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Neutrophils Require SHP1 To Regulate IL-1β Production and Prevent Inflammatory Skin Disease

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The regulation of neutrophil recruitment, activation, and disposal is pivotal for circumscribed inflammation. SHP1Y208N/Y208N mutant mice develop severe cutaneous inflammatory disease that is IL-1R dependent. Genetic reduction in neutrophil numbers and neutrophilic responses to infection is sufficient to prevent the spontaneous initiation of this disease. Neutrophils from SHP1Y208N/Y208N mice display increased pro–IL-1β production due to altered responses to MyD88-dependent and MyD88-independent signals. The IL-1R–dependent inflammatory disease in SHP1Y208N/Y208N mice develops independently of caspase 1 and proteinase 3 and neutrophil elastase. In response to Fas ligand, a caspase 1-independent inducer of IL-1β production, neutrophils from SHP1Y208N/Y208N mice produce elevated levels of IL-1β but display reduced caspase 3 and caspase 7 activation. In neutrophils deficient in SHP1, IL-1β induces high levels of pro–IL-1β suggesting the presence of a paracrine IL-1β loop. These data indicate that the neutrophil- and IL-1-dependent disease in SHP1Y208N/Y208N mice is a consequence of loss of negative regulation of TLR and IL-1R signaling. The Journal of Immunology, 2011, 186: 1131–1139.

The role of the innate immune system in many inflammatory diseases is being increasingly recognized, but the key cell types involved, and the relative roles played by microbes and endogenous danger signals as initiators and drivers of inflammatory disease, are controversial. A wealth of data now exists describing the role played within the innate immune system by the pathogen-sensing TLRs, nucleotide binding domain and leucine-rich repeat-containing proteins, and cytoplasmic DExD/H-box RNA helicases in the initiation of inflammation in response to infection and endogenous danger molecules. Although these pathogen-sensing molecules are likely to be critical for the initiation of inflammatory disease, other autocrine and paracrine proinflammatory cytokine loops may be required to propagate and sustain inflammatory disease in the absence of chronic infection (1). Proteins that serve to negatively regulate signaling immediately downstream of microbe-sensing receptors as well as proinflammatory cytokine signaling pathways are likely to be critical in preventing inflammatory disease (1). Dissecting the contributions of distinct cell types, microbes, and environmental factors that initiate and drive inflammation, as well as the genetic lesions underpinning disease susceptibility, will aid in the treatment of a broad range of inflammatory diseases.

Our previous work has demonstrated the key role played by microbes in the initiation of inflammatory skin disease and autoimmunity in SHP1Y208N/Y208N mice (2). Both microbes and MyD88-dependent IL-1R signaling were required for the manifestation of disease (2). However, it is not known how the microbe sensing and cytokine receptor signaling pathways integrate to drive disease. Although it is well established that the inflammatory disease is dependent on CD11b+ myeloid cells (3), it is not clear which specific myeloid cell type initiates and drives the disease, and the pathophysiological mechanism of IL-1 dependence in vivo remains unknown. In SHP1Y208N/Y208N mice with a Y208N mutation in the C-terminal Src homology 2 domain, TLR signaling and IL-1 production are normal in macrophages (2) suggesting that other cell types must be responsible for disease.

Much of our understanding of the role of SHP1 in chronic inflammatory and autoimmune disease has come from the analysis of two other SHPI mutant strains, motheaten (me) and motheaten viable (mev), which die at 3 and 9 wk of age, respectively, from pneumonitis (4, 5). Point mutations in me and mev promote aberrant splicing of the SHPI transcript resulting in reduced activity of the SHPI protein (6, 7). Both me and mev mice display elevated serum immunoglobulins, inflammation in the skin causing a motheaten appearance, increased myelopoiesis in the bone mar-

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The online version of this article contains supplemental material.

Abbreviations used in this article: FADD, Fas-associated death domain protein; Fasl, Fas ligand; hsp70, heat shock protein 70; PI, propidium iodide; rm, recombinant murine; SCF, stem cell factor.

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row, glomerulonephritis associated with granular deposits of Ig and complement, and male sterility associated with Leydig cell depletion in the testes and impaired spermatogenesis and testosterone production (4, 5). Compound RAG1 and SCID mutations in SHP1 mutant mice have demonstrated that B and T cells do not significantly contribute to the lethal pneumonitis, pulmonary fibrosis, and macrophage and granulocytic inflammatory lesions of me+ mice (8). TNF-neutralizing Abs reduce pulmonary fibrosis but do not prevent lethality (9). Treatment of me+ mice with anti-CD11b Ab alleviated the pneumonitis and foot inflammation, suggesting a role for macrophages and/or neutrophils in the pathophysiology of this disease (3).

SHP1 mutant mice display severe inflammatory lesions on the feet and autoantibody production but lack the lethal pneumonitis characteristic of me+ and me- mice (2) and thereby provide a valuable genetic tool to dissect inflammatory disease. The inflammation in SHP1Y208N/Y208N mice is triggered by microbes and are dependent on MyD88 and IL-1R but independent of TNF-α, Stat1, TRIF, and IFNs (2). The biochemical pathways and cell types that ultimately drive the inflammatory disease on the feet of SHP1 mutant mice have not been identified.

SHP1 has been implicated in the regulation of a diverse array of intracellular signaling molecules, cytokine receptors, Ig receptors, Ag receptors, and costimulatory molecules (10) although it is not clear which of these interactions mediated by SHP1 is essential to prevent severe inflammatory disease. Abu-Dayyeh et al. (11) proposed that SHP1 binds to multiple kinases, including Jak2, Jak3, TAK1, ERK1/2, p38, JNK, IL-1R-associated kinase 1, and Lyn, via a novel phosphorylation-independent kinase tyrosyl inhibitory motif. If correct, it is unlikely that any perturbation of a single protein interaction with SHP1 can completely explain the complex lethal inflammatory phenotype of SHP1 mutant mice. Analysis of the less complex, microbe-dependent inflammatory disease in SHP1Y208N/Y208N mice provides an avenue to identify the central pathways involved. We have genetically dissected this disease by introducing mutations into key signaling proteins. In this study, we report critical cellular mediators and biochemical pathways that initiate and drive inflammatory pathology in SHP1 mutant mice. We demonstrate that inflammatory disease in SHP1 mutant mice is mediated via excessive responses of neutrophils to IL-1 and TLR stimulation, supporting the presence of a paracrine loop of IL-1β production in vivo.

Materials and Methods

Mice

C57BL/6J mice were bred locally at The Scripps Research Institute (La Jolla, CA) or at The Walter and Eliza Hall Institute of Medical Research (Parkville, Victoria, Australia). G-CSF–deficient mice were obtained from Ian Wicks (The Walter and Eliza Hall Institute of Medical Research). MyD88-deficient mice were obtained from Shizuo Akira (Osaka University, Osaka, Japan). Ncf1 mutant mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Prmt3−/−/Ela2−/− mice were obtained from Dieter Jenne (Max Planck Institute of Neurobiology, Martinsried, Germany) (12). All experiments were carried out in accordance with the institutional animal care and use committee guidelines. Mice were housed under specific pathogen-free conditions with unlimited access to food and water.

Neutrophil purification

Bone marrow cells were isolated in HBSS supplemented with 0.075% BSA and 15 mM EDTA. Cells were overlayed on 78, 69, and 52% Percoll layers and centrifuged at 400 × g for 30 min. Neutrophils were removed from the 69/78% interface and resuspended in DMEM/10% FCS. The purity of neutrophil preparations was 96 ± 4% (mean ± SD, n = 14) as assessed by May–Grünwald Giemsa staining (Supplemental Fig. 2). Contaminating cells were composed of lymphocytes (3 ± 3%), monocytes (0.3 ± 0.6%), and nucleated RBCs (0.7 ± 0.9%). For biochemical assays, 0.4 × 10^6 to 1 × 10^6 neutrophils were stimulated with 100 ng/ml ultrapurified LPS (from Salmonella Minnesota R595; Axoxa), 100 ng/ml Pam3CSK4, 100 ng/ml Pam2CSK4 (Invivogen), 10 μg/ml lipoteichoic acid or 100 ng/ml Flag–Fas ligand (FasL; Axxora) cross-linked with anti-Flag Ab (Sigma) for the indicated times. For some experiments, 10 μM Q-V-D-Oph and 100 ng/ml FasL was added to neutrophils.

Cytokine ELISA and intracellular FACS

Cell-associated IL-1α and soluble IL-1β and TNF-α were measured by ELISA (eBioscience and Pharmingen). Intracellular staining for TNF-α was performed according to the manufacturer’s instructions (Pharmingen).

Preparation of cellular lysates and immunoblot

Lysates were prepared in 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 mM NaF, 1 mM sodium vanadate, 1 mM PMSF in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany). Neutrophil lysates were analyzed using SDS-PAGE and immunoblot to IL-1β (R&D Systems, Minneapolis, MN), SHP1 (Santa Cruz Biotechnology, Santa Cruz, CA), heat shock protein 70 (hsp70; Santa Cruz Biotechnology), cleaved caspase 7 (Chemicon), gelosin (Abcam), cleaved caspase 3, caspase 3, phospho-IκB, IκB, phospho-p38, p38, phospho-p42/44, and p42/44 (Cell Signaling).

Survival assays

Neutrophils were incubated in saline or G-CSF for up to 72 h. Neutrophil viability was assessed using annexin V and propidium iodide (PI) according to the manufacturer’s instructions (Pharmingen).

DNA ladderning

DNA was extracted from 6 × 10^5 bone marrow neutrophils using 50 mM Tris HCl (pH 8), 100 mM EDTA, 100 mM NaCl, 1% SDS, 1 mg/ml proteinase K, and SDS-Out Precipitation Reagent (Thermo Scientific). DNA was analyzed using agarose gel electrophoresis and ethidium bromide.

Wound repair

Mice were anesthetized with 4–5% isoflurane administered using a precision vaporizer and a regulated oxygen flow of 1–2 l/min. The incision

![FIGURE 1. SHP1Y208N/Y208N neutrophils mediate extensive tissue damage within 24 h of skin wounding. H&E-stained skin sections were examined microscopically from mice 3, 6, and 24 h after wounding. At 3 h, no difference was observed between sections from SHP1Y208N/Y208N and wild-type mice. A dramatic increase in neutrophil infiltration was noted in tissues adjacent to the wound site in SHP1Y208N/Y208N mutants 6 h after wounding. Extensive infiltration and destruction of dermal, fat, and muscle tissue was evident 24 h after wounding of the skin of SHP1Y208N/Y208N mutants. The arrows indicate areas of neutrophilic infiltration in the dermis. Original magnification ×200.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.0900348)
The following primer pairs were used to genotype other mutations that combined with Spin to create compound homozygous mutants: 

- **Myd88**<sup>−/−</sup>-<sup>5′-TGG CAT GCC TTC ATC ATG AAC C-3′</sup>, 5′-GTC AGA AAC AAC CAC CAT GC-3′, 5′-ATC GCC TTC TAT CGC CTT CTT GAG G-3′, G-CSF<sup>−/−</sup>, 5′-GTA TAA AGG CCC CCT GGA G-3′, 5′-TCC AGG GAC TTA AGC AGG AA-3′, 5′-CTG CAA GGC GAT TAA GTT GGC TAA-3′;  
- **Ncf1**<sup>−/−</sup>-<sup>5′-TGG AGG CCC CAT ATG TGG CAT CC-3′</sup>, 5′-CGA CAT ATT TCA TCA GTC TGC TTA-3′, 5′-GAA GAG GGA AGG GAG TCG CTG CTA-3′, 5′-CGG GAG CCG CTA TGC AAG GC-3′;  
- **Neutrophils**<sup>−/−</sup>-<sup>5′-GCC CTG CTG GAG CGG CGA-3′</sup>, 5′-AGC TCC CAT GCT GTG TTT C-3′, 5′-CCG CAG CAT GGA GGA CGA-3′.  

PCR amplicons from Ncf1 reactions were digested with Mspl to distinguish wild-type and mutant alleles.

### Results

**Response to tissue damage is compromised by infiltrating neutrophils in SHP1 mutant mice**

The cutaneous inflammatory disease in **SHP1**<sup>−/−</sup><sup>/</sup> mice is confined to the feet and is dependent on microbes (2). We hypothesized that the response of myeloid cells to minor injury and infection was likely to have initiated the pathology. To examine the response to minor skin injury in **SHP1**<sup>−/−</sup><sup>/</sup> mice, 3-mm punch biopsies of the skin were performed on the skin of the backs of the animals. Following sacrifice at 3, 6, and 24 h after the procedure, histological sections were analyzed. No abnormalities were observed in sections from **SHP1**<sup>−/−</sup><sup>/</sup> mice 3 h after wounding (Fig. 1), prior to the arrival of neutrophils at the wound site. In histological sections of skin biopsies from C57BL/6J control mice, subsequent neutrophil infiltration, inflammation, and tissue regeneration was limited to sites immediately adjacent to the site of wounding. In **SHP1**<sup>−/−</sup><sup>/</sup> mice, markedly increased neutrophil infiltration was evident at 6 h and significant neutrophil-associated pathological changes in the epidermal and dermal layers were present at 24 h after wounding in **SHP1**<sup>−/−</sup><sup>/</sup> mice (Fig. 1, Supplemental Fig. 1) similar to that previously described for spontaneous inflammatory disease in **SHP1**<sup>−/−</sup><sup>/</sup> mice (2).

**Neutrophils are critical for inflammatory disease in spin mice**

These data suggest that neutrophils played a key role in the initiation of severe inflammatory lesions on the feet of adult

### Table I. Normal myelopoiesis in bone marrow from **SHP1**<sup>−/−</sup><sup>/</sup> mice

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>Genotype</th>
<th>Colony Composition</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Blast</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Wild type</td>
<td>16±2</td>
</tr>
<tr>
<td><strong>SHP1</strong>&lt;sup&gt;−/−&lt;/sup&gt;&lt;sup&gt;/&lt;/sup&gt;</td>
<td>24±7</td>
<td>5±3</td>
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<tr>
<td>G-CSF</td>
<td>Wild type</td>
<td>15±5</td>
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<tr>
<td><strong>SHP1</strong>&lt;sup&gt;−/−&lt;/sup&gt;&lt;sup&gt;/&lt;/sup&gt;</td>
<td>13±8</td>
<td>0±0</td>
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<tr>
<td>M-CSF</td>
<td>Wild type</td>
<td>1±1</td>
</tr>
<tr>
<td><strong>SHP1</strong>&lt;sup&gt;−/−&lt;/sup&gt;&lt;sup&gt;/&lt;/sup&gt;</td>
<td>2±2</td>
<td>4±3</td>
</tr>
<tr>
<td>IL-3</td>
<td>Wild type</td>
<td>4±4</td>
</tr>
<tr>
<td><strong>SHP1</strong>&lt;sup&gt;−/−&lt;/sup&gt;&lt;sup&gt;/&lt;/sup&gt;</td>
<td>6±6</td>
<td>18±4</td>
</tr>
<tr>
<td>SCF</td>
<td>Wild type</td>
<td>6±6</td>
</tr>
<tr>
<td><strong>SHP1</strong>&lt;sup&gt;−/−&lt;/sup&gt;&lt;sup&gt;/&lt;/sup&gt;</td>
<td>7±7</td>
<td>14±4</td>
</tr>
<tr>
<td>SCF/IL-3/Epo</td>
<td>Wild type</td>
<td>13±3</td>
</tr>
<tr>
<td><strong>SHP1</strong>&lt;sup&gt;−/−&lt;/sup&gt;&lt;sup&gt;/&lt;/sup&gt;</td>
<td>17±2</td>
<td>26±12</td>
</tr>
</tbody>
</table>

Bone marrow cells (2.5 × 10<sup>5</sup>) were cultured in the presence of GM-CSF, G-CSF, M-CSF, IL-3, SCF, or combinations thereof. Cultures were enumerated at day 7 and then fixed and stained for differential counting at ×200 magnification. Results represent mean ± SD of colony number from three mice of each genotype.

- Blast, blast cell; G, granulocyte; GM, granulocyte-macrophage; Eo, eosinophil; Ep, erythropoietin; M, macrophage; Meg, megakaryocyte.
SHP1Y208N/Y208N mice, consistent with previous reports demonstrating a role for unspecified CD11b+ myeloid cells in the pathology of me and me’ mice (3). To examine a role for neutrophils in the inflammatory disease of SHP1Y208N/Y208N mice, we reduced neutrophil numbers by more than 50% (data not shown) in SHP1Y208N/Y208N mice by introducing a compound mutation in G-CSF. G-CSF is the dominant regulator of neutrophil production and neutrophil mobilization during emergency granulopoiesis (13). Fig. 2 shows that G-CSF deficiency prevents spontaneous inflammation in SHP1Y208N/Y208N mice, consistent with a critical role in the initiation and/or progression of disease for either G-CSF itself or G-CSF–induced neutrophils. To distinguish between these possibilities, we first investigated whether G-CSF signaling was abnormal in SHP1Y208N/Y208N mice, particularly as SHP1 has been implicated in negative regulation of cytokine-induced Jak/Stat signal transduction in neutrophils (14). No abnormality in G-CSF–induced Jak/Stat activation in SHP1Y208N/Y208N neutrophils was observed (data not shown), indicating that the Y208N mutation does not affect these cytokine signaling cascades. Consistent with this, the generation of neutrophils from myeloid progenitor cells in response to G-CSF stimulation, and other combinations of hematopoietic growth factors, was normal in SHP1Y208N/Y208N mice (Table I).

To exclude the possibility that G-CSF itself promotes inflammation by altering neutrophil responses to microbes, we examined responses to TLR ligands in vitro. Bone marrow neutrophils from wild-type mice were primed with cytokines (G-CSF, GM-CSF, IFN-γ, or saline) for 2 h and then stimulated with TLR ligands (LPS, Pam2CSK4, or Pam3CSK4) for 4 h. The production of TNF-α was used to assess responsiveness of neutrophils to TLR ligands. G-CSF did not increase the proportion of neutrophils that responded to TLR ligands or the magnitude of TNF-α production.

**FIGURE 3.** G-CSF does not enhance neutrophil responses to TLR ligands. A, Purified bone marrow neutrophils produce TNF-α in response to TLR ligands (LPS, Pam2CSK4, Pam3CSK4). Preincubation of the neutrophils for 2 h with G-CSF, GM-CSF, or IFN-γ alters the proportion of neutrophils that respond to the TLR ligand and the magnitude of production of TNF-α as assessed by intracellular FACS. No TNF-α staining was detected in neutrophils from TNF-α–deficient mice (data not shown). B, The flow cytometry data are summarized as mean ± SD of TNF-α+ neutrophils. n = 3 independent experiments. *p < 0.05 (G-CSF–, GM-CSF–, or IFN-γ–primed samples versus saline-primed samples). C, G-CSF does not increase IL-1β production from neutrophils. Purified bone marrow neutrophils were treated for 2 h with G-CSF, GM-CSF, or IFN-γ before activation with LPS for 4 h and FasL overnight. IL-1β levels were analyzed by ELISA. D, SHP1Y208N/Y208N neutrophils produce elevated levels of pro–IL-1β in response to LPS, Pam2CSK4, or Pam3CSK4. Neutrophils were incubated for 2 h with G-CSF, GM-CSF, or IFN-γ before activation with LPS for 4 h. Intracellular IL-1β was assessed by immunoblot using Abs specific for IL-1β. p42/44 is shown as a loading control. Representative data from three to four independent experiments. E, SHP1Y208N/Y208N neutrophils produce increased levels of TNF-α upon stimulation with TLR ligands (LPS, Pam2CSK4, Pam3CSK4). Preincubation of the neutrophils for 2 h with G-CSF significantly decreases the magnitude of production of TNF-α as assessed by ELISA compared with that of neutrophils preincubated for 2 h with saline. *p < 0.05 (SHP1Y208N/Y208N versus wild-type, mean ± SD, n = 4 independent experiments). *p < 0.05 for all comparisons of G-CSF–primed versus saline-primed samples for SHP1Y208N/Y208N and wild-type genotypes.
as assessed by intracellular accumulation of TNF (Fig. 3A, 3B). The level of pro–IL-1β induced by LPS, Pam3CSK4, or Pam3CSK4 was not increased by priming neutrophils with G-CSF but was significantly elevated by priming neutrophils with IFN-γ or GM-CSF (Fig. 3C, 3D). TNF-α production by wild-type and SHP1Y208N/Y208N neutrophils was reduced after exposure to G-CSF (Fig. 3E). However, independent of G-CSF, the magnitude of TNF-α and pro–IL-1β production was consistently greater by SHP1Y208N/Y208N neutrophils compared with that of controls (Fig. 3D, 3E). Collectively, these data suggest that the key abnormality is hyperresponsiveness of SHP1Y208N/Y208N neutrophils to microbial stimuli. Rather than having a direct role, G-CSF drives mobilization and recruitment of SHP1Y208N/Y208N neutrophils to the wound site, exacerbating the effects of the hyperresponsiveness to TLR ligands.

Abnormal TLR activation in SHP1Y208N/Y208N neutrophils

We have previously demonstrated a role for Myd88 and the IL-1R in both the inflammatory and autoimmune disease in SHP1 mutant mice (2). Yet when we examined macrophages, a cell considered pivotal to inflammatory disease in SHPI mutant mice (15), no abnormalities in LPS-induced signal transduction or in SHPI protein levels were found (2). In striking contrast, LPS-induced NF-κB and MAPK activation in purified SHP1Y208N/Y208N bone marrow neutrophils was increased in magnitude and SHP1 protein expression reduced (Figs. 4, 5A). Increases in the magnitude of IkB phosphorylation and degradation, as well as p38 and p42/44 phosphorylation, were evident in SHP1Y208N/Y208N neutrophils, culminating in an increase in pro–IL-1β and IL-1α production (Figs. 4, 5). Although MyD88 deficiency impaired LPS-induced signaling and accumulation of pro–IL-1β, a clear difference was noted in production of pro–IL-1β between SHP1Y208N/Y208N/Myd88−/− and Myd88−/− mutant mice, indicating a role for SHP1 in the regulation of MyD88-independent pathways (Fig. 4). IL-1β also induced more pro–IL-1β in SHP1Y208N/Y208N neutrophils compared with that of controls (Fig. 5B), suggesting that an autocrine or paracrine loop of IL-1β production could be responsible for the IL-1-dependent inflammation in SHP1Y208N/Y208N mice. Like SHP1Y208N/Y208N mutant macrophages, no abnormalities were detected in LPS-induced NF-κB or MAPK signaling pathways in SHP1Y208N/Y208N CD11c+ splenic dendritic cells (data not shown) highlighting the specificity of this signaling defect to the neutrophil lineage.

IL-1R-dependent inflammatory disease develops in the absence of caspase 1, protease 3, and neutrophil elastase

The mechanisms of processing of pro–IL-1β in neutrophils have not been extensively studied. In macrophages, monocytes, and dendritic cells, caspase 1 plays a central role in the production of bioactive IL-1β in response to tissue damage and a range of pathogens. To examine a role for caspase 1 and caspase 1-independent IL-1β production, we generated SHP1Y208N/Y208N mice deficient in caspase 1 or doubly deficient in protease 3 (Prm3−/−) and neutrophil elastase (Ela2−/−). Surprisingly, severe IL-1R–dependent inflammatory lesions developed in SHP1Y208N/Y208N/Caspase1−/− and SHP1Y208N/Y208N/Prm3−/−/Ela2−/− mice, indicating that alternative mechanisms are capable of processing pro–IL-1β in neutrophils or that functional redundancy exists between protease 3, neutrophil elastase, and caspase 1.

To explore potential mechanisms of IL-1β production, we examined the response of SHP1Y208N/Y208N neutrophils to Fas ligand (FasL/CD95L/Apo-1L), a potent caspase 1-independent inducer of pro–IL-1β processing and IL-1β production (16). FasL is known to initiate IL-1β production independently of caspase 1, although the protease responsible for this activity has not been resolved (16). It is known that SHP1 can bind CD95 (Fas/Apo-1) in neutrophils, but the cellular and biochemical consequences of this interaction have not been investigated (17). We hypothesized that the reduction of SHP1 in SHP1Y208N/Y208N neutrophils would lead to aberrant FasL-induced IL-1β production. SHP1 mutant neutrophils produced higher levels of IL-1β than those of wild-type neutrophils after LPS priming and FasL stimulation (wild-type, 111 ± 9 pg/ml IL-1β; SHP1Y208N/Y208N, 183 ± 36 pg/ml IL-1β; n = 4, p < 0.01). We also noted that neutrophils can process pro–IL-1β to the 17-kDa bioactive form upon incubation with LPS alone (Fig. 5D). Incubation of neutrophils with Q-VD-OPh, a caspase inhibitor, prevented FasL-induced processing of pro–IL-1β (Fig. 5D) and neutrophil apoptosis (data not shown). To examine whether SHP1Y208N/Y208N neutrophils display normal rates of apoptosis, we monitored neutrophil viability after incubation with saline or G-CSF. SHP1Y208N/Y208N neutrophils displayed higher rates of spontaneous apoptosis in response to G-CSF as shown by an annexinV−/PI− cells (Fig. 5E). Unlike wild-type cells, neither caspase 3 nor caspase 7 activation were detected in SHP1Y208N/Y208N neutrophils after stimulation by FasL (Fig. 6A). In addition, cleavage of the caspase 3 substrate, gelsolin, was evident in SHP1Y208N/Y208N neutrophils, but this
occurred at later time points and with reduced magnitude compared with that of wild-type neutrophils (Fig. 6B). These data demonstrate that SHP1 regulates G-CSF–induced neutrophil survival and is a critical physiological regulator of Fas signaling and FasL-induced IL-1β production.

The NADPH oxidase complex contributes to inflammatory disease in SHP1<sup>Y208N/Y208N</sup> mice

A fundamental characteristic of neutrophils is the ability to produce superoxide via activation of the NADPH oxidase complex. Kruger et al. (18) suggested that excessive H<sub>2</sub>O<sub>2</sub> production from SHP1<sup>mev/mev</sup> neutrophils may contribute to the inflammatory disease developing in SHP1<sup>mev/mev</sup> mice. To test this theory, we generated SHP1<sup>Y208N/Y208N</sup> mice deficient in Ncf1, a key cytosolic factor in the NADPH complex required for superoxide production. Consistent with previous assumptions, we found a reduced incidence of inflammatory disease in SHP1<sup>Y208N/Y208N</sup>Ncf1° mice compared with that of littermate controls (Fig. 7A). However, using coelenterazine, a superoxide dismutase-inhibitable luminescent reagent that specifically detects superoxide (19), we found no change in superoxide levels in neutrophils isolated from SHP1<sup>Y208N/Y208N</sup> mice (Fig. 7B). To test the effects of LPS and IL-1R hyperresponsiveness on SHP1<sup>Y208N/Y208N</sup> neutrophils, we primed neutrophils with LPS or IL-1β for 30 min prior to stimulation with fMLF. Again no significant differences in the magnitude or duration of superoxide production were evident in SHP1<sup>Y208N/Y208N</sup> neutrophils (Fig. 7B). This indicates that SHP1<sup>Y208N/Y208N</sup> neutrophils are not intrinsically defective in regulating superoxide production. The data suggest that the impairment of neutrophil function via elimination of NADPH oxidase activity reduces the incidence of inflammatory disease in SHP1<sup>Y208N/Y208N</sup> mice.

Discussion

IL-1 plays key physiological roles during infection and has been implicated in diverse pathophysiological conditions including...
periodic fever syndromes, asbestosis, type 2 diabetes, and dysregulated responses to influenza (20–25). During infection, the release of IL-1β contributes to the febrile response, induces inflammatory cytokine production, acute-phase protein synthesis, hypotension, neutrophil mobilization, and thrombocytosis (25). Despite its increasingly prominent role in diverse pathological conditions, relatively little is known about the key cell types and biological processes contributing to its release. In this study, we describe the role of neutrophils in the production of IL-1β and the key role that is played by SHP1 both in regulating responses to TLRs and in modulating caspase 1-independent pathways important for IL-1β production. We provide evidence of at least three abnormalities that may contribute to this neutrophil- and IL-1–dependent inflammatory disease in SHP1Y208N/Y208N mice: 1) excessive microbe-induced TLR signaling that enhances pro–IL-1β production; 2) altered Fas signaling in mutant neutrophils releasing bioactive IL-1β; and 3) excessive IL-1β–induced pro–IL-1β production establishing a pathological paracrine loop of IL-1β production. We propose that the integration of these three aberrant processes in SHP1 mutant mice is essential for the manifestation of severe inflammatory disease.

Fas ligand is a potent inducer of IL-1β production by neutrophils. Previous studies have demonstrated that FasL induces IL-1β production in neutrophils independently of caspase 1 activation (16), and it has been proposed that proteinase 3 and neutrophil elastase are responsible for processing pro–IL-1β in neutrophils (16, 26, 27). Our data demonstrate that proteinase 3 and neutrophil elastase are not the dominant mediators of IL-1β production in this IL-1R–dependent inflammatory disease in SHP1Y208N/Y208N mice. This indicates that other enzymes serve to process pro–IL-1β in neutrophils or that a redundancy of function allows caspase 1, proteinase 3, neutrophil elastase, or other processing enzymes to produce bioactive IL-1β. The observation that neutrophils can process pro–IL-1β to IL-1β upon incubation alone, coupled with increased rates of apoptosis of G-CSF–stimulated SHP1Y208N/Y208N neutrophils, suggests an important link in the formation of a paracrine loop by enabling the release of IL-1β from apoptotic SHP1Y208N/Y208N neutrophils.

Daigle et al. (17) described the association of SHP1 with Fas, but the biochemical consequences of an SHP1 deficiency on FasL-induced signaling in neutrophils were not reported. SHP1 also dephosphorylates Y397 and Y465 on caspase 8 via an interaction with Tyr310, leading to the activation of caspase 8 and apoptosis of myeloid cell lines (28). Our data are consistent with these data, showing that an SHP1 deficiency reduces caspase 3 and caspase 7 activation in response to Fas activation. However, IL-1β is still produced in response to stimulation by FasL, despite a defect in caspase activation, suggesting that residual activation of Fas/CD95 is sufficient to induce processing of pro–IL-1β. Fas-associated death domain protein (FADD) is an adapter molecule that asso-

![FIGURE 6. SHP1 controls caspase activation in response to FasL. A, Purified bone marrow neutrophils were stimulated with 100 ng/ml FasL for the indicated times. Cellular lysates were analyzed by immunoblot using Abs specific for cleaved caspase 3, caspase 3, or cleaved caspase 7. Data is representative of four independent experiments. B, Purified bone marrow neutrophils were stimulated with 100 ng/ml FasL for the indicated times. DNA fragmentation was analyzed by agarose gel electrophoresis. Cellular lysates were analyzed by immunoblot using Abs specific to caspase 3, gelsolin, or p42/44 as a loading control. Data are representative of four independent experiments.](http://www.jimmunol.org/doi/abs/1137)
Associates with Fas to initiate both caspase 8-dependent apoptosis and RIPK1-dependent cell death (29–31). The binding of SHP1 to phosphorylated Y291 on Fas (17) occurs at the interface of the Fas–FADD death domain complex and may facilitate the efficient recruitment of FADD, caspase 8, or RIPK1. Future studies are required to understand the role of SHP1 in the formation of the death-inducing signaling complex.

Our findings on the key role played by the NADPH oxidase in this IL-1R–dependent inflammatory disease warrant further investigation into the role played by this molecular complex on pro–IL-1β processing in neutrophils. It is not known if the NADPH complex modulates TLR and IL-1R signaling leading to pro–IL-1β production or if the complex modulates the undefined enzymatic complex required for pro–IL-1β processing in neutrophils. The impairment of NADPH oxidase activity in SHP1Y208N/Ncf1−/− mice may also affect the development of inflammatory disease via alterations in the activation of neutrophil proteases, neutrophil extracellular trap formation, or apoptosis (32–34).

The elucidation of biochemical and cellular mechanisms that regulate IL-1β production in neutrophils should stimulate a reevaluation of the role of neutrophils in IL-1–dependent autoimmune inflammatory diseases such as familial Mediterranean fever, which are characterized by neutrophil-rich lesions (35). The pathways that regulate SHP1 protein levels in neutrophils warrant investigation given that reductions in SHP1 protein are associated with psoriatic arthritis (36), multiple sclerosis (37), and leukemia (38–41). Future studies should seek to define the biochemical and cellular mechanisms that govern both the activation of neutrophils and their responses to apoptosis- and IL-1β–inducing stimuli such as FasL to understand how distinct pathways, such as pathogen recognition, cytokine signal transduction, and cell death pathways, integrate to drive inflammatory disease.

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Disclosures
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References