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J Immunol published online 11 March 2013
http://www.jimmunol.org/content/early/2013/03/10/jimmunol.1202045

Supplementary Material http://www.jimmunol.org/content/suppl/2013/03/11/jimmunol.1202045.DC1

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Signaling through Purinergic Receptors for ATP Induces Human Cutaneous Innate and Adaptive Th17 Responses: Implications in the Pathogenesis of Psoriasis

Meaghan E. Killeen,* Laura Ferris,* Erine A. Kupetsky,* Louis Falo, Jr.,* and Alicia R. Mathers**†

Human cutaneous dendritic cells (DCs) have the ability to prime and bias Th17 lymphocytes. However, the factors that stimulate cutaneous DCs to induce Th17 responses are not well known. Alarmins, such as ATP, likely play a pivotal role in the induction and maintenance of cutaneous immune responses by stimulating DC maturation, chemotaxis, and secretion of IL-1β and IL-6, Th17-biasing cytokines. In this study, using a well-established human skin model, we have demonstrated that signaling purinergic receptors, predominantly the P2X7 receptor (P2X7R), via an ATP analog initiate innate proinflammatory inflammation, DC17 differentiation, and the subsequent induction of Th17-biased immunity. Moreover, our results suggest a potential role for P2X7R signaling in the initiation of psoriasis pathogenesis, a Th17-dependent autoimmune disease. In support of this, we observed the increased presence of P2X7R in nonlesional and lesional psoriatic skin compared with normal healthy tissues. Interestingly, there was also a P2X7R variant that was highly expressed in lesional psoriatic skin compared with nonlesional psoriatic and normal healthy skin. Furthermore, we demonstrated that psoriatic responses could be initiated via P2X7R signaling in nonlesional skin following treatment with a P2X7R agonist. Mechanistic studies revealed a P2X7R-dependent mir-21 angiogenesis pathway that leads to the expression of vascular endothelial growth factor and IL-6 and that may be involved in the development of psoriatic lesions. In conclusion, we have established that purinergic signaling in the skin induces innate inflammation, leading to the differentiation of human Th17 responses, which have implications in the pathogenesis and potential treatment of psoriasis.

The skin is a very immunogenically active organ capable of triggering inflammation and potent T cell responses by appropriately responding to antigenic stimuli. The immunogenicity of skin correlates with a substantial number of resident DCs, including epidermal Langerhans cells (LCs) and dermal DCs (DDCs), which are both capable of activating naive T cells and biasing Th1 and Th17 immune responses (2–4).

In addition to cellular elements, the epidermis and dermis contain a vast network of regulatory cytokines, neuropeptides, and other endogenous factors with either proinflammatory or tolerogenic activities that contribute to the initiation and control of cutaneous inflammatory immune responses. One particular family of endogenous factors is the damage-associated molecular patterns, which signal cellular damage. Following trauma, endogenous damage-associated molecular patterns, termed alarmins, are released from damaged, stressed, or necrotic cells and initiate proinflammatory responses. Characteristics of alarmins are that they: 1) are released during nonprogrammed cell death; 2) are secreted by immune cells; 3) recruit and activate innate immune cells, including DCs; and 4) have regulatory capacities enabling them to promote tissue homeostasis and wound healing (5). However, when regulatory responses fail in autoimmune and inflammatory diseases such as psoriasis, rheumatoid arthritis, and systemic lupus erythematosus, alarmins also act as indicators of inflammation and disease severity (6). Thus, it is likely that alarmins also play a pivotal role in the induction and pathogenesis of such diseases by positive-feedback inflammatory mechanisms that break immunological self-tolerance and perpetuate the immune response (7). However, little is known regarding the mechanisms employed by alarmins in perpetuating inflammatory diseases, including the early progression of cutaneous psoriasis, a common accessible model.
of inflammatory diseases in which Th17 and Th1 cells are effectors.

ATP is a particularly interesting alarmin that via purinergic signaling induces DC maturation, chemotaxis, and secretion of IL-1β and IL-6, cytokines involved in the induction of Th17 immunity (8–14). Furthermore, ATP has been described as an adjuvant that induces an alternative DC maturation phenotype characterized by inhibition of IL-12 and stimulation of IL-23 production, which enhances and terminally differentiates Th17 responses (15, 16). In support of these findings, Atarashi et al. (17) demonstrated in mice that ATP secreted by mucosal commensal bacteria bias lamina propria Th17 responses by stimulating CD70(high)CD11c(low) colonic DCs. Additionally, several key studies have indicated that ATP plays a role in cutaneous inflammation and wound healing (18–22). For instance, Weber et al. (22) demonstrated that contact hypersensitivity responses dependent on IL-1β were inhibited in P2X7 receptor (P2X7R)−/− mice. Furthermore, P2X7R is functionally expressed on cutaneous keratinocytes and LCs (14, 19, 23, 24). Thus, we hypothesize that ATP signaling via P2X7R is functionally expressed on cutaneous keratinocytes and adaptive Th17 inflammatory responses in humans.

To explore our hypothesis, we began by treating human cutaneous explants and DCs with 2′(3′)-O-(4-benzoylbenzoyl) ATP (BzATP), an ATP analog and P2X7R agonist. In this regard, we determined that purinergic signaling provokes innate cutaneous inflammatory responses, DC17 differentiation, and Th17 responses. Moreover, our results further define a potential role for ATP in the induction of psoriasis by demonstrating the substantial presence of P2X7R in psoriatic skin. In conclusion, our results demonstrate that cutaneous inflammatory responses induced via purinergic signaling, predominantly P2X7R, have implications in the pathogenesis and potential treatment of inflammatory diseases, such as psoriasis.

Materials and Methods

Human tissues

Normal human skin was procured from healthy donors undergoing breast reduction or abdominoplasty at the University of Pittsburgh Medical Center, or tissues were procured from the National Disease Research Interchange (NDRI) or the Cooperative Human Tissue Network. Psoriatic lesional and nonlesional skin samples were obtained with informed consent through the University of Pittsburgh Medical Center Dermatology clinic or NDRI. Human peripheral blood samples (leukopacks) from healthy volunteers were acquired from the Central Blood Bank. All human samples were procured in accordance with Declaration of Helsinki protocols and University of Pittsburgh Institutional Review Board approval.

Isolation and culture of skin migratory DCs

Skin migratory DCs (smiDCs) were collected after migration from human skin explants as previously described (3, 25, 26). Briefly, a cutaneous mini knife (Integra-Padgett, Plainsboro, NJ) was used to prepare explants containing smiDCs. For these experiments, smiDCs were cultured at a concentration of 105–106 cells/well in 96-well round-bottom plates containing serum-free AIM V media supplemented with PBS, BzATP (350 μM), and/or KN-62 (1 μM) for 3 h. Cells were washed thoroughly and resuspended in AIM V media. Naive CD45RA+CD4+ T cells were then added to the cultures at a concentration of 1 × 106 cells/well. In some experiments, IL-1β or IL-23 Abs (R&D Systems) were added to the cultures. Depending upon the experimental protocol, cultures were incubated for 1–5 d. Cellular proliferation was measured in MLCs using the commercially available Quick Cell Proliferation Assay kit (BioVision, Mountain View, CA) according to the manufacturer’s instructions.

Flow cytometric analysis

The smiDCs and responder CD4+ T cells were stained by blocking with 10% normal donkey serum and then incubating cells with combinations of Abs against HLA-DR (BioLegend, San Diego, CA), langerin (Dendritics, Lyon, France), P2X7R (internal epitope; LifeSpan Biosciences, Seattle, WA), receptor for advanced glycation end products (RAGE; R&D Systems), CD86 (BioLegend), IL-23R (R&D Systems), CD25 (BD Biosciences, San Diego, CA), and CD4 (BD Biosciences). Internal P2X7R staining was followed by anti-rabbit–PerCP Ab (Jackson ImmunoResearch Laboratories, West Grove, PA), and biotinylated RAGE staining was followed by streptavidin–PerCP-Cy5.5 (BioLegend). Cells were fixed in 2% paraformaldehyde and analyzed using an LSR II flow cytometer (BD Immunocytometry Systems, San Jose, CA).

Immunofluorescent microscopy

Cross-sections of skin were prepared and immunofluorescently labeled as previously described (3). Briefly, biopsies and explants were embedded in Shandon Cryomatrix medium (Thermo Scientific, Waltham, MA) and snap frozen in prechilled methyl-butane (Sigma-Aldrich). The 8-μm cross-sections were cut using a cryostat, mounted onto slides pretreated with Vectabond (Vector Laboratories, Burlingame, CA), air-dried, fixed in 96% ethanol, and immunofluorescently labeled with combinations of Abs against P2X7R (internal epitope [LS-A9585], LifeSpan Biosciences), HLA-DR (BioLegend), DC-LAMP (R&D Systems), CD206 (BioLegend), inducible NO synthase (iNOS; R&D Systems), langerin (Dendritics), CD163 (BioLegend), and IL-23R (R&D Systems). Primary P2X7R Ab was followed by anti-rabbit–Cy3 and DC-LAMP, CD206, and iNOS primary Abs were followed by anti-mouse–Cy3 (secondary Abs were purchased from Jackson ImmunoResearch Laboratories, West Grove, PA), and biotinylated IL-23R was followed by streptavidin–PerCP-Cy5.5 (BioLegend). Cells were fixed in 2% paraformaldehyde and analyzed using an LSM II flow cytometer (BD Immunocytometry Systems, San Jose, CA).

Cutaneous injections

Twelve-millimeter punch biopsies prepared from epidermal–dermal explants and psoriatic biopsies were injected intradermally with 100 μl PBS, BzATP (350 μM), KN-62 (1 μM), mir-21 antagomirs ([Atg] 20 μM; Exiqon; Woburn, MA), and/or a combination of 10 μg/ml IL-1β and IL-6κR Abs (R&D Systems, Minneapolis, MN). Mice with psoriatic skin were procured in accordance with Declaration of Helsinki protocols and University of Pittsburgh Institutional Review Board approval. Wisconsin State University Institutional Review Board approval.
growth factor (VEGF; R&D Systems) ELISA kits following the manufacturer’s instructions. All ELISA plates were analyzed using SpectraMax 340PC, 384 plate reader (Molecular Devices). Assays were performed in duplicates, and the results are expressed as mean concentration ± SD.

Real-time qRT-PCR

Real-time qRT-PCR experiments were conducted using either total RNA or microRNA. Total RNA was extracted from cells and tissues using an RNeasy Plus Micro kit (Qiagen) according to the manufacturer’s instructions and quantified using the Qubit RNA Assay Kit (Invitrogen). For each RT assay, 10–100 ng RNA was converted to cDNA using the QuantiTect reverse transcription kit (Qiagen) with a modified protocol. Each 15 μl reaction consisted of 10 ng microRNA in 5 μl H2O, 1.5 μl reverse transcriptase, 1.5 μl 10× RT buffer, 0.15 μl 2′-deoxynucleoside 5′-triphosphate, and 0.2 μl RNase inhibitor. To this reaction mix, 13.3 μl 1:1 mix of the seven microRNA-specific primers were added. All cDNA samples were amplified using TaqMan Gene Expression Master mix (Applied Biosystems) and analyzed using the real-time Step One Plus sequence detection system (Applied Biosystems). In some instances, preamplification of small cDNA quantities was necessary and performed using the TaqMan PreAmp Master Mix (Applied Biosystems). Relative fold changes of RNA expression were calculated and normalized based on the 2^{-ΔΔCt} method.

FIGURE 1. Specific signaling through the P2X7R induces innate Th17-biasing cytokines. Bar graphs demonstrate the relative fold change in mRNA expression of IL-1β and IL-6 24 h following cutaneous injections with 350 μM of BzATP, 1 μM KN-62 (P2X7R antagonist), BzATP + KN-62 (control), or PBS (control; gray bar). Fold change was determined by relative qRT-PCR using the 2^{-ΔΔCt} method in which samples were normalized to GAPDH followed by PBS controls. Data expressed as mean ± SD of triplicates. Representative of three independent experiments. Asterisk indicates a significant difference compared with indicated treatment group: *p < 0.05.

FIGURE 2. Cutaneous proinflammatory innate immune responses induced ex vivo following signaling through the P2X7R. (A) Bar graphs demonstrate the relative fold change in mRNA expression of CXCL10, TNFR1, S100A7, CCL20, TNF-α, VEGF, NOS2, and TRAIL 24 and 72 h following cutaneous injections with 350 μM of BzATP ± 1 μM KN-62 normalized to 24 h PBS-injected controls (No Tx). (B) Examination of VEGF, S100A7, and NOS2 mRNA fold change, normalized to PBS control (No Tx), 72 h following treatment with BzATP ± IL-1β and IL-6Rα Abs. Fold change was determined using the relative qRT-PCR 2^{-ΔΔCt} method. Data expressed as mean ± SD of triplicates. Representative of three independent experiments. Asterisk indicates a significant difference compared with no Tx for each time point, unless otherwise indicated: *p < 0.05.
Western blot analysis

Psoriasis and normal cutaneous biopsies were minced and placed into prechilled RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, PMSF 10 mM, and 1× protease inhibitor mixture [Sigma-Aldrich]). Samples were then homogenized and clarified by centrifugation at 10,000 × g for 10 min. Lysates were mixed with Laemmli buffer (20% glycerol, 10% 2-ME, 5% SDS, 0.2 M Tris-HCl [pH 6.8], and 0.4% bromphenol blue) and boiled for 5 min. A total of 40 µg protein/lane was separated on a 12% SDS-PAGE gel followed by transfer to a polyvinylidene difluoride membrane (Bio-Rad), after which membranes were blocked with 5% nonfat dry milk in PBS/0.1% Tween-20 solution. Proteins were detected by overnight incubation with anti-P2X7R Ab (extracellular epitope [ab77413]; Abcam, Cambridge, MA) followed by anti-rabbit HRP-conjugated secondary Ab (Sigma-Aldrich) for 1 h at room temperature. Ab specificity was confirmed on a separate gel with a P2X7R peptide (Abcam), and blocking was performed according to the manufacturer’s instructions. Chemiluminescence reactions were carried out with ECL substrate (GE Healthcare, Piscataway, NJ) and exposed to film according to the manufacturer’s directions.

Results

Signaling via P2X7R stimulates innate cutaneous inflammation

To test our hypothesis that P2X7R signaling potentiates innate and adaptive Th17 immune responses in the skin, we used a well-established and physiological model of human skin epidermal–dermal explants, which allows for culturing human skin explants and for the isolation of a high number of smiDCs mobilizing from the skin to the skin draining lymph nodes via lymphatic vessels (25, 27). Our initial ex vivo experiments in which we injected cutaneous explants with BzATP confirmed human in vitro and murine results (8, 9, 12, 13, 17) by demonstrating a significant

FIGURE 3. Increased expression of inflammatory markers on cutaneous DCs following ex vivo stimulation with BzATP. Skin explants were injected with 350 µM of BzATP or PBS (No Tx). At 24 and 72 h following injections, skin sections were immunofluorescently labeled with langerin, HLA-DR, and DC-LAMP (A), CD206 (D), or iNOS (G). Dotted line represents epidermal–dermal junction, and ♦ indicates representative langerin+ cells that are expressing DC-LAMP, CD206, or iNOS. Merge panels include all three stains and DAPI nuclear counterstain. (B, C, F, and H) Quantitation of cells positive for HLA-DR, DC-LAMP, CD206, or iNOS. Bars indicate the mean ± SD of 11–15 replicates. (E) Characterization of CD206+ cells 72 h following BzATP treatment. CD206, red; CD163, green. White dots indicate representative DCs (CD206+CD163−), and ♦ indicates representative macrophages (CD206+CD163+). Original magnification ×40. Scale bars, 20 µm. Asterisks in bar graphs indicate a significant difference: *p < 0.05.
increase in the innate IL-1\(\beta\) and IL-6 transcripts in the skin 24 h following P2X7R stimulation compared with nontreated controls (Fig. 1). By 72 h following injections, IL-1\(\beta\) and IL-6 return to baseline levels (data not shown). To confirm that BzATP responses were occurring through P2X7R, we treated cutaneous explants with KN-62 (a specific P2X7R antagonist) or KN-62 plus BzATP and did not observe a significant difference compared with nontreated controls (Fig. 1). The study of P2X7R-dependent release of IL-1\(\beta\) has heavily focused on the ability of P2X7R signaling to induce the activation and assembly of the NALP3 inflammasome (28, 29). However, in this study, we have also determined that P2X7R signaling induces the increased expression of IL-1\(\beta\) mRNA in human skin.

We next expanded our studies to assess the expression levels of innate factors involved in cutaneous inflammation and autoimmune disorders. CXCL10 (Th1 chemokine) and TNFR1 (TNF-\(\alpha\) receptor) were significantly increased in cutaneous explants 24 h following BzATP injections compared with PBS-injected controls (Fig. 2A). Furthermore, 72 h following BzATP treatments, CXCL10 and TNFR1 mRNA levels remained significantly elevated, whereas S100A7, CCL20 (Th17 chemokine), TNF-\(\alpha\), VEGF, NOS2, and TRAIL were significantly upregulated (Fig. 2A). Additionally, the presence of KN-62 demonstrated a significant reduction in the BzATP-dependent increase in all innate factors examined in this study. To determine if the responses observed

**FIGURE 4.** Direct stimulation of human smiDCs with P2X7R agonist. smiDCs were cultured in the presence of the indicated treatments for 24 h and analyzed by ELISA (A) for the detection of secreted IL-1\(\beta\) and IL-6 in the culture supernatants and qRT-PCR (B) for the fold change of IL-23 and VEGF expression. Data are representative of three to eight independent experiments; bars represent the means ± SD from triplicates. Asterisks indicate a significant difference between indicated groups: *\(p < 0.05\). (C) smiDCs were stained with HLA-DR-, CD86-, IL-23R-, and langerin-Abs. Top panels are CD86 and HLA-DR expression; numbers represent percent positive and MFI within the gated region. Bottom panels signify langerin and IL-23R expression; numbers represent the percent positive within the respective quadrant. Flow cytometry data are one representative of four independent subjects with similar results. (D) Cutaneous explants were injected with 350 \(\mu\)M of BzATP and immunofluorescently labeled with IL-23R, langerin, and HLA-DR 72 h following treatment. Dashed line indicates epidermal–dermal junction. Original magnification ×40. Scale bar, 20 \(\mu\)m. Data are one representative of two independent experiments.
at 72 h were a direct result of P2X7R signaling or a secondary effect of early IL-1β and IL-6 secretion, we injected cutaneous explants with BzATP ± IL-1β- and IL-6Rα-neutralizing Abs. VEGF, S100A7, and NOS2 mRNA expression levels were significantly decreased in the Ab-treated group compared with BzATP treatment alone (Fig. 2B). Overall, these results demonstrate, for the first time, to our knowledge, that specifically signaling through the cutaneous P2X7R induces potent innate inflammatory responses in human skin directly and as a secondary response to IL-1β and IL-6.

**BzATP increases cutaneous DC maturation and inflammation**

We next examined the capability of extracellular ATP to activate cutaneous inflammatory DCs ex vivo by injecting explants with BzATP or PBS (No Tx control) and characterizing the DC populations by four-color immunofluorescence. At 24 h following BzATP injections, there was a significant increase in HLA-DR+ cells that was maintained at 72 h (Fig. 3A, 3B). Furthermore, 24 and 72 h following BzATP injections, there was a significant increase in the expression of DC-LAMP, a DC maturation marker highly expressed in inflammatory tissues (30, 31), on both epidermal and dermal DCs (Fig. 3A, 3C). CD206, a mannose receptor expressed by cutaneous DCs during severe inflammation (32), is also significantly increased by dermal cells, some of which are HLA-DR+ (Fig. 3D, 3F). Characterization of the CD206+ cells identified an abundance of two CD206+ populations, the CD206+CD163+ macrophages and CD206+CD163– DCs (Fig. 3E), both of which are increased in cutaneous inflammatory disorders (31–33). iNOS, a mediator of cutaneous inflammation secreted by DCs (34), was found to be significantly increased predominantly on dermal HLA-DR+ DCs following BzATP treatments (Fig. 3G, 3H). Further assessment found dermal populations of DC-LAMP+, CD206+, and iNOS+ cells that correspondingly express langerin and HLA-DR (Fig. 3A, 3D, 3G), which are likely inflammatory LCs migrating into the dermis. Of note, langerin has not been described on dermal DCs in human skin as in murine skin. These findings indicate that BzATP signaling stimulates cutaneous DCs to mature and differentiate into highly inflammatory DCs.

**Signaling directly through the P2X7R on smiDCs induces DC maturation**

Based on the above findings, it could not be discerned whether the maturation and differentiation of DCs is through direct DC signaling, stimulation of precursor DCs, or solely through keratinocyte stimulation. Thus, the next set of experiments was performed to address this question. Previous studies have confirmed in mice and humans that cutaneous LCs and keratinocytes express functional P2X7R (23, 24). Additionally, we determined that ~20% of total smiDCs and 33% of LCs maintained their P2X7R expression following migration, indicating that activated cutaneous DCs have the capacity to directly respond to P2X7R stimulation and that smiDCs can be used in P2X7R mechanistic studies (Supplemental Fig. 2).

We next examined the ability of P2X7R agonist to further increase smiDC maturation and terminal differentiation. To determine the optimal in vitro BzATP concentrations, we performed a titration experiment and examined IL-1β and IL-6 secretion and DC viability. Following treatment with 350 μM BzATP, IL-1β and IL-6 secretion was significantly increased by smiDCs and peaked at 3500 μM. IL-6 levels were also significantly increased by treatment with 350 μM BzATP and began to decrease at 3500 μM. Likewise, cellular viability began decreasing at the highest dose of 3500 μM BzATP (Fig. 4A, Supplemental Fig. 1). Thus, the 350 μM concentration is used throughout these experiments as the concentration that induces a maximal response in DCs without inducing cellular death. Additionally, smiDCs cultured in the presence of BzATP had significantly increased IL-23 and VEGF mRNA expression levels compared with smiDCs cultured in media alone (Fig. 4B). Next we determined that signaling was occurring through the P2X7R by culturing smiDCs in the presence of BzATP and/or KN-62. In the presence of KN-62, there was a significant decrease in the BzATP-dependent IL-1β, IL-6, IL-23, and VEGF levels compared with BzATP treatments alone, confirming that P2X7R was directly stimulated on smiDCs via BzATP (Fig. 4A, 4B, Supplemental Fig. 1D). Furthermore, in some cases KN-62 alone was able to suppress IL-1β and IL-6 below control levels indicating that endogenous ATP is present to some extent in DC cultures (Supplemental Fig. 1D).

**FIGURE 5.** Activation of smiDCs via P2X7R stimulates increased HMGB1 secretion and RAGE expression. smiDCs were stimulated overnight with BzATP and/or KN-62. (A) Following overnight culture, supernatants were collected and assessed for the presence of HMGB1 by ELISA. Data expressed as mean ± SD of replicates. Data are representative of four independent experiments. Asterisk indicates a significant difference between designated groups: *p < 0.05. (B) smiDCs were collected and examined by flow cytometry for the surface expression of RAGE, an HMGB1 receptor (filled histogram represents control Ab; dashed lined represents No Tx; gray line represents KN-62; and black line represents BzATP treatments). (C) RAGE expression on langerin+ cells; numbers represent the percent positive within the respective quadrant. Flow cytometry data are one representative of four independent experiments with similar results.
Using flow cytometry, we determined the maturation status of BzATP-stimulated smiDCs by assessing the expression levels of CD86 and HLA-DR. Following treatment with BzATP there was an increase in the mean fluorescence intensity (MFI) of CD86 and HLA-DR expression on the already highly activated migratory DCs (Fig. 4C). Furthermore, the percentage and MFI of CD86 and HLA-DR were decreased on smiDCs treated with KN-62 (Fig. 4C).

DC-secreted IL-23 is a key cytokine associated with the development of cutaneous inflammation and autoimmune diseases, through its terminal differentiation of Th17 responses, induction of IL-22, and perhaps via its adjuvant activity that directly stimulates DCs to present self-peptides (16, 35–38). Furthermore, IL-23R has been described to be upregulated on DCs present in psoriatic lesions (36). In this study, we determined that the expression of IL-23R on smiDCs was increased following BzATP treatment compared with PBS-treated controls and that the predominant population expressing IL-23R was langerin+ smiDCs (Fig. 4C, bottom panels). Importantly, the presence of KN-62 in smiDC cultures led to the downregulation of IL-23R (Fig. 4C, bottom panels). Consistent with these results, we observed IL-23R expression on LCs that have begun to migrate from the epidermis 72 h following cutaneous BzATP injections (Fig. 4D, arrows). However, in the skin several DDCs were also expressing IL-23R (Fig. 4D, asterisks), indicating that IL-23R+ DDCs are maintained in the skin following P2X7R stimulation or IL-23R is downregulated upon DDC migration. Overall, these experiments demonstrate that directly signaling through the P2X7R on cutaneous smiDCs is capable of further inducing maturation and differentiation of smiDCs.

**P2X7R signaling induces the release of HMGB1**

We next wanted to determine if P2X7R signaling of smiDCs could stimulate the release of other inflammatory alarmins, such as HMGB1.
For this, smiDCs were stimulated overnight in the presence or absence of BzATP and/or KN-62. Assessment of smiDC culture supernatants by ELISA demonstrated a significant increase in the amount of HMGB1 released following P2X7R stimulation compared with nontreated controls (Fig. 5A). Furthermore, KN-62 had the capacity to significantly inhibit the BzATP-induced response (Fig. 5A). The HMGB1 receptor, RAGE, is also increased on smiDCs following P2X7R signaling and decreased following KN-62 treatment (Fig. 5B, 5C). Moreover, RAGE is increased on langerin+ LCs in the presence of BzATP and decreased by KN-62 (Fig. 5C). Importantly, these data indicate that a potential role of ATP is to further trigger other alarmins, such as HMGB1, in an autocrine/paracrine loop, which induces and perpetuates inflammatory responses.

BzATP-differentiated smiDCs bias Th17 responses

Alloreactive CD4+ T cells stimulated with smiDCs proliferate and secrete significantly high levels of IFN-γ and IL-17 (3, 4). In the present work, we examined the ability of P2X7R signaling of smiDCs to further potentiate Th17 responses. Because CD3/CD28 activated CD4+ T cells secrete IL-17 when directly stimulated by BzATP (Supplemental Fig. 3), smiDCs were cultured with treatments for 3 h and washed well prior to being added to MLCs. In data not shown, we assessed the residual level of BzATP in smiDC cultures after 3 h of treatment and found that BzATP concentrations had returned to baseline levels. Following a 5-d incubation, the MLCs were significantly increased in CD4+ effector T cells expressing an intermediate level of CD25 (CD25\textsuperscript{int}) when cultured with BzATP-stimulated smiDCs (BzDCs) compared with nontreated smiDCs (NtDCs; Fig. 6A, 6B). Moreover, BzDCs have the capacity to potentiate Th17 differentiation compared with NtDCs, which was indicated by the increase in IL-17 secretion (Fig. 6C). However, due to the high variability inherent in the human model, the significant increase in IL-17 expression was not always consistent (Supplemental Table I); however, when we inhibited P2X7R signaling with KN-62, we did observe a more consistent decrease in the Th17 response (Fig. 6C, Supplemental Table I), indicating high levels of endogenous ATP present in the DC cultures, which potentially leads to negative feedback. Additionally, to address P2X7R signaling specificity, we confirmed that KN-62 had the capacity to inhibit the BzATP-dependent increase in IL-17 expression (Fig. 6C, Supplemental Table I). To verify that these findings were not dependent on an increase in T cell number, we also assessed the proliferation induced by smiDCs and did not see a significant difference in T cell proliferation among the treatment groups when examined by mitochondrial dehydrogenase activity (data not shown).

In addition to being a potent proinflammatory cytokine, IL-17 also appears to be an angiogenesis factor involved in tumor progression and autoimmune diseases via the induction of VEGF (39, 40). Thus, in addition to detecting a significant increase of VEGF in ex vivo skin and smiDCs following BzATP treatment (Figs. 2A, 4B), we also determined that VEGF was significantly increased in MLCs at both the mRNA (data not shown) and protein levels when naive T cells were stimulated by BzDCs compared with NtDCs (Fig. 6D). Finally, by neutralizing IL-1β or IL-23 in BzDC-stimulated MLCs, we determined that the P2X7R-dependent increase in Th17 differentiation was in part induced by IL-1β and IL-23 (Fig. 6E). Of note, inhibition of IL-6 signaling did not reduce the P2X7R-dependent Th17 responses in these cultures (data not shown).

To assess whether BzDCs affect Th17 differentiation at the transcriptional level, we examined the RORγt, which is a major transcription factor that drives Th17 differentiation. We clearly

![FIGURE 7.](http://www.jimmunol.org/) Lesional and nonlesional psoriatic skin exhibits enhanced P2X7R expression. (A) Human healthy donor and psoriatic nonlesional and lesional skin explants were immunofluorescently labeled with markers for P2X7R (red) and HLA-DR (green), plus DAPI (blue) nuclear counterstain. As a staining control, normal skin was stained with the secondary anti-rabbit–Cy3 Ab alone (2˚ Control). Original magnification ×40. Scale bars, 20 μm. Dotted line indicates the epidermal–dermal junction. Representative of four independent experiments. (B) Protein extracts were isolated from normal healthy skin and nonlesional and lesional psoriatic skin. Western blot analysis demonstrated the presence of two specific P2X7R variants, a 74- and 45-kDa protein (top panel). P2X7R specificity was confirmed with a blocking peptide (bottom panel). Three individual patients are presented, which are representative of seven individual donors examined. (C) Scatter plot demonstrates arbitrary units of P2X7R mRNA expression normalized to β2-microglobulin using the 2^\text{-ΔΔCT} method. Each dot represents an individual biopsy from normal, psoriatic nonlesional, or psoriatic lesional skin. Asterisk indicates a significant difference between indicated groups: *p < 0.05.
demonstrated a significant increase in the RORγt-encoding gene, RORC, in cultures stimulated by BzDCs compared with NtDCs (Fig. 6F). Additionally, there was a significant decrease in RORC expression when BzDCs were cultured in the presence of KN-62 (Fig. 6F). Finally, we assessed the expression levels of two microRNAs involved in T cell activation (mir-221) and Th17 differentiation (mir-326) (41, 42). MLCs stimulated by BzDCs expressed a significant increase in both mir-221 (2.44 ± 0.11-fold increase) and mir-326 (1.66 ± 0.07-fold increase) compared with naïve T cells stimulated with NtDCs (Fig. 6G). Additionally, KN-62 significantly suppressed mir-221 but had no effect on mir-326 (Fig. 6G). Overall, these results indicate that BzDCs were capable of potentiating Th17 differentiation at both the transcriptional and posttranscriptional levels, and thus, we can conclude that the direct P2X7R signaling of smiDCs leads to the initiation of a DC17 differentiation profile.

Lesional and nonlesional psoriatic skin expresses P2X7R

In a genetically predisposed environment, it has been suggested that alarmins could lead to the induction of psoriatic lesions by promoting a highly inflammatory positive-feedback loop (7); however, the role of alarmins in psoriasis pathogenesis has not been well addressed. In this context, ATP is a particularly appealing alarmin that, via P2X7R signaling, induces NF-κB activation and the IL-23/Th17 axis, both of which have been shown to be psoriasis-susceptibility pathways (17, 35, 43). Additionally, aberrant microRNA expression profiles have been observed in psoriasis, which have correlates within the P2X7R signaling pathway, such as mir-21 (44, 45). Therefore, P2X7R signaling links early inflammatory triggers with psoriasis susceptibility factors. We hypothesize that within a genetically susceptible microenvironment, cutaneous P2X7R signaling is a mechanism of psoriasis pathogenesis. To determine whether P2X7R has a role in initiating psoriatic lesions, we used immunofluorescence microscopy to compare the expression of P2X7R in the skin from healthy donors to that in lesional and nonlesional skin of patients with psoriasis. In Fig. 7, we observed by immunofluorescence the low expression of P2X7R in the epidermis of normal skin from patients without psoriasis (Fig. 7A). Expression is somewhat increased in the epidermis of nonlesional skin of patients with psoriasis and substantially increased in lesional skin from the same donor (Fig. 7A) (19). To confirm the expression of P2X7R in psoriatic skin, Western blot analysis was performed on cutaneous lysates. Similar to immunofluorescence data, the full-length canonical receptor represented by the expression of the 74-kDa P2X7R protein was slightly elevated in nonlesional samples and even less so in lesional samples (Fig. 7B, top panel). However, interestingly, we also detected a 45-kDa variant of the P2X7R that was highly expressed in all lesional samples examined, whereas it was only minimally expressed in normal tissues and nondetected in most nonlesional samples (Fig. 7B, top panel). We confirmed the specificity of these bands by using a blocking peptide, which inhibited P2X7R Ab binding (Fig. 7B, bottom panel). Further, analysis of the P2X7R at the mRNA level revealed that nonlesional tissues exhibited a significant increase in P2X7R mRNA compared with lesional samples, whereas no difference was observed in lesional samples compared with normal controls (Fig. 7C).

To begin to address the role of P2X7R signaling in the initiation of psoriasis pathogenesis, we injected nonlesional psoriatic explants ex vivo with BzATP or PBS (vehicle control) and examined the expression of VEGF, IL-6, and IL-23, factors positively associated with psoriasis pathogenesis (36, 46, 47). An assessment by qRT-PCR revealed a significant increase in VEGF, IL-6, and IL-23 transcript levels in nonlesional skin following injections with BzATP compared with nonlesional controls (Fig. 8). Importantly, there was no difference between nonlesional plus BzATP and lesional skin in the VEGF, IL-23, and IL-6 expression levels (Fig. 8). Furthermore, to address whether purinergic signaling is involved in perpetuation of psoriatic lesions, we injected lesional tissues with BzATP and determined that IL-23 is significantly increased compared with lesional controls, whereas VEGF and IL-6 were not increased (Fig. 8). Thus, these findings support the hypothesis that P2X7R signaling is involved in the initiation phase of psoriasis pathogenesis in addition to perpetuation of psoriatic lesions. Based on these findings, experimental murine models are now being developed to further explore this hypothesis.

We finally wanted to examine novel P2X7R-dependent signaling mechanisms involved in the induction of psoriatic cytokines. Interestingly, mir-21 is an angiogenic factor associated with psoriasis and that increases VEGF expression (44, 45, 48) (Fig. 9A).
To assess the capacity of P2X7R signaling to initiate the mir-21 pathway, we first injected normal human skin with BzATP and demonstrated a significant increase in mir-21 expression compared with PBS-injected controls (Fig. 9B). Next, we injected human cutaneous explants ex vivo with a mir-21 Atg, to specifically block mir-21 expression, in the presence or absence of BzATP. Consistent with our previous results in Figs. 1 and 2, cutaneous treatment with BzATP induced a significant increase of VEGF and IL-6 expression, which was significantly decreased by ~80% in the presence of the mir-21 Atg at both the protein and mRNA levels (Fig. 9C and data not shown, respectively). IL-6 and VEGF, to a lesser degree, were decreased in the PBS plus mir-21 Atg treatment group compared with PBS alone; however, not to the same level as the BzATP treatment group (Fig. 9C). Thus, we found that mir-21 stimulates the secretion of VEGF and IL-6 at a low level in the steady state and potently in a P2X7R-dependant manner. For the first time, to our knowledge, these results further define a mechanistic role for P2X7R signaling in inflammation and describe a potential mechanism for the induction of psoriasis by stimulating the P2X7R-dependent mir-21 angiogenesis pathway.

**Discussion**

Using human skin explants ex vivo and cutaneous DCs, we examined the inflammatory responses initiated by the cutaneous immune system following stimulation through the P2X7R. We demonstrated that P2X7R signaling in human cutaneous tissues induces innate and adaptive immune responses; specifically, responses leading to the differentiation of Th17 cells. Furthermore, we determined that P2X7R signaling is a potential mechanism involved in the initiation of psoriasis pathogenesis, perhaps through the induction of the mir-21 angiogenic network, a critical pathway involved in the pathogenesis of psoriasis (44, 45, 48, 49).

Key innate inflammatory markers, such as IL-6, IL-1β, and TNF-α, characteristic of psoriasis, are increased following cutaneous P2X7R stimulation. IL-6, secreted by endothelial cells and cutaneous DCs, is particularly important in the development of psoriasis for its capacity to block regulatory T cell functions (47).

TNF-α, a psoriasis susceptibility gene secreted by TNF/iNOS-producing DCs and effector T cells, has a critical role in psoriasis by in part initiating the expression of IL-1β and IL-8 (31, 50, 51). Moreover, TNF-α and IL-17 cooperate in the pathogenesis of psoriasis, as seen by the effect of TNF-α antagonism on IL-17 signaling and in their synergistic effect on the production of inflammatory cytokines by keratinocytes (50, 52). P2X7R stimulation also increases the cutaneous expression of iNOS, VEGF, CXCL10, S100A7, CCL20, and TRAIL, factors highly associated with inflammation and psoriasis compared with atopic dermatitis (31, 49, 51, 53, 54). Thus, the presence of P2X7R agonists, such as ATP, induces innate inflammatory mediators in the skin reminiscent of those observed in psoriatic lesions.

At least two downstream P2X7R signaling mechanisms have been described following P2X7R stimulation, which are inflammasome/IL-1β dependent and independent pathways (55). Barberà-Cremades et al. (55) described the P2X7R-dependent secretion of PGE2 as an inflammasome-independent mechanism mediated through intracellular calcium influx and MAPK signaling. Likewise, CXCL10 and TNFR1 are increased early in the skin following P2X7R activation likely through MAPK signaling (56, 57), whereas VEGF, iNOS, and S100A7 appear to be downstream of the inflammasome pathway (58). Therefore, we conclude that cutaneous immune responses induced following P2X7R signaling can occur through both inflammasome/IL-1β–dependent and –independent mechanisms.

DCs are significantly increased in psoriatic lesions, and many of the psoriasis pathogenic factors found in lesions, such as IL-23, TNF-α, and iNOS, are secreted by inflammatory DCs (31, 34, 36). The significant increase of HLA-DR+ DCs in the dermis of skin explants treated with the P2X7R agonist is remarkable considering the lack of blood supply to attract circulating inflammatory monocytes; therefore, ATP likely matures and differentiates resident monocytes and/or precursor DCs into inflammatory DCs (59). Cutaneous DC populations are also highly activated, expressing DC-LAMP, CD206, and iNOS following P2X7R injections. Moreover, direct P2X7R stimulation is capable of further maturing and

![Figure 9](http://www.jimmunol.org/)
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differentiating activated migratory DCs into DC17s as determined by the expression of IL-23R, IL-1β, IL-6, IL-23, iNOS, and VEGF. LCs are the cutaneous DC population capable of initiating Th17 responses (2, 3); thus, it is not surprising that inflammatory markers and alarmin receptors increased following BzATP treatment are coexpressed with langerin.

In murine macrophages, ATP is involved in the inflammasome-dependent release of the alarmin HMGB1, which acts as an inflammatory mediator and adjuvant stimulating DC activation, migration, and T cell-biasing functions (60–62). Specifically signaling through the P2X7R on cutaneous human DCs also induces the active release of HMGB1. Importantly, the downstream effects of HMGB1 signaling on DCs include the release of IL-6, IL-1β, and TNF-α (60, 63). The best-characterized receptor for HMGB1 is RAGE, which has been shown to be expressed on immature populations of DCs and that we demonstrate for the first time, to our knowledge, is upregulated on cutaneous DCs following P2X7R agonist treatment (60, 61, 64). Furthermore, S100A7, which is increased following P2X7R stimulation and induces VEGF secretion, has also been described as an agonist for RAGE (65). Thus, a mechanism of action of P2X7R signaling in human cutaneous DCs is to trigger downstream RAGE inflammatory responses. We hypothesize that ATP is also capable of directly stimulating the release of other alarmins, such as LL-37, IL-33, and heat-shock proteins.

In the presence of P2X7R agonist, cutaneous DCs express IL-1β and IL-23 to further potentiate Th17 responses, which are essential for inducing downstream effectors of psoriasis pathogenesis (66, 67). One such effector includes the secretion of VEGF, a potent inflammatory and angiogenesis factor that establishes a positive-feedback mechanism by further enhancing Th17 responses and tissue inflammation (39, 40, 46, 68). Overall, P2X7R signaling in healthy cutaneous tissues is capable of initiating inflammatory responses reminiscent of those observed in psoriasis lesions and thus warranted further examination of P2X7R signaling as a potential mechanism of the initiation of psoriasis pathogenesis.

This is the first report, to our knowledge, to demonstrate an increase in P2X7R expression in nonlesional skin and also the expression of P2X7R variants in psoriatic lesions. The increase of P2X7R in nonlesional skin suggests an important primary difference in the skin of patients with psoriasis and not just a secondary effect of inflammation. Interestingly, psoriasis lesions express both the 74-kDa full-length canonical P2X7R and a unique 45-kDa variant. There have been ∼11 splice variants identified for the P2X7R, indicated as P2X7RA–K, with P2X7RA representing the full-length form (69). Of particular interest is the P2X7RB variant, which: 1) is ~45 kDa in size; 2) is widely distributed among tissues; and 3) is lacking the intracellular C terminus that confers P2X7R signaling cytotoxicity (69, 70). Additionally, P2X7RB stimulates increased NFATc1 (NFAT2) activation, leading to enhanced cellular proliferation and decreased ATP-induced apoptosis (69, 70). It has been suggested that NFAT2 is involved in keratinocyte hyperproliferation in psoriasis (71, 72). Furthermore, reports have proposed that P2X7R variants are likely involved in human diseases (69, 73). Thus, we hypothesize that the increased expression of P2X7RB in psoriatic lesions is in part responsible for the increased proliferation and reduced apoptosis of lesional keratinocytes, contributing to the perpetuation of the psoriatic lesions.

In addition to increased levels of P2X7R, nonlesional psoriatic tissues also express significantly higher P2X7R transcripts compared with healthy controls and lesional samples. Correspondingly, mir-150, which triggers P2X7R mRNA instability, is decreased in nonlesional samples and returns to baseline levels in lesional psoriatic tissues (74, 75). These findings indicate that nonlesional psoriatic skin is poised to initiate psoriatic lesion formation following the release of danger signals and innate mediators by upregulating the surface expression of P2X7R. In support of this, P2X7R stimulation in nonlesional skin leads to the significant increase in VEGF, IL-23, and IL-6 expression comparable to levels found in psoriatic biopsies. Moreover, the VEGF and IL-6 pathways are induced by mir-21, which is increased in psoriatic tissues and in healthy skin following P2X7R signaling (44, 45, 48). Thus, it is tempting to speculate that ATP signaling through the P2X7R initiates an inflammatory cascade that is involved in the conversion of nonlesional to lesional psoriatic tissues by initiating the mir-21 pathway.

In summary, the factors that initiate and perpetuate psoriatic lesions from nonlesional skin are not known, but in this study, we have demonstrated that a possible trigger is the release of extracellular ATP from trauma or potentially the release of ATP from commensal bacteria (17). Overall, using normal human skin, we determined that signaling through purinergic receptors, particularly P2X7R, plays a role in the development of both innate and adaptive Th17 inflammatory immune responses in human skin. These findings have prompted us to further explore the P2X7R-dependent development of psoriasis pathogenesis in experimental murine models of psoriasis.

Acknowledgments
We thank Adriana Larregina for critical reading of the manuscript. We acknowledge use of tissues procured by the NDRI, Cooperative Human Tissue Network, and University of Pittsburgh Health Sciences Tissue Bank. Tissue sample imaging was performed in the Center for Biological Imaging.

Disclosures
The authors have no financial conflicts of interest.

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