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

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

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Chronic recurrent multifocal osteomyelitis (CRMO) in humans can be modeled in Pstpip2cmo 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 Pstpip2cmo 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 Pstpip2cmo 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 Pstpip2cmo 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), osteoporosis, Paget’s disease, arthritis, and periodontal disease, are increasingly per-vasive 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 (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 Pstpip2cmo 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 Pstpip2cmo 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

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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.

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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 Pstpip2cmo mice

The NLRP3 inflammasome plays a redundant role with caspase-8 to promote disease progression in Pstpip2cmo 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 Pstpip2cmo mice by analyzing cmo disease progression in NLRP3- and RIPK3-deficient Pstpip2cmo mice (Pstpip2cmoNlrp3−/−Ripk3−/−) and NLRP3- and AIM2-deficient Pstpip2cmo mice (Pstpip2cmoNlrp3−/−Aim2−/−). All mice with both genotypes (Pstpip2cmoNlrp3−/−Ripk3−/− and Pstpip2cmoNlrp3−/−Aim2−/−) developed disease similarly to Pstpip2cmo 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 lymphohemag 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 Pstpip2cmo mice.

Figure 1. RIPK3 and AIM2 are dispensable for disease progression in Pstpip2cmo mice. A, incidence of disease in WT (n = 5), Pstpip2cmoNlrp3−/− (n = 9), and Pstpip2cmoNlrp3−/−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), Pstpip2cmoNlrp3−/− (n = 10), and Pstpip2cmoNlrp3−/−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 Pstpip2cmo 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 Pstpip2cmo mice. First, we monitored disease progression in Pstpip2cmoNlrp3−/−Sykfl/flLysMcre mice and Pstpip2cmoNlrp3−/−Card9−/− mice. Although Pstpip2cmoNlrp3−/−Card9−/− mice did not show protection from disease, Pstpip2cmoNlrp3−/−Sykfl/flLysMcre mice displayed nearly complete protection (Fig. 2, A and B). Next we investigated whether deletion of SYK in Pstpip2cmo mice with intact NLRP3 would be sufficient to provide protection from disease. We found that myeloid-specific deletion of SYK alone in Pstpip2cmo mice (Pstpip2cmoSyrfl/flLysMcre) provided complete protection from disease (Fig. 2C). The structural bone lesions found by micro-CT and the popliteal lymphohemag observed in Pstpip2cmo, Pstpip2cmoNlrp3−/−, and Pstpip2cmoNlrp3−/−Card9−/− mice
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Figure 2. CARD9, but not SYK, is dispensable for disease progression in Pstpip2cmo mice. A, incidence of disease in WT (n = 5), Pstpip2cmoNlrp3−/− (n = 10), and Pstpip2cmoNlrp3−/−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), Pstpip2cmoNlrp3−/− (n = 8), and Pstpip2cmoNlrp3−/−Syk−/−LysMcre (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), Pstpip2cmo (n = 7), and Pstpip2cmoSyk−/−LysMcre (n = 13) mice over the experimental course and representative footpad images, footpad CT scans, and popliteal lymph nodes from these respective 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-
ate pro-IL-1β cleavage (22), AIM2 deficiency did not provide protection in Pstpip2<sup>cmo</sup> 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<sup>cmo</sup> 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<sup>cmo</sup> 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

Figure 3. SYK is involved in regulating the levels of pro-IL-1β and NF-κB in Pstpip2<sup>cmo</sup> 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<sup>cmo</sup>, and Pstpip2<sup>cmo</sup>Syk<sup>fl/fl</sup>LysM<sup>cre</sup> footpad lysates. B, immunoblot analysis of pro-IL-1β, Casp-8, Casp-1, p-SYK, t-SYK, and GAPDH in WT, Pstpip2<sup>cmo</sup>, and Pstpip2<sup>cmo</sup>Syk<sup>fl/fl</sup>LysM<sup>cre</sup> 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), and GAPDH in WT, Pstpip2<sup>cmo</sup>, and Pstpip2<sup>cmo</sup>Syk<sup>fl/fl</sup>LysM<sup>cre</sup> footpad lysates. D, immunoblot analysis of activated (cleaved) Casp-1, Casp-8, and GSDMD in WT, Pstpip2<sup>cmo</sup>, and Pstpip2<sup>cmo</sup>Syk<sup>fl/fl</sup>LysM<sup>cre</sup> 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<sup>cmo</sup>, and Pstpip2<sup>cmo</sup>Syk<sup>fl/fl</sup>LysM<sup>cre</sup> BMDMs treated with LPS + ATP or left untreated with medium. Representative blots from three independent experiments are shown.
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<sup>−/−</sup> 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 Ptpip2<sup>−/−</sup> 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 Ptpip2<sup>−/−</sup> mice indicate that SYK signaling potently mediates neutrophil recruitment in Ptpip2<sup>−/−</sup> 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 bony inflammation characterizing Ptpip2<sup>−/−</sup> 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 non-specificity 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 Ptpip2<sup>−/−</sup> 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

Ptpip2<sup>−/−</sup> (35), Nlrp3<sup>−/−</sup> (36), Ripk3<sup>−/−</sup> (37), Aim2<sup>−/−</sup> (38), Card9<sup>−/−</sup> (39), and Syk<sup>fl/fl</sup>LysMcre (25) mice have been described previously. Ptpip2<sup>−/−</sup>Nlrp3<sup>−/−</sup> mice were generated by crossing Ptpip2<sup>−/−</sup> and Nlrp3<sup>−/−</sup> mice. Then Ptpip2<sup>−/−</sup>Nlrp3<sup>−/−</sup>Ripk3<sup>−/−</sup>, Ptpip2<sup>−/−</sup>Nlrp3<sup>−/−</sup>Aim2<sup>−/−</sup>, Ptpip2<sup>−/−</sup>Nlrp3<sup>−/−</sup>Card9<sup>−/−</sup>, and Ptpip2<sup>−/−</sup>Nlrp3<sup>−/−</sup>Syk<sup>fl/fl</sup>LysMcre mice were generated by crossing Ptpip2<sup>−/−</sup>Nlrp3<sup>−/−</sup> mice onto Ripk3<sup>−/−</sup>, Aim2<sup>−/−</sup>, Card9<sup>−/−</sup>, and Syk<sup>fl/fl</sup>LysMcre backgrounds, respectively. Ptpip2<sup>−/−</sup>Syk<sup>fl/fl</sup>LysMcre mice were generated by crossing Ptpip2<sup>−/−</sup> and Syk<sup>fl/fl</sup>LysMcre mice. Ptpip2<sup>−/−</sup> 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% non-essential amino acids (Gibco), and 1% penicillin/streptomycin (Atlanta Biologicals). BMDMs were seeded at a concentration of 1 × 10<sup>6</sup> 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. 2842), 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 × 1344 mm matrix and a field of view of 30.04 × 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.

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**EDITORS’ PICK:** Targeting SYK prevents disease in Pstpip2<sup>cmo</sup> mice