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Inflammation in Mice Ectopically Expressing Human Pyogenic Arthritis, Pyoderma Gangrenosum, and Acne (PAPA) Syndrome-associated PSTPIP1 A230T Mutant Proteins*[S]

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Background: PAPA syndrome is an autoinflammatory disease linked to mutations in the *PSTPIP1* gene.
Results: Ectopic expression of mutant PSTPIP1 leads to elevated level of circulating proinflammatory cytokines.
Conclusion: Ectopic expression of mutant PSTPIP1 in mice partially recapitulates symptoms in human PAPA syndrome patients.
Significance: These observations provide the first genetic analysis elucidating the pathophysiological function of PSTPIP1.

Pyogenic Arthritis, Pyoderma Gangrenosum, and Acne Syndrome (PAPA syndrome) is an autoinflammatory disease caused by aberrant production of the proinflammatory cytokine interleukin-1. Mutations in the gene encoding proline serine threonine phosphatase-interacting protein-1 (PSTPIP1) have been linked to PAPA syndrome. PSTPIP1 is an adaptor protein that interacts with PYRIN, the protein encoded by the Mediterranean Fever (*MEFV*) gene whose mutations cause Familial Mediterranean Fever (FMF). However, the pathophysiological function of PSTPIP1 remains to be elucidated. We have generated mouse strains that either are PSTPIP1 deficient or ectopically express mutant PSTPIP1. Results from analyzing these mice suggested that PSTPIP1 is not an essential regulator of the Nlrp3, Aim2, or Nlrc4 inflammasomes. Although common features of human PAPA syndrome such as pyogenic arthritis and skin inflammation were not recapitulated in the mouse model, ectopic expression of the mutant but not the wild type PSTPIP1 in mice lead to partial embryonic lethality, growth retardation, and elevated level of circulating proinflammatory cytokines.

The proinflammatory cytokine interleukin-1β plays a pivotal role in the host response against infection. However, dysregulation of IL-1 signaling underlies a variety of acute and chronic inflammatory diseases (1). IL-1β and its closely related IL-18 are atypical cytokines, in that they are synthesized as non-functional cytosolic precursors. These precursors have to be processed into mature, biologically active forms. One of the most extensively studied IL-1 processing machineries is the inflammasome, a large protein complex formed in response to a myriad of inflammatory stimuli (2). Formation of the inflammasome induces the proximity of multiple procaspase-1 molecules that results in autocatalytic processing of procaspase-1 into its active form, which is then capable of cleaving the pro-IL-1β and IL-18 into mature biologically active cytokines (2).

Dysregulation of inflammasome activity is linked to a number of human autoinflammatory disorders, which are characterized by overproduction of IL-1β (3). Mutations in the *CIAS1* gene that encodes NLRP3 (also known as NALP3/Cryopyrin) are associated with three types of autoinflammatory diseases commonly called Cryopyrinopathies. Likewise, mutations in the *MEFV* gene that encodes PYRIN are associated with the most common autoinflammatory disease, Familial Mediterranean Fever (FMF) (4). PYRIN interacts with adaptor protein, apoptosis-associated Speck-like protein containing a CARD (ASC), and like NLRP3, triggers ASC oligomerization, activation of caspase-1, and IL-1β processing (5). Additional studies have also suggested that PYRIN may function as an anti-inflammatory agent by sequestering ASC (6). The exact function of

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PYRIN under physiological or infectious conditions, however, remains unclear.

The Pyogenic Arthritis, Pyoderma Gangrenosum, and Cystic Acne Syndrome (hereafter referred to as PAPAS,[6] OMIM604416), also known as Familial Recurrent Arthritis (FRA), is characterized by early onset, recurrent sterile arthritis and intense inflammation leading to joint destruction. Pyoderma gangrenosum characterized by purulent ulcerative skin lesions occurs in some patients, as does cystic acne (7). Monocytes from PAPAS patients produce significantly higher amount of IL-1β compared with those from normal subjects in response to LPS stimulation (8). Furthermore, PAPAS patients respond to anti-IL-1 therapy (9, 10). Taken together, these observations suggest that excessive production of IL-1 likely underlie the pathology of PAPAS.

Two mis-sense mutations, A230T and E250Q, in the gene encoding CD2-binding protein-1 (CD2BP1), now designated as Proline-Serine-Threonine Phosphatase-interacting Protein-1 (PSTPIP1), have been linked to PAPA syndrome (7). PSTPIP1 is an adaptor protein consisting of an N-terminal FER/CIP4 homologous domain (FCH), an intermediate coiled coil domain and a C-terminal SH3 domain. PSTPIP1 interacts with PEST-type protein tyrosine phosphatases (PEST-PTPs), and PYRIN. The two mutations responsible for PAPAS appear to diminish the interaction of PSTPIP1 with PEST-PTP. As a result, those mutant PSTPIP1 displayed increased phosphorylation and markedly increased interaction with PYRIN (8). Based on these observations, it was proposed that these PSTPIP1 mutants exert a dominant-negative effect on PYRIN and inhibit PYRIN anti-inflammatory activity, leading to increased production of IL-1β (7, 8). In contrast, Yu *et al.* reported that mutant PSTPIP1 engages PYRIN and ASC to form a novel type of inflammasome leading to caspase-1 activation (11). Like that of PYRIN, the patho-physiological function of PSTPIP1 remains largely enigmatic.

In the present study, we have generated mouse strains that either are PSTPIP1 deficient or ectopically express A230T mutant PSTPIP1 proteins. Our results demonstrated that PSTPIP1 is not an essential regulator of the well-characterized inflammasomes, nor is it involved in turpentine-induced inflammation in a mouse model of sterile inflammation, which is known to be an IL-1β-driven disease independent of caspase-1. Ectopic expression of PAPAS-associated mutant but not the wild type PSTPIP1 in mice lead to partial embryonic lethality, growth retardation, and elevated levels of inflammatory cytokines. However, these mice did not recapitulate the arthritis and skin inflammation features that are commonly found in human PAPA syndrome patients.

EXPERIMENTAL PROCEDURES

Mice and Turpentine Induced Inflammation—We generated a targeting vector to allow for conditional deletion of the *Pstpip1* gene in mouse using the *galK* selection system established by Neal Copeland's laboratory (12). Exons 4–11 of *Pstpip1* gene were flanked by two loxP sites through homologous recombination in C57BL/6 mouse embryonic stem (ES) cells. Independent mouse strains were derived from these ES cell clones. Mice heterozygous for the *Pstpip1 flox allele* were crossed with a cre deleter strain of mice (13) to generate a *Pstpip1*-deleted allele (that is the knock-out allele). Intercross of mice heterozygous for *Pstpip1*-deleted allele resulted in mice homozygous for the deleted allele, this is the *Pstpip1* knock-out strain of mice.

The Rosa-26-PSTPIP1 STOP floxed allele was generated following a strategy previously developed by Sasaki *et al.* (14). Namely, the Rosa-26 allele was targeted with a construct containing human PSTPIP1 cDNA preceded by a loxP flanked STOP cassette and marked by a signaling deficient truncated version of *hCD2* under the control of an internal ribosomal entry site (IRES) downstream of the inserted cDNA. Transgene transcription is controlled by a CAG promoter.

Turpentine-induced inflammation was carried out according to a protocol described by Fantuzzi *et al.* (15). Briefly, mice were injected subcutaneously in the right hind limb with 100 μl of turpentine. Blood was taken by tail bleeding at various time points after the injections, and serum was prepared. Mice were weighed just before and at 24 h intervals after turpentine injection for 13 days. All the animal care and procedures have been approved by the Institutional Animal Care and Use Committee (IACUC A3306-01) of the University of Massachusetts Medical School.

293T Cells Transfection and Luciferase Assay—cDNAs encoding wild type and mutant human PSTPIP1 were kind gifts of Dr. E. Alnemri. pCI-based expression plasmids for human pro-caspase-1 and ASC were from Millenium Pharmaceuticals. cDNAs encoding human or mouse Pyrin were purchased from Open Biosystems. MSCV retroviral vectors harboring cDNAs encoding human PSTPIP1, human ASC, mouse or human PYRIN along with human pro-caspase-1, ASC and that encoding a fusion protein of human pro-IL-1β with gaussia luciferase were co-transfected into 293T cells. 48 h later, luciferase activity was determined. An expression vector of *Renilla* luciferase (Promega) was included in the transfection to normalize the transfection efficiency.

Cell Culture, Flow Cytometry, Western Blotting, ELISA, and Histology—Bone marrow-derived macrophage and dendritic cell culture were prepared by culturing bone marrow cells in the presence of supernatants from L929 cells or recombinant GM-CSF for 8 days. On day 8, BMDM or BMDCs were harvested and plated at 2×10^5 cells/well in 96-well plates for ELISA or 2×10^6 cells/well in 12-well plate for immunoblotting. Cells were primed with ultrapure LPS (from *Escherichia coli* O111:B4, Invivogen) for 2 h, followed by stimulation with Nigericin, polydAdT (Sigma), or different pathogens. IL-1β p17 and Caspase-1 p10 immunoblots were conducted as described (16) with antibodies from Santa Cruz Biotechnology (caspase-1 p10) and R&D Systems (IL-1 β). The antibodies against β-actin and PSTPIP1 were from Sigma. ELISAs were performed with commercial kits from R&D Systems and according to the manufacturer's manual. hCD2 staining was performed on single cell suspensions with anti-hCD2-PE antibody from eBiosciences.

[6] The abbreviations used are: PAPAS, Pyogenic Arthritis, Pyoderma Gangrenosum, and Cystic Acne Syndrome; PSTPIP, proline-serine-threonine phosphatase-interacting protein; ES, embryonic stem; IRES, internal ribosomal entry site; MRI, Magnetic Resonance Image.

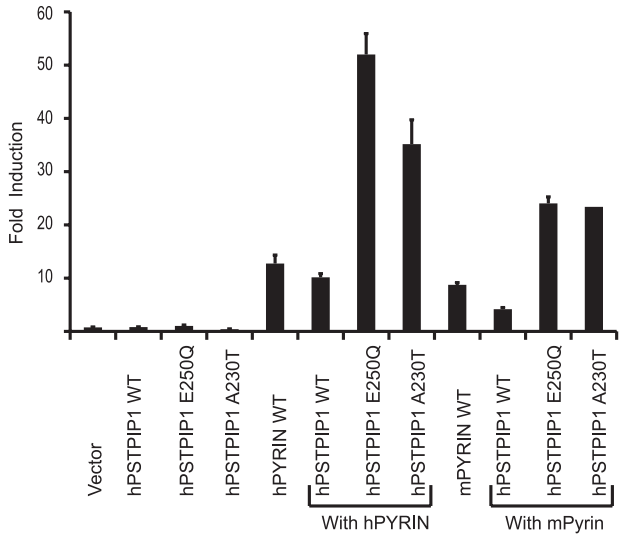

FIGURE 1. **Ectopic expression of mutant Pstpip1 proteins in 293 cells induces Pyrin dependent processing of murine pro-IL-1β.** MSCV retroviral vectors harboring cDNAs encoding human wild type or mutant PSTPIP1, human ASC, human and mouse PYRIN and pCI vectors harboring pro-caspase-1 and murine pro-IL-1β fused with gaussia luciferase reporter gene were transfected into HEK 293 cells along with a *Renilla* luciferase reporter plasmid. Luciferase activity of cell lysates was measured 48 h after transfection.

For histology, freshly isolated tissues were fixed in 10% formalin (Sigma-Aldrich), and 4 μm thick tissue sections were stained with hematoxylin/eosin.

RESULTS

Expression of PAPA-associated PSTPIP1 Mutant Proteins Induces Processing of Mouse pro-IL-1β by Mouse Caspase-1—Yu *et al.* (11) reported that in THP1 cells, human PAPA syndrome-associated mutant PSTPIP1 proteins can engage PYRIN and the adaptor protein ASC to activate caspase-1. Human and mouse PSTPIP1 proteins are 92% identical at the amino acid sequence level and conserved at the two PAPA syndrome-associated mutation sites A230 and E250. To test whether mutant human PSTPIP1 can engage mouse Pyrin to activate caspase-1, we co-transfected cDNAs encoding mutant human PSTPIP1, murine Pyrin, murine Asc, murine pro-caspase-1, and cDNAs encoding murine pro-IL-1β fused to a gaussia luciferase reporter into HEK 293 cells that lack endogenous inflammasome components. In this reconstituted system, activation of PYRIN inflammasomes can result in the cleavage of pro-IL-1β fusion proteins and activation of its luciferase activity (16). By measuring luciferase activity in cell lysates, our results showed that, indeed, mutant human PSTPIP1 can engage both murine and human PYRIN to activate pro-IL-1β processing (Fig. 1).

Generation of a Conditional Knock-out Allele of Pstpip1 in Mouse—To study the function of PSTPIP1, we established a conditional knock-out allele of mouse *Pstpip1* gene in which exons 4–11 were flanked by two loxP sites through homologous recombination in C57BL/6 mouse embryonic stem (ES) cells (Fig. 2*A*). The established allele will hereafter be referred to as the floxed (flanked by loxp site) *Pstpip1* allele (*Pstpip1 f/).* Independent mouse strains were derived from these ES cell clones and germline transmission was confirmed by Southern blot

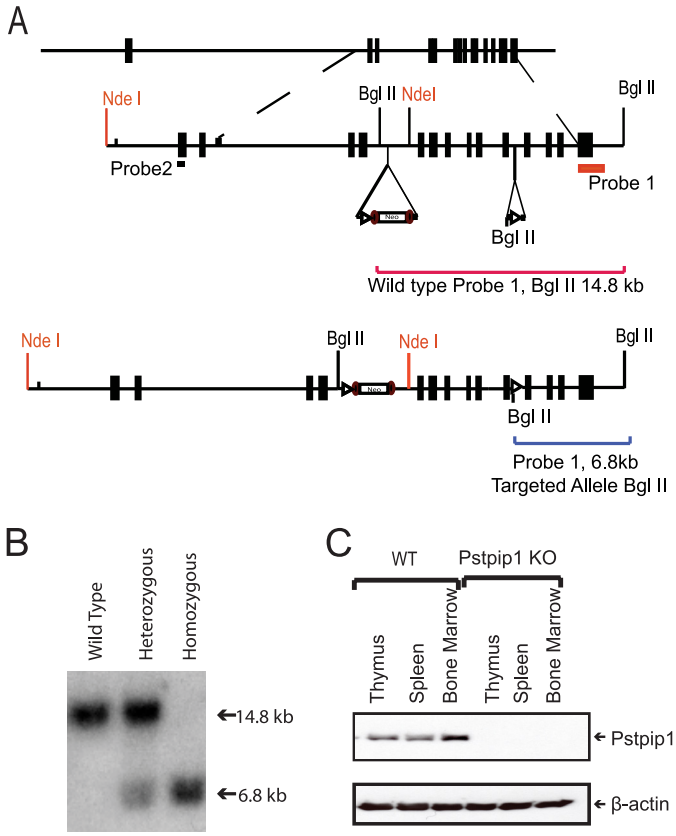

FIGURE 2. **Generation of a conditional knock-out allele of Pstpip1.** *A*, conditional mouse allele of Pstpip1 gene were established, in which exons 4–11 were flanked by two loxP sites through homologous recombination in C57BL6 mouse ES cells. This established allele will be hereafter referred to as the floxed (flanked by loxP sites) *Pstpip1* allele (*Pstpip1 f/*). ES cell clones carrying such floxed *Pstpip1* alleles were subsequently injected into C57BL6 blastocysts. Independent mouse strains were derived from the injection and germline transmission of the targeted *Pstpip1* allele was confirmed by Southern blotting analysis of tail DNA (*B*). Mice carrying the Pstpip1 f/allele were crossed to Cre deleter mice to generate the *Pstpip1*-deleted allele and were brought to homozygosity to generated Pstip1-deficient mice. The absence of Pstpip1 proteins was confirmed by Western blot of total cell lysate of lymphoid organs (*C*).

analysis of tail DNA samples (Fig. 2*B*). Mice heterozygous for *Pstpip1 f/* were crossed with a cre deleter, a strain of mice that harbor a CMV promoter-driven cre gene that is expressed early in embryo development and induces ubiquitous cre-mediated recombination (13), to generate a *Pstpip1*-deleted allele (that is the knock-out allele), here after referred to as *Pstpip1 del/* allele. Intercrossing of mice heterozygous for the *Pstpip1 del/* allele has given rise to a mouse strain that is homozygous for *Pstpip1 del* allele, this is the Pstpip1 knock-out (KO) strain. Western blot of total cell lysate from lymphoid organs has confirmed that, indeed, *Pstpip1* KO mice were deficient for Pstpip1 protein expression (Fig. 2*C*).

PSTPIP1 Is Not an Essential Regulator of Caspase-1-activating Nlrp3, Aim2, and Nlrc4 Inflammasomes—To examine the potential role of PSTPIP1 in regulating caspase-1 activating conventional inflammasomes, macrophages from wild type or *Pstpip1* KO mice were primed with LPS and stimulated with substances or pathogens that activate Nlrp3, Aim2, and Nlrc4 inflammasomes. We found that activation of Nlrp3, Aim2, or Nlrc4 inflammasomes was not affected by the absence of Pst-

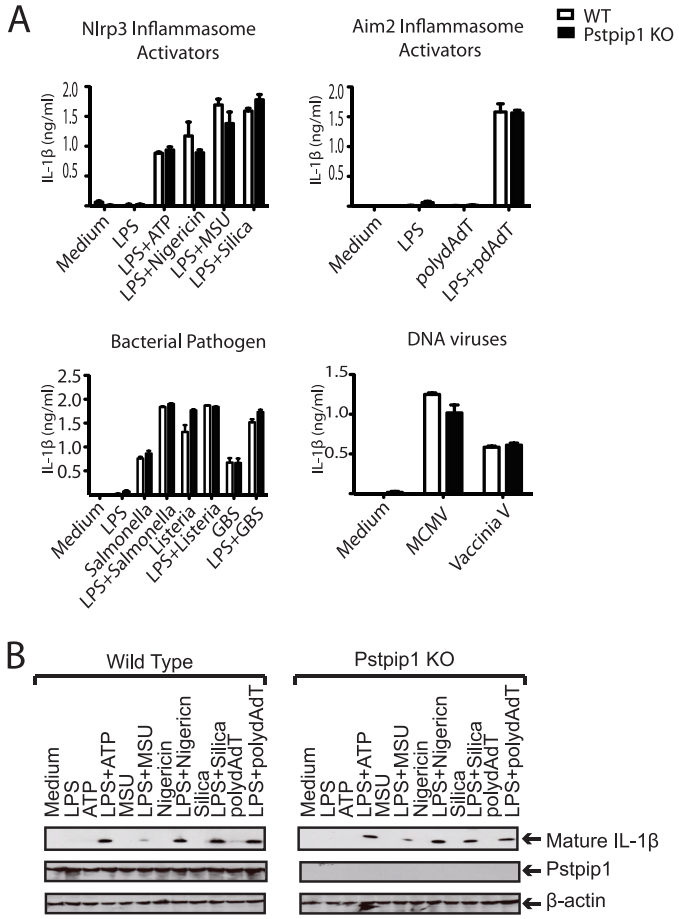

FIGURE 3. **Activation of conventional inflammasomes is not impaired in the absence of Pstpip1 proteins.** Bone marrow-derived macrophages were primed with 200 ng/ml pure LPS for 2 h followed by stimulation with stimulants that activate Nlrp3 (ATP 5 mM, Nigericin 5 μM, silica 500 μg/ml, MSU 500 μg/ml, *Gourp B streptococcus* (GBS) MOI 10), Aim2 (poly dA:dT 1.5 μg, Mouse Cytomega Virus (MCMV) MOI 10, Vaccina Virus MOI 5, *L. monocytogenes* MOI 5), or Nlrc4 (*S. typhymurium* MOI 5) inflammasomes, respectively, for additional 6 h. IL-1β in the culture supernatant was measured by ELISA (*A*), or supernatants were precipitated with methanol chloroform, and the presence of mature IL-1β was determined by Western blot.

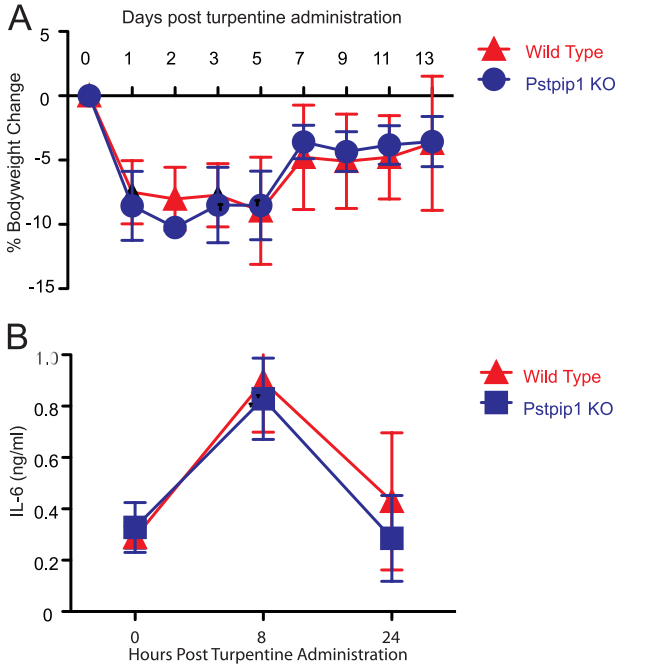

FIGURE 4. **Turpentine-induced inflammatory response is not affected in Pstpip1 knock-out mice.** Sex- and age-matched wild type (n = 10) and Pstpip1-deficient mice (n = 10) were injected s.c. with 100 μl of turpentine. Body weight was measured every day for 13 days after injection. Data are expressed as mean ± S. D. (*A*). Blood was drawn right before, 8 and 24 h post-turpentine injection. Circulating IL-6 levels were determined by ELISA. Data are expressed as mean ± S.D. (*B*).

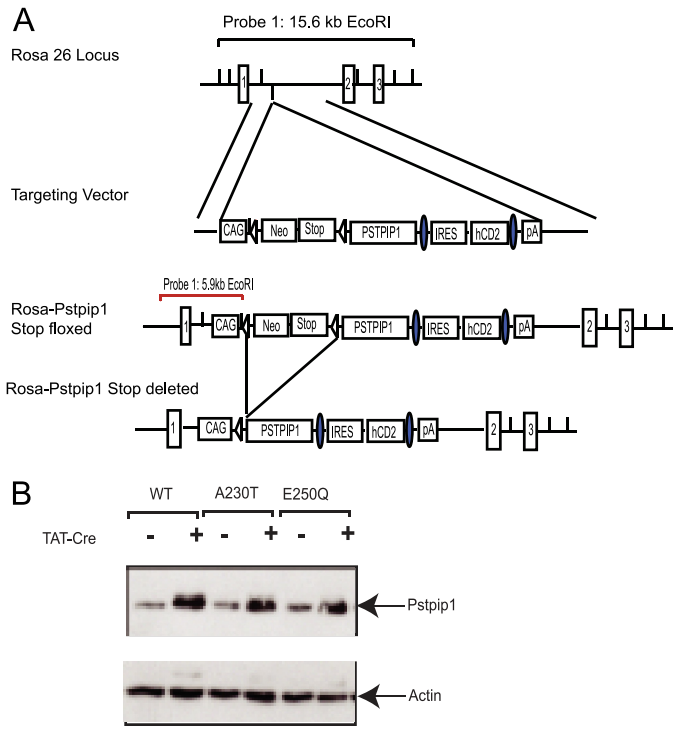

FIGURE 5. **Generation of Rosa-26-Pstpip1-expressing conditional allele.** cDNAs encoding wild type or A230T or E250Q mutant human PSTPIP1 proteins were inserted into Rosa-26 locus through homologous recombination in mouse ES cells. These targeted cDNAs were preceded by a transcriptional and translational loxP-flanked STOP signal. In this system, cre-mediated deletion of the STOP signal and subsequent expression of the conditional alleles can be monitored by the concomitant expression of the tailless human CD2 protein inserted downstream of the Pstpip1 cDNA controlled by an IRES (*A*). Treatment of targeted ES cells with TAT-Cre confirmed the ectopic expression of PSTPIP1 proteins (*B*). ●, Frt sites; -△-, loxP site.

pip1 proteins in macrophages (Fig. 3) or dendritic cells (data not shown).

The Turpentine-induced Inflammatory Response Is Not Affected in Pstpip1 KO Mice—Subcutaneous injection of turpentine induces local tissue damage resulting in a systemic acute phase response. This has been used as a well-characterized sterile inflammation model (17) that is dependent on IL-1β but not caspase-1 (15). Age- and sex-matched *Pstpip1* KO mice showed no difference in terms of body weight loss (Fig. 4*A*) and acute phase IL-6 production (Fig. 4*B*) after turpentine administration in comparison to wild type control mice suggesting that Pstpip1 is not required for turpentine-induced inflammation.

Establishment of a PSTPIP1 Rosa-26 Conditional Knockin Allele—In an effort to model the disease conditions of human PAPA syndrome in mouse, we used a conditional targeted mutagenesis technology that allows for the activation of genes of choice in a conditional manner. To this end, human PSTPIP1 cDNAs were inserted downstream of a transcriptional/translational loxP flanked STOP signal and targeted into the ubiqui-

A.

Litter #	# of Pups	Rosa26-Pstpip1 A230T del/+	Wild Type
1	4	0	3
2	10	0	10
3	2	0	2
4	6	0	6
5	8	0	8
6	7	0	7
7	3	2	1
8	4	0	4
9	4	0	4
10	5	1	4
11	6	1	5
Total	59	4	55

Litter #	# of Pups	Rosa26-Pstpip1 WT del/+	Wild Type
1	4	1	3
2	10	4	6
3	10	3	7
4	8	2	6
5	7	2	5
6	7	3	4
7	8	2	6
8	6	2	4
9	5	1	4
10	5	1	4
Total	70	21	49

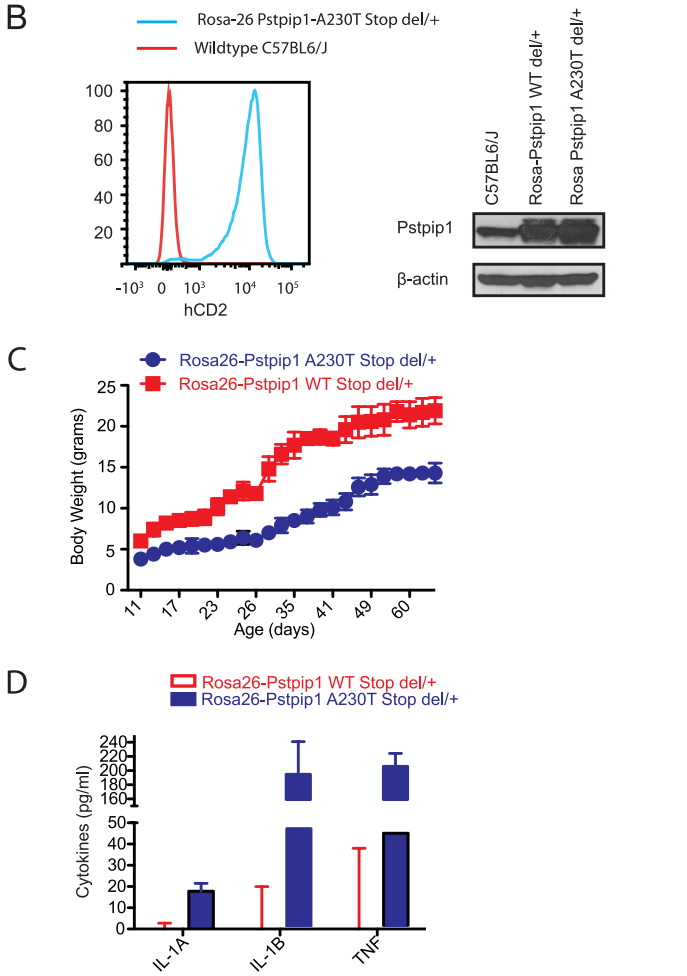

FIGURE 6. **A runty phenotype, elevated inflammatory cytokines, and normal hematopoietic cell populations in mice ectopically expressing Pstpip1 A230T mutant proteins.** Rosa-26 Pstpip1 A230T$^{STOP\ del/+}$ mice were not born in accordance to Mendelian ratio (*A*). Ectopic expression of human PSTPIP1 proteins were confirmed by Western blot of total cell lysate of splenocytes from Rosa-26 transgenic mice and by flow cytometry analysis of hCD2 expression (*B*). Body weight of mice ectopically expressing wild type or A230T mutant PSTPIP1 proteins was measured every other day or every 3 days 2 weeks after birth (*C*). Blood was drawn from mice ectopically expressing wild type or A230T mutant PSTPIP1 mutant proteins at the age of 6 months old, and circulating levels of IL-1α, IL-1β, and TNF were determined by ELISA (*D*).

tously expressed Rosa-26 locus. Expression of PSTPIP1 cDNAs is controlled by an upstream chicken gamma globulin (CAG) promoter. In this system, cre-mediated deletion of the STOP cassette and subsequent expression of the conditional PSTPIP1 allele can be monitored by the concomitant expression of the marker gene, a human *CD2* that is truncated for its cytoplasmic tail inserted downstream of the PSTPIP1 cDNA and controlled by an internal ribosomal entry site (IRES) (Fig. 5*A*). Human cDNAs encoding wild type, A230T, and E250Q mutant PSTPIP1 proteins together with IRES-hCD2 were targeted into Rosa-26 locus in C57BL/6 mouse ES cells. We designated the resultant alleles as Rosa-26-PSTPIP1 WT$^{STOP\ floxed}$ Rosa-26-PSTPIP1 A230T$^{STOP\ floxed}$ Rosa-26-PSTPIP1 E250Q$^{STOP\ floxed}$, respectively. To confirm that cre-mediated deletion of STOP signal results in expression of PSTPIP1, these targeted ES cells were treated with TAT-cre (18) to induce PSTPIP1 cDNA expression, Western blot of ES cell lysates showed increased amount of PSTPIP1 proteins in cre-treated targeted ES cell clones (Fig. 5*B*).

Growth Retardation, Partial Embryonic Lethality, and Elevated Level of Circulating Proinflammatory Cytokines in Rosa-PSTPIP1 A230T$^{STOP\ del/+}$ Mice—PAPA syndrome is a dominant disease, suggesting that the disease is caused by a gain-of-function mutation in *PSTPIP1* gene (7). To test whether ectopic expression of human PAPA syndrome associated mutant PSTIPT1 is sufficient to cause disease phenotypes in mice that mimic symptoms in human patients, we crossed mice harboring Rosa-26 PSTPIP1 WT$^{STOP\ floxed}$ or Rosa-26-PSTPIP1 A230T$^{STOP\ floxed}$ alleles to cre deleter (13) mouse strain expressing cre recombinase early in embryonic development, resulting in Rosa-26-PSTPIP1 WT$^{STOP\ deleted}$ or A230T$^{STOP\ deleted}$ alleles. Mice heterozygous for the Rosa-26-PSTPIP1$^{STOP\ deleted}$ allele are designated Rosa-26-PSTPIP1 WT or A230T$^{STOP\ del/+}$ mice. In these mice, ectopic PSTPIP1 wild type or A230T mutant proteins were ubiquitously expressed (13). Expression of the ectopic PSTPIP1 proteins was confirmed by both Western blot analysis of total cell lysates of splenocytes and for hCD2 expression on splenocytes from those mice (Fig. 6*B*). Rosa-26-PSTPIP1 A230T$^{STOP\ del/+}$ mice that ubiquitously express PSTPIP1 A230T mutant proteins were not born in accordance to Mendelian ratio, while mice ectopically expressing PSTPIP1 wild type proteins were not affected (Fig. 6*A*). Mice harboring a single PSTPIP1 A230T$^{STOP\ deleted}$ allele that were born alive, were smaller, and displayed growth retardation throughout development (Fig. 6*C*). These mice may also suffer from some neurological disorders that manifested as incessantly circling around their body axis giving the impression of chasing their tails (see supplemental movie). Magnetic Resonance Image (MRI) and histological section of the brain tissues revealed no dramatic changes in Rosa-26-PSTPIP1 A230T$^{STOP\ del/+}$ mice in comparison to the Rosa-26-PSTPIP1 WT$^{STOP\ del/+}$ mice (Fig. 7, *B* and *C*). Similar to observa-

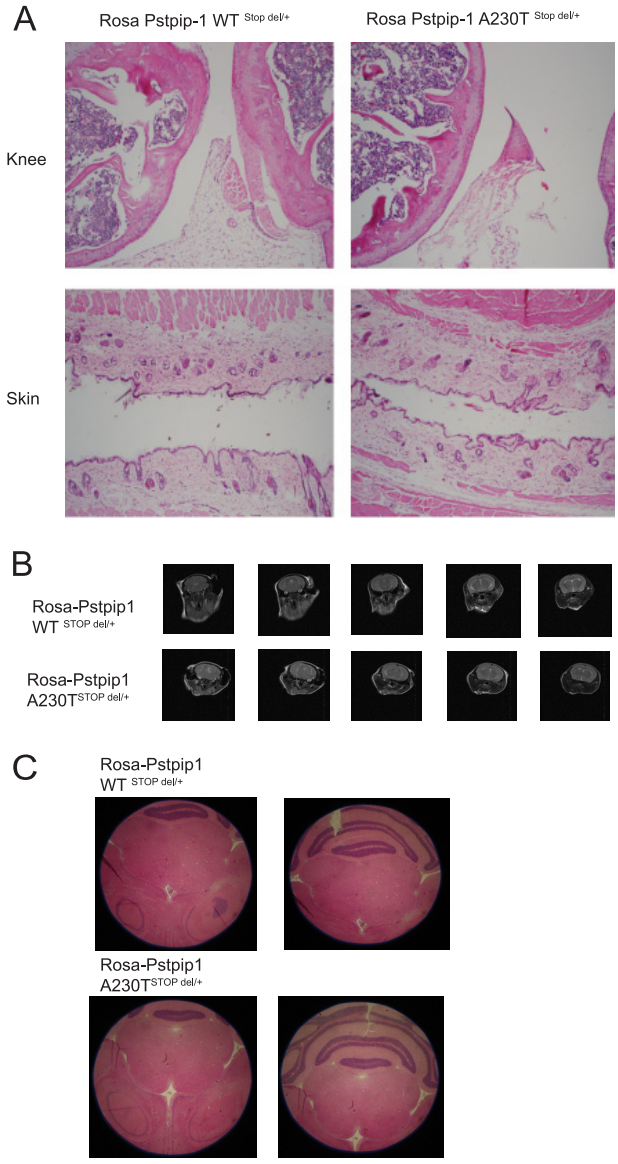

FIGURE 7. **No arthritis or inflammatory skin lesions found in mice ectopically expressing A230T mutant Pstpip1 proteins.** Hematoxylin and eosin-stained tissue sections of knee and skin showed no signs of inflammation (*A*). Magnetic Resonance Imaging of brains of Rosa-26-PSTPIP1 WT and A230T$^{STOP\ del/+}$ mice (*B*). H&E staining of brain sections from Rosa-26-PSTPIP1 WT and A230T$^{STOP\ del/+}$ mice (*C*).

tions in PAPA syndrome patients (8), mice harboring the mutant ectopically expressed PSTPIP1 alleles displayed higher level of proinflammatory cytokines IL-1α, IL-1β, and TNF in circulation compared with those ectopically expressing wild type PSTPIP1 (Fig. 6*D*). However, Rosa PSTPIP1 A230T mutants as well as their wild type controls did not display signs compatible with arthritis such as impaired mobility, swollen joints, or any skin lesions until the end of the observation period at 12 months of age. Consistent with these findings, analysis of hematoxylin eosin-stained tissue sections of the mice showed normal rodent joints and no synovial infiltration with mono- or polymorphonuclear cells, pannus formation, or joint destruction, as well as normal mouse skin without ulceration, necrosis, or inflammation (Fig. 7*A*).

Phenotypes in Rosa-26-PSTPIP1 A230T$^{STOP\ del/+}$ Mice Is Not Caused by Expression of Mutant PSTPIP1 Proteins in Hematopoietic Cells—Since mice ubiquitously expressing A230T mutant PSTPIP1 proteins are partially embryonic lethal, we crossed the Rosa-26 conditional PSTPIP1 alleles to VaviCre transgenic strain of mice, which would target the expression of ectopic PSTPIP1 specifically to hematopoietic tissues (19). This breeding led to Mendelian ratio of mice carrying Rosa-26 conditional alleles of both the wild type and the A230T mutant PSTPIP1. Flow cytometry analysis of anti-hCD2 stained total splenocytes and Western blot analysis of cell lysate of bone marrow-derived macrophages from these conditionally transgenic mice have confirmed ectopic expression of PSTPIP1 proteins (Fig. 8*B*). Whereas targeted expression of wild type PSTPIP1 proteins in Rosa-26-PSTPIP1 WT$^{STOP\ floxed/+}$VaviCre+ mice does not cause any abnormality, quite unexpectedly, mice that express A230T mutant PSTPIP1 proteins specifically in hematopoietic tissues in Rosa-26-PSTPIP1 A230T$^{STOP\ floxed/+}$VaviCre+ mice did not show any abnormality, either. Unlike the mouse strain that ubiquitously expresses A230T mutant PSTPIP1 proteins, Rosa-26-PSTPIP1 A230T$^{STOP\ floxed/+}$VaviCre+ mice showed normal body weight, and no behavior abnormalities (Fig. 8*A* and data not shown). Furthermore, bone marrow-derived macrophages established from Rosa-26-WT or A230T$^{STOP\ del/+}$ mice that ectopically express wild type or A230T mutant PSTPIP1 proteins did not produce excessive amount of proinflammatory cytokines when provoked with inflammasome-activating stimuli (Fig. 8*C*). These results suggest that the elevated level of proinflammatory cytokines in Rosa-26-PSTPIP1 A230T$^{STOP\ del/+}$ mice is not caused by hematopoietic cells. Consistent with these findings, sera from mice with targeted expression of A230T mutant proteins into hematopoietic cells did not show increased concentrations of proinflammatory cytokines in circulation (Fig. 8*D*).

DISCUSSION

PAPA syndrome falls into the category of autoinflammatory diseases (4) and appears to be caused by dysregulation of the IL-1β processing pathway (4, 20, 8). In addition, anakinra, an IL-1 receptor antagonist, was shown to be effective in treating a fraction of PAPAS patients (10). These results suggest that mutant PSTPIP1 may enhance the IL-1 processing and secretion pathway leading to disease conditions in human patients. Despite of extensive studies, it remains inconclusive how mutant PSTPIP1 regulates IL-1 processing and whether PSTPIP1 is a regulator of inflammasome activities (8, 11). In this study, we have generated a PSTPIP1-deficient mouse strain. In the absence of Pstpip1 proteins, mouse macrophages responded normally to stimuli that activate the Nlrp3, Nlrc4, and Aim2 inflammasomes (Fig. 3). These findings suggest that Pstpip1 is not required for activation of these caspase-1-activating inflammasomes.

In addition to the caspase-1-activating inflammasomes, mechanisms independent of caspase-1 exist that can lead to processing and secretion of IL-1β and inflammation. One example is the subcutaneous injection of turpentine. This is a well-characterized model of sterile inflammation that is mediated by IL-1β but independent of caspase-1 (15). In this model,

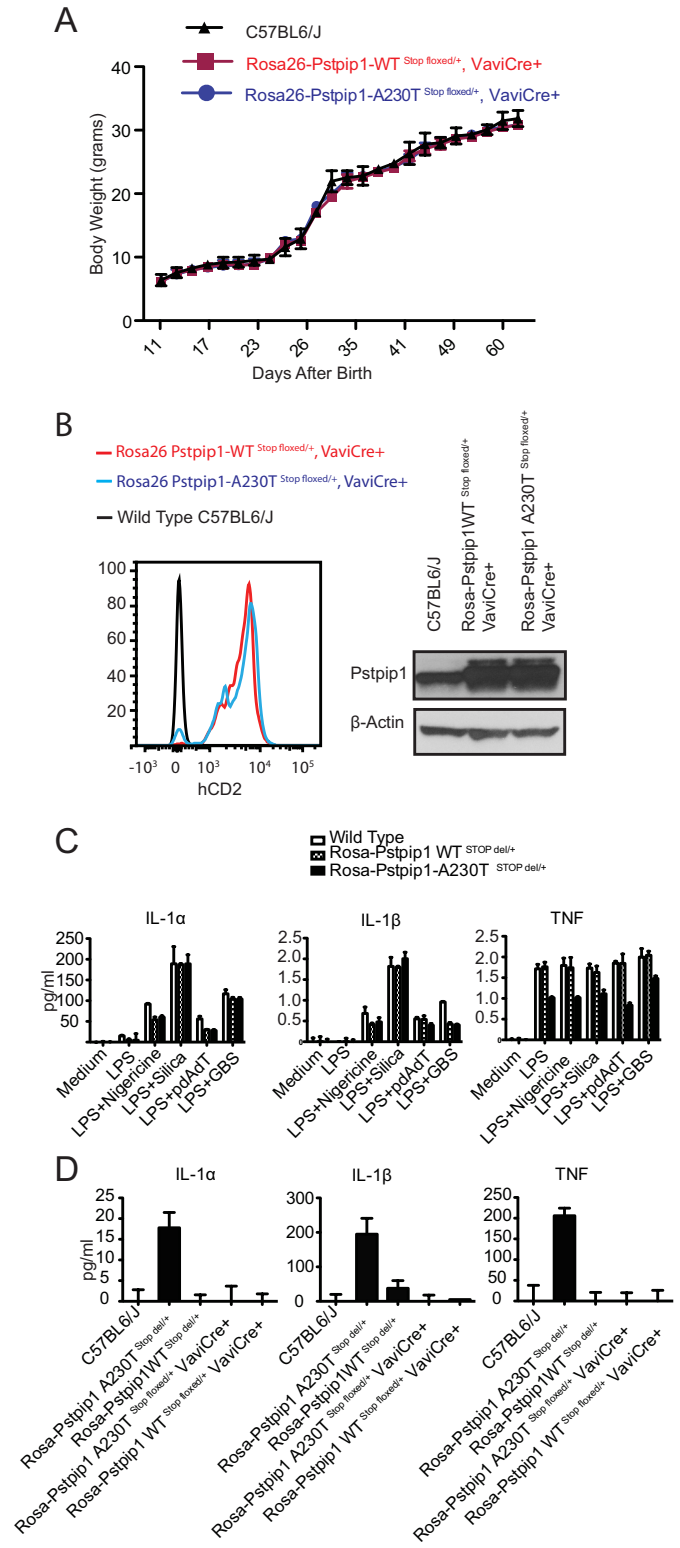

FIGURE 8. **The runty phenotype in Rosa-26-PSTPIP1 A230T$^{STOP\ del/+}$ mutant protein-expressing mice is not caused by mutant PSTPIP1 proteins in hematopoietic cells.** Body weight of mice ectopically expressing PSTPIP1 proteins specifically in hematopoietic cells was measured every 3 days. Data are expressed as mean ± S.D. (*A*). Ectopic expression of PSTPIP1 proteins was confirmed by Western blot and hCD2 expression on total splenocytes (*B*). Bone marrow- derived macrophage from mice ectopically expressing wild type or A230T mutant PSTPIP1 proteins primed with LPS (200 ng/ml) followed by stimulation with nigericin (5 μM,1 h), silica (500 μg/ml), poly dA:dT (3 μg), or GBS (MOI 10) for additional 6 h, IL-1β in culture supernatant were measured by ELISA (*C*). Blood was drawn from wild type C57BL/6, Rosa-PSTPIP1-WT$^{STOP\ del/+}$; Rosa-26-PSTPIP1 A230T$^{STOP\ del/+}$; Rosa-26-PSTPIP1 WT$^{STOP\ floxed/+}$, VaviCre+; Rosa-26-PSTPIP1 A230T$^{STOP\ floxed/+}$, VaviCre+ mice. Circulating IL-1α, IL-1β, and TNFα were determined by ELISA (*D*).

turpentine causes a systemic inflammatory response that results in acute phase body weight loss and elevated level of IL-6 in circulation secondary to IL-1β production. However, our results showed that inflammatory response was not affected in Pstpip1-deficient mice (Fig. 4), suggesting that Pstpip1 is not a critical regulator of this inflammatory pathway.

PAPA syndrome is a dominantly inherited disease suggesting that the disease-associated PSTPIP1 mutations are gain-of-function mutations (7, 11). This prompted us to think that ectopic expression of mutant PSTPIP1 proteins may be sufficient to cause disease conditions in mice mimicking human PAPAS. The establishment of Rosa-26 conditional alleles of both wild type and mutant PSTPIP cDNAs enabled us to dissect the disease mechanisms at the cellular level. The observation that mice ubiquitously expressing A230T mutant PSTPIP1 were not born according to Mendelian ratio suggests that some detrimental effect of mutant PSTPIP1, presumably related to its inflammatory nature, disrupted normal embryonic developmental processes. Furthermore, the few mice that were born, suffered from growth retardation with elevated levels of proinflammatory cytokines in the circulation, these phenotypes, in part, recapitulated the human disease symptoms. However, the most striking human disease features, namely, pyogenic arthritis, pyoderma gangrenosum, and acne were not identified in the A230T PSTPIP1 Rosa-26 transgenic mice (Fig. 7). One possible reason for this discrepancy could be the species difference. It has been suggested that mutant PSTPIP1 proteins exert its function through engaging pyrin (11). It is known that mouse pyrin are structurally different from the human pyrin that it lacks the B30.2/Spry domain (21). The precise function of pyrin as well as its C-terminal B30.2/Spry domains remains to be elucidated; however, it is possible that without the B30.2/Spry domain, mouse pyrin loses part, if not all, of its function in mediating the inflammatory effect of mutant PSTPIP1 proteins. Although we did observed that in the 293T over-expression system, mutant PSTPIP1 can engage mouse Pyrin to induce pro-IL-1β processing (Fig. 1), this phenomenon may be due to the high level expression of Pyrin proteins that is not attainable in physiological conditions. On the other hand, Demidowich *et al.* recently reported that not all human subjects carrying a mutant PSTPIP1 allele were affected by inflammatory symptoms. Even those patients who did develop diseases, they displayed symptoms with various expressivity (22). This incomplete penetrance in human patients suggests that other modifying genes or environmental factors may play a role in the pathogenesis of PAPAS.

Human autoinflammatory diseases are generally caused by dysregulation of innate immune signaling pathways, particularly that of IL-1β processing pathway. Myeloid cells, the first line innate immune responders, are the responsible cell types in many of such diseases, such as the Muckle Well syndrome caused by mutations in the *CIAS* gene encoding NLRP3 (23). We anticipated that PAPAS is also caused by dysfunctions in myeloid cells. Quite unexpectedly, when mutant PSTPIP1 pro-

teins were expressed specifically in hematopoietic cells in mice, phenotypes of partial embryonic lethality, growth retardation, and elevated proinflammatory cytokines were not emerging in these mice. These results suggested that ectopic expression of A230T mutant PSTPIP1 proteins in hematopoietic cells is not sufficient to promote the inflammatory phenotypes in mice (Fig. 8). Earlier reports showed that PBMCs from PAPA patients produces more IL-1β in response to LPS (8), however, this is in contradiction to a recent report where no increases in production of IL-1β or TNFα were discovered in LPS-stimulated PBMCs from PAPAS patients (22). Consistent with this later report, we found mouse macrophages carrying ectopically expressed A230T mutant PSTPIP1 proteins did not produce higher amount of proinflammatory cytokines as compared with wild type controls (Fig. 8).

Taken together, our present study showed that PSTPIP1 is not an essential regulator of the major types of caspase-1-activating inflammasomes, nor is it a critical regulator of the caspase-1 independent turpentine-induced inflammatory pathway. Ubiquitous, ectopic expression of mutant PSTPIP1 proteins in mice leads to partial embryonic lethality, growth retardation, and elevated levels of proinflammatory cytokines in the circulation. These phenotypes partially recapitulate symptoms of human PAPA syndrome, however, the major features of human disease, such as pyogenic arthritis and skin inflammation were not identified in the mouse disease model. Further studies are needed to elucidate the pathophysiological function of PSTPIP1.

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