SUPPLEMENTAL METHODS

Detailed clinical histories of patients with PAPA syndrome who received a JAK inhibitor

Patient 1 is an adult in their 30s with a familial A230T mutation in *PSTPIP1*. After patient fell at 4 years of age, their elbow became inflamed, tender, and required aspiration. At 11 years of age, they began to develop cystic acne lesions which were first treated with triamcinolone injections. By 13 years of age, the extensive cystic acne on their face and back required treatment with prednisone and isotretinoin.

At 14 years of age, this patient was referred to the National Institutes of Health and started on infliximab, given at the highest dose recommended for weight. Five years later, anakinra was started, and modest benefit was noted at a maximum dose of 500 mg daily. Later combination therapy with infliximab (10 mg/kg every four weeks), anakinra (400 mg daily), prednisone (17 mg daily), and methotrexate (10 mg weekly) was given. Anakinra was then changed to canakinumab (150 mg SC every 4 weeks). Despite these medications, patient developed recurrent PG lesions that required treatment with methylprednisolone and prednisone up to 60 mg daily. Other medications given without benefit included certolizumab, golimumab, and secukinumab.

In October 2019, this patient was prescribed tofacitinib (5 mg twice daily) in addition to infliximab (10 mg/kg every four weeks), canakinumab (150 mg every four weeks), and prednisone (50 mg daily). On tofacitinib, they have been able to taper off prednisone and decrease their dose of infliximab to 7 mg/kg every four weeks. They also have avoided hospitalization for more than 3 years.

Patient 2 is an adult in their 30s with a familial E250Q mutation in *PSTPIP1.* Since early childhood, they have had recurring episodes of monoarticular arthritis but have never had skin involvement. Methotrexate, leflunomide, colchicine, adalimumab, infliximab, and etanercept were tried with only partial improvement, and they remained on daily prednisone. Anakinra (100 mg daily) was the most effective, but they required a synovectomy at age 16 and another at age 19. At age 20, this patient was able to taper off prednisone for the first time in several years. However, a few months later, their disease flared aggressively with involvement of multiple joints.

At this patient's first NIH visit when they were 21 years old, CRP was 119 mg/L and ESR was 96 while receiving anakinra (400 mg daily), prednisone (7 mg daily), methotrexate (15 mg weekly), and methylprednisolone (250 mg weekly). Anakinra was increased to 600 mg daily and then golimumab was added. Methylprednisolone infusions were stopped, but they required prednisone up to 50 mg daily. They were later switched from golimumab to tocilizumab (8 mg/kg every 4 weeks) with the high dose of anakinra continued and were able to taper their dose of prednisone to 15 mg daily. Then anakinra was switched to canakinumab (up to 300 mg every 4 weeks), and tocilizumab was discontinued.

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On canakinumab and prednisone (15 mg daily), patient continued to have episodes of arthritis that required steroid injections and temporarily higher doses of prednisone. Tofacitinib (11 mg extended release daily) was begun in December 2021, and they began to feel better within days. Canakinumab was discontinued because of a misunderstanding, and they chose not to resume taking it. During 18 months of taking tofacitinib, this patient has had only one episode of arthritis, and their prednisone dose was gradually tapered to 11 mg daily.

Patient 3 is an adult in their 30s with a *de novo* E257G mutation in *PSTPIP1*. They have had many episodes of arthritis from infancy to young adulthood and have lost mobility in an elbow. They were treated with prednisone, methotrexate, colchicine, cyclosporin, and etanercept. Infliximab was reportedly effective for 1 ½ years. At 12 years of age, they began to have cystic acne. In their early 20s, they developed an abscess of the buttocks, and lesions spread bilaterally, drained, and scarred. Patient eventually required a wheelchair, because walking was too painful. Treatments tried included anakinra (100 mg daily), antibiotics, oral methylprednisolone, azathioprine, and abatacept.

After genetic testing was performed and the diagnosis of PAPA syndrome was made, this patient was seen at NIH at 23 years of age and prescribed golimumab, prednisone, and isotretinoin, the last of which was not tolerated. When anakinra was added, patient developed *Streptococcus anginosus* bacteremia and a lobar pneumonia. Anakinra was stopped, and the dose of golimumab was decreased. When they clinically worsened, the dose of golimumab was resumed and canakinumab was begun. However, they developed a fever, and four teeth were extracted for presumed dental abscesses. Their dose of golimumab was decreased and then stopped after they were admitted and found to have a large abscess of the gluteal muscle extending into the presacral space. This abscess was drained, and cultures grew *S. anginosus*. After this admission, they were treated with only canakinumab (300 mg every 4 weeks) and prednisone (20 mg daily). Patient continued to have some pain and drainage from the buttocks lesions. Canakinumab was discontinued for trials of monotherapy with tofacitinib, ruxolitinib, and ustekinumab, but these trials were associated with clinical worsening.

In April 2022, ruxolitinib (10 mg twice daily) was added to patient's regimen of canakinumab and prednisone. In less than 3 weeks, their CRP dropped from 77 to 10 mg/L**.** They reported increased energy, less frequent abscesses, and no further need for pain medication. However, their ESR remained elevated, and they continued to be anemic. Eight months later, their dose of ruxolitinib was increased to 15 mg twice daily.

Patient 4 is an adult in their 20s with a *de novo* E250Q mutation. This patient's first episode of arthritis occurred at 4 months of age. Skin abscesses often occurred after injuries. This patient required hospital admission numerous times. They have been followed at the NIH since 4 years of age. At 7 years old, they had an adverse reaction to infliximab and were prescribed adalimumab. Five years later, anakinra was added. When hospitalized locally, patient was often treated with antibiotics, which led to an infection with *Clostridium difficile*.

Adalimumab was switched to golimumab, and then anakinra was changed to canakinumab with the doses gradually increased. This resulted in a short-term improvement before flares of pyoderma gangrenosum lesions resumed and required methylprednisolone infusions followed by prednisone tapers.

When ruxolitinib (10 mg twice daily) was added to canakinumab (300 mg every 4 weeks) and golimumab (50 mg every 4 weeks), patient's CRP, ESR, and hematocrit normalized. Their skin lesions healed and have remained closed since ruxolitinib was begun 6 months ago.

Patient 5 is an adult in their 20s with a *de novo* A230T mutation in *PSTPIP1*. As a toddler, they developed a skin abscess that was culture-negative and recurrent. At 5 years of age, this patient fell and developed elbow swelling, fever, and elevated WBC count. Their elbow was aspirated, and the culture was negative. Arthritis recurred in this joint and was treated with antibiotics, 2 arthroscopic surgeries, and a capsular release. Several times patient developed abscesses over the surgical incision sites. Patient's mother researched her child's illness and suspected PAPA syndrome, which was confirmed by genetic testing when patient was about 9 years old. Patient was initially treated with colchicine, but a knee injury triggered an episode of arthritis which required a prolonged course of glucocorticoids.

This patient was first seen at NIH at 12 years of age and treated with anakinra and glucocorticoids intermittently. However, at 15 years of age, they began to have skin lesions involving the scalp and face. Golimumab (50 mg every 2 weeks) was started, and anakinra was used as needed for joint injuries. Patient chose to stop golimumab but resumed it after developing lesions of the chest, neck, and eyelid that required methylprednisolone infusions and prolonged courses of prednisone. Since starting ruxolitinib (10 mg twice daily) and continuing golimumab (50 mg twice monthly) 4 months ago, patient has developed only a few small papules that have healed, and they have tapered off prednisone.

Detailed clinical histories of patients with PSTPIP1-negative PAPA-like skin disease

Patient 1 is an adult in their 20s who had severe acne, hidradenitis suppurativa, and pyoderma gangrenosum lesions of the neck and upper chest. No variants were identified in *PSTPIP1*. Isotretinoin, antibiotics, and adalimumab (40 mg every week) were tried with minimal improvement. At this patient's first NIH visit at 18 years of age, CRP testing was 170 mg/L and sedimentation rate was 116 mm/hr. Golimumab (50 mg weekly), prednisone (25 mg daily), and anakinra (200 mg daily) were prescribed. On this regimen, the lesion on the left chest healed, but a new lesion developed on the right chest and required drainage. Patient was switched to canakinumab (150 mg every 4-6 weeks) without improvement. Isotretinoin (20 mg to 40 mg) was resumed, resulting in some improvement of acne. Ustekinumab (90 mg every 8 to 12 weeks) resulted in healing of skin lesions and allowed for prednisone to be tapered and discontinued.

Patient 2 is an adult in their 20s who has no variant identified in *PSTPIP1*. At 14 years of age, this patient developed arthralgias and severe nodulocystic acne that drained and healed with

atrophic scarring. They were treated unsuccessfully with adapalene, topical clindamycin, oral antibiotics, and high doses of isotretinoin. At this patient's first NIH visit at 15 years of age, they were prescribed golimumab (50 mg every 2 weeks) and a tapering course of prednisone. Although this medical regimen resulted in improvement, there remained some active draining lesions for which anakinra (100 mg daily) was prescribed to be used as needed. However, patient then developed painful cysts in an axilla and cystic lesions on a forearm. More than a year after starting golimumab, this patient developed psoriasis that became moderately severe despite topical treatments. Patient was begun on ustekinumab (45 mg every 8 to 12 weeks) and showed significant improvement of the acne, cystic lesions, and psoriasis.

Mice

WT C57BL/6J mice were obtained from the Jackson laboratory. *Pstpip1*^{KO} and *Pstpip1*^{A230T} mice were generated as described before with modifications [1]. The targeting construct was generated by inserting a 3.5 kb genomic fragment (5' arm) with a *neo*^{R} selection cassette flanked by two *loxP* sites and a 5.7 kb fragment (3' arm) carrying the A230T variant in exon 10 as shown in figure 1A. Linearized construct was introduced into mouse embryonic stem cells and screened for G418 resistance to make *Pstpip1*^{KO} mice. *Pstpip1*^{KO} mice were crossed with Cre-expressing mice for *neo*^{R} cassette removal to generate *Pstpip1*^{A230T} mice. Mice harboring a human B30.2 domain (*Mefv*$^{B30.2/B30.2}$) and *Mefv*-deficient (*Mefv*$^{-/-}$) mice have been previously described [1, 2]. Bone marrow-derived macrophages (BMDMs) were prepared as previously described [3]. Mouse blood was collected from the orbital sinus and centrifuged for serum collection or RBC-depleted cells were collected after ACK lysis buffer treatment. Mice used in each experiment were randomized by age and sex.

Human PBMC and CD14^{+} monocyte isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated by LSM-Lymphocyte Separation Medium (50494, MP Biomedicals) from freshly drawn peripheral venous blood of healthy controls or patients. CD14^{+} monocytes were isolated from PBMC using human CD14 MicroBeads (130-050-201, Miltenyi Biotec) on MS columns (130-042-201, Miltenyi Biotec).

Cell culture, retroviral transduction and stable cell line generation

U937 cells were obtained from ATCC (CRL-1593.2) and cultured in RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific). THP1-KO-NLRP3 cells were purchased from Invivogen (thp-konlrp3z). Retroviral transduction of U937 cells was performed as described previously with modification [4]. Wildtype PSTPIP1 tagged with C-terminal myc (RC203788, Origene) or PSTPIP1 with p.A230T, p.W232A, p.E250Q, p.Y345F, or ΔB30.2 mutation was cloned into pLNCX2 vector (631503, Takara) and then co-transfected into HEK293 cells with gag/pol (Addgene plasmid # 14887) and VSV.G (Addgene plasmid # 14888) which were gifts from Tannishtha Reya [5].

Stable cell lines were generated with wildtype PSTPIP1 or PSTPIP1 with p.A230T or p.E250Q mutation cloned into pEF1a-IRES-Neo (gift from Thomas Zwaka [6]; Addgene plasmid # 28019) modified with pEF1/myc-His A (V92120, Thermo Fisher Scientific) [7]. Plasmid was linearized with AseI (R0526L, New England Biolabs) then electroporated to U937 with Neon electroporation device (Thermo Fisher Scientific) at 1,400V, 10 ms x 3 pulses. Cells were selected with 800 μg/ml G418 for two weeks before they were used for experiments.

Knockdown assay

To knockdown target genes, siRNAs were electroporated into cells using Neon electroporation device (Thermo Fisher Scientific). For electroporation of U937 cell lines, 1×10^6 cells were resuspended in 100 μl R buffer with final concentration of 1μM siRNA. Cells were electroporated in 1400V for 10 ms, 3 pulses. For THP1-KO-NLRP3 cell lines, 1×10^6 cells were resuspended in 100 μl R buffer with final concentration of 1μM siRNA. Cells were electroporated in 1700V for 20 ms, 1 pulse. For $CD14^+$ monocytes, 1×10^6 cells were resuspended in 10 μl T buffer with final concentration of 1μM siRNA. Cells were electroporated in 2000V for 15 ms, 1 pulse. siRNAs (negative control siRNA 4390844, siMEFV s502557, siPSTPIP1 s17257, siAIM2 s18092, siNLRP3 s41555, siNLRC4 s33829) are from Thermo Fisher Scientific.

Inflammasome activation, enzyme-linked immunosorbent assay, and bead-based immunoassay

U937 cells were differentiated with 5 ng/ml PMA for 48 hours and then stimulated with 10 ng/ml ultrapure LPS (tlrl-3pelps, Invivogen) for six hours or primed with 20 ng/ml IFN-γ (300-02, Peprotech) for one day. PBMCs and $CD14^+$ monocytes were cultured in RPMI-1640 medium supplemented with 10% FBS and 20 ng/ml IFN-γ on Nunclon Sphera U96 bottom plate (174925, Thermo Fisher Scientific) for 24 hours. For *ex vivo* JAK inhibition, ruxolitinib or tofacitinib was treated in combination with IFN-γ. For the pyrin dephosphorylation, cells were cultured in media containing 1μM staurosporine for one hour. For pyrin inflammasome activation, THP1-KO-NLRP3 cells were differentiated and primed as above then treated with 10 ng/ml LPS and 4 μg/ml *C. difficile* toxin A (BML-G140-0050, Enzo Life Sciences) in serum-free RPMI for 6 hours. Cell culture supernatants were collected after treatment and analyzed. IL-1β and IL-18 were measured in cell culture supernatant by ELISA kit (SLB50, DL180, R&D Systems) according to the manufacturer's instruction. Serum IL-15 levels were measured with ELISA kit (41702, PBL Assay Science) according to the manufacturer's instruction. IL-18, IL-1β, TNF-α, and IL-6 were measured using Th1/Th2 Cytokine 11-Plex Human ProcartaPlex Panel (EPX110-10810-901, Thermo Fisher Scientific) according to the manufacturer's instruction.

Coimmunoprecipitation and immunoblot

U937 stable cell lines expressing PSTPIP1 tagged with C-terminal myc were lysed with lysis buffer (20 mM Tris pH 7.4, 50 mM NaCl, 0.5% IGEPAL-CA630) containing cOmplete Protease Inhibitor Cocktail EDTA-free tablet (Roche). Cell lysates were cleared by centrifuge then immunoprecipitated with Dynabeads Protein G (Thermo Fisher Scientific) bound with anti-myc antibody (4A6, Sigma Millipore). Beads were washed then eluted directly to Laemmli sample buffer. Immunoblots were prepared with Novex Tris-Glycine Gel Systems (Thermo Fisher Scientific) and probed overnight at 4 °C with antibodies to pyrin (1:2,000 dilution) [8], pyrin phospho-S242 (1:2,000 dilution; ab195975; abcam), myc (1:5000; 2040S, Cell Signaling Technology), PSTPIP1 (1:2,000 dilution) [8], 14-3-3ε (1:1,000 dilution; sc-23957; Santa Cruz Biotechnology), PKN1 (1:2,000 dilution; ab195264; abcam), PKN2 (1:1,000 dilution; 2612; Cell Signaling), pro-IL-1β (1:1,000 dilution; AF-201-NA; R&D Systems), pro-IL-18 (1:5,000 dilution; ab207324; abcam), NLRP3 (1:1,000 dilution; AG-20B-0014-C100, AdipoGen), AIM2 (1:250; ab93015; abcam), and NLRC4 (1:1000; 06-1125; Millipore). Secondary antibodies were blotted for 1 hour at room temperature (7074 and 7076, Cell Signaling Technology). Chemiluminescence images were digitally obtained using Odyssey Fc (LI-COR).

Immunohistochemistry

Tissue sections of formalin-fixed paraffin-embedded blocks of skin biopsies/excisions from PAPA patients, PAPA-like patients, and healthy donors were obtained from the NCI Laboratory of Pathology. The tissue sections were placed on coated glass slides and stained with anti-IFN-γ antibody (ab9657, abcam) at 1:500 dilution and anti-IFN-α antibody (21100-1, bio-techne) at 1:200 dilution by a third-party company (Histoserv, Inc., Germantown, MD). Stained slides were imaged on EVOS M5000 Imaging System (Thermo Fisher). Histology slides stained with anti-CD4, CD8, and CD56 antibodies were provided by NCI Laboratory of Pathology and scanned on NanoZoomer digital slide scanner (Hamamatsu).

Flow cytometry

RBC-lysed single cell suspensions were obtained from mouse peripheral blood and stained with fluorochrome-conjugated CD11b antibodies in BSA staining buffer (BioLegend). Cells were analyzed on a FACSCaliber flow cytometer (Becton Dickinson). The results were analyzed with FlowJo software (Becton Dickinson).

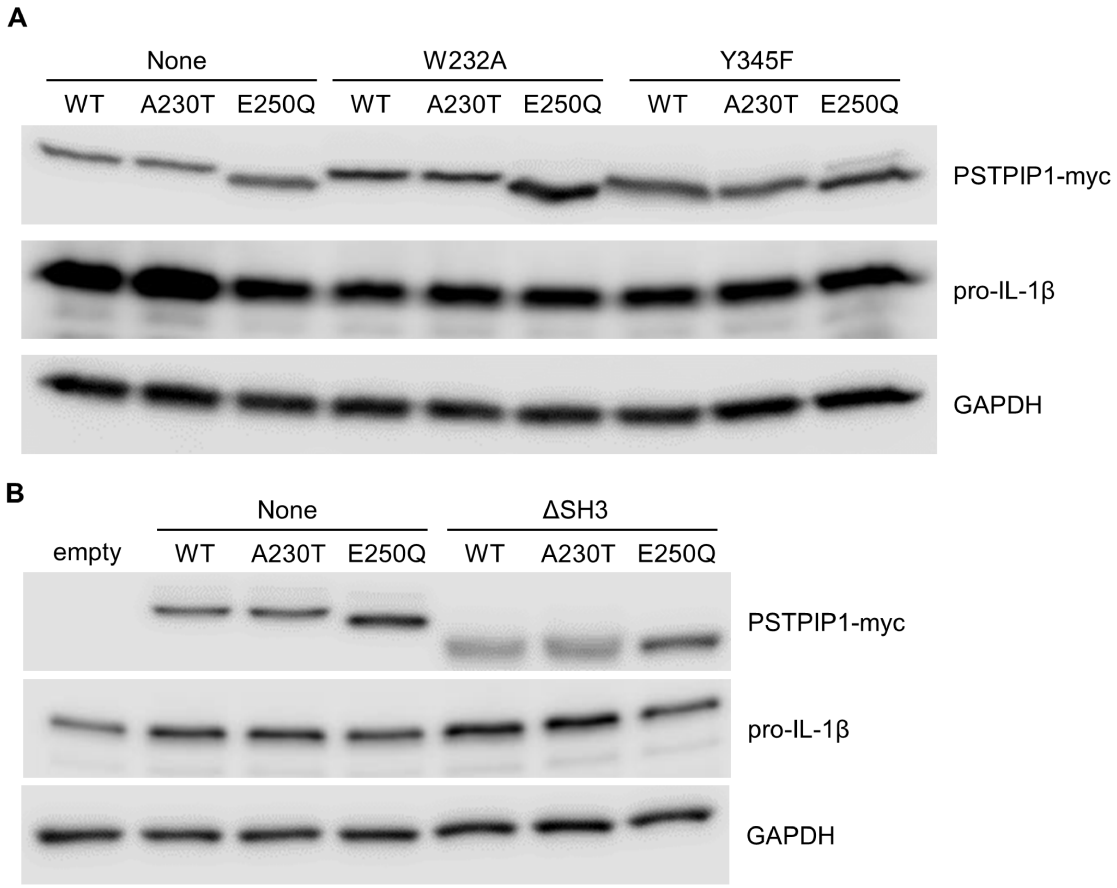
SUPPLEMENTAL DATA

Supplemental table 1. Summary of the clinical features of PAPA syndrome patients.

Patient number	PSTPIP1 variant	JAK inhibitor	Previous medications	Concurrent medications	Decreased prednisone dose	Clinical Improvement
1	A230T	Tofacitinib	certolizumab, golimumab, secukinumab, isotretinoin, anakinra, methotrexate	Infliximab, canakinumab	yes	No hospitalization for >3 years, PG lesions healed
2	E250Q	Tofacitinib	methotrexate, leflunomide, colchicine, adalimumab, infliximab, etanercept, golimumab, tocilizumab, anakinra	canakinumab (stopped)	yes	Only one episode of arthritis in 18 months
3	E257G	Ruxolitinib	methotrexate, colchicine, cyclosporin, etanercept, infliximab, anakinra, golimumab, isotretinoin, azathioprine, abatacept, ustekinumab, tofacitinib(alone), ruxolitinib (alone)	canakinumab	yes	Decreased frequency of abscesses, No longer required pain medication
4	E250Q	Ruxolitinib	infliximab, adalimumab, anakinra, isotretinoin	canakinumab, golimumab	yes	PG lesions healed
5	A230T	Ruxolitinib	anakinra, colchicine	golimumab	yes	PG lesions healed

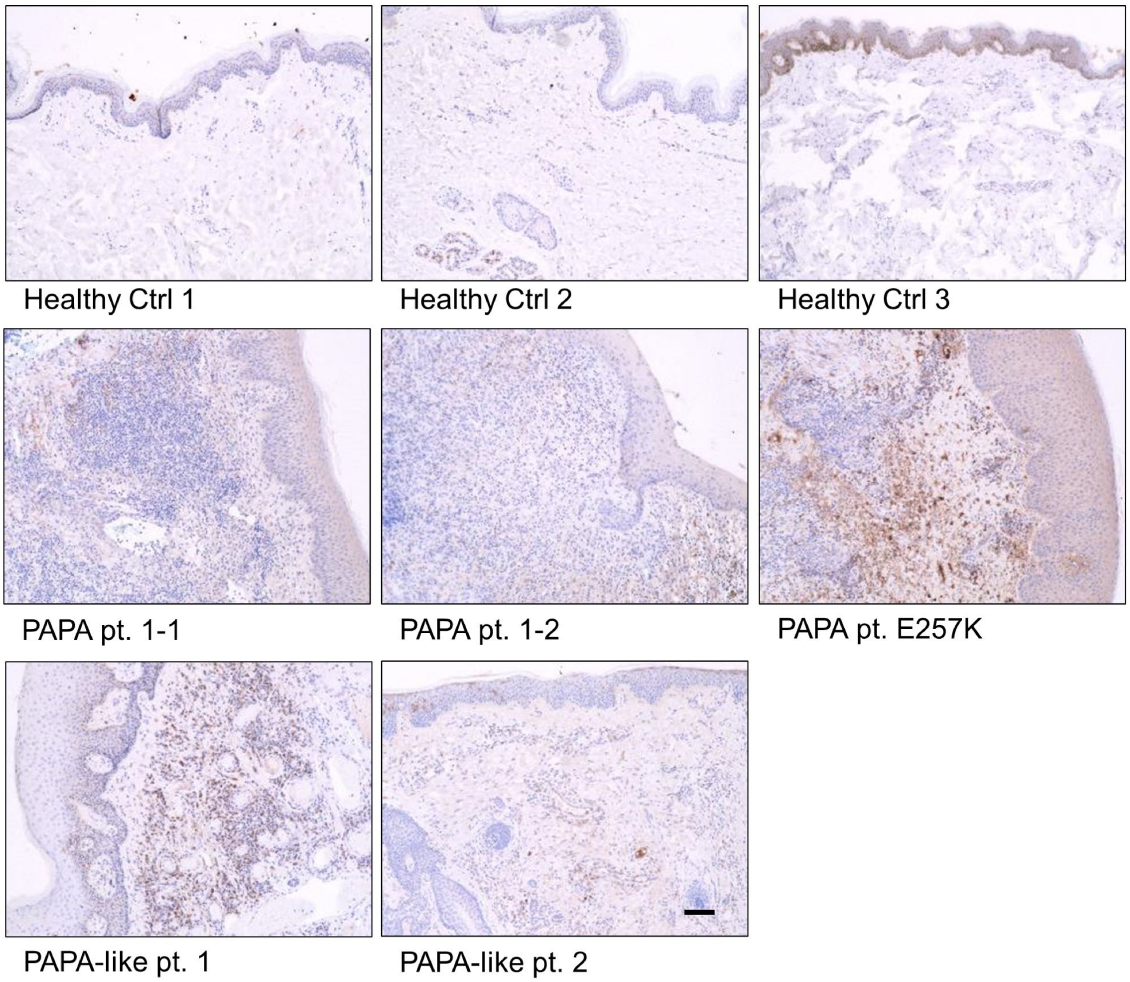
SUPPLEMENTAL FIGURES

Supplemental figure 1. Protein expression levels in retroviral-transduced U937 cell lines.



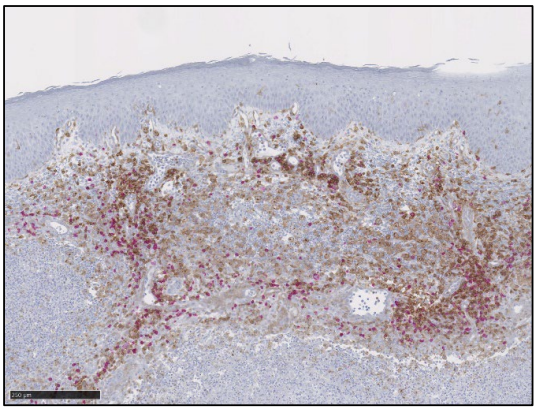
(A) Immunoblot analysis of PSTPIP1 and proIL-1β expression in cell lysates from retroviral-transduced U937 cell lines expressing WT or A230T and E250Q mutant PSTPIP1 proteins without or with secondary mutation, W232A or Y345F. (B) Immunoblot analysis of PSTPIP1 and proIL-1β expression in cell lysates from retroviral-transduced U937 cell lines expressing full length WT or A230T and E250Q mutant PSTPIP1 or SH3 domain-truncated WT or mutant PSTPIP1 proteins.

Supplemental figure 2. Expression of IFN-α2 in inflamed skin of PAPA patients and PAPA-like patients.

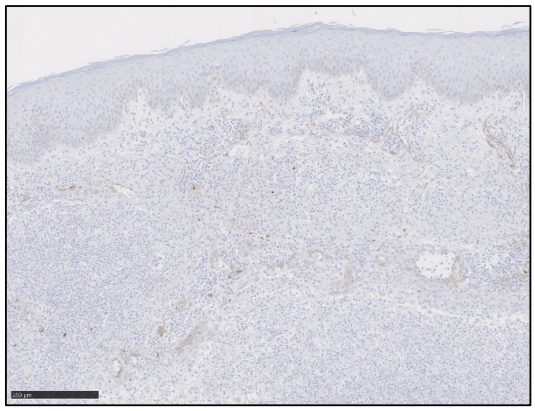


The skin biopsies of normal skin from three healthy donors (Healthy Ctrl 1, 2, and 3), affected skin from two PAPA patients (PAPA pt. 1 at two different points in time, three months apart, marked as 1-1 and 1-2, and a second now-deceased PAPA patient with the E257K *PSTPIP1* mutation who never received treatment with JAK inhibitors), and affected skin from two PAPA-like patients who were PSTPIP1-mutation negative (PAPA-like pt. 1 and 2) were stained with anti-human IFN-α2 antibody (DAB, brown color). Magnification ×10; scale bar, 100 μm; representative images shown.

Supplemental figure 3. Cellular infiltration in the inflamed skin of PAPA patients**.**



PAPA pt. 1 CD4 CD8 double staining



PAPA pt. 1 CD56 staining

The skin biopsies of inflamed skin from PAPA pt. 1 were double stained with anti-human CD4 and CD8 antibodies (stained in brown and red colors, respectively, left image), or stained with anti-CD56 antibody (stained in brown color, right image). Magnification ×10; scale bar, 250 μm; representative images shown.

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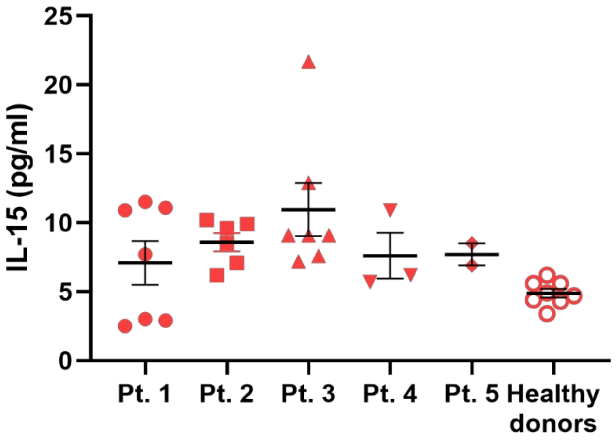
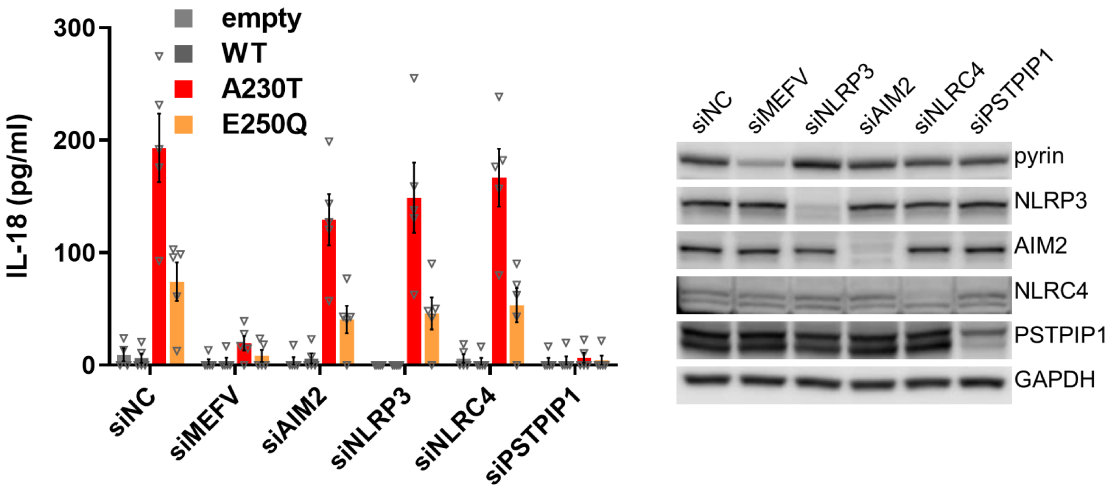
Supplemental figure 4. Elevated IL-15 in the blood of PAPA patients.



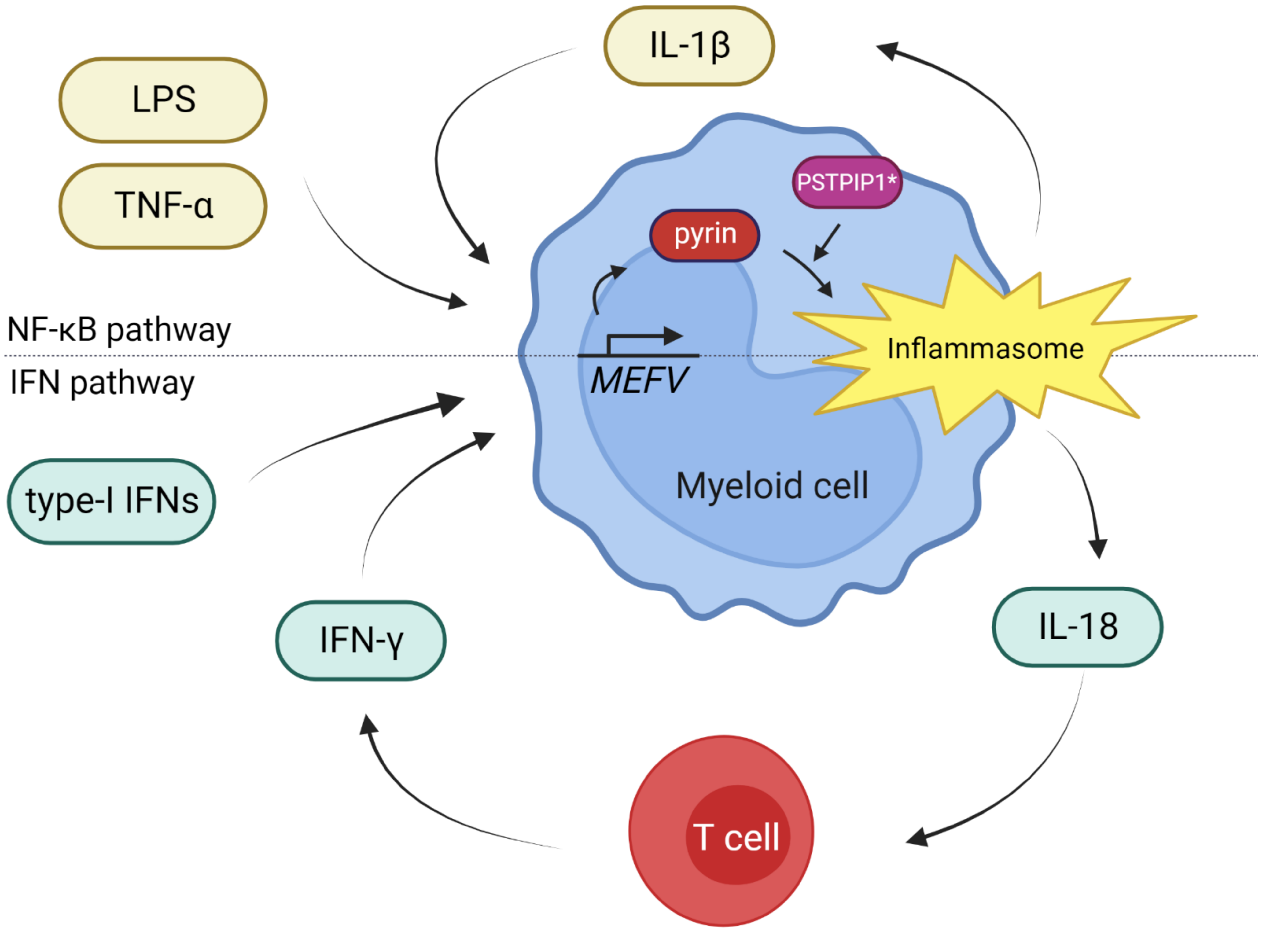
IL-15 was measured in serum and plasma of PAPA patients 1-5 at multiple time points and of healthy donors (N=8) by ELISA.

Supplemental figure 5. The pyrin inflammasome mediates PAPA-associated *PSTPIP1* mutation-induced IL-18 secretion by IFN-γ priming.



IL-18 measurements and immunoblot analysis of cell lysates from stable U937 cells lines expressing WT or PAPA-associated A230T and E250Q mutant PSTPIP1 proteins transiently transfected with negative control siRNA (siNC) or siRNA targeting the genes encoding pyrin, AIM2, NLRP3, NLRC4, and PSTPIP1, then primed with IFN-γ.

Supplemental figure 6. Proposed molecular pathogenesis of PAPA syndrome.



Both NF-κB and IFN signaling pathways induce *MEFV* gene expression. PSTPIP1 with PAPA-associated mutations (asterisk in figure) trigger non-canonical pyrin inflammasome activation in the absence of any additional activation signal. Pyrin inflammasome produces and releases active IL-1β and IL-18. T cells activated with IL-18 produce IFN-γ which further potentiates myeloid cells for more pyrin expression, forming a positive feedback loop in affected lesions. IFN-γ may also contribute to local inflammation. Graphic created with BioRender.com.

REFERENCES

1. Chae JJ, Komarow HD, Cheng J, et al. Targeted disruption of pyrin, the FMF protein, causes heightened sensitivity to endotoxin and a defect in macrophage apoptosis. Mol Cell 2003;11:591-604.
2. Chae JJ, Cho YH, Lee GS, et al. Gain-of-function Pyrin mutations induce NLRP3 protein-independent interleukin-1beta activation and severe autoinflammation in mice. Immunity 2011;34:755-68.
3. Park YH, Wood G, Kastner DL, et al. Pyrin inflammasome activation and RhoA signaling in the autoinflammatory diseases FMF and HIDS. Nat Immunol 2016;17:914-21.
4. Park YH, Remmers EF, Lee W, et al. Ancient familial Mediterranean fever mutations in human pyrin and resistance to Yersinia pestis. Nat Immunol 2020;21:857-67.
5. Reya T, Duncan AW, Ailles L, et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 2003;423:409-14.
6. Dejosez M, Levine SS, Frampton GM, et al. Ronin/Hcf-1 binds to a hyperconserved enhancer element and regulates genes involved in the growth of embryonic stem cells. Genes Dev 2010;24:1479-84.
7. Zheng C, Baum BJ. All human EF1alpha promoters are not equal: markedly affect gene expression in constructs from different sources. Int J Med Sci 2014;11:404-8.
8. Shoham NG, Centola M, Mansfield E, et al. Pyrin binds the PSTPIP1/CD2BP1 protein, defining familial Mediterranean fever and PAPA syndrome as disorders in the same pathway. Proc Natl Acad Sci U S A 2003;100:13501-6.