Neutrophil-Derived Proteases Escalate Inflammation through Activation of IL-36 Family Cytokines

Highlights

- Neutrophils can escalate inflammation via processing of extracellular cytokines
- Activated neutrophils liberate proteases that can process IL-36 family cytokines
- IL-36α, β, and γ are activated differentially by cathepsin G, elastase, or proteinase-3
- Cathepsin G activity is elevated in human psoriatic lesions

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In Brief

IL-36 cytokines require proteolytic processing for activation, but how this is achieved is unknown. Here, Henry et al. show that proteases liberated from activated neutrophils—cathepsin G, elastase, and proteinase-3—differentially process and activate all three IL-36 family members. Therefore, neutrophil-derived proteases can escalate inflammation through processing of extracellular cytokines.
Neutrophil-Derived Proteases Escalate Inflammation through Activation of IL-36 Family Cytokines

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SUMMARY
Recent evidence has strongly implicated the IL-1 family cytokines IL-36α, IL-36β, and IL-36γ as key initiators of skin inflammation. Similar to the other members of the IL-1 family, IL-36 cytokines are expressed as inactive precursors and require proteolytic processing for activation; however, the responsible proteases are unknown. Here, we show that IL-36α, IL-36β, and IL-36γ are activated differentially by the neutrophil granule-derived proteases cathepsin G, elastase, and proteinase-3, increasing their biological activity ~500-fold. Active IL-36 promoted a strong pro-inflammatory signature in primary keratinocytes and was sufficient to perturb skin differentiation in a reconstituted 3D human skin model, producing features resembling psoriasis. Furthermore, skin eluates from psoriasis patients displayed significantly elevated cathepsin G-like activity that was sufficient to activate IL-36β. These data identify neutrophil granule proteases as potent IL-36-activating enzymes, adding to our understanding of how neutrophils escalate inflammatory reactions. Inhibition of neutrophil-derived proteases may therefore have therapeutic benefits in psoriasis.

INTRODUCTION
Interleukin 1 (IL-1) family cytokines, which include the recently described IL-36α, β, and γ proteins, play major roles as initiators of inflammation and are frequently among the first cytokines produced in response to infection or injury (Kono and Rock, 2008; Sims and Smith, 2010; Afonina et al., 2015). IL-1 family cytokines are capable of triggering complex cascades of additional cytokine production from diverse cell types, such as resident tissue macrophages and dendritic cells as well as keratinocytes and endothelial cells lining local blood vessels (Towne et al., 2004; Dinarello, 2009; Vigne et al., 2011, 2012; Milovanovic et al., 2012). IL-36α, IL-36β, and IL-36γ are encoded by distinct genes, and evidence is accumulating rapidly to suggest that these cytokines play a key role in skin inflammation, particularly in psoriasis (Blumberg et al., 2007, 2010; Johnston et al., 2011; Marrakchi et al., 2011; Tortola et al., 2012; Towne and Sims, 2012; Farooq et al., 2013; Kanazawa et al., 2013). Individuals that carry hypomorphic mutations in the IL-36 receptor antagonist (IL-36RA) display a severe and highly debilitating form of psoriasis called generalized pustular psoriasis (Marrakchi et al., 2011; Farooq et al., 2013; Kanazawa et al., 2013). This suggests that deregulated IL-36 cytokine signaling is sufficient to drive aggressive skin inflammation and also that IL-36 is an important barrier cytokine. Analysis of IL-36 mRNA expression in skin biopsies from individuals with the most common form of psoriasis, psoriasis vulgaris, found dramatically elevated expression (100-fold) of all three IL-36 transcripts compared with non-lesional skin from the same individuals or non-affected controls (Blumberg et al., 2010; Johnston et al., 2011). Consistent with the idea that elevated IL-36 activity is an initiating event in psoriasis, transgenic expression of IL-36α in the mouse leads to a psoriasis-like condition at birth that can be exacerbated further with the skin irritant phorbol acetate (Blumberg et al., 2007). Moreover, application of a Toll receptor agonist (imiquimod) to the skin of humans and mice can provoke psoriasis outbreaks (Wu et al., 2004; Tortola et al., 2012) that are increased in severity in IL-36RA−/− mice (Tortola et al., 2012). Furthermore, imiquimod-induced psoriasis in mouse models is abolished completely on an IL-36RA−/− background (Tortola et al., 2012). Finally, transplantation of human psoriatic lesions onto immunodeficient (severe combined immunodeficiency) mice produces a psoriasis-like condition that is greatly improved through blocking the IL-36 receptor (Blumberg et al., 2010).

IL-36α, IL-36β, and IL-36γ are all generated as leaderless cytokines that lack biological activity (Towne et al., 2011). Therefore, proteolytic processing of IL-36 cytokines is required to unleash their pro-inflammatory activity, similar to other members of the IL-1 family, such as IL-1β and IL-18 (Afonina et al., 2015). Sims and colleagues (Towne et al., 2011) have shown that removal of a small number of residues from the N termini of IL-36α, IL-36β, and IL-36γ increases their biological activity by more than 10,000-fold. Because IL-36 cytokines appear to play a key role as initiators of inflammation in the skin barrier, inhibitors of IL-36 proteolytic activation may have considerable potential for the treatment of inflammatory skin conditions.
Truncated IL-36 Proteins Exhibit Biological Activity

To identify the proteases responsible for processing and activation of IL-36 cytokines, we established an IL-36 bioassay by stably transfecting HeLa cells with the human IL-36 receptor (IL-36R). Sims and colleagues (Towne et al., 2011) have reported that artificial truncation of IL-36α, β, and γ at particular N-terminal residues dramatically increases the activity of these proteins (Figure 1A). To confirm that HeLa<sup>IL-36R</sup> cells were IL-36-responsive, we created modified forms of IL-36α, β, and γ by inserting a caspase-3 cleavage motif, DEVD, proximal to the residues identified by Sims and colleagues (Towne et al., 2011) that are capable of generating biologically active IL-36 cytokines (Figure 1A). DEVD-modified IL-36α, β, and γ were processed readily by caspase-3 whereas their wild-type counterparts were not (Figure 1B). HeLa<sup>IL-36R</sup> cells failed to respond to either full-length or caspase-3-cleaved IL-36 cytokines, whereas HeLa<sup>IL-36R</sup> cells secreted multiple cytokines in response to caspase-3-processed, DEVD-modified forms of IL-36α, β, and γ but not full-length, unprocessed forms of these cytokines (Figures 1C and 1D). Furthermore, the spontaneously immortalized keratinocyte line HaCat, which naturally expresses the IL-36R, also responded to the caspase-3-cleaved IL-36<sup>DEVD</sup> forms. (Figure S1).
These data confirmed that proteolytic processing of IL-36α, β, and γ dramatically increases the biological activity of these cytokines and validated HeLaIL-36R cells as a bioassay to screen for proteases that naturally activate IL-36 cytokines.

Neutrophil-Derived Proteases Activate IL-36 Cytokines
One of the hallmarks of psoriatic lesions is persistent infiltration of the epidermis by neutrophils (Terui et al., 2000; Murphy et al., 2007; Ikeda et al., 2013). Neutrophil secretory granules contain three major serine proteases (elastase, cathepsin G, and proteinase-3) that are involved in bacterial killing as well as in the processing of certain cytokines and chemokines (Korkmaz et al., 2010). To explore whether neutrophil-derived proteases can process and activate IL-36 cytokines, we induced human peripheral blood neutrophils to degranulate with phorbol-12-myristate 13-acetate (PMA) (Brinkmann et al., 2004), thereby liberating granule proteases and generating reactive oxygen species (ROSs) (Figures 2A and 2B). Robust ROS production and protease activity was found in supernatants from PMA-treated neutrophils, as expected (Figure 2B). Purified full-length, wild-type IL-36α, β, and γ were then incubated with supernatants from untreated versus PMA-activated neutrophils, followed by assessment of IL-36 activity using HeLaIL-36R cells. As shown in Figure 2C, incubation of IL-36 cytokines in the presence of PMA-activated neutrophil supernatants resulted in robust activation of IL-36β and IL-36γ, whereas IL-36α was activated more modestly under the same conditions.

Identification of IL-36-Activating Proteases
To identify the specific protease(s) involved in IL-36α, β, and γ activation, we initially used a panel of broad-spectrum protease inhibitors. As Figure 2D illustrates, the serine protease inhibitor PMSF potently inhibited activation of IL-36α, β, and γ by activated (i.e., PMA-treated) neutrophil supernatants. A specific chemical inhibitor of cathepsin G (CatG inhibitor I) robustly inhibited the activation of IL-36β, whereas a chemical inhibitor of elastase (elastase inhibitor IV), which inhibits both elastase and proteinase-3, inhibited the activation of IL-36γ by activated neutrophil supernatants (Figure 2D; Figures S2A–S2C). Interestingly, IL-36α activation by neutrophil degranulates was inhibited by both elastase and cathepsin G inhibitors, suggesting that both of these enzymes contribute to the activation of this cytokine (Figure 2D). To further explore the identity of the IL-36 processing protease(s), we used a panel of protease inhibitors specific for either cathepsin G or elastase. As Figure 2E demonstrates, two different cathepsin G-specific inhibitors (CatG inhibitor I and FLF-chloromethylketone [FLF-cmk]) potently inhibited IL-36β activation by neutrophil supernatants but did not inhibit IL-36γ activation under the same conditions. In contrast, neutrophil supernatant-mediated IL-36γ activation was antagonized by an inhibitor of elastase-like activity (elastase inhibitor IV), whereas cathepsin G inhibitors did not antagonize activation of the latter (Figure 2E; Figures S2A–S2C). We also generated a biotin-conjugated form of the cathepsin G inhibitor, biotin-FLF-CMK, to ask whether this depleted the IL-36β-activating activity from neutrophil supernatants. As Figure 2F illustrates, biotin-FLF-CMK selectively depleted cathepsin G activity (FLF-thio-benzyl ester [FLF-sBzl] hydrolysis) from PMA-treated neutrophil supernatants but did not deplete elastase activity (AAPV-7-amino4-methyl-courmarin [AAPV-AMC] hydrolysis). Furthermore, FLF-CMK specifically inhibited purified cathepsin G but not purified elastase (Figure 2F, right). Consistent with this, biotin-FLF-CMK-depleted neutrophil supernatants failed to activate IL-36β but remained competent to activate IL-36γ (Figure 2G). Collectively, these data strongly suggest that cathepsin G processes and activates IL-36β and that an elastase-like activity (which could be either elastase itself or proteinase-3, which has a similar specificity to the latter protease) activates IL-36γ.

Purified Cathepsin G, Elastase, and Proteinase-3 Activate IL-36 Cytokines
We next compared the ability of purified cathepsin G, elastase, and proteinase-3 to process and activate IL-36 cytokines. The activity of all purified proteases was confirmed using synthetic substrate peptides (Figure 3A). As Figure 3B and Figure S3B demonstrate, and in agreement with our previous observations using primary human neutrophil supernatants, purified cathepsin G selectively promoted IL-36β activation, whereas both elastase and proteinase-3 preferentially activated IL-36γ. IL-36α was activated by incubation with either cathepsin G or elastase under the same conditions (Figure 3B). Because caspase-1 processes and activates the IL-1 family cytokines IL-1β and IL-18 we also explored whether caspase-1 or caspase-3 could process and activate IL-36 family cytokines. However, as Figure 3B illustrates, neither caspase activated any of the IL-36 cytokines. To confirm the above observations, we also titrated IL-36 cytokines over a wide concentration range in the presence and absence of a fixed concentration (100 nM) of cathepsin G, elastase, or proteinase-3. As Figure 3C shows, IL-36γ was again activated by cathepsin G or elastase and, to a lesser extent, by proteinase-3, with IL-36β activated preferentially by cathepsin G, whereas IL-36γ was activated by either elastase or proteinase-3 (Figure 3C). Furthermore, we also confirmed processing of IL-36 cytokines by purified neutrophil proteases or activated neutrophil degranulates by immunoblot analysis (Figures S3A and S3B). However, as Figures S3A and S3B demonstrate, although certain IL-1 family members (IL-1β) and IL-18 were also processed by neutrophil proteases, these processing events failed to generate biologically active cytokines. In contrast, IL-1α was both processed and activated by all three neutrophil-derived proteases, as reported previously (Afonina et al., 2011). Therefore, multiple (but not all) members of the extended IL-1 family are activated through processing by proteases liberated by activated neutrophils.

Elastase Is the Major IL-36γ-Processing Enzyme in Neutrophil Degranulates
Because both elastase and proteinase-3 were capable of robustly processing and activating IL-36γ (Figures 3B and 3C), we next sought to determine which of these proteases contributes the majority of the IL-36γ-processing activity in neutrophil degranulates. To address this issue, we immunodepleted elastase or proteinase-3 from activated neutrophil degranulates using specific antibodies. Immunodepletion of elastase led to a very significant reduction in AAPV-AMC hydrolysis (Figure 3D, top), whereas depletion of proteinase-3 had a much more...
modest effect in the same assay. Consistent with this, depletion of elastase robustly attenuated the activation of IL-36γ by neutrophil degranulates, suggesting that proteinase-3 plays a minor role in this context (Figure 3D, bottom). As expected from our earlier results, depletion of elastase or proteinase-3 did not affect processing of IL-36β, whereas inhibition of cathepsin G activity with a specific CatG inhibitor (CatG i) completely attenuated activation of IL-36β by neutrophil degranulates or purified cathepsin G (Figure 2E, left; Figure S3C). Taken together, these data argue that, although proteinase-3 can (in principle) process and activate IL-36β and IL-36γ when present at sufficient concentrations, at the concentrations found in neutrophil degranulates, elastase is the major IL-36γ-activating enzyme, and cathepsin G is the primary instigator of IL-36β activation.

Cell-Associated IL-36 Cytokines Are Cleaved and Activated by Neutrophil Proteases

To explore whether cell-derived, as opposed to recombinant, IL-36 cytokines were activated by neutrophil proteases, we expressed the full-length forms of these cytokines in HEK293T cells (Figure 4A). IL-36 cytokines were expressed as full-length proteins without any discernable cleavage products (Figure 4A). However, upon incubation of lysates from these cells with either cathepsin G (Figure 4B) or elastase (Figures 4C and 4D), proteolysis of IL-36 cytokines was detected. Furthermore, proteolysis of cell-derived IL-36β by cathepsin G, but not by elastase, resulted in robust biological activity (Figures 4E and 4H). Conversely, proteolysis of cell-derived IL-36γ by elastase, but not cathepsin G, potently activated this cytokine (Figures 4F and 4I). In accordance with previous results, proteolysis of cell-derived IL-36γ by either cathepsin G or elastase resulted in activation of the latter (Figure 4G). Collectively, these data demonstrate that neutrophil-derived proteases are potent instigators of IL-36-activation.

Mapping of Cathepsin G and Elastase Cleavage Sites within IL-36 Cytokines

To identify the cathepsin G cleavage site(s) within IL-36β that result in activation, we performed N-terminal Edman degradation sequencing of cathepsin G-treated IL-36β preparations. This analysis identified two candidate sites at Arg5 and Phe53 (Figure 5A). Therefore, we generated point mutations (R5A, F53A) at both of these sites to assess their role in cathepsin G-mediated activation of IL-36β. We initially tested each mutant for resistance to proteolysis by cathepsin G. As Figure 5B shows, mutation at Phe53 in IL-36β suppressed the appearance of the two major cleavage bands (~10 and ~4 kDa) but retained a third cleavage product (~17 kDa) just below the full-length protein. In contrast, mutation at Arg5 eliminated the faint ~17-kDa cleavage product but only partly affected the major cleavage products at ~10 and ~4 kDa (Figure 5B). However, as Figure 5C and Figures S4A–S4C illustrate, although mutation of Phe53 had no effect on cathepsin G-mediated IL-36β activation, mutation of Arg5 dramatically abolished activation of this cytokine. The R5A mutant also completely abolished proteinase-3-mediated IL-36β activation (Figure 5D). Furthermore, mutation of Arg5 also abolished activation of IL-36β by PMA-activated neutrophil supernatants (Figure 5D). Using a similar approach, we identified Val15 in IL-36γ as the residue that is cleaved by elastase to promote activation of the latter (Figures 5E–5G). Mutation of this residue (V15G) also dramatically attenuated activation of IL-36γ by elastase and proteinase-3 as well as by PMA-activated neutrophil degranulates (Figure 5H; Figure S4E). Finally, we identified Lys3 and Ala4 in IL-36α as the residues cleaved by cathepsin G and elastase, respectively (Figure 5I). The identities of the sites within IL-36 cytokines that are processed by all three neutrophil-derived proteases to activate the latter are summarized in Figure 5J.

Active IL-36β Induces the Expression of Numerous Pro-inflammatory Genes in Primary Keratinocytes

To further explore the biological effects of active IL-36, we conducted a gene expression analysis of cathepsin G-processed IL-36β in primary human keratinocytes. As Figure 6A demonstrates, active IL-36β induced robust expression of a diverse array of pro-inflammatory cytokines and chemokines from keratinocytes. Of particular note, IL-36 induced strong transcriptional upregulation of IL-17C (~50-fold within 8 hr), a cytokine that has been implicated as a key driver of the pathology observed in psoriasis and other inflammatory conditions.
Figure 3. Neutrophil-Derived Proteases Activate IL-36 Cytokines

(A) Hydrolysis of synthetic substrates (Ac-WEHD-AMC, Ac-DEVD-AMC, suc-FLF-sBzl, and Suc(oMe)-AAPV-AMC) by recombinant caspase-1 (1:20), caspase-3 (400 nM), cathepsin G (20 nM), elastase (50 nM), and proteinase 3 (100 nM).

(B) HeLaIL-36R cells were stimulated with IL-36α, β, or γ (500 pM) pre-incubated for 2 hr at 37°C with the indicated concentrations of recombinant caspase-1, caspase-3, or purified cathepsin G, elastase, or proteinase-3. IL-1β p17 (25 nM) served as a positive control for caspase titrations. After 24 hr, cytokine concentrations in the culture supernatants were determined by ELISA.

(C) HeLaIL-36R cells were stimulated with a titration of IL-36α, β, and γ pre-incubated for 2 hr at 37°C with fixed concentrations of purified cathepsin G (50 nM), elastase (100 nM), or proteinase-3 (100 nM). After 24 hr, cytokine concentrations in the culture supernatants were determined by ELISA.

(legend continued on next page)
IL-36 was sufficient to perturb skin differentiation with significant epidermal thickening and expanded stratum layers, similar to the hyperproliferative pathology of psoriasis (Figures 7A and 7B). These data suggest that active IL-36 cytokines may directly contribute to the pathological features of psoriasis through initiating not only the expression of a diverse array of pro-inflammatory cytokines and chemokines but also through stimulating the production of keratinocyte growth factors. In this context, it is highly relevant to note that previous studies have shown that the related cytokine IL-1α has been found to trigger production of keratinocyte growth factor (KGF) and epidermal growth factor (EGF) from fibroblasts (Maas-Szabowski et al., 2000). Although exploring this was outside of the scope of this study, it is highly plausible that IL-36 cytokines also exert at least some of their effects via fibroblasts as well as through effects on keratinocytes.

Psoriatic Human Skin Exhibits Elevated Cathepsin G-like Activity that Is Sufficient to Activate IL-36β

Neutrophil infiltration is a pathological hallmark of a wide range of inflammatory conditions, including inflammatory skin conditions such as psoriasis (Terui et al., 2000; Murphy et al., 2007; Amulic et al., 2012; Ikeda et al., 2013). Therefore, we sought to determine whether skin from patients with a common form of psoriasis (psoriasis vulgaris) exhibited elevated neutrophil protease activity. To explore this, we collected tape-stripped skin samples from healthy or psoriatic human volunteers, followed by elution of proteins from these samples and measurement of AAPV-AMC peptide (AAPVase) activity. Significantly, although none of the samples displayed elevated AAPVase activity, skin eluates from psoriatic individuals exhibited dramatically elevated levels of AAPVase activity (Figure 7C). Next we tested the ability of these skin eluates to activate exogenously added IL-36 cytokines. Consistent with the synthetic peptide hydrolysis results, IL-36β was activated robustly and selectively by eluates from psoriatic individuals (Figure 7D). In line with the absence of elevated AAPVase activity in any of the skin eluates, IL-36α and IL-36γ failed to be activated under the same conditions. Furthermore, a specific inhibitor of cathepsin G (CatG i) completely suppressed activation of IL-36β in eluates from psoriatic individuals, whereas inhibition of elastase had no effect (Figure 7D, right). These data provide preliminary evidence that cathepsin G levels are elevated in psoriatic skin and that this enzyme is responsible for the processing and activation of IL-36β in this context.

DISCUSSION

Here we report that IL-36 family cytokines are activated by neutrophil-derived granule proteases (Figure 5I). Biologically active IL-36 was found to induce a panoply of pro-inflammatory factors from primary human keratinocytes, many of which are known to be upregulated in lesional skin from psoriasis patients (Johnston et al., 2011; Ramirez-Carrozzi et al., 2011; Schonthaler et al., 2013). Active IL-36 was also sufficient to perturb differentiation in a human reconstituted 3D skin model, suggesting that one or more IL-36-induced factors may be directly responsible for the hyperproliferation and impaired differentiation observed in psoriatic skin. Consistent with our observations that cathepsin G can process and activate IL-36β, we have also found that cathepsin G activity is elevated dramatically in human psoriatic skin eluates and that this activity was capable of activating IL-36β.

Sequence Mapping of Neutrophil Cleavage Sites in IL-36 Cytokines

Although it is known, through deletion analysis, that IL-36 cytokines require processing at their N termini for activation (Towne et al., 2011), the proteases that naturally process and activate these cytokines have not been identified until now. We identified Arg5 as the critical residue required for IL-36β activation by cathepsin G. We also identified another cathepsin G cleavage site within IL-36β, Phe53, but found, through mutagenesis, that processing at this site was not required for activation. However,
replacement of Arg5 with an alanine (R5A) completely abolished the ability of cathepsin-G and proteinase-3 to activate IL-36β (Figure 5; Figure S4). Although Sims and colleagues (Towne et al., 2011) have shown that truncation of IL-36β after Gln4 greatly enhances biological activity, no protease has been identified that cleaves IL-36β at this site. We also mapped the
elastase and proteinase-3 cleavage sites within IL-36γ and identified Val15 as the critical residue required for processing of the latter by either protease. In contrast, Sims and colleagues (Towne et al., 2011) have reported previously that truncation of IL-36γ at Gln17 greatly enhanced biological activity. However, a protease capable of cleaving IL-36γ at Gln17 remains unknown.

**Neutrophils and Psoriatic Inflammation**

Immune cell infiltration is a hallmark of a number of skin-related inflammatory diseases. In particular, psoriatic plaques are heavily infiltrated with neutrophils, dendritic cells, macrophages, and T cells (Terui et al., 2000; Murphy et al., 2007; Nestle et al., 2009). Neutrophils are first responder cells in the innate immune system and play a critical role in the initial response to infection or tissue damage (Pham, 2006; Borregaard et al., 2007; Kolaczkowski and Kubes, 2013). Although release of neutrophil proteases can exert profound antimicrobial and protective effects during infection, these proteases can also provoke extensive tissue damage and exacerbate inflammation (Garver et al., 1986; Hubbard et al., 1991; Wiedow et al., 1992). Interestingly, the onset of psoriasis symptoms in affected individuals often follows a streptococcus A or viral infection (Telfer et al., 1992), which is associated with robust neutrophil and macrophage infiltration as well as necrosis of infected tissue. Psoriatic lesions can also develop as a result of physical injuries such as lacerations, burns, or surgical incisions (Weiss et al., 2002). Therefore, a feature that unites the above scenarios is that all are capable of triggering necrotic cell death in the epidermis, which results in neutrophil infiltration (Kono and Rock, 2008). As a consequence of necrosis, endogenous alarmins such as IL-36 and other IL-1 family cytokines are released into the extracellular space where they may become processed by proteases liberated from activated neutrophils. Therefore, damage to keratinocytes resulting in the liberation of IL-36 cytokines, either as a result of microbial infection or through tissue trauma, may play an important initiating role in psoriasis, especially in individuals lacking endogenous buffers of IL-36 activity, such as deficiency in the IL-36R antagonist (Marrakchi et al., 2011; Farooq et al., 2013; Onoufriodis et al., 2011; Kanazawa et al., 2013).

An interesting question that emerges from our data is why there is selectivity between neutrophil proteases for activation of particular IL-36 cytokines. One reason might be to preclude pathogen-encoded protease inhibitors from simultaneously blocking activation of all members of this family through neutralizing a single protease (Eggers et al., 2004). Another reason may be to prevent runaway escalation of inflammation through a single protease that simultaneously activates multiple members of the IL-1 family.

**Neutrophil-Derived Proteases as Amplifiers of Inflammation**

Although neutrophils are adept at engulfing and killing microbial agents by means of their granule enzymes, these destructive enzymes can also be released into the extracellular space either through degranulation or the formation of neutrophil extracellular traps (Brinkmann et al., 2004; Amulic et al., 2012). Although neutrophils are classically viewed as efficient microbial killers, these cells are also recruited abundantly to sites of sterile injury. This begs the question of why neutrophils are needed within a tissue in the absence of infection. Although neutrophil recruitment in these instances may serve as a safeguard against infection, it is also possible that these cells play an important role in coordinating inflammatory responses by amplifying the actions of cytokines, such as IL-36, that are released via tissue injury. Therefore, neutrophil recruitment may serve two major roles in immunity: pathogen elimination and amplification of immune responses through recruitment of other immune cells via the modulation of cytokine production. Moreover, previous studies have suggested that neutrophil proteases do indeed play an important immune-regulatory role that goes beyond their role in pathogen elimination (reviewed in...
Pham, 2006). The major neutrophil-derived proteases elastase, cathepsin G, and proteinase 3 have been implicated frequently in amplifying and sustaining inflammatory responses via processing of extracellular proteins, although no consistent mechanism has been identified to date (Adkison et al., 2002; Meyer-Hoffert and Wiedow, 2011; Lefranc¸ ais et al., 2012). Dipeptidyl peptidase 1 (DPPI)-deficient mice, which are defective in the activation of all major neutrophil serine proteases, exhibit dramatically reduced recruitment of neutrophils to inflammatory sites as well as a reduced incidence of certain experimental autoimmune diseases (Adkison et al., 2002; Pham, 2006 ). Furthermore, neutrophil recruitment to sites of injury or infection seems to be a pre-requisite for recruitment of macrophages and monocytes. These and other observations strongly suggest that neutrophil-derived proteases play important roles in amplifying inflammation, which may be explained by their ability to

![Image](https://example.com/image.png)

**Figure 6. Activated IL-36 Initiates a Global Pro-inflammatory Response from Primary Keratinocytes**

(A) Heatmap of IL-36β\text{CatG}-induced genes in primary keratinocytes at 8 hr.
(B) Primary keratinocytes were stimulated with IL-36β\text{FL} or IL-36β\text{CatG} (5 nM). At the indicated time points, the levels of cytokine mRNA transcripts were quantified by RT-PCR.
(C and D) Primary keratinocytes were stimulated for 24 h with either IL-36β\text{FL} or IL-36β\text{CatG} (5 nM). At the indicated time points, G-CSF, GM-CSF, IL-8, CXCL1, and CCL20 concentrations in the culture supernatants were determined by ELISA (C). Primary human keratinocytes were stimulated for 48 hr with either IL-36β\text{FL} or IL-36β\text{CatG} (5 nM) as shown. At the indicated time points, IL-17C concentrations in the culture supernatants were determined by ELISA (D).

Error bars represent the mean ± SEM of triplicate determinations from a representative experiment. All data shown are representative of at least three independent experiments. ***p < 0.0001, **p < 0.001, *p < 0.1, Student’s t test. See also Figure S5.
Figure 7. Active IL-36β Perturbs Differentiation in an Organotypic Skin Model, and Psoriatic Skin Displays Elevated Cathepsin G Protease Activity Sufficient to Activate IL-36β

(A) Organotypic skin reconstructs cultivated at the air-to-liquid interface were stimulated topically with either cathepsin G alone, IL-36βFL (2 nM), or IL-36βCatG (2 nM). Skin sections were stained with H&E against filaggrin, involucrin, and cytokeratin 10 and 14, as indicated, to display epidermal thickness and differentiation.

(B) Quantification of epidermal thickness using ImageJ software.

(C) Enzymatic activity in control (n = 6) or psoriatic skin (n = 6) elutes was measured using suc-FLF-εBzI and AAPV-AMC synthetic peptides to assess cathepsin G-like and elastase-like activity, respectively.

(D) Left: HeLaIL-36R cells were stimulated with IL-36α or IL-36γ (500 pM) pre-incubated for 2 hr at 37°C with control skin (n = 6) or psoriatic skin (n = 6) elutes. After 24 hr, cytokine concentrations in the culture supernatants were determined by ELISA. Right: HeLaIL-36R cells were stimulated with IL-36β (500 pM) pre-incubated (legend continued on next page)

Cell Reports 14, 708–722, February 2, 2016 © 2016 The Authors 719
process and activate members of the IL-36 family of cytokines, as we have shown here. Therefore, targeted inhibition of neutrophil-derived proteases may be beneficial in the treatment of inflammatory skin conditions such as psoriasis.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Polyclonal antibodies were generated against IL-36α, β, and γ proteins by repeated immunization of rabbits with the full-length recombinant IL-36 proteins (Biogenes). Anti-IL-1α (AHP281G) was obtained from AbD Serotec. Anti-IL-1β (MAb201) antibody was from R&D Systems. Anti-IL-18 (ab68435) antibody was obtained from Abcam. Anti-PCDGF (40-3400) was obtained from Thermo Fisher Scientific. Anti- elastase (clone NP57, sc-53388) was obtained from Santa Cruz Biotechnology. Anti-protease-3 (clone 6A8) was obtained from Euro Diagnostica. Protein A/G PLUS agarose (sc-2003) was obtained from Santa Cruz Biotechnology. The synthetic peptides Ac-DEVD-AMC, Ac-WEHD-AMC, and biotin-VAD-FMK were all purchased from Bachem. Suc(OMe)-AAPV-AMC was purchased from Peptanova. Biotin-VAD-FMK was purchased from ICN. The novel synthetic peptides biotin-FLF-CMK and z-FLF-CMK were synthesized by Boston Open Labs. The chemical inhibitors cathepsin G inhibitor I (219415) and elastase inhibitor IV (324759) were purchased from Calbiochem. Purified neutrophil-derived cathepsin G was purchased from Calbiochem. Purified neutrophil-derived elastase was purchased from Serva. Purified neutrophil-derived proteinase-3 was purchased from Enzo Scientific. Unless indicated otherwise, all other reagents were purchased from Sigma.

**Cell Culture**

HeLa cells were cultured in RPMI medium (Gibco) supplemented with 5% fetal calf serum (FCS). HaCaT cells were cultured in DMEM (GIBCO) supplemented with FCS (10%). Primary neonatal foreskin-derived keratinocytes P0 were purchased from Cell Systems and cultured in serum-free DermaLife K medium (Cell Systems). KG-1 cells were cultured in RPMI medium (Gibco) supplemented with 10% FCS, 10% sodium pyruvate, and 10% non-essential amino acids. The HeLa vector and HeLa IL-36R cell lines were generated by transfection with the pCX2Nempty or pCX2N-IL-1Rbp2 (IL-36R) plasmids, followed by selection using G-418 antibiotic (Sigma). IL-36R-overexpressing clones were expanded from a single cell. Clones were selected by demonstration of acquired optimal responsiveness to active forms of IL-36 via ELISA. The HeLa IL-36R:SEAP cell line was generated by transfection with the phi5fty2-SEAP plasmid (InvivoGen), followed by selection using zeocin antibiotic. Clones were expanded from a single cell and tested for secreted alkaline phosphatase (SEAP) production. All cells were cultured at 37°C in a humidified atmosphere with 5% CO₂.

**Expression and Purification of Recombinant Proteins**

Full-length IL-36α, β, and γ proteins were generated by cloning the human coding sequences in-frame with the poly-histidine tag sequence in the bacterial expression vector PET45b. Individual clones were sequence-validated. Protein was expressed by addition of 600 μM isopropyl (i-D-1-thiogalactopyranoside (IPTG)) to exponentially growing cultures of E. coli (BL21 strain), followed by incubation for 3 hr at 37°C. Bacteria were lysed by sonication, and poly-histidine tagged proteins were captured using nickel-nitrilotriacetic acid (NTA) agarose (QIAGEN), followed by elution into PBS (pH 7.2) in the presence of 100 mM imidazole. Modified forms of IL-36 that included a caspase-3-processing motif (DEVD) were cloned into the IL-36 sequences N-terminal to the known processing sites (Towne et al., 2011). All IL-36 mutants were expressed and purified in the same way. Recombinant poly-histidine-tagged caspase-1 and caspase-3 were expressed and purified as described previously (Walsh et al., 2011).

**Purification of Primary Neutrophils and Preparation of Degranulaties**

Primary human neutrophils were purified from donor whole blood using the Ficoll-Hypaque gradient method as described previously (Haslett et al., 1985). The purity of the cell preparations (>90%) was determined by H&E staining of cytopsins. To prepare degranulates, neutrophils (10⁶ per treatment) were stimulated in the presence or absence of 50 nM PMA in Hank’s balanced salt solution (HBSS)/0.25% BSA for 1–3 hr at 37°C in a humidified atmosphere with 5% CO₂. Supernatants were harvested and clarified by centrifugation at 4°C (10,000 × g for 5 min). Neutrophil degranulate aliquots were stored at −80°C.

**Protease Activity Assays**

Reactions (50 μl, final volume) were carried out in protease reaction buffer (50 mM HEPES [pH 7.2], 75 mM NaCl, and 0.1% 3-[(-cholamidopropyl)dimethy lammonio]-1-propanesulfonate [CHAPS] [2 mM DTT added only for caspases]) containing Ac-DEVD-ACF, Ac-WEHD-AMC, and Suc(OMe)-AAPV-AMC (50 μM, final concentration). Samples were measured by using an automated fluorimeter (SPARK 10M, Tecan) at wavelengths of 430 nm (excitation) and 535 nm (emission). For the suc-FLF-sBzl hydrolysis assay, the substrate was diluted to a final concentration of 300 mM in protease reaction buffer (50 mM HEPES [pH 7.2], 75 mM NaCl, 0.1% CHAPS, and 300 mM 5,5'-dithiobis nitrobenzoic acid [DTNB]). Cathepsin G hydrolyzes the synthetic substrate suc-FLF-sBzl with the release of the thiobenzyl group. The free thiobenzyl group reacts with DTNB and produces a chromophore (3,3',5,5'-tetramethylbenzidine [TMB]) that absorbs at 430 nm. Samples were measured by automated fluorimeter (SPARK 10M).

**Protease Cleavage Assays**

Reactions (40–100 μl, final volume) were carried out in protease reaction buffer (50 mM HEPES [pH 7.2], 75 mM NaCl, and 0.1% CHAPS) for 2 hr at 37°C. For IL-36 bioassays, IL-36 cytokines were typically cleaved at a 50-nM concentration and subsequently diluted onto target cells at a final concentration ranging from 0.25–2 nM.

**Tape Strip Samples from Control and Psoriatic Skin**

Fixomull (2 × 2 cm) adhesive tape strips were applied to healthy or psoriatic skin under firm pressure for 10 s. The tape strips were removed gently, placed in sterile 1.5-mL Eppendorf tubes, and eluted with protease reaction buffer (PRB) (50 mM HEPES [pH 7.2]/75 mM NaCl/0.1% CHAPS) under constant rotation for 1 hr at 4°C. Skin eluates were stored at −80°C. Enzymatic activity of control and psoriatic skin eluates was measured using the protease activity assays outlined above. Bioassays were conducted according to the protease cleavage assays outlined above.

**Gene Expression Microarray Analysis**

Primary human neonatal foreskin-derived keratinocytes (passage 3) were used for gene expression analysis. Primary keratinocytes were stimulated with full-length or cathepsin G-activated IL-36β for 8 hr. Cells were harvested with RNAprotect cell reagent (QIAGEN) and stored at −80°C. Analyses of samples were performed by IMGM Laboratories using the SurePrint G3 Human Gene Expression 8x60K v2 microarray using a one-color-based hybridization protocol. Gene expression analysis and the generation of heatmaps was conducted using software available from http://www.chibi.ubc.ca/matrix2png/bin/matrix2png.cgi.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.12.072.
AUTHOR CONTRIBUTIONS

C.M.H. designed and performed experiments, analyzed data, generated the figures, and wrote the figure legends. O.P.S. performed the experiments relating to patient skin samples and generated some figures. D.C. and I.S.A. performed experiments and generated some figures. D.K. performed the organotypic skin model experiments. S.J.M. conceived the study, designed and analyzed the experiments, supervised the study, and wrote the manuscript with contributions from C.M.H.

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