirr-deficient mice (Lech et al., 2009). Hyper activation of myeloid cells increased intrarenal cytokine and chemokine production and consequently, leukocyte recruitment, and renal injury. Renal ischemia/reperfusion studies with chimeric mice confirmed the primary role of Tir8/Sigirr in suppression of hematopoietic cell activation as compared to tubular epithelial cell, since lack of Tir8/Sigirr in hematopoietic cells largely reproduced the phenotype of renal IR injury seen in Tir8/Sigirr-deficient mice.

In the same line, Noris et al. (2009) showed that the early post transplant kidney inflammatory response was more severe in Tir8/Sigirr-deficient grafts, as shown by increased numbers of infiltrating neutrophils and macrophages and higher TNFα and chemokine expression, and was followed by an amplified adaptive immune response against donor antigens, so that acute allograft rejection occurred within 7–10 days after transplantation. Indeed, the lack of Tir8/Sigirr in the kidney graft led to amplification of all downstream effects of ischemia/reperfusion-induced TLRs/IL-1R-dependent inflammation in the graft. The expansion and maturation of DCs in the graft, Th1 T cell priming, and block of Treg development finally resulted in acute rejection, thus demonstrating a role for renal Tir8/Sigirr as a first-line regulation of allogeneic immune response *in situ* (Noris et al., 2009).

In contrast, lack of Tir8/Sigirr did not affect the intrarenal mRNA expression of pro-inflammatory chemokines, profibrotic mediators, or markers of renal fibrosis after unilateral ureter obstruction, nor the number of intrarenal macrophages and myofibroblasts or tissue remodeling in post obstructive kidneys (Skuginna et al., 2011). These results are in agreement with data showing that TLR2-, TLR9-, and MyD88-signaling do not significantly contribute to this model (Chowdhury et al., 2010).

ROLE OF TIR8/SIGIRR IN BRAIN INFLAMMATION

TIR8/SIGIRR is also present in brain (Polentarutti et al., 2003; Costelloe et al., 2008) where it is expressed on neurons, microglia, and astrocytes (Andre et al., 2005).

TIR8 was shown to play a functional role in suppressing microglial activation by LPS and overexpression of CD40 and ICAM-1 or the production of TNFα, IL-6, and other pro-inflammatory mediators, both *in vitro* and *in vivo*, in hippocampal tissue. The effect of LPS on exploratory behavior, the so called sickness behavior, as well as age-related neuroinflammation were also accentuated in Tir8/Sigirr-deficient mice and were associated with increased hippocampal expression of CD14 and TLR4, and NF-κB activation (Watson et al., 2010). In addition, in the absence of any external inflammatory stimulus, cognitive, and synaptic functions, such as novel object recognition, spatial reference memory, and long-term potentiation (LTP), were defective in Tir8/Sigirr-deficient mice and were associated with up-regulation of IL-1RI- and TLR4-mediated signal transduction in hippocampus (Costello et al., 2011; **Table 2**). IL-1ra, an anti-TLR4 antibody, and also the inhibition of JNK and NF-κB restored the decrease in synaptic functions in Tir8/Sigirr-deficient mice, demonstrating the key role of IL-1RI-and TLR4-activation by IL-1α and high mobility group box 1 (HMGB1), respectively, in this model. These findings highlighted the functional role of Tir8/Sigirr in regulating inflammatory mediated synaptic and cognitive decline, and described evidence of the key role of HMGB1 in this process (Costello et al., 2011).

Finally, it has been suggested that IL-36Ra has anti-inflammatory effects in the brain since it abrogated IL-1 and LPS-induced inflammatory responses specifically in glial cells and IL-1- or LPS-induced inhibition of LTP and the associated increase in IL-1β concentration. These effects of IL-36Ra depended on IL-4 production and were absent in mixed glia prepared from Tir8/Sigirr-deficient mice, suggesting that the anti-inflammatory effects of this cytokine are mediated, at least in part, by TIR8/SIGIRR (Costelloe et al., 2008).

ROLE OF TIR8/SIGIRR IN INTESTINAL CANCER

The connection between cancer and inflammation is well recognized in tumors epidemiologically linked to inflammatory processes. In addition, an inflammatory component is present in the microenvironment of most neoplastic tissues and includes the infiltration of white blood cells, prominently tumor-associated macrophages (TAM); the presence of inflammatory cytokines (e.g., TNFα, IL-1, IL-6, IL-23, IL-17, chemokines, such as CCL2); the occurrence of tissue remodeling and angiogenesis (Mantovani et al., 2008; Colotta et al., 2009; Biswas and Mantovani, 2010; Ben-Neriah and Karin, 2011). Two pathways link inflammation and cancer: in the intrinsic pathway, activation of different classes of oncogenes drives the expression of inflammation-related programs, which guide the construction of an inflammatory microenvironment, whereas in the extrinsic pathway, inflammatory conditions promote cancer development (e.g., colitis-associated cancer (CAC) of the intestine; Colotta et al., 2009). NF-κB is one of the key orchestrators of cancer-related inflammation (Ben-Neriah and Karin, 2011).

TIR8/SIGIRR was therefore a candidate player potentially involved in cancer-related inflammation and was first studied in a model of CAC, a colorectal disease that arises in patients suffering from chronic IBD, in particular Ulcerative Colitis. In the model of CAC induced by the pro-carcinogen Azoxymethane (AOM), followed by exposure to DSS, which causes chronic inflammation, Tir8/Sigirr-deficient mice exhibited increased susceptibility to intestinal carcinogenesis, in terms of number, size, and severity of lesions, in agreement with the increased susceptibility to inflammation (Garlanda et al., 2007b; Xiao et al., 2007; **Figure 3**; **Table 2**). Increased carcinogenesis was associated with increased permeability, local production of pro-inflammatory mediators, such as prostaglandin E_2, inflammatory cytokines and chemokines, and expression of genes involved in cell survival and proliferation (Bcl-xL and Cyclin D1) downstream of NF-κB. Gut epithelial cells played a pivotal role in mediating the regulatory functions of TIR8/SIGIRR, since TIR8/SIGIRR overexpression in gut epithelium rescued Tir8/Sigirr-deficient mice from developing severe CAC (Xiao et al., 2007). Studies with mice deficient of TLRs, ILRs, MyD88, or Tir8/Sigirr suggest that a fine balance of pro- and anti-inflammatory signals induced by commensal bacteria through TLRs- or ILRs-signaling is necessary for the homeostatic regulation of colon epithelium proliferation and apoptosis as well as for inflammatory responses, mechanisms of repair, and colitis-associated tumorigenesis (Rakoff-Nahoum et al., 2004; Araki et al., 2005; Salcedo et al., 2010). In the AOM-DSS CAC model, mucosal

damage induced by DSS causes exposure to commensal microbiota and enhanced migration of bacteria to the mesenteric lymph nodes (Vaishnava et al., 2008). Thus, in this model, Tir8/Sigirr plays a protective role probably by modulating the levels of TLRs signaling in the epithelial cells directly through its interaction with the TLRs that are activated by commensal bacteria. However, the control on pathways activated by TLRs signaling and involving ILRs can not be excluded. In addition, mediators downstream of NF-κB, such as IL-6, which has been shown to promote cancer growth in inflammation-associated cancer models through STAT3 activation, and chemokines, which promote leukocyte recruitment and angiogenesis in gastrointestinal neoplasia (Karin, 2006), were increased in Tir8/Sigirr-deficient mice treated with AOM and DSS.

The role of Tir8/Sigirr was recently investigated in the $Apc^{min/+}$ model, a spontaneous intestinal cancer model mimicking the Familial Adenomatous Polyposis syndrome, where loss of heterozygosity (LOH) of the tumor suppressor Apc is the exclusive genetic alteration leading to the tumor initiation in the $Apc^{min/+}$ mouse (Yamada et al., 2002). Akt-mTOR signaling is a critical pathway driving tumor initiation in the $Apc^{min/+}$ mouse, since it promotes cell cycle progression through posttranscriptional control of the key cell cycle regulators, such as Cyclin D1, cyclin E, and c-Mycand LOH of Apc (Aoki et al., 2003). Xiao et al. (2010) found that Tir8/Sigirr-deficiency in the $Apc^{min/+}$ mouse led to increased microadenoma formation, resulting in spontaneous colonic polyposis. Tir8/Sigirr-deficiency was associated with increased Akt-mTOR signaling and tumor initiation through its impact on cell proliferation and LOH of Apc in epithelium. This phenotype was dependent on the presence of commensal bacteria in the gut, implicating a critical role of TLRs signaling in colonic tumorigenesis in $Apc^{min/+}$ mice; however, hyperactivation of mTOR was also observed upon stimulation of epithelial cells with IL-1. Thus, this study suggests that Tir8/Sigirr is a tumor suppressor that controls colonic tumorigenesis by inhibiting IL-1- and TLRs-induced mTOR-mediated cell cycle progression and consequent genetic instability (Xiao et al., 2010).

ROLE OF TIR8/SIGIRR IN CHRONIC LYMPHOCYTIC LEUKEMIA

Chronic lymphocytic leukemia (CLL) is the most frequent leukemia in the western world and accounts for about 40% of all leukemias in adults over the age of 65 years. CLL is characterized by the accumulation of $CD5^+$ monoclonal B-cells in primary and secondary lymphoid tissues. Over time and because of unknown molecular events CLL may progress into an aggressive form characterized by a prolymphocytoid transformation; small cells are gradually replaced by clonally related, larger elements (prolymphocytes). Genetic defects are involved in the pathogenesis of the disease including repeated mutations in the MyD88, XPO1, and NOTCH1 genes (Puente et al., 2011; Rossi et al., 2012). Furthermore, stimuli originating from the microenvironment appear to contribute to the selection and expansion of the malignant clone (Ghia and Caligaris-Cappio, 2006) and CLL is emerging as a prototype of cancers where both genetic and micro environmental factors concur to the development, expansion and progression of the disease (Muzio et al., 2009a,b). Both normal and leukemic human B-cells express detectable levels of *TIR8/SIGIRR* mRNA (Muzio et al., 2009b); however, by PCR array analysis, malignant B-cells appeared to have low levels of mRNA (Arvaniti et al., 2011). Nevertheless, since protein levels of TIR8/SIGIRR may be differentially regulated (Veliz Rodriguez et al., 2012), it will be important to analyze TIR8/SIGIRR expression on the cell surface of normal and leukemic B-cells. As regarding to mouse models, an analysis was performed in TCL1 transgenic animals, a well characterized mouse model of CLL (Bichi et al., 2002). Both total and $CD19^+$ enriched splenocytes expressed detectable *Tir8/Sigirr* mRNA levels that were significantly lower in $CD19^+$ cells from TCL1 transgenic mice. Moreover, peritoneal B1 cells (which are marked by CD5 expression) expressed lower levels of *Tir8/Sigirr* mRNA compared with splenic B-cells suggesting that an intrinsic program of $CD5^+$ B-cells may regulate *Tir8/Sigirr* expression (Bertilaccio et al., 2011).

To address the functional involvement of TIR8/SIGIRR in CLL, *Tir8/Sigirr* was genetically inactivated in the TCL1 transgenic mouse model (Bertilaccio et al., 2011). The absence of Tir8/Sigirr did not modify normal B cell populations in wild type mice. In contrast, lack of Tir8/Sigirr in TCL1 transgenic mice accelerated appearance of monoclonal B-cell expansions and mouse life span was shortened. The morphology and phenotype of the mouse leukemic expansions reproduced the progression of human CLL into an aggressive phase characterized by the appearance of prolymphocytes (Bertilaccio et al., 2011; **Figure 3**; **Table 2**). Overall, these observations suggest an inhibitory role of TIR8/SIGIRR in CLL onset and progression. However, it is not clear whether TIR8/SIGIRR exerts its activity directly onto the malignant clone or indirectly through the tumor microenvironment, and which molecular mechanisms are involved. Indeed, different TLRs ligands can lead to either proliferation (TLR2, TLR4, TLR9; Tarnani et al., 2010), apoptosis (TLR3 and TLR9; Liang et al., 2010), or chemoresistance (TLR7 and TLR8; Cherfils-Vicini et al., 2010) of different cancer cells (Rakoff-Nahoum and Medzhitov, 2009). Examples of endogenous TLRs or ILRs ligands, which could constitutively activate cognate membrane receptors in the malignant clone or cells of the tumor microenvironment in the absence of TIR8/SIGIRR, are ILRs family cytokines such as IL-1, IL-18, IL-33, and TLRs ligands including PAMPs derived from intestinal microflora and/or DAMPs containing autoantigens. Thus, this study shows that unabated TLRs and/or ILRs stimulation is functionally involved in the development and progression of CLL, and that TIR8/SIGIRR plays a non-redundant role in controlling this process.

CONCLUDING REMARKS

Interleukin-1R like receptors and TLRs are key inflammatory receptors involved in inflammation and immunity recognizing IL-1 family ligands released in several conditions including sterile inflammation and cell death, or PAMPs and DAMPs, respectively. Available information is consistent with the view that TIR8/SIGIRR is a conserved and broadly expressed negative regulator of inflammation, tissue damage, autoimmunity, and cancer (**Figure 3**; **Table 2**), acting by negatively regulating ILRs- or TLRs-dependent signaling, possibly by interfering with the recruitment of TIR domain containing signaling molecules (**Figure 2**). Although TIR8/SIGIRR is still considered an orphan receptor, IL-36Ra has been proposed as brain-specific TIR8/SIGIRR ligand,

and the possibility of other TIR8/SIGIRR recognizing molecule(s) cannot be dismissed.

Members of the ILR family have been involved in T cell polarization and proliferation (Chan et al., 2001; Neighbors et al., 2001; Schmitz et al., 2005; Acosta-Rodriguez et al., 2007) and TIR8/SIGIRR has emerged as a negative regulator affecting Th1, Th2, and Th17 differentiation, which play a major role in autoimmunity and sterile inflammation (Garlanda et al., 2004, 2007b; Xiao et al., 2007; Bozza et al., 2008; Bulek et al., 2009).

Thus, strong genetic evidence in mice is consistent with a non-redundant regulatory function of TIR8/SIGIRR in pathogen-dependent as well as sterile inflammation, innate immunity, and polarized adaptive responses. Data supporting its relevance to human disease are still scarce, however, preliminary genetic evidence in humans and gene expression studies support the hypothesis that TIR8/SIGIRR could play a regulatory function also in human inflammatory conditions.

ACKNOWLEDGMENTS

The contributions of the European Commission (European Research Council project HIIS, MUGEN LSHG-CT-2005-005203, MUVAPRED LSH-CT-2003-503240), Ministero dell'Istruzione, dell'Università e della Ricerca [progetto FIRB RBLA039LSF (www.miur.it)], Associazione Italiana per la Ricerca sul Cancro (AIRC), Fondazione CARIPLO, and the Italian Cystic Fibrosis Research Foundation (http://www.fibrosicisticaricerca.it) are gratefully acknowledged.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 19 July 2012; paper pending published: 17 August 2012; accepted: 05 October 2012; published online: 29 October 2012.

Citation: Riva F, Bonavita E, Barbati E, Muzio M, Mantovani A and Garlanda C (2012) TIR8/SIGIRR is an interleukin-1 receptor/toll like receptor family member with regulatory functions in inflammation and immunity. Front. Immun. ***3****:322. doi: 10.3389/fimmu.2012.00322*

This article was submitted to Frontiers in Inflammation, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 18 September 2012
doi: 10.3389/fimmu.2012.00288

NLRP3-inflammasome activating DAMPs stimulate an inflammatory response in glia in the absence of priming which contributes to brain inflammation after injury

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Inflammation in the absence of infection (sterile inflammation) contributes to acute injury and chronic disease. Cerebral ischemia is a devastating condition in which the primary injury is caused by reduced blood supply and is therefore sterile. The cytokine interleukin-1β (IL-1β) is a key contributor to ischemic brain injury and central inflammatory responses. The release of IL-1β is regulated by the protease caspase-1, and its activating complex, the inflammasome. Of the known inflammasomes the best characterized, and one that is perceived to sense sterile injury is formed by a pattern recognition receptor called NOD-like receptor pyrin domain containing three (NLRP3). A key feature of NLRP3-inflammasome dependent responses *in vitro* in macrophages is the requirement of an initial priming stimulus by a pathogen (PAMP), or damage associated molecular pattern (DAMP) respectively. We sought to determine the inflammatory responses of NLRP3-activating DAMPs on brain derived mixed glial cells in the absence of an initial priming stimulus *in vitro*. In cultured mouse mixed glia the DAMPs ATP, monosodium urate, and calcium pyrophosphate dehydrate crystals had no effect on the expression of IL-1α or IL-1β and induced release only when the cells were primed with a PAMP. In the absence of priming, these DAMPs did however induce inflammation *via* the production of IL-6 and CXCL1, and the release of the lysosomal protease cathepsin B. Furthermore, the acute phase protein serum amyloid A (SAA) acted as a priming stimulus on glial cells resulting in levels of IL-1 expression comparable to those induced by the PAMP lipopolysaccharide. *In vivo*, after cerebral ischemia, IL-1 production contributed to increased IL-6 and CXCL1 since these cytokines were profoundly reduced in the ischemic hemispheres from IL-1α/β double KO mice, although injury-induced cytokine responses were not abolished. Thus, DAMPs augment brain inflammation by directly stimulating production of glial derived inflammatory mediators. This is markedly enhanced by DAMP-induced IL-1-release-dependent responses that require a sterile endogenous priming stimulus such as SAA.

Keywords: inflammation, caspase-1, priming, interleukin-1, NLRP3-inflammasome, cerebral ischemia

INTRODUCTION

Interleukin-1β (IL-1β) is a key pro-inflammatory cytokine that is central to the damaging inflammatory processes that accompany sterile disease (Dinarello, 2011). This is particularly true after an acute brain injury such as cerebral ischemia, or stroke, where IL-1β is established as a major contributor to damage (Brough et al., 2011). It is produced during disease or after an injury as an inactive precursor (pro-IL-1β) by cells of the innate immune system such as macrophages, or in diseases of the central nervous system (CNS), by microglia (Denes et al., 2008). In order for it to exert any biological effects it must be cleaved into an active molecule and released from the cell whereby it can act on the type I IL-1 receptor (IL-1RI) on responsive cells (Luheshi et al., 2009). A key protease required for the processing of pro-IL-1β is caspase-1. The activity of caspase-1 is regulated by its recruitment to multi-molecular scaffolds called inflammasomes following an inflammatory stress (Schroder and Tschopp, 2010). Inflammasomes are composed of a cytosolic pattern recognition receptor (PRR), pro-caspase-1, and, depending on the PRR, an adaptor molecule. The best characterized inflammasome forming PRR, and the one most implicated as a sensor of sterile injury, is NOD-like receptor pyrin domain containing three (NLRP3; Cassel and Sutterwala, 2010; Schroder and Tschopp, 2010). NLRP3 can be activated by a diverse array of disease associated molecules, where it oligomerizes with the adaptor ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) and caspase-1 to form the NLRP3-inflammasome, resulting in the processing of pro- to mature IL-1β and its release.

The release of IL-1β is considered to be a two step process (Hornung and Latz, 2010; Lopez-Castejon and Brough, 2011). IL-1β is not normally expressed and so its expression must be induced. Stimuli that do this are pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs; Chen and Nunez, 2010; Takeuchi and Akira, 2010). PAMPs are motifs

carried by pathogens, such as bacterial endotoxin (or lipopolysaccharide, LPS) of Gram negative bacteria, and DAMPs are endogenous molecules modified during disease or that are released by necrosis. Stimuli that prime macrophages in this way are however generally inefficient secretion stimuli for IL-1β and the primed cells are required to encounter an additional PAMP or DAMP stimulus that triggers formation of the inflammasome, activation of caspase-1, and subsequently the processing and secretion of IL-1β. This comes from an extensive literature on macrophages, and although there is evidence that microglia respond in a similar way to PAMP and DAMP stimulation (Brough et al., 2002; Halle et al., 2008), these responses have yet to be fully characterized. Not only are microglia a unique cell type originating from the yolk sac that are self-renewing throughout life (Ginhoux et al., 2010), but the brain is also protected by the BBB in the absence of injury, which keeps microglia isolated from potential blood-derived priming stimuli. In addition, inflammatory events triggered by DAMPs, other than IL-1 expression or release, have not been explored in glial cells previously.

The effects of NLRP3-activating DAMPs are commonly associated with IL-1 and on primed cells (Hornung and Latz, 2010). This is at least in part because NLRP3 expression is itself dependent upon priming (Bauernfeind et al., 2009). However, in many situations of acute injury, where there is rapid and localized loss of tissue (such as cerebral ischemia for example) there will be an abundance of DAMPs that may stimulate cells that have not been subject to an initial priming stimulus. LPS (often used to prime cells *in vitro*) is unlikely to be present *in vivo* during sterile injury and endogenous priming stimuli in the brain remain poorly characterized. DAMPs such as monosodium urate (MSU), and calcium pyrophosphate dehydrate (CPPD) crystals induce the production of the pro-inflammatory cytokine IL-6 in monocytes (Guerne et al., 1989) and osteoblast like cells (Bouchard et al., 2002) in the absence of a priming stimulus. MSU is also known to act as a potent adjuvant in driving adaptive immune responses independently of the NLRP3-inflammasome (Kool et al., 2011) and potentially dependent upon Syk kinase (Ng et al., 2008). Inflammatory responses in the brain (after cerebral ischemia for example) may be influenced by both local DAMPs and circulating inflammatory mediators once the breakdown of the BBB has occurred. As acute brain injury is associated with a marked central inflammatory response the aim of this research was to identify the inflammatory responses of glial cells, inflammatory cells of the CNS, to key mediators of sterile injury, NLRP3-activating DAMPs, in the absence of cell priming, and in the presence of a relevant endogenous priming stimulus.

MATERIALS AND METHODS

MATERIALS

DMEM culture media was purchased from Sigma (UK). Fetal bovine serum (FBS), glutamine, and a streptomycin/penicillin antibiotic solution were all purchased from Invitrogen (UK). Bacterial LPS (*Escherichia coli* 026:B6), Poly(IC), and ATP were purchased from Sigma (UK). MSU and CPPD crystals were from Invivogen (UK). Serum amyloid A (SAA) was purchased from PeproTech (UK). All primers for qPCR were purchased from Qiagen (UK).

MIDDLE CEREBRAL ARTERY OCCLUSION, PERFUSION, AND TISSUE HOMOGENIZATION

We induced cerebral ischemia by middle cerebral artery occlusion (MCAo) as described previously (Denes et al., 2010a). Briefly, C57BL/6J mice (Harlan Olac) or IL-1αβ-deficient (IL-1αβ double KO) mice, weighing 26–32 g were anesthetized with isoflurane and were subjected to MCAo for 60 min using an intraluminal filament (180 μm diameter, left side occluded) followed by 24 h reperfusion. After transcardial perfusion with saline, brains were collected, and homogenized as described previously (Chapman et al., 2009). Protein concentrations were calculated using BCA assay (Pierce/Thermo Fisher Scientific). Some mice were perfused with 4% paraformaldehyde (PFA), and following post-fixation in PFA and cryoprotection in sucrose, brain sections were cut on a sledge microtome for immunohistochemistry and cresyl-violet staining. All animal procedures were performed under the University of Manchester project license number (40/3076) and adhered to the UK Animals (Scientific Procedures) Act (1986).

IMMUNOHISTOCHEMISTRY

Immunostaining was performed on free-floating brain sections as described (Denes et al., 2010a). After blocking with 2% normal donkey serum in PBS containing 0.3% Triton X-100, rabbit anti-Iba1 (WAKO, Germany) and rat anti-CD45 (Serotec, UK) antibodies were incubated overnight. Antigens were visualized using appropriate fluorochrome (Alexa 594, Alexa 488)-conjugated donkey secondary antibodies (Invitrogen). Mounted brain sections were coverslipped with ProLong mounting medium (Invitrogen) and analyzed on an Olympus BX51 microscope using a Coolsnap ES camera (Photometrics) through MetaVue software (Molecular Devices). CD45 and Iba 1 positive cells were quantified by counting six separate sections from the ipsilateral and contralateral hemispheres (striatum and cortex). The numbers of Iba 1 positive cells expressing CD45 were recorded for both hemispheres.

CELL CULTURE

Mixed glia were cultured from C57Bl/6J at post-natal day 1–4 as described previously (Pinteaux et al., 2002). Whole brains were dissected into DMEM with 10% FBS v/v and 1% P/S. Meninges were removed and cells dissociated by trituration prior to seeding at a density equivalent to one brain/60 cm^2. The culture medium was changed twice a week until cultures reached confluency (14–20 days). These cultures are composed of 78% astrocytes, 12% O2A progenitor cells, and 10% microglia (Pinteaux et al., 2002). Cultures were treated with LPS (1 μg/ml), poly(IC; 50 μg/ml), ATP (5 mM), MSU (250 μg/ml), CPPD (250 μg/ml), or SAA (0.03–3 μg/ml) for 24 h. Cultures subjected to PAMP and DAMP stimulation were treated with LPS or SAA for 24 h followed by ATP, MSU, or CPPD for 1 h.

QUANTITATIVE REAL-TIME PCR

RNA was extracted from cultured mixed glia using the TRIzol® method (Invitrogen) and reverse transcribed to cDNA using MMLV reverse transcriptase according to the manufacturer's instructions (Invitrogen). Specific primers for IL-1β, IL-1α, caspase-1, NLRP3, ASC, iNOS, IL-6, TNFα, CXCL1, and CXCL12 were purchased from Qiagen (QuantiTech Primer Assays) and

qPCR was performed using Power SYBR® Green PCR mastermix (Applied Biosystems) and SDS v2.3 (Applied Biosystems). For each primer set the CT threshold was set manually to achieve a slope efficiency of >99% and a single product on melt curve analysis. RNA from LPS-treated J774 macrophages was used to create a standard curve and gene expression was calculated using the relative standard curve method. Data were normalized to expression levels of the housekeeping gene SDHA (QuantiTech Primer Assays, Qiagen) across each treatment and fold change was expressed relative to basal RNA levels from untreated mixed glia.

DETECTION OF CYTOKINES BY ELISA

Measurement of key inflammatory cytokines (IL-1β, IL-1α, IL-6, CXCL1) released into the culture supernatant or expressed in the lysate was performed using specific ELISAs (R&D Systems, UK) according to manufacturers guidelines.

DETECTION OF CYTOKINES BY ELISA AND CYTOMETRIC BEAD ARRAY

Measurement of cytokines released into the culture supernatant or expressed in the lysate was performed using specific ELISAs (R&D Systems, UK) according to manufacturer's guidelines. Key inflammatory cytokines (IL-1β, IL-1α, IL-6, CXCL1) were measured in all tissues examined using appropriate cytometric bead array (CBA) Flex Sets (BD Biosciences) according to the manufacturer's protocol.

FLOW CYTOMETRIC ANALYSIS

Cultured mixed glia were resuspended using 0.5 mM EDTA in PBS. The following fluorochrome-labeled monoclonal antibodies were applied according to manufacturer's instructions: PE conjugated anti-CD11c (1:100, eBioscience), APC conjugated anti-MHCII (1:200, eBioscience), FITC conjugated anti-CD11b (1:200, eBioscience), and PerCP-Cy5.5 conjugated anti-CD45 (1:500, eBioscience). The surface expression of these markers were analyzed using CyAn advanced flow cytometer (Beckman Coulter) and Summit v4.3 software (Dako). Microglia were gated for analysis based on co-expression of CD11b and CD45.

WESTERN BLOTTING

Following the experiment supernatants were harvested and prepared in sample buffer containing 1% β-mercaptoethanol. Samples were boiled and then electrophoresed on 12% SDS-acrylamide gels. Proteins were subsequently transferred onto nitrocellulose membrane and blotted using polyclonal sheep anti-mouse IL-1β (1:1000 in 5% milk, NIBSC, UK), or polyclonal goat anti-mouse cathepsin B (1:500, R&D Systems). The membrane was then stained with polyclonal rabbit anti-sheep IgG horse radish peroxidise (HRP) conjugate for IL-1β (1:2000 in 5% milk, Dako, UK), or with HRP-conjugated rabbit anti-goat IgG for cathepsin B (1:1000 in 5% milk, Dako, UK), with subsequent exposure using enhanced chemi-luminescence (ECL) reagents (Amersham, UK).

GEL ZYMOGRAPHY

Released gelatinase activity was assessed by gelatin-substrate zymography as previously described (Kleiner and Stetler-Stevenson, 1994). Briefly, serum free supernatants from treated cultured mixed glia were mixed with an equal volume of loading buffer (10% SDS, 50% glycerol, 400 mM Tris-HCl pH 6.8, 250 μg/ml bromophenol blue). All samples were loaded neat onto 8% zymography gels except for one of the ATP-treated supernatants (+*) which was diluted 1:3. Following electrophoresis, gels were washed in 2.5% Triton X-100 to remove SDS. Proteinases were renatured in activity buffer (50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$, 5 μM $ZnCl_2$, 0.02% NaN_3) for 96 h at 37°C prior to staining in 0.5% Coomassie Brilliant Blue R-250 in 40% methanol and 10% acetic acid for 1 h at rt. Gels were destained at rt in 10% acetic acid, 10% methanol until clear bands appeared. Molecular weight of bands was estimated against molecular weight markers (BioRad).

DATA ANALYSIS

All quantitative assessments were performed in a blinded manner. Unless stated otherwise, for two groups paired *t*-test (two-tailed), for three or more groups one-way or two-way analysis of variance (ANOVA) followed by Bonferonni's *post hoc* multiple- or paired-comparison were used. All data are expressed as mean ± SD. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

RESULTS

As discussed above, NLRP3 is proposed as a sensor of sterile injury and disease and recognizes a wide range of structurally diverse DAMPs (Cassel and Sutterwala, 2010). We selected three of the best characterized NLRP3-activating DAMPs for our investigation into the effects of cell priming. ATP activates NLRP3 *via* its activation of cell surface P2X7 receptors (Mariathasan et al., 2006). MSU and CPPD crystals, inflammatory drivers of gout, and pseudogout respectively also activate the NLRP3-inflammasome (Martinon et al., 2006). Uric acid crystals are also suggested to be a general inflammatory signal released from dead and dying cells (Kono et al., 2010). We initially looked at the effects of these NLRP3-activating DAMPs on cell priming itself. Primary cultures of mouse mixed glia (composed of astrocytes and microglia; Pinteaux et al., 2002) were treated with ATP, MSU, or CPPD crystals for 4 h after which lysates were harvested and analyzed by qPCR for the expression of markers of inflammatory cell priming (**Figure 1**). As a positive control cultures were treated with the PAMP LPS. We initially investigated the effects of these DAMPs and LPS on the expression of genes typically associated with the inflammasome and priming e.g., IL-1β (**Figure 1Ai**), IL-1α (**Figure 1Bi**), caspase-1 (**Figure 1Ci**), NLRP3 (**Figure 1Di**), and ASC (**Figure 1Ei**). In general, DAMPs had no effect on the expression of these genes except for CPPD crystals, where a significant increase in the expression of IL-1β and IL-1α was observed (**Figures 1Ai,Bi**). LPS stimulation increased the expression of all genes, including caspase-1 (**Figure 1C**) and NLRP3 (**Figure 1D**), but did not affect ASC, whose expression did not change with any treatment except with CPPD crystals where a significant decrease was observed (**Figure 1E**).

In order to determine whether these NLRP3-activating DAMPs were capable of stimulating a more general inflammatory response we extended our study on gene expression to include additional inflammatory genes such as iNOS (**Figure 1F**), IL-6 (**Figure 1G**), TNFα (**Figure 1H**), and CXCL1 (**Figure 1I**). Again, DAMPs had no effect, with the exception of CPPD crystals on the expression of iNOS (**Figure 1F**), IL-6 (**Figure 1G**), and CXCL1 (**Figure 1I**),

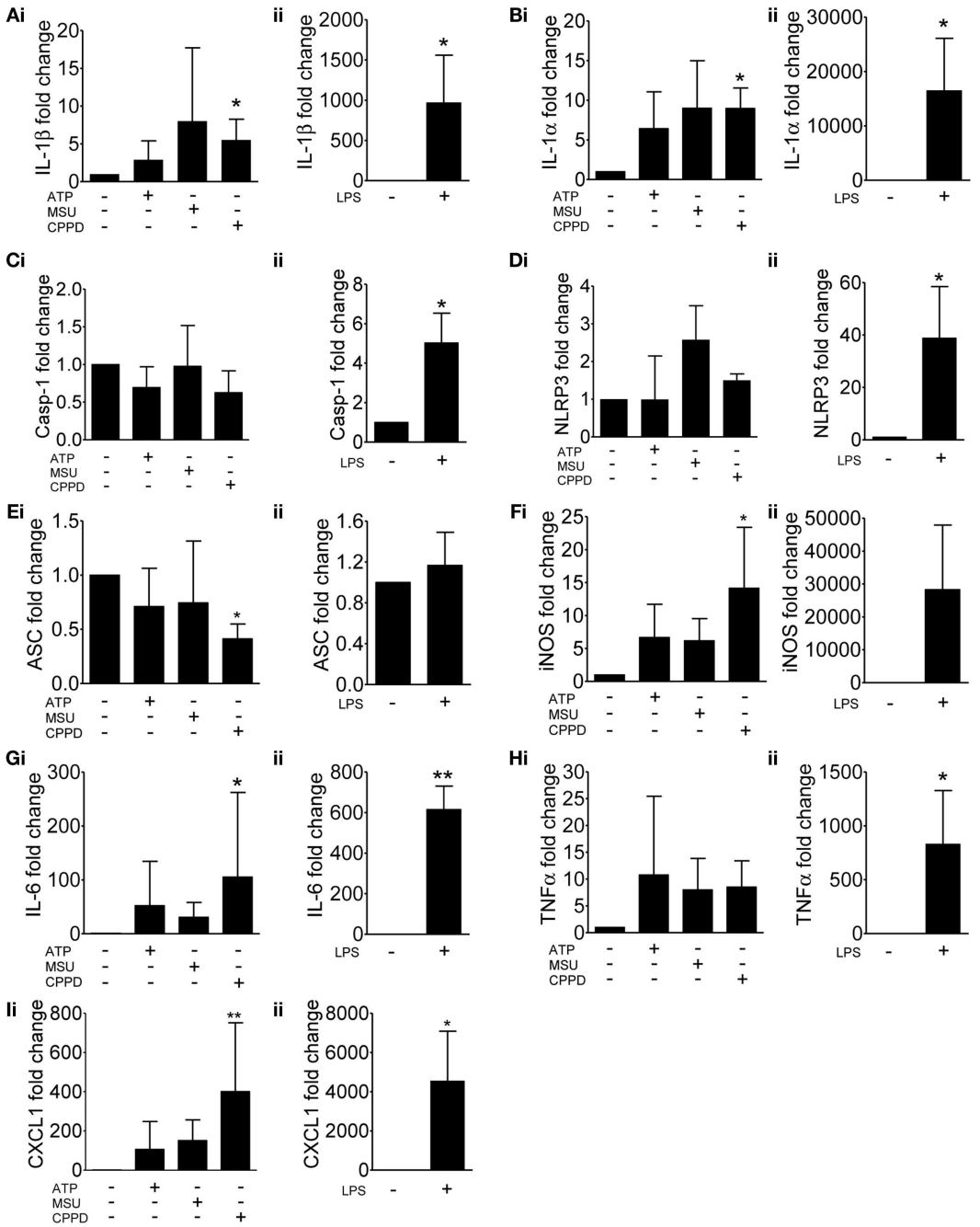

FIGURE 1 | Effects of DAMPs on the expression of pro-inflammatory genes in cultured mixed glia. mRNA levels of pro-inflammatory genes were measured by qPCR after 4 h exposure to the NLRP3-activating DAMPs ATP (5 mM), MSU, and CPPD (both 250 μg/ml) **(Ai)** or the PAMP LPS (1 μg/ml) **(Aii)**. Data were normalized to expression levels of the housekeeping gene SDHA across each treatment and fold change was expressed relative to basal RNA levels from untreated mixed glia. The genes analyzed were IL-1β **(A)**, IL-1α **(B)**, caspase-1 **(C)**, NLRP3 **(D)**, ASC **(E)**, iNOS **(F)**, IL-6 **(G)**, TNFα **(H)**, and CXCL1 **(I)**. Data are pooled samples from at least five separate experiments. $^{**}P < 0.01$, $^{*}P < 0.05$, vs. untreated.

whilst LPS induced a robust response across all genes tested. These data suggest that at the level of gene expression, and within the limits of the genes investigated in this study, and with the exception of CPPD, NLRP3-activating DAMPs have a negligible effect.

We then investigated protein levels in DAMP treated mixed glial cultures for IL-1β, IL-1α, IL-6, and CXCL1, which we have previously reported to be upregulated in response to focal ischemic injury in both the plasma and peripheral tissues, and in the brain (Chapman et al., 2009; Denes et al., 2010a; **Figure 2**). After 24 h

NLRP3-activating DAMPs induced no increase in the protein levels of IL-1β (**Figure 2Ai**) or IL-1α (**Figure 2Bi**), whilst MSU and CPPD did induce significant increases in the production of IL-6 (**Figure 2Ci**) and CXCL1 (**Figure 2Di**). Although raised at 24 h the effects of ATP on IL-6 and CXCL1 levels were not significant, but were when we investigated the earlier time point of 4 h (**Figure 2E**). LPS induced a robust increase in the levels of all proteins examined (**Figure 2**). The production of inflammatory mediators by DAMPs alone suggest that they are capable of inducing an inflammatory response, albeit not as robust when compared to the effects of typical PAMPs such as LPS. How these DAMPs act to induce this inflammatory response is not known. None of the DAMPs tested here are reported to act as ligands for toll-like receptor (TLR). However, as mentioned in the introduction MSU can induce Syk kinase signaling (Ng et al., 2008). These DAMPs also caused some cell death within our cultures (data not shown) and so it is possible that this contributed to the effects on cytokine production observed. Microglia can upregulate CD11c and MHCII markers on the cell surface after cerebral ischemia, resulting in a phenotype resembling that of dendritic cells (Felger et al., 2010). We investigated whether PAMPs and DAMPs can directly activate an antigen-presenting phenotype in microglia. Flow cytometry showed that LPS induced an activation of cell surface CD11c and MHCII in microglia, whilst DAMPs alone had no effect (**Figure 3**). These data suggest that the NLRP3-inflammasome activating DAMPs investigated here, when applied alone, do not act as a priming stimulus for microglial antigen presentation.

These DAMPs are known to engage the NLRP3-inflammasome and to activate caspase-1 resulting in the processing and release of IL-1β from primed cells. Although much of the literature on IL-1 secretion comes from work on peripheral macrophages, PAMP and DAMP-dependent IL-1 responses from microglia have been reported (e.g., Brough et al., 2002; Halle et al., 2008). The limited effects of these DAMPs observed so far was not due to their use at an insufficient concentration since priming of mixed glial cultures with LPS followed by treatment with ATP, MSU, or CPPD induced caspase-1 activation and the release of mature IL-1β (**Figure 4A**), and IL-1α (**Figure 4B**).

A key step during cerebral ischemia is the early breakdown of the BBB, although it is not known whether this is facilitated by the release of DAMPs from dying cells. Therefore we investigated the effects of DAMPs on the release of cathepsin B and of gelatinases which have been reported to be released following ATP-dependent activation of the P2X7 receptor in the absence of PAMP priming (Gu and Wiley, 2006; Lopez-Castejon et al., 2010), and which contribute to BBB damage after brain injury (Candelario-Jalil et al., 2009). In cultures of mixed glia ATP, MSU, CPPD, and LPS all induced the release of cathepsin B mature single chain (28–30 kDa) form (**Figure 5A**). Gelatin gel zymography of supernatants from cultures of mixed glia revealed that pro-MMP2 (72 kDa) was constitutively released under control conditions and that treatment of cultures with LPS, or with the DAMPs MSU or CPPD had no effect on released gelatinase activity (**Figure 5B**). P2X7 receptor activation in monocytes induces the release of active MMP9 (Gu and Wiley, 2006), and here in our cultures of mixed glia ATP-treatment induced a massive increase in released gelatinase activity (**Figure 5B**).

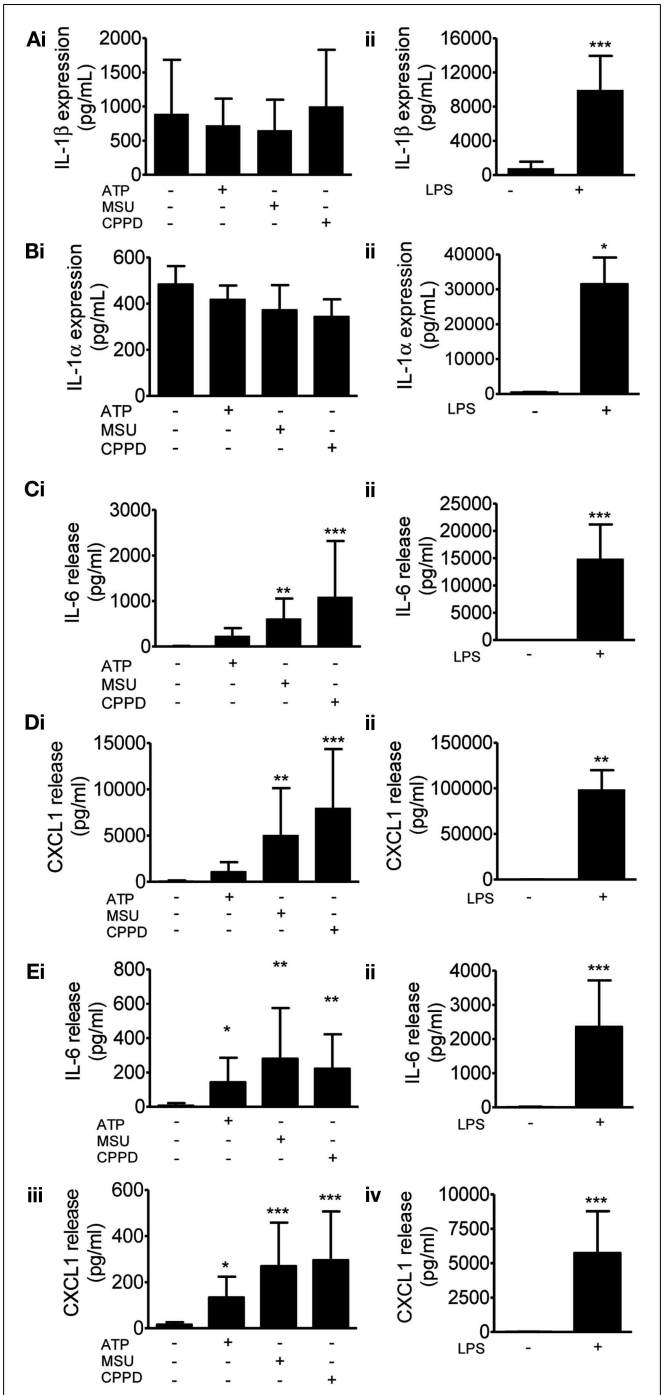

FIGURE 2 | Effects of DAMPs on pro-inflammatory protein levels in cultured mixed glia. Protein levels of pro-inflammatory mediators were measured by specific ELISAs after 24 h **(A–D)** or 4 h **(E)** exposure to the NLRP3-activating DAMPs ATP (5 mM), MSU, and CPPD [both 250 μg/ml; **(Ai)**] or the PAMP LPS [1 μg/ml; **(Aii)**]. The proteins analyzed were IL-1β **(A)**, IL-1α **(B)**, IL-6 **(C,E)**, CXCL1 **(D,E)**. Data are pooled samples from at least five separate experiments. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$, vs. untreated.

Although many DAMPs are reported to activate PRRs of the TLR family to prime inflammatory responses (Chen and Nunez, 2010), the priming stimulus for inflammasome function in the

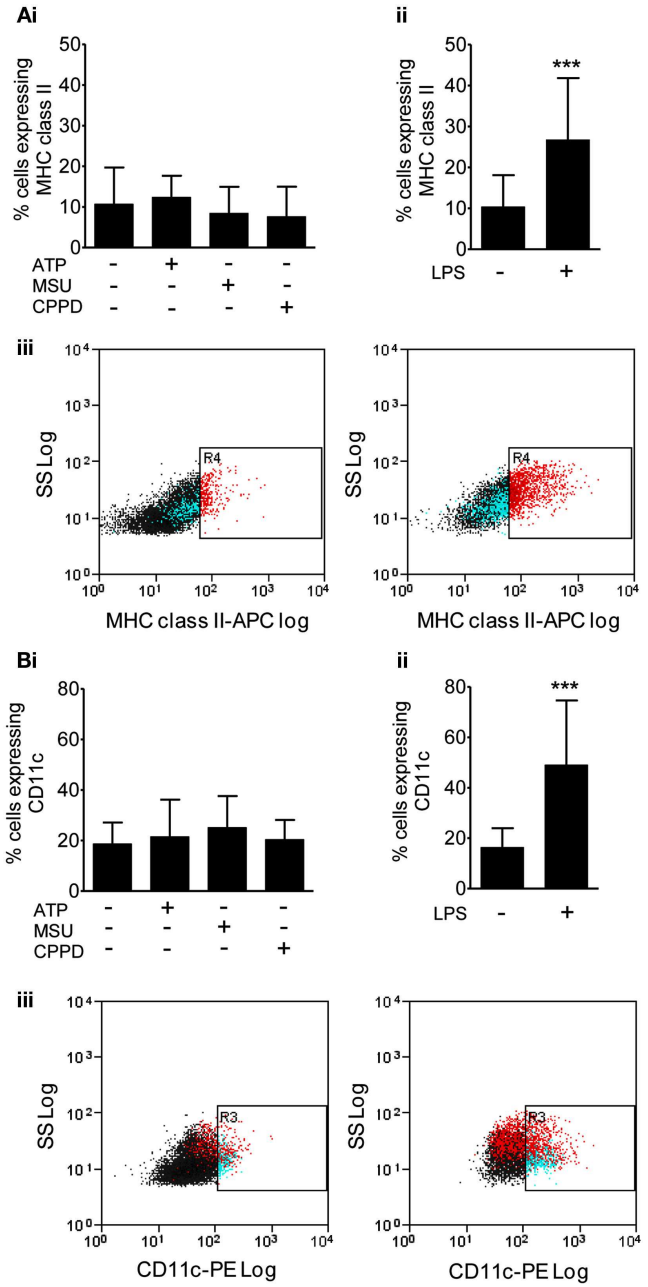

FIGURE 3 | Effects of DAMPs on surface marker expression in microglia cells. The expression of surface markers of microglia activation MHC class II **(A)**, and CD11c **(B)** were measured after 24 h exposure to the NLRP3-activating DAMPs ATP (5 mM), MSU, and CPPD [both 250 μg/ml; **(Bi)**] or the PAMP LPS [1 μg/ml; **(Bii)**]. Flow cytometry was used to quantify surface marker expression. Data are expressed as a percentage of cells that co-express CD45 and CD11b. Representative dot plots showing the CD45 and CD11b expressing cell population plus and minus LPS treatment are also shown **(Biii)**. Data are pooled samples from at least five separate experiments. $***P < 0.001$ vs. untreated.

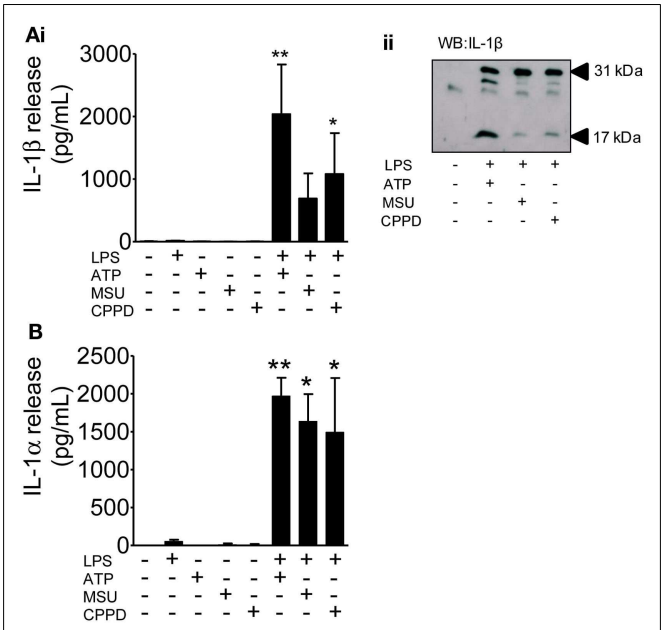

FIGURE 4 | The effects of PAMPs and DAMPs on the release of IL-1 from cultured mixed glia. Cultured mixed glia were treated (1 h) with the NLRP3-activating DAMPs ATP (5 mM), MSU, and CPPD (both 250 μg/ml) plus and minus a 24 h priming stimulus with the PAMP LPS (1 μg/ml). Release of both IL-1β **(A)** and IL-1α **(B)** were quantified by ELISA **(Ai)**, and processing of pro- (31 kDa) to mature (17 kDa) IL-1β in PAMP and DAMP treated cells was analyzed by Western blot **(Aii)**. Data are pooled samples from at least five separate experiments. $**P < 0.01$, $*P < 0.05$ vs. untreated.

brain *in vivo* is unknown. BBB breakdown takes place early after cerebral ischemia allowing the penetration of circulating inflammatory mediators into the brain. This coincides with systemic upregulation of acute phase proteins such as SAA, which acts as an alternative acute phase protein to CRP in mice and is upregulated in plasma as early as 4 h after MCAo (McColl et al., 2007). Four hours after MCAo in the mouse there is disruption of the BBB and microglia localized to these areas of focal BBB disruption express IL-1 (Luheshi et al., 2011), suggesting that plasma derived factors could provide the priming stimulus. Moreover, SAA is reported to prime NLRP3-inflammasome dependent responses in macrophages (Ather et al., 2011; Niemi et al., 2011), and in synovial fibroblasts (Migita et al., 2012), although its effect on brain glia is unknown. Treatment of mixed glial cultures with SAA (0.03–3 μg/ml, 24 h) induced the expression of IL-1β (**Figure 6A**) and IL-1α (**Figure 6B**). These cells were effectively primed for inflammasome dependent responses as the addition of the DAMP ATP (5 mM, 1 h) induced a significant release of IL-1β (**Figure 6C**). These data suggest the possibility that DAMP-dependent inflammasome responses in the brain may be primed by endogenous plasma constituents.

Since we had found that DAMPs mediated inflammation directly and also induced IL-1 release from primed glial cells *in vitro*, we aimed to investigate the effects of DAMPs on local pro-inflammatory responses induced by brain injury *in vivo* in the absence of IL-1. NLRP3-inflammasome activating DAMPs induce the release of both IL-1α and IL-1β from primed macrophages (Gross et al., 2012), and so to investigate the effects of DAMPs independently of IL-1 stimulated events we subjected WT and

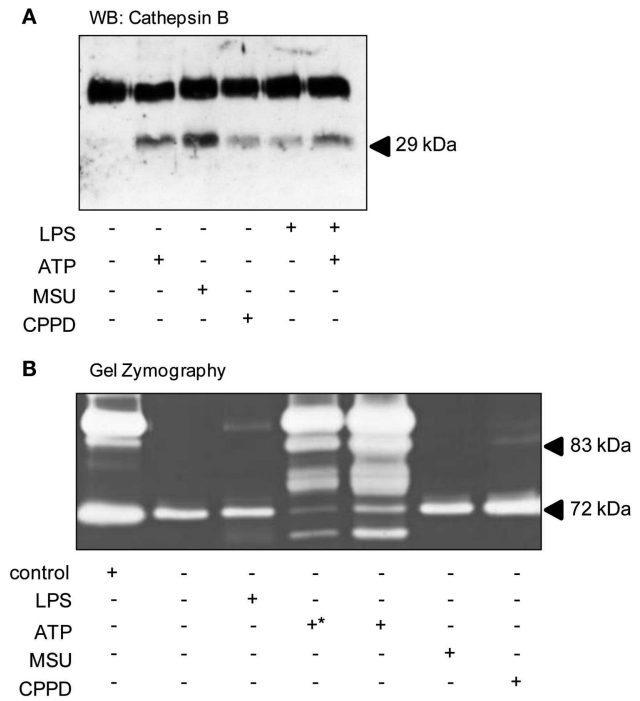

FIGURE 5 | DAMPs induce the release of proteases from cultured mixed glia. Cultured mixed glia were treated with the NLRP3-activating DAMPs ATP (5 mM), MSU, and CPPD (both 250 μg/ml) or the PAMP LPS (1 μg/ml) for 24 h. Supernatants were analyzed for the release of mature Cathepsin B (28–30 kDa) by Western blot **(A)**. To measure the release of martix metalloproteases by zymography all samples were loaded neat onto gels except for one of the ATP-treated supernatants (+*) which was diluted 1:3 **(B)**. Blots and gels shown are representative of at least five separate experiments.

IL-1α/β double KO mice to transient middle cerebral artery occlusion (tMCAo) a model of experimental stroke. Thus use of the IL-1α/β double KO mice allowed us to dissect DAMP-dependent responses independently of any IL-1-mediated inflammation. Following tMCAo there was a large lesion in the ipsilateral hemisphere of mice, suggesting that glial cells in this hemisphere would be exposed to DAMPs (**Figure 7Ai**). Within this area there was significant microglial cell activation when compared to the contralateral hemisphere, observed by increased expression of Iba1 and CD45 (**Figures 7Ai–iii**). Twenty four hours after MCAo IL-6 and CXCL1 were increased in the ipsilateral hemisphere independently of the presence of IL-1 ($P < 0.01$ and 0.001, respectively, two-way ANOVA) compared to the contralateral hemisphere (**Figures 7B,C**). WT mice demonstrated a higher level of increase (70-fold for IL-6 and 24-fold for CXCL1) than IL-1α/β KO mice (26-fold for IL-6 and 11-fold for CXCL1), but CXCL1 was still significantly upregulated in IL-1a/b KO mice in the ipsilateral hemisphere based on Bonferroni's *post hoc* comparison following two-way ANOVA ($P < 0.05$) and the interaction between genotype and hemispheric increase of CXCL1 was also significant (two-way ANOVA, $P < 0.01$; **Figures 7B,C**). IL-1β and IL-1α levels were undetectable in IL-1α/β KO mice and IL-1α displayed a significant increase after cerebral ischemia in the ipsilateral hemisphere (two-way ANOVA followed by Bonferroni's *post hoc* test, $P < -0.05$, not shown). These data are consistent with the *in vitro* data above, suggesting that DAMPs induce a priming-independent inflammatory response, and once expression and release of IL-1 takes place, this inflammatory response is markedly augmented in the brain *in vivo*.

DISCUSSION

Here, we have analyzed the pro-inflammatory effects of NLRP3-activating DAMPs in the absence/presence of priming stimuli *in vitro* in cultures of mixed glial cells. As a result of our investigations we propose that in acute brain injury DAMPs can contribute to brain inflammation *via* at least three mechanisms: by directly stimulating the production of glial derived pro-inflammatory mediators, by contributing to BBB injury through the release of various proteases, and by inducing IL-1 release from primed cells. The presence of IL-1 in turn, markedly augments damage-induced inflammatory responses *in vivo*.

Inflammation is recognized as a major contributor to the worsening of acute brain injury and inhibiting IL-1 with the receptor antagonist (IL-1Ra) is protective in experimental models of stroke (Brough et al., 2011), and has shown promise as a treatment in clinical trials (Emsley et al., 2005). Early after cerebral ischemia (4 h) the related IL-1 family member IL-1α is expressed by microglia in areas of brain that will become infarct (Luheshi et al., 2011), with subsequent expression of IL-1β occurring at later time points (24 h; Denes et al., 2008). Mice in which both IL-1α and IL-1β have been deleted (IL-1α/β double KO), but not single gene KOs, have markedly reduced damage in response to MCAo (Boutin et al., 2001).

Within an ischemic tissue there will be abundant levels of DAMPs that may serve to mediate local inflammatory responses. The inflammatory actions of DAMPs are most often reported as the ability to stimulate formation of the NLRP3-inflammasome, thus activating caspase-1 and inducing the release of IL-1β (Cassel and Sutterwala, 2010). However, *in vitro* DAMPs are only reported to achieve this after the cell, typically a macrophage, has been primed with a PAMP such as LPS (Bauernfeind et al., 2009; Hornung and Latz, 2010). In monocytes and synoviocytes (Guerne et al., 1989), and osteoblast like cells (Bouchard et al., 2002) release IL-6 occurs in response to the NLRP3-inflammasome activating DAMPs MSU and CPPD in the absence of priming. However, to-date, an investigation of the pro-inflammatory effects of NLRP3-activating DAMPs in glial cells, in the absence of priming, has not taken place. Thus, in this study we sought to determine the pro-inflammatory effects of NLRP3-activating DAMPs in the presence and absence of PAMPs on inflammatory cells from the brain. We used cultures of mixed glia, composed mainly of astrocytes and microglia (Pinteaux et al., 2002). Both microglia and astrocytes represent a source of IL-1 in culture (Brough et al., 2002; Bianco et al., 2009) and from our experiments it was not possible to identify the relative contribution of each cell type to released cytokine levels. Given their similarity to macrophages, microglia are generally considered to be the major source of IL-1 in the brain after an injury (Denes et al., 2010b), yet astrocytes are also known to express IL-1β following MCAo in mice (Denes et al., 2008). It is possible that a contribution from both cellular sources

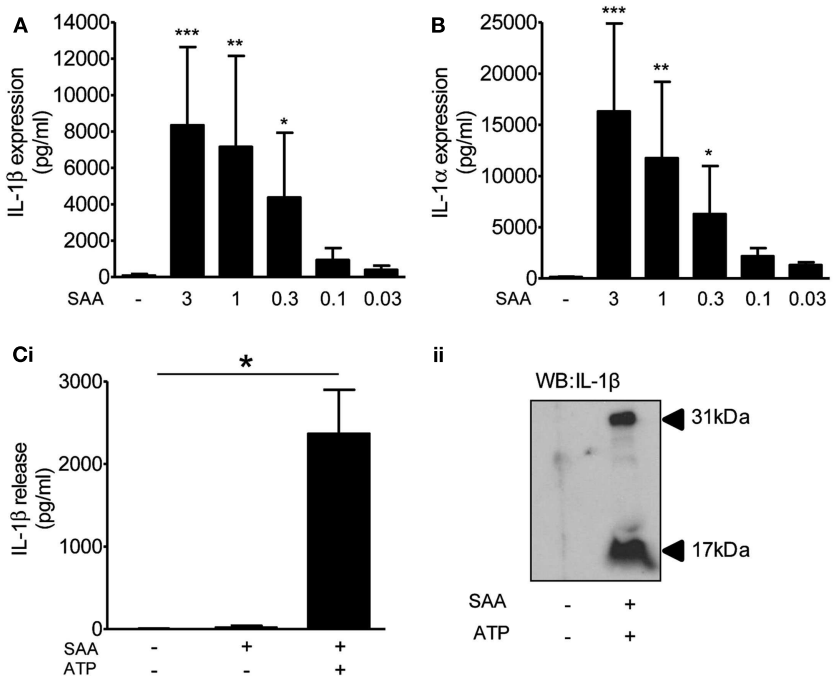

FIGURE 6 | The effects of DAMPs on the release of IL-1 from cultured mixed glia primed with serum amyloid A (SAA). Cultured mixed glia were treated with the indicated concentration of SAA for 24 h and the expression of IL-1β **(A)** and IL-1α **(B)** was measured in the cell lysate by ELISA. The NLRP3-activating DAMP ATP (5 mM) was added to the cultures after a 24 h priming stimulus with SAA (3 μg/ml) and released IL-1β was quantified by ELISA **(Ci)**. And processing of pro- (31 kDa) to mature (17 kDa) IL-1β was analyzed by Western blot **(Cii)**. Data are pooled samples from at least five separate experiments. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$ vs. untreated.

drives inflammation after brain injury, although further experiments with purified cell cultures, and animal models in which solely microglia or astrocytes can produce IL-1 are required to test this.

Entirely consistent with the literature on macrophages, we found that several well characterized DAMPs (ATP, MSU, and CPPD crystals), had no effect on IL-1β levels in the absence of prior PAMP priming (**Figures 1**, **2**, and **4**). The results for IL-1α mirrored the effects of DAMPs on IL-1β, i.e., the DAMPs had no effect except when added to a PAMP-primed cell when they induced release (**Figures 1**, **2**, and **4**). In contrast, we show that DAMPs induce IL-6 and CXCL1 release from glia *in vitro* in the absence of any priming stimulus, indicating that DAMPs have a direct pro-inflammatory effect on glial cells independently of PAMP-induced signals (**Figure 2**).

This priming-independent inflammatory response was also observed *in vivo* after MCAo in IL-1α/β double KO mice (**Figure 7**). The priming stimuli for IL-1-dependent responses in the brain after a stroke are not known. There are a number of possible candidates however. For example, after disruption of the BBB plasma derived molecules known to prime IL-1β responses such as minimally oxidized LDL, and which are associated with co-morbid diseases like type II diabetes, could prime glia at the lesion site (Masters et al., 2010). Other plasma derived molecules include acute phase reactants such as SAA which has also been reported to prime NLRP3-inflammasome dependent responses in macrophages (Ather et al., 2011; Niemi et al., 2011), and we know that 4 h following MCAo in the mouse plasma SAA levels are elevated (McColl et al., 2007). We showed that SAA was capable of priming glial cultures to express IL-1α and IL-1β (**Figure 6**). Analogous to the PAMP priming observed with LPS, SAA alone did not induce the release of IL-1, but IL-1β release did occur after a SAA-primed culture was treated with ATP (**Figure 6**). In addition to these examples it is possible that one of a plethora of DAMPs reported to activate TLRs could provide the priming stimulus (Piccinini and Midwood, 2010). However, our study serves to highlight that brain inflammatory cells can respond to endogenous priming stimuli to promote IL-1-dependent inflammatory responses.

We also discovered that treatment of cultured mixed glia with DAMPs induced the release of cathepsin B, and that the DAMP ATP induced a massive release of gelatinase activity in the absence of priming (**Figure 5**). ATP induced cathepsin B release from non-primed macrophages results in the *in vitro* degradation of extracellular matrix, suggesting its pro-inflammatory action (Lopez-Castejon et al., 2010). That MSU and CPPD crystals also induce the release of active cathepsin B from non-PAMP-primed cells (**Figure 5**) suggest that this could be a common inflammatory mechanism of DAMPs. Furthermore, treatment with a cathepsin B inhibitor *in vivo* is neuroprotective following stroke (Benchoua et al., 2004). The effects of DAMP-induced release of gelatinases such as MMP9 could be twofold. MMP9 is known to disrupt the integrity of the BBB following MCAo (McColl et al., 2008), and is also known to be neurotoxic in neuroinflammatory

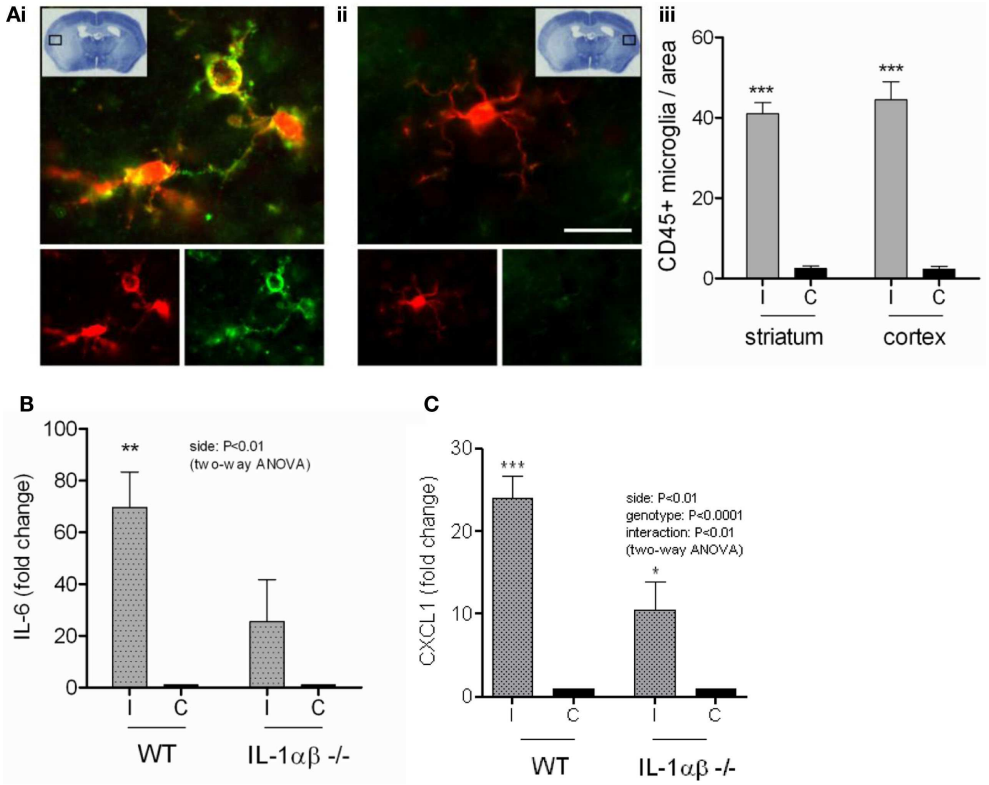

FIGURE 7 | The inflammatory response to cerebral ischemia. Cortical cell death occurs in the ipsilateral cortex after transient middle cerebral artery occlusion (tMCAo) [insert **(Ai)**]. Microglia in this region have a significantly more activated morphology **(Ai,iii)** as shown by immunofluorescent staining for Iba1 (red) and CD45 (green), compared to those from the same region of the contralateral hemisphere **(Aii,iii)**. At the protein level *in vivo*, IL-6 **(B)** and CXCL1 **(C)** increase significantly in the ipsilateral cortex of WT mice after tMCAo. IL-6 and CXCL1 protein levels are significantly attenuated in IL-1α/β KO mice **(B,C)**. Data are expressed as fold increase over the contralateral hemisphere, are from a minimum of three independent experiments and were analyzed by two-way ANOVA followed by a Bonferonni post hoc analysis. $^{**}P < 0.01$, $^{*}P < 0.05$.

in vitro models (Thornton et al., 2008). The consequence of these IL-1-independent DAMP effects would be increased production of inflammatory mediators and acute phase reactants, leukocyte recruitment, BBB disruption, and the influx of peripheral, systemic factors.

These data reveal how DAMPs induce inflammatory responses in the absence of any bacterial infection or products, and may be relevant to a range of sterile insults in addition to the model of brain injury used here. These data support a model where DAMPs released at a site of sterile injury induce the release of cytokines and proteases that are central to the establishment of an inflammatory response. DAMPs also induce the secretion of IL-1α and IL-1β from primed glia. In turn, the presence of IL-1 enhances sterile injury-induced inflammatory responses. It is not clear what primes microglia *in vivo* during ischemia but it could be one of a large number of factors, including plasma derived mediators such as SAA, and remains a subject for future investigation.

ACKNOWLEDGMENTS

The authors are grateful to the Wellcome Trust (David Brough, Gloria Lopez-Castejon), the BBSRC (Catherine Diane Savage), and the European Union's Seventh Framework Program (FP7/2008-2013) under Grant Agreements 201024 and 202213 (European Stroke Network; Adam Denes) for funding the research.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 May 2012; accepted: 28 August 2012; published online: 18 September 2012.

Citation: Savage CD, Lopez-Castejon G, Denes A and Brough D (2012) NLRP3-inflammasome activating DAMPs stimulate an inflammatory response in glia in the absence of priming which contributes to brain inflammation after injury. Front. Immun. ***3****:288. doi: 10.3389/fimmu.2012.00288*

This article was submitted to Frontiers in Inflammation, a specialty of Frontiers in Immunology.

frontiers in IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 03 September 2012
doi: 10.3389/fimmu.2012.00277

Monosodium urate crystals induce extracellular DNA traps in neutrophils, eosinophils, and basophils but not in mononuclear cells

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Neutrophil extracellular traps (NETs) are fibers of extracellular DNA released from neutrophils due to overwhelming phagocytic stimuli. The function of NETs is to trap and kill microbes to avoid spreading of potential pathogens. NETs are formed after encounter with various gram-positive and -negative bacteria but also in response to mediators causing sterile inflammation like interleukin-8 (IL-8), tumor necrosis factor (TNF), and phorbol myristate acetate (PMA). Here we show the formation of NETs (NETting) in response to monosodium urate (MSU) crystals as further model for sterile inflammation. We identified monocytes, neutrophils, and eosinophils as MSU phagocytosing cells. Basophils did not take up the crystals, instead they upregulated their activation marker CD203c after contact with MSU. Nevertheless, MSU crystals induced extracellular trap formation also in basophils, like in eosinophils and neutrophils, which phagocytose the crystals. In contrast, monocytes do not form NETs despite uptake of the MSU crystals. In contrast to the canonical stimuli like bacteria and PMA, MSU-induced NETosis was not abrogated by plasma. Our data show that MSU crystals induce extracellular DNA trap formation in all three granulocytes lineages (NETs, EETs, and BETs) but not in monocytes, and DNA externalization does not necessitate the uptake of the crystals.

Keywords: neutrophil extracellular traps, NETs, MSU, granulocytes, bacteria, PMA, inflammation

INTRODUCTION

Granulocytes play a crucial part in the innate immunity and can be subdivided into, neutrophils, eosinophils, and basophils with different features and functions for the immune response. Eosinophils develop from myeloid precursor cells in bone marrow stimulated by the cytokines IL-3, IL-5, and GM-CSF (Clark et al., 2007; von Kockritz-Blickwede et al., 2008; Brill et al., 2012). After maturation, eosinophils circulate in the blood and migrate to inflammatory sites in the tissues, or to helminth infections in response to chemokines like RANTES, and certain leukotrienes like LTB4. Following activation by type 2 cytokines released from Th2 cells, eosinophils produce ROS (Cline et al., 1968), growth factors (Rothenberg and Hogan, 2006) and cytokines (Crivellato et al., 2010), degranulate and release lipid mediators (Chirumbolo, 2012) and cytotoxic granule proteins (Popa-Nita and Naccache). Granule proteins from eosinophils include major basic protein, eosinophil cationic protein, and eosinophil peroxidase which can cause cell death in the target cell by induction of toxic pores and oxidative stress, but also may induce tissue damage and dysfunction (Crivellato et al., 2010). Major basic protein induces degranulation in mast cells and basophils (Zheutlin et al., 1984), which are together with eosinophils mediators of allergic responses and asthma pathogenesis.

Basophils are circulating granulocytes that orchestrate hypersensitivity (atopic) and anaphylactic reactions (Simons, 2010) and share a common lineage with tissue-dwelling mast cells. Recently basophils got into the focus due to their strategic role linking innate and acquired immunity. Besides promoting chronic inflammation in allergy (Mukai et al., 2009), basophils regulate Th2 cell function (Sokol and Medzhitov, 2010) immune cell memory and even serve as antigen-presenting cells (Maddur et al., 2010). In case of an infection, mature basophils degranulate and release histamine, proteoglycans (both prestored), the cytokines IL-3, IL-4, IL-6, IL-9, IL-13, IL-25, and GM-CSF, proteolytic enzymes, lipid mediators, and the chemokines MCP-1, MIP-1α, MIP-1β, and RANTES. However, basophils do not release the immunregulatory cytokines IFN-γ, IL-17, or IL-5 (Schroeder et al., 2009; Yamaguchi et al., 2009).

During the acute phase of infection neutrophils get attracted by chemotactic molecules, leave the blood vessels and migrate toward the site of infection (Massena et al., 2010). These phagocytic cells can incorporate microorganisms and kill them via antimicrobial proteins, proteolytic enzymes, and reactive oxygen species (ROS) (Clifford and Repine, 1982; Borregaard and Cowland, 1997). In addition, an alternative extracellular killing mechanism has been described when the phagocytic capacity is

Abbreviations: BET, basophil extracellular trap; DAPI, 4′-6-Diamidino-2-phenylindole; EET, eosinophil extracellular trap; FSc, forward scatter; IL, interleukin; LPS, lipopolysaccharide; MSU, monosodium urate; NET, neutrophil extracellular trap; PBMC, peripheral blood mononuclear cells; PFA, paraformaldehyde; PMA, phorbol myristate acetate; PMN, polymorphonuclear cells; ROS, reactive oxygen species; SSc, side scatter.

exhausted (Brinkmann et al., 2004; Urban et al., 2006). Activated neutrophils form extracellular fibers, called neutrophil extracellular traps (NETs), that are composed of loosened chromatin and granule proteins such as neutrophil elastase, cathepsin G and lactotransferrin (Urban et al., 2009). NETs immobilize the pathogens and, thereby, prevent microbial spreading (Brinkmann et al., 2004; Urban et al., 2006). NETs containing antimicrobial molecules efficiently kill microorganisms and contribute to an anti-microbial environment (Urban et al., 2006). The molecular binding mechanism is still unknown, but electrostatic interactions between the anionic surface of the microorganisms and cationic components of the NETs are discussed (Brinkmann and Zychlinsky, 2007). Formation of NETs (NETting) causes neutrophils' death, but NETosis is an active cell death mechanism distinct from apoptosis and necrosis and depends on the production of ROS by NADPH-oxidase (Fuchs et al., 2007). Blocking the respiratory burst by diphenylene iodonium (DPI) inhibits NETs formation. Patients suffering from chronic granulomatous disease (CGD) carry mutations in the phagocyte NADPH oxidase and are unable to generate ROS and to form NETs (Fuchs et al., 2007). After ROS production in activated neutrophils the nuclear membranes disintegrate generating vesicles and nuclear material as well as granular components are mixed (Brinkmann and Zychlinsky, 2007). Finally cells break off and release the NETs. NETting in granulocytes can be induced in response to various gram-positive and -negative bacteria as well as fungi and parasites but also by sterile mediators as interleukin-8 (IL-8), tumor necrosis factor (TNF) and phorbol myristate acetate (PMA) (von Kockritz-Blickwede and Nizet, 2009).

The deposition of monosodium urate (MSU) crystals in tissues and joints causes gouty arthritis. When uric acid, the final product of the human purine metabolism, exceeds the limit of solubility (70 μg/ml), it crystallizes as sodium containing MSU (So, 2007; Schorn et al., 2011). The uptake of MSU crystals is accompanied by the release of pro-inflammatory cytokines and chemokines from granulocytes and monocytes (Schorn et al., 2010).

In our study we employed MSU as further model for sterile inflammation. We analyzed the effect of MSU crystals on in all three granulocytes lineages (neutrophils, eosinophils, and basophils) and mononuclear cells (monocytes, B cells, and T cells). We observed that monocytes, neutrophils, and eosinophils take up MSU crystals. Although basophils did not phagocytose MSU crystals, they upregulated their lineage marker CD203c after contact with MSU. Furthermore, we identified neutrophils, eosinophils, and basophils as cells capable to externalize nuclear DNA after incubation with MSU crystals. In contrast to NETting induced by PMA and bacteria, the formation of MSU-dependent NETs was not inhibited by plasma.

MATERIALS AND METHODS

ISOLATION OF PMN, PBMC, EOSINOPHILS, AND BASOPHILS FROM HUMAN WHOLE BLOOD

Venous blood was taken from human blood donors in full agreement with institutional guidelines. Heparinized whole blood (20 U/ml) was centrifuged at 3400 g for 10 min (Rotina 46, Hettich) for the generation of autologous plasma. Peripheral blood mononuclear cells (PBMC) and polymorphonuclear neutrophils (PMN) were purified by density gradient centrifugation using Lymphoflot (Bio-Rad). The PBMC fraction was purified from platelets by centrifugation through a cushion of fetal bovine serum (Invitrogen GmbH). Erythrocytes were eliminated by hypotonic lysis. Basophils were negatively isolated from the PBMC fraction via indirect magnetic cell sorting employing a Basophil Isolation Kit (Miltenyi Biotech). The purity of the isolated basophils was verified by CD123/CD303 staining. Eosinophils were achieved from the PMN fraction employing an Eosinophil Isolation Kit (Miltenyi Biotech) according to the standard protocol. The purity of the isolated eosinophils was assured by quantifying $CD16^-CD49d^+$ cells. Viable cells were counted in a Neubauer counting chamber and cell density was adjusted to 2×10^6 cells/ml in 100% autologous plasma.

FLUORESCENCE MICROSCOPY

Whole blood cells or isolated cells were incubated for 2–5 h with 1 mg/ml or 200 μg/ml MSU crystals, respectively. Cytospins were prepared after lysing erythrocytes and non-ingested crystals by the TQprep Workstation (Beckman Coulter). The nuclear and extranuclear DNA of the samples was stained with 1 μg/ml 4′-6-Diamidino-2-phenylindole (DAPI; Invitrogen GmbH) for 30 min. After washing, the samples were analyzed by fluorescence microscopy using standard filter sets. For a better demonstration NETs and cells were artificially colored by morphology of their nucleus/DNA distribution. NETs were colored in green, polymorphonuclear cells in red, mononuclear cells in blue and MSU crystals in yellow or white.

For the detection of nuclear histone H3 in NETs, a monoclonal anti-human histone H3 antibody (1.5 μg/ml) produced in mice (Abcam) was incubated with cytospins for 30 min at room temperature, washed twice with PBS and incubated with goat anti-mouse IgG-FITC (Southern Biotech), washed with PBS and analyzed by fluorescence microscopy.

ANALYZES OF MSU PHAGOCYTOSING CELLS

Whole blood cells were incubated with 1 mg/ml MSU crystals for 1 h at 37°C. The phagocytosis of MSU crystals was determined by analyzing the SSc change in flow cytometry. We identified the following cell types by morphological properties (FSc, SSc) and by cell specific surface markers: $CD14^+$ monocytes, $CD16^+$ neutrophils, $CD16^-CD49d^+$ eosinophils, $CD123^+CD203^+$ basophils, $BDCA\text{-}2^+CD11c^-$ plasmacytoid dendritic cells (pDC), $CD3^+CD56^-$ T cells, $CD3^-CD56^-$ B cells, $CD3^-CD56^+$ natural killer (NK) cells, and $CD3^+Cd56^+$ natural killer T cells (NKT cells).

PLASMA CONCENTRATION DEPENDENCY OF NETting PMN

PMN were isolated from human venous blood as described previously. We investigated 5×10^6 cells/ml in 0% plasma (100% R0), 20% plasma (+80% R0), and 100% plasma, whereas R0 was composed of RPMI 1640 medium (Gibco) with 1% penicillin/streptomycin (Gibco), 1% hepes (Merck), and 1% glutamine (Gibco). NETs formation was stimulated by 100 nM PMA, 2×10^7 bacteria (heat inactivated streptococcus pneumoniae), 200 μg/ml MSU crystals or R0 as control for 4 h at 37°C. After preparation of cytospins, the DNA was stained with 1 μg/ml

DAPI and the NETs formation was analyzed by fluorescence microscopy.

QUANTIFICATION OF CYTOKINES/CHEMOKINES

The concentration of certain chemokines/cytokines was analyzed in culture supernatants of 2×10^6 isolated eosinophils, basophils and neutrophils per milliliter in autologous plasma after 18 h incubation with 200 μg/ml MSU crystals or PBS as control employing multiplex bead technology (eBioscience). Quantification of cytokines/chemokines was gained by cytofluorometry.

CYTOSPINS

2×10^5 cells were centrifuged onto glass slides (ThermoFisher) at 850 g for 10 min (Rotina 46, Hettich) using cytospin cuvettes. The supernatant was removed and the slides were centrifuged for additional 5 min at 2000 g. The DNA of PFA-fixed cells was stained with 1 μg/ml DAPI and analyzed by fluorescence microscopy using standard filter sets.

PREPARATION OF MSU CRYSTALS

A solution of 10 mM uric acid and 154 mM NaCl (both from Merck KGaA) was adjusted to pH 7.2 and agitated for 3 days for the production of MSU crystals. For sterilization the needle-shaped crystals were washed with ethanol, dried under sterile conditions and heated at 180°C for 2 h. The crystals were stored in 40 mg/ml in sterile PBS. In whole blood assays we used 1 mg/ml and in analysis with isolated cells we used 200 μg/ml MSU crystals. The limit of solubility of MSU crystals is 70 μg/ml.

LYSIS OF ERYTHROCYTES, SOLUBILIZATION OF CRYSTALS, AND FLOW CYTOMETRY

Erythrocytes and non-ingested MSU crystals were automatically lysed using a TQprep Workstation (Beckman Coulter) before measurement with a Gallios™ cytofluorometer (Beckman Coulter). The cytofluorometric data were analyzed with the Kaluza software (Beckman Coulter). Electronic compensation was used to eliminate bleed-through fluorescence.

STATISTICAL ANALYSIS

We performed statistical analyzes with SPSS PASW statistics 18. The results are represented as mean ± SEM of at least three and up to five independent experiments. Student's *t*-test or an analysis of variance for repeated measurements was used. The alpha level of all the tests or the P value was set at 0.05.

RESULTS

MSU CRYSTALS INDUCE FORMATION OF EXTRACELLULAR TRAPS BY GRANULOCYTES

After incubation of whole blood with MSU, we detected extranuclear DNA in the cultures (**Figure 1A**). Employing transmission light microscopy of cytospins we observed that crystal-speared phagocytes (**Figure 1A**, left) were surrounded by externalized DNA (**Figure 1A**, right). After identification of NETs formation by blood cells after contact with MSU crystals, we analyzed the NETting capacity of isolated PMN and PBMC. **Figure 1B** shows that neutrophils ejected NETs (right) after phagocytosis of MSU crystals (left). The residual MSU crystals are trapped and immobilized by the NETs. In contrast to PMN, isolated PBMC did not respond with the formation of extracellular traps, suggesting that the latter skill is confined to granulocytes (**Figure 1C**). Furthermore, the existence of NETs induced by MSU crystals was confirmed by histon H3 staining (**Figures 2A–D**).

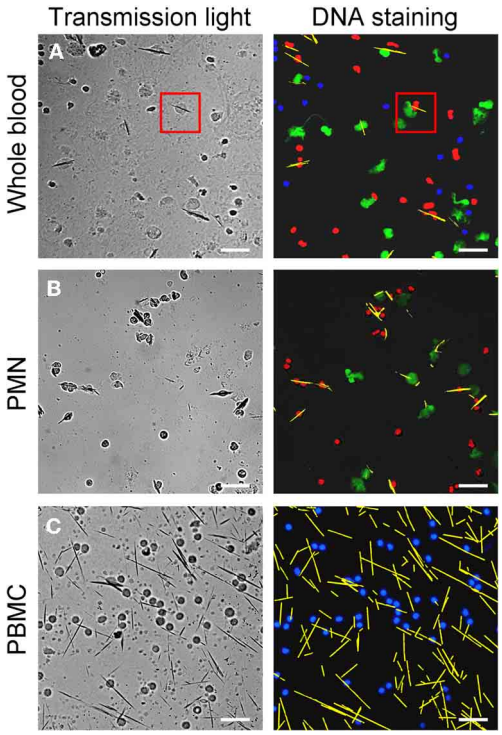

FIGURE 1 | MSU crystals induce NET formation in PMN but not in PBMC. Whole blood cells **(A),** isolated PMN **(B)** and isolated PBMC **(C)** were incubated with MSU crystals. Cytospins were prepared and stained for nuclear and extranuclear DNA. The transmission microscopy (left) shows phagocytes which have ingested MSU crystals. The DNA staining (right) shows NETting granulocytes in whole blood cells (**A**, red square) and isolated PMN **(B)**. No MSU-induced extracellular DNA was observed in isolated PBMC **(C)**. In **(A)** and **(B)** erythrocytes and free MSU crystals were dissolved by acid treatment. **(A–C)** NETs were artificial colored in green, PMN in red, mononuclear cells in blue and MSU crystals in yellow. All experiments were performed at least 3 times. Scale bars,100 μm.

MONOCYTES, NEUTROPHILS, AND EOSINOPHILS TAKE UP MSU CRYSTALS

Next we analyzed the cell types able to ingest MSU crystals. For this purpose, we incubated whole blood with MSU crystals and subsequently stained the cells with marker antibodies to identify the cell types involved in MSU clearance. The cell types were identified in flow cytometer according to their morphology (forward and side scatter reflect information about cell size and granularity, respectively) and surface markers (**Figure 3**). The uptake of MSU crystals by monocytes (61.2%), neutrophils (47.3%), and eosinophils (50.1%) was reflected by a drastically increased side scatter (SSc) of cells with the surface markers CD14, CD16, and CD49d, respectively. In contrast, basophils ($CD203c^+$ cells) did

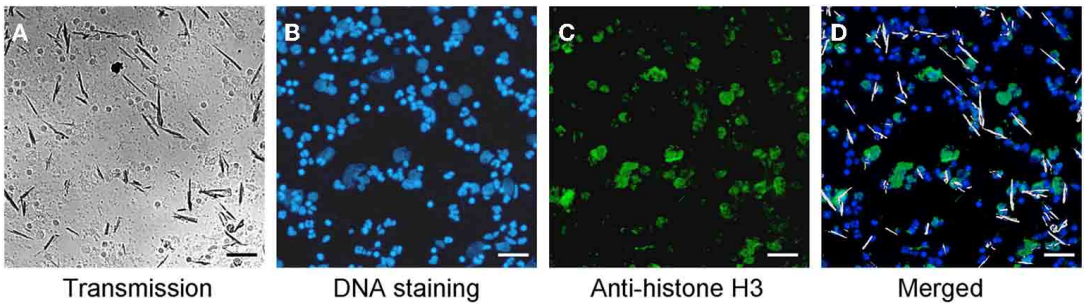

FIGURE 2 | MSU induced NETs contain histon H3. (A–D) Whole blood cells were incubated with MSU crystals. Cytospins were prepared and stained for nuclear and extranuclear DNA **(B)** and histon H3 **(C)**. The merged picture **(D)** shows the co-localization of DNA and histon H3. Erythrocytes and free MSU crystals were dissolved by acid treatment. The MSU crystals were artificial colored in white. All experiments were performed at least 3 times. Scale bars, 100 μm.

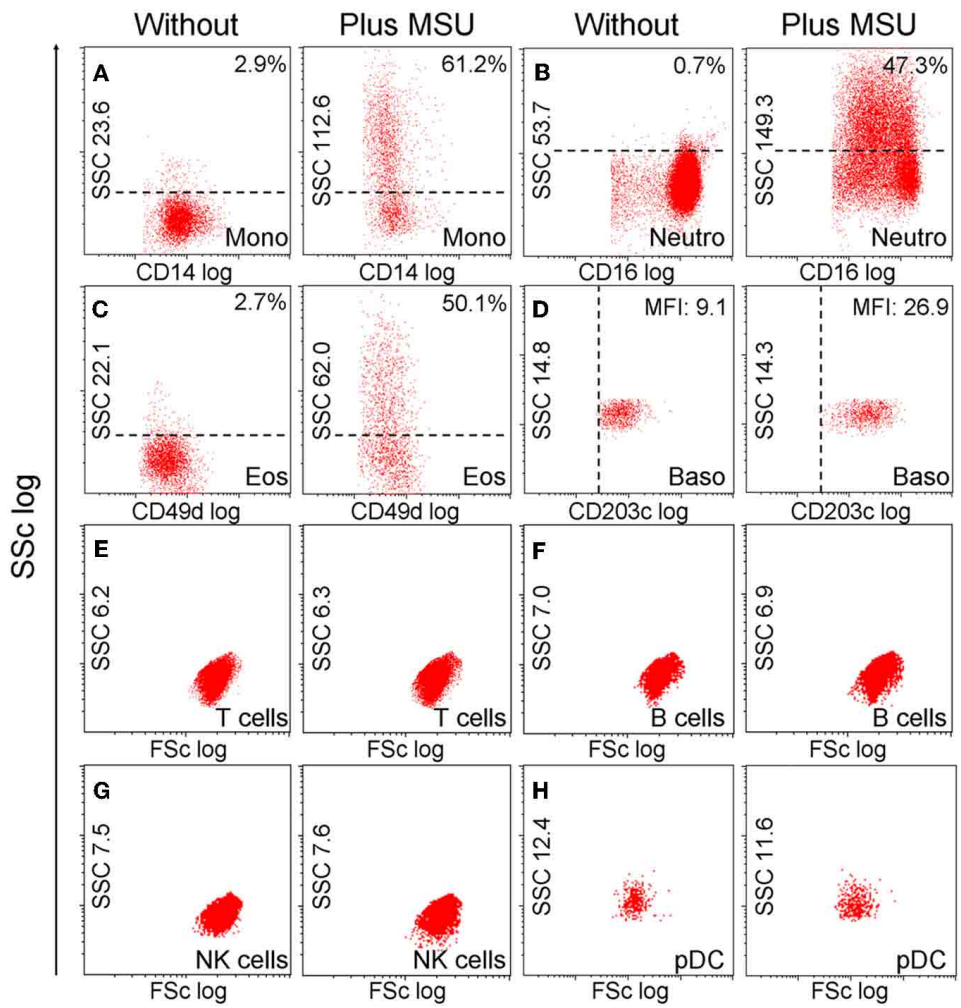

FIGURE 3 | Monocytes, neutrophils and eosinophils ingest MSU crystals. (A–H) Whole blood cells were incubated with MSU crystals. After lysis of the erythrocytes the morphology (forward scatter, side scatter) of the leukocytes was characterized by flow cytometry. The individual leukocyte populations were defined by cell lineage-specific surface markers. The uptake of crystals was reflected by dramatic increases of the cells' SSc values after phagocytosis. Monocytes **(A)** neutrophils **(B)** and eosinophils **(C)** ingested MSU crystals. Basophils **(D)** did not take up the crystals, but upregulated their surface activation marker CD203c. T cells **(E)** B cells **(F)** NK cells **(G)** and pDCs **(H)** did not increase the side scatter in response to MSU crystals. SSc, side scatter; FSc, forward scatter; Mono, monocytes; neutro, neutrophils; eos, eosinophils; baso, basophils; NK, natural killer cells; pDC, plasmacytoid dendritic cells. All experiments were performed at least three times.

not increase their side scatter but upregulated their lineage marker CD203c after incubation with MSU crystals. B cells, T cells, NK cells, pDC, (**Figure 3**) and NKT cells (not shown) did not respond to MSU crystals with an increase in the SSc.

NEUTROPHILS, EOSINOPHILS, AND BASOPHILS FORM EXTRACELLULAR TRAPS AFTER INCUBATION WITH MSU CRYSTALS

Neutrophils have been described to externalize nuclear DNA that forms NETs in response to various stimuli. Since the NET induction by MSU was faster and more efficient than canonical activators of NETting we analyzed the potential to externalize nuclear DNA of neutrophils, eosinophils, basophils, and monocytes in response to MSU. To this end we employed isolated neutrophil, eosinophil, and basophil granulocytes as well as PBMC to study the clearance of MSU. **Figure 4** shows that neutrophils (**Figure 4A**), eosinophils (**Figure 4B**) and basophils (**Figure 4C**) release extracellular DNA after culture in the presence of MSU crystals. Monocytes did not form extracellular traps despite the uptake of crystals (not shown). Furthermore, MSU crystals induced the release of IL-8 in the supernatants of eosinophils. In contrast, neutrophils released huge amounts of IL-6 and IL-8 during NET formation (**Figure 4D**). Since basophils are well known to degranulate spontaneously during prolonged culture the baseline cytokine levels were very high and no cytokine response to MSU was to be observed (not shown). The formation of extracellular traps by eosinophils was specific for MSU crystals; silica crystals did not induce EET structures (**Figure 4E**).

NETting OF GRANULOCYTES INDUCED BY MSU IS NOT SENSITIVE TO PLASMA

The induction of PMA induced NETs is reportedly inhibited even by low concentrations of plasma and is fully abrogated by high plasma concentrations (> 20%). Therefore, we analyzed the NETs formation by PMN in the presence of plasma (**Figure 5**). We incubated isolated PMN with PMA or bacteria, both canonical stimuli for NETs formation, or with MSU crystals in medium containing 0%, 20%, or 100% plasma. PMA, bacteria and MSU crystals induced NET formation in the absence of plasma as revealed by DNA staining. NETs formation induced by bacteria and PMA was drastically reduced in the presence of 20% or 100% plasma. In contrast, MSU crystals induced large NETs by PMN even in the presence of pure plasma. The size and density of the NET aggregates was even higher in the presence of increasing amounts of plasma (**Figure 5**).

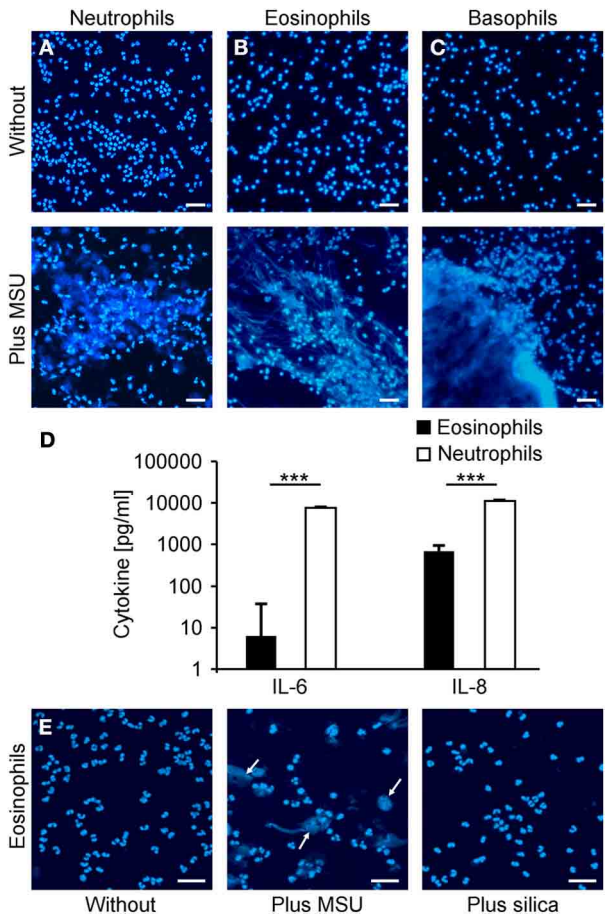

FIGURE 4 | Neutrophils, eosinophils and basophils form extracellular traps after incubation with MSU. (A–C) Isolated neutrophils **(A)**, eosinophils **(B)** or basophils **(C)** in plasma were incubated without (upper) or with MSU crystals (lower). Cytopsins were prepared and stained for extranuclear DNA. The DNA staining showed extracellular trap formation and clumping in neutrophils, eosinophils and basophils. **(D)** Cytokine/chemokine induction of eosinophils and neutrophils were analyzed after MSU incubation. Eosinophils produce traces of IL-6 and intermediate amounts of IL-8 after MSU incubation. Neutrophils release high amounts of IL-6 and IL-8 after incubation with MSU crystals. Values of mock-treated cells served as baseline. **(E)** Isolated eosinophils were incubated without (left), with MSU crystals (middle), or with silica crystals (right). The DNA staining shows extracellular trap formation (arrows) only in samples with MSU crystals. All experiments were performed at least three times. Scale bars, 100 μm; *** $P < 0.001$.

DISCUSSION

Neutrophils have been identified by several groups to produce extracellular DNA traps and consequently these structures have been named "neutrophil extracellular traps." Recent data indicate that this phenomenon is not restricted to neutrophils but is also executed by other cells (Guimaraes-Costa et al., 2012). Beside neutrophils, mast cells and eosinophils have already been described to produce extracellular traps (von Kockritz-Blickwede et al., 2008). In this manuscript we describe a systematic analysis of the ability of blood leukocytes to release extracellular DNA in response to MSU crystals, a strong stimulus of sterile inflammation. In accordance with previous data investigating canonical inducers of NET formation, like PMA, bacteria, and LPS (Fuchs et al., 2007) we observed that PBMC do not release NETs after culture with MSU crystals.

We found that neutrophils, eosinophils, and basophils react differentially in response to MSU crystals. Neutrophils and eosinophils both ingest MSU and release nuclear DNA that forms extracellular fibers. However, in whole blood not all cells may get in direct contact to the crystals within the time frame of the assay (1 h), and do not increase their side scatter. After

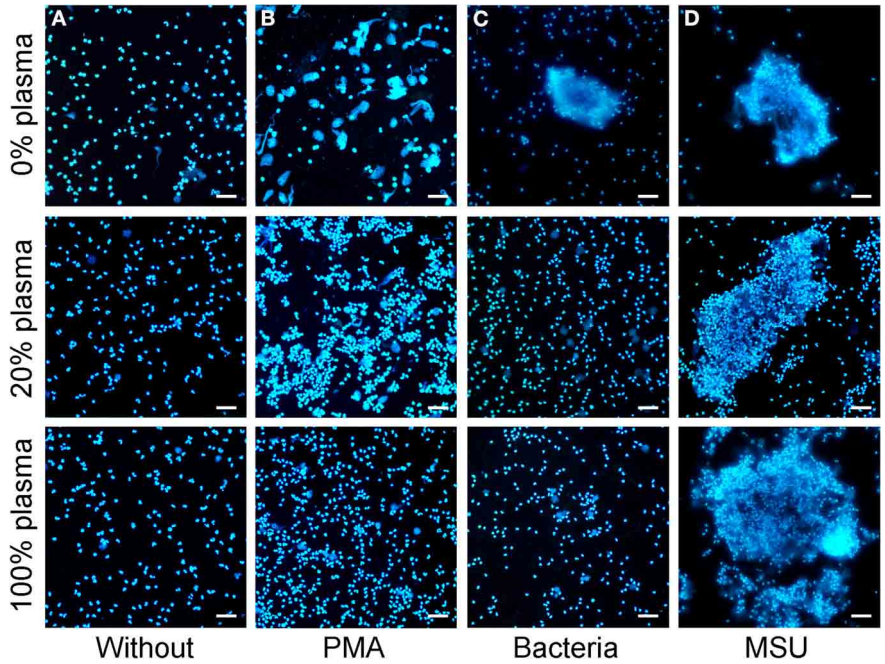

FIGURE 5 | MSU induced NETting of neutrophils is not inhibited by plasma. (A–D) Isolated neutrophils were incubated without stimuli **(A)**, with PMA **(B)**, with bacteria **(C)**, or with MSU crystals **(D)** in the presence of different concentrations of plasma (0% upper; 20% middle, 100% lower). High plasma concentrations inhibit NETting induced by PMA **(B)** and by bacteria **(C)**. MSU induced NETting **(D)** is not inhibited and rather increased by high concentrations of plasma. All experiments were performed at least three times. Scale bars, 100 μm.

longer incubations, the NETosis sequesters the crystals in dense clots. Interestingly, the basophils did not ingest the MSU, but got activated as shown by expression of the activation marker CD203c. Two hours after contact to the MSU crystals, all three granulocytes subtypes formed clusters composed of externalized DNA densely associated with the crystals. In the blood these aggregates were specific for granulocytes and were never seen with other cell types such as monocytes or lymphocytes. Interestingly, as noticed in previous works the formation of extracellular traps was independent of the ingestion of MSU crystals.

Neutrophils are the major leucocyte population of blood. Within minutes following trauma, neutrophils migrate toward the site of inflammation, where they are involved in the frontline immune defense, in phagocytosis of pathogens and in the formation of pus (Weiss, 1989). Neutrophil phagocytosis is strongly enhanced by opsonization of their prey (Burnett et al., 1993). After internalization they may kill the microbes by the formation of ROS and hydrolytic enzymes. Alternatively, neutrophils may degranulate and release an assortment of proteins from specific, azurophilic and tertiary granules (Kumar and Sharma, 2010). In 2004, Brinkmann and colleagues described a surprising observation: certain modes of neutrophil activation cause the release of net-like DNA structures into the extracellular space (Brinkmann et al., 2004). These represent a third mechanism for trapping and killing microbes independent of phagocytosis. This mechanism mainly works extravascularly since NET formation and agglutination of bacteria within the vessels may carry the risk for thrombotic events (Brill et al., 2012) Therefore, it is not surprising that plasma is a strong inhibitor for NETting (Fuchs et al., 2007). Under extreme conditions like sepsis, rapid intravascular NET formation has been observed in the liver sinusoids and the pulmonary capillaries, where it occurs at the expense of endothelial injury (Clark et al., 2007). Similar to this life-threatening condition, the stimulus exerted by MSU crystals also overcomes the inhibitory activity of plasma. Whether this contributes to the pathogenesis of gout is currently under investigation.

Eosinophils ingest inert polystyrene particles and bacteria although less efficiently than neutrophils. The concomitants of phagocytosis, including transcriptional activity, secretion of enzymes and degranulation are similar in neutrophils and eosinophils (Cline et al., 1968). Eosinophils are involved in the fight against helminth infections and are also elevated in the presence of certain parasites. A role for eosinophils in other biological processes has been reported for e.g., allograft rejection and neoplasia (Rothenberg and Hogan, 2006). Therefore, the formation of extracellular traps may contribute to the function of the eosinophils by immobilizing their targets (Guimaraes-Costa et al., 2012). Similar to neutrophils, eosinophils release cytokines during the formation of the extracellular DNA traps. Here, a bias by contaminating neutrophils in the eosinophil preparation is unlikely since the cytokine profiles of eosinophils and neutrophils differed considerably.

Like neutrophils and eosinopils, basophils are motile cells that migrate into inflamed tissues. Similar to mast cells basophils are prone to orchestrate tissue healing and inflammation-related angiogenesis; the latter through the action of vascular endothelial

growth factors and their receptors (Crivellato et al., 2010). The failure to identify basophil-derived cytokines and chemokines induced by MSU crystals is due to the well-established tendency of these cells for spontaneous degranulation (Chirumbolo, 2012), which resulted in high baseline levels of these mediators blurring MSU specific effects.

The uptake of MSU crystals by phagocytes has been shown to be dependent on heat-labile serum-factors and divalent cations (Schorn et al., 2010). In eosinophils, a catapult-like release of mitochondrial DNA after stimulation with lipopolysaccharide, complement or eotaxin has been described, which, reportedly did not end up in cell death (Nizet and Rothenberg, 2008). This is in striking contrast to the formation of extracellular DNA aggregated by eosinophils after coculture with MSU crystals. Here the externalization of nuclear DNA clearly indicated cell death. We also found an upregulation in the release of proinflammatory cytokines by eosinophils after challenge with MSU. These crystals, being responsible for gout, robustly externalized DNA in all subtypes of granulocytes e.g., NETs, EETs, and BETs for neutrophils, eosinophils and basophils, respectively.

Compared with stimuli like PMA or bacteria, which elicit NETs only in conditions of low plasma, MSU provoked NETs also in the presence of full plasma. We argue that the stimulus exerted by MSU is more robust than those from the canonical inducers bacteria and PMA. However, the exact definition of the differences needs further examination. The wide extension of the MSU-induced NETs is most likely due to the fact that the crystals act as scaffold stabilizing the extracellular DNA. Neutrophils are known to be critical for gouty inflammation (Popa-Nita and Naccache, 2010), and NETs have been identified in cells from the synovial fluid (Mitroulis et al., 2011). Pro-inflammatory cytokines carrying danger signals, and protolytical enzymes attached to the NETs may promote tissue injury and inflammation in gouty arthritis. A role of NETs has also been described for the pathogenesis of SLE and asthma, suggesting an importance of these extracellular DNA structures in the amplification of inflammatory responses (Hakkim et al., 2010). In the case of SLE, the well-established deficiency for the clearance of dying cells and nuclear remnants also includes the dismantling of NETs (Janko et al., 2008; Muñoz et al., 2010).

ACKNOWLEDGMENTS

This project was supported by the Interdisciplinary Center for Clinical Research (IZKF) at the University Hospital of the University of Erlangen-Nuremberg, project A41 (Christine Schorn, Martin Herrmann), by the Masterswitch project of the European Union (Georg Schett, Martin Herrmann), by Deutsche Forschungsgemeinschaft (SFB643-TP B5) (Christina Janko), by the training Grant GK SFB 643 from the DFG (Christine Schorn, Christina Janko), and the K. und R. Wucherpfennigstiftung (Martin Herrmann).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 13 June 2012; paper pending published: 04 July 2012; accepted: 15 August 2012; published online: 03 September 2012.

*Citation: Schorn C, Janko C, Latzko M, Chaurio R, Schett G and Herrmann M (2012) Monosodium urate crystals induce extracellular DNA traps in neutrophils, eosinophils, and basophils but not in mononuclear cells. Front. Immun. **3**:277. doi: 10.3389/fimmu.2012.00277*

This article was submitted to Frontiers in Inflammation, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 08 March 2013
doi: 10.3389/fimmu.2013.00050

Mechanisms of disease: inflammasome activation and the development of type 2 diabetes

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Over the recent past, the importance of aberrant immune cell activation as one of the contributing mechanisms to the development of insulin-resistance and type 2 diabetes (T2D) has been recognized. Among the panoply of pro-inflammatory cytokines that are linked to chronic metabolic diseases, new data suggests that interleukin-1β (IL-1β) may play an important role in initiating and sustaining inflammation-induced organ dysfunction in T2D. Therefore, factors that control secretion of bioactive IL-1β have therapeutic implications. In this regard, the identification of multiprotein scaffolding complexes, "inflammasomes," has been a great advance in our understanding of this process. The secretion of bioactive IL-1β is predominantly controlled by activation of caspase-1 through assembly of a multiprotein scaffold, "inflammasome" that is composed of NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) ASC (apoptosis associated speck-like protein containing a CARD) and procaspase-1. The NLRP3 inflammasome appears to be an important sensor of metabolic dysregulation and controls obesity-associated insulin resistance and pancreatic beta cell dysfunction. Initial clinical "proof of concept" studies suggest that blocking IL-1β may favorably modulate factors related to development and treatment of T2D. However, this potential therapeutic approach remains to be fully substantiated through phase-II clinical studies. Here, we outline the new immunological mechanisms that link metabolic dysfunction to the emergence of chronic inflammation and discuss the opportunities and challenges of future therapeutic approaches to dampen NLRP3 inflammasome activation or IL-1β signaling for controlling type 2 diabetes.

Keywords: inflammation, T cells, adipocytes, glyburide, macrophages, pycard, IL-1β, caspase-1 apoptosis

INTRODUCTION

With a disease rate of 8.3% and cost of $174 billion, there is no debate that diabetes is a highly prevalent and costly life-long disease (Dall et al., 2010; Centers for Disease Control and Prevention, 2011). Whereas type 1 diabetes is characterized by autoimmune destruction of pancreatic islets, Type 2 diabetes (T2D) has been described as an autoinflammatory disorder, characterized first by insulin resistance in peripheral tissues followed by beta cell failure, including decreased islet size and insulin production (McGonagle and McDermott, 2006).

T2D is clearly associated with obesity, and clinical progression of this disease has been linked to chronic low-grade inflammation due to activation of immune cells. However, up until recently, the identity of specific immunological sensors that are triggered in response to metabolic dysfunction to produce a state of inflammation was not fully understood. The underlying clinical rationale to identify the immunological triggers of metabolically driven inflammation has been to develop approaches to therapeutically target the immune sensors and break the feed-forward cycle of organ dysfunction and development of diabetes. Among several sites of inflammation in metabolic diseases, adipose tissue is a large contributor to circulating proinflammatory cytokines during obesity. Initially, tumor necrosis factor-α (TNFα) was considered a possible therapeutic target because its adipose tissue expression was increased in multiple rodent-obesity models and TNFα decreased insulin signaling in insulin sensitive tissues (Hotamisligil et al., 1993, 1994a,b; Hotamisligil and Spiegelman, 1994). Accordingly, *Tnf* mRNA expression was shown to be increased in adipose tissue of obese hyperinsulinemic human subjects (Hotamisligil et al., 1995). Furthermore, weight loss-induced improvement in insulin-sensitivity was associated with reduction in TNF suggesting that this pro-inflammatory cytokine impairs insulin-action. (Hotamisligil et al., 1995). Consistent with these clinical findings, mechanistic studies using *Tnfα* gene knockout mice or neutralization of TNF with antibodies improved glycemia in obese mice and rats, respectively, making it a potential therapeutic target for T2D (Hotamisligil et al., 1994a; Uysal et al., 1997). Despite overwhelming evidence in favor of TNF having a critical role in regulating inflammation and insulin-action (Hotamisligil et al., 1993, 1994a,b, 1995; Hotamisligil and Spiegelman, 1994; Peraldi et al., 1996; Uysal et al., 1997; Liu et al., 1998), the translation of basic research findings with TNF targeted neutralization approaches to diabetes care in humans has had disappointing results with both acute

and chronic treatment (Ofei et al., 1996; Paquot et al., 2000; Di Rocco et al., 2004; Wascher et al., 2011). Further studies to enhance the delivery and tissue availability of TNF targeted treatments are being pursued to improve treatment outcomes. Here, we discuss clinically relevant, novel "inflammasome" mechanisms that regulate interleukin-1β (IL-1β) and IL-18 driven pro-inflammatory cascades. We discuss the current experimental evidence and potential future therapeutic strategies to target the "inflammasome" pathway in prevention and treatment of diabetes.

THE NLRP3 INFLAMMASOME REGULATES IL-1β SECRETION DURING METABOLIC STRESS

Given the recent developments in understanding inflammation as a mediator of disease progression, an understanding of factors related to IL-1β regulation is in order. IL-1β is a proinflammatory cytokine that is implicated in the pathogenesis of many inflammatory diseases including diabetes, rheumatoid arthritis and genetic auto-inflammatory disorders (Dinarello, 2011). Although IL-1β is produced by many cell types, it is predominately produced by monocytes, macrophages, and neutrophils (Dinarello, 2011). While most cytokines are regulated at the transcriptional (gene regulation) level, IL-1β is further regulated at the protein level, being stored as an inactive pro-form, which must be cleaved by the IL-1β processing cysteine protease, caspase-1 (**Figure 1**) for its secretion and activation (Dinarello, 2011). As an additional level of control, caspase-1 is also stored in an inactive state which in turn is activated within large cytosolic multiprotein complexes termed "inflammasomes" upon receiving specific signals (Schroder and Tschopp, 2010). Classically, the inflammasome driven caspase-1 activation and IL-1β secretion occurs as innate immune cells like macrophages engulf bacterial, fungal, and viral proteins. The inflammasome activation is therefore a vital immune response to protect the host against numerous pathogens (Schroder and Tschopp, 2010). Interestingly, new evidence suggest that inflammasome activation may be important in chronic diseases such as obesity and diabetes where low

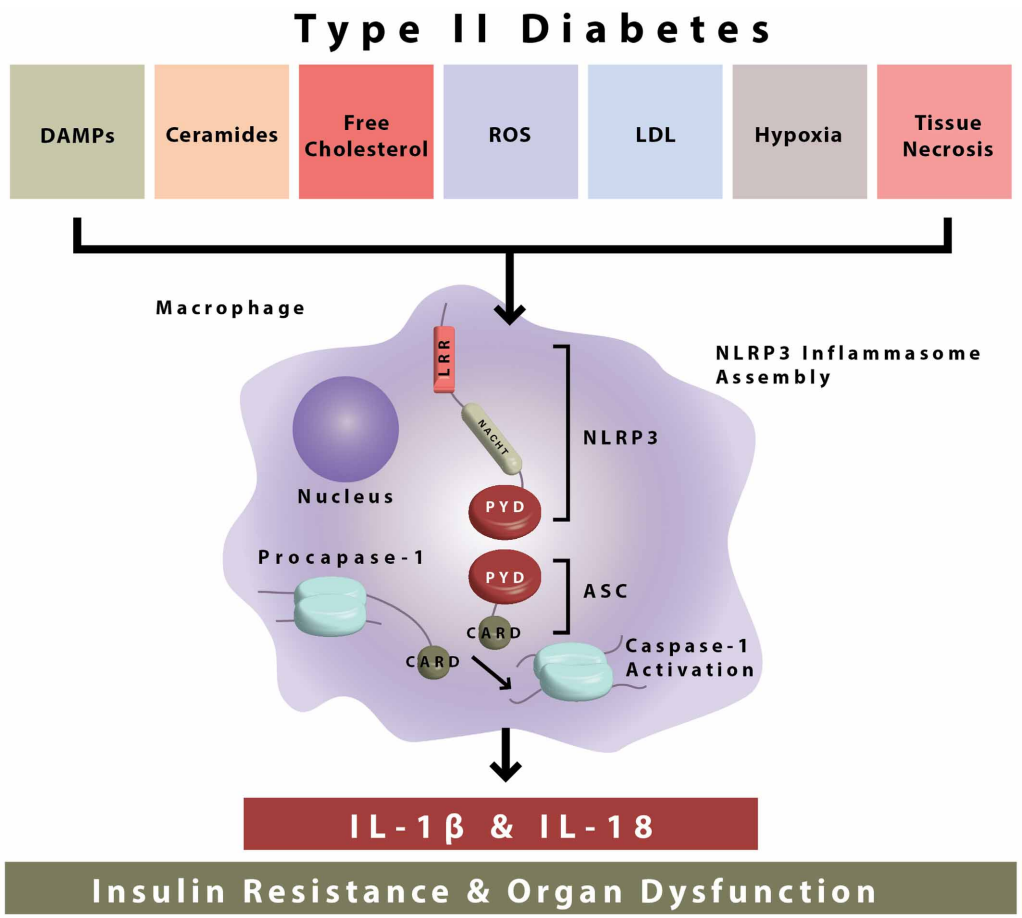

FIGURE 1 | NLRP3 inflammasome activation during type II diabetes. The NLRP3 inflammasome is composed of NLRP3, ASC, and procaspase-1. During type II diabetes there is increased accumulation of endogenous danger-associated molecular patterns (ceramides, free cholesterol, etc.) which are sensed by the NLRP3 inflammasome. After sensing of DAMPs, inflammasome assembly occurs by interactions in the protein domains of NLRP3 ASC and procaspase-1. This leads to caspase-1 activation and the secretion of bioactive IL-1β and IL-18, which go on to cause insulin resistance and organ dysfunction.

grade inflammation occurs without overt infection (Schroder and Tschopp, 2010).

The NLRP3 inflammasome is formed through the interaction of several cellular proteins. As demonstrated, these key proteins are identified as NLRP3 (for nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) ASC (apoptosis associated speck-like protein containing a CARD), and procaspase-1 (**Figure 1**). NLRP3 is expressed predominantly in circulating monocytes and tissue macrophages (Guarda et al., 2011). The NLRP3 inflammasome components contain conserved protein domains and interaction of these domains on inflammasome proteins (i.e., homotypic protein–protein interactions) leads to inflammasome assembly. Thus, the pyrin domain of NLRP3 interacts with the pyrin domain of ASC, and the CARD (caspase activation recruitment domain) of ASC interacts with the CARD domain of procaspase-1 (**Figure 1**). In this way, the NLRP3 inflammasome is formed leading to the cleavage of procaspase-1 to its enzymatically activated form (**Figure 1**).

Activation of the NLRP3 inflammasome by bacteria that produce pore forming toxins (Mariathasan et al., 2006), viruses (Muruve et al., 2008; Allen et al., 2009), and fungi (Gross et al., 2009) and the resultant release of IL-1β and IL-18 plays a critical role in host defense. Interestingly, the NLRP3 inflammasome can also be activated in response to accumulation of endogenous damage associated molecular patterns (DAMPs) that are of non-microbial origin and cause "sterile inflammation" (**Figure 1**). NLRP3 inflammasome activating metabolic "danger signals" include, urate, cholesterol crystals, extracellular ATP, certain fatty acids and islet amyloid peptides (**Figure 1**). Growing recognition of the NLRP3 inflammasome pathway in triggering sterile inflammation, i.e., inflammation of non-infectious origin has put this innate immune sensor at the crossroads of metabolic disease and inflammation (Wen et al., 2012).

The accumulation of DAMPs during chronic inflammatory diseases is hypothesized to contribute to systemic inflammation and disease pathogenesis. During the pathogenesis of T2D, the NLRP3 inflammasome has been proposed to sense and mediate downstream inflammatory events of "glucotoxicity" (Zhou et al., 2010), islet amyloid polypeptide (Masters et al., 2010), lipid intermediates (i.e., ceramides) (Vandanmagsar et al., 2011), and fatty acids (Wen et al., 2011). The mechanism of inflammasome activation that links these events remains ambiguous, but it may be that these danger signals converge on similar signaling pathways resulting in inflammasome activation.

Mitochondrial damage may be the common pathway between these stimuli because reactive oxygen species appear to be necessary for NLRP3 inflammasome activation, and changes in the redox state of the cell may be a common mediator between danger signals and inflammasome activation (Jin and Flavell, 2010). Treatment of macrophages with LPS and ATP leads to increased reactive oxygen species, mitochondrial damage, and release of mtDNA, a DAMP, into the cytosol (Nakahira et al., 2011). Although mitochondrial DNA is sensed by the AIM2 inflammasome, it also serves as a co-activator of caspase-1 in conjunction with NLRP3 activation by LPS and ATP (Nakahira et al., 2011). Autophagy, a process by which cells remove damaged organelles, buffers inflammasome activation by removing damaged mitochondria, and limiting ROS production and mtDNA escape into the cytosol, and is activated by NLRP3 inflammasome activators (Shi et al., 2012). Moreover, the inflammasome components NLRP3 and ASC are ubiquitinated and subsequently degraded by autophagy, and pro-IL-1β is degraded by autophagy as well (Harris et al., 2011; Shi et al., 2012). Thus, there is a complex interplay between autophagic maintenance of mitochondria and inflammasome proteins that controls inflammasome activation.

Activated caspase-1 proceeds to cleave pro-IL-1β, pro-IL-18, and other undefined substrates. IL-1β signaling then occurs through the IL-1 receptor I (IL1R1), and leads to the activation of the transcription factor nuclear factor-Kappa-Beta (NFkB) and the expression of inflammatory genes (**Figure 1**) Though lymphocytes are considered a primary target of IL-1β and IL-18, the receptors for both these inflammasome-dependent cytokines are ubiquitously expressed in many different types of cells and tissues, including high expression in pancreatic islets (Boni-Schnetzler et al., 2009). IL-1β signaling is inhibited by IL-1 receptor antagonist (IL-1RA) and decoy receptors, i.e., IL-1R2. Decoy receptors appear to bind IL-1 but do not cause IL-1 signaling. IL-1 signaling is necessary for the inflammatory response, but is highly regulated due to the negative effects of chronic inflammation to body tissues and organs. Regulation of IL-1 signaling is maintained in healthy individuals, but appears to be elevated during chronic proinflammatory disease states, which makes this pathway a valuable therapeutic target in T2D.

REDUCED NLRP3 INFLAMMASOME ACTIVATION INCREASES INSULIN SENSITIVITY AND IMPROVES GLUCOSE HOMEOSTASIS

The NLRP3 inflammasome, as described in **Figure 1**, has emerged as a key regulator of glucose and insulin homeostasis. Specifically, pre-clinical studies have shown that the genetic deletion of Nlrp3 and Asc in high-fat diet fed mice results in improved glucose tolerance and enhanced insulin sensitivity (Stienstra et al., 2011; Vandanmagsar et al., 2011; Wen et al., 2011). Along with systemic improvements in glucose and insulin homeostasis, the gene knockout animals, i.e., *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ have decreased circulating IL-18, and reduced adipose tissue IL-1β, markers of caspase-1 activation (Vandanmagsar et al., 2011). The pleiotropic effects of inhibition of NLRP3 inflammasome in obesity are evident by improved insulin signaling in adipose tissue, liver, and skeletal muscle and increased insulin secretion in the pancreas [**Figure 2**; (Stienstra et al., 2011; Vandanmagsar et al., 2011; Wen et al., 2011)].

ADIPOSE TISSUE

Adipose is a complex tissue consisting of adipocytes, immune cells, vasculature, and stromal cells. Macrophages are recruited to adipose tissue during obesity and represent the largest population of NLRP3 expressing cells in fat (Weisberg et al., 2003). Recent studies in animal models demonstrate that obesity is associated with progressive caspase-1 activation in adipose tissue

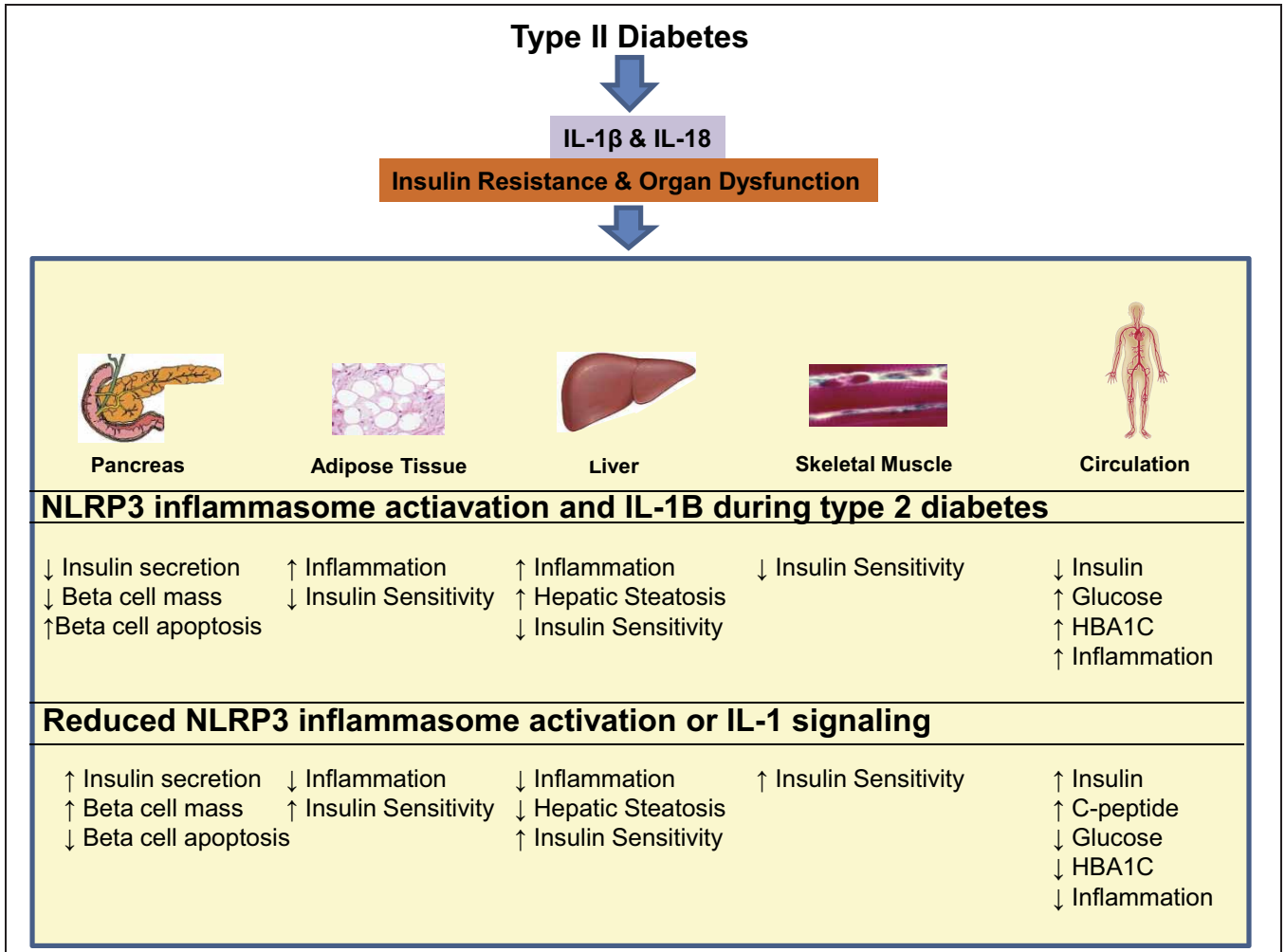

FIGURE 2 | The consequences of NLRP3 inflammasome activation during type II diabetes and possible benefits of NLRP3 and IL-1 targeted therapies. NLRP3 inflammasome activation during type II diabetes results in the production of IL-1β and IL-18, which go on to cause insulin resistance and organ dysfunction in the pancreas, adipose tissue, liver, skeletal muscle, and circulation. NLRP3 and IL-1β targeted therapy may have potential to reduce local tissue inflammation and systemic inflammation resulting in systemic improvement in insulin secretion, insulin sensitivity as well as organ function.

(Vandanmagsar et al., 2011). Consistent with the causal role of NLRP3 inflammasome activation in the development of inflammation, deletion of Nlrp3 in mice, prevents obesity-induced caspase-1 activation.

The NLRP3 inflammasome instigates inflammation and causes leukocytosis (i.e., increased immune cell infiltration) in visceral adipose tissue during obesity. Consistent with this, the $CD4^+$ and $CD8^+$ T cell leukocyte sub populations are specifically reduced in visceral adipose tissue of obese *Nlrp3*$^{-/-}$ mice (Vandanmagsar et al., 2011). This reduction in T cells in response to reduced NLRP3 inflammasome activation is attributable to a decrease in effector-memory T cell subtype presence in the adipose tissue (Vandanmagsar et al., 2011). Together with prior studies, these data suggests that approaches that reduce the number of activated T cell populations in adipose tissue lowers inflammation and improves insulin-action (Feuerer et al., 2009; Nishimura et al., 2009; Winer et al., 2009; Yang et al., 2010).

Visceral adipose tissue macrophages isolated from obese *Nlrp3*$^{-/-}$ mice have reduced expression of the proinflammatory cytokine *Tnf*-α and chemokines (*Ccl20* and *Cxcl1*) involved in lymphocyte recruitment. These results demonstrate the pleiotropic effects of the NLRP3 inflammasome on activation and recruitment of adipose tissue leukocytes. The functional consequences of reduced inflammation in *Nlrp3* and *Asc* deficient mice are improved insulin signaling both in fat and other insulin sensitive tissues (Vandanmagsar et al., 2011; Wen et al., 2011).

Inflammation plays a causal role in insulin resistance, and in rodent models targeting inflammatory cytokine production through genetic and pharmacological approaches results in improvements in insulin signaling (Olefsky and Glass, 2010; Kanneganti and Dixit, 2012). After insulin binds to the insulin receptor, insulin initiates signaling cascades that activate downstream pathways, notably PI3K-AKT and the mitogenic MAP kinase-ERK pathways (Biddinger and Kahn, 2006). In adipose tissue of obese *Nlrp3*$^{-/-}$ mice, phosphorylation of AKT is enhanced

indicating greater insulin signaling (Vandanmagsar et al., 2011). The reduction of IL-1 signaling also improves adipose tissue insulin sensitivity in a similar way. Adipose tissue explants from high-fat-fed IL-1 receptor null mice exhibit improved insulin signaling compared to wild type animals, including increased glucose transport, AKT phosphorylation, and increased gene expression of proteins involved with insulin signaling and glucose uptake (*Irs-1* and *Glut4*) (McGillicuddy et al., 2011). These results demonstrate the strong immune and metabolic consequences of NLRP3 inflammasome activation and IL-1 signaling during obesity.

Not only does caspase-1 activation influence whole adipose tissue insulin sensitivity, but it may have direct effects on adipocyte growth, differentiation and metabolism (Stienstra et al., 2010). Interestingly, human and mouse adipocyte cell lines express the caspase-1 protein, and its expression is increased over the course of adipocyte differentiation (Stienstra et al., 2010). Caspase-1 inhibition results in increased expression of favorable adipogenic (*Pparγ*) and metabolic markers (*Glut4* and *adiponectin*) in 3T3-L1 adipocytes (Stienstra et al., 2010). Given low expression of *Nlrp3* and *Asc* in adipocytes (Vandanmagsar et al., 2011), the significance of adipocyte-derived IL-1β remains ambiguous because macrophages are the predominant cellular sources of IL-1β.

Caspase-1 activates multiple protein substrates other than IL-1β and IL-18, so the exact contribution of downstream mediators of NLRP3 inflammasome activation remains unclear. IL-1β treated 3T3-L1 adipocytes have reduced capacity to differentiate into mature adipocytes, and exhibit insulin resistance and reduce glucose uptake (Lagathu et al., 2006; Jager et al., 2007; Stienstra et al., 2010). Surprisingly, IL-18 does not appear to have an effect on 3T3-L1 adipocyte differentiation or the expression of adipogenic genes in spite of its known pro-inflammatory properties (Stienstra et al., 2010). Given that IL-18 promotes differentiation of T cells into activated pro-inflammatory T-helper1 (T_H1) IFNγ producing cells (Okamura et al., 1995), it is likely that NLRP3 inflammasome mediated IL-18 secretion induces adipose tissue inflammation via T cell activation (Vandanmagsar et al., 2011; Wen et al., 2011).

SKELETAL MUSCLE AND LIVER

Skeletal muscle is a large metabolically active tissue and accounts for the majority of insulin stimulated glucose disposal. As indicated by improved performance on glucose and insulin tolerance tests, obese *Nlrp3*$^{-/-}$ mice exhibit increased skeletal muscle insulin signaling (Vandanmagsar et al., 2011; Wen et al., 2011). The effects on skeletal muscle are most likely driven through decreased adipose tissue and systemic inflammation because there are not high concentrations of Nlrp3 expressing cells within skeletal muscle, however, local macrophages could be influencing this process.

The liver is also a major contributor to glucose homeostasis by generating glucose through gluconeogenesis. The liver also becomes insulin resistant during the development of T2D, and this is associated with increases in the levels of hepatic steatosis. IL-1β may contribute to this process, given that cultured liver cells treated with IL-1β exhibit decreased insulin response (Nov et al., 2010). *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ knockout mice exhibit enhanced liver AKT activation in response to insulin challenge compared to high-fat fed WT mice (Vandanmagsar et al., 2011; Wen et al., 2011). Along with improvements of liver insulin sensitivity, obese *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice exhibit reduced hepatic steatosis compared to wild type controls (Stienstra et al., 2011; Vandanmagsar et al., 2011). Notably, recent studies also show that NLRP3 inflammasome is required for the maintenance of gut epithelial integrity. In response to methionine-choline deficiency (a model of NASH in mice), NLRP3 inflammasome deficient animals develop exaggerated fatty liver disease due to microbial pathogen-associated molecular patterns leakage into the liver via the portal circulation and activation of the pro-inflammatory response via the Toll-like receptors 4 and 9 (Henao-Mejia et al., 2012). In the same study, *Asc*$^{-/-}$ mice were also noted to have increased weight gain and glucose intolerance during high fat feeding, which were reversible upon administration of antibiotics. This is in contrast with other findings that indicate *Asc*$^{-/-}$ mice have increase insulin sensitivity and glucose tolerance (Youm et al., 2011). T2D has been associated with increased circulating endotoxin concentration, but it is unclear whether this is cause or consequence in disease pathogenesis (Pussinen et al., 2011).

NLRP3 INFLAMMASOME AND INSULIN SECRETION

Pancreatic islets, macrophages and dendritic cells may all be sources of IL-1β in the pancreas. Cultured pancreatic islets are thought to produce low levels of IL-1β (Arnush et al., 1998). Macrophages and dendritic cells also reside in the pancreas, and macrophages are increased in rodent models of T2D and in patients with T2D (Ehses et al., 2007). It has been shown that IL-1β treatment alone or in combination with IFN-γ induces beta cell death in cell culture, although the exact mechanism by which this occurs is debated (Mandrup-Poulsen et al., 1986; Collier et al., 2011). Consistent with those results, blocking IL-1β action on isolated beta cells using IL-1RA (IL-1 receptor antagonist) improves beta cell survival (Ardestani et al., 2011). IL-1β production in the pancreas is likely mediated by NLRP3 inflammasome-dependent activation of caspase-1 (Youm et al., 2011) as also outlined in **Figure 1**. In support of this concept, mice that lack NLRP3 inflammasome components (*Nlrp3*, *Asc*) have increased pancreatic islet size in response to chronic high-fat diet, resulting in increased insulin response to glucose challenge despite improvements in peripheral insulin sensitivity (Youm et al., 2011). Additionally, reduction of Nlrp3 inflammasome activation in chronically obese mice protects the pancreatic beta cells against cell death (Youm et al., 2011). These findings suggest that reduction in Nlrp3 inflamamsome activity may protect the pancreatic islet from caspase-1 mediated inflammatory death. TXNIP may be a crucial mediator connecting beta cell death and inflammasome activation by linking glucotoxicity and ER stress to NLRP3 inflammasome activation (Zhou et al., 2010). Global TXNIP$^{-/-}$ mice recapitulate an insulin sensitive phenotype very similar to that of NLRP3 ablation (Yoshihara et al., 2010). In pancreatic beta cells, TXNIP ablation reduces glucotoxicity, ER Stress and the subsequent inflammatory and apoptotic responses (Zhou et al., 2010; Oslowski et al., 2012). TXNIP serves as a signaling node linking ER stress, IL-1B production

and beta cell apoptosis. The consequences of pancreatic beta cell TXNIP and inflammasome activation *in vivo* are unclear because beta cell and myeloid cell specific knockouts have not been used to address this issue. Consistent with the important role of IL-1β in the pancreas, *Il1r1*$^{-/-}$ mice that are deficient in IL-1β signaling exhibit improved insulin-secretion in response to glucose challenge (McGillicuddy et al., 2011). Thus, lowering Nlrp3 inflammasome activation may protect against the transition from insulin-resistance to an overt type 2 diabetic stage by mechanisms that involve protection from loss of insulin-producing beta cells. It is presently unclear whether persistent Nlrp3 inflammasome activation causes the transition from insulin-resistance to islet decompensation and development of overt T2D.

THERAPEUTIC IMPLICATIONS

Initial studies in humans suggest that NLRP3 inflammasome activation in obesity could be important in development and treatment of insulin-resistance and diabetes [**Figure 2**; (Larsen et al., 2007; Vandanmagsar et al., 2011; Goossens et al., 2012)]. Lee et al. recently published a study on inflammasome activation in blood monocytes isolated from type 2 diabetic, drug naive patients ($n = 47$) and healthy controls ($n = 57$) (Lee et al., 2013). This study determined that both during basal and inflammasome activating conditions (stimulation with free fatty acids, ATP, or urate) blood monocytes from patients with T2D have greater caspase-1 activation and secretion of the caspase-1 activated proteins, IL-1β and IL-18. Inflammasome activation can occur in response to diverse cellular stresses including reactive oxygen species, the unfolded protein response and altered autophagy. In the context of this experiment, hyperglycemia in these T2D patients resulted in elevated ROS production and greater inflammasome activation. Knockdown of ASC or NLRP3 using RNA interference abrogated the response to DAMPs demonstrating specificity to this pathway in T2D patients (Lee et al., 2013). This study provides evidence that the Nlrp3 inflammasome activation in T2D patients contributes toward the chronic pro-inflammatory state.

Gossens et al. designed a study to assess the gene expression of *Nlrp3* and T-cell markers in subcutaneous adipose tissue from lean and obese subjects, and to determine if these genes were associated with glucose homeostasis measured by the hyperinsulinemic-euglycemic clamp (Goossens et al., 2012). Obese subjects had increased body weight, body fat%, adipocyte diameter, fasting glucose, and insulin and glucose infusion rate during the hyperinsulinemic-euglycemic clamp test. The expression of cellular markers of inflammasome activation, i.e., *Nlrp3*, *caspase-1*, and T cell markers, were positively associated with increased expression of inflammatory genes. Furthermore, *caspase-1* and *Il-18* gene expression, and the ratio of *Tbx21*/*Cd3*ε expression, a marker of pro-inflammatory T-helper 1 cells, were negatively correlated with glucose infusion rate. Consistent with relevance of this pathway in diabetes treatment, *Il-1*β, *Nlrp3*, and *Asc* gene expression is reduced after 1 year of weight loss in obese type 2 diabetic patients, and these gene expression changes were positively correlated with improvements in glycemia (Vandanmagsar et al., 2011). Such associations are further supported by evidence from human adipose tissue explants that high glucose levels induce proinflammatory gene expression (*Il-6*, *Il-8*, and *Il-1*β), increase intracellular pro-IL1β and secretion of bioactive IL-1β (Koenen et al., 2011). Taken together, these studies indicate that the NLRP3 inflammasome components are expressed in human adipose tissue, are responsive to high glucose concentrations, and associate with markers of glycemia.

Given the key role of the inflammasome in possibly mediating many of the factors associated with progression to T2D, and in control of the condition, it is imperative to test interventions that may favorably interdict on this system. In humans, there has been interest in a specific IL-1 receptor antagonist. Anakinra, IL-1 receptor antagonist, competes with IL-1β for binding to IL1R1 and has received attention as an agent that may have efficacy on glycemic control. Larsen et al. conducted a double-blind, parallel-group trial in which anakinra ($n = 34$) or placebo ($n = 33$) was administered subcutaneously once/day for 13 weeks (Larsen et al., 2007). The study population were patients with T2D, >27 body mass index, glycated hemoglobin >7.5% and had no change in medication type or dose over the course of the study. As expected, subjects in the anakinra treatment group had increased circulating IL-1RA concentration compared to placebo, 1256 ± 958 and 0.6 ± 0.4 μg/L, respectively. Anakinra treatment resulted in significant reduction in glycated hemoglobin % measured by the difference between baseline and 4 or 13 weeks in the treatment versus placebo groups (Larsen et al., 2007). As reported, at 13 weeks, in the anakinra group, the glycated hemoglobin level was 0.46% points lower than in the placebo group ($P = 0.03$).

To explain changes in glycated hemoglobin concentration, beta-cell secretory function and insulin sensitivity were measured at 13 weeks. Anakinra treatment lowered the proinsulin:insulin ratio and increased C-peptide concentration in response to oral or intravenous glucose, all measures were presented as change from baseline. Thus, anakinra treatment increased circulating IL-1RA and the insulin secretory capacity of the pancreas, but had no effect on insulin sensitivity. In agreement with no effect on insulin action, insulin-regulated gene expression in skeletal muscle, serum adipokine levels, and body-mass index were found to be similar in the two study groups. Similar results were achieved from a study on patients with prediabetes that showed enhanced beta-cell function during OGTT in response to anakinra treatment, in the absence of increased insulin sensitivity (van Asseldonk et al., 2011). Blockade of IL-1 with anakinra does improve glycemia and beta-cell secretory function and reduces markers of systemic inflammation.

A follow up study on the diabetics from the Larsen et al. was performed after the initial anakinra treatment was removed. Interestingly, this relatively short term (13 weeks) anakinra treatment had long term effects after the treatment was removed. Thirty-nine weeks after the treatment, the anakinra treated group had an improved blood proinsulin:insulin ratio, decreased C-reactive protein and IL-6 concentrations, but no differences in hemoglobin A1c (Larsen et al., 2009). Within this study there were responders and non-responders. Responders had lower starting serum IL-1RA concentrations and a higher group frequency of

a specific gene polymorphism (SNP rs4251961 allele C), which is associated with low circulating IL-1RA (Larsen et al., 2009), indicating that IL-1RA may be beneficial for only specific populations. Reduced IL-6 concentrations may also be responsible for a portion of improvements of pancreatic function with anakinra treatment in that study. A trial utilizing the IL-6 monoclonal antibody Actemra (tocilizumab) during T2D could test this hypothesis.

In summary, the findings thus far indicated that IL-1 blockade with specific agents induces improvement of pancreatic insulin secretory function and reduces markers of systemic inflammation lasting 39 weeks after treatment withdrawal. Given these results, it is of interest to speculate that IL-1β specific monoclonal antibodies will have beneficial effects in T2D. Monoclonal antibodies directed against IL-1β may be favorable compared with anakinra due to the short half-life of anakinra, which requires daily injections, and specificity to IL-1β. While anakinra antagonizes IL-1R1 signaling by both IL-1β and IL-1α, neutralization of IL-1β through monoclonal antibodies may be favorable because IL-1α signaling still remains. Intact, IL-1α signaling may also reduce the efficacy of monoclonal antibodies because inflammasome activators can cause the secretion of IL-1α in addition to IL-1β (Gross et al., 2012). Proof of concept for monoclonal antibodies has been obtained. In a phase 1 study, gevokizumab (XOMA 052), a monoclonal antibody against IL-1β, showed beneficial effects on glycemic control and beta-cell function. Specifically, studies were conducted that evaluated single intravenous infusion or subcutaneous injection of placebo or IL-1β targeted monoclonal antibody at 0.01–3.0 or 0.03–0.3 mg/kg, respectively. One month after antibody administration, there were no significant differences in HbA1c, but at 2 and 3 months a significant reduction was noted in the intermediate doses (0.03–1.0 mg/kg) compared to the combined low dose (0.01 mg/kg) and placebo group (Cavelti-Weder et al., 2012). As expected, intravenous administration of gevokizumab resulted in greater circulating antibody concentrations than subcutaneous administration. Subcutaneous delivery of the 0.03 and 0.3 mg/kg antibody doses at day 0, 14, and 28 did not result in a substantial improvement over a single administration at day 0 alone. It may be that microvascular damage that occurs during T2D is limiting tissue blood perfusion and for this reason the circulating antibody concentrations do not reflect the increased number of doses. A phase 2 trial was done using gevokizumab in 421 patients, but beneficial effects on glycated hemoglobin and glycemia were not observed, while reduction in C-reactive protein was observed (DeGuzman, 2011). The capacity of gevokizumab to improve glucose homeostasis is currently unclear, but like anakinra it may be effective in specific populations. As indicated in the study by Larsen et al., there may be a subset of the population that responds more favorably to IL-1β targeted therapy. The stronger effects of anakinra could be due to inhibition of both IL-1β and IL-1α signaling. It is important to recognize that inflammation is an ongoing process and that IL-1β targeted therapies will reduce inflammation at the time of administration, but prior inflammatory damage incurred during the lifespan may not be readily reversible. Thus, the timing of the intervention may be key to IL-1β targeted therapy and prevention of pancreatic damage may be more useful than treatment of chronic pancreatic inflammation. Importantly, given the NLRP3 inflammasome is an upstream activator of caspase-1 and IL-1, the inhibition of exaggerated NLRP3 inflammasome activation by future drugs may have better therapeutic outcomes in T2D.

Another intriguing question is whether any of the currently available agents we have at our disposal for use in T2D have any effect on the inflammasome. The cellular energy sensor, AMPK, is known to influence caspase-1 activation. As discussed earlier, the NLRP3 inflammasome is activated by fatty acids, notably palmitate, but this effect is blocked by administration of AICAR, an AMPK agonist. Metformin is a known AMPK activator and 2m of metformin treatment reduces NLRP3 inflammasome activation in peripheral blood mononuclear cells from type 2 diabetic patients. In addition to metformin, the sulfonylurea glyburide has been identified as an NLRP3 inflammasome inhibitor, while other sulfonylurea drugs do not inhibit inflammasome activation (Lamkanfi et al., 2009). This is an interesting finding considering the concerns hypoglycemia, weight gain, and secondary failures with use of sulfonylureas. In certain circumstances, particularly sepsis, glyburide can act as an anti-inflammatory and is associated with reduce mortality in lipopolysaccharide challenged rodents, and observationally in type 2 diabetics with melioidosis compared to patients with no diabetes (Lamkanfi et al., 2009; Koh et al., 2011). This effect was limited to glyburide treatment and was not associated with either metformin or insulin treatment. It is presently unclear if the use of glyburide at doses lower than those typically used clinically will have positive effects on beta cell survival and this question may deserve a more thorough investigation. Compared to IL-1 targeted therapies, glyburide may provide the additional benefit of inhibiting the NLRP3 inflammasome and cleavage of many caspase-1 substrates. It would be useful to directly assess the effect of low-dose glyburide on markers of inflammasome activation in diabetics or pre-diabetics. As discussed above, its effects may be more useful for the prevention of pancreatic damage rather than an intervention after damage has already occurred.

Because IL-1 is important for innate immune signaling, blocking its action with IL-1RA could have negative effects on the immune response. In the study by Larsen et al., there were minimal adverse effects of anakinra, with the predominant negative effect being an increase in injection site reactions (Larsen et al., 2007). In a larger study of anakinra treatment for rheumatoid arthritis there was no increase in adverse events besides injection site reactions (Nuki et al., 2002). IL-1 neutralization may provide a path to improving pancreatic function, with minimal adverse effects. However, improvements in drugs targeting this pathway are required because of the modest effects that have been seen thus far.

CONCLUSION

IL-1β is an influential immune modulator. Uncontrolled activation of the NLRP3 inflammasome during disease states and increased IL-1β synthesis, secretion, and signaling can lead to inflammatory disease. Initial clinical studies that dampen IL-1β signaling have shown promise in controlling diabetes but detailed

evidence to support use of IL-1 neutralizing antibodies in diabetic patients has thus far not yielded successful results. Notably, once cleaved, caspase-1 can regulate the activity of several proteins other than just IL-1β and IL-18 (Agard et al., 2010). New data shows that high-fat diet feeding-induced caspase-1 can deactivate Sirt1 and leads to insulin-resistance (Chalkiadaki and Guarente, 2012). Activation of Sirt1 by resveratrol and other small molecules improves glycemic control (Lagouge et al., 2006; Feige et al., 2008). Thus given inflammasome activation can impair several metabolically relevant signaling proteins such as Sirt1, additional studies are required to test whether specific inflammasome or caspase-1 inhibitors offer better therapeutic alternatives than IL-1β inhibition as treatment for diabetes. Considering that the pancreatic damage that occurs during diabetes is an ongoing inflammatory process that may not be readily reversible, an early preventive strategy targeting NLRP3 inflammasome may prove useful in diabetes and its complications.

ACKNOWLEDGMENTS

Vishwa D. Dixit is supported in part by US National Institutes of Health (NIH) grants AG31797, DK090556, and the Pennington Foundation. Ryan W. Grant is supported by the NIH T32 training grant DK064584-10S1. We thank William Cefalu for useful discussion and Timothy Nguyen for help with graphic design.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 November 2012; accepted: 10 February 2013; published online: 08 March 2013.

*Citation: Grant RW and Dixit VD (2013) Mechanisms of disease: inflammasome activation and the development of type 2 diabetes. Front. Immunol. **4**:50. doi: 10.3389/fimmu.2013.00050*

This article was submitted to Frontiers in Inflammation, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 07 August 2012
doi: 10.3389/fimmu.2012.00246

Sterile inflammation – do innate lymphoid cell subsets play a role?

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The recent identification of several novel innate lymphoid cell (iLC) subsets has increased our understanding of the mechanisms which link the innate and adaptive immune systems. While the contribution of these subsets toward the pathogenesis of human disease remains largely to be determined, it seems likely that they will play a particularly important role in sterile inflammatory settings where the innate response is seen as a critical mediator of inflammation. Several recent studies have highlighted the role of endogenous damage-associated molecular patterns such as IL-33, IL-1α, and IL-1β in promoting lymphoid cell responses. This review discusses the influence of such endogenous danger signals on novel iLCs such as lymphoid tissue-inducer cells, innate type 2 helper cells, and γδ T cells and explores how these responses may contribute to the development of an inflammatory response in a sterile setting.

Keywords: sterile inflammation, innate lymphoid cell, γδ T cell, type 1 response, type 2 response

INTRODUCTION

Acute inflammation directed by the innate immune response has evolved to efficiently combat infection and is critical to host defense. However, in the absence of overt infection, such innate mechanisms may also be activated as a result of tissue injury resulting from metabolic or exogenous sources (Rock et al., 2010). These events lead to what is termed a sterile inflammatory response which, if not appropriately resolved, can lead to the development of chronic inflammation which underlies or exacerbates a variety of human diseases.

The driving signals of sterile inflammation are known as damage-associated molecular patterns (DAMPs) and while distinct from their pathogen-derived counterparts, they elicit their effects through many of the same signaling pathways and networks (Bianchi, 2007). As a result, much of what we know about the mechanisms involved in sterile inflammation has stemmed from investigations of the innate inflammatory response to infection. Indeed, as a result of collateral damage often observed during the innate response to infection, DAMPs can also contribute to inflammation in non-sterile settings (Yang and Oppenheim, 2009; Yang et al., 2009; Andersson and Tracey, 2011). Most studies on the sterile inflammatory response have focused on the sources of these endogenous DAMPs, such as necrotic cells (Krysko et al., 2011). However, there is a growing awareness of the influence DAMPs play in orchestrating sterile inflammation through activating different cell types (Nace et al., 2012). This review will focus on the effects of specific DAMP activity on recently defined innate lymphoid cell (iLC) populations and how these distinct populations of cells play a role in orchestrating subsequent inflammatory events relevant to human disease.

DAMAGE-ASSOCIATED MOLECULAR PATTERNS

Damage-associated molecular patterns (DAMPs) are proinflammatory mediators largely activated under conditions of cellular stress or injury which may ultimately result in cell death. Stimuli which elicit these responses can range from sterile particulates derived from either aberrant metabolism, e.g., cholesterol crystals in atherosclerosis (Duewell et al., 2010) or external toxins, e.g., asbestos (Dostert et al., 2008). Similarly, unprogramed cell death, such as that observed under ischemic conditions or chemotherapeutic treatment strategies, leads to a loss of cellular integrity which can result in the exposure of DAMPs and the development of sterile inflammation (Arslan et al., 2011; Awong et al., 2012). In recent times, the identity of several DAMPs has been revealed and their effects on the generation of the sterile immune response described. As this review will focus on how these signals specifically influence iLCs responses, a number of the most relevant DAMPs are described below.

IL-1 α/β

The proinflammatory effects of both IL-1α and IL-1β have long been recognized. Although both DAMPs share the same IL-1 receptor, and thus appear to elicit very similar effects, their expression and activity are distinctly regulated (Dinarello, 2009). IL-1β gene expression is induced upon stimulation by numerous inflammatory signals but is normally translated in its inactive proform. Prior to its release from cells IL-1β undergoes maturation through proteolytic cleavage by caspase-1 in events mediated by an inflammasome involving the proteins NLRP3 and ASC (Li et al., 1995; Lamkanfi and Dixit, 2009). Critically, activation of the signaling events required for the appropriate assembly and activation of this NLRP3 inflammasome are known to be mediated by a wide range of sterile particulates and metabolites implicating IL-1β as central to numerous sterile inflammatory conditions (Martinon et al., 2006; Dostert et al., 2008; Masters et al., 2010). Unlike IL-1β, IL-1α appears to be constitutively expressed by a wider range of cell types and although it also undergoes proteolytic cleavage, is active in its unprocessed form. Although IL-1α

does not appear to require inflammasome activity for its activation it is known to be released in large amounts upon cell death through necrosis and has been found play a central role in driving neutrophil recruitment as part of the sterile inflammatory response (Chen et al., 2007).

The effects of IL-1 activity on both the innate and adaptive immune responses have been extensively characterized (Dinarello, 2009), and there is accumulating evidence that these DAMPs play an important role in driving iLC subset responses. These events are likely to play a significant role in driving neutrophil recruitment under sterile conditions and are discussed in greater detail below.

IL-33

IL-33 is another member of the IL-1 family of cytokines which is exposed upon cellular injury and acts upon its cognate receptor ST2 in complex with the IL-1RAcP (Schmitz et al., 2005). Although originally thought to be post transcriptionally regulated by caspase-1-mediated cleavage, in a manner similar to IL-1β, it is now apparent that IL-33 exhibits biological activity in its full-length uncleaved form and is released upon necrotic cell death to exert its biological activity in a similar manner to IL-1α (Liew et al., 2010). As well as acting as a classical "alarmin," intracellular IL-33 can also act as a transcriptional repressor although the specific genes and pathways which it targets in this role are not well-defined (Carriere et al., 2007; Ali et al., 2011). Release of IL-33 is strongly implicated as an instructive signal in the development of type 2 immune responses and as such it is thought to play an important role in the allergic response (Schmitz et al., 2005). More recently it has been demonstrated that IL-33 has profound effects on innate type 2 helper cells and nuocytes and may play a central role in driving type 2 immunity under sterile settings (Kim et al., 2012; Mirchandani et al., 2012). The influence of IL-33 on iLC populations in the context of sterile inflammation is discussed in further detail below.

EFFECTS OF DAMPs ON INNATE LYMPHOID CELL SUBSETS

The effects of DAMP signaling on cells of the innate immune system such as dendritic cells and macrophages have been extensively described elsewhere (Chen and Nunez, 2010). Similarly, since their earliest identification there have been numerous studies detailing how endogenous DAMPs can directly influence the adaptive T cell response. For example, HMGB1, CpG containing DNA motifs, and hsp60 have all been described to directly influence activated T cell responses (Dumitriu et al., 2005; Zanin-Zhorov et al., 2006; LaRosa et al., 2007). In more recent times there has been an intense interest in identifying novel lymphoid cell subsets which are thought to play an important role in bridging the gap between the innate and adaptive immune responses. These subsets are largely segregated by their ability to rapidly express effector cytokines more commonly associated with adaptive T helper cell responses such as IL-17 related cytokines or IL-13 and IL-4. Perhaps unsurprisingly, this has led to significant advances in understanding the role of these subsets in both autoimmune and allergic disease settings (Cai et al., 2011; Barlow et al., 2012; Pantelyushin et al., 2012). However there is significant evidence to suggest that such subsets may also play a central role in inflammation under sterile settings. In particular, the observation that proinflammatory cytokines normally associated with adaptive T cell responses are elevated in certain auto-inflammatory disease settings, in the absence of any identified sources of antigenic stimulation, indicates that iLCs may play an important role (Lasiglie et al., 2011).

INNATE LYMPHOID CELL SUBSETS IMPLICATED IN STERILE INFLAMMATION

γδ T CELLS

Arguably the most extensively described iLC populations are γδ T cells, which comprise about 5% of the overall T cell population. They differ from conventional αβ T cells in that they express invariant γ and δ chains as part of their T cell receptor and are resident predominantly at mucosal sites (Cua and Tato, 2010). γδ T cells appear to lack the requirement for conventional antigen presentation and this has contributed to the hypothesis that these cells act as tissue-resident immune sentinel cells. Of particular relevance to sterile inflammatory conditions are the recent observations from both mouse and human studies that specific subsets of γδ T cells respond to IL-R1 stimulation by expressing significant levels of IL-17A (Sutton et al., 2009; Ness-Schwickerath et al., 2010; Caccamo et al., 2011). These cells which constitutively express CCR6 and the transcription factor RORγT may act as an important instructive signal to promote the generation of adaptive Th17 type responses but have also been implicated as important mediators of disease in their own right (Sutton et al., 2009). In particular, γδ T cells which express IL-17A in response to IL-1α/β play an important role in driving neutrophilia and dermal inflammation in psoriasis in both mouse models and human patients (Cai et al., 2011; Laggner et al., 2011; Pantelyushin et al., 2012). Specifically, human $V\gamma9V\delta2^+$ cells, which are induced to express IL-17A in the presence of IL-1β, have been implicated as playing a prominent role in psoriasis (Laggner et al., 2011). As neutrophilic skin inflammation is a prominent feature of cryopyrin-associated inflammatory syndrome (CAPS), which are autoinflammatory disorders, associated with inflammasome hyperactivity and increased IL-1β processing, it is tempting to speculate that $V\gamma9V\delta2^+$ T cells may play a role in mediating these events (Kolivras et al., 2011). Indeed, in a recent study, significantly elevated levels of IL-17A was found in the serum of CAPS patients and although $CD4^+$ Th17 cells were implicated as the source of IL-17A, the status of γδ T cell subsets was not examined (Lasiglie et al., 2011). Similarly, characterizations of Nlrp3 gene-targeted mice harboring mutations which mimic those causing disease in humans have demonstrated significant skewing toward a Th17 type response. Of particular interest in this regard, Meng et al. (2009) have demonstrated that mice expressing a R258W mutation (corresponding to the R260W mutation found in Muckle–Wells disease patients) exhibit spontaneous skin inflammation associated with neutrophil inflammation and a Th17 dominated response. As this phenotype bears a remarkable similarity to psoriasis, in which γδ T cells are a prominent source of IL-17 related cytokines, it raises the possibility that γδ T cells may also play an important role in skin manifestations among CAPS patients.

While it has been established that caspase-1-dependent IL-1β activity can promote expression of IL-17A by γδ T cells in autoimmune disease settings (Lalor et al., 2011), whether this

occurs under sterile settings has not been investigated. We have found that the presence of IL-1β with IL-23 can induce significant rapid expression of IL-17A by murine γδ T cells *in vivo* under sterile settings and in the absence of exogenous antigen. In contrast, $CD3^+$ γδ TCR^- cells do not express significant levels of IL-17A under these conditions (**Figure 1**). As both IL-1β and IL-23 have been shown to be expressed at higher levels by monocyte derived dendritic cells from CAPS patients (Lasiglie et al., 2011), this further indicates that γδ T cell subsets may provide an important innate source of IL-17 related cytokines in autoinflammatory disease.

LYMPHOID TISSUE-INDUCER CELLS

Another prominent innate lymphoid subset characterized by their ability to quickly and efficiently express IL-17 and/or IL-22 are lymphoid tissue-inducer cells (LTi; Takatori et al., 2009). LTi cells require the activity of the RORγT transcription factor for their development and were initially identified for their role in driving lymphoid tissue development during embryogenesis. More recently, LTi have been found to play important roles in innate immune responses at mucosal surface such as the intestine and the skin where they have been implicated as playing an important role in responding to both pathogenic and commensal bacteria (Ivanov et al., 2006; Pantelyushin et al., 2012). However there is also emerging data indicating that these cells can play a prominent role in sterile inflammatory settings.

As well as the autoinflammatory syndromes referred to above, which result from genetic abnormalities, "sterile" inflammation can also occur as a result of therapeutic regimens designed to target specific cell populations for depletion. Treatments such as chemotherapy and irradiation often result in undesirable effects on the development of a healthy immune system with serious consequences for the patient. Recently, it has been demonstrated that LTi cells play a central role in promoting appropriate thymic

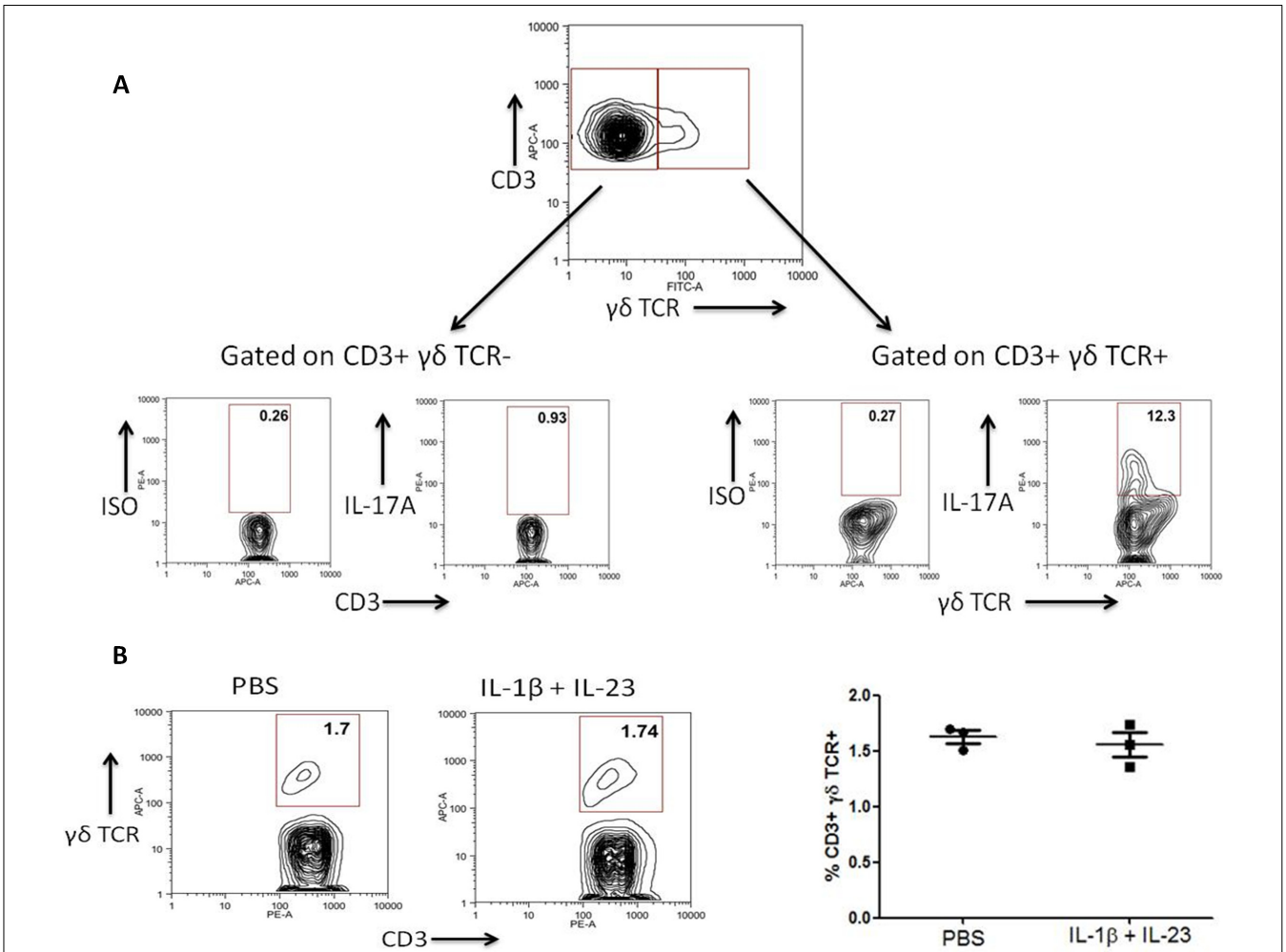

FIGURE 1 | IL-1β and IL-23 induce rapid expression of IL-17A by γδ T cells *in vivo*. C57Bl/6 mice were injected into the hind footpad with IL-1β (50 ng) and IL-23 (50 ng) or PBS. After 4 h cells were harvested from the popliteal lymph nodes and treated for a further 4 h with brefeldin A without *ex vivo* stimulation. Cells were stained for expression of CD3, γδ TCR and intracellular IL-17A or isotype control and examined by flow cytometry. **(A)** Data shown as IL-17A expression by live $CD3^+γδTCR^-$ and $CD3^+γδTCR^+$ cells is representative of four separate experiments. **(B)** The percentage frequency of $CD3^+γδTCR^+$ cells is unaltered after injection with IL-1β and IL-23. Data representative of three individual mice.

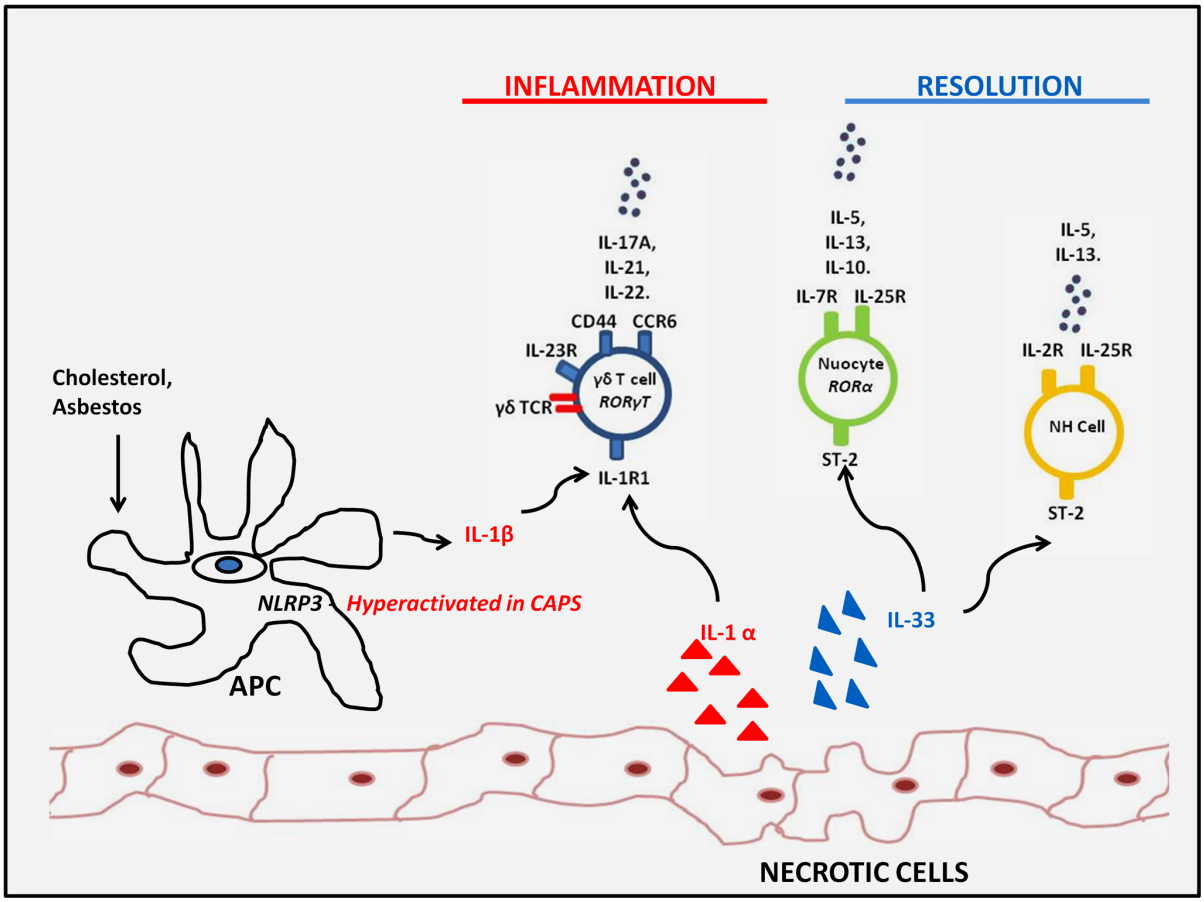

FIGURE 2 | Innate lymphoid subsets responding to sterile inflammation. Innate lymphoid subsets such as γδ T cells and LTi cells may respond to increased IL-1β expression as a result of either genetic- or particulate-driven inflammasome activation or IL-1α exposed upon tissue damage. These DAMPs drive the innate expression of IL-17 family members which may contribute to sterile inflammation or IL-22 which can promote tissue regeneration. On the other hand, IL-33 exposed upon tissue damage, drives type 2 innate responses by nuocytes and NH cells which can have a proresolving influence and dampen sterile inflammatory responses observed in obesity and atherosclerotic plaques.

regeneration in such sterile inflammatory settings (Dudakov et al., 2012). These effects are mediated largely through expression of IL-22 which promotes epithelial repair and tissue regeneration and highlight the role of iLCs in promoting the resolution of "sterile" inflammation (Dudakov et al., 2012). As an important source of IL-17A at mucosal sites, LTi cells have also been found play an important role in driving acute inflammatory responses to an array of both infectious and chemical stimuli (Ivanov et al., 2006; Pantelyushin et al., 2012). Although whether these cells can specifically respond to direct stimulation with DAMPs such as IL-1α/β (or indeed IL-33) has yet to be fully investigated, it is possible that they may also play an important role in bridging innate and adaptive immunity under sterile settings.

INNATE TYPE-2 CELLS

The recent discovery of a number of subsets of innate immune cells expressing ST-2 and responding to IL-33 has advanced our understanding of this cytokine and further expanded its emerging role as an alarmin (Liew et al., 2010). One such cell type, the nuocyte, is an innate type 2 cell which was originally identified as an IL-25 responsive non-T/non-B, γ-common chain-dependent cell which provided IL-4, IL-5, and abundant amounts of IL-13 at the onset of helminth infection (Neill et al., 2010). Interestingly, nuocytes are also expanded upon treatment with IL-33 and cells treated in this manner also secreted IL-6, IL-10, and GM-CSF (Moro et al., 2010).

The ability of nuocytes to respond to IL-33 in this manner implicates a role for these cells in the immune response to cellular stress. It is established that tissue damage caused by factors including high free fatty acids and oxidative stress commonly leads to necrosis and release of IL-33 (Moussion et al., 2008; Cayrol and Girard, 2009). Whilst no direct role for nuocytes has been described under such conditions, it is tempting to speculate that these cells could potentially provide an important source for at least some of the cytokines expressed in these settings, including IL-5, IL-10, and most notably IL-13 (Miller et al., 2010). For example, in atherosclerotic lesions, where severe tissue damage and necrosis are involved, IL-33 is thought to play a protective role through its ability to switch what is a type 1 dominated response toward a less inflammatory type 2 response. IL-33 increases the

levels of IL-4, IL-5, and IL-13 and reduces incidence of F4/80 positive macrophages and CD3 positive cells found in atherosclerotic lesions (Miller et al., 2008). These observations raise the interesting possibility that nuocytes could potentially provide a source of these cytokines and play a protective role in cardiac disease.

The natural helper (NH) cell is another recent addition to the expanding number of innate type 2 lymphoid cell populations (Koyasu and Moro, 2011). These cells are capable of producing large amounts of IL-5 and IL-13 when stimulated by IL-33 in the presence of IL-2, IL-7, or thymic stromal lymphopoietin (TSLP; Halim et al., 2012). Interestingly, and in contrast to the other described innate type 2 populations, NH cells are predominantly found in fat-associated lymphoid clusters where it is thought that they play important roles in maintenance of homeostasis in adipose tissues (Moro et al., 2010). Moreover, IL-33 and ST-2 expression in adipose tissue has been reported to play a protective role in obesity-driven sterile inflammation through promotion of a type 2 environment. These events were found to occur in association with switching of the macrophage response from a M1 to M2 phenotype raising the possibility that NH cells could also contribute to the regulation of the sterile inflammatory response to obesity by acting as an important orchestrator of the type 2 response (Miller, 2011).

Nuocytes and NH cells are two phenotypically similar cell subsets which respond to IL-33 and appear to play pathogenic roles in models of allergic and parasitic inflammation. However with the emerging importance of IL-33 as an endogenous danger signal further investigation into what role these subsets may play in mediating type 2 immunity under some settings of sterile inflammation could prove beneficial.

CONCLUDING REMARKS

Although the innate immune response to DAMPs is critical to the initiation of sterile inflammation, this often occurs in association with enhanced expression of effector cytokines more commonly associated with adaptive T helper cell responses. The recent identification of several iLC subsets which can respond rapidly to innate stimuli by expressing such cytokines, in the absence of any obvious requirement for antigenic stimulation, indicates that these subsets may be important mediators of inflammation under sterile conditions. Interestingly, the studies highlighted above, demonstrate that these cells can play both proinflammatory and proresolving roles depending on the inflammatory setting and the effector cytokines which are expressed (**Figure 2**). Further investigation will likely reveal whether, as well as being critical regulators at the interface between innate and adaptive immunity under settings of infection and autoimmunity, these cells are also important mediators of sterile inflammation.

ACKNOWLEDGMENTS

This work was supported by grants from the National Children's Research Centre, Dublin, European Research Council Marie Curie, and Science Foundation Ireland to Patrick T. Walsh.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 July 2012; paper pending published: 14 July 2012; accepted: 24 July 2012; published online: 07 August 2012.
*Citation: Russell SE and Walsh PT (2012) Sterile inflammation – do innate lymphoid cell subsets play a role? Front. Immun. **3**:246. doi: 10.3389/fimmu.2012.00246*
This article was submitted to Frontiers in Inflammation, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 28 January 2013
doi: 10.3389/fimmu.2013.00009

Endometriosis, a disease of the macrophage

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Endometriosis, a common cause of pelvic pain and female infertility, depends on the growth of vascularized endometrial tissue at ectopic sites. Endometrial fragments reach the peritoneal cavity during the fertile years: local cues decide whether they yield endometriotic lesions. Macrophages are recruited at sites of hypoxia and tissue stress, where they clear cell debris and heme-iron and generate pro-life and pro-angiogenesis signals. Macrophages are abundant in endometriotic lesions, where are recruited and undergo alternative activation. In rodents macrophages are required for lesions to establish and to grow; bone marrow-derived Tie-2 expressing macrophages specifically contribute to lesions neovasculature, possibly because they concur to the recruitment of circulating endothelial progenitors, and sustain their survival and the integrity of the vessel wall. Macrophages sense cues (hypoxia, cell death, iron overload) in the lesions and react delivering signals to restore the local homeostasis: their action represents a necessary, non-redundant step in the natural history of the disease. Endometriosis may be due to a misperception of macrophages about ectopic endometrial tissue. They perceive it as a wound, they activate programs leading to ectopic cell survival and tissue vascularization. Clearing this misperception is a critical area for the development of novel medical treatments of endometriosis, an urgent and unmet medical need.

Keywords: endometriosis, alternatively activated macrophages, angiogenesis, Tie-2 expressing macrophages, iron, phagocytosis, hypoxia

INTRODUCTION

Endometriosis is a common condition, affecting a rather large fraction of menstruating women, characterized by the hormone-dependent persistence and growth of vascularized endometrial tissue at ectopic sites, typically the pelvis, with pain and reduced fertility (Lebovic et al., 2001; Giudice and Kao, 2004; Berkley et al., 2005; Bulun, 2009; Luisi et al., 2009; Giudice, 2010; Simoens et al., 2012). Lesions are thought to originate from endometrial fragments shed during menstruations, which reach via the Fallopian tubes the peritoneal cavity. The event, which is likely to physiologically occur in most women, endometriotic and non-endometriotic, is referred to as "retrograde menstruation" (Sampson, 1927). Supporting evidence is mostly indirect. Endometriosis for example only takes place in species that menstruate, such as primates: this suggests that menstruation is required for the disease (D'Hooghe and Debrock, 2002). Shed endometrium is thought to initially adhere to endoabdominal structures, in particular the peritoneal wall and ovaries. Adhesion probably occurs quite commonly in healthy, non-endometriotic women. In *in vitro models*, adhesion requires a relatively short time and is apparently actively supported by mesothelial cells (Lucidi et al., 2005; Nair et al., 2008), with an apparently healthy peritoneum that thus play an important facilitatory role in the further development of endometriotic lesions (Fassbender et al., 2011).

A further necessary step for lesions establishment is the capacity of ectopic endometrial cells to invade the underlying basement membrane. The molecular bases of this phenomenon are partially elucidated. Endometrial tissue has invades even intact serosal membranes, indicating that a previously disrupted peritoneum is not a requirement (Nair et al., 2008).

Invasion in a prerequisite for the organization of the ectopic endometrial cells in tridimensional cysts but is not sufficient: novel vessels are also necessary. Novel vessels originate through sprouting from the adjacent vasculature or incorporation of circulating precursor endothelial cells at sites of vascularization (Becker et al., 2011; Laschke et al., 2011a,b). They ensure the transport of oxygen and nutrients, the excretion of catabolites and the maintenance of fluid balance.

All together the persistence of ectopic endometriotic tissue is associated to uncontrolled growth, invasion of adjacent tissues, defective apoptosis, neoangiogenesis, and sustained local inflammatory responses. These features are not tissue-autonomous, but depend on the peculiar features of the innate immune response to the auto-transplantation of a hormone-regulated tissue at novel sites. Of importance, several differences exist between the eutopic endometrium of women with and without endometriosis including invasive properties and resistance to apoptosis, as well as between ectopic and autologous eutopic endometrium of endometriotic patients (Novembri et al., 2011). This suggests that the inflammatory peritoneal environment influences the behavior of endometrial ectopic cells.

Macrophages in particular are master regulators of the innate response to injured, infected, and neoplastic tissues. As such they are in charge of selecting the appropriate response to restore homeostasis: they include on one hand the identification and destruction of pathogens, infected or transformed cells; on the other hand the activation, proliferation, and differentiation of

precursor/stem cell and the generation of neovessel. The perception of the role of macrophages in endometriosis has grown in the last years: in this review we will discuss the most recent data in the literature that name macrophages as defendants, with the charge of contributing to cause human endometriosis.

METHODS

Literature searches were performed in PubMed, Scopus, and ISI Web of Knowledge databases for publications focusing on the state and functions of macrophages in human and murine endometriosis. The searches included the key words "endometriosis," "ectopic endometrial lesions," and "endometrium" were matched with the key words "macrophages," "Tie-2 expressing macrophages," "angiogenesis," "hypoxia," "innate immunity," and "ovarian cancer." The bibliographic research encompasses studies on rodents, non-human primates and humans. No limits were set for publication dates.

ENDOMETRIUM AND ANGIOGENESIS

Organ vasculature comprises an integrated system of arteries, arterioles, capillaries, venules, and veins that ensures blood circulation and the transport of oxygen and other gases to target tissues. Blood vessels that form during growth and development generally arise by the sprouting from pre-existing vessels, via a process referred to as angiogenesis (Potente et al., 2011). Angiogenesis seldom occurs in the adult organism in normal tissues under physiologic conditions.

Endometrium represents an exception: the tightly regulated variation of ovarian steroids estrogen and progesterone concentrations cyclically triggers the remodeling of the organ vasculature, with angiogenesis and lymphangiogenesis (Girling and Rogers, 2009). Estrogens during the proliferative stage of the menstrual cycle cause the rapid growth of the vasculature. Progesterone during the secretory phase controls the maturation of the capillary sub-epithelial plexus and the development/coiling of spiral arterioles. Finally, the swift hormone withdrawal triggers the endometrial repair in the perimenstrual period.

The molecular mechanisms involved in hormone-regulated remodeling of eutopic endometrial vessels are being actively investigated. The vascular endothelial growth factor (VEGF) family and associated receptors and the angiopoietin–tyrosine kinase with Ig and epidermal growth factor homology domain (angiopoietin/Tie-2) system play an important role, since they connect hormonal levels to vessel remodeling (Girling and Rogers, 2009; Mints et al., 2010; Elsheikh et al., 2011; Lash et al., 2012).

Peritoneal macrophages are a well-characterized source of VEGF and ovarian steroids regulate the production of the growth factor (McLaren et al., 1996). Estrogens act on various macrophage signaling pathways, influencing in particular those related to the ability to sustain the recruitment of inflammatory cells and the remodeling of inflamed tissues, such as mitogen-activated protein kinase (MAPK), phosphatidylinositide-3-kinase/protein kinase B (PI3K/AKT), and nuclear factor-kappa B (NF-κB): as a consequence, a deregulated response to steroids might influence the survival of ectopic endometrial cells and promote the vascularization of the lesions (Cakmak et al., 2009; Pellegrini et al., 2012).

Premenstrual progesterone withdrawal possibly represents the crucial event: during the late secretory phase the demise of the corpus luteum results in the abrupt fall of bioactive progesterone. This triggers an acute inflammatory response with destruction of the upper "functional" layers of the human endometrium (for a thorough and well-written review, see Maybin et al., 2011a): endometrial prostaglandin (PG), PGE2 and PGF2α, are synthesized that act as potent vasoconstrictor on the spiral arterioles. PG production, spiral arteriole vasoconstriction, and local hypoxia in turn regulate the production of chemokines, such as IL-8 (CXCL8) and CXC chemokine ligand 12 (CXCL12) stromal cell derived factor (SDF-1).

Inflammation and chemokine production results in the attraction of neutrophils, that actively contribute to the destruction of the functional layer of the endometrium (Kaitu'u-Lino et al., 2007): typically tissue breakdown is associated with loss of integrity of endometrial vessel and blood cells extravasation; endometrial fragments and blood are then flushed from the uterus to the vagina with overt menstruation.

The inflammatory response has a double-edged action: besides triggering the regulated destruction of the tissue, it initiates and guides the endometrial repair.

Progesterone withdrawal indeed causes a transient constriction of spiral arteries in the top layers of the endometrium, which is supposed to cause hypoxia (Fan et al., 2008). Hypoxia is the stimulus that triggers vessel remodeling in injured and regenerating tissues as well as in tumors. It elicits an adaptive response, which is largely mediated by the hypoxia-inducible transcription factor-1alpha (HIF1α): under hypoxic conditions HIF1α translocates to the nucleus where it enhances and accelerates the transcription of genes with appropriate response elements, including angiopoietin 2 (Ang-2) CXCL12 and VEGF (Wu et al., 2007, 2011; Liu et al., 2011a; Maybin et al., 2011b,c; Wong et al., 2011; Henriet et al., 2012; Sarkar et al., 2012). Other signals induced by hypoxia and PG in epithelial cells and macrophages, such as connective tissue growth factor, have been convincingly suggested to play a role in endometrial repair (Maybin et al., 2012).

Events downstream HIF1α nuclear translocation are not apparently relevant for tissue destruction (Maybin et al., 2011a): in contrast they are required for attraction of macrophages and for their regenerative action in the hypoxic tissue (Du et al., 2008; Wong et al., 2011; Sarkar et al., 2012). For example ablation of HIF1α in tumor models is associated with decreased CXCL12/SDF1α levels and a less effective recruitment of bone marrow-derived macrophages in the tumors (Du et al., 2008).

Of critical importance for the environmental changes associated to endometriosis (see below), the vasculature of ectopic lesions remains dependent on the systemic concentration of hormones. As such, these structures undergo a cyclic remodeling, with synchronized tissue destruction and bleeding. This has been well-established since the pioneering work of Markee (1978), who has described that endometrial fragments transplanted in the eye anterior chamber of rhesus monkeys implant connect to the iris vasculature and grow. However, the tissue abruptly regresses after hormone withdrawal: cyclic destruction of the ectopic tissue is associated to coiling of spiral arterioles, vasoconstriction episodes, and bleeding.

The insight on the physiological control of eutopic endometrium is likely to be crucial to understand why endometriosis occurs. During menstruation red blood cells, hemoglobin, and leucocytes accumulate in the peritoneal fluid of most healthy women (Bokor et al., 2009): this indicates that retrograde menstruation is a fairly common event. Interestingly, retrograde menstruation appears not to be *per se* more frequent or abundant in endometriotic women (Bokor et al., 2009): therefore, the presence of endometrial tissue in the peritoneal cavity is not sufficient to cause endometriosis (Bulun, 2009). The role of the genetic background and/or of environmental factors deserves better attention and will be a hot topic for researchers in the next years.

MACROPHAGES ARE VERSATILE CELLS

Macrophages have been originally identified as an integral component of the mononuclear phagocyte system (MPS). They derive from bone marrow progenitors that enter the bloodstream as monocytes. Within a relatively short time they reach peripheral tissues where they yield resident macrophages or antigen-presenting cells, including dendritic cells (DCs): the MPS contributes both to pathogen elimination and to housekeeping functions (van Furth and Cohn, 1968; Murray and Wynn, 2011). Macrophages are professional phagocytes, since they are endowed with the molecular machinery necessary to internalize and dispose of extracellular particulate substrates, including microbes or endogenous constituents, such as apoptotic cells or senescent erythrocytes.

Dedicated pattern-recognition receptors (PRRs) are non-clonally expressed by innate immune cells and by macrophages in particular (Palm and Medzhitov, 2009). PRRs have been initially thought to have undergone evolutionary selection because of their ability to selectively identify molecular structures referred to as pathogen-associated molecular patterns (PAMPs) expressed by large classes of microbes (Janeway, 1992). After PAMP recognition, activation of PRRs recruits tightly coordinated events, including: (i) the production of various cytokines that attract and activate leukocytes (Nathan, 2002), arming them for neutralization/elimination of invading pathogen; (ii) the activation of an acute phase response, with the production of conserved soluble PRRs, such as pentraxins (Manfredi et al., 2008). Acute phase proteins in turn tune leukocyte activation and quench their ability to collaterally damage the tissue.

Besides microbial PAMPs, endogenous moieties trigger the PRR activation: damage-associated molecular pattern (DAMP) comprise an array of heterogeneous molecules that are released during cell and tissue necrosis and that via PRR activation elicit inflammation and prompt tissue regeneration even in the context of sterile injuries (Maroso et al., 1996; Bianchi, 2007; Lotze et al., 2007; Rubartelli and Lotze, 2007; Urbonaviciute et al., 2008; Bianchi and Manfredi, 2009; Manfredi and Rovere-Querini, 2010; Zhang et al., 2010a; Castiglioni et al., 2011; Liu et al., 2011b).

Pattern-recognition receptors represent a crucial asset for the phagocytic ability of macrophages: they on one hand interact with the phagocytic substrate and on the other hand activate specific signaling cascades within the phagocyte. The characteristics of the signaling events are crucial to determine which array of soluble signals is produced.

An example is given by the signal transduction associated with the best-characterized family of PRRs, the Toll-like receptors (TLR): TLR 3, 7, and 9 are preferentially expressed in endosomes, where they have access to viral constituents. As a consequence, they activate PI3K/mTOR/S6K with downstream production of antiviral and pro-inflammatory cytokines, such as type 1 interferons: the resulting response eventually leads to virus elimination.

TLR4, which is expressed at the plasma membrane, together with other extracellular moieties such as MD-2 and CD14, recognizes gram-negative bacteria lipopolysaccharide. Its recruitment results, *via* MyD88-dependent and -independent pathways, in NF-kB nuclear translocation and in the recruitment of the kinase pathways with an eventual production of inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6: the response is crucial for the control of the infection (Kawai and Akira, 2011). The activation of other PRRs, such as those that recognize dying cells phagocytic tags, prompts the production of immunosuppressive cytokines, such as IL-10 and transforming growth factor β (TGF-β) and of factors implicated in the tissue regeneration and repair (Huynh et al., 2002; Ravichandran, 2011).

Thus, PRRs decipher the code associated to an infected or injured tissue, and thus enable macrophages to activate the innate/acquired immune response that is more functional for restoring homeostasis (Brancato and Albina, 2011; Murray and Wynn, 2011). Conversely, macrophages extensively reprogram their functional properties in response to PAMPs and DAMPs (Nau et al., 2002; Martinon et al., 2010; London et al., 2011).

During sterile inflammation, which is by definition driven by DAMPs exclusively, macrophages play crucial and non-redundant actions in matrix remodeling and angiogenesis, which are required for effective tissue healing (Vezzoli et al., 2010; Bosurgi et al., 2011; Castiglioni et al., 2011; Rock et al., 2011; Vezzoli et al., 2011).

Macrophages have a rather heterogeneous array of characteristics, which possibly reflects the diverse functions they exert within the microenvironment: they become effector cells that kill invading pathogens, such as mycobacteria or *Leishmania major* and dramatically modify the environment via the production of cytokines and reactive oxygen (ROS) and nitric oxide (NO) species (Darrah et al., 2007; Mylonas et al., 2009). Such macrophages are referred to as classically activated/inflammatory macrophages, or M1 cells (**Figure 1**).

Macrophages also undergo a distinct activation program ("alternative activation"): as a consequence they tune inflammatory responses and adaptive immunity, scavenge debris and promote angiogenesis, tissue remodeling, and repair (Mantovani et al., 2004; Gordon and Taylor, 2005). Macrophages within regenerating tissues are often alternatively activated, also termed "M2 cells" (**Figure 1;** Daley et al., 2010; Gordon and Martinez, 2010; O'Brien et al., 2010; Schwartz, 2010; Brancato and Albina, 2011; Cairo et al., 2011; Corna et al., 2010; David and Kroner, 2011; Harel-Adar et al., 2011; Jaeschke, 2011; London et al., 2011; Wang and Harris, 2011) In contrast, uncontrolled classical activation in general results in defective healing and persistent inflammation (Gordon, 2003; Sindrilaru et al., 2011) Recent elegant studies suggest that neural stem cells in a model of murine sterile spinal cord injury possibly direct physically interact with endogenous

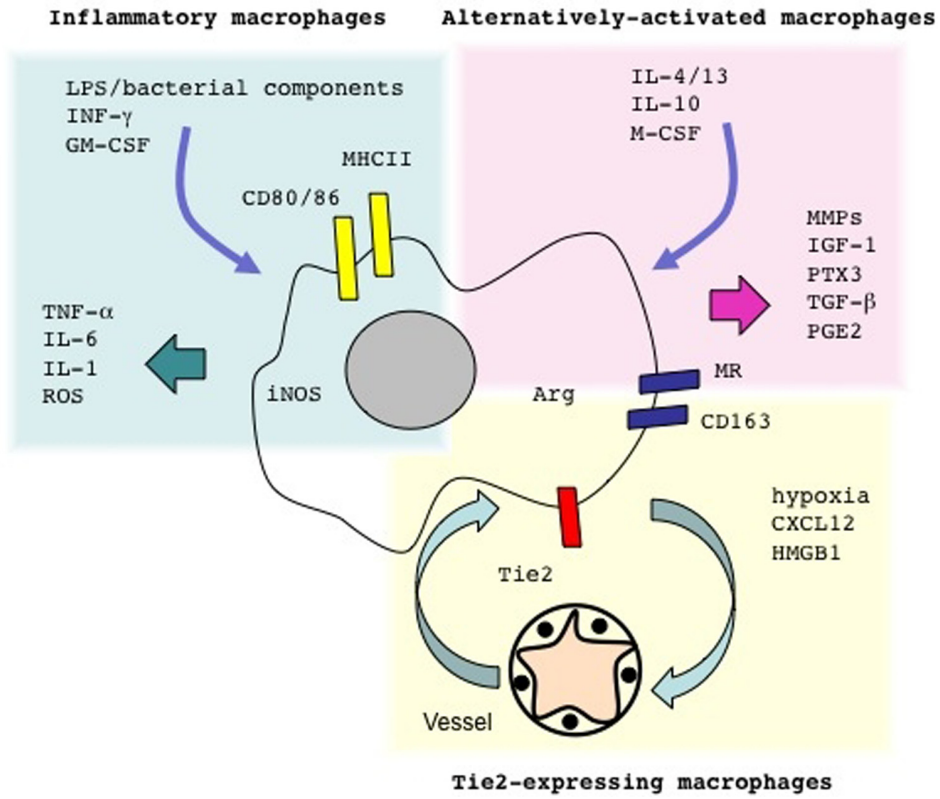

FIGURE 1 | Macrophages come in different flavors. Microbial components such as lipopolysaccharide (LPS) and/or cytokines such as IFN-γ or GM-CSF elicit conventionally activated macrophages. These cells secrete inflammatory cytokines and generate molecules involved in the anti-bacterial response, such as reactive oxygen species (ROS). Treatment with different cytokines like IL-4 and/or IL-13, IL-10 and/or M-CSF supports the activation of alternative activated macrophages. Local hypoxia, possibly in conjunction with chemotactic signals such as CXCL12 and/or DAMPs, such as HMGB1, recruit from the blood precursors that yield Tie-expressing macrophages in the inflamed tissues. The expression of specific arrays of membrane receptors and the generation of soluble and gaseous messengers reflect the macrophage activation programs. LPS, lipopolysaccharide; IFN-γ, interferon-γ; GM-CSF, granulocyte macrophage-colony stimulating factor; M-CSF, macrophage-colony stimulating factor; MHCII, major histocompatibility complex class II; CD163, haptoglobin–hemoglobin complex receptor; CD206, mannose receptor; TGF-β, transforming growth factor β; PGE2, prostaglandin E2; ROS, reactive oxygen species.

macrophages, an event that modulates expression levels of inflammatory cell transcripts *in vivo*, with a shift from "classically activated" (M1-like) macrophages to cells that facilitating the healing or regeneration of the lesion (Cusimano et al., 2012). This is not an exception due to the environment of the spinal cord: a similar shift occurs in injured skeletal muscles, apparently facilitated by the signals derived from the apoptotic substrates (Arnold et al., 2007).

Recombinant cytokines can be used to activate macrophage precursors. Alternatively activated macrophages can be propagated *in vitro* by exposing monocytes or bone marrow precursors to low concentrations of macrophage-colony stimulating factor (M-CSF) in the presence of IL-4, IL-13, or IL-10 (Gordon, 2003; Mantovani et al., 2004). Exposure to microbial components in the presence of γIFN or to granulocyte macrophage-colony stimulating factor (GM-CSF) elicits classically activated, inflammatory macrophages.

Specific subpopulations of macrophages are preferentially involved in guiding angiogenesis (Qian and Pollard, 2010; De Palma and Naldini, 2011). Macrophages that express the Tie-2 receptor (TEM or Tie-2-expressing monocytes/macrophages) sustain neoangiogenesis in a variety of experimental tumor models (**Figure 1**). Circulating monocytes in normal conditions express limited amounts of Tie-2: however, they substantially up-regulate it after homing to hypoxic tissue, where they yield a subset of perivascular macrophages (Du et al., 2008; De Palma and Naldini, 2009; Squadrito and De Palma, 2011). Angiopoietins, cytokines that regulate the quiescent and the angiogenic microvasculature, are known ligands of Tie-2: *in vivo* studies in which TEMs had been specifically depleted demonstrate that they are required to support angiogenesis and accelerate tumor growth (De Palma et al., 2003, 2005), indicating that TEMs interaction with the angiogenic vasculature plays a non-redundant role.

The model that apparently better fit with the data obtained in these diverse systems regards macrophages as guardians of the tissue integrity, that are in charge of: (i) perceiving clear and actual injury in the tissue; (ii) clearing dying cells and tissue debris; (iii) regulating neovessel generation and extracellular matrix remodeling; (iv) sustaining stem and progenitor cells migration, proliferation, survival, and differentiation (Lolmede

et al., 2009; Stappenbeck and Miyoshi, 2009; Sun et al., 2009; Vezzoli et al., 2010; Zhang et al., 2010b; Ehninger and Trumpp, 2011; Hara et al., 2011; London et al., 2011).

Macrophage activation is effective at enforcing regenerative and vascular responses that conduce to tissue repair with restitutio ad integrum when original injuries are intense and short lasting. In contrast, it is likely to be detrimental when the cause(s) of homeostasis disruption cannot be eliminated. We speculate that this applies to human endometriosis.

ENDOMETRIOSIS, HYPOXIA, AND MACROPHAGES

Angiogenesis is a prerequisite for endometriotic lesions to establish and to grow *in vivo*. Endometriotic lesion neovascularization involves both conventional sprouting angiogenesis and actual vasculogenesis (Laschke et al., 2011a): the process shares several features with the vessel remodeling process that take play in injured tissues and is required for wound healing (Potente et al., 2011). It is physiologically dependent on macrophage activation, since the depletion of macrophages in experimental models of wound healing jeopardizes VEGF generation and disturbs neovascularization (van Amerongen et al., 2007; Martinez et al., 2008). Reduced/delayed differentiation of myofibroblasts, re-epithelialization, collagen deposition, and cell proliferation also ensue macrophage depletion and neovessel disruption (Mirza et al., 2009).

A large body of evidence indicates that macrophages are responsible for the angiogenic switch, i.e., the increase in the density of vessels that often characterizes the benign-to-malignant transition in cancer, being involved both in the initial establishment of the vasculature and in the subsequent remodeling of the vessels (Pollard, 2008). Their action is mediated via several pathways, that lead to: (i) the production of matrix metalloproteinases (MMPs) that are required for the release of VEGF bound to the extracellular matrix at hypoxic sites, thus increasing the availability of the bioactive growth factor; and (ii) the expression of VEGF family members, which in turn behave as attractors of myeloid cells, including macrophages. Of interest, an association between endometriosis and VGEF-A gene polymorphisms has been reported, even if caution should be used because of the effect of possible confounding factors.

In experimental models of endometriosis, early phases of lesion establishment are characterized by a transient hypoxia, which results in the up-regulation of HIF1α, with downstream expression of VEGF (Wu et al., 2007, 2011; Becker et al., 2008; Lin et al., 2012). Limited ischemia of the endometrium in the early and middle secretory phase occurs: the event is apparently associated with the up-regulated expression of VEGF in the late secretory phase of the menstrual cycle in endometriotic women (Donnez et al., 1998). Interestingly, endometrial fragments from women in which a transient ischemia had been induced by repeated clamping/declamping of the uterine artery transplanted onto the chick embryo chorioallantoic membrane demonstrated higher VEGF expression and better survival: this mechanism could facilitate implantation/establishment of endometrium at ectopic sites (Ren et al., 2011). Moreover, response to ischemia is likely to play a role in established lesions of endometriotic patients: the relative expression of HIF1α and VEGF differ at various sites within endometriotic lesions, possibly accounting for some of their heterogeneous histological characteristics (Goteri et al., 2004, 2010).

It is important to underline that the vascular response to ischemia depends on the recruitment of activated macrophages at lesions sites (e.g., see Machado et al., 2010). Macrophages are indeed necessary to license lesions for growth and spreading (Bacci et al., 2009; Haber et al., 2009; Capobianco et al., 2011 and see below).

MACROPHAGES AS A KEY TO THE SUSCEPTIBILITY TO ENDOMETRIOSIS

The data discussed above indicate that endometriosis depends on the ability of endometrial tissue at ectopic sites to attract macrophages and to be as a consequence licensed for survival, angiogenesis, growth, and spreading (**Figures 2 and 3**). Macrophages physiologically convey these licensing signals in the eutopic endometrium, which undergoes physiological destruction as a consequence of progesterone withdrawal**.**

Infiltrating macrophages are a consistent feature of endometriotic lesions. Independent lines of evidence indicate that they undergo activation as a consequence of signals generated within ectopic lesion (Lebovic et al., 2001; Zhang et al., 2006; Herrmann Lavoie et al., 2007; Lawson et al., 2007; Minici et al., 2007; Galleri et al., 2008; Lousse et al., 2008) or possibly of the lack of hormone-regulated anti-inflammatory signals in the ectopic but none in the eutopic endometrium (Novembri et al., 2011). However, it is unclear whether these changes in eutopic endometrium are primary defects or consequences of the development of endometriosis (Novembri et al., 2011). Ectopic endometriotic lesions are histologically similar to their putative eutopic precursors. However, several genes are differentially expressed in ectopic and eutopic of endometriotic and non-endometriotic patients and significant biochemical differences exist (Meola et al., 2010). These differences might stem from epigenetic modifications that occur in the eutopic endometrium of patients with endometriosis. Differential methylation and expression of genes involved in the regulation of implantation, such as *HOXA10* and *PR-B,* have been for example reported in the eutopic endometrium of patients with endometriosis compared with controls (Stilley et al., 2012).

In endometriotic lesions the macrophage NF-kB-dependent pathway is engaged (Lousse et al., 2008) with transactivation of responsive gene elements that control angiogenesis and tissue remodeling (Hagemann et al., 2008; Timmer and Nizet, 2008). NF-kB inhibition decreases the *in vivo* growth of experimental lesions, indicating that its activation plays a particularly relevant role (Lousse et al., 2008, 2009).

The stimuli involved in the activation of macrophages are so far unclear. It is extremely unlikely that microbial structures (PAMPs) are involved. As such, the signals involved in the macrophages activation must be released in sterile conditions, like it happens for multiorgan trauma, which causes the systemic inflammatory response syndrome or at specific sites, as a consequence of ischemia/reperfusion or of immune mediated events, like in the case of allograft rejection. The endogenous sterile triggers that are responsible for sterile inflammation in these conditions are

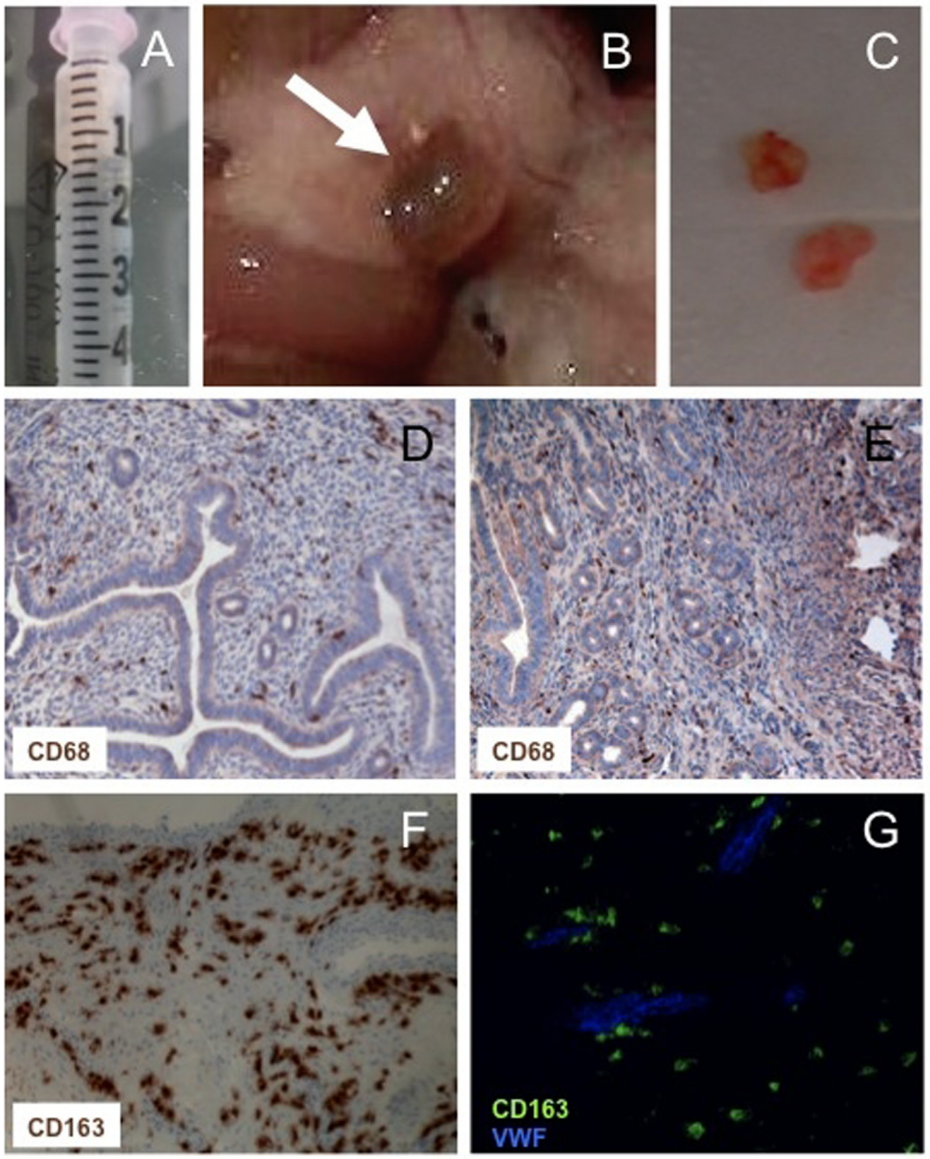

FIGURE 2 | Macrophages in experimental and human endometriosis. Endometrial fragments (maximal diameter <1 mm) derived from estradiol benzoate-treated mice **(A)** injected intra-peritoneally yield endometriotic lesions **(B)** that can be excised and processed for disease assessment **(C)** or immunohistochemical evaluation **(D–G)**. $CD68^+$ macrophages are abundant in human and experimental lesions **(D,E)**. Most macrophages within human lesions express markers of alternative activation, such as the hemoglobin/haptoglobin CD163 scavenger receptor **(F)**. $CD163^+$ (green) vWF- (blue) pro-angiogenic Tie2-expressing macrophages surround the neoformed vessels **(G)**.

apparently equally effective as microbial PAMPs and rely on similar molecular pathways; NF-kB nuclear translocation and activation is a clear example (Palumbo et al., 2007).

Once ectopic lesions are established, tissue remnants that are generated as a consequence of cyclic response to progesterone withdrawal accumulate in the peritoneal cavity since they cannot be flushed out with the menstrual blood. Cell remnants have a potentially noxious action on living tissues: as such, they require to be disposed by active phagocytosis. This is in general a relatively silent event, with macrophages recognizing and phagocytosing scattered cells that die in the midst of living tissues because of their physiological tissue turnover, releasing in the meantime immunosuppressive and anti-inflammatory signals. However, the challenge with a relatively ample load of cells dying in a synchronized fashion may exert a paradoxically opposite effect, representing a further threat to tissue integrity and immune tolerance (Rovere et al., 1998, 2000; Manfredi et al., 2002; Capobianco et al., 2011). Specifically the release of intracellular moieties endowed with inflammatory and adjuvant properties results in the activation of macrophages, which in turn release cytokines and chemokines, thus enforcing a positive feed-back forward loop (Zhang et al., 2010a; Castiglioni et al., 2011). The persistence of cells dying as

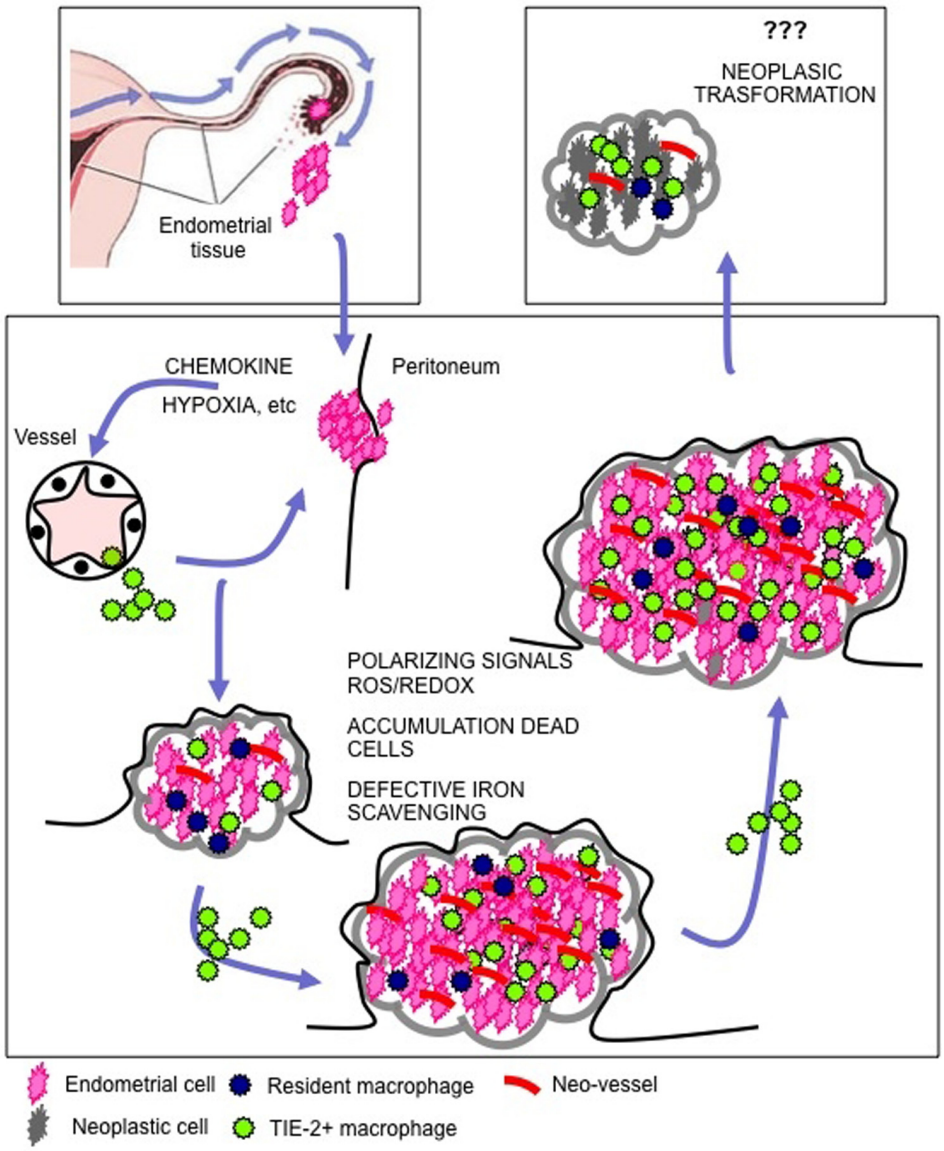

FIGURE 3 | Macrophages drive endometriosis. Endometrial cells reach the peritoneal cavity as a consequence of retrograde menstruation. The recruitment of macrophages at the site of endometrial cell engraftment is a limiting step for the lesion to organize and to growth. Local hypoxia and the production of chemokines play a major role in the recruitment of macrophages. Once endometriosis is established, the cyclic death of endometrial cells as a consequence of progesterone withdrawal leads to the release of cell debris, erythrocytes, and heme-bound iron in the peritoneal cavity. Recruited macrophages perceive ongoing cell death and tissue damage: in endometriotic patients they activate a reparative/regenerative/angiogenic program that is required for lesion maintenance, growth and spreading. In a subgroup of patients endometriosis is a preneoplastic condition. The persisting tissue healing action of macrophages that keep interfering with the physiological apoptosis while prompting proliferation of epithelial cells might set the scenario in which genetic alterations accumulate.

results of progesterone withdrawal within endometriotic lesions could cyclically activate infiltrating macrophages, thus sustaining the inflammation associated to the disease.

Relatively little is known on the ability of peritoneal macrophages to dispose of endometrial remnants, even if an apparent impairment in the ability to phagocytose particulate substrate has been reported (Chuang et al., 2009). The defect has been associated with a defect in the expression and the function of the class B scavenger receptor CD36, a well recognized player in the recognition and clearance of apoptotic cells, in endometriosis: such defect could be possibly related to the action of PG (Chuang et al., 2010). However, it is difficult to verify whether such defect is upstream or downstream the persistent inflammation of the peritoneal cavity associated to the disease.

Ectopic endometrium breakdown causes hemorrhage, with extravasation and persistence of aging red blood cells within the lesions; moreover erythrocytes accumulate into the pelvis because of retrograde menstruation (Defrere et al., 2008, 2012; Lousse

et al., 2009, 2012; Capobianco et al., 2010). The transfer of human menstrual effluent in immunodeficient mice results in the generation of iron deposits, which are similar to those observed in patients (Defrere et al., 2006), demonstrating that the specific features of the peritoneal microenvironment do not allow an effective processing of red blood cells, in contrast to other professional hemocatheretic tissues, such as the spleen.

The clearance of red blood cells and of apoptotic remnants has similar molecular constrains and often relies on common phagocytic tags/receptors pairs, which are in general expressed by professional phagocytes, such as macrophages (Ravichandran, 2011). The peritoneal fluid of endometriotic women indeed contains iron-loaded macrophages and higher fluid concentrations of the metal, which are possibly associated with the severity of the disease manifestations (Lousse et al., 2012). The iron in the fluid possibly derives from the lysis of red blood cells that that are not effectively cleared by macrophages, and as a consequence release hemoglobin. This moiety may as well bind to haptoglobin and be internalized by macrophages via the CD163 hemoglobin/haptoglobin scavenger receptor. In endometriosis patients this pathway is either originally defective or peritoneal macrophages become insufficient/unable to deal with the amounts of hemoglobin present in the liquid. The concentration of free iron within endometriotic cysts has also been investigated and found to be high. Metal accumulation was reflected by a higher oxidative stress, which in turn favors epigenetic modifications, prompting the evolution of ectopic tissue in *bona fide* neoplastic lesions (Yamaguchi et al., 2008, 2010).

Macrophages are also able to directly recognize senescent erythrocytes, internalize and digest them, an event which in the bone marrow is necessary for the active recycling of heme-iron for effective erythropoiesis (Li and Ginzburg, 2010; Ganz, 2012). Peritoneal macrophages are known to accumulate iron (Lousse et al., 2009). So far relatively little information is available on the characteristics of the macrophages disposal of red blood cells and heme-iron within endometriotic lesions, in particular when progesterone withdrawal jeopardizes the integrity of the vessel walls.

All together iron homeostasis is disrupted in the peritoneal cavity of patients with endometriosis: the accumulation of unscavenged iron associates with the increased generation of ROS and with a persistently activated pathway of the NF-kB: alterations of this pathway have been reported in eutopic endometrium of patients with endometriosis, suggesting a possible causative role in the natural history of the disease (Ponce et al., 2009). Interestingly polymorphism of the NFKB1 promoter have been described that are significantly associated with an increased risk to develop endometriosis (Zhou et al., 2010).

The peritoneal environment, whose characteristics are modulated by a vast and complex array of regulatory peptides and growth factors (Petraglia et al., 2008; Florio et al., 2009), could further promote and amplify the function of alternatively activated macrophages. Immune and non-immune cells for example produce urocortin neuropeptides, especially under inflammatory stimuli and hypoxic conditions (Buhler et al., 2009; Imperatore et al., 2010). Neuropeptides have been involved in the resolution/termination of the innate response, possibly via regulation of the production of inflammatory cytokines and of endothelial permeability (Di Comite et al., 2007, 2009). It has been recently shown the various members of the urocortin families are differentially expressed in ectopic and eutopic endometrium: their expression is finely regulated throughout the menstrual cycle in the eutopic tissue, while virtually no menstrual-cycle-related changes were found in endometriotic lesions (Novembri et al., 2011). Intriguingly, urocortin down-regulates secretion by activated macrophages of the best-characterized DAMP signal, the high-mobility group box 1 (HMGB1) nuclear protein, suggesting that macrophages are major targets in its inhibitory activity (Chorny and Delgado, 2008). HMGB1 constitutively promotes angiogenesis and is used by inflammatory macrophages to attract vessel-associated stem cells and to favor tissue remodeling (Campana et al., 2009; Lolmede et al., 2009). HMGB1 physically associates with CXCL12 and specifically modulates the CXCL12/CXC chemokine receptor 4 (CXCR4) pathway (Zhao et al., 2007; Campana et al., 2009; Schiraldi et al., 2012), i.e., a crucial chemotactic pathway involved in the recruitment of phagocytes and of stem cells at sites of hypoxia. Other endogenous negative signals regulating HMGB1 action have been described (Bianchi and Manfredi, 2009), but their involvement in the pathogenesis of endometriosis have not so far been extensively studied.

THE DEVELOPMENT OF ENDOMETRIOSIS DEPENDS ON MACROPHAGES

Animal models represent a useful tool to study *in vivo* early steps of the natural history of endometriosis, which would be impossible to tackle in patients. Small animals and rodents in particular have several advantages and have prompted in the last decades a substantial in our insight of the pathogenesis of the disease: they are relatively inexpensive, inbred animals are available, including genetically modified models, in which the endometrial tissue can be transferred and relevant biological events evaluated in reference to the characteristics of the developing lesions (Becker et al., 2006; Tirado-Gonzalez et al., 2012). Lesions in murine models are surgically induced, for example by micro-laparatomic techniques (Nisolle et al., 2000) or induced by injection of endometrial tissue of various origin within the peritoneal cavity (Somigliana et al., 1999, 2001; Fainaru et al., 2008). The latter approach, as originally described, rely on the transfer of fragments of endometrial tissue harvested from syngeneic donor mice and recapitulates important aspects of the disease, comprising hormone-dependence, ability to escape immune surveillance, and characteristics of the neovascularization of ectopic endometrium (Somigliana et al., 1999). Endometriotic lesions are known to originate only in some mouse strains, a feature that resembles the heterogeneous sensitivity to the diseases of the human population.

It must however be underlined that these models have substantial disadvantages (Tirado-Gonzalez et al., 2010): in particular, rodents do not menstruate and thus do not *per se* develop endometriosis. As a consequence biological events are missing that are substantially influence the natural history of the diseases, such as the cyclic disruption of the ectopic endometrium as a consequence of variation of the systemic concentration of sexual hormones (discussed above) and the accumulation of iron in the lesions and the peritoneal cavity.

We have used an experimental mouse model (**Figure 2**) to verify whether macrophages are actually required and influence

the establishment of endometriotic lesions. We have depleted macrophages by the intraperitoneal injection of clodronate encapsulated into liposomes: this system results in the selective targeting of clodronate to macrophages, that are killed without undue toxicity on cells belonging to other lineages (Van Rooijen and Sanders, 1994; Bacci et al., 2009; Cottone et al., 2011). We have observed that in the absence of macrophages, syngeneic endometrium retains the ability to adhere to the peritoneal layer and to infiltrate the serosal membrane. However, ectopic lesions fail to grow in these conditions. The pharmacological depletion of macrophages at later times, when endometriotic lesions have already established and organized, "congeal" them: vessels do not extend to the lesions core, which stop growing and do not develop a well-organized glandular and stromal architecture (Bacci et al., 2009).

The data suggest that the recruitment of macrophages into the lesions is not only an early event in the lesion development, but a necessary step for the successful establishment of endometriotic lesions. Interestingly, a similar differential sensitivity of the early phases of the diseases, which are not affected, versus the later growth neovascularization and spreading of lesions characterizes immunodeficient mice that do not express the Tgfb1 transplanted with human eutopic endometrial tissue (Hull et al., 2012). Members of the TGF-β family have been implicated both in the initiation of menstruation and in repair of eutopic endometrium (Omwandho et al., 2010). Moreover, TGF-β is critically expressed in endometriotic lesions (Omwandho et al., 2010).

Transforming growth factor β locally increases the expression of the PAR2 gene with possible downstream increased secretion of IL-6 from endometriotic stromal cells thus suggesting an upstream role of the cytokine in coordinating the cascade of inflammatory signals of the disease (Saito et al., 2011). TGF-β is a key factor produced by macrophages challenged with apoptotic cell remnants (Xiao et al., 2008), which as such could represent a relevant source of the cytokine.

Macrophage dependence is not limited to mice, since initiation and growth of endometriotic lesions are both jeopardized in a rat model of the disease after depletion of peritoneal macrophages by local injection of liposomal alendronate (Haber et al., 2009). Of interest, in this model the degree of lesion infiltration by macrophages is directly correlated with the peritoneal concentrations of TNF-α, while those of the CC chemokine ligand 2 (CCL2)/monocyte chemoattractant protein 1 (MCP-1) chemokine are negatively correlated with the degree of macrophage infiltration (Haber et al., 2009). As discussed above, macrophages deliver trophic and anti-apoptotic signals. These effects in the early phase after endometrium injection are likely to be more relevant (Lin et al., 2006), before novel vessels have established (Eggermont et al., 2005; Grummer, 2006; Becker et al., 2008), which promote the survival of ectopic cells in an hypoxic environment (Lin et al., 2006).

REPARATIVE AND PRO-ANGIOGENETIC MACROPHAGES IN ENDOMETRIOSIS

Macrophages from patients with endometriosis and mice with implanted endometriotic lesions (but not peritoneal macrophages from non-endometriotic patients or from control mice) express typical markers of alternative activation, in particular high levels of scavenger receptors, CD163 and CD206 (Bacci et al., 2009). CD206 belongs to the C-type lectin superfamily is a well-characterized PRR and contributes to remove or inactivate inflammatory signals (Allavena et al., 2004). CD163 mediates endocytosis of haptoglobin-hemoglobin complexes, with degradation of heme-iron components that can be recycled for erythropoiesis (Kristiansen et al., 2001; Borda et al., 2008), a feature that may be particularly important in a disease in which disturbance of macrophage iron homeostasis appear particularly important (see above). Macrophage polarization results in differential iron management in both human and mice, with classically activated M1 macrophages that are characterized by iron sequestration, which operates as a bacteriostatic mechanism (Cairo et al., 2011).

In contrast alternatively activated M2 macrophages are endowed with the ability to effectively internalize and recycle the metal, with is reflected by a larger intracellular labile iron pool (Corna et al., 2010; Recalcati et al., 2010). The ability to uptake and recycle the metal to bystander cells could be relevant for the repair of tissues in which large amount of heme-iron are expressed, such as the skeletal muscle or the myocardium (Brunelli and Rovere-Querini, 2008; Corna et al., 2010) and may conversely be involved in sustaining the proliferation of neoplastic cells and the growth of neoplastic lesions (Recalcati et al., 2010; Cairo et al., 2011). It is tempting to speculate that the skewing of endometriotic macrophages toward alternative activation results in a more effective transfer of the metal to epithelial cells, with the effect to support the growth and the spreading of the lesions. This model would nicely fit with the increased extracellular concentration of iron in the peritoneal fluid of patients with endometriosis, as well as with the relative overload of macrophages with the metal (Defrere et al., 2012).

Macrophages are alternatively activated in the inflammatory peritoneal fluid or in the endometriotic lesions, both in patients and experimental animals (Bacci et al., 2009). This suggests that this program is important for the natural history of the disease. To experimentally address this possibility, we have set up a model of cell transfer with various polarized macrophage populations in mice in which the endogenous macrophage population had been previously depleted (see above). In this system, alternatively activated macrophages strongly enhance the growth of endometriotic lesions. In contrast mice injected with conventionally activated inflammatory macrophages develop minute lesions, that do not grow and are characterized by a severely disrupted glandular and stromal architecture (Bacci et al., 2009).

Given the critical role of lesions neovascularization in their outcome, we have focused on the possible role of TEMs, the best-characterized population of macrophages involved in angiogenesis: this subset has mainly been characterized in tumor models (De Palma and Naldini, 2011) but it has convincingly been suggested to play a role even in non-neoplastic conditions, in particular in the physiological development of embryos (Pucci et al., 2009). We have observed that human TEMs infiltrate areas surrounding endometriotic novel vessels (Capobianco et al., 2011). To obtain a mouse model in which TEMs, and not Tie2-expressing endothelial cells, are targeted we have transplanted in wild-type

recipients bone marrow progenitor cells expressing a suicide gene under the Tie2 promoter/enhancer. In this system, TEMs effectively infiltrated endometriotic lesions, whose growth abruptly abates after TEM depletion. Of interest, in the absence of TEMs endothelial cells caspase 3 is activated. This results in disruption of vessel integrity and of glandular architecture, suggesting a paracrine action of signals delivered by TEMs on the survival of endothelial cells within the neovessel wall.

ENDOMETRIOSIS AS A PRENEOPLASTIC CONDITION: A ROLE OF MACROPHAGES?

Endometriotic tissue comprises non-transformed cells only. However, it shares several features with neoplasms (uncontrolled growth, invasion of adjacent tissues, defective apoptosis, sustained local inflammatory responses). Conversely, endometriosis increases the risk of ovarian cancer, in particular invasive low-grade serous, clear-cell, and endometrioid subtypes of the neoplasm (Yamaguchi et al., 2008, 2010; Pearce et al., 2012). The molecular bases of the association are not completely defined. As discussed above, macrophages are physiologically recruited in injured tissues, where they activate the neo-angiogenic switch, sustain resistance to apoptotic stimuli and stimulate the proliferation and invasion of precursor cells, in order to prompt tissue regeneration. Macrophages recruited in the endometriotic lesions activate a similar program: they interfere with physiological apoptosis while prompt proliferation, thus setting the scenario in which genetic alterations accumulate. This action may contribute to the evolution of lesions toward atypical endometriosis and metaplasia, which in turn fosters the development of borderline and finally fully malignant ovarian cancer (Wei et al., 2011). Further studies are required to experimentally address this possibility.

CONCLUSION

Endometriosis represents an immunological unique scenario, since it derives from the auto-transplantation of endometrial cells at distant sites. Recent studies have identified lesional macrophages as responsible for the outcome of the auto-transplantation, since their unrestrained activation toward a reparative phenotype allows the survival, the neovascularization, and the growth of the lesions. This event possibly depends on the transfer at distant sites of inflammatory regenerative mechanisms that are physiologically activated to repair the eutopic endometrium during the menstrual cycle. Clarifying the molecular mechanisms of the misperception of macrophages is a critical area for the development of novel medical treatments of endometriosis, an urgent and unmet medical need.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 08 October 2012; accepted: 07 January 2013; published online: 28 January 2013.

Citation: Capobianco A and Rovere-Querini P (2013) Endometriosis, a disease of the macrophage. Front. Immun. ***4****:9. doi: 10.3389/fimmu.2013.00009*

This article was submitted to Frontiers in Inflammation, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 10 September 2012
doi: 10.3389/fimmu.2012.00286

DAMP signaling in fungal infections and diseases

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Fungal infections and diseases predominantly affect patients with deregulated immunity. Compelling experimental and clinical evidence indicate that severe fungal diseases belong to the spectrum of fungus-related inflammatory diseases. Some degree of inflammation is required for protection during the transitional response occurring temporally between the rapid innate and slower adaptive response. However, progressive inflammation worsens disease and ultimately prevents pathogen eradication. The challenge now is to elucidate cellular and molecular pathways distinguishing protective vs. pathogenic inflammation to fungi. In addition to fungal ligands of pattern recognition receptors (pathogen-associated molecular patterns, PAMPs), several host-encoded proteins, the damage-associated molecular patterns (DAMPs), are released during tissue injury and activate innate recognition receptors. DAMPs have been shown to regulate inflammation in fungal diseases. The DAMP/receptor for advanced glycation end-products axis integrated with the PAMP/Toll-like receptors axis in the generation of the inflammatory response in experimental and clinical fungal pneumonia. These emerging themes better accommodate fungal pathogenesis in the face of high-level inflammation seen in several clinical settings and point to DAMP targeting as a novel immunomodulatory strategy in fungal diseases.

Keywords: DAMPs, PAMPs, fungal diseases, inflammation, immunoregulation

INTRODUCTION

Fungi are associated with a wide spectrum of diseases in humans and animals, ranging from acute self-limiting pulmonary manifestations and cutaneous lesions in immunocompetent individuals to severe inflammatory diseases and life-threatening invasive infections in immunocompromised patients. Most fungi are ubiquitous in the environment and humans are exposed by inhaling spores. The ability to colonize almost every niche within the human body involves specific reprogramming events to adapt to environmental conditions (Cooney and Klein, 2008). In the case of commensals, such as *Malassezia* spp. and *Candida albicans*, co-evolution with their mammalian hosts for millions of years implicates the existence of complex mechanisms of immune adaptations and, likewise, of sophisticated mechanisms to antagonize immunity.

Indeed, most fungi are considered harmless in the context of normal host responses indicating that a stable pathogen–host interaction is common for microorganisms with inherently low virulence. This implicates that a high degree of coexistence occurs between fungi and their mammalian hosts, which deviates into overt disease only under specific conditions, most prominently deficits in resistance and tolerance mechanisms, respectively, defined as the ability to limit pathogen burden or the damage caused by the infection and/or the host (Romani, 2011).

INFLAMMATION: THE GOOD, THE BAD, AND THE UGLY

As in autoimmunity and chronic inflammation, an imbalance between pro- and anti-inflammatory signals may prevent successful host/fungal interaction, thus leading to infection and disease (Romani and Puccetti, 2007). Indeed, despite the occurrence of severe fungal infections in immunocompromised patients, clinical evidence indicate that fungal diseases also occur in the setting of an heightened inflammatory response, in which immunity occurs at the expense of host damage and pathogen eradication (Perfect, 2012). It is known that aberrant stimulation of Toll-like receptors (TLRs) by damage-associated molecular patterns (DAMPs) may result in increased expression of cytokines, chemokines, and proteases, perpetuating a vicious inflammatory cycle that constitutes the hallmark chronic inflammatory human diseases (Srikrishna and Freeze, 2009; Piccinini and Midwood, 2010). We have recently described an additional mechanism by which host inflammation may favor fungal infectivity and promotes the transition from fungal commensalism to infection. Fungal sensing of the mammalian cytokine interleukin (IL)-17A induced artificial nutrient starvation conditions in *C. albicans* and *Aspergillus fumigatus*, two major human fungal pathogens, resulting in increased adhesion and filamentous growth that clinically translates in a dramatic increment of biofilm formation and fungal virulence (Zelante et al., 2012). Thus, commensals or ubiquitous fungi have evolved a contingency-based system during co-evolution to guarantee their persistence in an inflammatory host environment. The main implication of these findings is that, at least in specific clinical settings, it is a heightened inflammatory response that likely compromises a patient's ability to eradicate infection, and not an "intrinsic" susceptibility to infection that determines a state of chronic or intractable diseases (Romani and Puccetti, 2007). The conceptual principle highlighting a truly bipolar nature of the inflammatory process in infection is

best exemplified by the occurrence of severe fungal infections in patients with immune reconstitution syndrome, an entity characterized by localized and systemic inflammatory reactions, worsening disease in opportunistic and non-opportunistic infections, that are associated with immunological recovery (Gupta and Singh, 2011; Perfect, 2012). Additionally, a high incidence of fungal infections and sensitization to *Aspergillus* spp. has been described in the hyper-IgE syndrome in which increased levels of pro-inflammatory gene transcripts have been found (Antachopoulos et al., 2007; Holland et al., 2007). Therefore, paradoxically, increased inflammatory innate response may predispose to either fungal infections or deregulated immune responses to the fungus. Thus, fungal diseases represent an important paradigm in immunology since they can result from either the lack of recognition or over-activation of the inflammatory response.

INNATE RECOGNITION OF FUNGI

VIA PATHOGEN-ASSOCIATED MOLECULAR PATTERNS

Multiple cell populations and cell-signaling pathways are involved in the antigen-independent recognition of the fungus by the innate immune system. Applying systems biology approaches to this complex process has resulted in a better appreciation of the intricate cross-talk provided by temporal changes in mediators, metabolites, and cell phenotypes underlining the coordinated processes (Santamaria et al., 2011). Pattern recognition receptors (PRRs) for fungal pathogen-associated molecular patterns (PAMPs) include TLRs, C-type lectin receptors, nucleotide oligomerization domain-like receptors (NLRs), and NALP3 inflammasome (Romani, 2011; **Figure 1**). Both murine and human studies have confirmed the association of susceptibility to fungal infections and diseases with genetic deficiency of selected PRRs (Cunha et al., 2011a). By varying the composition with the morphotype, growth stage, environment sensing, and fungal species, the cell wall provides the prime sources of PAMPs that, as such, are ideal targets for recognition as non-self by mammalian cells. To achieve optimal activation of antigen-specific adaptive immunity, it is first necessary to activate the pathogen-detection mechanisms of the innate immune response. However, by hyper-inducing pro-inflammatory cytokines, facilitating tissue damage, or impairing protective immunity, PRR activation itself is

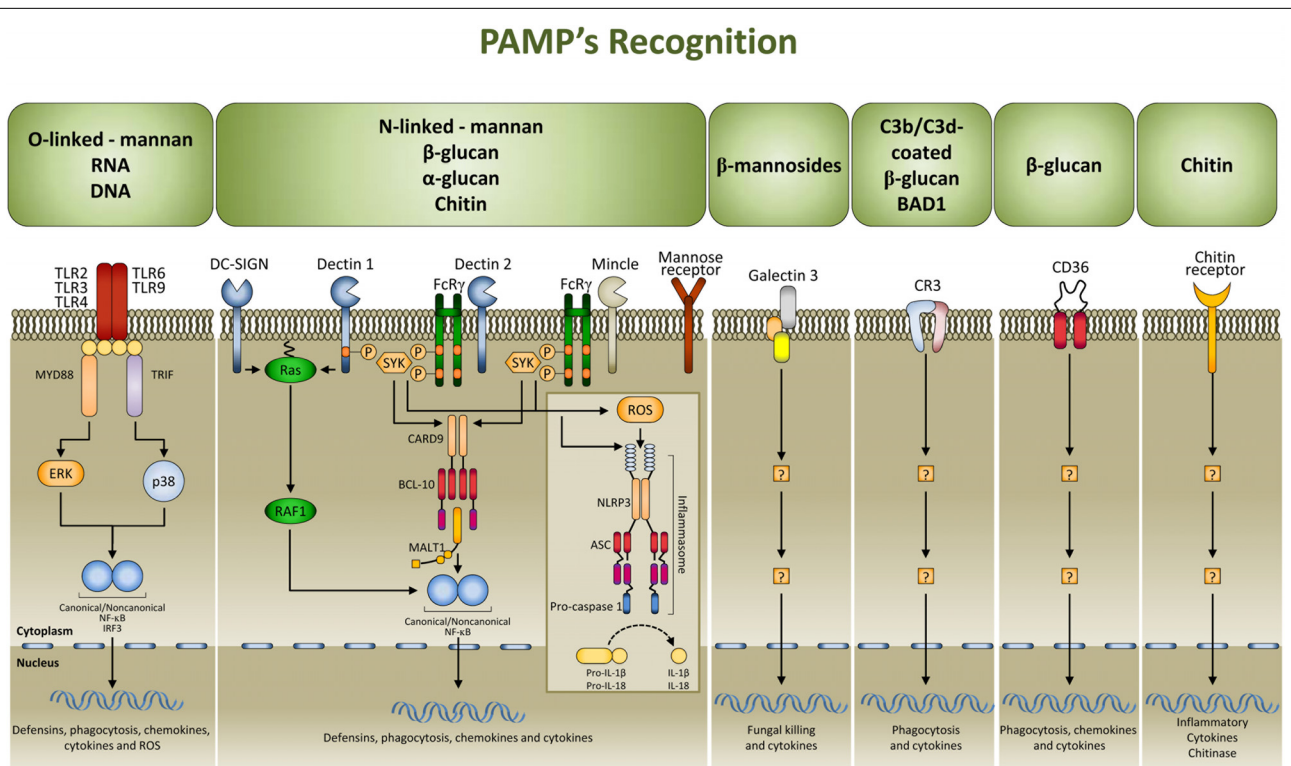

FIGURE 1 | Signaling pathways in fungal pathogen-associated molecular patterns (PAMPs) recognition. PAMPs are recognized by pattern recognition receptors (PRRs). The major PRRs are Toll-like receptors (TLRs); C-type lectin receptors [CLRs; such as dectin 1, dectin 2, DC-specific ICAM3 grabbing non-integrin (DC-SIGN), mincle, and the mannose receptor], galectin family proteins (such as galectin 3), and the class B scavenger receptor CD36. TLRs and CLRs activate multiple intracellular pathways upon binding to specific fungal PAMPs, including β glucans [especially β (1,3)-glucans with varying numbers of β (1,6) branches], chitin, a polymer of *N*-acetylglucosamine, mannans linked to proteins through N or O linkages, β (1,2) linked oligomannosides, and fungal nucleic acids. These signals activate canonical or non-canonical nuclear factor-κB (NF-κB) and the NOD, LRR- and pyrin domain-containing 3 (NLRP3) inflammasome, and this culminates in the production of defensins, chemokines, cytokines, and reactive oxygen species (ROS). CR3, complement receptor 3; ASC, apoptosis-associated speck-like protein containing a CARD; BCL 10, B cell lymphoma 10; CARD9, caspase recruitment domain-containing protein 9; ERK, extracellular signal-regulated kinase; FcRγ, Fc receptor γ chain; IL, interleukin; IRF3, IFN-regulatory factor 3; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; MYD88, myeloid differentiation primary response protein 88; SYK, spleen tyrosine kinase.

a double-edged sword and this may explain why PMNs, although essential in initiation and execution of the acute inflammatory response and subsequent resolution, may act as double-edged swords, as the excessive release of oxidants and proteases may be responsible for injury to organs and fungal sepsis (Romani and Puccetti, 2007).

VIA A DUAL SENSOR SYSTEM

There is a growing suspicion that there are additional types of innate immune sensing mechanisms that are not based on pattern recognition but rather on other principles. It is likely that the kind of principles involved are similar to the "guard theory" described in the plant immunity field by Dangl and Jones (2001) whereby the immune system senses the consequences of some stereotypic function of a pathogen or virulence factor. That is, instead of directly sensing the microbial structure, these sensors detect unusual suspicious activities associated with the microbial virulence apparatus. This kind of sensing has been proven to complement pattern recognition mechanisms in fungal infections. Indeed, during inflammation, host- and fungal-derived proteases trigger the activation of protease-activated receptors (PARs), a family of G-protein-coupled receptors (Shpacovitch et al., 2007). It has been shown that activation of TLRs by fungi unmasks an essential and divergent role for PAR1 and PAR2 in downstream signaling and inflammation (**Figure 2**). TLRs activated PARs and triggered distinct signal transduction pathways involved in inflammation and immunity to *C. albicans* and *A. fumigatus*. Inflammation was promoted by PAR1 activation in response to *Candida* and by PAR2 inhibition in response to *Aspergillus*. This occurred by TLR regulation of PAR signaling. Thus, after recognition by TLRs, PARs may become activated to sense proteolytic virulence factors and tissue injury, to mediate inflammatory responses and modulate the activity of TLRs (Moretti et al., 2008). This implicates that fungal recognition by TLRs may be licensed by DAMP recognition (further discussed below). Conceptually, the model is consistent with a binary signaling pathway of mammalian recognition of fungi as observed in *Drosophila*. A fungal protease used by the entomopathogenic fungus *Beauveria bassiana* to digest the cuticle was shown to activate the Toll pathway by inducing the maturation of Persephone into an active protease (Gottar et al., 2006). Thus, many of the mechanisms used by the innate immune system in animals show surprising parallels with those of immunity in plants. Ultimately, sensing pathogen and virulence is a dual sensor system to detect fungi that works throughout evolution.

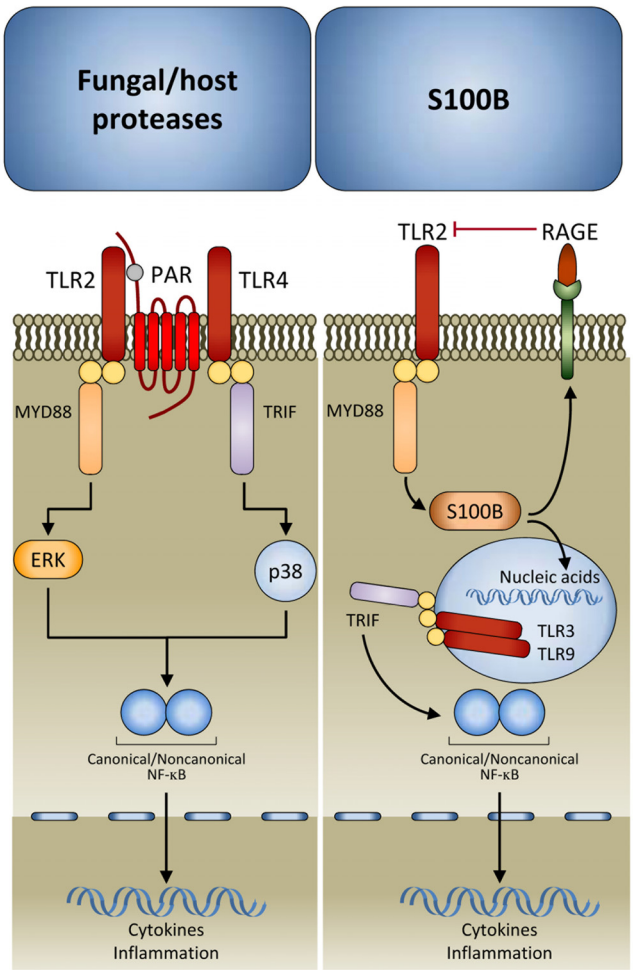

FIGURE 2 | Damage-associated molecular patterns (DAMP) signaling in response to fungi. After TLR activation, protease-activated receptors (PARs) sense proteolytic virulence factors and tissue injury and contribute to fungal recognition through a dual sensor system. In addition, the DAMP, S100B, through the spatio-temporal integration of signals from TLRs and RAGE, allows the immune system to discriminate between pathogen-derived and endogenous danger signals (see text for details). TRIF, TIR domain-containing adaptor protein inducing IFN-β; MYD88, myeloid differentiation primary response protein 88; RAGE, receptor for advanced glycation end-products.

VIA DAMAGE-ASSOCIATED MOLECULAR PATTERNS

Inflammation results from recognition of PAMPs and DAMPs (Gallucci and Matzinger, 2001). Despite the identification of specific signaling pathways negatively regulating responses to PAMPs or DAMPs (Bianchi, 2007), the unexpected convergence of molecular pathways responsible for recognition of PAMPs and DAMPs raised the question of whether and how the host discriminates between PAMPs and DAMPs and the relative contribution of either one to inflammation, immune homeostasis, and mechanisms of repair during infection. It seems clear that the strategy of innate immunity to identify and specifically respond to the presence of a broad class of pathogens and injuries is based on the existence of a set of PRRs that can discriminate between PAMPs and DAMPs. The combined activation of these different receptors by their specific ligands triggers signaling pathways that can be complementary, synergistic, or antagonistic (Lee and Kim, 2007; Trinchieri and Sher, 2007). DAMPs such as the high mobility group box 1 protein and S100 proteins represent important danger signals that, although primarily intracellular, may mediate inflammatory responses through autocrine/paracrine interactions with the receptor for advanced glycation end-products

(RAGE), a multiligand receptor of the immunoglobulin superfamily (Schmidt et al., 2001; Donato, 2007; Sparvero et al., 2009). Integral to the biology of RAGE and its ligands is their upregulation and increased accumulation in multiple biological and disease settings. The ability to activate expression programs that encode innate immune responsive genes confers to RAGE a central role in chronic inflammatory diseases. Engagement of RAGE converts a brief pulse of cellular activation to sustained cellular dysfunction, eventually leading to inflammation and tumor promotion. However, because RAGE is expressed in multiple, distinct cell types, including immune cells, and both murine and human RAGE gene undergoes extensive splicing with distinct splice isoforms being uniquely distributed in different tissues (Kalea et al., 2009), it is not surprising that diverse signal transduction and effector pathways may be impacted by RAGE depending on sites, ligands and time course of ligand–RAGE stimulation (Donato, 2007).

A mechanism that discriminates between fungi- and danger-induced immune responses via the spatio-temporal integration of signals from TLRs and RAGE has recently been described (**Figure 2**). The mechanism exploits a previously unrecognized role for the S100B/RAGE axis that, in sensing danger, plays a critical and unanticipated role as a fine modulator of inflammation in experimental (Sorci et al., 2011) and human (Cunha et al., 2011b) fungal pneumonia. By forming complexes with various TLR ligands, S100B exhibited promiscuous activities at the extracellular and intracellular levels. It inhibited TLR2 via RAGE, through a paracrine epithelial cells/neutrophil braking circuit, and this accounted for its anti-inflammatory activity in infection. However, the ability of S100B to bind nucleic acids resulted in the activation of intracellular TLRs converging on TRIF and eventually resolving danger-induced inflammation via transcriptional down-regulation of S100B gene expression. Thus, in addition to the notion that danger signals may terminate overactive immune responses (Sitkovsky and Ohta, 2005), our study reveals that a pathogen-induced signal may also terminate unnecessary danger-induced injury. A genetically determined hyper-function of the DAMP signaling was indeed associated with invasive aspergillosis in hematopoietic stem cell transplanted patients (Cunha et al., 2011b) and with symptomatic *Candida* vaginitis (Yano et al., 2012). Conceptually, our findings raise the intriguing possibility that the host may have developed mechanisms to ameliorate the response to PAMPs via DAMPs. This is also exemplified by signaling through TLR3. In addition to activation by viral double-stranded RNA, TLR3 can be activated by endogenous mRNA released by necrotic cells (Kariko et al., 2004), a mechanism by which epithelial injury may lead to inflammation. TLR3 plays a non-redundant role in the induction of immunological tolerance in fungal infections (De Luca et al., 2007, 2010), a finding highlighting the contribution of sensing danger in response to fungi. On a translational level, targeting DAMP signaling may be of therapeutic benefit in high-risk patients. We have recently found that a hyper-function of the DAMP signaling underlies inflammation in response to airborne fungi in mice with cystic fibrosis and obtained a proof-of-concept demonstration that the inhibition of RAGE could be of therapeutic benefit in these mice (unpublished observations). Together, these findings confirm that an injudicious response to PAMPs may result in a hostile environment to beneficial commensal fungi. Therefore, discrimination between pathogenic forms, causing cellular damage and tissue injury, and innocuous commensals may be critical in maintaining the balance and tissue homeostasis.

DANGER SENSING IN RESPONSE TO FUNGI: THE PIVOTAL ROLE OF INFLAMMASOMES

Among PRRs, the cytosolic NLR family member NLRP3 (also known as NALP3, cryopyrin, and CIAS1) is a key player in host defense against *C. albicans* (Gross et al., 2009; Hise et al., 2009; Joly et al., 2009; Joly and Sutterwala, 2010). A role for NALP3 has also been shown in response to *A. fumigatus* (Said-Sadier et al., 2010). NLRs are a family of intracellular proteins with a tripartite modular structure that contain a central nucleotide-binding oligomerization domain, a C-terminal LRR, and an N-terminal effector-binding domain that shares structural similarity with a subclass of plant disease resistance genes. Several NLRs (NALPs and IPAF subfamilies) form multi-protein complexes termed inflammasomes which activate inflammatory procaspases and the subsequent processing and secretion of IL-1β, IL-18, and IL-33 (**Figure 1**). As intracellular receptors, the NLR family is in a prime location to detect danger signals associated with host stress and may therefore play a critical role in recognizing the transition from commensal to pathogen (Joly and Sutterwala, 2010). The activation of the NLRP3 inflammasome result in the activation of caspase-1 and processing and secretion of IL-1β that mediates strong innate antifungal responses and regulates Th1/Th17 cell activation (Bellocchio et al., 2004; Vonk et al., 2006; Bozza et al., 2008; Hise et al., 2009; van de Veerdonk et al., 2011). As a matter of fact, mice deficient for IL-1R (the receptor for IL-1β) are resistant and mice with hyper-functioning of the IL-1β signaling are susceptible to candidiasis. In addition, polymorphisms in the gene coding for NLRP3 have been associated with recurrent vulvovaginal candidiasis (Lev-Sagie et al., 2009), a finding consistent with the notion that deregulated NLRP3 inflammasome activation is associated with both heritable and acquired inflammatory diseases.

Currently, three models exist to explain NLRP3 inflammasome activation: (a) lysosomal disintegration and release of its content by phagocytosed material; (b) induction of reactive oxygen species production at mitochondrial membranes; and (c) potassium efflux by membrane channels or ionophoric compounds (Mankan et al., 2012). While it is accepted that *C. albicans* live yeasts induce caspase-1-mediated IL-1β secretion in a NLRP3-dependent manner (Gross et al., 2009; Hise et al., 2009; Joly et al., 2009; Kumar et al., 2009), the precise pathways involved in NLRP3 inflammasome activation are yet to be defined. Although hyphae did not possess stimulatory properties, the ability of *C. albicans* to transition from yeast to hyphal form was essential for NLRP3 activation (Joly et al., 2009). Thus, irrespective of the nature of the ligands that activate NLRP3 in *C. albicans* infection, sensing danger by the NLRP3 inflammasome, and likely NLRC4 (Tomalka et al., 2011), is an inherent component of the host control of fungal opportunism and infectivity.

CONCLUSION

It is now clear that several DAMPs are vital danger signals that alert the immune system to tissue damage upon fungal infections. However, PRR activation by DAMPs may initiate positive feedback loops where increasing tissue damage perpetuates pro-inflammatory responses leading to chronic inflammation. Indeed, DAMPs have been implicated in fungal diseases where excessive inflammation plays a key role in pathogenesis, including fungal pneumonia in transplanted patients and recurrent vulvovaginal candidiasis. Dissection of the reciprocal regulation between DAMP and PAMP signaling pathways, and the relative contribution of either one, in fungal diseases may increase our understanding of the pathogenesis of these diseases with the ultimate goal of developing new strategies that target DAMPs to selectively modulate the immune response to fungi.

ACKNOWLEDGMENTS

We thank Dr. Cristina Massi Benedetti for digital art and editing. Supported by the Specific Targeted Research Project ALLFUN (FP7–HEALTH–2009 Contract number 260338) and the Italian Grant Application 2010 Fondazione per la Ricerca sulla Fibrosi Cistica (Research Project FFC#21/2010) with the contribution of funded Francesca Guadagnin, Coca Cola light Tribute to Fashion and Delegazione FFC di Belluno). Agostinho Carvalho and Cristina Cunha were financially supported by fellowships from Fundação para a Ciência e Tecnologia (contracts SFRH/BPD/46292/2008 and SFRH/BD/65962/2009, respectively).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 July 2012; accepted: 25 August 2012; published online: 10 September 2012.

*Citation: Cunha C, Carvalho A, Esposito A, Bistoni F and Romani L (2012) DAMP signaling in fungal infections and diseases. Front. Immun. **3**:286. doi: 10.3389/fimmu.2012.00286*

This article was submitted to Frontiers in Inflammation, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 23 October 2012
doi: 10.3389/fimmu.2012.00321

The entry of fetal and amniotic fluid components into the uterine vessel circulation leads to sterile inflammatory processes during parturition

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Pro-inflammatory cytokines play an important role during the process of human parturition. The focus of this review was to explore the contribution of biological, biochemical, and genetic changes in the onset of term labor. This article reviews the English-language literature on inflammatory, hormonal, and immunological factors in an effort to identify the molecular basis of human parturition. The majority of the genes and proteins up-regulated in parturition at term are related to four functional categories, mechanical stretch-mediated damage-associated molecular patterns (DAMPs) activation, response to immunity, induction of inflammatory signaling, and progressive uterine myometrial contractility and resultant term birth. Mechanical stretch could promote the entry of amniotic fluid components into the uterine vessel circulation that is the common physiologic mechanism at term prior to labor. The fetal or amniotic fluid-derived DAMPs could activate the immune system. The inflammatory mediators are produced by infiltrating activated leukocytes and by the reproductive tissues themselves such as myometrium, and subsequently lead to uterine contractions. This review supports the sterile inflammation hypothesis that there are at least two phases of human parturition: the initial wave of the entry of amniotic fluid components into uterine vasculatures would be followed by the second big wave of subsequent myometrial contraction.

Keywords: parturition, inflammation, stretch, amniotic fluid, myometrium

INTRODUCTION

Dramatic advances of molecular analysis and biological profiling represent an opportunity to improve an in-depth understanding of human parturition. Parturition is characterized by the activation of innate immune and neuroendocrine mechanisms. Pregnancy is a unique immunological state in which a balance of immune tolerance and suppression may participate in the regulation of the host immune response and protection of the fetus. Oxytocin and corticotropin-releasing hormone are important neuroendocrine pathways involved in parturition (Petraglia et al., 2010). Thus, endocrine–immune interaction controls conditioning of the myometrium and plays as a prelude to the onset of labor.

Furthermore, accumulating evidence suggests that ascending intrauterine infection results in premature birth and high fetal mortality (Martius and Eschenbach, 1990). There has been an increased awareness of the role of infection and inflammation at the time of parturition. Bacterial infection and sterile inflammation (a physiological process) are key mechanisms of human preterm and term labor, respectively. Several studies focused on the feedforward loop in which, near the end of pregnancy, the pro-inflammatory cytokine–prostaglandin (PG) axis activates the uterus (Golightly et al., 2011). Excessive inflammation produces uterine contractile stimulants leading to labor and delivery.

Despite considerable research and progress in the technology of reproduction, the causes of the initial events driving parturition remain obscure. We review the contemporary literature on sterile inflammation that support mechanism of parturition at term.

STUDY METHODOLOGY

The present study reviews the literature for biological studies of human parturition. Data pertaining to *in vitro* and *in vivo* studies were included. A computerized literature search was performed to identify relevant studies reported in the English language. All abstracts from Medline electronic database were reviewed to identify papers for full-text review. The web-based database were searched, combining the keywords "genome-wide," "proteomics," "onset," "labor," "term," "myometrium," "cervix," "amniotic fluid," "TLR," "inflammation," "immunity" "leukocytes," "cytokine," "complement," and "NF-kappaB" with "parturition." Additionally, references in each study were searched to identify potentially missed studies. Target publications are mainly reports on human studies and animal models, as well as basic studies in gene and protein expression systems. Abstracts were not included, since they do not undergo a stringent peer review process.

PARTURITION AFFECTED BY THE STATUS OF IMMUNITY

Alterations in maternal immunity, peripheral tolerance and feto-maternal tolerance of uteroplacental unit have been seen during pregnancy, the so-called "immunological paradox" (Spencer et al., 2008). In humans, there are key players in the regulation of the pathway involved in suppression of immune responses. NK

cells, T regulatory (Treg) cells, the Th1/Th2 shift and complement system had a key role in the suppression of immune responses (Kokubu et al., 2005; Guerin et al., 2009). NK cells were critical for the success of pregnancy and closely involved in parturition (Kokubu et al., 2005). Treg cells favored fetal development and escaped from the host immune system through suppression of the activation of immune cells including antigen presenting cells, $CD4^+$ and $CD8^+$ T lymphocytes. Treg cell numbers were considered to increase early in pregnancy and then began to decline at parturition and decreased in the postpartum period (Norris et al., 2011). These specific immune systems might be necessary to achieve maternal alloantigen tolerance during pregnancy. This phenomenon is referred to as the Th1/Th2 shift. Down-regulation of the Th1 response and Th2 predominance were associated with successful pregnancy maintenance (Sykes et al., 2012). Th1 cells secreted pro-inflammatory cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). The Th1 cytokine-mediated Toll-like receptor (TLR) activation contributed to the inflammatory response in the initiation of labor.

In contrast, pregnancy hormones such as progesterone, estradiol, leukemia inhibitory factor, and PGD_2 promoted the Th2 cell profiling by modulating a direct tie between hormone and immune function. Th2 cells produced interleukin (IL)-4, IL-5, IL-13, and IL-10. IL-10 is an anti-inflammatory cytokine produced primarily by decidual macrophages and overexpressed at the maternal–fetal interface and is crucial for dampening inflammation. In animal experiments using rhesus monkeys, this cytokine blocked IL-1β-induced preterm labor (Sadowsky et al., 2003). IL-10 also inhibited IL-1β-induced decreases in placental PG dehydrogenase (PGDH) expression (Pomini et al., 1999). There was a dynamic spatial and temporal expression pattern for IL-10 in human placenta: IL-10 was expressed in the first and second trimester placental tissues. In term tissue, however, this cytokine diminished before the onset of labor (Hanna et al., 2000). IL-10 down-regulated the expression of Th1 pro-inflammatory cytokines through suppression of activation of the transcription factor nuclear factor-kappaB (NF-κB), a driver gene for inflammation-related signaling pathway. These data support the requirement of the Th1/Th2 shift for successful parturition.

In addition, one of the initial responses of this innate immunity may be an activation of the complement cascade. Complement can generate biologically active products, which trigger inflammation. Normal pregnancy was characterized by an increase in anaphylatoxin C5a in the maternal circulation in the third trimester, suggesting that the complement system is activated. C5a up-regulated pro-inflammatory and pro-labor mediators, including pro-inflammatory cytokines (IL-6 and IL-8), cyclooxygenase (COX)-2, PGE_2 and $PGF_2\alpha$, matrix metalloproteinase (MMP)-9, and 8-isoprostane in human gestational tissues via the C5a receptor (CD88)-mediated NF-κB activation (Lappas et al., 2012). Abundant research has demonstrated complement activation in an innate immunity of human parturition (Gallery et al., 1981; Benson et al., 2006; Benson, 2007; Soto et al., 2009; Gonzalez et al., 2011; Kato et al., 2012; Lappas et al., 2012), but has yet to investigate whether complement activation is the result of fetal antigen leaking into the maternal circulation.

MECHANICAL STRETCH OF UTERINE MYOMETRIUM AT TERM

Many investigators have analyzed genome-wide transcriptomes and proteomics of the reproductive tissues at different stages of parturition. At term, uterine myometrium, including myometrial smooth muscle cells and fibroblasts, was stretched by growing fetuses. Molecular mechanism mediating stretch-induced signaling pathways has been elucidated. Cyclic mechanical stretch induced an increase in secretion of pro-inflammatory cytokines in myometrial smooth muscle cells compared to non-stretch controls (Sooranna et al., 2004; Kendal-Wright et al., 2010; Hua et al., 2012). This increase in cytokine production correlated with activation of NF-κB (Mendelson, 2009). Mechanical stretch also stimulated COX-2 expression through activation of the activated protein (AP)-1 system (Sooranna et al., 2004). Thus, cyclic stretch and release in myometrial smooth muscle cells stimulated a robust activation of NF-κB and AP-1. Some of the important genes up-regulated in human myometrium during term labor were monocyte chemotactic protein-1 (MCP-1, also known as C-C chemokine motif ligand 2, CCL-2), IL-8, and TNF-α. MCP-1 was a member of a large chemokine family and displayed chemotactic activity for monocytes/macrophages, as well as promoting macrophage activation (Esplin et al., 2005). MCP-1 expression was enhanced by mechanical stretch of the uterine myometrium. IL-8 and TNF-α, whose expression was specifically restricted to myometrium after the onset of labor, were potential candidates of contraction-associated cytokines. In contrast, both IL-1β and IL-6 were present in term myometrium before and during labor, suggesting that these cytokines were involved in the preparation or conditioning for the synchronized contractions of labor (Sehringer et al., 2000). IL-1β participated in the regulation of the myometrial contractions via an increase in PGs production (Hertelendy et al., 1993). IL-1β was synthesized as an inactive precursor, pro-IL-1β, and then cleaved into the active form through cytosolic protein complexes termed "inflammasomes" (Gotsch et al., 2008). The placenta expressed the inflammasomes. Cellular stress in response to inflammatory conditions accounted for activation of the inflammasomes, which occurred during labor. The previous elegant review discussed the role of the inflammasomes system and their potential to contribute to the pathogenesis of preterm birth (Abrahams, 2011). Unfortunately, we have very little understanding of their function in normal pregnancy and the onset of term labor.

These data suggest that transduction of the stretch signal in myometrial smooth muscle cells involves alteration of the gene expression signature. Activation of NF-κB and AP-1 increased expression of several genes implicated in the control of immunity and inflammation (Mendelson, 2009; Khanjani et al., 2011). MCP-1 locally mediated leukocyte migration into uterine myometrial tissues. Myometrial smooth muscle cells can play a role as immune cells and participate in the sterile inflammation at term (Khanjani et al., 2011; Shynlova et al., 2012). Taken together, mechanical stretch-induced NF-κB/AP-1 activation, which occurs prior to labor, modulates the expression of numerous inflammation-associated genes that are directly or indirectly involved in the positive feedback loop during parturition.

THE ENTRY OF FETAL AND AMNIOTIC FLUID COMPONENTS INTO THE UTERINE VESSEL CIRCULATION

Prior to labor, there were prominent changes in the myometrial fibers that increase the distance between muscle layers and promoted edema. These cells exhibited such morphology as shearing, shrinkage, and apoptosis. Endothelial cell damage in the uterine myometrium were very common at term prior to labor. The vascular lumen of endothelial cells contained fibrin and platelet thrombi, microparticles, desquamated endothelial cells, amniotic squamous cells, and mucoid material (Leong et al., 2008). The entry of amniotic fluid components into the uterine vessel circulation might be the common physiologic mechanism. Histologically, these changes were present in myometrial tissues obtained during labor at term, providing a mechanism by which fetal and amniotic fluid components may access myometrial cells (Leong et al., 2008). In addition, small amount of fetal red cells were normally detectable in peripheral blood of the mother in all pregnancies, indicating that fetal cells can enter the maternal circulation (Ahmed and Abdullatif, 2011). The presence of not only intact fetal cells but also fetal-origin nucleic acids (cell-free fetal DNA and RNA) in maternal blood has been identified. Cell-free fetal nucleic acids afford the opportunity for the promising prenatal genetic testing. Part of the fetal DNA fragments derived from the placenta. These data support that a substantial amount of fetal antigens might be transported to the uterine vasculature and maternal circulation at term prior to labor.

Changes in the recognition and adaptation to a set of foreign antigens would be a mechanism of the onset of labor. The maternal responses to an alloantigen challenge were reduced during pregnancy (Spencer et al., 2008), while, alloantigens resulted in immune-mediated fetal rejection in the term parturition. Recently, pattern recognition receptors (PRRs) responsive to unique molecules, termed pathogen-associated molecular patterns (PAMPs), have received considerable attention as possible contributors to the onset of preterm labor. Microorganisms have PAMPs that were recognized by PRRs such as TLRs, Nod-like receptors (NLRs), and the inflammasomes (Tang et al., 2012). PRRs recognized not only PAMPs, but also host-derived danger signals "alarmin" or damage-associated molecular patterns (DAMPs) derived from damaged tissue. In general, DAMPs are known to be cell-derived immunity. The fetal DNA found in the maternal circulation could act as DAMPs through PRRs such as TLR9 or AIM2 (absent in melanoma 2; Barber, 2011). The TLR9 has an ability to bind structurally highly conserved microbial molecules such as CpG motif-containing DNA and subsequently initiates the production of Th1 pro-inflammatory cytokines and chemokines (Barber, 2011). AIM2 acts as a DNA sensor in innate immunity and mediates inflammatory responses involving IL-1β. AIM2 also triggered the assembly of the inflammasomes. Cell-free fetal DNA would mediate innate immune signaling that provides an important step toward initiation of parturition.

Furthermore, hyaluronan, a component of the extracellular matrix, was a component of the DAMPs associated with NLRs. Intra-amniotic hyaluronan levels were elevated in pregnancies. Hyaluronan was released into the extracellular milieu and also amniotic fluid where it modulates immune activity. Yet, this hypothesis has not been proved.

These initial events prior to labor hint at a possible causative role. During pregnancy and prior to labor, women were tolerant of their semi-allogeneic fetal components: the maternal immune system came into contact with trophoblasts and other semi-allogeneic components, including amniotic fluid, fetal cells, and cell-free fetal DNA. The modulation of cell-mediated immunity caused by a substantial amount of DAMPs at term prior to labor may be responsible for the increased susceptibility to parturition.

INFILTRATION OF LEUKOCYTES IN UTERINE MYOMETRIUM AND CERVIX

An accumulating body of evidence has demonstrated that uterine myometrial contraction coincident with the onset of term labor was accompanied by the massive influx of leukocytes in all regions of uterine myometrium, amnion, choriodecidua, and cervix following spontaneous labor compared with non-laboring tissues (Thomson et al., 1999; Keski-Nisula et al., 2000; Osman et al., 2006; Gomez-Lopez et al., 2011; Hamilton et al., 2012; Shynlova et al., 2012). Histological analysis demonstrated that, in the myometrium, tissue macrophages, neutrophils, and T lymphocytes massively increased coincident with the onset of labor at term (Thomson et al., 1999; Keski-Nisula et al., 2003; Osman et al., 2006). Marked myometrial inflammation was not associated with the prediction of pathological conditions such as infection (Keski-Nisula et al., 2003). The influx of fetal leukocytes into the myometrium has been implicated in the initiation of parturition in mice (Kim et al., 2006). During human labor, however, fetal macrophages from the amniotic cavity or the chorioamniotic membranes did not migrate into the myometrium (Kim et al., 2006). Leukocytes into the myometrium was a maternal origin. The uterus at term was infiltrated with inflammatory cells, which was subsequently associated with advanced labor and uterine contraction, because pro-inflammatory cytokines such as TNF-α can stimulate uterine smooth muscle cell contractility (Keski-Nisula et al., 2003; Yellon et al., 2003; Fitzgibbon et al., 2009; Kamel, 2010; Lee et al., 2012). Inflammatory cells orchestrate processes required for initiation of the myometrial contraction (Thomson et al., 1999; Osman et al., 2003).

The uterine cervix must be disorganized before, during and after parturition, via release of proteolytic enzymes and followed by a tissue repair postpartum. Leukocyte density increased two- to threefold between the first trimester of pregnancy and term, prior to the onset of labor (Spencer et al., 2008). A marked increase in macrophage density was observed after the onset of labor (Keski-Nisula et al., 2000; Timmons et al., 2009; Kamel, 2010). This occurred during the course of cervical softening and effacement (Bokström et al., 1997). In contrast, neutrophils specifically increased in the postpartum period and were involved in the postpartum tissue repair (Thomson et al., 1999; Hamilton et al., 2012).

Taken together, leukocyte infiltration is a complex process involving at least two steps, including the first step, mechanical stretch of uterine myometrium without involvement of resident macrophages, and then the second step, the entry of fetal and amniotic fluid alloantigens into the maternal circulation. These steps might drive myometrial chemokine expression primarily via activation of NF-κB, which in turn results in a prominent leukocyte

infiltration into the uterus. DAMPs such as fetal antigens activated infiltrated macrophages via the TLR/AIM2/NF-κB pathway. The infiltration of myometrium (before the onset of labor) and cervix (in the postpartum) with activated leukocytes has been associated with the initiation of parturition and a rapid repair postpartum, respectively.

INFLAMMATORY GENE REGULATORY NETWORKS IN PARTURITION

Several investigators used a network mining algorithm to identify tightly connected gene expression pathways that were frequently present in microarray data sets samples. Global gene expression analyses and single gene approaches revealed that human labor involved the infiltration of specific leukocyte subsets and the secretion of autocrine and paracrine mediators, including NF-κB, pro-inflammatory cytokines (IL-1, IL-6, TNF-α), chemokines (IL-8/CXCL-8, MCP-1/CCL-2), IL-1 receptor accessory protein (IL-1RAP), TLRs, COX-2, contraction-associated proteins (CAPS), oxytocin receptor (OXTR), connexin-43 (CX-43), the prostaglandin F receptor (FP), hypoxia-inducible factor (HIF), thrombospondin 1 (TSP-1), MMP-2, and MMP-9 (Vora et al., 2010; Li et al., 2011; Lim et al., 2012). The majority of these genes were downstream targets of NF-κB and PRRs such as TLRs. Spontaneous term labor group is associated with increasing activation of the NF-κB signaling network relative to the term no labor cohort (Vora et al., 2010). Therefore, sterile inflammation has underlied parturition at term and the TLR/NF-κB axis in macrophages is an essential pathway.

ENDOCRINE AND PROSTANOID PATHWAYS

Myometrial contractility has been the predominant focus for the mechanism that contributes to regulation of development of parturition and initiates labor. The endocrine status affects the process of parturition. The potential factors included PGs, oxytocin,

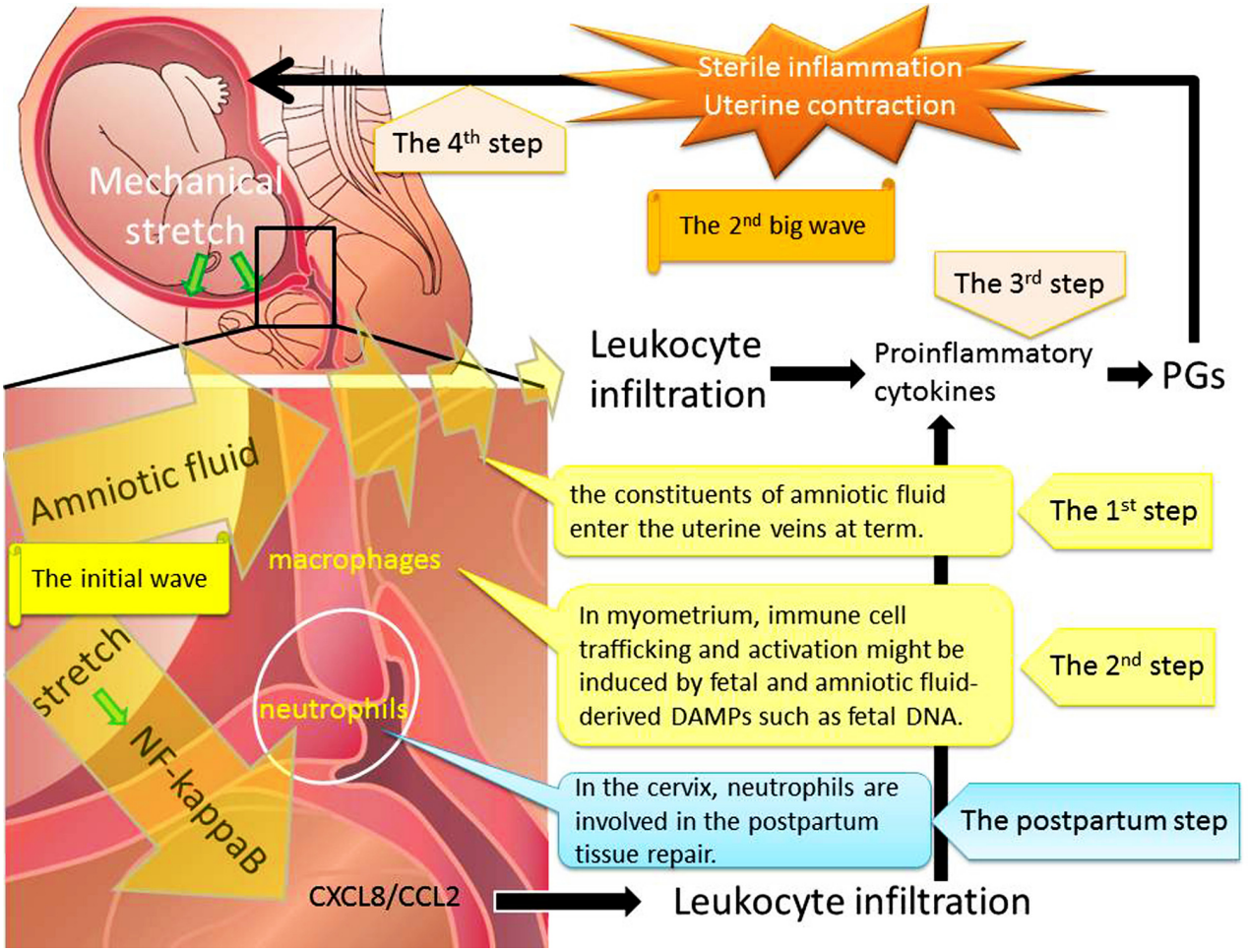

FIGURE 1 | Mechanical stretch and subsequent leukocyte infiltration are essential for labor onset at term. *Step 1*: Mechanical stretch synergistically augmented leukocyte infiltration and production of pro-inflammatory chemokines via the NF-κB pathway. *Step 2*: The entry of amniotic fluid components into uterine vasculatures promoted myometrial infiltration with macrophages and neutrophils. *Step 3*: Infiltrating leukocytes could induce expression of pro-inflammatory cytokines and PGs through the activation of NF-κB. *Step 4*: The final step is the progressive uterine contractility and resultant term birth. We hypothesized that the initial wave of mechanical stretch and leukocyte infiltration would be followed by the second big wave of sterile inflammation and subsequent myometrial contraction.

nitric oxide, COX-2, cytokines, as well as endocrine mediators such as estrogen, progesterone, corticotrophin releasing hormone, and cortisol. Activation of contractile genes (e.g., COX-2, OXTR) was directly promoted by transcription factors NF-κB and AP-1. Such changes in prostanoids and COX-2 pathways seem to be inflammation-mediated physiological responses at the later stages of parturition. It is likely that all these factors were involved in the feedforward loop during parturition (Christiaens et al., 2008; Golightly et al., 2011).

SUMMARY

This review focuses on the contribution of biological, biochemical, and genetic changes during each phase of activation in the process of parturition. This process consists of four steps (**Figure 1**). The first step is mechanical stretch of myometrium which can promote the entry of amniotic fluid components into the uterine vessel circulation at term prior to labor. The constituents of amniotic fluid include fetal and amniotic fluid-derived DAMPs such as cell-free fetal DNA and RNA. The second step consists of immune cells trafficking and activation which might be induced by fetal and amniotic fluid components. The third step is inflammatory reactions with the release of Th1 cytokines and down-regulation of the Th2 response. This seemingly irreversible step consists of the enhanced production of PGs and endocrine mediators. The final step is the progressive uterine contractility and resultant term birth. Therefore, immune cell trafficking and activation induced through myometrial stretch-mediated DAMP activation are part of the initial mechanism that immediately enhances uterine myometrial contractility and initiates parturition.

In conclusion, there are at least two phases of human parturition: the initial wave of the entry of amniotic fluid components into uterine vasculatures would be followed by the second big wave of sterile inflammation by infiltrating leukocytes and subsequent myometrial contraction.

ACKNOWLEDGMENTS

Grant support: Supported by Grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan to the Department of Obstetrics and Gynecology, Nara Medical University (Hiroshi Kobayashi).

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 08 August 2012; accepted: 03 October 2012; published online: 23 October 2012.

Citation: Kobayashi H (2012) The entry of fetal and amniotic fluid components into the uterine vessel circulation leads to sterile inflammatory processes during parturition. Front. Immun. ***3****:321. doi: 10.3389/fimmu.2012.00321*

This article was submitted to Frontiers in Inflammation, a specialty of Frontiers in Immunology.