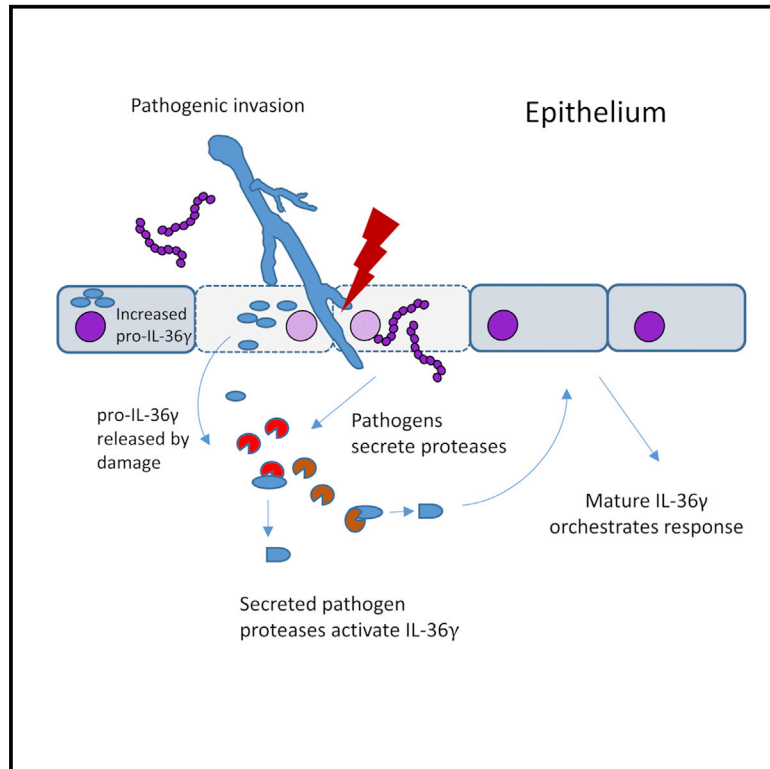


The Proinflammatory Cytokine IL-36 γ Is a Global Discriminator of Harmless Microbes and Invasive Pathogens within Epithelial Tissues

Graphical Abstract



Authors

Thomas Macleod, Joseph S. Ainscough, Christina Hesse, ..., Sarah Herrick, Miriam Wittmann, Martin Stacey

Correspondence

m.wittmann@leeds.ac.uk (M.W.),
m.stacey@leeds.ac.uk (M.S.)

In Brief

Macleod et al. demonstrate that the epithelial cytokine IL-36 γ discriminates between pathogens and harmless commensals at epithelial barriers. IL-36 γ is upregulated by epithelial tissue following microbial challenge, but it is only released and activated by destructive pathogenic microbes, whereupon it initiates an immune response.

Highlights

- Epithelial pathogens induce expression and release of IL-36 γ
- Proteases secreted by several epithelial pathogens activate IL-36 γ
- The *A. fumigatus* and *S. pyogenes* virulence factors Asp F13 and SpeB activate IL-36 γ
- IL-36 γ is a global sensor of pathogen-derived proteases during epithelial infection



Article

The Proinflammatory Cytokine IL-36 γ Is a Global Discriminator of Harmless Microbes and Invasive Pathogens within Epithelial Tissues

Thomas Macleod,¹ Joseph S. Ainscough,¹ Christina Hesse,^{2,3} Sebastian Konzok,^{2,3} Armin Braun,^{2,3} Anna-Lena Buhl,⁴ Joerg Wenzel,⁴ Paul Bowyer,⁵ Yutaka Terao,⁶ Sarah Herrick,⁷ Miriam Wittmann,^{1,8,9,*} and Martin Stacey^{1,10,*}

¹Faculty of Biological Sciences, School of Molecular and Cellular Biology, University of Leeds, Leeds LS2 9JT, UK

²Fraunhofer Institute for Toxicology and Experimental Medicine, Nikolai-Fuchs-Straße 1, 30625 Hannover, Germany

³Member of the German Center for Lung Research (DZL), Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH) Research Network, Hannover, Germany

⁴Department of Dermatology and Allergy, University of Bonn, Bonn 53012, Germany

⁵Division of Infection, Immunity & Respiratory Medicine, University of Manchester, Manchester M13 9PL, UK

⁶Division of Microbiology and Infectious Diseases, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

⁷Faculty of Biology Medicine and Health, School of Biological Sciences, University of Manchester and Manchester Academic Health Science Centre, Manchester M13 9PL, UK

⁸Faculty of Medicine and Health, Leeds Institute of Rheumatic and Musculoskeletal Medicine, University of Leeds, Leeds LS2 9JT, UK

⁹Leeds Biomedical Research Centre, National Institute for Health Research, Leeds Teaching Hospitals, Leeds, UK

¹⁰Lead Contact

*Correspondence: m.wittmann@leeds.ac.uk (M.W.), m.stacey@leeds.ac.uk (M.S.)

<https://doi.org/10.1016/j.celrep.2020.108515>

SUMMARY

Epithelial tissues represent vital interfaces between organisms and their environment. As they are constantly exposed to harmful pathogens, innocuous commensals, and environmental microbes, it is essential they sense and elicit appropriate responses toward these different types of microbes. Here, we demonstrate that the epithelial cytokine interleukin-36 γ (IL-36 γ) acts as a global discriminator of pathogenic and harmless microbes via cell damage and proteolytic activation. We show that intracellular pro-IL-36 γ is upregulated by both fungal and bacterial epithelial microbes; yet, it is only liberated from cells, and subsequently processed to its mature, potent, proinflammatory form, by pathogen-mediated cell damage and pathogen-derived proteases. This work demonstrates that IL-36 γ senses pathogen-induced cell damage and proteolytic activity and is a key initiator of immune responses and pathological inflammation within epithelial tissues. As an apically located epithelial proinflammatory cytokine, we therefore propose that IL-36 γ is critical as the initial discriminator of harmless microbes and invasive pathogens within epithelial tissues.

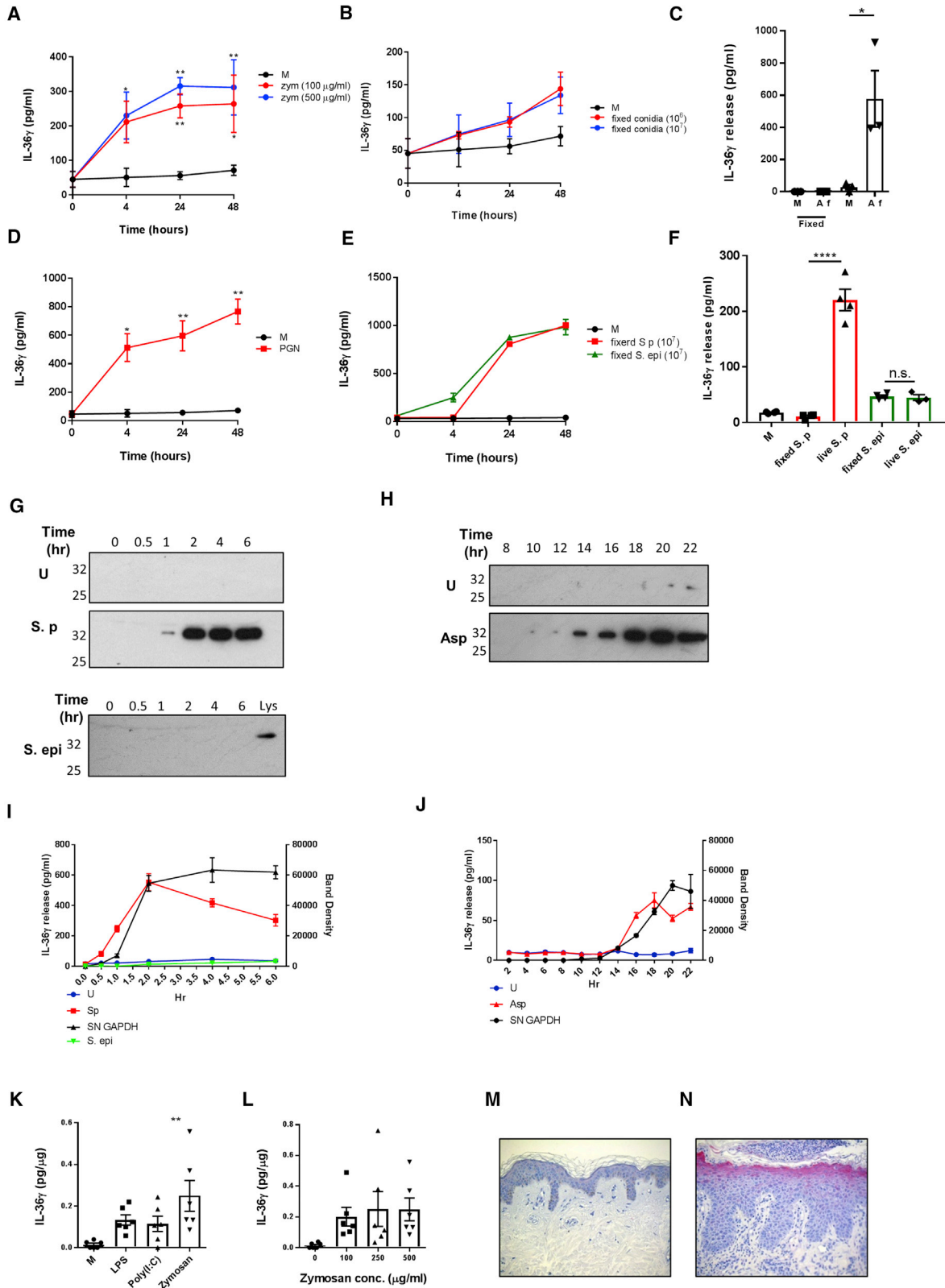
INTRODUCTION

As crucial interfaces between the body and its environment, epithelial sites such as the skin, gut, and lungs are constantly challenged by ubiquitous exogenous microbes. One mechanism by which the host can potentially discriminate harmful pathogens from commensals is via pathogenic damage. As host tissue can be a non-permissive environment for microbial growth, pathogens often produce cytotoxic virulence factors that assist colonization by facilitating immunosuppression and nutrient acquisition. Damaging virulence factors result in the release of cellular components known as alarmins such as interleukin-1 α (IL-1 α) and high mobility group box 1 (HMGB1), which are normally sequestered within healthy cells. Upon release, these function to activate tissue-resident inflammatory cells and induce cytokine production that recruits inflammatory cells to the site of infection and skews the adaptive immune system toward an appropriate response to ultimately clear infection (Bianchi

et al., 2017; Kono et al., 2010; Eigenbrod et al., 2008; Oppenheim and Yang, 2005; Yang et al., 2017).

Interestingly, a significant number of epithelial pathogens have evolved proteolytic enzymes for nutrient acquisition and the colonization of extracellular matrix (ECM)-rich epithelial tissues (Singh et al., 2012). For example, the clinically significant bacterial pathogen *Streptococcus pyogenes*, an etiological agent of pharyngitis, cellulitis, and erysipelas, and *Aspergillus fumigatus*, which causes invasive aspergillosis with fatality rates in excess of 50% in immunocompromised individuals (Lin et al., 2001), both produce ECM-degrading proteases (Tamura et al., 2004; Burns et al., 1996; Iadarola et al., 1998). Given that these proteases are important mediators of invasion and that the ability for a microbe to invade can be the difference between a pathogen and a commensal, it is postulated that proteases are often essential mediators of pathogenicity. It would therefore be beneficial to the host to identify the presence of such proteolytic virulence factors, enabling immediate recognition of a pathogenic presence.





(legend on next page)

The IL-36 cytokines (IL-36 α , IL-36 β , IL-36 γ , receptor antagonist [RA]) are a recently characterized group of cytokines belonging to the IL-1 superfamily (Dunn et al., 2001). While many IL-1 cytokines can be found expressed throughout the body, the IL-36 cytokines are predominately expressed in epithelial tissue such as the skin, lungs, gut, and mucosa, particularly at apical locations, suggesting an important role in barrier immune function (Dunn et al., 2001; Gabay and Towne, 2015; Boutet et al., 2016). Indeed, it is now well established that IL-36 γ is a proinflammatory mediator highly expressed in psoriasis and is involved in the initiation and maintenance of pathological inflammation (Johnston et al., 2011; He et al., 2013; Berekmeri et al., 2018; Bridgewood et al., 2018). However, this cytokine is also a critical mediator of immune responses to several classes of invading pathogens at epithelial barriers (Kovach et al., 2017; Gao et al., 2018). As a proinflammatory protein, IL-36 γ induces expression of a range of antimicrobial peptides such as cathelicidin (LL-37), human beta defensins 2 and 3, and S100 proteins as well as several cytokines and chemokines such as itself and IL-1 α/β , IL-8, IL-17, and CCL20, cytokines typically associated with an immune response to extracellular pathogens (Foster et al., 2014; Johnston et al., 2011; Carrier et al., 2011). Intratracheal administration of IL-36 γ in mice leads to an influx of neutrophils and macrophages, in addition to T helper 1 (Th1) and Th17 cells, crucial for the orchestration of an adaptive immune response to invading pathogens (Ramadas et al., 2011; Carrier et al., 2011). Furthermore, several studies have shown expression of IL-36 γ is elevated upon challenge by numerous invasive bacterial and fungal pathogens, including *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Aspergillus fumigatus*, and *Candida albicans*, and subsequent studies have implicated IL-36 γ involvement in initiating an inflammatory response following infection by such organisms. Indeed, in mouse models of bacterial pneumonia and mucosal candidiasis, deficiency of IL-36 signaling leads to increased mortality and fungal burdens (Kovach et al., 2017; Gresnigt et al., 2013; Verma et al., 2018; Braeugelmann et al., 2018).

As with other IL-1 family cytokines, IL-36 γ is produced as an inactive precursor that requires precise N-terminal processing to become biologically active. The activating cleavage event occurs between Gln₁₇ and Ser₁₈, nine amino acids upstream of the conserved IL-1 motif, and deviation from this site by a single amino acid reduces biological activity more than 1,000-fold (Towne et al., 2011). Unlike the well-characterized IL-1 β and IL-18 cytokines, this activation has been shown to be an inflam-

masome-independent process, which, in psoriasis, is mediated via the endogenous proteases cathepsin S and neutrophil elastase (Ainscough et al., 2017; Henry et al., 2016). However, the mechanisms by which IL-36 γ is released and activated during microbial infection remain unknown.

Given the importance of proteolytic virulence factors in establishing pathogenic invasion, and the importance of proteolytic processing in the activation of IL-36 γ at epithelial barriers, we examined the effects of pathogen-mediated release and proteolytic activation of IL-36 γ . The work presented here provides evidence that IL-36 γ acts as a link between pathogenic proteolytic activity and initiation of an immune response in epithelial tissue. We demonstrate that IL-36 γ is upregulated by microbes, released by pathogenic damage, and processed into its bioactive form by several proteases from important fungal and bacterial epithelial pathogens. We therefore propose that IL-36 γ is an epithelial alarmin that acts as a global early sensor of pathogenic invasion, enabling the innate immune response to discriminate harmful from harmless microbes. This work builds a more complete picture of how IL-36 signaling is initiated during microbial infection and demonstrates the importance of IL-36 cytokines in immune defense at epithelial barriers.

RESULTS

Epithelial Pathogens Upregulate and Release IL-36 γ

IL-36 γ is expressed at epithelial barriers and has been shown to be upregulated in response to a number of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), β -glucans, and poly(I:C). While mechanisms of release of IL-36 γ are currently unclear, one possibility is that IL-36 γ remains in the cytoplasm until cellular damage results in release of the immature protein into the surrounding extracellular space, where it is available for activation by extracellular proteases.

To investigate the effect pathogenic challenge has on IL-36 γ expression and release, the oral buccal epithelial cell line TR146 was stimulated with varying concentrations of zymosan and peptidoglycan for 0–48 h (Figures 1A and 1D). Measurement of lysate IL-36 γ by ELISA showed both zymosan- and peptidoglycan-induced expression of IL-36 γ . TR146 cells were also treated with a varying amount of fixed *A. fumigatus* conidia, *S. pyogenes*, and the harmless commensal *Staphylococcus epidermidis* for 0–48 h, and lysate IL-36 γ was measured by ELISA (Figures 1B and 1E). Again, an increase in IL-36 γ expression was observed upon stimulation with all fixed microbes.

Figure 1. Epithelial Pathogens Induce Expression and Release of IL-36 γ

(A–N) TR146 cells (10^5 per well) were treated with M, zym (A; 100 and 500 $\mu\text{g}/\text{mL}$), or fixed *Aspergillus* conidia (B; 10^6 and 10^7) for 0–48 h, and lysate IL-36 γ was measured by ELISA (n = 3). (C) TR146 cells were treated with M or with either live or fixed *Aspergillus* conidia (10^7) for 24 h, and supernatant IL-36 γ was measured by ELISA (n = 3). TR146 cells were treated with PGN (D; 10 $\mu\text{g}/\text{mL}$), fixed *S. p.*, or fixed *S. epi* (10^7) for 0–48 h, and lysate IL-36 γ was measured by ELISA (n = 3). TR146s (F) were treated for 24 h with M or with either live or fixed *S. p.* and *S. epi.*, and supernatant IL-36 γ was measured by ELISA (n = 4). TR146 cells were subjected to media change with antibiotic-free DMEM and then left U, inoculated with either *S. p.* (10^7) or *S. epi.* (10^7) (supernatant GAPDH compared with Lys) for 0–6 h (G, S, p; I, S, epi) or Asp (10^6) for 0–22 h (H and J). Supernatant was collected from cells at the indicated time points and measured for GAPDH by western blot (G and H) and IL-36 γ by ELISA (I and J) (n = 3). GAPDH band intensity was analyzed in ImageJ software and plotted against IL-36 γ concentration (I and J). Precision-cut lung slices were treated with PAMPs (K) and zym (L; 100–500 $\mu\text{g}/\text{mL}$). Lysate IL-36 γ was measured by ELISA (n = 6). Anti-IL-36 γ immunohistochemistry staining of sections from healthy (M) and *T. rubrum*-infected (N) skin. Western blots are representative of three individual experiments. A one-way ANOVA was used to determine statistical significance of differences between treatment groups. *p < 0.05, **p < 0.01, and ****p < 0.0001. Data shown are mean \pm SEM. Abbreviations are as follows: Asp, *A. fumigatus* conidia; Lys, lysate GAPDH; PGN, peptidoglycan; M, media alone; S, epi, *S. epidermidis*; S, p, *S. pyogenes*; U, uninfected; zym, zymosan.

However, while IL-36 γ expression was evident in the lysate, little or no corresponding increase was observed in the supernatant of stimulated cells, with IL-36 γ often undetectable (Figure S1). TR146 cells were then treated with either fixed or viable *A. fumigatus* conidia, *S. pyogenes*, or *S. epidermidis* (Figures 1C and 1F). Measurement of IL-36 γ in supernatants from cells treated with live *A. fumigatus* and *S. pyogenes*, both of which are pathogens, showed a significant increase in extracellular IL-36 γ compared with their fixed counterparts, indicating release of the cytokine only occurred when cells were inoculated with viable pathogens. Strikingly, cells treated with the commensal *S. epidermidis* did not release IL-36 γ into their culture supernatant, suggesting the live commensal was unable to induce IL-36 γ release.

Given that extracellular IL-36 γ was only observed following inoculation with live proliferating pathogens and that bacterial and fungal growth can be destructive to cells, it was hypothesized that cellular damage was responsible for release of IL-36 γ . To test this, TR146 cells were infected with either *A. fumigatus*, *S. pyogenes*, or *S. epidermidis*, and the release of the cytosolic housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was monitored alongside that of IL-36 γ . As shown in Figures 1G and 1H, extracellular GAPDH was present only in the pathogen-infected samples, and release of both GAPDH and IL-36 γ coincided over time (Figures 1I and 1J), suggesting release of IL-36 γ may occur as a result of membrane damage. We did not observe the release of either IL-36 γ or GAPDH from cells infected with the commensal *S. epidermidis*, suggesting this commensal is not inducing release of IL-36 as it is not inducing cellular membrane damage.

To examine the relevance of IL-36 γ in other types of epithelial tissues, the expression of IL-36 γ in response to PAMP stimulation was examined in cultured human lung tissue using *ex vivo* precision-cut lung slices (Temann et al., 2017). Slices were treated with LPS, poly(I:C), and zymosan at the indicated concentrations, and lysate IL-36 γ was assessed by IL-36 γ ELISA. As shown in Figure 1G, all PAMPs tested induced increased expression of IL-36 γ , with zymosan inducing the strongest expression (Figures 1K and 1L). Again, no measurable IL-36 γ was detectable in the supernatants of the precision-cut lung slices. Furthermore, immunohistochemistry on sections from healthy and *Trichophyton rubrum*-infected skin shows IL-36 γ is highly expressed in the uppermost layers of the epidermis following infection by *T. rubrum* (Figures 1M and 1N).

IL-36 γ Is Activated by Proteases Derived from a Range of Human Pathogens

As many epithelial pathogens are known to express proteases to facilitate invasion and nutrient acquisition, the effects of culture filtrates from *A. fumigatus*, *S. pyogenes*, *T. rubrum*, and *Staphylococcus aureus* on the processing of IL-36 γ were investigated.

Recombinant pro-IL-36 γ possessing an N-terminal small ubiquitin-like modifier (SUMO) tag was incubated with culture filtrates from *A. fumigatus*, *S. pyogenes*, *T. rubrum*, and *S. aureus* at 37°C for 1 h. Resolution by SDS-PAGE revealed that incubation with the culture filtrates from all pathogens tested results in truncation of IL-36 γ (Figures 2A–2D). Analysis of the truncated products by liquid chromatography-mass spectrometry (LC-

MS) identified the prominent species as possessing a mass of 17,031 Da in all of the culture filtrates (Figure S2). This species corresponds to the predicted mass of the highly active mature IL-36 γ S18 (illustrated in Figure 2). In order to confirm that N-terminal SUMO was not having an effect on cleavage, the culture filtrate incubations were repeated using recombinant pro-IL-36 γ without N-terminal SUMO and analyzed by LC-MS. Again, the prominent species possessed a mass corresponding to mature IL-36 γ S18 in all culture filtrates (Figure S2).

Culture filtrate-incubated pro-IL-36 γ was tested for biological activity by utilizing an activity assay previously developed (Ainscough et al., 2017). Culture filtrates incubated with or without pro-IL-36 γ were added to IL-36 γ -sensitive HaCaT cells in the presence or absence of IL-36RA and monitored for IL-8 expression. While addition of either culture filtrate or pro-IL-36 γ alone elicited only very low amounts of IL-8 secretion, the addition of culture filtrate and pro-IL-36 γ induced strong responses (Figures 2E–2H). Furthermore, this response is shown to be IL-36 specific as addition of IL-36RA significantly reduced IL-8 secretion. These observations indicate several pathogens across different domains of life secrete proteases that activate IL-36 γ .

In addition to human (h)IL-36 γ , we also examined the susceptibility of mouse (m)IL-36 γ to activation by these epithelial pathogens to test whether this activation is evolutionarily conserved. While hIL-36 γ and mL-36 γ are structurally similar, the primary amino acid sequences surrounding the cleavage site required for generating biologically active IL-36 γ are distinct.

As with hIL-36 γ , mL-36 γ was incubated with culture filtrate of *A. fumigatus*, *S. pyogenes*, and *T. rubrum* at 37°C for 1 h prior to resolution by SDS-PAGE. As with hIL-36 γ , cleavage was observed in all samples (Figures 3A–3C). While addition of pro-mIL-36 γ or culture filtrate alone to mL-36-sensitive mouse embryonic fibroblasts (MEFs) did not induce significant secretion of mL-6, addition of culture-filtrate-incubated pro-mIL-36 γ induced strong mL-6 secretion (Figures 3D–3F). LC-MS analysis again confirmed that IL-36 γ was cleaved to the proactive form of murine IL-36 γ (G13).

Activation of IL-36 γ by *S. pyogenes* Is Dependent upon Virulence Factor SpeB

Upon identifying culture filtrates extracted from several important human epithelial pathogens activated IL-36 γ , we next endeavored to identify what was responsible for the activation. We proceeded with *S. pyogenes* and *A. fumigatus* as these pathogens are the most destructive and medically important of those originally tested.

In order to identify the *S. pyogenes* protease responsible for cleavage and activation of IL-36 γ , SUMO-pro-IL-36 γ was incubated with *S. pyogenes* culture filtrate in the presence of the broad-range Ser inhibitor PMSF, Cys inhibitor E64, and metalloproteinase inhibitor EDTA for 1 h at 37°C. As shown in Figure 4A, while cleavage was unaffected by addition of PMSF and EDTA, addition of the Cys protease inhibitor E64 completely inhibited cleavage. These samples were also tested for biological activity. Initially, protease inhibitors were added to cells with IL-36 γ S18 to ensure they did not generate false negative results. A pan-protease inhibitor cocktail including E64, PMSF, and EDTA (PI) added to HaCaT cells in combination with IL-36 γ S18 had no

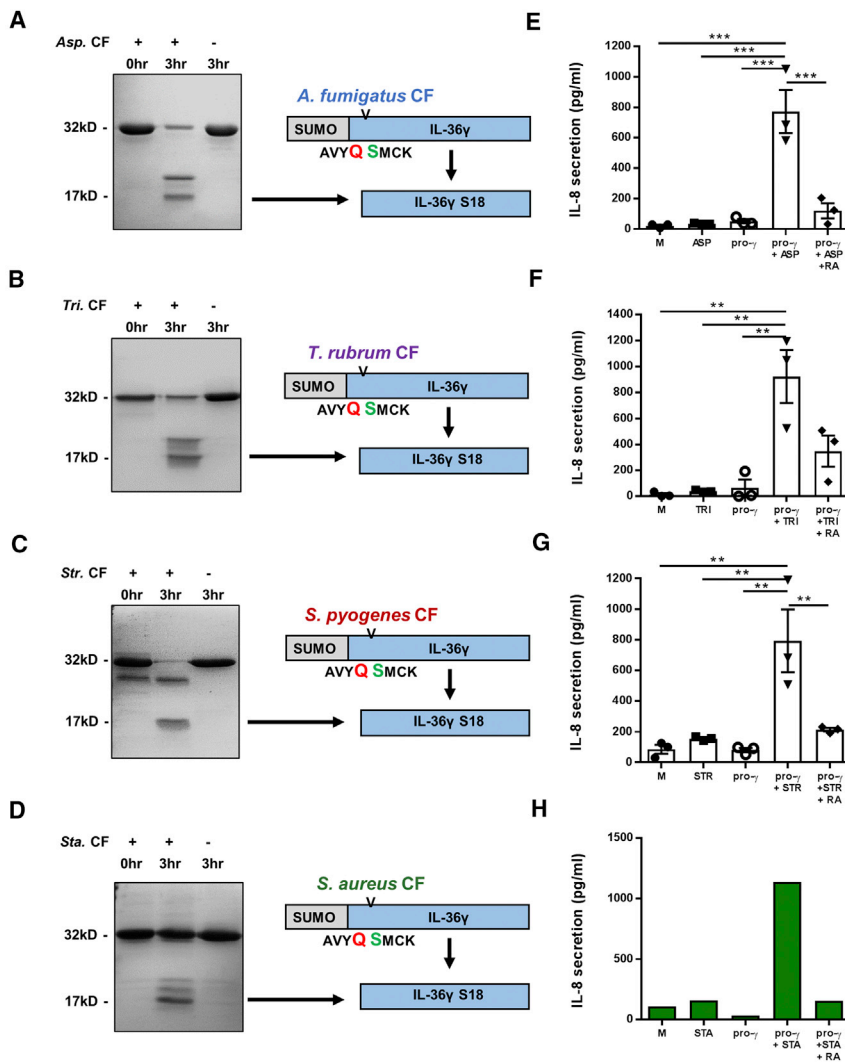


Figure 2. Human IL-36 γ Is Activated by Proteases Derived from a Range of Human Pathogens

(A–H) SUMO-tagged human IL-36 γ (1 μ g) was incubated at 37°C for 0 or 3 h, with or without 2 μ L of either Asp. CF (A), Tri. CF (B), Str. CF (C), or Sta. CF (D). Samples were analyzed by Coomassie-blue-stained SDS-PAGE gel. Cleaved products were also analyzed by mass spectrometry, with diagrams depicting the IL-36 γ truncation generated in response to each CF. In addition, HaCaT cells (10⁵ per well) were incubated for 24 h with M; pro- γ (10 nM); CF; a combination of pro- γ and CF; or a combination of pro- γ , CF, and IL-36RA (50 nM; E–H) (E–G, n = 3; H, n = 1). A one-way ANOVA was used to determine statistical significance of differences between treatment groups. **p < 0.01 and ***p < 0.001. Data shown are mean \pm SEM. Abbreviations are as follows: Asp. CF, *A. fumigatus* culture filtrate; pro- γ , pro-IL-36 γ ; Sta. CF, *S. aureus* culture filtrate; Str. CF, *S. pyogenes* culture filtrate; Tri. CF, *T. rubrum* culture filtrate.

by SDS-PAGE showed truncation of IL-36 γ (Figure 4F). Furthermore, addition of SpeB and pro-IL-36 γ to HaCaT cells resulted in secretion of IL-8 that could be inhibited by addition of IL-36RA (Figure 4G). Addition of SpeB or pro-IL-36 γ alone resulted in little or no IL-8 secretion. These results therefore suggest IL-36 γ is activated by SpeB secreted by *S. pyogenes*. Finally, the cleavage of pro-IL-36 γ by SpeB was interrogated by LC-MS, which identified the prominent species with a mass corresponding to that of IL-36 γ S18 (illustrated in Figure 4F).

To assess whether mIL-36 γ is also susceptible to activation by SpeB, the above-mentioned experiments were repeated with recombinant pro-mIL-36 γ . Cleavage assays showed that while wild-type *S. pyogenes* culture filtrate truncated mIL-36 γ , Δ SpeB culture filtrate did not (Figure 4H). Furthermore, stimulation of MEFs with Δ SpeB culture-filtrate-incubated mIL-36 γ did not elicit an IL-36-dependent response (Figure 4I). Finally, incubation of pro-mIL-36 γ with recombinant SpeB resulted in rapid truncation of pro-mIL-36 γ (within 5 min) that was identified by LC-MS as biologically active mature mIL-36 γ (G13) (Figure 4J). Addition of SpeB-processed pro-mIL-36 γ to MEFs also induced a strong response (Figure 4K). These results indicate both hIL-36 γ and mIL-36 γ are processed by SpeB to generate biologically active IL-36 γ .

Aspergillus fumigatus Virulence Factor Asp F13 (Alp1) Activates Released IL-36 γ in a Cell-Based Infection Assay

A similar approach was utilized to identify the activating protease produced by *A. fumigatus*. SDS-PAGE analysis of culture-filtrate-incubated SUMO-pro-IL-36 γ indicated that cleavage

significant effect on IL-8 secretion, indicating the protease inhibitors themselves would not inhibit IL-36 γ activity (Figure 4B). Addition of the culture-filtrate-incubated samples to HaCaT cells revealed IL-36 γ truncated in the presence of PMSF and EDTA both had IL-36 γ -specific activity, while the addition of E64 prevented IL-36 γ -mediated IL-8 secretion (Figure 4C). These results indicate a Cys protease secreted by *S. pyogenes* is responsible for the activation of IL-36 γ .

During infection, *S. pyogenes* abundantly secretes the virulence factor and Cys protease SpeB. We therefore examined whether SpeB might also be responsible for the activation of IL-36 γ by incubating SUMO-pro-IL-36 γ with culture filtrate from a SpeB-deficient mutant strain of *S. pyogenes* (Δ SpeB). Analysis by SDS-PAGE showed that in contrast to wild-type *S. pyogenes*, IL-36 γ did not undergo cleavage upon incubation with Δ SpeB *S. pyogenes* culture filtrate, and addition of the incubated sample to HaCaT cells did not elicit a strong IL-8 secretion (Figures 4D and 4E). These results suggest SpeB is responsible for processing of IL-36 γ . For confirmation, SUMO-pro-IL-36 γ was incubated with recombinant SpeB at 37°C for 1 h. Analysis

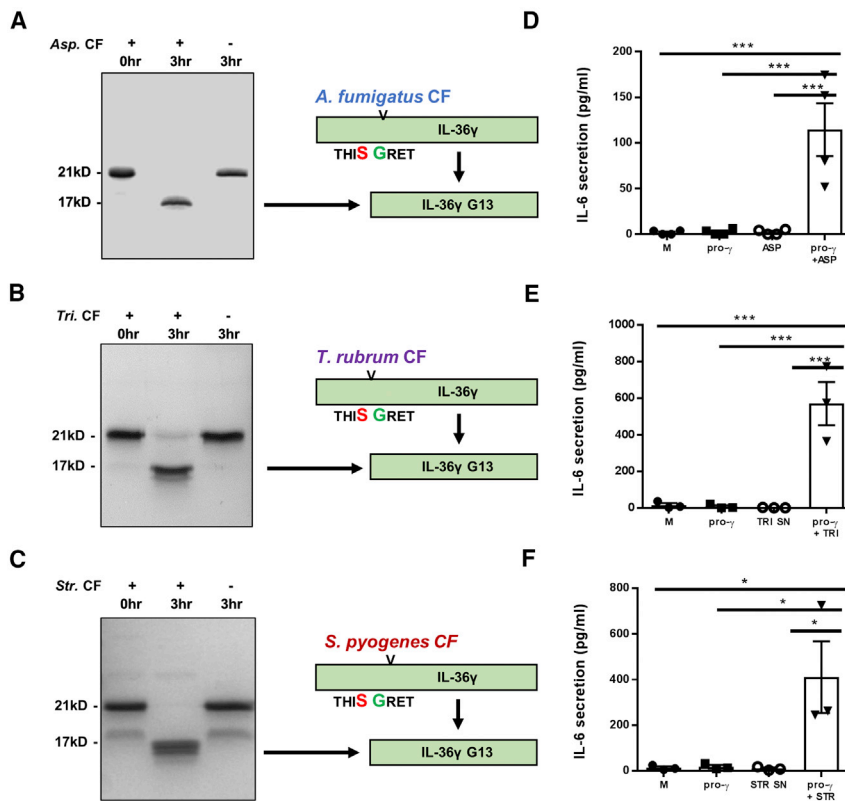


Figure 3. Mouse IL-36 γ (mIL-36 γ) Is Also Activated by Proteases Derived from a Range of Pathogens

(A–F) SUMO-tagged mIL-36 γ (1 μ g) was incubated at 37°C for 0 or 3 h, with or without 1 μ L of either Asp. CF (A), *Tri.* CF (C), or *Str.* CF (E). Samples were analyzed by Coomassie-blue-stained SDS-PAGE gel. Cleaved products were also analyzed by mass spectrometry, with diagrams depicting the IL-36 γ truncation generated in response to each CF. In addition, MEF cells (10^5 per well) were incubated for 24 h with M, pro- γ (10 nM), CF, or a combination of pro- γ and CF (B, D, and F). A one-way ANOVA was used to determine statistical significance of differences between treatment groups. * $p < 0.05$ and *** $p < 0.001$. Data shown are mean \pm SEM (n = 3).

IL-36 γ -induced IL-8 secretion could be inhibited by addition of IL-36RA, whereas Asp F5-incubated pro-IL-36 γ had no effect (Figures 5E and 5F). These results suggest IL-36 γ is truncated and activated by *A. fumigatus* Ser protease Asp F13 (confirmed by MS; Figure S3).

We also tested whether Asp F13 and Asp F5 were capable of activating mIL-36 γ . Recombinant pro-mIL-36 γ was incubated with wild-type, Asp F13 $^{-/-}$, and Asp F5 $^{-/-}$ *Aspergillus* culture filtrate.

was unaffected when conducted in the presence of Cys protease inhibitor E64 and metalloproteinase inhibitor EDTA; however, the addition of Ser inhibitor PMSF completely ablated cleavage (Figure 5A). Testing these incubations for biological activity by addition to HaCaT cells reflected the results observed by SDS-PAGE. When added in combination with *Aspergillus* culture filtrate and pro-IL-36 γ , the addition of E64 and EDTA had no effect on IL-36 γ activity; however, the addition of PMSF significantly ablated IL-36 γ -mediated IL-8 secretion (Figure 5B). These results implicate a Ser protease in the activation of IL-36 γ .

As the secreted Ser protease Asp F13 has been identified as a major virulence factor secreted during infection, it provided a good candidate protease as an activator of IL-36 γ . Therefore, culture filtrate from an Asp F13-deficient strain of *A. fumigatus* was examined against the wild type. As a negative control, a deficient strain of the virulence factor metalloprotease Asp F5 (Mep) was also examined. SUMO-pro-IL-36 γ was incubated with wild-type, Asp F5 $^{-/-}$, and Asp F13 $^{-/-}$ *A. fumigatus* culture filtrate. SDS-PAGE resolution of the samples showed both wild-type and Asp F5 $^{-/-}$ culture filtrates truncated IL-36 γ , while Asp F13 $^{-/-}$ did not (Figure 5C). Stimulation of HaCaT cells with wild-type and Asp F5 $^{-/-}$ culture-filtrate-incubated pro-IL-36 γ causes strong IL-8 secretion, while stimulation with Asp F13 $^{-/-}$ culture-filtrate-incubated pro-IL-36 γ had little effect on IL-8 secretion (Figure 5D). Finally, for confirmation, SUMO-IL-36 γ was incubated with recombinant Asp F13 and Asp F5 and analyzed by SDS-PAGE and activity assay. Rapid cleavage was observed with recombinant Asp F13, but not Asp F5. Moreover, the addition of the incubated proteins to HaCaT cells revealed Asp F13-truncated

SDS-PAGE analysis showed both wild-type and Asp F5 $^{-/-}$ culture filtrate truncated pro-mIL-36 γ , whereas Asp F13 $^{-/-}$ did not (Figure 5G). Addition of the incubated proteins to MEFs showed that in contrast to wild-type- and Asp F5 $^{-/-}$ -culture-filtrate-incubated mIL-36 γ , Asp F13 $^{-/-}$ -culture-filtrate-incubated pro-mIL-36 γ had no biological activity (Figure 5I). Finally, as with hIL-36 γ , mIL-36 γ was incubated with recombinant Asp F13 and Asp F5. Analysis by SDS-PAGE showed that unlike Asp F5, Asp F13 rapidly processed mIL-36 γ , and subsequent addition to MEFs revealed the Asp F13-processed mIL-36 γ to be biologically active (Figures 5H and 5J). These results indicate Asp F13 processes both hIL-36 γ and mIL-36 γ to a biologically active mature cytokine.

Finally, after establishing that Asp F13 can activate recombinant IL-36 γ , we utilized a cell-based infection assay to examine whether *Aspergillus* would also release and activate IL-36 γ in an Asp F13-dependent fashion, or whether pathogen-mediated cell damage in the absence of Asp F13 is in itself enough to induce activation of IL-36 γ . A stable HEK293 cell line expressing C-terminal FLAG-tagged pro-IL-36 γ (pro γ F-293) was generated as outlined in the STAR Methods (Figure S4). The pro γ F-293 cells were inoculated with conidia of wild-type and mutant strains of *A. fumigatus* for 24 h, and the expressed IL-36 γ in the supernatant was immunoprecipitated by its C-terminal FLAG tag. Western blot analysis showed IL-36 γ was released into the supernatant by all strains of *Aspergillus*. Furthermore, supernatant from wild-type- and Asp F5 $^{-/-}$ -infected pro γ F-293 cells contained truncated IL-36 γ , shown by activity assay to be biologically active (Figures 6A and 6B). Supernatant from cells infected

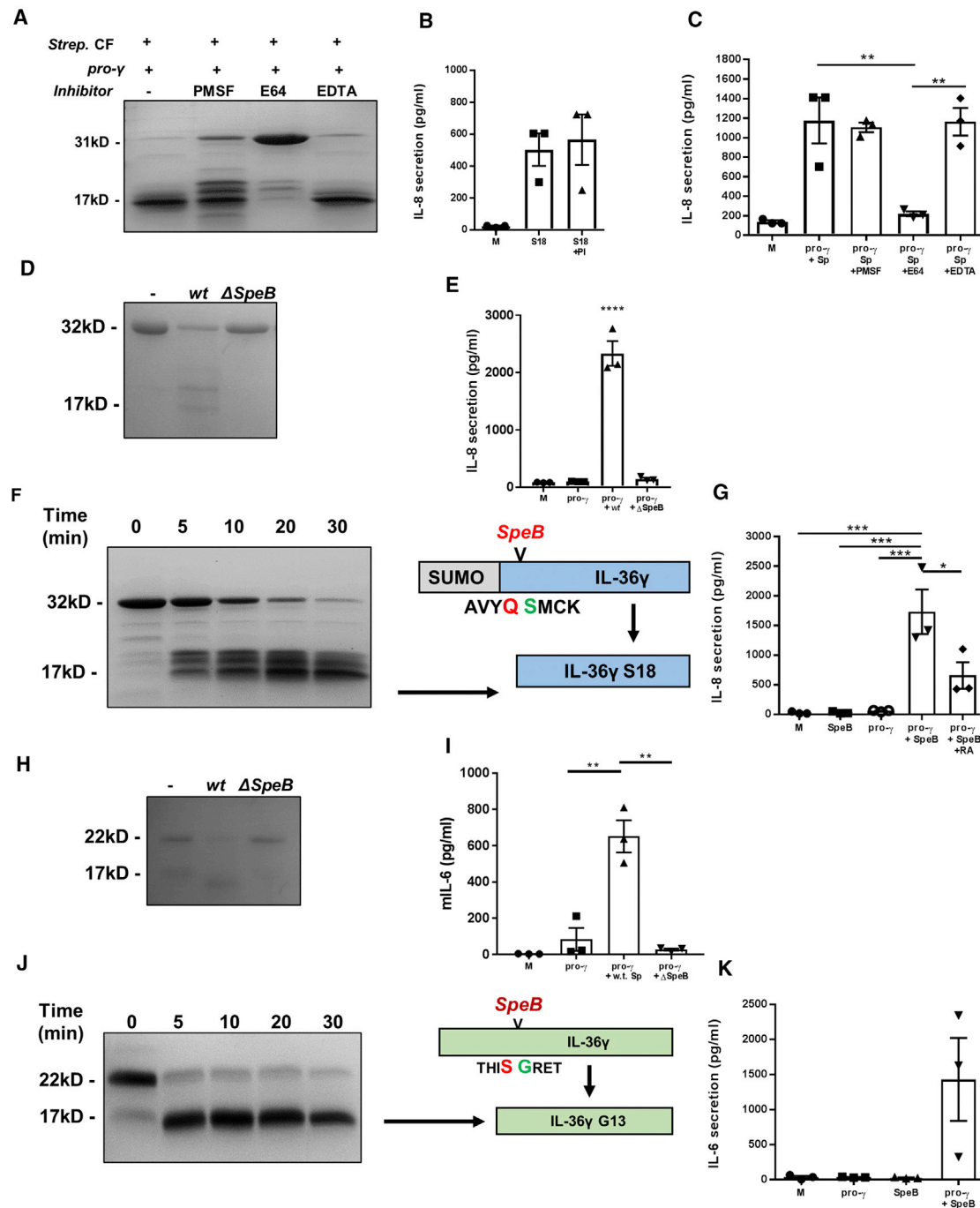


Figure 4. Activation of IL-36 γ by *S. pyogenes* Is Dependent on SpeB

(A–K) (A) SUMO-tagged human IL-36 γ (1 μ g) was incubated at 37°C for 3 h with *Strep.* CF, with and without the Ser inhibitor PMSF (10 mM), the Cys protease inhibitor E64 (20 μ M), or metalloproteinase inhibitor EDTA (5 mM). (B) HaCaT cells (10⁵ per well) were incubated for 24 h with M or S18 (10 nM), with and without a broad-range protease inhibitor that includes PMSF, E64, and EDTA (PI). (C–K) HaCaT cells (10⁵ per well) were incubated for 24 h with M, pro- γ , and *S. pyogenes* CF (Sp; 1 μ L), pro- γ and *S. pyogenes* CF with PMSF (0.1 mM), E64 (0.2 μ M), or EDTA. SUMO-tagged human IL-36 γ (1 μ g; D) or mouse pro- γ (H) was incubated at 37°C for 3 h with wt and Δ SpeB *S. pyogenes* CF. HaCaT cells (E) or MEFs (I) (10⁵ per well) were incubated for 24 h with M, pro- γ (10 nM), or pro- γ and wt (wt Sp) or Δ SpeB *S. pyogenes* CF. HaCaT cells were stimulated with human pro- γ , while MEFs were stimulated with mouse pro- γ . SUMO-tagged human IL-36 γ (1 μ g; F) or mouse pro- γ (J) was incubated at 37°C with 20 ng of recombinant SpeB for 0–30 min. Cleaved products were also analyzed by mass spectrometry, with diagrams depicting the IL-36 γ truncation generated. HaCaT cells (G) and MEFs (K) were stimulated with M, pro- γ , recombinant SpeB, or pro- γ with SpeB (and pro- γ +

(legend continued on next page)

with Asp F13^{-/-} did contain pro-IL-36 γ , indicating cell damage induced release of the cytokine; yet, IL-36 γ remained in its pro-form and when tested by activity assay did not show any biological activity. These results indicate in the context of an infection, IL-36 γ is released in its inactive form as a result of cell damage and then activated by Asp F13 secreted by *A. fumigatus* during infection.

DISCUSSION

The mechanisms surrounding IL-36 γ release from cells are not completely understood. As leaderless proteins, the IL-36 cytokines do not enter the canonical secretory system following synthesis, and unlike IL-1 β and IL-18, the underlying mechanisms of release are unclear. Indeed, in the majority of experimental systems examined, induced IL-36 cytokines appear to be retained as intracellular cytokines. This is also true of the IL-1 family alarmins IL-33 and IL-1 α , which function as proinflammatory mediators following release as a result of cellular damage. In this study, we demonstrated that while microbial stimulation of epithelial cells increased intracellular stores of IL-36 γ , the cytokine was only released when incubated with live pathogens. The live commensal *S. epidermidis* did not induce release of IL-36 γ despite increasing its intracellular expression (Figure 1). Indeed, release of IL-36 γ induced by pathogenic microbes paralleled that of the cytosolic housekeeping protein GAPDH, suggesting that in the context of infection the major mechanism of IL-36 γ release may be a result of pathogen-induced membrane damage or necrosis rather than through an active response to microbial challenge, perhaps similarly to IL-33 and IL-1 α . It therefore seems likely that known cytotoxic components such as pore-forming toxins secreted by the pathogens would be responsible for release of IL-36 γ . The use of lytic factors to destroy host cells for nutrient acquisition and immune evasion is a common feature of many invasive pathogens, and both *S. pyogenes* and *A. fumigatus* produce cytotoxins such as streptolysin O and Asp-hemolysin, respectively, during infection (Singh et al., 2012; Nilsson et al., 2006; Wartenberg et al., 2011). It may therefore be likely that numerous pathogens will have the ability to release IL-36 γ from epithelial cells. Furthermore, by incubating recombinant pro-IL-36 γ with pathogen culture filtrates, we demonstrated that the epithelial pathogens *A. fumigatus*, *S. pyogenes*, *T. rubrum*, and *S. aureus* all produce proteases that process recombinant IL-36 γ into its mature form (Figure 2), suggesting once released from epithelial tissue IL-36 γ will undergo activation, as was demonstrated with *A. fumigatus* (Figure 6). Therefore, IL-36-mediated signaling is likely to occur shortly after infection-induced damage, initiating and orchestrating an immune response directed against extracellular pathogens. As the epithelial microbes tested spanned both fungi and bacteria associated with infection at different epithelial sites, and given the apical expression of IL-36 γ , we believe these results indicate that IL-36 γ is broadly sensitive to pathogen-derived

proteases, and its release and activation following pathogen-mediated damage implicate IL-36 γ as an epithelial alarmin.

The results were further supported by the fact that recombinant mIL-36 γ was activated by the same culture filtrates (Figure 3). This was somewhat surprising as bioactive forms of hIL-36 γ and mIL-36 γ were generated by disparate classes of pathogen-derived proteases despite the requirement of precise cleavage (human Gln₁₇-Ser₁₈, mouse Tyr₁₂-Gly₁₃) and non-conserved primary amino acid sequences proximal to the cleavage sites. Structural information obtained from the crystal structure of hIL-36 γ shows the N terminus to be a flexible, exposed region that protrudes from the compact core of the protein's IL-1 domain and would therefore be more accessible for processing (Günther and Sundberg, 2014). Structural alignment of multiple species suggests that despite the differences in primary amino acid sequences, the exposed flexible N terminus is a common feature; therefore, protease sensitivity is likely to be an evolutionarily conserved phenomenon. Furthermore, analogous protease sensitivity has been recently described for other IL-1 family members, most notably IL-33, shown to be sensitive to activation by numerous allergen proteases. Unlike IL-36 γ , however, protease sensitivity of IL-33 triggers type II immune responses (Cayrol et al., 2018). Additionally, while the other IL-36 cytokines were not extensively tested in this study, we did demonstrate that IL-36 α also undergoes activation following incubation with *A. fumigatus* and *T. rubrum* culture filtrate (Figure S5). Having three functionally similar cytokines with structurally distinct cleavage sites may prove advantageous in the detection of invasive pathogens by enabling sensitivity to a wider range of pathogens. It has also been proposed that the recent evolution of multiple IL-36 genes from an ancestral IL-1 gene could provide a system that would resist microbial immune evasion (Jensen, 2017). Indeed, multiple pathogens, particularly DNA viruses, are known to prevent IL-1 activity via cytokine binding proteins, increased cytokine degradation, and inhibition of cytokine-activating proteases (Richards et al., 2014). The existence of three IL-36 agonists would therefore circumvent similar immune evasion strategies (Jensen, 2017).

Identifying virulence factors secreted by pathogens as activators of IL-36 γ has implications for the role microbial infection may have in inflammatory conditions. As several non-infectious inflammatory conditions have microbial etiologies, these observations may provide a mechanism for the initiation of inflammation via microbe-induced IL-36-mediated signaling. In the clinical setting, it is well established that streptococcal throat infection is the major triggering factor of guttate psoriasis and that IL-36 γ is abundantly expressed in the outermost skin layers of psoriatic individuals (D'Erme et al., 2015; Telfer et al., 1992). In some patients the connection between streptococcal infection and psoriasis flare is so strong that guidelines recommend tonsillectomy (Simões et al., 2015). As streptococcal SpeB is the most highly secreted protein produced during infection and is known to interact with host proteins with both pro- and anti-inflammatory

SpeB with IL-36RA; G). HaCaT cells were stimulated with human IL-36 γ , while MEFs were stimulated with mIL-36 γ . Samples were analyzed by Coomassie-blue-stained SDS-PAGE gel (A, D, F, H, and J) or measured by ELISA (B, C, E, G, I, and K). A one-way ANOVA was used to determine statistical significance of differences between treatment groups. *p < 0.05, **p < 0.01, and ***p < 0.001. Data shown are mean \pm SEM (n = 3). Abbreviations are as follows: S18, IL-36 γ S18; *Strep.* CF, *S. pyogenes* culture filtrate; wt, wild type.

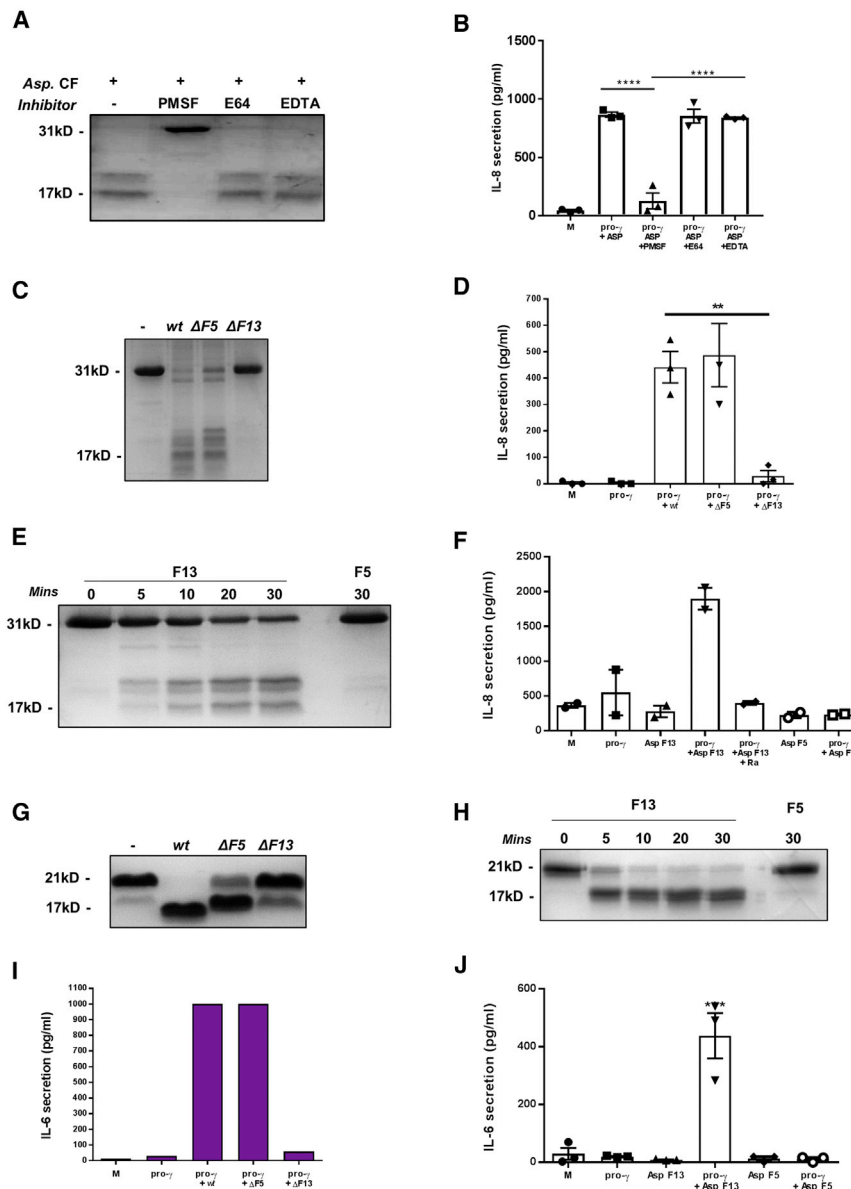


Figure 5. Activation of IL-36 γ by *Aspergillus* Is Dependent on the Virulence Factor Asp F13

(A–J) (A) SUMO-tagged human IL-36 γ (1 μ g) was incubated at 37°C for 3 h with Asp. CF, with and without the Ser inhibitor PMSF (10 mM), the Cys protease inhibitor E64 (20 μ M), or metalloproteinase inhibitor EDTA (5 mM). (B–J) HaCaT cells (10^5 per well) were incubated for 24 h with M, or pro- γ (10 nM) and Asp. CF filtrate (1 μ L), with and without PMSF (0.1 mM), E64 (0.2 μ M), or EDTA. SUMO-tagged human IL-36 γ (1 μ g; C) or mouse pro- γ (G) was incubated at 37°C 3 h with wt, Δ F5, or Δ F13 Asp. CF. HaCaT cells (D) or MEFs (I) (10^5 per well) were incubated for 24 h with M, pro- γ (10 nM), or pro- γ and wt, Δ F5, or Δ F13 Asp. CF. HaCaT cells were stimulated with human pro- γ , while MEFs were stimulated with mouse pro- γ . SUMO-tagged human IL-36 γ (1 μ g; E) or mouse pro- γ (H) was incubated at 37°C with 20 ng of recombinant F13 or F5 for 0–30 min. HaCaT cells (F) and MEFs (J) were stimulated with M, pro- γ , recombinant F13, recombinant F5, pro- γ with F13, or pro- γ with F5. HaCaT cells were stimulated with human IL-36 γ , while MEFs were stimulated with mIL-36 γ . Samples were analyzed by Coomassie-blue-stained SDS-PAGE gel (A, C, E, G, and H) or measured by ELISA (B, D, F, I, and J). A one-way ANOVA was used to determine statistical significance of differences between treatment groups. **p < 0.01, ***p < 0.001, and ****p < 0.0001. Data shown are mean \pm SEM (B, D, and J, n = 3; F, n = 2; I, n = 1). Abbreviations are as follows: Δ F5, Asp F5 $^{-/-}$; Δ F13, Asp F13 $^{-/-}$; F5, Asp F5; F13, Asp F13.

consequences, the data presented here may well provide a mechanistic link between the proteolytic activation of IL-36 γ by *S. pyogenes* SpeB and the initiation of guttate psoriasis (Nelson et al., 2011; Elliott, 1945). Published data have also shown that IL-36 γ is expressed in other inflammatory skin diseases associated with microbial infection including hidradenitis suppurative, eczema, and tinea, suggesting a potential causative role (Di Caprio et al., 2017; Hessam et al., 2018; Otobe et al., 2018). IL-36 γ is also upregulated in inflamed intestinal tissue as a result of stimulation from microbiota (Russell et al., 2016; Nishida et al., 2016). With a heavy microbial presence in the gut and increased expression of IL-36 cytokines, it seems plausible tissue damage and production of microbial proteases in inflammatory bowel disease might facilitate initiation of inflammation through liberation and activation of IL-36 cytokines. In

addition to its pathological role, IL-36 signaling in the gut has been demonstrated to be critical in both barrier defense and wound repair (Ngo et al., 2018). IL-36 signaling is a potent inducer of IL-23—a cytokine that, in turn, is a significant inducer of IL-22—providing a bridge between immune activation and epithelial repair. Although not examined in this study, the protease sensitive nature of IL-36 cytokines also provides a potential sensing mechanism for gut parasites such as helminths. These parasites are well documented to secrete a variety of proteases and cause significant epithelial damage (Caffrey et al., 2018; McKay et al., 2017). The proteolytic activation of IL-36 γ in the gut may therefore provide a potential mechanism to facilitate an inflammatory response and subsequent wound repair.

Epithelial barriers such as the skin, lungs, and gut perform multiple essential physiological functions and represent critical interfaces between an organism and its environment. However, due to the ubiquitous nature of commensal microbes and potentially damaging pathogens, these sites must be able to mount robust immune responses toward pathogens, while at the same time prevent detrimental inflammatory responses toward harmless commensals. The results obtained in this study have

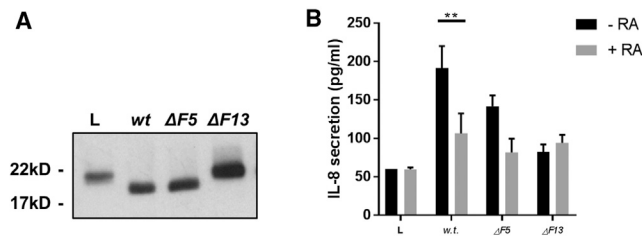


Figure 6. Human Pathogen *A. fumigatus* Induces the Release and Activation of Endogenous IL-36 γ

(A) The pro γ F-293 cells were inoculated with wt, Δ F5, or Δ F13 *A. fumigatus* conidia for 24 h. Supernatants were removed from cells, and FLAG-tag-immunoprecipitated IL-36 γ was analyzed by western blot alongside pro γ F-293 lysate (L).

(B) HaCaT cells were stimulated with 50 μ L of pro γ F-293 supernatant after 24 h incubation following mechanical lysis (L), inoculation with wt, Δ F5, Δ F13 *A. fumigatus* conidia in the presence or absence of 1 μ g/mL IL-36RA (RA). Harvested supernatants were tested for IL-8 concentration by ELISA. A one-way ANOVA was used to determine statistical significance of differences between treatment groups. * $p < 0.05$ and ** $p < 0.01$. Data shown are mean \pm SEM (n = 4).

demonstrated a scenario in which IL-36 γ is induced, released from cells via pathogenic damage, and subsequently activated as a direct result of secreted proteolytic virulence factors by invasive epithelial pathogens. Given the characteristics of IL-36 γ as an apically located epithelial initiator of inflammation and its sensitivity to pathogen-derived proteases, we believe this work demonstrates that IL-36 γ functions as a global epithelial alarmin and broad sensor of pathogenic infection. This provides a critical mechanism whereby host organisms can discriminate invasive pathogens from harmless microbes. In addition to describing a general sensor of pathogenic presence, this work illustrates the potential for microbial infection to act as a trigger for IL-36-mediated pathological inflammation in susceptible individuals and helps build a more complete picture of how IL-36-mediated signaling is actually initiated in the context of epithelial infection, giving us a greater understanding of immune defenses at epithelial barriers.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
 - Lead Contact
 - Materials Availability
 - Data and Code Availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 - Cell lines
 - Microbial strains and culture conditions
- METHOD DETAILS
 - Reagents
 - Generation of recombinant proteins
 - Activity assays
 - Culture filtrate production

- Infection assays
- Immunohistochemistry
- ELISA
- FLAG Immunoprecipitation
- LC-MS
- Gel electrophoresis and western blotting
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2020.108515>.

ACKNOWLEDGMENTS

This work was supported by the Fraunhofer Cluster of Excellence Immune-Mediated Diseases (CIMD) and Fraunhofer International Consortium for Anti-Infective Research (iCAIR). We thank D. Jonigk and G. Warnecke (Medical School, Germany) and L. Wilkens and H.-G. Fieguth (KRH Clinics Hannover, Germany) for pathological support and collaboration in lung tissue supply. This article also presents independent research supported by the National Institute for Health Research (NIHR) Leeds Biomedical Research Centre (BRC). The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health. The authors gratefully acknowledge the Medical Research Council (MRC) (MR/M01942X/1) and British Skin Foundation (044_R_19) for funding.

AUTHOR CONTRIBUTIONS

T.M., J.S.A., C.H., S.K., and A.B. undertook experimental work. M.S. and M.W. contributed equally to study design and supervision. P.B., Y.T., and S.H. provided vital reagents. All authors contributed to writing the manuscript, with T.M. taking the leading role.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: February 14, 2020

Revised: October 5, 2020

Accepted: November 19, 2020

Published: December 15, 2020

REFERENCES

- Ainscough, J.S., Macleod, T., McGonagle, D., Brakefield, R., Baron, J.M., Alase, A., Wittmann, M., and Stacey, M. (2017). Cathepsin S is the major activator of the psoriasis-associated proinflammatory cytokine IL-36 γ . *Proc. Natl. Acad. Sci. USA* *114*, E2748–E2757.
- Berekmeri, A., Lutzko, A., Alase, A., Macleod, T., Ainscough, J.S., Laws, P., Goodfield, M., Wright, A., Helliwell, P., Edward, S., et al. (2018). Detection of IL-36 γ through noninvasive tape stripping reliably discriminates psoriasis from atopic eczema. *J. Allergy Clin. Immunol.* *142*, 988–991.e4.
- Bianchi, M.E., Crippa, M.P., Manfredi, A.A., Mezzapelle, R., Rovere Querini, P., and Venereau, E. (2017). High-mobility group box 1 protein orchestrates responses to tissue damage via inflammation, innate and adaptive immunity, and tissue repair. *Immunol. Rev.* *280*, 74–82.
- Boutet, M.A., Bart, G., Penhoat, M., Amiaud, J., Brulin, B., Charrier, C., Morel, F., Lecron, J.C., Rolli-Derkinderen, M., Boureille, A., et al. (2016). Distinct expression of interleukin (IL)-36 α , β and γ , their antagonist IL-36Ra and IL-38 in psoriasis, rheumatoid arthritis and Crohn's disease. *Clin. Exp. Immunol.* *184*, 159–173.
- Braegelmann, J., Braegelmann, C., Bieber, T., and Wenzel, J. (2018). Candida induces the expression of IL-36 γ in human keratinocytes: implications for a

- pathogen-driven exacerbation of psoriasis? *J. Eur. Acad. Dermatol. Venereol.* **32**, e403–e406.
- Bridgwood, C., Fearnley, G.W., Berekmeri, A., Laws, P., Macleod, T., Pon-nambalam, S., Stacey, M., Graham, A., and Wittmann, M. (2018). IL-36 γ Is a Strong Inducer of IL-23 in Psoriatic Cells and Activates Angiogenesis. *Front. Immunol.* **9**, 200.
- Burns, E.H., Jr., Marciel, A.M., and Musser, J.M. (1996). Activation of a 66-kilo-dalton human endothelial cell matrix metalloprotease by *Streptococcus pyogenes* extracellular cysteine protease. *Infect. Immun.* **64**, 4744–4750.
- Caffrey, C.R., Goupil, L., Rebello, K.M., Dalton, J.P., and Smith, D. (2018). Cysteine proteases as digestive enzymes in parasitic helminths. *PLoS Negl. Trop. Dis.* **12**, e0005840.
- Carrier, Y., Ma, H.L., Ramon, H.E., Napierata, L., Small, C., O'Toole, M., Young, D.A., Fouser, L.A., Nickerson-Nutter, C., Collins, M., et al. (2011). Inter-regulation of Th17 cytokines and the IL-36 cytokines in vitro and in vivo: implications in psoriasis pathogenesis. *J. Invest. Dermatol.* **131**, 2428–2437.
- Cayrol, C., Duval, A., Schmitt, P., Roga, S., Camus, M., Stella, A., Burlet-Schiltz, O., Gonzalez-de-Peredo, A., and Girard, J.P. (2018). Environmental allergens induce allergic inflammation through proteolytic maturation of IL-33. *Nat. Immunol.* **19**, 375–385.
- D'Erme, A.M., Wilsmann-Theis, D., Wagenpfeil, J., Hölzel, M., Ferring-Schmitt, S., Sternberg, S., Wittmann, M., Peters, B., Bosio, A., Bieber, T., and Wenzel, J. (2015). IL-36 γ (IL-1F9) is a biomarker for psoriasis skin lesions. *J. Invest. Dermatol.* **135**, 1025–1032.
- Di Caprio, R., Balato, A., Caiazza, G., Lembo, S., Raimondo, A., Fabbrocini, G., and Monfrecola, G. (2017). IL-36 cytokines are increased in acne and hidradenitis suppurativa. *Arch. Dermatol. Res.* **309**, 673–678.
- Dunn, E., Sims, J.E., Nicklin, M.J., and O'Neill, L.A. (2001). Annotating genes with potential roles in the immune system: six new members of the IL-1 family. *Trends Immunol.* **22**, 533–536.
- Eigenbrod, T., Park, J.H., Harder, J., Iwakura, Y., and Núñez, G. (2008). Cutting edge: critical role for mesothelial cells in necrosis-induced inflammation through the recognition of IL-1 alpha released from dying cells. *J. Immunol.* **181**, 8194–8198.
- Elliott, S.D. (1945). A Proteolytic Enzyme Produced by Group a Streptococci with Special Reference to Its Effect on the Type-Specific M Antigen. *J. Exp. Med.* **81**, 573–592.
- Foster, A.M., Baliwag, J., Chen, C.S., Guzman, A.M., Stoll, S.W., Gudjonsson, J.E., Ward, N.L., and Johnston, A. (2014). IL-36 promotes myeloid cell infiltration, activation, and inflammatory activity in skin. *J. Immunol.* **192**, 6053–6061.
- Gabay, C., and Towne, J.E. (2015). Regulation and function of interleukin-36 cytokines in homeostasis and pathological conditions. *J. Leukoc. Biol.* **97**, 645–652.
- Gao, N., Me, R., Dai, C., Seyoum, B., and Yu, F.X. (2018). Opposing Effects of IL-1Ra and IL-36Ra on Innate Immune Response to *Pseudomonas aeruginosa* Infection in C57BL/6 Mouse Corneas. *J. Immunol.* **201**, 688–699.
- Gresnigt, M.S., Rösler, B., Jacobs, C.W., Becker, K.L., Joosten, L.A., van der Meer, J.W., Netea, M.G., Dinarello, C.A., and van de Veerdonk, F.L. (2013). The IL-36 receptor pathway regulates *Aspergillus fumigatus*-induced Th1 and Th17 responses. *Eur. J. Immunol.* **43**, 416–426.
- Günther, S., and Sundberg, E.J. (2014). Molecular determinants of agonist and antagonist signaling through the IL-36 receptor. *J. Immunol.* **193**, 921–930.
- He, Q., Chen, H.X., Li, W., Wu, Y., Chen, S.J., Yue, Q., Xiao, M., and Li, J.W. (2013). IL-36 cytokine expression and its relationship with p38 MAPK and NF- κ B pathways in psoriasis vulgaris skin lesions. *J. Huazhong Univ. Sci. Technolog. Med. Sci.* **33**, 594–599.
- Henry, C.M., Sullivan, G.P., Clancy, D.M., Afonina, I.S., Kulms, D., and Martin, S.J. (2016). Neutrophil-Derived Proteases Escalate Inflammation through Activation of IL-36 Family Cytokines. *Cell Rep.* **14**, 708–722.
- Hessam, S., Sand, M., Gambichler, T., Skrygan, M., Rüdell, I., and Bechara, F.G. (2018). Interleukin-36 in hidradenitis suppurativa: evidence for a distinctive proinflammatory role and a key factor in the development of an inflammatory loop. *Br. J. Dermatol.* **178**, 761–767.
- Iadarola, P., Lungarella, G., Martorana, P.A., Viglio, S., Guglielminetti, M., Kor-zus, E., Gorrini, M., Cavarra, E., Rossi, A., Travis, J., and Luisetti, M. (1998). Lung injury and degradation of extracellular matrix components by *Aspergillus fumigatus* serine proteinase. *Exp. Lung Res.* **24**, 233–251.
- Jensen, L.E. (2017). Interleukin-36 cytokines may overcome microbial immune evasion strategies that inhibit interleukin-1 family signaling. *Sci. Signal.* **10**, eaan3589.
- Johnston, A., Xing, X., Guzman, A.M., Riblett, M., Loyd, C.M., Ward, N.L., Wahn, C., Prens, E.P., Wang, F., Maier, L.E., et al. (2011). IL-1F5, -F6, -F8, and -F9: a novel IL-1 family signaling system that is active in psoriasis and promotes keratinocyte antimicrobial peptide expression. *J. Immunol.* **186**, 2613–2622.
- Kono, H., Karmarkar, D., Iwakura, Y., and Rock, K.L. (2010). Identification of the cellular sensor that stimulates the inflammatory response to sterile cell death. *J. Immunol.* **184**, 4470–4478.
- Kovach, M.A., Singer, B., Martinez-Colon, G., Newstead, M.W., Zeng, X., Mancuso, P., Moore, T.A., Kunkel, S.L., Peters-Golden, M., Moore, B.B., and Standiford, T.J. (2017). IL-36 γ is a crucial proximal component of protective type-1-mediated lung mucosal immunity in Gram-positive and -negative bacterial pneumonia. *Mucosal Immunol.* **10**, 1320–1334.
- Lin, S.J., Schranz, J., and Teutsch, S.M. (2001). Aspergillosis case-fatality rate: systematic review of the literature. *Clin. Infect. Dis.* **32**, 358–366.
- Macleod, T., Doble, R., McGonagle, D., Wasson, C.W., Alase, A., Stacey, M., and Wittmann, M. (2016). Neutrophil Elastase-mediated proteolysis activates the anti-inflammatory cytokine IL-36 Receptor antagonist. *Sci. Rep.* **6**, 24880.
- McKay, D.M., Shute, A., and Lopes, F. (2017). Helminths and intestinal barrier function. *Tissue Barriers* **5**, e1283385.
- Namvar, S., Warn, P., Farnell, E., Bromley, M., Fraczek, M., Bowyer, P., and Herrick, S. (2015). *Aspergillus fumigatus* proteases, Asp f 5 and Asp f 13, are essential for airway inflammation and remodelling in a murine inhalation model. *Clin. Exp. Allergy* **45**, 982–993.
- Nelson, D.C., Garbe, J., and Collin, M. (2011). Cysteine proteinase SpeB from *Streptococcus pyogenes* - a potent modifier of immunologically important host and bacterial proteins. *Biol. Chem.* **392**, 1077–1088.
- Ngo, V.L., Abo, H., Maxim, E., Harusato, A., Geem, D., Medina-Contreras, O., Merlin, D., Gewirtz, A.T., Nusrat, A., and Denning, T.L. (2018). A cytokine network involving IL-36 γ , IL-23, and IL-22 promotes antimicrobial defense and recovery from intestinal barrier damage. *Proc. Natl. Acad. Sci. USA* **115**, E5076–E5085.
- Nilsson, M., Sørensen, O.E., Mörgelin, M., Weineisen, M., Sjöbring, U., and Herwald, H. (2006). Activation of human polymorphonuclear neutrophils by streptolysin O from *Streptococcus pyogenes* leads to the release of proinflammatory mediators. *Thromb. Haemost.* **95**, 982–990.
- Nishida, A., Hidaka, K., Kanda, T., Imaeda, H., Shioya, M., Inatomi, O., Bamba, S., Kito, K., Sugimoto, M., and Andoh, A. (2016). Increased Expression of Interleukin-36, a Member of the Interleukin-1 Cytokine Family, in Inflammatory Bowel Disease. *Inflamm. Bowel Dis.* **22**, 303–314.
- Oppenheim, J.J., and Yang, D. (2005). Alarmins: chemotactic activators of immune responses. *Curr. Opin. Immunol.* **17**, 359–365.
- Otobe, S., Sugaya, M., Nakajima, R., Oka, T., Takahashi, N., Kabasawa, M., Miyagaki, T., Asano, Y., and Sato, S. (2018). Increased interleukin-36 γ expression in skin and sera of patients with atopic dermatitis and mycosis fungoides/Sézary syndrome. *J. Dermatol.* **45**, 468–471.
- Ramadas, R.A., Ewart, S.L., Medoff, B.D., and LeVine, A.M. (2011). Interleukin-1 family member 9 stimulates chemokine production and neutrophil influx in mouse lungs. *Am. J. Respir. Cell Mol. Biol.* **44**, 134–145.
- Richards, K.H., Doble, R., Wasson, C.W., Haider, M., Blair, G.E., Wittmann, M., and Macdonald, A. (2014). Human papillomavirus E7 oncoprotein increases production of the anti-inflammatory interleukin-18 binding protein in keratinocytes. *J. Virol.* **88**, 4173–4179.
- Russell, S.E., Horan, R.M., Stefanska, A.M., Carey, A., Leon, G., Aguilera, M., Statovci, D., Moran, T., Fallon, P.G., Shanahan, F., et al. (2016). IL-36 α

- expression is elevated in ulcerative colitis and promotes colonic inflammation. *Mucosal Immunol.* **9**, 1193–1204.
- Simões, J.F., Ribeiro, J., Ferreira, B.R., and Paiva, S. (2015). The role of tonsillectomy in psoriasis treatment. *BMJ Case Rep.* **2015**, bcr2014206899.
- Singh, B., Fleury, C., Jalalvand, F., and Riesbeck, K. (2012). Human pathogens utilize host extracellular matrix proteins laminin and collagen for adhesion and invasion of the host. *FEMS Microbiol. Rev.* **36**, 1122–1180.
- Tamura, F., Nakagawa, R., Akuta, T., Okamoto, S., Hamada, S., Maeda, H., Kawabata, S., and Akaike, T. (2004). Proapoptotic effect of proteolytic activation of matrix metalloproteinases by *Streptococcus pyogenes* thiol proteinase (Streptococcus pyrogenic exotoxin B). *Infect. Immun.* **72**, 4836–4847.
- Telfer, N.R., Chalmers, R.J., Whale, K., and Colman, G. (1992). The role of streptococcal infection in the initiation of guttate psoriasis. *Arch. Dermatol.* **128**, 39–42.
- Temann, A., Golovina, T., Neuhaus, V., Thompson, C., Chichester, J.A., Braun, A., and Yusibov, V. (2017). Evaluation of inflammatory and immune responses in long-term cultured human precision-cut lung slices. *Hum. Vaccin. Immunother.* **13**, 351–358.
- Terao, Y., Mori, Y., Yamaguchi, M., Shimizu, Y., Ooe, K., Hamada, S., and Kawabata, S. (2008). Group A streptococcal cysteine protease degrades C3 (C3b) and contributes to evasion of innate immunity. *J. Biol. Chem.* **283**, 6253–6260.
- Towne, J.E., Renshaw, B.R., Douangpanya, J., Lipsky, B.P., Shen, M., Gabel, C.A., and Sims, J.E. (2011). Interleukin-36 (IL-36) ligands require processing for full agonist (IL-36 α , IL-36 β , and IL-36 γ) or antagonist (IL-36Ra) activity. *J. Biol. Chem.* **286**, 42594–42602.
- Verma, A.H., Zafar, H., Ponde, N.O., Hepworth, O.W., Sihra, D., Aggor, F.E.Y., Ainscough, J.S., Ho, J., Richardson, J.P., Coleman, B.M., et al. (2018). IL-36 and IL-1/IL-17 Drive Immunity to Oral Candidiasis via Parallel Mechanisms. *J. Immunol.* **201**, 627–634.
- Wartenberg, D., Lapp, K., Jacobsen, I.D., Dahse, H.M., Kniemeyer, O., Heinekamp, T., and Brakhage, A.A. (2011). Secretome analysis of *Aspergillus fumigatus* reveals Asp-hemolysin as a major secreted protein. *Int. J. Med. Microbiol.* **301**, 602–611.
- Yang, D., Han, Z., and Oppenheim, J.J. (2017). Alarmins and immunity. *Immunol. Rev.* **280**, 41–56.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Polyclonal goat anti-IL-36 γ antibody	R and D Systems	Cat# BAF2320; RRID:AB_2280258
Monoclonal mouse anti-FLAG M2 antibody	Sigma-Aldrich	Cat# F3165; RRID:AB_259529
Bacterial and Virus Strains		
BL21-CodonPlus (DE3)-RIL <i>E. coli</i>	Agilent	Cat# 230245
Biological Samples		
		NA
Chemicals, Peptides, and Recombinant Proteins		
Human IL-36 γ	Ainscough et al., 2017 ; This lab	PMID: 28289191
Human IL-36 γ S18	Ainscough et al., 2017 ; This lab	PMID: 28289191
Human IL-36 α	Ainscough et al., 2017 ; This lab	PMID: 28289191
Human IL-36RA V2	Ainscough et al., 2017 ; This lab	PMID: 28289191
Mouse IL-36 γ	This paper	NA
Mouse IL-36 γ G13	This paper	NA
Mouse IL-36 α	This paper	NA
Protease Inhibitor Cocktail	ThermoFischer	Cat# A32955
PMSF	Merck Millipore	Cat# 52332
E64	Merck Millipore	Cat# 324890
Critical Commercial Assays		
IL-8 ELISA	BioLegend	431504
IL-36 γ ELISA	Berekmeri et al., 2018 ; This lab	PMID: 29782895
Deposited Data		
Experimental Models: Cell Lines		
TR146	ECACC	Cat# 10032305, RRID:CVCL_2736
HEK293T	ATCC	Cat# CRL-3216, RRID:CVCL_0063
HaCaT	CLS	Cat# 300493/p800_HaCaT, RRID:CVCL_0038
pro γ F-293	This paper	NA
Experimental Models: Organisms/Strains		
<i>Streptococcus pyogenes</i>	Terao et al., 2008 ; Provided by Prof. Yutaka Terao, Niigata University	PMID: 18160402
<i>Streptococcus pyogenes</i> Δ <i>speB</i>	Terao et al., 2008 ; Provided by Prof. Yutaka Terao, Niigata University	PMID: 18160402
<i>Staphylococcus aureus</i>	ATCC	Cat# 6534
<i>Staphylococcus epidermidis</i>	ATCC	Cat# 12228
<i>Aspergillus fumigatus</i>	Namvar et al., 2015 ; Provided by Dr Sarah Herrick, University of Manchester	PMID: 25270353
<i>Aspergillus fumigatus</i> Asp F5 ^{-/-}	Namvar et al., 2015 ; Provided by Dr Sarah Herrick, University of Manchester	PMID: 25270353
<i>Aspergillus fumigatus</i> Asp F13 ^{-/-}	Namvar et al., 2015 ; Provided by Dr Sarah Herrick, University of Manchester	PMID: 25270353
<i>Trichophyton rubrum</i>	ATCC	Cat# 28188
Software and Algorithms		
GraphPad Prism 7	GraphPad	https://www.graphpad.com

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Martin Stacey (M.Stacey@leeds.ac.uk).

Materials Availability

All proteins and cell lines generated and used in this study are available on request from the Lead Contact, Martin Stacey.

Data and Code Availability

This study did not generate or analyze datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines

TR146, HEK293T, and HaCaT cells were used in this study. The cell line pro γ F-293 was also generated from HEK293 cells in the following method. cDNA of pro-IL-36 γ was cloned into pcDNA3.1 vector containing a G418 resistance gene using reverse primers containing a C-terminal FLAG tag to generate the fusion protein pro-IL-36 γ -FLAG. The vector was linearized prior to transfection into HEK293 cells and stable transfectants were selected using G418 (500 μ g/ml). Stable transfectants were subject to limiting dilution to generate monoclonal colonies, which were bulked and screened for strong IL-36 γ expression by ELISA. Successful production of FLAG-tagged IL-36 γ was confirmed by western blot (Figure S1).

All cells were cultured in FCS-supplemented culture medium (DMEM; Life technologies), containing 400 μ g/ml penicillin/streptomycin and 10% FCS (Life Technologies) at 37°C in a 5% CO₂ incubator.

Microbial strains and culture conditions

A wild-type *S. pyogenes* strain and its isogenic Δ speB mutant were isolated and generated as previously described (Terao et al., 2008). *S. pyogenes* were cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY). *S. aureus* and *S. epidermidis* were cultured in 2YT media. *A. fumigatus* and its Asp F 13 and Asp F 5 mutants were generated as previously described (Namvar et al., 2015). *A. fumigatus* and *T. rubrum* were cultured with Sabouraud dextrose or agar. Conidia were harvested from agar by washing with 0.05% Tween-20 in PBS.

METHOD DETAILS

Reagents

The protease inhibitors PMSF and E64 were purchased from Merck Millipore. Total protease inhibitor cocktail was obtained from Thermo Fischer. For Western-blot analysis, the primary antibodies used were a biotinylated goat anti-IL-36 γ antibody and mouse anti-FLAG M2. HRP-conjugated avidin and HRP-conjugated anti-mouse were used for detection.

Generation of recombinant proteins

cDNA of full length, pro human and mouse IL-36 γ , IL-36 α , IL-36RA V2, human IL-36 γ S18 and mouse IL-36 γ G13 were cloned into a Champion pET SUMO expression vector (Invitrogen, UK). Proteins were expressed in BL21-CodonPlus (DE3)-RIL *E. coli* overnight at 25°C and soluble proteins purified via Ni²⁺-affinity and size exclusion chromatography. Proteins were further purified by Ni²⁺-affinity chromatography prior to overnight cleavage of N-terminal SUMO by the Ulp1 protease, followed by subsequent ion exchange and size exclusion chromatography into 20 mM Tris pH7.4, 300 mM NaCl as previously described (MacLeod et al., 2016; Ainscough et al., 2017).

Activity assays

Activity assays were performed as previously described (Ainscough et al., 2017). Briefly, HaCaT cells were plated at 2x10⁵ cells/well (24-well plate) in complete culture media, and cultured to 90% confluence. The media was then removed and replaced with fresh media. For most activity assay experiments, indicated treatments were added and cells incubated for 24 hours at 37°C. For activity assay experiments involving protease inhibitors, samples were preincubated with protease inhibitors for 3 hours at 4°C before being added to HaCaT cells. For the activity assay experiments involving cell supernatants from treated pro γ F-293 cells, supernatants were centrifuged for removal of microbial debris and preincubated with total protease inhibitors for 3 hours at 4°C prior to addition to HaCaT cells. Following incubation, cell supernatants were removed and frozen at -80°C.

Culture filtrate production

5 mL of appropriate growth medium was inoculated with microbes and incubated at 37°C for 24 hours. Cultures were then centrifuged, and supernatants were removed and filtered.

Infection assays

pro γ F-293 s were plated in 6 well plates and grown to 90% confluence. Cells were washed 2x in PBS and media replaced with infection assay medium (PBS, 1 g/L dextrose, 100 mg/L MgCl₂, 100 mg/L CaCl₂, 30 mM HEPES). Cells were inoculated with the indicated amount of pathogen and incubated at 37°C 5% CO₂ for the time indicated. Supernatants were removed and treated according to FLAG immunoprecipitation or activity assay.

Immunohistochemistry

Formalin-fixed, paraffin-embedded skin sections were stained using standard hematoxylin and eosin, as well as periodic acid Schiff staining. IL-36 γ protein expression was analyzed by IHC using the monoclonal mouse IgG1 anti-human-IL-36 γ antibody ab156783 (Abcam Inc., Cambridge, MA) without pretreatment with a dilution of 1:500. Visualization was performed using the REAL staining kit (DAKO, Hamburg, Germany) with Fast Red as chromogen (Braegelmann et al., 2018).

ELISA

Supernatants were analyzed for IL-8 protein using a specific ELISA kit from Biolegend (San Diego, CA). Supernatants and lysates were analyzed for IL-36 γ protein using an in-house monoclonal based ELISA previously described (Berekmeri et al., 2018). IL-8 ELISA was performed following the manufacturer's instructions. IL-36 γ ELISA was performed as follows. Immunosorbent 96-well ELISA plates were coated with 2 μ g/mL capture antibody in PBS at 4°C overnight. Plates were then washed with 0.1% Tween 20/PBS and blocked for 1 hour in 2% BSA in 0.1% Tween-20/PBS. Samples were incubated subsequently for 1 hour at room temperature before washing and incubation with 1 μ g/mL biotinylated detection antibody for 1 hour. Plates were then washed and incubated with streptavidin–horseradish peroxidase (BioLegend, London, United Kingdom) for 20 minutes. After washing, TMB was used as a chromogenic substrate (Thermo Scientific). The reaction was stopped with 2N H₂SO₄, and OD was measured at 450 nm. A standard curve was obtained from a 7-point serial dilution of protein standard and used to calculate IL-36 γ concentrations (Berekmeri et al., 2018). The lower limits of accurate detection for IL-8 and IL-36 γ were 15.6 pg/ml and 24 pg/ml respectively.

FLAG Immunoprecipitation

Supernatants were centrifuged at 10000 g for removal of microbial and cellular debris and total protease inhibitor added to working concentration. M2-conjugated gel was washed and reconstituted to equivalent volume in PBS before adding 20 μ l of M2-conjugated gel per ml of supernatants. Supernatants were then mixed for 24h at 4°C before pelleting of the M2-conjugated gel by gentle centrifugation. The pellet was washed in PBS, boiled in SDS-loading dye and subsequent analysis by western blot.

LC-MS

1 μ g of protein was loaded onto a MassPREP micro desalting column (Waters) and washed 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.

Gel electrophoresis and western blotting

Samples were diluted in sample buffer (50 mM Tris HCl pH 6.8, 2% SDS, 10% glycerol, 0.02% bromophenol blue) and heated at 90°C for 5 min. Samples were resolved on a 15% (SUMO-tagged proteins) or 17% (immunoprecipitated samples) polyacrylamide gel and either stained with Coomassie or proteins transferred to a nitrocellulose membrane for western blotting. IL-36 γ was detected using anti-IL-36 γ antibody and avidin-HRP secondary. Proteins were visualized using enhanced chemiluminescence reagents (Sigma, UK).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using the software Graphpad Prism 7. Statistical details of experiments can be found in the figure legends. 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. Differences were considered statistically significant when $p < 0.05$.

Cell Reports, Volume 33

Supplemental Information

**The Proinflammatory Cytokine IL-36 γ Is a Global
Discriminator of Harmless Microbes and Invasive
Pathogens within Epithelial Tissues**

Thomas Macleod, Joseph S. Ainscough, Christina Hesse, Sebastian Konzok, Armin Braun, Anna-Lena Buhl, Joerg Wenzel, Paul Bowyer, Yutaka Terao, Sarah Herrick, Miriam Wittmann, and Martin Stacey

Supplementary Information

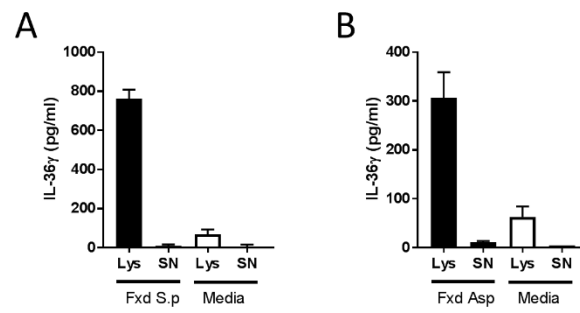


Fig. S1: TR146 cells do not release IL-36 γ following non-viable pathogen stimulation, related to Figure 1. TR146 cells (10^5 per well) were treated with fixed *S. pyogenes* (A; Fxd S.p), fixed *A. fumigatus* conidia (B; Fxd Asp) or media alone for 24 hours. Lysate and supernatant IL-36 γ concentration measured by ELISA. Data shown are mean \pm SEM (n = 3).

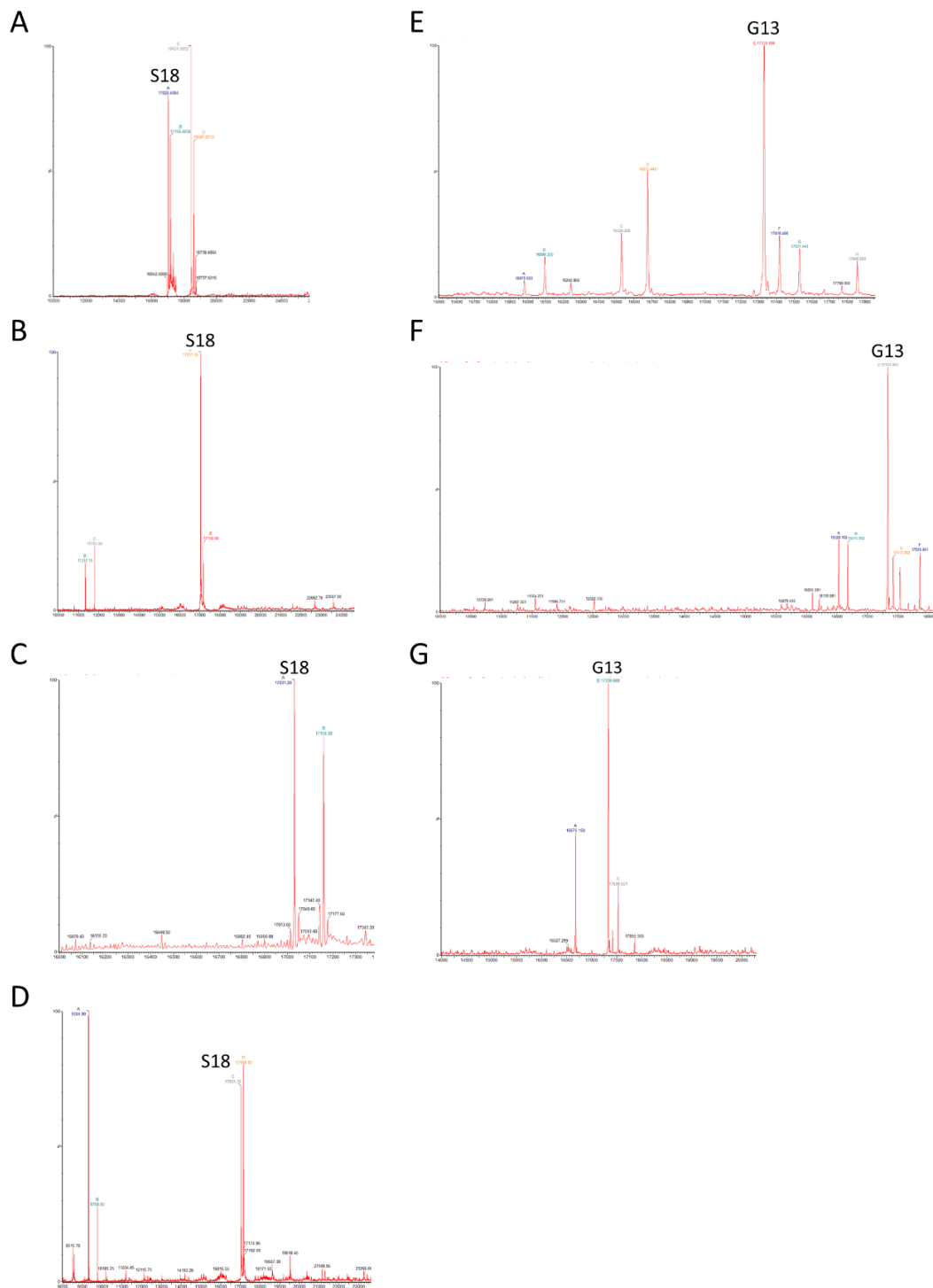


Fig. S2: Liquid chromatography-mass spectrometry traces of IL-36 cleavage, related to Figure 2. 2 μ g of recombinant full length IL-36 proteins were incubated with culture filtrates or recombinant proteases for 1 hour at 37C and analysed by liquid chromatography-mass spectrometry as outlined in methods and materials. Liquid chromatography traces show resulting cleavage products from hIL-36 γ + *A. fumigatus* (A), hIL-36 γ + *T. rubrum* (B), hIL-36 γ + *S. pyogenes* (C), hIL-36 γ + *S. areus* (D), mL-36 γ + *A. fumigatus* (E), mL-36 γ + *T. rubrum* (F), mL-36 γ + *S. pyogenes* (G). Peaks corresponding to the active IL-36 truncations are depicted above and labelled with their respective N-terminal amino acids. hIL-36 γ S18 (S18; 17031 Da), mL-36 γ G13 (G13; 17331 Da).

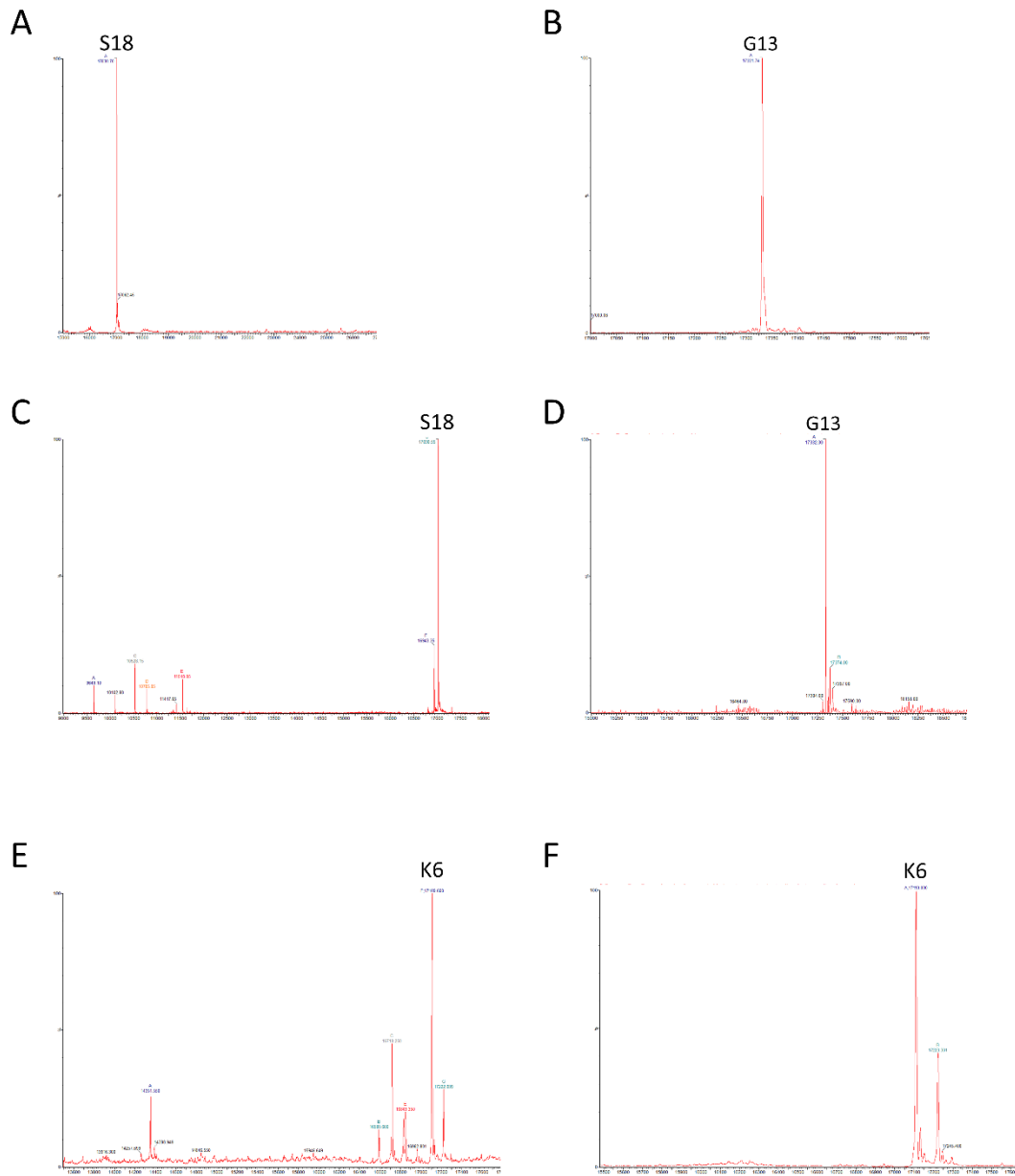


Fig. S3: Liquid chromatography-mass spectrometry traces of IL-36 cleavage, related to Figure 2. 2 μg of recombinant full length IL-36 proteins were incubated with culture filtrates or recombinant proteases for 1 hour at 37C and analysed by liquid chromatography-mass spectrometry as outlined in methods and materials. Liquid chromatography traces show resulting cleavage products from hIL-36 γ + SpeB (**A**), mLIL-36 γ + SpeB (**B**), hIL-36 γ + Asp F13 (**C**), mLIL-36 γ + Asp F13 (**D**), hIL-36 α + *A. fumigatus* (**E**), hIL-36 α + *T. rubrum* (**F**). Peaks corresponding to the active IL-36 truncations are depicted above and labelled with their respective N-terminal amino acids. hIL-36 γ S18 (S18; 17031 Da), mLIL-36 γ G13 (G13; 17331 Da), hIL-36 α K6 (K6; 17113).

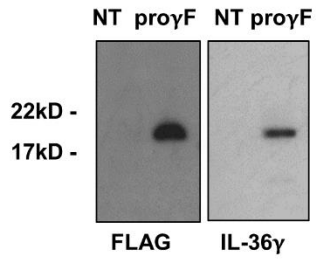


Fig. S4: Confirmation of *FLAG*-tagged *IL-36γ* expression by stable *proγF*-293 cell line, related to STAR Methods. Lysates of non-transfected 293 cells (NT) and stable *proγF*-293 cells (*proγF*) were analysed for expression of *FLAG*-tagged *IL-36γ* by western blot.

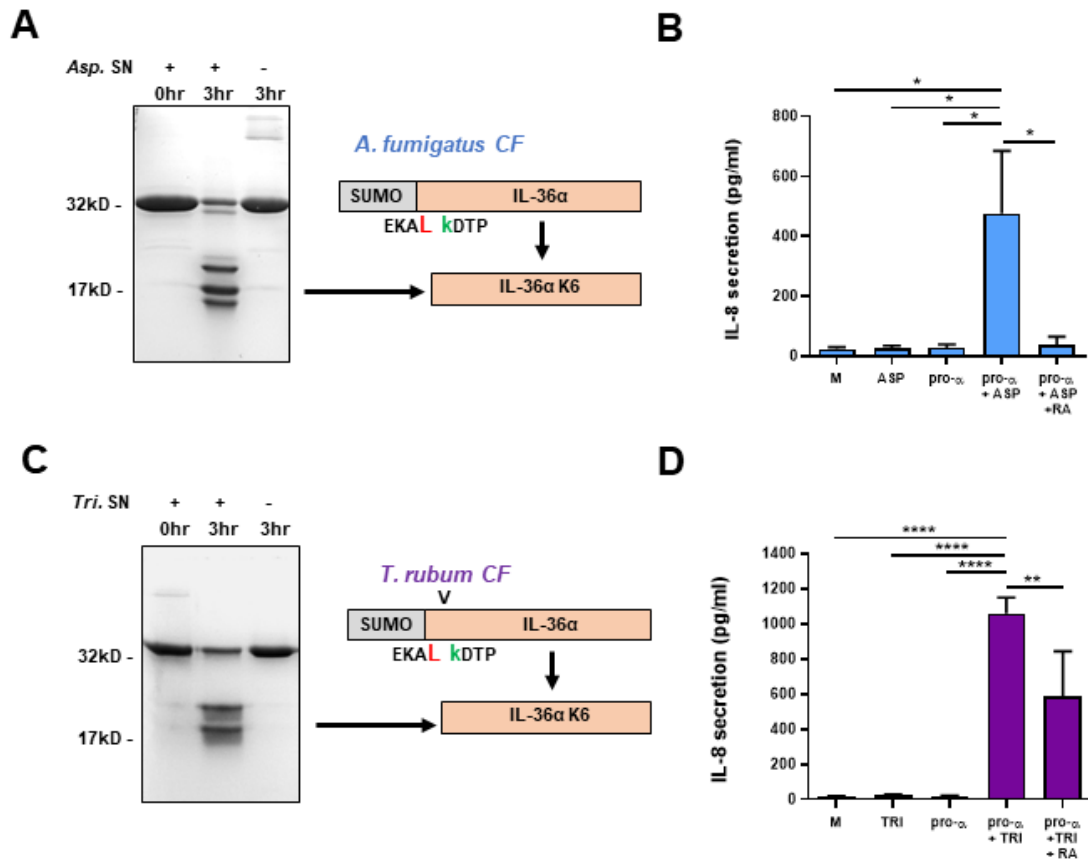


Fig. S5: IL-36 α is also cleaved and activated by *Aspergillus fumigatus* and *Trichophyton rubrum*, related to Figure 2. 1 μ g of SUMO-tagged human IL-36 α was incubated at 37°C for 0 hours or 3 hours, with or without 2 μ l of either *A. fumigatus* culture filtrate (Asp. CF; **A**) or *T. rubrum* culture filtrate (*Tri.* CF; **C**). Samples were analysed by Coomassie stained SDS-PAGE gel. Cleaved products were also analysed by mass spectrometry, with diagrams depicting the IL-36 α truncation generated in response to each culture filtrate. In addition, HaCaT cells (10^5 per well) were incubated for 24 hours with media alone (M), pro-IL-36 α (pro- α ; 10 nM), culture filtrate, a combination of pro-IL-36 α and culture filtrate, or a combination of pro-IL-36 α , culture filtrate and IL-36RA (50 nM; **B**, **D**). A one-way ANOVA was used to determine statistical significance of differences between treatment groups. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Data shown are mean \pm SEM (**B**; $n=3$, **D**; $n=4$).