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ATP, IL-17 and IL-23 INFLAMMATORY CIRCUITS ACTIVATE Neutrophil Extracellular Traps (NETs) in the DEVELOPMENT of PSORIASIFORM DERMATITIS

TESIS DOCTORAL

Presentada por:
JULIO ALEXANDER DIAZ PEREZ

Dirigida por:
ISABEL GUILLEN SALAZAR

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I would like to thank my wife Paola. Her support, encouragement, patience and love.

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INDEX OF CONTENTS

INDEX OF FIGURES

ABSTRACT

Psoriasis vulgaris is a chronic inflammatory cutaneous disease that affects approximately 2% of the US population. Psoriasis occurs when a genetically susceptible individual has a trigger stimulus that initiates cell selection, proliferation and damage. During the past four decades, predictive gene signatures, important participating cells and specific signaling mediators have been identified. A better understanding of these pathogenic events have lead to more effective, targeted methods to prevent, diagnose and treat this disease. However, more advances are required in early disease. Several triggers have been proposed as initiator events for psoriasis, including alarmins such as ATP. ATP is a particularly interesting alarmin that, via 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. However, the role of alarmins in psoriasis mechanistic pathogenesis have not been well addressed. Here I report that 2'(3')-O-(4-Benzoylbenzoyl) adenosine 5'-triphosphate, an ATP analog and P2X7R agonist, in the presence of an ATPase inhibitor induced psoriasiform dermatitis in mice characterized by acanthosis, increased vascularity, parakeratosis, microabscess formation, and increased inflammation and inflammatory infiltrates. The induced inflammatory response is largely dependent on the IL-1β/NLRP3 inflammasome pathway and neutrophils extracellular traps. In conclusion, my results demonstrate that cutaneous inflammatory responses induced via alarmin signaling through the P2X7R have implications in the pathogenesis and potential treatment of inflammatory diseases, such as psoriasis.

KEY WORDS: Psoriasis, Pathogenesis, Neutrophil, P2X7 receptor, Purinergic receptors, ATP, Alarmins.

RESUMEN

La psoriasis vulgar es una enfermedad cutánea inflamatoria crónica que afecta aproximadamente al 2% de la población de EE. UU. La psoriasis ocurre cuando un individuo genéticamente susceptible tiene un estímulo desencadenante que inicia la selección, proliferación y daño celular. Durante las últimas cuatro décadas, se han identificado biomarcadores de genes predictivos, células participantes importantes y mediadores de señalización específicos. Una mejor comprensión de estos eventos patógenos podría conducir a métodos efectivos y específicos para prevenir, diagnosticar y tratar esta enfermedad. Se han propuesto varios desencadenantes como eventos iniciadores de la psoriasis, incluidas alarminas como el ATP. El ATP es una alarmina particularmente interesante que, a través de la señalización via receptor P2X7 (P2X7R), induce la activación de NF-κB y el eje IL-23 / IL-17, los cuales han demostrado que son vías de patogénesis en la psoriasis. Sin embargo, el papel de las alarminas en la patogénesis de la psoriasis no se ha abordado bien. Aquí informo que 2 '(3') - O- (4-Benzoilbenzoil) adenosina 5'-trifosfato, un análogo de ATP y agonista de P2X7R, en presencia de un inhibidor de ATPasa induce dermatitis psoriasiforme en ratones caracterizada por acantosis, aumento de la vascularización, paraqueratosis, formación de microabscesos y aumento de la inflamación y los infiltrados inflamatorios. La respuesta inflamatoria inducida depende en gran medida de la vía del inflamasoma IL-1β / NLRP3 y de las trampas extracelulares de neutrófilos. En conclusión, mis resultados demuestran que las respuestas inflamatorias cutáneas inducidas a través de la señalización purinérgica a través del P2X7R tienen implicaciones en la patogénesis y el tratamiento potencial de enfermedades inflamatorias, como la psoriasis.

PALABRAS CLAVE: Psoriasis, patogénesis, neutrófilo, receptor P2X7, receptores purinérgicos, ATP, alarminas.

ABBREVIATIONS LIST:

2’(3’)-O-(4-Benzoylbenzoyl) adenosine 5’-triphosphate (BzATP)

P2X7 receptor (P2X7R)

quantitative RT-PCR (qRT-PCR)

Imiquimod (IMQ)

Ecto-nucleoside triphosphate diphosphohydrolases (E-NTDPase)

Antigen presenting cells (APCs)

Loci for psoriasis (PSORS)

Pityriasis rubra pilaris (PRP)

Single nucleotide polymorphisms (SNPs)

Vascular endothelial growth factor (VEGF)

Interleukin (IL)

Regulatory T (T-reg) cells

Dermal dendritic cells (DDCs)

Human papillomaviruses (HPV)

Human endogenous retroviruses (HERVs)

Transforming growth factor (TGF)

Interferon (IFN)

Nitric oxide (NO)

Src-family protein tyrosine kinases (SFKs)

Epidermal growth factor (EGF)

Keratinocyte growth factor (KGF)

Insulin growth factor 1 (IGF-1)

Hypoxia inducible factor-1 (HIF-1)

1. INTRODUCTION

Psoriasis is a chronic, recurring, inflammatory skin disease that affects approximately 1 to 3% of the general population [1,2]. This disease is only observed in humans; other animals do not develop it spontaneously [2] Murine models that exhibit some features of psoriasis after genetic or immune manipulations are imperfect, as they do not represent the full clinico-pathological spectrum of the disease [2,3]. This seem to be related with the differences between human and mice skin (Figure 1 and Table 1). However, relevant information to understand psoriasis has been produced with the use of these models [3,4]. Importantly, numerous advances in the identification of key players in the disease development have opened a door of hope for psoriatic patients [2,5].

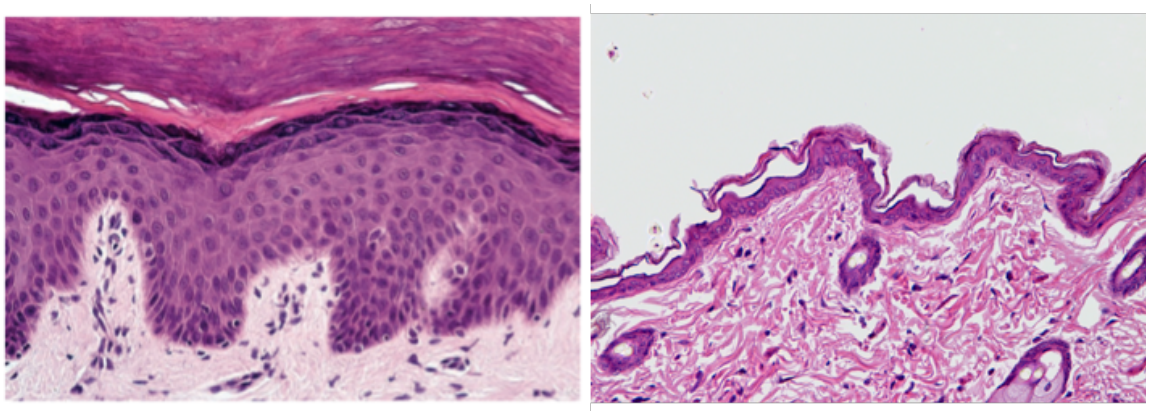


Fig 1. Differences between human (left) and mouse (right) skin. Mouse skin is thinner, lacks of sweet glands, and have increased number of hair follicles.

The clinical presentation of the disease is variable, and several subtypes have been described such as: guttate, inverse, seborrheic, erythrodermic, pustular, palmoplantar, and plaque forms [5]. Lesions begin at any age and generally are well demarcated, erythematous, scaly and disfiguring [5,6] (Figure 2).

Criteria	Human	Mouse
Epidermis of body (thin) skin	Thick (50–100 μm)	Thin (10–15 μm)
Epidermis of thick skin (plantar/ palmer surfaces)	Thick (300–400 μm) Palms of hands, soles of feet	Tail (70–80 μm), footpad (150–400 μm), muzzle (20–30 μm), eyelid (50–60 μm)
Hair types: body	Vellus Terminal	Guard Auschene Zigzag Awl
Specialized hair types	Cilia (eyelashes) Eyebrows Pubic Facial (beard, mustache) Axillary Ear hair	Cilia (eyelashes) Vibrissae (specialized somatosensory organ with blood-filled sinus around the muzzle, eyes, and feet) Perianal Tail hair Ear hair (inner and outer pinnae are different)
Emergence of hair	19–21 weeks gestation	5 days postpartum
Hair cycle	Mosaic pattern	Wave pattern of whole body as neonates Wave pattern within zones in adults
Tail and tail hairs	No (no tail)	Yes
Sebaceous glands	Yes	Yes
Modified sebaceous glands	Meibomian glands Ceruminous glands	Meibomian glands Zymbal gland Preputial gland Clitoral gland (in females) Perianal gland (in males)
Eccrine glands	Found on entire body including soles and palms	Limited to footpads
Apocrine sweat glands	Axilla, genitoanal region,	Mammary glands present in most skin including limbs

Tab 1. Differences between human and mice skin. From: Sundberg JP, Ichiki T. Genetically engineered mice handbook. Boca Raton: CRC Press; 2005.

The main histopathologic characteristics are acanthosis with parakeratosis without hyperkeratosis due to rapid proliferation of keratinocytes, elongation of rete ridges, decreased or absent granular cell layer, suprapapillary thinning, Munro micro-abscesses, increased mitotic figures, dermal congestion, angiogenesis as well as a mixed dermal/epidermal infiltrate of lymphocytes, macrophages, mast cells, neutrophils and innate lymphoid cells (Figure 3) [5,7]. The disease is associated with arthritis, type 2 diabetes, systemic lupus erythematous, myopathy, vitiligo, cutaneous T-cell lymphoma, enteropathy, spondylitic heart disease, and Crohn's disease [1,2,5,6,7]. All of these co-morbidities are associated with inflammatory cell deregulation [6,7,8,9,10].

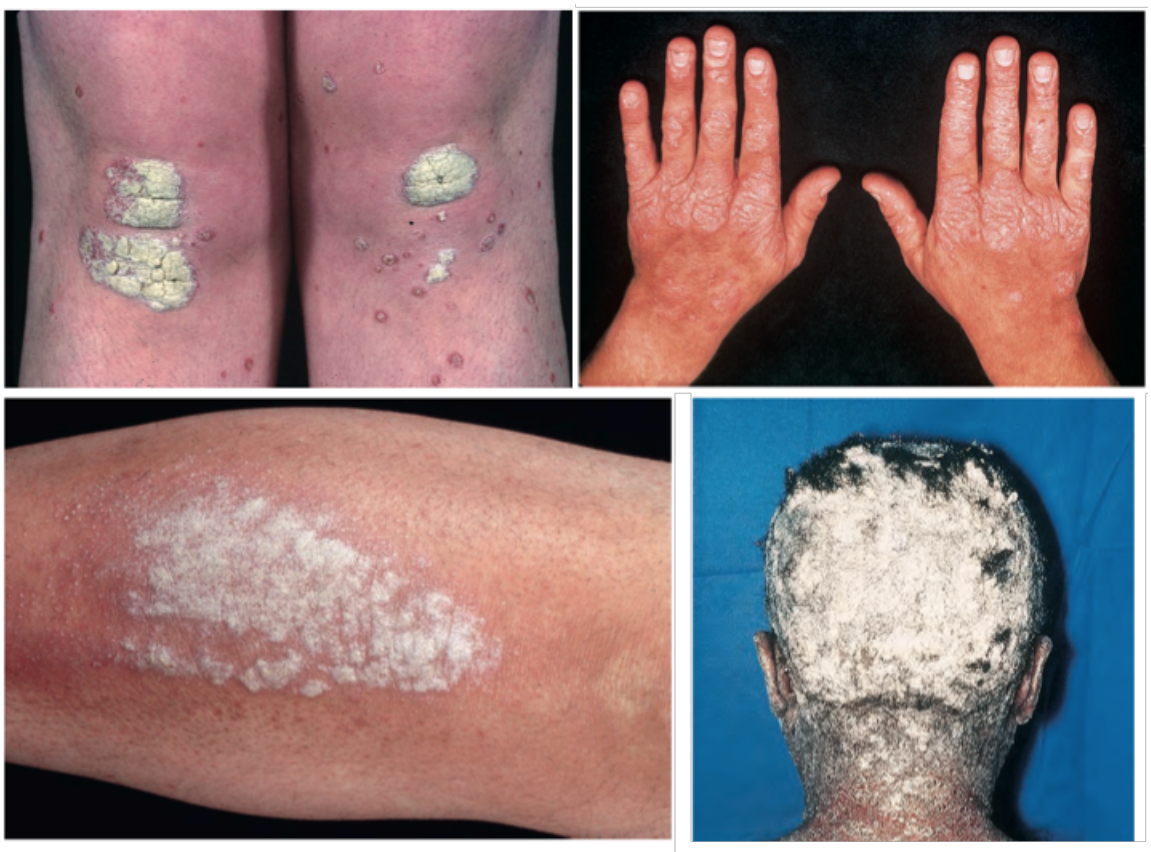


Fig 2. Variable clinical manifestations of classical well developed psoriasis that is manifested with scaly dry plaques on extensor surfaces.

Currently, evidence indicates that an imbalance in the activity of multiple cells after injury plays the main role in the development of psoriasis [2,7]. This deregulation seems to be the consequence of impaired signaling between keratinocytes and immune cells [8,11,12]. The crucial importance of this finding has been proved through the striking response of the disease to targeted biological therapies [2,8]. Microbial agents and trauma are the best known initiating factors in the development of the disease [2,8]. Epidermal stem cells generate the initial stimulus/stimuli that trigger/s keratinocytes and antigen presenting cells (APCs) to create the signaling cascade that generates psoriatic lesions [12]. Variations in the pathogenic events lead to the development of clinical (Figure 4A) and histologic variants of the disease (Figure 4B).

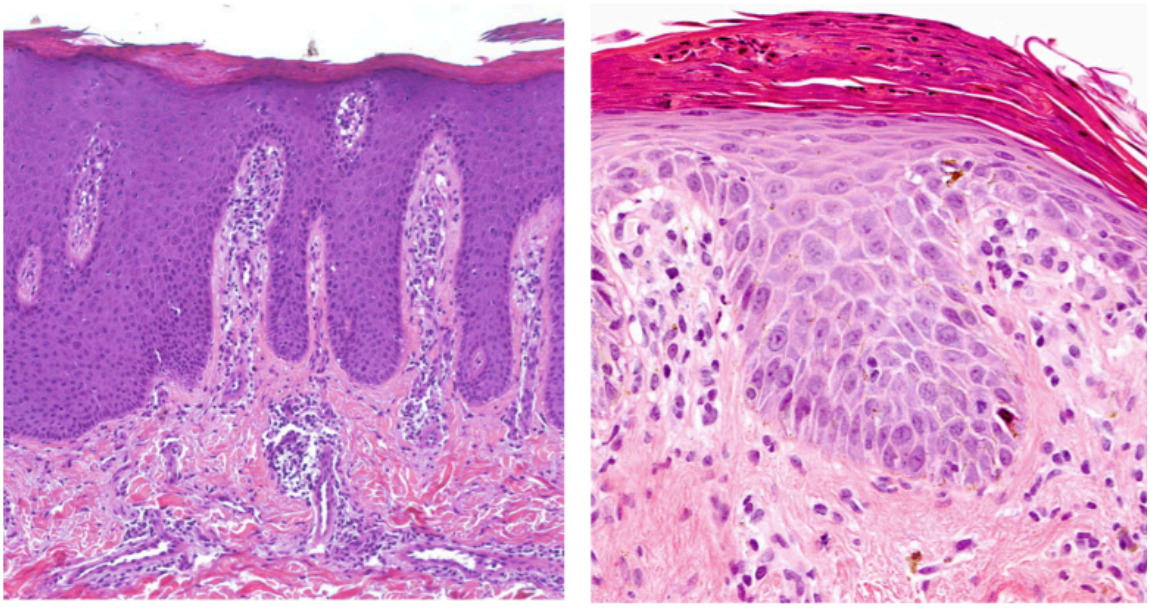


Fig 3. Histopathology of well developed classic psoriasis. The classic view includes the presence of elongated acanthosis, with parakeratosis, and neutrophil rich infiltrate.

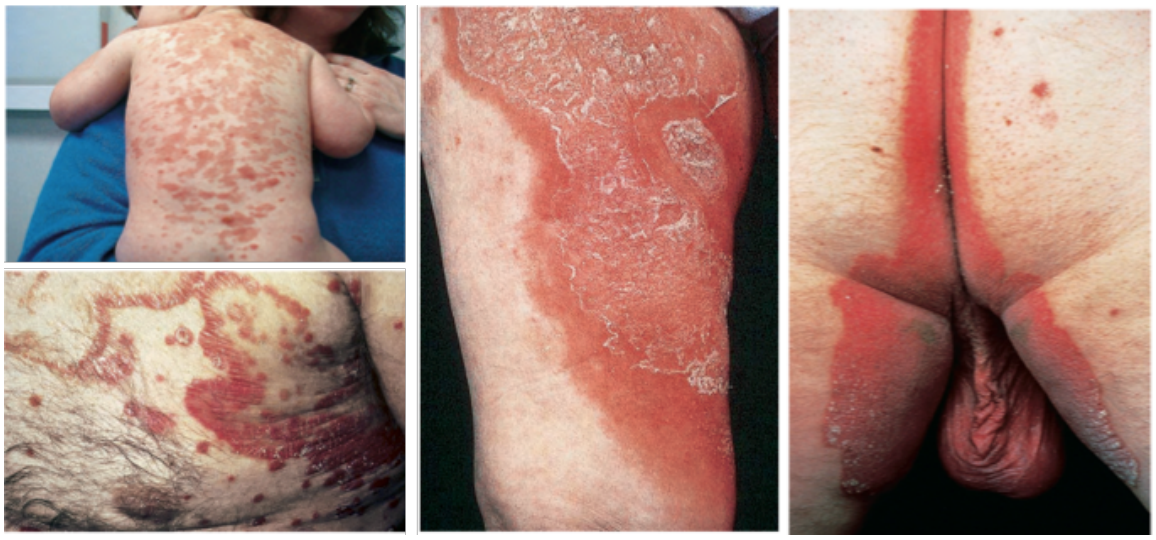


Fig 4A. Clinical variants of psoriasis that include pediatric presentation, flexural, pustular and inverse involvement.

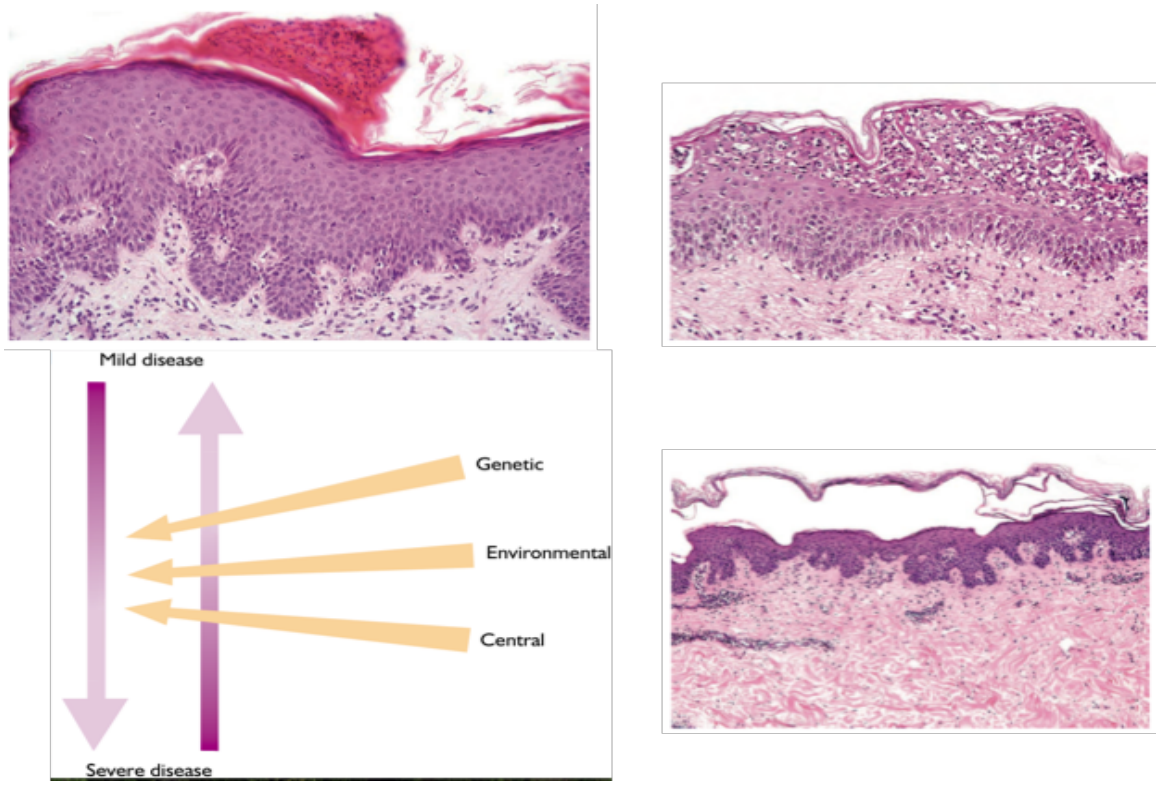


Fig 4B. Histologic variants of psoriasis that could be dependent of the timeline of development and the pathogenic mechanism.

1.1 Predisposing genes

Evidence to support genetic basis in the development of psoriasis dates back to 1954, when Lomholt described the presentation in residents of the Faroe Islands. He observed features that were indicative of hereditary traits associated with development of the disease. Similarly, Farber and Nall, in 1974, observed that 70% of monozygotic twins developed the disease when their twin was also affected [13,14,15,16]. Nine susceptibility loci for psoriasis (PSORS) have been recognized using genome-wide scans [1,13,14]. PSORS1 locus encodes the HLA class I gene, including HLAC6w (chromosome 6p21.3), the gene variant with highest relative risk for psoriasis (ratio of 23.1 in homozygous and 8.9 in heterozygotes) [14]. HLA-B*27, HCR*WWCC and CDSN*5 are also located in PSORS1 [17,18]. PSORS2 is located in 17q24–q25 [17]. SLC9A3R1, and NAT9 genes are located in PSORS2 [19]. PSORS3 is located on 4q [17]. PSORS4 loci located on 1cen-q21 encode the genes for the chemotactic proteins S100A8 and S100A9 [19]. PSORS5 encode cation/chloride cotransporters on 3q21 [20]. JunB is localized in PSORS6 [19,20]. The antagonistic regulator of Jun B, cJun, is located in PSORS7 (1p) [20,21]. PSORS8 and PSORS9 are mapped in 4q31 [21]. More recently, subjects with CARD14 mutations have been described to strongly display characteristics of both psoriasis and PRP.

1.2 Selective regulators

miRNAs and single nucleotide polymorphisms (SNPs) regulate the expression of proteins encoded by PSORS genes [22]. miR-203, mir-21, and miR-146a are overexpressed in psoriasis [23]. miR-203 activity is associated with down-regulation in cytokine signaling 3 (SOCS-3). SOCS-3 is involved in regulation of inflammatory cells and keratinocytes through STAT3 and PIM1 [24]. mir-21 plays a relevant role regulating

vascular endothelial growth factor (VEGF) and interleukin (IL)-6 pathways [25]. miR-146a is abundant in regulatory T (T-reg) cells, dermal dendritic cells (DDCs) and mast cells [23]. miR-125b, miR-424, miR-99a, miR-31 are also associated with psoriasis [25]. rSNPs down regulates SLC9A3R1 and NAT9 genes. Products of these genes downregulate the immune response. The inhibition of the inhibition allows for the development of auto-reactive T cells and the deregulation of T cell signaling, which are major events in the pathogenesis of psoriasis [22]. SNPs also regulate the angiogenic stimuli in psoriasis through VEGF modulation [17].

1.3 Physical trauma

Psoriasis regularly develops in cutaneous locations with frequent physical trauma, such as extensor surfaces. The induction of lesions after trauma is referred as the Köbner phenomenon in honor of Heinrich Köbner (1838-1904), who described this manifestation in several pathologic skin entities [5,7,26]. Physical injury liberates numerous inflammatory mediators known as alarmins, including IL-1, IL-6, ATP, LL37 (cathelicidin) and TNF-alpha, initiating the pathogenic cascade of cell interactions seen in psoriasis [12,27].

1.4 Microorganisms

Infections are also initiator factors for psoriasis [8]. Group A streptococcal infection (A, C and G) is the most recognized [28]. Antigenic mimicry between the streptococcal M-protein and human keratinocyte keratins is the trigger event [29,30]. Human type-1 keratins, such as K17, and the streptococcal M6 protein show strong sequence homology, and they both bind to HLA-Cw6 [29]. This effect induces the expression of heat shock proteins (HSPs), specifically HSP60 and HSP65, by keratinocytes [31,32,33]. HSPs stimulate gamma-delta T-cell receptors and Toll-like receptors (TLRs) 1, 2 and 4

[[32,34]. Also, HSP expression increases T-cell adhesion to fibronectin [33,35,36]. Other microorganisms associated with psoriasis include bacteria (S. aureus), fungi (Malassezia sp. and Candida sp.) and viruses, such as human papillomaviruses (HPV5 and HPV36) and human endogenous retroviruses (HERVs) [37,38,39].

An important remark is that, after psoriatic lesions are established, numerous antimicrobial peptides are secreted by keratinocytes [40]. B-defensin-2 (encoded by DEFB4), psoriasin (S100A7), calgranulin (S100A8 and S100A9), small proline-rich region proteins (SPRR) and LCE protein are commonly upregulated in psoriatic lesions [13]. This effect is mediated by IL-23, and it is the responsible for the low frequency of super infections observed in psoriatic lesions [13].

1.5 Epidermal stem cells activation

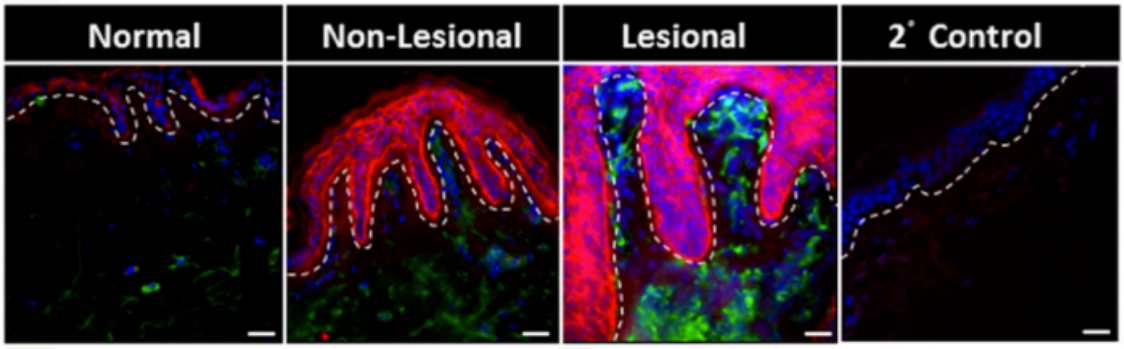
After injury produced by the initiator factors, epidermal stem cells are activated. This effect is supported by tumor protein p63, MYC, integrin-b1, insulin-like growth factor, epidermal growth factor and transforming growth factor (TGF)-alpha signaling pathway, and negatively controlled by TGF-beta signaling [33,41,42].

1.6 Immune cells activation

Almost all cutaneous cell types are implicated in the development of psoriasis. Signaling between cutaneous resident APCs, keratinocytes and T-cells is highly active in the disease. DDCs play a key role in the disease promoting T-cell activation and proliferation [43,44]. ATP, through P2X7R and TNF-alpha, activates cutaneous DDCs via e-cadherin (Figure 5) [44]. DDCs stimulate other DDCs and T-cells by producing IL-23 and neuropeptides in mostly a local process independent of lymph node activity [3]. Thus,

therapy targeting DDCs is promising [31]. IL-23 promotes the maintenance of T-cells by inducing the production of IL-17 and IL-22 [3,30].

Figure 5. Lesional and nonlesional psoriatic skin exhibits enhanced P2X7R expression



Tissue macrophages are also significant in psoriasis initiation [8,45]. Both macrophage and DDC activation and function are NF-kB dependent [13,45]. NF-kB activity in psoriasis is regulated by miR-31 [13,46,47]. It also liberates large amounts of interferon (IFN)-alpha and nitric oxide (NO) in psoriasis [48,49]. IFN-alpha promotes upregulation of MHC class I expression, induces cross-presentation of self-Ags to CD8+ T-cells, and activates T-cells [50]. Once local cutaneous T-cells are activated, a complex network of signaling ensues, creating a vicious cycle to promote T cell expansion and activation [22,51]. T- helper 1 (Th1), T-helper 17 (Th17) and T-reg cells are increased in psoriatic lesions [52,53]. These cells are also correlated with the disease severity and treatment response [54,55]. IL-1 stimulates Th1 cells proliferation [56]. Th17 cells participation is mediated by IL-23 and IL-22 [3]. IL-23, a cytokine made by macrophages and DDCs, plays a key position in the development of autoimmunity driving the expansion of Th17 cells from naive T-cells in co-stimulation with IL-6 and TGF-alpha [3]. IL-22 and IL-4 downregulate IL-23 liberation from phagocytic cells and induce keratinocyte proliferation via phosphorylation of the Stat3 transcription factor [3,8,41]. IL-22 also induces IL-1, IL-6, and TNF-alpha gene expression that favor Th17 differentiation [30,57]. Th17 cells are

the main producers of IL-22, and they lack the IL-22 receptor [58]. Blockade of IL-22 activity leads to a decrease of IL-1 and IL-6 gene expression and prevents further Th17 differentiation and, consequently, the progression of disease [59]. Treg cells in psoriasis are dysfunctional; they do not inhibit cytotoxic T-cells [30,60]. IL-6 signaling regulates this dysfunction through the expression of CD39 in Treg cells (56). CD39+ Treg cells are functionally impaired to inhibit IL-17 secretion [61,62] The development of Th1, Th17 and T-reg cells from naïve CD4+ T cells is induced by TGF-β, IL-1, IL-6 and IL-12 [63]. Finally, γδ T-cells and NK cells are also known to play an essential role in psoriasis by producing IL-17 [3,64]. The participation of these cells is mediated by CXCR3, CXCR4, CCR5, CCR6, IL-23 secreted by APCs and keratinocytes regulating the cytotoxic activity in psoriatic lesions through the release of cytotoxic granules [30,65].

1.7 Inflammatory cells recruitment and tissue damage

IL-22 stimulates the expression of antimicrobial chemotactic proteins in the skin, including defensins and cathelicidins [66,67]. These proteins enhance cutaneous inflammation by activating and recruiting immune cells, mainly CD4+ cells, CD8+ cells, neutrophils, macrophages, and dendritic cells [68]. Chemotactic proteins S100A8 (calgranulin A) and S100A9 (calgranulin B) are almost invariably over-expressed in psoriasis [69]. Both S100A8 and S100A9 are induced through the downregulation of JunB/AP-1 and c-Jun, which is mediated by IL-22 [33,70]. IL-22 neutralization significantly reduces the skin expression of chemotactic proteins, preventing further recruitment and activation of immune cells [33,71,72]. Principally T-cells are observed in the epidermis and in the dermal-epidermal interface [11]. If the T-cell stimuli are intense, as is seen in psoriatic arthritis, T-cell clones are produced: mainly CD8+ cells induced by ADAMTSL5 [73]. CD8+ T-cells in psoriatic epidermis target and destroy melanocytes

using Granzyme B [17,73]. Neutrophils are recruited by CXCL1, CXCL2, IL-8 and IL-18 [74]. They secrete IL-17, AMPs and elastase [8,75].

1.8 Keratinocyte proliferation

The cytokines produced by Th1, Th17 and T-reg cells induce the activation of Src-family protein tyrosine kinases (SFKs), STAT3 signaling and Janus tyrosine kinases, promoting keratinocyte proliferation through TP63 and RUNX1 [76]. This effect is mediated by the expression of TGF-alpha an epidermal growth factor (EGF) receptor ligand, keratinocyte growth factor (KGF), IL-18, IL-22, SOCS-3 and insulin growth factor 1 (IGF-1) [77]. The expression of these growth factors is restricted to the lower spinous layers in psoriasis, the same location where high numbers of proliferative keratinocytes are observed, as proven by cyclin-D1, GLUT-1 and Ki67 expression [78,79,80]. The proliferative activity is also commanded by: amphiregulin, interferon (INF)-gamma, tumor necrosis factor (TNF)-alpha, VEGF, IL-2 and IL-1 [81,82,83]. These last cytokines act as chemoattractants for intra-epidermal neutrophils and T-cells [75], further perpetuating the inflammatory activity.

1.9 Angiogenesis and vascular changes

A prominent angiogenic tissue reaction is characteristic of psoriasis. Vascular proliferation, enlargement of lymphatic vessels and congestion are typical histopathologic characteristics [27,40]. Hypoxia inducible factor-1 (HIF-1), VEGF and nitric oxide play a significant role in the progression of these characteristics (41). HIF-1 alpha is produced by keratinocytes and stimulate VEGF and iNOS to enhance angiogenesis [84,85]. The expression of VEGF is upregulated, and increased VEGF protein expression is strongly correlated with disease severity [86]. K14-VEGF transgenic mice that express VEGF164 in the epidermis develop psoriasis-like disease

[40]. Furthermore, VEGF induces blood vessel hyperpermeability, leading to the congestion and tissue edema that is typical of psoriasis [87]. This effect probably results from increased expression of adhesion molecules such as E- and P-selectin [59]. Anti-angiogenic therapy is also promising in psoriasis management [88].

1.10 Systemic manifestations

10–40% of psoriatic patients develop psoriatic arthritis [89]. Psoriatic arthritis usually, but not always, presents after the onset of cutaneous disease [90]. Nail changes are observed in approximately half of psoriatic patients [91]. Nail changes and psoriatic arthritis are strongly associated [90,91]. Psoriatic arthritis is a highly destructive form of arthritis associated with increased activity of RANK-positive myeloid osteoclast cells [92]. TNF-alpha signaling through TNFR1 and IL-23 are key in the development of these osteoclast cells in psoriatic arthritis [33]. miR-146a inhibits the expression of IRAK-1 and TRAF-6 proteins that regulate TNF-alpha signaling [23,93]. HLA-Cw6 and RAPTOR also participate as genetic regulators in psoriatic arthritis via KIK and mTOR [28,90,94]. The development of nail changes is related to the close proximity of the nail folds to the ‘‘entheseal unit’’ of the distal inter-phalangeal joint region, explaining the strong association between nail changes and joint involvement in psoriasis [95,96].

2. HYPOTHESIS

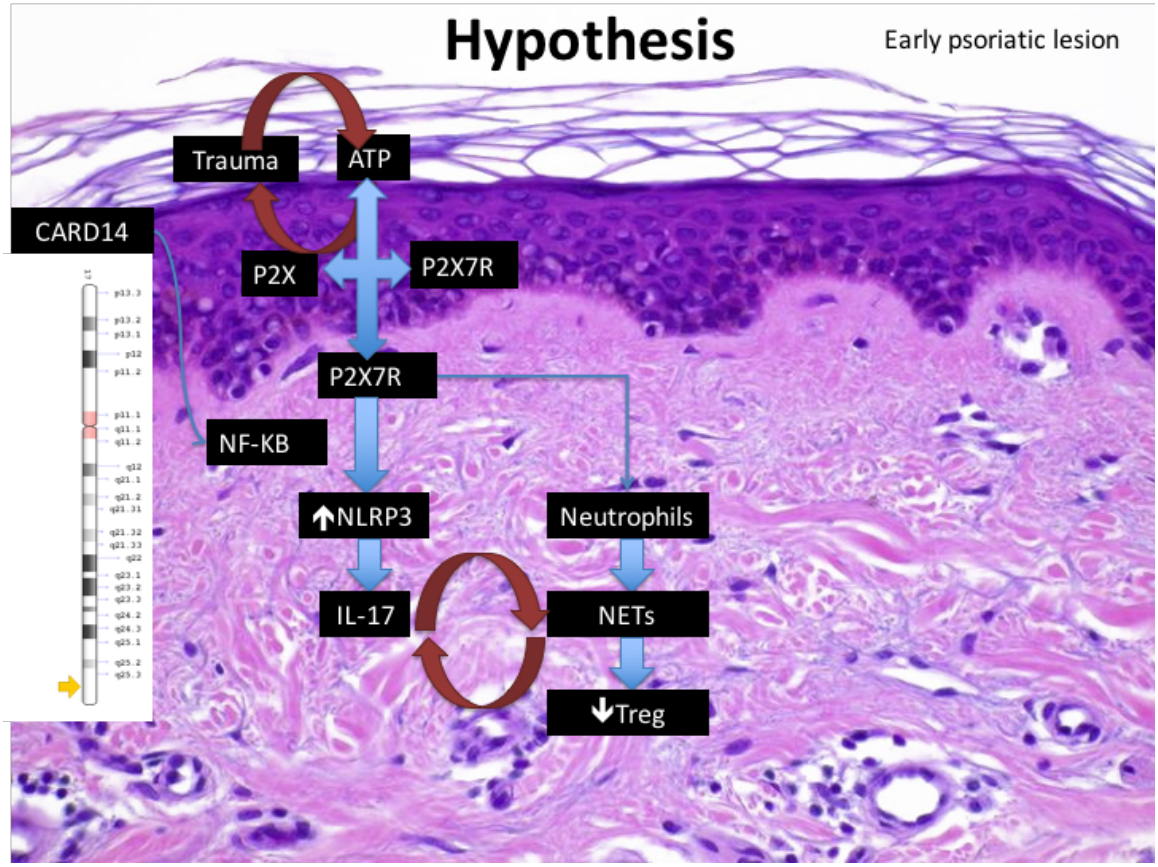
Psoriasis lesions are induced as a result of an imbalance in both innate and adaptive immune responses and aberrant keratinocyte proliferation and differentiation. Additionally, psoriasis is a cell-mediated disease in which Th17 cells were thought to be the main effectors [97]. However, recent studies in human psoriasis patients and in mouse models of psoriasis suggest that innate γδ T-cells, neutrophils, mast cells, and innate lymphoid cells (ILCs) are the cells secreting the majority of IL-17 compared to adaptive Th17 cells [98-106].

2.1 Working hypothesis

Several triggers have been proposed as initiator events for psoriasis, including IL-1, IL-6, CAMP/LL-37 (cathelicidin), TNF-α, and alarmins [106]. Alarmins are damage-associated molecular patterns that act as danger-signals, inducing innate and adaptive inflammatory responses. Following 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 [107, 108]. Supporting this is the Koebner phenomenon in which lesions frequently develop at sites of trauma. Moreover, ATP, an alarmin, is a neurological co-transmitter in both the peripheral and central nervous system [109]. The sympathetic nervous system releases ATP during conditions of stress, thereby linking stress and the exacerbation of psoriasis [109, 110]. However, the role of alarmins in psoriasis pathogenesis has not been well addressed. In this context, ATP is a particularly interesting alarmin that, via 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 [111-115]. In addition to ATP, another alarmin, LL-37, an antimicrobial peptide

secreted by keratinocytes in psoriatic lesions is also an agonist of the P2X7R [116, 117]. Several key studies have indicated that ATP plays a role in cutaneous inflammation and wound healing [117-123]. For instance, Weber et al. demonstrated that contact hypersensitivity responses dependent on IL-1β were inhibited in P2X7R -/- mice [124]. We and others have shown that P2X7R is highly upregulated in psoriatic lesions [120, 124]. Moreover, we determined that purinergic signaling in a human *ex vivo* model provokes innate cutaneous inflammatory responses, DC17 differentiation, and Th17 responses [124]. Thus, we hypothesize that cutaneous P2X7R signaling is an early trigger of psoriasis pathogenesis that can be a target for therapeutics (Figure 6).

Figure 6. Hypothesis.



3. OBJECTIVES

Here I study that intradermal injections of 2'(3')-O-(4-Benzoylbenzoyl) adenosine 5'-triphosphate (BzATP), an ATP analog and P2X7R agonist, in the presence of an ATPase inhibitor induced psoriasiform dermatitis characterized by acanthosis, increased vascularity, parakeratosis, microabscess formation, and increased inflammation and inflammatory infiltrates. I look for mechanistic evidence that the inflammatory response induced following P2X7R signaling is dependent on the IL-1β/NLRP3 inflammasome pathway and neutrophils.

2. Materials and Methods

2.1. Mice

C57Bl/6 wild type mice were purchased from Jackson Laboratories (Bar Harbor, Maine). P2X7R -/- mice were purchased from Jackson Laboratories, NLRP3 -/- mice were obtained from Lexicon Genetics Incorporated (The Woodlands, TX), and IL-23 -/- mice were obtained from Genentech (San Francisco, CA) and were all bred and housed in the University of Pittsburgh animal facility. All mice used in this study were on the C57Bl/6 background. Male and female mice were used between the ages of 6 and 12 weeks. Mice were housed under specific-pathogen-free conditions and treated according to the NIH guide for the care and use of laboratory animals.

2.2 Human samples

Non lesional and lesional skin from patients with psoriasis was collected via shave biopsy. Appropriate IRB approval was obtained from the University of Pittsburgh.

2.3. Experimental design

Mice received two separate 100 µL intradermal injections of BzATP alone [(350 µM); Sigma-Aldrich; St. Louis, MO)], a selective P2X7R agonist, in combination with Sodium polyoxotungstate [POM1 (3.2 mg/kg; Tocris Bioscience; Bristol, United Kingdom)], an E-NTDPase/ATPase inhibitor, or PBS (vehicle control), daily on shaved backs for 4 consecutive days. In some experiments mice also received A438079 (80 µmol/kg [29]; Tocris Bioscience), a competitive P2X7R antagonist that is inactive at other P2 receptors. In separate experiments lesional development was induced in mice by daily intradermal injections of 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 the application of

Imiquimod [IMQ (5% Aldara); 3M Pharmaceuticals; St. Paul, MN)] was topically administered daily for 5 days.

2.3. Antibody (Ab) treatments

Mice were 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 started on day -1 of BzATP injections.

2.4. Histology and Immunohistochemistry

Cutaneous tissue samples were collected and placed in 4% phosphate buffered formalin and paraffin-embedded. Sections (4 µm) used for histology were deparaffinized, rehydrated, and stained with hematoxylin and eosin. Sections used for immunohistochemistry were prepared utilizing the heat-induced epitope retrieval technique by being immersed first in 10-mmol/L sodium citrate buffer (pH 6) and heated to 90 °C for 10 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 then sections were blocked with 0.5% BSA. Samples were incubated with rat anti-CD31 antibody (clone ER-MP12; ThermoFisher) followed by biotinylated donkey anti-rat IgG secondary Ab (Jackson ImmunoResearch Laboratories). 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 counter-stained with hematoxylin. Samples were evaluated by a board certified dermatopathologist with a Zeiss microscope using a digital micrometer where required. Quantification of vascularity was based on CD31 staining utilizing ImageJ software (NIH; Bethesda, MD).

2.5. Immunofluorescence

To characterize cutaneous cellular infiltrate, cross-sections of mouse back skin were prepared and stained as previously described [30]. Briefly, frozen cross-sections were embedded in Tissue-Tek OCT (Miles Laboratories; Elkhart, IN) and snap frozen in pre-chilled methyl-butane (Sigma-Aldrich). Cryostat sections (8 μm) were mounted onto slides pre-treated with Vectabond (Vector Laboratories; Burlingame, CA), and fixed in 96% EtOH. Tissue sections were blocked with PBS 10% normal goat or donkey serum and the avidin/biotin blocking kit (Vector). Cross-sections were immunofluorescently labeled with MHC class II: Alexa488 (clone M5/114.15.2; BD Biosciences; San Jose, CA), GR-1: Alexa 647 (clone RB6-8C5; Biolegend; San Diego, CA), and F4/80: Biotin (clone BM8; Biolegend) followed by SA-Cy3 (Jackson ImmunoResearch; West Grove, PA). Nuclei were counter-stained with 4'6-diamidino-2-phenylindole 2HCl (DAPI; Molecular Probes; Eugene, OR). Images were acquired using an Olympus Provis AX-70 microscope system (Olympus) with FluoView 500 software.

2.6. Tissue Cytokines

At indicated times, skin samples (4 mm) were collected and minced with a scalpel 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 utilizing 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.

2.7. Quantitative RT-PCR (qRT-PCR)

Real-time qRT-PCR experiments were conducted using total RNA, which was isolated

using TRIzol reagent (Invitrogen). For each RT assay, 2 μg RNA was converted to cDNA utilizing 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, TNFα, and S100A9 (Applied Biosystems). Endogenous control was GusB. All cDNA samples were amplified with the Veriquest PCR Master Mix (Affymetrix; Santa Clara, CA) and analyzed using the real-time Step One Plus sequence detection system (Applied Biosystems). Relative fold changes of RNA expression were calculated and normalized based on the $2^{-\Delta\Delta Ct}$ method.

2.8. Flow cytometry

10 mm sections of skin were collected and minced with a scalpel 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 (Gibco) for 45 min at 37℃. 10 mM EDTA was added for an additional 5 min at room temperature. To make a single-celled suspension samples were passed over a 70 μm cell-strainer followed by a 40 μm cell-strainer (Corning). Cells were blocked with Fc block (CD16/CD32; BD Biosciences) and 10% donkey serum then stained using CD3:BUV395 (clone 145-2C11; BD Biosciences), CD127:PE (clone A7R34; eBiosciences), CD45.2:PerCP/Cy5.5 (clone 104; BD Biosciences), CD11b: V450 (clone M1/70; BD Biosciences), CD11c: PE/Cy7 (clone HL3; BD Biosciences), Ly6C: Alexa 488 (clone HK1.4; Biolegend), Ly6G: Alexa 647 (clone IA8; Biolegend), IL-17a: V450 (clone TC11-18H10; BD Biosciences), and Lineage: Fitc (CD3/GR-1/CD11b/B220/Ter-119; Biolegend). Cells were fixed in 2% paraformaldehyde and measured using an LSR II flow cytometer (BD Immunocytometry Systems, San Jose, CA). FlowJo (Tree Star, Ashland, OR) software was used for analysis. Populations were initially gated on live cells in the forward versus side scatter and by CD45 vs eFluor 780 viability dye (eBioscience, San Diego, CA). Gates were then

set based on negative controls, single-staining, and fluorescence minus one controls.

2.9. Statistics

Results from multiple different groups were compared using a one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison post-hoc test. Comparison of two means was performed by a 2-tailed Student's T-test. Data was analyzed using GraphPad Prism 5 software (GraphPad Software; San Diego, CA). A p value < 0.05 was considered statistically significant.

3.0 RESULTS

3.1 P2X7R signaling is involved in the development of a psoriasiform phenotype induced by rIL-23.

Previous studies have demonstrated that P2X7R expression is increased in psoriatic lesions [120, 124]. Thus, to test our hypothesis that P2X7R signaling in the skin has a role in the development and maintenance of psoriatic lesions we induced two acute models of psoriasis (rIL-23 and IMQ) in P2X7R -/- mice.

The rIL-23 and IMQ, a TLR7/8 agonist, models of psoriasis share many features of human psoriasis and have been utilized successfully in multiple strains of mice to provide valuable insight into the pathogenesis of psoriasis [127, 128]. Recombinant IL-23 was injected intradermally or IMQ was topically applied daily to WT ($P2X7R^{+/+}$) and $P2X7R^{-/-}$ mice for 5 days. In the acute rIL-23 and IMQ models, psoriasiform dermatitis develops in WT mice, with marked epidermal hyperplasia, increased inflammatory infiltrates, parakeratosis, and microabscess formation (Figure 7A, left panels).

Consistent with our hypothesis, in P2X7R -/- mice rIL-23 does not promote a psoriasis-like phenotype. Conversely, in the IMQ model, P2X7R -/- mice have considerable cutaneous inflammatory infiltrate and epidermal hyperplasia (Figure 7A). These findings are consistent with those recently described by Ronald Sluyter (personal communication). Together this data suggests that the rIL-23 model utilizes the P2X7R pathway to induce a psoriasis-like phenotype and the IMQ model does not utilize this pathway.

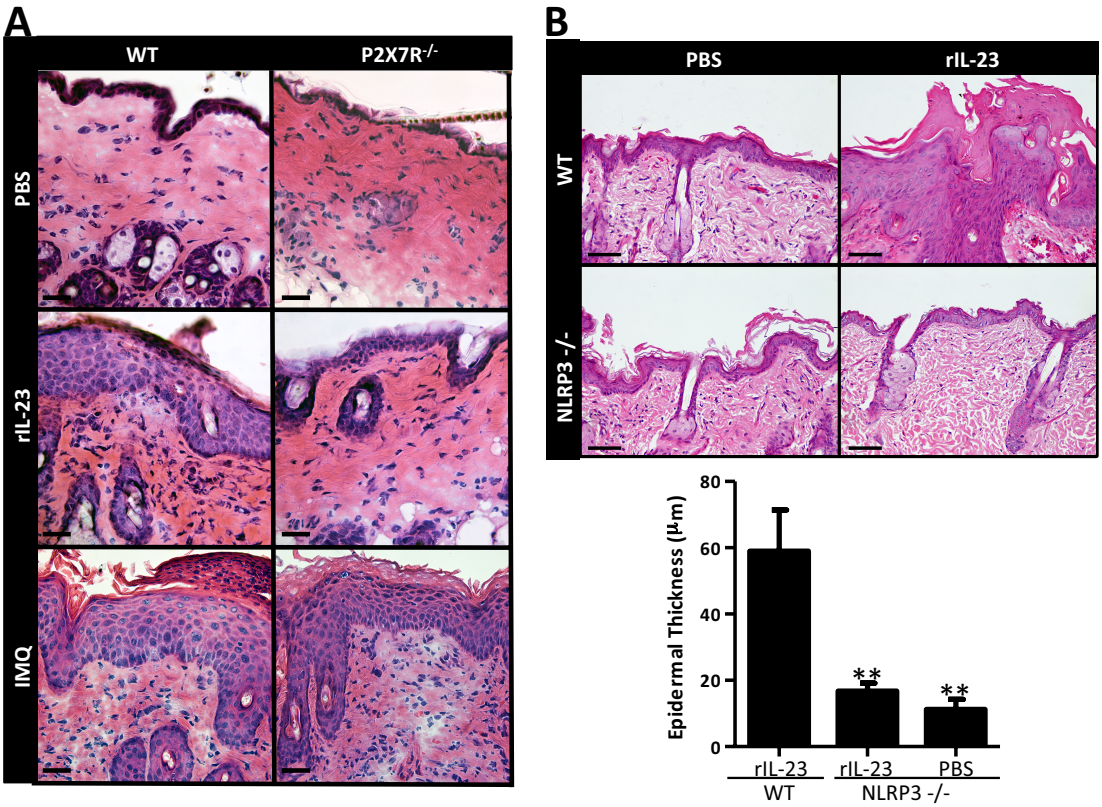


Fig 7. P2X7R signaling is necessary for the induction of acute psoriasiform inflammation induced by rIL-23 but not IMQ. (**A**) rIL-23 and IMQ were utilized to induce inflammation in P2X7R -/- mice or age-matched WT mice. On day 6 skin samples were collected and stained with H&E to assess the histological phenotype. Three mice per treatment group. Measure bar = 50 µm. (**B**) WT or NLRP3 -/- mice were injected daily for 5 days with rIL-23 or PBS control. On day 6 skin samples were collected and stained with H&E to assess histological phenotype, measure bar = 100 µm. Epithelial thickness was quantitated. Bars represent the mean ± SEM of 4-5 mice, 10 HPF were averaged for each mouse. Asterisks indicate a significant difference compared to WT mice treated with rIL-23, ** = $p < 0.01$. One representative of two independent experiments with 4-5 mice per treatment group.

Inflammasome activation and production of mature IL-1β is a prototypical pathway activated by P2X7R signaling [129]. However, the contribution of the inflammasome to the development of psoriasis in the rIL-23 model has not been described. To determine if inhibition of the rIL-23 model of psoriasis in the P2X7R -/- mice was due, in part, to the absence of the P2X7R/NLRP3 pathway, we induced the rIL-23 model in NLRP3 -/- mice. In NLRP3 +/+ mice, rIL-23 induced a characteristic psoriasiform phenotype with epidermal thickening, parakeratosis, and microabscess formation (Figure 7B). However,

in NLRP3 -/- mice injected with rIL-23 there was a loss of the psoriasiform phenotype and a significant decrease in the epidermal thickness, compared to NLRP3 +/+ mice (Figure 7B). Interestingly, studies have previously demonstrated that IMQ is capable of inducing inflammation in NLRP3 -/- mice [130], which is consistent with our finding that IMQ is capable of inducing inflammation in the P2X7R -/- mice (Figure 7A). These data demonstrating that the rIL-23 model is dependent on the NLRP3 inflammasome pathway and IMQ is not, provide a potential explanation for why rIL-23 is not capable of inducing psoriasiform inflammation in P2X7R -/- mice and IMQ can induce a potent inflammatory response. Overall, the lack of inflammation in the rIL-23/P2X7R -/- model was highly encouraging and prompted the further investigation of P2X7R signaling in psoriasis.

3.2 P2X7R signaling induces inflammatory skin changes compatible with early psoriasiform dermatitis.

To determine if directly signaling through the P2X7R could lead to the development of psoriasis-like lesions we intradermally injected mice daily for 4 d with 350 μM of BzATP, a selective P2X7R agonist that exhibits 5-10 fold greater potency over ATP. Titration studies were performed to determine the appropriate dose of BzATP (data not shown). Intradermal injections of BzATP induced the development of parakeratosis and a significant increase in epidermal hyperplasia compared to PBS vehicle control (Figure 8A and B). However, BzATP alone did not induce a full-fledge psoriasiform dermatitis. BzATP, like ATP, is hydrolyzed by ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDase) into the anti-inflammatory adenosine molecules and therefore adenosine is likely blocking the development of a psoriasis-like phenotype. Studies conducted by Michaud et al. [131] demonstrated that intratumoral administration of a selective NTPDase inhibitor lead to the increase in extracellular ATP and restored

chemotherapeutic responses. Thus, in conjunction with BzATP mice were injected with POM1, an inhibitor of E-NTPDases [132]. BzATP + POM1 initiates the development of a full psoriasiform inflammatory response, characterized in these slides by prominent parakeratosis, microabscess formation, and a significant increase in epidermal thickness compared to PBS and BzATP alone (Figure 8A and B). In the dermis the level of vascularization was increased with the vertical elongation, activation, and congestion of blood vessels in mice treated with BzATP + POM1, compared to PBS control, as determined by histopathological examination and CD31 expression (Figure 8C). Furthermore, hair follicle miniaturization with sebaceous gland hypoplasia and infundibulum dilation, classical signs of psoriasis in human scalp [133, 134] were observed in the hairy skin of these mice. This response is an acute inflammatory response that begins to resolve after 4 days of treatment.

While BzATP is considered a selective P2X7R agonist, BzATP still exhibits partial agonist activity at other purinergic receptors, P2X1 and P2Y1 receptors. Thus, to confirm that the observed response is occurring through the P2X7R we utilized A438079 (A4), a competitive P2X7R antagonist that is inactive at other P2 receptors [125, 135]. For this study, mice were treated with BzATP + POM1 or BzATP + POM1 + A4 daily for 4 days. Treatments with A4 block the inflammatory response eliminating acanthosis, parakeratosis, and microabscess formation induced by BzATP + POM1 (Figure 8D). Likewise, when P2X7R -/- mice are treated with BzATP + POM1 an inflammatory response does not develop and epidermal thickness is significantly decreased compared to WT mice (data not shown). Importantly, these findings strongly support our hypothesis that directly signaling through the P2X7R can induce the development of a psoriasis-like response. Moreover, our studies exclude changes secondary to drug reactions and other types of hypersensitivity reactions that could mimic psoriasis.

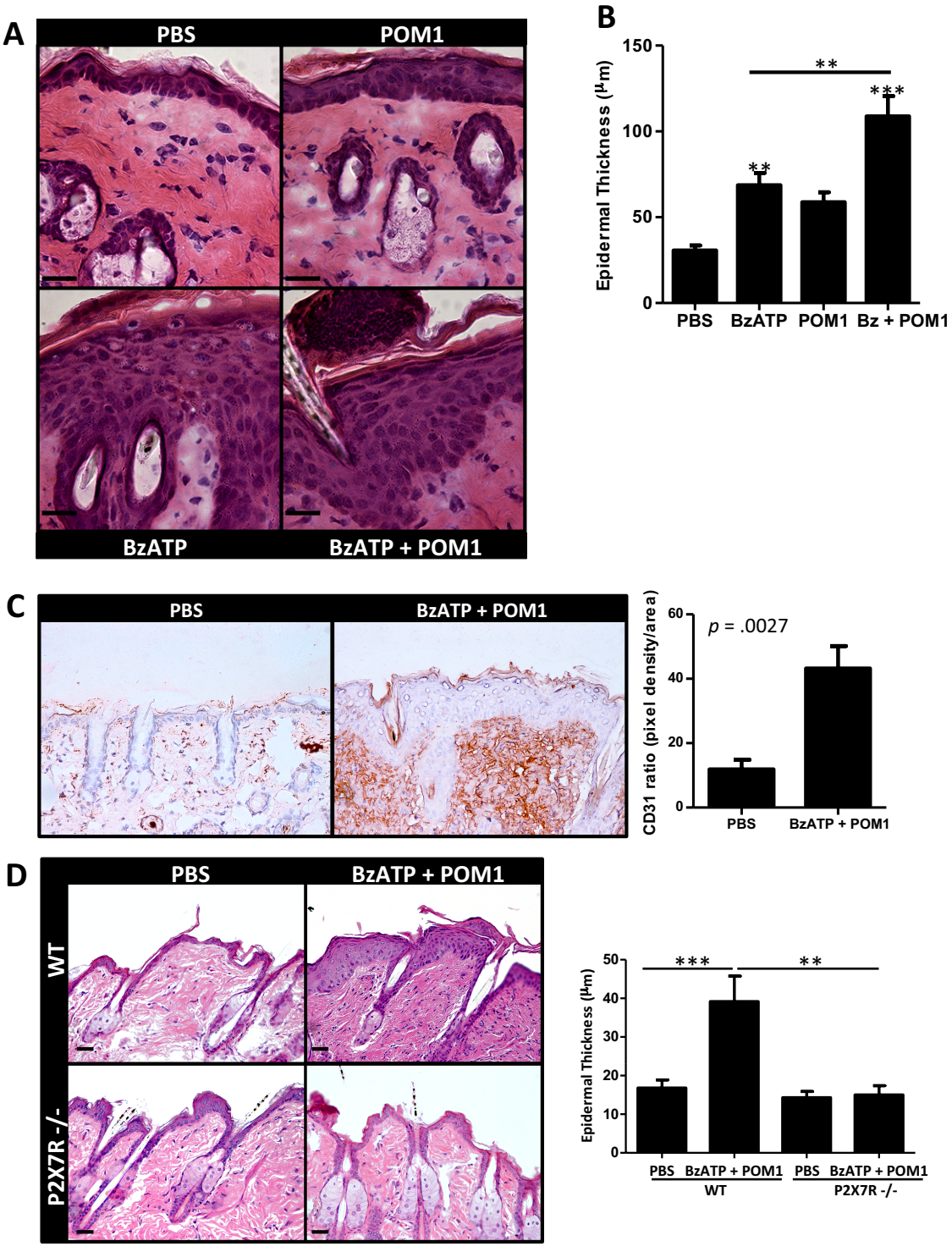

A
PBS
POM1
BzATP
BzATP + POM1
B
Epidermal Thickness (μm)
150
100
50
0
**
***
**
PBS
BzATP
POM1
Bz + POM1
C
PBS
BzATP + POM1
CD31 ratio (pixel density/area)
60
40
20
0
p = .0027
PBS
BzATP + POM1
D
PBS
BzATP + POM1
WT
P2X7R -/-
Epidermal Thickness (μm)
50
40
30
20
10
0
***
**
PBS
BzATP + POM1
PBS
BzATP + POM1
WT
P2X7R -/-

Fig 8. Signaling via the P2X7R induces a psoriasis-like inflammatory phenotype in mice. C57Bl/6 mice were injected daily for 4 d with BzATP ± POM1, an inhibitor of NTPDases, or PBS (vehicle control). (**A**) On day 5 skin samples were collected and stained with H&E to assess histological phenotype, measure bar = 50 μm. (**B**) Epidermal thickness was quantitated, bars are the mean ± SEM, three independent measurements were averaged from each mouse (n=10 from multiple independent experiments). (**C**) On day 5 skin samples were collected and immunohistochemically stained with CD31 (brown) and counter-stained with hematoxylin. The density of CD31 expression was quantitated and expressed and graph represents the ratio of pixel density/area ± SEM (n = 6-7 mice). (**D**) P2X7R -/- or WT mice were injected daily for 4 d with BzATP ± POM1 or PBS. On day 5 skin samples were collected and stained with H&E to assess histological phenotype, measure bar = 100 μm. Epidermal thickness was quantitated, bars are the mean ± SEM, ten independent measurements were averaged from each mouse (n = 4-6). One representative of two independent experiments. Asterisks indicate a significant difference compared to PBS control unless otherwise indicated. **= $p < 0.01$ and *** = $p < 0.001$.

We have observed that BzATP treatments must be injected very superficially approximate to the epidermal layer or the inflammatory response induced will be more characteristic of panniculitis. Thus, to confirm our observations, we utilized a microneedle array (MNA) technology that can be designed to deposit its contents, in this case our treatment groups, very close to the epidermis. Interestingly, unlike BzATP injections, MNA/BzATP was capable of inducing a potent psoriasiform dermatitis, characterized by a significant increase in acanthosis, parakeratosis, and microabscess formation, similar to that observed with MNA/BzATP+POM1. Thus, by directly targeting the P2X7R expressed on keratinocytes BzATP is capable of enacting an inflammatory response before becoming hydrolyzed into adenosine. However, some inflammation was observed in the blank MNA treatment group.

3.3. Signaling through the P2X7R induces an increase in inflammatory infiltrates that impart a critical role on the development of the psoriasiform dermatitis phenotype.

To assess the inflammatory infiltrates induced following P2X7R signaling, cutaneous cross-sections were collected on day 5 and examined by immunofluorescence. Tissues

were stained with antibodies specific for F4/80 (a macrophage marker), MHC-class II (a marker for antigen presenting cells), and GR-1 (a granulocyte marker). By day 5, compared to the PBS treatment group, there was an observed increase in inflammatory infiltrates in the BzATP treatment group, infiltrates were highly enriched for cells expressing MHC-class II (Figure 9A). Compared to both the PBS and BzATP treatment groups, BzATP + POM1 induced a marked increase in inflammatory infiltrates into the papillary dermis and the superficial perivascular areas that are $F4/80^{+}$, MHC class II^{+}, and $Gr\text{-}1^{+}$ (Figure 9A). To further delineate and quantitate the infiltrate flow cytometry was performed on cutaneous signal-cell suspensions on day 5. Based on the gating strategy previously described by Pommier et al. [136], it was determined that $LY6C^{high}CD11c^{-}$ inflammatory monocytes were increased, though due to experimental variations did not reach statistical significance, while $LY6G^{high}$ $CD11c^{-}$ neutrophils, and $LY6C^{high}CD11c^{+}$ inflammatory DCs were significantly increased following BzATP + POM1 treatments, compared to PBS controls (Figure 9B).

Psoriasis is a chronic inflammatory skin disease dependent on the IL-23/IL-17 axis [41]. Studies in murine models of psoriasis have demonstrated that innate γδ T-cells, neutrophils, and innate lymphoid cells (ILCs) are the cells secreting the majority of IL-17 compared to adaptive Th17 cells [98-100, 102, 103]. However, it is not known which cells are responding to exogenous ATP and P2X7R signaling in order to induce the psoriasiform response. Therefore, to determine which cell populations are important for the induction of the inflammatory response and to further identify the inflammatory cells secreting IL-17, flow cytometry was utilized. First, the production of IL-17 was confirmed, mice treated with BzATP + POM1 had a significant increase in cutaneous IL-17-secreting cells, compared to PBS alone (Figure 10A, top panels). Further characterization determined that the cells expressing IL-17 were $CD45^{+}CD3^{-}CD127^{-}$ $Lineage^{+}$ (Figure 10A, bottom panels). In this regard, there was a significant increase in

$CD45^{+}CD3^{-}CD127^{-}Lineage^{+}IL\text{-}17^{+}$ cells following BzATP + POM1 treatments compared to PBS alone (Figure 10A, bottom panels).

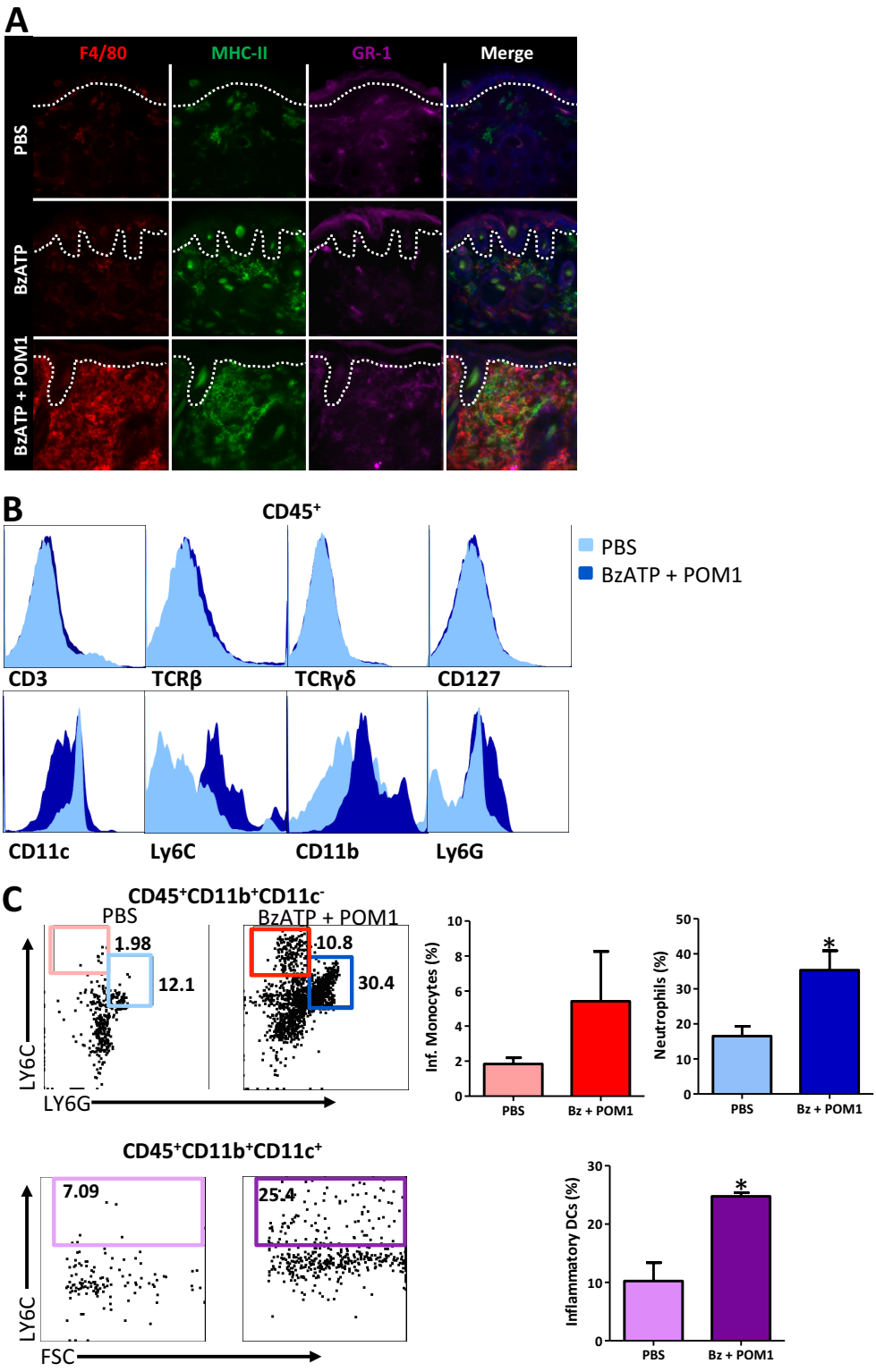
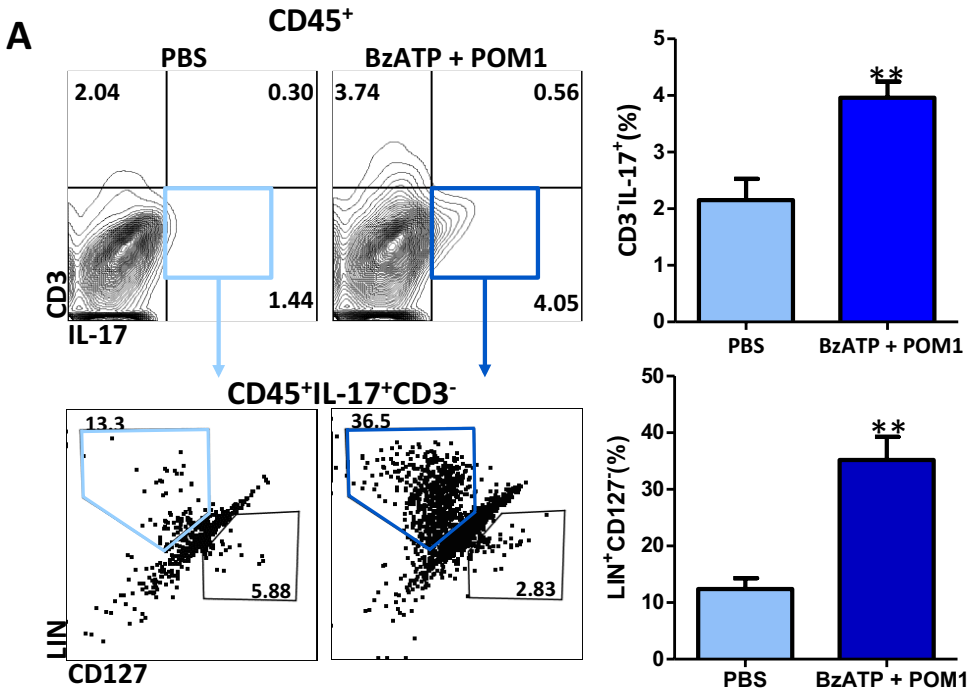


Fig 9. Inflammatory monocytes, inflammatory DCs, and neutrophils are increased in the skin following stimulation through the P2X7R. C57Bl/6 mice were injected daily for 4 d with BzATP ± POM1 or PBS (vehicle control). (**A**) On day 5 skin sections were collected and immunofluorescently labeled with F4/80 (red), MHC-II (green), and GR-1 (purple) specific Abs. Merged panels include all three stains and DAPI nuclear counter-stain. Dashed line indicates epidermal-dermal junction. (**B**) Expression of CD3, TCRβ, TCR γδ, CD127, CD11c, CD11b, LY6C, and LY6G on cutaneous inflammatory infiltrates following PBS or BzATP + POM1 treatments on day 5. (**C**) A more in-depth phenotypic analysis of cutaneous infiltration was performed by flow cytometry. Signal-cell suspensions were assessed for $CD45^+CD11b^+CD11c^-Ly6C^+$ inflammatory monocytes, $CD45^+CD11b^+CD11c^-Ly6G^+$ neutrophils (top panels) and $CD45^+CD11b^+CD11c^+Ly6C^+$ inflammatory DCs (bottom panels). In the right panels the bars represent the mean ± SEM of the percentage of each population indicated. Four mice per treatment group were divided into two tubes for staining. One representative of two independent experiments. *= $p < 0.05$

The markers utilized eliminated Th17 cells ($CD3^+$), γδ T-cells ($CD3^+$), and ILCs ($CD127^+$) as producers of IL-17 following P2X7R signaling. Within the lineage population are neutrophils, which have been demonstrated to express IL-17 [101, 138] and are significantly increased in the skin following BzATP + POM1 (Figure 9A and B). Therefore, to determine if the inflammatory response induced following P2X7R stimulation is dependent on neutrophils in this model, mice were treated with LY6G Ab to deplete neutrophils. Consistent with previous studies, BzATP + POM1 induced a psoriasis-like phenotype with a significant increase in epidermal thickness (Figure 10C). Mice treated with BzATP + POM1 in the presence of LY6G Ab did not develop a prominent psoriatic phenotype. Nevertheless, there is still a significant increase in epidermal thickness in the BzATP + POM1 + LY6G Ab treatment group, compared to PBS controls (Figure 10C). However, this increase does not attain the degree of thickness observed in mice treated with BzATP + POM1. Interestingly, there is a significant increase in mitotic keratinocytes in both the BzATP + POM1 and the BzATP + POM1 + LY6G Ab treatment groups compared to PBS controls (Figure 10C). Overall, this data indicates that neutrophils contribute to the development of the acute psoriasiform dermatitis induced following P2X7R signaling, in part, by secreting IL-17.

Moreover, keratinocytes promote inflammation by directly responding to BzATP treatments, as determined by the significant increase of keratinocytes in mitosis.



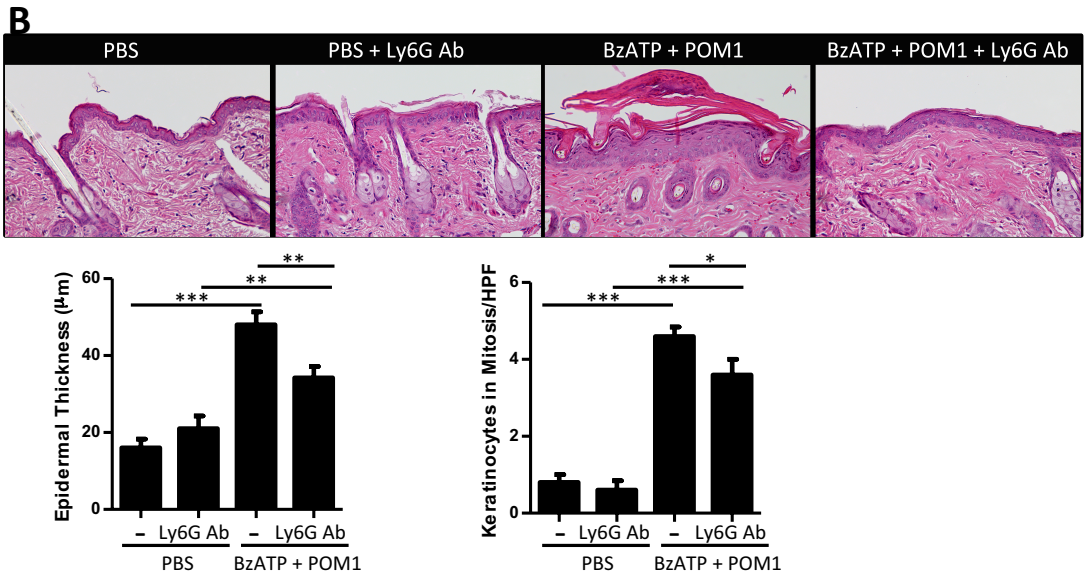


Fig 10. Acute inflammation is induced by IL-17 secreting neutrophils. C57Bl/6 mice were injected daily for 4 d with BzATP + POM1 or PBS (vehicle control). (**A**) Injection site skin was collected on day 5 and single-cell suspensions were stained with CD45-, IL-17-, CD3-, and Lineage (LIN)-specific Abs, 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 ± SEM (n=3-5) of the percentage of $CD3^-IL\text{-}17^+$ cells. The bottom left panels were further gated on the $CD3^-IL\text{-}17^+$ cells (red and green boxes). The bottom right graph represents the mean ± SEM (n=3-5) of the percentage of the LIN^+CD127^- cells. One representative of two independent experiments. Asterisks indicate a significant difference compared to PBS treatment, **= $p < 0.01$. (**B**) In addition to BzATP + POM1, mice were also treated with LY6G antibodies. On day 5 skin samples were collected and stained with H&E to assess histological phenotype, measure bar = 100 µm. Epithelial thickness and keratinocyte mitosis was quantitated. Bars represent the mean ± SEM of 5 mice, 10 HPF were averaged for each mouse. One representative of two independent experiments with 4-5 mice per treatment group and two injection sites per mouse. Asterisks indicate a significant difference between indicated groups.*= $p < 0.05$,**= $p < 0.01$, and *** = $p < 0.001$.

3.4. The role of innate inflammatory cytokines induced following cutaneous signaling through the P2X7R.

To evaluate the role of the innate cytokines induced following P2X7R signaling, mice were first treated with BzATP in the presence or absence of POM1, and skin was evaluated for changes in gene expression by qRT-PCR 12 h and 72 h following initial treatment. At the 12 h time point there was a significant increase in IL-1β, IL-1α, and S100A9 in the BzATP + POM1 treatment group compared to PBS control (Figure 11A). Additionally, there is a trend for increased TNFα that does not quite reach statistical significance by an ANOVA. By 72 h following injections there was a significant increase in IL-6 in the BzATP + POM1 treatment group compared to PBS control and also at this time point the significant increase in IL-1β is sustained (Figure 11B). However, IL-17 mRNA was not detect at any time point. We next expanded out studies to assess the protein expression by Luminex technologies at 72 h and 120 h into the treatment regimen. Compared to PBS control, IL-1α, IL-23, and S100A9 were significantly increased at 72 h and IL-6 was significantly increased at 120 h in the BzATP + POM1 treatment group (Figure 11C). These data are consistent with *ex vivo* studies demonstrating the capacity of P2X7R signaling to induce the expression of characteristic psoriasis cytokines [124].

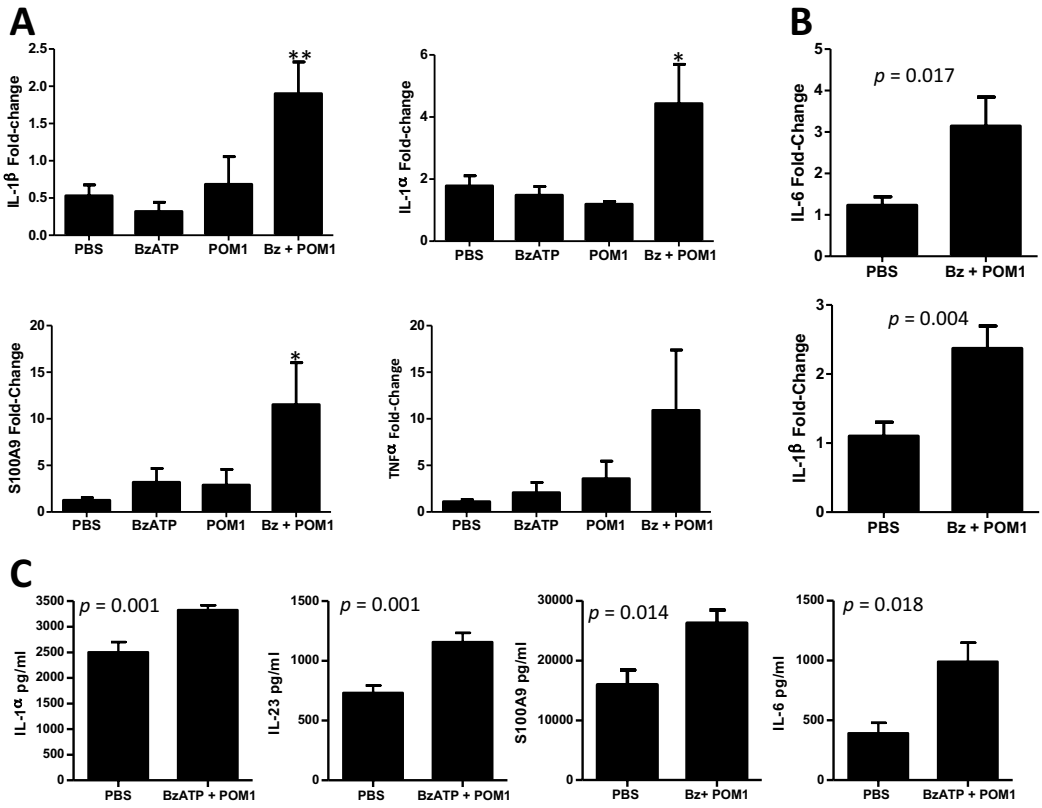


Fig 11. Innate cytokines are induced following cutaneous signaling through the P2X7R. (**A and B**) Bar graphs demonstrate the relative fold change in mRNA expression of IL-1β, IL-1α, S100A9, TNF-α, and IL-6 (**A**) 12h (IL-1β, IL-1$^{\alpha}$, S100A9 and TNFα) and **(B)** 72 hr (IL-1β and IL-6) following cutaneous injections with BzATP ± POM1 normalized to PBS injected controls. Fold-change was determined using the relative qRT-PCR $2^{-\Delta\Delta Ct}$ method. **(C)** Bar graphs represent the protein concentration of IL-1α and IL-23 at 72 h and S100A9 and IL-6 at 120 h. **(A-C)** Data are expressed as mean ± SEM of 4-10 individual mice, each sample was ran in triplicate or RT-PCR and duplicate for Luminex protein samples. Asterisk indicates a significant difference compared to PBS from multiple comparison groups, * = $p < 0.05$ and ** = $p < 0.01$. For only two groups the p value is indicate.

One of the downstream effectors of P2X7R signaling is IL-23 (Figure 11C) [18, 28], which enhances and terminally differentiates Th17 responses and activates innate IL-17 secreting cells, such as γδ Tcells, ILCs, and neutrophils [114, 139, 140]. Thus, to determine if the IL-23 pathway contributes to the development of the psoriasis dermatitis induced by BzATP + POM1 injections, IL-23 -/- mice were injected with BzATP + POM1.

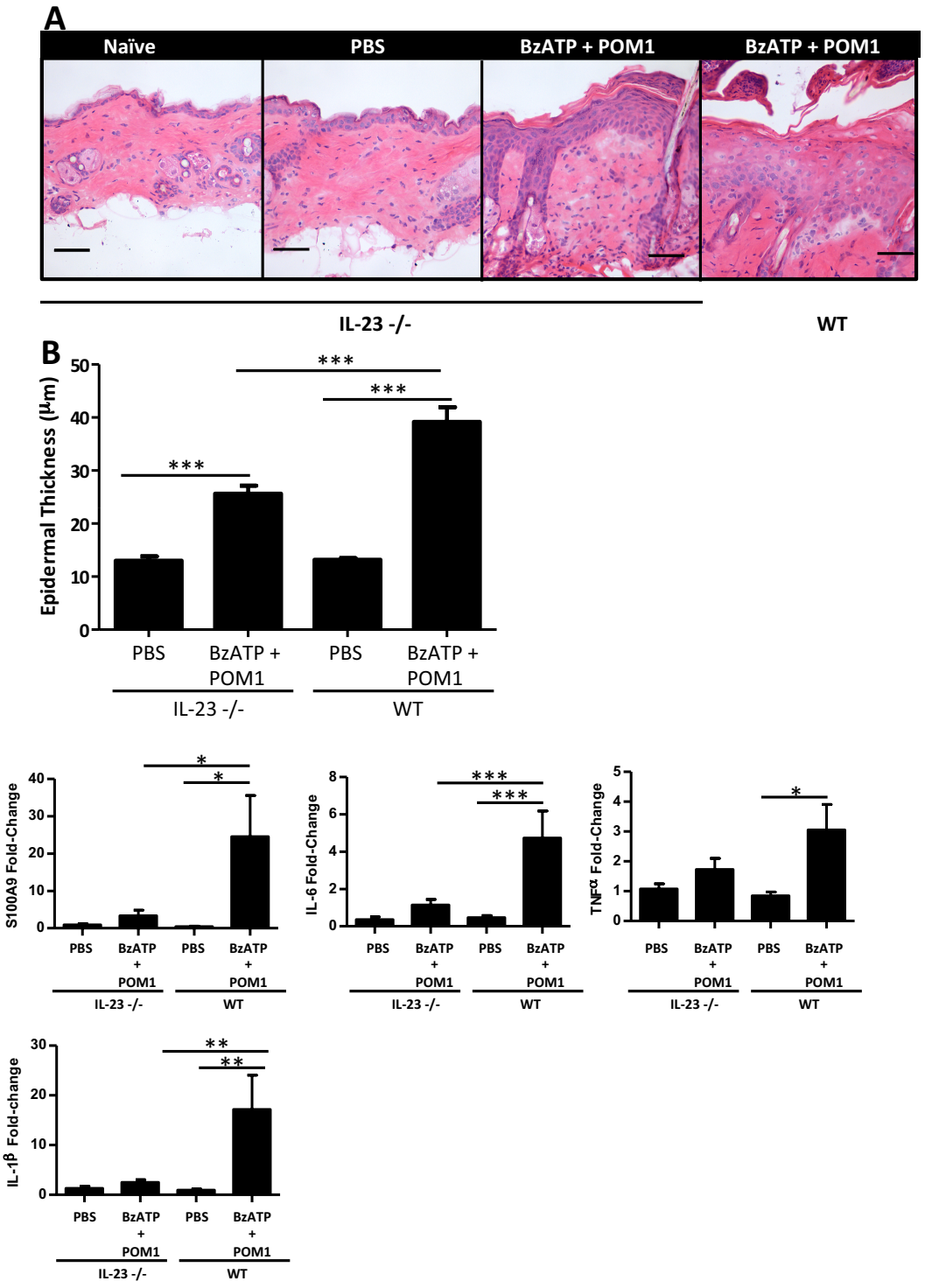

A
Naïve
PBS
BzATP + POM1
BzATP + POM1
IL-23 -/-
WT
B
Epidermal Thickness (µm)
***
***
***
PBS
BzATP + POM1
PBS
BzATP + POM1
IL-23 -/-
WT
S100A9 Fold-Change
IL-6 Fold-Change
TNFα Fold-Change
IL-1β Fold-change
PBS
BzATP + POM1
IL-23 -/-
WT

Fig 12. The acute psoriasiform inflammation induced by P2X7R signaling is potentiated by IL-23 but not dependent on IL-23. IL-23 -/- mice and age-matched C57Bl/6 WT mice were injected with BzATP + POM1 daily for 4 days. On day 5 skin samples were collected and stained with H&E to assess histological phenotype, measure bar = 100 µm. (**B**) Epithelial thickness was quantitated. Bars represent the mean ± SEM of 4-5 mice, 10 HPF were averaged for each mouse. Asterisks indicate a significant difference between indicated groups, *** = $p < 0.001$. One representative of three independent experiments with 4-5 mice per treatment group and two injection sites per mouse.

In the absence of IL-23, BzATP + POM1 was still capable of inducing an inflammatory psoriasis-like phenotype with the presence of significant epidermal thickening, parakeratosis, and microabscess formation (Figure 12A and B). Though the inflammatory response did not tend to be as potent in the IL-23 -/- mice as in the IL-23 +/+ mice (Figure 12A and B). Thus, we can conclude that IL-23 can potentiate the inflammatory response induced following P2X7R stimulation but it is not necessary, likely due to the multifactorial nature of psoriasis.

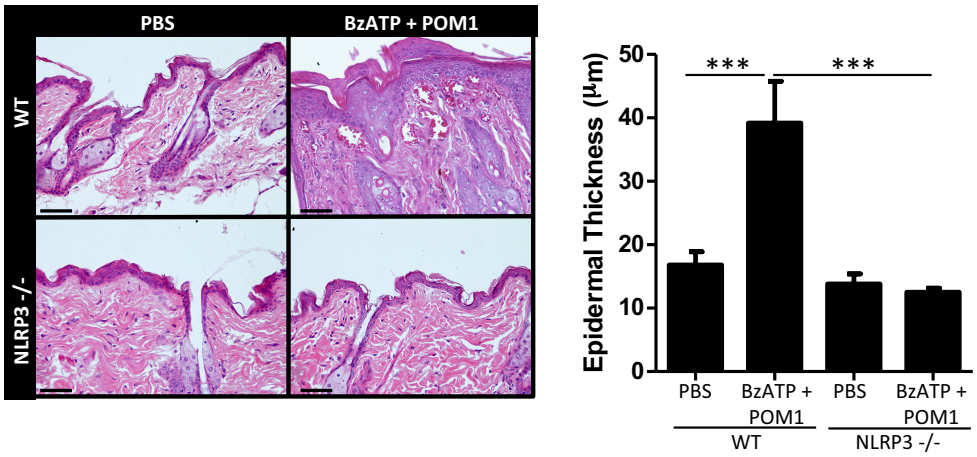


Fig 13. The psoriasiform inflammation induced following P2X7R stimulation or rIL-23 injections is dependent on the NLRP3 inflammasome. NLRP3 -/- or WT C57Bl/6 mice were injected daily for 4 days with BzATP + POM1 or PBS. On day 5 skin samples were collected and stained with H&E to assess histological phenotype, measure bar = 100 µm. Epithelial thickness was quantitated. Bars represent the mean ± SEM of 4-5 mice, 10 HPF were averaged for each mouse. Asterisks indicate a significant difference between indicated groups, *** = $p < 0.001$. One representative of three independent experiments with 4-5 mice per experiment and two injection sites per mouse.

Upstream of IL-23 is the NLRP3 inflammasome/IL-1β pathway. Studies support a role for an aberrant inflammasome/IL-1β pathway in psoriasis [141-145]. Moreover, IL-1β is a key differentiation cytokine for Th17 cells. While inflammasome activation is a prototypical pathway activated by P2X7R signaling, P2X7R signaling can induce both inflammasome-dependent and -independent responses. Therefore, the inflammasome/IL-1β pathway was assessed by treating NLRP3 -/- mice with BzATP + POM1. In the NLRP3 -/- mice, inflammation is not induced when mice were treated with BzATP + POM1, compared to NLRP3 +/+ mice (Figure 13). Consistent with the histological observations, there was a significant decrease in epidermal thickness in the NLRP3 -/- mice compared to NLRP3 +/+ mice. Therefore, these studies indicate that the psoriasiform response induced following P2X7R signaling is dependent on the NLRP3 inflammasome pathway (Figure 14). These studies are consistent, demonstrating that the rIL-23 model of psoriasis was also dependent on the NLRP3 inflammasome.

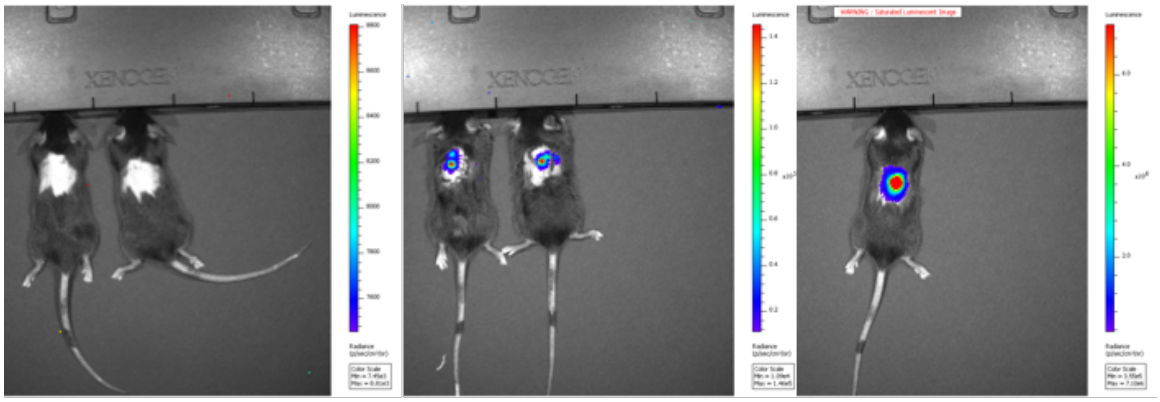


Fig 14. P2X7R and inflammasome in rIL-23 model of psoriasiform dermatitis.

Finally, we translated our results performing similar experiments in human non-lesional skin engrafted in mice obtaining similar results (Figure 15 and 16).

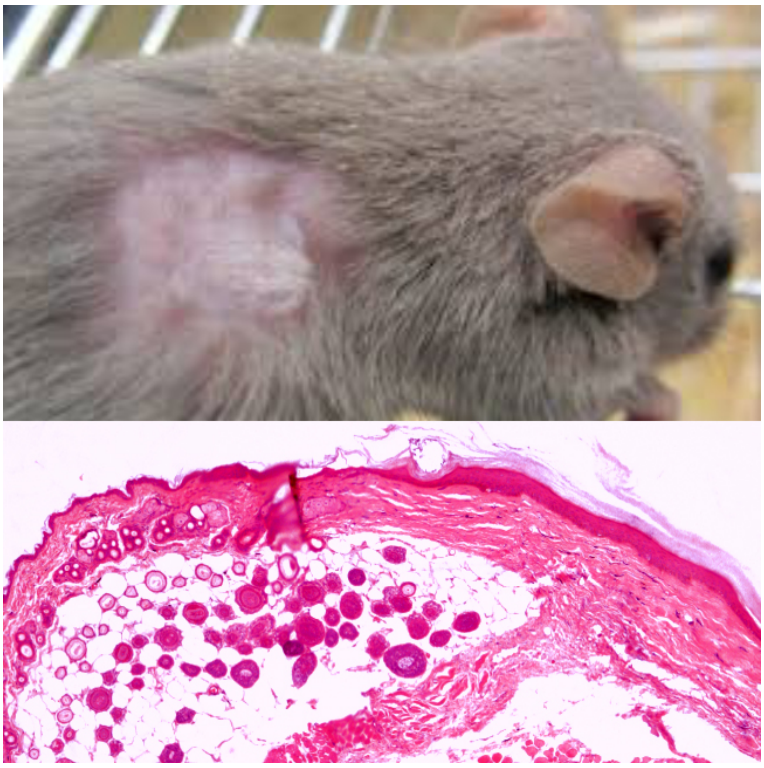


Fig 15. Transplanted human skin into mice.

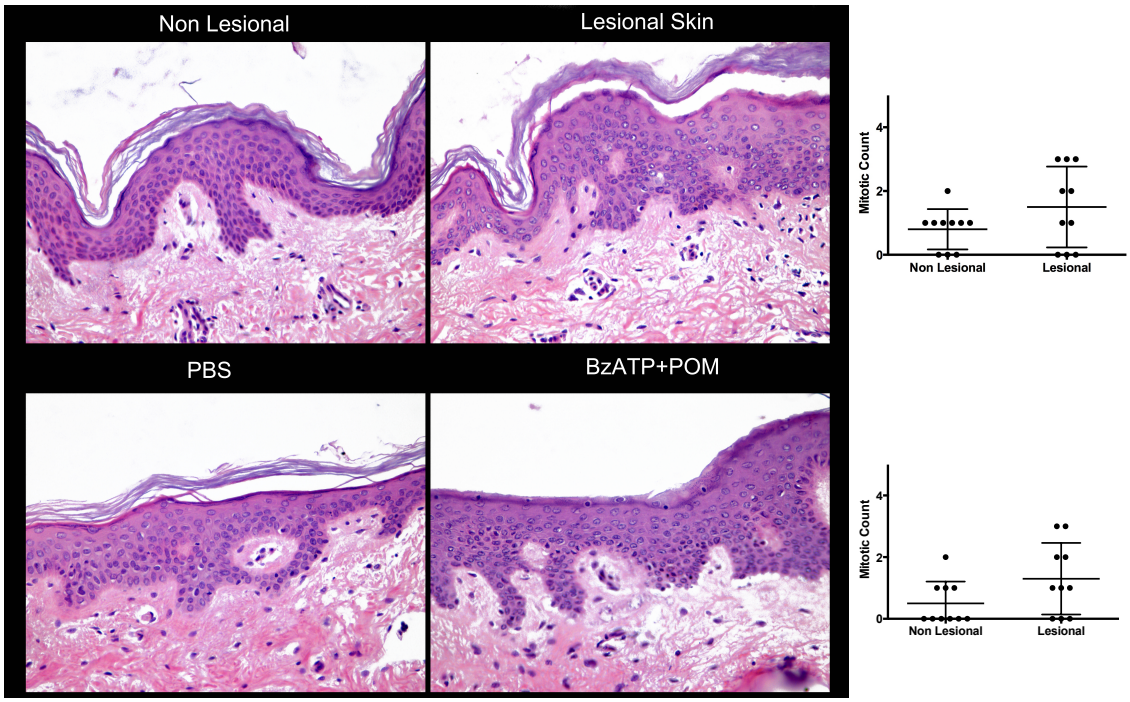


Fig 16. The engrafted non lesional human skin from a psoriatic patient into mice show features of Psoriasiform dermatitis after challenge with BzATP.

4.0. DISCUSSION

Psoriasis has no cure. To date there are a variety of topical and systemic treatments for psoriasis patients; however, these treatments are not effective in all patients, relapses tend to occur, and less than 40% of patients are satisfied with their therapies [146, 147]. Improvement in our scientific knowledge of psoriasis is necessary to advance our understanding of the pathogenesis of psoriasis so that more physiologically oriented psoriasis therapies maybe elucidate. Psoriasis is dependent on environmental and genetic influences that render the skin susceptible to an imbalance in pro-inflammatory cytokines, chemokines, and growth factors stimulating aberrant immunity and epidermal keratinocyte functions. Several triggers have been proposed as initiator events for psoriasis, including IL-1, IL-6, CAMP/LL-37 (cathelicidin), TNFα, and alarmins, such as ATP [106].

Following 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 [107, 108]. Supporting this is the Koebner phenomenon in which lesions frequently develop at sites of trauma. However, the role of alarmins in psoriasis pathogenesis has not been well addressed. Here we demonstrate that signaling through the P2X7R induced an acute innate inflammatory response and the proliferative activity of keratinocytes leading to the development of acanthosis, prominent parakeratosis/hyperkeratosis, and the formation of microabscesses.

There was also the characteristic expansion of dermal vascularization and an increase in innate inflammatory infiltrates into the papillary dermis, such as neutrophils, inflammatory monocytes, and inflammatory DCs [117, 158-160]. Moreover, following

P2X7R signaling the cytokine profile, including IL-1β, IL-1α, IL-6, IL-23, S100A9, TNFα, and IL-17, was distinctive of a psoriasiform response [106, 107, 159]. Thus, through the cutaneous injections of alarmins we have demonstrated that signaling through the cutaneous P2X7R for ATP leads to the development of a psoriasiform inflammatory response that mirrors the pathophysiologic immune and histopathologic phenotype of psoriasis in humans. Indicating that ATP released following trauma and/or stress can lead to the development of psoriatic lesions in a susceptible microenvironment.

The P2X7R-dependent inflammatory response began to quickly resolve after day 4 of treatments. Moreover, by this time point we did not observe the increase of Tcells into the inflamed skin. Studies by Nakajima et al. [150] demonstrated that in the absence of adaptive T cells, innate IL-1R signaling can still lead to the development of a psoriasis-like inflammatory response, consistent with our observations following P2X7R stimulation. 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 and alternates with an adaptive autoimmune response [152]. Thus, the response observed following the cutaneous injections of BzATP + POM1 mirror the innate autoinflammatory phase of psoriasis. However, in our model, the development of the adaptive inflammatory response may be inhibited by the eventual hydrolysis of BzATP into the anti-inflammatory adenosine, the desensitization of the P2X7R, or the absence of a genetic component relative to psoriasis. Studies are underway to determine if signaling through the P2X7R can ultimately lead to the adaptive autoimmune phase by utilizing genetically predisposed models, overexpressing P2X7R, and variable dosing techniques, studies which are beyond the scope of this manuscript.

We observed the significant increase in cutaneous IL-17-secreting neutrophils following P2X7R stimulation, consistent with the development of an autoinflammatory response and that neutrophils are a predominant cell type expressing IL-17 in psoriasis, [101, 138, 152]. Mechanistic studies depleting neutrophils confirmed their importance in the development of the P2X7R-dependent innate inflammatory response. However, IL-17 mRNA was not detected at the time points tested. It is possible that we missed an early transcript expression but studies examining psoriatic lesions have indicated that neutrophils, like mast cells, have preformed IL-17 that is released by degranulation and through ETosis [101, 138].

Thus, in the studies presented herein the neutrophils that are migrating into the cutaneous sites of inflammation are likely releasing preformed IL-17 that can induce a potent inflammatory response. However, IL-17 is involved in inducing the recruitment of neutrophils to the skin by triggering the release of cytokines and chemokines from keratinocytes [153, 154, 155]. Therefore, we question how neutrophils are being attracted to the skin if they are the cells expressing IL-17. In this respect, studies have suggested that ATP can act as a find-me signal directly attracting neutrophils functionally expressing P2X7R to sites of stress or trauma, where ATP is being released [156,157]. Furthermore, IL-1β released by activated keratinocytes or antigen presenting cells can also lead to cutaneous neutrophil recruitment [125, 158]. Da Silva et al. [125] demonstrated that P2X7R is necessary for cutaneous neutrophil migration in a model of irritant contact dermatitis. Importantly, in the studies by da Silva and those presented herein, the neutrophil accumulation appeared to be dependent on IL-1β.

P2X7R is involved in the release of active IL-1β through the activation of the NLRP3 inflammasome. Karmakar et al. [155] demonstrated that stimulation through the P2X7R

on neutrophils leads to the activation of the NLRP3 inflammasome and the secretion of active IL-1β. Consistent with our findings that inflammation was abrogated in NLRP3 -/- mice. Indicating that P2X7R signaling occurs through the NLRP3 inflammasome pathway, which has been indicated as important for autoimmune inflammation. Therefore, there appears to be a positive feedback mechanism in which keratinocytes and/or APCs are triggered by P2X7R stimulation to secrete IL-1β, which, in turn, enhances the accumulation of IL-17-secreting neutrophils at sites of stress or trauma. The IL-17 will then further act on keratinocytes to promote the expression of inflammatory cytokines, chemokines, and anti-microbial peptides leading to the perpetuation of the inflammatory response in a genetically susceptible microenvironment [164-167].

Conclusions

IL-23 is important for the expression of IL-17 from both innate and adaptive IL-17-secreting cells and therapeutics targeting IL-23 have been successfully utilized in patients with psoriasis. Moreover, the IL-1β pathway, which we have demonstrated to be important for the P2X7R-dependent psoriasis-like dermatitis, further promotes the IL-23 pathway. However, when IL-23 was knocked out in our mouse model we still observed a psoriasiform inflammatory response though not as prominent as in wildtype mice. It is possible that the IL-23 knockout mice have developed a compensatory mechanism or that the acute inflammatory response induced following P2X7R stimulation relies more heavily on the upstream IL-1β pathway.

Though in human trials IL-1β inhibition has not been highly successful, suggesting that the IL-1β pathway is not critical for thedevelopment of psoriatic lesions. However, because IL-1β is such an early responder its effects may already be enacted and set into motion early in the lesional development before treatments start; thus, IL-1β therapeutics may have a more substantial role as a maintenance therapeutic to prevent the development of future flair-ups. While P2X7R is also an early pathway upstream of IL-1β there are still multiple other downstream effects including the expression of IL-6, IL-23, IL-17, and S100A9 proteins that would also be targeted with P2X7R antagonists.

Together the results presented herein demonstrate that cutaneous inflammatory responses induced via purinergic signaling through the P2X7R have implications in the pathogenesis and potential treatment of inflammatory diseases, such as psoriasis.

Conclusiones

IL-23 es importante para la expresión de IL-17 a partir de células secretoras de IL-17 tanto innatas como adaptativas, y se han utilizado con éxito terapias dirigidas a IL-23 en pacientes con psoriasis. Además, la vía de la IL-1β, que hemos demostrado que es importante para la dermatitis similar a la psoriasis dependiente de P2X7R, promueve aún más la vía de la IL-23. Sin embargo, cuando se eliminó la IL-23 en nuestro modelo de ratón, todavía observamos una respuesta inflamatoria psoriasiforme, aunque no tan prominente como en los ratones de tipo salvaje. Es posible que los ratones con inactivación de IL-23 hayan desarrollado un mecanismo compensatorio o que la respuesta inflamatoria aguda inducida después de la estimulación de P2X7R se base más en la vía de IL-1β corriente arriba.

Aunque en ensayos con seres humanos, la inhibición de IL-1β no ha tenido mucho éxito, lo que sugiere que la vía de IL-1β no es crítica para el desarrollo de lesiones psoriásicas. Sin embargo, debido a que la IL-1β es un respondedor tan temprano, es posible que sus efectos ya se hayan promulgado y puesto en movimiento temprano en el desarrollo de la lesión antes de que comiencen los tratamientos; por tanto, las terapias de IL-1β pueden tener un papel más sustancial como una terapia de mantenimiento para prevenir el desarrollo de futuros ataques. Si bien P2X7R también es una vía temprana cadena arriba de IL-1β, todavía hay muchos otros efectos cadena abajo que incluyen la expresión de proteínas IL-6, IL-23, IL-17 y S100A9 que también serían dirigidas con antagonistas de P2X7R.

Juntos, los resultados presentados en este documento demuestran que las respuestas inflamatorias cutáneas inducidas mediante la señalización purinérgica a través de P2X7R tienen implicaciones en la patogénesis y el tratamiento potencial de enfermedades inflamatorias, como la psoriasis.

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