Extracellular ATP and IL-23 Form a Local Inflammatory Circuit Leading to the Development of a Neutrophil-Dependent Psoriasiform Dermatitis

Julio A. Diaz-Perez¹, Meaghan E. Killeen¹, Yin Yang¹, Cara D. Carey¹, Louis D. Falo Jr.¹,² and Alicia R. Mathers¹,³

Psoriasis is a chronic inflammatory skin disease dependent on the IL-23/IL-17 axis, a potent inflammatory pathway involved in pathogen clearance and autoimmunity. Several triggers have been proposed as initiators for psoriasis, including alarmins such as adenosine triphosphate. However, the role of alarmins in psoriasis pathogenesis and cutaneous inflammation has not been well addressed. Studies show that signaling through the P2X7 receptor (P2X7R) pathway underlies the development of psoriasiform inflammation. In this regard, psoriasiform dermatitis induced by IL-23 is dependent on P2X7R signaling. Furthermore, direct activation of the P2X7R is sufficient to induce a well-characterized psoriasiform dermatitis. Mechanistic studies determined that P2X7R-induced inflammation is largely dependent on the IL-1β/NLRP3 inflammasome pathway and neutrophils. In conclusion, this work provides basic mechanistic insight into local inflammatory circuits induced after purinergic P2X7R signaling that are likely involved in the pathogenesis of many inflammatory diseases, such as psoriasis.


INTRODUCTION
Psoriasis vulgaris is a chronic inflammatory cutaneous disease that affects approximately 2%–3% of the population. Psoriasis is categorized as a psoriasiform dermatitis with histopathological manifestations including epidermal acanthosis, hyperkeratosis, parakeratosis, microabscess formation, vascular expansion, and infiltration of leukocytes into both dermis and epidermis. Several triggers have been proposed as initiator events for psoriasis, including IL-1, IL-6, CAMP/LL-37, TNF-α, and alarmins (Alwan and Nestle, 2015). Alarmins are damage-associated molecular patterns that act as danger signals, inducing innate and adaptive inflammatory responses. After trauma, alarmins are released from damaged, stressed, or necrotic cells. In a genetically predisposed environment, it has been suggested that alarmins could lead to the induction of psoriatic lesions by promoting a positive inflammatory feedback loop (Dumitriu et al., 2005; Gallucci et al., 1999). Supporting this is the Koebner phenomenon, in which lesions frequently develop at sites of trauma where alarmins are locally released. However, the role of alarmins in psoriasis pathogenesis has not been well addressed. In this context, extracellular adenosine triphosphate (ATP) is a particularly interesting alarmin that, via purinergic P2X7 receptor (P2X7R) signaling, induces NF-κB activation and the IL-23/IL-17 axis, both of which have been shown to be psoriasis susceptibility pathways (Atarashi et al., 2008; Di Virgilio et al., 2017; Nair et al., 2009). Moreover, the sympathetic nervous system releases ATP during times of stress, thereby linking stress and the exacerbation of psoriasis (Burnstock, 2009; Stohl et al., 2013). Several key studies also support a role for ATP in cutaneous inflammation. For instance, contact hypersensitivity responses were inhibited in P2X7R−/− mice and potentiated in the presence of ATPγS, an ATP analog (Granstein et al., 2005; Weber et al., 2010). We and others have shown that P2X7R is highly up-regulated in psoriatic lesions in both human and mouse models (Geraghty et al., 2017; Killeen et al., 2013; Pastore et al., 2007). Finally, P2X7R signaling in a human ex vivo skin model provokes the expression of innate cutaneous inflammatory cytokines, DC17 differentiation, and T helper (Th) type 17 responses (Killeen et al., 2013). Thus, we hypothesize that cutaneous P2X7R signaling is an early trigger of psoriasis pathogenesis.

RESULTS AND DISCUSSION
To test our hypothesis, we induced two acute models of psoriasis (rIL-23 and imiquimod [IMQ]) in P2X7R−/− mice. Recombinant murine IL-23 was injected intradermally (i.d.) or IMQ was topically applied daily to C57BL/6 (wild type [WT]) and P2X7R−/− mice. In the acute rIL-23 and IMQ
models, psoriasiform dermatitis develops in WT mice, with marked epidermal thickening, increased inflammatory infiltrates, parakeratosis, and microabscess formation (Figure 1a, and see Supplementary Figure S1 online). In line with our hypothesis, rIL-23 did not promote a psoriasis-like phenotype in P2X7R-/- mice (Figure 1a, and see Supplementary Figure S1). Conversely, in the IMQ model, P2X7R-/- mice have considerable cutaneous inflammation and epidermal thickening (Figure 1a, and see Supplementary Figure S1), consistent with recent findings (Geraghty et al., 2017). Together these data suggest that the P2X7R pathway is necessary in the rIL-23 model to induce a psoriasis-like phenotype but that the P2X7R pathway is dispensable for the IMQ model.

We next sought to determine how IL-23 was stimulating the P2X7R pathway. To accomplish this, we cutaneously injected mice with rIL-23 and using in vivo bioluminescence imaging showed a significant release of ATP in mice injected with rIL-23 compared with vehicle control (Figure 1b). Thus, these data indicate that IL-23 can induce the cutaneous secretion of ATP, leading to the activation of purinergic signaling.

ATP/P2X7R interactions initiate activation of the NLRP3 inflammasome and cleavage of pro-IL-1β to its active form (Di Virgilio et al., 2017). To determine if the level of ATP released after rIL-23 injections was biologically significant, we injected rIL-23 into NLRP3-/- mice. In WT mice, rIL-23 induced a characteristic psoriasiform phenotype. However, in NLRP3-/- mice injected with rIL-23, the psoriasiform phenotype was lost, and there was a significant decrease in epidermal thickness compared with WT mice (Figure 1c). Overall, these data indicate that rIL-23 leads to the secretion of biologically relevant levels of ATP. Moreover, to our knowledge, the contribution of the inflammasome to the development of psoriasis in the rIL-23 model has been previously unreported.

Studies have previously shown that inflammation induced by IMQ is independent of the NLRP3 inflammasome (Rabeony et al., 2015; Walter et al., 2013), which is consistent with our finding that IMQ is capable of inducing inflammation in P2X7R-/- mice (Figure 1a). Furthermore, because the IMQ-dependent inflammatory response is independent of the P2X7R/IL-1β/NLRP3 inflammatory circuit, the IL-1α or IL-36 pathways are likely involved in the induction of the IL-23/IL-17 axis in the IMQ model of psoriasis (Rabeony et al., 2015; Walter et al., 2013). The mechanisms that lead to psoriasis pathogenesis are poorly understood, and likely multiple different pathways lead to the same disease phenotype based on genetic predisposition of the patient. Moreover, mice do not naturally develop psoriasis and because of the major differences between mice and humans, murine models represent only various features of the psoriatic disease. Thus, even though the IMQ model does not signal through the P2X7R pathway, the IMQ model of psoriasis still shares many features of human psoriasis and, when used appropriately, will continue to provide valuable insight into the pathogenesis of psoriasis (Swindell et al., 2017).

To determine if direct P2X7R signaling is sufficient for the development of psoriasis-like lesions, we i.d. injected mice with 2’(3’)-O-(4-benzoylbenzoyl) adenosine 5’-triphosphate (BzATP), an ATP analog and a potent P2X7R agonist. Titration studies were conducted to determine the appropriate dose of BzATP (see Supplementary Figure S2 online). Intradermal injections of BzATP induced the development of parakeratosis and a significant increase in epidermal hyperplasia compared with vehicle control (Figure 2a). However, BzATP alone did not induce a full-fledged psoriasiform dermatitis. BzATP, like ATP, is hydrolyzed by ecto-nucleoside triphosphate diphosphohydrolases into the anti-inflammatory adenosine molecule (Kukley et al., 2004). Thus, in conjunction with BzATP, mice were injected with POM1, an inhibitor of ecto-nucleoside triphosphate diphosphohydrolases. BzATP plus POM1 initiates the development of a full psoriasis-like inflammatory response, characterized by prominent parakeratosis, microabscess formation, and a significant increase in epidermal thickness compared with PBS and BzATP or POM1 alone (Figure 2a). Additionally, in the dermis the level of vascularization was significantly increased in mice treated with BzATP plus POM1, compared with PBS, as determined by histopathological examination and CD31 expression (Figure 2b). The development of cutaneous inflammation was not different between female and male mice (data not shown). Finally, the psoriasiform dermatitis induced after P2X7R signaling is an acute inflammatory response that begins to resolve after 4 days of treatment (data not shown), likely due to the absence of a genetic predisposition. For instance, the genetic predisposition of psoriasis patients to produce excessive IL-1β is supported, in part, by a gain-of-function mutation in the NLRP3 gene (rs10733113) (Carlström et al., 2012). Thus, P2X7R stimulation of this highly active NLRP3 inflammasome in psoriasis patients may lead to pathological levels of IL-1β that cannot be achieved or sustained naturally in mice.

BzATP is a potent P2X7R agonist, but it exhibits partial agonist activity at other purinergic receptors. Thus, to confirm that the observed response occurs through the P2X7R, we used A438079 (A4), a competitive P2X7R antagonist that is inactive with WT mice, inflammation was not induced when mice were treated with BzATP plus POM1 (see Supplementary Figure S3 online). Likewise, when P2X7R-/- mice were treated with BzATP plus POM1, an inflammatory response did not develop, and epidermal thickness was significantly decreased compared with WT mice (Figure 2c). These findings strongly support our hypothesis that signaling directly through the P2X7R can induce the development of a psoriasis-like response.

Studies support a role for an aberrant inflammasome/IL-1β pathway in psoriasis (Carlström et al., 2012). Moreover, the NLRP3 inflammasome/IL-1β pathway is a prototypical downstream pathway of the P2X7R; however, P2X7R signaling can induce both inflammasome-dependent and -independent responses. Therefore, the relationship between the inflammasome/IL-1β pathway and P2X7R signaling was assessed by treating NLRP3-/- mice with BzATP plus POM1. In the NLRP3-/- mice, inflammation was not induced when mice were treated with BzATP plus POM1, compared with WT mice (Figure 2d). Consistent with the histological observations, there was not a significant increase in epidermal thickness in the NLRP3-/- mice compared with WT mice (Figure 2d). Therefore, these data indicate that the psoriasiform response induced after P2X7R signaling is dependent on
the NLRP3 inflammasome pathway. Moreover, these studies are consistent with those in Figure 1, showing that the rIL-23 model of psoriasis was dependent on the NLRP3 inflammasome.

The role of innate cytokines induced after P2X7R signaling was subsequently evaluated in mice treated with BzATP in the presence or absence of POM1. Relevant cutaneous changes were observed in transcript expression at 12 hours and 72 hours after initial treatment. At the 12-hour time point there was a significant increase in IL-1β, IL-1α, and S100A9 in the BzATP plus POM1 treatment group compared with controls (Figure 3a). Additionally, there was a trend for increased TNF-α that did not reach statistical significance. By 72 hours there was a significant increase in IL-6 in the BzATP plus POM1 treatment group compared with the control group (Figure 3b). The significant increase in IL-1β was also sustained in the skin for 72 hours (Figure 3b). We next expanded our studies to assess protein expression. At 72 hours and 120 hours, significant changes in protein expression were detected. Compared with controls, IL-1α, IL-23, and S100A9 were significantly increased at 72 hours, and IL-6 was significantly increased at 120 hours in the BzATP plus POM1 treatment group (Figure 3c). These data are consistent with our ex vivo studies showing the capacity of P2X7R signaling to induce the expression of characteristic psoriasiform cytokines (Killeen et al., 2013).

Psoriasis is dependent on environmental and genetic influences that render the skin susceptible to an imbalance in proinflammatory cytokines, chemokines, and growth factors stimulating aberrant immunity and keratinocyte functions. Whether keratinocytes have a direct role in inducing lesions through the stimulation of an aberrant immune response or if they are targets of an imbalanced immune response is not completely understood. To determine whether P2X7R expression and stimulation is necessary on keratinocytes and/or hematopoietic cells, we generated chimeric mice in which WT bone marrow was transferred into lethally irradiated P2X7R-/- mice compared with nonirradiated WT mice or when WT bone marrow was transferred into WT recipient mice and stimulated with BzATP plus POM1 (Figure 4a). Conversely, when P2X7R-/- bone marrow was transferred
Figure 2. Signaling via P2X7R induces a psoriasis-like inflammatory phenotype in mice dependent on NLRP3 inflammasome. C57BL/6 mice were injected daily for 4 days with BzATP with or without POM1, or PBS (vehicle control). (a) On day 5, skin samples were collected and stained with hematoxylin and eosin to assess histological phenotype. Scale bar = 50 µm. Epidermal thickness was quantitated. Bars are the mean ± standard error of the mean; 10 independent HPF measurements were averaged from each mouse (n = 10 combined from multiple independent experiments). (b) On day 5, skin samples were collected and immunohistochemically stained with CD31 (brown) and counterstained with hematoxylin (blue). The density of CD31 expression was quantitated, and the
Figure 3. Innate cytokines are induced after cutaneous signaling through the P2X7R. (a, b) Bar graphs show the relative fold change in mRNA expression of IL-1β, IL-1α, S100A9, TNF-α, and IL-6 (a) 12 hours (IL-1β, IL-1α, S100A9, and TNF-α) and (b) 72 hours (IL-1β and IL-6) after cutaneous injections with BzATP with or without POM1 normalized to PBS-injected controls. (c) Bar graphs represent the protein concentration of IL-1α and IL-23 at 72 hours and S100A9 and IL-6 at 120 hours. (a–c) Data are expressed as mean ± standard error of the mean (n = 4–10 mice) combined from two independent experiments. Each sample was run in triplicate for reverse transcriptase–PCR and duplicate for Luminex protein samples. Asterisk indicates a significant difference compared with PBS from multiple comparison groups, *P < 0.05 and **P < 0.01. Bz, 2'(3')-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate; BzATP, 2'(3')-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate; P2X7R, P2X7 receptor; PBS, phosphate buffered saline; POM1, sodium polyoxotungstate; WT, wild type.
Figure 4. P2X7R-dependent cutaneous inflammation is dependent on hematopoietic cells. (a) Lethally irradiated WT or P2X7R<sup>−/−</sup> mice were reconstituted with total BM from WT or P2X7R<sup>−/−</sup> mice. Congenic mice were treated with BzATP plus POM1 or PBS. On day 5, skin samples were collected and stained with hematoxylin and eosin. Scale bar = 100 μm. Bar graph represents the mean increase ± standard error of the mean in epidermal thickness; 10 independent high-powered field measurements were averaged from each mouse (n = 6–16 mice combined from four independent experiments). (b) WT mice were injected daily for 4 days with BzATP with or without POM1 or PBS. On day 5, skin sections were collected and immunofluorescently labeled with F4/80 (red), MHC-II (green), and GR-1 (LY6C/LY6G) (white) specific antibodies. Merged panels include all three stains and DAPI nuclear counterstain. Dashed line indicates epidermal-dermal junction. Scale bar = 50 μm. (c) Phenotypic analysis of cutaneous infiltration was performed on day 5 by flow cytometry. Single-cell suspensions were assessed for CD45<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>−</sup>LY6C<sup>+</sup> inflammatory monocytes, CD45<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>−</sup>LY6G<sup>+</sup> neutrophils (top panels),
into WT recipient mice, there was a significant reduction in the development of inflammation and epidermal thickness compared with WT controls (Figure 4a). Overall, these data show that hematopoietic cells expressing P2X7R are essential for the development of psoriasiform dermatitis in mice after stimulation with exogenous P2X7R agonists.

To determine which hematopoietic cells are important for the development of cutaneous inflammation, the inflammatory infiltrate induced after P2X7R signaling was assessed. By day 5, compared with the PBS treatment group, in the BzATP group infiltrates were highly enriched for cells expressing MHC-class II (Figure 4b). Compared with both the PBS and BzATP treatment groups, BzATP plus POM1 induced a marked increase into the papillary dermis and the superficial perivascular areas of F4/80+ (macrophages), MHC class II+ (antigen presenting cells), and LY6C/LY6G+ (granulocytes) (Figure 4b). Cutaneous single-cell suspensions of the infiltrate were prepared and further characterized on day 5 by flow cytometry. Initially, we examined general cellular markers typically present in psoriatic lesions. We did not observe an increase in total CD3+ T cells, γδ T cells, γδ T cells, or CD127+ innate lymphoid cells (see Supplementary Figure S4 online). We detected a decrease in CD11c+ dendritic cells (see Supplementary Figure S4 online), likely due to dendritic cells migrating from the skin to the draining lymph nodes. Finally, there was an increase in LY6C+, CD11b+, and LY6G+ cells (see Supplementary Figure S4). To further delineate and quantify the cutaneous cells increased in the skin, we used a gating strategy previously described (Pommier et al., 2013). It was determined that LY6C<sub>high</sub>CD11b<sub>high</sub>CD11c<sup>+</sup> inflammatory monocyes, LY6G<sub>high</sub>CD11b<sub>high</sub>CD11c<sup>+</sup> neutrophils, and LY6C<sub>high</sub>CD11b<sub>high</sub>CD11c<sup>+</sup> inflammatory dendritic cells were significantly increased after BzATP plus POM1 treatments compared with controls (Figure 4c).

IL-17 is a main effector cytokine in psoriasis that indirectly and directly induces keratinocyte proliferation and hyperplasia along with the secretion of inflammatory cytokines and chemokines, forming a self-amplifying feed-forward response (Hawkes et al., 2017). To ascertain if IL-17A is increased in the skin after P2X7R stimulation, we first determined that the IL-17A mRNA transcript levels are increased after treatment with BzATP plus POM1, compared with controls (Figure 5a). Furthermore, the production of IL-17A was confirmed by flow cytometry; mice treated with BzATP plus POM1 had a significant increase in cutaneous IL-17A—secreting cells compared with PBS alone (Figure 5b, top panels). Studies in murine models of psoriasis have shown that γδ T cells, neutrophils, and innate lymphoid cells are the cells secreting the majority of IL-17A compared with adaptive Th17 cells (Cai et al., 2011; Gladiator and LeibundGut-Landmann, 2013; Mabuchi et al., 2011; Pantelyushin et al., 2012; Sutton et al., 2012). However, in our model we determined that the cells expressing IL-17A were CD45<sup>+</sup>CD3<sup>+</sup>CD127<sup>+</sup>Linage<sup>+</sup> (Figure 5b, bottom panels). In this regard, there was a significant increase in CD45<sup>+</sup>CD3<sup>+</sup>CD127<sup>+</sup>Linage<sup>+</sup> cells after BzATP plus POM1 treatment compared with control (Figure 5b, bottom panels). The markers used identified Th17 cells (CD3<sup>+</sup>γδ T cells (CD3<sup>+</sup>), and innate lymphoid cells (CD127<sup>+</sup>) as producers of IL-17A after P2X7R signaling. Within the lineage population are neutrophils, which have been shown to express IL-17A (Lin et al., 2011; Reich et al., 2015) and are significantly increased in the skin after BzATP plus POM1 injections (Figure 4b and c). To further confirm that neutrophils were expressing IL-17A after BzATP plus POM1 treatments, we used an IL-17A fate-tracking reporter mouse (IL-17<sup>F<sub>Y</sub></sup>FP) (Hiroya et al., 2011; Huppler et al., 2015). For this, IL-17<sup>F<sub>Y</sub></sup>FP mice were treated with BzATP plus POM1, and cutaneous cross-sections were stained with LY6G-specific antibodies. We show that neutrophils in the epidermal microabscess were capable of expressing IL-17A in our model of psoriasiform dermatitis (Figure 5c). Thus, these data indicate that neutrophils are an early infiltrate expressing IL-17A in the skin after P2X7R stimulation, which is consistent with the finding that neutrophils are a predominant cell type expressing IL-17A in psoriasis (Lin et al., 2011; Reich et al., 2015).

Finally, we sought to directly determine if neutrophils are required for the development of P2X7R-dependent inflammation. To accomplish this, mice were treated with a LY6G antibody (Ab) to deplete neutrophils. Consistent with previous data, BzATP plus POM1 induced a psoriasis-like phenotype with a significant increase in epidermal thickness (Figure 5d). Mice treated with BzATP plus POM1 in the presence of a LY6G Ab did not develop a prominent psoriatic phenotype, compared with PBS controls (Figure 5d). Thus, these data indicate that neutrophils are necessary for the development of the psoriasiform dermatitis induced after P2X7R signaling. Likewise, neutrophils have been associated with the pathophysiology of psoriasis, and studies have shown that an increase in neutrophil extracellular traps (NETs) and NEtosis are positively correlated with disease severity (Hu et al., 2016).

Although we detected IL-17A expression only in neutrophils, we are not ruling out the possibility that other cell populations, such as T cells, express IL-17A in psoriatic disease. Our results indicate that innate inflammation induced after P2X7R stimulation is capable of initiating a psoriasiform dermatitis in the absence of T-cell activation. In this regard, psoriasis has recently been described as a bimodal immune response with the initiation of lesions being developed by an innate autoinflammatory response that later develops into an adaptive autoimmune response (Christophers et al., 2014). The response observed after the cutaneous injections of BzATP plus POM1 reflect the innate autoinflammatory phase of psoriasis. Recent studies suggest that IL-17<sup>+</sup> resident memory T cells are important in the adaptive phase of human psoriasis, but resident memory T cells are virtually absent in naive specific pathogen-free laboratory mice, which may explain why P2X7R stimulation does not lead to the chronic adaptive phase observed in human disease (Beura et al., 2016; Clark, 2015).
CONCLUSIONS
The present study shows that ATP signaling through P2X7R is necessary to develop a psoriasis-like inflammatory response induced by IL-23. A finding that has emerged from these studies is that IL-23 leads to the active secretion of biologically relevant levels of ATP necessary for P2X7R activation. Moreover, directly signaling through cutaneous P2X7R leads to the development of a psoriasiform inflammatory response that mirrors the pathophysiologic immune response and histopathologic phenotype of psoriasis in humans. These data also suggest that ATP released after trauma and/or stress can result in the development of a psoriatic Koebner response in a susceptible microenvironment. Finally, this study supports a basic immunology model in which IL-23 and P2X7R signaling form a positive feedback loop that facilitates the development of psoriatic dermatitis (see Supplementary Figure S5 online).

MATERIALS AND METHODS

Mice
C57BL/6 and P2X7R−/− mice were purchased from Jackson Laboratories (Bar Harbor, ME). NLRRP3−/− mice were obtained from Lexicon Genetics Incorporated (The Woodlands, TX). IL-23−/− mice were obtained from Genentech (San Francisco, CA), and IL17ACreRosa26ReYFP (IL-17eYFP) mice were a kind gift from Sarah Gaffen; all were bred and housed in the University of Pittsburgh animal facility. Male and female mice were used between the ages of 6 and 12 weeks. Mice were housed under specific pathogen-free conditions. All mice were treated according to the National Institutes of Health guide for the care and use of laboratory animals, and experiments and protocols were approved by the University of Pittsburgh’s institutional animal care and use committee.

Experimental design
Mice were injected i.d. with 100 μl of either BzATP (350 μmol/L, unless otherwise indicated) (Sigma-Aldrich, St. Louis, MO), or BzATP in combination with POM1 (3.2 mg/kg) (Tocris Bioscience, Bristol, UK), with or without A438079 (A4; 80 μmol/kg; Tocris, Minneapolis, MN) in PBS (vehicle control) at two sites daily on the shaved backs for 4 consecutive days. In some experiments, mice were also intraperitoneally injected every other day with 0.1 mg rat anti-mouse LY6G Ab (clone 1A8; BioXcell, Lebanon, NH) dissolved in 200 μl PBS. Injections of LY6G Ab were initiated on day −1 of BzATP injections. In separate experiments, lesional development was induced in mice by daily i.d. injections of murine rIL-23 (500 ng) (eBiosciences, San Diego, CA), with or without A438079 (A4; 80 μmol/kg; Tocris, Minneapolis, MN) in PBS (vehicle control) at two sites daily on the shaved backs for 4 consecutive days. In some experiments, mice were also intraperitoneally injected every other day with 0.1 mg rat anti-mouse LY6G Ab (clone 1A8; BioXcell, Lebanon, NH) dissolved in 200 μl PBS. Injections of LY6G Ab were initiated on day −1 of BzATP injections. In separate experiments, lesional development was induced in mice by daily i.d. injections of murine rIL-23 (500 ng) (eBiosciences, San Diego, CA) or Miltenyi Biotec, Auburn, CA) in 100 μl of PBS on the shaved backs for 5 days, or 62.5 mg of IMQ (5% Aldara; 3M Pharmaceuticals, St. Paul, MN) was topically administered daily for 5 days.

In vivo detection of ATP
In vivo detection of ATP was performed with bioluminescence imaging as previously described (Di Virgilio et al., 2016; Weber et al., 2010). In brief, mice were injected i.d. daily with rIL-23 or PBS (negative control) on days 1–4. On day 4, 2 × 10⁶ HEK293-pmeLuc cells, a kind gift from Francesco Di Virgilio (Pellegratti et al., 2008) were injected i.d. in 100 μl of DMEM at the same injection site as rIL-23. At 24 hours after cellular injections, mice were treated with a final dose of rIL-23 or PBS. At 4 hours after rIL-23 injections, some mice were treated i.d. with ATP in 100 μl (positive control, 1 mmol/L) followed by intraperitoneal injections of α-luciferin (3mg/mouse) in PBS. Twenty minutes later, mice were imaged with the IVIS 200 Luminometer (Perkin Elmer, Waltham, MA). The acquisition time was 3 minutes/ acquisition, and binning was 4.

Generation of bone marrow-chimeric mice
Mice were gamma-irradiated with two separate exposures to 550 rads of whole body irradiation 6 hours apart for a total of 1,100 rads. Within 24 hours, mice were intravenously reconstituted with 1 × 10⁷ undepleted bone marrow cells. Mice were fed Uniprim chow (Envigo, Madison, WI) for 1 week after irradiation and used in experiments 6–8 weeks after bone marrow reconstitution.

Histology and immunohistochemistry
Cutaneous tissue samples were collected and processed for hematoxylin and eosin staining and immunohistochemistry. For this, tissues were blocked with 3% H2O2 and then with 0.5% bovine serum albumin. Samples were incubated with rat anti-CD31 antibody (ThermoFisher, Grand Island, NY) followed by biotinylated donkey anti-rat IgG secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunoreactivity was detected by incubation with a 3,3′-diaminobenzidine peroxidase substrate kit according to manufacturer’s instructions (Vector Laboratories, Burlingame, CA). Sections were then counterstained with hematoxylin. Quantification of vascularity was based on CD31 staining using ImageJ software (National Institutes of Health, Bethesda, MD).

Immunofluorescence
Cross-sections of mouse back skin were prepared and stained as previously described (Mathers et al., 2009). Sections were immunofluorescently labeled with major histocompatibility complex class II: Alexa488 (BD Biosciences, San Jose, CA), GR-1 (Ly6C/Ly6G): Alexa 647 (BioLegend, San Diego, CA), and F4/80: Biotin (BioLegend) followed by SA-Cy3 (Jackson ImmunoResearch). Nuclei were counterstained with DAPI (Molecular Probes, Eugene, OR).

Tissue cytokines
At indicative time points, skin samples (4 mm) were collected, minced, and placed into Cell Lysate Buffer (RayBiotech, Norcross, GA) supplemented with protease inhibitors. Lysates were diluted 1:2, and cytokine concentrations were measured in duplicate using Luminex technology with the Fluorokine Multianalyte Profiling kit according to manufacturer’s instructions (R&D Systems, Minneapolis, MN). Samples were read on a Bio-Plex 200 system (BioRad, Hercules, CA) using the Bioplex 6.1 software.

Quantitative reverse transcriptase–PCR
Real-time quantitative reverse transcriptase–PCR experiments were conducted using total RNA, which was isolated using TRIzol reagent (Invitrogen, Waltham, MA). For each reverse transcriptase assay, 2 μg RNA was converted to cDNA using RNA to cDNA High Capacity Master Mix (Applied Biosystems, Carlsbad, CA). Gene expression was determined with the following TaqMan assays: IL-6, IL-1α, IL-1β, TNF-α, IL-17A, and S100A9 (Applied Biosystems). Endogenous control was GusB. All cDNA samples were run in triplicate, amplified with the Veriquest PCR Master Mix (Affymetrix, Santa Clara, CA), and analyzed using the real-time StepOnePlus sequence detection system (Applied Biosystems). Relative fold changes were calculated based on the 2−ΔΔCt method.

Flow cytometry
Skin biopsy samples (10 mm) were collected, minced, and then enzymatically digested in 1 mg/ml Collagenase D (Roche, Indianapolis, IN), 1 mg/ml DNase (Roche), 10 mg/ml hyaluronidase (Sigma-Aldrich), and 0.1% BSA in IMDM (ThermoFisher) for 45 minutes at
Figure 5. Acute inflammation induced after P2X7R stimulation is dependent on neutrophils. (a) Bar graph shows the relative fold change in mRNA expression of IL-17 72 hours after cutaneous injections with BzATP plus POM1. Data are expressed as mean ± standard error of the mean (n = 17–21 mice). The P-value is indicated. (b) C57BL/6 mice were injected with BzATP plus POM1 or PBS. Skin was collected on day 5, and single-cell suspensions were stained with CD45-, IL-17-CD3-, and Lineage (LIN)-specific Abs and viability dye, then analyzed by flow cytometry. The top left panels were gated on live CD45+ cells, and the top right graph represents the mean ± standard error of the mean (n = 3–5 mice) of the percentage of CD3+IL-17+ cells. The bottom left panels were further gated on the CD3+IL-17+ cells (light blue and dark blue boxes). The bottom right graph represents the mean ± standard error of the mean (n = 3–5 mice) of the percentage of the LIN+CD127+ cells. One representative of two independent experiments. (c) IL-17eYFP (green) mice were injected with BzATP plus POM1, and on day 5 cutaneous sections were collected and immunofluorescently labeled with LY6G (red). Dashed line indicates epidermal-dermal junction. Scale bar = 50 μm. (d) BzATP plus POM1 or PBS was injected in the presence or absence of LY6G-specific antibodies. On day 5, skin samples were collected and stained with hematoxylin and eosin. Scale bar = 100 μm. Epidermal thickness was quantitated. Bars represent mean ± standard error of the mean (n = 5 mice); 10 independent high-powered field measurements were averaged from each mouse. One representative of two independent experiments. Asterisks indicate a significant difference compared with PBS treatment unless otherwise indicated. *P < 0.05, **P < 0.01, ***P < 0.001. Ab, antibody; BzATP, 2′(3′)-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate; KO, knockout; MHC, major histocompatibility complex; P2X7R, P2X7 receptor; PBS, phosphate buffered saline; POM1, sodium polyoxotungstate; WT, wild type.
37 °C. Next, 10 mmol/L EDTA was added for an additional 5 minutes at room temperature. To make a single-celled suspension, samples were passed over a cell strainer (Corning, Corning, NY). Cells were blocked with Fc block (CD16/CD32) (BD Biosciences) and 10% donkey serum, then stained with antibodies listed in the Supplemental Methods online. The lineage stain includes CD3/GR-1/CD11b/B220/ Ter-119 antibodies. Populations were initially gated on live cells in the forward versus side scatter and by CD45 versus eFluor 780 viability dye (eBioscience). Gates were then set based on negative controls, single-staining, and fluorescence minus one controls.

Statistics
Multiple groups were compared using a one-way analysis of variance followed by Newman-Keuls multiple comparison post hoc test, or a two-way analysis of variance was used followed by a Bonferroni post hoc test if two affecting factors were present. Comparison of two means was performed by a two-tailed Student t test. A P value less than 0.05 was considered statistically significant.

ACKNOWLEDGMENTS
We thank Tina Sumpter and Daniel Kaplan for critical reading of the manuscript. Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under award number R01AR067746 (to ARM.). This project used the UPCI Cancer Biomarkers Facility; LumineX Core Laboratory that is supported in part by award P30CA047904 and the Hillman In Vivo Imaging Facility that is supported in part by award P30CA047904. The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

ORCID
Alicia Mathers: http://orcid.org/0000-0001-6931-8605

CONFLICT OF INTEREST
LDF has financial interest in SkinJect and Brainstage. The other authors state no conflict of interest.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.05.018.

REFERENCES


