Cathepsin S is the major activator of the psoriasis-associated proinflammatory cytokine IL-36γ

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The proinflammatory cytokine IL-36γ is highly expressed in epithelial cells and is a pivotal mediator of epithelial inflammation. In particular, IL-36γ is strongly associated with the inflammatory skin disease psoriasis. As with other IL-1 cytokines, IL-36γ is expressed as an inactive precursor and must be processed by specific proteases to become bioactive. Our aim therefore was to identify proteases capable of IL-36γ activation and explore the importance of this activation in psoriasis. Using a keratinocyte-based activity assay in conjunction with small-molecule inhibitors and siRNA gene silencing, cathepsin S was identified as the major IL-36γ-activating protease expressed by epithelial cells. Interestingly, cathepsin S activity was strongly up-regulated in samples extracted from psoriasis patients relative to healthy controls. In addition, IL-36γ–Ser18, identified as the main product of cathepsin S-dependent IL-36γ cleavage, induced psoriasisform changes in human skin-equivalent models. Together, these data provide important mechanistic insights into the activation of IL-36γ and highlight that cathepsin S-mediated activation of IL-36γ may be important in the development of numerous IL-36γ-driven pathologies, in addition to psoriasis.

Significance

IL-36γ is a potent cytokine that drives and orchestrates inflammation. It is strongly expressed at barrier tissues such as the skin and is particularly relevant to inflammatory diseases that affect these tissues, including psoriasis. IL-36γ is expressed as an inactive precursor that requires precise N-terminal truncation (9 amino acids upstream of a conserved A-X-D motif) was also required for IL-36α, IL-36β, and IL-36γ to gain biological activity (25). Specifically, activation of IL-36α was demonstrated when cleaved at amino acid K6, IL-36β at R5, and IL-36γ at S18. In these in vitro studies, precise cleavage was found to increase receptor affinity over 10,000-fold, suggesting that processing is likely to be significant in an in vivo setting.

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The interleukin (IL)-1 family cytokines are fundamental regulators of the innate immune system and orchestrate multiple inflammatory responses (1, 2). IL-1 cytokines are produced rapidly following infection or injury and are capable of potently inducing a range of beneficial proinflammatory processes, including additional cytokine expression, antigen-presenting cell migration, and leukocyte activation and infiltration (3–5). The aberrant expression and regulation of IL-1 cytokines is associated with a broad range of immunoopathologies, ranging from autoimmune to inborn immune disorders (6–8). Therefore, a greater insight into the regulation and function of IL-1 cytokines is not only of academic interest but also of significant therapeutic importance.

IL-36α, IL-36β, and IL-36γ are agonistic cytokines and the most recently discovered of the IL-1 family (9). Interestingly, there is growing evidence to suggest that these cytokines are important for the development of several inflammatory disorders, including psoriasis (10). In psoriatic lesions, the IL-36 cytokines have been shown to be among the most specific and highly up-regulated mRNAs relative to other inflammatory skin diseases and healthy controls (11–13). Moreover, hypomorphic mutations in the IL-36 receptor antagonist (IL-36Ra) cause the severe and potentially lethal subtype of psoriasis called pustular psoriasis in a number of cohorts (14, 15). Mouse models further support these observations, showing that IL-36 overexpression in keratinocytes results in a transient inflammatory skin condition resembling psoriasis (16). In addition, IL-36 receptor-deficient mice have been found to be resistant to Imiquimod-induced psoriasisform dermatitis (17). Interestingly, recent studies have also demonstrated a role for the IL-36 receptor in mucosal wound healing, suggesting that IL-36 cytokines have an important physiological role in epithelial repair and homeostasis as well as the more established, pathological role in the initiation and maintenance of psoriatic inflammation (18).

Unlike many other cytokines, IL-1 family members are expressed without a signal peptide and therefore are not secreted via the classical secretory pathway (19). In addition, most IL-1 cytokines are expressed as inactive precursors and must be processed to become biologically active (20). The activation of IL-1β and IL-18, for instance, is dependent upon caspase-1, a proteolytic enzyme that is regulated by large multiprotein inflammasome complexes (21, 22). This cascade can be induced by a range of endogenous and exogenous triggers and serves as an important sensor of danger (23). In contrast, IL-1α does not contain a caspase-1 cleavage motif and is instead processed by the calcium-dependent protease calpain (24). In short, these IL-1–processing proteases are central to the activation of IL-1 family cytokines and thus are essential mediators of inflammation. In a previous report, it was demonstrated that a precise N-terminal truncation (9 amino acids upstream of a conserved A-X-D motif) was also required for IL-36α, IL-36β, and IL-36γ to gain biological activity (25). Specifically, activation of IL-36α was demonstrated when cleaved at amino acid K6, IL-36β at R5, and IL-36γ at S18. In these in vitro studies, precise cleavage was found to increase receptor affinity over 10,000-fold, suggesting that processing is likely to be significant in an in vivo setting.

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IL-36 activating proteases in the context of psoriatic inflammation, where the therapeutic potential is evident (26). IL-36γ is of particular interest here, as this cytokine is the only IL-36 cytokine constitutively expressed in the skin (13) and is the most strongly up-regulated in psoriasis plaques (12). In a recent report by Henry et al., it was found that the neutrophil-derived proteases neutrophil elastase and proteinase 3 were capable of activating IL-36γ (27). Although the findings of this report are of interest, the expression of these IL-36γ-activating proteases by neutrophils would likely be limited to sites of acute inflammation. Furthermore, despite neutrophil proteases generating IL-36γ activity, the study showed that the majority of cleavage by neutrophil proteases generated inactive forms of IL-36γ and only minor undetectable amounts of bioactive cytokines were responsible for activity. Due to their lack of specificity and expression profile, it is therefore unlikely that these proteases represent the major processors of IL-36γ within barrier tissues. In this study, we show that IL-36γ processing is independent of caspase-1 and that the cysteine protease cathepsin S precisely cleaves and activates IL-36γ. Both intracellular and secreted extracellular cathepsin S activity was detectable in a range of skin resident cells, including fibroblasts and keratinocytes, suggesting that this protease represents the major regulator of IL-36γ at barrier tissues. Furthermore, it was also shown that cathepsin S levels and activity are elevated in psoriatic skin lesions and that IL-36γ Ser(S)15, the product of cathepsin S-dependent IL-36γ processing, drives hyperkeratosis in skin equivalents. Together, these data demonstrate that the precise cleavage of IL-36γ by cathepsin S is critical in the activation of its bioactivity and that this processing may be pivotal in the generation of a psoriatic phenotype.

Results

Epithelial Cell-Derived Proteases Activate IL-36γ. Initial experiments confirmed that the addition of truncated IL-36γ (IL-36γ-S18) induces the secretion of the proinflammatory cytokine IL-8 in a human keratinocyte cell line (HaCaT cells; Fig. L4). In contrast, the addition of full-length IL-36γ (IL-36γ FL) had no effect on IL-8 levels, supporting previous evidence suggesting that IL-36 cytokines require processing to elicit their proinflammatory activity (25). These effects were shown to be IL-36 dependent, as they were ablated by addition of the IL-36Ra. Importantly, these results also validated the use of HaCaT cells as a bioassay for measuring IL-36γ processing.

It is well established that other IL-1 cytokines, most notably IL-1β, can be cleaved and activated by caspase-1 (21). By incubating IL-36γ FL or SUMO-tagged IL-36γ FL (both polyhistidine tagged) with recombinant caspase-1 and then running the samples on an anti-His Western blot, it was confirmed that caspase-1 does not cleave IL-36γ FL or SUMO-tagged IL-36γ FL, suggesting that this caspase is not capable of IL-36γ activation (Fig. 1A). Although some IL-36-activating proteases have been identified previously, we propose that these do not represent the major tissue resident activators of IL-36γ. Recent evidence suggests that IL-36 cytokines play important roles in inflammatory responses at epithelial surfaces. Therefore, we hypothesized that IL-36γ-activating proteases may also be expressed within the cells of such barrier tissues. To test this hypothesis, the epithelial cell line A549 was lysed using a hypotonic buffer and incubated with HaCaTs for 24 h. Neither the addition of the lysate nor IL-36γ FL alone had an effect on IL-8 expression (Fig. 1C). However, when both the lysate and IL-36γ FL were added together, there was a modest but significant increase in IL-8 expression, suggesting that there are IL-36γ-activating proteins within the lysate. Importantly, the addition of IL-36Ra ablated this effect, demonstrating that the increase in IL-8 expression was due to IL-36γ activity.

To determine the subcellular localization of the IL-36γ- processing activity, the A549 cell lysates were separated into cytosolic and lysosomal fractions and the activity assay was repeated. First, successful separation of cellular fractions was demonstrated by Western blot analysis. Here it was shown that the cytosol fraction contained the cytosol marker GAPDH but not the lysosome marker LAMP-1 (lysosomal-associated membrane protein 1), whereas the lysosomal fraction contained LAMP-1 but not GAPDH (Fig. 1D). In the activity assay experiments, the addition of both IL-36γ FL and A549 cytosol had no effect on IL-8 levels, suggesting that the IL-36γ-processing activity did not reside within this fraction (Fig. 1E). In contrast, the addition of both the lysosomal fraction and IL-36γ FL caused a strong and significant up-regulation in IL-8 secretion, relative to both IL-36γ FL alone and lysosome alone-treated cells. Again, this effect was almost completely ablated when IL-36Ra was added, excluding nonspecific effects on IL-8 secretion. To investigate whether there is IL-36γ processing in other cell types, the above experiment was repeated using lysosomal fractions from HaCaT cells (Fig. 1F), primary human fibroblasts (Fig. 1G), and keratinocytes (Fig. 1H). As observed with A549 cells, the incubation of cells with both IL-36γ FL and lysosome caused a significant and IL-36γ-specific up-regulation in IL-8 secretion, relative to samples treated with IL-36γ FL or lysosome alone. Together these data highlight that IL-36γ-activating cytokines are expressed ubiquitously, at least within the lysosomes of cells in barrier tissues.

Activation of IL-36γ Is Dependent on Cathepsin S. The lysosome contains a broad range of proteases, most of which are either serine or cysteine proteases (28). To identify the IL-36γ-activating protease, the class of this protease was first determined using broad-range molecular inhibitors. As previously shown, the incubation of HaCaT cells with both IL-36γ FL and A549 cell lysosome caused a significant increase in IL-8 secretion (Fig. 2A). Although this IL-8 secretion was unaffected by addition of the serine protease inhibitor AEBSF [4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride], IL-8 secretion was almost completely ablated by the addition of the cysteine protease inhibitor E64. Importantly, E64 had no effect on IL-36γ S18-induced IL-8 secretion, demonstrating that the inhibitor does not simply inhibit IL-8 secretion. Thus, it was concluded that the IL-36γ-activating lysosomal protease is a cysteine protease/s.

In the activity assay used, HaCaT cells were cultured in a neutral-buffered media, and therefore, the IL-36γ-activating lysosomal protease must be both stable and active at pH 7. Whereas most lysosomal cysteine proteases only function at an acidic pH, cathepsin S can function at a neutral pH (29), and so this protease was identified as a candidate IL-36γ-activating lysosomal protease. To test this, HaCaT cells were incubated with A549 cell lysosome and IL-36γ FL, both in the absence and presence of the specific cathepsin S inhibitor cathepsin S inhibitor (CATSi). IL-8 secretion induced in response to lysosome and IL-36γ FL was completely abrogated by the addition of CATSi (Fig. 2B). Again, the inhibitor had no effect on S18-induced IL-8 secretion, highlighting that the CATSi functions to inhibit IL-36γ FL processing specifically. To support these findings and rule out any nonspecific effects of the CATSi, the activity assay was repeated using lysosome extracted from A549 cells that had been transfected with cathepsin S siRNA or scrambled siRNA. Down-regulation of cathepsin S lysosomal protein expression in the cathepsin S siRNA-transfected cells was significant, relative to the scrambled siRNA control (Fig. 2C). Importantly, the down-regulation in cathepsin S expression resulted in a strong and significant decrease in the lysosome and IL-36γ FL-induced IL-8 secretion, again relative to the scrambled siRNA control (Fig. 2D). Therefore, there is robust evidence to suggest that the activation of IL-36γ by lysosomal proteases is dependent on cathepsin S.

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Secreted Cathepsin S Activates IL-36γ. It is well established that cathepsin S can be secreted by a range of different cell types. Thus, we postulated that cathepsin S may also function extracellularly to induce IL-36γ FL activation. In this investigation, cathepsin S was shown to accumulate in the supernatants of A549 cells, primary keratinocytes, and primary fibroblasts (Fig. 3A). To test whether this secreted cathepsin S could activate IL-36γ, supernatants were collected and added to HaCaT cells. In these experiments, 0.1 mM Dithiothreitol (DTT) was added to the supernatants to facilitate cathepsin S activity. Importantly, the addition of IL-36γ FL to DTT-supplemented OptiMEM did not have any effect on IL-8 production, relative to DTT-supplemented OptiMEM alone, highlighting that the de novo cathepsin S production by HaCaT cells is not significant enough to...

Fig. 1. Epithelial cell proteases activate IL-36γ. (A) HaCaT cells (10^5 per well) were incubated with media alone (U), IL-36γ FL (36γ; 10 nM), IL-36γ S18 (S18; 10 nM), or both IL-36γ S18 and IL-36Ra (RA; 50 nM) for 24 h. In addition, 1 μg of SUMO-tagged recombinant IL-36γ (suIL-36γ), IL-36γ FL, or pro–IL-1β (all polyhistidine tagged) was incubated with recombinant caspase-1 at 37 °C for 6 h. (B) These samples were then analyzed by Western blot using an anti–His-HRP antibody. A protein marker lane on each gel was used to determine molecular weight. (C) HaCaT cells (10^5 per well) were also incubated for 24 h with media alone, IL-36γ FL, whole-cell lysates from A549 cells (W), or a combination of IL-36γ FL and whole-cell lysates with and without IL-36Ra (50 nM). (D) HaCaT cells (10^5 per well) were incubated for 24 h with IL-36γ FL (10 nM), the cytosolic fraction of A549 cells (C), a combination of IL-36γ FL and cytosol with and without IL-36Ra (10 nM), the lysosome fraction from A549 cells (L), or a combination of IL-36γ FL and lysosome with and without IL-36Ra. HaCaT cells (10^5 per well) were also incubated with IL-36γ FL (10 nM), the lysosome fraction of either HaCaT cells (F), primary fibroblasts (G), or primary keratinocytes (H), or a combination of IL-36γ FL and lysosome with and without IL-36Ra (50 nM). (A, C, and E–H) The supernatants from these experiments were analyzed for the presence of IL-8 using cytokine-specific ELISA. (D) The cytosolic and lysosomal fractions from A549 cells were analyzed by Western blot using either an anti–LAMP-1 or anti-α-tubulin antibody. A one-way ANOVA was used to determine statistical significance of differences between treatment groups. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data shown are mean ± SEM (n = 3).
affect IL-8 secretion (Fig. 3B). Here, the addition of both IL-36γ FL with either A549 cell-conditioned media (Fig. 3B), keratinocyte-conditioned media (Fig. 3C), or fibrobласт-conditioned media (Fig. 3D) caused an increase in IL-8 secretion, relative to IL-8 secretion induced by the addition of conditioned media alone. These data indicate that there is IL-36γ FL processing activity within the secretome of A549 cells, primary keratinocytes, and primary fibroblasts. Of note, activity was ablated by the addition of IL-36Ra, proving that the increase in IL-8 secretion was specifically due to the processing of IL-36γ FL.

To determine whether this extracellular IL-36γ FL processing activity was dependent on cathepsin S, A549 cells were transfected with cathepsin S siRNA or scrambled siRNA. Down-regulation of cathepsin S protein levels was observed in the supernatant of the cathepsin S siRNA-transfected cells and was found to be significantly lower than cathepsin S levels in supernatants extracted from the scrambled siRNA-treated cells (Fig. 3E). Importantly, the down-regulation of cathepsin S resulted in a strong and significant decrease of IL-8 secretion induced by IL-36γ FL in combination with conditioned media, relative to the scrambled siRNA control (Fig. 3F). Therefore, these results demonstrate that secreted cathepsin S also activates IL-36γ FL.

**Recombinant Cathepsin S Cleaves and Activates IL-36γ.** To explore the nature of cathepsin S-dependent IL-36γ cleavage, recombinant cathepsin S and recombinant IL-36γ FL were used. In these experiments, the recombinant IL-36γ FL was expressed with an N-terminal SUMO tag so that IL-36γ FL processing could be visualized by electrophoresis. When both IL-36γ FL and cathepsin S were incubated together at 37 °C, IL-36γ FL was rapidly cleaved (within 5 min) into a protein of ~17 kDa (Fig. 4A). The identity of the cleaved protein was interrogated by mass spectrometry and was found to have a mass of 17,031, corresponding exactly to the predicted mass of the active form IL-36γ cleaved between residue glutamine 17 and serine 18 (Fig. 4A). This was also confirmed via N-terminal sequencing.

To demonstrate that recombinant cathepsin S can activate IL-36γ FL, both proteins were incubated together or separately for 10 min at 37 °C, and the products of this incubation were added to cultured HaCaT cells. Although the addition of IL-36γ FL or cathepsin S did not have any effect on IL-8 secretion when added individually, the addition of both together caused a strong and significant up-regulation in IL-8 secretion, supporting previous evidence suggesting that cathepsin S potently activates IL-36γ FL (Fig. 4B). Finally, it was important to demonstrate that cathepsin S could activate native as well as recombinant IL-36γ FL. It was well established that keratinocytes constitutively express IL-36γ (12). To determine whether cathepsin S can activate the IL-36γ contained within primary keratinocytes, HaCaT cells were incubated with either cathepsin S alone, the cytosolic fraction of primary keratinocyte alone, or both cytosolic fraction and cathepsin S. Whereas the addition of cathepsin S alone did not affect on IL-8 secretion, the addition of the cytosolic fraction caused a strong up-regulation in IL-8 secretion, suggesting that there are IL-8-inducing factors in this fraction (Fig. 4C). Importantly, IL-8 secretion was significantly higher when cells were incubated with both cathepsin S and cytosolic fraction together.

![Activation of IL-36γ](image)

**Fig. 2.** Activation of IL-36γ is dependent on cathepsin S. (A) HaCaT cells (10⁵ per well) were incubated for 24 h with media alone, IL-36γ S18 (S18; 10 nM), or IL-36γ FL (FL; 10 nM) with the lysosome fraction of A549 cells, all with and without the cysteine protease inhibitor E64 (0.2 μM) or the serine inhibitor AEBSF (2 μM). (B) In these experiments, the inhibitors were preincubated with the IL-36γ S18 or lysosome at 4 °C for 3 h to give an initial concentration of 0.1 mM for AEBSF and 10 μM for E64. HaCaT cells (10⁵ per well) were also incubated for 24 h with media alone, IL-36γ S18 (10 nM), or IL-36γ FL (10 nM) with the lysosome fraction of A549 cells, all with and without the cathepsin S inhibitor (CATS; 100 ng/mL). (C) Again, the CATS was preincubated with the IL-36γ S18 or lysosome at 4 °C for 3 h to give an initial concentration of 5 μg/mL. To inhibit expression of cathepsin S within A549 lysosomes, A549 cells were transfected with cathepsin S siRNA (CATS) or scrambled siRNA (SCRAM; both 25 nM) for 24 h and lysosome fractions extracted. The lysosome fractions were analyzed for the presence of cathepsin S using a specific ELISA. (D) Finally, HaCaT cells (10⁵ per well) were incubated for 24 h with the lysosome of SCRAM-transfected or CATS-transfected A549 cells, both with or without IL-36γ FL (10 nM). (A, B, and D) The supernatants from these experiments were analyzed for the presence of IL-8 using cytokine-specific ELISA. A one-way ANOVA (A, B, and D) or unpaired t test (C) was used to determine statistical significance of differences between treatment groups. **P < 0.01; ***P < 0.001. Data shown are mean ± SEM (n = 3).
relative to cells incubated with the cytosolic fraction alone, suggesting that cathepsin S can activate the native IL-36γ protein contained within the cytosol of primary keratinocytes. Importantly, the addition of IL-36Ra completely abrogated this effect, demonstrating that the observed effect was IL-36 dependent. In these experiments, the addition of IL-36Ra did not completely inhibit IL-8 production, suggesting that there are proinflammatory factors present in the cytosol other than active IL-36.

**Neutrophil Proteases Do Not Cleave IL-36γ into the Potent IL-36γ S18 Isoform.** As discussed previously, a recent study by Henry et al. has shown that the neutrophil proteases cathepsin G, neutrophil elastase, and proteinase 3 all cleave IL-36γ, with neutrophil elastase and proteinase 3 increasing the activity of the protein (Henry et al., 2016) (27). Thus, it was important to assess the relevance of cathepsin S-dependent IL-36γ in the context of these neutrophil proteases. In this study, SUMO-tagged IL-36γ was incubated with recombinant cathepsin G, neutrophil elastase, or proteinase 3 for 30 min at 37 °C. The processing of IL-36γ was then visualized by electrophoresis (Fig. 5A) and the identity of the cleaved products analyzed by N-terminal sequencing.

**Fig. 3.** Secreted cathepsin S activates IL-36γ. A549 cells, primary keratinocytes, or primary fibroblasts were incubated in six-well plates until confluent, at which point the media was replaced with OptiMEM and cells incubated for a further 24 h. (A) Supernatants were analyzed for the presence of cathepsin S using cytokine-specific ELISA. HaCaT cells (10⁵ per well) were incubated for 24 h with 0.1 mM DTT-supplemented conditioned media from A549 cells (B), primary keratinocytes (C), and primary fibroblasts (D). Cells were incubated with either media alone, with IL-36γ FL (10 nM), or with both IL-36γ FL and IL-36Ra (50 nM). (B–D) Supernatants were analyzed for the presence of IL-8 using cytokine-specific ELISA. To inhibit expression of cathepsin S, A549 cells were transfected with cathepsin S siRNA (CATS) or scrambled siRNA (SCRAM; both 25 nM) for 24 h and the supernatants extracted. (E) Supernatants were analyzed for the presence of cathepsin S using a specific ELISA. (F) Finally, HaCaT cells (10⁵ per well) were incubated for 24 h with the supernatants from SCRAM-transfected or CATS-transfected A549 cells, either with or without IL-36γ FL (10 nM). (F) The supernatants from these experiments were analyzed for the presence of IL-8 using cytokine-specific ELISA. A one-way ANOVA (B–D and F) or unpaired t test (E) was used to determine statistical significance of differences between treatment groups. *P < 0.05; **P < 0.01. Data shown are mean ± SEM (n = 3).

**Fig. 4.** Recombinant cathepsin S can cleave and activate IL-36γ. We incubated 2 μg of SUMO-tagged IL-36γ with 20 ng of recombinant cathepsin S at 37 °C for 30 min. Several time points were taken and analyzed by coomassie-stained SDS/PAGE gel. (A) Diagram depicts the truncation generated by cathepsin S cleavage identified by mass spectrometry analysis. (B) HaCaT cells (10⁵ per well) were incubated for 24 h with media alone, IL-36γ (10 nM), cathepsin S (Cat S; 10 ng/mL), or a combination of IL-36γ and cathepsin S. (C) In addition, HaCaT cells were incubated with media alone, the cytosol of keratinocytes, cathepsin S (10 ng/mL), a combination of cytosol and cathepsin S, or a combination of cytosol, cathepsin S, and IL-36Ra (50 nM). (B and C) Supernatants were analyzed for the presence of IL-8 using a specific ELISA. A one-way ANOVA was used to determine statistical significance of differences between treatment groups. *P < 0.05. Data shown are mean ± SEM (n = 3).
From these analyses, it was shown that proteinase 3 and cathepsin G truncated IL-36γ to Y16 and Q17, respectively, whereas elastase generated both truncations. To compare the activity of these IL-36γ truncations to IL-36γ S18 (the truncation produced by cathepsin S), recombinant versions of these proteins were added to HaCaT cells and incubated for 24 h (Fig. 5B). Here, it was shown that S18 induced a potent expression of IL-8 at all concentrations tested. In contrast, the Y16 truncation and the Q17 truncation had no effect on IL-8 expression. To support these data, HaCaT cells were incubated with IL-36γ FL and various concentrations of either recombinant cathepsin S, cathepsin G, neutrophil elastase, or proteinase 3 (Fig. 5C). Here IL-8 secretion was markedly higher when the cells were incubated with cathepsin S, further demonstrating that the product of cathepsin S-dependent IL-36γ processing is a much more potent inducer of inflammation, relative to the products of neutrophil protease-dependent IL-36γ processing.

To investigate whether the processing of IL-36γ by neutrophil proteases has a down-regulatory effect on the activation of IL-36γ by cathepsin S, HaCaT cells were incubated for 24 h with IL-36γ and various concentrations of cathepsin S, both with and without the neutrophil proteases cathepsin G, neutrophil elastase, and proteinase 3 (Fig. 5D). In these experiments, the addition of neutrophil proteases had a down-regulatory impact upon IL-8 expression, suggesting that neutrophil proteases serve to down-regulate the activation of IL-36γ by cathepsin S. To determine whether neutrophil proteases also down-regulate the IL-8 expression induced by IL-36γ S18, HaCaT cells were incubated for 24 h with various concentrations of IL-36γ S18, both in the presence and absence of neutrophil proteases (Fig. 5E). Again, the addition of neutrophil proteases reduced the IL-8 expression induced by IL-36γ S18. In contrast with previous studies, these data suggest that neutrophil proteases serve to dampen IL-36γ-mediated proinflammatory responses by nonspecifically processing both the full-length and active cytokines into inactive or only partially active truncations.
and Cathepsin S Are Strongly Up-Regulated in Psoriasis.

In psoriatic lesions, the presence and activity of the IL-36γ family member S18, or IL-36Ra. Intriguingly, the addition of IL-36γ S18 caused psoriasiform changes including stratum corneum thickening (hyperkeratosis) and epidermal cornification, the process whereby living keratinocytes differentiate into nonliving corneocytes (Fig. 7A). The thickness of the stratum corneum was measured and shown to be significantly thicker in the S18-treated samples (Fig. 7B). IL-8 secretion was also measured and shown to be significantly higher in the S18-treated samples, suggesting that S18 drives proinflammatory and differentiation-related changes in the epidermal compartment (Fig. 7C).

IL-36γ and Cathepsin S Are Strongly Up-Regulated in Psoriasis. Previous studies have shown that IL-36γ RNA is strongly up-regulated in psoriatic biopsies. Importantly, using the tape stripping methodology, we were able to demonstrate the corresponding increase in IL-36γ protein in psoriatic lesions, relative to healthy controls (Fig. 6D). However, as we were unable to distinguish the active and inactive forms of IL-36γ in psoriatic lesions, the presence and activity of the IL-36γ-activating protease cathepsin S by skin resident cells was investigated. Cathepsin S secretion was up-regulated by the psoriasis-associated cytokines TNFα or IFNγ in primary fibroblasts and was strongly up-regulated by IFNγ in primary keratinocytes (Fig. 6A). Cathepsin S secretion was up-regulated further by the addition of both TNFα and IFNγ together, suggesting that these cytokines have a synergistic effect on cathepsin S expression. Neither IL-17 nor IL-22 nor IL-17 had an effect on cathepsin S secretion in these experiments. Interestingly, a previous study using cathepsin S staining of biopsies has suggested that cathepsin S may be up-regulated in psoriatic lesions (30). To consolidate and quantify these findings, cathepsin S protein levels were analyzed in tape-strip samples acquired from the skin of psoriasis patients and healthy controls. Here, cathepsin S levels and activity were significantly higher in the samples acquired from psoriasis patients, suggesting that cathepsin S is up-regulated in psoriasis (Fig. 6B and C). Together, these data serve to show that both cathepsin S and IL-36γ are strongly up-regulated in psoriatic lesions, implicating the processing of IL-36γ by cathepsin S as a component of psoriatic inflammation.

IL-36γ S18 Causes Hyperkeratosis in a Skin-Equivalent Model. Having shown that cathepsin S activates IL-36γ by cleaving it into IL-36γ S18 and having shown that both cathepsin S and IL-36γ are strongly up-regulated in psoriasis, it was important to investigate whether IL-36γ S18 drives any changes that culminate in a psoriatic phenotype. To address this, reconstituted human epithelium skin equivalents were treated with either growth media alone, IL-36γ S18, or IL-36Ra. Intriguingly, the addition of IL-36γ S18 caused psoriasiform changes including stratum corneum thickening (hyperkeratosis) and epidermal cornification, the process whereby living keratinocytes differentiate into nonliving corneocytes (Fig. 7A). The thickness of the stratum corneum was measured and shown to be significantly thicker in the S18-treated samples (Fig. 7B). IL-8 secretion was also measured and shown to be significantly higher in the S18-treated samples, suggesting that S18 drives proinflammatory and differentiation-related changes in the epidermal compartment (Fig. 7C).
these changes were blocked when the samples were treated with the Ra, confirming that the effects observed were dependent on IL-36γ S18. This would explain the S18-induced thickening of the stratum corneum and concomitant loss of normal differentiation pattern.

**Discussion**

Activated IL-1 family cytokines play a fundamental role in innate immune responses (31, 32). Thus, the proteases involved in IL-1 cytokine activation are considered to be critical mediators of inflammation. The importance of cytokine processing is highlighted by regulatory defects in caspase-1 activation, which cause the recurrent, systemic, and severe inflammatory episodes observed in cryopyrin-associated periodic syndromes and other autoinflammatory diseases such as Familial Mediterranean Fever (2, 33). The correct balance of inflammation versus tissue maintenance is also of particular relevance at cutaneous and epithelial borders, which are constantly exposed to complex pathogenic and other environmental stresses (34). This is evident in the rare autoinflammatory diseases deficiency of the IL-1Ra (DIRA) and deficiency of the IL-36Ra (DITRA), where patients present with severe cutaneous skin eruptions (35). As well as its apparent pathological role in inflammation, IL-36γ is also involved in wound repair within epithelial compartments (9, 36, 37). Thus, a comprehensive understanding of IL-36γ activation in the epithelium is imperative. In this investigation, we show that cathepsin S is the major IL-36γ-activating protease produced by skin resident cell types, implicating this protease as a central mediator of IL-36γ-driven cutaneous inflammation.

The mechanisms involved in the processing and secretion of IL-1 cytokines are of longstanding academic interest, because of both their clinical significance and their evident complexity (38, 39). Despite this interest, many of the processes involved are still poorly understood. In general, most IL-1 cytokines require two independent signals for secretion: an initial signal to induce expression of the precursor and a second signal to drive cleavage and secretion (40). Most of the proteases that process IL-1 precursors have been identified as cysteine proteases, and thus, many of the current models of IL-1 secretion indicate that processing occurs before secretion (21, 24, 41). In contrast, cathepsin S is readily secreted by a range of skin resident cells, suggesting that this IL-36γ-activating protease is abundantly expressed in the extracellular space of epithelial tissue. Given that IL-36γ is constitutively expressed in keratinocytes and is further induced by microbial-derived stimuli (42), we hypothesize that IL-36γ released following necrotic cell death is rapidly activated by extracellular cathepsin S. Therefore, IL-36γ may act as an important damage-associated molecular pattern (DAMP), akin to IL-1α and IL-33 mediating inflammation and wound repair within epithelial compartments (43).

Although necrotic cell death is likely to be an important route for IL-36γ release and activation, it may not be the only route. Many IL-1 cytokines have various mechanisms of release, and thus, it is probable that IL-36γ also has multiple pathways of activation, especially given that cathepsin S is not secreted by the cells of many other tissues. Intriguingly, previous studies have shown that a fraction of cytosolic pro-IL-1α is sequestered into early lysosomes and secreted in monocytes (44). Given that cathepsin S is contained within lysosomes, it is tempting to speculate that IL-36γ may also be sequestered into these secretory lysosomes. As lysosomal cathepsin S has been shown to activate IL-36γ, this process would not only facilitate IL-36γ release but would also allow for concomitant activation. In a previous study by Henry et al., it was proposed that the neutrophil proteases elastase and proteinase 3 are responsible for driving IL-36γ-dependent inflammatory responses (27). In more detail, it was suggested that the trauma-induced necrosis at the epidermis caused both IL-36γ release and neutrophil recruitment, culminating in a neutrophil protease-dependent activation of IL-36γ. Although it is clear that the exposure of IL-36γ to neutrophil proteases results in the generation of inflammatory responses, this research questions the relative importance of neutrophil protease-dependent IL-36γ cleavage in an in vivo setting, especially in light of the evidence herein. We show that the neutrophil protease-dependent cleavage of IL-36γ results in the production of truncations that are significantly less potent than the S18 truncation. Moreover, whereas neutrophil-dependent IL-36γ activation requires cellular recruitment followed by subsequent activation and degranulation (45), cathepsin S is strongly expressed at the site of IL-36γ release and so can instantaneously cleave the cytokine following its release. Thus, our data suggest that cathepsin S is the major protease responsible for driving IL-36γ–dependent responses in the skin. Interestingly, neutrophil proteases down-regulate cathepsin S–mediated IL-36γ activation, suggesting that neutrophil proteases may actually serve to dampen IL-36γ–driven inflammation at epithelial borders. This is supported by previous work in our laboratory, which demonstrated that neutrophil proteases activate IL-36Ra (46).

In recent studies, a role for IL-36γ in the development of psoriasis has been postulated, not least because IL-36γ has been identified as one of the most strongly and specifically up-regulated genes in psoriatic lesions (12–14). Therefore, having demonstrated a role for cathepsin S in IL-36γ activation and having demonstrated that the expression levels of both proteins are elevated in psoriatic lesions, it is highly likely that cathepsin S plays an important role in the pathogenesis of psoriasis. Furthermore, given that the product of cathepsin S–dependent IL-36γ cleavage induces stratum corneum thickening (hyperkeratosis) in a skin-equivalent model, the results herein suggest that the activation of IL-36γ by cathepsin S may even be pivotal in driving a psoriatic phenotype. This hypothesis is supported by previous work that shows that IL-36 signaling is required in the Imiquimod-induced model of psoriasis (17). Future studies, involving inducible cathepsin S overexpression or ablation in mice, should provide further credence to these findings and thus provide a basis for future in vivo investigations and therapeutic targeting.

At present, anti-TNFα, anti-IL-17, and anti-IL-23 treatment of severe psoriasis patients has been shown to be of high clinical benefit, with higher efficacy regarding IL-23/IL-17 pathway blockage (47). IL-36γ has been shown to be induced by both IL-17 and TNF, and they act synergistically to up-regulate its expression and secretion. Therefore, targeting downstream IL-36γ could potentially be more beneficial than targeting IL-17 or TNF alone (48). Psoriasis susceptibility genes that impact on the NF-κB pathway (e.g., CARD14, A20) have also been suggested to cause higher IL-36 expression and activity (49). Once expressed and activated, IL-36 acts in a positive feedback manner, activating skin tissue cells including keratinocytes and fibroblasts. Thus, under circumstances of limited IL-36Ra availability and/or excessive activity, this research questions the therapeutic value of IL-36Ra in psoriasis. Future studies, involving inducible cathepsin S overexpression or ablation in mice, should provide further credence to these findings and thus provide a basis for future in vivo investigations and therapeutic targeting.

To conclude, this study demonstrates that cathepsin S is the major protease of IL-36γ at barrier tissues. These findings are not only significant from a mechanistic perspective but may also provide new therapeutic strategies for the treatment of psoriasis, especially as both IL-36γ and cathepsin S are strongly up-regulated in psoriatic lesions. These results now pave the way...
for in vivo studies to investigate the extent to which cathepsin S activation of IL-36γ contributes to the maintenance of epithelial homeostasis and to the initiation and development of psoriasis.

Methods

Reagents and Antibodies. The protease inhibitors E64 and CATSi were purchased from Merck Millipore. AEBSF was obtained from Sigma. Human recombinant cathepsin S was purchased from Bio Vision. Cathepsin S siRNA and scrambled siRNA were purchased from Dharmacon. For Western blot analysis, the primary antibodies were a rabbit anti-SUMO antibody (AB14405; AbCam), a mouse anti-GAPDH antibody (GT239; Genetex), a mouse anti–LAMP-1 antibody (SC18822; Santa Cruz Biotechnology), and a goat anti-IL-36γ antibody (BAP2894; R&D Systems). HRP-conjugated anti-rabbit antibodies used were anti-rabbit IgG antibody (64405; Southern Biotech), an anti-mouse IgG antibody (A9917; Sigma), and an anti-goat antibody (A3919; Sigma). Human recombinant pro-IL–1β was purchased from Sino Biological, and human recombinant caspase-1 was purchased from Biovision. The human recombinant proteins TNFα, IFNγ, IL-17, and IL-22 were all purchased from R&D Systems.

Generation of Recombinant Proteins. To generate IL-36 fusion proteins possessing N-terminal SUMO domains, cDNA of IL-36γ FL, IL-36RA V2, and IL-36γ S18 proteins were cloned into a Champion pET SUMO expression vector (Invitrogen). Proteins were subsequently expressed in BL21-CodonPlus (DE3)-RIL Escherichia coli overnight at 25 °C and soluble proteins purified via Ni²⁺-affinity and size exclusion chromatography. Proteins used for stimulations were purified by Ni²⁺-affinity chromatography before overnight cleavage of N-terminal SUMO by the Ulp1 protease, followed by subsequent ion exchange and size exclusion chromatography into 20 mM Tris, pH 7.4, 300 mM NaCl.

Caspase-1 Activity Assay. We diluted 1 μg of SUMO-tagged recombinant IL-36γ S18 (biologically active form), IL-36γ FL, or pro–IL-1β (all polyhistidine tagged) in assay buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 100 μg/mL glycerol, 100 μg/mL LAMP-1) and incubated it with recombinant caspase-1 (4 units) at 37 °C for 6 h.

Cell Lysis and Fractionation. Whole-cell lysates, lysosomal extracts, and cytosolic extracts were generated from 10⁴ growing cells. Cells were harvested by centrifugation at 800 × g. To generate whole-cell lysates, cells were resuspended in 500 μL cell extract buffer (20 mM Hepes, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM PMSF, and 0.1% Triton X-100) and incubated for 20 min on ice. Cells were then transferred to a Dounce-type homogenizer and homogenized with 15 strokes of a 8-type pestle. The homogenate was centrifuged at 1,000 × g for 10 min to remove the nuclei and other cellular debris. The supernatant was then collected and centrifuged at 15,000 × g to separate the lysosome from the cytosol. The cytosol was then frozen down at −80 °C and the lysosome resuspended in 500 μL cell extract buffer. Following a 20-min incubation on ice and repeated freeze thaw cycles, the lysosomal fraction was centrifuged at 15,000 × g for 30 min and supernatants frozen at −80 °C.

IL-36 Cleavage Assay. For activity assay experiments, HaCaTs were plated at 10⁵ cells per well (24-well plate) in complete culture media and incubated for 24 h. The media was then removed and replaced with OptiMEM (Life Technologies). For most activity assay experiments, indicated treatments were added in conjunction with OptiMEM and cells incubated for 24 h at 37 °C. For activity assay experiments involving protease inhibitors, lysosomal fractions were preincubated with E64, AEBSF, or CATSi for 3 h at 4 °C before being added to HaCaT cells. For the activity assay experiments involving cell supernatants, A549 cells, primary keratinocytes, or primary fibroblasts were plated at 0.5 × 10⁶ cells per well (six-well plate) in complete culture media. Once confluent, the media was replaced with OptiMEM and cells incubated for a further 24 h. Following incubation, the conditioned media was removed and frozen at −80 °C. In preparation for the activity assay, the conditioned media was supplemented with 0.1 mM DTT. The activity assay for these experiments was performed as previously described, the only difference being that the media on the HaCaT cells was replaced with the conditioned media and not OptiMEM. Following incubation, cell supernatants were removed and frozen at −80 °C.

ELISA. Supernatants were analyzed for IL-8 protein using a specific ELISA kit from Biolegend. Supernatants and lysates were analyzed for cathepsin S protein using a specific ELISA kit from R&D Systems. ELISAs were performed following the manufacturer’s instructions. The lower limits of accurate detection for IL-8 and cathepsin S were 15.6 pg/mL. For the conditioned media experiments, levels of IL-8 in the media before the activity was deduced from levels recorded after the activity assay to give an accurate measure of de novo IL-8 synthesis.

Molecular Mass Confirmation by LC–MS. We loaded 1 μg protein onto a Mascprep micro desalting column (Waters) and washed it for 5 min with 10% (vol/vol) acetonitrile/0.1% formic acid. Following a 1-min gradient to 85% (vol/vol) acetonitrile/0.1% formic acid, the protein was eluted into a Xevo G2-XS QToF (Waters) using electrospray ionization for molecular mass measurement.

Tape Stripping. All samples were collected with written informed consent in place of the patients, taken in accordance with the Declaration of Helsinki (REC 14/NE/1199). Healthy volunteers were also recruited from the University of Leeds and written consent was also sought (BIOC09-001). Research was approved by the North-East York Research Ethics Committee. The severity of lesions was clinically assessed before sample collection using a square adhesives discs of 3.8 cm² (Cuderm). Samples from nonlesional skin were taken preferably from the ventral lower arm area. Location of lesional samples was guided by lesion appearance. Only nonerosive, nonoozing lesions were tape stripped. The tapes were placed on the skin for 5 s with gentle pressure. The first tape was discarded, and 10 subsequent tapes collected were put in an empty container and immediately stored on dry ice for transportation or storage at −80 °C until processing for protein extraction. To extract the protein, tapes were placed in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, 5 mM EDTA, 1 mM PMSF, 1% Triton X-100) for 30 min at 4 °C. Samples were sonicated three times for 20 s with a 20-s interval on ice.

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between each sonication. Samples were centrifuged for 10 min at 15,000 × g. Supernatants were removed and frozen at –80°C.

**BCA.** Total protein concentrations were quantified using a reducing agent compatible BCA assay (Biovision). The BCA assay was performed following the manufacturer’s instructions.

**Cathepsin S Activity Assay.** Samples (1 μg total protein per sample) were analyzed for cathepsin S activity using a fluorometric kit obtained from Biovision. The activity assay was performed following the manufacturer’s instructions.

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**Statistics.** Statistical analysis was performed using the software Graphpad Prism 6. Data were analyzed by one-way ANOVA to determine overall differences, and a Tukey post hoc test was performed to determine statistically significant differences between treatment groups. Data were also analyzed using an unpaired t test.

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