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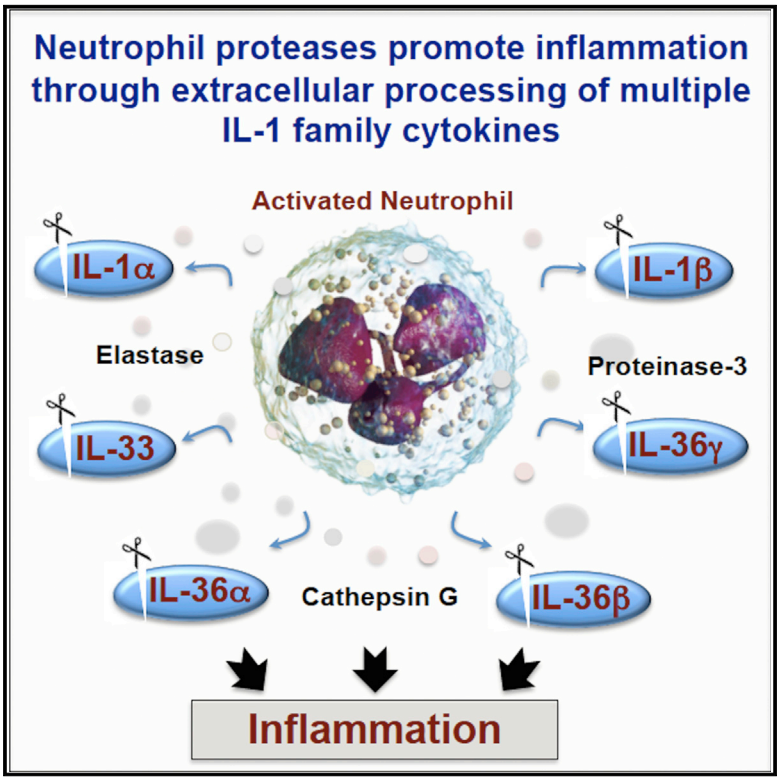
Extracellular Neutrophil Proteases Are Efficient Regulators of IL-1, IL-33, and IL-36 Cytokine Activity but Poor Effectors of Microbial Killing

Graphical Abstract



Authors

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In Brief

Here, Clancy et al. show that proteases released by activated neutrophils into the extracellular space exhibited poor antimicrobial activity but were potent modulators of IL-1α, IL-1β, IL-33, IL-36α, IL-36β, and IL-36γ activation states. Thus, neutrophils play a key role in modulating inflammatory responses through processing of multiple IL-1 family cytokines.

Highlights

- Activated neutrophils release multiple proteases into the extracellular space
- Extracellular neutrophil proteases modulate the activity of IL-1 family cytokines
- Neutrophil proteases process cytokines 100-fold better than they kill microbes
- Neutrophils play an immunoregulatory role in addition to their role as phagocytes

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Extracellular Neutrophil Proteases Are Efficient Regulators of IL-1, IL-33, and IL-36 Cytokine Activity but Poor Effectors of Microbial Killing

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SUMMARY

Neutrophil granule proteases are thought to function as anti-microbial effectors, cooperatively hydrolyzing microorganisms within phagosomes, or upon deployment into the extracellular space. However, evidence also suggests that neutrophil proteases play an important role in the coordination and escalation of inflammatory reactions, but how this is achieved has been obscure. IL-1 family cytokines are important initiators of inflammation and are typically released via necrosis but require proteolytic processing for activation. Here, we show that proteases liberated from activated neutrophils can positively or negatively regulate the activity of six IL-1 family cytokines (IL-1α, IL-1β, IL-33, IL-36α, IL-36β, and IL-36γ) with exquisite sensitivity. In contrast, extracellular neutrophil proteases displayed very poor bactericidal activity, exhibiting 100-fold greater potency toward cytokine processing than bacterial killing. Thus, in addition to their classical role as phagocytes, neutrophils play an important immunoregulatory role through deployment of their granule proteases into the extracellular space to process multiple IL-1 family cytokines.

INTRODUCTION

Neutrophils play a critical role in microbial killing predominantly through phagocytosis and destruction of microorganisms via the contents of their specialized granules. However, the relative importance of neutrophil granule proteases, versus reactive oxygen, in the microbial killing process has been debated, with evidence suggesting that neutrophil proteases are dispensable for killing of fungal and bacterial pathogens (Pham et al., 2004; Segal, 2005; Vethanayagam et al., 2011). Activated neutrophils are also well known to liberate their granule constituents into the extracellular space when faced with non-phagocytosable particles, or large numbers of dead cells, but what purpose this serves is far from clear. It is often speculated that neutrophil degranulation is a strategy employed to kill microbes extracellularly (Faurschou and Borregaard, 2003; Lacy and Eitzen, 2008; Kolaczkowska and Kubes, 2013); however, there is surprisingly little evidence to support this view. Although neutrophils are regarded primarily as anti-microbial phagocytes, these cells are also robustly recruited to sterile wounds and rapidly achieve very high concentrations (~10^9/mL) at such sites (Chen et al., 2007; Kono and Rock, 2008; Lämmermann et al., 2013). What role neutrophils play in the context of sterile injury—where their anti-microbial effectors functions are not required and indeed can exacerbate tissue damage—is unresolved but seems more likely to imply an immunoregulatory role rather than an anti-microbial one.

In support of the idea that neutrophils play a role in the coordination of sterile immune reactions, previous studies have implicated neutrophils as key amplifiers of inflammatory reactions, even in sterile contexts (Rock and Kono, 2008; Amulic et al., 2012; Kolaczkowska and Kubes, 2013). Mice deficient in multiple neutrophil granule proteases (cathepsin G, elastase, proteinase-3) exhibit dramatically reduced inflammatory reactions and are highly protected from the development of several autoimmune conditions, as well as lipopolysaccharide (LPS)-induced endotoxic shock (Adkison et al., 2002; Hu and Pham, 2005; Pham, 2006). This suggests that neutrophil granule proteases play roles in inflammation beyond the destruction of engulfed pathogens (Belaaouaj et al., 1998; Reeves et al., 2002; Fu et al., 2017). Similarly, mice deficient in cathepsin C (DPPI), which is required to activate all neutrophil granule proteases, also display dramatic protection from collagen-induced arthritis and endotoxic shock (Adkison et al., 2002; Hu and Pham, 2005; Tkalcevic et al., 2000). This suggests that neutrophils, via the proteases contained within their specialized granules, serve as important amplifiers of inflammation, although the substrates cleaved to achieve this remain obscure.

Interleukin-1 (IL-1) family cytokines are instrumental as initiators and amplifiers of inflammation and are frequently among the first cytokines produced in response to infection or injury due to their constitutive expression in barrier tissues (Sims and Smith, 2010; Lukens et al., 2012; Afonina et al., 2015; Martin, 2016). IL-1 family members are capable of triggering complex cascades of additional cytokine production from diverse cell

types, such as resident tissue macrophages and dendritic cells, epidermal keratinocytes, as well as endothelial cells lining local blood vessels (Garlanda et al., 2013; Afonina et al., 2015). Due to the broad-spectrum nature of their effects, members of the IL-1 family therefore act as important initiators of inflammatory reactions. The IL-1 family is composed of seven bona fide pro-inflammatory cytokines including: IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ (Sims and Smith, 2010; Johnston et al., 2011). However, the majority of these cytokines are produced as inactive precursor proteins that require proteolytic processing to achieve their fully active state (Afonina et al., 2015). Although caspase-1 is well known to process and activate IL-1β and IL-18 (Thornberry et al., 1992; Gu et al., 1997), and multiple additional proteases have been implicated in the processing of other members of the extended IL-1 family (Hazuda et al., 1990, 1991; Black et al., 1988; Afonina et al., 2011; Macleod et al., 2016; Henry et al., 2016), no common route to regulating the activation state of IL-1 family cytokines has emerged to date.

IL-1 family cytokines do not possess secretory signals and increasing evidence suggests that these cytokines are predominantly released via necrosis (Rider et al., 2011; Chen et al., 2007; Lüthi et al., 2009; Cullen et al., 2015; Henry et al., 2016). Thus, IL-1 family cytokines behave as classical "danger signals," released from their host cells upon cell-death-associated membrane rupture. As noted earlier, peripheral blood neutrophils are among the first cells recruited to necrotic lesions, reaching very high densities within 30 min of wounding and frequently degranulate upon encounter with large particles, such as aggregates of dead cells (Chen et al., 2007; McDonald et al., 2010; Lämmermann et al., 2013). This suggests that neutrophils, via the proteases contained within their specialized granules, may be key regulators of IL-1 family processing and activation, helping to coordinate the early events that promote recruitment of additional immune cells to sites of cell death. Indeed, we have recently reported that IL-36α and IL-36γ are processed and activated by elastase, and IL-36α and IL-36β are activated by cathepsin G (Henry et al., 2016). These observations, coupled with sporadic reports on the activation of individual IL-1 family members, such as IL-1α and IL-33 by individual neutrophil granule proteases (Afonina et al., 2011; Lefrançais et al., 2012; Afonina et al., 2015), suggest that the latter proteases may play a key role as amplifiers of inflammatory cytokine activation states through processing and activating multiple members of the extended IL-1 family. However, because previous studies have examined processing of individual IL-1 family members with purified proteases, in isolation, at relatively arbitrary concentrations, it remains unclear whether neutrophil-derived proteases act as physiological regulators of IL-1 family activation states when deployed collectively, as occurs during degranulation. In addition, the relative potency of neutrophil granule proteases as cytokine processing enzymes, as compared with their antibacterial activities, has not been addressed.

Here, we have explored the ability of the mixture of granule proteases liberated by activated human peripheral blood neutrophils, as well as purified neutrophil proteases (elastase, cathepsin G, proteinase-3), to process and activate all pro-inflammatory IL-1 family cytokines (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ). We show that physiologically relevant (low nanomolar) concentrations of the latter proteases enhanced or suppressed the activity of six members of the extended IL-1 family, the sole exception being IL-18. In sharp contrast, extracellular neutrophil proteases exhibited very poor killing activity against multiple bacterial strains (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Bacillus subtilis*), even at concentrations 100-fold greater than those required to process IL-1 family cytokines. These observations identify neutrophil-derived proteases as key regulators of IL-1 family cytokine activity states upon secretion into the extracellular space and challenge our perception of neutrophils as predominantly anti-microbial effectors. Neutrophils may play a central role in regulating inflammatory reactions, either positively or negatively, by modulating IL-1 family cytokine activity through deployment of their granule proteases in response to necrotic cells. As a consequence, neutrophil proteases may represent highly promising therapeutic targets for the treatment of inflammatory diseases.

RESULTS

Supernatants from Activated Neutrophils Contain Multiple Proteases

Sporadic reports have implicated individual neutrophil proteases in the processing of single IL-1 family members, but the impact of these proteolytic events on the bioactivity of the latter cytokines was not explored in many of these studies (Black et al., 1988; Hazuda et al., 1990; Afonina et al., 2011; Henry et al., 2016). Furthermore, most previous studies on processing of IL-1 family members utilized individual proteases in isolation, whereas, under physiological conditions of neutrophil degranulation, cytokines are far more likely to encounter multiple granule proteases simultaneously.

To explore the combinatorial effects of neutrophil granule proteases on the processing of IL-1 family members under physiological conditions, we initially prepared supernatant fractions from control (i.e., untreated) versus activated (i.e., phorbol 12-myristate 13-acetate [PMA]-treated) primary human neutrophil populations (Figure 1A), which results in the liberation of multiple proteases into the extracellular space. As expected, we detected robust cathepsin G activity (as indicated by Phe-Leu-Phe-thiobenzyl ester [FLF-sBzl] hydrolysis; Figure 1B, left panel), as well as elastase/proteinase-3 activity (as indicated by hydrolysis of Ala-Ala-Pro-Val [AAPV]-amido-4-methylcouramin [AMC]; Figure 1B, right panel), in the supernatants of PMA-activated neutrophils. Addition of small-molecule inhibitors of cathepsin G or elastase to activated neutrophil supernatants specifically inhibited Phe-Leu-Phe-thiobenzyl esterase (FLF-ase) or AAPV-ase activities, respectively (Figure 1C). Thus, supernatants from activated neutrophils contain multiple protease activities, as expected (Pham, 2008; Henry et al., 2016). Using purified cathepsin G and elastase, we quantified protease concentrations in neutrophil degranulate supernatants by comparing dilutions of degranulate supernatants to known amounts of cathepsin G and elastase (Figure S1). Using this approach, we estimated the concentration of cathepsin G and elastase/proteinase-3 in activated neutrophil degranulate supernatants (prepared at 10^7 cells/mL) to be ~100 and ~200 nM, respectively (Figure S1).

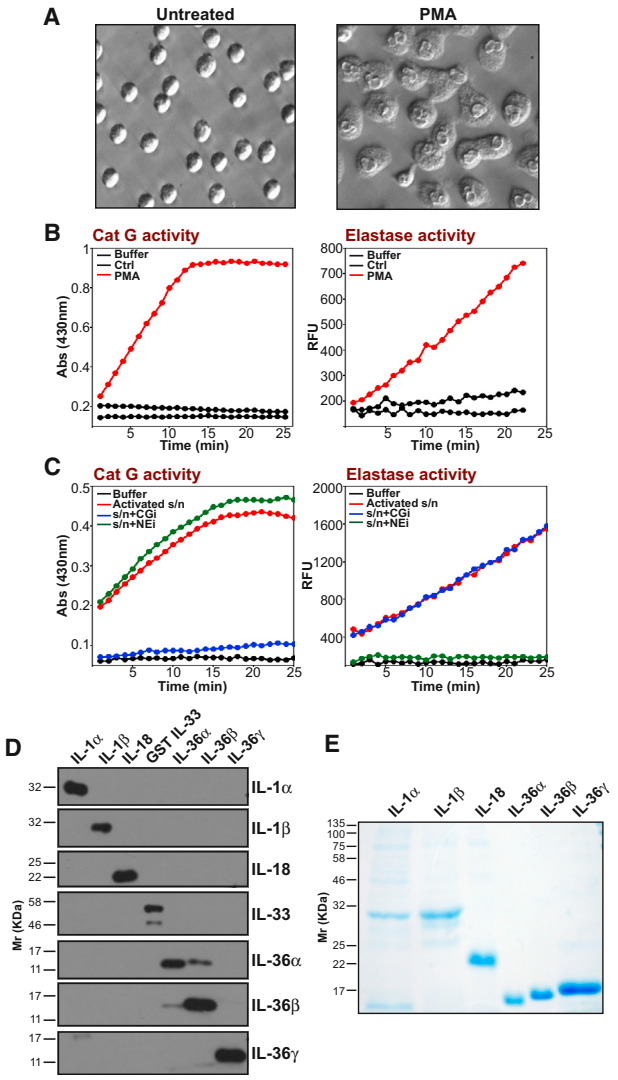


Figure 1. Activated Neutrophils Liberate Multiple Proteases upon Degranulation

(A) Primary human neutrophils isolated from peripheral blood were stimulated with PMA (100 nM) for 2 hr. Cells were visualized by phase-contrast microscopy.

(B) Primary human neutrophils (1×10^7 cells/mL) were stimulated with PMA as in (A). Cathepsin G (Cat G) and elastase enzymatic activity in neutrophil degranulate supernatants was measured by suc-FLF-sBzl and AAPV-AMC hydrolysis assay, respectively, over 25 min.

(C) Neutrophil degranulate supernatants were incubated with Cat G inhibitor I (CGi, 10 μM) or elastase inhibitor IV (NEi, 10 μM) for 30 min. After 30 min, Cat G and elastase enzymatic activity in the supernatants was measured by suc-FLF-sBzl and AAPV-AMC hydrolysis assay over 25 min.

(D) Recombinant IL-1 family cytokines (10 ng) were electrophoresed on SDS-PAGE gels. IL-1 family proteins were immunoblotted as indicated using specific antibodies.

(E) Recombinant IL-1 family cytokines (5 μg) were electrophoresed on SDS-PAGE gel and visualized with Coomassie blue staining.

Because the purity and bioactivity of IL-1 family cytokines from commercial sources is highly variable, we expressed and purified all members of the extended IL-1 family in *E. coli.* As Figures 1D and 1E show, all cytokines were successfully expressed and were found to be >95% pure. In subsequent experiments, all cytokines were used at equimolar amounts.

Neutrophil-Derived Proteases Process and Activate IL-1α but Not IL-1β

We have previously shown that purified elastase can process and enhance the basal activity of IL-1α ~10-fold through cleaving this cytokine after Ala^{101} (Afonina et al., 2011; Figure 2A); however, it is not known whether the other major neutrophil granule proteases, cathepsin G and proteinase-3, can also process and activate IL-1α. As shown in Figure 2B, supernatants from activated human neutrophils robustly processed and activated IL-1α, over a wide range of dilutions, leading to a dramatic increase in the activity of this cytokine. Importantly, neutrophil degranulate on its own had no effect on cytokine production by HeLa cells in the absence of co-incubated IL-1α (Figure 2B). Furthermore, incubation of IL-1α with purified elastase, cathepsin G, or proteinase-3 also resulted in activation of this cytokine by all three proteases at low nanomolar concentrations (Figures 2D and 2F). Thus, all three neutrophil granule proteases process and activate IL-1α, either alone, or in combination under physiological conditions.

Although processing and activation of IL-1β by caspase-1 has been intensively studied, previous reports have also suggested that this cytokine is processed by elastase, but whether this processing leads to IL-1β activation remains unclear (Hazuda et al., 1990; Guma et al., 2009; Figure 2A). In sharp contrast to our observations with IL-1α, incubation of purified full-length IL-1β with supernatants from activated neutrophils failed to activate the latter, despite evident processing (Figure 2C). By comparison, incubation of IL-1β with purified caspase-1 resulted in robust activation of this cytokine, as expected (Figure 2C). We also incubated pro-IL-1β with purified elastase, cathepsin G, or proteinase-3 at a range of concentrations and once again failed to see activation of this cytokine, despite evident processing by several of these proteases (Figures 2E and 2G). Thus, although neutrophil-derived proteases all process IL-1α and IL-1β under physiological conditions of neutrophil degranulation, this only leads to activation of the former but not the latter.

Active IL-1β Is Inactivated by Elastase and Cathepsin G

Because neutrophil proteases processed but did not activate pro-IL-1β, we considered the possibility that these proteases might inactivate mature p17 IL-1β, produced through processing via caspase-1. To explore this possibility, we generated recombinant truncated p17 IL-1β (Figures 3A and 3B), which was constitutively active (Figure 3C), as expected. As Figure 3D demonstrates, incubation of mature p17 IL-1β with supernatants from activated neutrophils led to a dramatic inactivation of this cytokine. Similarly, this effect was also seen through co-incubation of mature p17 IL-1β with nanomolar concentrations of purified elastase or cathepsin G (Figure 3E). Thus, although neutrophil proteases can process both pro- as well as mature IL-1β, this resulted in inactivation of this cytokine under conditions where IL-1α was robustly activated.

Neutrophil Proteases Activate IL-33 but Not IL-18

Similar to IL-1β, IL-18 is also processed and activated by caspase-1 (Gu et al., 1997; Figure 4A). However, an isolated report has also

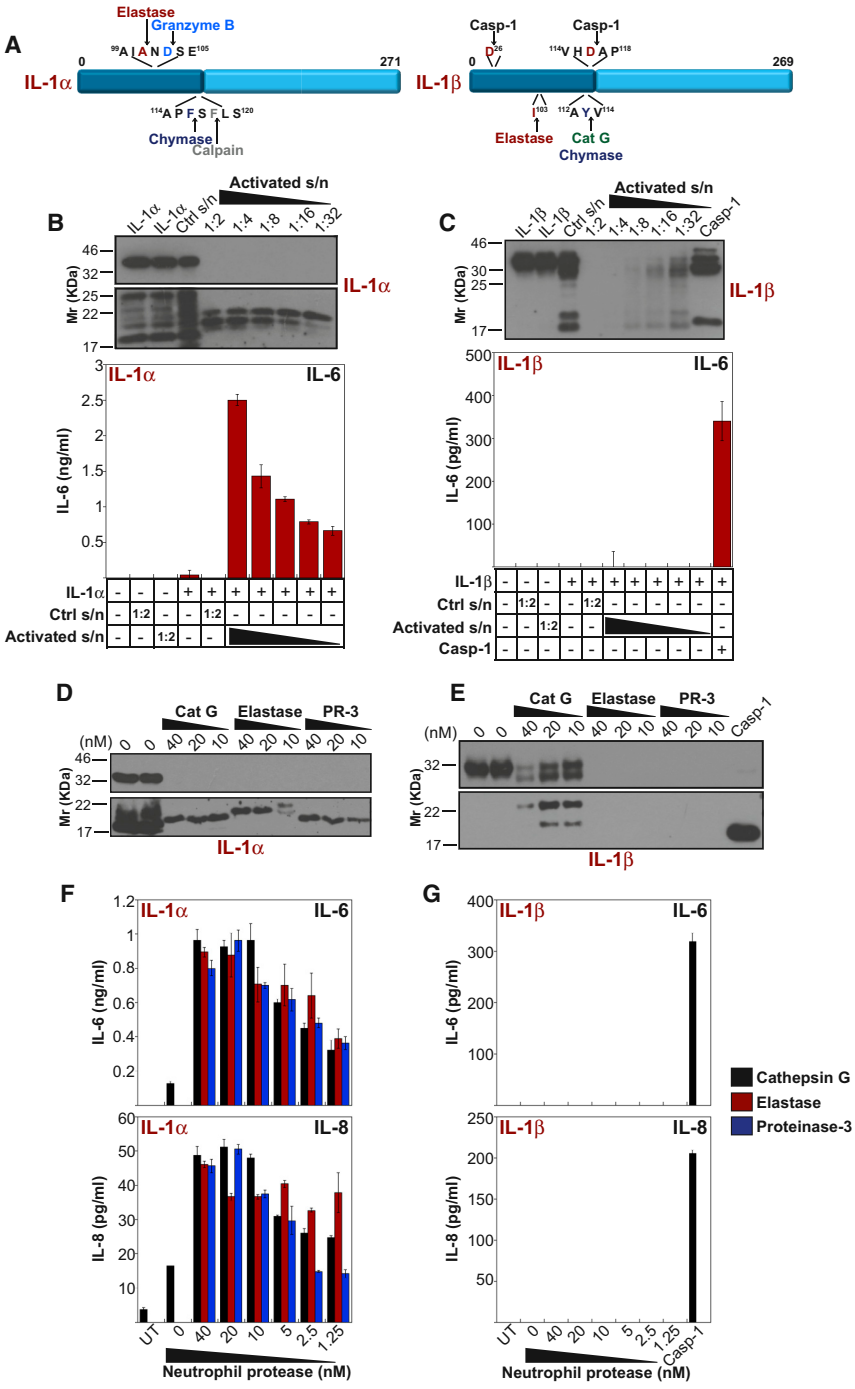


Figure 2. Neutrophil Granule Proteases Process and Activate IL-1α but Fail to Activate IL-1β

(A) Schematic of IL-1α and IL-1β indicating processing sites by neutrophil proteases and other activating enzymes such as granzyme B and caspase-1.

(B) Recombinant IL-1α (50 nM) was incubated for 2 hr at 37°C with 1:2 serial dilutions of either control (Ctrl) or PMA-activated neutrophil degranulate. Processing of IL-1α by neutrophil degranulate was analyzed by immunoblot. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-1α (1 nM). After 18 hr, IL-6 concentration in the culture supernatant was measured by ELISA.

(C) Recombinant IL-1β (50 nM) was incubated for 2 hr at 37°C with control or PMA-activated degranulate. Recombinant caspase-1 was used as a positive control for IL-1β processing and activation. Processing of IL-1β by neutrophil degranulate was analyzed by immunoblot. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-1β (1 nM). After 18 hr, IL-6 concentration in the cell-culture supernatant was measured by ELISA.

(D) IL-1α (50 nM) was incubated for 2 hr at 37°C with purified neutrophil proteases, cathepsin G (Cat G), elastase, or proteinase-3, as indicated. IL-1α processing was analyzed by immunoblot.

(E) IL-1β (500 nM) was incubated for 2 hr at 37°C with purified neutrophil proteases, as above in (D). Caspase-1 was used as positive control for IL-1β processing. Processing was analyzed by immunoblot.

(F) IL-1α (50 nM) was incubated for 2 hr at 37°C with purified Cat G, elastase, or proteinase-3, as indicated. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-1α (1 nM), as indicated. After 18 hr, IL-6 and IL-8 concentrations in the cell-culture supernatant were measured by ELISA.

(G) IL-1β (50 nM) was incubated for 2 hr at 37°C with purified Cat G, elastase or proteinase-3, or caspase-1, as indicated. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-1β (1 nM), as indicated. After 18 hr, IL-6 and IL-8 concentrations in the cell-culture supernatant were measured by ELISA. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment.

suggested that proteinase-3 may also be capable of promoting the activation of this cytokine (Sugawara et al., 2001). However, we failed to find activation of pro-IL-18 through exposure of the latter to supernatants from activated neutrophils or to individual neutrophil-derived proteases (Figures 4B, 4D, and 4F), whereas caspase-1 readily activated this cytokine under the same conditions.

We also assessed the processing and activation of pro-IL-33 by neutrophil proteases, as this cytokine is reportedly activated by elastase and cathepsin G (Figure 4A; Lefrançais et al., 2012). However, as can be seen from Figure 4C, while full-length IL-33 displayed robust activity, supernatants from activated neutrophils clearly inactivated IL-33, which was somewhat surprising given previous reports using purified elastase and cathepsin G (Lefrançais et al., 2012). However, it has been suggested that proteinase-3 can process and inactivate IL-33 (Bae et al., 2012). Thus, it was possible that proteinase-3 was opposing the activation of IL-33 by cathepsin G and/or elastase when all three proteases were present simultaneously. Consistent with this idea, we found that, whereas the basal activity of IL-33 was robustly enhanced through incubation with purified elastase or cathepsin G as previously reported (Figures 4E and 4G), incubation with proteinase-3 led to inactivation of this cytokine (Figure 4G). Thus, although elastase and cathepsin G are

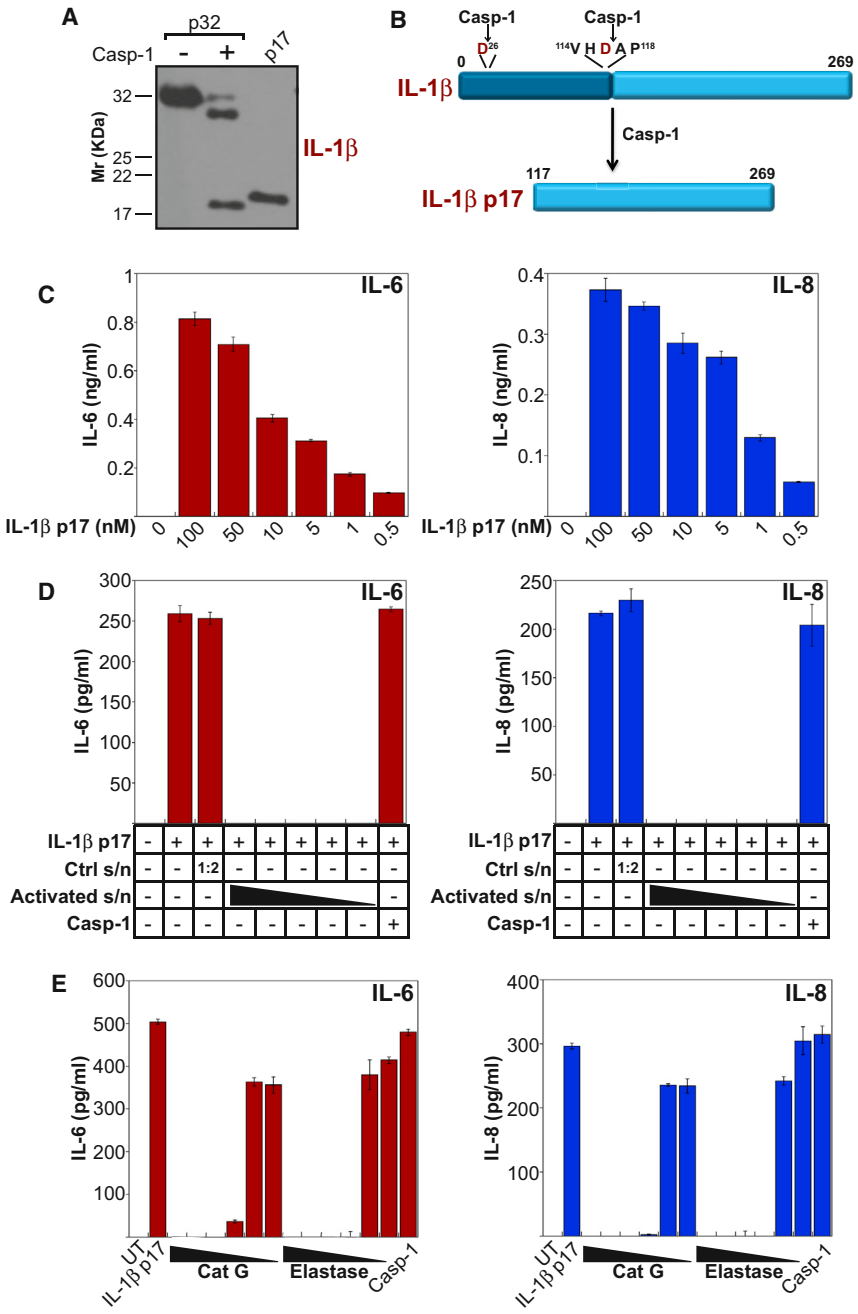


Figure 3. Neutrophil Granule Proteases Inactivate Mature p17 IL-1β

(A) Recombinant full-length IL-1β (50 nM) was incubated, or not, with caspase-1 for 2 hr at 37°C. Reactions were electrophoresed and immunoblotted, as indicated, alongside recombinant truncated IL-1β p17 (50 nM).

(B) Schematic of IL-1β processing by caspase-1 to generate the active IL-1β p17 fragment. Caspase-1 processing sites are indicated.

(C) HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were treated with a titration of recombinant IL-1β p17, as indicated. After 18 hr, IL-6 and IL-8 concentration in the cell-culture supernatants was measured by ELISA.

(D) Recombinant IL-1β p17 (50 nM) was incubated for 2 hr at 37°C with control (Ctrl) or PMA-activated neutrophil degranulate. Recombinant caspase-1 was used as a control. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-1β p17 (1 nM), as indicated. After 18 hr, IL-6 and IL-8 concentration in cell-culture supernatants was measured by ELISA.

(E) Recombinant IL-1β p17 (500 nM) was incubated with a titration of cathepsin G or elastase (50–0.25 nM, serial dilutions) for 2 hr, 37°C. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-1β p17 (5 nM), as indicated. After 18 hr, IL-6 and IL-8 concentration in cell-culture supernatants was measured by ELISA. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment.

able to enhance the activity of IL-33 in the absence of proteinase-3, the net effect of exposure of IL-33 to all three proteases collectively (as is the situation in activated neutrophil degranulates) is to suppress rather than enhance the activity of this cytokine.

Proteinase-3 Opposes Cat G- or Elastase-Mediated Enhancement of IL-33 Activity

Because proteinase-3 inactivated full-length IL-33, we also explored whether this protease could suppress the enhanced activity of IL-33 seen after prior exposure to cathepsin G or elastase. As shown in Figures S2A and S2B, this was indeed the case, with proteinase-3 eliminating the enhanced activity of IL-33 seen upon exposure to cathepsin G or elastase when these proteases were present at equimolar concentrations. In contrast, none of the purified proteases inactivated IL-18 that was previously processed and activated by caspase-1 (Figure S2C). These data explain how neutrophil granule proteases inactivate IL-33 when all three proteases are present collectively (Figure 4C) but also argue that IL-18 is not subject to regulation (either positively or negatively) by the latter proteases.

All Members of the IL-36 Cytokine Family Are Processed and Activated by Neutrophil-Derived Proteases

We have recently reported that neutrophil-derived proteases differentially process and activate all three IL-36 cytokines (Henry et al., 2016; Figure 5A). In agreement with this, incubation of all three IL-36 cytokines with supernatants from activated neutrophil degranulates resulted in robust activation of the latter (Figures 5B–5D). As Figure 5E shows, IL-36α was activated by elastase and cathepsin G, whereas IL-36β was preferentially activated by cathepsin G (and to a lesser extent by proteinase-3) and IL-36γ by either elastase or proteinase-3. Thus, all three IL-36 sub-family cytokines are processed and activated by neutrophil-derived proteases, either alone or under conditions of neutrophil degranulation where all three proteases are present simultaneously. Thus, neutrophil

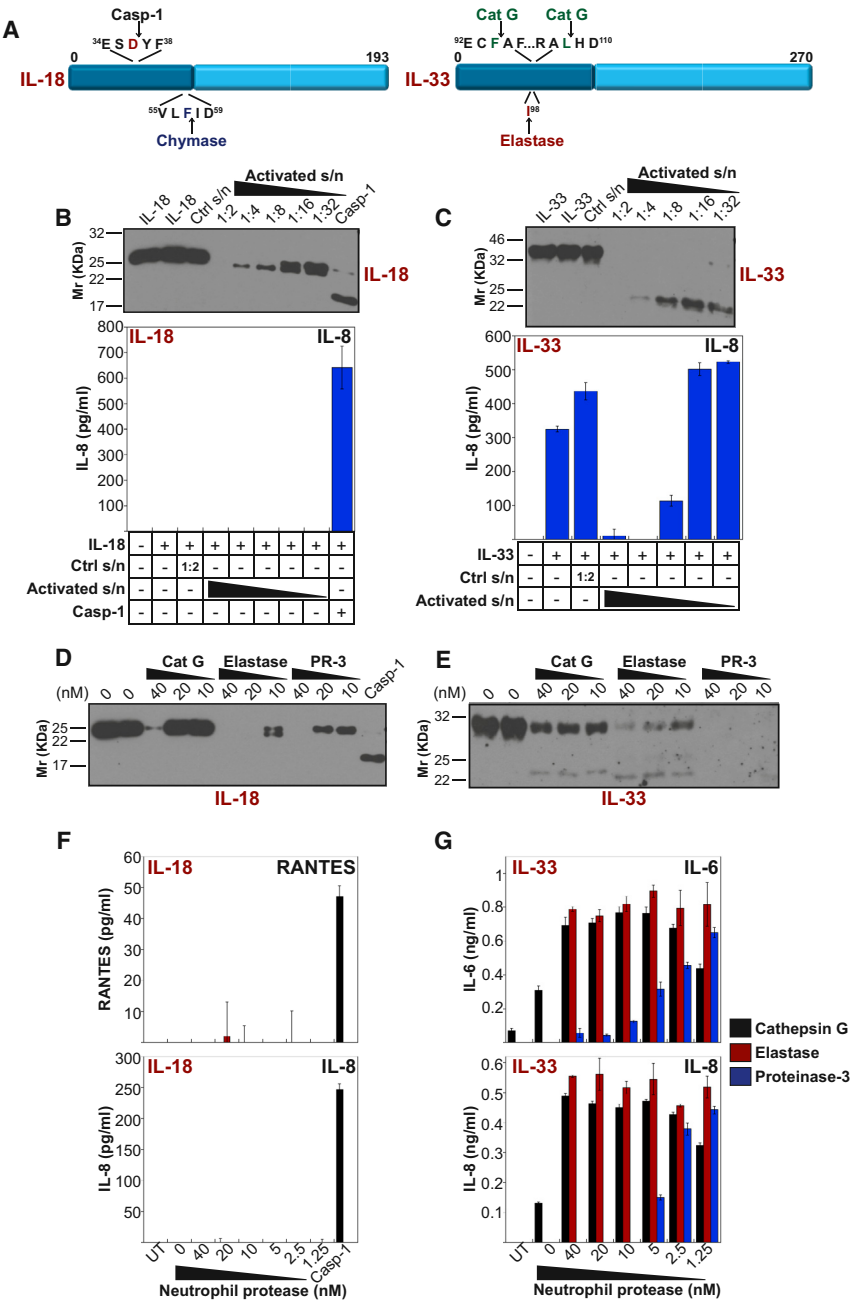


Figure 4. IL-33 Is Activated by Purified Elastase or Cathepsin G but Inactivated in Response to Neutrophil Degranulate Supernatant

(A) Schematic of IL-18 and IL-33 proteins indicating processing sites by neutrophil proteases. Caspase-1 processing site in IL-18 is also highlighted.

(B) Recombinant IL-18 (50 nM) was incubated for 2 hr at 37°C with serial dilutions of control or PMA-activated neutrophil degranulate. Caspase-1 was used as a positive control for IL-18 processing and activation. IL-18 processing was analyzed by immunoblot. KG-1 cells were stimulated with IL-18 (1 nM), as indicated. After 24 hr, IL-8 concentration in cell-culture supernatants was measured by ELISA.

(C) *In vitro*-transcribed/translated full-length IL-33 was incubated for 2 hr at 37°C with serial dilutions of control or PMA-activated neutrophil degranulate. IL-33 processing was analyzed by immunoblot. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells overexpressing IL-33R (ST2) were stimulated with IL-33 (0.031 μL), as indicated. After 24 hr, IL-8 in cell-culture supernatants was measured by ELISA.

(D) IL-18 (500 nM) was incubated for 2 hr at 37°C with purified neutrophil proteases, cathepsin G (Cat G), elastase, or proteinase-3, as indicated. Caspase-1 was used as a positive control for IL-18 cleavage. IL-18 processing was analyzed by immunoblot.

(E) *In vitro*-transcribed/translated IL-33 was incubated for 2 hr at 37°C with purified neutrophil proteases, cathepsin G (Cat G), elastase, or proteinase-3, as indicated. IL-33 processing was analyzed by immunoblot.

(F) IL-18 (50 nM) was incubated for 2 hr at 37°C with Cat G, elastase or proteinase-3, or caspase-1, as indicated. KG-1 cells were stimulated with IL-18 (1 nM), as indicated. After 24 hr, IL-8 and regulated on activation, normal T cell expressed and secreted (RANTES) concentrations in cell-culture supernatants were measured by ELISA.

(G) IL-33 was treated as in (E). HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells overexpressing IL-33R were stimulated with IL-33 (0.031 μL), as indicated. After 24 hr, IL-6 and IL-8 in cell-culture supernatants were measured by ELISA. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment.

granule proteases can modulate the activation state of six of the seven pro-inflammatory cytokines within the extended IL-1 family (Figure 5F).

Neutrophil Granule Proteases Modulate Sterile Inflammatory Responses *In Vivo*

To explore the physiological relevance of neutrophil proteases in the regulation of inflammatory responses *in vivo*, we used a mouse model of sterile inflammation induced by administration of alum into the peritoneal cavity of C56BL/6 mice. Alum-induced sterile inflammation has previously been shown to be dependent on IL-1 activity (Oleszycka et al., 2016). To explore the role of neutrophil proteases in the context of Alum-induced sterile inflammation, we administered the latter in the presence or absence of small-molecule inhibitors of Cat G, or elastase, or a combination of both inhibitors. As Figure 6A shows, injection of alum into the peritoneal cavity induced massive recruitment of neutrophils and monocytes within 24 hr, as compared with injection of PBS alone (Figure 6A). However, recruitment of immune cells to the peritoneal cavity (including monocytes, macrophages, neutrophils, eosinophils, natural killer (NK) cells, and other immune cell types) was robustly suppressed in the presence of the combination of cathepsin G and elastase inhibitors, as compared with alum alone, or either inhibitor

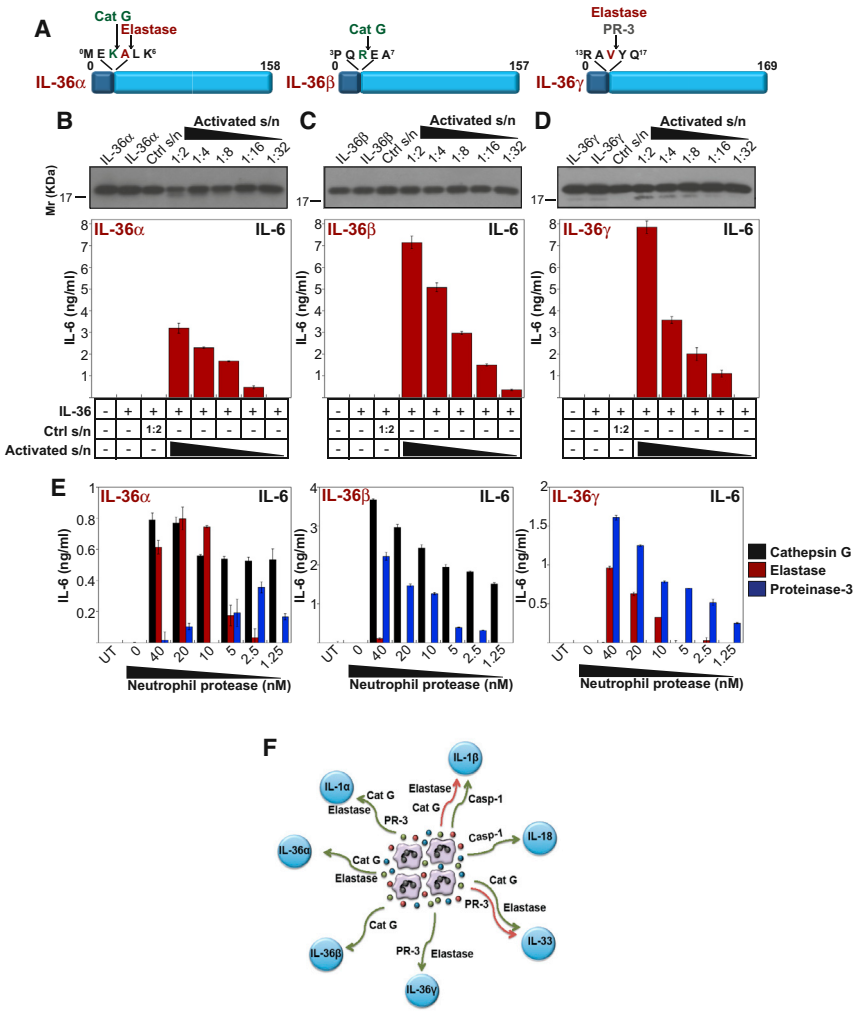


Figure 5. IL-36 Cytokines Are Activated by Neutrophil Granule Proteases

(A) Schematic of IL-36α, IL-36β, and IL-36γ proteins indicating processing sites by neutrophil proteases.

(B–D) Recombinant IL-36α (B), IL-36β (C), and IL-36γ (D) were incubated (at 50 nM) with control or PMA-activated neutrophil degranulate for 2 hr, 37°C. Processing of IL-36 cytokines by neutrophil degranulates was analyzed by immunoblot. HeLa$^{IL-36R-SEAP}$ cells were then stimulated with IL-36α, IL-36β, or IL-36γ (1 nM final concentration), as indicated. After 18 hr, IL-6 and IL-8 concentrations in cell-culture supernatants were measured by ELISA.

(E) Recombinant IL-36α, IL-36β, or IL-36γ (all at 50 nM) were incubated with cathepsin G, elastase, or proteinase-3, as indicated, for 2 hr, 37°C. HeLa$^{IL-36R-SEAP}$ cells were then stimulated with IL-36α, IL-36β, or IL-36γ (1 nM final concentration), as indicated. After 18 hr, IL-6 and IL-8 concentrations in cell-culture supernatants were measured by ELISA. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment.

(F) Schematic of modulation of IL-1 family cytokine activation states by neutrophil proteases. Green arrows denote cytokine activation, while red arrows denote inactivation.

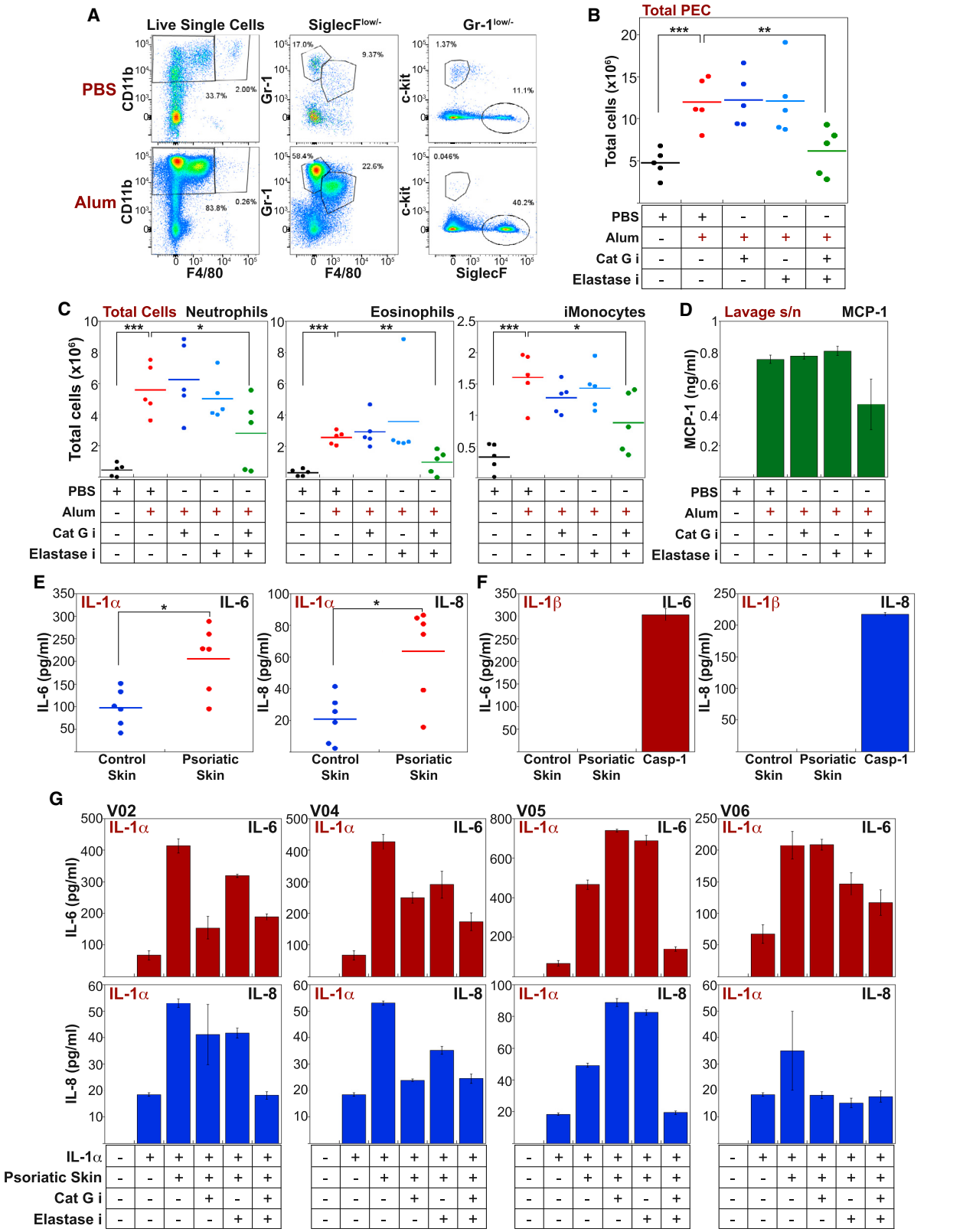
alone (Figure 6B). Looking at individual cell types: recruitment of neutrophil, inflammatory monocyte, and eosinophil numbers were all suppressed in the presence of cathepsin G and elastase inhibitors (Figure 6C). Moreover, levels of MCP-1 (one of the chemokines induced by IL-1 family cytokines) in peritoneal lavages were also suppressed by inhibition of cathepsin G and elastase activity (Figure 6D). Taken together, these results indicate that the enzymatic activities of neutrophil proteases play a key role in amplifying alum-induced sterile inflammation, consistent with our *in vitro* observations concerning their ability to process and activate multiple IL-1 family cytokines.

Endogenous Human Neutrophil Proteases Modulate IL-1 Cytokine Activity *Ex Vivo*

Neutrophil influx and elevated neutrophil protease activity is associated with a wide range of inflammatory diseases including psoriasis and inflammatory lung conditions (Amulic et al., 2012). We have previously shown that skin eluates from psoriasis patients have elevated cathepsin G activity, versus healthy control skin, and efficiently process and activate IL-36β (Henry et al., 2016).

To further explore this, we tested the ability of skin eluates from healthy volunteers or psoriatic individuals to activate exogenously added IL-1α or IL-1β. As Figure 6E illustrates, IL-1α was robustly and selectively activated by psoriatic skin eluates. Conversely, IL-1β failed to be activated by skin eluates from psoriatic patients and was only activated by caspase-1, as shown previously (Figure 6F). Furthermore, specific inhibition of cathepsin G and elastase activity suppressed IL-1α activation by psoriatic skin eluates (Figure 6G). These data indicate that psoriatic skin lesions contain sufficient levels of neutrophil protease activity to robustly process and enhance IL-1α activity.

We also obtained bronchoalveolar lavage fluids (BALFs) from patients with alpha-1-antitrypsin (α1AT) deficiency, chronic obstructive pulmonary disease (COPD), and non-cystic fibrosis bronchiectasis, as well as healthy volunteers, to investigate whether IL-1 family cytokines are processed in these disease conditions. Previous studies have shown that BALF samples from these patients contain high concentrations of proteases, including elastase, due to invasion of the lungs by neutrophils and other innate immune cells (Bergin et al., 2012). As shown in Figure S3A, BALF samples from α1AT-deficient patients robustly activated IL-36β, while IL-36γ activity was potently enhanced by lung lavages from α1AT-deficiency and bronchiectasis patients. Inhibition of neutrophil proteases, particularly elastase, in bronchiectasis BALF samples abrogated IL-36γ activation; however, activation of this cytokine by α1AT-deficient patient samples appeared to be neutrophil protease



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independent (Figure S3B). Similarly, IL-1α was robustly activated by lung lavage samples from α1AT-deficient, COPD, and bronchiectasis patients in a neutrophil protease-dependent manner (Figures S3C and S3D). Thus, neutrophil proteases capable of processing and activating IL-1 cytokines are released extracellularly in inflammatory diseases *in vivo*.

Neutrophil Granule Proteases Exhibit Poor Antibacterial Activity

Although neutrophil granule proteases are typically regarded as microbicidal agents, the relative importance of neutrophil-derived reactive oxygen species versus granule proteases in this context has been debated (Pham et al., 2004; Segal, 2005; Reeves et al., 2002; Vethanayagam et al., 2011). Previous studies have shown that purified neutrophil proteases can indeed kill bacteria, but only when used at micromolar concentrations (Belaaouaj et al., 1998, 2000; Standish and Weiser, 2009; Stapels et al., 2015). Such concentrations are unlikely to be attainable upon degranulation, where the extracellular concentrations of cathepsin G and elastase are ~100–200 nM upon activation of neutrophils at 10^7 cell/mL (Figure S1) but are much more likely to be achievable within phagolysosomes. Thus, we next explored the ability of activated neutrophil degranulates and purified neutrophil proteases to kill *E. coli*, *B. subtilis*, *S. aureus*, and *S. typhimurium* under conditions identical to those used to explore IL-1 family cytokine processing.

As Figures 7A and 7B illustrate, we found that supernatants from activated neutrophils (prepared at 10^7 cell/mL) exhibited little killing activity against three of the four bacterial species, with the exception of *B. subtilis*. Penicillin/streptomycin killed all four bacterial species very effectively, as expected. Moreover, although neutrophil degranulates exhibited cytotoxic activity against *B. subtilis*, addition of cathepsin G and elastase/proteinase-3 inhibitors to the latter supernatants failed to suppress killing of *B. subtilis* to any significant degree (Figure 7C). This suggests that other granule constituents, such as defensins, were responsible for much of the killing seen (Figure 7B). In agreement with the foregoing observations, incubation of all four bacterial species with purified cathepsin G or elastase, at concentrations up to 2 μM, also failed to achieve significant bacterial killing (Figures 7D and 7E). The exception to the latter was cathepsin G, which killed *B. subtilis* at concentrations above 50 nM. Thus, extracellular neutrophil-derived proteases exhibited surprisingly weak bactericidal activity as compared with their ability to process and activate multiple IL-1 family cytokines.

Collectively, these data argue that, upon liberation into the extracellular space, neutrophil granule proteases play a major role as modulators of the activation state of cytokines within the extended IL-1 family (Figure 5F), the only exception being IL-18 (Figure 4). In contrast, these proteases were inefficient at killing several bacterial species, either alone or in combination, under the same conditions. This suggests that degranulated neutrophil proteases may play a key role as regulators of inflammatory reactions, rather than as effectors of microbial killing.

DISCUSSION

Here, we have shown that the neutrophil granule proteases, elastase, cathepsin G, and proteinase-3, individually and collectively modulate the activity state of multiple IL-1 family cytokines, by processing and activating IL-1α, IL-33, IL-36α, IL-36β, and IL-36γ at physiologically relevant concentrations down to 1–2 nM. We also found that active IL-1β and IL-33 could be inactivated by neutrophil-derived proteases, suggesting that neutrophils might participate in the polarization of immune responses toward particular effector states through modulation of IL-1 cytokine activity status. We have also demonstrated the importance of neutrophil protease-dependent regulation of inflammation *in vivo* using alum-induced recruitment of immune cells into the peritoneal cavity in the presence or absence of neutrophil protease inhibitors. Furthermore, we have extended these findings to human inflammatory conditions by demonstrating that levels of neutrophil proteases found at sites of skin and lung inflammation are sufficient to robustly activate IL-1α and IL-36 cytokines. Contrary to the idea that neutrophil proteases are predominantly anti-microbial enzymes, we found that these proteases possess relatively poor antimicrobial properties in the extracellular space, either collectively or individually, relative to their ability to process IL-1 family cytokines. However, the key issue is likely to be the protease concentrations that are attainable in phagolysosomes, versus the extracellular space. Thus, neutrophil proteases may serve different roles intracellularly versus extracellularly, with their role as

Figure 6. Neutrophil Proteases Regulate Inflammation and Cytokine Processing *In Vivo* and in Tissue Samples from Inflammatory Patients

C56BL/6 mice (five mice per group) were injected i.p. with alum (1 mg) or alum in combination with cathepsin G inhibitor (100 μM) or elastase inhibitor (100 μM), either alone or in combination for 24 hr.

(A) Representative gating strategy for $CD11b^+$ cells, neutrophils ($CD11b^+Gr\text{-}1^+F4/80^-$), inflammatory monocytes ($CD11b^+Gr\text{-}1^+F4/80^+$), mast cells ($Gr\text{-}1^{low}c\text{-}kit^+$), and eosinophils ($Gr\text{-}1^{low}SiglecF^+$).

(B) After 24 hr, mice were sacrificed and the peritoneal cavity was washed with 5 mL PBS. Total cellularity was scored by microscopy.

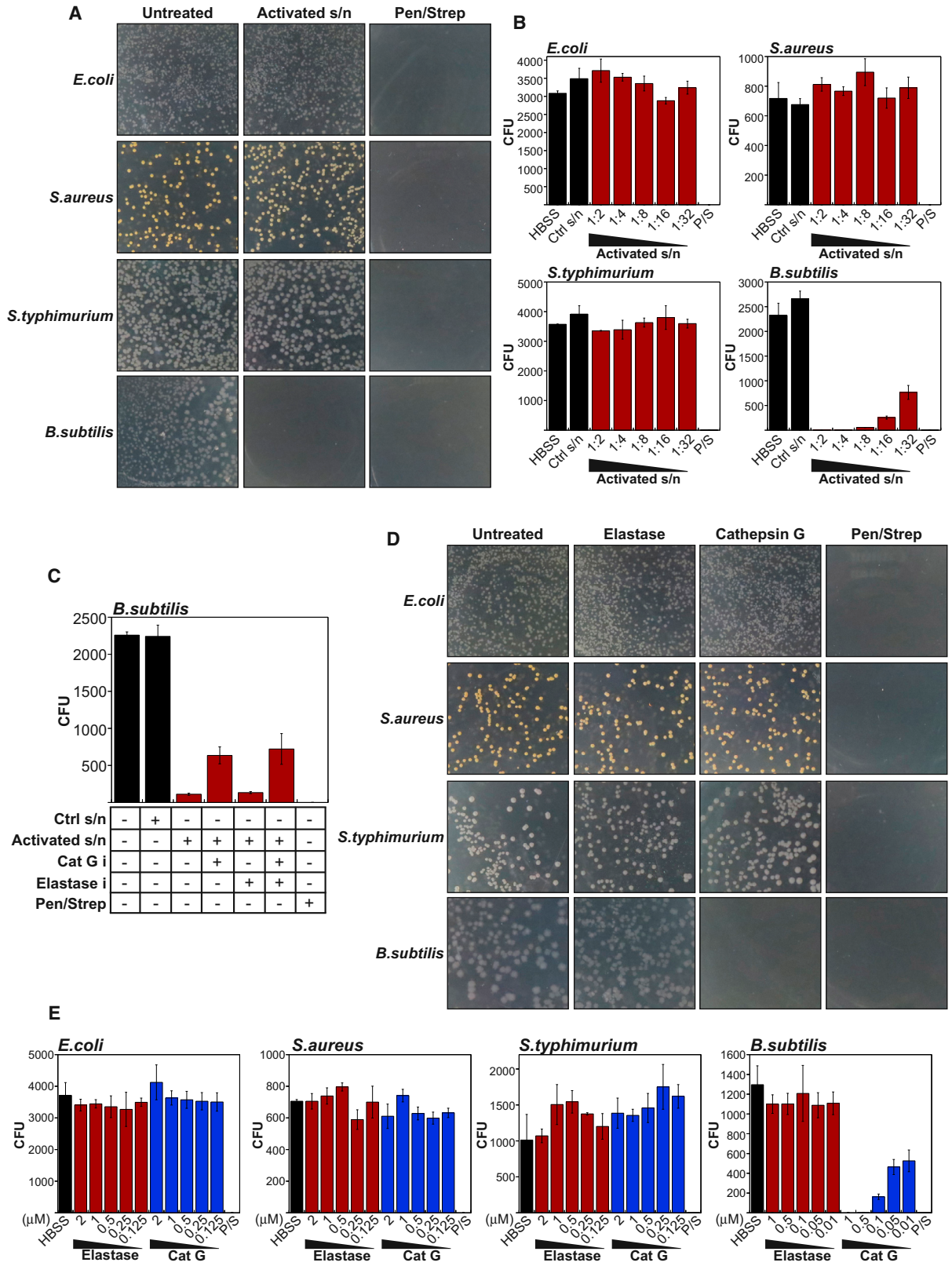
(C) Peritoneal infiltrates were immunostained and quantified by flow cytometry. Population numbers were then generated from total cellularity (B).

(D) MCP-1 concentration in peritoneal lavage supernatants was measured by ELISA.

(E) Recombinant IL-1α (50 nM) was incubated with control skin (n = 6) or psoriatic skin (n = 6) eluates for 2 hr, 37°C. $HeLa^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-1α (1 nM). After 18 hr, IL-6 and IL-8 concentrations in cell-culture supernatants were measured by ELISA.

(F) Recombinant IL-1β (50 nM) was incubated with control skin (n = 6) or psoriatic skin (n = 6) eluates for 2 hr, 37°C. Caspase-1 was used as a positive control for IL-1β activation. $HeLa^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-1β (1 nM). After 18 hr, IL-6 and IL-8 concentrations in cell-culture supernatants were measured by ELISA.

(G) IL-1α (50 nM) was incubated with psoriatic skin eluates (volunteer [V] 02, 04, 05, 06) pretreated with cathepsin G inhibitor (10 μM) or elastase inhibitor (10 μM), either alone or in combination. $HeLa^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-1α (1 nM). After 18 hr, IL-6 and IL-8 concentrations in cell-culture supernatants were measured by ELISA. Error bars represent the mean ± SEM. *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$ by Student's t test.



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microbicidal enzymes being more relevant intracellularly, and their cytokine-processing function predominating upon liberation into the extracellular space. This suggests that degranulation of neutrophils to liberate their arsenal of proteases may serve a predominantly immune regulatory role, rather than an antimicrobial one. Given that six of the seven members of the extended IL-1 family were activated or inactivated by neutrophil granule proteases, our observations also argue that these enzymes act as global regulators of IL-1 family activation states.

Our data also provide a likely explanation for why mice deficient in neutrophil serine proteases, or DPPI (cathepsin C), the protease responsible for activation of all three neutrophil proteases, are defective in inflammatory responses, and are protected from collagen-induced arthritis and LPS-induced shock (Tkalcevic et al., 2000; Adkison et al., 2002). Protease-deficient neutrophils also exhibit dramatically impaired recruitment to inflammatory sites, although migration toward chemotactic substances was not impaired (Tkalcevic et al., 2000; Adkison et al., 2002; Young et al., 2004). This is consistent with the idea that, upon arrival at a site of tissue damage where IL-1 family cytokines have been released into the extracellular space, neutrophil proteases are instrumental in promoting further neutrophil and macrophage recruitment to the inflammatory site through processing and activating one or more IL-1 family cytokines to promote local production of chemokines as well as dilation of local blood vessels. In the absence of these proteases, this amplification may fail to occur thereby attenuating the inflammatory response. Indeed, this appears to be the case in sterile inflammatory responses to alum. Alum injection in the peritoneum induces local tissue damage and results in IL-1α-dependent immune cell recruitment (Oleszycka et al., 2016), which was abrogated in the presence of specific inhibitors against cathepsin G and elastase. Thus, neutrophil protease activity is involved in this context in amplifying alum-induced inflammation by promoting immune cell influx to the site of damage, most likely through processing IL-1 family cytokines, particularly IL-1α.

Although the conventional view is that neutrophil proteases are key effectors of microbial killing, these enzymes exhibit surprisingly low potency in this regard, with previous studies indicating anti-bacterial killing activity only at micromolar concentrations (Shafer and Onunka, 1989; Belaaouaj et al., 1998, 2000; Standish and Weiser, 2009). While such concentrations are likely achievable within the tight confines of a phagosome, a number of observations also argue that protease-deficient neutrophils do not display a general defect in their ability to eradicate bacterial or fungal pathogens (Pham et al., 2004; Vethanayagam et al., 2011; Sørensen et al., 2014). Neutrophils from individuals with Papillon-Lefevre individuals, that lack DPPI/cathepsin C and are therefore devoid of neutrophil serine protease activity, are not significantly impaired in bacterial killing and only a small cohort of Papillon-Lefèvre syndrome (15%–20%) suffer from serious infections (Pham et al., 2004; Sørensen et al., 2014; Roberts et al., 2016). This suggests a level of redundancy in neutrophil antimicrobial effectors and suggests that neutrophil proteases may not be the most important granule proteins required for antibacterial activity. Similarly, mice doubly deficient in elastase and cathepsin G do not exhibit major defects in clearing common bacterial and fungal pathogens, whereas those deficient in NADPH oxidase ($p47^{Phox-/-}$) were severely impaired by comparison (Vethanayagam et al., 2011). This is at odds with the idea that reactive oxygen is not involved in direct killing of pathogens but is instead required for activation of neutrophil granule proteases within phagolysosomes (Reeves et al., 2002; Segal, 2005).

Whatever the role that neutrophil granule proteases play in the killing of engulfed microorganisms, it seems unlikely that these enzymes would be effective anti-microbial agents at the concentrations achievable upon degranulation into the extracellular space, which is in the 100 to 200 nM range at neutrophil concentrations of 10^7 per mL. Indeed, although neutrophils have long been known to undergo degranulation (Faurschou and Borregaard, 2003; Lacy and Eitzen, 2008), what purpose this serves is largely speculative. More recent studies also suggest that these cells also form neutrophil extracellular traps (NETs), which also exposes their protease content to the extracellular space (Brinkmann et al., 2004; Papayannopoulos and Zychlinsky, 2009; Kaplan and Radic, 2012). Indeed, proteomics studies suggest that proteases represent ~10% of the protein component of NETs (Urban et al., 2009). However, it is telling that much of the anti-bacterial properties of NETs have been ascribed to histones and other NET components such as DNA and calprotectin rather than to their proteases (Brinkmann et al., 2004; Urban et al., 2009; Halverson et al., 2015). Indeed, we have also recently shown that neutrophil proteases exposed on NETs can robustly activate IL-1α and IL-36 cytokines, suggesting that NETs can also serve as platforms for extracellular cytokine activation (Clancy et al., 2017).

Processing of IL-1β and IL-18 via caspase-1 has attracted considerable attention over the past 20 years (Thornberry et al., 1992; Gu et al., 1997), but other members of the IL-1 family have previously been suggested to be processed and activated by different proteases, including calpain, elastase, cathepsin G,

Figure 7. Neutrophil Serine Proteases Exhibit Poor Antimicrobial Activity in the Extracellular Space

(A) *E. coli*, *S. aureus*, *S. typhimurium*, and *B. subtilis* were incubated with control or activated neutrophil degranulate supernatant for 2 hr at 37°C. Penicillin/streptomycin (Pen/Strep) was used as a positive control in antimicrobial assays. Bacteria were diluted appropriately and plated on agar plates in triplicate. Images of representative bacterial plates were taken after overnight incubation at 37°C.

(B) Quantification of CFU counts from (A). Colony forming units (CFUs) were counted after overnight incubation at 37°C.

(C) Activated neutrophil degranulate supernatant was incubated with Cat G inhibitor I (CGi, 10 μM) or elastase inhibitor IV (NEi, 10 μM) for 30 min, followed by incubation with *B. subtilis* for 2 hr, 37°C. Bacteria were plated on agar plates in triplicate, and CFUs were counted after overnight incubation at 37°C.

(D) *E. coli*, *S. aureus*, *S. typhimurium*, and *B. subtilis* were incubated with a titration of purified neutrophil elastase or cathepsin G. Penicillin/streptomycin (Pen/Strep) was used as a positive control in antimicrobial assays. Bacteria were diluted appropriately and plated on agar plates in triplicate. Images of representative bacterial plates were recorded after overnight incubation at 37°C.

(E) Quantification of CFU counts from (A). CFUs were counted after overnight incubation at 37°C. Error bars represent the mean ± SEM of duplicate or triplicate determinations from a representative experiment.

proteinase-3, mast cell chymase, granzyme B, and granzyme A, among others (Hazuda et al., 1990; Black et al., 1988; Joosten et al., 2009; Netea et al., 2015; Afonina et al., 2015). However, many of these studies examined processing of individual IL-1 family members by individual proteases, in isolation, and frequently at non-physiological concentrations (Hazuda et al., 1990, 1991; Black et al., 1988; Joosten et al., 2009; LeFrançais et al., 2012). Because many of the latter proteases are deployed simultaneously (e.g., in the context of degranulation), their combined activities on specific protein substrates may result in effects that differ markedly from those seen when the effects of such proteases are studied in isolation. For example, although purified neutrophil elastase has been reported to process and activate IL-1α, IL-1β, and IL-33 (Hazuda et al., 1990; Lefrançais et al., 2012; Afonina et al., 2011), elastase is rarely released in the absence of the two other major neutrophil proteases, cathepsin G, and proteinase-3. Therefore, studying the effects of neutrophil proteases on specific substrates collectively, rather than in isolation, is arguably more physiologically relevant and may result in biological effects that are different from those seen with purified proteases used in isolation.

Taken together, our data suggest that a major role of activated neutrophils at inflammatory sites is to regulate and shape the inflammatory response by delivering pro-inflammatory proteases to these sites. Upon deployment through degranulation, neutrophil proteases can positively or negatively influence inflammatory reactions by modulating the activity of multiple IL-1 family cytokines released from necrotic cells. Neutrophil proteases may therefore represent promising therapeutic targets for the treatment of conditions, such as psoriasis and arthritis, characterized by extensive neutrophil influx.

EXPERIMENTAL PROCEDURES

Reagents

Polyclonal antibodies were generated against IL-36α, β, and γ proteins by repeated immunization of rabbits with the full-length recombinant IL-36 proteins (Biogenes, Germany). Anti-IL-1α (AHP281G) was obtained from AbD Serotec (UK). Anti-IL-1β (MAB201) antibody was from R&D Systems (UK). Anti-IL-18 (ab68435) antibody was obtained from Abcam. Anti-IL-33 (Nessy-1) antibody was obtained from Enzo Life Sciences (UK). Suc(oMe)-AAPV-AMC was purchased from Peptanova (Germany); suc-FLF-sBzl was purchased from Bachem (Germany). Chemical inhibitors CatG inhibitor I (219372) and Elastase Inhibitor IV (324759) were purchased from Calbiochem (UK). Purified neutrophil-derived cathepsin G was purchased from Calbiochem (UK). Purified neutrophil-derived elastase was purchased from Serva (Germany). Purified neutrophil-derived proteinase-3 was purchased from Enzo (UK). Alhydrogel (alum) was purchased from Brenntag Biosector (Frederikssund, Denmark). Unless otherwise indicated, all other reagents were purchased from Sigma (Ireland).

Cell Culture

HeLa$^{IL\text{-}36R}$ cell lines were generated by transfection with pCXN2.IL-1Rrp2 (IL-36R) plasmids followed by selection using G-418 antibiotic (Sigma), as described previously (Henry et al., 2016). HeLa$^{IL\text{-}36R\text{-}SEAP}$ cell line was generated by transfection with pNifty2-secreted alkaline phosphatase (SEAP) plasmid (InvivoGen) followed by selection using zeocin antibiotic. Clones were expanded from single cells using limiting dilution cloning. Clones were selected by demonstration of acquired optimal responsiveness to active forms of IL-36 via SEAP production and ELISA. KG-1 cells were cultured in RPMI media (GIBCO), supplemented with 10% fetal calf serum (FCS), 10% sodium pyruvate, and 10% non-essential amino acids. All cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Animal Studies

C56BL/6 mice were purchased from Charles River Laboratories. Mice were injected intraperitoneally (i.p.) with 1 mg of Alhydrogel. After 24 hr, peritoneal exudate cells (PECs) were collected by washing the peritoneal cavity with 5 mL of ice-cold PBS. Cells were pelleted by centrifugation (400 × *g*, 5 min, 4°C), supernatants were collected for cytokine analysis, and PECs were analyzed for cellular infiltration as described previously (Oleszycka et al., 2016). Animal experiments were carried out in accordance with the regulations of the European Union and Irish Department of Health, and all procedures performed were conducted under animal license number B100/3321 and were approved by the Trinity College Dublin Animal Research Ethics Committee (Ethical Approval Number 091210).

Expression and Purification of Recombinant IL-1 Family Proteins

Full-length IL-1α was expressed and purified as described previously (Afonina et al., 2011). Full-length IL-1β, IL-18, IL-36α, β, and γ proteins were generated by cloning the human coding sequence in frame with the poly-histidine tag sequence in the bacterial expression vector pET45b and purified as described in Supplemental Experimental Procedures.

Purification of Primary Neutrophils and Preparation of Degranulates

Primary human neutrophils were purified from donor blood using the plasma-Percoll gradient method as described previously (Henry et al., 2016). To prepare degranulates, neutrophils (10^7 cells per treatment) were stimulated in the presence or absence of 100 nM PMA in Hank's balanced salt solution (HBSS)/0.25% BSA for 2 hr at 37°C in a humidified atmosphere with 5% CO_2. Supernatants were harvested and clarified by centrifugation and stored at −80°C.

Protease Cleavage Assays

Reactions (40–100 μL final volume) were carried out in protease reaction buffer (50 mM HEPES [pH 7.4], 75 mM NaCl, 0.1% CHAPS) for 2 hr at 37°C. For IL-1 family cytokine bioassays, IL-1 cytokines were typically cleaved at a 50 nM concentration and subsequently diluted onto target cells at a final concentration ranging from 1 to 5 nM.

BALF Analysis

BALF samples from control individuals or patients with alpha-1-antitrypsin deficiency, COPD, or non-cystic fibrosis bronchiectasis were collected as described previously (Reeves et al., 2010). Informed patient consent was obtained, and ethical approval was granted by the Beaumont Hospital Institutional Review Board.

Psoriatic Skin Eluate Analysis

Skin eluates from control and psoriatic skin were prepared as described previously (Henry et al., 2016). Protease cleavage assays using skin eluates were carried out as outlined above.

In Vitro Antimicrobial Assays

Escherichia coli, *Staphylococcus aureus*, *Salmonella typhimurium*, or *Bacillus subtilis* was incubated with neutrophil degranulate supernatants or purified neutrophil proteases in a total volume of 20 μL in HBSS for 2 hr, at 37°C. Penicillin/streptomycin (100 U/mL penicillin/100 μg/mL streptomycin, Sigma) was used as a positive control for all assays. Bacteria were diluted appropriately and immediately spread on agar plates in triplicate. Colony-forming unit (CFU) numbers were determined after overnight incubation at 37°C.

Statistical Analysis

Statistical analysis was assessed by two-tailed paired Student's t test. Asterisk(s) indicate significance, *$p \leq 0.1$, **$p \leq 0.01$, ***$p \leq 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.02.062.

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AUTHOR CONTRIBUTIONS

D.M.C. performed experiments, analyzed data, generated the figure panels, and wrote the figure legends. G.P.S. and C.M.H. purified recombinant proteins and performed experiments. H.B.T.M. and E.C.L. performed in vivo mouse experiments. E.P.R. and N.G.M. provided human bronchoalveolar lung lavage samples. S.J.M. conceived the study, designed and analyzed experiments, supervised the study, and wrote the manuscript with contributions from D.M.C.

DECLARATION OF INTERESTS

G.P.S., C.M.H., and S.J.M. have filed US and European patents relating to inhibition of IL-36 proteolytic processing for the treatment and/or reduction of inflammation (PCT/EP2015/071446).

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