NON-CANONICAL IL-1β PROCESSING VIA CASPASE-8 IN MURINE
DENDRITIC CELLS AND MACROPHAGES

by

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LIST OF ABBREVIATIONS

AIM2- Absent in melanoma 2
AMC- Aminomethylcoumarin
ASC- Apoptosis-associated speck-like protein containing a C-terminal CARD
ATP - Adenosine triphosphate
BMDC- Bone marrow derived dendritic cell
BMDM- Bone marrow derived macrophage
BSA - Bovine serum albumin
BSS- Basal salt solution
CARD- Caspase recruitment domain
cIAP- Cellular inhibitor of apoptosis protein
DAMP – Damage-associated molecular pattern
DC - Dendritic cell
DED- Death effector domain
DMSO- Dimethylsulfoxide
Dox- Doxorubicin
DRP1- Dynamin-related protein 1
DSS- Disuccinimidyl suberate
ELISA - Enzyme-linked immunosorbent assay
FADD- Fas-associated death domain
FLIP- FLICE-inhibitory protein
ICE- Interleukin-converting enzyme
IL-1- Interleukin-1
IL-1R- Interleukin-1 receptor
IRAK- IL-1 receptor associated kinase
IRF- Interferon regulatory factor
LCS-1- Lung cancer screen-1
LPS – Lipopolysaccharide
LRR- Leucine-rich repeat
MHC- Major histocompatibility complex
MLKL- Mixed lineage kinase domain-like
MyD88- Myeloid differentiation primary response gene (88)
MSU- Monosodium urate
NLRP3- Nod-like receptor family, pyrin domain containing 3
NOD- Nucleotide-binding oligomerization domain
Ox- Oxaliplatin
Pam₃CSK₄- N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)- propyl]-[R]-cysteiny-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine
PAMP – Pathogen associated molecular pattern
Panx1- Pannexin-1
PBS - Phosphate buffered saline
PGAM 5- Phosphoglycerate mutase family member 5
PI - Propidium iodide
PARP- Poly ADP-ribose polymerase
Poly dA:dT- poly(deoxyadenylic-deoxythymidylic) acid
PYD- Pyrin domain
RIP- Receptor-interacting protein
ROS- Reactive oxygen species
Smac- Second mitochondrial-derived activator of caspases
SOD- Superoxide dismutase
STS- Staurosporine
TIR- Toll-interleukin 1 receptor
TIRAP- TIR-domain-containing adapter protein
TLR – Toll-like receptor
TNF- Tumor necrosis factor
TRAM- TRIF-related adaptor molecule
TRIF- TIR-domain-containing adapter inducing interferon-β
Ac-YVAD-cmk- acetylated Tyr-Val-Ala-Asp-chloromethylketone
z-IETD-fmk- Z-Iso-Glu-Tyr-Asp-fluoromethylketone
z-VAD-fmk- Z-Val-Ala-Asp-fluoromethylketone
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Non-canonical IL-1β Processing via Caspase-8 in Murine Dendritic Cells and Macrophages

Abstract

by

CHRISTINA ANTONOPOULOS BUZZY

Dysregulated IL-1β production has been implicated in a host of disease settings, ranging from autoinflammatory syndromes to diabetes and Alzheimer’s disease. The first-identified interleukin-converting enzyme or ‘ICE’ was caspase-1. The assembly of cytosolic, multi-protein complexes called inflammasomes canonically triggers the activation of caspase-1. Following proximity-induced autocatalysis of procaspase-1 dimers, activated caspase-1 molecules proteolytically catalyze the conversion of the pro-inflammatory cytokines, proIL-1β and proIL-18, into their mature cytokines, IL-1β and IL-18. As will be discussed in the following chapters, the coordination of IL-1β cytokine generation is under the regulation of the individual proteins which comprise the particular cytosolic, multi-protein complex.

Caspase-8 has recently emerged as an alternative ICE in response to microbially-induced inflammation for the generation of mature IL-1β and IL-18. My dissertation studies contribute to this rapidly growing field in multiple unique facets. First, in the genetic absence of caspase-1, caspase-8 is recruited to an ASC-containing platform for IL-1β processing in response to nigericin in dendritic cells (DCs). Besides acting as a
direct IL-1β convertase, caspase-8 also regulates the activation of caspase-1 induced by nigericin in LPS-primed control DCs, adding an additional layer of complexity to IL-1β regulation. In the context of anti-cancer treatment, the commonly used pro-apoptotic chemotherapeutic drug, doxorubicin (Dox), induces a non-canonical IL-1β processing platform, consisting of RIP1/FADD/caspase-8 and is driven by the downregulation of the cellular inhibitor of apoptosis protein 1 (cIAP1). Enzymatically active caspase-8 then promotes IL-1β maturation within this specific rioptosome complex. Of particular interest is how this inflammatory signaling pathway may contribute to cancer pathogenesis or amelioration during treatment with Dox.

My data additionally demonstrate the intertwined nature of inflammatory and cell death signaling pathways. The activity of IL-1β convertases, caspase-1 and caspase-8, dictates which mode of cell death a TLR4-primed macrophage/DC will undergo and is highly stimulus-dependent. Consequently, IL-1β signaling is closely associated with pyroptosis when a caspase-1 platform is engaged and apoptosis when a caspase-8 platform is engaged. Herein this thesis, I discriminate between caspase-1- and caspase-8-based signaling pathways in murine dendritic cells and macrophages for the proteolytic maturation of IL-1β.
CHAPTER 1

Introduction

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Overview

Interleukin-1β is a pleiotropic cytokine that plays a vital role in the regulation of the immune system and is mainly produced by the activation of large, cytosolic multi-protein complexes termed inflammasomes in myeloid leukocytes. Following inflammasome assembly, interleukin-converting enzymes are activated, proIL-1β is processed, and biologically active IL-1β is released and binds to the IL-1R to modulate both innate and adaptive immune processes, including the immune response to cancer. In this thesis, I evaluated the factors that discriminate between canonical (caspase-1-mediated) versus non-canonical (caspase-8-mediated) IL-1β processing pathways in murine DCs and macrophages. My data support a model wherein TLR4-activated DCs treated with cancer chemotherapy drugs leads to decreased cIAP1 (cellular inhibitor of apoptosis protein 1) expression and the engagement of a TRIF-dependent RIP1/FADD/caspase-8 signaling platform for caspase-8-dependent IL-1β processing (Chapter 3). My studies also illuminate a role for caspase-8-dependent IL-1β processing via an NLRP3/ASC/caspase-8 platform in caspase-1-deficient DCs (Chapter 4). Additionally, I discuss how the anti-lung cancer drug, LCS-1, targets multiple proteins
within the NLRP3 inflammasome pathway to inhibit IL-1β processing (Chapter 5).

Finally, I discuss how to translate and dissect the involvement of the caspase-8-based IL-1β signaling pathway in a chemotherapeutic clinical scenario (Chapter 6).

I. **Physiological Role for IL-1β in Immune Cell Signaling**

**IL-1β in Inflammatory Diseases**

Inflammation is a biological response to pathogens and danger molecules and results in the production and secretion of cytokines, of which the interleukin family of cytokines comprise a vast majority. PAMPs and DAMPs engage ligand-specific TLR receptors on leukocytes and result in the production and secretion of pro-inflammatory cytokines, most notably, IL-1β, IL-6, and TNFα. IL-1β processing possesses the following features: 1) it requires NF-κB-dependent upregulation of the precursor cytokine, proIL-1β; 2) it requires a finely orchestrated mechanism of activation, which will be described in the following section, Inflammasomes; and 3) it contains a leaderless peptide sequence and consequently, is secreted via non-classical export mechanisms (1, 2). Myeloid leukocytes produce the vast majority of IL-1β but non-hematopoietic cells, such as endothelial and epithelial cells, also express inflammasome proteins and alternative mechanisms to induce IL-1β processing and maturation. The overproduction of IL-1β has been associated with chronic disease triggers including, cholesterol crystals in atherosclerosis, sustained elevated blood sugar levels in diabetes-related retinopathy and neuropathy, the formation of amyloid beta fibers in Alzheimer’s disease, and inflammatory bowel disease pathogenesis (3-6). Interestingly, both isoforms of IL-1, IL-
1α and IL-1β, bind to the IL-1R; however, while both proIL-1α and IL-1α are biologically active and can activate the IL-1R, only processed IL-1β can activate the IL-1R.

The derailing of IL-1β signaling in the innate immune system has given rise to a group of disorders called Systemic Autoinflammatory Diseases (SAIDs). This broad range of heterogeneous disorders is characterized by the dysregulated production of pro-inflammatory cytokines, such as IL-1β and also TNFα, and the unpredictable occurrence of multi-organ inflammation, which occurs in the absence of autoreactive T cells and autoantibodies (7). Several gain-of-function mutations in the most widely characterized inflammasome protein, NLRP3, are responsible for the Cryopyrin Associated Periodic Syndromes (CAPS), including Familial Cold Autoinflammatory Syndrome (FCAS), Muckle Well’s Syndrome (MWS) and chronic infantile neurological cutaneous and articular syndrome (CINCA). These are classified as hereditary periodic fever syndromes and are attributed to the constitutive assembly of NLRP3 inflammasomes and the overproduction of IL-1β, which can be triggered by physical and emotional stressors, such as cold temperatures in the case of FCAS. Another well-known example of these diseases includes Familial Mediterranean Fever (FMF), in which a mutation in the MEFV gene responsible for encoding the inflammatory protein, Pyrin, results in periodic acute fever, arthritis, and other symptoms. Depending on the disease and severity, IL-1R antagonist therapies, such as Anakinra, have provided relief for some IL-1β–driven pathologies but little relief for others. Understanding the detailed, molecular pathways that lead to IL-1β production may aid in the design of targeted therapies to alleviate IL-
1β–driven periodic fever syndromes and chronic inflammatory diseases.

The predominant cell types that give rise to the production of IL-1β consist of DCs and macrophages; however, other cell types process IL-1β, notably in the context of various disease settings. Neutrophils and vascular endothelial cells, which express NLRP3 and ASC, are capable of producing IL-1β via caspase-1-dependent inflammasome activation, while other cell types possess alternative, caspase-1-independent enzyme-mediated mechanisms for processing IL-1β and are described in the section, Alternative Proteases. Netosis, a form of neutrophil cell death, and the formation of neutrophil extracellular traps (NETs) are increased in neutrophils in a subset of lupus patients. NETs activate immunostimulatory molecules on the endothelium that cause dysregulated inflammation in autoimmune Systemic Lupus Erythematosus (SLE). Macrophages from lupus patients are highly susceptible to inflammasome stimulation following exposure to NETs or LL-37, an antibacterial protein present on the surface of NETs (8). Moreover, vascular endothelial cells have been shown to activate inflammasomes via the canonical, caspase-1-dependent pathway in response to stimulation of the xenobiotic receptor, PXR (Pregnane X Receptor) (9). PXR acts as a dominant transcription factor to control innate immune responses such as TLR and NOD receptor expression and particularly, NLRP3. NLRP3 inflammasome activation in non-myeloid-derived cells has also been reported to exacerbate inflammation associated with diabetic nephropathy (10). Thus, IL-1β is a biologically important cytokine, which modulates inflammatory signaling in multifarious disease settings.
**IL-1β in Tumor Immunity**

Innate immune signaling in tumor-resident dendritic cells (DC) and macrophages can contribute to the efficacy of chemotherapeutic agents by engaging multiple T-cell-mediated adaptive immune responses that limit the expansion of chemotherapy-resistant tumor cells (11). IL-1β has emerged as a particularly relevant macrophage/DC-derived cytokine that – depending on tumor type and micro-environmental milieu – can drive either the beneficial limitation of tumor growth via immunogenic T-cell pathways or the maladaptive potentiation of tumor progression. The latter actions of IL-1β may involve: 1) local release of paracrine growth factors from tumor stromal cells; 2) enhanced tumor angiogenesis; or 3) accumulation of myeloid-derived suppressor cells (MDSC) that counteract anti-tumor immunosurveillance (reviewed in (11, 12)). Given these contrasting roles for IL-1β in tumor growth and anti-tumor therapy, it is important to define the diversity of signaling pathways that facilitate IL-1β production by macrophages/DC within the context of diverse cancer chemotherapeutic regimens.

Previous studies have identified two distinct, but not mutually exclusive, pathways (Figure 1). The intended function of most chemotherapeutic agents to directly initiate apoptotic or necrotic death signaling cascades in rapidly dividing cancer cells also results in extracellular accumulation of tumor-derived macromolecules (e.g., high mobility group B1 protein /HMGB1) and small molecule metabolites (e.g., ATP). These act as danger-associated molecular patterns (DAMPS) for engagement of the canonical NLRP3→ASC→caspase-1 inflammasome cascade that facilitates secretion of bioactive IL-1β from tumor-resident macrophages/DC. This indirect pathway of IL-1β production via a paracrine tumor cell→myeloid cell axis has been described for multiple
Macrophages, DCs, and myeloid-derived suppressor cells (MDSC) comprise the major cellular source of IL-1β within tumor microenvironments. In the context of cancer chemotherapy, locally produced IL-1β can support anti-tumor immune responses by directly (or indirectly via IL-17 secreted by γδ T cells) polarizing tumor antigen-reactive CD8+ T cells into effector cells that kill or suppress chemotherapy-resistant cancer cells. However, IL-1β may also directly (or indirectly via IL-17) support tumor growth by stimulating growth factor release from tumor stromal cells or by enhancing angiogenesis and tumor vascularization. Production and secretion of biologically active IL-1β involves the convergence of so-called ‘signal 1’ and ‘signal 2’ cascades. The former involves NF-κB-dependent upregulation proIL-1β, while the latter involves regulated assembly of caspase-containing signaling complexes that catalyze IL-1β processing. Previous studies have identified two pathways by which cancer chemotherapeutic agents induce activation of caspase-1 inflammasomes and consequent IL-1β processing in tumor-resident myeloid leukocytes. The first is an indirect axis whereby mediators (e.g., ATP) released from dying cancer cells act as paracrine agonists for myeloid leukocyte receptors (e.g., P2X7R) and signaling cascades that regulate assembly of caspase-1 inflammasome complexes. The second mechanism involves direct stimulatory effects of certain chemotherapeutic agents on apoptotic or necrotic signaling cascades in myeloid leukocytes that converge on caspase-1 inflammasome signaling. A newly reported third pathway (in red) indicates some chemotherapeutic drugs can synergize with TLR4 signaling to stimulate assembly of caspase-8 ripoptosome complexes, which act as alternative, non-canonical platforms for the proteolytic maturation of IL-1β.
Figure 1.1
chemotherapeutic drugs (e.g., oxaliplatin, doxorubicin, mitoxantrone) (13). A second pathway involves direct stimulatory effects of certain chemotherapeutic agents on the inflammasome-mediated IL-1β processing machinery in macrophages/DC. This is consistent with the ability of NLRP3 to act as a sensor of an extraordinarily diverse range of cell stress stimuli that involve perturbed homeostasis of the plasma membrane, lysosome, or mitochondrial compartments (14). This direct pathway of IL-1β production via an autonomous myeloid cell axis has been observed in response to a subset of chemotherapeutic or pro-apoptotic drugs that can trigger mitochondrial dysfunction (e.g., doxorubicin, staurosporine) or lysosomal dysfunction (gemcitabine, 5-fluorouracil) in different myeloid leukocytes (15-17).

II. IL-1β Processing Platforms

Inflammasomes

1) Priming Signals

As previously mentioned, proIL-1β requires NF-κB-dependent upregulation. Experimentally, the lipopeptide, Pam3CysK4, or more commonly, the cell wall component of gram-negative bacteria, LPS, engages TLR2 or TLR4 receptors, respectively, and utilizes MyD88 and/or TRIF innate immune signaling pathways to induce NF-κB-dependent gene transcription (Fig. 1.2). A central dogma of inflammasome signaling is the requirement for a priming step (signal 1) before the activation stimulus (signal 2) to induce inflammasome assembly. While this priming step is necessary for upregulation of both proIL-1β and NLRP3, Lin et al. show that caspase-1 activation, which occurs upstream of IL-1β processing, occurs quite rapidly with
Figure 1.2  TLR4 induces MyD88 and TRIF signaling pathways

LPS-induced TLR4 receptor signaling is analogous to combined downstream TLR2 and TLR3 receptor signaling pathways following ligation with the lipopeptide, Pam3CSK4, and Poly I:C, a synthetic dsRNA, respectively. The TIR domain associates with TIRAP for MyD88 induction of NF-κB-dependent pro-inflammatory cytokines. TIR also associates with TRAM for TRIF induction of NF-κB and IRF3/7 transcription.
Figure 1.2
*simultaneous* priming (TLR) and activation (ATP) stimuli even in the presence of the translational inhibitor, cycloheximide (18). They also demonstrate the differential requirement for IRAK1/IRAK4 and MyD88/TRIF in simultaneously-primed and activated versus sequentially primed and activated BMDM. Fernandes-Alnemri *et al.* corroborated that the early-phase transcription-independent activation of the NLRP3 inflammasome is dependent on IRAK1 and MyD88 and partially IRAK1-dependent when solely activating the TRIF pathway (19). Physiologically this temporal concept is important to consider because the encounter of these stimuli *per se* most likely occurs simultaneously. Teleologically, this early-phase caspase-1 activation with simultaneous signal 1 and signal 2 stimuli is thought to have developed in order to quickly contain the spread of intracellular microorganisms via caspase-1-dependent pyroptotic mechanisms, while the delay in proIL-1β protein synthesis and IL-1β maturation aids in enforcing a potent inflammatory response. Notably, this begs the critical question as to which physiological ligands constitute the endogenous inflammasome ‘priming’ signals?

Several plausible host-derived or microbial priming candidates exist, including the TLR4 ligand and nuclear-derived protein, HMGB1, the release of heat shock proteins, hyaluronan, β-defensins, autocrine IL-1β, and the escape of gut-derived organisms (20-22). Recently, the anaphylotoxin, C5a, was proposed as an endogenous priming signal. An *et al.* utilized human monocytes to demonstrate that upon C5aR engagement, proIL-1β levels are up-regulated, and C5aR activation is required for uric acid crystal-induced IL-1β production (23). Overall, others have reported endogenous host-derived ligands for TLR2 and TLR4; interestingly, many of the ligands have been described as “PAMP-sensitizing molecules” or “PAMP-binding molecules” rather than bona fide TLR ligands.
2) Stimulation, Formation, and Assembly of Inflammasomes

Activation of TLRs or receptors for proinflammatory cytokines, including IL-1β, induces the NF-κB-dependent expression of proIL-1β (31-33 kD) as a cytosolic, biologically inactive precursor protein and induces the expression of the inflammasome scaffolding protein, NLRP3. The canonical cleavage and processing of proIL-1β into the mature IL-1β cytokine (17 kD) is catalyzed by caspase-1, via a pathway regulated by multi-protein inflammasome signaling complexes. Prior to its subclassification within the caspase family of cysteine proteases, caspase-1 was originally defined as the ‘interleukin-converting enzyme.’ Caspase-1 is constitutively expressed as a 45 kD inactive precursor in macrophages, DCs, and other cell types. Following appropriate signal 1 and signal 2 stimulation, inflammasome platforms assemble resulting in the autocatalytic activation of procaspase-1 dimers to form a p20/p10 heterotetramer. Caspase-1 activation in inflammasome-competent cells also induces a unique form of caspase-1/11-dependent cell death termed pyroptosis, which rapidly eliminates the replicative niche of intracellular microbes.

The most intensively studied inflammasome, the NLRP3 inflammasome, comprises an oligomeric complex, which consists of NLRP3, the ‘scaffolding/seeding protein’, ASC, the ‘adapter protein’, and the canonical IL-1β convertase, procaspase-1, that rapidly assemble in response to diverse stress stimuli such as increased ROS (24), mitochondrial dysfunction (16), perturbation of intracellular ion homeostasis (particularly K⁺ efflux) (25-27), disruption of lysosomal membrane integrity (28), and activation of deubiquitinases (29-31). The NLRP3 protein contains a C-terminal LRR domain, nucleotide-binding NACHT domain, and an N-terminal PYR domain. Through
homotypic protein-protein interactions, NLRP3 recruits the adapter protein, ASC, via its PYD domain, and finally ASC recruits caspase-1 via its CARD domain (Fig 1.3). More specifically, inflammasomes assemble in response to PAMPs and sterile DAMPs, including soluble and particulate material, such as extracellular ATP, double-stranded DNA/RNA, anthrax lethal toxin, asbestos, silica particles, MSU crystals, prions, bacteria, fungi, heme (32) and other stimuli. These stimuli induce the assembly of many types of inflammasome complexes that differ in their core scaffolding protein (NLRP3, NLRP1, NLRC4, AIM2, pyrin (33), while they all lead to the activation of ‘ICE’s’ that proteolytically process not only proIL-1β but also proIL-18 into their mature, biologically active forms.

Inflammasomes are highly regulated macromolecular platforms and biophysical data demonstrate that ASC inflammasomes form long, ASC prion-like filaments that precipitate into detergent-insoluble lysate compartments following centrifugation and will be assayed as a means of inflammasome assembly or ‘ASC oligomerization’ in subsequent chapters. Lu et al. propose a mechanism whereby NLRP3 and AIM2 act to nucleate PYD filaments of ASC. Next, ASC filaments nucleate CARD filaments of caspase-1, leading to proximity-induced autocatalytic caspase-1 activation, the process of which is termed nucleation-induced polymerization (34). On the other hand, Cai et al. propose a ‘prion-like polymerization mechanism’ whereby PYD and CARD act as prion proteins because functionally, the yeast prion domain replaces PYD and CARD and induces intact cell signaling (35). Similar to Lu et al., Cai and colleagues conclude that recombinant PYD fibers induce ASC polymerization and caspase-1 activation. To further the notion of ‘seeding’ and nucleation-induced polymerization, ASC specks have
Figure 1.3  Protein-protein interactions between NLRP3, ASC, caspase-1, and caspase-8 within inflammasome complexes

NLRP3 associates with ASC via PYD-PYD domain interactions; ASC interacts with caspase-1 via CARD-CARD interactions; the PYD domain of ASC also can interact with the DED of caspase-8. Following activation of the NLRP3 inflammasome, long ASC-ASC filaments assemble, resulting in the formation of prion-like structures.
Figure 1.3
recently been reported to be released extracellularly and mediate intercellular communication due to lysosome-mediated macrophage ingestion of ASC specks. Franklin et al. detect significant amounts of externalized ASC in immortalized murine macrophages in addition to ASC in the serum of patients with FCAS. This also correlated with increased extracellular IL-1β, substantiating that the release of ASC specks serve as danger signals to sustain the inflammatory response (36). Similar to ASC externalization, Baroja-Mazo et al. report that NLRP3 is secreted with ASC specks in response to LPS and nigericin/ATP/MSU/E. Coli/poly dA; dT/ or anthrax lethal toxin stimulation in BMDM, presumably via passive release during sustained stimulation (37). In contrast, they report that ASC is released rapidly (within 20 min) via pyroptotic mechanisms. The dynamic, diverse, and wide-spread utility of inflammasome complex formation to elicit IL-1 family cytokine secretion (IL-1β and IL-18) reflects a critical innate and adaptive immune response to protect the host from potentially harmful signals.

Activation of caspase-1 (via inflammasome activation) and/or caspase-11 leads to pyroptotic cell death, resulting in osmotic swelling and lysis of the cell. A non-canonical caspase-11 inflammasome pathway exists, which is independent of NLRP3, ASC, and caspase-1. Whereas both caspase-1 and caspase-11 activate pyroptosis, only caspase-1 activation leads to processing of IL-1β and IL-18, but caspase-11 induces the release of IL-1α and HMGB1. Mouse caspase-11 is the murine ortholog of human caspase-4, while mice do not express caspase-5. Unlike procaspase-1, procaspase-11 expression must be induced via TLR/NF-kB/interferon-induced mechanisms. TLR4→TRIF-stimulation induces the expression of IRF3/7-regulated genes, creating an autocrine feedback mechanism via type 1 interferon signaling. Consequently, when the canonical
NLRP3/ASC/caspase-1 inflammasome is first primed with LPS, procaspase-11 expression is induced with this primary signal, leading to coincident upregulation of proIL-1β. The caspase-11 inflammasome is activated by cytosolic delivery of LPS with Gram-negative bacteria such as *Citrobacter rodentium*, *Escherichia coli*, *Vibrio cholerae*, and *Salmonella typhimurium* (38). Importantly, it is the lipid A component of LPS that binds and activates murine caspase-11 and human caspase-4 (39). Alternately, transfection of LPS into the cell is able to activate the caspase-11 pathway. The precise mechanism of lytic cell death is currently an active area of research. The quest for identifying the pyroptotic channel(s) that result in perturbed ion homeostasis and consequent lysis is under current investigation by Hana Russo in our lab.

3) **Ion Flux in Inflammasome Activation**

Besides the aforementioned mechanisms of lysosome destabilization and ROS-induced triggers for NLRP3 inflammasome activation, the efflux of K⁺ ions remains a long-standing, predominant mechanism for NLRP3 inflammasome assembly. Kahlenberg and Dubyak characterized that ATP-induced P2X7R activation and K⁺ efflux activated the inflammasome in LPS-primed murine macrophages; P2X7R is a non-selective cation channel, and ATP ligation leads to the efflux of K⁺ and simultaneous influx of Ca²⁺ and Na⁺ in murine macrophages (40). Sustained stimulation of P2X7R induces the formation of a large nonselective pore that is permeable to large organic and inorganic molecules (≤ 800 Da). At the onset of my thesis, it was hypothesized that the hemi-channel, pannexin-1 (Panx1), associated with the P2X7R to form a large ion-conducting pore following inflammasome activation based on observations by others that
dye influx was suppressed in the presence of Panx1 inhibitors following P2X7R activation (41, 42). However, my preliminary experiments in addition to publications by others (43) confirmed that pannexin-1 is not necessary for inflammasome activation. Currently, the contribution of ion flux signals during NLRP3 activation is under investigation in our lab by Michael Katsnelson, including determining the effects of Ca$^{2+}$ and Na$^+$ gradient perturbations upon inflammasome activation. While several groups report that perturbations in Ca$^{2+}$ and Na$^+$ concentrations are involved in the activation of the NLRP3 inflammasome (27, 44), it is clear that it is highly stimulus-dependent, indicating that the flux of select ions may be more important with certain inflammasome stimuli than with others. Verhoef et al. also reported the inhibitory effects of extracellular chloride on the ability to activate IL-1β via P2X7R stimulation (45).

Globally, because of their ability to induce K$^+$ efflux, short-term stimulation (30 min) of the P2X7R with extracellular ATP or stimulation with the Streptomyces hygroscopicus-derived potassium ionophore, nigericin, a H$^+/K^+$ antiporter, are robust NLRP3 inflammasome activation stimuli and will be utilized throughout this thesis as central experimental stimuli.

**Caspase-1-independent IL-1β Processing Pathways**

1) *Alternative Proteases*

In addition to processing of IL-1β by inflammasome-dependent caspase-1 activation, numerous other enzymes have been reported to cleave proIL-1β into mature IL-1β. Serine proteases, matrix metalloproteases, as well as cathepsins can mediate IL-1β processing. For example, neutrophils utilize elastase to cleave IL-1β as shown by the
observation that IL-1β release from *Pseudomonas aeruginosa*-infected neutrophils was suppressed in elastase knockout mice (46). In addition, various cathepsin isoforms have also been implicated in IL-1β maturation. For example, peripheral blood mononuclear cells infected with *Listeria monocytogenes* induced phagolysosomal rupture and release of cathepsin B to mediate IL-1β processing (47). Cathepsin B was also implicated in IL-1β processing in response to the acute phase protein, serum amyloid A, in murine and human macrophages (48). Kono *et al.* report that cathepsin C is important for the sterile inflammatory response to silica crystals. Consequently, cathepsin C-deficient mice display dampened inflammatory responses because cathepsin C is important for activating leukocyte-derived serine proteases that process IL-1β (49). Lastly, microglial cells process IL-1β via cathepsin D under acidic environmental conditions or lactic acidosis and results in the release of 20 kD rather than 17 kD IL-1β (50).

2) **Caspase-8 as an “Interleukin-Converting Enzyme” and its Non-apoptotic Roles**

Additional IL-1β convertases include the classically-defined initiator caspase, caspase-8, which is best characterized for its multiple roles in the regulation of cell death via apoptosis or necroptosis (51). Canonically, caspase-1 has been the predominant ICE in the activation of the NLRP3 inflammasome signaling complex. However, recent studies indicate that ASC inflammasomes recruit caspase-8 to the signaling platform and that caspase-8 can act as an ‘ICE’ for processing and generating mature 17kD IL-1β in response to a diverse range of PAMPs and DAMPs (52-56). Maelfait *et al.* first observed that activation of the TRIF (TIR domain-containing adapter-inducing interferon-β)
signaling pathway by TLR3 or TLR4 induced a caspase-8 signaling pathway that, when combined with cyclohexamide-mediated inhibition of protein translation, was sufficient to drive efficient IL-1β processing even in caspase-1 knockout macrophages (52). A subsequent study found that treatment of LPS-primed macrophages or DCs with Smac (Second mitochondrial-derived activator of caspases)-mimetic drugs triggered a robust maturation of IL-1β that was mediated in part by caspase-8 (53). Also, the engagement of Fas (CD95) signaling in WT or caspase-1 knockout macrophages was shown to induce IL-1β and IL-18 processing via caspase-8 (54). A Fas-induced caspase-8 cascade was also identified as a major pathway for IL-1β and IL-18 production in Listeria-infected peritoneal exudate cells isolated from B6 mice (55). Recently, it was shown that the assembly of RIP1/RIP3 kinases and caspase-8-containing complexes triggers the activation of caspase-1 and IL-1β processing induced by Yersinia via the Yersinia Outer protein J (YopJ) protein in TLR4-stimulated BMDM and importantly, caspase-8 activation was shown to activate caspase-1 processing (57, 58). In the enterica serovar Typhimurium infection model, an NLRC4/ASC/caspase-8/caspase-1 pathway is engaged in BMDM, whereby active caspase-1 and caspase-8 are both recruited to the complex as observed by fluorescence microscopy (59, 60). Additionally, Candida albicans and mycobacterial species trigger Syk kinase/ASC/caspase-8 activation for processing of IL-1β in BMDM via dectin-1, an extracellular sensor that detects the presence of carbohydrate ligands (61, 62). Gurung et al. showed that genetic deletion of FADD or caspase-8 severely hampered the ability of LPS-primed BMDM to produce mature IL-1β in response to extracellular ATP or nigericin (63). Another study provided evidence that the absence of the caspase-8 paralog, c-FLIP, significantly impaired IL-1β maturation.
and release (64). Caspase-8 along with its interacting partners, FADD and c-FLIP, possess pleiotropic roles in immune cell, and more specifically, inflammasome signaling. With respect to cell death signaling, Sagulenko et al. observed the induction of an apoptotic versus pyroptotic cell death in Casp1/11−/− BMDM versus WT BMDM, respectively, following engagement of either NLRP3 or AIM2 inflammasome activation (65). This caspase-1-independent form of cell death coincided with caspase-8-dependent processing and release of IL-1β, the mechanism of which will be discussed in Chapter 4. Overall, these recent studies raise the important question as to the discrimination between caspase-1 and caspase-8-based signaling platforms.

Besides acting as an IL-1β convertase, caspase-8 has been shown to additionally regulate the priming and assembly steps of NLRP3 inflammasome signaling (63, 66). Gurung et al. showed that in a RIPK3-deficient background, caspase-8 deficiency results in the suppression of both priming and activation of caspase-1 and caspase-11-based NLRP3 inflammasomes in BMDM (63). Additionally, DC-specific expression of caspase-8 prevents systemic autoimmunity as DC-specific caspase-8 knockout mice present with splenomegaly, lymphadenopathy, proteinuria, and detectable levels of dsDNA and ssDNA-reactive IgG auto-antibodies (67). Although the cell types of focus in this thesis are primarily DCs and macrophages, the non-apoptotic role of caspase-8 have also beeen assessed in T and B lymphocytes. LPS treatment of purified B cells from conditional caspase-8 B cell knockout mice (bcas8−/−) indicate that caspase-8 is required for B-cell activation and proliferation (68). Also in this study, caspase-8 was shown to associate with IKKαβ to induce NF-κB activation and upregulation of key transcriptional genes. In bcas8−/− mice, cytokine genes were downregulated, showing an
important role for caspase-8 in transcriptional priming of inflammatory genes in TLR4-stimulated B cells. Similarly, in a report by Kataoka et al., caspase-8 was shown to cleave FLIP(L); FLIP(L) p43 was able to bind TRAF2 and induce NF-κB activation via p65 phosphorylation and translocation of NF-κB into the nucleus following TLR4 stimulation in HEK293 cells (69). The duration of stimulation was discussed in the aforementioned B cell study as a factor in procaspase-8’s ability to modulate NF-κB signaling and tracks with my finding that the duration of the NF-κB stimulus dictates the contribution of a caspase-8-mediated NF-κB-dependent loss in target gene expression (e.g., proIL-1β) (Fig. 3.10). Similarly, Allam et al. observed a defect in proIL-1β expression following LPS stimulation of Casp8−/−Rip3−/− BMDM and reduced circulating serum IL-1β in response to LPS injection in Casp8−/−Rip3−/− mice compared to WT mice (66). Nevertheless, defining the non-apoptotic roles of pro-caspase-8, particularly as they pertain to inflammasome signaling, remain elusive.

3) Ripoptosomes and Cell Death

Necroptosis, a regulated form of necrosis, is a specialized form of cell death in cells expressing RIP1 and/or RIP3. Necroptosis, like pyroptosis, is characterized by oncosis as a consequence of cellular ion perturbation (70). Experimentally, necroptosis is induced in macrophages and DCs following simultaneous TLR4 or death receptor family stimulation with combined caspase-8 inhibition using the pan-caspase inhibitor, zVAD, or the caspase-8 selective inhibitor, IETD. While pyroptosis is activated selectively in caspase-1-expressing cells, necroptosis is engaged in diverse cell types. Following necroptotic stimulation, a RIP1/RIP3-containing complex or ‘rioptosome’ assembles
cytosolically, and its formation results in the activation of mitochondrial proteins, the signaling of which is under intense exploration (71). The necroptotic signaling pathway was first characterized in tumor cells stimulated with death-receptor family ligands, such as TNF, FasL, TRAIL and combined caspase-8 inhibition. Canonically, the membrane-bound TNFR complex I trimerizes upon TNF stimulation. Secondarily, a cytosolic TNFR complex II forms and recruits RIP1 and RIP3. The enzymatic activity of caspase-8 determines the mode of cell death: 1) active caspase-8 will cleave and inactivate RIPK1 to induce caspase-8-dependent apoptosis or 2) inactive caspase-8 will allow RIPK1 to remain catalytically active to phosphorylate RIPK3. Active RIPK3 phosphorylates mitochondrial MLKL. The pseudokinase, MLKL then activates PGAM5 and in turn, PGAM5 activates DRP-1 to induce mitochondrial fission.

Another layer of ripoptosome regulation includes the recruitment of cFLIP isoforms, cFLIP_L (long) and cFLIP_S (short), which dimerize with caspase-8 and alter the catalytic state of caspase-8, thus dictating the cell’s ability to necroptose. Additionally, cIAPs are E3 ubiquitin ligases and regulate the expression of proteins within ripoptosomes (e.g. RIPs). When mitochondrial-derived Smac/Diablo, an endogenous IAP ligand, is released during intrinsic apoptosis, it binds cIAPs and prevents cIAPs from inhibiting executioner cell death mechanisms. cIAPs contain baculoviral IAP repeats motifs that are conserved throughout the IAP family of proteins and are encoded by the BIRC3 gene in humans. Many isoforms of cIAPs exist including cIAP1, cIAP2, and XIAP. XIAP is the best characterized of the three IAP isoforms and inhibits executioner caspase-3/7/9 activity. Lack of XIAP expression clinically manifests into X-linked lymphoproliferative syndrome type 2 (XLP-2). cIAP1 has been shown to target RIP1 for
K63-mediated degradation by the proteasome, while cIAP2 possesses a less defined role.

In addition to necroptosis, another programmed form of cell death includes apoptosis. Apoptosis, an immunologically silent mode of cell death, can be activated via extrinsic or intrinsic mechanisms and is centrally regulated by the activation of caspases, a family of cysteine proteases that are expressed as zymogens with minimal protease activity. Activation of caspase-1 and caspase-8 have been previously discussed in this chapter in the context of inflammatory signaling for cytokine processing. However, caspases are traditionally known for their roles in the induction of apoptosis and are categorized into initiator and executioner (or effector) caspases. Procaspases-8 and -9 are initiator caspases, and their activation serves to cleave and activate executioner caspases, procaspases-3 and -7. Extrinsic apoptosis involves signaling via death receptors (e.g. FasR, TRAIL-R, TNFR, etc). Activated and trimerized death receptors induce the formation of a death-inducing signaling complex (DISC) and lead to the activation of caspase-8, allowing caspase-8 to act as an initiator caspase to activate caspases-3 and -7. Caspases-3/7 then cleave cellular proteins, including PARP, to repair damaged DNA. In contrast, intrinsic apoptosis requires a cell stressor (e.g. infection, radiation, genotoxic stress, increased intracellular Ca\(^{2+}\), etc), which activates the release of cytochrome c from the mitochondria. Cytochrome c release is tightly regulated by Bcl-2 family proteins. Once the BH3-only family proteins are activated, this allows for the oligomerization of the BH4 proteins, Bak and Bax, in the mitochondrial outer membrane (72). Bak/Bax forms a pore and facilitates mitochondrial outer membrane permeabilization and consequently, the release of cytochrome c into the cytosol. Cytochrome c then binds to the apoptosome, consisting of caspase-9 and the adapter molecule, APAF-1, and caspase-
9 activation results in caspase-3/7 activity. Morphologically, apoptotic cells undergo shrinkage, karyorrhexis, karyolysis, and blebbing and are distinguishable from necroptotic or pyroptotic cells that osmotically lyse and promote inflammatory responses.

Goals of the thesis research

The goal of this thesis was to further explore IL-1β processing pathways with proapoptotic chemotherapeutic drugs and with conventional NLRP3 inflammasome stimuli in myeloid DCs and macrophages. Given the initial premise that chemotherapy drug-treated tumor cells release DAMPs that activate the NLRP3 inflammasome in DCs, I sought to recapitulate this scenario. However, I observed a direct effect of these proapoptotic agents on IL-1β processing (Chapter 3). By using both pharmacological agents and knockout mouse models, I mechanistically defined a TRIF-dependent ripoptosome/caspase-8 mediated IL-1β processing pathway engaged in doxorubicin and staurosporine-treated DCs that was independent of caspase-1 and correlated with decreased expression of the anti-apoptotic protein, cIAP1. Because formation of this ripoptosome complex required many hours of stimulation (~12 h), I utilized a conventional NLRP3 activator, nigericin, to determine whether this ripoptosome/caspase-8-mediated IL-1β processing pathway was engaged with sustained stimulation (> 2 h). My data reveal that while an NLRP3/ASC/caspase-1 inflammasome is engaged in WT DCs with acute (and sustained) stimulation, sustained nigericin stimulation induced the assembly of an NLRP3/ASC/caspase-8 inflammasome complex in Casp1/11−/− DCs for processing of IL-1β. By examining the role of caspase-8 in this signaling complex, I also validated a caspase-8-induced caspase-1 activation mechanism (Chapter 4). Overall, these studies seek to understand the interplay between caspase-1 versus caspase-8-based signaling platforms.
CHAPTER 2

General Methods

Reagents

Key reagents and their sources were: *Escherichia coli* LPS serotype O1101:B4 (List Biological Laboratories), Pam3CSK4 (Invivogen), Ac-YVAD-cmk (Bachem), z-IETD-fmk (R&D), zVAD-fmk (Tocris, Abcam, or APExBio), recombinant murine GM-CSF (Peprotech), recombinant murine TNFα (Peprotech), nigericin sodium salt (Sigma-Aldrich and Tocris), doxorubicin (LC Laboratories or Sigma-Aldrich), staurosporine (LC Laboratories), UCN-01 (Enzo), necrostatin-1 (Tocris), Imject Alum (Thermo Scientific), and suberic acid bis (N-hydroxysuccinimide ester) (Sigma-Aldrich). Oxaliplatin, cisplatin, TRIzol reagent, nigericin, and ATP were purchased from Sigma-Aldrich. Anti-caspase-1 (p10) rabbit polyclonal (sc-514), anti-actin goat polyclonal (sc-1615), anti-FADD goat polyclonal (sc-6036), anti-ASC rabbit polyclonal (sc-22514-R), and all HRP-conjugated secondary antibodies (Abs) were from Santa Cruz Biotechnology. Anti-caspase-1 (p20) mouse monoclonal (AG-20B-0042) was from AdipoGen. The monoclonal 3ZD anti-IL-1β Ab, which recognizes both 31 kDa/29 kDa proIL-1β and 17 kDa mature IL-1β in western blot analysis, was provided by the Biological Resources Branch, National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). Other antibodies included anti-cIAP1 mouse monoclonal (1E1-1-10) from Enzo, anti-RIP mouse monoclonal (38-RIP) from BD Biosciences, and anti-NLRP3 mouse monoclonal (Cryo-2) from AdipoGen. Anti-caspase-7 (9492), anti-caspase-8 (4927), and anti-PARP (9542) rabbit polyclonal Abs were from Cell Signaling. Other
antibodies included anti-caspase-8 mouse monoclonal (ALX-804-448) from Enzo and anti-NLRP3 mouse monoclonal (MAB7578) from R&D. Murine IL-1β DuoSet ELISA kit was from R&D Systems, and the murine TNFα ELISA kit was from BioLegend. Cell Titer-Glo Luminescent Viability Assay Kit was from Promega. Alamar blue Reagent was from Invitrogen. EnzChek Caspase-3 Assay Kit and DEVD-cho were from Invitrogen. RT² SYBR Green/ROX qPCR Master Mix (PA-012) and predesigned qPCR primers for murine IL-1β (PPM03109E), murine TNFα (PPM03113F), and murine GAPDH (PPM02946E) were from SA Biosciences. Transcriptor first strand cDNA synthesis kit was from Roche.

**Murine models**

Wild-type C57BL/6 mice were purchased from Taconic and Jackson Laboratories. Mice lacking both caspase-1 and caspase-11 on the C57BL/6 background (Casp1⁻/⁻ Casp11⁻/⁻) have been previously described (73-75). Asc⁻/⁻, Trif⁻/⁻, and Nlrp3⁻/⁻ Nlrc4⁻/⁻ double-knockout mouse strains (C57BL/6 background) were obtained from Eric Pearlman (Case Western Reserve University), while the Nlrp3⁻/⁻ Nlrc4⁻/⁻ double-knockout mouse strains originated from Shizuo Akira (Osaka University in Osaka, Japan). Femoral and tibial bones for BMDC cultures were also isolated from Casp8⁻/⁻ Rip3⁻/⁻, Casp8⁺/⁺ Rip3⁺/⁺ (also referred to as Rip3⁻/⁻ in Chapter 4) and control Casp8⁺/⁺ Rip3⁺/⁺ mice which have been described previously (76). All experiments and procedures involving mice were approved by the Institutional Animal Care and Use Committees of Case Western Reserve University.
Isolation, culture, and experimental testing of bone-marrow derived dendritic cells (BMDC) and macrophages (BMDM)

Bone marrow from 9-12 week old mice was isolated by minor modification of previously described protocols (77). Mice were euthanized by CO₂ inhalation. Femura and tibiae were removed, briefly sterilized in 70% ethanol, and PBS was used to wash out the marrow cavity plugs. The bone marrow cells were resuspended in DMEM (Sigma Aldrich), supplemented with 10% bovine calf serum (HyClone Laboratories), 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Lonza), 15 ng/ml GM-CSF, plated onto 150-mm dishes, and cultured in the presence of 10% CO₂. On day 3 post-isolation, 80% of the non-adherent population was removed and centrifuged at 300 g for 5 min at room temperature, and fresh medium was applied. Five days post-isolation, the resulting loosely adherent BMDC were collected, resuspended to a cell density of ~1 × 10⁶/ml in the above differentiation medium, and plated into 6-well (2 ml/well), 12-well (1 ml/well), 24-well (0.5 ml/well), or 96-well (0.1 ml/well) plates as needed for particular experiments, and used between days 7 and 10 post-isolation. For some experiments, BMDM were generated from the isolated bone marrow. BMDM were cultured in DMEM supplemented with 25% sterile filtered conditioned medium from L929 fibroblasts (a source of M-CSF) or 20 ng/ml recombinant MCSF. BMDM media additionally contained 10% bovine calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. BMDM were plated onto 150-mm dishes and cultured in the presence of 10% CO₂. BMDM media was replaced with fresh media on days 3 and 6 post-isolation, plated on new dishes on day 7 (using a 4 mg/ml lidocaine and
10 mM EDTA solution in PBS, pH7.4 for a 10 minute incubation to lift the adherent macrophages from the plate), and utilized within next the 3-4 days.

For experimental tests, plated BMDC were centrifuged at 300 x g for 5-10 min, and the differentiation medium was removed and replaced with low-serum DMEM (0.1% bovine calf serum plus penicillin, streptomycin, and L-glutamine). The cells were equilibrated for 15 min at 37°C in 10% CO₂ prior to addition of test reagents. BMDC were routinely primed with 0.1-1 µg/ml LPS for 4 h to activate TLR4 signaling prior to treatment with indicated concentrations of Dox, STS (or other chemotherapeutic drugs) (2-18 h), 10 µM nigericin (0.5-6 h), or 240-480 µg/ml alum (6 h). The LPS was present throughout the secondary stimulus. In some experiments, cells were primed with 0.2-2 µg/ml Pam₃CSK₄ to induce TLR2 rather than TLR4 signaling cascades. Stimulation of NLRP3/caspase-1 inflammasome signaling by either P2X7 receptor activation or nigericin treatment was routinely used as a positive control by supplementing the medium of LPS-primed BMDC with either 5 mM ATP or 10 µM nigericin for the final 30 min of test incubations. Where indicated, the BMDC cultures were treated with various pharmacological inhibitors (1-100 µM Ac-YVAD-cmk, 50-100 µM z-VAD-fmk, 1-100 µM z-IETD-fmk, 100 µM DEVD-cho, 50 µM necrostatin-1) either before or after the LPS priming steps.

**ELISA measurements for IL-1β or TNFα release**

BMDC were seeded in 24-well plates. Extracellular media samples were removed and centrifuged at 10,000 g for 15 s to pellet floating BMDC. The supernatants
were then assayed for murine IL-1β or murine TNFα by standard ELISA (R&D Systems for IL-1β or BioLegend for TNFα) according to the manufacturer’s protocol. All test conditions for ELISA-based experiments were performed in duplicate.

**Preparation of detergent-soluble and detergent-insoluble cell lysate fractions, processing of extracellular media, and western blot protocols**

In Chapter 3, BMDC (4-5 x 10^6 in 60 mm culture dishes) were stimulated for 2 h with or without LPS (1 µg/ml) or TNFα (50 ng/ml) in the absence or presence of 50 µM zVAD in DMEM containing 10% bovine calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2mM L-glutamine. Other dishes were primed with LPS (1 µg/ml) for 4 h and then stimulated for an additional 3-8 h with 10 µM Dox in the absence or presence of 50 µM zVAD–volumes for the Chapter 3 approach will be indicated in parentheses following the description of the Chapter 4 approach for this method. In Chapter 4, BMDC were seeded in 6-well plates and treated as indicated in the text of the figure legends prior to processing of the extracellular medium and separation of the detergent-soluble lysate supernatant fraction from the detergent-insoluble lysate pellet.

Incubations were terminated by removal of supernatant medium for sedimentation and isolation of detached cells. Detached cells were centrifuged at 400 x g for 5 min and washed with 1 ml of ice-cold PBS. Whole cell detergent lysates were prepared by addition of 56 µl (85 µl) of RIPA lysis buffer (0.5% Na deoxycholate, 0.1% SDS, 1% Igepal CA630 in PBS, pH 7.4 plus protease inhibitor cocktail) to the adherent cells on the dish and incubated on ice for 5 min. Lysed adherent cells were scraped with a rubber
policeman, pooled with detached cells, and extracted for an additional 10 min on ice. The whole cell lysates were separated into detergent-soluble and detergent-insoluble fractions by centrifugation at 15,000 x g for 15 min at 4°C. SDS sample buffer (18-20 µl) was added to the detergent-soluble fractions, while 56 µl of RIPA lysis buffer (supplemented with 5mM MgCl₂) was added to the detergent-insoluble lysate pellet. The approach used in Chapter 3 results consisted of subjecting the insoluble lysate pellet to 5 x freeze-thaw cycles (dry ice/ethanol and 37°C heating blocks) and vortexing after each freeze-thaw cycle, but this approach was not used in Chapter 4 results because the freeze-thaw step was not required to obtain the detergent-insoluble lysate pellet. Instead, the insoluble lysate pellet was subjected to intense vortexing. Then, each sample was DNase-treated (2 units/sample) and incubated on ice for 10 min prior to addition of SDS sample buffer (12 µl) and extraction at 100°C for 5 min (Chapters 3 and 4).

Extracellular medium samples were concentrated by trichloroacetic acid precipitation/acetone washing, and, detergent-soluble cell lysates were prepared by detergent-based extractions as described previously (73) prior to standard processing by SDS-PAGE, transfer to PVDF membranes, and western blot analysis. Primary antibodies (Abs) for western blotting were used at the following concentrations: 5 µg/ml for IL-1β, 1 µg/ml for caspase-1 (Santa Cruz), 1 µg/ml for actin, 0.1 µg/ml for caspase-7, 1.2 µg/ml for caspase-8 (Cell Signaling), 0.05 µg/ml for PARP, 2 µg/ml for cIAP, 1 µg/ml for NLRP3 (AdipoGen), 0.4 µg/ml for RIP, 1 µg/ml for FADD, 1 µg/ml for caspase-1 (AdipoGen), 1 µg/ml for caspase-8 (Enzo), 2 µg/ml for NLRP3 (R&D), and 0.4 µg/ml for ASC. Where indicated, western blots were probed with anti-caspase-1 and anti-IL-1β
Abs simultaneously. HRP-conjugated secondary Abs were used at a final concentration of 0.13 μg/ml. Chemiluminescent images of the developed blots were detected, stored, and quantified using a FluorChemE processor (Cell Biosciences).

ASC oligomerization assay using the cross-linking reagent, disuccinimidyl suberate (DSS)

BMDC were seeded in 6-well plates (2x10^6 cells/well). After stimulation, culture supernatants were collected and separated from the pelleted cells. Cell lysates were prepared using RIPA lysis buffer and centrifuged at 6,000 rpm at 4°C for 10 min. The sample pellets were washed 1-2 x with PBS and then resuspended to a final concentration of 2 mM DSS in PBS followed by brief vortexing and incubation for 30 min at room temperature. The cross-linked pellets were centrifuged at 6,000 rpm for 10 min followed by a brief 5s high-speed centrifugation to pellet the cross-linked material. The DSS solution was decanted, and the cross-linked pellets were resuspended in 30µl of SDS sample buffer followed by extraction at 100°C for 5 min. A 12% SDS-polyacrylamide gel was used to run the samples, and western blots were performed and probed for ASC to determine ASC oligomer formation for monomeric (24 kD), dimeric (48 kD), and oligomeric (>48 kD) ASC.
**Cell viability assays**

For both cell viability assays, BMDC were seeded in 96-well plates (1x10^5 cells/well).

**Intracellular ATP measurement as an index of cell viability**

Viability of BMDC was measured by quantifying intracellular ATP content using the Cell Titer-Glo Luminescent Viability Assay (Promega). Luminescence was quantified in relative light units (RLU) using a BioTek Synergy HT plate-reader and normalized to the values measured in control, untreated BMDC.

**Alamar blue redox metabolism**

Viability of BMDC was assayed using the metabolic redox sensor dye, alamar blue reagent, to monitor the time-dependent conversion of resazurin to resorufin at excitation wavelength of 540nm and emission wavelength of 620nm. Experiments were timed such that all stimulations ended simultaneously, and alamar blue (10 µl/well) was added and incubated with the cells for 1 hr at 37°C. Fluorescence was quantified in relative light units (RLU) using a BioTek Synergy HT plate-reader and normalized to the values measured in control, untreated BMDC.

**Caspase-3/7 activity assays**

BMDC seeded in 12-well plates were primed for 4 h with LPS and then incubated with various chemotherapeutic agents plus or minus 100 µM DEVD-cho for an additional 2-18 h prior to preparation of cell lysates for quantification of accumulated caspase-3/7 activity by EnzChek Fluorescence Caspase-3 assay kits (Invitrogen).
**qPCR analyses**

\(\text{Casp}^8^{+/+} \text{ Rip}^3^{+/+}, \text{Casp}^8^{-/-} \text{ Rip}^3^{-/-}, \text{ and Casp}^8^{+/+} \text{ Rip}^3^{-/-}\) BMDC were treated with 1 \(\mu\)g/ml LPS for 1 or 4 h prior to extraction of total RNA by TRIZol reagent. Transcriptor First Strand cDNA Synthesis kit was used to generate first-strand cDNA from the purified RNA. IL-1\(\beta\), TNF\(\alpha\), and GAPDH transcripts were quantified using a StepOne-Plus Real-Time PCR System (Applied Biosystems) with reactions performed in 25 \(\mu\)l containing RT\(^2\) SYBR Green/ROX qPCR Master Mix (12.5\(\mu\)l), 1:100 dilutions of RT product, and 1 \(\mu\)M PCR primer pair stock that were run in triplicate. Amplification cycle conditions were 95\(^\circ\)C for 10 minutes followed by 40 cycles of (95\(^\circ\)C, 15 sec; 55\(^\circ\)C, 30-40 sec; and 72\(^\circ\)C, 30 sec.). Expression of IL-1\(\beta\) or TNF\(\alpha\) was calculated using the \(\Delta\Delta C_t\) method using StepOne software v. 2.1 with values normalized to GAPDH expression.

**Propidium iodide influx assay**

BMDC were seeded in 24-well plates (5x10\(^5\) cells/well). After LPS priming, the culture medium was removed from each well, washed 1 x with PBS, and replaced with 0.5 ml/well of a balanced salt solution (130 mM NaCl, 5mM KCl, 1.5 mM CaCl\(_2\), 1mM MgCl\(_2\), 25mM HEPES, 0.1% bovine serum albumin and 5 mM glucose, pH 7.4) containing 1 \(\mu\)g/ml propidium iodide. The plate was placed into a Synergy HT plate reader (BioTek) preheated to 37\(^\circ\)C. Baseline fluorescence (540 nm excitation \(\rightarrow\) 620 nm emission at 1 min intervals) was recorded for 10 min. Cells were then stimulated with 10 \(\mu\)M nigericin for another 4 h and the changes in fluorescence were recorded at 1 min
intervals. Assays were terminated by permeabilization of the BMDC with 1% triton X-100 to quantify maximum fluorescence of the propidium²⁺/DNA complexes. An increase in propidium fluorescence correlates with pyroptosis driven by NLRP3 inflammasome and caspase-1 activation. Propidium²⁺ binding to DNA occurs when there is either 1) gating of a propidium²⁺-permeable plasma membrane channel/pore or 2) frank cell lysis that releases cellular DNA. The time-dependent increases in fluorescence induced by nigericin were normalized to the maximum fluorescence measured in triton X-100-permeabilized cells after subtraction of basal intrinsic fluorescence.

**Data processing and analysis**

All experiments were repeated multiple times with separate BMDC preparations. Figures illustrating western blot results are from representative experiments. As indicated, figures illustrating IL-1β ELISA, cell viability, or caspase activity results represent either the mean (± SE) of data from several identical experiments, the mean (± SE) where each condition was performed in triplicate, quadruplicate, or more within a single experiment, or the average (± range) of duplicate samples from single representative experiments. Experiments with 3 or more repeats were analyzed by one-way ANOVA with Bonferroni post-test comparison using Prism 3.0 software. The absolute magnitudes of maximal IL-1β release as quantified by ELISA could vary by ~2-fold between different BMDC preps. Thus, for some experimental series, IL-1β release measured in different conditions or genotypes was normalized to the maximal release for each BMDC preparation, and the normalized values from several identical experiments were then used to generate means (± SE) for evaluation by ANOVA.
CHAPTER 3

Pro-apoptotic Chemotherapeutic Drugs Induce Non-canonical Processing and Release of IL-1β via Caspase-8 in Dendritic Cells

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Summary

In this study, we assessed the relative contributions of caspase-1 and caspase-8 to the processing and release of IL-1β in LPS-primed dendritic cells stimulated with the chemotherapeutic drug, doxorubicin, or the pro-apoptotic agent, staurosporine. Our observations support a model wherein TLR4 activation induces upregulation of proIL-1β together with the TRIF-dependent assembly of a latent caspase-8 signaling platform. Pro-apoptotic agents, such as Dox or STS, act to license such caspase-8 signaling complexes for maximal caspase-8-mediated cleavage of proIL-1β, in part by depleting cIAPs. Comparative analyses of DCs isolated from control mice versus Casp1/11−/− or Casp8−/−Rip3−/− mice indicated that caspase-8 acts as the predominant IL-1β converting enzyme in response to engagement of TLR4 signaling when the intrinsic apoptotic pathway is triggered by chemotherapeutic agents.
Introduction

The regulation of IL-1β signaling, both in the innate and adaptive immune senses, influences the immunogenic response during cancer progression and therapy (78, 79). Depending on context, IL-1β can contribute to beneficial anti-tumor immune responses or maladaptive responses such as neovascularization leading to tumor survival. Much attention has been focused on the mechanisms of IL-1β production within particular tissue niches including tumor microenvironments. NLRP3/caspase-1 inflammasomes play a central role in the regulation of IL-1β production within tumor loci or in response to chemotherapeutic drugs. In tumor-bearing mice treated with oxaliplatin, NLRP3-dependent IL-1β release from dendritic cells (DCs) occurs via paracrine activation of P2X7 receptors (P2X7R) in response to ATP released from dying tumor cells (5). In this model, DC-derived IL-1β contributes to the tumor-specific immune response by polarizing tumor antigen-reactive CD8+ T cells into IFN-γ-producing cytotoxic T cells (Fig. 1.1) (80). Two other drugs, gemcitabine and 5-fluorouracil, have been shown to elicit NLRP3-dependent IL-1β production in response to the accumulation of cytosolic cathepsin B in myeloid-derived suppressor cells (MDSC) with consequent promotion of tumor growth and vascularization (Fig. 1.1) (81). Other reports have described the ability of pro-apoptotic chemotherapeutic agents, such as doxorubicin and staurosporine analogs, to directly activate NLRP3- and caspase-1-dependent IL-1β processing in ex vivo LPS-primed macrophages via mechanisms correlated with ribotoxic stress or increased mitochondrial dysfunction (Fig. 1.1) (16, 82). Several reports of non-canonical IL-1β processing via caspase-8 indicate that pro-inflammatory cytokine production may be mediated by caspase-8, potentially in signaling complexes such as ripoptosomes,
known to control apoptotic or necroptotic death cascades. In this study, we explore the effects of various pro-apoptotic chemotherapeutic drugs on pro-inflammatory signaling and cell death mechanisms.

**Results**

*Pro-apoptotic chemotherapeutic drugs induce the release of IL-1β in LPS-primed murine bone marrow-derived dendritic cells (BMDC)*

Figure 3.1A illustrates the ability of several pro-apoptotic or chemotherapeutic drugs (12 h stimulation) to induce IL-1β processing and release in LPS-primed BMDC. Robust accumulation of extracellular IL-1β (10-20 ng/ml over 12 h) was elicited in response to: 1) doxorubicin (Dox), an anthracycline inhibitor of topoisomerase II; 2) staurosporine (STS), a broad-specificity kinase inhibitor commonly used to induce intrinsic apoptosis (83, 84); and 3) UCN-01, a 7-OH staurosporine analog and experimental therapeutic for hematopoietic and solid tumors (85, 86). The IL-1β release was comparable in magnitude to that elicited by ATP activation of P2X7 receptors for 30 min. In contrast, oxaliplatin and cisplatin, two widely-used platinum-based chemotherapeutic agents, did not stimulate significant IL-1β production. The ability of Dox, STS, or UCN-01, but not the platinum reagents, to trigger IL-1β release correlated with the relative efficacies of the drugs to induce apoptosis of LPS-primed BMDC as indicated by the intracellular accumulation of active caspase-3/7 (Fig. 3.1B). These results are consistent with the studies of Sauter *et al.* (82) and Shimada *et al.* (87) who reported the abilities of Dox and STS, respectively, to stimulate an NLRP3- and caspase-1-dependent IL-1β processing response in LPS-primed murine macrophages. It is
Figure 3.1 Pro-apoptotic chemotherapeutic drugs induce the release of IL-1β in LPS-primed murine BMDC

(A) BMDC were primed with LPS (1µg/ml) for 4 h prior to stimulation for 12 h with a panel of pro-apoptotic agents including staurosporine (STS, 5 µM), UCN-01 (10 µM), doxorubicin (Dox, 10 µM), oxaliplatin (Ox, 25 µM) and cisplatin (CDDP, 25 µM). The extracellular medium was collected and assayed for IL-1β by ELISA. BMDC were primed with LPS for 15.5 h prior to ATP (5mM) stimulation for 30 min. Results are the mean ± range of two experiments. (B) The kinetics of drug-induced caspase-3/7 activity in LPS-primed BMDC was measured by proteolytic cleavage of the DEVD-AMC substrate. The concentrations of each drug used were the same as described in (A). Results are from a single experiment. (C) The kinetics of IL-1β release from LPS-primed and Dox-stimulated (10 µM) WT BMDC were assayed by ELISA. Results are the mean ± SE from 4-8 experiments. (D) LPS-primed BMDC were stimulated with varying doses of Dox for 12 h. Results are the mean ± SE of 3 experiments.
Figure 3.1
important to note that LPS was present throughout the duration of drug stimulation to facilitate sustained TLR4 signaling. We characterized the kinetics (Fig. 3.1C) and concentration-response relationship (Fig. 3.1D) for Dox-stimulated IL-1β release. After a lag phase of 4 h, 10 µM Dox induced a progressive increase in IL-1β production over the subsequent 8 h with a plateau at times ≥ 12 h. Increased IL-1β accumulation (at 12 h) occurred over a narrow range of [Dox] with a threshold at 1 µM and maximal effect at 10 µM; concentrations >10 µM resulted in an attenuated response due likely to a more rapid loss of BMDC viability. Although 1 µg/ml LPS was routinely used for TLR4 activation, equivalent Dox-induced IL-1β release was observed in BMDC primed with 10 or 100 ng/ml LPS while 1 ng/ml LPS was sub-threshold for supporting the response to Dox (Fig. 3.2). Western blot analysis (Fig. 3.3) of BMDC stimulated with 10 µM Dox revealed that: 1) the time-dependent accumulation of extracellular IL-1β assayed by ELISA reflected the release of mature (17 kDa) IL-1β rather than unprocessed proIL-1β; 2) the release of mature IL-1β was preceded by the processing and release of the p10 subunit of active caspase-1; and 3) the proteolytic maturation of IL-1β was temporally correlated with the proteolytic processing and release of the p20 subunit of caspase-7, another defined substrate for caspase-1 in myeloid lineage leukocytes.

*Doxorubicin induces caspase-1 independent processing and release of IL-1β in LPS-primed BMDC*

Given the strong temporal association of the accumulation of mature IL-1β and active caspase-1 p10 subunit in the Dox-treated BMDC (Fig. 3.3), we hypothesized that
**Figure 3.2** IL-1β release in response to varying concentrations of LPS followed by doxorubicin or ATP stimulation

WT and Casp1/11−/− BMDC were treated with 0, 1, 10, or 1000 ng/ml of LPS for 4 h and then co-stimulated with Dox (10 µM) for another 12 h or with ATP (5mM) for 30 min. Results are the mean ± SE of 3 experiments.
Figure 3.2
**Figure 3.3** Doxorubicin induces the release of IL-1β in LPS-primed murine BMDC

WT BMDC were LPS-primed and Dox-stimulated (10 µM), and the extracellular medium and cell lysates were collected and processed for western blot analysis for detection of IL-1β, caspase-1, and caspase-7. BMDC were primed with LPS for 5.5 h prior to ATP (5mM) stimulation for 30 min. The data are representative of results from 3 experiments.
Figure 3.3

Intracellular

- β-actin (45 kDa)
- procasp1 (45 kDa)
- procasp7 (34 kDa)
- proIL-1β (33 kDa)
- casp1 (10 kDa)
- casp7 (20 kDa)
- IL-1β (17 kDa)

Extracellular

LPS only + ATP 2 h 4 h 8 h 12 h 18 h + Dox

- β-actin (45 kDa)
- procasp1 (45 kDa)
- procasp7 (34 kDa)
- proIL-1β (33 kDa)
- casp1 (10 kDa)
- casp7 (20 kDa)
- IL-1β (17 kDa)
the IL-1β processing reflected Dox-induced activation of the NLRP3/caspase-1 inflammasome pathway. This was tested by directly comparing (in the same experiments), the kinetics and magnitudes of Dox-induced IL-1β release in LPS-primed wildtype (WT) versus Casp1/11<sup>−/−</sup> BMDC. To our surprise, we observed that the rate and magnitude of mature IL-1β production were only modestly delayed (by ~ 2 h) and decreased (by ~20%) in the Casp1/11<sup>−/−</sup> BMDC as monitored by either western blot or ELISA (Fig. 3.4 A, B). Efficient ablation of caspase-1 signaling in the knockout cells was confirmed by: 1) the absence of p45 procaspase-1 and the p10 subunit of active caspase-1 in the cell lysates and extracellular medium; and 2) suppression of P2X7 receptor-stimulated IL-1β maturation and release. As with WT cells, 1 ng/ml LPS was sub-threshold for supporting Dox-induced IL-1β release in the Casp1/11<sup>−/−</sup> BMDC, and equivalent secretion was observed when 10 μM Dox was combined with 10, 100, or 1000 ng/ml LPS (Fig. 3.2). The observation that WT, Asc<sup>−/−</sup>, and Nlrp3<sup>−/−</sup> Nlrc4<sup>−/−</sup> BMDC (all LPS-primed) exhibited similar magnitudes of IL-1β release in response to 12 h stimulation with 10 μM Dox further indicated the presence of an alternative inflammasome-independent pathway for IL-1β processing (Fig. 3.5A). As with the Casp1/11<sup>−/−</sup> cells (Fig. 3.4B inset), the Asc<sup>−/−</sup> or Nlrp3<sup>−/−</sup> Nlrc4<sup>−/−</sup> BMDC exhibited a reduced IL-1β release at 8 h of Dox stimulation but the differences did not reach statistical significance at p<0.05. In contrast, ATP-stimulated IL-1β production was completely suppressed in the same Asc<sup>−/−</sup> and Nlrp3<sup>−/−</sup> Nlrc4<sup>−/−</sup> BMDC preparations. Production of mature IL-1β in response to Dox was also observed in WT LPS-primed BMDC treated with YVAD, an inhibitor of caspase-1 activity (Fig. 3.5 B, C).
**Figure 3.4** Doxorubicin induces caspase-1 independent processing and release of IL-1β in LPS-primed BMDC

(A) LPS-primed (1 µg/ml) WT or *Casp1/11−/−* BMDC were stimulated with Dox (10 µM) for 2-18 h, and western blot analysis of IL-1β and caspase-1 from cell lysates and extracellular supernatants was performed. BMDC were LPS-primed for 5.5 h followed by 30 min of ATP (5mM) stimulation. Results are representative of 3 identical experiments. (B) The release of IL-1β from LPS-primed (1ug/ml) WT or *Casp1/11−/−* BMDC stimulated or not with 10 µM Dox for 12 h was assayed by ELISA. IL-1β release was normalized to WT BMDC treated with LPS + Dox for 12 h and expressed as the mean ± SE of 5 experiments. The differences between WT and *Casp1/11−/−* BMDC were not significant (n.s, P > .05) by Student’s t-test. Inset: Kinetics of Dox-induced IL-1β release (normalized to WT BMDC treated with LPS + Dox for 12 h) in LPS-primed WT versus *Casp1/11−/−* BMDC as described in part (A). Results are the mean ± SE of 3 experiments.
Figure 3.4
Figure 3.5  Doxorubicin-induced IL-1β release is independent of ASC and NLRP3/NLRC4 and is YVAD-insensitive

(A) WT, Asc<sup>−/−</sup>, and Nlrp3<sup>−/−</sup> Nlrc4<sup>−/−</sup> BMDC were primed with LPS for 4 h before stimulation with Dox for 4, 8, or 12 h and assayed for release IL-1β release by ELISA. Parallel samples were primed with LPS for 15.5 h prior to ATP (5mM) stimulation for 30 min. IL-1β release was normalized to WT BMDC treated with LPS + Dox for 12 h and expressed as the mean ± SE of 3-5 experiments. ***P < .001 or n.s by ANOVA. (C) Western blot analysis of LPS-primed (1µg/ml) WT or Casp1/11<sup>−/−</sup> BMDC stimulated with 10 µM Dox for 12 h or 5mM ATP for 30 min in the presence or absence of the caspase-1 inhibitor, YVAD (50 µM). BMDC were LPS-primed for 7.5 h followed by ATP (5mM) stimulation for 30 min. The data are representative of results from 2 experiments. (B) Concentration-response relationship for Dox-stimulated IL-1β release in the presence or absence of YVAD in LPS-primed BMDC. The curve depicted without YVAD treatment is the same as shown in Figure 3.1D. Results are the mean ± SE of 3 experiments.
Figure 3.5

A. LPS-primed BMDC

IL-1β Release Normalized to WT BMDC
(100 = LPS + Dox : 12h)

Untreated  LPS only  + Dox (4h)  + Dox (8h)  + Dox (12h)  + ATP

WT  Asc-/-  Nlrp3-/-  Nlrc4-/-

n.s  n.s  n.s  n.s  ***

B. LPS-primed BMDC

IL-1β (pg/ml)

0  5000  10000  15000  20000  25000

0.1  1  10  100

+ Dox (12h)  + Dox + YVAD (12h)

LPS-primed BMDC

Dox (µM)

C. WT  Casp1/11-/-  YVAD

LPS only  + ATP, 30 m  + Dox, 12 h
YVAD only modestly reduced (by ~15%) the IL-1β release stimulated by maximally effective (10 μM) Dox while producing a ~50% suppression of the response to submaximal (3 μM) Dox and complete inhibition of P2X7 receptor-induced IL-1β release. Inclusion of YVAD during Dox treatment of Casp1/11−/− BMDC did not reduce IL-1β maturation. Using a murine bone marrow-derived macrophage (BMDM) experimental model, Sauter et al. (82) reported that the ability of shorter term Dox exposure (8 h) to stimulate IL-1β processing was almost completely suppressed in macrophages isolated from Asc−/−, Nlrp3−/−, or Casp1−/− mice. We found that more prolonged (12 h) Dox treatment also induced caspase-1–independent IL-1β release in murine BMDM (Fig. 3.6A). We similarly observed that LPS + Dox elicited IL-1β release in the RAW264.1 murine macrophage cell line (Fig. 3.6B) that lacks expression of functional ASC (45, 88) and cannot process IL-1β in response to canonical inflammasome agonists such as ATP. These data suggest that intracellular signals generated during the initial phases of Dox-induced apoptosis in DCs and macrophages elicit modest assembly of NLRP3/ASC/ caspase-1 inflammasomes but that this canonical pathway is superseded by a caspase-1-independent pathway for IL-1β processing driven by signals which develop with sustained apoptotic stress.

As indicated in Fig. 3.1B, Dox treatment induces peak accumulation of apoptotic executioner caspase-3/7 activity within 4–8 h, which precedes the major phase of IL-1β processing and release. We tested whether active caspase-3/7 comprises a necessary upstream signal for the caspase-1-independent IL-1β processing by including the caspase-3/7 inhibitor DEVD during LPS + Dox stimulation. DEVD had no inhibitory
Figure 3.6 Macrophages also produce IL-1β in response to LPS and doxorubicin

(A) WT and Casp1/11−/− BMDM were treated with LPS (1 μg/ml) for 4 h prior to co-stimulation with Dox (10 μM) for an additional 4, 8, or 12 h. Alternatively, BMDM were treated with LPS for 15.5 h prior to stimulation with ATP (5mM) for 30 min. Results are from one experiment.  (B) RAW 264.7 macrophages were primed with LPS for 4 h and co-stimulated with either Dox for an additional 12 h or with ATP for 30 min. Results are from one experiment.
Figure 3.6

A. LPS-primed BMDM

- WT
- Casp1/11-/-

B. RAW Macs

- Untreated
- LPS only
- Dox 12h
- ATP 30m
Figure 3.7  Dox-induced accumulation of active apoptotic executioner caspases can be dissociated from its stimulatory action on non-canonical IL-1β processing and release.

(A) The release of IL-1β was measured in LPS-primed WT or Casp1/11−/− BMDC stimulated ± 10μM Dox for 12 h in the presence or absence of DEVD (50μM). IL-1β release was normalized to WT BMDC treated with LPS + Dox for 12 h. Results are the mean ± range of 2 experiments. (B) Caspase-3/7 activity was measured in LPS-primed WT and Casp1/11−/− BMDC ± 10 μM Dox in the presence or absence of DEVD. Casp3/7 activity was normalized to WT BMDC treated with LPS + Dox for 8 h. Results are the mean ± range of 2 experiments.
Figure 3.7
effect on LPS + Dox-stimulated IL-1β release in wildtype or Casp1/11−/− BMDC (Fig. 3.7A) but completely suppressed the increased caspase-3/7 activity stimulated by Dox in parallel BMDC samples (Fig. 3.7B). Thus, Dox-induced accumulation of active apoptotic executioner caspases can be dissociated from its stimulatory action on non-canonical IL-1β processing and release.

**TLR4 activation coupled with caspase-8 inhibition induces RIP1/RIP3-dependent necroptosis and release of unprocessed proIL-1β, while TLR4 activation coupled with Dox treatment induces caspase-8-dependent apoptosis**

We hypothesized that caspase-8 was the alternative IL-1β processing enzyme activated in Dox-treated BMDC given its demonstrated ability to act as an IL-1β convertase in other models of macrophage innate immune response (52-54, 89). However, testing this by utilizing the pan-caspase inhibitor, zVAD, or the caspase-8 selective inhibitor, IETD, in LPS-primed BMDC is complicated by the risk of unleashing RIP1/RIP3-dependent necroptotic death downstream of TLR4 activation (90, 91).

Consistent with the induction of necroptosis under such conditions, we observed a time-dependent accumulation of extracellular proIL-1β in BMDC within 4 h after initiation of the co-treatment with LPS and zVAD (Fig. 3.8A). The ability of zVAD to completely inhibit the caspase-1-mediated IL-1β processing response to P2X7 receptor activation verified the efficacy of zVAD as a pan-caspase inhibitor in these experiments. The LPS + zVAD-induced release of proIL-1β was completely suppressed by the RIP1 inhibitor, necrostatin-1 (Nec-1), consistent with a role for necroptosis in the release of extracellular proIL-1β. Similarly, combined LPS + IETD treatment of WT BMDC, either with or
Figure 3.8 TLR4 activation coupled with caspase-8 inhibition induces RIP1/RIP3-dependent necroptosis and release of unprocessed proIL-1β

(A) Western blot analysis of IL-1β and caspase-1 in cell lysates and extracellular medium from WT BMDC treated with LPS (1μg/ml) and zVAD (50 μM) for 2-8 h with or without necrostatin-1 (Nec-1, 50 μM). (B) Western blot analysis of IL-1β and caspase-1 in cell lysates and extracellular medium from WT BMDC treated with LPS for 4 h prior to stimulation with Dox (10 µM) in the presence or absence of IETD (100 μM) for 12 h. In panels A and B, BMDC were primed with LPS for 7.5 h prior to 30 min of ATP (5mM) stimulation. The data are representative of results from 2-3 experiments.
Figure 3.8
without Dox stimulation (12 h), resulted in the accumulation of extracellular proIL-1β (Fig. 3.8B). In contrast, IETD completely suppressed the accumulation of extracellular mature IL-1β triggered by LPS + Dox. The induction of necroptosis in BMDC co-treated with LPS + zVAD or LPS + IETD (versus LPS alone) was also verified by observing decreased cell viability (as indicated by total intracellular ATP content) and the ability of Nec-1 to prevent this loss of viability (Fig. 3.9A).

We also assessed the roles of caspase-8 in the LPS + zVAD-induced death response in WT, Casp8\(^{-/-}\) Rip3\(^{-/-}\) and Casp8\(^{+/+}\) Rip3\(^{+/+}\) BMDC. Casp8\(^{+/+}\) mice undergo mid-gestational death due to unrestrained necroptosis, but this phenotype is reversed in mice that additionally lack RIP3 (Casp8\(^{-/-}\) Rip3\(^{-/-}\)) (76, 92). Consistent with this phenotype, the decrease in BMDC viability induced by combined LPS + zVAD was absent in Casp8\(^{-/-}\) Rip3\(^{-/-}\) or Casp8\(^{+/+}\) Rip3\(^{+/+}\) BMDC (Fig. 3.9B). The ability of Dox treatment alone to elicit similar decreases in BMDC viability, independent of caspase-8 and/or RIP3, was consistent with initiation of the intrinsic apoptotic cascade with likely engagement of caspase-9 as the initiator caspase. Kinetic analysis revealed that Dox alone triggered a rapid decrease (50% within 4 h) in the viability of WT or Casp1/11\(^{-/-}\) BMDC (Fig. 3.9C) that was suppressed in LPS-primed cells, consistent with a protective effect of NF-κB-dependent anti-apoptotic gene expression. However, sustained (≥ 8 h) co-treatment with LPS + Dox resulted in decreased viability indicating engagement of an alternative regulated cell death pathway. The similar kinetics and magnitude of this response to LPS + Dox in WT and Casp1/11\(^{-/-}\) BMDC argued against involvement of pyroptosis. In contrast, the rescued viability in Casp8\(^{-/-}\) Rip3\(^{-/-}\) cells, but not in WT or
Figure 3.9 Necrostatin-1 inhibits necroptosis induced by LPS + zVAD or LPS + IETD, and TLR4 activation coupled with Dox treatment induces caspase-8-dependent apoptosis in BMDC

(A) WT BMDC were co-treated with LPS + zVAD or LPS + IETD in the presence or absence of Nec-1, and cell viability was assessed by measuring intracellular ATP content. Results are from a single experiment with each condition performed in quadruplicate.

(B) WT, Casp8−/− Rip3−/−, and Casp8+/− Rip3−/− BMDC were treated with LPS (16 h), Dox (12h), or LPS for 4 h prior to Dox or zVAD for 12 h, and cell viability was assessed. Results are from a representative experiment (of 2 similar experiments) performed in quadruplicate. ***P < .001 by ANOVA.

(C) WT and Casp1/11−/− BMDC were LPS-primed or not for 4 h prior to stimulation or not with Dox for 4, 8, or 12 h, and cell viability was assessed. Results are from a representative experiment of 2 similar experiments with each condition performed in triplicate.
Figure 3.9
Casp8<sup>+/−</sup> Rip3<sup>−/−</sup> cells, indicated that TLR4 activation coupled with Dox treatment mediated caspase-8-dependent apoptosis (Fig. 3.9B).

Doxorubicin induces caspase-8 dependent IL-1β processing and release in LPS-primed BMDC

We also utilized the Casp<sup>8−/−</sup> Rip3<sup>−/−</sup> and Casp<sup>8+/+</sup> Rip3<sup>−/−</sup> BMDC to assess the role of caspase-8 as the alternative IL-1β processing enzyme stimulated by LPS + Dox. We first determined how canonical NLRP3/caspase-1 inflammasome signaling pathways are operative in the knockout BMDC. LPS triggered rapid (within 1 h) and quantitatively equivalent accumulation of IL-1β mRNA (and TNFα mRNA, data not shown) in WT (Casp<sup>8+/+</sup> Rip3<sup>+</sup>), Casp<sup>8−/−</sup> Rip3<sup>−/−</sup>, and Casp<sup>8+/+</sup> Rip3<sup>−/−</sup> BMDC (Fig. 3.10A). LPS priming for 8 h induced similar accumulation of proIL-1β protein in the three cell types (Fig. 3.10C). Inclusion of nigericin or ATP as canonical NLRP3 stimuli during the final 30 min of the 8 h LPS treatment periods also resulted in robust release of mature IL-1β in each BMDC genotype, albeit at modestly lower levels in the Casp<sup>8−/−</sup> Rip3<sup>−/−</sup> cells (Fig. 3.10 B and C). When the total LPS treatment time was extended to 16 h (Fig. 3.10D) or 18 h (Fig. 3.12A), we observed that the proIL-1β protein levels were markedly reduced in Casp<sup>8−/−</sup> Rip3<sup>−/−</sup> BMDC relative to those in WT or RIP3-knockout cells at these time points, or in Casp<sup>8−/−</sup> Rip3<sup>−/−</sup> cells at 8 h post LPS. In contrast, LPS stimulation for 16 h induced equivalent TNFα secretion in the three BMDC genotypes (Fig. 3.11C). Thus, the combined absence of caspase-8 and RIP3 apparently results in signals (or loss of sustaining signals) that result in gradual attenuation of on-going TLR4-driven transcription and translation of proIL-1β. Consistent with this model, inclusion of
Figure 3.10  Doxorubicin induces caspase-8 dependent IL-1β processing and release in LPS-primed BMDC despite the deficiency in proIL-1β translation in Casp8−/− Rip3−/− BMDC post 8h of LPS stimulation

(A) ProIL-1β mRNA (normalized to GAPDH) was measured by qPCR in WT, Casp8−/− Rip3−/−, and Casp8+/+ Rip3−/− BMDC treated with LPS (1 µg/ml) for 1 h or 4 h. Results are from a single experiment. (B) BMDC of the indicated genotypes were treated with LPS (1 µg/ml) for 7.5 h prior to simulation ± ATP (5 mM) for an additional 30 min; IL-1β release was assayed by ELISA. Results are the mean ± range of 2 experiments. (C) Western blot analysis of proIL-1β, procaspase-1, and procaspase-8 in cell lysates (intra) and mature IL-1β and caspase-1 p10 subunit in extracellular (extra) supernatants of WT, Casp8−/− Rip3−/−, and Casp8+/+ Rip3−/− BMDC treated with LPS for 8 h and stimulated ± nigericin (10 µM) for the final 30 min of the LPS treatment period. Data are representative of results from 2 experiments. (D) Western blot analysis of pro-IL-1β and procaspase-1 in cell lysates (intra) and mature IL-1β and caspase-1 p10 subunit in extracellular (extra) supernatants of WT, Casp8−/− Rip3−/−, and Casp8+/+ Rip3−/− BMDC treated with LPS for a total of 16 h and stimulated with either Dox (10 µM) for the final 12 h, or with ATP (5 mM) or nigericin (10 µM) for the final 30 min, of the LPS treatment period. Results are representative of 3 similar experiments.
Figure 3.10
Figure 3.11  Prolonged LPS stimulation (16h total) suppresses nigericin and doxorubicin-induced IL-1β release but not TNFα secretion in Casp8<sup>−/−</sup> Rip3<sup>−/−</sup> BMDC

(A, B) WT, Casp8<sup>−/−</sup> Rip3<sup>−/−</sup>, and Casp8<sup>+/+</sup> Rip3<sup>−/−</sup> BMDC were treated with LPS (1 μg/ml) for a total of 16 h and stimulated with Dox (10 μM) for the last 12 h (A) or nigericin (10 μM) for the last 30 min (B) of the LPS treatment period. IL-1β release was assayed by ELISA and normalized to WT stimulated with LPS + Dox (A) or WT stimulated with LPS + nigericin (B). Results are the mean ± SE of 3 experiments for (E) or the mean ± SE for 2 experiments (F) ***P < .001 or not significant (n.s) by ANOVA. (C) TNFα release (by ELISA) from WT, Casp8<sup>−/−</sup> Rip3<sup>−/−</sup>, and Casp8<sup>+/+</sup> Rip3<sup>−/−</sup> BMDC treated ± LPS (1 μg/ml) for 16 h. Results are the mean ± range of 2 experiments.
Figure 3.11
**Figure 3.12** IL-1β processing is suppressed during pharmacological inhibition of caspase-8 or RIPK1 in WT and Casp1/11−/− BMDC treated with LPS and doxorubicin

(A) Western blot analysis of proIL-1β, procaspase-1, and NLRP3 expression in WT, Casp8−/− Rip3−/−, and Casp8+/− Rip3−/− BMDC treated for 18 h with 0, 10, 100, or 1000 ng/ml of LPS. Results are representative of 2 experiments. (B) Western blot analysis of procaspase-8, procaspase-1, and proIL-1β in cell lysates (intra) and mature IL-1β, caspase-8 p18 subunit, and caspase-1 p10 subunit in the extracellular supernatants (extra) from WT or Casp1/11−/− BMDC treated with LPS (1 µg/ml) for 4 h prior to co-stimulation with Dox (10 µM) for another 2-18 h or with LPS for 5.5 h prior to ATP (5mM) stimulation for 30 min. The data are representative of results from 3 experiments. (C) WT and Casp1/11−/− BMDC were treated with LPS (1 µg/ml) for a total of 16 h and stimulated ± Dox (10 µM), ± IETD (100 µM), ± Nec-1 (50 µM) for the final 12 h of the LPS treatment period. IL-1β release was assayed by ELISA and normalized to the samples stimulated with LPS + Dox. Results are the mean ± SE of 3 experiments. ***P < .001 by ANOVA.
A. LPS treatment time: 18h

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<th>Casp8&lt;sup&gt;−/−&lt;/sup&gt; Rip3&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Casp8&lt;sup&gt;+/−&lt;/sup&gt; Rip3&lt;sup&gt;−/−&lt;/sup&gt;</th>
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B. LPS (ng/ml)

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<td>casp1 (10 kDa)</td>
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C. LPS-primed BMDC

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<td>Normalized to WT BMDC</td>
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Figure 3.12
nigericin or ATP during the final 30 min of 16 h LPS treatment periods resulted in markedly reduced levels of mature IL-1β production in Casp8<sup>−/−</sup> Rip3<sup>−/−</sup> BMDC as assayed by western blot (Fig. 3.10D) and ELISA (Fig. 3.11B). Notably, the ability of nigericin to elicit production and release of caspase-1 p10 subunit by Casp8<sup>−/−</sup> Rip3<sup>−/−</sup> BMDC was suppressed when these cells were treated with LPS for 16 h (Fig. 3.10D) but not for 8 h (Fig. 3.10C); the former effect was correlated with the reduced ability to sustain elevated levels of TLR4-dependent NLRP3 protein expression (Fig. 3.12A).

These analyses of canonical NLRP3 inflammasome signaling provide an important context for comparison and interpretation of the non-canonical IL-1β release responses to LPS + Dox in WT, Casp8<sup>−/−</sup> Rip3<sup>−/−</sup>, and Casp8<sup>+/+</sup> Rip3<sup>−/−</sup> BMDC. IL-1β release was completely suppressed in Casp8<sup>−/−</sup> Rip3<sup>−/−</sup> BMDC compared to WT BMDC treated with LPS and Dox as assayed by ELISA (Fig. 3.11A) and western blot analysis (Fig. 3.10D). The LPS + Dox-induced IL-1β release was attenuated by ~50-60% in Casp8<sup>+/+</sup> Rip3<sup>−/−</sup> cells, suggesting a possible modulatory role for RIP3 in the response to Dox. It is important to note that the Dox treatment in these experiments was initiated after 4 h of LPS priming and maintained during the next 12 h of LPS stimulation. Despite the gradual decrease in TLR4-dependent proIL-1β protein expression in Casp8<sup>−/−</sup> Rip3<sup>−/−</sup> BMDC, the cells contained high levels of this cytokine precursor during the initial several hours of Dox treatment. Thus, the complete inhibition of Dox-induced mature IL-1β release in these cells most likely reflects the absence of caspase-8 as a major non-canonical IL-1β converting enzyme rather than reduced levels of the proIL-1β substrate.
Consistent with a role for caspase-8 as an IL-1β converting enzyme, procaspase-8 processing and accumulation of mature extracellular caspase-8 was detected in WT and Casp1/11−/− BMDC treated with LPS + Dox for >4 h (Fig. 3.12B). Interestingly, the clearance of procaspase-8 induced by Dox occurred more rapidly in Casp1/11−/− BMDC compared to WT BMDC. With recognition of the caveats in using pharmacological inhibitors of caspase-8 to assess its role in IL-1β processing in LPS-primed BMDC, we observed that the ability of LPS + Dox to stimulate extracellular accumulation of ELISA-measurable IL-1β was greatly suppressed by IETD in both WT and Casp1/11−/− BMDC (Fig. 3.12C). The RIP1 inhibitor Nec-1 also suppressed these IL-1β release responses. These results implicate caspase-8 signaling platforms, possibly in RIP1-containing ripoptosomes, as key mediators of LPS + Dox stimulated non-canonical IL-1β processing.

*LPS + Dox-induced IL-1β release is TRIF-dependent and correlated with IAP downregulation and recruitment of caspase-8/FADD to a detergent-insoluble compartment*

Maelfait et al. demonstrated that the ability of TLR4 and TLR3 activation (combined with cyclohexamide-mediated suppression of protein turnover) to induce caspase-8 maturation of IL-1β was compromised in macrophages from TRIF-knockout mice (52). Kaiser and Offermann found that TRIF possesses a RHIM (receptor interacting protein (RIP) homotypic interaction motif) domain that facilitates association with RIP1 (and RIP3) (93). Thus, activation of TLR4 in macrophages and DCs results in NF-κB-dependent upregulation of proIL-1β, via both the MyD88 and TRIF adapters, as
well as the assembly of latent RIP1/ FADD/ caspase-8 ripoptosome complexes, orchestrated by the TRIF adapter (Fig. 3.13A). Because TLR4 activation drives the NF-κB-mediated upregulation of NLRP3 expression (Fig. 3.12A) (94), LPS-treated cells will also be primed for induction of canonical caspase-1-mediated IL-1β maturation in the presence of appropriate signal 2 stress stimuli. In contrast, while TLR2 activation can also trigger the NF-κB-dependent upregulation of proIL-1β and NLRP3 that facilitates utilization of caspase-1 as an IL-1β converting enzyme, this pathway will not engage TRIF-dependent signaling cascades, such as those leading to possible assembly of RIP1/ FADD/ caspase-8 ripoptosomes (Fig. 3.13A). To test the differential contributions of MyD88- versus TRIF-signaling to LPS + Dox-induced IL-1β processing, WT and Trif⁻/⁻ BMDC were primed either with the TLR4 agonist, LPS, or the lipopeptide TLR2 agonist, Pam₃CSK₄, for 4 h before stimulation with Dox for 12 h. LPS + Dox-induced IL-1β maturation and release as assayed by western blot (Fig. 3.13B) and ELISA (Fig. 3.13C and D) was significantly reduced in Trif⁻/⁻ BMDC. Prolonged LPS treatment resulted in lower levels of proIL-1β in the Trif⁻/⁻ cells (Fig. 3.13B), but this partial reduction in proIL-1β substrate contrasted with the near-complete suppression of IL-β maturation in response to Dox. Inclusion of YVAD did not further suppress the residual LPS + Dox-triggered IL-1β release observed in Trif⁻/⁻ BMDC (Fig. 3.13D). Although Pam₃CSK₄ and LPS induced equivalent accumulation of proIL-1β in WT cells (Fig. 3.13B), combined stimulation by Pam₃CSK₄ + Dox resulted in ~4-fold less production of mature IL-1β compared to LPS + Dox treatment (Fig. 3.13C). Regardless, the magnitude of Pam₃CSK₄
**Figure 3.13** LPS + Dox-induced IL-1β release is TRIF-dependent

(A) A model for parallel pathways of canonical NLRP3 inflammasome activation and TRIF-induced caspase-8 signaling complexes that may mediate IL-1β processing induced by LPS + doxorubicin. (B) Western blot analysis of proIL-1β and procaspase-1 in cell lysates (intra) and mature IL-1β and caspase-1 p10 subunit in extracellular supernatants (extra) from WT and Trif−/− BMDC stimulated Pam3CSK4 (200 ng/ml) ± Dox (10 µM) or LPS (1 µg/ml) ± Dox for 12 h. The data are representative of 2 experiments. (C, D) IL-1β release (by ELISA) from WT or Trif−/− BMDC treated with Pam3CSK4 (200 ng/ml) or LPS (1 µg/ml) for a total of 16 h and stimulated ± Dox (10 µM) (C, D) or Dox + YVAD (50 µM) (D) for the final 12 h of the LPS or Pam3CSK4 treatment periods). Data in (C) are representative of results from 2 experiments. Data in (D) are the mean ± SE of 3 experiments. **P < .01 or not significant (n.s) by ANOVA.
Figure 3.13
+ Dox-induced IL-1β release was similar in Trif−/− and WT BMDC. These data support a role for TRIF in mediating a TLR4-dependent activation of caspase-8 and consequent IL-1β maturation when combined with Dox.

We next explored the mechanism by which Dox may license the activation of caspase-8 within the presumed signaling platforms assembled in response to the TLR4-TRIF cascade. Tenev et al. reported that genotoxic stressors such as etoposide (a topoisomerase II inhibitor like doxorubicin) facilitate ripoptosome assembly and caspase-8-mediated cell death in non-myeloid tumor cells by inducing the degradation of cellular Inhibitor of Apoptosis Proteins (cIAPs) (95). Furthermore, Vince et al. showed that Smac mimetics, which induce degradation of cIAPs, activated IL-1β processing in LPS-primed BMDC via a caspase-8 pathway that required coordinate down-regulation/inhibition of all three major IAPs including cIAP1, cIAP2, and XIAP (53).

We assessed the levels of cIAP1 in WT and Casp1/11−/− BMDC treated (or not) with LPS only, Dox only, or LPS + Dox. The amount of cIAP1 was greatly reduced in LPS + Dox-treated BMDC and attenuated in the LPS only or Dox only treatment conditions compared to untreated cells (Fig. 3.14A). As a positive control, we showed that Dox treatment reduced cIAP1 levels in Jurkat leukemic T cells.

We initially assessed whether LPS + Dox stimulated the formation of RIP1/ FADD/caspase-8 ripoptosome complexes in BMDC by adapting co-immunoprecipitation protocols developed for the characterization of such platforms in tumor cells or non-myeloid cell types challenged with pro-apoptotic stimuli, TLR3 agonist, or TNFα (95-97). WT BMDC were treated with various combinations of LPS, Dox, and zVAD, or
TNFα ± zVAD, prior to generation of whole lysates by standard detergent extraction, removal of detergent-insoluble material by centrifugation, incubation of the detergent-soluble lysates with anti-FADD, collection of anti-FADD immune complexes, and western blot analysis of the immune complexes for caspase-8, FADD, and RIP1. ZVAD is commonly used in such analyses to stabilize caspase-8-containing signaling complexes (95-97). No caspase-8 or RIP1 was observed in FADD immunoprecipitates prepared from cells treated with any combination of those stimuli. However, we consistently found that the detergent-soluble faction (i.e., the fraction subjected to subsequent immunoprecipitation) of lysates from BMDC treated for 2 h with LPS + zVAD or TNFα + zVAD contained markedly lower levels of procaspase-8 and FADD (Fig. 3.14B). To our surprise, analysis of the detergent-insoluble lysate pellets from cells stimulated with LPS + zVAD or TNFα + zVAD revealed robust enrichment of 57 kDa procaspase-8 and 45 kDa partial cleavage fragment of caspase-8, as well as lower amounts of 18 kDa processed caspase-8 subunit; FADD was also enriched in the same detergent-insoluble fractions. This redistribution of caspase-8 and FADD was not observed in lysates from cells treated with only zVAD (data not shown) or in BMDC that were primed with LPS for 4 h followed by treatment with Dox for an additional 8 h (Fig. 3.14B) or 3 h (data not shown). However, inclusion of zVAD during the Dox treatment did facilitate caspase-8 and FADD partitioning into the insoluble lysate pellets (Fig. 3.14B). Analysis of RIP1 recruitment to these pellets was limited by the finding that total RIP1 levels in BMDC (but not BMDM or J774.1 murine macrophages) were below the detection limit of the antibody used (Fig. 3.16C). These data indicate that TLR4 activation in BMDC induces the recruitment of caspase-8 and FADD to signaling platforms that may underlie non-
**Figure 3.14** LPS + Dox-induced IL-1β release correlates with IAP downregulation and recruitment of caspase-8/ FADD to a detergent-insoluble compartment

(A) Western blot analysis of cIAP1 (asterisk denotes a non-specific band), proIL-1β, procaspase-1, and actin in cell lysates from WT BMDC or *Casp1/11* −/− BMDC treated ± LPS (1 µg/ml) for 4 h prior to stimulation ± Dox (10 µM) for 12 h. As a positive control, lysates from control or Dox-treated Jurkat leukemic T cells were also analysed. Results are from a single experiment. **(B)** WT BMDC were treated with LPS (1 µg/ml) or TNFα (50 ng/ml) for 2 h in the presence or absence of zVAD (50 µM). Alternatively, BMDC were treated with LPS for 4 h prior to stimulation with Dox (10 µM) or Dox + zVAD for another 8 h. Cell lysates were separated into detergent-soluble versus detergent-insoluble fractions for western blot analysis of caspase-8 and FADD. Results are representative of 2 experiments.
Figure 3.14

A.

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<tr>
<td>Dox</td>
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**Intracellular**

- clAP1 (62 kDa)
- β-actin (45 kDa)
- procasp1 (45 kDa)
- proIL-1β (33 kDa)

B.

**Detergent-soluble fraction**

- procasp8 (57 kDa, 45 kDa)
- casp8 (18 kDa)
- FADD (27 kDa)

**Detergent-insoluble fraction**

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*WT Casp1/11−/− Jurkat Intracellular*
canonical IL-1β processing when combined with pro-apoptotic stimuli that down-regulate IAPs.

*Staurosporine induces caspase-8-dependent IL-1β processing and release in LPS-primed BMDC*

Staurosporine (STS) has been extensively studied with regard to its ability to induce a very rapid apoptotic response in most cell types via incompletely understood mechanisms that include suppression of multiple pro-survival kinases (98, 99), disruption of mitochondrial integrity (87), and allosteric modulation of caspase-9 independently of Apaf-dependent apoptosome assembly (100). Shimada et al. demonstrated that STS also stimulates a rapidly developing NLRP3- and caspase-1 dependent IL-1β processing response in LPS-primed murine macrophages via a mechanism linked to disruption of mitochondrial integrity (87). Fig. 3.15A shows that STS triggers a similarly rapid (near-maximal within 4 h) increase in IL-1β processing and release in LPS-primed WT BMDC. Notably, STS also induced a robust accumulation of mature extracellular IL-1β in Casp1/11−/− BMDC. The STS-stimulated processing and release of IL-1β in both WT and Casp1/11−/− cells was temporally correlated with production and release of the p18 subunit of active caspase-8 (Fig. 3.15A). As with LPS + Dox treatment, LPS + STS-induced clearance of procaspase-8 occurred more rapidly in Casp1/11−/− BMDC compared to WT BMDC even though mature 18 kD caspase-8 accumulation appeared to be equivalent. ELISA measurements indicated that STS-induced IL-1β release (at 4 h) was non-significantly attenuated by ~25% in either Casp1/11−/− BMDC (Fig. 3.15B) or in WT BMDC treated with YVAD (data not shown). STS-induced IL-1β release from
Figure 3.15 Staurosporine induces caspase-8-dependent IL-1β processing and release in LPS-primed BMDC

(A) Western blot analysis of procapase-8, procaspase-1, and proIL-1β in cell lysates (intra) and mature IL-1β, caspase-8 p18 subunit, and caspase-1 p10 subunit in the extracellular supernatants (extra) from WT or Casp1/11−/− BMDC were treated ± LPS (1 µg/ml) for 4 h and then co-stimulated with STS (5 µM) for an additional 2-18 h or with LPS for 5.5 h prior to ATP (5mM) stimulation for 30 min. The data are representative of results from 3 experiments. (B) LPS-primed (1 µg/ml, 4h) WT and Casp1/11−/− BMDC were stimulated ± STS (5 µM) for 4 h; IL-1β release was assayed by ELISA and normalized to WT BMDC treated with LPS + STS (4 h). Results are the mean ± SE of 4-5 experiments. The differences between WT and Casp1/11−/− BMDC were not significant (n.s, P > .05) by Student’s t-test.
**Figure 3.15**

A.

- **WT**
  - Intra: LPS only, 2 h, 4 h, 8 h, 24 h
  - Extra: LPS only, 2 h, 4 h, 8 h, 24 h

- **Casp1/11−/−**
  - Intra: LPS only, 2 h, 4 h, 8 h, 24 h
  - Extra: LPS only, 2 h, 4 h, 8 h, 24 h

- **Proteins**
  - Caspase 8 (57 kDa)
  - Caspase 1 (45 kDa)
  - Prolactin-1β (33 kDa)
  - Caspase 8 (18 kDa)
  - IL-1β (17 kDa)
  - Caspase 1 (10 kDa)

B.

- **IL-1β Release**
  - Normalized to WT BMDC
  - LPS treatment time: 8 h

- **Graph**
  - WT
  - Casp1/11−/−
  - n.s
  - LPS + STS (4h)

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**Figure 3.15**
Figure 3.16  IL-1β processing is reduced in Casp8<sup>−/−</sup> Rip3<sup>−/−</sup> BMDC and during pharmacological inhibition of caspase-8 or RIPK1 in WT and Casp1/11<sup>−/−</sup> BMDC treated with LPS and staurosporine

(A) WT BMDC were primed with LPS (1 µg/ml, 4 h) prior to stimulation ± STS (5 µM, 4 h) in the presence or absence of IETD (100 µM) or Nec-1 (50 µM); IL-1β release was assayed by ELISA. Results are from a single representative experiment of 3 experiments.

(B) WT, Casp8<sup>−/−</sup> Rip3<sup>−/−</sup>, and Casp8<sup>+/−</sup> Rip3<sup>−/−</sup> BMDC were treated ± LPS (1 µg/ml) for 4 h prior to co-stimulation ± STS (5 µM) for an additional 4 h. Results are from a single experiment with each condition was performed in duplicate. (C) Upper panel: Western blot analysis of cIAP1 of lysates from untreated WT BMDC or WT BMDC treated with STS (5 µM, 4 h), Dox (10 µM, 12 h), or oxaliplatin (Ox) (25 µM, 18 h). Lower panel: Western blot analysis of cIAP1, PARP (full length and cleaved forms), and RIP1 in lysates in control versus STS-treated (5 µM, 4 h) BMDC, BMDM, and J774 murine macrophages. Results are from a single experiment.
**Figure 3.16**
either WT (Fig. 3.16A) or Casp1/11−/− (data not shown) BMDC was markedly suppressed by IETD and partially (~50%) decreased by Nec-1 (Fig. 3.16A). A 75% reduction in STS-triggered IL-1β secretion was also observed in Casp8−/− Rip3−/− BMDC (Fig. 3.16B). Similarly to treatment with 12 h of Dox, a 4 h exposure to STS suppressed the expression of cIAP1 in WT BMDC (Fig. 3.16C, upper panel). In contrast, 18 h of oxaliplatin induced no change in cIAP1 expression relative to untreated BMDC. Additionally, a 4-h treatment with STS was sufficient to produce marked depletion of cIAP1 levels in other murine myeloid cell types including BMDC, BMDM, and the J774.1 murine macrophage line (Fig. 3.16C, lower panel). Rapid STS-triggered depletion of cIAP1 in these cells was correlated with apoptotic induction as indicated by depletion of intact 116 kDa PARP (poly-ADP-ribosyltransferase) and accumulation of 89 kDa PARP cleavage product. STS treatment also decreased RIP1 protein levels in these detergent-solubilized cell lysate samples. Despite the complexity of STS action, these data indicate that this apoptosis inducer also triggers caspase-8 dependent IL-1β processing and release in LPS-primed BMDC.

Discussion

This study describes a novel and robust mechanism of caspase-8-dependent processing of IL-1β induced by the chemotherapeutic drug, Dox, in LPS-primed BMDC. This release did not require caspase-1 based on the fact that mature IL-1β was produced by Casp1/11−/− BMDC as well as by WT BMDC treated with YVAD (Figs. 3.4, 3.5 B and C). The pro-apoptotic agent, STS, triggered caspase-1 independent IL-1β release in LPS-primed BMDC even more rapidly than Dox (Fig. 3.15 A and B). Dox and STS-induced
IL-1β production in LPS-primed WT BMDC was attenuated in the presence of the caspase-8 inhibitor, IETD, or the RIP1 inhibitor, Nec-1, while Dox- and STS-induced IL-1β release was greatly suppressed in LPS-primed \textit{Casp8}^{−/−}\textit{Rip3}^{−/−} BMDC, implicating a caspase-8 signaling platform for IL-1β processing in TLR4-activated DCs (Figs. 3.10-3.12 and 3.15-3.16). The marked attenuation of LPS + Dox-induced IL-1β release from \textit{Trif}^{−/−} BMDC relative to WT cells supported an important role for TRIF in coupling TLR4 stimulation to caspase-8 activation and non-canonical IL-1β processing (Fig. 3.13). In the presence of a pan-caspase inhibitor, TLR4 activation induced recruitment of caspase-8 and FADD to a detergent-insoluble compartment (Fig. 3.14) and also triggered RIP1-dependent necroptotic death (Figs. 3.8-3.9). The ability of both Dox and STS to trigger IL-1β release in LPS-primed BMDC was correlated with the degradation of cIAP1 (3.14A) which, together with cIAP2 and XIAP, is known to restrain ripoptosome activity by destabilizing such complexes. Taken together, these results support a model wherein TLR4 drives TRIF-dependent assembly of caspase-8 ripoptosomes that are licensed as IL-1β converting enzyme platforms in the presence of pro-apoptotic stimuli, likely via modulated expression of IAPs and/or other factors (\textit{e.g.} cFLIP) which control ripoptosome assembly, stability, and activity. However, a rapidly expanding literature is identifying roles for caspase-8 as a component of multiple signaling complexes or pathways linked to innate immune response (54, 55, 65, 89, 101). Thus, ripoptosomes may comprise only one of several caspase-8-based pathways that participate in non-canonical IL-1β processing.
Relationships between caspase-1 and caspase-8 signaling in IL-1β processing and release

Consistent with the reported role for the NLRP3 inflammasome in LPS + Dox-induced IL-1β production by bone marrow-derived macrophages (82), we observed reduced IL-1β release at the early (≤ 8 h) stages of LPS + Dox stimulation in BMDC lacking caspase-1 (Fig.3.4), ASC (Fig. 3.5A), or NLRP3 (Fig. 3.5A). However, when Dox stimulation was sustained, the relative contribution of this NLRP3/caspase-1 cascade to IL-1β accumulation was superseded by an alternative pathway that was attenuated in the absence of caspase-8 or TRIF (Figs. 3.5A, 3.10D, 3.11A, and 3.13). Robust proteolytic maturation of caspase-1 per se was invariably observed in WT BMDC treated with LPS + Dox (Figs. 3.1, 3.3, and 3.4) or LPS + STS (Fig. 3.15) despite the fact that caspase-1 was dispensable for maximal IL-1β processing. We initially expected that the activation of caspase-1 and caspase-8 in response to Dox or STS comprised independent IL-1β processing pathways operating in parallel. However, our observations and recent studies by others suggest a linked but asymmetric relationship in the ability of pro-apoptotic stimuli to activate the caspase-1 versus caspase-8 pathways coupled to IL-1β maturation and release. Although the caspase-8 pathway was strongly activated by LPS + Dox in BMDC that lack caspase-1, caspase-11, ASC, or NLRP3 (Figs. 3.4 and 3.5A), activation of caspase-1 or caspase-1 mediated IL-1β processing was greatly reduced in Casp8−/-Rip3−/- BMDC and attenuated in cells lacking only RIP3 (Fig. 3.10D). This was mediated in part by the reduced ability of the Casp8−/-Rip3−/- cells to sustain high expression of proIL-1β and NLRP3 protein during prolonged TLR4 activation (Figs. 3.10D).
3.10D and 3.12A). Notably, the ability of ATP or nigericin, as rapidly acting signal 2 stimuli for NLRP3 inflammasome assembly, to trigger caspase-1 processing (Fig. 3.10C) and IL-1β release (Fig. 3.10B and C) was normal in Casp8−/−Rip3−/− BMDC during the earlier (<8 h) stages of TLR4 activation but markedly reduced when the Casp8−/−Rip3−/− cells were primed with LPS for longer durations (>12 h) before being challenged with nigericin or ATP (Figs. 3.10B and 3.11B). These data indicate that caspase-8 in DCs and macrophages can act directly as an alternative IL-1β converting enzyme and indirectly as a modulator of the expression of proIL-1β and NLRP3. Although the underlying mechanism for this latter role of caspase-8 remains to be defined, the dependence on prolonged TLR4 activation suggests feedback regulation by autocrine or paracrine pathways that fine-tune ongoing transcription, translation, and turnover of proIL-1β and NLRP3, and possibly other proinflammatory gene products.

Other investigators have recently described complex roles for caspase-8 in the direct and indirect regulation of IL-1β production (53, 102). Kang et al. observed that accumulation of active RIP3 and RIP1 in BMDC isolated from mice with a DC-restricted deletion of Casp8 was sufficient to license rapid assembly of NLRP3 inflammasomes in response to TLR4 activation in the absence of a signal 2 stimulus, such as ATP or nigericin (102). This ability of caspase-8 deletion to potentiate TLR4 induction of NLRP3 inflammasomes was independent of significant RIP1/RIP3-induced cell death. Kang et al. additionally noted that caspase-8 and FADD were co-immunoprecipitated with NLRP3 in lysates from LPS-stimulated WT BMDC. We found that caspase-8 and FADD were recruited to a detergent-insoluble compartment in DCs treated with LPS and...
zVAD (Fig. 3.14B). Vince et al. characterized additional interactions between caspase-8, RIP3, and NLRP3 signaling in their model of Smac-mimetic-induced IL-1β accumulation by murine BMDM and BMDC (53). Activation of RIP3 was correlated with production of reactive oxygen species that stimulated IL-1β processing via both caspase-1 and caspase-8. Those investigators also reported recruitment of caspase-8 and RIP1 to detergent-insoluble fraction in lysates of the Smac-mimetic stimulated BMDM. Another study found that caspase-8 interacted with ASC and co-localized with AIM2/ASC specks during cell death triggered by Francisella tularensis infection of caspase-1-deficient BMDM (101). Finally, Sagulenko et al. have described a direct interaction between the death effector domain (DED) of procaspase-8 and the pyrin domain of ASC that can occur in context of NLRP3- or AIM2-inflammasome activation in macrophages (65).

These various findings underscore complex interactions between caspase-8 and proteins/pathways associated with canonical caspase-1 inflammasome assembly and activity. Our data suggest that pro-apoptotic agents, in conjunction with TLR4 activation, elicit a temporally defined hierarchy of reactions that utilize various combinations of TRIF, FADD, RIP1/3, and conventional inflammasome proteins for the assembly of caspase-8 and caspase-1 platforms that catalyze IL-1β processing. Although both Dox and STS induced proteolytic processing of caspase-1 and caspase-8 (Figs. 3.12B and 3.15A), STS stimulated these responses more rapidly with significant activation of caspase-1 activation preceding that of caspase-8. This more rapid engagement of the caspase-1 pathway may underlie the lower efficacy of IETD or caspase-8 deletion in attenuating STS-induced IL-1β processing (Figs. 3.16 A and B)
relative to Dox-induced IL-1β production (Figs. 3.11A and 3.12C). The relative rate at which a particular “signal 2” stimulus triggers activation of the NLRP3/caspase-1 pathway likely determines whether the more slowly developing caspase-8 pathway will or will not comprise a major route for IL-1β processing. ATP acting directly via P2X7 non-selective cation channel receptors, and nigericin, acting as a direct K+ ionophore, trigger dissipation of the normal trans-plasma membrane K+ gradient within minutes and the resulting decrease in cytosolic [K+] elicits exceptionally rapid (complete within 30 min) and efficient assembly of NLRP3 inflammasomes (Figs. 3.3, 3.5C, 3.10 C-D, and 3.12B) (27). Thus, very robust processing of proIL-1β occurs prior to any substantial activation of caspase-8. STS also triggers K+ efflux-dependent NLRP3 activation (16), but the K+ efflux likely occurs as a secondary and more slowly induced consequence of mitochondrial dysfunction and reduced ATP generation. As a result, STS-induced activation of caspase-1 occurs only modestly faster than induction of the caspase-8 pathway (Fig. 3.15A). Finally, because changes in mitochondrial function (and apoptotic signaling) are even more slowly induced by Dox, the activation of both the caspase-1 and caspase-8 pathways develop over similar time periods.

**Pro-apoptotic stimuli and IAPs as regulators of IL-1β production**

We observed a strong correlation between the relative efficacies of pro-apoptotic agents to induce IL-1β production and their abilities to stimulate apoptotic executioner caspase-3/7 activity in DCs (Fig. 3.1 A and B). Perturbation of mitochondrial function comprises one possible link by which pro-apoptotic agents can engage both the caspase-1 and caspase-8 pathways for IL-1β production. STS elicits exceptionally rapid changes in
mitochondrial function and apoptotic progression in macrophages (87) and most other cell types by incompletely understood mechanisms. Given its ability to inhibit topoisomerase II, Dox can induce conventional DNA damage-dependent apoptosis in tumor cells and other rapidly dividing cells (103). In quiescent or slowly proliferating cells (e.g., cardiomyocytes), Dox also triggers apoptosis via activation of iron-dependent redox cycling reactions in mitochondria that result in accumulation of reactive oxygen species (ROS) (103). Accumulation of ROS together with release of mitochondrial DNA has been linked to assembly of the NLRP3 inflammasome pathway for IL-1β production (3). However, another hallmark of the intrinsic apoptotic pathway is the release of Smac from dysfunctional mitochondria. Binding of Smac to IAPs either directly inhibits their ability to bind to caspases, as with XIAP, or induces their autoubiquitination and proteasome-mediated clearance as with the cIAP1/cIAP2 E3 ubiquitin ligases (104). The cIAPs target pro-apoptotic proteins and components of the ripoptosome, including RIP1 and RIP3, for ubiquitin-mediated degradation (Fig. 3.13A). Genotoxic stressors, such as etoposide, or the degradation of cIAPs using Smac mimetics, promote ripoptosome assembly (53, 95). We found that Dox and STS, but not oxaliplatin, induced marked downregulation of cIAP1 levels in BMDC (Figs. 3.14A and 3.16C); this likely comprises one mechanism by which these agents trigger caspase-8 activation when combined with TLR4 stimulation. A limitation of our study is that we assayed only changes in cIAP1 levels. Vince et al. found that the ability of Smac-mimetics to maximally induce IL-1β production by LPS-primed BMDM or BMDC required coordinated targeting of cIAP2 and XIAP, in addition to cIAP1. Thus, it will be important to assess the effects of Dox and other pro-apoptotic agents on the expression/ activity of cIAP2 and XIAP in future
studies of non-canonical IL-1β production. In addition to IAPs, other E3 ubiquitin ligases may contribute to caspase-8 regulation in our model. Jin et al. identified a critical role for cullin-3 in mediating the polyubiquitination, aggregation, and full activation of caspase-8 during TRAIL-mediated apoptosis of cancer cells (105). Notably, those investigators also observed the recruitment of caspase-8 and FADD to a detergent-insoluble compartment similar to our findings in LPS + zVAD-stimulated BMDC (Fig. 3.14B).

The role of cIAPs in inflammasome assembly and caspase-1 signaling is more complex. Labbé et al. found that cIAP1 and cIAP2 were required for efficient caspase-1 activation and IL-1β processing in response to multiple stimuli for NLRP3 and NLRC4 inflammasome assembly (106). This reflected a direct interaction of the cIAPs with caspase-1 that results in an activating and non-degradative K63-linked ubiquitination of caspase-1. In contrast, Vince et al. showed that the loss of cIAPs following stimulation with Smac mimetics promoted RIP3-dependent caspase-1 and caspase-8 activation for IL-1β processing (53). These nominally conflicting observations are particularly germane given the E3 ubiquitin ligase activity of IAPs and recent findings that the ubiquitination status of NLRP3 (29-31) and caspase-1 (106) strongly modulate the assembly and activity of caspase-1 inflammasomes. Our observations that the caspase-8 pathway progressively predominates over the caspase-1 pathway during Dox and STS treatment may reflect the opposing consequences of IAP suppression on these signaling cascades. Progressive loss of the cIAPs may act to bias the signaling network by reversing the stimulatory effect of K63-ubiquitination on caspase-1 while enhancing
rioptosome assembly/activity. Further investigation is required to assess the differential
roles of IAPs and other E3 ubiquitin ligases in the engagement of the canonical and non-
canonical pathways for IL-1β processing by various cell death and inflammatory stimuli.

*Roles for TRIF, RIPs, and other adapters in regulating caspase-8 mediated processing of
IL-1β*

The ability of LPS + Dox to stimulate IL-1β maturation and release was greatly reduced in *Trif*−/− BMDC (Fig. 3.13B-D). The absence of TRIF could reduce the contribution from the TLR4→TRIF→IRF3/7→IFN-β→caspase-11 signaling cascade that amplifies NLRP3/caspase-1 inflammasome activation (Fig. 3.13A) (107). However, another major role for TRIF is recruitment of RIP1 via RHIM domain interactions with consequent induction of RIP1/FADD/caspase-8 complexes. The assembly, activity, and stability of caspase-8-containing ripoptosomes are additionally modulated by association with isoforms of cFLIP (cellular FLICE inhibitory protein), a non-catalytic paralogue of caspase-8 (51). Maximal ripoptosome-associated caspase-8 activity (sufficient to drive apoptosis) is restrained via association of caspase-8 with cFLIP<sub>L</sub> (long isoform).

However, the low-level ripoptosome-associated caspase-8 activity of the cFLIP<sub>L</sub>-containing heterodimeric complex is sufficient to either cleave RIP1 and/or RIP3 directly or to eliminate another process that impacts function and thereby suppresses the assembly of the necrosome complexes necessary for necroptosis. When the activity of this heterodimeric complex is completely inhibited, either by pharmacological inhibition of caspase-8 or when the complex contains cFLIP<sub>s</sub> (short isoform), the suppression of RIP1 and RIP3 is relieved; RIP1 then phosphorylates RIP3 which regulates an incompletely
understood necroptotic signaling cascade (92, 96). Inhibition of RIP1 kinase activity by Nec-1 blocks this cascade. This latter necroptotic pathway was operative in our BMDC experimental model as indicated by morphology as well as the loss of DC viability in response to LPS stimulation in combination with either pan-caspase inhibition or selective caspase-8 inhibition (Fig. 3.9A). The inhibitory effects of Nec-1 on LPS + Dox or STS-induced IL-1β release illustrated in Figs. 3.12C and 3.16A further suggested that this IL-1β production involves a RIP1-regulated pathway for engagement of caspase-8. The accumulation of extracellular IL-1β was temporally correlated with caspase-8 activation in WT and Casp1/11−/− BMDC stimulated with Dox (Fig. 3.12B) or STS (Fig. 3.15A) and markedly suppressed in Casp8−/−Rip3−/− BMDC (Figs. 3.10D, 3.11A, and 3.16B).

There are several limitations in our experimental results and their implications regarding the possible involvement of RIP1/FADD/caspase-8 ripoptosomes as the major signaling platform for non-canonical IL-1β processing. The genetic support for caspase-8 as the alternative IL-1β converting enzyme utilized cells deficient in both caspase-8 and RIP3. Experiments with cells lacking only caspase-8 would eliminate potentially confounding effects of additional RIP3 ablation on the Dox-stimulated responses. However, unlike Casp8−/−Rip3−/− BMDC, cells deficient in only RIP3 were characterized by only modestly attenuated IL-1β processing and release in responses to LPS + Dox (Figs. 3.10D and 3.11A) or LPS + STS (Fig. 3.16B). This suggests that the absence of caspase-8 rather than RIP3 underlies the very strong suppression of IL-1β production in Casp8−/−Rip3−/− cells. Our results also suggest stimulus-specific roles for RIP1 because
Nec-1 almost completely suppressed Dox-stimulated IL-β production (Fig. 3.12C) but only partly attenuated the response to STS (Fig. 3.16A). However, an additional caveat is that Nec-1 inhibits indoleamine 2,3-dioxygenase (IDO), a known immunomodulatory enzyme (108). Finally, the isolation of RIP1, FADD, and caspase-8 in co-precipitating immune complexes would provide stronger biochemical support for ripoptosomes as an underlying signaling platform for non-canonical IL-1β processing. Studies of ripoptosome assembly have predominantly utilized cancer cells or established cell lines that may express higher levels of the interacting signaling proteins (36-38). Moreover, the accumulation of RIP1/FADD/caspase-8 ripotosomes as soluble protein complexes within the cytosol may be an intermediate state superseded by the recruitment or aggregation of such complexes into higher-order macromolecular ensembles. This possibility is supported by our observations (Fig. 3.14B) and those of Vince et al. (53), demonstrating the recruitment of caspase-8 into a detergent-insoluble compartment of BMDC treated with LPS + zVAD or BMDM treated with LPS + Smac mimetic, respectively. Additional studies are necessary to address how RIP1, RIP3, and cFLIP isoforms modulate the ability of various pro-apoptotic stimuli to induce specific caspase-8 signaling complexes that mediate processing of IL-1β.

*Context-dependent modes of dendritic cell death triggered by doxorubicin*

The differential viabilities of Casp8−/− Rip3−/−, Casp8+/+ Rip3−/−, and control Casp8+/+ Rip3+/+ BMDC treated with Dox only, LPS only, or LPS + Dox indicated that Dox may engage either a conventional caspase-9-mediated intrinsic apoptotic program in the absence of TLR4 co-stimulation or a caspase-8 mediated apoptosis in the context of
TLR4 activation (Fig. 3.9B). The viability of \textit{Casp8}\textsuperscript{−/−}/\textit{Rip3}\textsuperscript{−/−} BMDC treated with LPS + Dox, but not Dox only, was sustained thereby implicating caspase-8-dependent apoptosis as the mode of cell death in this instance. Pyroptosis was eliminated as a major mode of cell death or IL-1β release under LPS + Dox treatment conditions because similar time courses of reduced viability (Fig. 3.9C) and extracellular IL-1β accumulation (Fig. 3.4B) were observed in WT and caspase-1-deficient BMDC. Dox-induced accumulation of active executioner caspase-3/7 activity could also be dissociated from the parallel IL-1β processing response to this pro-apoptotic agent (Fig. 3.7). Notably, mature IL-1β rather than proIL-1β was the predominant form in the extracellular medium of cells treated with LPS + Dox (Fig. 3.3). This indicated that the production of mature IL-1β and its release from the DCs was tightly coordinated with the decrease in cell viability orchestrated by apoptotic signaling. In contrast, induction of necroptosis by LPS + zVAD or LPS + IETD resulted in a massive release of proIL-1β that was suppressed by Nec-1 (Fig. 3.8A).

Relevance to the anti-tumor chemotherapeutic and immunogenic actions of doxorubicin

It will be important to determine whether doxorubicin and related anthracyclines can engage this caspase-8 mediated pathway for IL-1β production under in vivo conditions wherein these agents exert their anti-tumor chemotherapeutic and immunogenic actions. In this regard, two questions are particularly germane. First, might dendritic cells or macrophages within tumor loci be exposed to the micromolar doxorubicin concentrations that trigger this signaling cascade in cell culture conditions? Both the tumor cells and immune cells within tumor sites can be exposed to high local
concentrations of chemotherapy drugs depending on the method and site of drug delivery. There is increasing development of new modes of doxorubicin therapy that involve tumor-directed delivery via encapsulation within liposomes or conjugation with nanoparticles and antibody complexes (109, 110). Such particulate-based therapies can result in phagocytosis of Dox-containing liposomes or conjugates by tumor-resident DCs and macrophages. The second question concerns possible in vivo mediators within the tumor microenvironment that might act as TLR4 agonists to facilitate the TRIF/RIP1/FADD interactions that are also required for caspase-8 activation. Several studies have identified HMGB1 released from dying tumor cells or other host cells as a relevant DAMP agonist for TLR4 that supports activation of IL-1β processing and release (22, 80, 111-113). Moreover, advanced stages of cancer progression or cytotoxic cancer therapies can result in compromised barrier function of the gut epithelia and increased circulating levels of PAMPs derived from commensal bacteria (114, 115). This study further validates the importance of exploring the direct effects of pro-apoptotic chemotherapeutic drugs on IL-1β production by tumor-resident immune cells in addition to previously described models wherein other DAMPs, such as ATP, released from dying chemotherapy-treated tumor cells initiate canonical inflammasome signaling (116). Moreover, the overall consequences of the myeloid-driven IL-1β production response to various cancer chemotherapy drugs may vary with the stage of tumor progression (117). For example, IL-1β production elicited in the early stages of cancer therapy may be advantageous to the tumor-bearing host in initiating an IL-1β-dependent immunogenic anti-tumor response (80), but at more advanced stages, may contribute to tumor growth (81). Overall, better understanding of the relative contributions of various IL-1β
processing pathways elicited by cancer chemotherapeutics may aid in the development of refined therapeutic approaches that limit tumor progression.
CHAPTER 4

Caspase-8 as a Regulator and Effector of NLRP3 Inflammasomes in Murine Dendritic Cells

The content in this chapter will be submitted for review to the *J Immunol*.

**Summary**

Current therapies for IL-1β-driven inflammatory diseases involve IL-1R antagonism, but pharmacological inhibition of IL-1β convertases may also aid in mitigating IL-1β-associated pathologies. We and others have recently described the induction of non-canonical IL-1β processing via caspase-8 recruited to ripoptosome signaling platforms in myeloid cells. In this study, we describe how NLRP3/ASC inflammasome platforms can also recruit caspase-8 to drive IL-1β processing in bone marrow-derived dendritic cells (BMDC). Sustained stimulation (>2 h) of TLR4-primed *Casp1/11/^+^* BMDC with the canonical NLRP3 agonist nigericin results in a delayed phase of robust IL-1β processing and release mediated by caspase-8. In contrast, sustained nigericin treatment did not induce IL-1β processing in *Nlrp3/^−^* or *Asc/^−^* BMDC. Inhibition of caspase-1 or caspase-8 activity in wildtype (WT) BMDC similarly attenuated the nigericin-induced IL-1β release responses suggesting that caspase-8 plays a dual role as both an activator of caspase-1 and a direct IL-1β-converting enzyme. Notably, IL-1β processing in response to prolonged nigericin stimulation was also markedly reduced in *Casp8/^−^* *Rip3/^−^* BMDC but not in *Rip3/^−^* BMDC. Cell viability was
also preserved in nigericin-treated Casp8<sup>−/−</sup>Rip3<sup>−/−</sup> cells, emphasizing critical roles for caspase-8 in both inflammasome signaling and its conventional role as a cell death regulator. Biochemical analyses indicated that caspase-8 is recruited into detergent-insoluble protein complexes in nigericin-stimulated cells. Our data suggest that, in the presence of caspase-1, caspase-8 predominantly acts as an initiator caspase to facilitate caspase-1 processing; however, in the absence of caspase-1, the inflammasome-associated caspase-8 directly acts as an executioner caspase and major IL-1β converting protease. Understanding the molecular events leading to caspase-8-based cytokine processing in the presence of attenuated caspase-1 function may shape pharmacological targeting of particular inflammasome signaling pathways involved in various inflammatory diseases.

**Introduction**

IL-1β is a proinflammatory cytokine that requires NF-κB-dependent upregulation of its pro-cytokine form and proteolytic processing into its mature, biologically active form in order to activate the IL-1 receptor and mediate inflammation. Regulation and activation of this cytokine in macrophages and dendritic cells has focused on caspase-1, the predominant interleukin-1-converting enzyme (ICE), downstream of the NLRP3 inflammasome signaling complex—the best characterized sentinel for the activation and processing of IL-1β. However, recent studies have reported activation of caspase-8 as an ICE for processing and generating mature 17 kD IL-1β in response to a variety of PAMPs and DAMPs (52–56). The assembly of RIP1/RIP3 kinases and caspase-8-containing complexes triggers the activation of caspase-1 and IL-1β processing induced by *Yersinia pestis* via the YopJ protein in TLR4-stimulated BMDM (57, 58). In the *Salmonella*
_enterica serovar Typhimurium_ infection model, an NLRC4/ASC/caspase-8/caspase-1 pathway is engaged whereby active caspase-1 and caspase-8 are both recruited to the complex as observed by fluorescence microscopy in BMDM (59, 60). Additionally, candida and mycobacterial species trigger Syk kinase-dependent caspase-8 activation in BMDM via dectin-1, an extracellular sensor that detects the presence of microbial carbohydrate ligands (61, 62). Thus, findings from several examples of microbial infection or innate immune response support a model in which caspase-8 is recruited to multi-protein signaling platforms that contribute to the proteolytic maturation of caspase-1 and/or IL-1β.

Caspase-8 has been extensively characterized as an initiator caspase involved in death receptor-mediated apoptosis (e.g. Fas-R, TNF-R, TRAIL-R) and a suppressor of necroptosis (51). This latter role for caspase-8 in limiting RIP3-mediated necroptosis and the full viability of _Casp8<sup>−/−</sup>Rip3<sup>−/−</sup>_ mice (76) underscores the pleiotropic functions of caspase-8. The role(s) of caspase-8 as an ICE and/or a regulator of inflammasome signaling have motivated studies to define the underlying mechanisms. Some recent reports have indicated that caspase-8 regulates the priming and assembly steps of NLRP3 inflammasome signaling (63, 66). Gurung _et al._ showed that genetic deletion of FADD or caspase-8 markedly attenuated the ability of LPS-primed BMDM to produce mature IL-1β in response to extracellular ATP or nigericin (63). We and others have reported that the absence of caspase-8 reduced the sustained accumulation of proIL-1β and NLRP3 protein in LPS-primed BMDC and BMDM (66, 118). Interestingly, Allam _et al._ demonstrated that global NFκB activation was not impaired in _Casp8<sup>−/−</sup>Rip3<sup>−/−</sup>_ BMDM such that degradation of IκBα was equivalent to that in control BMDM, even though
serum IL-1β levels in response to LPS injection were reduced in Casp8<sup>−/−</sup> Rip3<sup>−/−</sup> mice (66). Another study reported that the absence of c-FLIP, a caspase-8 paralog which controls caspase-8 activity, significantly impaired IL-1β maturation and release (64). Caspase-8 along with its interacting partners, FADD and c-FLIP, possess pleiotropic roles in immune cells, particularly in signaling cascades for cell death and IL-1β processing. With respect to cell death signaling, Sagulenko and colleagues reported pyroptotic cell death in control BMDM versus apoptotic cell death in Casp1/11<sup>−/−</sup> BMDM upon engagement of either NLRP3 or AIM2 inflammasomes (65). However, IL-1β processing and release responses were not fully characterized in that study. We recently described a TLR4/TRIF-dependent RIP1/FADD/caspase-8-dependent signaling platform for non-canonical caspase-8-mediated IL-1β processing in BMDC co-treated with LPS and pro-apoptotic chemotherapeutic drugs; the induced IL-1β maturation was correlated with decreased cIAP1 expression and apoptotic DC death (118). Overall, these studies raise questions regarding the cross-talk between caspase-1 and caspase-8-based signaling platforms in the processing and release of IL-1β, as well as the direction of different cell death pathways.

In this study, we compared how the canonical NLRP3 agonist nigericin stimulates inflammasome signaling and IL-1β production in WT versus Casp1/11<sup>−/−</sup> BMDC. These experiments uncovered an NLRP3/ASC/caspase-8-dependent IL-1β processing pathway that required prolonged (>2 h) nigericin stimulation in caspase-1-deficient DC in contrast to the very rapid (<0.5 h) engagement of the NLRP3/ASC/caspase-1 pathway for IL-1β maturation in WT DCs. Based on both pharmacological inhibition and genetic knockout models, we conclude that caspase-8 acts as an alternative ICE in response to sustained
nigericin stimulation in Casp1/11−/− BMDC. However, pharmacological inhibition of caspase-8 in nigericin-stimulated WT BMDC markedly attenuates the processing of caspase-1 as well as IL-1β. These results suggest that both caspase-1 and caspase-8 are recruited to NLRP3/ASC inflammasomes. In the presence of caspase-1, the recruited caspase-8 appears to predominantly facilitate caspase-1 processing and activation and thus potentiate caspase-1-mediated IL-1β maturation. In the absence of caspase-1, the inflammasome-associated caspase-8 directly acts as the major IL-1β converting protease. The study further emphasizes the complex dynamics of intracellular multi-protein complex assembly involved in the processing and maturation of IL-1β and provides alternative mechanisms for IL-1β production in ultimately shaping the recruitment of immune cells to the site of tissue injury or damage.

**Results**

*Sustained nigericin stimulation induces delayed processing and release of mature IL-1β in LPS-primed Casp1/11−/− murine bone marrow-derived dendritic cells (BMDC)*

Consistent with previous reports (25, 40, 119), nigericin-induced NLPR3/ASC/caspase-1 inflammasome activation and IL-1β release was maximal within 30 min in WT BMDC (Fig. 4.1A and B) and this rapid response to nigericin was completely suppressed in Casp1/11−/− BMDC. However, a delayed phase of IL-1β processing and release was observed during sustained (>2 h min) stimulation of these knockout cells with nigericin. This slower accumulation of mature IL-1β by Casp1/11−/− BMDC reached a plateau within 4 to 6 h and the peak magnitude was ~ 33% of that observed in WT cells; the absolute amount of caspase-1 independent IL-1β secretion from 5 x 10⁵ BMDC was ~20
Figure 4.1 Sustained nigericin stimulation induces delayed processing and release of mature IL-1β in LPS-primed Casp1/11−/− murine BMDC

(A) WT and Casp1/11−/− BMDC were primed with LPS (100 ng/ml) for 4 h prior to stimulation with nigericin (10 µM) for 0.5 h, 1 h, 2 h, 4 h, or 6 h, and the extracellular medium was collected and assayed for IL-1β by ELISA. BMDC which were stimulated for 30 min with nigericin were primed with LPS for 5.5 h. Results are the mean ± SE of 16 experiments. The differences in IL-1β release between WT and Casp1/11−/− BMDC treated with LPS and nigericin at all timepoints were significant (P < .001), by one-way ANOVA analysis and Bonferroni post-test. (B) BMDC were stimulated as in (A), and the extracellular media and cell lysates were collected and processed for western blot analysis for detection of IL-1β, caspase-1, caspase-8 and NLRP3. The data are representative of results from 3 experiments.
Figure 4.1
ng/ml/6 h (Fig. 4.1A). The export of mature 17 kD IL-1β from Casp1/11−/− BMDC during sustained nigericin treatment was temporally correlated with enhanced proteolytic processing and release of the mature 18 kD caspase-8 subunit (Fig. 4.1B). In contrast, WT BMDC released significantly less 18 kD caspase-8 despite the comparable kinetics of intracellular procaspase-8 clearance. We also observed the robust appearance of a ~29 kD cleavage product of proIL-1β during acute stimulation with nigericin (0.5-2 h) in Casp1/11−/− BMDC (Fig. 4.1B). The canonical caspase-1 cleavage site for proIL-1β at residues C116 and D117 generates 17 kD mature IL-1β. We identified a potential cleavage site of murine proIL-1β at S16/D17 which may be additionally targeted by caspase-8 to generate the 29 kD fragment prior to cleavage at the canonical C116/D117 locus (Fig 4.2). Only minor accumulation of this 29 kD IL-1β product was observed in WT BMDC.

ASC-based inflammasomes form large protein aggregates that can be sedimented by low-speed centrifugation of detergent-solubilized cell extracts followed by cross-linking to indicate the extent of ASC oligomerization (53). Nigericin induced similarly rapid rates and extents of ASC oligomerization in both WT and Casp1/11−/− BMDC (Fig. 4.3A) consistent with previous studies (19). As an additional index of caspase-1-based inflammasome activation, we assayed the accumulation of propidium²⁺ dye which tracks with induction of caspase-1 pyroptosis. Intracellular accumulation of the normally impermeant propidium²⁺ via increased plasma membrane permeability facilitates its intercalation with DNA resulting in increased 540→620 nm fluorescence. Fig. 4.3B shows that the nigericin-induced increases in propidium²⁺ accumulation in both WT versus Casp1/11−/− cells were temporally correlated with the kinetics of IL-1β processing.
(Fig. 4.1B). While WT BMDC were characterized by robust influx of propidium$^{2+}$ during the first 30 min of stimulation, significant accumulation of fluorescent propidium$^{2+}$/DNA complexes in the Casp1/11$^{-/-}$ BMDC required >2 h of sustained nigericin treatment. We also assessed the metabolic viability of BMDC treated with nigericin in the presence or absence of LPS priming by measuring redox-dependent changes in Alamar blue metabolism. A rapidly induced decrease in Alamar blue redox turnover (within 60 min) was evident in LPS + nigericin-treated WT BMDC (Fig. 4.3C). In contrast, decreases in Alamar blue metabolism in Casp1/11$^{-/-}$ BMDC viability were delayed by >2 h after LPS + nigericin treatment. These results are consistent with the findings of Sagulenko et al. who reported that nigericin induces rapid pyroptotic death of LPS-primed WT BMDC but apoptotic death of LPS-primed Casp1/11$^{-/-}$ BMDC (65).

*Sustained nigericin-induced processing and release of IL-1$\beta$ is dependent on NLRP3 and ASC and correlates with caspase-8 activation and cell death in murine BMDC*

Given the robust activation of caspase-8 during sustained nigericin stimulation of the Casp1/11$^{-/-}$ BMDC, we hypothesized that procaspase-8 is recruited to NLRP3/ASC inflammasome platforms. Nigericin-induced IL-1$\beta$ processing and release at all timepoints (0.5, 2, 4, 6 h) was completely suppressed in Nlrp3$^{-/-}$ and ASC$^{-/-}$ BMDC as indicated by both western blot analysis and (Fig. 4.4) and ELISA quantification (Fig. 4.5A). Notably, caspase-8 activation was also suppressed at all timepoints during nigericin stimulation of Nlrp3$^{-/-}$ and ASC$^{-/-}$ BMDC, suggesting that NLRP3 and ASC are essential components of the relevant inflammasome platform for caspase-8. We also
Figure 4.2 Caspase-1 and predicted caspase-8 cleavage sites in proIL-1β to generate 17 kD IL-1β and 29 kD proIL-1β

This is the murine proIL-1β amino acid sequence (269 AA, 31 kD) obtained from the UniProtKB/Swiss-Prot database. The first arrow indicates the probable cleavage site for caspase-8 in red (FDSD-between S16 and D17) to generate 29 kD proIL-1β, and the second arrow indicates the caspase-1/caspase-8 cleavage site in blue (LVCD-between C116 and D117) to generate 17 kD mature IL-1β.
**Figure 4.3** Sustained nigericin-induced ASC oligomerization, propidium uptake, and cell viability in WT and Casp1/11⁻/⁻ BMDC

(A) Detergent-insoluble lysates from WT and Casp1/11⁻/⁻ BMDC treated with LPS and nigericin as described in (A) were cross-linked with disuccinimidyl suberate (DSS), and samples were run on a 12% polyacrylamide gel. Western blot analysis for detection of monomeric (mono.), dimeric (di.), and oligomeric (oligo.) ASC was performed.  

(B) Propidium dye influx was assessed in WT and Casp1/11⁻/⁻ BMDC treated with LPS (4 h) followed by 4 h of nigericin (arrow indicates the addition of nigericin). Propidium dye fluorescence is expressed as a percentage of maximal plasma membrane permeablization with triton X-100 treatment for each condition. The results are a representative graph of 2 identical experiments performed.  

(C) The viability of WT and Casp1/11⁻/⁻ BMDC stimulated as described in (A) was measured using the redox potential indicator dye, alamar blue. Results are the mean ± SE of 2-3 experiments.
Figure 4.3
Figure 4.4 Sustained nigericin-induced processing and release of IL-1β is dependent on NLRP3 and ASC and correlates with caspase-8 activation in murine BMDC

WT, Asc−/−, and Nlrp3−/− BMDC were primed with LPS (100 ng/ml) for 4 h before stimulation with nigericin for 0.5 h, 2 h, 4 h, or 6 h. The extracellular media and cell lysates were collected and processed for western blot analysis for detection of IL-1β, caspase-1, and caspase-8. The data are representative of results from 3 separate experiments.
Figure 4.4
Figure 4.5 Sustained nigericin-induced IL-1β release, ASC oligomerization, and loss of cell viability in WT, Nlrp3<sup>−/−</sup> and Asc<sup>−/−</sup> BMDC

(A) BMDC were stimulated as in (A), and the extracellular medium was collected and assayed for IL-1β by ELISA. BMDC were primed with LPS for 5.5 h prior to nigericin stimulation for 30 min. Results are the mean ± SEM of 3 experiments. (B) Detergent-insoluble lysate fractions were cross-linked with DSS, and ASC oligomerization was assayed by Western blot analysis in WT, Asc<sup>−/−</sup>, and Nlrp3<sup>−/−</sup> BMDC stimulated with LPS alone or LPS plus a 0.5 h, 2 h, 4 h, or 6 h nigericin stimulus. (C) BMDC were primed with LPS in low-serum-containing medium (0.1% calf serum) followed by stimulation with nigericin either in a NaCl-based buffered saline solution (BSS) or in a high K<sup>+</sup>-containing (130mM KCl) BSS, and the amount of IL-1β released was quantified by ELISA. Results are from a single experiment with each condition performed in triplicate. (D) The viability of WT, Asc<sup>−/−</sup>, and Nlrp3<sup>−/−</sup> BMDC stimulated with LPS (4 h) or LPS (4h) followed by a 3-4 h nigericin stimulus was assayed using the redox potential indicator dye, alamar blue. Viability is expressed as a percentage relative to untreated BMDC. Results are the mean ± SE of 7 independent wells per condition from 2 experiments. ***P < .001 by ANOVA.
Figure 4.5
verified the absence of ASC oligomerization with both acute and sustained nigericin stimulation in Nlrp3\(^{-/-}\) and ASC\(^{-/-}\) BMDC (Fig. 4.5B).

Because a reduction in cytosolic \([K^+]\) is a well-characterized signal for NLRP3 inflammasome assembly, we assayed the caspase-1-independent IL-1\(\beta\) production response to nigericin in a test medium containing increased extracellular \(K^+\) (130 mM KCl versus the physiological 5 mM KCl) to disrupt the normal gradient for \(K^+\) efflux. The ability of high \(K^+\) medium to completely suppress IL-1\(\beta\) release in both WT and Casp1/11\(^{-/-}\) BMDC during acute or sustained nigericin stimulation supports the involvement of a common NLRP3 inflammasome platform in both cell types (Fig. 4.5C). The metabolic viability of WT BMDC treated with LPS + nigericin (but not LPS only) decreased within 3-4 h, but this was not observed in the absence of either NLRP3 or ASC (Fig. 4.5D). Thus, the decrease in BMDC viability during acute or sustained stimulation with nigericin is critically dependent on NLRP3 and ASC (65).

**IL-1\(\beta\) release during sustained nigericin stimulation is similarly reduced by the absence of caspase-1 or caspase-8 expression and the pharmacological inhibition of either caspase**

We hypothesized that caspase-8 was the alternative IL-1\(\beta\) processing enzyme in nigericin-treated Casp1/11\(^{-/-}\) BMDC given its robust activation upon sustained nigericin stimulation (> 2 h) (Fig. 4.1B) and absence of activation in Nlrp3\(^{-/-}\) and Asc\(^{-/-}\) BMDC (Fig. 4.4). We assessed the relative contributions of caspase-1 versus caspase-8 during sustained (6 h) nigericin stimulation by comparing IL-1\(\beta\) release in LPS-primed WT, Casp1/11\(^{-/-}\), and Casp8\(^{-/-}\)Rip3\(^{-/-}\) BMDC in the absence or presence of pharmacological
Figure 4.6 YVAD and IETD concentration-response curves for nigericin-induced IL-1β processing and release in LPS-primed WT, Casp1/11−/−, and Casp8−/− Rip3−/− BMDC

(A, B) WT, Casp1/11−/−, and Casp8−/− Rip3−/− BMDC were primed with LPS (100 ng/ml) for 4 h and then stimulated with nigericin (10 µM) for 6 h in the presence or absence of varying doses of the caspase-1 inhibitor, YVAD, or the caspase-8 inhibitor, IETD, and the extracellular medium was collected and assayed for IL-1β by ELISA. Results are the mean ± SE from 6 independent wells for each condition and expressed as a percentage of WT BMDC treated with LPS + nigericin (6h). ***P < .001 or not significant (n.s) by ANOVA. (C, D) BMDC were treated exactly as described in Figs. 3A-B, but IL-1β release is expressed as a percentage of each genotype treated with LPS + nigericin (6h). (E, F) The kinetics of IL-1β release were assayed by ELISA from LPS-primed (100 ng/ml) and nigericin-stimulated WT and Casp1/11−/− BMDC in the presence or absence of YVAD (10 µM) or IETD (20 µM). Results are the mean ± SE of 3 experiments.
inhibitors that target caspase-1 (YVAD-fmk) or caspase-8 (IETD-fmk). In Fig. 4.6A and B, the absolute magnitudes of IL-1β release in the three DC genotypes are normalized to the maximal response in WT cells in the absence of YVAD or IETD. In Fig. 4.6C and D, the same data are plotted with each genotype’s response normalized to the maximal response for that genotype in the absence of YVAD or IETD. The genetic absence of either caspase-1 or caspase-8 produced similar reductions (60-70%) in the IL-1β release response to sustained (6 h) nigericin stimulation (Fig. 4.6A), thus indicating contributions of both caspases to the overall IL-β maturation process. Notably, the potency of YVAD was markedly greater in WT and Casp8<sup>−/−</sup>Rip3<sup>−/−</sup> BMDC (IC<sub>50</sub> < 1 µM) than in Casp1/11<sup>−/−</sup> BMDC (IC<sub>50</sub> ~ 10 µM) (Fig. 4.6A and C). The ability of higher YVAD concentrations to further suppress IL-1β release in the Casp1/11<sup>−/−</sup> cells indicated cross-inhibition of caspase-8 activity with increasing [YVAD] (Fig. 4.6C). In contrast to the differential potency of YVAD in WT and Casp8<sup>−/−</sup>Rip3<sup>−/−</sup> cells versus Casp1/11<sup>−/−</sup> cells, the potency and efficacy of IETD in suppressing nigericin-stimulated IL-1β release (IC<sub>50</sub> ~ 5 µM) was similar in all three genotypes of BMDC (Fig. 4.6B and D). As with the inhibitory actions of YVAD in caspase-1-null DC, the ability of IETD to further suppress IL-1β release in the caspase-8-null DC indicated cross-inhibition of caspase-1 activity with increasing [IETD] (Fig. 4.6D). However, IETD was more potent and efficacious than YVAD in inhibiting the IL-1β release response in the Casp1/11<sup>−/−</sup> BMDC (compare Fig. 4.6C and D).

The Fig. 4.6 concentration-response data with Casp8<sup>−/−</sup>Rip3<sup>−/−</sup> and Casp1/11<sup>−/−</sup> BMDC allowed us to select concentrations of YVAD (10 µM) and IETD (20µM) that,
respectively, produced at least 80% suppression of the IL-1β release driven by caspase-1 (in the absence of caspase-8) or by caspase-8 (in the absence of caspase-1). We used YVAD and IETD at these concentrations to further characterize the roles of caspase-8 in regulating nigericin-stimulated NLRP3 inflammasome signaling in the presence or absence of caspase-1. Multiple indices of NLRP3 inflammasome activity stimulated by acute (30 min) or sustained (6 h) nigericin treatment in WT BMDC were markedly and similarly attenuated in the presence of 20 µM IETD or 10 µM YVAD; these included release of total IL-1β (by ELISA, Figs. 4.6E and F), release of 17 kD mature IL-1β (by western blot, Fig. 4.7B), and extracellular accumulation of caspase-1 p20 subunit (Fig. 4.7B). The ability of 20 µM IETD to attenuate the rapidly induced phase of NLRP3-dependent IL-1β processing and release in WT cells was not due to suppression of proximal ASC oligomer formation (Fig. 4.7A). Indeed, similarly rapid accumulation of ASC oligomers in response to nigericin was observed in WT and Casp1/11−/− BMDC in the absence or presence of 20 µM IETD, 10 µM YVAD, or 50 µM zVAD pan-caspase inhibitor. This indicates that suppression of caspase-8 enzymatic activity in WT cells attenuates IL-1β processing at the level of caspase-1 activation rather than NLRP3 inflammasome assembly. Notably, treatment of Casp1/11−/− BMDC with 20 µM IETD (Figs. 4.6E and 4.7B), but not 10 µM YVAD (Figs. 4.6F and 4.7B), produced complete suppression of nigericin-stimulated IL-1β release as measured by ELISA or western blot. IETD, but not YVAD, also suppressed the early intracellular generation of the 29 kD IL-1β cleavage product in nigericin-stimulated Casp1/11−/− cells (Fig. 4.7B). Intracellular accumulation of mature p17 IL-1β was observed at 1 h post-nigericin stimulation in
Figure 4.7  Caspase-8 activates caspase-1 in WT BMDC and acts as an IL-1β convertase in Casp1/11−/− BMDC

(A) Detergent-insoluble lysate fractions were cross-linked with DSS, and ASC oligomerization was assayed in WT and Casp1/11−/− BMDC stimulated with LPS alone (5 h) plus 30 min nigericin in the presence or absence of IETD (20 µM), YVAD (10 µM), and zVAD (50 µM). Results are representative of 2 similar experiments performed. (B) LPS-primed WT or Casp1/11−/− BMDC were stimulated with nigericin for 0.5 h or 6 h in the presence or absence of YVAD (10 µM) or IETD (20 µM), and western blot analysis of IL-1β, caspase-1, and caspase-8 from cell lysates and extracellular supernatants was performed. BMDC treated with 30 min of nigericin were LPS-primed for 5.5 h. Results are representative of 3 experiments performed.
Figure 4.7
Figure 4.8 Nigericin induces intracellular accumulation of 29 kD proIL-1β in LPS- and Pam₃CysK₄-primed Casp1/11⁻/⁻ BMDC and subsequent generation of mature intracellular 17 kD IL-1β and export only in LPS-primed BMDC.

BMDC were primed with LPS or Pam₃CysK₄ for 4 hr prior to stimulation with nigericin for 0.5 h, 1 h, 2 h, or 4 h. Western blot analysis was performed and probed for IL-1β.
Casp1/11−/− BMDC, but release of p17 IL-1β to the extracellular compartment required an additional 60 min of incubation (Fig. 4.8).

The western blot analyses of intracellular caspase-8 levels during nigericin stimulation illustrated in Figs. 4.1B, 4.4, and 4.7B utilized standard whole cell lysates prepared by detergent extraction followed by brief centrifugation to sediment the nuclei and insoluble cytoskeleton. Because oligomerized ASC complexes in nigericin-stimulated BMDC distribute into this detergent-insoluble fraction (Fig. 4.9A), we reasoned that caspase-8 recruited to such ASC complexes might be also be present in this compartment. Fig. 4.9B shows that procaspase-8 is localized in both the detergent-soluble and detergent-insoluble lysate fractions of LPS-primed WT and Casp1/11−/− BMDC. Nigericin stimulation induced significant redistribution of procaspase-8 from these intracellular compartments to the extracellular medium, and this was correlated with accumulation of the p18 subunit of cleaved caspase-8. The extracellular accumulation of caspase-8/p18 was enhanced in the nigericin-stimulated Casp1/11−/− cells and temporally correlated with near-complete loss of procaspase-8/p55 from the detergent-insoluble compartment and accumulation of 45 kDa caspase-8 in that fraction. 20 μM IETD by itself did not alter the distribution of caspase-8 in the various intracellular or extracellular fractions of LPS-primed BMDC but did completely suppress the nigericin-stimulated production of extracellular caspase-8/p18. The presence of 50 μM zVAD licensed necroptosis in LPS-treated BMDC (both WT and Casp1/11−/−) as indicated by the near complete redistribution of procaspase-8 into the detergent-insoluble intracellular and the extracellular compartments. The LPS+zVAD-treated cells were also characterized by a prominent accumulation of the detergent-insoluble 45 kD caspase-8
**Figure 4.9** Caspase-8 and ASC localize to a detergent-insoluble lysate compartment

WT and Casp1/11−/− BMDC were treated with LPS (4 h) and nigericin (6 h) in the presence or absence of zVAD (50 μM) or IETD (20 μM). The extracellular media, detergent-soluble lysate fractions, and detergent-insoluble lysate fractions were collected and processed for western blot analysis of ASC (A) and caspase-8 (B). Results are from a representative experiment.
Figure 4.9
product. Our observation that 20 μM IETD did not recapitulate these latter effects of zVAD indicated IETD at this concentration did not license necroptosis in the LPS-primed DC. Taken together, these analyses based on genetic, pharmacological, and biochemical approaches support a role for caspase-8 in the regulation of nigericin-stimulated IL-1β release in either the presence or absence of caspase-1.

**IL-1β processing, caspase-1-activation, and cell death in nigericin-stimulated WT, Casp8−/− Rip3−/− and Rip3−/− murine DC**

We next compared the response to acute (30 min) and sustained (6 h) nigericin stimulation in WT, Casp8−/−Rip3−/− and Rip3−/− BMDC by western blot analysis. Caspase-8-knockout mice are embryonic lethal due to unrestrained necroptosis and require co-deletion of RIP3 to rescue viability (76, 92). We observed attenuated mature 17 kD IL-1β release in Casp8−/−Rip3−/− BMDC compared to WT and Rip3−/− BMDC in response to both acute and sustained nigericin stimulation (Figs. 4.10A and 4.6A/B). Not surprisingly, similar caspase-1 activation, as measured by the release of the enzymatically active p20 caspase-1 subunit (Fig. 4.10A) and as measured by nearly equivalent influxes of propidium iodide (Fig. 4.11A), occurred in Casp8−/−Rip3−/− as in WT and Rip3−/−, thus revealing that caspase-1 acts as the IL-1β convertase in caspase-8-deficient cells. For further characterization of inflammasome activation, western blot analysis of detergent-insoluble lysate fractions (± DSS) showed that both procaspase-8, ASC, and to a lesser extent, caspase-1, relocalize from the detergent-soluble lysate fraction to the detergent-insoluble pellet fraction in LPS primed and nigericin (and zVAD)-stimulated WT BMDC (Fig. 4.10B). We probed for NLRP3 in the pellet but did not observe any signal (data not
Figure 4.10  IL-1β processing and ASC oligomerization in LPS-primed and nigericin/zVAD stimulated WT, Casp8−/− Rip3−/− and Rip3−/− murine BMDC

(A) WT, Casp8−/− Rip3−/− and Rip3−/− BMDC were LPS primed for 4 hr and then stimulated with either nigericin for 0.5 h or 6 h or stimulated with the pan-caspase inhibitor, zVAD, for 3h. The extracellular media and detergent-soluble cell lysates were collected and processed for western blot analysis for detection of IL-1β, caspase-1, caspase-8, and ASC. Results are representative of 2 experiments performed.  (B) Cells were stimulated as in (A) and detergent-insoluble lysates (± DSS) were collected and processed for detection of monomeric, dimeric, and oligomeric ASC.
Figure 4.10
shown). Moreover, we observed a diminution in ASC oligomerization in $\text{Casp8}^{-/-} \text{Rip3}^{-/-}$ BMDC compared to WT and $\text{Rip3}^{-/-}$ BMDC (Fig. 4.10B), which was enhanced in the acute nigericin stimulation. With sustained nigericin stimulation in $\text{Casp8}^{-/-} \text{Rip3}^{-/-}$ BMDC, ASC oligomer formation was comparable to WT BMDC.

In assaying the responses of $\text{Casp8}^{-/-} \text{Rip3}^{-/-}$ and $\text{Rip3}^{-/-}$ BMDC, we utilized the necroptotic stimulus, LPS + zVAD (3 h), to gauge cell death as a functional positive control for $\text{Rip3}^{-/-}$ BMDC. Necroptotic signaling involves the activation of RIP3, which leads to MLKL and PGAM5 activation to induce mitochondrial destabilization (71). Additionally, MLKL has been shown to insert into the plasma membrane, as well as the mitochondrial membrane, during necroptosis, forming a permeation pore (120). As a consequence, cellular ionic disequilibrium precedes the necroptotic morphological characteristics of cell swelling, lysis, and release of intracellular contents, including unprocessed proIL-1$\beta$ (Fig. 4.10A). The release of 31kD proIL-1$\beta$ in response to LPS +zVAD was substantially reduced in $\text{Casp8}^{-/-} \text{Rip3}^{-/-}$ and $\text{Rip3}^{-/-}$ BMDC compared to WT BMDC because of the inability of the cells to necroptose. Also, we observed equivalent propidium influx profiles of WT, $\text{Casp8}^{-/-} \text{Rip3}^{-/-}$, and $\text{Rip3}^{-/-}$ BMDC after 30 min of LPS + nigericin stimulation, indicating that caspase-1 is activated (Fig. 4.11A). As shown in Fig. 4.11B, while WT BMDC displayed a decrease in viability with sustained LPS + nigericin stimulation (comparable to Fig. 4.3C) due to pyroptosis, $\text{Casp8}^{-/-} \text{Rip3}^{-/-}$ BMDC, surprisingly, remained viable, and $\text{Rip3}^{-/-}$ BMDC displayed an intermediate loss of viability.
Figure 4.11 Propidium influx and cell viability in LPS-primed and nigericin/zVAD stimulated WT, Casp8$^{-/-}$ Rip3$^{-/-}$ and Rip3$^{-/-}$ murine dendritic cells

(A) WT, Casp8$^{-/-}$ Rip3$^{-/-}$ and Rip3$^{-/-}$ BMDC were LPS primed and stimulated with nigericin (10 µM) for 40 min (arrow indicates the addition of nigericin), and the influx of propidium was quantified by fluorescence. (B) WT, Casp8$^{-/-}$ Rip3$^{-/-}$, and Rip3$^{-/-}$ BMDC were primed with LPS (100 ng/ml) for 4 h prior to stimulation with nigericin (10 µM) for 1 h, 2 h, 4 h, or 6 h. Cell viability was assayed using alamar blue, an indicator of mitochondrial redox metabolism. Results are representative of 3 similar experiments performed.
Figure 4.11
**TLR2 priming followed by nigericin stimulation and TLR4 priming followed by alum stimulation each induce caspase-1-independent IL-1β processing**

Next, we asked whether the NLRP3/ASC/caspase-8-dependent mechanism of IL-1β processing occurred with other signal 1 and signal 2 inflammasome stimuli. Hence, we utilized the TLR2 lipopeptide, Pam₃CysK₄ (4 h), to stimulate NF-κB signaling followed by nigericin, and indeed, IL-1β processing was observed by ELISA in Casp1/11⁻/⁻ BMDC, albeit on a smaller scale than with LPS priming (Fig. 4.12A). The export of mature IL-1β was observed in Casp1/11⁻/⁻ BMDC primed with LPS but not with Pam₃CysK₄ (Fig. 4.12B). Given that the nigericin stimulus takes hours to induce IL-1β processing in Casp1/11⁻/⁻ BMDC, we chose the NLRP3 inflammasome stimulus, alum (6 h), with which to compare to nigericin stimulation. We also observed a caspase-1-independent processing of IL-1β induced by alum (Fig. 4.12C). Alum stimulation varies from nigericin in that it induces lysosomal destabilization and cell death in response to phagocytosis of alum particulates versus nigericin, which triggers dissipation of the normal trans-plasma membrane K⁺ gradient within minutes. The viability of alum-stimulated (6 h) LPS-primed BMDC, as measured by the alamar blue reagent, did not vary from LPS-only treated BMDC in either WT or Casp1/11⁻/⁻ BMDC (Fig. 4.12D).

Overall, this caspase-8-dependent pathway can be triggered with other signal 1 and signal 2 stimuli and requires hours to engage the pathway in caspase-1-deficient DCs.
**Figure 4.1** TLR2 priming followed by nigericin stimulation and TLR4 priming followed by alum stimulation each induce caspase-1-independent IL-1β processing

(A) WT and Casp1/11−/− BMDC were primed with Pam3CSK4 (2 µg/ml) or LPS (100 ng/ml) for 4 h prior to stimulation with nigericin for 0.5 h or 6 h. Results are the mean ± SE from 3 independent wells for each condition. (B) WT and Casp1/11−/− BMDC were stimulated as described in (A), and western blot analysis was performed for the detection of IL-1β and caspase-1. (C) WT and Casp1/11−/− BMDC were primed with LPS (100 ng/ml) for 4 h prior to stimulation with alum (240 µg/ml, n=2 and 480 µg/ml, n=3) for 6 h, and the extracellular medium was collected and assayed for IL-1β by ELISA. Results are expressed as the mean ± SE of 5 experiments. (D) WT and Casp1/11−/− BMDC were primed with LPS and either stimulated or not with alum (480 µg/ml) for 6 h. Mitochondrial redox metabolism was measured using alamar blue. Results are representative of one experiment.
Figure 4.12
Discussion

This study describes a mechanism in which nigericin induces the NLRP3/ASC-dependent activation of caspase-8 for IL-1β processing in the genetic absence of the canonical ICE, caspase-1. During sustained nigericin stimulation (>2 h) in Casp1/11−/− BMDC, IL-1β processing and release occurred simultaneously with the activation and release of mature caspase-8 (18 kD) (Figs. 4.1 and 4.3). This release required the presence of ASC and NLRP3 based on the observations that no mature IL-1β was produced and secreted during sustained nigericin stimulation in Nlrp3−/− and Asc−/− BMDC (Fig. 4.4 and 4.5). Nigericin-induced IL-1β production in LPS-primed Casp1/11−/− BMDC was attenuated in the presence of the caspase-8 inhibitor, IETD, while nigericin-induced IL-1β release was suppressed in LPS-primed Casp8−/−Rip3−/− BMDC, implicating a caspase-8 signaling platform for IL-1β processing in TLR4-activated DCs (Figs. 4.6, 4.7, 4.9 and 4.10). Nigericin stimulation in TLR4-primed BMDC induced: 1) recruitment of ASC and caspase-8 to a detergent-insoluble compartment in both WT and Casp1/11−/− BMDC (Fig. 4.7C); 2) caspase-8 activation and IL-1β release in Casp1/11−/− BMDC (6 h nigericin); 3) caspase-1 activation and IL-1β release WT BMDC (6 h nigericin); and 4) apoptosis in Casp1/11−/− BMDC versus pyroptosis in WT BMDC (Fig. 4.3C). Importantly, the catalytic activity of caspase-8 did not affect ASC oligomerization in WT BMDC following LPS and nigericin stimulation, but in the presence of IETD, the accumulation of active caspase-1 was suppressed, indicating that active caspase-8 may act at the level of caspase-1 activation leading to IL-1β processing rather than the assembly of the NLRP3/ASC inflammasome. The vaccine adjuvant, alum, another well-
defined NLRP3 activator, also induced IL-1β processing in LPS-primed Casp1/11−/− BMDC (Fig. 4.12C). Taken together, this caspase-1-independent inflammasome platform is engaged with diverse, sustained stimuli and may serve as an alternative pathway by which IL-1β can be processed in the absence of caspase-1. Most importantly, this alternative pathway further unveiled the modulatory role of caspase-8 in caspase-1-competent cells as an activator of caspase-1 as has been recently reported with Yersinia infection models (57, 58).

*Biophysical recruitment of caspase-8 and caspase-1 to an NLRP3/ASC inflammasome and caspase-8-induced caspase-1 activation*

Herein, we report that ASC and caspase-8 are both recruited to detergent-insoluble compartments upon LPS priming and nigericin (6 h) stimulation. However, during short-term, canonical inflammasome activation (LPS + nigericin 0.5 h), ASC also relocalizes to the detergent-insoluble lysate compartment along with procaspase-1, proIL-1β and procaspase-8 (Fig. 4.7). This mobilization of caspase-8 during canonical inflammasome activation may reflect the biophysical configuration of the inflammasome assembly process, as has been previously reported, suggesting that not only is caspase-1 recruited to the inflammasome, but also caspase-8 (59). It is evident that if caspase-1 is available to process IL-1β, then it will act as the predominant ICE. However, when caspase-1 is genetically absent, caspase-8 acts as the ICE to proteolytically process proIL-1β into its biologically active form. Studies by various other groups have characterized the formation of large, prion-like inflammasome structures (34, 35) and defined the interaction between the PYD of ASC and the DED of caspase-8 (65),
substantiating that the ASC inflammasome is able to recruit caspase-8 to the complex (Fig. 1.3). Also, in the genetic absence of caspase-8, ASC oligomer formation is not as efficient with a 0.5 h nigericin stimulus compared to WT BMDC, while prolonged nigericin stimulation (6 h) in caspase-8-deficient BMDC appears to mimic WT BMDC (Fig. 4.10B). This observation suggests that either pro-caspase-8 acts as a signaling protein for NLRP3 inflammasome assembly, which is consistent with what other groups have reported (63), or there may be a reduction in NLRP3 levels. However, pharmacological inhibition of caspase-8 activity had no effect on ASC oligomer formation, emphasizing the pleiotropic role of caspase-8 as an ICE and pro-caspase-8 as a signaling molecule. In addition, the data (Figs. 4.6 and 4.7) points toward the paradigm proposed by Weng et al. and Philip et al. whereby caspase-8 activates caspase-1 (57, 58).

An important question to consider is why it requires on the order of hours (> 2 h) to engage the caspase-8 inflammasome pathway in Casp1/11−/− BMDC. The role of caspase-8 in extrinsic cell death-mediated pathways and/or its modulation of NF-κB-dependent translational signaling may alter the dimerization of caspase-8 with non-catalytic caspase-8 paralogs, cFLIP’s, in the cytosol. Consequently, it may take this length of time (> 2 h) for caspase-8 to dissociate from its interacting partners, relocalize, and/or to be recruited in sufficient quantities to the NLRP3/ASC inflammasome complex in Casp1/11−/− BMDC, while WT BMDC may require only a basal amount of caspase-8 expression in order to activate caspase-1.

An additional unique feature of the NLRP3/ASC/caspase-8 inflammasome complex includes the accumulation of 29 kD pro-IL-1β. As shown in Figs. 4.1B and 4.8,
29 kD proIL-1β is present with LPS priming alone in both WT and Casp1/11−/− BMDC, but the appearance of the 29 kD band is more pronounced with extended nigericin stimulation in Casp1/11−/− BMDC. Taken together, western blot data show that 29 kD proIL-1β is generated first followed by subsequent cleavage to generate intracellular 17 kD IL-1β in Casp1/11−/− BMDC one hour post nigericin stimulation (Figs. 4.7B and 4.8). Upon close examination and comparison of the relative amounts of proIL-1β and mature IL-1β intracellularly and extracellularly, there are varying levels within the two compartments. It appears that proIL-1β intracellularly far exceeds the amount that is processed and released to the extracellular environment. Recent studies have discussed the ubiquitination and proteasome-mediated degradation of proIL-1β (122, 123), which may also apply to NLRP3. During sustained nigericin stimulation, we did not detect the presence of NLRP3 in the detergent-insoluble lysate compartment (data not shown), potentially due to proteasome-mediated degradation of NLRP3. Similar experiments were performed with alum as the secondary inflammatory stimulus; however, the amount of mature IL-1β was significantly less than with nigericin, and no caspase-8 processing was observed by western blot (Fig. 4.11A and B), possibly due to cathepsin-mediated protein degradation. Alum stimulation is known to induce lysosomal destabilization and the release of cathepsins to activate the NLRP3 inflammasome and process proIL-1β in WT BMDM. The use of pharmacological inhibitors was assayed with alum stimulation, and in the presence of IETD, alum-induced IL-1β release was suppressed in Casp1/11−/− BMDC (data not shown).
**Interconnection between inflammation and cell death**

In this study, various genetic knockout models were utilized and each genetic background dictated the type of cell death pathway engaged by the same stimulus (LPS + Nig or LPS + zVAD). In the genetic absence of caspase-1, nigericin-treated LPS primed BMDC lost viability as measured by alamar blue, but instead of undergoing pyroptosis (caspase-1-dependent cell death) as the WT BMDC did, they underwent apoptosis (caspase-3/7-dependent cell death), consistent with Sagulenko et al. (Fig. 4.3C) (65). In addition, propidium uptake was observed in both WT and Casp1/11+/− BMDC, but Casp1/11+/− BMDC influx was delayed by 2-4 h compared to WT BMDC (Fig. 4.3B). The fact that propidum influx still occurred in Casp1/11+/− BMDC suggests that another catalytically active enzyme, possibly caspase-8, may be responsible for the gating of the propidium permeable channel in the genetic absence of caspase-1. If caspase-8 can substitute for caspase-1 for the purpose of IL-1β processing, then caspase-8 may induce an alternative form of cell death that mimics pyroptosis, potentially a caspase-8-mediated pyroptotic pathway. However, preliminary data from our lab using zVAD to monitor propidium uptake in response to LPS and nigericin in Casp1/11+/− BMDC is not inhibited. Further studies using IETD and specialized genetic models (see Pathophysiological relevance) must be performed.

The necroptotic stimulus, LPS + zVAD, was utilized as a positive control for RIP3-knockout BMDC, and indeed, the necroptotic release of extracellular proIL-1β that was observed in WT BMDC was suppressed in Casp8+/− Rip3+/− and Rip3+/− BMDC (Fig. 4.10A). Propidium fluorescence was observed in all three genotypes treated with LPS
and nigericin (30 min) (Fig. 4.11A). Interestingly, only in WT BMDC did propidium uptake begin approximately 40 minutes post LPS+ nigericin stimulation in the presence of zVAD, suggestive of a putative necroptosis-mediated channel because no propidium influx was observed in Casp8\(^{-/-}\) Rip3\(^{-/-}\) and Rip3\(^{-/-}\) BMDC, and the cells cannot undergo pyroptosis or apoptosis in the presence of zVAD.

**Necroptosis and inflammasome signaling**

Necroptosis is a coordinately regulated cell death pathway, which converges on the activation of RIP1/RIP3 signaling proteins to activate mitochondrial fission and cell death. Necroptosis can be induced in macrophages and DCs with LPS treatment followed by simultaneous caspase-8 inhibition with the pan-caspase inhibitor, zVAD, or caspase-8 selective inhibitor, IETD. Progression to necroptosis involves cellular swelling, as a result of ionic disequilibrium, and induces the release of intracellular molecules to the extracellular environment. The efflux of K\(^+\) from myeloid cells is a well-characterized mechanism for inflammasome activation. The observation that LPS + zVAD induced ASC oligomerization in WT BMDC suggests that necroptosis-induced K\(^+\) efflux induces inflammasome activation (Fig. 4.13). Inhibition of RIPK1 with necrostatin-1 resulted in abrogation of ASC oligomerization in WT BMDC, implicating necroptosis in inducing ASC oligomerization. ASC oligomerization was also suppressed in necroptosis-induced Nlrp3\(^{-/-}\) BMDC. The disruption of ionic equilibrium, including K\(^+\) efflux, may act as a “recognition pattern” similar to a PAMP for its PRR. Recently, cytosolic delivery of the synthetic TLR3 dsRNA ligand, Poly I:C, induced MAVS-dependent K\(^+\) efflux and consequent activation of the NLRP3 inflammasome (121), a prime example of how changes in cytosolic ionic milieu trigger the assembly and
activation of inflammatory signaling complexes. This indicates that ASC oligomerization occurs in response to necroptotic induction, presumably due to the efflux of K⁺ ions. Treatment of BMDC with LPS + zVAD in the presence of high K⁺-containing medium and assaying ASC oligomerization by western blot analysis would indicate the plausibility of this presumption. Also, it would be important to quantify levels of intracellular K⁺ using atomic absorbance spectroscopy following LPS + zVAD stimulation in WT BMDC to determine if there is a correlation between ASC oligomerization and decreases in intracellular K⁺ during necroptotic induction.

Pathophysiological relevance

During caspase-1 inhibition with YVAD in WT BMDC (Fig. 4.7B), 29 kD proIL-1β was not generated intracellularly following LPS + acute nigericin (0.5 h) treatment as in Casp1/11⁻/⁻ BMDC. On one hand, generation and release of active caspase-8 was suppressed during pharmacological inhibition of caspase-1 with YVAD in WT BMDC, and on the other hand, generation and release of active caspase-1 was suppressed during pharmacological inhibition of caspase-8 with IETD in WT BMDC (Figs. 4.7B and C), indicating cross-selectivity of YVAD and IETD for their specified target caspases. Notably, in the genetic absence of caspase-8, caspase-1 can still be activated, albeit at a
**Figure 4.13** Necroptosis induces inflammasome assembly in murine dendritic cells

WT and *Nlrp3*-/- BMDC were primed with LPS for 4 h followed by stimulation with zVAD (50µM) or nigericin (10 µM) for 3 h in the presence of absence of the RIPK1 inhibitor, necrostatin-1 (Nec-1), applied 30 min before the zVAD or nigericin stimulus. Detergent-insoluble cell lysates were crosslinked with DSS and ASC oligomerization was detected by western blot analysis.
lesser degree than in WT BMDC. Approximately 1/3 the amount of IL-1β was produced by caspase-8-knockout BMDC compared to WT BMDC stimulated with LPS and nigericin (6 h) as quantified by ELISA (Fig. 4.6A and B). The ideal approach would include the utilization of a Casp1−/−Casp8−/−Rip3−/− triple knockout genetic model and/or siRNA-mediated knockdown of caspase-1 in Casp8−/−Rip3−/− BMDC and siRNA-mediated knockdown of caspase-8 in Casp1−/− BMDC to compare the IL-1β responses in all three genetically targeted manipulations. Moreover, we observed enhanced potency of IETD for WT (IC₅₀ ~ 3 µM) versus Casp1/11−/− BMDC (IC₅₀ ~ 6 µM), potentially due to caspase-8’s role as an ICE, caspase-1 activator, and/or unclear role in the priming stage of NLRP3 inflammasome signaling, as reported by Gurung et al. (63). Furthermore, in the genetic absence of caspase-8, ASC oligomerization was not as efficient compared to WT or Rip3−/− BMDC following acute nigericin stimulation, indicating that caspase-8 may additionally modulate inflammasome assembly upstream of IL-1β processing.

Overall, we are beginning to uncover the multiple functions of procaspase-8 and caspase-8 in inflammatory signaling, while many of the same proteins that participate in cell death/necroptotic signaling platforms are also recruited to cytosolic inflammatory complexes that converge upon IL-1β processing (e.g FADD and cFLIP). Knowing the various pathways by which IL-1β is processed and released will be important in IL-1R-mediated pharmaceutical drug targeting.
CHAPTER 5

Superoxide Dismutase in Inflammasome Activation

Introduction

Chapter 3 focused on characterizing the caspase-8-dependent pathway for IL-1β processing and secretion in BMDC stimulated with LPS and doxorubicin. In this section, I present my preliminary work on determining the effect of the small molecule anti-lung cancer compound, LCS-1, on IL-1β processing in murine DCs. This drug has been shown to reduce the growth of lung adenocarcinoma cell lines via inhibition of cytoprotective superoxide dismutase 1 (SOD1) signaling. SOD1 is a metalloenzyme that catalyzes the dismutation of superoxide radicals into molecular oxygen and water to eliminate reactive oxygen species (ROS). Meissner et al. demonstrated that caspase-1 is reversibly inhibited by glutathionylation of cysteine residues in SOD1+/− macrophages stimulated with LPS and nigericin, indicating that SOD1 activity regulates caspase-1 activation (124). Interestingly, under hypoxic conditions, glutathionylation of redox-sensitive cysteine residues, Cys397 and Cys362, of caspase-1 was reversed.

Results

In the following experiments, I assessed the ability of LCS-1, a recently identified small molecule inhibitor of SOD1, to inhibit caspase-1 activity in BMDC (125). Post-translational modification (e.g., glutathionylation) of caspase-1 leading to caspase-1 inhibition provides a physiological context in which caspase-1 activity is regulated. In utilizing LCS-1, I additionally wanted to assess the effect of this cancer drug on both
canonical caspase-1 signaling and non-canonical caspase-8 signaling, which mediate IL-1β processing. Initial characterization included the use of an assay for pyrptotic channel activation using the propidium dye influx assay as an index of caspase-1 activation following stimulation of WT BMDC with LPS and nigericin in the presence or absence of LCS-1. Figure 5.1A shows a dose-dependent decrease in propidium influx with LCS-1. For subsequent assays, a concentration of 10 μM LCS-1 was utilized to effectively inhibit caspase-1. A summer medical student, Kerly Guerrero, assisted me in verifying that LCS-1 inhibits SOD activity at the indicated concentration of 10 μM LCS-1 using a SOD activity assay (data not shown). LCS-1 also inhibited IL-1β secretion induced by 30 min or 6 h of nigericin stimulation in WT and Casp1/11−/− BMDC (Fig. 5.1B and C). However, LCS-1 inhibited IL-1β secretion in Casp1/11−/− BMDC treated with nigericin for 6 h with increased potency compared to WT BMDC treated with nigericin for 30 min or 6 h (IC50 of 1 μM versus 3 μM). This difference in effective inhibition suggested that LCS-1 also inhibits caspase-8 activation because of Chapter 4 findings that caspase-8 acts as an IL-1β convertase in the genetic absence of caspase-1. Western blot analysis (Fig. 5.2) indeed confirmed my hypothesis; LCS-1 inhibited caspase-8 processing and consequently, caspase-8-mediated IL-1β maturation in Casp1/11−/− BMDC stimulated with LPS and nigericin (6h). Additionally, I examined the effect of LCS-1 on ASC oligomer formation, and the presence of LCS-1 inhibited ASC oligomerization in response to LPS and 30 min of nigericin stimulation (data not shown).
**Figure 5.1** LCS-1 inhibits nigericin-induced propidium influx and both caspase-1 and caspase-8-mediated IL-1β processing pathways in BMDC

(A) WT BMDC were primed with LPS for 4 hr and LCS-1 (1, 3, 10, 30 μM) was added 10-20 min prior to nigericin stimulation for 30 min followed by permeabilization of all cell membranes with triton X-100. (B, C) WT and Casp1/11−/− BMDC were treated as described in (A) in addition to stimulating cells for 6 h with nigericin. IL-1β release from supernatants was quantified by ELISA.
A. Equilibration Nigericin ± LCS-1 addition

Red: +Nig
Blue: + LCS-1 (1uM) + Nig
Green: + LCS (3uM) + Nig
Teal: + LCS-1 (10uM) + Nig
Purple: + LCS-1 (30uM) + Nig

Nigericin addition
Triton X-100 addition

± LCS-1

B. LPS-primed WT BMDC + 30m Nig

C. LPS-primed BMDC + 6h Nig (10uM)

Figure 5.1
**Figure 5.2** LCS-1 inhibits caspase-1 and caspase-8 activation in LPS-primed BMDC

WT and Casp1/11^-/- BMDC were LPS primed (100 ng/ml) for 4 h and subsequently stimulated with nigericin for 30 min or 6 h in the presence or absence of the SOD1 inhibitor, LCS-1 (10μM). Lysates and supernatants were collected for western blot analysis.

![Western Blot Analysis Figure](image-url)
**Discussion**

The LRR domain of NLRP3 consists of multiple cysteine residues, and it is hypothesized that just as SOD1-deficient mice display glutathionylation on the cysteine-reactive residues of caspase-1, LCS-1 may bind the cysteine-rich residues of NLRP3. It is hypothesized that LCS-1 inhibits NLRP3 and consequent ASC oligomerization by binding to cysteine-rich residues on the LRR domain of NLRP3, consistent with cysteine-reactive chemistry induced via the aforementioned glutathionylation event in SOD1<sup>−/−</sup> macrophages. Fig. 5.3 depicts how LCS-1 may inhibit not only SOD-1 but also caspase-1, caspase-8, and NLRP3. Thus, the NLRP3 inflammasome comprises a novel target for anti-inflammatory actions of the SOD1-directed therapeutic agent, LCS-1, by inhibiting both caspase-1 and caspase-8 inflammasome pathways in addition to NLRP3 (Fig. 5.3). Knowing the effects LCS-1 possesses on suppressing inflammatory signaling pathways may shape clinical regimens to treat lung cancer patients. Future studies consist of examining: 1) the glutathionylation status of caspase-1 by measuring the levels of reduced (GSH) and oxidized (GSSG) caspase-1 via incorporation of biotin-tagged glutathione ethylester (BioGEE) in DCs/macrophages; 2) the accumulation of ROS in the presence or absence of LCS-1 to evaluate the efficacy of LCS-1 inhibition on SOD activity; and 3) the viability of DCs and macrophages treated with LCS-1 to assess the cytotoxicity of this small molecular inhibitor.
**Figure 5.3** LCS-1 targets three proteins in NLRP3 inflammasome signaling

A schematic showing the multiple NLRP3 inflammasome targets of the SOD1 inhibitor, LCS-1: NLRP3, caspase-1, and caspase-8.
CHAPTER 6

Discussion and Future Directions

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The previous chapters have described the assembly of two very different caspase-8-containing platforms that converge on the processing and release of IL-1β. The one involves TLR4 and doxorubicin-induced activation of a TRIF→RIP1/ FADD/ caspase-8 signaling axis (Chapter 3), while the other involves the formation of an NLRP3/ASC/ caspase-8 signaling platform (Chapter 4). Notably, the chemotherapeutic-drug induced ripoptosome/caspase-8 signaling complex correlated with a downregulation of cIAP1 expression, nigericin-induced NLRP3/ASC/caspase-8 complex occurred in caspase-1-deficient DCs, and caspase-8 activation facilitated caspase-1 in response to nigericin treatment of WT DCs. Interestingly, both doxorubicin- and nigericin-induced caspase-8 platforms correlated with the induction of cell death signaling in BMDC. Using biochemical assays, pharmacological inhibitors, and various genetic knockout models, we have been able to identify the contribution of caspase-8 to IL-1β in chemotherapy-induced signaling in DCs, to caspase-1 competent DCs, and to caspase-1-deficient cells. Our data herein indicate that these IL-1β processing pathways may play roles in DCs/macrophages within the tumor microenvironment and in cells that express NLRP3 and ASC but do not express caspase-1. However, multiple research questions remain and merit further discussion as well as application to the clinical realm.
Caspase-8-dependent IL-1β Production in the Tumor Microenvironment

In Chapter 3, we describe a third, mechanistically distinct signaling cascade by which IL-1β production can be induced by chemotherapeutic drugs (118). Although this pathway also involves direct actions on macrophages/DCs, it is independent of the NLRP3→ASC→caspase-1 inflammasome axis and reflects engagement of a RIP1→FADD→caspase-8 ripoptosome axis (Figure 1.1). These findings add to the growing literature regarding important roles for caspase-8 as both an alternative IL-1β converting enzyme and a regulator of canonical inflammasomes (14). Notably, this caspase-8 ripoptosome axis for IL-1β production was triggered by some common chemotherapeutic/pro-apoptotic agents (doxorubicin, staurosporine) but not by others (oxaliplatin, cisplatin). The ability of the various drugs to activate the caspase-8 pathway was correlated with their efficacy to downregulate expression of cIAPs. Release of mitochondrial Smac (Second mitochondrial-derived activator of caspases) suppresses the E3 ubiquitin ligase function of cIAPs with consequent inhibition of ubiquitin-mediated degradation of ripoptosomes (126). Downregulated cIAP1 levels in doxorubicin-treated DCs appeared to be critical for preservation of the FADD/caspase-8 complex; this corroborates previous studies implicating cIAP downregulation in IL-1β processing triggered by Smac-mimetic compounds (127).

The identification of a caspase-8 pathway for IL-1β production has several implications in the context of cancer chemotherapy. First, toll-like receptor 4 (TLR4) signaling in the tumor-resident macrophages/DCs of cancer patients undergoing chemotherapy may be activated by commensal bacteria-derived endotoxins that leak across compromised gut epithelial barriers or by tumor-derived DAMPs, such as
HMGB1. This active TLR4 signaling may synergize with drugs, such as doxorubicin, to engage the RIP1/FADD/caspase-8 ripoptosome axis in vivo. Second, in contrast to caspase-1 which is predominantly expressed in myeloid leukocytes, caspase-8 is ubiquitously expressed. The caspase-8 pathway for IL-1β processing may thus be engaged in non-myeloid stromal cell types of tumors, such as epithelial cells, endothelial cells and fibroblasts, or some cancer cell types per se, which do not express high levels of caspase-1 but may express proIL-1β in particular inflammatory contexts.

Why do certain chemotherapeutic drugs activate IL-1β processing in BMDC while other genotoxic chemotherapeutic drugs do not?

Ongoing studies indicate that the ability of particular chemotherapeutic drugs to drive caspase-8-mediated IL-1β processing correlates with their ability to elicit pro-apoptotic signaling cascades in macrophages/DCs, even in the context of the NF-κB signals that are required for production of proIL-1β. This is likely to be relevant given the prominent role of NF-κB in transactivation of anti-apoptotic/pro-survival genes. In Figure 6.1, LPS priming of BMDC delays the loss in viability of oxaliplatin, cisplatin, mitoxantrone, and etoposide-treated DCs in contrast to doxorubicin, which induces a significant decrease in viability in the presence or absence of LPS priming. Importantly, Dox, a topoisomerase II inhibitor, is the only drug of the five tested in Fig. 6.1 to induce IL-1β release in BMDC (data not shown). An amalgamation of my data ascribes Dox’s ability to induce IL-1β release to the drug’s ability to induce cell death in inflammatory DCs rather than its mechanism of action as a topoisomerase II inhibitor because the topoisomerase inhibitors, etoposide and mitoxantrone, did not induce IL-1β release in
murine BMDC. In contrast, tumor cells are sensitive to platinum agents (e.g., oxaliplatin and cisplatin) and topoisomerase inhibitors (128). Tumor cells proliferate rapidly in comparison to macrophages/DCs, and most chemotherapeutic agents act on cancer cells by inducing molecular injuries (e.g., DNA-damage) resulting in the transcriptional or post-transcriptional activation of pro-apoptotic proteins (e.g., pro-apoptotic Bcl-2 family members). A key issue is to discriminate how a particular drug may differentially integrate NF-κB-driven pro-inflammatory versus anti-apoptotic gene expression in malignant cells versus tumor-resident immune cells. Moreover, given the key role of organellar dysfunction in the activation of NLRP3 inflammasomes and ripoptosomes (14, 126), the analysis of the effects of chemotherapeutic agents on mitochondrial, lysosomal, and plasma membrane integrity will likely provide new insights into the multiple pathways underlying IL-1β production in different tumor models.

How are DAMPs released from dying cells?

In Chapter 1, the release of tumor-derived DAMPs to activate the NLRP3 inflammasome in murine tumors using the chemotherapeutic platinum agent, oxaliplatin, was discussed. In general, the rate of tumor cell proliferation and death both act as rheostats to set the inflammatory tone of the tumor microenvironment. Rapidly dying cells release DAMPs that activate nearby cells to sustain the inflammatory response. However, an interesting question that remains is the mechanism of how DAMPs are released. Besides stochastic lytic permeabilization of the plasma membrane or regulated export pathways (e.g., secretory vesicles/lysosomes, exosomes, autophagic vesicles, etc),
**Figure 6.1** Inflammatory dendritic cells are resistant to conventional chemotherapeutic drug-induced cell death with the exception of doxorubicin.

WT BMDC were primed with LPS for 4 h followed by treatment with etoposide (10 μM), mitoxantorone (1μM), oxaliplatin (50μM), cisplatin (25μM), and doxorubicin (10μM) for 18-24 hr. Cell viability was assayed using the alamar blue reagent.
the gating of ion channels have been shown to mediate the flux of metabolites for cellular communication. Similar to the pore formed by Bak/Bax insertion into the mitochondrial membrane during apoptosis, MLKL forms a pore during necroptosis. The helical MLKL protein inserts into the mitochondrial membrane during necroptosis, forming a permeation pore, and can mediate the flux of molecules up to 10 kD (129, 130). Further characterization of this pore and others may explain the mode of DAMP release from dying cells. Alternatively, pannexin-1, a mammalian ortholog of the invertebrate innexin gap junction protein, has been shown to mediate the release of nucleotides from Jurkat T cells via apoptotic induction of a caspase-3/7-mediated cleavage of the C-terminus to gate the channel open (131). My colleague, Andrea Boyd Tressler, recently reported the involvement of Panx1 in ATP release following exposure to the chemotherapeutic/proapoptotic drugs, doxorubicin, etoposide, and staurosporine (132). Thus, Panx1 may also be a candidate channel in myeloid cells through which DAMPs efflux to activate the inflammasome in DCs and macrophages.

What is next for caspase-8-mediated inflammasome signaling?

Numerous examples in the literature point toward NLRP3/ASC/caspase-1 dependent IL-1β activation. However, the contributions of caspase-8 as an ICE and procaspase-8 as a signaling molecule are just beginning to be explored in disease models of autoimmunity and diabetes (133, 134). More importantly, cells other than macrophages and DCs that express NLRP3 and ASC may contribute to IL-1β maturation and release. Preliminary unpublished studies from a collaborative group indicate that specifically polarized Th17 cells, which express NLRP3 and ASC, are capable of
processing IL-1β via a caspase-8-dependent mechanism. Within the past several years caspase-8 has explosively emerged in non-apoptotic signaling roles (e.g., besides its conventional role as an initiator caspase in classical apoptotic signaling.) Thus, further exploration using genetic knockouts of caspase-8 with the Casp8<sup>−/−</sup>/Rip3<sup>−/−</sup>, Casp8<sup>−/−</sup>/FADD<sup>−/−</sup>, Casp8<sup>−/−</sup>/Rip1<sup>−/−</sup>, and Casp8<sup>−/−</sup>/FADD<sup>−/−</sup>/Rip1<sup>−/−</sup> double knockout mice may be utilized with the caveats of knowing the mouse model limitations, as discussed in Chapters 3 and 4. Herein we learn that assessing the genetic absence of an enzyme is functionally distinct from pharmacologically inhibiting its proteolytic activity, especially when the zymogenic form of the protein also acts as a signaling molecule. Undoubtedly, researchers in the inflammasome field are further investigating the formation of ASC filaments and the recruitment of caspase-8 to the NLRP3/ASC inflammasome. Importantly, how do caspase-1 and caspase-8 three-dimensionally interact with large signaling platforms such as inflammasomes?

*Caspase-8 in the clinical setting*

The relevance of this novel caspase-8 platform may be assayed in the clinic by immunohistochemical analysis of caspase-8 in biopsied tumors before and after treatment with a doxorubicin chemotherapeutic regimen. Additionally, it would be interesting to determine which proteins interact with caspase-8 by examining sequence homology in the human THP-1 monocyte cell line. cFLIPs are known to regulate the activity of murine caspase-8, but less is known about caspase-8 regulatory proteins in humans. Inflammasome regulation in humans differs from mice in that the human protein CARDINAL regulates NLRP3 inflammasome activation in humans, while no murine
homolog of CARDINAL exists. Humans also express endogenous inflammasome inhibitors identified as COP and POP proteins, which consist of CARD-only or PYD-only domains that bind ASC and caspase-1 proteins (135). Also, it would be worth examining whether caspase-8 polymorphisms are present in humans, particularly in the active site of caspase-8. Performance of *in vitro* enzyme assays mixing recombinant proIL-1β with mutated caspase-8 protein would address proIL-1β cleavability.

Notably, the higher single dosage of doxorubicin utilized to treat murine DCs (10 µM) in Chapter 3 experiments differ from the longer term, lower-dose chemotherapeutic regimen prescribed by physicians in cancer clinics. It would be interesting to assess IL-1β processing in murine BMDC treated for a longer term (> 18 h) and lower dose (~ 10 nm) with Dox. It has been reported that the TLR2/TLR9/MyD88 signaling pathway is important for the acute inflammatory response in an acute doxorubicin-induced peritonitis mice (136). Assessing the *in vitro* effect of low-dose Dox treatment of BMDC would indicate the signaling pathways which are engaged in DCs. An additional layer of complexity involves the use of combination chemotherapeutic regimens involving two or more anti-neoplastic drugs. Undoubtedly, the type of cancer and genetic make-up of the patient will dictate the efficacy of particular chemotherapeutic regimens.

Overall, this thesis defined two distinct caspase-8-mediated mechanisms by which DCs and macrophages process IL-1β. Utilization of these unconventional IL-1β signaling pathways may be relevant to both tumor-resident DCs/macrophages that sense high local concentrations of chemotherapeutic drugs as well as relevant to cells which lack caspase-1 expression but must produce IL-1β locally. Importantly, the implication that caspase-8 activates caspase-1 in control DCs is evidence of the complex regulation
of inflammasomes. Understanding the multiple ways in which IL-1β is generated will likely provide insight for developing therapeutic strategies to target this cytokine.
October 30, 2014

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these agents is a necessary and common feature of their activity. *J. Biol. Chem.* 269: 15195-15203.


apoptosis is dispensable for NLRP3 inflammasome activation but non-apoptotic caspase-8 is required for inflammasome priming. *EMBO Rep* 15: 982-990.


exosomes requires the ASC/NLRP3 inflammasome but is independent of caspase-1. *J. Immunol.* 182: 5052-5062.


