Unprocessed Interleukin-36α Regulates Psoriasis-Like Skin Inflammation in Cooperation With Interleukin-1

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Generalized pustular psoriasis is a severe skin disease characterized by epidermal hyperplasia, neutrophil-rich abscesses within the epidermis, and a mixed inflammatory infiltrate in the dermis. The disease may be caused by missense mutations in the IL-36 receptor antagonist, IL-36Ra. Curiously, the related IL-1Ra has therapeutic effects in some of these latter patients. Here, using an experimental mouse model of psoriasiform skin inflammation, we demonstrate *in vivo* connections between IL-36 and IL-1 expression. After disease initiation, IL-36α-deficient mice exhibited dramatically diminished skin pathology, including absence of epidermal neutrophils, reduced keratinocyte acanthosis, and less dermal edema. In contrast, IL-36β and IL-36γ knockout mice developed disease indistinguishable from that of wild-type mice. The endogenous IL-36α was not processed through proteolysis. Although IL-36α expression was strongly induced in an IL-1 signaling-dependent manner during disease, expression of IL-1α was also dependent upon IL-36α. Hence, after being upregulated by IL-1α, IL-36α acts through a feedback mechanism to boost IL-1α levels. Analyses of double knockout mice further revealed that IL-36α and IL-1α cooperate to promote psoriasis-like disease. In conclusion, IL-1α and IL-36α form a self-amplifying inflammatory loop *in vivo* that in patients with insufficient counter regulatory mechanisms may become hyper-engaged and/or chronic.

Journal of Investigative Dermatology (2015) **135,** 2992–3000; doi:10.1038/jid.2015.289; published online 13 August 2015

INTRODUCTION

Psoriasis encompasses a number of non-infectious inflammatory conditions of the skin (Raychaudhuri *et al.*, 2014). Generalized pustular psoriasis (GPP) is the most severe form involving not only skin inflammation, but also systemic symptoms such as fever and malaise. GPP skin inflammation is characterized by reddening of the skin and formation of epidermal pustules filled with neutrophils. When large areas of the skin are affected by GPP, the condition can be life-threatening. Palmoplantar pustulosis affects specifically the hands and feet, and like GPP involves formation of neutrophil-containing pustules in the epidermis. The most common form of psoriasis is plaque psoriasis. This disease is characterized by red plaques of inflamed skin, which are often scaly owing to dysregulated differentiation of the epidermis. Although pustules do not form in plaque psoriasis *per se*, increased neutrophil recruitment into the epidermis is still observed. Typically, these neutrophils cluster together within the stratum corneum of the epidermis and are known as Munro's microabscesses.

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Abbreviation: GPP, generalized pustular psoriasis

Received 26 March 2015; revised 22 June 2015; accepted 5 July 2015; accepted article preview online 23 July 2015; published online 13 August 2015

The etiology of the different types of psoriasis is poorly understood; however, a seminal advancement of our knowledge was made in 2011, when missense mutations within the gene, *IL36RN*, encoding the IL-36 receptor antagonist (IL-36Ra) were identified in patients with GPP (Marrakchi *et al.*, 2011; Onoufriadis *et al.*, 2011). IL-36Ra is a natural inhibitor of three related cytokines: IL-36α, IL-36β, and IL-36γ (formerly known as IL-1F6, IL-1F8, and IL-1F9, respectively). The physiological function of the IL-36 cytokines remains unknown; however, they were linked to psoriasis pathology even before the discovery of the *IL36RN* mutations. Early studies using transgenic mice overexpressing IL-36α in keratinocytes revealed skin inflammation with some resemblance to psoriasis (Blumberg *et al.*, 2007). Furthermore, several studies have observed increased IL-36α and IL-36γ mRNA expression in plaque psoriatic skin (reviewed in Jensen, 2010).

Imiquimod is a ligand for toll-like receptor 7 (Colak *et al.*, 2014) and adenosine receptors (Schön *et al.*, 2006; Kan *et al.*, 2012), and has therapeutic effects in humans against basal cell carcinomas, actinic keratoses, and warts caused by human papillomaviruses. A known side effect of the drug is psoriasiform skin inflammation (Gilliet *et al.*, 2004; Wu *et al.*, 2004; Fanti *et al.*, 2006; Rajan and Langtry, 2006; van der Fits *et al.*, 2009; Patel *et al.*, 2011). This has been exploited to develop a mouse model of psoriasis, which has rapidly become very popular owing to its strong resemblance to the human condition (reviewed in Flutter and Nestle, 2014). Using the imiquimod-induced psoriasiform skin inflammation model, we previously demonstrated that IL-1R1 signaling, via

the chemokines CXCL1 and CXCL2, has an essential role in recruiting neutrophils to the epidermis (Uribe-Herranz *et al.*, 2013). Furthermore, IL-1, here referring to both IL-1α and IL-1β, promoted psoriasis-like epidermal hyperplasia via IL-1α as the dominant form of the two cytokines expressed in the model (Uribe-Herranz *et al.*, 2013). Given the recently identified genetic link between IL-36 and excessive epidermal recruitment of neutrophils (Marrakchi *et al.*, 2011; Onoufriadis *et al.*, 2011) in addition to the overexpression of IL-36 in plaque psoriasis (Jensen, 2010), we here examined the role of the IL-36 cytokines and their interplay with the IL-1 axis in the imiquimod-induced psoriasis mouse model.

RESULTS

IL-36α, but not IL-36β or IL-36γ, is essential for the development of psoriasiform skin disease

Although the IL-36 cytokines have been linked to inflammatory responses, their physiological functions remain unknown. Curiously, there are three agonist IL-36 cytokines, but only one receptor, IL-36R. With the long-term goal of elucidating the role of this apparent redundancy, we assembled a portfolio of knockout (KO) mice representing each individual IL-36 cytokine (Supplementary Table S1 online and Supplementary Figures S1 online). The strains have no apparent defects or obvious phenotypes. This is in agreement with studies involving IL-36R$^{-/-}$ mice (Blumberg *et al.*, 2007, 2010; Tortola *et al.*, 2012; Vigne *et al.*, 2012; Lamacchia *et al.*, 2013). Because IL-36 signaling has recently been linked to GPP (Marrakchi *et al.*, 2011; Onoufriadis *et al.*, 2011), we examined the role of each individual IL-36 cytokine in the imiquimod-induced psoriasis model (Figure 1 and Supplementary Figure S3 online). Somewhat surprisingly, we found that while ablation of neither IL-36β nor IL-36γ affected the imiquimod-induced phenotype, IL-36α KO mice exhibited a dramatically reduced phenotype compared with wild-type mice (Figure 1 and Supplementary Figure S3 online). Externally, the IL-36α KO skin appeared thinner, less red, and less scaly (Figure 1a). In agreement with these observations, histological and immunohistochemical analyses revealed reduced epidermal acanthosis (Figure 1c and e and Supplementary Figure S3a online) and dermal edema (Figure 1c and Supplementary Figure S3c online) in IL-36α KO mice compared with wild type. These results demonstrate that IL-36α, but not IL-36β or IL-36γ, has a significant role in driving the imiquimod-associated psoriasiform skin pathology.

Formation of neutrophil-rich microabscesses in the epidermis is IL-36α-dependent

A characteristic feature of psoriasis is diffuse neutrophilic dermal inflammation and especially formation of epidermal

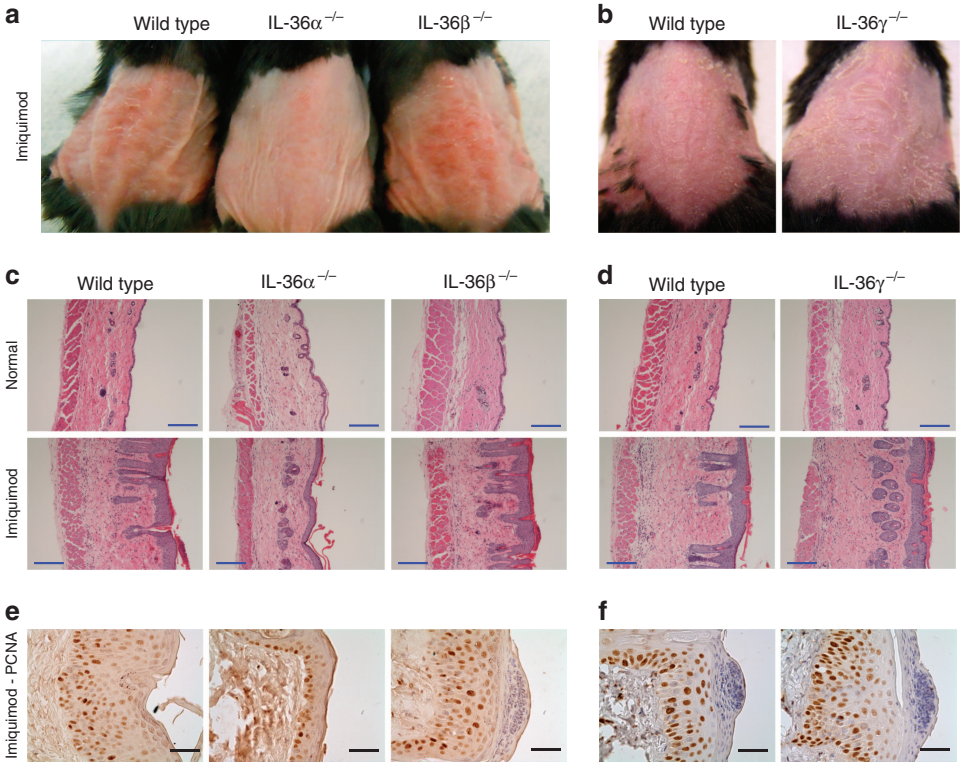

Figure 1. IL-36α has a significant role in psoriasiform skin disease induced by imiquimod. Wild-type, IL-36α$^{-/-}$, IL-36β$^{-/-}$, and IL-36γ$^{-/-}$ mice were treated with imiquimod for 4 days. The day after the last application anesthetized (**a**) or killed (**b**) mice were photographed and skin collected after being killed. Control mice were denuded and left untreated until skin collection. Skin was examined by H&E staining (**c** and **d**) and immunohistochemistry for PCNA (**e** and **f**). Representative images from five (IL-36α$^{-/-}$ and IL-36β$^{-/-}$) and three (IL-36γ$^{-/-}$) independent imiquimod experiments are shown. Blue scale bars = 200 μm. Black scale bars = 50 μm. H&E, hematoxylin and eosin; PCNA, proliferating cell nuclear antigen.

abscesses containing neutrophils (Raychaudhuri *et al.*, 2014). Our previous studies of IL-1 signaling in the imiquimod model demonstrated that IL-1 has a crucial role in the formation of such abscesses (Uribe-Herranz *et al.*, 2013). Similar analyses here revealed that IL-36α KO mice had diminished dermal infiltration by neutrophils and fewer epidermal microabscesses than wild-type mice (Figure 2 and Supplementary Figure S4 online). No differences between wild-type and either IL-36β- or IL-36γ-deficient mice could be detected (Figure 2 and Supplementary Figure S4 online). This, in agreement with the above-described IL-36α-dependent phenotypes, demonstrates that of the three IL-36 cytokines, IL-36α is the primary driver of neutrophil recruitment to the epidermis in the utilized skin inflammation model.

Neutrophil recruitment to IL-36α-deficient epidermis can be rescued through CXCL1

The neutrophil chemotactic CXCL1 is expressed at elevated levels in psoriasis (see Uribe-Herranz *et al.*, 2013 for reference) and human IL-36α stimulates CXCL1 production by human keratinocytes (Foster *et al.*, 2014). Using mouse keratinocytes, we observed that mouse IL-36α induced CXCL1 mRNA (Figure 3a) and protein (data not shown) in a transient and concentration-dependent manner. Furthermore, *in vivo* levels of CXCL1 secreted from inflamed skin were lower in IL-36α KO mice compared with wild type (Figure 3b). In the imiquimod model, we have shown that neutrophils are recruited to the epidermis in an IL-1-dependent manner via, for example, CXCL1 (Uribe-Herranz *et al.*, 2013). In the IL-36α KO mice, topical application of physiologically relevant levels of CXCL1 (Figure 3b) caused the formation of inflammatory foci in the skin (Figure 3c) and recruitment of neutrophils to the epidermis and dermis (Figure 3d). The latter demonstrates that the migratory capacity of the neutrophils is intact in the IL-36α KO mice. Overall, these observations suggest that IL-36α promotes neutrophil recruitment, at least in part, through CXCL1.

IL-36α is produced and secreted at high levels in imiquimod-treated skin

To explore the specific involvement of IL-36α, but not IL-36β or IL-36γ, in regulating imiquimod-induced skin inflammation, we examined the expression of these. All three IL-36 mRNAs were significantly upregulated in response to topical imiquimod treatment (Supplementary Figure S5a and c online), with IL-36α being the most dramatically induced (≅60-fold). IL-36α protein could be detected readily in culture medium from explanted skin after only two imiquimod applications (Figure 4a and Supplementary Figure S5d online). Similar IL-36β and IL-36γ secretion was not detected (data not shown). Hence, the specific involvement of IL-36α, but not IL-36β nor IL-36γ, in the examined skin inflammation model (Figure 1) likely reflects the dramatic induction of IL-36α gene expression (Supplementary Figure S5a online) and the subsequent correspondingly high protein levels achieved (Figure 4a and Supplementary Figure S5d online).

We previously reported that imiquimod induces expression of IL-1α and IL-1β both *in vitro* and *in vivo* (Uribe-Herranz *et al.*, 2013). In comparison, we here found that the IL-36α, but not IL-18, mRNA is induced at higher concentrations (Supplementary Figure S6 online) than those activating IL-1α and IL-1β gene expression (Uribe-Herranz *et al.*, 2013).

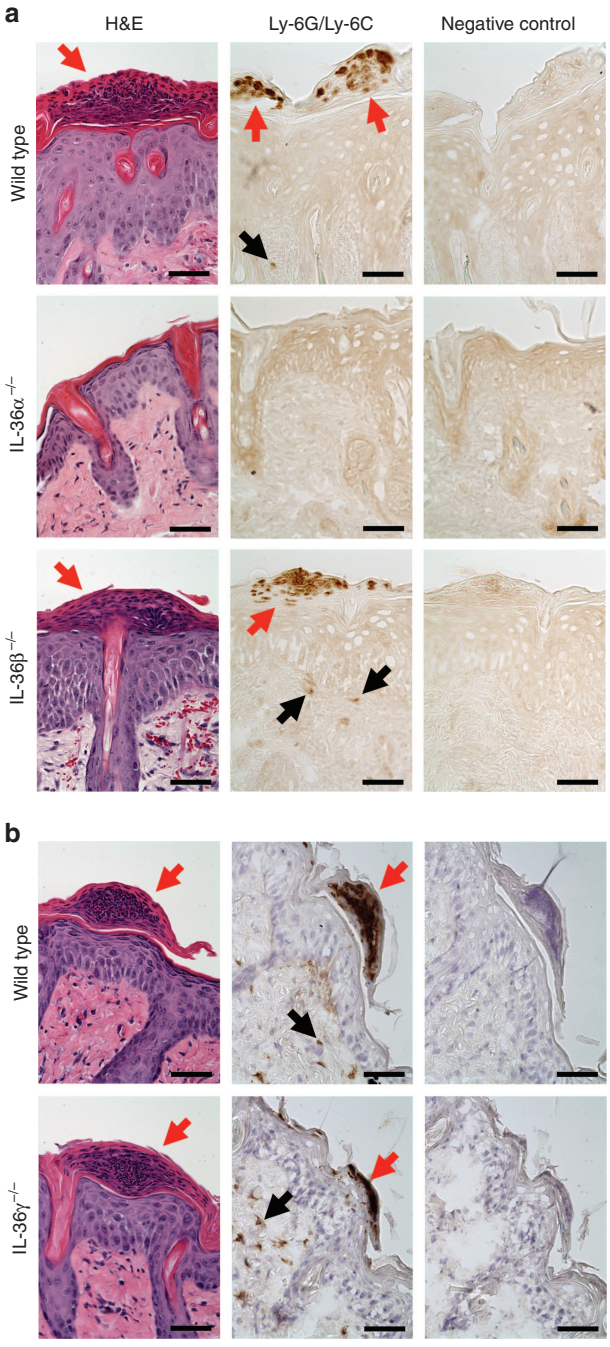

Figure 2. IL-36α, but not IL-36β or IL-36γ, is required for neutrophil recruitment. Wild-type (**a** and **b**), IL-36α$^{-/-}$ (**a**), IL-36β$^{-/-}$ (**a**) and IL-36γ$^{-/-}$ (**b**) mice were treated with imiquimod for 4 days as described in Figure 1. Skin was collected the day after the last imiquimod application and examined by H&E staining or immunohistochemistry for neutrophils (Ly-6G/Ly-6C). Red arrows indicate Munro's microabscesses (clusters of neutrophils) at the top of the epidermis. Black arrows indicate individual neutrophils within the dermis. Representative images from the experiments also analyzed in Figure 1 and Supplementary Figure S3 online are shown. Scale bars = 50 μm. H&E, hematoxylin and eosin.

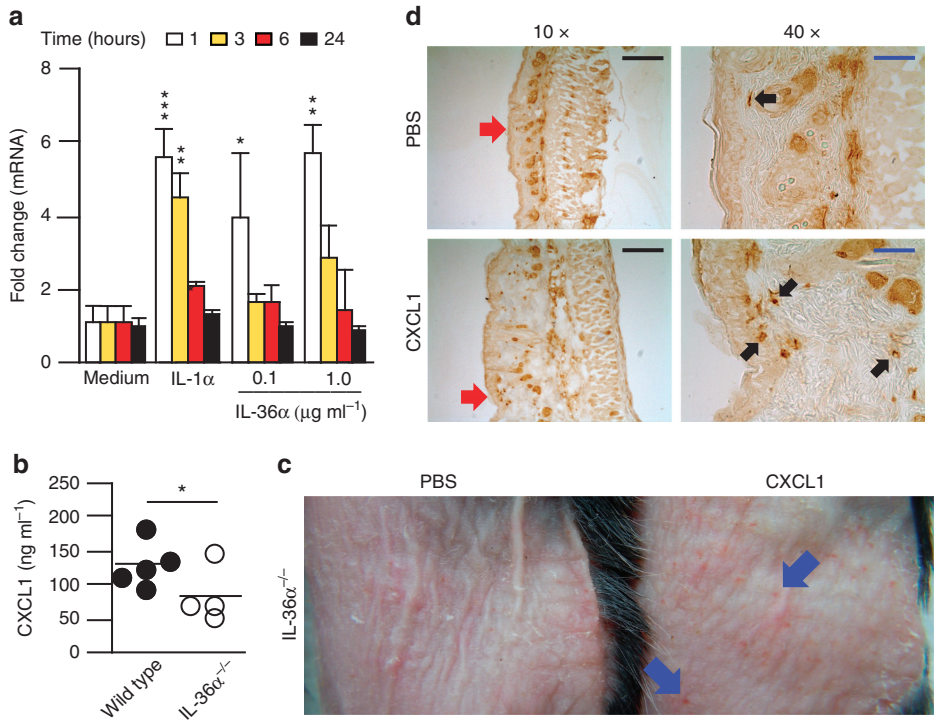

Figure 3. IL-36α may promote neutrophil recruitment through CXCL1. (**a**) Mouse primary keratinocytes were treated with medium only, IL-1α (10 ng ml^{-1}) or IL-36α (as indicated). CXCL1 mRNA levels were determined using real-time PCR (means+SD). (**b**) Wild-type (black circles, $n=5$) and IL-36α$^{-/-}$ (white circles, $n=4$) mice were treated with imiquimod four times and 4-mm skin biopsies explanted the day after the last drug application. Production of CXCL1 was examined by ELISA. (**c** and **d**) IL-36α$^{-/-}$ mice were treated with imiquimod+PBS or imiquimod+25 ng CXCL1 per mouse twice. External skin appearance was photodocumented (**c**) and neutrophil recruitment examined using immunohistochemistry for Ly-6G/Ly-6C (**d**). Blue arrows, examples of focal inflammation (red spots). Black arrows, examples of neutrophils (Ly-6G/Ly-6C-positive cells). Red arrows, regions enlarged in ×40 images. Black bars = 200 μm. Blue bars = 50 μm. *$P<0.05$; **$P<0.01$; ***$P<0.001$. PBS, phosphate-buffered saline.

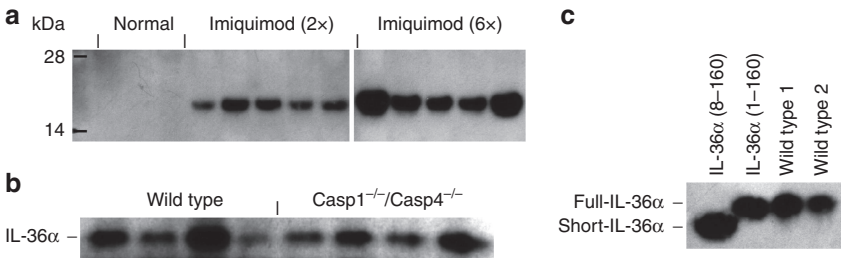

Figure 4. Topical imiquimod treatment leads to dramatic induction and secretion of unprocessed IL-36α in an inflammatory caspases-independent manner. (**a**) Wild-type mice ($n=3$–5 per time point) were treated with imiquimod as indicated. Skin was collected and used for 4-mm explant cultures. Levels of secreted IL-36α were examined by western blotting. (**b**) Wild-type and Casp1$^{-/-}$/Casp4$^{-/-}$ mice ($n=4$ per group) were treated with imiquimod four times and IL-36α secretion examined. (**c**) Two independent samples of secreted proteins (wild type 1 and wild type 2) were analyzed by western blotting next to full-length recombinant IL-36α (IL-36α (1–160)) and predicted processed short recombinant IL-36α (IL-36α (8–160)) in EpiLife medium.

Despite the dramatic induction of the IL-36α mRNA (Supplementary Figure S6 online), we were not able to detect IL-36α protein production (data not shown). This is in agreement with our reported observations that these drug levels are cytotoxic to cultured keratinocytes (Uribe-Herranz *et al.*, 2013). Given the topical application of imiquimod in the here-used mouse model, it is difficult to assess the drug concentrations experienced by the epidermal keratinocytes; however, it is possible that imiquimod has a significant role as a direct activator of IL-36α gene expression in this model.

IL-36α is released in an inflammatory caspases-independent manner

A curious characteristic of IL-1 and IL-36 cytokines is that they lack signal peptides for conventional protein secretion (reviewed in Jensen, 2010). The release mechanism for IL-1β,

which is the best characterized, often involves the inflammasomes and the inflammatory caspases caspase-1 and caspase-4 (Shin and Brodsky, 2015). In macrophages, imiquimod induces IL-1β secretion through NLRP3 inflammasome-mediated activation of caspase-1 (Kanneganti *et al.*, 2006). We previously established that the release of IL-36γ from cells treated with double-stranded RNA is dependent upon the inflammatory caspases (Lian *et al.*, 2012), whereas IL-1α release during herpes simplex virus-1 infection is caspase-1/-4–independent (Milora *et al.*, 2014). Hence, we examined whether IL-36α release in the here-utilized imiquimod model was dependent or independent of the inflammatory caspases. Interestingly, our data revealed that IL-36α release is independent of caspase-1 and -4 (Figure 4b). This suggests an IL-36α release mechanism distinct from that controlling IL-1β.

IL-36α does not undergo processing *in vivo*

On the basis of size, the IL-36 cytokines were predicted upon discovery to be synthesized as mature proteins unlike the related IL-1β, which requires proteolytic cleavage for activation and extracellular release (Shin and Brodsky, 2015). Full-length recombinant IL-36 proteins, expressed in bacteria, are active, albeit only at high concentrations (see Jensen, 2010 for reference). Through genetic engineering, it has been demonstrated that truncation of 17 amino acids from the N-terminus of IL-36γ led to enhanced activity (Towne *et al.*, 2011). Curiously, removal of fewer, or more, amino acids did not have similar effects. Consequently, it was proposed that the IL-36 cytokines require proteolytic processing, at very specific sites, to be activated (Towne *et al.*, 2011). By sequence comparison, processing of mouse IL-36α was predicted to involve seven amino acids ((Towne *et al.*, 2011), shown in blue in Supplementary Figure S1b online). To determine whether IL-36α is processed *in vivo*, we examined the molecular weight of secreted endogenous IL-36α compared with full-length and truncated (predicted) recombinant proteins. Interestingly, we found that endogenously secreted IL-36α migrated identically to full-length recombinant IL-36α (Figure 4c). Consequently, we conclude that IL-36α is released as an unprocessed protein.

IL-36α expression is induced by IL-1 *in vitro* and *in vivo*

In vitro studies have established that IL-1β can stimulate expression of the human IL-36α mRNA in bronchial epithelial cells (Chustz *et al.*, 2011). In a similar manner, we found that mouse IL-1α induced IL-36α gene expression in mouse keratinocytes (Figure 5a). We previously demonstrated that IL-1 signaling, via IL-1R1 and primarily IL-1α, has an essential role in initiating skin disease in the imiquimod model (Uribe-Herranz *et al.*, 2013). Hence, we hypothesized that IL-1α signaling could be involved in driving the inducible expression of IL-36α found *in vivo* (Figure 4 and Supplementary Figure S5 online). Consistent with this hypothesis, we observed significantly lower levels of IL-36α secretion from inflamed IL-1R1-deficient skin than wild type (Figure 5b). Hence, IL-1α is an important regulator of IL-36α expression *in vivo*.

IL-36α regulates IL-1α in a feedback loop

Ex vivo studies of peripheral blood mononucleated cells from a single patient with GPP and a healthy control revealed potentiated activation of IL-1α gene expression in GPP cells in response to IL-36α (Onoufriadis *et al.*, 2011). Using primary mouse keratinocytes, we observed rapid induction of the IL-1α mRNA in response to IL-36α (Figure 5c). In the mouse model of psoriasis we used, we observed progressive upregulation of IL-1α mRNA during disease development (Supplementary Figure S7 online). This is in agreement with our previously reported data demonstrating upregulation of IL-1α protein (Uribe-Herranz *et al.*, 2013). The induction of IL-1α correlated with increased IL-36α mRNA and protein expression (Figure 4 and Supplementary Figure S5 online), and we therefore examined whether the inducible IL-1α expression was dependent, at least in part, upon IL-36α. Indeed, we found that levels of IL-1α released from imiquimod-treated skin were significantly lower in the absence of IL-36α than in the presence of IL-36α (Figure 5d). Levels of IL-18 (Supplementary Figure S8 online), but not IL-1β and IL-17A (not shown), were also found to be IL-36α-dependent. Hence, we conclude that once expression of IL-36α is initiated, via IL-1R1 signaling (Figure 5a and b), IL-36α feeds back to induce IL-1α expression (Figure 5c and d).

IL-1α and IL-36α cooperate to promote epidermal hyperplasia, dermal edema, and chemokine production

Previously, we showed that IL-1R1 signaling has a significant role in initiating epidermal hyperplasia and neutrophil recruitment through CXCL1 in response to imiquimod (Uribe-Herranz *et al.*, 2013). As IL-36α expression is induced via IL-1R1 (Figure 5a and b), we wondered whether IL-36α acts downstream of IL-1 in a sequential linear pathway or whether the two cytokines act together when present at the same time. To address this, we generated IL-36α and IL-1R1 double KO mice and compared epidermal hyperplasia, dermal edema, and CXCL1 expression with that induced in single KO mice (Figure 5e and g). In IL-36α$^{-/-}$/IL-1R1$^{-/-}$ mice, the thickness of both the epidermis (Figure 5e) and the dermis (Figure 5f) was significantly thinner than that in single KO mice. Additionally, production of the neutrophil chemotactic CXCL1 was reduced in the IL-36α$^{-/-}$/IL-1R1$^{-/-}$ mice compared to single KO mice (Figure 5g). This demonstrates that once the expression of IL-36α is induced, IL-1α and IL-36α act together *in vivo* to promote psoriasiform skin disease.

DISCUSSION

The discovery of missense mutations in *IL36RN* causing generalized GPP (Marrakchi *et al.*, 2011; Onoufriadis *et al.*, 2011) implicates the overzealous IL-36 signaling in the disease pathology. Here, we show that in the imiquimod-induced psoriasiform skin disease model, IL-36α is the primary form of IL-36 driving skin pathology, including epidermal hyperplasia, dermal inflammation, and neutrophil recruitment to the epidermis (Figures 1 and 2 and Supplementary Figure S4 online). The active form of IL-36α is, against expectations, not proteolytically processed in connection with its release from cells (Figure 4c). Our study

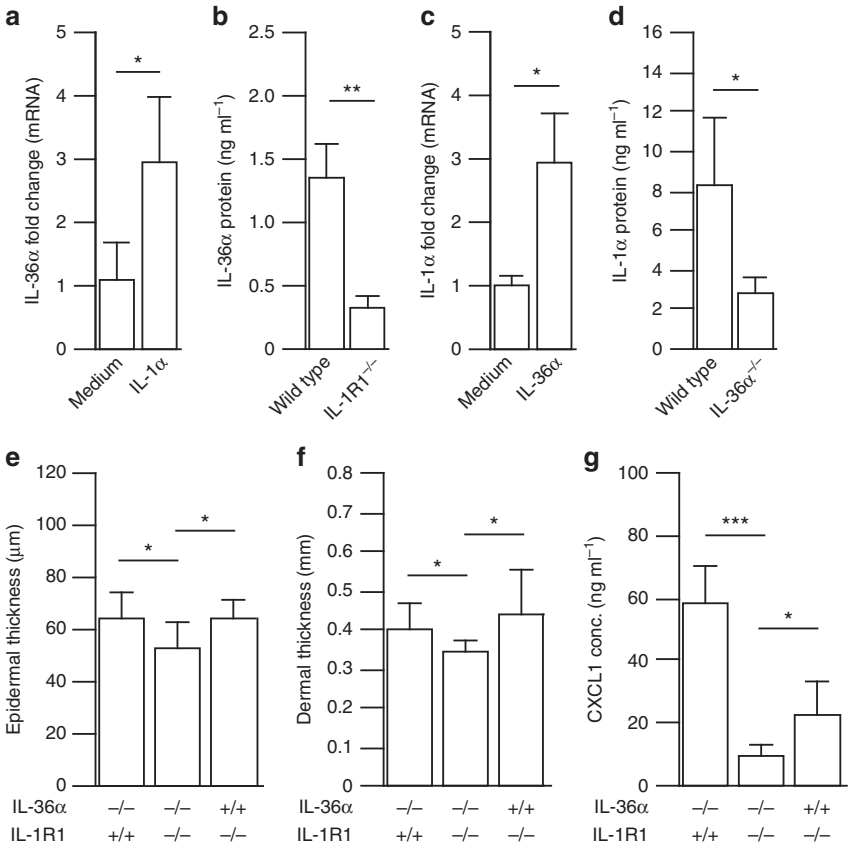

Figure 5. IL-36α and IL-1 cooperate to drive psoriasiform skin inflammation through mutual regulation. (**a** and **c**) Mouse primary keratinocytes were treated with medium only, IL-1α (10 ng ml^{-1}) or IL-36α (1 μg ml^{-1}) for 1 hour. IL-36α (**a**) and IL-1α (**c**) mRNA levels were evaluated using real-time PCR. (**b**) Wild-type ($n=4$) and IL-1R1 KO ($n=3$) mice were treated with imiquimod two times. Skin was explanted and maintained for another 24 hours. Levels of IL-36α secreted into the medium were determined by ELISA. (**d**) Wild-type ($n=4$) and IL-36α$^{-/-}$ ($n=4$) mice were treated with imiquimod four times. Treated skin was explanted and levels of IL-1α secreted into the medium determined by ELISA. (**e**–**g**) IL-36α ($n=6$), IL-1R1 ($n=7$) and IL-36α/IL-1R1 double KO ($n=7$) mice were treated with imiquimod four times. Epidermal (**e**) and dermal (**f**) thickness was examined using H&E staining and ImageJ. (**g**) Levels of CXCL1 secretion were determined by ELISA. Data points are shown as mean+SD. *$P<0.05$; **$P<0.01$; ***$P<0.001$. H&E, hematoxylin and eosin; KO, knockout.

further reveals that elevated expression of both IL-36α and IL-1α are intricately linked during disease, as each cytokine is involved in regulating expression of the other (Figure 5a and d). Through this mutual induction, IL-36α and IL-1α act together to boost the pathogenic function of the other cytokine's signaling pathway (Figure 5e and g).

Anakinra, recombinant human IL-1Ra, has been successfully used off label to treat individual cases of GPP associated with IL-36Ra missense mutations (Rossi-Semerano *et al.*, 2013; Hüffmeier *et al.*, 2014). Analyses of cytokine production by peripheral blood mononuclear cells from a patient with GPP revealed elevated IL-1α production in response to IL-36α compared with a healthy control sample (Onoufriadis *et al.*, 2011). In the *in vivo* model we used, IL-1α is indeed a downstream target of IL-36α signaling (Figure 5d). Interestingly, we also find that IL-36α expression is dependent upon IL-1 signaling (Figure 5b), suggesting feedback mechanisms between the IL-1 family cytokines (Supplementary Figure S9 online). Owing to this inter-regulation, IL-1α and IL-36α form a self-amplifying cycle (Supplementary Figure S9 online), which may run out of control in the absence of proper regulatory mechanisms such as the IL-1Ra and IL-36Ra. This cycle may not only explain the often sudden and dramatic disease flares seen in patients with inflammatory diseases, for example GPP, but may also pinpoint the chronic nature of these conditions. As such, this cycle (Supplementary Figure S9 online) may represent an ideal therapeutic target to restore homeostatic balance.

In addition to a direct feedback loop between IL-1α and IL-36α (Figure 5a and d and Supplementary Figure S8 online), additional factors may contribute to further amplification of the pathogenic process. The IL-1 and IL-36 cytokines regulate their own expression (Carrier *et al.*, 2011; Uribe-Herranz *et al.*, 2013 and references therein); hence, they further fuel the process (Supplementary Figure S9 online). A second connected cycle of amplification may involve other cytokines (Supplementary Figure S9 online) as IL-36 expression is directly induced in keratinocytes by IL-17A, IL-22, and TNF-α (Carrier *et al.*, 2011), and expansion of IL-17 producing γδ T cells is IL-36-dependent (Tortola *et al.*, 2012). Interestingly, IL-36R KO mice exhibit a milder imiquimod-induced phenotype than mice deficient of factors from the well-known IL-23/

IL-17/IL-22 pathogenic axis, suggesting that the IL-36 system has an additional distinct activity (Tortola *et al.*, 2012). The here-identified IL-1α/IL-36α loop may be this activity (Supplementary Figure S9 online). While Torlola *et al.* did not observe an effect of IL-1R1 ablation upon epidermal acanthosis, our previous (Uribe-Herranz *et al.*, 2013) and the here-present study did find such an effect. This apparent discrepancy may be due to the different application sites. Tortola *et al.* applied imiquimod to the ears, whereas we apply it to the back as in the original model report (van der Fits *et al.*, 2009). The acanthosis observed in the ears is significantly milder (Tortola *et al.*, 2012) than that observed in the back skin (van der Fits *et al.*, 2009) (Figures 1 and 2 and Supplementary Figure S3 online). Hence, the IL-1R1-dependent phenotype may be missed in the ear-version of the model. Consequently, caution should be taken when comparing studies utilizing the imiquimod mouse model of skin inflammation.

Keratinocytes appear to be the source of IL-36 in at least GPP (Marrakchi *et al.*, 2011). Although we readily detected IL-1-dependent IL-36α expression in the imiquimod model (Figure 5b), we have not been able to detect protein production in keratinocyte cultures. This could suggest that signals, additional to IL-1, are required to achieve the *in vivo* observed levels. Such additional factors are also suggested by our *in vivo* data demonstrating only a partial reduction in IL-36α expression in IL-1R1 KO mice (Figure 5b) compared with the dramatic full induction (Figure 4a and Supplementary Figure S5 online). IL-17 A may be one such factor as it activates IL-36 mRNA expression *in vitro* (Carrier *et al.*, 2011). Although we did not observe a difference in IL-17A levels at the here-examined time-point associated with the skin phenotype, IL-17A is known to be transiently induced in the model (van der Fits *et al.*, 2009); hence, IL-17A could contribute to the IL-36α induction at an earlier time point. Imiquimod cream may also directly induce IL-36α in a manner distinct, or similar to, that previously described for IL-1α (Uribe-Herranz *et al.*, 2013; Walter *et al.*, 2013). The latter is a deviation from human psoriasis pathology where the disease triggers are poorly understood. Identification of initiator factors in both the imiquimod model and human pathology may represent new therapeutic and preventive targets.

In response to inflammatory signals, IL-1β is synthesized as a pro-protein. This pro-IL-1β is cleaved by caspase-1, an independent signaling event, to gain functional activity (reviewed in Jensen, 2010). On the basis of genetic engineering, it has been suggested that the IL-36 cytokines require similar proteolytic cleavage to be activated (Towne *et al.*, 2011). Our studies here demonstrate that IL-36α is released from skin cells in an unprocessed form (Figure 4c). This unprocessed IL-36α appears to be active, as dramatically reduced skin pathology is observed in IL-36α-deficient mice (Figures 1 and 2 and Supplementary Figure S4 online). Although it was previously shown that IL-36α gained activity upon removal of seven or five amino acids from the N-terminus (mouse (Supplementary Figure S1B online, blue highlight) vs. human), the full-length IL-36α, unlike IL-1β, still has signaling capacity (reviewed in Jensen, 2010). The observed *in vivo* activity of unprocessed IL-36α reported here may be due to high local production of the cytokine leading to a microenvironment conducive for the relatively low activity of full-length IL-36α. However, it is a possibility that under conditions different from the here-studied skin inflammation, IL-36α can undergo processing to enhance its activity, and that the protease involved is not activated in the here-utilized model.

Interestingly, *in vitro* processing of IL-36γ appears to be associated with keratinocyte differentiation, as it is induced by high calcium concentrations (Li *et al.*, 2014). As epidermal differentiation is dysregulated in psoriasis and associated with aberrant calcium levels (Menon and Elias, 1991), it is possible that the IL-36 processing protease is not activated in psoriatic skin. If IL-36γ activity is strictly dependent upon processing, then lack of cleavage could explain the absence of a phenotypic effect in our IL-36γ$^{-/-}$ mice (Figure 1). IL-36γ is well known to be upregulated in psoriatic skin (reviewed in Jensen, 2010), and it has been proposed as a biomarker to distinguish psoriasis from other inflammatory skin conditions (D'Erme *et al.*, 2015). If IL-36γ is to be considered a therapeutic target and not just a biomarker, it will be critical to determine whether IL-36γ is, aside from upregulated, responsible for disease pathology and/or progression. Such activity may be dependent upon site-specific processing and consequently, it would be very interesting to determine whether IL-36γ is processed in healthy and diseased human skin.

In summary, we have linked IL-1α and IL-36α in an *in vivo* feedback loop (Supplementary Figure S9 online). Such a loop could explain both sudden and dramatic disease onset as seen in, for example GPP, and the chronic nature of psoriatic diseases. As such, this self-amplifying cycle (Supplementary Figure S9 online) represents a promising therapeutic target; however, as discussed above, many questions remain unresolved. Through further studies, an improved insight into the mechanisms whereby IL-36 and keratinocytes initiate and maintain psoriatic disease may reveal novel therapeutic strategies and targets.

MATERIALS AND METHODS

Mice

IL-1R1 and Casp1/Casp4 deficient mice were obtained from the Jackson Laboratory. *Il1f6*$^{+/-}$ mice were generously donated by GlaxoSmithKline and backcrossed onto the C57BL/6 background. *Il1f9*$^{+/-}$ and *Il1f8*$^{+/-}$ mice were procured from the Mutant Mouse Regional Resource Center (University of North Carolina) and Knockout Mouse Project University of California Davis (project ID: VG13041), respectively. Mice were interbred to generate KO (single or double) and wild-type mice (all on the C57BL/6 background). Homozygous founders were used for further breeding. All experimental mice were bred in house in a specific pathogen-free animal facility. All housing, breeding, and experimental procedures involving mice were approved by the Temple University Institutional Animal Care and Use Committee and in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. Experimental mice (male and female)

were used at age 8–10 weeks and matched for sex in each independent experiment.

Genotyping

Mice were genotyped using a previously described protocol (Jensen *et al.*, 2006). PCR was performed using primers listed in Supplementary Table S2 online.

Flow analyses

Blood was collected by cardiac puncture and erythrocytes lysed with RBC lysis buffer (eBioscience, San Diego, CA). Neutrophils, monocytes, and lymphocytes were identified based on forward and side scatter using a FACSCanto Cell Analyzer (BD Biosciences, San Jose, CA).

Induction of psoriasiform skin inflammation

Mice were denuded by shaving and subsequent treatment with depilating cream. Imiquimod cream (5%, 62.5 mg, Medicis, Scottsdale, AZ) was applied daily to a 6 cm^2 area as indicated for each experiment. Skin was collected the day after the last application unless indicated otherwise.

Quantification of epidermal and dermal thickness

Phosphate-buffered saline-buffered formaldehyde (4%)-fixed skin was examined by standard hematoxylin and eosin staining. For each skin specimen, three independent images were acquired. Epidermal and dermal thickness was measured in three independent locations using ImageJ. An average thickness of the epidermis and dermis was calculated for each tissue. In most experiments, including those involving double KOs, the measurements were performed blinded to the strain genotype.

Immunohistochemistry

Neutrophils were detected using rat anti-mouse Ly-6G/Ly-6C antibody (BD Biosciences) as previously described (Uribe-Herranz *et al.*, 2013). Proliferating cell nuclear antigen was visualized using the PCNA staining kit (Life Technologies, Frederick, MD) according to the manufacturer's instructions.

Real-time RT-PCR

RNA was isolated using the RNeasy Plus Universal system (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was reverse-transcribed and cDNA analyzed as previously described (Sanmiguel *et al.*, 2009). Additional primers are listed in Supplementary Table S2 online. Actin was used as the housekeeping gene for ΔΔCt analyses.

Protein preparations

For analyses of secreted protein levels, standardized 4- mm circular skin biopsies (Miltex, York, PA) were placed in 200 µl EpiLife medium supplemented with EDGF (Life Technologies) for 24 hours. Explant medium was collected for ELISA analyses. Total protein was extracted from skin in previously described lysis buffer (Jensen and Whitehead, 2003) using a Bio-Gen PRO200 homogenizer equipped with interchangeable Multi-Gen 7XL probes (PRO Scientific, Oxford, CT). Protein levels were determined using Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA).

Cell cultures

Human and mouse primary keratinocytes were obtained and maintained as previously described (Uribe-Herranz *et al.*, 2013). Mouse cells were treated with mouse cytokines (IL-1α (Peprotech, Rocky Hill, NJ) and IL-36α (aa 8–160) (R&D Systems, Minneapolis, MN)) as indicated. Water-soluble imiquimod was from InvivoGen.

ELISA

Sandwich ELISAs (Peprotech (CXCL1, IL-1α) and R&D Systems (IL-17A and IL-18)) were performed according to the manufacturer's instructions. Direct ELISAs for mouse IL-36 were performed as previously described (Lian *et al.*, 2012). Individual IL-36 cytokines were detected by sequential incubation with: (i) goat anti-IL-36α (AF2297, R&D Systems), or goat anti-IL-36β (AF2298, R&D Systems) and (ii) appropriate species-specific horseradish peroxidase-conjugated secondary antibodies. The detection limit for the mouse IL-36β ELISA was ≈400 pg ml^{-1} (IL-36α levels were detected in the 500–10,000 pg ml^{-1} range).

Western blotting

Proteins were separated by PAGE, transferred to polyvinylidene difluoride membranes and detected by enhanced chemiluminescence using anti-IL-36α (AF2297), anti-IL-36γ (custom synthesized at Genscript, Piscataway, NJ) or anti-GAPDH (FL-335, Santa Cruz, Dallas, TX) and appropriate secondary antibodies.

Statistical analyses

All experiments were performed at least three times unless indicated otherwise. Data shown are arithmetic means ± standard deviations (SD) unless indicated otherwise. Statistical significance was calculated using analysis of variance or students *t* test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We wish to express our gratitude to numerous investigators at GlaxoSmithKline, many of who remain unknown to us, who made the transfer of founders for the unpublished IL-36α KO mouse strain possible. We thank Samantha L. Miller for help with skin thickness measurements. This study was supported in part by an award (AR053672) from the National Institute of Arthritis, Musculoskeletal and Skin Diseases to LEJ.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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