The nonreceptor tyrosine kinase SYK drives caspase-8/NLRP3 inflammasome-mediated autoinflammatory osteomyelitis

Received for publication, August 12, 2019, and in revised form, November 5, 2019 Published, Papers in Press, November 12, 2019, DOI 10.1074/jbc.RA119.010623

Tejasvi K. Dasari[‡§1], Rechel Geiger[‡1], Rajendra Karki[‡], Balaji Banoth[‡], Bhesh Raj Sharma[‡], Prajwal Gurung[‡¶], Amanda Burton[‡], and Thirumala-Devi Kanneganti[‡2]

From the [‡]Department of Immunology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105, the [§]School of Medicine, Baylor College of Medicine, Houston, Texas 77030, and the [¶]Inflammation Program, University of Iowa, Iowa City, Iowa 52241

Edited by Dennis R. Voelker

Chronic recurrent multifocal osteomyelitis (CRMO) in humans can be modeled in *Pstpip2^{cmo}* mice, which carry a missense mutation in the proline–serine–threonine phosphatase–interacting protein 2 (*Pstpip2*) gene. As *cmo* disease in mice, the experimental model analogous to human CRMO, is mediated specifically by IL-1β and not by IL-1α, delineating the molecular pathways contributing to pathogenic IL-1β production is crucial to developing targeted therapies. In particular, our earlier findings support redundant roles of NLR family pyrin domain-containing 3 (NLRP3) and caspase-1 with caspase-8 in instigating *cmo*. However, the signaling components upstream of caspase-8 and pro-IL-1β cleavage in *Pstpip2^{cmo}* mice are not well-understood. Therefore, here we investigated the signaling pathways in these mice and discovered a central role of a nonreceptor tyrosine kinase, spleen tyrosine kinase (SYK), in mediating osteomyelitis. Using several mutant mouse strains, immunoblotting, and microcomputed tomography, we demonstrate that absent in melanoma 2 (AIM2), receptor-interacting serine/threonine protein kinase 3 (RIPK3), and caspase recruitment domain–containing protein 9 (CARD9) are each dispensable for osteomyelitis induction in *Pstpip2^{cmo}* mice, whereas genetic deletion of *Syk* completely abrogates the disease phenotype. We further show that SYK centrally mediates signaling upstream of caspase-1 and caspase-8 activation and principally up-regulates NF-κB and IL-1β signaling in *Pstpip2^{cmo}* mice, thereby inducing *cmo*. These results provide a rationale for directly targeting SYK and its downstream signaling components in CRMO.

Autoinflammatory bone diseases, including chronic recurrent multifocal osteomyelitis (CRMO),[3] osteoporosis, Paget's disease, arthritis, and periodontal disease, are increasingly pervasive contributors to severe chronic pain, physical disabilities, and morbidity (1). CRMO is primarily a pediatric chronic inflammatory bone disease, with at least 80% of patients experiencing primary symptoms, including osteomyelitis and debilitating bone pain (2). Treatment of CRMO is currently limited to nonsteroidal anti-inflammatory drugs with escalation to corticosteroids or bisphosphonates for pain relief (3). However, all current therapeutic options have limited specificity to the pathophysiology underlying CRMO.

To study the molecular mechanisms underpinning disease manifestation, CRMO in humans can be modeled in mice that carry the L98P missense mutation in the *Pstpip2* gene. Proline–serine–threonine phosphatase–interacting protein 2 (PSTPIP2), a Fes/CIP4 homology domain and Bin-Amphiphysin-Rvs (F-BAR) family protein involved in regulating membrane and cytoskeletal dynamics (4), is encoded by *Pstpip2* on chromosome 18 in both humans and mice and is predominantly expressed in the myeloid lineage (5). The L98P mutation in mice is termed *chronic multifocal osteomyelitis (cmo)*, and *Pstpip2^{cmo}* mice are phenotypically characterized by autoinflammatory disease involving the bones and skin, resulting in osteomyelitis and bone deformities. The bone lesions in both *cmo* disease and CRMO are associated with increased IL-1 signaling, osteoclast-mediated resorption, and an elevation of osteoclast precursors (6), but the specific inflammatory pathways critical for disease are not known.

IL-1β has been established as the principle driver of dysregulated cellular homeostasis, extracellular matrix composition, proinflammatory cytokine production, and osteolysis in a diverse array of autoinflammatory, hematologic, and bone diseases, including osteoarthritis (7) and multiple myeloma (8). Inhibition of IL-1β and IL-1 receptor (IL-1R) signaling has been shown to completely protect against disease in *Pstpip2^{cmo}* mice (9), suggesting that inhibition of IL-1β, IL-1R, or their upstream regulators could provide significant benefit to patients with autoinflammatory bone disease. It is known that caspase-1–mediated cleavage of pro-IL-1β is activated by the nucleotide-binding oligomerization domain–like receptor family, pyrin domain–containing 3 (NLRP3) inflammasome (10), and previous studies have established a redundant role of caspase-1 or NLRP3 with caspase-8 in mediating this cleavage and disease

This work was supported by National Institutes of Health Grants CA163507, AR056296, AI124346, and AI101935 and by the American Lebanese Syrian Associated Charities (to T.-D. K.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Dept. of Immunology, St. Jude Children's Research Hospital, MS #351, 570 St. Jude Place, Ste. E7004, Memphis TN 38105-2794. Tel.: 901-595-3634; Fax 901-595-5766; E-mail: Thirumala-Devi.Kanneganti@StJude.org.

[3] The abbreviations used are: CRMO, chronic recurrent multifocal osteomyelitis; SYK, spleen tyrosine kinase; ASC, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain; CT, computed tomography; LPS, lipopolysaccharide; BMDM, bone marrow-derived macrophage; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; GSDMD, gasdermin D.

progression (11, 12). However, the signaling cascade involved in caspase-8 activation remains not well-understood.

The nonreceptor tyrosine kinase SYK is a central regulatory molecule in innate immune Toll-like receptor and nucleotide-binding oligomerization domain–like receptor signaling pathways (13, 14) and inflammatory cytokine secretion (15). SYK is also known to play a role in activating caspase-8, resulting in IL-1β processing (16). Based on the involvement of SYK in the caspase-8 pathway and the importance of caspase-8 in mediating *cmo* disease, we sought to determine the role of SYK signaling in regulating *cmo* disease. Here we discovered the mechanistic basis underpinning SYK-dependent induction of autoinflammatory osteomyelitis. Specifically, we show that SYK critically up-regulates the pro-IL-1β production responsible for *cmo* disease progression and proinflammatory NF-κB signaling, which contributes to pro-IL-1β up-regulation.

Results

RIPK3 and AIM2 are dispensable for disease progression in Pstpip2^{cmo} mice

The NLRP3 inflammasome plays a redundant role with caspase-8 to promote disease progression in *Pstpip2^{cmo}* mice, indicating that NLRP3 is an upstream regulator of caspase-1 activation (12), but our understanding of the upstream regulation of caspase-8 activation remains incomplete. Although caspase-8 deficiency is embryonically lethal, caspase-8 – deficient mice can be completely rescued by deleting receptor-interacting serine/threonine kinase 3 (RIPK3) (17–19). In addition, reduced IL-1β production and abolished caspase-8 activation in *Ripk3$^{-/-}$* bone marrow–derived dendritic cells suggest that RIPK3 is required for caspase-8 activation and subsequent release of IL-1β (20). Absent in melanoma 2 (AIM2) acts as an inflammasome sensor for cytosolic DNA, and it activates caspase-1 through the adaptor protein apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC). AIM2 induces caspase-8 activation in caspase-1–deficient macrophages in the context of several bacterial infections, including *Burkholderia* (21), *Francisella* (22), and *Legionella* (23). Given their established functions in caspase-8 activation under various conditions, we explored the roles of RIPK3 and AIM2 in mediating caspase-8 activation in *Pstpip2^{cmo}* mice by analyzing *cmo* disease progression in NLRP3- and RIPK3-deficient *Pstpip2^{cmo}* mice (*Pstpip2^{cmo}Nlrp3$^{-/-}$Ripk3$^{-/-}$*) and NLRP3- and AIM2-deficient *Pstpip2^{cmo}* mice (*Pstpip2^{cmo}Nlrp3$^{-/-}$Aim2$^{-/-}$*). All mice with both genotypes (*Pstpip2^{cmo}Nlrp3$^{-/-}$Ripk3$^{-/-}$* and *Pstpip2^{cmo}Nlrp3$^{-/-}$Aim2$^{-/-}$*) developed disease similarly to *Pstpip2^{cmo}* mice (Fig. 1, *A* and *B*). Microcomputed tomography (micro-CT) scans of the inflamed areas revealed an extensive reduction in bone density and structural malformation in the feet of these mice (Fig. 1, *A* and *B*). Further, massive lymphomegaly was observed in the popliteal lymph nodes draining inflamed footpads (Fig. 1, *A* and *B*). These data suggest that RIPK3 and AIM2 are dispensable for disease progression in *Pstpip2^{cmo}* mice.

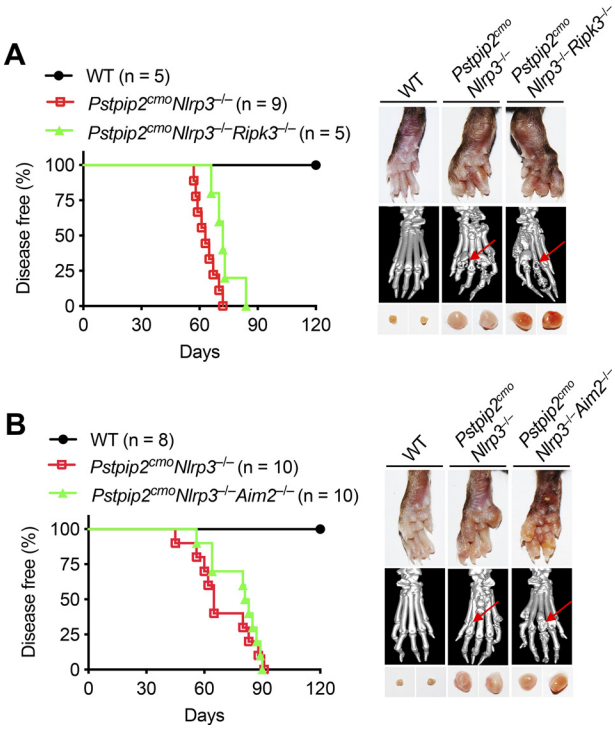

Figure 1. RIPK3 and AIM2 are dispensable for disease progression in *Pstpip2^{cmo}* mice. *A*, incidence of disease in WT (n = 5), *Pstpip2^{cmo}Nlrp3$^{-/-}$* (n = 9), and *Pstpip2^{cmo}Nlrp3$^{-/-}$Ripk3$^{-/-}$* (n = 5) mice over the experimental course and representative footpad images, footpad CT scans, and popliteal lymph nodes from these respective mice. *B*, incidence of disease in WT (n = 8), *Pstpip2^{cmo}Nlrp3$^{-/-}$* (n = 10), and *Pstpip2^{cmo}Nlrp3$^{-/-}$Aim2$^{-/-}$* (n = 10) mice over the experimental course and representative footpad images, footpad CT scans, and popliteal lymph nodes from these respective mice.

SYK, but not CARD9, is required for inflammatory disease progression in Pstpip2^{cmo} mice

In addition to the role of SYK in innate immune signaling pathways (13, 14) and inflammatory cytokine secretion (15), recent evidence has indicated the involvement of SYK in a diverse range of biological functions, including cellular adhesion, platelet activation, and osteoclast maturation (24). The SYK adaptor protein caspase recruitment domain–containing protein 9 (CARD9) is expressed primarily in lymphoid tissues and contributes to innate immune signaling in response to fungal, viral, and bacterial infections (25–27). Given that SYK and CARD9 are involved in caspase-8 activation and subsequent IL-1β processing in bone marrow–derived dendritic cells during fungal infection (16), we explored the respective contributions of SYK and CARD9 to disease progression in *Pstpip2^{cmo}* mice. First, we monitored disease progression in *Pstpip2^{cmo}Nlrp3$^{-/-}$Syk$^{fl/fl}$LysM^{cre}* mice and *Pstpip2^{cmo}Nlrp3$^{-/-}$Card9$^{-/-}$* mice. Although *Pstpip2^{cmo}Nlrp3$^{-/-}$Card9$^{-/-}$* mice did not show protection from disease, *Pstpip2^{cmo}Nlrp3$^{-/-}$Syk$^{fl/fl}$LysM^{cre}* mice displayed nearly complete protection (Fig. 2, *A* and *B*). Next we investigated whether deletion of SYK in *Pstpip2^{cmo}* mice with intact NLRP3 would be sufficient to provide protection from disease. We found that myeloid-specific deletion of SYK alone in *Pstpip2^{cmo}* mice (*Pstpip2^{cmo}Syk$^{fl/fl}$LysM^{cre}*) provided complete protection from disease (Fig. 2*C*). The structural bone lesions found by micro-CT and the popliteal lymphomegaly observed in *Pstpip2^{cmo}*, *Pstpip2^{cmo}Nlrp3$^{-/-}$*, and *Pstpip2^{cmo}Nlrp3$^{-/-}$Card9$^{-/-}$* mice

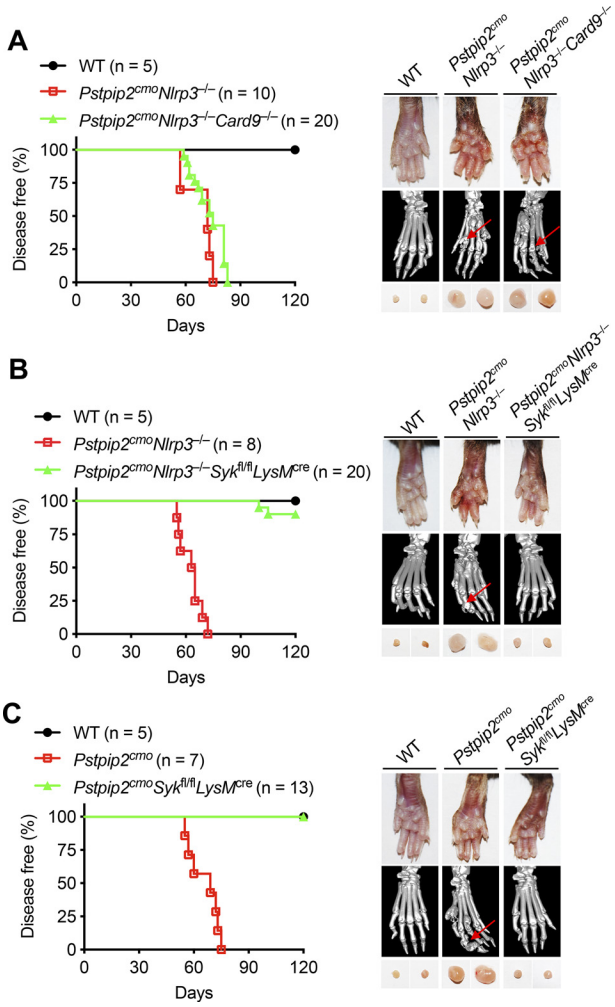

Figure 2. CARD9, but not SYK, is dispensable for disease progression in *Pstpip2^{cmo}* mice. *A*, incidence of disease in WT ($n = 5$), *Pstpip2^{cmo}Nlrp3$^{-/-}$* ($n = 10$), and *Pstpip2^{cmo}Nlrp3$^{-/-}$Card9$^{-/-}$* ($n = 20$) mice over the experimental course and representative footpad images, footpad CT scans, and popliteal lymph nodes from these respective mice. *B*, incidence of disease in WT ($n = 5$), *Pstpip2^{cmo}Nlrp3$^{-/-}$* ($n = 8$), and *Pstpip2^{cmo}Nlrp3$^{-/-}$Syk$^{fl/fl}$LysM^{cre}* ($n = 20$) mice over the experimental course and representative footpad images, footpad CT scans, and popliteal lymph nodes from these respective mice. *C*, incidence of disease in WT ($n = 5$), *Pstpip2^{cmo}* ($n = 7$), and *Pstpip2^{cmo}Syk$^{fl/fl}$LysM^{cre}* ($n = 13$) mice over the experimental course and representative footpad images, footpad CT scans, and popliteal lymph nodes from these respective mice.

were rescued in *Pstpip2^{cmo}Nlrp3$^{-/-}$Syk$^{fl/fl}$LysM^{cre}* and *Pstpip2^{cmo}Syk$^{fl/fl}$LysM^{cre}* mice (Fig. 2, *A–C*). Taken together, these data suggest that SYK functions upstream of both caspase-1 and caspase-8 in inducing *cmo* disease, that SYK is sufficient and necessary for *cmo* disease induction, and that NLRP3 and CARD9 are dispensable for *cmo* disease progression.

SYK mediates cmo disease by promoting proinflammatory signaling but not inflammasome activation

Disease in *cmo* mice is mediated by the cytokine IL-1β (9). To investigate the role of SYK in regulating IL-1β up-regulation in *cmo*, we first measured pro-IL-1β expression and SYK activation in the footpads of WT and *Pstpip2^{cmo}* mice. Footpads from *Pstpip2^{cmo}* mice had increased pro-IL-1β expression and SYK activation with respect to those of WT mice (Fig. 3*A*). Myeloid-specific deletion of SYK in *Pstpip2^{cmo}* mice reduced the expression of pro-IL-1β in footpads to a level similar to that of WT mice without affecting the expression of caspase-1 or caspase-8 (Fig. 3*A*). Consistent with these data, the expression of pro-IL-1β induced by lipopolysaccharide (LPS) treatment was increased in bone marrow-derived macrophages (BMDMs) isolated from *Pstpip2^{cmo}* mice relative to that of BMDMs from WT mice (Fig. 3*B*). The increased pro-IL-1β expression in *Pstpip2^{cmo}* mice correlated with activation of SYK. Myeloid-specific deletion of SYK in *Pstpip2^{cmo}* mice abolished the increased induction of pro-IL-1β in BMDMs upon LPS stimulation relative to *Pstpip2^{cmo}* BMDMs without affecting the expression of caspase-1 and caspase-8 (Fig. 3*B*). These findings suggest a primary role for SYK in mediating pro-IL-1β production and *cmo* disease progression.

We next sought to identify additional intracellular signaling pathways mediated by SYK signaling that contribute to induction of pro-IL-1β expression and excessive inflammation in *Pstpip2^{cmo}* mice. Recent evidence has demonstrated that the mitogen-activated protein (MAP) kinases ASK1 and ASK2 centrally regulate NF-κB and downstream MAP kinases, including JNK, ERK, and p38, to drive autoinflammatory disease progression in the *Ptpn6^{spin}* mouse model of neutrophilic dermatosis (28). We hypothesized that NF-κB and MAP kinase signaling promote *cmo* disease progression and that SYK plays a role in regulating this signaling. Although there was more activation of NF-κB and ERK in the footpads of *Pstpip2^{cmo}* mice compared with WT mice, JNK and p38 were activated similarly (Fig. 3*C*). However, deletion of SYK reversed the elevated NF-κB but not ERK activation in *Pstpip2^{cmo}* mice, suggesting that NF-κB plays an important role downstream of SYK to mediate persistent inflammation in *cmo* disease.

Furthermore, SYK has been shown to regulate inflammasome activation and IL-1β maturation downstream of dectin-1 signaling (16). We therefore asked whether SYK regulates both NLRP3 inflammasome and caspase-8 activation upstream of IL-1β production. We observed similar caspase-1 and caspase-8 cleavage in BMDMs derived from WT, *Pstpip2^{cmo}*, and *Pstpip2^{cmo}Syk$^{fl/fl}$LysM^{cre}* mice in response to the classical NLRP3 inflammasome trigger LPS + ATP, which was further supported by the similar gasdermin D (GSDMD) activation observed among these genotypes (Fig. 3*D*). In addition, we further noticed that SYK deficiency did not affect the expression of pro-IL-1β, NLRP3, and ASC, all of which are crucial components of inflammasome signaling (Fig. 3*E*). These data suggest that SYK does not regulate the caspase-1 and caspase-8 activation mediated by the classical NLRP3 trigger. Overall, our data indicate that SYK regulates NF-κB signaling, but not inflammasome activation, for the induction of pro-IL-1β to mediate disease progression in *Pstpip2^{cmo}* mice.

Discussion

Cmo has been shown to be mediated by pathological IL-1β production downstream of NLRP3/caspase-1 and caspase-8 (9, 11, 12). Disease progression occurs despite single deficiency of either caspase-1 or caspase-8 (12), which suggests that the caspases function as part of distinct complexes that are independently activated. Although caspase-1 and caspase-8 have both been shown to colocalize with AIM2/ASC speck to medi-

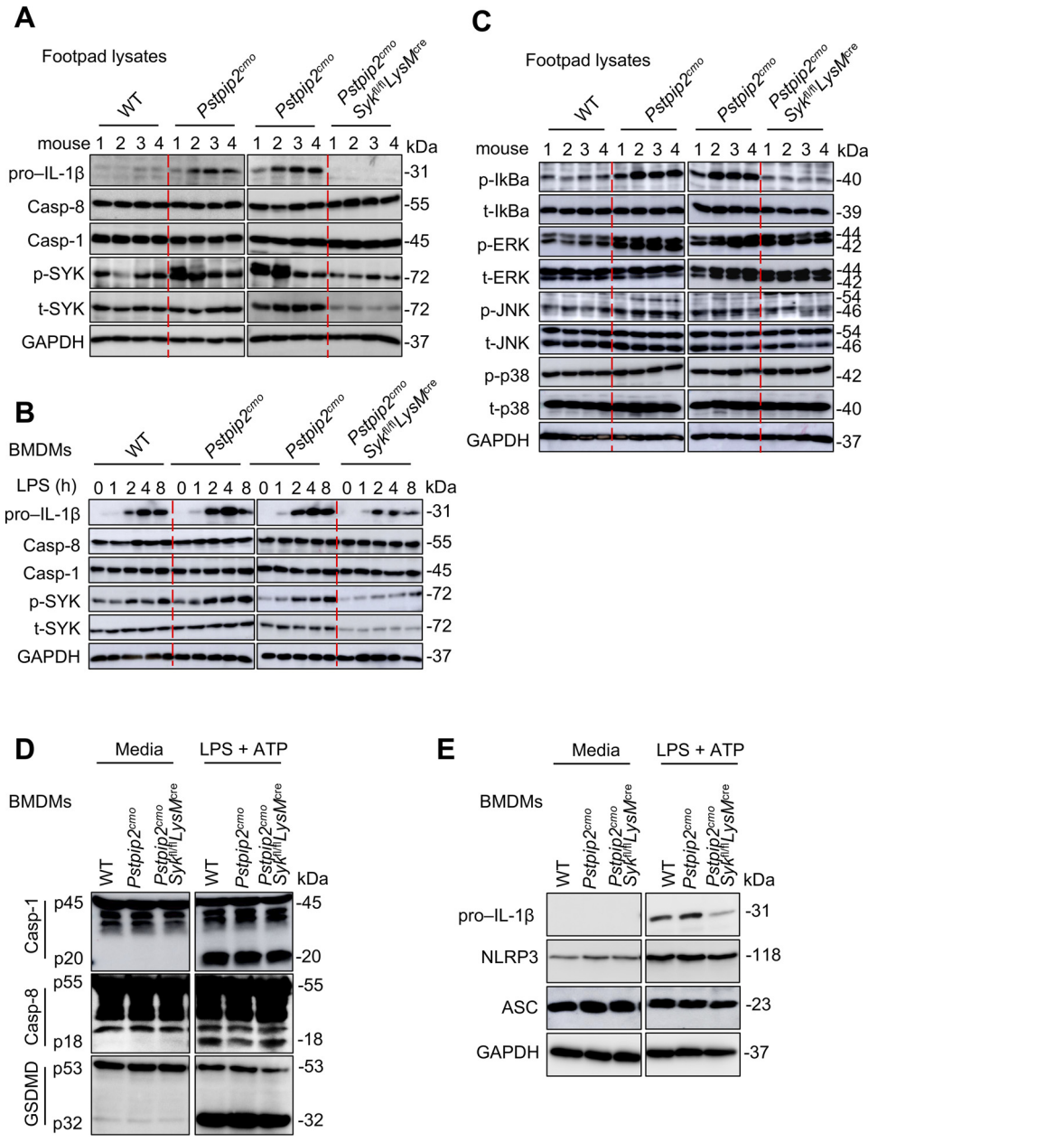

Figure 3. SYK is involved in regulating the levels of pro-IL-1β and NF-κB in *Pstpip2^{cmo}* mice. *A*, immunoblot analysis of pro-IL-1β, caspase-8 (Casp-8), caspase-1 (Casp-1), phospho-SYK (p-SYK), total SYK (t-SYK), and GAPDH in WT, *Pstpip2^{cmo}*, and *Pstpip2^{cmo}Syk$^{fl/fl}$LysM^{cre}* footpad lysates. *B*, immunoblot analysis of pro-IL-1β, Casp-8, Casp-1, p-SYK, t-SYK, and GAPDH in WT, *Pstpip2^{cmo}*, and *Pstpip2^{cmo}Syk$^{fl/fl}$LysM^{cre}* BMDMs at the indicated time points after LPS treatment. *C*, immunoblot analysis of phospho-IκBα (p-IκBα), total IκBα (t-IκBα), phospho-ERK (p-ERK), total ERK (t-ERK), phospho-JNK (p-JNK), total JNK (t-JNK), phospho-p38 (p-p38), total p38 (t-p38), and GAPDH in WT, *Pstpip2^{cmo}*, and *Pstpip2^{cmo}Syk$^{fl/fl}$LysM^{cre}* footpad lysates. *D*, immunoblot analysis of activated (cleaved) Casp-1, Casp-8, and GSDMD in WT, *Pstpip2^{cmo}*, and *Pstpip2^{cmo}Syk$^{fl/fl}$LysM^{cre}* BMDMs treated with LPS + ATP or left untreated with medium. *E*, immunoblot analysis of the inflammasome components pro-IL-1β, NLRP3, ASC, and GAPDH in WT, *Pstpip2^{cmo}*, and *Pstpip2^{cmo}Syk$^{fl/fl}$LysM^{cre}* BMDMs treated with LPS + ATP or left untreated with medium. Representative blots from three independent experiments are shown.

ate pro-IL-1β cleavage (22), AIM2 deficiency did not provide protection in *Pstpip2^{cmo}* mice, further supporting that, in *cmo* disease, caspase-1 and caspase-8 operate and are activated independently in distinct complexes. In this study, we demonstrated that deficiency of SYK in *Pstpip2^{cmo}* mice prevented the induction of osteomyelitis. SYK signaling upstream of caspase-1 and caspase-8 to promote pro-IL-1β production centrally mediates *cmo* disease induction. Thus, it is interesting that deficiency of the SYK adaptor protein CARD9 did not provide protection in *Pstpip2^{cmo}* mice. In addition to promoting pro-IL-1β synthesis, SYK, but not CARD9, has been shown to regulate NLRP3 inflammasome activation during fungal infection (29). This suggests that the CARD9 pathway selectively transduces SYK signaling to promote pro-IL-1β synthesis but not inflammasome activation. Additionally, several reports have highlighted the role of SYK in regulation of the NLRP3- and caspase-8-mediated inflammasomes (16, 29, 30). However, our data with the canonical NLRP3 trigger LPS + ATP did not reveal a dependence of caspase-1 and caspase-8 processing on SYK, suggesting an exclusively diverse but specific role for SYK in mediating *cmo* disease. In this regard, SYK primarily acts as a pivotal regulator of pro-IL-1β synthesis but not as a regulator of

inflammasome activation; however, these two processes both converge toward the production of active IL-1β. Recent evidence has also established central roles for the NLRP3 inflammasome and IL-1β signaling in several additional related disorders of nonbacterial osteomyelitis, including Majeed syndrome; synovitis, acne, pustulosis, hyperostosis, and osteitis syndrome; and deficiency of IL-1R antagonist (3, 9, 12). Our findings provide an important context for evaluating the role of SYK in mediating these related autoinflammatory bone disorders and for the therapeutic potential of SYK inhibitors in this disease spectrum.

The central regulatory role of SYK is not confined to IL-1β–mediated autoinflammatory disease. We have reported previously that SYK licenses MyD88 to induce IL-1α–mediated inflammatory disease in *Ptpn6*^{spin} mice (31). Similarly, we observed increased activation of SYK in the absence of PSTPIP2, suggesting that PSTPIP2 functions to suppress SYK signaling. However, the regulatory mechanisms behind SYK activation by PSTPIP2 require further investigation. Recent evidence has established that PSTPIP2 interacts with SHIP1, which is encoded by *Ptpn6* (32), suggesting that SHIP1 may be able to modulate SYK activation through its phosphatase activity.

SYK signaling is known to be activated downstream of various cell surface receptors, including CD74, integrins, C-type lectin receptors (dectin-1 and dectin-2), and Fc receptors (27). Identification of the specific triggers of SYK activation in these *Pstpip2^{cmo}* mice would further clarify the signaling mechanism and provide a deeper understanding of the progression of *cmo* disease. SYK signaling has also been strongly associated with the recruitment of neutrophils to areas of inflammation (33). The marked reductions in inflammation and lymphomegaly seen in SYK-deficient *Pstpip2^{cmo}* mice indicate that SYK signaling potentially mediates neutrophil recruitment in *Pstpip2^{cmo}* mice. Although T cell dysregulation has been associated with inflammatory bone diseases, previous studies have characterized the osteomyelitis in *cmo* disease by increased neutrophil numbers without T cell abnormalities (9, 34). As neutrophils have been implicated as major contributors to IL-1β production in *cmo* (11), our findings suggest that SYK-mediated recruitment and activation of neutrophils may also play a role in promoting the boney inflammation characterizing *Pstpip2^{cmo}* mice. Previous studies have shown that inhibition of signaling pathways highly associated with caspase-8 activation and inflammatory bone disease, such as tumor necrosis factor signaling, fails to protect against *cmo* disease (9, 12). This also indicates that current guidelines for the therapeutic use of tumor necrosis factor inhibitors in the subset of patients with CRMO and concurrent autoimmune diseases may not be effective in treating CRMO. Therapeutic options for the largely pediatric and adolescent CRMO population are limited by nonspecificity and inadequate control of pain and disease progression, which can result in physical disabilities or permanent deformities. As genetic deletion of *Syk* in the myeloid compartment of *Pstpip2^{cmo}* mice resulted in complete prevention of disease induction and progression, SYK and its downstream signaling components represent promising novel therapeutic targets in CRMO.

Experimental procedures

Mice

Pstpip2^{cmo} (35), *Nlrp3*$^{-/-}$ (36), *Ripk3*$^{-/-}$ (37), *Aim2*$^{-/-}$ (38), *Card9*$^{-/-}$ (39), and *Syk*$^{fl/fl}$*LysM*^{cre} (25) mice have been described previously. *Pstpip2^{cmo}Nlrp3*$^{-/-}$ mice were generated by crossing *Pstpip2^{cmo}* and *Nlrp3*$^{-/-}$ mice. Then *Pstpip2^{cmo}Nlrp3*$^{-/-}$ *Ripk3*$^{-/-}$, *Pstpip2^{cmo}Nlrp3*$^{-/-}$*Aim2*$^{-/-}$, *Pstpip2^{cmo}Nlrp3*$^{-/-}$ *Card9*$^{-/-}$, and *Pstpip2^{cmo}Nlrp3*$^{-/-}$*Syk*$^{fl/fl}$*LysM*^{cre} mice were generated by crossing *Pstpip2^{cmo}Nlrp3*$^{-/-}$ mice onto *Ripk3*$^{-/-}$, *Aim2*$^{-/-}$, *Card9*$^{-/-}$, and *Syk*$^{fl/fl}$*LysM*^{cre} backgrounds, respectively. *Pstpip2^{cmo}Syk*$^{fl/fl}$*LysM*^{cre} mice were generated by crossing *Pstpip2^{cmo}* and *Syk*$^{fl/fl}$*LysM*^{cre} mice. *Pstpip2^{cmo}* mice were purchased from The Jackson Laboratory and were on the BALB/c background. All other mutant mice were on the C57BL/6 background. Littermate controls were utilized to evaluate the influence of genetic deletions on immune responses, IL-1β regulation, and *cmo* disease progression. All mice were kept in the Animal Resource Center at St. Jude Children's Research Hospital. Animal studies were conducted according to protocols approved by the St. Jude Animal Care and Use Committee.

Cell culture and stimulation

Primary BMDMs were grown for 5 to 6 days in Iscove's modified Dulbecco's medium (Gibco) supplemented with 10% FBS (Atlanta Biologicals), 30% L929-conditioned medium, 1% nonessential amino acids (Gibco), and 1% penicillin/streptomycin (Sigma). BMDMs were seeded at a concentration of 1×10^6 cells onto 12-well plates. After incubating overnight, cells were stimulated with LPS (100 ng/ml, InvivoGen) for the indicated time (0–8 h) or treated with LPS + ATP (LPS, 4 h; ATP (5 mM, Roche), 30 min) (38) before cell harvest.

Western blotting

For immunoblotting, BMDMs and footpad protein lysates were prepared by tissue homogenization in radioimmune precipitation assay lysis buffer supplemented with a protease inhibitor mixture (Roche) and PhosSTOP (Roche). A Pierce BCA protein assay kit was used to quantify samples. A total of 40 μg of protein was resolved using SDS-PAGE and transferred onto PVDF membranes (40). The membranes were blocked in 5% skim milk before primary antibodies were added and incubated overnight at 4 °C. Afterward, membranes were incubated with HRP-tagged secondary antibodies for 1 h at room temperature. Primary antibodies were anti-GAPDH (Cell Signaling Technology, catalog no. 5174), anti-IL-1β (Cell Signaling Technology, catalog no. 12507), anti-phospho-ERK1/2 (Cell Signaling Technology, catalog no. 9101), anti-total ERK1/2 (Cell Signaling Technology, catalog no. 9102), anti-phospho-p38 (Cell Signaling Technology, catalog no. 9211), anti-total p38 (Cell Signaling Technology, catalog no. 9212), anti-phospho-IκBα (Cell Signaling Technology, catalog no. 2859), anti-total IκBα (Cell Signaling Technology, catalog no. 9242), anti-phospho-SYK (Cell Signaling Technology, catalog no. 2717), anti-total SYK (Cell Signaling Technology, catalog no. 2712), anti-phospho-JNK (Cell Signaling Technology, catalog no. 9251), anti-total JNK (Cell Signaling Technology, catalog no. 9252), anti-caspase-1 (Adipogen, catalog no. AG-20B-0044-C100),

anti-ASC (Adipogen, catalog no. AG-25B-0006-C100), anti-NLRP3 (Adipogen, catalog no. AG-20B-0014-C100), anti-gasdermin D (Abcam, catalog no. Ab155233), and anti-caspase-8 (Adipogen, catalog no. AG-20T-0138-C100). Secondary HRP antibodies were purchased from Jackson ImmunoResearch Laboratories.

Micro-CT

A Siemens Inveon μCT scanner (Siemens Healthcare) was used to capture micro-CT images. Mouse footpads were imaged with a 672 $\times$ 1344 mm matrix and a field of view of 30.04 $\times$ 60.08 mm with one bed position. Projections were obtained at 80 peak kilovoltage and 500 μA (1050-ms exposure, 1000-ms settle time) over half rotation (440 projections), giving an isotropic resolution of 44.7 μm. Inveon Research Workplace software was used to process the data.

Statistical analysis

Each experiment was repeated at least twice before inclusion in the manuscript. The log-rank (Mantel–Cox) test was used to compare statistical significance between survival curves in the two groups.

Author contributions—T. K. D., R. G., R. K., B. B., B. R. S., P. G., and A. B. data curation; T. K. D., R. G., R. K., B. B., B. R. S., P. G., and A. B. formal analysis; T. K. D., R. G., R. K., B. B., B. R. S., P. G., and A. B. investigation; T. K. D. and R. K. writing-original draft; T. K. D., R. G., R. K., B. B., B. R. S., P. G., A. B., and T.-D. K. writing-review and editing; R. K. and T.-D. K. supervision; T.-D. K. conceptualization; T.-D. K. funding acquisition.

Acknowledgments—We thank Nicole Lantz for help with animal husbandry and Rebecca Tweedell, Ph.D., for scientific editing and writing support. We also extend thanks to Dr. Jieun Kim in the St. Jude Small Animal Imaging Center for help with acquiring and analyzing the micro-CT data.

References

1. Crowson, C. S., Matteson, E. L., Myasoedova, E., Michet, C. J., Ernste, F. C., Warrington, K. J., Davis, J. M., 3rd, Hunder, G. G., Therneau, T. M., and Gabriel, S. E. (2011) The lifetime risk of adult-onset rheumatoid arthritis and other inflammatory autoimmune rheumatic diseases. *Arthritis Rheum.* **63,** 633–639 CrossRef Medline
2. Jurik, A. G. (2004) Chronic recurrent multifocal osteomyelitis. *Semin. Musculoskelet. Radiol.* **8,** 243–253 CrossRef Medline
3. Hofmann, S. R., Kapplusch, F., Girschick, H. J., Morbach, H., Pablik, J., Ferguson, P. J., and Hedrich, C. M. (2017) Chronic recurrent multifocal osteomyelitis (CRMO): presentation, pathogenesis, and treatment. *Curr. Osteoporos. Rep.* **15,** 542–554 CrossRef Medline
4. Roberts-Galbraith, R. H., and Gould, K. L. (2010) Setting the F-BAR: functions and regulation of the F-BAR protein family. *Cell Cycle* **9,** 4091–4097 CrossRef Medline
5. Stern, S. M., and Ferguson, P. J. (2013) Autoinflammatory bone diseases. *Rheum. Dis. Clin. North Am.* **39,** 735–749 CrossRef Medline
6. Chitu, V., Nacu, V., Charles, J. F., Henne, W. M., McMahon, H. T., Nandi, S., Ketchum, H., Harris, R., Nakamura, M. C., and Stanley, E. R. (2012) PSTPIP2 deficiency in mice causes osteopenia and increased differentiation of multipotent myeloid precursors into osteoclasts. *Blood* **120,** 3126–3135 CrossRef Medline
7. Daheshia, M., and Yao, J. Q. (2008) The interleukin 1β pathway in the pathogenesis of osteoarthritis. *J. Rheumatol.* **35,** 2306–2312 CrossRef Medline
8. Li, Y., Li, N., Yan, Z., Li, H., Chen, L., Zhang, Z., Fan, G., Xu, K., and Li, Z. (2016) Dysregulation of the NLRP3 inflammasome complex and related cytokines in patients with multiple myeloma. *Hematology* **21,** 144–151 CrossRef Medline
9. Lukens, J. R., Gross, J. M., Calabrese, C., Iwakura, Y., Lamkanfi, M., Vogel, P., and Kanneganti, T. D. (2014) Critical role for inflammasome-independent IL-1β production in osteomyelitis. *Proc. Natl. Acad. Sci. U.S.A.* **111,** 1066–1071 CrossRef Medline
10. Karki, R., and Kanneganti, T. D. (2019) Diverging inflammasome signals in tumorigenesis and potential targeting. *Nat. Rev. Cancer* **19,** 197–214 Medline
11. Lukens, J. R., Gurung, P., Vogel, P., Johnson, G. R., Carter, R. A., McGoldrick, D. J., Bandi, S. R., Calabrese, C. R., Vande Walle, L., Lamkanfi, M., and Kanneganti, T. D. (2014) Dietary modulation of the microbiome affects autoinflammatory disease. *Nature* **516,** 246–249 CrossRef Medline
12. Gurung, P., Burton, A., and Kanneganti, T. D. (2016) NLRP3 inflammasome plays a redundant role with caspase 8 to promote IL-1β-mediated osteomyelitis. *Proc. Natl. Acad. Sci. U.S.A.* **113,** 4452–4457 CrossRef Medline
13. Lin, Y. C., Huang, D. Y., Chu, C. L., and Lin, W. W. (2010) Anti-inflammatory actions of Syk inhibitors in macrophages involve non-specific inhibition of Toll-like receptors-mediated JNK signaling pathway. *Mol. Immunol.* **47,** 1569–1578 CrossRef Medline
14. Lin, Y. C., Huang, D. Y., Chu, C. L., Lin, Y. L., and Lin, W. W. (2013) The tyrosine kinase Syk differentially regulates Toll-like receptor signaling downstream of the adaptor molecules TRAF6 and TRAF3. *Sci. Signal.* **6,** ra71 Medline
15. Hara, H., Tsuchiya, K., Kawamura, I., Fang, R., Hernandez-Cuellar, E., Shen, Y., Mizuguchi, J., Schweighoffer, E., Tybulewicz, V., and Mitsuyama, M. (2013) Phosphorylation of the adaptor ASC acts as a molecular switch that controls the formation of speck-like aggregates and inflammasome activity. *Nat. Immunol.* **14,** 1247–1255 CrossRef Medline
16. Gringhuis, S. I., Kaptein, T. M., Wevers, B. A., Theelen, B., van der Vlist, M., Boekhout, T., and Geijtenbeek, T. B. (2012) Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1β via a noncanonical caspase-8 inflammasome. *Nat. Immunol.* **13,** 246–254 CrossRef Medline
17. Gurung, P., and Kanneganti, T. D. (2015) Novel roles for caspase-8 in IL-1β and inflammasome regulation. *Am. J. Pathol.* **185,** 17–25 CrossRef Medline
18. Oberst, A., Dillon, C. P., Weinlich, R., McCormick, L. L., Fitzgerald, P., Pop, C., Hakem, R., Salvesen, G. S., and Green, D. R. (2011) Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature* **471,** 363–367 CrossRef Medline
19. Kaiser, W. J., Upton, J. W., Long, A. B., Livingston-Rosanoff, D., Daley-Bauer, L. P., Hakem, R., Caspary, T., and Mocarski, E. S. (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature* **471,** 368–372 CrossRef Medline
20. Moriwaki, K., Bertin, J., Gough, P. J., and Chan, F. K. (2015) A RIPK3-caspase 8 complex mediates atypical pro-IL-1β processing. *J. Immunol.* **194,** 1938–1944 CrossRef Medline
21. Bast, A., Krause, K., Schmidt, I. H., Pudla, M., Brakopp, S., Hopf, V., Breitbach, K., and Steinmetz, I. (2014) Caspase-1-dependent and -independent cell death pathways in *Burkholderia pseudomallei* infection of macrophages. *PLoS Pathog.* **10,** e1003986 CrossRef Medline
22. Pierini, R., Juruj, C., Perret, M., Jones, C. L., Mangeot, P., Weiss, D. S., and Henry, T. (2012) AIM2/ASC triggers caspase-8-dependent apoptosis in *Francisella*-infected caspase-1-deficient macrophages. *Cell Death Differ.* **19,** 1709–1721 CrossRef Medline
23. Mascarenhas, D. P. A., Cerqueira, D. M., Pereira, M. S. F., Castanheira, F. V. S., Fernandes, T. D., Manin, G. Z., Cunha, L. D., and Zamboni, D. S. (2017) Inhibition of caspase-1 or gasdermin-D enable caspase-8 activation in the Naip5/NLRC4/ASC inflammasome. *PLoS Pathog.* **13,** e1006502 CrossRef Medline
24. Mócsai, A., Humphrey, M. B., Van Ziffle, J. A., Hu, Y., Burghardt, A., Spusta, S. C., Majumdar, S., Lanier, L. L., Lowell, C. A., and Nakamura, M. C. (2004) The immunomodulatory adapter proteins DAP12 and Fc receptor γ-chain (FcRγ) regulate development of functional osteoclasts

through the Syk tyrosine kinase. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 6158–6163 CrossRef Medline

25. Malik, A., Sharma, D., Malireddi, R. K. S., Guy, C. S., Chang, T. C., Olsen, S. R., Neale, G., Vogel, P., and Kanneganti, T. D. (2018) SYK-CARD9 Signaling axis promotes gut fungi-mediated inflammasome activation to restrict colitis and colon cancer. *Immunity* **49,** 515–530.e5 CrossRef Medline
26. Wagener, M., Hoving, J. C., Ndlovu, H., and Marakalala, M. J. (2018) Dectin-1-Syk-CARD9 signaling pathway in TB immunity. *Front. Immunol.* **9,** 225 CrossRef Medline
27. Mócsai, A., Ruland, J., and Tybulewicz, V. L. (2010) The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat. Rev. Immunol.* **10,** 387–402 CrossRef Medline
28. Tartey, S., Gurung, P., Dasari, T. K., Burton, A., and Kanneganti, T. D. (2018) ASK1/2 signaling promotes inflammation in a mouse model of neutrophilic dermatosis. *J. Clin. Invest.* **128,** 2042–2047 CrossRef Medline
29. Gross, O., Poeck, H., Bscheider, M., Dostert, C., Hannesschläger, N., Endres, S., Hartmann, G., Tardivel, A., Schweighoffer, E., Tybulewicz, V., Mocsai, A., Tschopp, J., and Ruland, J. (2009) Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* **459,** 433–436 CrossRef Medline
30. Lin, Y. C., Huang, D. Y., Wang, J. S., Lin, Y. L., Hsieh, S. L., Huang, K. C., and Lin, W. W. (2015) Syk is involved in NLRP3 inflammasome-mediated caspase-1 activation through adaptor ASC phosphorylation and enhanced oligomerization. *J. Leukoc. Biol.* **97,** 825–835 CrossRef Medline
31. Gurung, P., Fan, G., Lukens, J. R., Vogel, P., Tonks, N. K., and Kanneganti, T. D. (2017) Tyrosine kinase SYK licenses MyD88 adaptor protein to instigate IL-1α-mediated inflammatory disease. *Immunity* **46,** 635–648 CrossRef Medline
32. Drobek, A., Kralova, J., Skopcova, T., Kucova, M., Novák, P., Angelisová, P., Otahal, P., Alberich-Jorda, M., and Brdicka, T. (2015) PSTPIP2, a protein associated with autoinflammatory disease, interacts with inhibitory enzymes SHIP1 and Csk. *J. Immunol.* **195,** 3416–3426 CrossRef Medline
33. Van Ziffle, J. A., and Lowell, C. A. (2009) Neutrophil-specific deletion of Syk kinase results in reduced host defense to bacterial infection. *Blood* **114,** 4871–4882 CrossRef Medline
34. Grosse, J., Chitu, V., Marquardt, A., Hanke, P., Schmittwolf, C., Zeitlmann, L., Schropp, P., Barth, B., Yu, P., Paffenholz, R., Stumm, G., Nehls, M., and Stanley, E. R. (2006) Mutation of mouse Mayp/Pstpip2 causes a macrophage autoinflammatory disease. *Blood* **107,** 3350–3358 CrossRef Medline
35. Chitu, V., Ferguson, P. J., de Bruijn, R., Schlueter, A. J., Ochoa, L. A., Waldschmidt, T. J., Yeung, Y. G., and Stanley, E. R. (2009) Primed innate immunity leads to autoinflammatory disease in PSTPIP2-deficient cmo mice. *Blood* **114,** 2497–2505 CrossRef Medline
36. Karki, R., Man, S. M., Malireddi, R. K. S., Gurung, P., Vogel, P., Lamkanfi, M., and Kanneganti, T. D. (2015) Concerted activation of the AIM2 and NLRP3 inflammasomes orchestrates host protection against *Aspergillus* infection. *Cell Host Microbe* **17,** 357–368 CrossRef Medline
37. Newton, K., Sun, X., and Dixit, V. M. (2004) Kinase RIP3 is dispensable for normal NF-κBs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. *Mol. Cell Biol.* **24,** 1464–1469 CrossRef Medline
38. Karki, R., Lee, E., Place, D., Samir, P., Mavuluri, J., Sharma, B. R., Balakrishnan, A., Malireddi, R. K. S., Geiger, R., Zhu, Q., Neale, G., and Kanneganti, T. D. (2018) IRF8 regulates transcription of NAIPS for NLRC4 inflammasome activation. *Cell* **173,** 920–933 e913 CrossRef Medline
39. Gross, O., Gewies, A., Finger, K., Schäfer, M., Sparwasser, T., Peschel, C., Förster, I., and Ruland, J. (2006) Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature* **442,** 651–656 CrossRef Medline
40. Sharma, B. R., Karki, R., Lee, E., Zhu, Q., Gurung, P., and Kanneganti, T. D. (2019) Innate immune adaptor MyD88 deficiency prevents skin inflammation in SHARPIN-deficient mice. *Cell Death Differ.* **26,** 741–750 CrossRef Medline