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Interrupting an IFN-γ-dependent feedback loop in the syndrome of pyogenic arthritis with pyoderma gangrenosum and acne

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ABSTRACT

Objectives To study the molecular pathogenesis of PAPA (pyogenic arthritis, pyoderma gangrenosum and acne) syndrome, a debilitating hereditary autoinflammatory disease caused by dominant mutation in *PSTPIP1*.

Methods Gene knock-out and knock-in mice were generated to develop an animal model. THP1 and retrovirally transduced U937 human myeloid leukaemia cell lines, peripheral blood mononuclear cells, small interfering RNA (siRNA) knock-down, site-directed mutagenesis, cytokine immunoassays, coimmunoprecipitation and immunoblotting were used to study inflammasome activation. Cytokine levels in the skin were evaluated by immunohistochemistry. Responsiveness to Janus kinase (JAK) inhibitors was evaluated ex vivo with peripheral blood mononuclear cells and in vivo in five treatment-refractory PAPA patients.

Results The knock-in mouse model of PAPA did not recapitulate the human disease. In a human myeloid cell line model, PAPA-associated *PSTPIP1* mutations activated the pyrin inflammasome, but not the NLRP3, NLRC4 or AIM2 inflammasomes. Pyrin inflammasome activation was independent of the canonical pathway of pyrin serine dephosphorylation and was blocked by the p.W232A *PSTPIP1* mutation, which disrupts pyrin-PSTPIP1 interaction. IFN-γ priming of monocytes from PAPA patients led to IL-18 release in a pyrin-dependent manner. IFN-γ was abundant in the inflamed dermis of PAPA patients, but not patients with idiopathic pyoderma gangrenosum. Ex vivo JAK inhibitor treatment attenuated IFN-γ-mediated pyrin induction and IL-18 release. In 5/5 PAPA patients, the addition of JAK inhibitor therapy to IL-1 inhibition was associated with clinical improvement.

Conclusion PAPA-associated *PSTPIP1* mutations trigger a pyrin-IL-18-IFN-γ positive feedback loop that drives PAPA disease activity and is a target for JAK inhibition.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ *PSTPIP1* mutations cause a dominantly inherited syndrome of pyogenic arthritis with pyoderma gangrenosum and acne (PAPA). The PSTPIP1 protein binds to pyrin, the protein mutated in familial Mediterranean fever. PAPA-associated mutations in PSTPIP1 cause increased binding affinity to pyrin and the release of IL-1β and IL-18 from leucocytes. However, the molecular pathophysiology of PAPA syndrome is poorly understood, and PAPA patients are relatively refractory to targeted therapies as compared with patients with other IL-1-mediated diseases.

WHAT THIS STUDY ADDS

⇒ This is the first study to demonstrate that *PSTPIP1* mutations activate the pyrin inflammasome, but not the NLRP3, NLRC4 or AIM2 inflammasomes, by a non-canonical mechanism independent of pyrin phosphorylation. This non-canonical pyrin inflammasome activation induces a positive feedback loop through the release of IL-1β and IL-18 that drives IFN-γ in lesional skin, which further induces pyrin expression. Ex vivo, Janus kinase (JAK) inhibitors block this vicious circle and JAK inhibitors had a beneficial effect in 5/5 treatment-refractory PAPA patients.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study introduces the pyrin inflammasome-IL-18-IFN-γ feedback loop as a possible conceptual basis for several autoinflammatory disorders characterised by high IL-18 production. It lays the foundation for future studies to investigate the safety and efficacy of IL-18 and JAK inhibitors in these disorders, given the initial success of JAK inhibitors in PAPA patients.

INTRODUCTION

Pyogenic arthritis, pyoderma gangrenosum and acne (PAPA) syndrome (OMIM #604416) is an autosomal dominant autoinflammatory disease first described in 1997,[1] possibly antedated by individual case reports.[2] Wise *et al* demonstrated that PAPA syndrome is caused by mutations in *PSTPIP1* (also known as *CD2BP1*).[3] PAPA syndrome is characterised by early-onset neutrophil-mediated

inflammation, leading to recurrent sterile arthritis and skin manifestations that include ulcerations, pyoderma gangrenosum (PG) and severe cystic acne.[4]

PSTPIP1 belongs to the Fes/CIP4 homology-Bin/Amphiphysin/Rvs (F-BAR) protein family, members of which are localised in the cell membrane and induce dynamic changes in membrane curvature.[5] The F-BAR domain located on the N-terminus of PSTPIP1 also serves as a homodimerisation domain, and the dimeric surface of PSTPIP1 plays an important role in its interaction with other proteins, such as PTP-PEST, PTP-HCSF and LYP.[6] At the C-terminus of PSTPIP1 is an SRC homology 3 (SH3) domain that mediates interaction with the polyproline motifs of c-Abl, CD2 and Wiskott-Aldrich syndrome protein (WASP).[7] PSTPIP1 has been associated with numerous cellular activities that involve cytoskeletal rearrangement, including filopodia formation,[8] podosome disassembly in osteoclasts and macrophages,[9 10] and immunological synapse formation in lymphocytes.[11] PSTPIP1 also directly interacts with the inflammasome protein, pyrin.[12]

Inflammasomes are multiprotein complexes that are assembled in direct or indirect response to damage-associated or pathogen-associated molecular patterns.[13] Once activated, inflammasome sensor proteins such as NLRP3 and pyrin recruit the adaptor molecule ASC to form specks and activate caspase-1, which catalyses the cleavage of proIL-1β and proIL-18 to their biologically active fragments, and the cleavage of gasdermin D into pore-forming fragments that allow cytokine egress and promote pyroptosis. Inflammasome activation is regulated at the transcriptional, post-transcriptional and post-translational levels. Aberrant activation of inflammasomes under sterile conditions may lead to autoinflammatory disease.

Pyrin is expressed in myeloid cells, including monocytes, macrophages and neutrophils. *MEFV*, the gene encoding pyrin, is an inducible gene that can be stimulated by multiple signals, such as LPS, TNF-α and types 1 and 2 interferon.[14] The inactivation of RhoA signalling triggers pyrin inflammasome activation.[15 16] In the presence of RhoA activity, the downstream effector kinases PKN1 and PKN2 maintain phosphorylation of two serine residues, 208 and 242, in human pyrin. 14-3-3 interacts with the phosphorylated serine residues on pyrin to block pyrin inflammasome activation. The loss of RhoA signalling, and subsequently the loss of PKN kinase activity, may lead to serine dephosphorylation and activation of the pyrin inflammasome.[16]

Multiple human autoinflammatory diseases are caused by aberrant activation of the pyrin inflammasome. Mutations in the C-terminal pyrin B30.2 domain lead to familial Mediterranean fever (FMF).[17 18] Hypomorphic mutations in mevalonate kinase impair RhoA geranylgeranylation, leading to impaired RhoA signalling, in the hyperimmunoglobulin D with periodic fever syndrome (HIDS; also known as mevalonate kinase deficiency, MKD).[16] In pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND), missense mutations that replace serine 242 prevent 14-3-3 binding to pyrin.[19] Mutation in the actin-regulatory gene *WDR1* causes a syndrome of periodic fever with immunodeficiency and thrombocytopenia (PFIT), with high serum levels of IL-18 and likely pyrin inflammasome activation.[20] C-terminal mutations in *CDC42* have been associated with pyrin-dependent neonatal onset pancytopenia, autoinflammation, rash and hemophagocytic lymphohistiocytosis (NOCARH syndrome).[21–23]

Several years prior to the elucidation of the pyrin inflammasome, we demonstrated that endogenous pyrin and PSTPIP1 are coexpressed in monocytes and granulocytes, and could be coprecipitated in cell lines.[12] PAPA syndrome-associated mutations in PSTPIP1 increased pyrin binding and augmented IL-1β production by patient leucocytes and transfected cell lines. Others demonstrated that mutant PSTPIP1 induced ASC oligomerisation in a pyrin-dependent manner,[24] and IL-1β targeted therapy was shown to be effective for the treatment of PAPA syndrome.[25–27]

We recently reported that total and free IL-18 levels in the serum of PAPA syndrome patients are elevated comparable to that of patients with *NLRC4*-gain-of-function mutations, and higher than that of patients with FMF, irrespective of PAPA disease activity.[28] Here, we examine the effect of PSTPIP1 mutations in the context of a more comprehensive understanding of inflammasome biology than previous studies, we provide evidence that IFN-γ drives a positive feedback loop in PAPA syndrome, and we explore the possibility of interrupting this amplification loop by targeting the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) signalling pathway.

METHODS

Detailed clinical histories of the five treatment-refractory PAPA patients who were treated with JAK inhibitors, and of two patients with PAPA-like skin disease but without *PSTPIP1* mutations who were not treated with JAK inhibitors, are provided in online supplemental methods.

Mice used in this study were maintained at the National Human Genome Research Institute animal facility. Mouse blood was collected, and serum was isolated and used for immunoassays. Bone marrow cells were harvested from the femurs and tibiae of euthanised mice and differentiated to macrophages. Fully differentiated macrophages were primed and stimulated for inflammasome activation. Further detailed animal procedures including the development of mouse models can be found in online supplemental methods.

Human peripheral blood mononuclear cells (PBMCs) were isolated from patients and healthy donors. PBMCs were further purified to isolate $CD14^+$ monocytes. Isolated cells were primed with IFN-γ for pyrin expression. *PSTPIP1*-expressing U937 stable cell lines were generated by retroviral transduction or by electroporation of *PSTPIP1*-expressing plasmids. Cell lines were differentiated and primed for pyrin expression. Cell culture supernatants were collected for immunoassays and multiplex cytokine panels, and whole cell lysates for immunoblot and coimmunoprecipitation. Staurosporine was used to induce pyrin dephosphorylation. For knockdown studies, siRNAs were electroporated into cells. A full version of the methods, which includes immunohistochemistry, ex vivo JAK inhibition and the complete list of materials used in the study, is provided in online supplemental methods.

RESULTS

Inability to demonstrate a disease phenotype in knock-in mice

To study the pathogenesis of PAPA syndrome in vivo, we generated a *Pstpip1* gene knock-out (KO) mouse, designated as $Pstpip1^{-/-}$ hereafter, and a knock-in (KI) carrying a common PAPA-associated mutation, A230T (figure 1A). Neither heterozygous ($Pstpip1^{A230T/+}$) nor homozygous ($Pstpip1^{A230T/A230T}$) KI mice exhibited typical features such as skin or joint inflammation seen in patients with PAPA syndrome (figure 1B). In addition, bone marrow-derived macrophages (BMDM) from $Pstpip1^{A230T/A230T}$ or $Pstpip1^{-/-}$ mice did not demonstrate inflammasome activation in response to lipopolysaccharide (LPS) priming (figure 1C). As PSTPIP1 has been associated with pyrin in

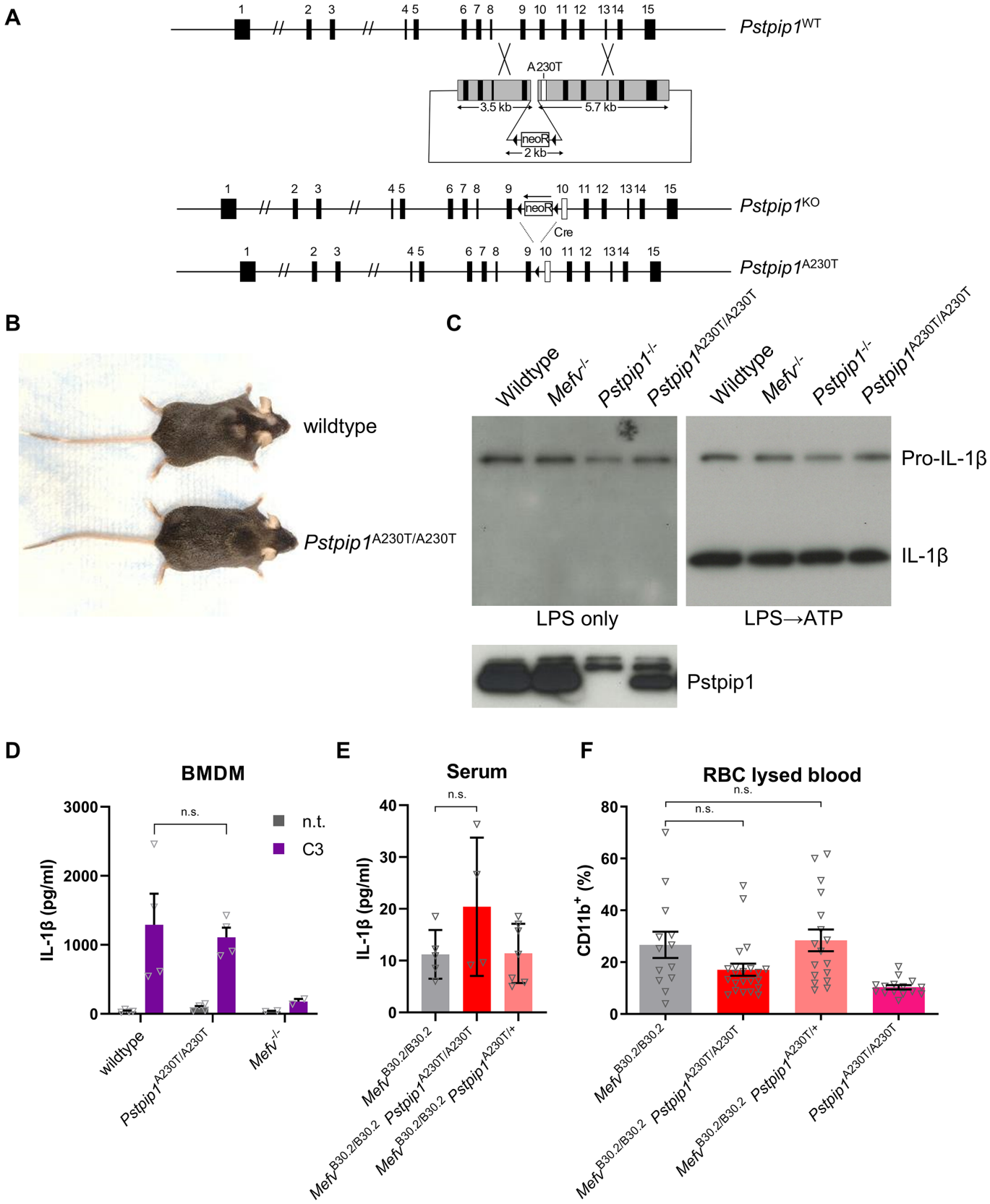

Figure 1 *Pstpip1*$^{A230T/A230T}$ KI mice do not develop an autoinflammatory phenotype. (A) Strategy for generating *Pstpip1*^{KO} and *Pstpip1*^{A230T} mice. (B) Gross appearance of age-matched and gender-matched wild-type and homozygous *Pstpip1*$^{A230T/A230T}$ KI mice. (C) Immunoblot analysis of LPS-primed BMDMs from WT, *Mefv*$^{-/-}$, *Pstpip1*$^{-/-}$ and *Pstpip1* $^{A230T/A230T}$ mice with or without following ATP treatment to trigger the NLRP3 inflammasome as a positive control. (D) IL-1β measurements from LPS-primed BMDMs of WT, *Pstpip1* $^{A230T/A230T}$ and *Mefv*$^{-/-}$ mice with or without *Clostridium botulinum* C3 toxin for pyrin inflammasome activation. (E, F) IL-1β measurements of serum (E) and flow cytometry of peripheral blood cells for CD11b^{+} population (F) from *Mefv*$^{B30.2/B30.2}$, *Mefv*$^{B30.2/B30.2}$*Pstpip1* $^{A230T/A230T}$, *Mefv*$^{B30.2/B30.2}$*Pstpip1* $^{A230T/+}$ and *Pstpip1* $^{A230T/A230T}$ mice. P values were calculated with Mann-Whitney U test. BMDM, bone marrow-derived macrophages; LPS, lipopolysaccharide; n.s. not significant.

previous studies, we examined the BMDMs of *Pstpip1*$^{A230T/A230T}$ for pyrin inflammasome activation induced by the RhoA-inactivating *Clostridium botulinum* C3 toxin. IL-1β release from C3 toxin-treated *Pstpip1*$^{A230T/A230T}$ BMDMs was comparable to the wild-type (WT) (figure 1D). The results indicate that in the mouse, the A230T mutation of PSTPIP1 does not cause a phenotype recapitulating human PAPA syndrome, consistent with a previous study of mice harbouring the mutant human *PSTPIP1* transgene.[29]

Hypothesising that the pyrin inflammasome is not activated by A230T mutant *Pstpip1* in the mouse because the murine pyrin protein lacks the B30.2 domain, we crossed *Pstpip1*$^{A230T/A230T}$ mice with KI mice harbouring the WT B30.2 domain of human pyrin (*Mefv*$^{B30.2/B30.2}$), which have a mild inflammatory phenotype.[16] *Pstpip1*$^{A230T/A230T}$*Mefv*$^{B30.2/B30.2}$ mice showed a similar degree of mild inflammatory phenotypes to *Mefv*$^{B30.2/B30.2}$ mice (figure 1E,F). Thus, to date, we have not been able to model PAPA syndrome in mice.

Pyrin inflammasome activation with PAPA-associated *PSTPIP1* mutations

Since PAPA syndrome is an autoinflammatory disease mediated by myeloid cells, we next attempted to study PSTPIP1 mutations in human myeloid lines. We generated retroviral-transduced U937 human myeloid leukaemia cells expressing WT or PAPA-associated A230T and E250Q mutant PSTPIP1 proteins (online supplemental figure 1A). Cells expressing either mutant PSTPIP1 protein secreted IL-1β with LPS priming alone without specific inflammasome activators; PSTPIP1 mutant cells released significantly more IL-1β than cells expressing WT PSTPIP1 or non-expressing vector control (figure 2A).

To identify the inflammasome(s) that mediate IL-1β secretion from PSTPIP1 mutant cells, we knocked down the genes encoding key inflammasome proteins in the established U937 cell lines using small interfering RNA (siRNA). IL-1β release was significantly diminished only in the *MEFV*-knockdown (figure 2B), which indicates that the PAPA-induced IL-1β secretion is independent of the AIM2 (absent in melanoma 2), NLRP3 or NLRC4 inflammasomes, but dependent on the pyrin inflammasome. Conversely, we examined whether PSTPIP1 is required for pyrin inflammasome activation by RhoA inactivation. In LPS-primed monocytic THP1 cells, treatment with the RhoA inactivating toxin TcdA induced IL-1β secretion, which is blocked by knockdown of *MEFV* but not by knockdown of *PSTPIP1*, indicating that WT PSTPIP1 is not necessary for TcdA-induced human pyrin inflammasome activation (figure 2C).

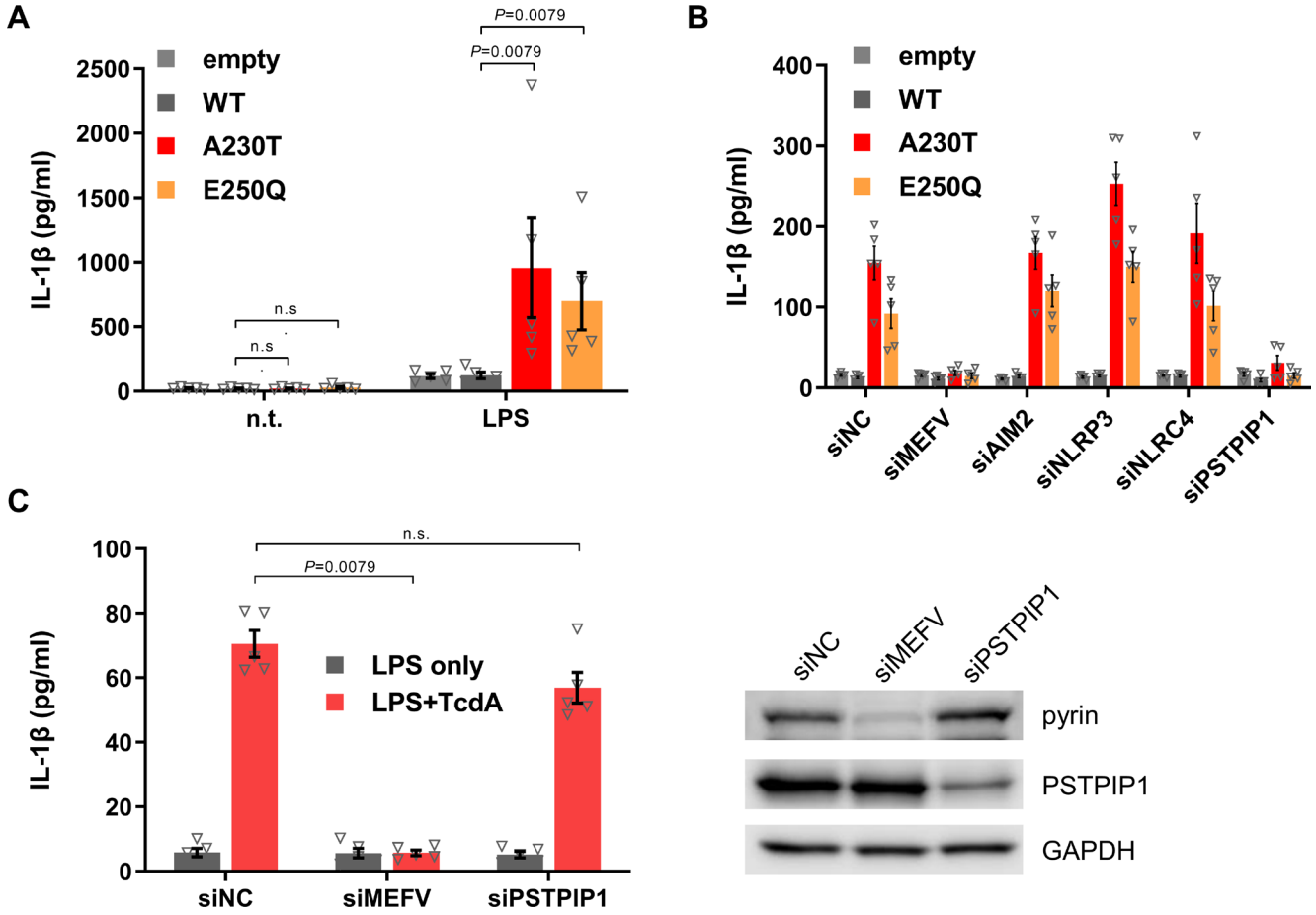

Figure 2 PAPA-associated *PSTPIP1* mutations activate the pyrin inflammasome. (A) IL-1β measurements of cell culture supernatants from LPS-primed retroviral-transduced U937 cell lines expressing WT or PAPA-associated A230T and E250Q mutant PSTPIP1 proteins. (B) IL-1β measurements of cell culture supernatants from cell lines from (A) transiently transfected with negative control siRNA with no substantial sequence similarity to mouse or human gene sequences (siNC) or siRNA targeting the genes encoding pyrin, AIM2, NLRP3, NLRC4 or PSTPIP1, then primed with LPS. (C) IL-1β measurements of cell culture supernatants and immunoblot analysis of cell lysates from THP1-KO-NLRP3 cells transiently transfected with siNC or siRNA targeting the genes encoding pyrin or PSTPIP1, then treated with LPS with or without *Clostridium difficile* toxin A (TcdA). Results are from at least five independent experiments. P values were calculated with Mann-Whitney U test. LPS, lipopolysaccharide; n.s. not significant; n.t, no treatment; PAPA, pyogenic arthritis, pyoderma gangrenosum and acne.

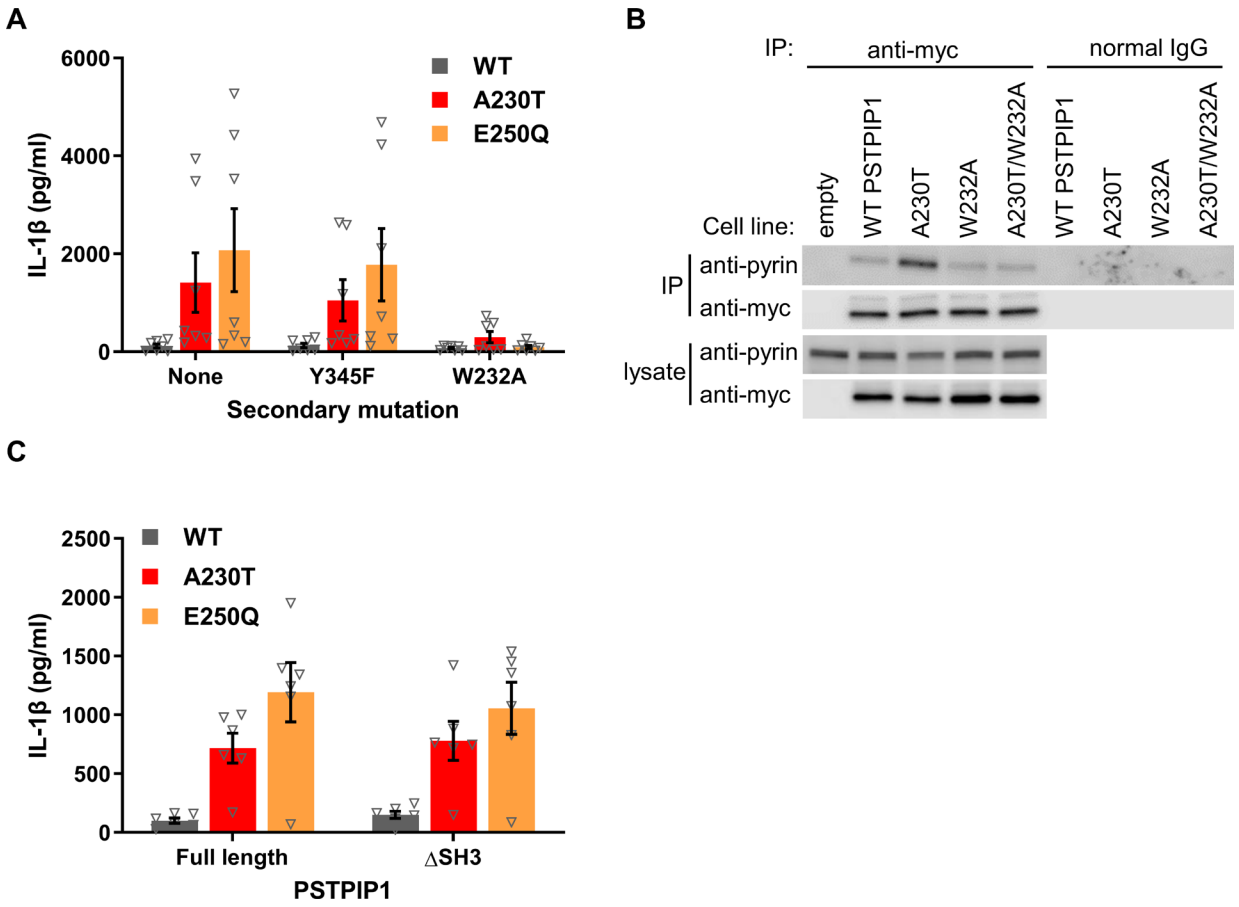

Figure 3 PAPA-associated mutant PSTPIP1 activates the pyrin inflammasome through increased interaction. (A) IL-1β measurements of cell culture supernatants from LPS-primed retroviral-transduced U937 cell lines expressing WT or A230T or E250Q mutant PSTPIP1 proteins without (first three columns) or with secondary mutation, Y345F (second three columns) or W232A (last three columns). (B) Immunoblot analysis of pyrin-PSTPIP1 interaction in lysates of retroviral transduced U937 cells expressing myc-tagged WT or A230T, W232A or A230T/W232A mutant PSTPIP1 proteins assessed before (lysate) and after immunoprecipitation (IP) with antibody to myc or normal IgG. (C) IL-1β measurements of cell culture supernatants from LPS-primed retroviral-transduced U937 cell lines expressing full length WT or A230T or E250Q mutant PSTPIP1 (first three columns) or SH3 domain-truncated WT or mutant PSTPIP1 proteins (second three columns). All IL-1β measurements are from at least five independent experiments. The immunoprecipitation result is representative of three independent experiments. LPS, lipopolysaccharide; PAPA, pyogenic arthritis, pyoderma gangrenosum and acne.

PSTPIP1-pyrin interaction and inflammasome activation

We next examined the correlation between the pyrin-PSTPIP1 interaction and pyrin inflammasome activation. We previously reported that the W232 residue is directly involved in the interaction of WT PSTPIP1 with pyrin, and that the Y345 residue is important for the interaction of phosphorylated WT PSTPIP1 with pyrin.[12] To interrogate the interaction of mutant PSTPIP1 with pyrin, we generated U937 cells expressing PSTPIP1 with the W232A or Y345F mutation in the presence of the PAPA-associated A230T or E250Q mutation. There was no impairment of IL-1β secretion in cells expressing PSTPIP1 with the A230T/Y345F or E250Q/Y345F double mutation in comparison with cells with the A230T or E250Q single mutation, respectively. On the other hand, in cells expressing A230T/W232A or E250Q/W232A mutant PSTPIP1, IL-1β secretion was significantly decreased (figure 3A). These results suggest that PAPA-induced pyrin inflammasome activation is mediated by the direct interaction of mutant PSTPIP1 with pyrin but is independent of PSTPIP1 phosphorylation at tyrosine 345. Consistent with our previous studies, we observed that the interaction of pyrin with A230T mutant PSTPIP1 was significantly increased relative to WT PSTPIP1, and the interaction was completely abolished when W232 was mutated (figure 3B).

The C-terminal SH3 domain (amino acids 360–416) of PSTPIP1 is involved in the interaction with pyrin as well as interactions with WASP, ABL and CD2.[7 12] To test the role of the SH3 domain in the activation of the pyrin inflammasome, we generated U937 cells expressing A230T or E250Q SH3-truncated PSTPIP1 (ΔSH3) (online supplemental figure 1B). There was no reduction of IL-1β secretion in cells with mutant ΔSH3 in comparison with mutant full-length PSTPIP1 proteins (figure 3C). This result suggests that the SH3 domain of PSTPIP1 and other SH3-associated PSTPIP1 binding proteins have no role in PAPA-induced pyrin inflammasome activation.

IFN-γ expression in the skin of PAPA patients

IL-18 is a potent inducer of IFN-γ production.[30] We hypothesised that pyrin inflammasome-induced IL-18 processing may increase IFN-γ production in PAPA patients. Although circulating IFN-γ was noted to be elevated in one study of PAPA patients,[31] the level of CXCL9, a chemokine induced by IFN-γ and often used as a biomarker for IFN-γ, was not elevated in a second report,[28]

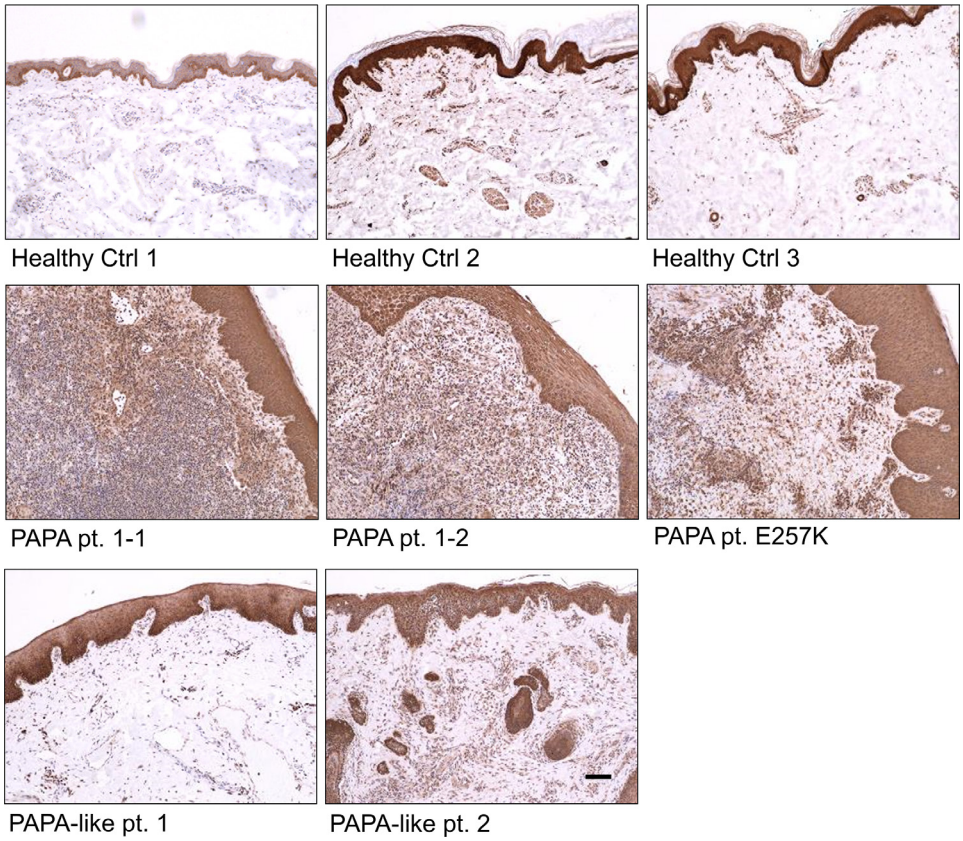

Figure 4 IFN-γ is highly expressed on the skin of PAPA patients (dense dermal inflammatory infiltrate showed strong and diffuse staining of IFN-γ). The skin biopsies of normal skin from three healthy donors (Healthy Ctrl 1, 2 and 3), inflamed skin from two PAPA patients (PAPA pt. 1 at two different points in time, 3 months apart, marked as 1–1 and 1–2, and a second now-deceased PAPA patient with the E257K *PSTPIP1* mutation who never received treatment with JAK inhibitors), and inflamed skin from two PAPA-like patients who were PSTPIP1-mutation negative (PAPA-like pt. 1 and 2) were stained with anti-human IFN-γ antibody (DAB, brown colour). Magnification×10; scale bar, 100 µm; representative images shown. JAK, Janus kinase; PAPA, pyogenic arthritis, pyoderma gangrenosum and acne.

suggesting that estimates of circulating IFN-γ activity might be confounded by the severity or activity of disease in the patients sampled. These data also suggest a possible localised effect of IFN-γ and are consistent with the absence of macrophage activation syndrome (MAS) in PAPA patients. Therefore, we examined the expression of IFN-γ in skin biopsies of PAPA patients, since the skin is a major locus of disease activity, and it has been reported that signalling pathways for IFN-γ producing cells, cytotoxic T cells and NK cells, are upregulated in the skin of PAPA patients.[31] By immunohistochemistry, we observed that the level of IFN-γ was highly increased in the dermis of perilesional skin from PAPA patients in comparison with the skin from healthy controls (figure 4). Unlike the results obtained for IFN-γ, however, there was little or no consistent expression of a type-I IFN, IFN-α2 (online supplemental figure 2), which also induces pyrin expression.[14]

To determine whether the high level of IFN-γ in the dermis is unique to PAPA patients, we examined skin from patients with idiopathic refractory PG (PAPA-like patients), who do not have *PSTPIP1* mutations and display severe skin manifestations comparable to PAPA syndrome with normal serum IL-18 levels.[28] Only a modest amount of IFN-γ was seen in skin biopsies from PAPA-like patients (figure 4), which suggests that the increased expression of IFN-γ in the dermis is a specific feature of PAPA syndrome.

IFN-γ can be produced by various types of cells.[32] We further investigated the identity of infiltrating cells in PAPA patient skin lesions. We found massive infiltration of $CD4^+$ and $CD8^+$ T cells, but not many $CD56^+$ NK cells (online supplemental figure 3). IL-18 induces IFN-γ in synergy with other cytokines such as IL-12 or IL-15.[33] We detected modestly elevated levels of IL-15 (online supplemental figure 4) but not IL-12 (data not shown) in the blood of PAPA patients.

IFN-γ induces pyrin expression and IL-18 secretion in myeloid cells with PAPA-associated *PSTPIP1* mutations

We previously reported that IFN-γ induces *MEFV* gene expression in human leucocytes.[14] ProIL-18, which is constitutively present in some myeloid cells and keratinocytes, is a substrate of caspase-1. Since mutant PSTPIP1 activates the pyrin inflammasome, we hypothesised that the induction of pyrin alone would trigger IL-18 processing in PAPA. Therefore, we examined IL-18 secretion in the U937 model by priming cells with IFN-γ. The priming increased pyrin protein levels in U937 cells (figure 5A). Minimal IL-18 secretion was observed from cells expressing A230T PSTPIP1, but not from cells with WT or E250Q PSTPIP1, without IFN-γ priming (figure 5B). However, cells with both A230T or E250Q mutant PSTPIP1 secreted substantially higher IL-18 than cells with WT PSTPIP1 when treated with IFN-γ (figure 5B). In our ex vivo experimental system, IFN-γ treatment substantially increased the level of pyrin protein in $CD14^+$ monocytes from healthy controls and PAPA patients (figure 5C). However, in response to IFN-γ, monocytes from PAPA patients only released significantly more IL-18 than healthy controls, implying the activation of inflammasome.

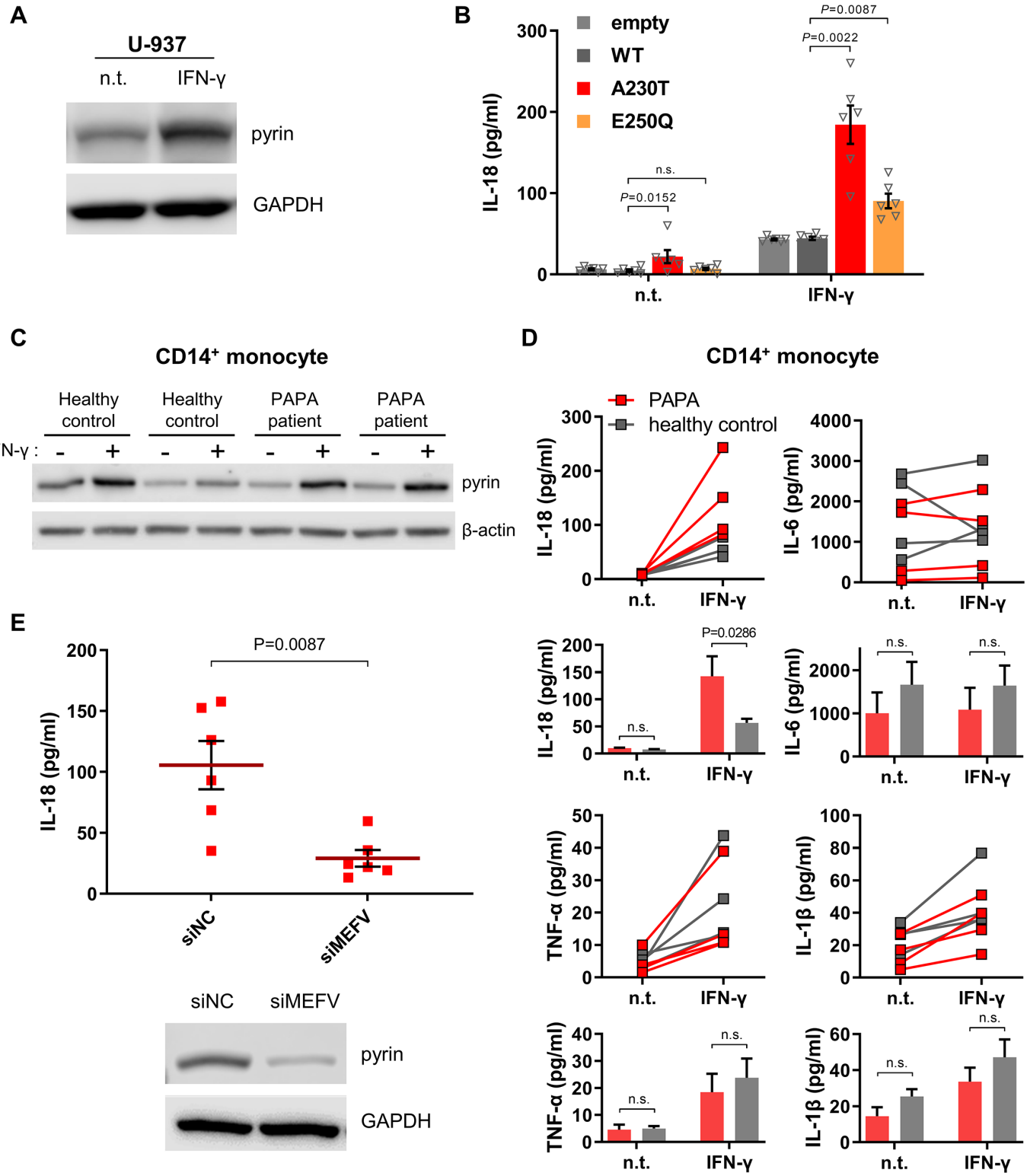

Figure 5 IFN-γ induces pyrin expression and IL-18 secretion in myeloid cells with PAPA-associated *PSTPIP1* mutations. (A) Immunoblot analysis of pyrin expression in cell lysates of U937 cells with or without IFN-γ treatment. (B) IL-18 measurements of cell culture supernatants from stable U937 cells expressing WT or A230T or E250Q mutant PSTPIP1 proteins with or without IFN-γ treatment. (C) Immunoblot analysis of pyrin expression in cell lysates of CD14$^+$ monocytes from two healthy controls and two PAPA patients with or without IFN-γ treatment. (D) Bead-based multiplex analysis of IL-18, IL-6, TNFα and IL-1β levels in cell culture supernatants released by ex vivo cultured CD14$^+$ monocytes from PAPA patients or healthy controls unprimed or primed with IFN-γ (n=4 each group). (E) IL-18 measurements of cell culture supernatants from CD14$^+$ monocytes of PAPA patients transiently transfected with siNC or si*MEFV* then primed with IFN-γ. P values were calculated with Mann-Whitney U test. n.s. not significant; n.t. no treatment; PAPA, pyogenic arthritis, pyoderma gangrenosum and acne.

While IFN-γ treatment increased the production of other proinflammatory cytokines as well, only the increase in IL-18 levels reached statistical significance between healthy controls and PAPA patients. IL-1β was not primed in this experimental setting and thus did not show a significant difference (figure 5D). We confirmed that PAPA mutation-associated IL-18 secretion is also dependent on the pyrin inflammasome in U937 cell lines and monocytes of patients (online supplemental figure 5 and figure 5E, respectively). Taken together, these data suggest a positive feedback loop linking PAPA-mediated pyrin inflammasome activation, IL-18 processing, and dermal IFN-γ levels, which probably amplifies inflammation.

PAPA-associated mutations activate the pyrin inflammasome without affecting pyrin dephosphorylation

Pyrin inflammasome assembly is suppressed by pyrin phosphorylation at Ser208 and Ser242 and subsequent binding of 14-3-3 proteins to the phosphorylated residues.[16 19 34] To test how mutant PSTPIP1 affects this known pyrin regulatory mechanism, we examined pyrin's interaction with 14-3-3. Unexpectedly, we found that the 14-3-3 interaction was not altered by mutant PSTPIP1 (figure 6A). Moreover, we found no difference in the phosphorylation of pyrin Ser242 between cells expressing WT and mutant PSTPIP1 (figure 6B). Staurosporine activates pyrin inflammasome by inhibiting PKN activity.[16] Staurosporine treatment significantly boosted IL-18 processing in cells expressing mutant PSTPIP1 (figure 6C). However, we failed to see any difference in pyrin phosphorylation between WT and mutants, and the Ser242 residue was uniformly dephosphorylated in all cells within the staurosporine-treated group (figure 6B). Monocytes from PAPA patients also displayed Ser242 phosphorylation comparable to healthy controls, and staurosporine dephosphorylated pyrin in all treated cells (figure 6D). These results demonstrate that pyrin inflammasome activation by PAPA-associated mutant PSTPIP1 is not mediated by pyrin dephosphorylation or dissociation of 14-3-3.

JAK inhibition and PAPA syndrome

The foregoing results suggest that the cutaneous inflammation of PAPA syndrome is amplified by a positive feedback loop involving pyrin, IL-18 and dermal IFN-γ. Consistent with this hypothesis, IL-18 secretion from IFN-γ-treated U937 cells expressing the A230T PSTPIP1 mutation was significantly decreased in a dose-dependent manner by ruxolitinib or tofacitinib, inhibitors for the JAK-STAT pathway that transduces signals from the IFN-γ receptor (figure 7A). As expected, treatment with JAK inhibitors reduced IFN-γ-induced pyrin expression in U937 cells (figure 7B). We confirmed the inhibitory effect of JAK inhibitors on pyrin inflammasome activation ex vivo in PAPA patients' leucocytes. As observed in U937 cell lines, JAK inhibitors suppressed IL-18 secretion (figure 7C) as well as pyrin expression in PBMCs of PAPA patients (figure 7D). Ruxolitinib was more effective in suppressing pyrin inflammasome activation than tofacitinib in this ex vivo system.

Five of five unrelated refractory PAPA patients have shown positive clinical outcomes (figure 7E). Patient 1 has a familial A230T mutation. Despite concomitant treatment with canakinumab and infliximab, the patient required up to 60 mg of prednisone daily and long hospitalisations for extensive wounds. On tofacitinib, the patient tapered off prednisone and avoided hospitalisation for more than 3 years. Patient 2 carries a familial E250Q mutation. On 15 mg of prednisone daily and canakinumab, the patient continued to have intermittent episodes of arthritis that required steroid injections and increased oral steroids. On tofacitinib alone, the patient was able to reduce the dose of prednisone to 11 mg/day and had fewer episodes of arthritis.

Patient 3 has a de novo mutation, E257G, causing severe disease with anaemia and extensive wounds on the buttocks. Therapy with dual biologics, canakinumab and golimumab, was not tolerated because of bacteraemia. On high doses of canakinumab and 20 mg of prednisone daily, the patient continued to have inflammation, anaemia, and cysts developing intermittently within his wounds. On canakinumab and ruxolitinib, the patient reported fewer cysts, more energy, and less pain. Patient 4 has a de novo E250Q mutation and extensive, recurring PG lesions despite dual biologic treatment with canakinumab and adalimumab (figure 7F). These recurrences required high doses of intravenous methylprednisolone for control and would often recur as glucocorticoids were tapered. After ruxolitinib was begun, PG lesions remained healed despite discontinuation of prednisone (figure 7G). Patient 5 has a de novo A230T mutation and developed intermittent PG lesions despite golimumab. High doses of parenteral methylprednisolone followed by long tapering courses of prednisone were required. After starting ruxolitinib, the patient was able to taper off prednisone without the development of significant skin lesions. More detailed clinical information is available in online supplemental methods and summarised in online supplemental table 1.

DISCUSSION

Although the genetic basis of PAPA syndrome was discovered over 20 years ago,[3] and PSTPIP1 was identified as a pyrin-binding protein shortly thereafter,[12] the molecular pathophysiology of PAPA syndrome remains poorly understood, and targeted monotherapy remains elusive. By demonstrating that mutant PSTPIP1 interacts with the pyrin inflammasome in a previously unrecognised way that creates an IFN-γ-dependent feedback loop, this manuscript provides the conceptual basis for a novel therapeutic approach to PAPA syndrome.

The first major finding of this paper is the demonstration, using a U937 myeloid human cell line model, that, without additional stimuli, PAPA-associated gain-of-function PSTPIP1 mutations cause activation of the pyrin inflammasome, but not the NLRP3, NLRC4 or AIM2 inflammasomes. Earlier work demonstrated increased IL-1β release by cell lines transfected with mutant PSTPIP1, relative to WT PSTPIP1, in COS-7L cells cotransfected with human pyrin, ASC and caspase-1.[12] Similarly, LPS-stimulated monocytes from patients with PAPA syndrome secreted markedly increased IL-1β, relative to healthy control monocytes.[12] However, those studies antedated the detailed molecular understanding of the NLRP3, NLRC4, AIM2 or pyrin inflammasomes. The current analysis definitively places PAPA syndrome on the growing list of disorders of the pyrin inflammasome[13] that includes FMF, PAAND, HIDS/MKD, PFIT and NOCARH syndrome.[21 23]

The recognition of PAPA syndrome as a pyrin inflammasomopathy provided the context for experiments demonstrating that PAPA-associated PSTPIP1 mutations activate the pyrin inflammasome by a non-canonical mechanism. We and others have demonstrated that the pyrin inflammasome is usually held in check by RhoA-dependent PKN1/2-induced phosphorylation at pyrin residues 208 and 242, leading to pyrin sequestration by 14-3-3 molecules.[16 19 34] Pyrin 'senses' microbial toxins that inactivate RhoA by its release from PKN1/2 phosphorylation and 14-3-3 binding, thus allowing pyrin inflammasome assembly.

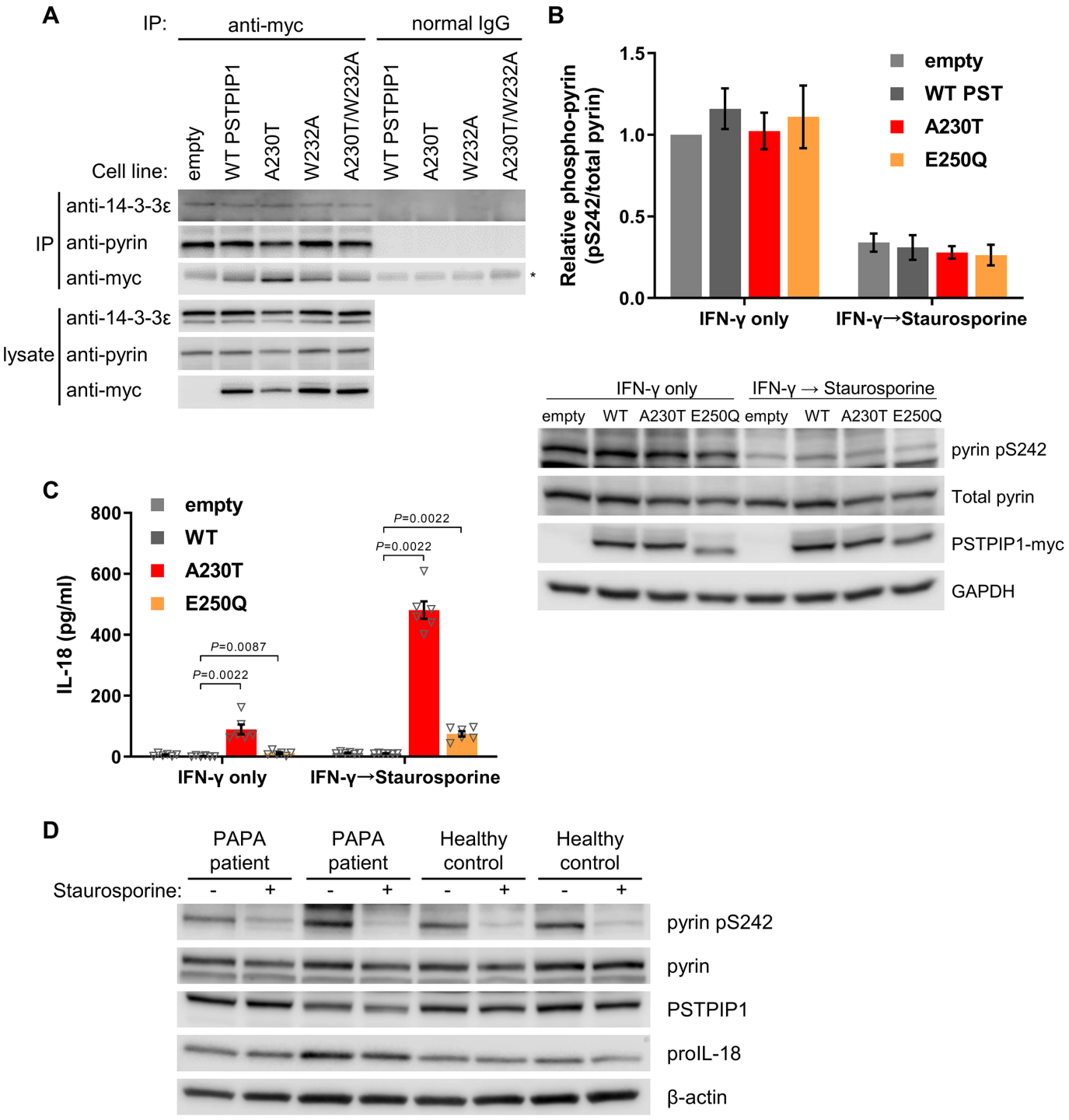

Figure 6 PAPA-associated mutations activate the pyrin inflammasome without affecting pyrin dephosphorylation. (A) Immunoblot analysis of pyrin-14-3-3ε interaction in lysates of stable U937 cells expressing myc-tagged WT or A230T, W232A or A230T/W232A mutant PSTPIP1 proteins assessed before (lysate) and after immunoprecipitation (IP) with antibody to myc or normal IgG. (B) Measurements of pyrin phosphorylation in cell lysates from stable U937 cells expressing myc-tagged WT, A230T or E250Q mutant PSTPIP1 proteins after IFN-γ treatment or IFN-γ followed by staurosporine treatment. Cell lysates were immunoblotted with antibodies against pyrin phospho-Ser242 and total pyrin. Band intensities of phosphorylated pyrin were quantified and normalised against total pyrin and plotted (top) from three independent immunoblot analyses (bottom, a representative immunoblot). (C) IL-18 measurements of cell culture supernatants from (B). IL-18 measurements were from at least five independent experiments. (D) Immunoblot analysis of pyrin phosphorylation and expression of PSTPIP1 and proIL-18 in cell lysates of $CD14^+$ monocytes from two healthy controls and two PAPA patients after IFN-γ treatment or IFN-γ followed by staurosporine treatment. P values were calculated with the Mann-Whitney U test. PAPA, pyogenic arthritis, pyoderma gangrenosum and acne.

Here, we demonstrate that canonical pyrin inflammasome activation is neither dependent on PSTPIP1 nor is mutant-PSTPIP1-associated pyrin inflammasome activation dependent on pyrin dephosphorylation or 14-3-3 release. However, a PSTPIP1 mutation known to prevent its binding to pyrin blocked PSTPIP1-associated pyrin inflammasome activation, underscoring the

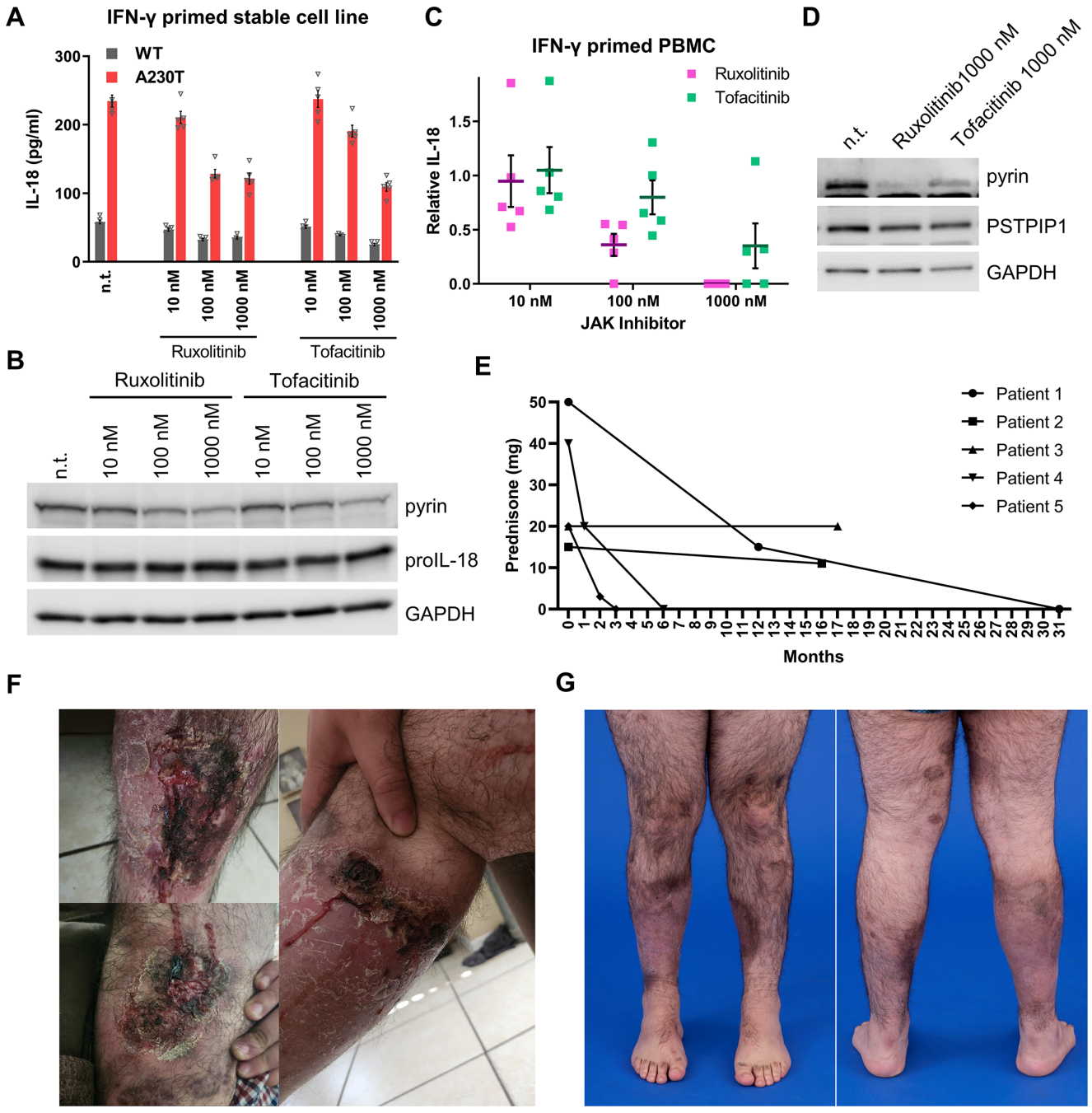

Figure 7 JAK inhibition suppresses PAPA syndrome-mediated inflammation. (A) IL-18 measurements of cell culture supernatants from stable U937 cells expressing WT or A230T mutant PSTPIP1 proteins after IFN-γ treatment and various doses of ruxolitinib or tofacitinib. (B) Immunoblot analysis of pyrin or proIL-18 expression in cell lysates from (A). (C) IL-18 measurements of cell culture supernatants from PBMCs of PAPA patients after IFN-γ treatment and various doses of ruxolitinib or tofacitinib. The IL-18 levels measured from cells treated with ruxolitinib or tofacitinib were normalised to the IL-18 levels measured from cells without ruxolitinib or tofacitinib treatment. (D) Immunoblot analysis of pyrin or PSTPIP1 expression in cell lysates from (C). (E) Daily prednisone doses of patients at baseline prior to starting a JAK inhibitor and at follow-up visits months later. (F) Several pyoderma gangrenosum skin lesions, characterised by poorly healing cutaneous ulcers with undermined edges, on the legs of patient 4 prior to starting ruxolitinib. (G) Six months after starting ruxolitinib, the cutaneous leg ulcers of patient 4 have healed with residual dermal scars. JAK, Janus kinase; PAPA, pyogenic arthritis, pyoderma gangrenosum and acne; PBMCs, peripheral blood mononuclear cell.

importance of physical interaction. Previous work from our laboratory indicates an important role for the B-box and coiled-coil domains of pyrin in PSTPIP1 binding,[12] thus adding to evidence that this region of the pyrin protein plays an important regulatory role.[35–38]

We hypothesised that differences between the coiled-coil domains of human and mouse pyrin might also explain our inability to develop a KI mouse model of PAPA syndrome. Even though there is a high level of homology between human and murine PSTPIP1 in the region where PAPA-associated mutations reside in humans, and even though we developed a mouse line that harbours a human pyrin B30.2 domain to overcome the lack of a B30.2 domain in endogenous mouse pyrin, mouse pyrin is noteworthy for its differences with human pyrin in the

coiled-coil domain. We found that the PAPA-associated A230T KI *Pstpip1* mutation binds mouse Pyrin more avidly than WT (not shown), indicating that if the differences in the pyrin coiled-coil domains are the explanation for the inability to develop a mouse model, it is not merely because of a failure of murine Pyrin and Pstpip1 to interact.

Given the possibility of non-canonical pyrin inflammasome activation dependent on the binding of mutant PSTPIP1 to the central coiled-coil domain of pyrin, we sought evidence of aberrant systemic or localised IL-1β or IL-18 in PAPA syndrome. Previous data from our group demonstrated markedly increased total and free IL-18 in the blood of PAPA patients but not in the blood of patients with idiopathic PG.[28] Therefore, in the current manuscript, we hypothesised a positive feedback loop in which the non-canonically activated pyrin inflammasome releases excessive amounts of IL-18, which induces IFN-γ, a known inducer of pyrin.[14] Failing to find high levels of IFN-γ or its biomarker CXCL9 in the blood, by immunohistochemistry we found high levels of IFN-γ in lesional PAPA skin that were not present in the skin lesions of idiopathic PG patients. Supporting its role in pathogenesis, ex vivo IFN-γ priming of monocytes from PAPA patients led to increased levels of IL-18, but not IL-1β, IL-6 or TNF-α in a pyrin-dependent manner. Our data further substantiate the possibility of a feedback loop (online supplemental figure 6) that should be, and is, amenable to treatment with JAK inhibitors. Clinical data from 5/5 PAPA patients on JAK inhibitors in combination with IL-1β-blockade treatment (4/5) are encouraging, notwithstanding the fact that the current generation of JAK inhibitors may disrupt the signalling of cytokines and growth factors beyond IFN-γ. We propose a therapeutic strategy that includes targeting the IFN-γ-pyrin-IL-18 axis in addition to conventional IL-1β-targeted therapy for the treatment of PAPA syndrome.

Adult-onset Still's disease (AOSD) and systemic juvenile idiopathic arthritis (sJIA) are diseases also marked by distinctly high levels of IL-18.[39 40] Patients with AOSD and sJIA may develop MAS, a life-threatening condition driven by IFN-γ.[41] Despite the shared involvement of IL-18 and IFN-γ, the phenotypic features of PAPA syndrome differ from AOSD and sJIA in many ways. Polyarticular arthritis may be seen in AOSD and sJIA, while PAPA syndrome usually targets one or two joints at a time. The skin is only mildly affected as an evanescent salmon-coloured rash in AOSD and sJIA. We are unaware of any reported case of MAS in PAPA. These clinical differences may derive from different inflammasomes being involved in the IL-18 production in the two diseases, or may be due to the anatomic localisation of PAPA syndrome to the skin and joints as compared with the more systemic involvement of AOSD and sJIA. Nevertheless, recent reports of successful treatment of refractory AOSD and sJIA with JAK inhibitors emphasise molecular similarities among these three conditions.[42 43] Emapalumab, a monoclonal antibody against IFN-γ that has been successfully used in treating MAS in AOSD and sJIA, could potentially be another therapeutic option for PAPA.[44]

We are currently examining the response of PAPA patients to JAK inhibition in a more systematic manner, given the strong functional foundation and promising clinical data reported in this manuscript. We are cautiously optimistic that the recognition of IFN-γ-dependent signalling represents a watershed in the treatment of PAPA syndrome.

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