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Neutrophil-derived proteases act as global regulators of IL-1 family cytokine activation states

Thesis submitted to Trinity College Dublin
for the degree of Doctor of Philosophy

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2016

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PUBLICATIONS

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ABBREVIATIONS

AAPV	Ala-Ala-Pro-Val
AFC	7-amino-4-trifluoromethylcoumarin
AMC	7-amino-4-methylcoumarin
ANCA	Anti-neutrophil cytoplasmic antibody
ASC	Apoptosis-associated speck-like protein containing CARD
ATP	Adenosine triphosphate
BPI	Bactericidal/permeability-increasing protein
BSA	Bovine serum albumin
CAPS	Cryopyrin-associated periodic syndromes
CGD	Chronic granulomatous disease
CGi	Cathepsin G inhibitor I
COPD	Chronic obstructive pulmonary disease
CRISPR	Clustered regularly-interspaced short palindromic repeats
CRMO	Chronic recurrent multifocal osteomyelitis
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
CYLD	Cylindromatosis
DAMP	Damage-associated molecular pattern
DEVD	Asp-Glu-Val-Asp
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPI	Dipeptidyl peptidase I
DTT	Dithiothreitol
DUB	Deubiquitinase
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELANE	Elastase gene
ELISA	Enzyme-linked immunosorbent assay
ENA-78	Epithelial-derived neutrophil-activating protein 78 (CXCL5)
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FLF-sBzL	Phe-Leu-Phe-thiobenzyl ester
fMLP	N-formylmethionine-leucyl-phenylalanine

G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GPP	Generalised pustular psoriasis
GST	Glutathione S-transferase
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid
HMGB1	High mobility group box 1
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
IFN	Interferon
IKK	IκB kinase
IL	Interleukin
IL-18BP	IL-18 binding protein
IL-1R	Interleukin-1 receptor I
IL-1Ra	IL-1 receptor antagonist
IL-1RAcP	IL-1 receptor accessory protein
IL-36R	IL-36 receptor
IL-36Ra	IL-36 receptor antagonist
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRAK	Interleukin-1 receptor-associated kinase
kDa	Kilodalton
LAD	Leukocyte adhesion deficiency
LB	Luria Bertani broth
LDG	Low-density granulocytes
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
LUBAC	Linear ubiquitin chain assembly complex
MAC1	Macrophage-1 antigen
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NEi	Neutrophil elastase inhibitor IV
NEMO	NFκB essential modulator
NET	Neutrophil extracellular trap
NF-HEV	Nuclear factor in high endothelial venules
NFDM	Non-fat dry milk

NFκB	Nuclear factor kappa-light-chain enhancer of activated B cell
NGAL	Neutrophil gelatinase-associated lipocalin
Ni-NTA	Nickel-nitrilotriacetic acid
NK	Natural killer cell
NLR	Nod-like receptor
NLS	Nuclear localisation sequence
NOD	Nucleotide-binding oligomerisation domain
Oligo	Oligonucleotide
PAD4	Protein arginine deiminase 4
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PAR	Protease-activated receptor
PASI	Psoriasis area severity index
PBS	Phosphate-buffered saline
PIPES	Piperazine-N-N'-bis(2-ethanesulphonic acid)
PKC	Protein kinase C
PLS	Papillon-Lefévre syndrome
PMA	Phorbol 12-myristate 13-acetate
Poly(I:C)	Polyinosinic:polycytidylic acid
PRB	Protease reaction buffer
PRR	Pathogen recognition receptor
PSGL1	P-selectin glycoprotein ligand-1
RAGE	Receptor for advanced glycation endproducts
RFU	Relative fluorescence units
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SCN	Severe congenital neutropenia
SDF-1α	Stromal cell-derived factor 1α
SDS	Sodium dodecyl sulphate
SEAP	Secreted embryonic alkaline phosphatase
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosus
SLP1	Secretory leukocyte protease inhibitor 1
SUMO	Small ubiquitin-like modifier
TAB	TAK1 binding protein
TACE	TNFα converting enzyme
TAK1	TGFβ-activated kinase 1
TAM	Tumour-associated macrophage

TE	Tris-EDTA
TEV	Tobacco etch virus
TGF	Transforming growth factor
TIR	Toll/IL-1 receptor homology domain
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
TRAF6	TNF receptor associated factor 6
TRAIL	TNF-related apoptosis-inducing ligand
Ulp1	Ubiquitin-like-specific protease 1
UT	Untreated
VEGF	Vascular endothelial growth factor
WT	Wild-type

SUMMARY

IL-1 family cytokines are pleiotropic cytokines capable of eliciting robust pro-inflammatory cytokine and chemokine secretion from a broad variety of cell types and tissues. Recent evidence has strongly implicated IL-36 type cytokines, members of the IL-1 family, as key initiators of inflammation in the skin barrier. IL-36 sub-family cytokines belong to the extended IL-1 family and, similar to most members of this family, are expressed as inactive precursors that require proteolytic processing for activation. In chapter 3, we describe the development of a method for the production of biologically active IL-36 through introduction of a caspase cleavage motif, DEVD, within the N-termini of these cytokines. Caspase-3-processed ^{DEVD}IL-36 cytokines exhibited robust biological activity on a range of responsive cell types, including primary keratinocytes. These modified forms of IL-36 were used to elucidate molecules involved in the IL-36 receptor signalling pathway. Here, we identify that MyD88, IRAK4, TRAF6 and p65 are essential mediators of IL-36 signalling, a pathway closely related to IL-1 receptor signalling. We also identify the deubiquitinase OTULIN as a negative regulator of IL-36 signalling. By targeting and degrading linear ubiquitin chains required for optimal IL-36 signalling, OTULIN serves to limit IL-36-induced cytokine and chemokine production.

Robust neutrophil infiltration and elevated neutrophil activity is associated with a range of inflammatory diseases and autoimmune conditions. Neutrophils have long been implicated in playing an important role in the coordination and escalation of inflammatory reactions, possibly via their arsenal of granule proteases. Consistent with this, mice deficient in neutrophil granule proteases exhibit a profound defect in recruitment to inflamed sites and are protected from certain autoimmune diseases. Despite the potential importance of this role, no clear picture has emerged as to how neutrophils achieve this. Members of the extended IL-1 family (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ) are important instigators and amplifiers of inflammation, especially at barrier surfaces. However, while the majority of IL-1 family members require proteolytic processing for activation, no common route to regulating the activation state of these cytokines has emerged to date. In chapter 4, we show that proteases liberated from activated neutrophils can positively or negatively regulate the activity of six members of the IL-1 cytokine family. This suggests that neutrophils, through deploying their granule-derived proteases into the extracellular space at sites of infection and injury, serve as key regulators of the bioactivity of IL-1 family cytokines, thereby playing an important role in the coordination of inflammatory reactions.

Activated neutrophils can undergo a mode of regulated cell death that results in the extrusion of chromatin into the extracellular space, thereby acting as extracellular traps for microorganisms. Neutrophil extracellular traps (NETs) contain DNA decorated with histones as well as antimicrobial proteins and the neutrophil granule proteases, elastase, cathepsin G and proteinase-3. NET-associated factors are thought to enhance the anti-microbial properties of these structures and localisation of anti-microbial factors on NETs may serve to increase their local concentration. Because neutrophil-derived proteases have been implictated in the processing and activation of certain cytokines and chemokines, we explored whether NETs could serve as a platform to process and activate pro-inflammatory IL-36 cytokines. In chapter 5, we show that NETs potently processed and activated IL-36α, IL-36β and IL-36γ through NET-associated elastase and cathepsin G. These data suggest that NET structures can act as sites of cytokine activation.

The data presented in the course of this thesis points to an important immunomodulatory role of neutrophils in the regulation of the activity state of IL-1 family cytokines. These immune cells can promote and potentiate inflammation by modulating the activity of multiple IL-1 family members and the interactions between neutrophils and IL-1 cytokines may play an important role during infection, inflammatory diseases and cancer.

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Chapter 1

Introduction

1.1 Inflammation

1.1.1 The inflammatory response

Inflammation serves as the body's first defence against bacterial or viral infections, or tissue damage, and allows the affected tissue to return to homeostasis. The inflammatory response displays a number of key characteristics including local swelling (edema), redness (due to capillary dilation), pain and heat. The local effects of inflammation are orchestrated by a complex variety of inflammatory mediators produced by immune cells and barrier epithelia in response to an invading pathogen or tissue destruction (Medzhitov, 2008).

Upon infection or injury, tissue resident macrophages, mast cells and neutrophils from peripheral blood are key instigators in initiating inflammation (Delves et al., 2016). Macrophages and mast cells recognise and respond to microbes or tissue injury by innate immune receptors such as the Toll-like receptors (TLRs), through recognition of conserved components of pathogens called PAMPs (pathogen-associated molecular patterns) and damage-associated release of host derived molecules called 'danger signals'. Activated macrophages and mast cells secrete a plethora of inflammatory mediators including cytokines (TNF, IL-1, IL-6), chemokines (IL-8, MCP-1, CCL20), and vasoactive amines (histamine), as well as causing complement activation. These inflammatory mediators coordinate to induce vasodilation and permeability of surrounding blood vessels, thus allowing recruitment and infiltration of neutrophils to the site of infection or injury. Upon arrival at the site of infection, neutrophils are further activated and unleash a battery of toxic agents to destroy invading pathogens (Amulic et al., 2012; Medzhitov, 2008).

Once immune cells have become activated and their arsenals of toxic antimicrobial effectors have been released, it is unavoidable that some collateral damage to surrounding healthy tissue will occur. Persistent or prolonged inflammatory responses are highly detrimental to the host, with chronic inflammation associated with diseases such as cancer, cardiovascular disease and autoimmunity, among many others (Rock et al., 2010). For this reason, it is of utmost importance that inflammatory responses are tightly regulated to minimise damage to bystander host tissue (Kono and Rock, 2008; Medzhitov, 2008).

1.1.2 Cell death and inflammation

The immune system is a sophisticated defence mechanism comprising germline encoded innate immunity and the acquired adaptive immune system (Delves et al., 2016). While the crosstalk and cooperation between layers of the immune system is elaborate, its primary function is recognising foreign materials, coordinating their removal and instigating tissue repair. Thus, a major role of the immune system is identifying what is foreign (nonself) from host-derived factors (self).

The link between tissue damage and inflammation has long been observed. In her seminal paper, Polly Matzinger put forward the danger model, suggesting that the immune system responds to damage rather non-self entities alone (Matzinger, 1994, 2002). This theory proposed that, in addition to conserved components of pathogens (i.e. PAMPs), immune cells can respond to 'danger signals' or 'alarmins' released from damaged or necrotic cells which can mount an appropriate inflammatory response. Thus, the way in which a cell dies is of paramount importance to how it is sensed by the immune system (Kono and Rock, 2008; Matzinger, 1994).

Every day millions of cells are turned over in healthy adult tissues (Raff et al., 1993). In multicellular organisms, aged, damaged and excess cells are eliminated by a programmed mode of death known as apoptosis as part of a natural process to maintain tissue homeostasis (Kerr et al., 1972). Apoptosis constitutes the most common form of cell death in the body and occurs during development, tissue remodeling and as part of the immune response. During apoptosis, cells are dismantled from within in a highly regulated fashion and are quickly cleared to avoid damage and disruption to neighbouring cells (Taylor et al., 2008). Apoptotic cells are widely considered to be 'immunologically silent', which permits disposal of unwanted cells without any perturbations to the surrounding tissue environment (Martin et al., 2012).

Conversely, necrosis is the unregulated destruction of cells by injury or infection. Necrosis can occur for a number of reasons including burns, lacerations, compression injury, toxins or pathogens and involves the rapid and complete rupture of cells and the non-specific release of cellular material. Necrotic cells are thought to elicit immune responses through release of endogenous danger signals, also called damage-associated molecular patterns (DAMPs) (Chen et al., 2007; Kono and Rock, 2008; Martin, 2016).

Over the last decade, the identification of potential DAMPs has been an active area of investigation. Indeed, a number of potential danger signals have been determined

including classical pro-inflammatory cytokines (IL-1α, IL-33), uric acid crystals, genomic DNA, ATP, and the chromatin-binding protein HMGB1 (Martin, 2016). In particular, IL-1 family members represent excellent candidates for danger signals as they are highly pro-inflammatory cytokines capable of eliciting potent inflammatory responses. Furthermore, IL-1 family cytokines are highly expressed in barrier tissues, including the skin and lung epithelial cells, and are expressed as leaderless proteins that lack a signal peptide for conventional secretion (Carta et al., 2013). Indeed, the only known mode of release of these cytokines thus far is through necrotic cell death. (Cayrol and Girard, 2014; Chen et al., 2007; Luthi et al., 2009; Moussion et al., 2008; Rider et al., 2011; Cullen et al., 2015).

1.2 IL-1 family cytokines

1.2.1 Overview of the IL-1 family

Interleukin-1α and interleukin-1β (IL-1α and IL-1β), as their names suggest, were the first interleukins identified. Since their discovery, the IL-1 family has grown to 11 members, with pro-inflammatory and anti-inflammatory activity (Fig 1.1). IL-1 family cytokines share 12-54% sequence homology and all members contain a characteristic barrel-like single β trefoil structure (Jensen, 2010). Nine of the eleven family members are located on chromosome 2 (IL-18 is found on chromosome 11, while IL-33 is on chromosome 9), and probably arose from duplication of a common ancestral gene. IL-1 cytokines signal through a closely related set of receptors to activate NFκB and MAPK signalling pathways to promote inflammation. IL-1 family cytokines have been implicated in disease progression in many inflammatory disorders including gout, rheumatoid arthritis and genetic diseases such as cryopyrin-associated periodic syndromes (CAPS) (Dinarello, 2009; Lukens et al., 2012). Because of their potent pro-inflammatory effects, IL-1 family cytokines are under tight regulation by three receptor antagonists, namely IL-1 receptor antagonist (IL-1Ra), IL-36 receptor antagonist (IL-36Ra), IL-18 binding protein (IL-18BP) and the recently described IL-38, which has been claimed to act as a receptor antagonist for the IL-36 receptor (Dinarello, 2009; Sims and Smith, 2010).

1.2.2 IL-1α and IL-1β

IL-1 is the archetypal and most extensively studied cytokine of the IL-1 family. IL-1 is an apical cytokine and exerts effects on virtually all cells and organs and can mediate a vast array of biological activities including promoting fever, activation of the acute phase response, leukocyte recruitment and activation, angiogenesis and antibody synthesis (Afonina et al., 2015; Garlanda et al., 2013). The pro-inflammatory effects of IL-1 are

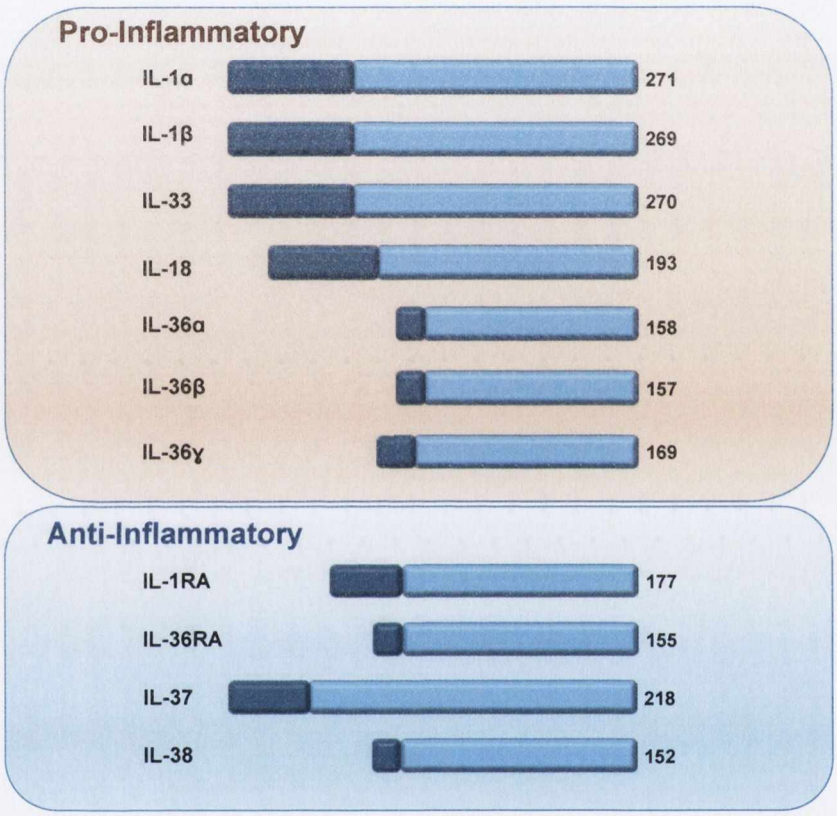

Figure 1.1 Members of the extended IL-1 family
The IL-1 family of cytokines is made up of 11 members. All IL-1 family members are grouped according to their pro- or anti-inflammatory activity.

mediated by 2 distinct proteins, IL-1α and IL-1β, which bind to the same receptor, the IL-1 receptor (IL-1R) (Auron et al., 1984; Lomedico et al., 1984; March et al., 1985).

IL-1α is constitutively produced as a 31 kDa precursor protein and is highly expressed by keratinocytes, endothelial cells, fibroblasts, and epithelial cells. IL-1α precursor exhibits biological activity and can bind to and activate IL-1R in its full-length form (Mosley et al., 1987). IL-1α lacks a signal peptide and is released during necrotic cell death where it functions as an alarmin to propagate an immune response during sterile inflammation (Chen et al., 2007; Rider et al., 2011). The pro-piece of IL-1α contains a nuclear localisation sequence (NLS) that allows IL-1α to translocate to the nucleus. Cytosolic IL-1α translocates to the nucleus during apoptosis, which may act as a mechanism to sequester the pro-inflammatory effects of IL-1 during apoptotic cell death (Cohen et al., 2010; Wessendorf et al., 1993).

In contrast, the IL-1β precursor is biologically inactive, with expression limited to hematopoietic cells such as monocytes and macrophages. IL-1β requires processing by caspase-1 to generate the mature 17 kDa form and unleash its biological activity (Black et al., 1988; Mosley et al., 1987). Similar to pro-IL-1β, caspase-1 is also expressed as an inactive precursor that requires formation of the multiprotein inflammasome complex for activation. The inflammasome complex comprises NOD-like receptor proteins (NLRs) such as NLRP3, NLRP1 or NLRC4 and the adaptor protein ASC to recruit caspase-1 and promote its autoactivation (Lamkanfi and Dixit, 2014). Activated caspase-1 processes and activates pro-IL-1β, resulting in release of the mature active cytokine (Martinon et al., 2002).

1.2.3 IL-18

Interleukin-18 was originally identified as an IFNγ-inducing factor and is now recognized as a multifaceted cytokine capable of augmenting both innate and adaptive immune responses (Nakamura et al., 1989; Ushio et al., 1996). Similar to IL-1β, IL-18 is expressed as an inactive 23 kDa precursor protein that requires cleavage by caspase-1 for full biological activity (Ghayur et al., 1997; Gu et al., 1997). Pro-IL-18 is constitutively expressed in macrophages, dendritic cells, keratinocytes, intestinal epithelial cells, synovial fibroblasts, and osteoblasts (Afonina et al., 2015; Arend et al., 2008). Its major role is the robust induction of IFNγ production, T cell proliferation and NK cell activation in response to pathogens, particularly in the clearance of intracellular pathogens. However IL-18 can also amplify the innate immune response by inducing cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), TNFα,

IL-6, and IL-8. IL-18 signals through the IL-18 receptor to initiate signalling. IL-18 activity is regulated by a high affinity binding protein IL-18 binding protein (IL-18BP) which is present in the serum of healthy humans at a 20-fold molar excess compared to IL-18 (Dinarello, 1999; Dinarello et al., 2013).

Deregulated IL-18 activity has been implicated in a number of autoimmune and inflammatory diseases such as arthritis, inflammatory bowel disease and psoriasis. Deletion of IL-18 in mice leads to obesity, insulin resistance and atherosclerosis, resembling elements of metabolic syndrome in humans (Arend et al., 2008; Dinarello et al., 2013).

1.2.4 IL-33

IL-33 was initially discovered as a nuclear factor highly expressed in high endothelial venules (thus previously annotated NF-HEV). Akin to IL-1α, IL-33 contains an NLS sequence and can translocate to the nucleus and associate with chromatin (Baekkevold et al., 2003). IL-33 is constitutively expressed in epithelial barrier cells and plays a major role in type-2 innate immunity and allergic responses by promoting production of Th2 cytokines (IL-4, IL-5 and IL-13), maturation of mast cells and eosinophil expansion (Cayrol and Girard, 2014; Luthi et al., 2009).

IL-33 was identified as the ligand for the orphan IL-1 family receptor ST2 which associates with IL-1 accessory protein (IL-1RAcP) to activate pro-inflammatory signalling. Target cells expressing the ST2 receptor include Th2 cells, mast cells, eosinophils, basophils and NK cells (Cayrol and Girard, 2014; Dinarello, 2009; Schmitz et al., 2005). Administration of IL-33 in mice confirmed its potent pro-inflammatory effects with striking histological changes in the lung and small intestine, enlargement of the spleen, eosinophil infiltration and elevated expression of Th2 associated cytokines (Schmitz et al., 2005; Luthi et al., 2009).

1.2.5 IL-36 family cytokines

IL-36α, IL-36β and IL-36γ are encoded by distinct genes and all three cytokines signal through the IL-1Rrp2/IL-1RAcP complex (Towne et al., 2011). Similar to IL-1, IL-36 cytokine signalling is regulated via a receptor antagonist (IL-36Ra), which binds IL-36R but does not transduce a signal (Towne et al., 2004). IL-36 ligands are highly expressed in skin and barrier tissue. Recently Sims and colleagues demonstrated that full-length IL-36 cytokines display little biological activity, with high amounts of recombinant unprocessed IL-36 required to elicit a response (Debets et al., 2001; Towne et al., 2004). However, artificial truncation of IL-36 cytokines at specific

residues within their N-termini greatly increased the biological activity of these cytokines (Towne et al., 2011). This suggests that, similar to IL-1β and IL-18, IL-36 cytokines are produced as leaderless proteins that require proteolytic processing for activity.

Accumulating evidence has implicated IL-36 cytokines as key inflammatory mediators in a variety of skin disorders, particularly psoriasis. Psoriasis is a common inflammatory skin disorder that affects ~2% of the population and is characterized by red, raised, scaly plaques. Keratinocyte hyperproliferation and impaired differentiation occurs, leading to skin thickening and scaling, resulting in breakages in the skin barrier. Psoriatic skin is also associated with marked infiltration of immune cells such as neutrophils and macrophages in the epidermis (Gottlieb, 2005; Lowes et al., 2007; Nestle et al., 2009).

All three IL-36 cytokines have been shown to be elevated in psoriatic lesional skin compared to non-lesional skin or healthy controls (D'Erme et al., 2015; Johnston et al., 2011). Furthermore, a recent study from our laboratory has identified neutrophil proteases, cathepsin G, elastase and proteinase-3 as potent activators of IL-36 family cytokines, resulting in dramatically enhanced activity of these proteins (Henry et al., 2016).

1.2.6 IL-1 signalling

IL-1 family members signal through closely related, heterodimeric receptor complexes to activate NFκB and MAPK pathways and promote inflammation. The IL-1 receptor (IL-1R) family is comprised of 11 members that transduce signals in response to their cognate cytokine ligands. IL-1 family receptors contain three extracellular immunoglobulin domains and a Toll/IL-1 receptor (TIR) cytoplasmic domain. The TIR domain is highly conserved and critical for signalling. This domain is also present within Toll-like receptors, highlighting the similarity between IL-1R and TLR signalling and the central role of the TIR domain in the response to both microbial invasion and endogenous pro-inflammatory signals (O'Neill and Dinarello, 2000; Martin, 2016). Activation of these receptors recruits the IL-1R accessory protein (IL-1RAcP), a co-receptor crucially required for signalling and results in the formation of four different receptor complexes: the IL-1 receptor (IL-1RI/IL-1RAcP), IL-33 receptor (ST2/IL-1RAcP), IL-36 receptor (IL-36R/IL-1RAcP) and the IL-18 receptor. The IL-18R is the only receptor complex that does not require IL-1RAcP for signalling (Garlanda et al., 2013; Thomas et al., 2012).

Activation of these receptor complexes by their corresponding ligands allows the recruitment of the adaptor protein MyD88 (Medzhitov et al., 1998; Wesche et al., 1997). MyD88 is a key component in IL-1 receptor signalling, with MyD88-deficient mice displaying defects in IL-1 signalling resulting in reduced NFκB and JNK activation and a decrease in IL-1-induced acute phase proteins (Adachi et al., 1998). MyD88, in turn, recruits IL-1 receptor-associated kinase (IRAK) 1 and IRAK4. IRAK4 phosphorylates IRAK1, allowing recruitment of the adaptor protein TRAF6 (Cao et al., 1996; Wietek and O'Neill, 2002). The TAK1/TAB complex associates with TRAF6, leading to activation of NFκB and MAP kinases and the production of pro-inflammatory cytokines and chemokines downstream (Fig. 1.2) (Sims and Smith, 2010; Weber et al., 2010).

Due to the highly pro-inflammatory and pleiotropic activity of IL-1 family cytokines, their signalling is tightly regulated to prevent uncontrolled inflammation. This regulation is mediated by receptor antagonists and decoy receptors. IL-1Ra binds to IL-1RI with similar affinity to IL-1 cytokines but prevents the recruitment of IL-1RAcP, thus inhibiting downstream signalling and subsequent cytokine induction. Interestingly, IL-1Ra is the only IL-1 family member that encodes a classical signal peptide for conventional secretion through the ER/Golgi pathway and is upregulated in response to IL-1 signalling, thereby acting as part of a negative feedback loop to quench inflammation (Sims and Smith, 2010). Another member of the IL-1R family, IL-1RII, also binds IL-1 cytokines but lacks the intracellular TIR domain and therefore acts as a decoy receptor. IL-1RII can also recruit IL-1RAcP, sequestering it at the inactive receptor complex to further prevent signalling (Garlanda et al., 2013).

IL-18 activity is also regulated by a decoy receptor, IL-18BP. IL-18BP is a soluble receptor with high affinity for IL-18, thus preventing its interaction with cell-surface IL-18R and inhibiting pro-inflammatory signalling. IL-18BP is normally found in the circulation at 20-fold higher molar excess than IL-18, however this ratio may become unbalanced during inflammation (Dinarello et al., 2013). An alternatively spliced, soluble form of ST2 regulates IL-33 activity. IL-36α, IL-36β and IL-36γ activity is regulated by IL-36Ra, which shares greater than 50% homology to IL-1Ra, and binds IL-36R to prevent activation. Similar to IL-36 ligands, IL-36Ra is inactive in its full-length form and requires removal of Met1 to unleash its antagonist activity (Debets et al., 2001; Macleod et al., 2016; Towne et al., 2011).

1.2.7 The role of ubiquitination in IL-1 receptor signalling

Ubiquitination is a post-translational protein modification involving the addition of ubiquitin moieties to a target protein to regulate a variety of cellular functions

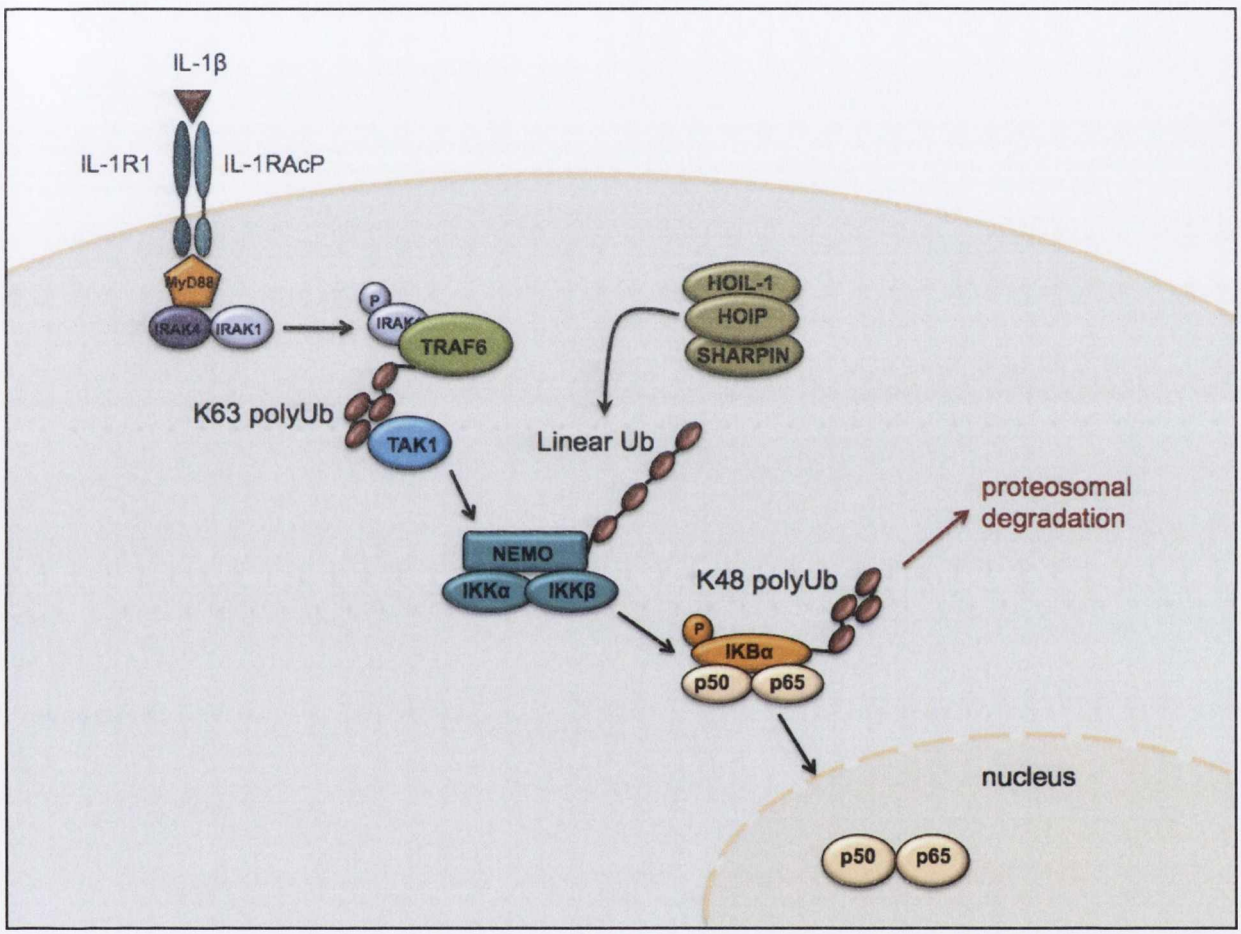

Figure 1.2 IL-1 receptor signalling
Schematic of IL-1 receptor signalling after IL-1 cytokine ligation. IL-1 signalling is tightly regulated by Lys-48 (degradative) and Lys-63 and linear chain ubiquitination (signalling), in tandem with deubiquitinase enzymes.

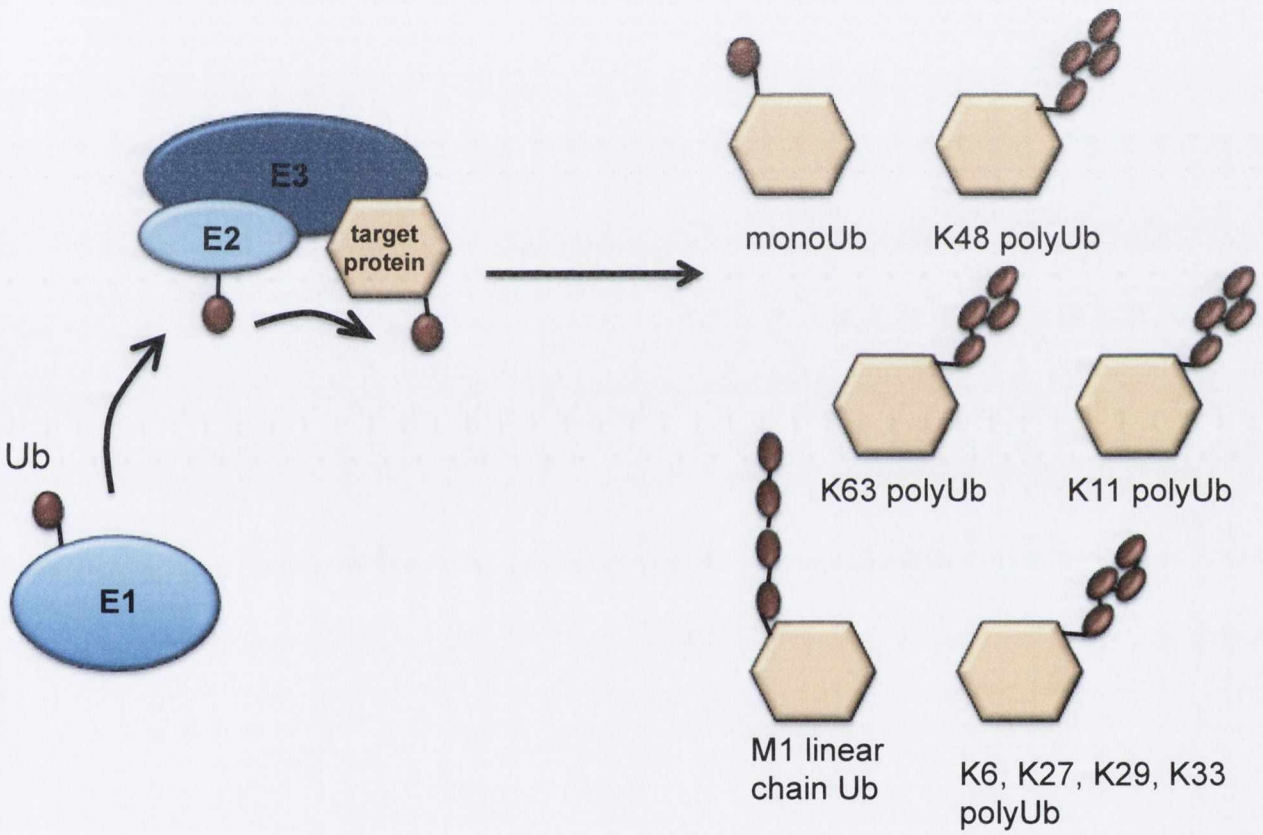

Figure 1.3 Ubiquitination and polyubiquitin linkages
Ubiquitination is a post-translational protein modification that results in the regulation of a variety of cellular functions. Ubiquitin is attached to target proteins by three enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligases). The type of ubiquitin chain determines what cellular function it will regulate, for example, K48 chains target proteins for degradation by the proteosome, while K63 and linear ubiquitin chains are involved in signalling pathways.

(Komander and Rape, 2012). Ubiquitin is a universally expressed low molecular weight protein (8.6 kDa). Ubiquitination is carried out by three distinct enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s). Firstly, ubiquitin is activated by E1s in an ATP-dependent manner and subsequently transferred to the E2 enzyme. An E3 ubiquitin ligase then mediates the conjugation of ubiquitin to the target protein. This cycle can be repeated to conjugate more and more ubiquitin moieties to form polyubiquitin chains. The type of ubiquitin linkage will determine the cellular function mediated by that particular ubiquitin chain (Fig. 1.3) (Chen, 2005; Tokunaga, 2013; Komander and Rape, 2012). For example, the polyubiquitination of Lys48 (K48) promotes proteosomal degradation of the target protein, while K63 and linear ubiquitination (M1) are associated with signal transduction (Tokunaga, 2013).

Activation of IL-1R is tightly regulated by ubiquitination, particularly Lys63-, Lys48- and linear polyubiquitin chains (Chen, 2005; Wertz and Dixit, 2010; Tarantino et al., 2014). As discussed previously, activation of the IL-1R recruits MyD88, IRAK1/4 and TRAF6 (Cohen, 2014). In concert with the E2 complex Ubc13-Uev1, the E3 ubiquitin ligase activity of TRAF6 promotes K63-linked autoubiquitination. These K63 polyubiquitin chains allow the recruitment of the TAK1/TAB complex through TAB binding directly to ubiquitin chains. K63 polyubiquitin chains also facilitate the recruitment of the LUBAC complex, which in turn promotes methionine-1 (Met1)-linked linear ubiquitin chain formation on NEMO, causing IKK activation and phosphorylation of IκB (Emmerich et al., 2013; Tokunaga et al., 2009). Phosphorylated IκB is then decorated with K48 ubiquitin linkages, promoting IκB degradation and translocation of NFκB subunits to the nucleus where they initiate transcription of pro-inflammatory cytokines and chemokines. (Fig 1.2) (Chen, 2005; Corn and Vucic, 2014; Fiil and Gyrd-Hansen, 2014; Sims and Smith, 2010).

Ubiquitination is regulated by deubiquitinase enzymes (DUBs) that oppose the activity of E3 ligases (Komander and Rape, 2012). DUBs can cleave ubiquitin chains from target proteins to prevent proteosomal degradation or signal transduction. DUBs can also act as editing enzymes by modifying the number of ubiquitin linkages or changing the type of linkage on a target protein. Important DUBs involved in the regulation of NFκB activation are A20, CYLD and the recently described OTULIN deubiquitinase. OTULIN antagonises the assembly of linear ubiquitination and regulates pro-inflammatory signalling induced by TNF and NOD receptors (Fiil et al., 2013; Keusekotten et al., 2013; Rivkin et al., 2013). Linear ubiquitination by the LUBAC complex has also been described as an important mediator of IL-1 signalling, with Met1

ubiquitin chains formed on existing K63 polyubiquitin chains. These hybrid ubiquitin chains may allow the recruitment of different kinases concomitantly, thereby allowing cross-activation and accelerating IL-1 signalling (Emmerich et al., 2013). Similarly, A20 primarily targets K63 chains to inhibit NFκB signalling in a negative feedback loop (Harhaj and Dixit, 2011; Wertz et al., 2015). CYLD can also remove both K63 and linear ubiquitin chains from TRAF6, TAK1 and NEMO to control NFκB activation (Chen, 2005; Harhaj and Dixit, 2011).

1.2.8 IL-1 family cytokines require proteolytic processing for activation

A key feature of IL-1 family cytokines is their requirement for proteolytic processing to release their biological activity. The majority of these cytokines are synthesised as inactive precursor proteins that require processing in their N-termini to achieve their fully active state (Afonina et al., 2015; Martin, 2016). IL-1β, IL-18 and the IL-36 cytokine subfamily are all expressed as inactive proforms that require proteolytic cleavage to enhance their bioactivity. Conversely, IL-1α and IL-33 are biologically active as full-length proteins, but this activity can be further enhanced upon removal of portions of their N-terminal regions (Fig 1.4) (Afonina et al., 2011; Mosley et al., 1987; Towne et al., 2011; Henry et al., 2016).

1.2.9 Caspase-1-dependent activation of IL-1 cytokines

The generation and release of biologically active IL-1β and IL-18 relies on the activity of caspase-1. Caspase-1 is also synthesized as an inactive zymogen and undergoes autoactivation as part of the inflammasome complex (Lamkanfi and Dixit, 2014; Martinon et al., 2002). The inflammasome is a cytosolic, multi-protein complex made up of caspase-1, the adaptor protein ASC and a nucleotide-binding oligomerisation domain-like receptor (NLR) which acts as a sensor for PAMPs or alarmins (Latz et al., 2013). Numerous different types of inflammasome have been described based on the NLR protein they contain and what they respond to (Lamkanfi and Dixit, 2014). The NLRP3 inflammasome has been the most widely studied of these complexes to date.

By now, it has been well established that two signals are required for inflammasome activation and subsequent IL-1β activation and release. The first signal (signal I) is mediated by PAMPs (typically LPS) and serves to transcriptionally upregulate NLRP3 and precursor IL-1β, while the second (signal II) induces processing and release of mature IL-1β. Signal II agents can be hugely diverse stimuli derived from microbes (peptidoglycan, flagellin, pore-forming toxins) or from the host itself (uric acid crystals, ATP) (Agostini et al., 2004; Mariathasan et al., 2006). The uniting feature of all these stimuli has been debated, but a decrease in intracellular K^+ is a common effect of many

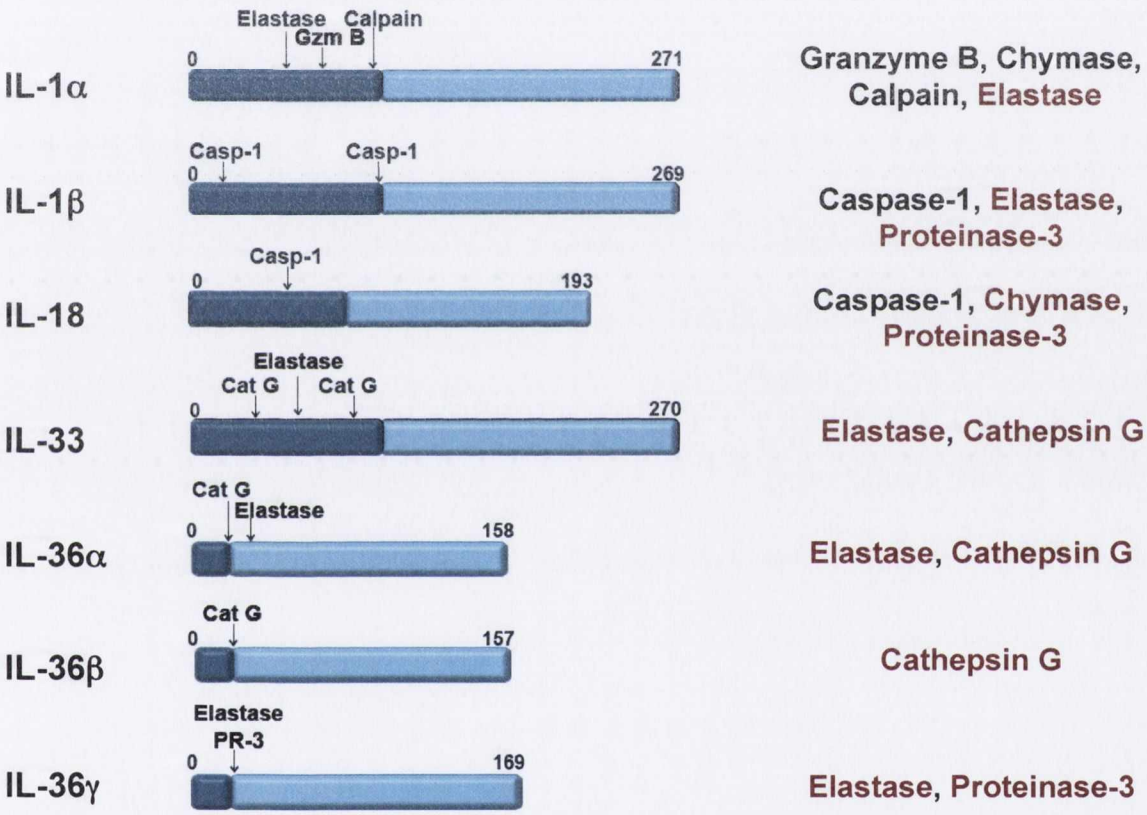

Figure 1.4 IL-1 cytokines require proteolytic processing for activation
A key feature of IL-1 family cytokines is their requirement for proteolytic processing to achieve their fully active state. The majority of these cytokines are expressed as inactive precursors that require processing to release their bioactivity. Some of the major proteases involved in the proteolytic regulation of IL-1 family members are listed here. Sporadic reports have implicated neutrophil granule proteases (highlighted in red) in the processing and activation of multiple IL-1 family cytokines and may represent a unified route to IL-1 cytokine activation.

of these agents (Munoz-Planillo et al, 2013; Cullen et al, 2015). The requirement for two signals for IL-1β activation and release adds another layer of complexity and added regulation to prevent unwanted or excessive IL-1β activity. A recent study from our laboratory identified that all inflammasome signal II stimuli that we examined could trigger necrosis and subsequently, active IL-1β release (Cullen et al, 2015). The IL-18 precursor is constitutively expressed, however activation of IL-18 by caspase-1 is under similar regulation by the inflammasome complex (Latz et al., 2013).

IL-33 is biologically active in its full-length form, however, initial reports proposed that IL-33 could also be cleaved by caspase-1 to enhance its basal activity (Schmitz et al., 2005). Subsequent studies revealed that IL-33 was not a physiological substrate for caspase-1 but instead could be processed by caspase-3 and caspase-7, leading to the inactivation of this cytokine. This may represent a mechanism to suppress IL-33 activity during apoptotic cell death to prevent an immune response and maintain homeostasis (Cayrol and Girard, 2009; Luthi et al., 2009)

While the processing and activation of IL-1β and IL-18 by caspase-1 has been widely studied, intermittent reports also suggest that this may not be the only protease involved in their maturation (Dinarello, 2009; Netea et al., 2015; Afonina et al., 2015).

1.2.10 Inflammasome-independent processing and activation of IL-1 family cytokines

Sporadic reports over the last 20 years have implicated a wide variety of proteases, predominantly neutrophil serine proteases cathepsin G, elastase and proteinase-3 in the maturation of IL-1 cytokines independent of caspase-1. Other proteases including granzyme A, mast cell chymase, chymotrypsin and matrix metalloproteinases have also been implicated in IL-1β processing (Dinarello, 2009; Irmler et al., 1995; Netea et al., 2015; Schonbeck et al., 1998).

Early studies showed that IL-1β could be processed by numerous proteases including cathepsin G and elastase, yielding cleavage fragments that were similar in size to mature caspase-1-processed p17 IL-1β (Black et al., 1988; Hazuda et al., 1989; Hazuda et al., 1990; Hazuda et al., 1991). Similarly, early studies also showed that IL-1α could be cleaved by cathepsin G (Hazuda et al., 1991). However, these studies examined individual IL-1 cytokines in combination with single proteases, in isolation, and often under non-physiological conditions. While these studies suggest that neutrophil proteases can cleave IL-1β, the functional outcomes of these processing events were not investigated. Therefore, it remains unclear whether these reported

processing events activate IL-1β. Elastase and proteinase-3 have also been shown to enhance IL-1β secretion from THP-1 cells and neutrophils, however whether or not the IL-1β released was biologically active was not examined (Coeshott et al., 1999). An isolated report has also suggested that IL-18 can be activated in a caspase-1-independent manner by neutrophil proteinase-3 (Sugawara et al., 2001).

Inflammasome-independent activation of IL-1β has also been reported in a number of *in vivo* models. Fantuzzi and colleagues provided some of the first evidence that IL-1β may be activated independently of caspase-1 during turpentine-induced inflammation (Fantuzzi et al., 1997). Subcutaneous injection of turpentine induces an IL-1β-dependent sterile inflammatory response and local tissue damage resulting in fever, loss of body weight and the induction of cytokines and acute phase proteins. IL-1β-deficient mice are protected against turpentine-induced inflammation, however, surprisingly, caspase-1-deficient mice responded similarly to wild-type mice, developing a robust inflammatory response. This suggests that IL-1β is processed and activated by alternative proteases in caspase-1-deficient mice, however the authors do not directly investigate the activity of IL-1β from caspase-$1^{-/-}$ mice or identify the proteases responsible for its activation in this context (Fantuzzi et al., 1997). In addition, in an IL-1-dependent mouse model of arthritis, casp-$1^{-/-}$ mice responded similarly to their wild-type counterparts and displayed similar disease progression. This effect was suggested to be mediated by neutrophil proteases, as a neutrophil elastase inhibitor ameliorated disease symptoms and lowered IL-1β levels (Guma et al., 2009; Joosten et al., 2009). More recently, the role of IL-1β in osteomyelitis was also studied. Mice with mutations in PSTPIP2 develop bone inflammation similar to symptoms seen in human chronic recurrent multifocal osteomyelitis (CRMO). This phenotype was found to be IL-1R and IL-1β-dependent but caspase-1 and NLRP3-independent, again, suggesting inflammasome-independent activation of IL-1β (Cassel et al., 2014; Lukens et al., 2014).

Furthermore, isolated reports have emerged linking neutrophil proteases to the processing and activation of other members of the IL-1 family. IL-1α exhibits biological activity as a full length protein, however this activity can be significantly enhanced upon proteolysis by calpain, granzyme B and neutrophil elastase (Afonina et al., 2011). IL-33 is also active as a full length precursor protein but can be further activated by neutrophil elastase and cathepsin-G to potentiate its pro-inflammatory effects (Lefrancais et al., 2012). Conversely, IL-33 activity has been shown to be inactivated by proteinase-3, as well as caspase-3 and caspase-7 (Bae et al., 2012; Luthi et al., 2009). IL-36 cytokines

have been heavily implicated in the pathogenesis of psoriasis, however the proteases responsible for their activation were unidentified, until recently. As previously mentioned, our laboratory have recently discovered that neutrophil-derived proteases are key activators of IL-36 family cytokines and robustly process all three of the IL-36 cytokine subfamily and potently enhance their biological activity (Henry et al., 2016). Therefore, mounting evidence points to neutrophil-derived proteases as key mediators of IL-1 family cytokine activation and may represent a unified route to processing of cytokines within this family. Inflammasome-independent processing of IL-1 family cytokines by neutrophil-derived proteases may play an important role in inflammatory diseases that are characterised by extensive neutrophil influx such as psoriasis and arthritis (Netea et al., 2015).

1.2.11 IL-1 family cytokines in inflammation and disease

As previously discussed, IL-1 family members are apical cytokines that can exert potent pro-inflammatory effects on numerous cell types and tissues throughout the body. Because of this, IL-1 family cytokines are key players in sterile inflammatory diseases and autoinflammatory disorders including rheumatoid arthritis, psoriasis, gout, neurodegenerative disorders and atherosclerosis, as well as systemic autoinflammatory diseases such as CAPS.

IL-1 has been implicated in the pathophysiology of a number of neurodegenerative disorders including Alzheimer's disease, stroke and Parkinson's disease. In Alzheimer's disease, amyloid-β accumulation in the brain leads to NLRP3 inflammasome activation and IL-1β production, contributing to the elevated levels of IL-1 typically associated with this disease (Halle et al., 2008; Lukens et al., 2012). IL-33 has also been associated with Alzheimer's disease, however its role seems to be protective rather than contributing to disease progression. IL-33 levels are markedly reduced in Alzheimer's patients and a recent study has shown that IL-33 can rescue memory deficits and amyloid-β accumulation by activating microglia to enhance phagocytosis and the induction of anti-inflammatory cytokines (Fu et al., 2016; Lukens et al., 2012). Similarly, IL-1 levels are also elevated in stroke patients following neuronal cell death. IL-1 signalling is also associated with cartilage damage and the production of joint damaging agents such as matrix metalloproteinases in rheumatoid arthritis and gout (Dinarello, 2011). Aberrant inflammasome activation and increased systemic IL-1β and IL-18 activity is a hallmark of a number of autoinflammatory diseases such as CAPS, Familial Mediterranean fever and familial cold-induced autoinflammatory syndrome. The importance of controlling IL-1 activity is reflected in IL-1Ra-deficient mice. These animals develop spontaneous inflammatory responses,

damage to blood vessels and joint damage. Interestingly, these mice also display increased susceptibility and enhanced tumour development following exposure to carcinogens (Garlanda et al., 2013). Human patients with mutations resulting in non-functional IL-1Ra have also been described. Deficiency of the IL-1 receptor antagonist (DIRA) manifests in severe systemic inflammation with pustular skin lesions and bone inflammation (Aksentijevich et al., 2009; Reddy et al., 2009).

IL-33 activity has been associated with asthma and allergic reactions. IL-33 levels are elevated in asthmatic patients and administration of IL-33 to mouse lungs induces inflammation and airway hyperresponsiveness. Genome-wide association studies have linked IL-33 and ST2 genes to asthma, suggesting they may be associated with a susceptibility to this disorder (Liew et al., 2010; Oboki et al., 2010). As previously described, IL-36 cytokines are highly expressed in the skin and have been linked to psoriasis. Mice transgenic for IL-36α in skin keratinocytes exhibit features of a psoriasis-like inflammatory skin condition that can be further exacerbated by the skin irritant, phorbol ester or by combined deletion of IL-36Ra (Blumberg et al., 2010; Blumberg et al., 2007). Similarly, application of the TLR7 agonist Imiquimod to mouse skin results in psoriatic plaque formation that severely worsens when IL-36Ra is deleted. Conversely, IL-36R deficient mice are completely protected against imiquimod-induced psoriasis (Tortola et al., 2012). IL-36 proteins are highly expressed in skin keratinocytes and can be further upregulated in response to IL-17 and TNF, key mediators in psoriasis (Carrier et al., 2011; Johnston et al., 2011)

Successful treatment of inflammatory diseases with receptor antagonists or neutralizing antibodies to IL-1 family members underlines the importance of the role of these cytokines in these diseases. IL-1 blocking treatments include anakinra, a recombinant form of IL-1Ra; canakinumab, a monoclonal antibody specific for IL-1β and rilanocept, a soluble decoy receptor also known as IL-1Trap. Anakinra has been widely successful in the treatment of rheumatoid arthritis, while canakinumab and rilanocept are used to treat CAPS and other related genetic inflammatory disorders (Afonina et al., 2015; Dinarello, 2011). Despite the success of anakinra, there are problems associated with this treatment, notably the short half-life of this recombinant protein and the requirement for daily administration. As detailed already, IL-1 cytokine activation can be mediated by both inflammasome-dependent and –independent mechanisms, therefore treatments should be developed based on this. The discovery of MCC950, a new NLRP3 inflammasome specific inhibitor, is a promising potential therapy for a range of autoinflammatory diseases. MCC950 blocked caspase-1 activation and suppressed IL-1β activation leading to a protective effect in mouse models of CAPS and multiple

sclerosis (Coll et al., 2015). While MCC950 and other similar inflammasome inhibitors could be an effective treatment for disorders exacerbated by caspase-1-dependent activation of IL-1 cytokines, there may be little effect in the treatment of diseases characterized by massive neutrophil inflammation and subsequent inflammasome-independent processing of IL-1 cytokines.

IL-36Ra also plays an important role in limiting inflammation, particularly in the skin. Indeed, generalised pustular psoriasis (GPP), the most severe and often life-threatening form of the disease is associated with loss-of-function mutations in IL-36Ra (Farooq et al., 2013; Marrakchi et al., 2011; Onoufriadis et al., 2011). IL-36 cytokines released from damaged cells during development or stochastic tissue damage may contribute to the progression of psoriasis. Antagonistic IL-36Ra activity is required to block aberrant IL-36 receptor signalling during this normal wear and tear of the skin, however. loss of IL-36Ra activity results in unopposed IL-36 cytokine activity, NFκB and MAPK activation and pro-inflammatory cytokine release, resulting in GPP (Lowes et al., 2014). Thus, antagonism of IL-36 signalling may represent an attractive therapeutic target for psoriasis treatment. Given the apical nature of IL-36 signalling in the inflammatory cascade and the relatively limited expression of IL-36 cytokines to skin and barrier tissues, significant therapeutic benefits may be associated with targeting this cytokine family, without unwanted systemic effects associated with treatments targeting TNFα or IL-17.

1.3 Neutrophils

1.3.1 The life of a neutrophil

Phagocytosis by macrophages and microphages was first discovered by Elie Metchnikoff during pioneering experiments involving challenging starfish larvae with rose thorns to stimulate immune responses (Kaufmann, 2008; Delves et al., 2016). We now know microphages as neutrophils, widely regarded as the first responders of the immune system, responding to damage or infection within minutes. Neutrophils constitute the most abundant leukocytes, accounting for 50-70% of circulating cells. Neutrophils are continuously generated in bone marrow from myeloid precursors, before being released into the blood stream (Delves et al., 2016).

The process of neutrophil development in the bone marrow from haematopoietic stem cells is known as granulopoiesis. In this process, bone marrow resident stem cells differentiate to myeloblasts, proliferating cells that are committed to becoming granulocytes. These myeloblasts further mature to neutrophils and are readily

mobilised upon infection or injury (Amulic et al., 2012; Kruger et al., 2015). Neutrophil maturation and release are tightly regulated to prevent aberrant neutrophil activity. G-CSF is an important mediator involved in neutrophil development and regulates neutrophil proliferation, survival and development. Accordingly, mice deficient in G-CSF or its receptor have dramatic, albeit not complete, neutropenia. Neutrophil release into the blood stream is controlled by CXCL2 and CXCR4. Neutrophils released from the bone marrow can either enter the blood stream or enter neutrophil pools found in the spleen, liver and lungs. While the exact role of these neutrophil pools remains unclear, they may act as reservoirs of mature neutrophils to be rapidly mobilised in response to infection or damage (Kolaczkowska and Kubes, 2013; Kruger et al., 2015).

Neutrophils are short lived cells, typically surviving only 6-8 hours in circulation, however their longevity increases upon activation. Neutrophils can be defined by two distinct characteristics: the shape of their nucleus and their specific granules (Amulic et al., 2012; Kolaczkowska and Kubes, 2013). The nucleus displays a lobular appearance, with separated lobes joined by chromatin, giving rise to their alternative name: polymorphonuclear leukocytes. Neutrophil granules are formed during maturation from myeloblasts and involve the formation of vesicles from the Golgi apparatus to form granular structures. Neutrophil granules contain a plethora of proteases, antimicrobial peptides and other degradative enzymes effective at killing invading pathogens (Amulic et al., 2012; Borregaard et al., 2007; Faurschou and Borregaard, 2003).

During infection or injury, neutrophils migrate from the bloodstream towards the site of infection along chemotactic gradients. As they move through peripheral tissues towards the site of infection, neutrophils become progressively activated and undergo a striking transition that enables these cells to exert powerful anti-microbial effects. Activated neutrophils are highly phagocytic and can also release a panoply of soluble factors into the extracellular space such as reactive oxygen species (ROS) and proteases contained within their specialised granules (Amulic et al., 2012; Kolaczkowska and Kubes, 2013; Pham, 2006).

1.3.2 Neutrophil recruitment and activation

Neutrophil recruitment from the blood stream occurs rapidly and is immediately initiated after infection or injury. The neutrophil recruitment cascade comprises a number of consecutive steps including: tethering, rolling, adhesion, crawling and finally transmigration out of the blood vessel (Kolaczkowska and Kubes, 2013). At sites of infection or host damage, pro-inflammatory signals accumulate and stimulate

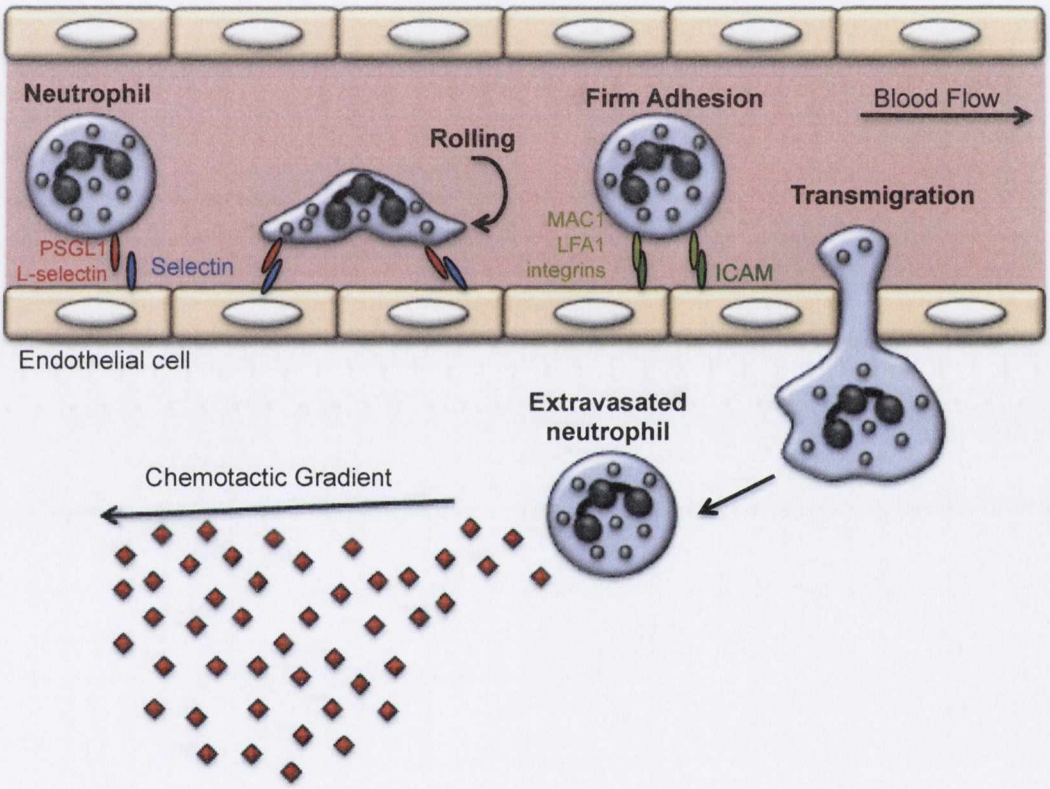

Figure 1.5 Neutrophil recruitment from blood flow
Schematic of neutrophil recruitment and activation. Neutrophils constitutively express PSGL1 and L-selectin to bind to activated endothelial cells. Neutrophils roll along vessel walls before undergoing firm adhesion and transendothelial migration. Extravasated neutrophils move towards the site of injury or infection along chemotactic gradients.

endothelial cells near the injured site. These signals include bacterial-derived PAMPs (for example, LPS or fMLP) or host-derived factors (TNFα, IL-1, histamine). Activated endothelial cells upregulate a variety of adhesion molecules including P-selectin and E-selectin. Neutrophils continuously scope and probe blood vessels and can recognise and bind to selectin proteins. Neutrophils constitutively express the glycoprotein P-selectin glycoprotein ligand 1 (PSGL1) and L-selectin, both of which are crucial for recognising activated endothelial cells. Endothelial selectins tether neutrophils to the vessel wall and initiate neutrophil 'rolling' along a chemotactic gradient. Chemokines decorating the endothelium or pro-inflammatory cytokines present in the inflamed tissue prime neutrophils to undergo full activation and maximal degranulation and NADPH oxidase activation upon arrival at the damaged site. After rolling, integrin-mediated 'firm adhesion' occurs, whereby LFA-1 and MAC-1 integrins expressed by neutrophils bind to ICAM1 and ICAM2 on endothelial cells. Neutrophil movement stops and cells undergo transendothelial migration. Migration can occur paracellularly (between cells) or transcellularly (through cells) (Fig 1.5) (Amulic et al., 2012; Kolaczkowska and Kubes, 2013; Mocsai, 2013)

Once out of the bloodstream, the neutrophil must now navigate to the site of infection, through the collagen-rich basement membrane. This process may be mediated by granule-derived proteases, or by preferential migration through less-dense regions of the basement membrane (Kolaczkowska and Kubes, 2013). Neutrophils follow chemotactic gradients formed by signals released from the site of infection including IL-8, fMLP and complement factor C5a. Upon reaching the peak of the chemotactic gradient, neutrophils have arrived at their destination and can proceed to fully activate and release their arsenal of antimicrobial weapons. Neutrophils coordinate pathogen removal through phagocytosis, release of granule contents and neutrophil extracellular trap formation (Amulic et al., 2012; Segal, 2005). Activated neutrophils also secrete pro-inflammatory chemokines and cytokines (such as IL-1, IL-8, MCP-1, TNFα, VEGF) to trigger further monocyte and neutrophil recruitment as well as T cell infiltration and to initiate a wound healing response (Borregaard et al., 2007). Because of the potential harm to host cells, it is imperative that neutrophils are rapidly cleared after eliminating microbes and resolving infection. Activated neutrophils are cleared by apoptosis to resolve inflammation and prevent tissue damage. Apoptotic neutrophils are phagocytosed by macrophages in a process known as efferocytosis to prevent further inflammation (Kruger et al., 2015).

1.3.3 Neutrophil microbial killing

Neutrophils are potent phagocytes that engulf and kill invading pathogens quickly and effectively to limit the spread of an infection. Neutrophils exert their antimicrobial effects through both oxidative and non-oxidative mechanisms (Fig 1.6) (Segal, 2005). Phagocytosis is a major mechanism employed by innate immune cells to eliminate microbes. Phagocytosis occurs within minutes of encountering a pathogen and is mediated by opsonic receptors such as Fcγ receptors, C-type lectins and complement receptors (Amulic et al., 2012). Microbes are engulfed in a phagocytic vacuole which fuses with neutrophil granules to release anti-microbial effector proteins into the phagosome. Concurrent with degranulation in the phagosome, the NADPH oxidase complex assembles on the phagosomal membrane to generate ROS. Thus, neutrophil oxidative and non-oxidative microbial killing mechanisms work in tandem to eliminate invading pathogens.

When phagocytosis is initiated, NADPH oxidase is activated and generates superoxide by reducing molecular oxygen. Superoxide rapidly dismutates to hydrogen peroxide which is targeted by myeloperoxidase (MPO) to generate highly reactive species. ROS can be further converted to hypochlorous acid and chloramines and contribute to microbial killing (Amulic et al., 2012; Segal, 2005). Indeed, loss-of-function mutations in NADPH oxidase is associated with chronic granulomatous disease (CGD), characterised by increased susceptibility to infection due to decreased microbial killing by CGD neutrophils (Bianchi et al., 2011; Kruger et al., 2015).

Non-oxidative microbial killing is mediated by proteases, antimicrobial peptides and other toxic molecules released when neutrophil granules fuse to the phagolysosome (containing the engulfed pathogen). There are four types of neutrophil granules: azurophilic (primary), specific (secondary), gelatinase (tertiary) and secretory granules (Fig 1.7). Neutrophil granules are formed consecutively during maturation and can be classified by their contents, as well as their propensity for exocytosis after neutrophil activation (Korkmaz et al., 2010). Secretory granules are the first granules released after neutrophil activation and contain a variety of receptors required for proper neutrophil extravasation and chemotaxis. Tertiary granules contain high levels of matrix metalloproteinase 9 (also known as gelatinase B), while specific granules contain a variety of antimicrobial molecules including lysozyme, defensins and lactoferrin. Azurophilic granules are equipped with the major ammunition of the neutrophil and contain MPO, defensins, lysozyme, bactericidal/permeability-increasing protein (BPI), as well as the serine proteases cathepsin-G, elastase and proteinase-3

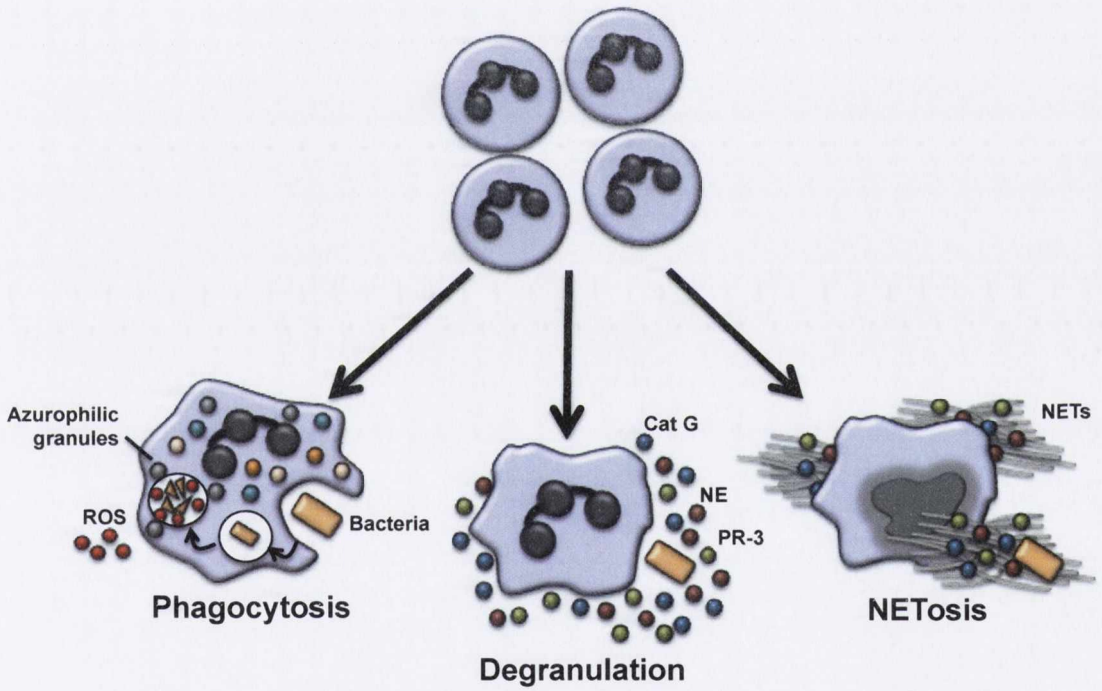

Figure 1.6 Neutrophil anti-microbial mechanisms
Schematic of neutrophil anti-microbial strategies. Neutrophils can kill microbes through oxidative and non-oxidative mechanisms including phagocytosis, release of neutrophil proteases and antimicrobial peptides during degranulation and through the release of neutrophil extracellular traps.

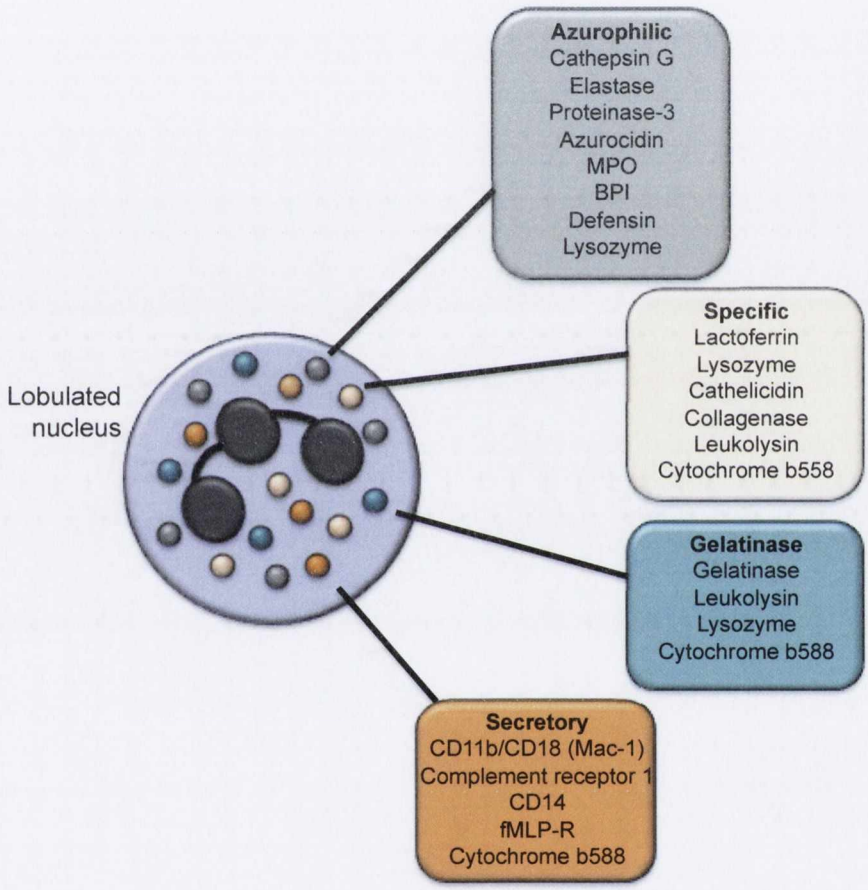

Figure 1.7 Neutrophil granule components
Schematic of neutrophil granule constituents. Neutrophils contain four types of granules: azurophilic, specific, gelatinase and secretory granules. Neutrophil granules are classified by their contents and the timing of their synthesis during neutrophil differentiation, as well as their propensity for exocytosis.

and the pseudoprotease azurocidin (Borregaard et al., 2007; Faurschou and Borregaard, 2003).

Neutrophils contain three major types of antimicrobial effectors: cationic proteins and peptides, enzymes, and proteins that disrupt microbial homeostasis by interfering with their nutrient uptake. The full range of these deadly effector molecules are stored in neutrophil granules, primarily in the azurophilic and specific granules (Amulic et al., 2012; Gabay et al., 1989). Neutrophil cationic peptides include defensins and cathelicidins. Neutrophils mainly express α-defensins that target microbial membranes and display antimicrobial activity against a wide range of bacteria and fungi (Faurschou and Borregaard, 2003). hCAP-18 is the only cathelicidin expressed in humans and represents a major constituent of specific granules. Proteinase-3 cleaves the full-length hCAP-18 protein to liberate LL-37, a potent antimicrobial cleavage fragment, effective against a broad range of gram-positive and gram-negative bacteria (Sorensen et al., 2001; Turner et al., 1998). Neutrophils also express antimicrobial cationic proteins, one of which, bactericidal/permeability-increasing protein (BPI) is highly effective in targeting gram-negative bacteria. BPI binds with high affinity to LPS to neutralise it and increases microbial membrane permeability (Gazzano-Santoro et al., 1992). Neutrophil enzymes involved in microbial killing include the potent antimicrobial enzyme lysozyme, as well as the serine proteases cathepsin G, elastase and proteinase-3. The role of these proteases in microbial killing and inflammation will be explored in depth later. Neutrophils also express a range of proteins that contribute to microbial killing by interfering with microbial nutrient uptake. These include lactoferrin, an iron-chelating protein and calprotectin, which chelates zinc. Additionally, neutrophil gelatinase-associated lipocalin (NGAL) binds to bacterial siderophores to further deprive bacteria of iron uptake (Faurschou and Borregaard, 2003; Miethke and Skerra, 2010). These are just some examples of the broad range of antimicrobial effectors deployed by neutrophils to eliminate invading pathogens and underline the importance of these front line cells in host defence.

Indeed, the importance of neutrophil activity in infectious and non-infectious disease is reflected in patients with neutrophil defects. Severe congenital neutropenia (SCN) affects 1 in 200,000 individuals, leading to reduced neutrophil numbers due to a defect in neutrophil differentiation. These patients suffer severe bacterial and fungal infections. The majority of SCN cases are caused by mutations in the *ELANE* gene, encoding neutrophil elastase and require lifelong G-CSF treatment to promote neutrophil maturation. Similarly, leukocyte adhesion deficiencies (LAD) and WHIM syndrome also lead to severe neutropenia and increased susceptibility to infection as

neutrophils cannot leave the circulation or the bone marrow to reach the site of infection or injury (Kruger et al., 2015).

1.3.4 Neutrophil serine proteases

The neutrophil serine proteases cathepsin G, elastase and proteinase-3 are stored in the azurophilic granules and play important roles in microbial killing and inflammation. Cathepsin-G, elastase and proteinase-3 are members of the chymotrypsin superfamily of serine proteases and share a high degree of homology (at the DNA and protein level) with granzymes, mast cell chymase, and tryptase (Korkmaz et al., 2010; Pham, 2006). These proteases contain a catalytic triad of histidine, aspartic acid and serine. Azurocidin, a closely related serine protease lacks the catalytic serine residue, rendering it inactive, however it does display antimicrobial activity (Campanelli et al., 1990; Pham, 2006). Neutrophil elastase, proteinase-3 and azurocidin genes are found clustered on chromosome 19 in humans, while the cathepsin G gene is located on chromosome 14 and it is thought that these proteases evolved from a common ancestor through gene duplication (Korkmaz et al., 2010).

Cathepsin G, elastase and proteinase-3 are synthesised as inactive zymogens, requiring two separate N-terminal processing steps for activity. This N-terminal cleavage is mediated by dipeptidyl peptidase I (DPPI) and is crucial for full protease activation (Adkison et al., 2002). After synthesis, an N-terminal signal peptide is removed by a signal peptidase, leaving two amino acid residues at the N-terminus. This pro-dipeptide is cleaved by DPPI to produce the active enzymes. While this processing is critical for protease activation, it also serves to target the proteases to the appropriate granules. In the absence of DPPI cleavage, proforms of these proteases are constitutively secreted or degraded. Loss-of-function mutations in DPPI are the driver of Papillon-Lefévre syndrome, a genetic disorder characterised by skin thickening and severe periodontitis (Pham, 2006).

Under normal conditions, neutrophil protease activity is counteracted by endogenous protease inhibitors. Serpins are high molecular weight (40-50 kDa) endogenous serine protease inhibitors that form complexes with neutrophil proteases to suppress their activity. Serpins act as pseudo-substrates that bind to the catalytic pocket of these proteases in a covalent complex, ultimately resulting in the inactivation of both molecules. Elastase and proteinase-3 are inhibited by α_1-antiproteinase inhibitor and elafin, while cathepsin G is inhibited by α-chymotrypsin. Elastase and cathepsin G activity is also impeded by secretory leukocyte protease inhibitor (SLP1) (Korkmaz et al., 2008). However, at inflammatory sites these proteases likely escape inhibition due

to the high local concentration of proteases. ROS generated at inflammatory sites may also inactivate the latter inhibitors to allow unimpeded extracellular protease activity (Ossanna et al., 1986; Pham, 2008).

1.3.5 Neutrophil serine proteases as antimicrobial enzymes

As previously discussed, neutrophil serine proteases found in the azurophilic granules are key antimicrobial enzymes involved in intracellular pathogen killing in the phagosome (Gabay et al., 1989; Segal, 2005). Direct microbicidal activity of elastase, cathepsin G and proteinase-3 have been demonstrated *in vitro* against a broad range of pathogens. Cathepsin G can directly kill *Staphylococcus aureus*, but surprisingly, the catalytic activity of this enzyme was not required for its antimicrobial activity (Shafer and Onunka, 1989). Purified neutrophil elastase was shown to be directly bactericidal against *Klebsiella pneumoniae* and *Escherichia coli* but not *S. aureus*, suggesting that elastase activity is required for gram-negative bacterial killing (Belaaouaj et al., 2000; Belaaouaj et al., 1998). A more recent report has also suggested that purified elastase and cathepsin G, as well as neutrophil degranulate supernatant from fMLP-activated neutrophils, can kill *Streptococcus pneumoniae* (Standish and Weiser, 2009). Neutrophil elastase also contributes to microbial killing by targeting and degrading bacterial virulence factors. Elastase cleaves OmpA on the outer membrane of *E. coli*, interfering with bacterial membrane stability and resulting in bacterial clearance (Belaaouaj et al., 2000). Elastase also targets virulence factors of *Shigella flexneri, Salmonella enterica*, and *Yersinia enterocolitica*. This study highlights the importance of elastase activity in the phagosome to promote bacterial killing as loss of elastase activity allows *Shigella* to escape the phagosome, increasing bacterial survival (Weinrauch et al., 2002).

The importance of neutrophil serine proteases in bacterial killing was confirmed in studies using mice deficient in these enzymes. Previously, it was thought that ROS was the key mediator involved in intracellular microbial killing, however, mice deficient in cathepsin G and elastase have been shown to have increased susceptibility to, and mortality following *S. aureus* infection, despite normal neutrophil maturation and function (Reeves et al., 2002). Similarly, elastase and cathepsin G deficient mice had dramatically reduced survival probability following sublethal doses of *Aspergillus fumigatus* compared to wild-type mice that were completely unaffected by challenge with this fungus (Tkalcevic et al., 2000). Elastase knockout mice are also highly susceptible to *K. pneumoniae*, displaying worse clinical symptoms and higher mortality than their wild-type counterparts (Belaaouaj et al., 1998). While these studies suggest that mice deficient in neutrophil proteases display increased sensitivity to pathogenic

insults, it remains unclear whether this is due to direct proteases action on the microbes. Alternatively, these proteases may exert indirect effects by modulating IL-1 cytokine activity which may contribute to microbial killing in these models. Although neutrophil proteases are considered important antimicrobial mediators, the sheer number and variety of bactericidal effectors stored in neutrophil granules points to a level of redundancy in this system. Indeed, individuals with Papillon-Lefévre syndrome (PLS) have only residual levels of neutrophil protease activity but do not display severe defects in microbicidal activity and only a small cohort of PLS patients suffer from serious systemic infections. This suggests that other neutrophil anti-microbial effectors can compensate for diminished protease activity to clear invading pathogens (Pham, 2006).

As well as their important intracellular role in the clearance of microbes during phagocytosis, neutrophil proteases are also released extracellularly through limited exocytosis of azurophilic granules. While the extracellular antimicrobial efficacy of these proteases is still debated, accumulating evidence suggests that these enzymes promote and potentiate inflammation. Previous studies have also demonstrated that neutrophil proteases can be expressed on the plasma membrane during neutrophil activation. Membrane-bound proteases retain their enzymatic activity and this process may serve as a mechanism to protect proteases from inactivation by extracellular serpins (Csernok et al., 1994; Owen et al., 1995).

1.3.6 Neutrophil-derived proteases as instigators of inflammation

As well as their established roles in microbial killing, increasing evidence implicates neutrophil serine proteases as pro-inflammatory mediators. Massive neutrophil infiltration is a key characteristic of many inflammatory diseases including chronic obstructive pulmonary disease (COPD), cystic fibrosis, acute lung injury, rheumatoid arthritis and psoriasis (Amulic et al., 2012; Korkmaz et al., 2010). In inflammatory diseases, neutrophils accumulate and secrete proteases, contributing to tissue destruction and inflammation. Increased neutrophil protease activity can be detected in bronchoalveolar lavage fluid in patients with COPD and cystic fibrosis, while elastase activity is found in synovial fluid of rheumatoid arthritis patients and contributes to cartilage damage (Afonina et al., 2011; Korkmaz et al., 2010; Wright et al., 2010). Accordingly, inhibition of neutrophil protease activity has beneficial effects in a range of inflammatory diseases (Pham, 2006).

The use of neutrophil protease deficient mice has confirmed the importance of these enzymes in the protection against infectious diseases. However, these studies also

demonstrated the somewhat unappreciated role of these proteases in inflammation. Mice deficient in neutrophil proteases display dramatically reduced inflammatory responses and are protected from a number of inflammatory insults. Mice lacking cathepsin G and elastase are protected against LPS-induced endotoxic shock (Tkalcevic et al., 2000). The absence of neutrophil elastase protects mice from experimentally-induced bullous pemphigoid, an autoimmune, inflammatory skin disease characterised by blisters and the presence of autoantibodies (Liu et al., 2000). Mice deficient in all three neutrophil proteases through the deletion of DPPI also display dramatic protection against experimentally-induced arthritis (Adkison et al., 2002).

Neutrophil proteases were first identified as degradative enzymes that could process and break down extracellular matrix components. Indeed, elastase was first identified as a processor of the ECM component elastin. Furthermore, all three neutrophil serine proteases cleave elastin, fibronectin, collagen and numerous other matrix and plasma proteins (Korkmaz et al., 2010; Korkmaz et al., 2008). Because of their broad and overlapping substrate range, neutrophil proteases were considered non-specific, redundant and destructive enzymes that negligently degrade and damage host tissue. While neutrophil protease activity certainly contributes to inflammation and host injury, accumulating data suggests that this process is not entirely random and that these proteases actively regulate immune responses by specifically modulating inflammatory mediators and cell signalling (Bank and Ansorge, 2001; Afonina et al., 2015).

1.3.7 Neutrophil serine proteases modulate cytokine and chemokine activity

Accumulating evidence suggests that neutrophil serine proteases can process extracellular cytokines and chemokines to modulate inflammation. Proteinase-3 can process IL-8 to generate truncated forms of this chemokine that are more potent chemoattractants towards neutrophils (Padrines et al., 1994). Similarly, cathepsin G processes ENA-78 (also known as CXCL5), a neutrophil stimulating cytokine, to enhance its elastase releasing activity and chemotactic activity (Nufer et al., 1999). Cathepsin G also processes CCL15, increasing its chemotactic activity on monocytes to promote monocyte recruitment to inflammatory sites (Richter et al., 2005). Neutrophil serine proteases also contribute to inflammation by targeting progranulin, a ubiquitously expressed protein with anti-inflammatory activity. Proteinase-3 and elastase-mediated processing of progranulin results in the loss of its anti-inflammatory activity, and instead generates a pro-inflammatory cleavage product (Kessenbrock et al., 2011; Kessenbrock et al., 2008).

Neutrophil serine proteases can also negatively regulate immune responses by inactivating pro-inflammatory mediators. All three neutrophil serine proteases process and inactivate IL-6, suppressing the pro-inflammatory effects of this cytokine (Bank et al., 1999; McGreal et al., 2010). Proteolysis of RANTES by cathepsin G removes three residues at the N-terminus and results in decreased chemotactic activity (Lim et al., 2006). Furthermore, elastase has been shown to deactivate SDF-1α, a chemotactic factor for T cells, monocytes and dendritic cells, and reduce T cell transendothelial migration *in vitro* (Rao et al., 2004). Inactivation of signalling proteins by neutrophil proteases may represent a method to dampen and resolve inflammation.

Neutrophil-derived proteases also regulate the activity of the potent pro-inflammatory cytokine, TNFα. TNFα is expressed as a membrane-bound protein that requires proteolytic processing by TNFα converting enzyme (TACE) to release the soluble active cytokine. Studies utilising TACE-deficient fibroblasts have shown that neutrophil proteases, cathepsin G and elastase, can liberate membrane-bound TNFα to increase levels of the soluble, active protein (Mezyk-Kopec et al., 2005). *In vitro* data also suggested that proteinase-3 could process TNFα, however no functional analyses were carried out (Mezyk-Kopec et al., 2005; Robache-Gallea et al., 1995). Conversely, neutrophil elastase and cathepsin G can negatively regulate TNFα activity by processing the mature cytokine to suppress its immunostimulatory and cytotoxic activity. Moreover, elastase mediates shedding of the TNF receptor from neutrophil membranes, providing additional control of TNF signalling (Bank and Ansorge, 2001; Pham, 2006; Porteu et al., 1991; Scuderi et al., 1991).

Previous studies have also linked neutrophil proteases to the regulation of growth factor signalling. Neutrophil elastase has been implicated in the activation of the epidermal growth factor receptor (EGFR), albeit indirectly. Elastase releases membrane-bound TGFα through proteolytic processing which in turn activates EGFR to promote cell proliferation. Elastase treatment promoted keratinocyte proliferation *in vitro* and epidermal hyperproliferation *in vivo*, in an EGFR and TGFα dependent manner, at concentrations of elastase similar to those found in psoriatic lesions (Meyer-Hoffert et al., 2004; Rogalski et al., 2002; Wiedow and Meyer-Hoffert, 2005). Elastase also stimulates mucous production from lung epithelial cells in an EGFR and TGFα dependent manner, possibly contributing to lung inflammation (Kohri et al., 2002). Other studies have suggested that neutrophil proteases can directly activate cell surface receptors such as integrins, protease-activated receptors (PARs) and TLRs, however opposing effects of activation and deactivation of receptors have been documented and direct binding of neutrophil proteases to these receptors have not

been shown. Previous reports have suggested that elastase can induce production of IL-8 through TLR4 activation, however the levels of IL-8 induction were marginal and again, binding of elastase to TLR4 was not shown (Devaney et al., 2003; Korkmaz et al., 2010; Pham, 2006). It is more likely that cytokine production induced by neutrophil proteases occurs indirectly through processing of intermediate proteins.

As previously discussed, neutrophil serine proteases have also been implicated in the processing and activation of IL-1 family members. Proteinase-3 and elastase have previously been claimed to process and activate IL-1β and IL-18 (Black et al., 1988; Coeshott et al., 1999; Joosten et al., 2009; Sugawara et al., 2001). Cathepsin G and elastase can potently enhance IL-33 activity, while elastase can increase IL-1α activity (Afonina et al., 2011; Lefrancais et al., 2012). A recent report from our group identified neutrophil serine proteases as major activators of IL-36 cytokines. Human keratinoctyes respond to active IL-36 by inducing a battery of pro-inflammatory cytokines and chemokines including IL-17C, G-CSF, IL-8 and IL-36γ, thereby acting as a positive feedback loop to further promote inflammation. Active IL-36β perturbs proper keratinocyte differentiation in an organotypic skin model and neutrophil protease activity from psoriatic skin lesions can also activate IL-36 cytokines, indicating that the processing of IL-36 cytokines by neutrophil proteases may contribute to keratinocyte hyperplasia and epidermal disorder in psoriasis (Henry et al., 2016). A more recent study has shown that elastase can process IL-36Ra to release its antagonistic, anti-inflammatory activity (Macleod et al., 2016). Incubation with activated neutrophil supernatant or purified neutrophil elastase results in the removal of Met1 residue from IL-36Ra and allows it to function as a receptor antagonist, blocking IL-8 and CCL20 production from keratinocytes, fibroblasts and an epidermal skin model. This suggests that elastase can promote the anti-inflammatory activity of IL-36Ra to dampen inflammation, however the importance of this in psoriasis or other inflammatory conditions with high IL-36 activity is yet to be determined (Macleod et al., 2016).

1.3.8 Neutrophils promote recruitment of leukocytes during inflammation

As discussed, neutrophil granule proteases can modulate local inflammation by processing extracellular cytokines and chemokines. However, neutrophil granule proteins can also propagate inflammation by inducing the recruitment and activation of other immune cells such as monocytes and macrophages, dendritic cells and T cells (Soehnlein et al., 2009).

Upon stimulation at an inflammatory site, neutrophils produce a broad range of cytokine and chemokines *de novo* in response to bacterial antigens and pro-inflammatory

mediators including LPS and TNFα. Neutrophils express a variety of chemokines including IL-8, CXCL1, MCP-1, MIP-1α among many others that can induce the recruitment of further neutrophils, monocytes, macrophages, dendritic cells, natural killer (NK) cells and T cells to the tissue (Tecchio et al., 2014). In addition, neutrophil serine proteases process and further enhance the chemotactic activity of these proteins in the extracellular milieu to further promote immune cell infiltration. Neutrophil granule proteins such as cathepsin G, azurocidin, LL-37 and defensins are also directly chemotactic for monocytes and promote enhanced phagocytosis and ROS production from these cells (Soehnlein et al., 2008a; Soehnlein et al., 2008b). In turn, macrophages recruited to inflammatory sites suppress further neutrophil recruitment and activity and phagocytose apoptotic neutrophils to resolve inflammation and return to tissue homeostasis (Amulic et al., 2012).

Neutrophils also promote recruitment of dendritic cells by producing chemokines such as CCL3. Neutrophils induce dendritic cell activation through direct cell-cell interactions or by granule proteins such as α-defensins and LL-37 (Amulic et al., 2012; Mocsai, 2013).

Crosstalk between neutrophils and T cells occurs during acute and chronic inflammation and during tumour defence. Neutrophils can activate T cells directly or by indirect interactions with dendritic cells. T cell recruitment to an inflammatory site is mediated by chemokine production from neutrophils and other tissue resident cells. Neutrophils produce IL-12 upon activation, a potent T cell stimulating cytokine (Bliss et al., 1999; Mantovani et al., 2011). Previous studies have suggested that neutrophils may act as additional antigen-presenting cells and express MHC class II and co-stimulatory molecules including CD83 in the presence of T cells (Muller et al., 2009). This elaborate immune regulation works both ways, with T cell-derived IFNγ capable of promoting neutrophil survival and activation. Neutrophils can also act in an immunosuppressive manner by releasing arginase, nitric oxide synthase and ROS, all of which can inhibit T cell activation (Amulic et al., 2012; Muller et al., 2009).

Overall, accumulating evidence suggests that neutrophils are key modulators of immune responses, acting as the first responders to host damage or infection. Neutrophils can promote inflammation by modulating local signalling through the action of their granule proteins, particularly cathepsin G, elastase and proteinase-3. Complex interactions occur between neutrophils and other immune cells to potentiate inflammation and activate adaptive immunity.

1.3.9 Neutrophil extracellular traps

Neutrophils kill pathogens by phagocytosis and degranulation, however, neutrophils can also contribute to the elimination of extracellular microbes without engulfing them. An additional antimicrobial mechanism of neutrophils is the release of intracellular components in the form of neutrophil extracellular traps (NETs). NETs were first described by Zychlinsky and colleagues in 2004 as a novel mechanism for bacterial clearance by neutrophils. NETs are mesh-like structures comprised of decondensed chromatin and DNA fibres decorated with antimicrobial peptides and neutrophil granule proteins (Brinkmann et al., 2004). These web-like structures have a cloud-like appearance and cover large areas, much larger than the cell they originate from. NETs form physical barriers to trap pathogens and prevent bacterial dissemination, as well as exposing high extracellular concentrations of antimicrobial effectors. NET formation has been detected in humans and mice, as well as other vertebrates and insects, suggesting that extracellular traps may represent a conserved antimicrobial mechanism (Brinkmann and Zychlinsky, 2012; Kaplan and Radic, 2012).

Using a proteomics approach, a recent study identified NET-bound components including nuclear, granular and cytoplasmic proteins. Histones are the most abundant NET component, making up ~70% of NET-associated proteins, followed by neutrophil elastase (~5%). NETs also contain other neutrophil serine proteases proteinase-3 and cathepsin G, albeit at lower abundance, as well as the antimicrobial effectors lysozyme, lactoferrin and azurocidin (Urban et al., 2009). NETs limit microbial spread and contribute to microbial killing but have also been implicated in autoimmune and inflammatory diseases by externalising granule proteins.

1.3.10 NETosis

Activated neutrophils undergo an unusual and novel form of cell death, termed NETosis, which culminates in the expulsion of chromatin into the extracellular space (Fuchs et al., 2007). Upon activation, neutrophils flatten and stick down firmly to the substratum. Numerous NET-inducing stimuli have been identified including cytokines, such as TNFα, IL-8 and IFNγ, TLR ligands or complement receptors, or direct activation by bacteria (Brinkmann and Zychlinsky, 2012). Once activated, chromatin decondenses and neutrophils lose their lobulated nucleus. Granules disintegrate and the nuclear envelope disaggregates, causing the cytoplasm and nucleus to mix. This process is dependent on elastase and myeloperoxidase. Elastase translocates to the nucleus and may be involved in histone processing. Indeed, elastase-deficient mice are impaired in NET formation and human neutrophils from donors completely deficient in MPO also fail to form NETs (Brinkmann and Zychlinsky, 2012; Metzler et al., 2011;

Papayannopoulos et al., 2010). Finally, the cell membrane ruptures and NETs are released (Fig 1.8) (Fuchs et al., 2007).

NETosis is distinct from apoptosis and necrosis and apoptotic or necrotic stimuli do not lead to NET formation, even after prolonged stimulation (Fuchs et al., 2007). NADPH oxidase and ROS are critical mediators of NETosis. Neutrophils from CGD patients fail to make NETs and are susceptible to recurrent infections, however, addition of exogenous hydrogen peroxide rescues NET formation indicating that ROS is important in this process (Bianchi et al., 2011; Brinkmann and Zychlinsky, 2012). Histone citrullination by PAD4 and the Raf-MEK-ERK signalling pathway are also thought to be important regulators of NETosis (Hakkim et al., 2011; Kruger et al., 2015). Interestingly, recent studies have also suggested that viable neutrophils can also form NETs in a non-lytic manner and retain phagocytic and crawling capabilities (Yipp et al., 2012; Yousefi et al., 2009). Extracellular traps have also been observed in other granulocytic cell types including eosinophils and mast cells (Kaplan and Radic, 2012).

1.3.11 Neutrophil extracellular traps contribute to microbial killing

NETs exert antimicrobial functions by trapping and killing bacteria, preventing microbial dissemination and by inactivating virulence factors. NETs have been shown to trap gram-positive and gram-negative bacteria, fungi, viruses and protozoa and may be particularly effective against pathogens that are too big to engulf such as fungal hyphae. NETs are decorated with numerous antimicrobial proteins including lysozyme, BPI, defensins and granule proteases which contribute to killing of pathogens trapped in close proximity to these proteins (Brinkmann and Zychlinsky, 2012). Histones, the most abundant NET component, are potent antimicrobial proteins that can directly kill pathogens and the DNA backbone itself has also been shown to be an effective antimicrobial agent (Brinkmann et al., 2004; Halverson et al., 2015). NETs inactivate virulence factors from *Shigella flexneri* and *Staphylococcus aureus* through action of their granule proteases (Brinkmann et al., 2004). Neutrophils can protect against fungal infection with *Aspergillus nidulans* and *Candida albicans* through the action of NET-bound calprotectin, a complex of S100A8 and S100A9 found in high abundance on trap structures (Urban et al., 2009).

The importance of NETs in immunity is underscored by evolutionary conservation of this process (Kaplan and Radic, 2012). Concomitantly, pathogens have also evolved mechanisms to evade the antimicrobial effects of NETs. Bacteria stick to NETs through charge interactions, however, encapsulated bacteria can avoid being trapped (Wartha et al., 2007). In addition, many pathogens, such as *Streptococcus pyogenes,*

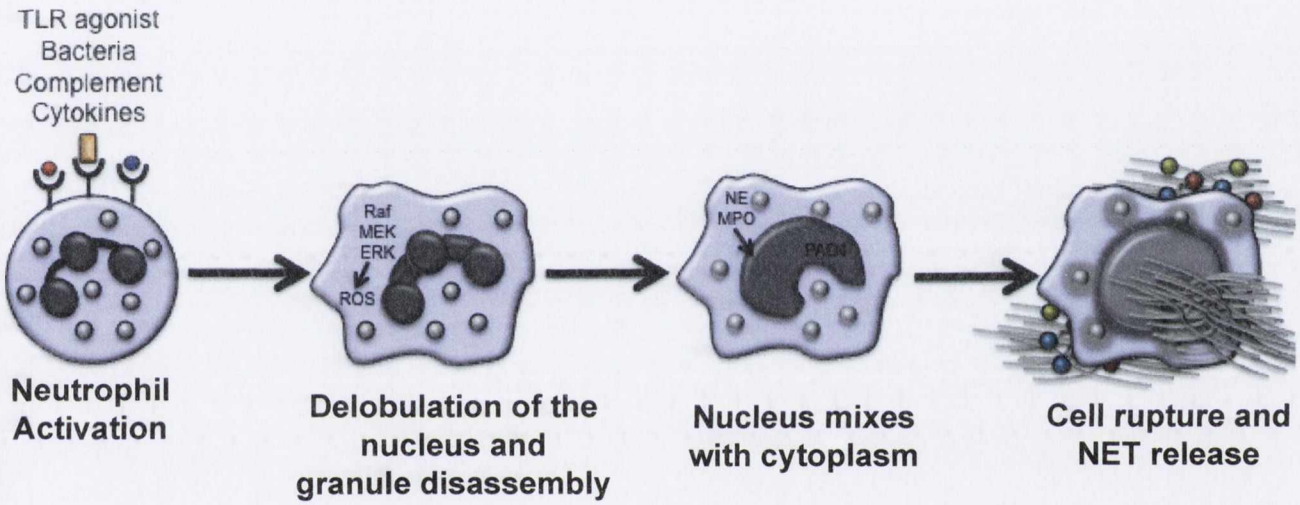

Figure 1.8 NETosis
Schematic of neutrophil extracellular trap formation. Neutrophils respond to TLR agonists, bacteria, cytokines, complement etc. Cells lose their lobulated nucleus, granules and nuclear envelope disintegrates,causing the cytoplasm and nucleus to mix. This process is regulated by Raf-MEK-ERK pathway, ROS and histone citrullination mediated by PAD4. Ultimately, the cell membrane ruptures and NETs are releasesd.

Streptococcus pneumoniae and *Staphylococcus aureus* encode endonucleases capable of degrading the DNA backbone of NET structures, thus enhancing the invasiveness of these pathogens (Beiter et al., 2006). Microbes may also evade neutrophil-mediated killing by expressing catalase enzymes that block the oxidative burst and ROS required for proper NET formation and neutrophil function (Papayannopoulos and Zychlinsky, 2009).

NETs contain a broad range of antimicrobial effectors, however their role in direct extracellular microbial killing remains controversial. NETs have been shown to bind and kill a variety of pathogens including *S. aureus*, *Shigella flexneri* and *C. albicans*, among many others (Brinkmann et al., 2004; Urban et al., 2006). However, in the case of *S. pneumoniae* and *Mycobacterium tuberculosis*, these pathogens attach to NETs but are not killed, suggesting that NET-mediated trapping alone may be sufficient to limit the spread and infectious efficacy of these pathogens (Beiter et al., 2006; Papayannopoulos and Zychlinsky, 2009). Thus, while NETs represent an important neutrophil antimicrobial mechanism and can indeed contribute to the control of microbial dissemination and infection, whether or not this occurs by direct microbial killing in the extracellular space is still debated (Menegazzi et al., 2012).

1.3.12 Neutrophils in autoinflammatory and autoimmune diseases

Neutrophil recruitment, activation and NETosis must be tightly regulated to prevent uncontrolled, detrimental neutrophil activity. Failure to clear neutrophils from an injured site or prolonged or aberrant neutrophil activity can lead to chronic inflammation and host damage. Neutrophil accumulation and NET release has been found in many inflammatory and autoimmune disorders including systemic lupus erythematosus (SLE), ANCA vasculitis, rheumatoid arthritis, psoriasis, acute lung inflammation, atherosclerosis and gout (Branzk and Papayannopoulos, 2013; Brinkmann and Zychlinsky, 2012; Yipp and Kubes, 2013).

Neutrophil influx is found in numerous lung inflammatory disorders including cystic fibrosis and COPD. Chronic neutrophil infiltration in the lungs of cystic fibrosis and COPD patients promote tissue damage and organ dysfunction. Moreover, NETs have been found in the sputum of cystic fibrosis patients and contribute to sputum viscosity and lung damage (Amulic et al., 2012; Brinkmann and Zychlinsky, 2012). High levels of neutrophils and their associated NETs are also found in synovial fluid from rheumatoid arthritis patients and contribute to tissue and cartilage damage by releasing proteases and other granule proteins. Cytokine and chemokine production by activated neutrophils in synovial fluid also leads to joint damage (Wright et al., 2010).

Several neutrophil and NET-bound proteins such as proteinase-3, MPO and histones are targets for antineutrophil cytoplasmic antibodies (ANCAs) in SLE and vasculitis (Garcia-Romo et al., 2011; Kessenbrock et al., 2009). Wegener's granulomatosis is a type of vasculitis characterised by the presence of ANCAs against proteinase-3 and MPO. ANCAs recognise and bind proteinase-3, promoting neutrophil activation and degranulation and exacerbation of inflammation (Amulic et al., 2012). Furthermore, the antimicrobial peptide LL-37 exposed on NET structures in complex with DNA can stimulate plasmacytoid dendritic cells to secrete type I IFNs, potent pro-inflammatory mediators in autoimmune disease, particularly SLE (Kaplan and Radic, 2012; Lande et al., 2007).

Immune cell infiltration is a hallmark of a number of skin-related inflammatory diseases. In particular, psoriatic plaques are heavily infiltrated with neutrophils, dendritic cells, macrophages, and T cells (Murphy et al., 2007; Nestle et al., 2009; Terui et al., 2000). IL-17 and IL-23 are key cytokines involved in the pathogenesis of psoriasis, and a recent study identified mast cells and neutrophils as the predominant cell types expressing IL-17 in psoriatic skin lesions. Neutrophils isolated from psoriasis patients underwent spontaneous NET formation, with IL-17 detected on NET structures (Lin et al., 2011). Thus, the role of neutrophils in the pathogenesis of psoriasis is becoming more appreciated, as previously it was thought that T cells and other immune cells were the main effectors of this disease.

1.4 Aims of this study

As outlined above, IL-1 family members are important pro-inflammatory cytokines that serve to potentiate inflammatory responses after injury or infection. The IL-36 subfamily of IL-1 cytokines have been recently described as being important mediators of skin inflammation, particularly in psoriasis (Blumberg et al., 2010; Blumberg et al., 2007; Johnston et al., 2011; Towne and Sims, 2012). IL-36 cytokines require proteolytic processing for activation, however the proteases responsible for IL-36 activation were previously unknown (Towne et al., 2011). Here, we describe a method for the production of biologically active IL-36 cytokines through the insertion of a caspase-3 cleavage motif (DEVD) within the N-termini of these cytokines.

IL-36α, β and γ signal through the IL-36 receptor (formerly denoted IL-1Rrp2) to activate NFκB and MAP kinase signalling pathways, however the downstream mediators of this process are not known. We sought to elucidate mediators of IL-36 receptor signalling through use of small molecule chemical inhibitors and RNA

interference strategies. IL-36 receptor downstream signalling targets may represent potential targets for therapeutic intervention for inflammatory skin conditions.

As noted earlier, sporadic reports have implicated neutrophil serine proteases in IL-1 family processing, but it remains unclear whether this is a common route to activation of all members of the IL-1 family (Afonina et al., 2011; Black et al., 1988; Hazuda et al., 1990; Netea et al., 2015). Thus, we explored the idea that neutrophil-derived proteases may act as global regulators of IL-1 family activation. Using supernatants from activated human neutrophils, as well as individual purified neutrophil proteases, we investigated the ability of these proteases to process and activate all members of the extended IL-1 family: IL-1α, IL-1β, IL-18, IL-33 and IL-36α, β and γ.

As neutrophil-derived proteases and other granule proteins are also extruded extracellularly as part of NET structures, we also explored the possibility that NETs can act as sites of IL-1 family cytokine activation. We examined the ability of NET-bound neutrophil proteases elastase, cathepsin G and proteinase-3 to activate IL-36 cytokines. NETs may represent a stable platform for extracellular cytokine activation by neutrophil-derived proteases. Neutrophil proteases are released extracellularly during degranulation, however these proteases may be rapidly diffused throughout the inflammatory site and surrounding tissue. Containment of proteases on NETs may concentrate and increase their local activity and allow association with circulating cytokines and chemokines to promote their activation.

Chapter 2

Materials and Methods

2.1 Purification of plasmid DNA

2.1.1 Bacterial strains

E. coli strain DH5α was used to amplify and prepare plasmid DNA. *E. coli* BL21-CodonPlus(DE3)-RIL or BL21 ClearColi were used to express recombinant proteins under the control of the T7 promoter.

2.1.2 Preparation of competent bacteria

Bacteria for DNA transformation were prepared using the Inuoe method. DH5α bacteria were streaked on LB agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar). Single colonies were inoculated in 3 ml LB and cultured overnight, 280 rpm, 37 °C. Overnight cultures were diluted into 50 ml SOB medium (2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$). Cultures were grown at room temperature, under agitation to an OD_{600} of 0.4-0.6 nm. Bacteria were harvested by centrifugation at 2,500 g for 10 minutes. Cells were resuspended in 20 ml Inuoe transformation buffer (10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250mM KCl, pH 6.7) and incubated for 10 minutes on ice. Cells were centrifuged again and resuspended in 4 ml transformation buffer. DMSO was added to a final concentration of 7 %. Cells were divided into 200 µl aliquots and frozen stocks were kept at -70 °C.

2.1.3 Transformation of competent bacteria by heat shock

Bacteria were thawed and 50 µl was dispensed into 13 ml bug culture tubes. 1 µl of plasmid DNA was added and bacteria were incubated on ice for 30 minutes to allow plasmid DNA to coat the cells. Cells were heat-shocked for 40 seconds at 42 °C and immediately transferred to ice for 2 minutes. 450 µl of LB medium was added and bacteria were grown at 37 °C for 1 h, 220 rpm to allow expression of the antibiotic resistance cassette. 100 µl was plated on LB agar containing an appropriate antibiotic and incubated overnight at 37 °C.

2.1.4 Plasmid Purification – miniprep

Single colonies from transformed bacterial plates were inoculated in 3 ml LB with antibiotic and grown overnight at 37 °C, 280 rpm. 1 ml of culture was centrifuged at 20,800 g for 1 minute. Supernatant was removed and the cell pellet was resuspended in 100 µl of P1 buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA). Bacteria were lysed with 200 µl of P2 buffer (1 % SDS, 0.2 M NaOH) followed by 200 µl of P3 buffer (3 M KAc, pH 5.5) to precipitate genomic DNA. Precipitated DNA was pelleted by centrifugation at 20,800 g for 15 minutes. Supernatant containing plasmid DNA was harvested and 0.7

x volume DNA-grade isopropanol was added for 20-30 minutes to precipitate plasmid DNA. DNA was centrifuged at 20,800 g for 10 minutes and washed in 1 ml of ice-cold 70 % ethanol. The pellet was air-dried and DNA was then resuspended in 10 µl DNase/RNase-free water, followed by 10 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8). 2 µl plasmid DNA was run on 0.6 % agarose gel to check purity and yield. Gels were stained in ethidium bromide for 15-20 minutes and visualized under UV light.

2.1.5 Plasmid Purification – midiprep

50-100 µl of bacterial starter cultures were inoculated in 50 ml LB with antibiotics and grown overnight. The culture was pelleted at 2,500g for 15 minutes. The cell pellet was resuspended in 4 ml of P1 buffer. Bacteria were lysed with 4 ml of P2 buffer for 1 minute on ice. 4 ml of P3 buffer was added and incubated for 5 minutes on ice to precipitate genomic DNA. Precipitated DNA was pelleted at 15,000 g for 15 minutes at 4 °C. A Qiagen Q100 column was equilibrated with 4 ml of Qiagen QBT buffer. Supernatant was applied to the column and allowed to flow through. DNA bound to the column was washed twice with 10 ml of Qiagen QC buffer and then eluted with 5 ml Qiagen QF buffer. Plasmid DNA was precipitated with 0.7 x volumes of DNA-grade isopropanol for 20-30 minutes. DNA was pelleted for 30 minutes at 15,000 g, 4 °C and washed twice with 1 ml ice-cold ethanol. DNA was air-dried and resuspended in 100 µl DNase/RNase-free water, followed by 100 µl TE buffer. Plasmid DNA was run on 0.6 % agarose gel to check purity and yield.

2.2 Recombinant proteins

2.2.1 Expression and Purification of recombinant proteins

His-tagged proteins were expressed from pET45b vector which encode an N-terminal His^6 tag and are driven by the T7 promoter. GST-tagged proteins were expressed from pGEX vector, which encodes an N-terminal GST tag. Plasmids were transformed into BL21 ClearColi strain or DH5α for GST-tagged proteins and inoculated into 3 ml LB cultures with ampicillin. After overnight growth, cultures were diluted to 500 ml in LB with ampicillin and grown to an OD_{600} of 0.4-0.6 nm. Full-length IL-1α, IL-1β, IL-18 and IL-36α, β and γ were expressed by addition of 600 µM IPTG to exponentially growing cultures of BL21 ClearColi, followed by incubation for 3 h at 37 °C. Truncated p17 IL-1β was expressed by addition of 200 µM IPTG to exponentially growing cultures of ClearColi followed by incubation for 90 min at room temperature. Caspase-1 and caspase-3 was expressed and purified as described previously (Walsh et al., 2011). After protein induction, cells were pelleted at 7,500 rpm for 10 minutes at 4 °C.

2.2.2 Purification of His-tagged proteins

Bacterial pellets were frozen and then resuspended in TM lysis buffer (50 mM Tris-HCl, pH 8.5, 10 mM β-mercaptoethanol, 1 mM PMSF, 2 μg/ml aprotinin, 10 mg/ml leupeptin) to 2 % of the original culture volume. Bacteria were lysed by sonication using 6 x 30 second pulses at 60 % amplitude in a Branson sonifier. Insoluble material was removed by centrifugation at 15,000 g for 15 minutes at 4 °C. 50-100 μl Ni-NTA agarose was added to supernatants and incubated under rotation at 4 °C for 3 h to capture His-tagged proteins. Bound protein was washed three times in 1 ml Imidazole wash buffer (20 mM Tris-HCl, pH 8.5, 10mM β-mercaptoethanol, 10 % glycerol, 20 mM imidazole). His-tagged proteins were eluted from the resin by addition of 250 μl of Imidazole elution buffer (PBS, pH 7.2, 100 mM imidazole) for 30 minutes at 4 °C. Elutions were repeated 3 times. Purified proteins were aliquoted into 10-20 μl aliquots and frozen at -70 °C. For IL-1α and IL-1β purification, sodium sarkosyl was added to the bacterial lysate to a final volume of 0.25 %. Bacteria were lysed by sonication and sarkosyl was subsequently sequestered by addition of TritonX-100 to a final volume of 0.5 % and captured using nickel-NTA agarose, as above.

2.2.3 Purification of GST-tagged proteins

Bacterial pellets were resuspended in NETN buffer (20 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5 % NP-40) at 2 % of the original culture volume. Cells were lysed by sonication and insoluble material was removed by centrifugation. 50-100 μl glutathione-sepharose resin was added to supernatants and incubated under rotation at 4 °C for 3 h to capture GST-tagged proteins. Bound proteins were washed 3 times in 1 ml ice-cold PBS. GST-tagged proteins were eluted in PBS containing 10 mM glutathione under rotation at room temperature.

2.2.4 In vitro transcription/translation

^{35}S-labelled IL-33 was synthesised using a coupled *in vitro* transcription/translation system (Promega, UK). His-tagged IL-33 in pET45b plasmid template was added to a reaction containing rabbit reticulocyte lysate, amino acid mix (minus methionine), T7 RNA polymerase and ^{35}S-methionine (10 mCi/ml, Amersham). Reactions were set up on a 25 μl scale as follows:

12.5 μl rabbit reticulocyte lysate

1 μl TNT reaction buffer

1 μl amino acid mix (minus methionine)

2 μl ^{35}S methionine

1 μg Qiagen-pure template plasmid DNA

1 μl RNasin

1 μl T7 polymerase

ddH_2O to 25 μl

Reactions were carried out for 2 h at 30 °C.

2.3 Protein detection and analysis

2.3.1 SDS-PAGE electrophoresis

Proteins were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for analysis. Protein samples were denatured by boiling in SDS loading buffer (2 % SDS, 50 mM Tris-HCl, pH 6.8, 10 % glycerol, 2.5 % β-mercaptoethanol). Proteins were loaded on 12 % SDS-PAGE gels and electrophoresed at 55 V through the stacking gel, followed by 75 V through the resolving gel. Proteins were visualised by Coomassie Blue staining (45 % methanol, 10 % acetic acid, 0.25 % Coomassie Brilliant Blue). Radio-labelled methionine protein gels were fixed for 2 h in fixative solution (45 % methanol, 10 % acetic acid), followed by incubation for 10 minutes in Amplify reagent and vacuum drying onto Whatman paper for 1 h. Gels were exposed to autoradiography film (Santa Cruz) at -70 °C for 12-48 h.

2.3.2 Western Immunoblotting

Protein samples were prepared using SDS loading buffer, boiled for 7 minutes and electrophoresed on 12 % SDS-PAGE gels. Proteins were then transferred onto 0.2 μm nitrocellulose membrane at 40 mA overnight. Membranes were blocked for 1 h (5 % NFDM, 0.05 % sodium azide in Tris-buffered saline, Tween-20, TBST). The indicated proteins were probed using specific antibodies, typically diluted 1:1000. Membranes were washed 3 times in TBST and then incubated with the relevant HRP-conjugated secondary antibody (Jackson Labs, USA) diluted 1:2000. Membranes were again washed and proteins were visualized with SuperSignal West Pico (Thermo Scientific) and exposure to autoradiography films.

2.4 Cellular assays

2.4.1 Cell Culture

$HeLa^{IL36R}$ cell lines were generated by transfection with pCXN2.IL-1Rrp2 (IL-36R) plasmid followed by selection using G418 antibiotic (Sigma). IL-36R over-expressing clones were expanded from a single cell. $HeLa^{IL36R\text{-}SEAP}$ cell line was generated by

transfection of stable HeLa^{IL36R} over-expressing clones with pNifty2-SEAP plasmid (InvivoGen) followed by selection using Zeocin antibiotic. Clones were expanded from a single cell using limiting dilution cloning. Clones were selected by demonstration of acquired optimal responsiveness to active forms of IL-36 via SEAP production and ELISA. HaCaT cells were cultured in DMEM (Gibco) supplemented with FCS (10%). Primary neonatal foreskin-derived keratinocytes were purchased from Cell Systems (Germany) and cultured in serum-free Dermalife K media (Cell Systems, Germany). 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.

2.4.2 Bioactivity assays

Cells were plated at 5 x 10^5 in 24-well plates (VWR). After overnight incubation, cells were stimulated with IL-1 family cytokines, as indicated. Cells were incubated with stimuli for 18-24 h and cell supernatants were subsequently collected. Cytokines and chemokines were measured from cell culture supernatants using specific ELISA kits (R&D systems). All cytokine assays were carried out using duplicate or triplicate samples from each culture.

2.4.3 Detection of cytokines and chemokines by ELISA

Cytokines and chemokines were measured from cell culture supernatants using commercially available specific paired ELISA kits (R&D Systems) including: human IL-6, IL-8, CXCL1, CCL20, VEGF, G-CSF, GM-CSF, RANTES, IL-1β. Briefly, 50 µl of capture antibody was coated per well on a 96 well plate according to manufacturer's instructions, and incubated overnight at room temperature. Wells were washed three times with 150 µl wash buffer (0.05% Tween-20 in PBS, pH 7.2), then blocked for 1 h in reagent diluent (1% BSA in PBS, pH 7.2). Samples were diluted as required in reagent diluent and 50 µl of each sample was added to wells and incubated for 2 h at room temperature. Appropriate standards were prepared for each ELISA assay according to manufacturer's instructions. Wells were washed again three times, followed by 50 µl of biotin-conjugated detection antibody incubated for 2 h. Wells were washed three times and 50 µl streptavidin-HRP was added to wells and incubated for 30 minutes in the dark. Finally, wells were washed three times, followed by addition of 50 µl TMB substrate solution. The reaction was stopped with 30 µl of 2 N sulphuric acid and absorbance was read at 450 nm on an ELISA plate reader (TECAN). All cytokine assays were carried out using duplicate or triplicate samples from each culture.

2.4.4 Protease cleavage assays

Reactions containing recombinant IL-1 proteins and neutrophil proteases were carried out in protease reaction buffer (50 mM HEPES, pH 7.4, 75 mM NaCl, 0.1 % CHAPS, 2 mM DTT) for 2 h at 37 °C. IL-1 family cytokines were typically cleaved at 50 nM. Full length or processed IL-1 family cytokines were subsequently diluted onto target cells (5 x 10^4 cells/well) at a final concentration ranging from 1 nM to 10 nM for 18-24 h.

2.4.5 Measurement of NFκB activation by SEAP assay

HeLa^{IL36R}SEAP cells were plated at 5 x 10^4 cells/well in a tissue culture dish in RPMI supplemented with 5 % heat-inactivated FCS. Cells were treated, as indicated and cell culture supernatants were harvested and secreted alkaline phosphatase in the medium was detected using Quantiblue reagent (InvivoGen). 10 µl cell culture supernatant was incubated with 190 µl pre-warmed QuantiBlue reagent and incubated for 15 min at 37 °C. Absorbance readings were taken at 620 nm with a microplate reader (Infinite F50, TECAN) after 15 min to 6 h incubation to assess SEAP activity.

2.4.6 RNA interference

HeLa^{IL36R} were transfected with 200 nM siRNA using nucleofection (Amaxa). For each transfection cells (5 x 10^5 cells/cuvette) were resuspended in 100 µl of transfection solution (5 mM KCl, 15 mM $MgCl_2$, 20 mM HEPES, 150 mM Na_2HPO_4/NaH_2PO_4, pH 7.2). Cells were nucleofected with 200 nM siRNA using Amaxa programme I-013. Cells were diluted in 10 ml of medium and plated at the required density. Cells were incubated for 48 h after nucleofection followed by IL-36 or IL-1β stimulation for 18-24 h. siRNA sequences were as follows:

MyD88 #1: sense 5'-GGCACCUGUGUCUGGUCUA-3'
MyD88 #2: sense 5'-GGGCAUCACCACACUUGAU-3'
IRAK4 #1: sense 5'-GCAGCAAUGGUUGACAUUA-3'
IRAK4 #2: sense 5'-GCCCAGACAGUCAUGACUA-3'
TRAF6 #1: sense 5'-CAGCGCUGUGCAAACUAUAUA-3'
TRAF6 #2: sense 5'-CAUUAAGGAUGAUACAUUAUU-3'
TAK1 #1: sense 5'-GGAGAUCGAGGUGGAAGAG-3'
TAK2 #2: sense 5'-UUGCUUAUCUUACACUGGA-3'
TAK3 #3: sense 5'- GGCAAAGCAACAGAGUGAAUCUGG-3'
NEMO #1: sense 5'-AACAGGAGGUGAUCGAUAAUU-3'
NEMO #2: sense 5'-GAAUGCAGCUGGAAGAUCUUU-3'
NEMO #3: sense 5'- GGAAGAGCCAACUGUGUGA-3'
NEMO #4: sense 5'-ACAGGAGGUGAUCGAUAAG-3'

HOIP #1: sense 5'-GGUACUGGCGUGGUGUCAAGUUUAA-3'

HOIP #2: sense 5'-CACCACCCUCGAGACUGCCUCUUCU-3'

FADD #1: sense 5'-GAUUGGAGAAGGCUGGCUC-3'

FADD #2: sense 5'-GAACUCAAGCUGCGUUUAU-3'

TRADD #1: sense 5'-GGAGGAUGCGCUGCGAAAUUU-3'

TRADD #2: sense 5'-CUGGCUGAGCUGGAGGAUG-3'

RIPK1 #1: sense 5'- GGAGCAAACUGAAUAAUGAAGAGCA-3'

Caspase-8 #1: sense 5'-GUUCCUGAGCCUGGACUAC-3'

Caspase-8 #2: sense 5'-AACUACCAGAAAGGUAUACCU-3'

XIAP #1: sense 5'-GUGGUAGUCCUGUUUCAGC-3'

XIAP #2: sense 5'-GCAGUUGACAAGUGUCCCA-3'

p65 #1: sense 5'-GAUUGAGGAGAAACGUAAA-3'

p65 #2: sense 5'-GCCCUAUCCCUUUACGUCA-3'

p100 #1: sense 5'-CAGCCUAAGCAGAGAGGCU-3'

p100 #2: sense 5'-CAUGAAGAUUGAGCGGCCU-3'

2.4.7 Transfection of mammalian cells using Genejuice®

HeLa^{IL36R} cells were transfected by the Genejuice® method. Cells were plated at 1 x 10^5 cells/well on 6-well plate. Cells were transfected using the Genejuice reagent (Merck, Germany) according to manufacturer's protocol. Briefly Genejuice (1 µl/well) was added to RPMI and incubated for 10 min. 100 µL Genejuice-RPMI mixture was added dropwise to plasmid DNA in 96 well plates and incubated for 15 min for the DNA-Genejuice complexes to form. Genejuice-DNA complexes were then added to cells. Cells were incubated with Genejuice-DNA complexes for 24 hours before cell-based assays were performed. Reporter plasmid (eGFP) was used to indicate transfected cells.

2.5 Neutrophil preparations

2.5.1 Isolation of neutrophils

Primary neutrophils were purified from peripheral human blood using discontinuous plasma-Percoll gradients as described by Haslett et al (1985). Briefly, peripheral blood from healthy volunteers was collected in lithium heparinized tubes (Greiner). Blood was pooled in 50 ml tubes containing 4.4 ml of 5 % sodium citrate. Blood was centrifuged at 300 g for 20 minutes. The platelet-rich plasma was removed and centrifuged at 2500 g for 15 minutes to obtain platelet-poor plasma (PPP). To the remaining blood, 5 ml of 6 % dextran (mol. wt. 500,000, Sigma) was added and volume was topped up to 50 ml with 0.9 % saline solution, mixed gently and stood for 30 – 40 minutes to allow

erythrocyte sedimentation to occur. During dextran sedimentation, a stock solution of Percoll (Sigma) was prepared in 0.9 % saline in a ratio of 9:1 (vol/vol) Percoll/saline. After 30 – 40 minutes, the leukocyte-rich plasma was aspirated and centrifuged at 280 g for 6 minutes. The resulting pellet was resuspended in 3 ml PPP and transferred to 13 ml sterile tube containing 2 ml freshly prepared 42 % Percoll in PPP underlayed with 2 ml 51 % Percoll in PPP. The gradients were centrifuged for 10 minutes at 280 g. Neutrophils are found at the interface between the 42 % and 51 % gradients and extend into the 51 % gradient to just above the erythrocyte pellet. The neutrophil fraction was washed twice in HBSS/0.25 % BSA (without calcium or magnesium) and pelleted at 250 g for 5 minutes. After washing, neutrophils were resuspended in HBSS/0.25 % BSA with calcium and magnesium and plated for assays. Purity of cell populations was determined by Hematoxylin and Eosin staining of cytospins.

2.5.2 Activation of neutrophils with PMA

To prepare neutrophil degranulate supernatant, cells (1 x 10^7 cells/ml) were stimulated, or not with 100 nM PMA in HBSS/0.25 % BSA for 2 h at 37 °C in a humidified atmosphere with 5 % CO_2. Supernatants were harvested and clarified by centrifugation. Neutrophil degranulate supernatant was stored at -80 °C. To induce neutrophil extracellular trap formation, neutrophils (5 x 10^6 – 1 x 10^7 cells/ml) were stimulated, or not with 100 nM PMA in HBSS/0.25 % BSA for 4 h at 37 °C.

2.5.3 IL-36 cytokine activation by neutrophil extracellular traps

Neutrophils (1 x 10^7 cells/well) were stimulated, or not with 100 nM PMA in HBSS/0.25 % BSA for 2 h at 37 °C. After 4 h, neutrophil degranulate supernatant was removed and cells were washed three times with 2 ml PBS to remove residual soluble protease activity released during degranulation. IL-36α, IL-36β or IL-36γ (50 nM) was added to wells in 1 ml in HBSS/0.25 % BSA and incubated for 30 minutes at 37 °C to allow cytokine activation. IL-36 was harvested and centrifuged at 500 g for 5 minutes before being titrated onto $HeLa^{IL\text{-}36R\text{-}SEAP}$ cells to measure biological activity. For assays using DNase I, neutrophils were treated and washed as before to give NETs, followed by incubation with DNase I (20 – 40 U/ml, Sigma) for 30 minutes at 37 °C. DNase I was removed and cells were washed again with PBS followed by treatment with IL-36β (5 nM) for 30 minutes at 37 °C. Similar assays were carried out using cathepsin G inhibitor and elastase inhibitor (5 μM), followed by IL-36 cytokine treatment.

2.5.4 Fluorimetric analysis of enzymatic activity

Reactions were carried out in protease reaction buffer (50 mM HEPES, pH 7.4, 75 mM NaCl, 0.1 % CHAPS, 2 mM DTT) containing 50 μM Ac-DEVD-AFC to measure

caspase-3 activity or Suc(oMe)-AAPV-AMC to measure elastase activity. Samples were measured using an automated fluorimeter (SparkM, TECAN) at wavelengths of 430 nm (excitation) and 535 nm (emission). For suc-FLF-sBzL assay to measure cathepsin-G activity, substrate was diluted to a final concentration of 300 μM in protease reaction buffer (50 mM HEPES, pH 7.4, 75 mM NaCl, 0.1% CHAPS, 300 μM DTNB). Samples were measured using an automated fluorimeter (SparkM, TECAN) at an absorbance wavelength of 430 nm.

2.5.5 Neutrophil extracellular trap immunostaining

Neutrophils ($5 \times 10^5 - 2 \times 10^6$ cells/well) were plated on coverslips coated with 0.001 % Poly-L-Lysine (Sigma) and treated with 100 nM PMA in HBSS/0.25 % for 4 h at 37 °C. After 4 h, cell supernatant was removed and neutrophils were fixed with 4 % paraformaldehyde for 15 minutes. Cells were blocked with 2 % BSA (in PBS, pH 7.4) for 1 h to reduce non-specific binding of antibodies. Specific primary antibodies were diluted in 2% BSA and typically used at 1:100 – 1:500 dilutions. Primary antibodies were incubated for 2 h at room temperature. Cells were washed 3 times with 2 ml of PBS. Cells were incubated with relevant secondary antibodies diluted 1:1000 in 2 % BSA for 1 h at room temperature. Cells were washed again with PBS, followed by incubation with Hoechst for 10 minutes. Coverslips were mounted on slides with 5 – 10 μl of Slow Fade (Molecular Probes). Cells were visualised and analysed using a laser scanning confocal microscope (Olympus FV1000) using a 488 nm Argon laser (green fluorescence) and a 405 nm LD laser (Hoechst). Confocal images were acquired as z stacks on Fluoview 1000 V.1 software.

2.5.6 Cytokine induction from neutrophils

To measure cytokine induction from neutrophils in response to TNFα, LPS or IL-1 family cytokines, neutrophils were isolated as before and plated at 1×10^7 cells/ml in RPMI containing 5 % human serum (MP Bio). Neutrophils were treated with TNFα, LPS or IL-1 family cytokines, as indicated. IL-1 family proteins were expressed and purified in ClearColi bacteria to eliminate endotoxin contamination from protein preparations. Cytokine concentrations in cell culture supernatants were measured by ELISA at 24 h and 48 h after treatment.

Chapter 3

Downstream pathways involved in IL-36 receptor signalling

3.1 Introduction

IL-1 family cytokines, which include the recently described IL-36α, IL-36β, and IL-36γ proteins, play major roles as initiators of inflammation and are frequently among the first cytokines produced in response to infection or injury (Afonina et al., 2015; Kono and Rock, 2008; Lukens et al., 2012; Sims and Smith, 2010; Martin, 2016). IL-1 family cytokines can initiate complex cascades of additional cytokine production from diverse cell types (Debets et al., 2001; Dinarello, 2009; Milovanovic et al., 2012; Towne et al., 2004; Vigne et al., 2011; Vigne et al., 2012). IL-36α, IL-36β, and IL-36γ are encoded by distinct genes and accumulating evidence suggests that these cytokines play a key role in skin inflammation, particularly in psoriasis (Blumberg et al., 2010; Blumberg et al., 2007; Farooq et al., 2013; Frey et al., 2013; Johnston et al., 2011; Kanazawa et al., 2013; Marrakchi et al., 2011; Onoufriadis et al., 2011; Tortola et al., 2012; Towne and Sims, 2012). Members of the IL-1 family are typically among the most upstream cytokines to be released upon infection or cell damage and most likely represent the key damage-associated molecular patterns (DAMPs) as a consequence of their release during necrosis (Afonina et al., 2015; Cullen et al., 2015; Martin, 2016).

IL-36α, IL-36β, and IL-36γ are all generated as leaderless cytokines that lack biological activity and are not secreted via the canonical ER-Golgi secretion route. Thus, proteolytic processing of IL-36 cytokines is required to unleash their pro-inflammatory activity, similar to other members of the IL-1 family, such as IL-1β and IL-18 (Towne et al., 2011). Sims and colleagues have shown that removal of a small number of residues from the N-termini of IL-36α, IL-36β, and IL-36γ increases their biological activity by greater than 10,000-fold (Towne et al., 2011). However, the proteases responsible for activation of IL-36 cytokines were unknown until recently.

To facilitate investigations into the biological activity of the IL-36 cytokine subfamily, a simple and efficient approach for the production of biologically active IL-36 cytokines is desirable. Here, we describe a novel strategy for the production of large amounts of soluble active recombinant IL-36 cytokines through insertion of a caspase-3 cleavage motif (DEVD) within the N-termini of these cytokines, followed by processing by purified recombinant caspase-3. This approach enabled us to process and activate human IL-36 cytokines with exquisite specificity, leading to a dramatic increase in biological activity. Here, we show that caspase-3-processed recombinant ^{DEVD}IL-36 is highly active on a range of cell types, including primary keratinocytes, and elicits robust production of multiple cytokines and chemokines from target cells. We also established

a convenient reporter cell line HeLa$^{IL\text{-}36R}$ overexpressing the IL-36 receptor, as well as an NFκB-SEAP reporter plasmid, which will be useful as a screening tool to identify molecules involved in IL-36 activation and downstream signalling. Using purified forms of IL-36, we also generated polyclonal antibodies against all three IL-36 cytokines that displayed specificity for individual IL-36 proteins.

IL-36 ligands have been shown to signal through the previously orphan IL-1 family receptor, IL-1 receptor-related protein 2 (IL-1Rrp2). Initial studies were carried out by overexpressing IL-1 receptors in Jurkat cells to investigate the requirements for IL-36 signalling. Overexpression of IL-1Rrp2, but not IL-1R, IL-18R or ST2, was shown to be required for IL-36 responsiveness in a number of cell lines (Debets et al., 2001; Towne et al., 2004). IL-1Rrp2 activation by IL-36α, β and γ recruits IL-1R accessory protein (IL-1RAcP) and leads to NFκB, JNK and ERK activation, with similar kinetics to IL-1R activation by IL-1β (Towne et al., 2004). IL-1Rrp2 and all three IL-36 proteins are highly expressed in human skin and epithelial tissues, suggesting that this signalling pathway may be important in skin inflammatory diseases (Debets et al., 2001; Towne et al., 2004; Towne and Sims, 2012).

To date, there has been little investigation into the downstream mediators of IL-36 signalling after IL-1Rrp2 receptor activation. Because of the requirement for IL-1RAcP and the similarity in signalling kinetics, IL-36R signal transduction is assumed to be similar to that for IL-1R. Elucidation of IL-36R downstream signalling constituents may yield attractive targets for therapeutic intervention for a range of inflammatory conditions, including psoriasis and other inflammatory skin disorders. Here, we use small molecule inhibitors as well as RNA interference to target potential molecules involved in IL-36 receptor signalling, such as MyD88, IRAKs and TRAF6. We also investigated the role of linear ubiquitination in the regulation of IL-36 receptor signalling by targeting the deubiquitinase OTULIN, which removes linear ubiquitin chains and which has previously been shown to be important in the regulation of TNF and NOD receptor signalling (Fiil et al., 2013; Keusekotten et al., 2013).

3.2 Results

3.2.1 Generation of DEVD-modified IL-36 cytokines

Previous studies have shown that the full-length forms of IL-36α, IL-36β, and IL-36γ exhibit little or no bioactivity (Towne et al., 2011), similar to other members of the extended IL-1 family, but become active upon removal of small stretches of N-terminal amino acids. For example, truncation of IL-36α after Leu5 has been shown to enhance

its activity 10,000-fold. Similarly, truncation of IL-36β after Gln4 and IL-36γ after Gln17 also unleashed the activity of the latter cytokines (Towne et al., 2011). However, the proteases that naturally process at these sites to activate these cytokines have not been reported to date.

To facilitate activation of recombinantly expressed IL-36 cytokines for further study of their biological activity, we generated insertions within the N-terminal region of all three IL-36 coding sequences to insert the known caspase-3 cleavage motif, DEVD (Fig. 3.1). Caspase-3 is a relatively specific endoprotease that cleaves after aspartic acid (Asp) residues contained within specific tetrapeptide motifs that are not commonly found at spatially accessible sites within the majority of proteins (Timmer et al., 2009). Thus, insertion of caspase-3 cleavage sites permits the generation of highly precise protein truncations to eliminate purification tags, autoinhibitory domains, or other unwanted protein domains prior to use in biological assays.

The DEVD motifs were inserted at regions within IL-36 cytokines previously identified by Sims and colleagues to result in liberation of robust biological activity upon proteolysis at these sites (Towne et al., 2011). We then transformed BL-21 (RIL) strain *E. coli* with pET45b-based IL-36 expression plasmids, followed by induction of protein expression for 3 h at 37 °C. As shown in Fig. 3.2, all three IL-36 cytokines were strongly expressed under these conditions and were readily purified as soluble proteins from the supernatant fraction of sonicated bacterial cell lysates. Proteins were washed and eluted from nickel-NTA affinity matrix and yields were estimated at ~10 μg per mL of bacterial culture (Fig. 3.2).

3.2.2 Processing of DEVD-modified IL-36 by purified caspase-3

To facilitate processing of DEVD-modified IL-36 cytokines, we expressed and purified recombinant caspase-3 as previously reported (Walsh et al., 2008). Purified caspase-3 was highly active as demonstrated by its ability to cleave the synthetic caspase substrate DEVD-AFC (Fig. 3.3 A). We next incubated DEVD-modified IL-36β with recombinant caspase-3 over a range of concentrations from 1 μM to 200 nM and, as shown in Fig. 3.3 B, C, this resulted in cleavage of the DEVD-modified form of IL-36 β (^{DEVD}IL-36 β) to a slightly smaller product consistent with removal of the N-terminal residues as desired.

3.2.3 DEVD-modified IL-36 cytokines are biologically active

To ask whether caspase-3-cleaved DEVD-modified IL-36 cytokines were biologically active, we titrated full-length versus caspase-3-cleaved forms of ^{DEVD}IL-36 α, β and γ

onto HeLa cells that were stably transfected with the human IL-36 receptor. As shown in Figure 3.4 A, whereas all three full-length ^{DEVD}IL-36 cytokines were completely inactive in their unprocessed forms, processing of ^{DEVD}IL-36 cytokines with caspase-3 resulted in highly robust biological activity as demonstrated by the production of high levels of IL-6, IL-8 and CXCL1 by HeLa$^{IL\text{-}36R}$ cells. Importantly, caspase-3 alone had no effect on cytokine production by HeLa cells (Fig. 3.4 B), excluding the unlikely possibility that addition of the latter protease to the IL-36 preparations could be the source of increased cytokine activity.

We also examined some of the intracellular signal transduction events seen in response to IL-36. As seen in Figure 3.5, treatment of HeLa$^{IL\text{-}36R}$ cells with biologically active IL-36 resulted in the rapid phosphorylation and proteolytic processing of the NFκB inhibitor IκB, in tandem with phosphorylation of the p65 subunit of NFκB as well as phosphorylation of p38 MAPK. Collectively, the above data clearly demonstrate that removal of the N-terminal region of IL-36 cytokines through insertion of a tetrapeptide caspase cleavage motif permitted the simultaneous removal of the poly-histidine purification tag as well as the N-terminal region responsible for autoinhibition of IL-36 family cytokines, leading to the generation of highly active IL-36.

3.2.4 Generation of a reporter cell line for the assessment of IL-36 activity

Because IL-36 promotes NFκB activation (Fig. 3.5), we also generated a convenient reporter cell line, based on the NFκB-dependent expression of secreted alkaline phosphatase (SEAP), to assess IL-36 activity. HeLa cells stably overexpressing IL-36R were transfected with a NFκB-SEAP reporter plasmid and cells were selected using the antibiotic Zeocin in combination with limiting dilution selection. A number of clones were screened for NFκB-inducible SEAP expression, and a single clone was selected for further study (Fig 3.6 A, B). HeLa$^{IL\text{-}36R}$ cells stably transfected with the NFκB-SEAP reporter exhibited robust SEAP activity upon treatment with caspase-3-cleaved ^{DEVD}IL-36 (Fig 3.6 C). As expected, HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells also secreted multiple cytokines in response to IL-36 treatment (Fig 3.6 D). This reporter line will be useful for future studies aimed at identifying components of IL-36 signal transduction events and also for screening of inhibitors of IL-36 signalling.

3.4.5 Transformed and primary keratinocytes are responsive to IL-36

Because the preceding tests of the biological activity of IL-36 cytokines were conducted on cells overexpressing the IL-36 receptor, we also asked whether cells that naturally express the IL-36R were IL-36 responsive. As noted in the introduction, IL-36 is strongly linked with psoriasis, which is thought to result from excessive inflammation in

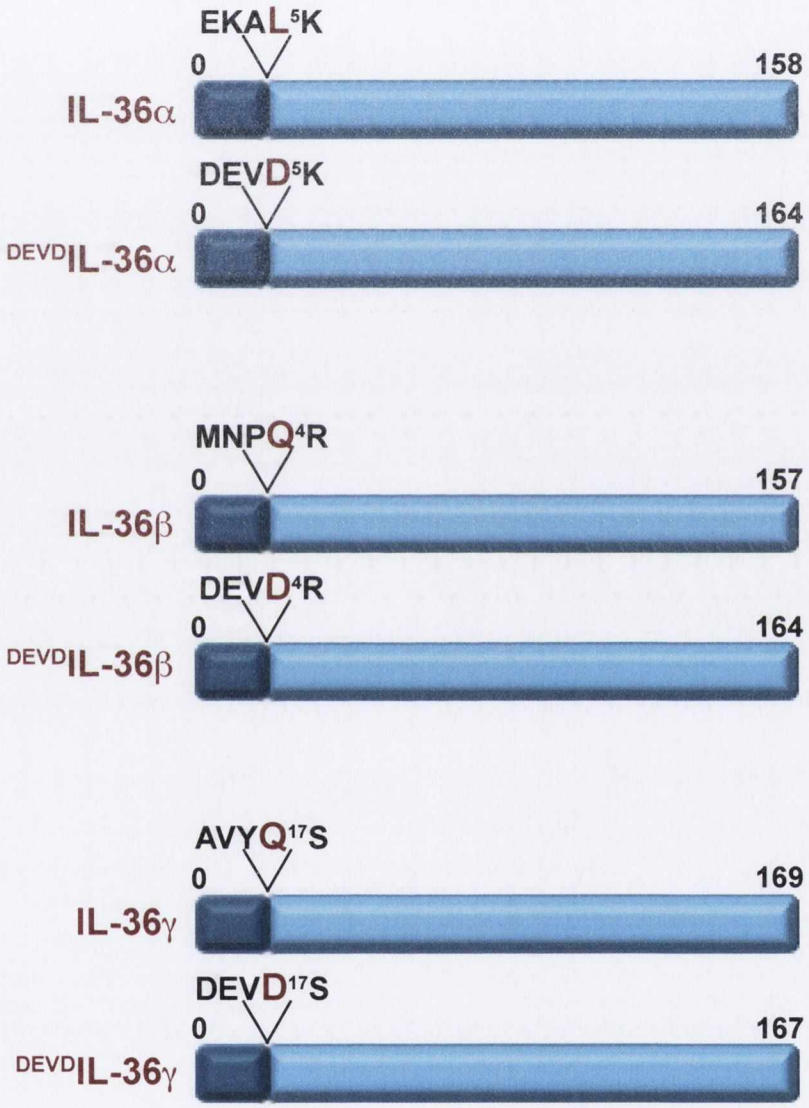

Figure 3.1 Insertion of DEVD cleavage motifs into the N-terminal regions of IL-36 cytokines
Schematic of modified forms of IL-36α, IL-36β and IL-36γ where a caspase-3-processing motif (DEVD) was inserted into the IL-36 sequence, N-terminal to the known artificial processing sites (Towne et al., 2011).

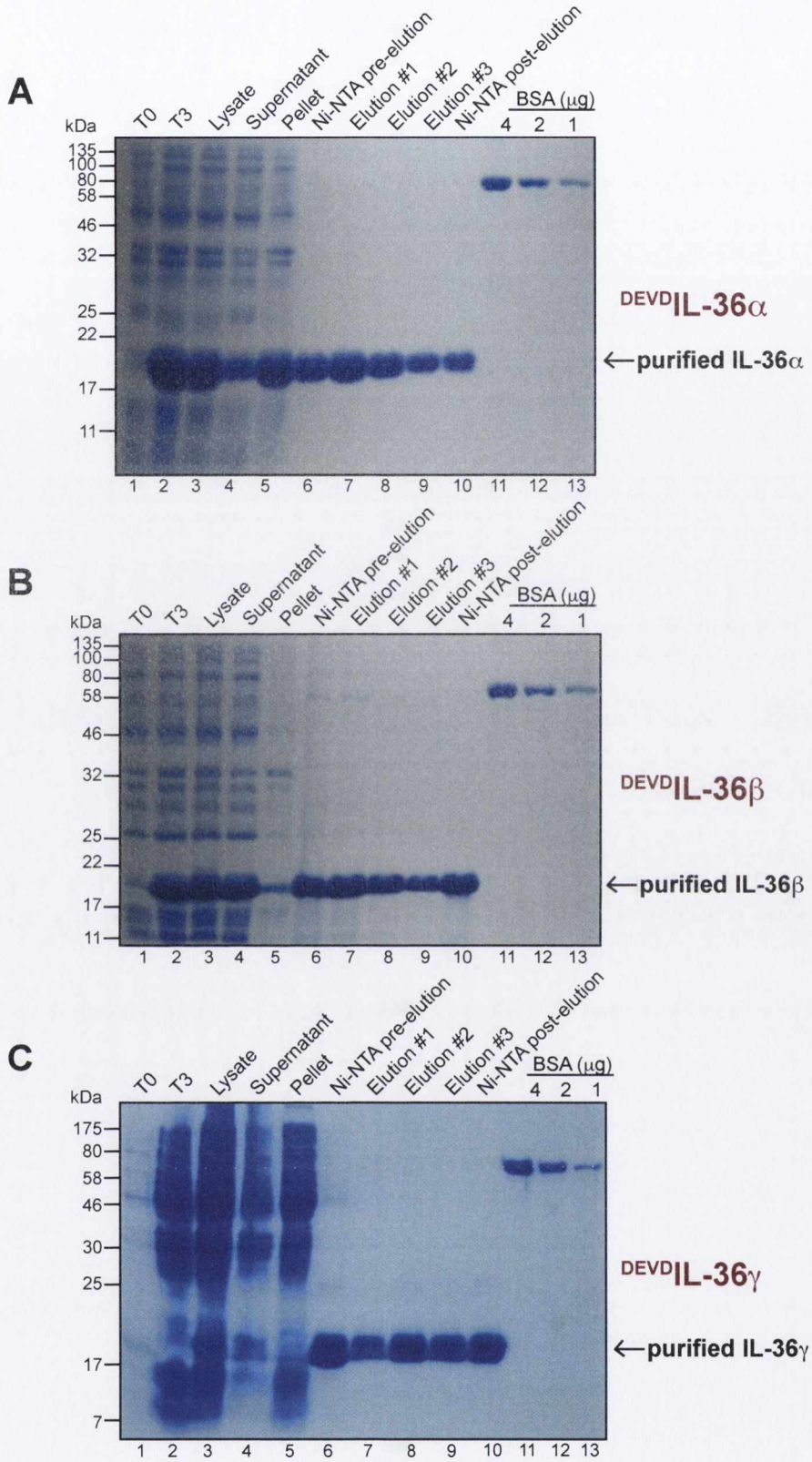

Figure 3.2 Recombinant DEVD-modified IL-36 expression and purification from bacteria
A. SDS-PAGE gel of samples taken at indicated stages of bacterial expression and purification of ^{DEVD}IL-36α from a 250 ml culture of BL21-CodonPlus(DE3)-RIL bacteria. Samples of bacterial lysate were taken before (T0) (lane 1), or after (T3) (lane 2), induction of protein expression. Note the appearance of the ~18kDa ^{DEVD}IL-36α band after induction of expression. Equivalent volumes of bacterial lysate after sonication (Lysate) (lane 3), the clarified bacterial supernatant (Supernatant) (lane 4) and the insoluble material removed (Pellet) (lane 5) were also run. A sample of Ni-NTA agarose before and after protein capture was run alongside 10 µl samples of each elution fraction. **B.** SDS-PAGE gel of samples taken at various stages of bacterial purification of ^{DEVD}IL-36β. **C.** SDS-PAGE gel of samples taken at various stages of bacterial expression and purification of ^{DEVD}IL-36γ. 10 µl samples of each elution fraction were run. Samples were run alongside indicated amounts of BSA for estimation of protein concentration. Molecular weight markers are shown (kDa).

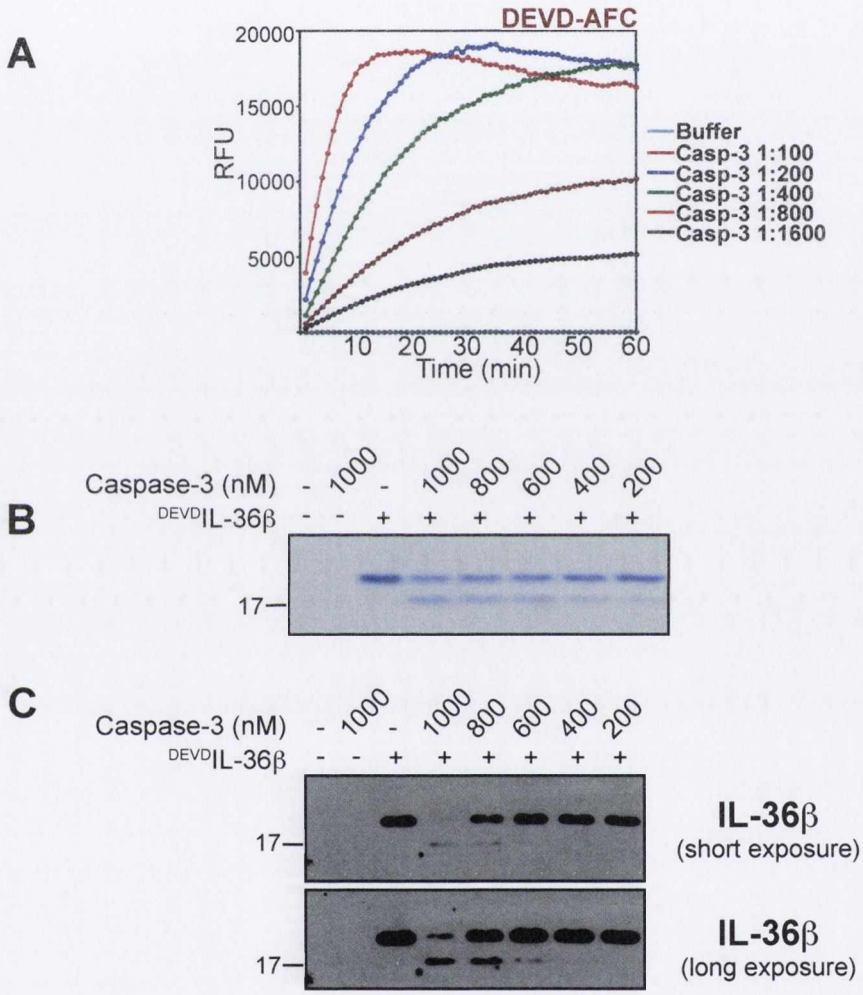

Figure 3.3 Caspase-3 cleaves DEVD-modified IL-36
A. Hydrolysis of the synthetic substrate Ac-DEVD-AMC (50 μM) by recombinant caspase-3 was monitored over the indicated time-course. **B.** Recombinant full-length ^{DEVD}IL-36β (100 ng/ml) was incubated at 37 °C for 2 h, either alone or in the presence of indicated concentrations of recombinant caspase-3. Proteolysis was analyzed by SDS-PAGE gel electrophoresis. **C.** Full-length ^{DEVD}IL-36β (100 ng/ml) was incubated at 37 °C for 2 h, either alone or in the presence of indicated concentrations of recombinant caspase-3, followed by analysis by immunoblot.

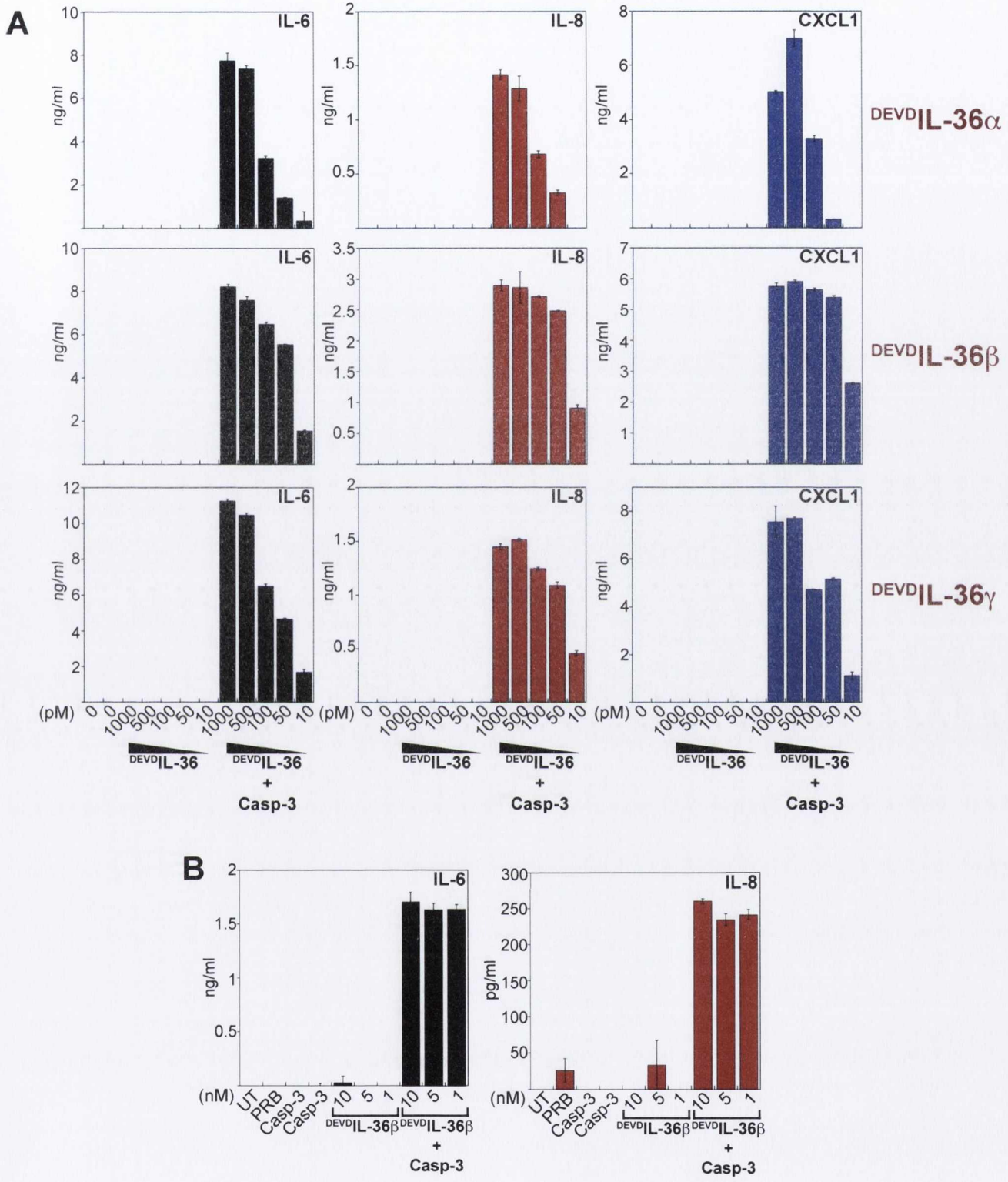

Figure 3.4 Truncated IL-36 proteins exhibit robust biological activity
A. ^{DEVD}IL-36α, ^{DEVD}IL-36β and ^{DEVD}IL36γ (250 nM) were incubated either alone or in the presence of caspase-3 for 2 h at 37 °C. HeLa cells stably overexpressing IL36R (5x10^4 cells per well) were stimulated with a titration of ^{DEVD}IL-36, as indicated. After 18 h, cytokine concentrations in the culture supernatants were determined by ELISA. **B.** ^{DEVD}IL-36β (5 mM) was incubated either alone or in the presence of caspase-3 for 2 h at 37 °C. Protease reaction buffer (PRB) or caspase-3 alone were used as negative controls. HeLa cells stably overexpressing IL36R (5x10^4 cells per well) were stimulated with a titration of ^{DEVD}IL-36, as indicated. After 18 h, cytokine concentrations in the culture supernatants were determined by ELISA. Error bars represent the mean +/- SEM of duplicate determinations from a representative experiment.

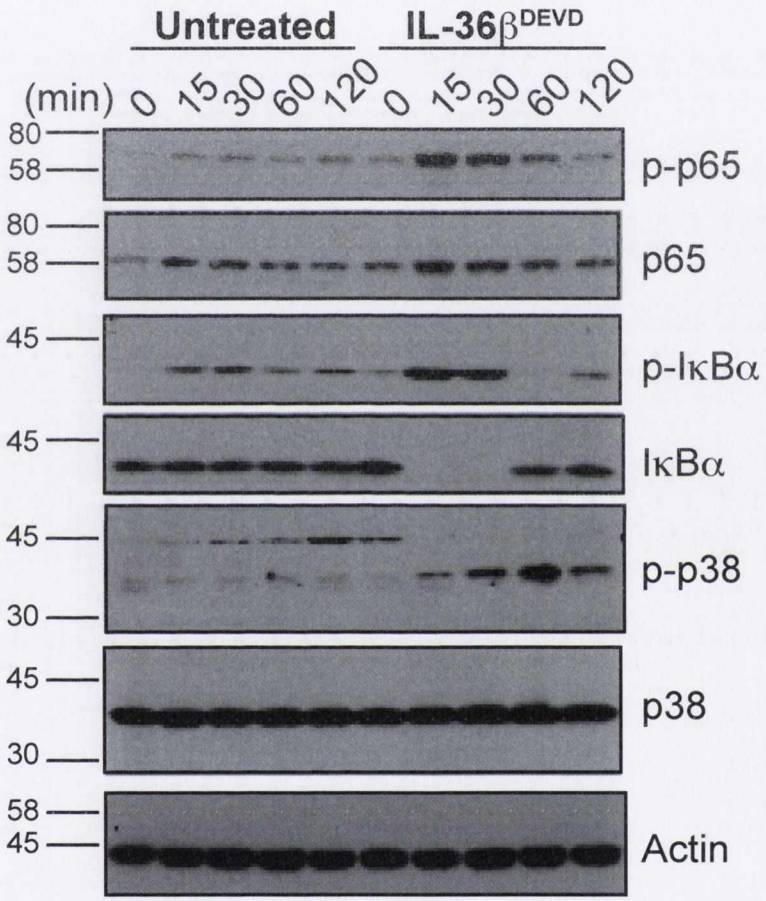

Figure 3.5 IL-36 activates NFκB and MAPK signalling pathways
HeLa$^{IL36R\text{-}SEAP}$ cells ($1x10^6$ cells per 6 cm plate) were stimulated, or not, with 5 nM of active ^{DEVD}IL-36β. At indicated timepoints, cell lysates were made in 200 μl SDS loading buffer. Whole-cell lysates were analysed by immunoblot with the indicated antibodies.

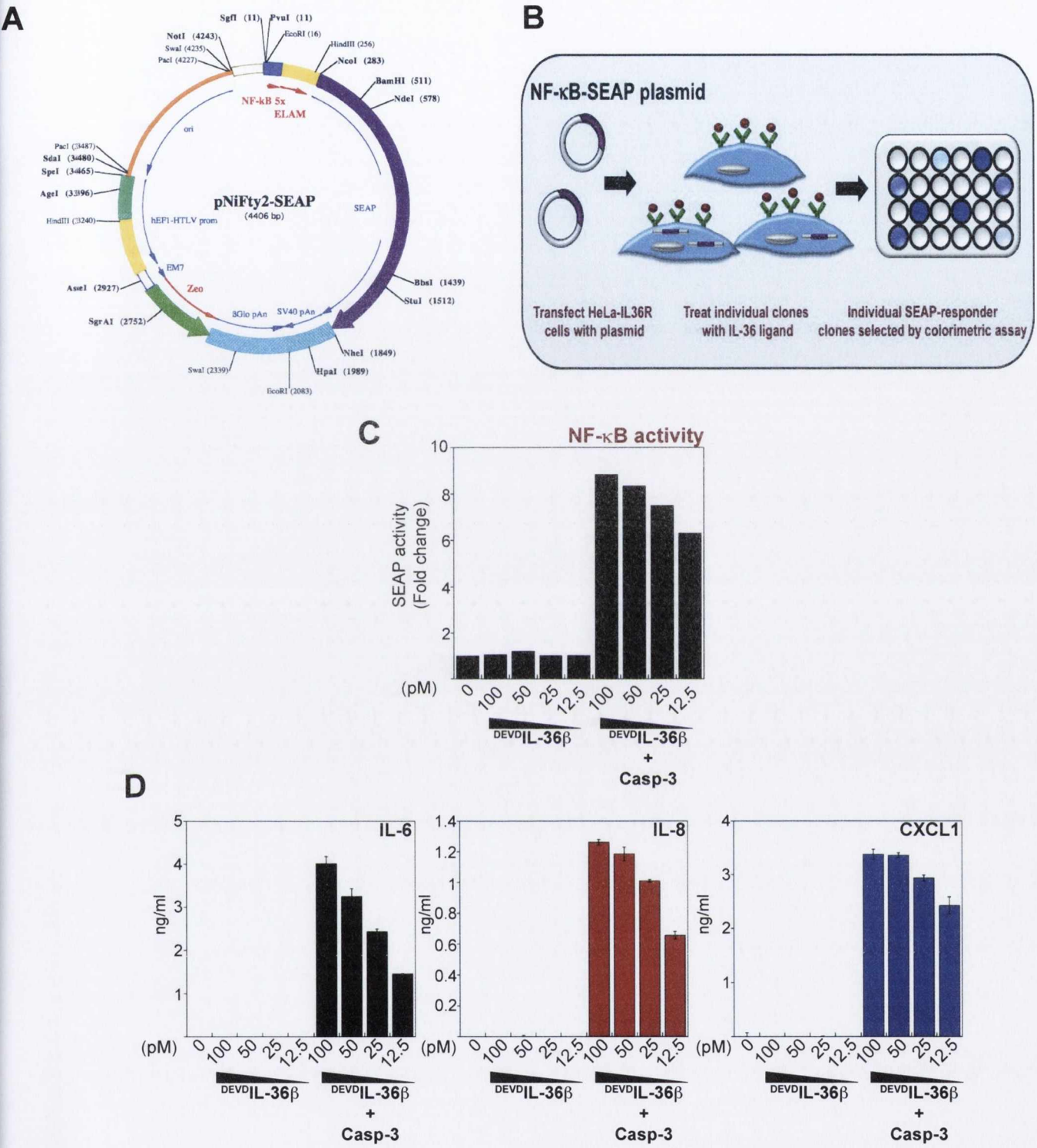

Figure 3.6 Establishment of HeLa^{IL36R} NFκB SEAP reporter cell line

A. Schematic of pNiFty2-SEAP plasmid map used to establish NFκB reporter cell line (InvivoGen). **B.** HeLa$^{IL36R\text{-}SEAP}$ cell line was generated by transfection of 5 μg pNiFty2-SEAP plasmid followed by selection using Zeocin antibody (InvivoGen). SEAP overexpressing 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. **C.** HeLa^{IL36R} cells stably expressing the NF-κB SEAP reporter plasmid were stimulated with a titration of ^{DEVD}IL-36β, as indicated. After 18 h, cell culture supernatant was incubated with Quantiblue reagent to detect secreted alkaline phosphatase in the medium. Absorbance was read at 620 nm after 4 h incubation with Quantiblue reagent. **D.** Cytokine concentrations in the culture supernatants from (C) were determined by ELISA. Error bars represent the mean +/- SEM of duplicate determinations from a representative experiment.

the epidermal layers of the skin, driven in part through the release of inflammatory mediators from keratinocytes. Thus, we asked whether transformed HaCaT keratinocytes, as well as primary human keratinocytes were responsive to caspase-3-cleaved DEVD-modified IL-36 cytokines. As shown in Fig. 3.7, both cell types were highly responsive to caspase-3-cleaved, but not full-length, ^{DEVD}IL-36, once again confirming that caspase-3-processed ^{DEVD}IL-36 is highly active and can be readily produced in large amounts for the exploration of IL-36 function in cellular and molecular studies. Furthermore, these data also show that insertion of a simple tetrapeptide caspase cleavage motif is a useful strategy for the facile removal of purification tags or protein domains that interfere with protein solubility of activity.

3.4.6 Generation of polyclonal antibodies against individual IL-36 proteins

We also used recombinant IL-36 proteins to immunize rabbits with individual IL-36 proteins (i.e. α, β or γ) to generate polyclonal antibodies capable of recognizing the latter cytokines (Fig. 3.8 A). As Fig. 3.8 B illustrates, this approach yielded antibodies that were highly specific for IL-36β and IL-36γ, with little cross-reactivity against each other, as well as antibodies that were relatively specific for IL-36α. Moreover, the IL-36γ polyclonal antibody was capable of detecting endogenous levels of IL-36γ from primary human keratinocytes (Fig. 3.8 C). These antibodies will be useful for investigations exploring the factors that influence IL-36 expression in healthy versus inflamed tissues.

3.2.7 Use of small molecule inhibitors to investigate IL-36R downstream signalling

IL-36 cytokine family signals through IL-1Rrp2 receptor, now known as IL-36 receptor, however the downstream signalling events occurring after receptor ligation have thus far not been fully elucidated. Molecules recruited to the IL-36 receptor after activation may present potential targets for therapies, especially in skin inflammation. To investigate the downstream signalling events after IL-36 receptor activation, we used small molecule inhibitors to target likely candidate pathways that may mediate IL-36 signalling (Fig. 3.9). We hypothesised that IL-36 signalling may be similar to its related family members, IL-1α and IL-1β which signal through the well-investigated IL-1 receptor.

3.2.8 NFκB inhibition

As shown in Fig. 3.5 and 3.6, DEVD-modified IL-36 can robustly activate NFκB and promote IκB degradation and subsequently induce cytokine and chemokine production.

To confirm the crucial role of the NFκB pathway in IL-36 receptor signalling, we used the NFκB inhibitor BAY-11-7082 which selectively and irreversibly inhibits IκBα phosphorylation. HeLa^{IL36R} cells were pretreated with BAY-11-7082, followed by treatment with active ^{DEVD}IL-36β. Biological activity was measured by the level of cytokine induction in culture media, with mature IL-1β p17 used as a positive control. High concentrations of BAY-11-7082 caused cell death (Fig 3.10 A), by blocking the pro-survival role of NFκB, as previously reported (Rauert-Wunderlich et al., 2013). BAY-11-7082 was titrated on HeLa cells over a range of concentrations to find optimal doses that block NFκB activation, without accompanying cell death. Inhibition of NFκB potently suppressed IL-6 and IL-8 secretion from HeLa^{IL36R} cells in response to active ^{DEVD}IL-36, highlighting the importance of NFκB in IL-36 receptor signalling. A similar inhibition of cytokine induction was seen in response to active IL-1β (Fig 3.10 B).

3.2.9 MAP kinase inhibition

We next investigated whether MAPK signalling pathways are involved downstream of the IL-36 receptor upon IL-36 cytokine ligation using small molecule MAP kinase inhibitors. The chemical inhibitor UO126 is a selective dual inhibitor of MEK1 and MEK2 protein kinases, also known as ERK. UO126 had no effect on cell viability, even at high concentrations of the inhibitor (Fig 3.11 A). Inhibition of ERK robustly reduced the levels of IL-6 and IL-8 released after IL-36β treatment, suggesting ERK kinases are involved in IL-36 receptor signalling. ERK inhibition also strongly antagonised signalling through the IL-1 receptor, with diminished cytokine induction when cells are treated with mature IL-1β in combination with the UO126 inhibitor (Fig 3.11 B).

As shown in Fig. 3.5, active ^{DEVD}IL-36β promotes activation of p38 MAPK through phosphorylation of p38. Thus, we also looked at the contribution of p38 MAPK in IL-36R signalling using two chemical inhibitors of p38, SB202190 and SB203580. SB202190 and SB203580 selectively bind to the ATP pocket of p38 to inhibit the kinase activity. Neither SB202190 nor SB203580 p38 inhibitor were cytotoxic to HeLa^{IL36R} cells at any concentration of inhibitor used (Fig 3.12 A, C). SB202190 greatly reduced IL-36β-mediated IL-6 secretion, and also IL-8, albeit to a lesser extent. SB202190 had a much greater effect on IL-1β signalling and associated cytokine secretion, with a robust reduction in IL-6 and IL-8 when cells were pretreated with the p38 inhibitor (Fig 3.12 B).

Similar results were seen with the SB203580 p38 inhibitor. IL-36β-induced IL-6 was reduced when p38 was inhibited, however there was little effect on IL-8. In contrast, a

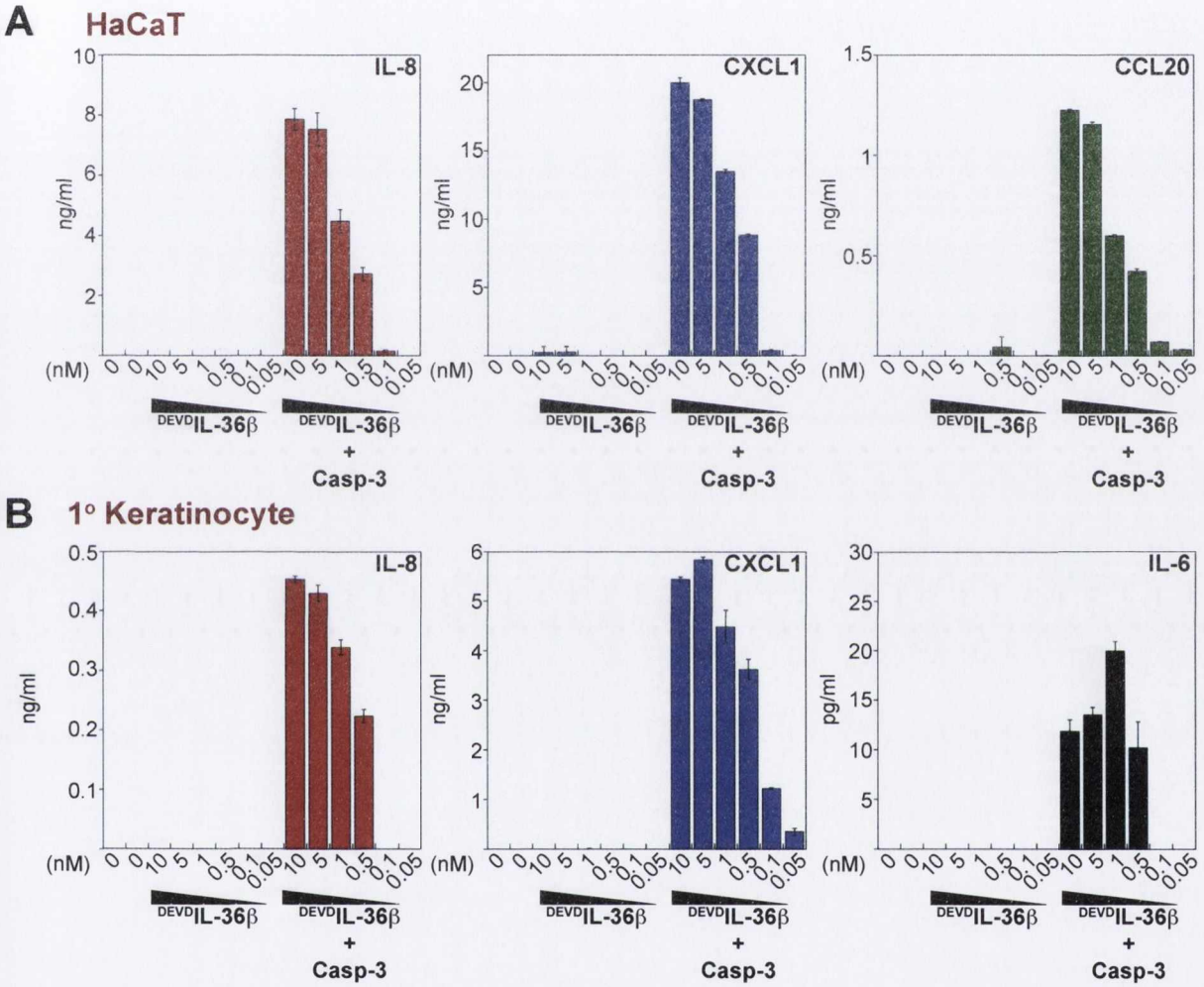

Figure 3.7 Truncated IL-36 proteins exhibit biological activity on keratinocytes
(A) ^{DEVD}IL-36β (250 nM) was incubated, either alone, or with caspase-3 (400 nM) for 2 h at 37 °C. HaCaT cells (5x10^4 cells per well) were stimulated with ^{DEVD}IL-36β, as indicated. After 18 h, cytokine concentrations in the culture supernatants were determined by ELISA. **(B)** Primary human keratinocytes (4x10^4 cells per well) were stimulated with ^{DEVD}IL-36β, as indicated. After 18 h, cytokine concentrations in the supernatants were determined by ELISA. Error bars represent the mean +/- SEM of duplicate determinations from a representative experiment.

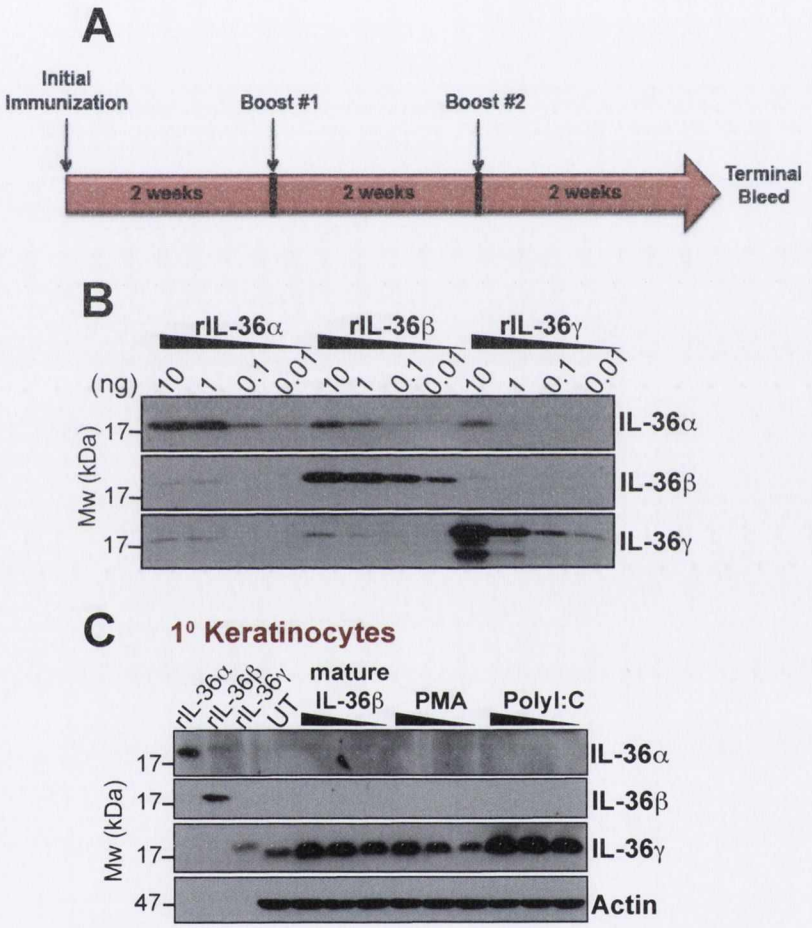

Figure 3.8 Generation of polyclonal antibodies against human IL-36 proteins
A. Schematic of the immunization protocol for generation of IL-36 polyclonal antibodies. **B.** Western blot analysis of the specificity of IL-36 polyclonal antibodies tested against indicated amounts of recombinant IL-36 proteins. **C.** Primary keratinocytes were treated with a titration of mature IL-36β (10, 5, 2.5 nM), PMA (40, 20, 10 nM) and Poly(I:C) (100, 50, 25 μg/ml). Lysates were prepared and analysed by immunoblot using IL-36 polyclonal antibodies described in (B). Recombinant IL-36 (500 pg) serves as a positive control for each immunoblot.

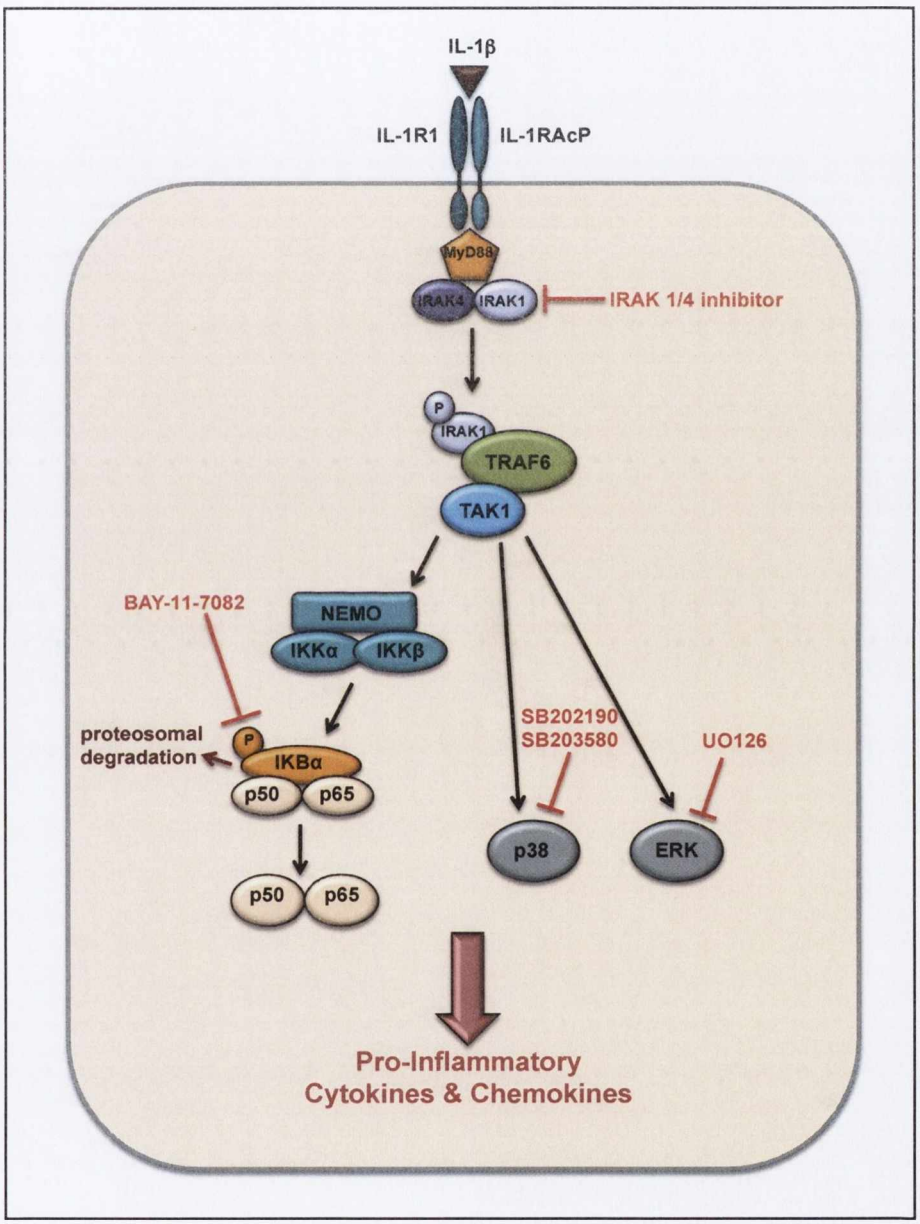

Figure 3.9 Use of small molecule inhibitors to investigate IL-36R downstream signalling
Schematic of the small molecule inhibitors used to investigate the downstream signalling events after IL-36 receptor activation. Small molecule inhibitors targeting likely candidate pathways such as NFκB and MAP kinases were used, as indicated.

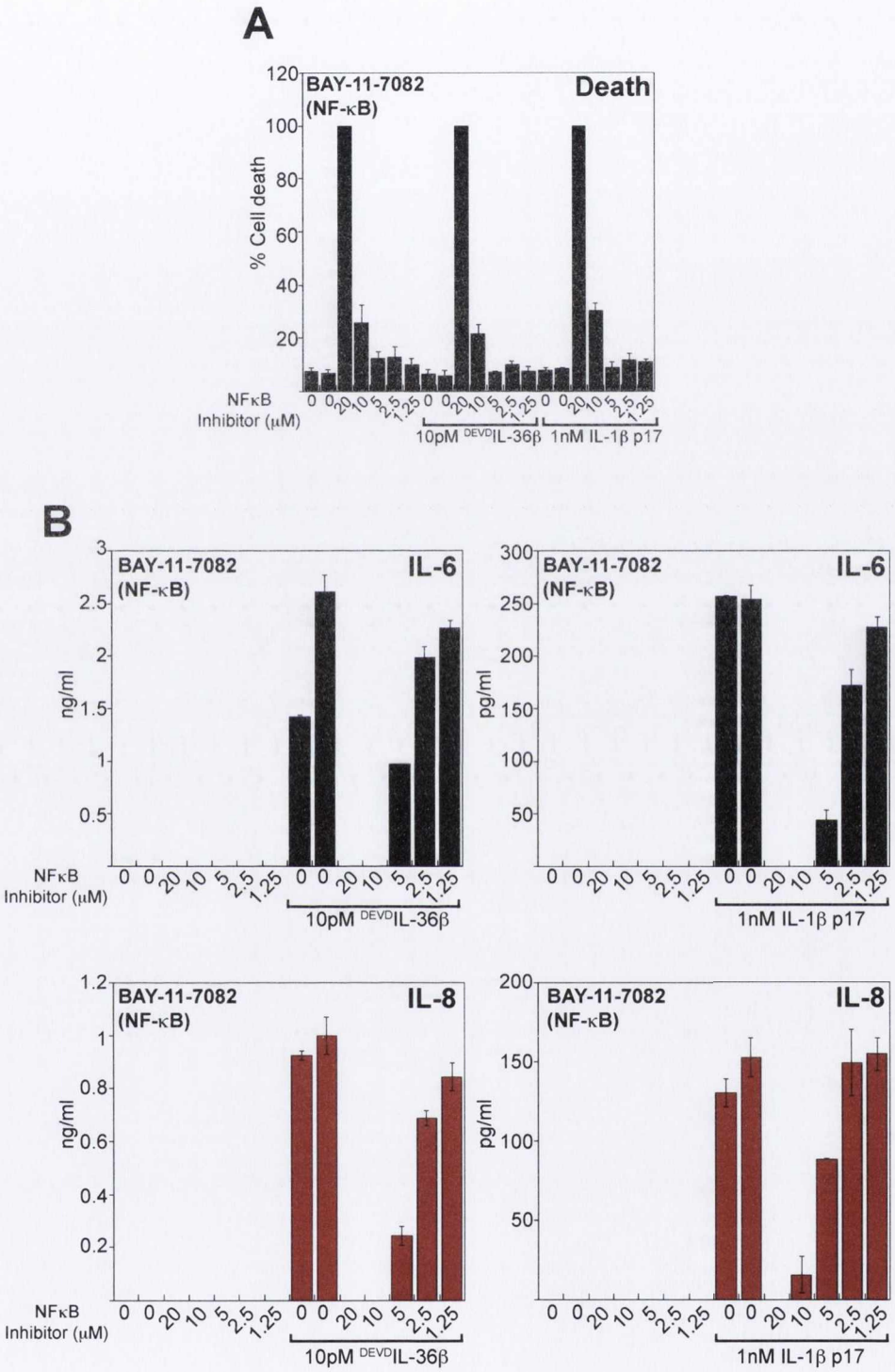

Figure 3.10 NFκB inhibition blocks IL-36-induced cytokine production
A. HeLa^{IL36R} cells were preincubated with NFκB inhibitor BAY 11-7082, as indicated, for 1 h. Cells were then treated with active ^{DEVD}IL-36β or active IL-1β p17, as indicated. After 24 h, cell death was counted by morphological analysis. **B.** After 24 h, cytokine concentrations in cell culture supernatants was measured by ELISA.

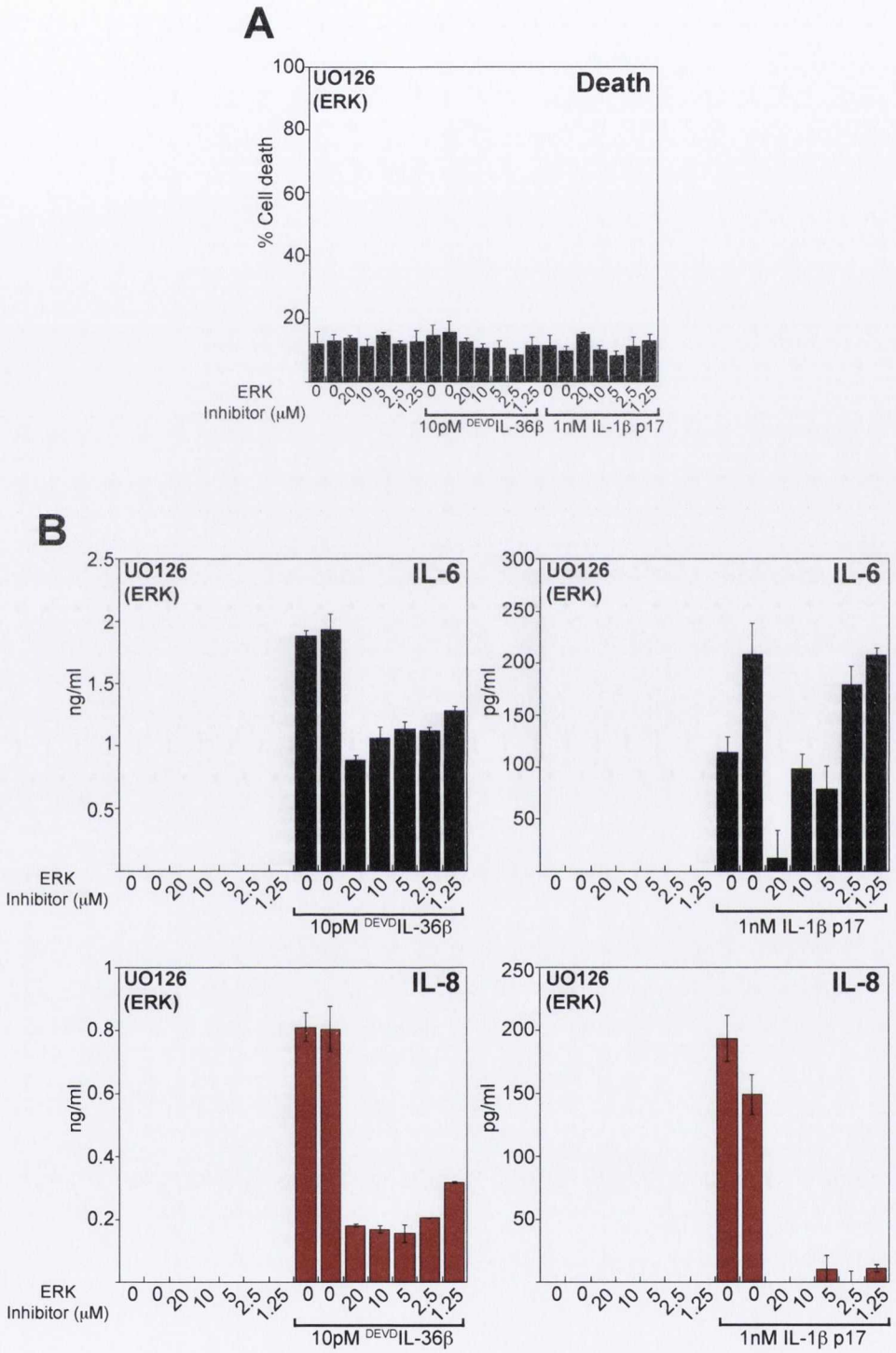

Figure 3.11 ERK inhibition blocks IL-36-induced cytokines

A. HeLa^{IL36R} cells were preincubated with ERK inhibitor UO126, as indicated, for 1 h. Cells were then treated with active ^{DEVD}IL-36β or active IL-1β p17, as indicated. After 24 h, cell death was counted by morphological analysis. **B.** After 24 h, cytokine concentration in culture supernatants were measured by ELISA.

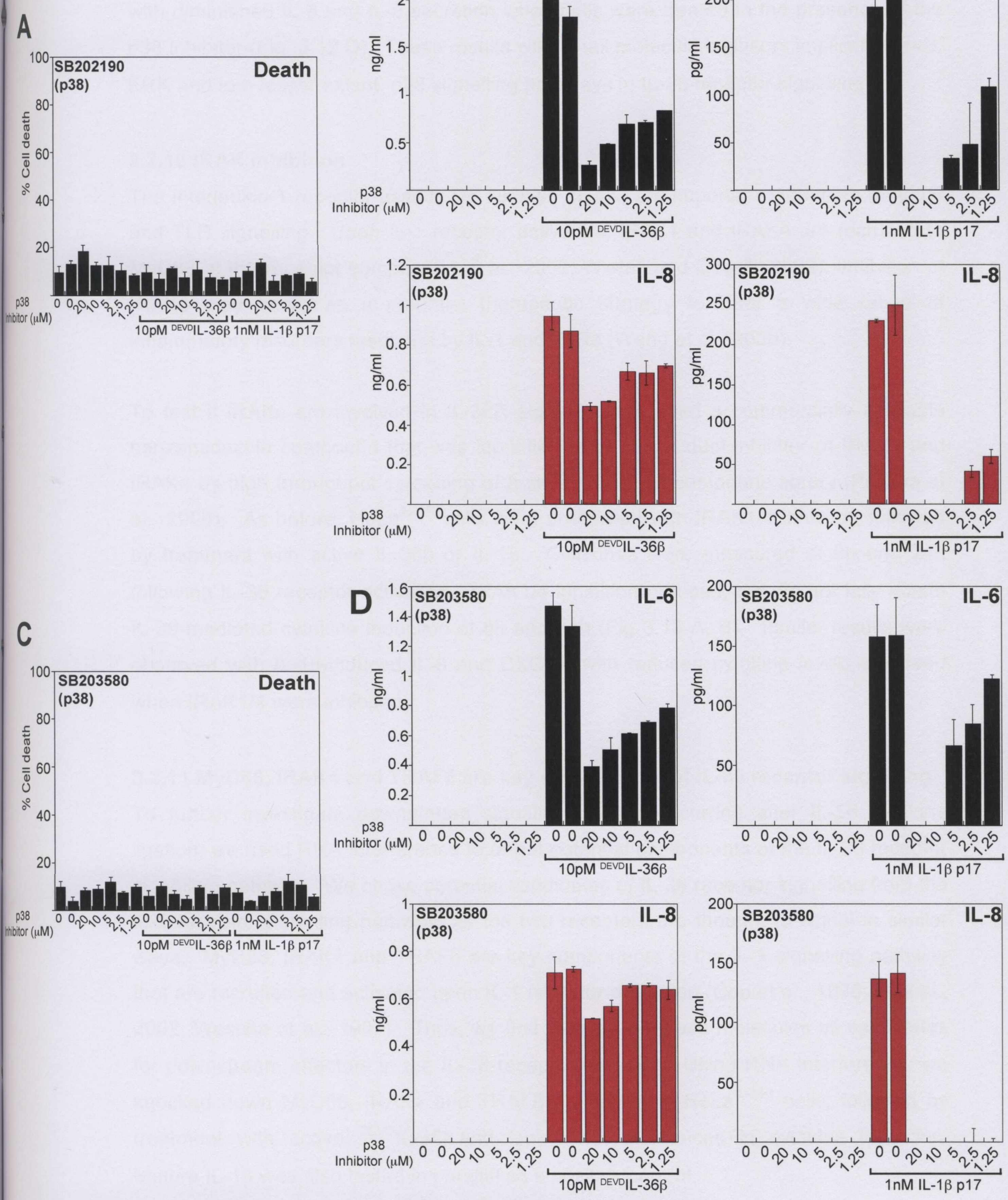

Figure 3.12 p38 inhibition partially blocks IL-36-induced cytokines

A. HeLa^{IL36R} cells were preincubated with p38 inhibitor SB202190, as indicated for 1 h. Cells were then treated with active ^{DEVD}IL-36β or active IL-1β p17. After 24 h, cell death was counted by morphological analysis. **B.** After 24 h, cytokine concentration in culture supernatants were measured by ELISA. **C.** Cells were preincubated with p38 inhibitor SB203580, followed by treatment with active ^{DEVD}IL-36β or active IL-1β p17. Cell death was counted at 24 h. **D.** After 24 h, cytokine concentration in culture supernatants were measured by ELISA.

much more pronounced effect of SB203580 was seen on the IL-1β signalling pathway, with diminished IL-6 and IL-8 secretion when cells were treated in the presence of the p38 inhibitor (Fig. 3.12 D). These results with small molecule inhibitors implicate NFκB, ERK and to a lesser extent, p38 signalling pathways in IL-36 receptor signalling.

3.2.10 IRAK inhibition

The interleukin-1 receptor-associated kinases (IRAK) are important regulators of IL-1R and TLR signalling. Upon IL-1 receptor activation, IRAK1 and IRAK4 are recruited to MyD88 at the receptor complex (Li et al., 2002; Wietek and O'Neill, 2002). Inhibition of IRAKs may serve as a potential therapeutic strategy to treat a wide range of inflammatory disorders mediated by IL-1 and TLRs (Wang et al., 2009).

To test if IRAKs are involved in IL-36R signalling, we used a commercially available benzamidazole compound that was identified as a potent dual inhibitor of IRAK1 and IRAK4 by high throughput screening of a small molecule compound library (Powers et al., 2006). As before, $HeLa^{IL36R}$ cells were pretreated with IRAK1/4 inhibitor, followed by treatment with active IL-36β or IL-1β. Cytokines were measured at 8h and 24h following IL-36 receptor activation. IRAK1/4 inhibition reduced, but did not fully ablate IL-36-mediated cytokine induction at 8h and 24h (Fig 3.13 A, B). Similar results were observed with IL-1β-induced IL-8 and CXCL1, with reduced cytokine levels observed when IRAK1/4 were inhibited.

3.2.11 MyD88, IRAK4 and TRAF6 are key components of IL-36 receptor signaling

To further investigate downstream signalling events occurring after IL-36 cytokine ligation, we used RNA interference to target potential components of the IL-36 receptor signalling pathway. We chose potential candidates of IL-36 receptor signalling from the IL-1 receptor signalling pathway, as the two receptors are thought to signal in similar ways. MyD88, IRAK4 and TRAF6 are key components of the IL-1 signalling pathway that are recruited and activated upon IL-1 receptor activation (Cao et al., 1996; Li et al., 2002; Wesche et al., 1997). Thus, we first focused on these molecules as candidates for downstream effectors in the IL-36 receptor pathway. Using RNA interference, we knocked down MyD88, IRAK4 and TRAF6 molecules in $HeLa^{IL-36R}$ cells, followed by treatment with active ^{DEVD}IL-36β and looked for differences in cytokine induction. Mature IL-1β was also tested in parallel as a positive control.

Knockdown of MyD88 completely attenuated IL-36 and IL-1-mediated IL-6 and IL-8, suggesting a crucial role for this adaptor in IL-36 receptor signalling (Fig 3.14 A). IRAK4 knockdown resulted in a partial ablation of IL-36-induced cytokines (Fig 3.14 B).

Similarly, knockdown of the adaptor TRAF6 also blunted IL-6 and IL-8 production in response to IL-36β (Fig 3.14 C).

The adaptor protein TRAF6 recruits the TAK/TAB complex, which in turn associates with NEMO to activate NFκB through translocation of the p65/p50 heterodimer to the nucleus to promote gene transcription. RNA interference was used to knockdown protein levels of NEMO, TAK1 and p65 in HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells to investigate their importance in IL-36 receptor signalling. As seen before, the requirement for MyD88 in IL-36 receptor signalling is absolute, with ^{DEVD}IL-36-induced cytokines being completely suppressed upon MyD88 ablation by RNAi. Knockdown of the NFκB subunit p65 also potently suppressed ^{DEVD}IL-36-induced cytokines, again highlighting the importance of the NFκB pathway in IL-36 receptor signalling (Fig. 3.15 A). NEMO and TAK1 knockdown was not as effective as MyD88 or p65 knockdown and only partially ablated ^{DEVD}IL-36-induced IL-6 and IL-8, despite good knockdown efficiency of these proteins, as confirmed by immunoblot of whole cell lysates (Fig. 3.15 B). Similar results were seen with IL-1β-mediated cytokine induction, with MyD88 and p65 completely ablating IL-6 and IL-8 in response to IL-1β p17, while NEMO and TAK1 had minimal effects on cytokine induction (Fig 3.15 A).

3.2.12 Downstream mediators of death receptor signalling are not involved in IL-36 signal transduction

As well as looking at candidates from the IL-1 receptor pathway, we also sought to investigate the role of novel mediators in IL-36 receptor signalling. To this end, we focused on mediators involved in TNF, TRAIL and Fas receptor signalling as potential members of the IL-36 receptor cascade. MyD88 knockdown was used as a positive control for all experiments, as we have already confirmed the crucial role for MyD88 in IL-36 signalling (Fig 3.14 and 3.15). Knockdown of FADD, TRADD, RIPK1, caspase-8, or cullin-3 had no effect on NFκB activation by ^{DEVD}IL-36β, thereby ruling out their involvement in IL-36 receptor signalling (Fig 3.16 A, B). A possible role for non-canoncial NFκB activation in IL-36 signalling remains obscure as NIK knockdown could not be validated by immunoblot. XIAP knockdown resulted in partial ablation of IL-36β-mediated NFκB activation. However, although both siRNAs knock XIAP down to an extent, they have opposing effects on IL-36-induced SEAP activity, suggesting that the contribution, if any, of XIAP to this pathway requires further investigation (Fig 3.16 B).

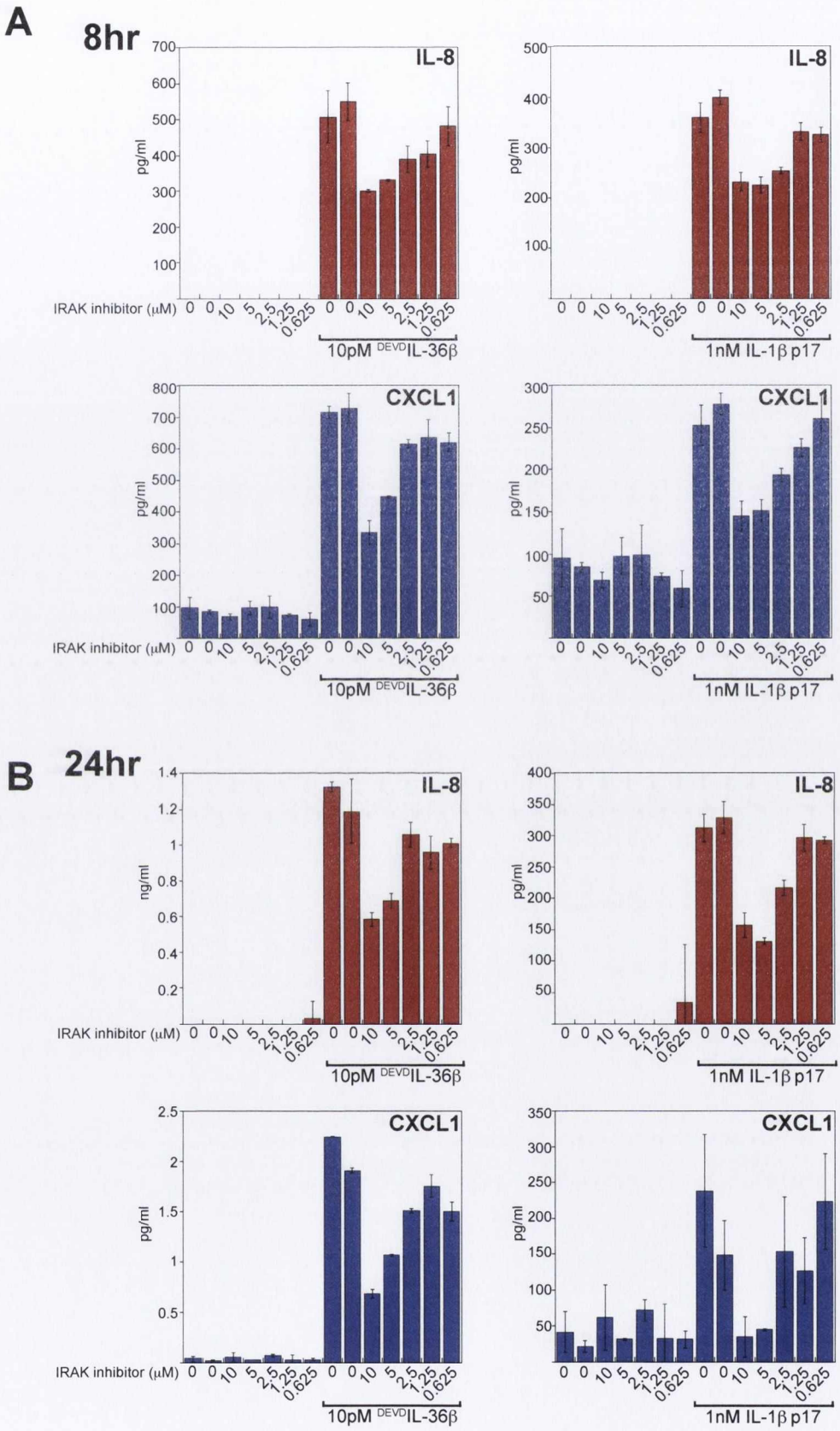

Figure 3.13 IRAK inhibition partially ablates IL-36-induced cytokines
A. HeLa^{IL36R} cells were preincubated with IRAK1/4 inhibitor, as indicated for 1 h. Cells were then treated with active ^{DEVD}IL-36β or active IL-1β p17. Cytokine concentration in culture supernatants were measured by ELISA after 8 h, or **B.** 24 h.

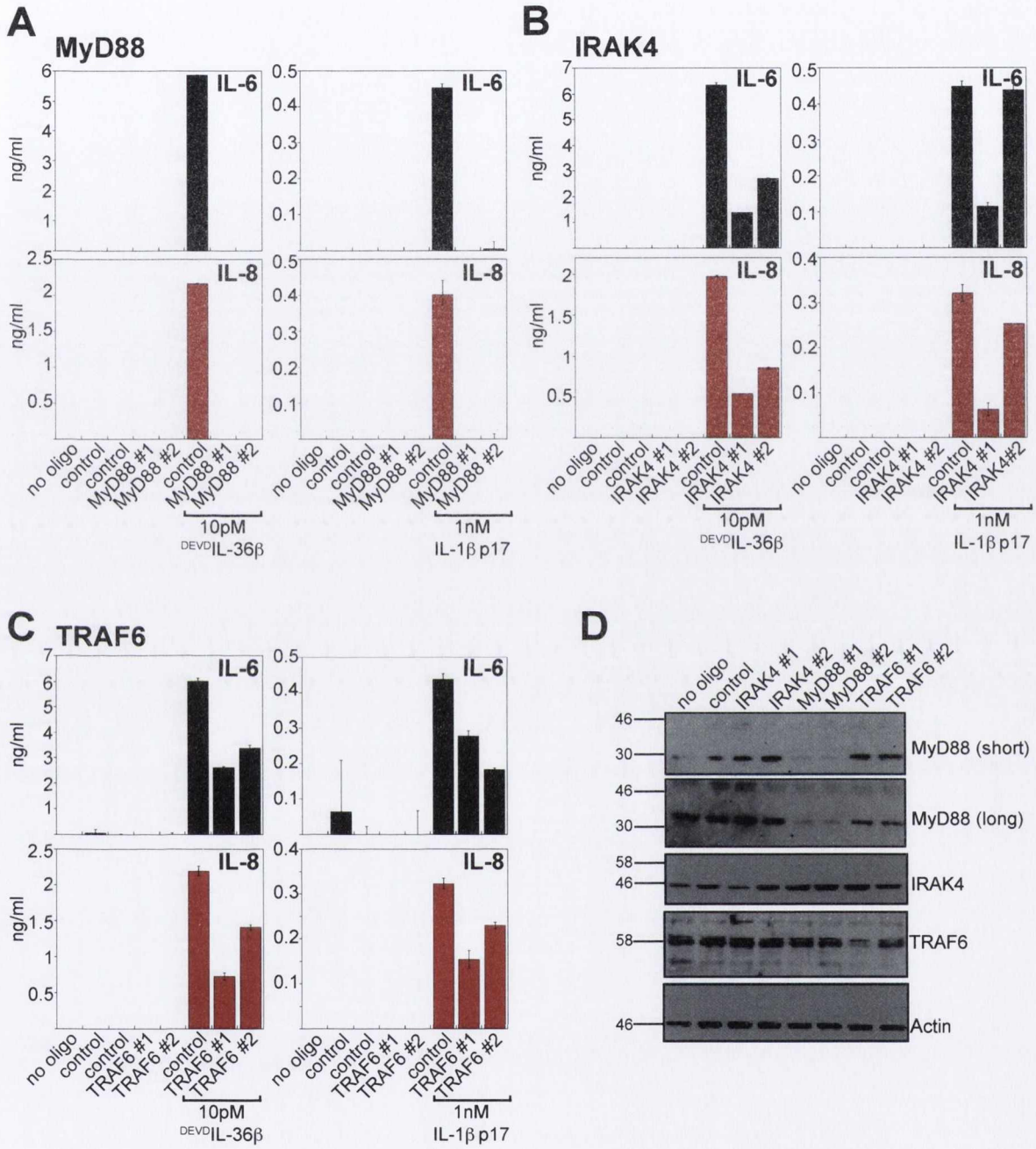

Figure 3.14 MyD88, IRAK4 and TRAF6 are key components of IL-36 receptor signalling
A. HeLa^{IL36R} cells were nucleofected with siRNAs (200nM) targeting MyD88, **B.** IRAK4 or **C.** TRAF6. After 48 h, cells were treated with active ^{DEVD}IL-36β or IL-1β p17, as indicated. Cytokine concentration in culture supernatants was measured by ELISA after 18 h. **D.** Whole-cell lysates were analysed by immunoblot to determine knockdown efficiency.

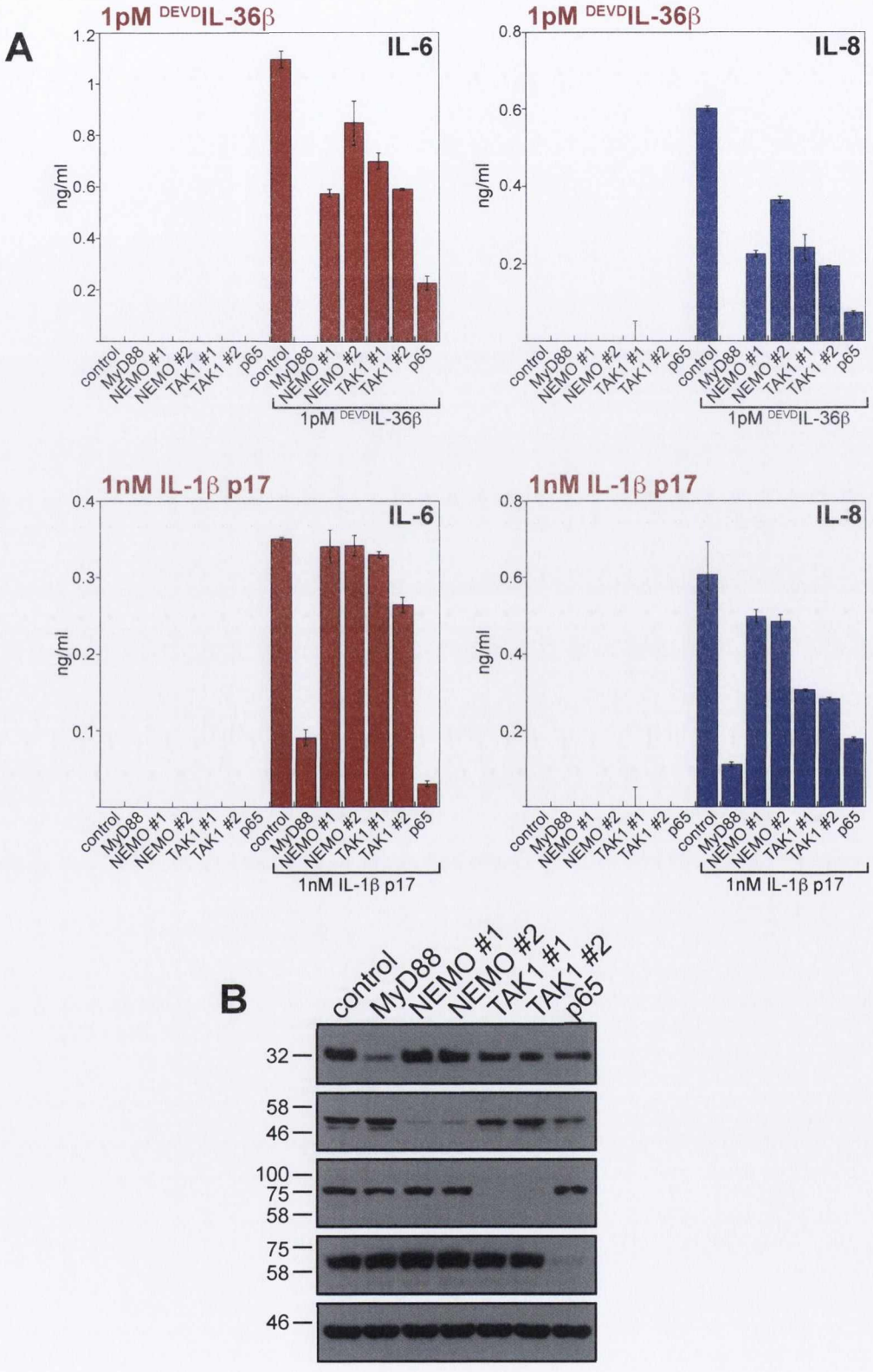

Figure 3.15 p65-mediated NFκB activation is crucial for IL-36 receptor signalling
A. HeLa$^{IL36R\text{-}SEAP}$ cells were nucleofected with siRNAs, as indicated. After 48 h, cells were left untreated or treated with active ^{DEVD}IL-36β or IL-1β p17, as indicated. IL-6 and IL-8 concentrations in cell culture supernatants were measured by ELISA after 18 h. **B.** Lysates from **A.** were analysed by immunoblot with specific antibodies to check knockdown efficiency.

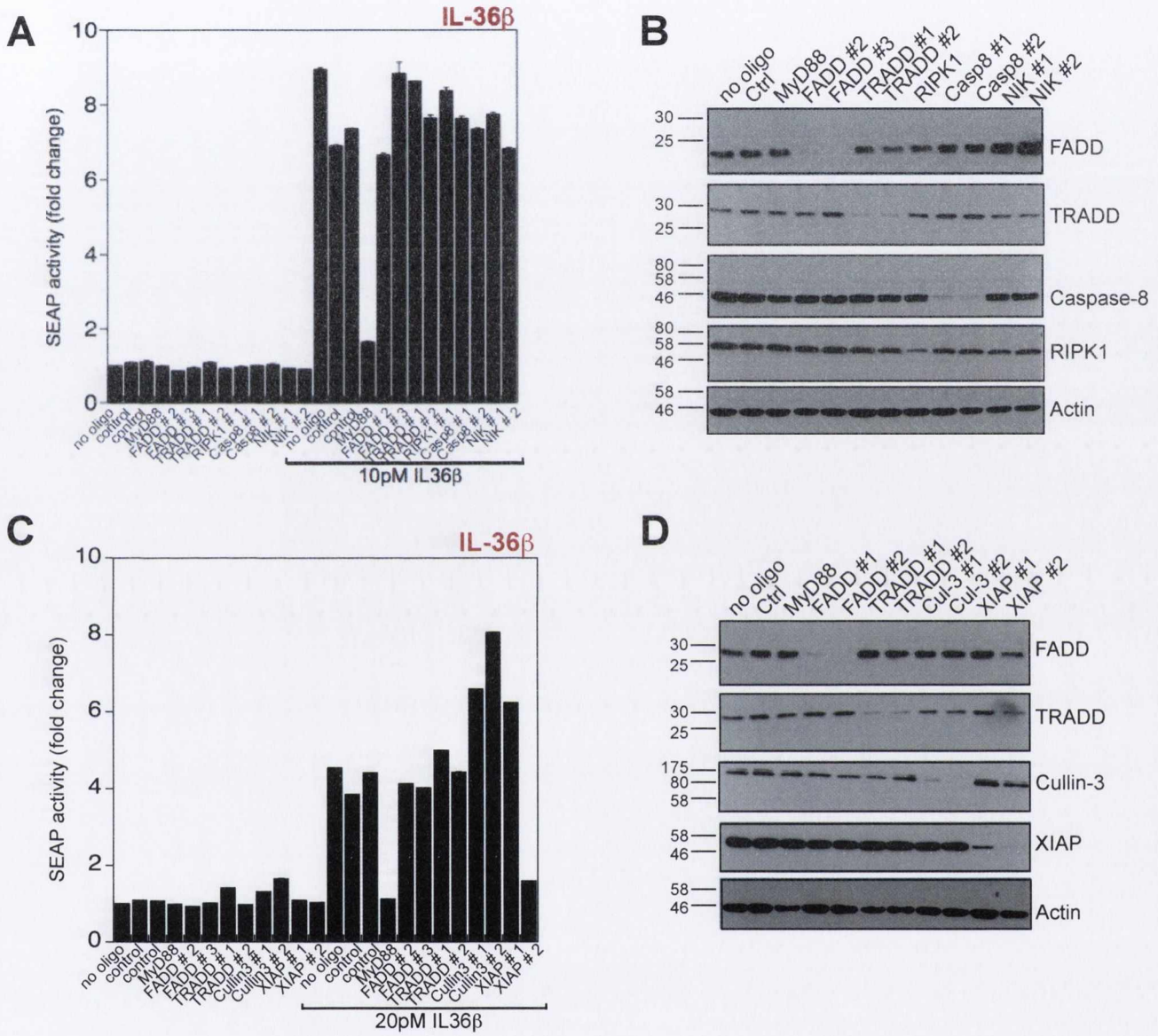

Figure 3.16 Mediators of death receptor signalling are not involved in IL-36R signal transduction
A. HeLa$^{IL36R\text{-}SEAP}$ cells were nucleofected with siRNAs, as indicated. After 48 h, cells were treated with active ^{DEVD}IL-36β and NFκB activation was measured by SEAP release via QuantiBlue assay after 18 h. **B.** Lysates from **A.** were analysed by immunoblot to check knockdown efficiency. **C, D.** HeLa$^{IL36R\text{-}SEAP}$ cells were nucleofected with siRNAs, as indicated. After 48 h, cells were treated with active ^{DEVD}IL-36β and NFκB activation was measured by SEAP release via QuantiBlue assay after 18 h.

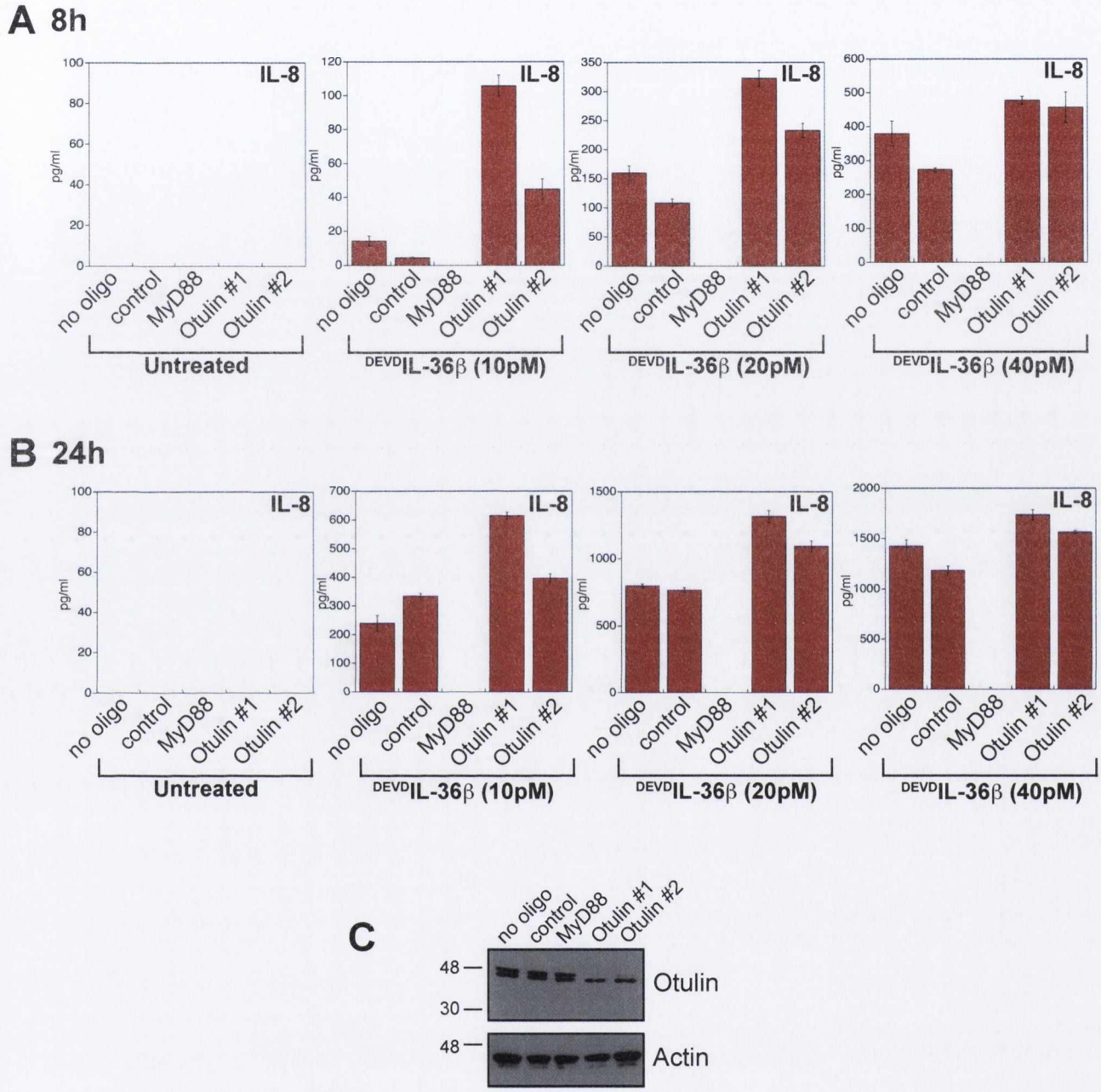

Figure 3.17 OTULIN knockdown enhances IL-36-induced cytokines

A. HeLa$^{IL36R\text{-}SEAP}$ cells were nucleofected with siRNAs, as indicated. After 48 h, cells were treated, or not, with increasing concentrations of active ^{DEVD}IL-36β. Cytokine concentration in the culture supernatants was measured by ELISA after 8 h or **B.** 24 h. **C.** Knockdown efficiency was analysed by immunoblot of whole cell lysates.

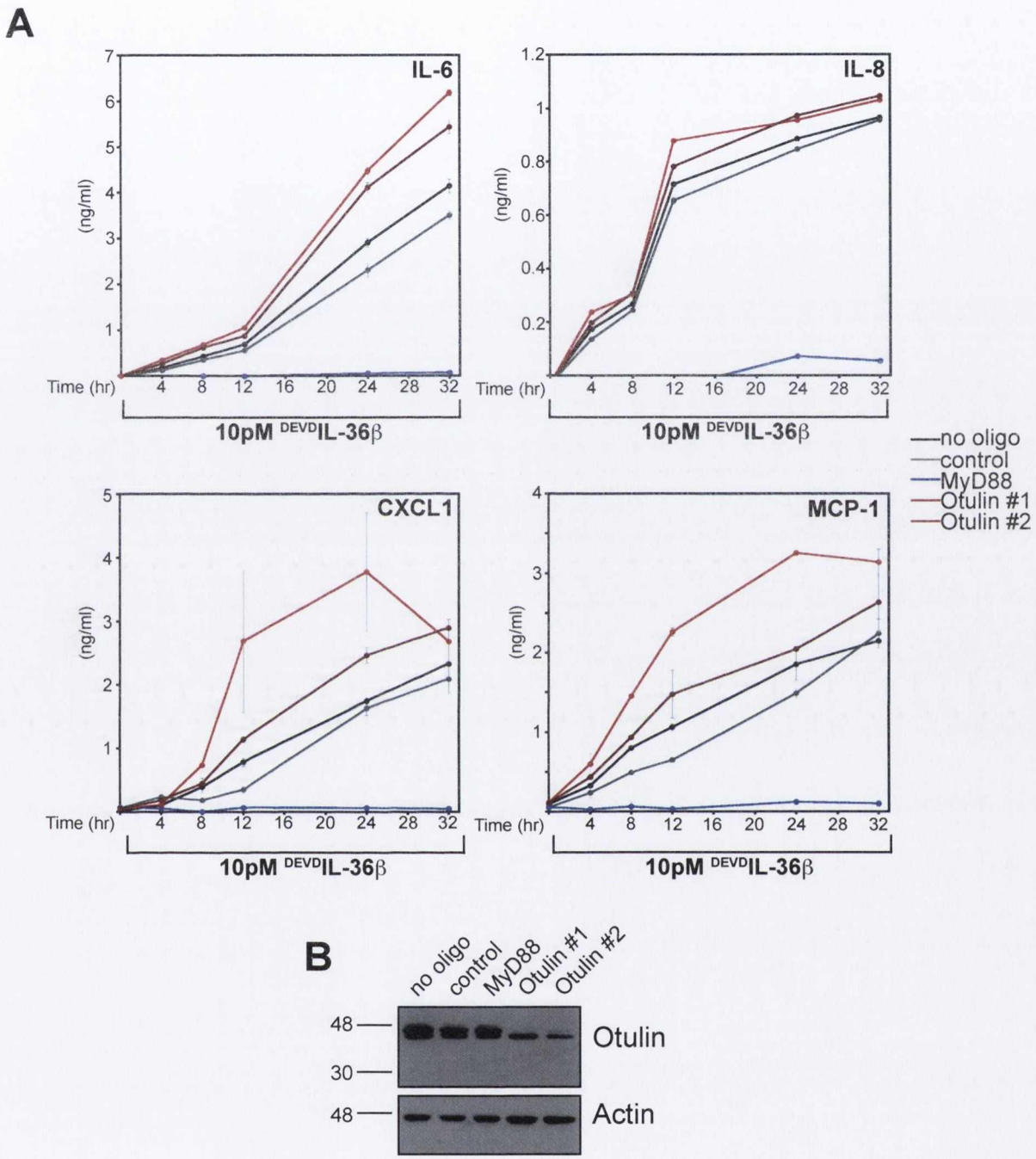

Figure 3.18 Suppression of OTULIN activity enhances production of IL-36-induced cytokines
A. HeLa$^{IL36R\text{-}SEAP}$ cells were nucleofected with siRNAs, as indicated. After 48 h, cells were treated, or not, with active ^{DEVD}IL-36β. Cell culture supernatants were harvested at indicated timepoints and cytokine concentrations in the supernatants was measured by ELISA. **B.** Knockdown efficiency was analysed by immunoblot.

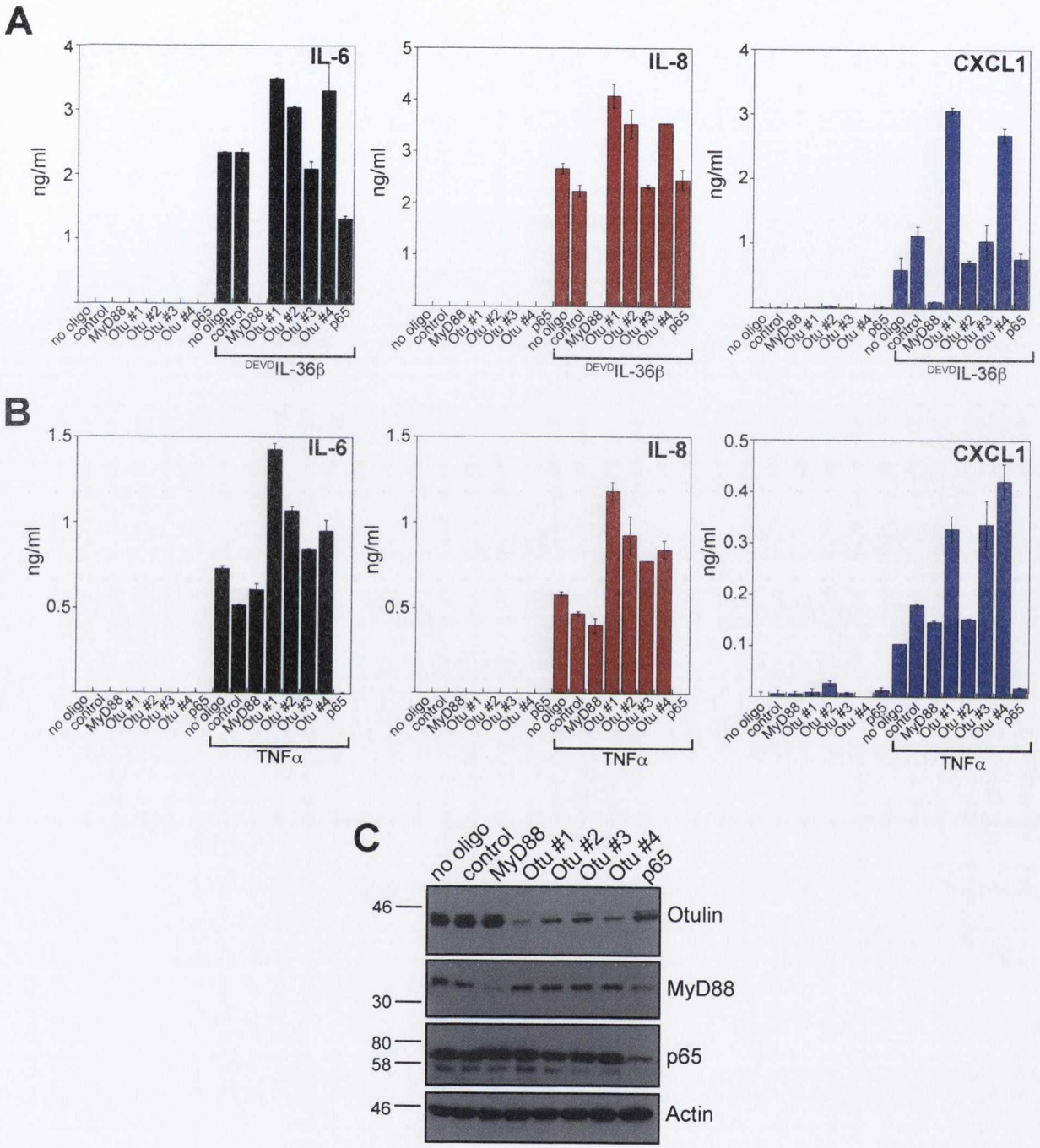

Figure 3.19 OTULIN negatively regulates IL-36 and TNF signalling

A. HeLa$^{IL36R\text{-}SEAP}$ cells were nucleofected with siRNAs, as indicated. After 48 h, cells were treated with active ^{DEVD}IL-36β (5 pM). Cytokine concentration in the culture supernatants was measured by ELISA after 18 h. **B.** HeLa$^{IL36R\text{-}SEAP}$ cells were nucleofected with siRNAs, as indicated. After 48 h, cells were treated with TNFα (5 ng/ml). Cytokine concentration in the culture supernatants was measured by ELISA after 18 h. **C.** Knockdown efficiency was analysed by immunoblot of whole cell lysates.

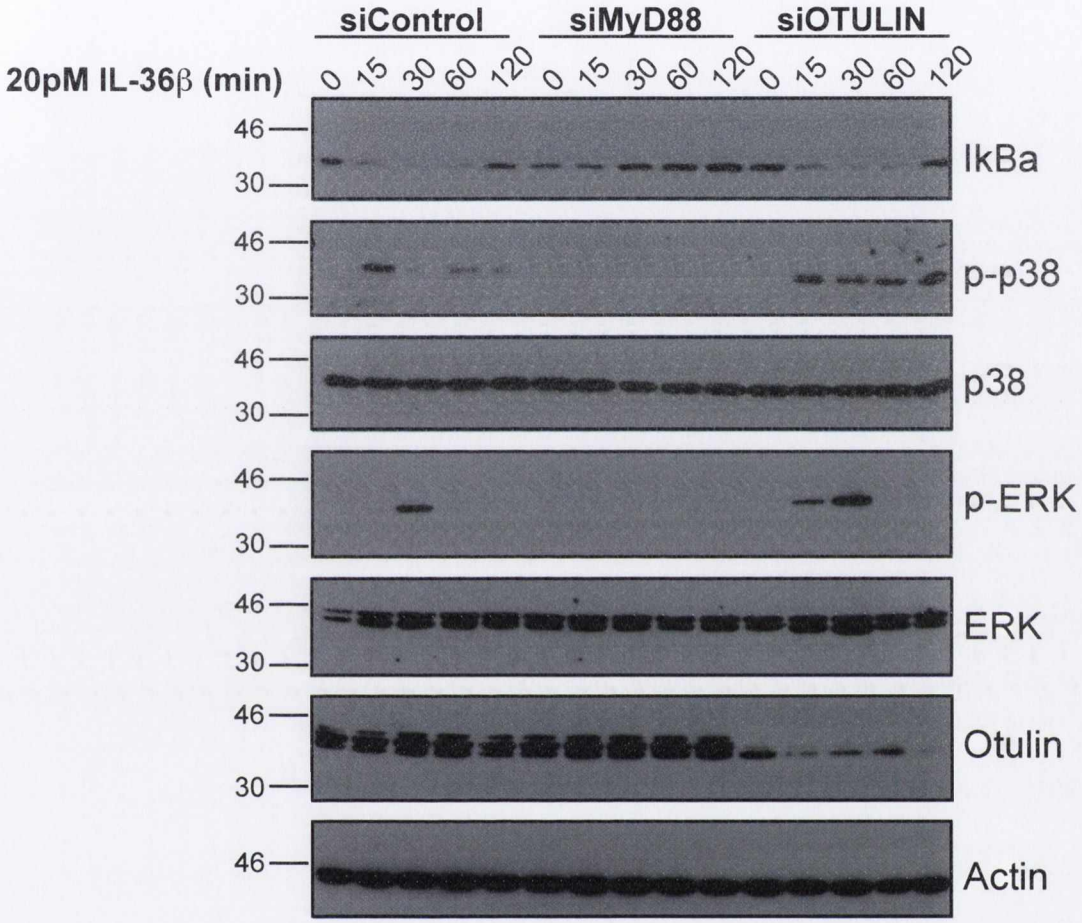

Figure 3.20 OTULIN knockdown enhances MAPK activation
HeLa$^{IL36R\text{-}SEAP}$ cells were nucleofected with siRNAs, as indicated. After 48 h, cells were treated with 20 pM active ^{DEVD}IL-36β. Cell lysates were made at indicated timpoints. Whole-cell lysates were analysed by immunoblot with the indicated antibodies.

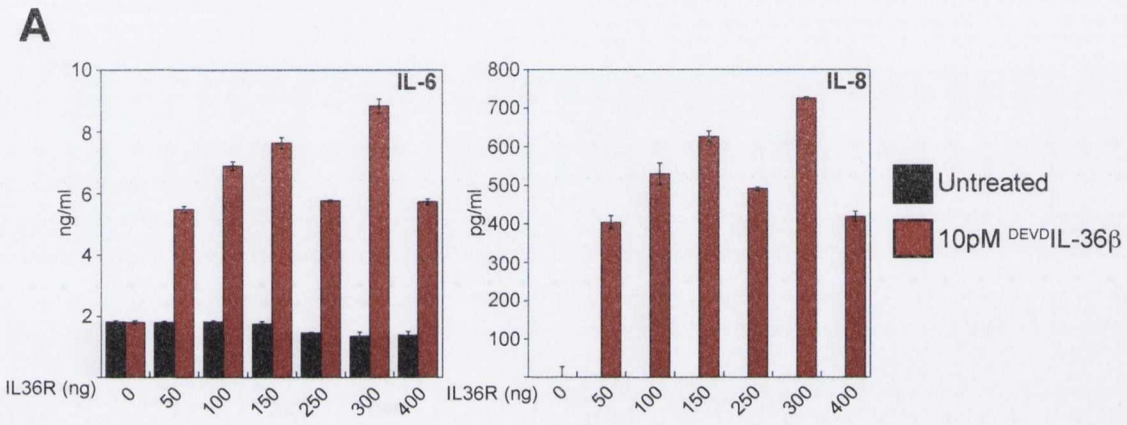

Figure 3.21 IL-36R overexpression in HeLa cells
HeLa cells were transfected with IL-36R plasmid, as indicated. After 24 h, cells were treated, or not, with active ^{DEVD}IL-36β. Cytokine concentration in culture supernatants was measured by ELISA after 18 h.

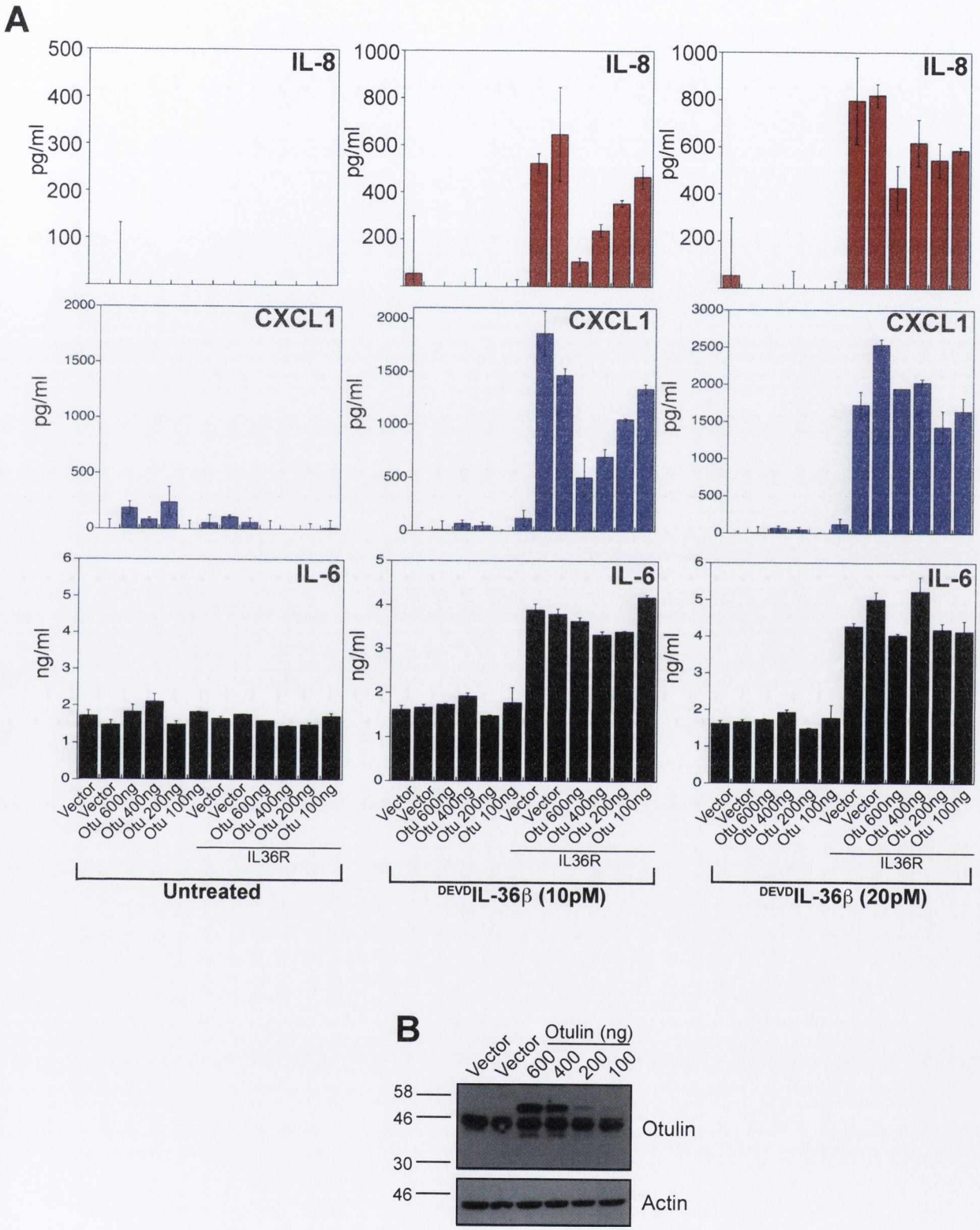

Figure 3.22 OTULIN overexpression disrupts IL-36 receptor signalling
A. HeLa cells were co-transfected with OTULIN plasmid, as indicated, and IL-36R (400 ng). After 24 h, cells were treated with active ^{DEVD}IL-36β. Cytokine concentrations were measured by ELISA. **B.** Immunoblot analysis of OTULIN overexpression in HeLa cells.

3.2.13 OTULIN deubiquitinase regulates signalling in response to IL-36R activation

As previously discussed, IL-1α and IL-1β bind to IL-1R and activate a signal cascade leading to NFκB and MAPK activation and pro-inflammatory cytokine expression. Activation of IL-1R is tightly regulated by ubiquitination, particularly Lys63-, Lys48- and linear polyubiquitin chains. The assembly of ubiquitin chains is regulated by deubiquitinase enzymes, thus further refining inflammatory signalling cascades. The deubiquitinase FAM105B/OTULIN has been recently described as a specific deubiquitinase for Met1-linked linear ubiquitin chains and has been shown to be a critical mediator in limiting pro-inflammatory responses following immune receptor activation (Emmerich et al., 2013; Fiil et al., 2013; Keusekotten et al., 2013).

To examine the involvement of linear ubiquitination in IL-36 signalling, we knocked down OTULIN deubiquitinase in HeLa^{IL36R} cells and explored whether cytokine induction was augmented following IL-36 treatment. Knockdown of MyD88 was used as a positive control. IL-36-mediated IL-8 induction was completely ablated when MyD88 was removed from the system. In contrast, OTULIN knockdown increased levels of IL-8 secretion at 8h and 24h following IL-36β treatment (Fig 3.17 A, B). This suggests that linear ubiquitination is associated with IL-36-mediated cytokine induction, and that OTULIN deubiquitinase represses the inflammatory response by regulating linear ubiquitination chain formation. Similar results were observed over a timecourse of IL-36 treatment, with increased levels of IL-6, IL-8, CXCL1 and MCP-1 observed after OTULIN knockdown (Fig 3.18)

As in figure 3.16 and 3.17, knockdown of OTULIN using a number of different siRNAs enhanced IL-6, IL-8 and CXCL1 induction in response to active IL-36β, while MyD88 knockdown fully attenuates IL-36 activity (Fig 3.19 A). Previously published work has implicated OTULIN as a regulator of TNFα signalling (Fiil et al., 2013; Keusekotten et al., 2013). Similar results were obtained using TNFα as an activating stimulus. Knockdown of OTULIN enhanced TNFα-induced IL-6, IL-8 and CXCL1, while knockdown of p65 ablated all cytokine induction in this system (Fig 3.19 B).

RNAi-mediated depletion of MyD88 blocks IκBα degradation as well as phosphorylation of p38 and ERK in response to active ^{DEVD}IL-36β, thus blocking activation of NFκB and MAP kinases and subsequent cytokine induction. Conversely, RNAi-mediated depletion of OTULIN enhanced phosphorylation of p38 and ERK MAP kinases compared with cells transfected with control siRNA in response to active IL-36β, however IκBα degradation was unaffected (Fig 3.20). Enhanced MAP kinase activation

in response to OTULIN knockdown may account for enhanced cytokine secretion upon ^{DEVD}IL-36 treatment.

3.2.14 OTULIN overexpression inhibits IL-36 signalling

Wild-type HeLa cells were transiently transfected with IL-36R in combination with OTULIN. Thus, any cell capable of responding to IL-36 should also have elevated OTULIN levels allowing us to selectively look at the effect of OTULIN overexpression on IL-36 activation. HeLa cells expressing the IL-36R were highly responsive to active IL-36β, whereas wild-type cells failed to respond (Fig 3.21). IL-36R-expressing HeLa cells robustly induce IL-8 and CXCL1 following IL-36β stimulation, however combined overexpression of OTULIN partially blocks this (Fig 3.22). However, no effect on IL-6 secretion was observed, suggesting that this is a selective effect. This data contributes to the idea of OTULIN as a negative regulator of IL-36 signalling by disrupting linear ubiquitin chains required for signalling.

3.3 Discussion

Here, we have shown that the insertion of a caspase-3 cleavage motif (DEVD) into IL-36 cytokines enabled us to produce highly active and soluble forms of these cytokines upon processing with caspase-3. These data also confirmed that proteolytic processing of IL-36α, β and γ dramatically increase the biological activity of these cytokines. We established a HeLa$^{IL\text{-}36R}$ bioassay to screen for proteases that naturally activate IL-36 cytokines, as well as a convenient reporter cell line, HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells to rapidly detect NFκB activation by IL-36 cytokines. We also investigated the downstream signalling cascade activated upon IL-36 receptor ligation by its cognate ligands, and identified MyD88, IRAK4, TRAF6 and p65 as key signalling components of this pathway which are absolutely required for pro-inflammatory cytokine and chemokine induction in response to IL-36 cytokines. Additionally, we identified the importance of linear ubiquitination in IL-36 receptor signalling and regulation of this pathway by the deubiquitinase OTULIN.

3.3.1 Production of biologically active IL-36 family cytokines through insertion of N-terminal caspase cleavage motif

IL-36 cytokines require N-terminal processing for activation, however the proteases responsible for this activation were unknown, until recently (Towne et al., 2011; Henry et al., 2016). Thus, the insertion of DEVD motifs allowed us to potently activate IL-36 cytokines using caspase-3 to generate truncations at sites previously identified to liberate biological activity within these cytokines (Towne et al., 2011). Caspases have

exquisite specificity for cleavage after aspartic acid residues and so can selectively and efficiently process the DEVD tetrapeptide which is not commonly found in many proteins, especially within surface exposed loop regions prone to proteolysis. The DEVD peptide was originally identified from the caspase cleavage site within PARP, a DNA repair enzyme, targeted by caspase-3 during apoptosis (Nicholson et al., 1995). Derivatives of DEVD are frequently used to assess caspase-3 enzymatic activity by fluorimetric methods and as inhibitors of caspase-3 activity (Walsh et al., 2008). Here, we have demonstrated a novel use of the DEVD motif as a fusion tag to generate proteins that are susceptible to proteolysis by caspase-3 (Clancy et al., 2016).

Similar strategies have been previously used by other groups to purify biologically active forms of IL-36Ra and IL-38 (Hu et al., 2015; Towne et al., 2011). IL-36Ra requires removal of the N-terminal Met1 residue to act as an antagonist of IL-36 signalling but exhibits little activity when this residue is present (Towne et al., 2011). Towne *et al.* used a factor Xa protease-removable N-terminal GST tag to generate truncated IL-36Ra. However, one potential drawback of this approach is that GST, being a relatively large fusion partner (26 kDa), may introduce solubility and folding problems in proteins tagged using this strategy. Furthermore, removal of the GST tag by factor Xa has been reported to be relatively ineffective and non-specific, reducing yields of the desired cleavage product (Jenny et al., 2003; Young et al., 2012).

Small ubiquitin-related modifier (SUMO) fusion tags have also been used to purify N-terminally truncated IL-36 ligands (Towne et al., 2011). SUMO is a relatively small protein tag (~11 kDa) often used as a solubility enhancer for protein purification by promoting proper folding and stability. After purification, the SUMO moiety can be removed by the highly specific SUMO protease Ulp1. However, unlike the GST tag, SUMO requires an additional affinity tag for purification of the fusion protein, usually a polyhistidine tag (Young et al., 2012). Hu *et al.* recently described the purification of IL-38 as a TEV fusion protein. TEV protease, from the tobacco etch virus, is more stringent than factor Xa and other commonly used fusion tag proteases like thrombin or enterokinase. Wild-type TEV protease can be deactivated by autolysis and expression of the protease is hindered by solubility issues, however, mutated forms of the protease have been developed for increased stability and expression yields (Kapust et al., 2001). Inclusion of the small caspase-3-specific peptide tag DEVD acts as a simple and reliable alternative for accurate removal of purification tags and inhibitory domains within proteins with minimal disruption to the fusion partner (Clancy et al., 2016).

3.3.2 IL-36 cytokines require proteolytic processing to release biological activity

As discussed in the introduction, IL-1 family cytokines are generated as inactive precursors, requiring proteolytic processing within their N-termini to release bioactivity (Afonina et al., 2015; Mosley et al., 1987). It is interesting that removal of very small numbers of residues from the N-termini of IL-36 family cytokines have such dramatic effects on the biological activities of these cytokines. For example, the IL-36 N-termini may party occlude the receptor binding domain through steric clashes, removal of which permits a more stable interaction with the IL-36R complex. Alternatively, proteolysis of IL-36 cytokines may induce a conformational change in these proteins that increases their affinity for the IL-36 receptor, thereby increasing biological potency. Interestingly, Hazuda and colleagues have reported that IL-1α and IL-1β undergo profound conformational changes upon removal of their N-termini and, as a consequence, the mature regions of these molecules switch from a proteinase K-sensitive to a proteinase K-insensitive state (Hazuda et al., 1991). This change is most likely reflected in an altered conformation that increases affinity for the IL-1 receptor.

Recently, the crystal structure of IL-36γ was solved, making it the first reported structure of an IL-36 ligand. Truncated IL-36γ, beginning at Ser18 corresponding to the active form identified previously by Towne *et al.*, was used to solve the structure of this cytokine. It was shown that IL-36γ contained the typical β-trefoil fold found in all other IL-1 family cytokines, made up of 12 β strands connected by 11 loops (Gunther and Sundberg, 2014). While the mechanism of IL-36 cytokine agonism is largely similar to that of IL-1β, there were some distinct differences in specific interactions between each ligand and their respective receptor complexes. Using the IL-36γ structure, Gunther and Sundberg identified β strands 11 and 12 (β11/12) and β4/5 as important sites for binding to the IL-36 receptor and for IL-36γ activity. Charge interactions are also important mediators of IL-36 ligand binding to the IL-36 receptor (Gunther and Sundberg, 2014). This study determines the structure of artificially truncated IL-36γ and identifies areas important for IL-36γ activity in β strands located towards the C-terminal of the protein sequence. While these sites may be important for IL-36γ activity in its biologically active state, this study does not address the autoinhibitory effect of the N-terminal of IL-36γ. The further resolution of IL-36 ligand structures, both in their full-length, as well as their processed forms by naturally occurring proteases, will help to elucidate how exactly these molecules interact with the IL-36R/IL-1RAcP receptor complex.

3.3.3 IL-36 receptor signalling is similar to IL-1 receptor downstream signalling

Using small molecule inhibitors and RNA interference we sought to elucidate the downstream mediators of IL-36 receptor signalling. We initially targeted known kinases and adaptor molecules involved in IL-1 receptor signalling, which has been extensively studied. Indeed, the results detailed here confirm that the TIR adaptor MyD88 is crucial for IL-36 receptor signalling. MyD88 is a proximal mediator of IL-1 signalling recruited to the IL-1 receptor complex upon ligand binding. Ablation of MyD88 by RNA interference completely blocked cytokine induction in response to IL-36 cytokines, underlining the crucial role of this adaptor in IL-36 receptor signalling. This result was confirmed in a recent publication showing that MyD88 and Tollip are required for IL-36 receptor signalling (Saha et al., 2015).

Knockdown of IRAK4, TRAF6 and p65 also ablated IL-36-induced cytokines, implicating their involvement in IL-36 receptor pathway. NEMO and TAK1 knockdown only partially suppressed IL-36-induced cytokines, despite effective knockdown of these proteins as confirmed by immunoblot (Fig. 3.14). Due to potential levels of redundancy in IL-36 receptor signalling, combined targeting of NEMO and TAK1 or the whole TAK1/TAB complex may be required to fully suppress cytokine induction in response to IL-36 ligands.

Here, we have also established a convenient reporter cell line $HeLa^{IL\text{-}36R}$, which overexpresses the NFκB responsive SEAP reporter plasmid. Activation of NFκB signalling by IL-36 induces the release of secreted alkaline phosphatase into culture medium that is highly stable and can be readily detected by colorimetric assay. This allows the rapid and continuous monitoring of NFκB activation without the need to disrupt or lyse cell populations. This bioassay permits the rapid screening of compounds that may naturally antagonize IL-36 cytokine activity.

These results suggest that IL-36 receptor signalling follows a similar pathway to that of the IL-1 receptor, involving MyD88, IRAKs, TRAF6 and the TAK1 complex, culminating in NFκB and MAPK activation. Dysregulation of IL-1 family members have been implicated in skin inflammation and mediate a variety of disorders including psoriasis and atopic dermatitis. However, treatment options for these diseases are often unsatisfactory. IL-36 cytokines are key players in the pathogenesis of psoriasis, thus targeting IL-36 signalling pathways may be an effective therapy for skin inflammation (Jensen, 2010).

IRAK or TRAF6 may represent attractive therapeutic targets as they appear to be

important mediators of IL-1 and TLR signalling. Interestingly, a number of IRAK4 kinase inhibitors have been developed for use in treating a range of inflammatory diseases mediated by TLRs and IL-1 family cytokines including rheumatoid arthritis, inflammatory bowel disease and SLE (Wang et al., 2009). Individuals deficient in IRAK4 have been described and show impaired responses to IL-1R, IL-18R and TLR stimulation. Despite this, adults deficient in IRAK4 activity are not susceptible to chronic infection, although can be prone to certain pyrogenic infections at younger ages (Wang et al., 2009). Thus, modulation of IRAK4 activity may provide therapeutic benefits for a range of inflammatory skin diseases, without the systemic immune suppression seen with anakinra (IL-1Ra) or anti-TNFα therapies.

3.3.4 Linear ubiquitination is involved in the regulation of IL-36 receptor signalling

IL-1 receptor signalling is tightly regulated by ubiquitination, particularly Lys-48, Lys-63 and linear polyubiquitination. This ubiquitination is in turn modulated by deubiquitinase enzymes which can deconstruct polyubiquitin chains. The deubiquitinase FAM105B/OTULIN is a recently described modulator of Met1-linked linear ubiquitin chains and has been shown to limit pro-inflammatory responses following TNF receptor or NOD receptor activation (Fiil et al., 2013; Fiil and Gyrd-Hansen, 2014; Keusekotten et al., 2013). Here, we sought to investigate the role of linear ubiquitination in IL-36 receptor signalling.

Knockdown of OTULIN enhanced IL-36 signalling, while overexpression of this deubiquitinase blocked IL-36-induced IL-8 and CXCL1, suggesting that OTULIN can modulate IL-36 signalling by regulating linear ubiquitin chain formation by the LUBAC complex. However, the modulation of IL-36-induced cytokines by OTULIN knockdown was not as effective as that for TNFα-induced cytokines and varying results were obtained depending on the siRNA used (Fig 3.16 - 3.18). Knockdown of OTULIN using CRISPR technology may give more definitive results as all OTULIN protein would be deleted from cells. The deubiquitinase CYLD has also been reported to target and disrupt linear ubiquitin chains and so may also be involved in the regulation of IL-36 signalling (Hrdinka et al., 2016). Additionally, combined knockdown of OTULIN, CYLD and the A20 deubiquitinase may give greater effects as A20 has been shown to be important in regulating NFκB signalling (Harhaj and Dixit, 2011). Indeed, both the deubiquitinase and the E3 ligase activities of A20 have been implicated in the regulation of NFκB activation, with a recent study identifying the importance of A20 binding to the C-terminal of NEMO to keep pro-inflammatory NFκB signalling in check (Zilberman-Rudenko et al., 2016).

Previous reports have highlighted the importance of linear ubiquitination by the LUBAC complex and its regulation by the OTULIN deubiquitinase in the regulation of TNF and NOD2 receptor signalling (Fiil et al., 2013; Fiil and Gyrd-Hansen, 2014; Keusekotten et al., 2013). Results shown here suggest that linear ubiquitination is also important for IL-36 receptor signalling. As the IL-36 receptor signalling pathway closely resembles that for IL-1 receptor and also TLR receptor signalling, this suggests that linear ubiquitination and OTULIN may also be important mediators in these pathways.

Chapter 4

Neutrophil granule proteases act as global regulators of IL-1 family cytokine activation states

4.1 Introduction

Peripheral blood neutrophils are the first responder cells recruited to necrotic lesions and rapidly achieve very high concentrations (~10^9/mL) in the vicinity of even sterile wounds (Kono and Rock, 2008; Lammermann et al., 2013; McDonald et al., 2010). However, it is not clear what role neutrophils play in the context of sterile injury, as these cells are typically viewed predominantly as phagocytes, adept as engulfment of bacteria and other microorganisms. Previous studies have shown that 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. This is at odds with the idea that neutrophil granule proteases play a predominantly anti-microbial role in coordinating the destruction of engulfed pathogens (Belaaouaj et al., 1998; Pham, 2006; Reeves et al., 2002; Weinrauch et al., 2002). Similarly, mice deficient in cathepsin C (DPPI), which is required to process and activate all neutrophil 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 instigators and amplifiers of inflammation, although the precise targets of neutrophil proteases in this context remains obscure.

IL-1 family members are capable of triggering complex cascades of additional cytokine production from diverse cells types, such as resident tissue macrophages and dendritic cells, epidermal keratinocytes, as well as endothelial cells lining local blood vessels (Afonina et al., 2015; Garlanda et al., 2013). Due to the broad-spectrum nature of their effects, members of the IL-1 family therefore act as important amplifiers of inflammatory reactions. 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; Mosley et al., 1987; Towne et al., 2011). Although caspase-1 is well known to process and activate IL-1β and IL-18 (Gu et al., 1997; Thornberry et al., 1992), multiple additional proteases have been implicated in the processing of other members of the extended IL-1 family (Afonina et al., 2011; Black et al., 1988; Hazuda et al., 1990; Hazuda et al., 1991; Henry et al., 2016), however, 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 only released upon necrosis (Cayrol and Girard,

2014; Chen et al., 2007; Cullen et al., 2015; Luthi et al., 2009; Rider et al., 2011). 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 minutes of wounding, and frequently degranulate upon encounter with large particles. 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.

Processing of IL-1β and IL-18 via caspase-1 has attracted considerable attention over the past 20 years (Fantuzzi and Dinarello, 1999; Gu et al., 1997; Thornberry et al., 1992), but other members of the IL-1 family have been reported to be processed and activated by different proteases, including: calpain, elastase, cathepsin G, proteinase-3, mast cell chymase, granzyme B, and granzyme A, among others (Afonina et al., 2015; Black et al., 1988; Coeshott et al., 1999; Hazuda et al., 1990; Joosten et al., 2009; Netea et al., 2015). However, many of the latter studies examined processing of individual IL-1 family members by individual proteases, in isolation, and frequently at non-physiological concentrations (Black et al., 1988; Hazuda et al., 1990; Hazuda et al., 1991; Joosten et al., 2009; Lefrancais et al., 2012). Because neutrophil 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 (Afonina et al., 2011; Hazuda et al., 1990; Lefrancais et al., 2012), 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, may result in biological effects that are different from those seen with purified proteases alone.

We have recently found that the least well characterized members of the extended IL-1 family, IL-36α, IL-36β and IL-36γ, which are encoded by distinct genes, are all processed and activated by the mixture of proteases that are released upon activation and degranulation of primary human neutrophils (Henry et al., 2016). These observations, coupled with sporadic reports on the activation of individual IL-1 family members, such as IL-33 and IL-1α (Afonina et al., 2011; Lefrancais et al., 2012; Netea et al., 2015), by neutrophil proteases now suggests 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 the majority of previous studies have examined processing of single IL-1 family members with purified proteases in isolation, it remains unclear whether neutrophil-derived proteases act as global regulators of IL-1 family activation states when deployed collectively.

Here, we have explored the ability of supernatants from 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 the latter proteases act to enhance or suppress the activity state of six of the seven members of the extended IL-1 family. These observations identify neutrophil-derived proteases as key regulators of IL-1 family cytokine activation states and challenge the conventional view of neutrophils as 'mere' microbial killers.

4.2 Results

4.2.1 Supernatants from activated neutrophils contain multiple proteases

Sporadic reports have implicated individual neutrophil proteases in the processing and activation of individual IL-1 family members (Afonina et al., 2011; Black et al., 1988; Coeshott et al., 1999; Hazuda et al., 1990; Henry et al., 2016; Joosten et al., 2009). However, the majority of these studies have utilized individual neutrophil granule proteases, whereas under physiological conditions substrates are much 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, we first isolated neutrophils from human peripheral blood and induced them to degranulate using the PKC activator phorbol 12-myristate 13-acetate (PMA) (Fig. 4.1 A). Activated neutrophils undergo striking morphological changes, including flattening and tight adherence to the cell culture dish (Fig. 4.1 B).

Treatment of neutrophils with PMA resulted in the liberation of multiple proteases into the extracellular space. As expected, we detected dramatic increases in cathepsin G activity (as indicated by FLF-sBzL hydrolysis), as well as elastase and proteinase-3 activity (as indicated by hydrolysis of AAPV-AMC) in the supernatants of PMA activated neutrophils (Fig 4.1 C). Addition of cathepsin G or elastase inhibitors to activated neutrophil supernatants completely abolished FLF-ase or AAPV-ase activity, as expected (Fig. 4.1 D). Thus, supernatants from activated neutrophils contain multiple proteases as previously demonstrated (Korkmaz et al., 2010; Pham, 2008).

Because the purity and bioactivity of cytokines from commercial sources is highly variable, we also expressed and purified all members of the extended IL-1 family in ClearColi BL-21(DE3) strain *E. coli*. ClearColi BL-21 bacteria encode genetic deletions that results in modified lipopolysaccharide (LPS) molecule to allow protein expression and purification free from endotoxin contamination (Mamat et al., 2013). As Fig 4.2 shows, all proteins were successfully expressed and were found to be >95% pure. All cytokines were used at equimolar amounts in subsequent experiments. To measure the effect of neutrophil proteases on IL-1 cytokines, recombinant IL-1 proteins were incubated with neutrophil degranulate supernatants or purified neutrophil proteases. These reactions were then used to treat HeLa cells and cytokine induction was measured by ELISA to check for enhanced IL-1 activity (Fig. 4.3).

4.2.2 Neutrophil-derived proteases process and activate IL-1α

While full-length IL-1α can bind to the IL-1 receptor and exhibits biological activity, increased IL-1α activity has also been reported after processing by several different proteases. Previous reports have shown that elastase can process and enhance the basal activity of IL-1α ~10-fold by cleaving this cytokine at Ala101 (Afonina et al., 2011) (Fig. 4.4 A), however it is unclear whether cathepsin G or proteinase-3 can also process and activate IL-1α.

Recombinant full-length IL-1α was incubated with supernatants from untreated or PMA-activated neutrophils and IL-1α activity was measured using a HeLa$^{IL\text{-}36R}$ bioassay (Fig. 4.3). Supernatants from activated neutrophils robustly processed full-length IL-1α, over a wide range of dilutions, leading to a robust increase in the activity of this cytokine (Fig. 4.4 B, C). This is consistent with previous observations with elastase (Afonina et al., 2011).

Furthermore, incubation of IL-1α with purified elastase, cathepsin G or proteinase-3 from human neutrophils also resulted in robust processing and activation of this cytokine by all three proteases at low nanomolar concentrations (Fig 4.5 A, B). Thus, all three neutrophil granule proteases process and activate IL-1α, either together or in isolation.

4.2.3 Neutrophil-derived proteases do not activate IL-1β

IL-1β and IL-18 are pro-inflammatory cytokines that undergo activation by caspase-1 under the regulation of the inflammasome complex. While inflammasome activation and IL-1β regulation by caspase-1 have been comprehensively studied, there are some

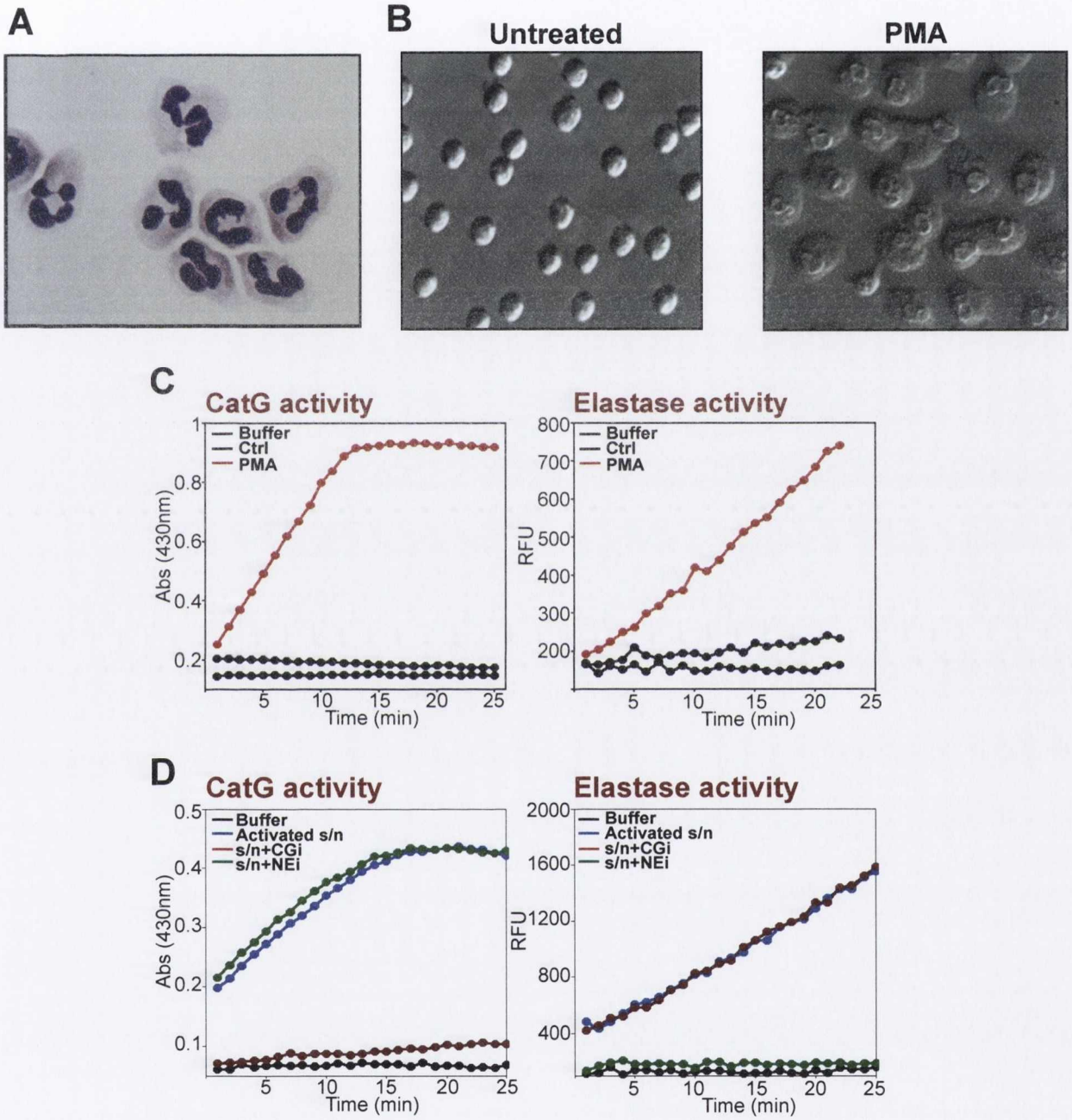

Figure 4.1 Activated neutrophil supernatants contain protease activity
A. Primary human neutrophils isolated from peripheral blood were stimulated with PMA (100 nM) for 2 h. Cells were visualised by phase contrast microscopy. **B.** Primary human neutrophils ($1x10^7$ cells/ml) were stimulated with PMA 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 minutes. **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 treatment, Cat G and elastase enzymatic activity in the supernatants was measured by suc-FLF-sBzl and AAPV-AMC hydrolysis. Part **C.** was contributed by Graeme Sullivan

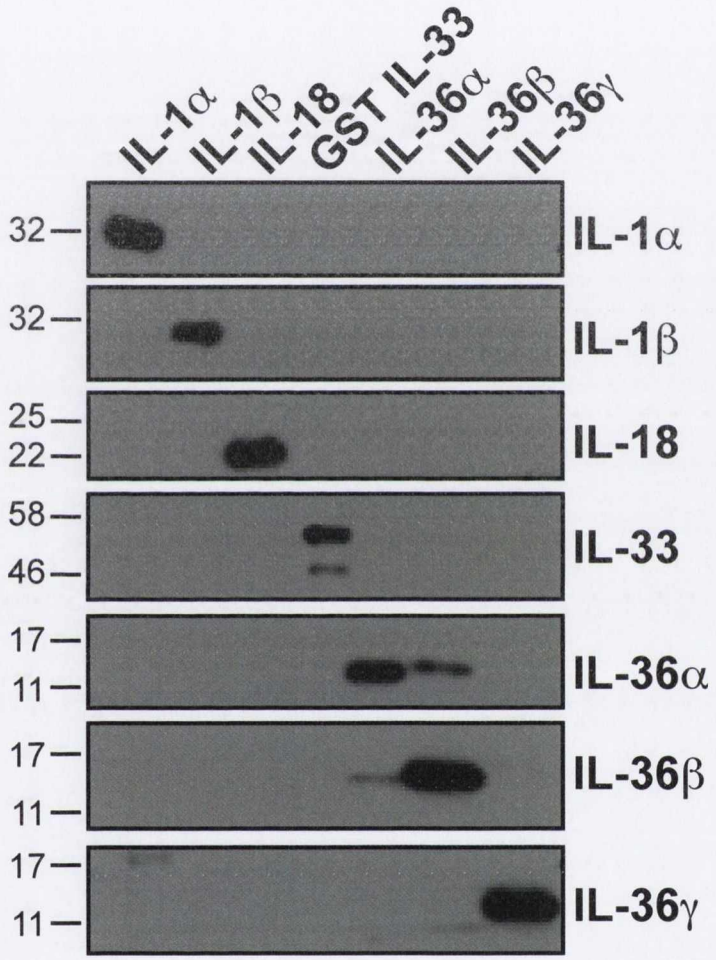

Figure 4.2 Purification of recombinant IL-1 family proteins from bacteria
Recombinant IL-1 family cytokines were expressed and purified as detailed in the experimental methods. Recombinant IL-1 family cytokines (10 ng) were electrophoresed on SDS-PAGE gels. IL-1 family proteins were immunoblotted as indicated using specific antibodies.

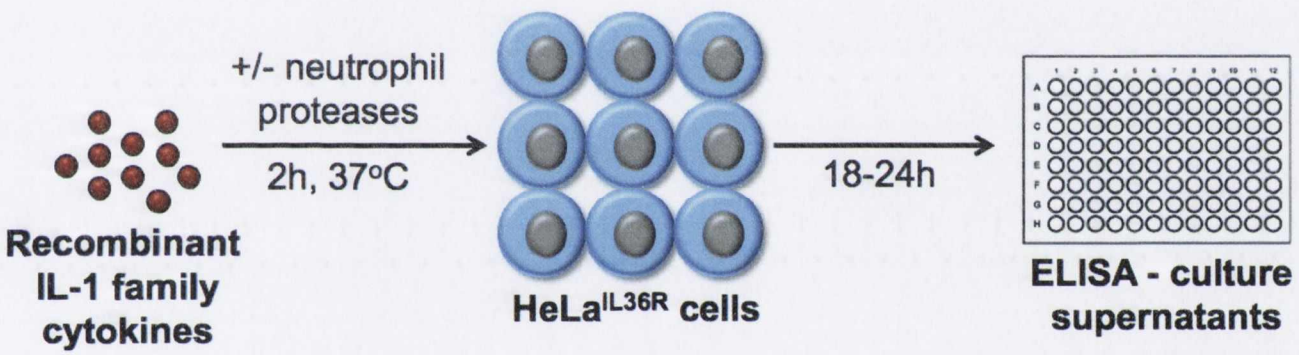

Figure 4.3 Layout of IL-1 cytokine activity assay

Schematic showing the layout of biological activity assays on HeLa$^{IL\text{-}36R}$ cells to measure the effect of neutrophil proteases on IL-1 cytokines. Recombinant IL-1 family proteins (typically 50 nM protein) were incubated with neutrophil degranulate supernatants or purified neutrophil proteases for 2 h, 37 °C. Reactions were then diluted on to HeLa$^{IL\text{-}36R}$ cells and incubated for 18-24 h. Cytokine concentration in cell culture supernatants was measured by ELISA.

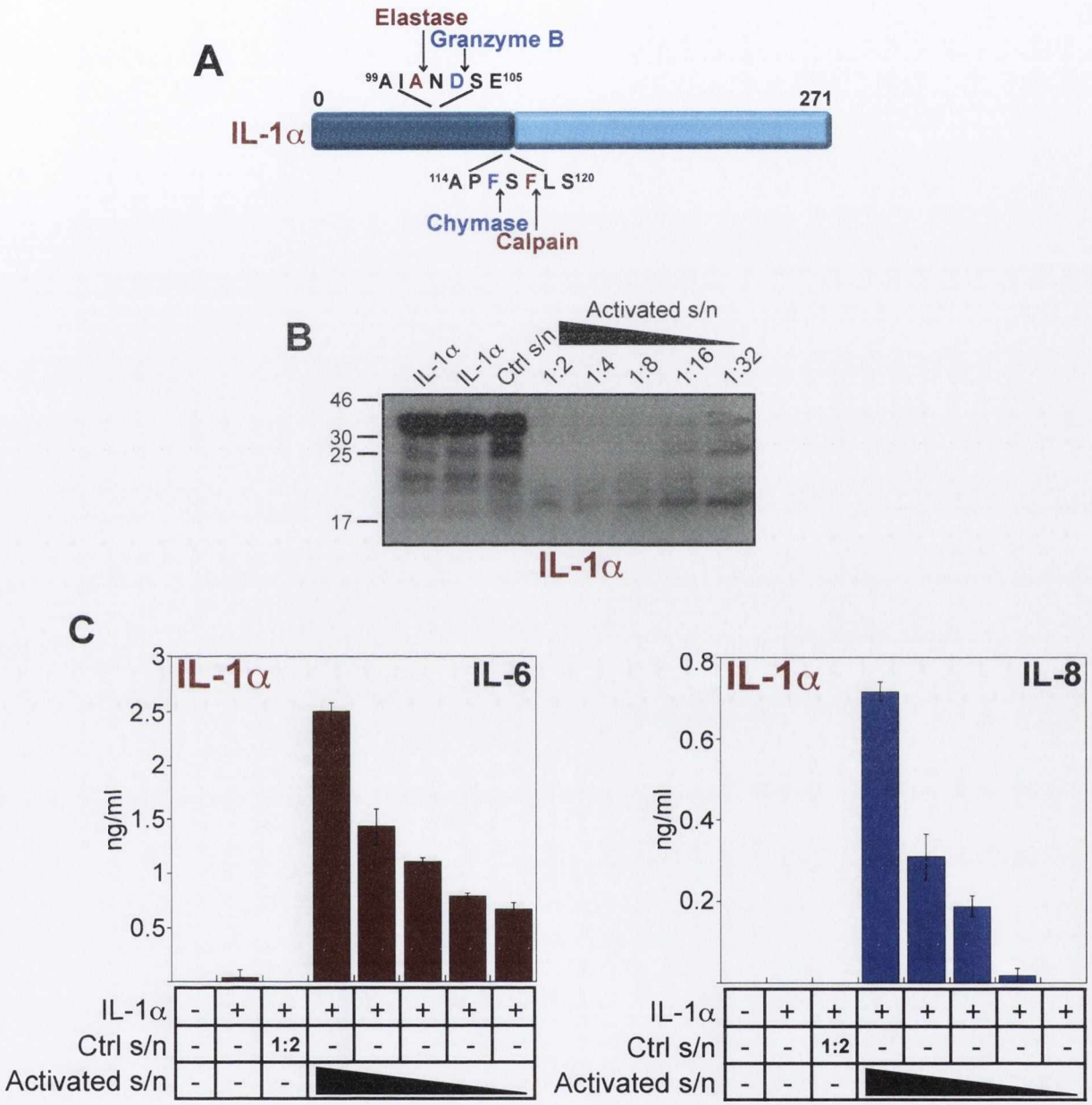

Figure 4.4 Activated neutrophil degranulates processes and activates IL-1α
A. Schematic of full-length IL-1α indicating processing sites by elastase, granzyme B, chymase and calpain. **B.** Recombinant IL-1α (50 nM) was incubated for 2 h at 37 °C with 1:2 serial dilutions of either control (ctrl) or PMA-activated neutrophil degranulate. Processing of IL-1α was analysed by immunoblot. **C.** HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-1α (1 nM). After 18 h, IL-6 and IL-8 concentrations in the culture supernatant was measured by ELISA. Error bars represent the mean +/- SEM of triplicate determinations from a representative experiment.

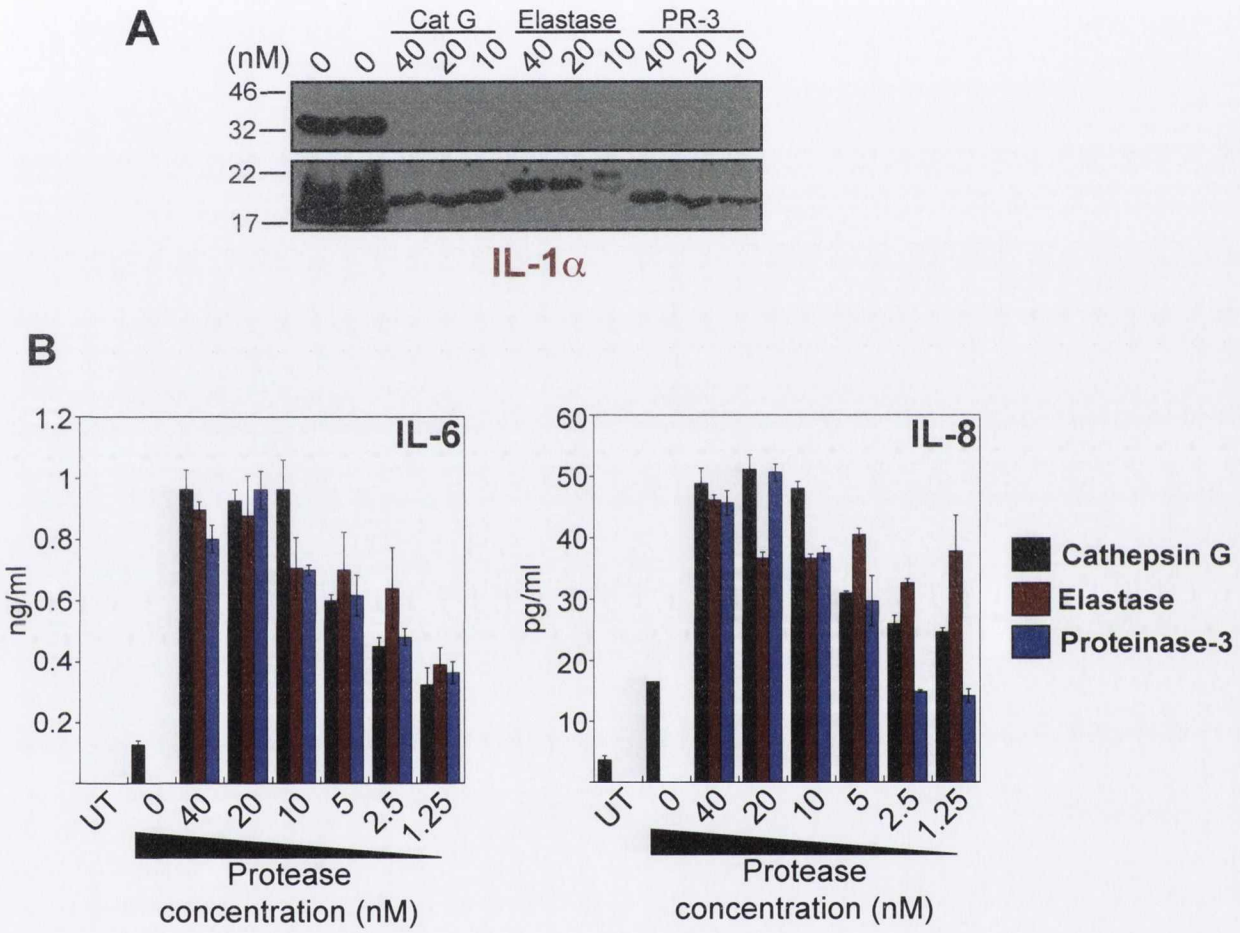

Figure 4.5 Purified neutrophil proteases activate IL-1α
A. IL-1α (50 nM) was incubated for 2h, 37 °C with purified neutrophil proteases: cathepsin G, elastase and proteinase-3, as indicated. IL-1α processing was analysed by immunoblot. **B.** HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-1α (1 nM), as indicated. After 18 h, IL-6 and IL-8 concentrations in cell culture supernatants was measured by ELISA.

reports suggesting IL-1β can be activated by inflammasome-independent mechanisms. Sporadic reports have suggested that IL-1β is processed by elastase and cathepsin G at amino acid residues slightly upstream of the caspase-1 cleavage site (Fig. 4.6 A) (Black et al., 1988; Hazuda et al., 1990).

Full-length IL-1β was incubated with activated neutrophil degranulate supernatant and activity was measured using the HeLa bioassay, as before. In stark contrast to our observations with IL-1α, incubation of purified full-length IL-1β with supernatants from activated neutrophils failed to activate this cytokine, despite evident processing (Fig. 4.6 B, C). In sharp contrast, incubation of IL-1β with purified caspase-1 resulted in robust activation of this cytokine, as expected (Fig. 4.6 C).

We also incubated full-length pro-IL-1β with purified elastase, cathepsin G or proteinase-3 and once again, failed to see any activation of this cytokine (Fig. 4.7 A, B). Again, IL-1β was robustly activated by caspase-1. It appears that incubation of IL-1β with these proteases leads to partial or full degradation of this cytokine (Fig. 4.7 A). Thus, neutrophil-derived proteases activate IL-1α but not IL-1β.

4.2.4 Active IL-1β is inactivated by elastase and cathepsin G

Because neutrophil proteases processed, but did not activate pro-IL-1β, we also considered the possibility that these proteases could inactivate mature p17 IL-1β, produced through processing via caspase-1 (Fig. 4.8 A). Thus, we generated recombinant p17 IL-1β, which was constitutively active in a HeLa$^{IL\text{-}36R}$ bioassay, as expected (Fig. 4.8 B).

However, incubation of mature p17 IL-1β with supernatants from activated neutrophils led to a dramatic abolition of the activity of this cytokine (Fig. 4.9 A). This effect was also seen through co-incubation of mature p17 IL-1β with purified elastase or cathepsin G (Fig 4.9 B). Incubation of mature p17 IL-1β with caspase-1 does not further modulate the activity of this cytokine. 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-1β was clearly activated.

4.2.5 Neutrophil proteases do not modulate IL-18 activity

Similar to IL-1β, IL-18 is also processed and activated by caspase-1 (Gu et al., 1997), however neutrophil and mast cell proteases have been implicated in IL-18 processing through inflammasome-independent mechanisms. IL-18 has been reported to be

processed by mast cell chymase, however, the cleavage product generated has slightly less biological activity than its caspase-1-cleaved counterpart (Fig 4.10 A)(Omoto et al., 2006). An isolated report has also suggested that proteinase-3 may also be capable of promoting the activation of this cytokine (Sugawara et al., 2001).

Full-length IL-18 was incubated with activated neutrophil degranulate and activity was measured using the IL-18-responsive macrophage cell line KG-1. Activated neutrophil degranulate supernatants process IL-18 to a slightly smaller fragment than the full-length protein (Fig. 4.10 B). Similar to IL-1β, activated neutrophil degranulate did not activate pro-IL-18, whereas incubation with caspase-1 resulted in robust activation (Fig. 4.10 C). Similarly, exposure of pro-IL-18 to individual neutrophil-derived proteases also failed to activate this cytokine. Analysis by immunoblot revealed that IL-18 is poorly processed by individual neutrophil proteases, and may in fact be degraded at higher concentrations of these proteases, particularly elastase (Fig. 4.11 A, B).

We also investigated whether neutrophil proteases could modulate the activity of caspase-1-activated IL-18. As seen previously, incubation of IL-18 with caspase-1 potently enhanced the activity of this cytokine. However, when caspase-1-activated IL-18 was co-incubated with purified neutrophil cathepsin G, elastase or proteinase-3, the activity was not modulated by these proteases (Fig. 4.12), suggesting that neutrophil proteases are not involved in the regulation of IL-18 activity.

4.2.6 IL-33 can be both activated and inactivated by neutrophil proteases

We also assessed the processing and activation of pro-IL-33 by neutrophil proteases, as this cytokine has been reported to be activated by elastase and cathepsin G (Fig. 4.15 A) (Lefrancais et al., 2012). HeLa$^{IL\text{-}36R}$ cells used previously were rendered IL-33 responsive by transient transfection of the IL-33 receptor (IL-33R, also known as ST2). Cells were highly responsive to full-length IL-33, but only when IL-33R was present (Fig. 4.13 A). GST-tagged IL-33 was purified from bacteria and used in activity assays with neutrophil degranulate supernatants, as before. Full-length IL-33 displayed robust activity in its unprocessed form but surprisingly, activated neutrophil degranulate supernatant inactivated IL-33 activity over a range of dilutions (Fig. 4.13 B, D). IL-33 appears to be degraded by activated neutrophil degranulate, as seen by immunoblot (Fig. 4.13 C).

Similar experiments were carried out using IL-33 generated by *in vitro* transcription/translation. His-tagged IL-33 was very selectively and efficiently expressed in a rabbit reticulocyte lysate system and exhibited strong biological activity

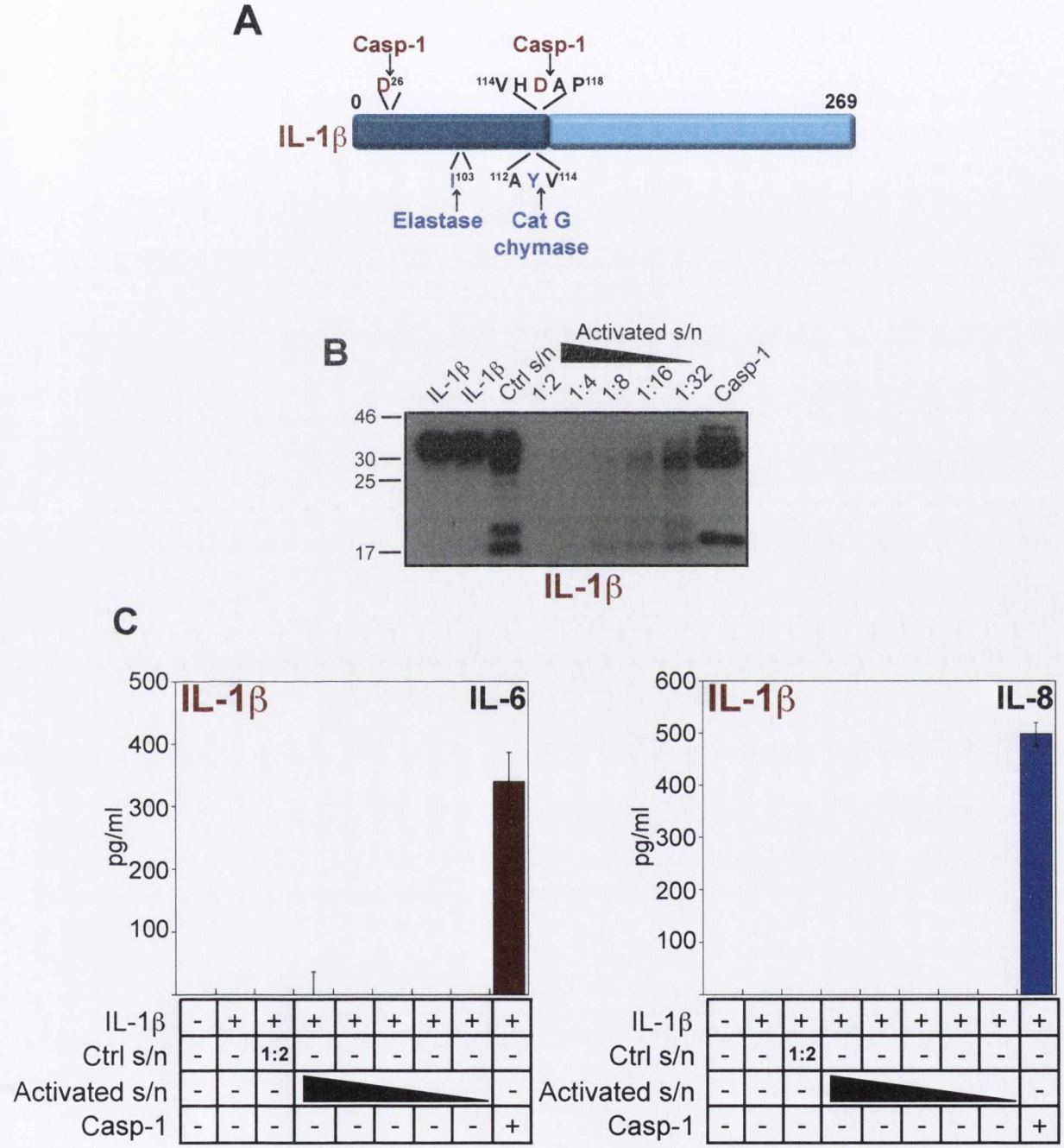

Figure 4.6 Activated neutrophil degranulate fail to activate IL-1β

A. Schematic of full-length IL-1β indicating processing sites by caspase-1 and putative processing sites by elastase, cathepsin G and chymase. **B.** Recombinant IL-1β (50 nM) was incubated for 2 h at 37 °C with control (ctrl) or PMA-activated neutrophil degranulate. Recombinant caspase-1 was used as a positive control for IL-1β processing and activation. Processing of IL-1β was analysed by immunoblot. **C.** HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-1β (1 nM). After 18 h, IL-6 and IL-8 concentrations in the culture supernatant was measured by ELISA.

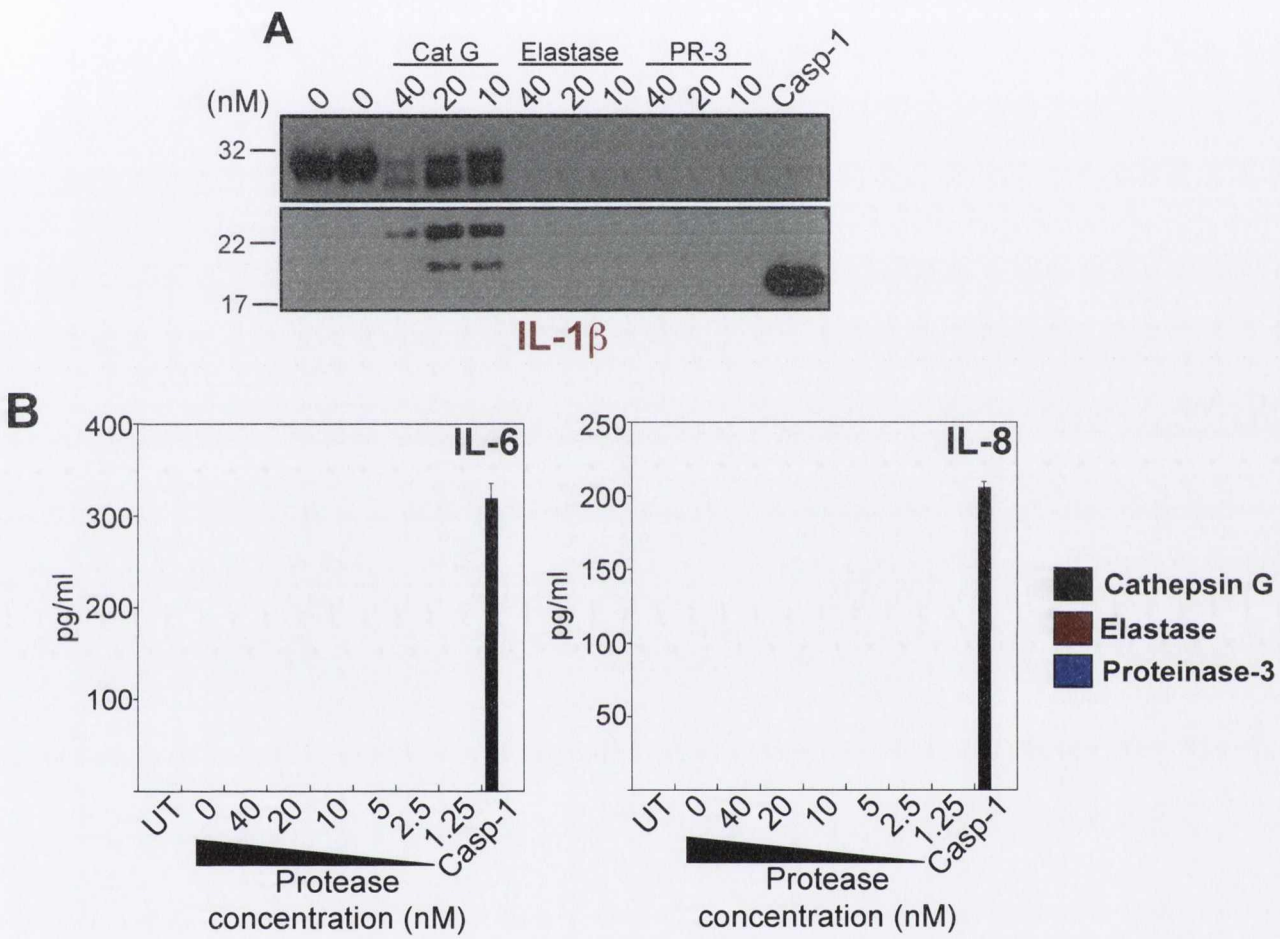

Figure 4.7 Individual neutrophil proteases do not activate IL-1β

A. IL-1β (500 nM) was incubated for 2h, 37 °C with purified neutrophil proteases: cathepsin G, elastase and proteinase-3, as indicated. IL-1β processing was analysed by immunoblot. **B.** HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-1β (1 nM), as indicated. After 18 h, IL-6 and IL-8 concentrations in cell culture supernatants was measured by ELISA.

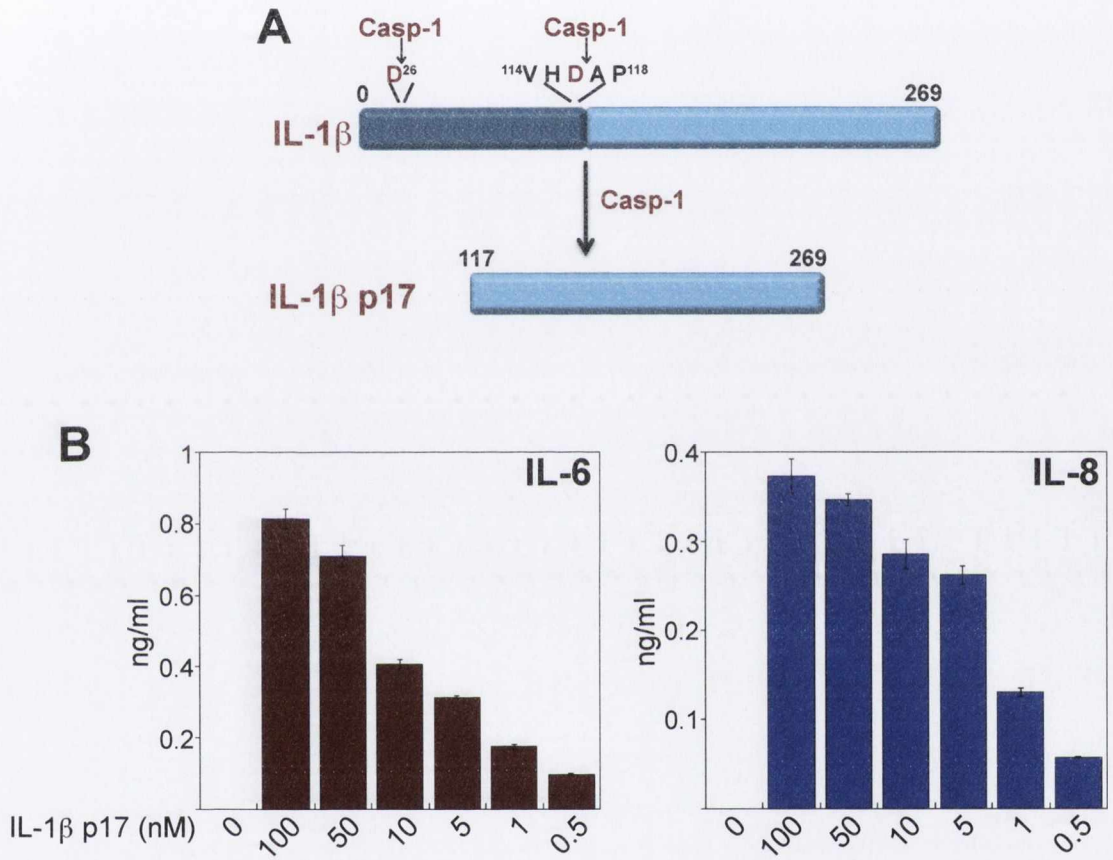

Figure 4.8 Truncated p17 IL-1β is constitutively active
A. Schematic of IL-1β processing by caspase-1 to generate the active IL-1β p17 fragment. Caspase-1 processing sites are indicated. **B.** HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with a titration of recombinant IL-1β p17, as indicated. After 18 h, IL-6 and IL-8 concentrations in the culture supernatant was measured by ELISA.

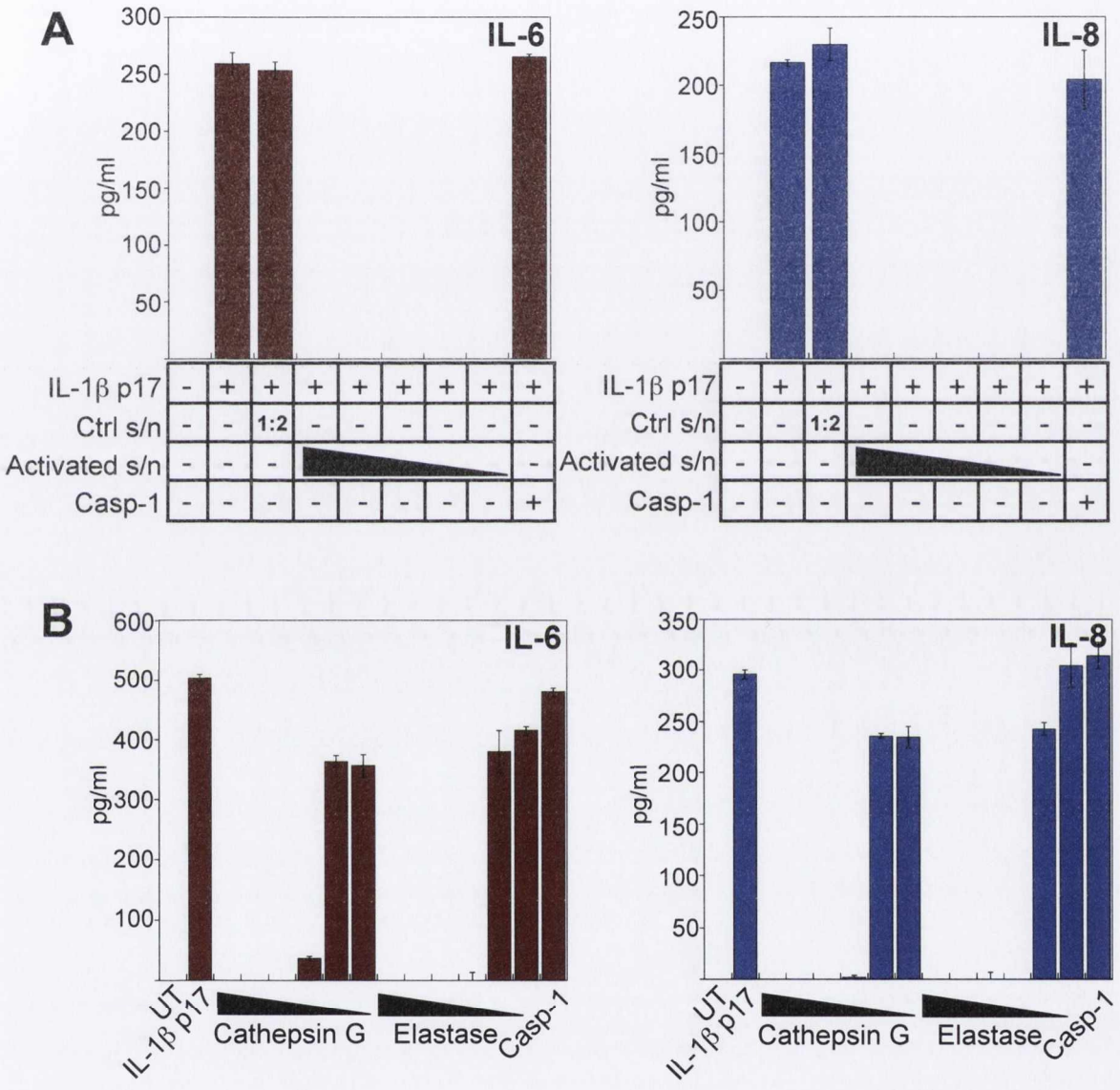

Figure 4.9 Neutrophil proteases inactivate mature IL-1β p17
A. Recombinant IL-1β p17 (50 nM) was incubated for 2 h at 37 °C with control (ctrl) or PMA-activated neutrophil degranulate. Recombinant caspase-1 was used as a control. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-1β p17 (1 nM). After 18 h, IL-6 and IL-8 concentration in cell culture supernatant was measured by ELISA. **B.** Recombinant IL-1β p17 (500 nM) was incubated for 2 h at 37 °C with a titration of cathepsin G or elastase (40 nM - 1.25 nM). Recombinant caspase-1 was used as a control. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-1β p17 (5 nM). After 18 h, IL-6 and IL-8 concentration in cell culture supernatant was measured by ELISA.

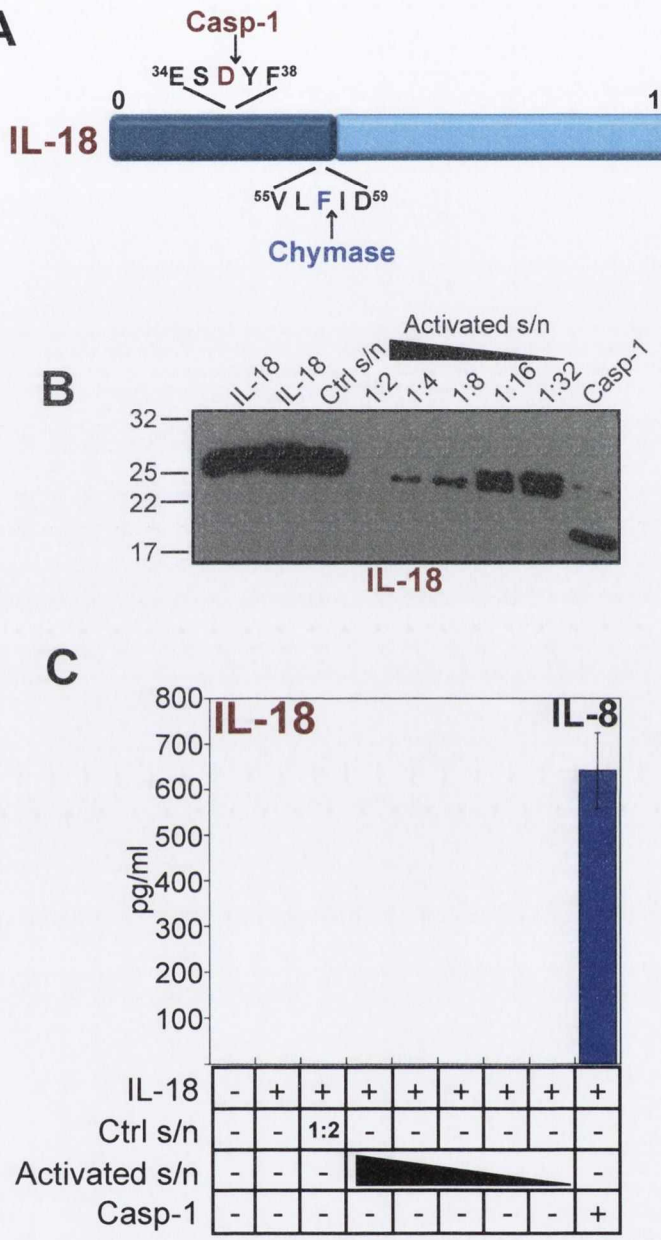

Figure 4.10 Neutrophil degranulate supernatants do not activate IL-18
A. Schematic of full-length IL-18 indicating processing sites by neutrophil proteases. Caspase-1 processing site is also highlighted. **B.** Recombinant IL-18 (50 nM) was incubated for 2 h at 37 °C with control (ctrl) or PMA-activated neutrophil degranulate. Recombinant caspase-1 was used as a control. KG-1 cells were stimulated with IL-18 (1 nM). After 18 h, IL-8 concentration in cell culture supernatant was measured by ELISA. Part **C.** was contributed by Graeme Sullivan.

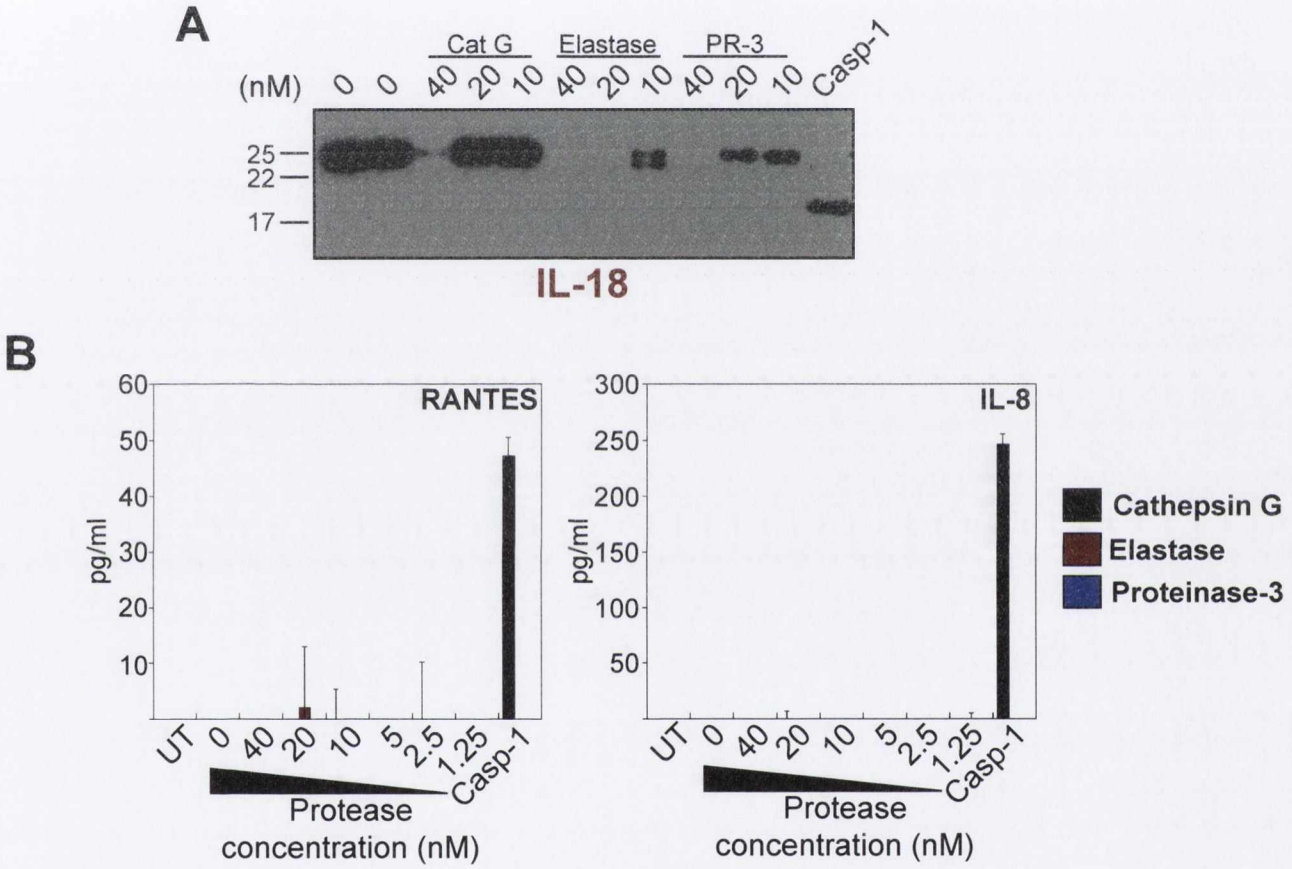

Figure 4.11 Purified neutrophil proteases do not activate IL-18

A. IL-18 (500 nM) was incubated for 2 h at 37 °C with purified neutrophil proteases: cathepsin G, elastase or proteinase-3, as indicated. Caspase-1 was used as a positive control for IL-18 cleavage. IL-18 processing was analysed by immunoblot. **B.** IL-18 (50 nM) was incubated for 2 h at 37 °C with a titration of neutrophil proteases, as indicated. Recombinant caspase-1 was used as a control. KG-1 cells were stimulated with IL-18 (1 nM). After 18 h, IL-8 and RANTES concentration in cell culture supernatant was measured by ELISA.

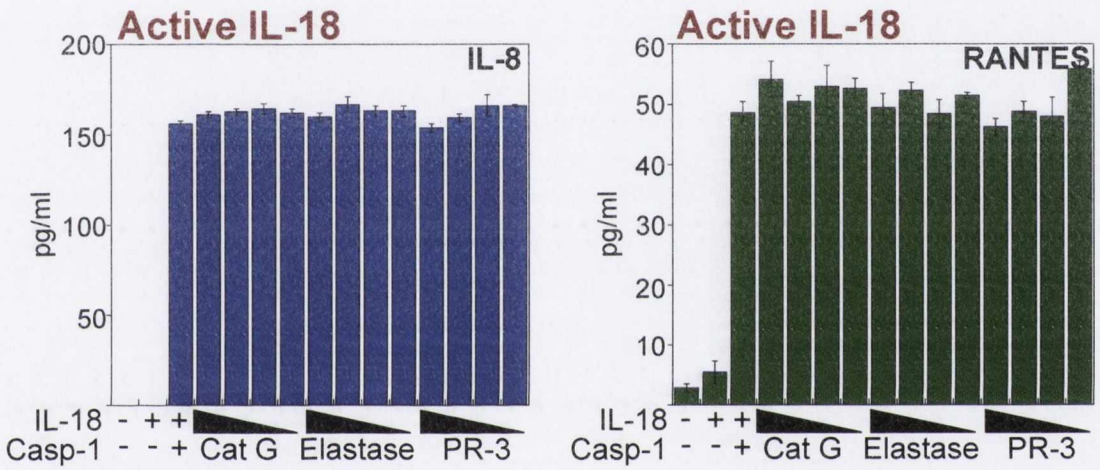

Figure 4.12 Neutrophil proteases do not modulate the activity of active IL-18
Recombinant IL-18 (50 nM) was incubated with caspase-1 with, or without, a titration of cathepsin G, elastase or proteinase-3 (40 nM - 5nM). KG-1 cells were subsequently stimulated with IL-18 (2 nM), as indicated. IL-8 and RANTES concentrations in cell culture supernatants was measured by ELISA after 18 h.

in its full-length, unprocessed form (Fig. 4.14 A, B). However, supernatants from activated neutrophils again clearly processed and inactivated IL-33 at high concentrations, which was somewhat surprising given previous reports (Fig. 4.15 B, C). A more recent study has suggested that proteinase-3 can inactivate IL-33 (Bae et al., 2012). Thus, it was possible that proteinase-3 could oppose the activation of cathepsin G and elastase when all three proteases were present together. Consistent with this idea, we found that whereas the basal activity of IL-33 was robustly enhanced by purified elastase or cathepsin G as previously reported (Fig. 4.16 A, B), incubation with proteinase-3 lead to rapid inactivation of this cytokine (Fig. 4.16 B).

However, lower concentrations of proteinase-3 also enhance IL-33 activity (Fig. 4.16 B), suggesting that the effect of neutrophil proteases on IL-33 activity may be dose dependent. Similar results were also seen with neutrophil degranulate supernatants, as higher dilutions of activated degranulate supernatants also enhanced IL-33 activity (Fig. 4.15 C). Thus, although elastase and cathepsin G are able to enhance the activity of IL-33 when present in the absence of proteinase-3, the net effect of these proteases on IL-33 activity may reflect the concentration of neutrophil proteases present in vivo at an inflammatory site. At early stages of an inflammatory response, neutrophils are present in lower numbers and the concentration of neutrophil proteases is still relatively low, IL-33 activity may be enhanced to potentiate inflammation. However, as more neutrophils are recruited to the inflamed site and more proteases are released via degranulation, the relative amount of proteinase-3 versus cathepsin G or elastase increases, resulting in IL-33 inactivation.

4.2.7 Proteinase-3 suppresses cathepsin G- or elastase-mediated enhancement of IL-33 activity

Because proteinase-3 inactivated full-length IL-33, we also explored whether this protease could also suppress the enhanced activity of IL-33 seen after exposure to cathepsin G or elastase. As shown in Fig. 4.17, this was indeed the case, with proteinase-3 readily eliminating the enhanced activity of IL-33 seen upon exposure to cathepsin G or elastase when these proteases were present at equimolar concentrations (Fig. 4.17 A, B). These data explain how neutrophil granule proteases inactivate IL-33 when all three proteases are present collectively. This indicates that the balance of proteases present at an inflammatory site will determine the functional effect on IL-33 and underscores the importance of using a physiological system where multiple proteases are present simultaneously, as opposed to individual proteases in isolation.

4.2.8 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 (Fig. 4.18 A) (Henry et al., 2016). In agreement with this, incubation of all three IL-36 cytokines with activated neutrophil supernatants resulted in robust activation of these proteins (Fig. 4.18 B). IL-36 ligands were also potently activated by purified neutrophil cathepsin G, elastase and proteinase-3 (Fig. 4.19). IL-36α was activated by elastase and cathepsin G, while IL-36β was preferentially activated by cathepsin G and IL-36γ by elastase and proteinase-3. Thus, all three IL-36 sub-family cytokines are processed by neutrophil-derived proteases, either alone or in combination.

Collectively, these data argue that neutrophil granule proteases play a major role as modulators (both positively as well as negatively) of the activation state of the majority of pro-inflammatory cytokines within the extended IL-1 family, with the only exception being IL-18 (Fig. 4.20). Our observations provide a molecular perspective on how neutrophil-derived proteases contribute to amplifying inflammatory reactions through direct processing and activation of multiple IL-1 family cytokines that are released upon tissue damage.

4.3 Discussion

Here, we have shown that neutrophil-derived serine proteases can act as modulators of IL-1 family cytokine activation. These results show that neutrophil-derived proteases can potently activate IL-36 cytokines and robustly enhance IL-1α activity. Addition of neutrophil proteases liberates IL-1α and IL-36 activity and robustly potentiates their pro-inflammatory potential. Conversely, neutrophil proteases do not activate IL-1β and in fact, inactivate the mature p17 IL-1β generated by caspase-1 cleavage. Similarly, neutrophil-derived proteases do not modulate IL-18 activation, with effective processing and activation of IL-18 only seen upon incubation of this cytokine with caspase-1. Neutrophil-derived proteases have been shown to have contradictory effects on IL-33 activation. While cathepsin G and elastase have been shown to activate IL-33 and enhance its biological activity, proteinase-3 has been shown to inactivate this cytokine (Bae et al., 2012; Lefrancais et al., 2012). Our results with purified neutrophil proteases mirror these reports. However, previous studies had not addressed the effect of neutrophil degranulate supernatants on IL-33 activity, when all three proteases are present together. Neutrophil degranulate supernatants inactivate IL-33 activity, and this was shown to be due to proteinase-3-mediated suppression of IL-33 activity (Fig. 4.17).

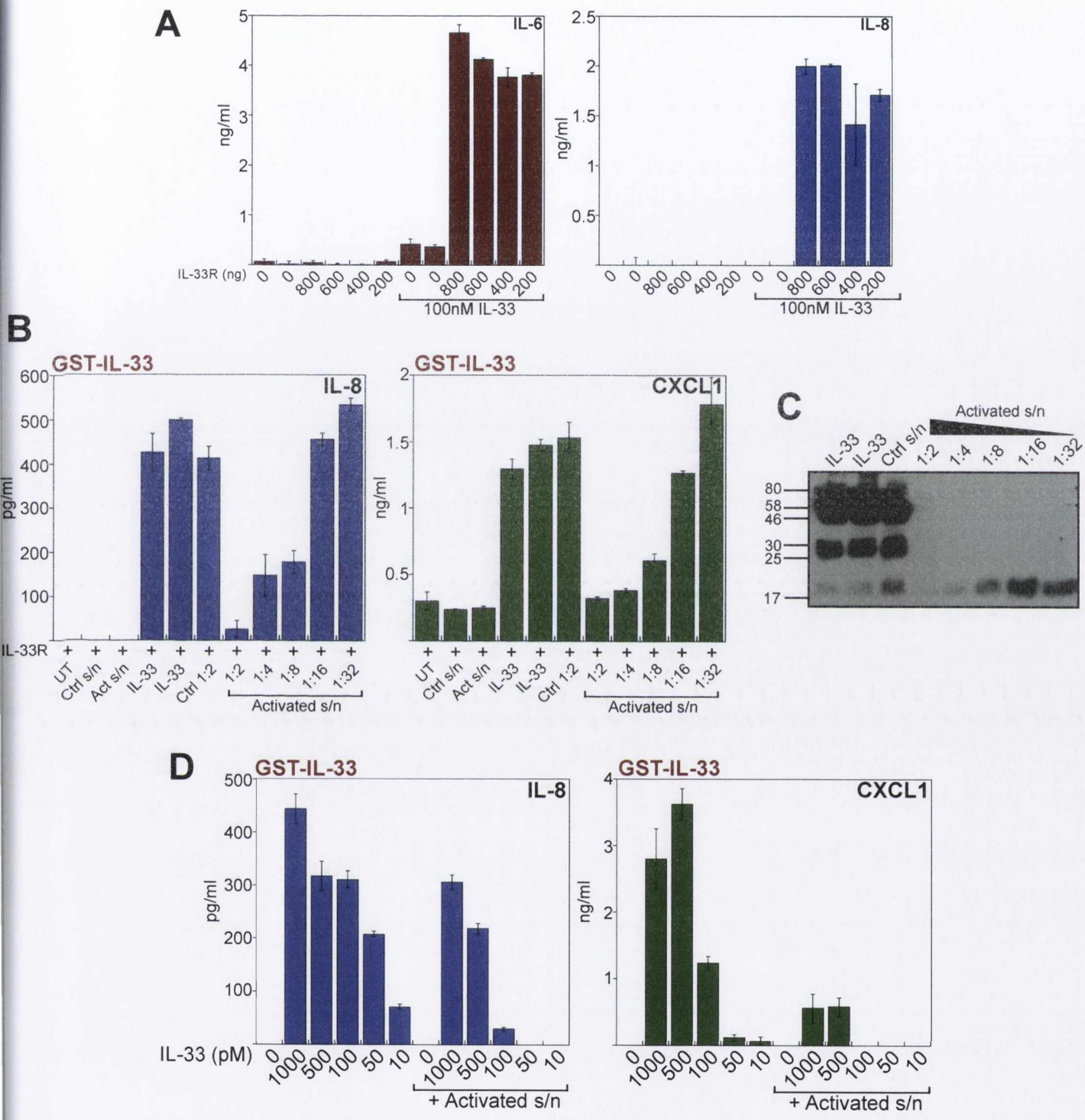

Figure 4.13 Neutrophil degranulate supernatants inactivate GST-IL-33
A. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were transfected with IL-33R, as indicated. After 24 h, cells were treated, or not, with 100 nM GST-IL-33. Cytokine concentration in the supernatants was measured by ELISA after 24 h. **B.** GST-IL-33 (50 nM) was incubated for 2 h at 37 °C with a titration of control or PMA-activated neutrophil degranulate supernatant. HeLa$^{IL\text{-}36\text{-}SEAP}$ cells were transfected with IL-33R and stimulated with GST-IL-33 (1 nM), as indicated. Cytokine concentration in culture supernatants was measured by ELISA after 24 h. **C.** GST-IL-33 (50 nM) was incubated with neutrophil degranulates, as before, and processing was analysed by immunoblot. **D.** GST-IL-33 was incubated, or not, with PMA-activated neutrophil degranulate. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells transfected with IL-33R were stimulated with a titration of GST-IL-33, as indicated. Cytokine concentration in culture supernatants was measured by ELISA after 24 h.

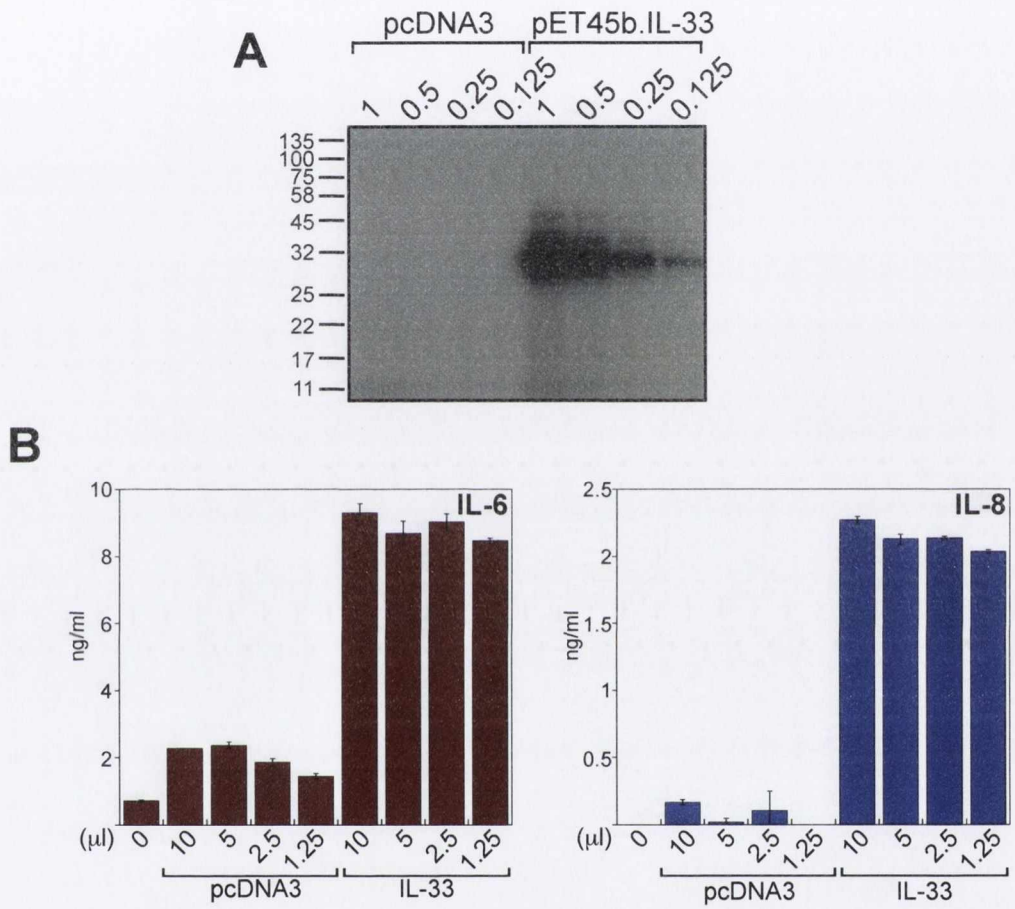

Figure 4.14 IL-33 generated by in vitro transcription/translation is active on HeLa cells overexpressing IL-33R

A. His-tagged full-length IL-33 in pET45b plasmid template was in vitro transcribed/translated in a rabbit reticulocyte lysate system. pcDNA3 empty vector was used as a control for ITT reactions. Samples of ITT reactions were subject to analysis by SDS-PAGE/fluorography, as indicated. **B.** HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells transfected with IL-33R were treated with a titration of in vitro transcribed/translated full-length IL-33, as indicated. IL-6 and IL-8 concentration in cell culture supernatants was measured by ELISA.

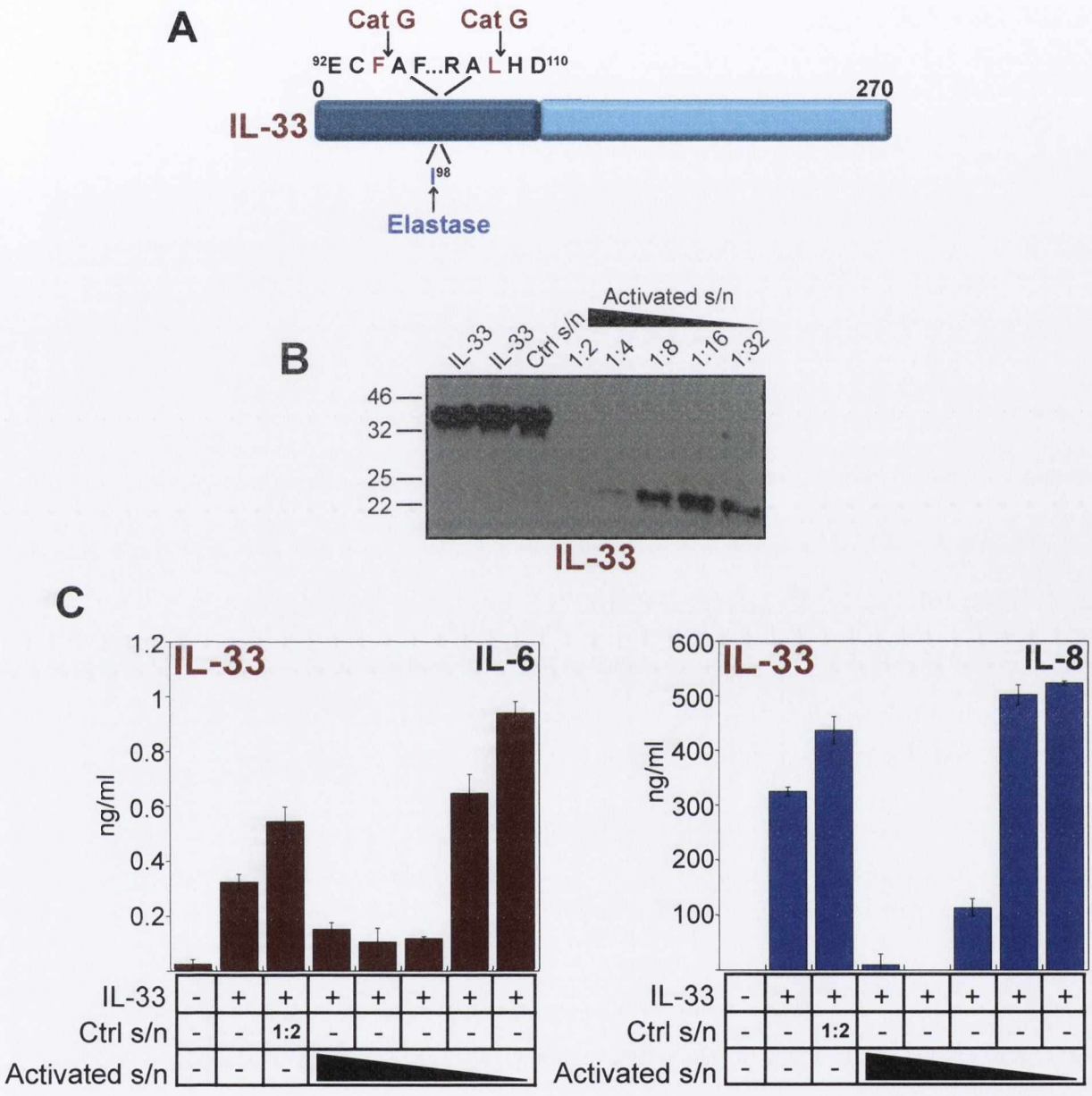

Figure 4.15 Activated neutrophil degranulate supernatants abolish IL-33 activity
A. Schematic of full-length IL-33 protein indicating processing sites by neutrophil proteases cathepsin G and elastase. **B.** In vitro transcribed/translated IL-33 (1 μl ITT) was incubated for 2 h at 37 °C with control or PMA-activated neutrophil degranulate. IL-33 processing was analysed by immunoblot. **C.** In vitro transcribed/translated IL-33 (0.125 μl ITT) was incubated for 2 h at 37 °C with control or PMA-activated neutrophil degranulate. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells overexpressing IL-33R were stimulated with IL-33, as indicated. After 24 h, IL-6 and IL-8 in cell culture supernatants was measured by ELISA.

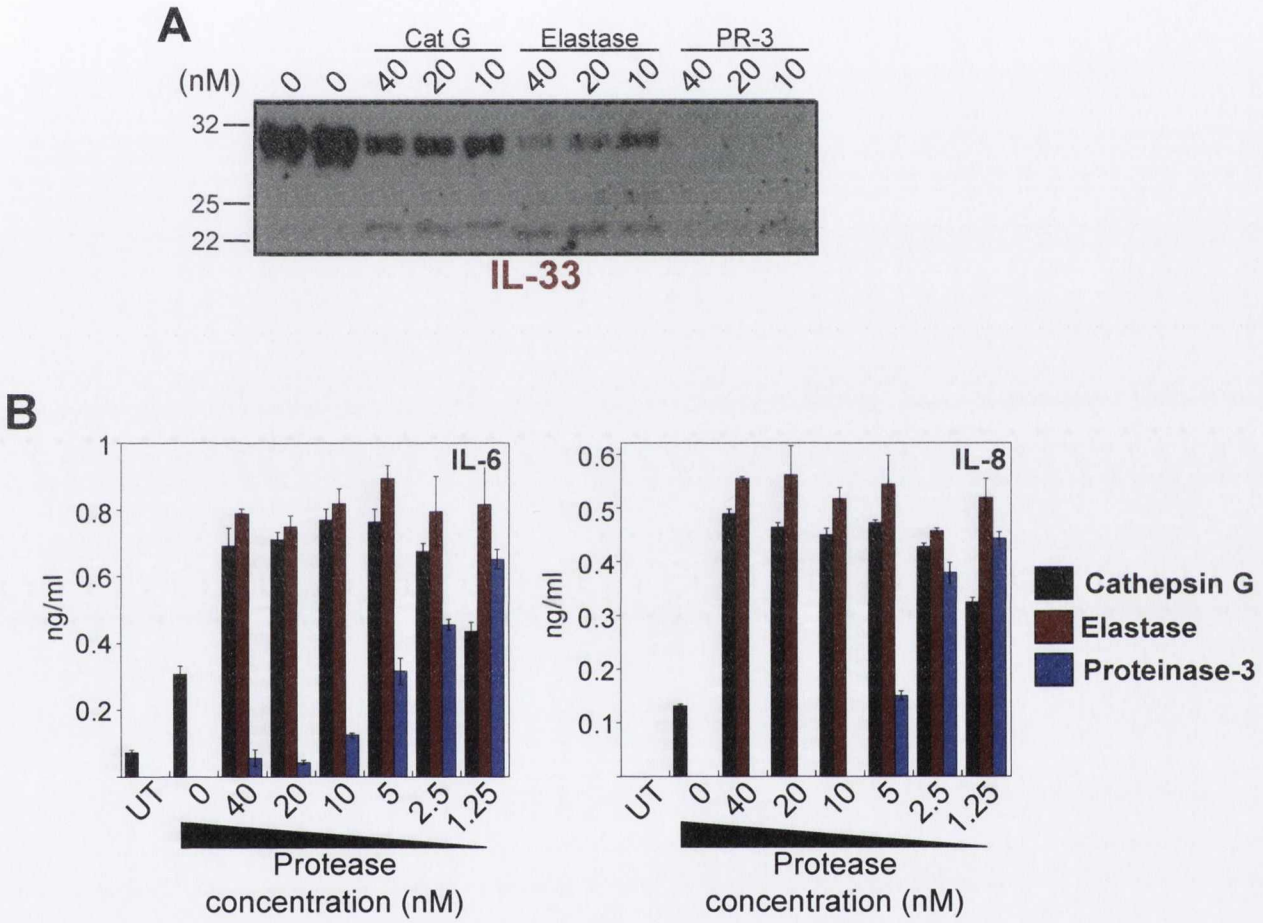

Figure 4.16 IL-33 is activated by purified cathepsin G and elastase but inactivated by proteinase-3

A. In vitro transcribed/translated IL-33 (1 μl ITT) was incubated for 2 h at 37 °C with purified cathepsin G, elastase or proteinase-3, as indicated. IL-33 processing was analysed by immunoblot. **B.** In vitro transcribed/translated IL-33 (0.125 μl ITT) was incubated for 2 h at 37 °C with purified cathepsin G, elastase or proteinase-3, as indicated. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells overexpressing IL-33R were stimulated with IL-33 and cytokine concentration in cell culture supernatants was measured by ELISA after 24 h.

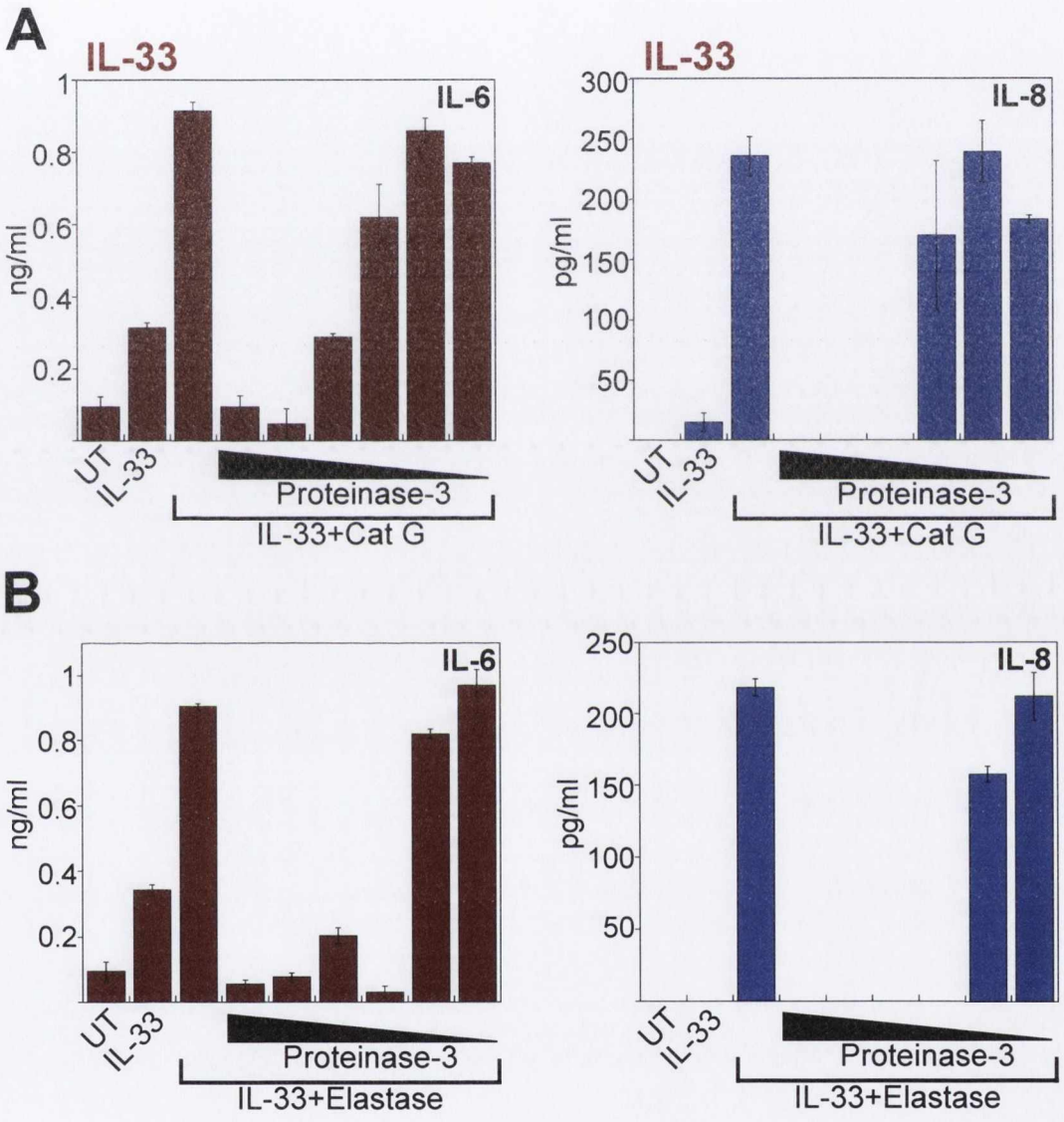

Figure 4.17 Proteinase-3 inactivates mature IL-33
In vitro transcribed/translated IL-33 (0.125 μl ITT) was incubated with a fixed concentration of **A.** cathepsin G (40 nM) or **B.** elastase (40 nM) with, or without, a titration of proteinase-3 (40 nM to 1.25 nM) for 2 h, 37 °C. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells overexpressing IL-33R were stimulated as indicated. After 18 h, IL-6 and IL-8 concentrations in cell culture supernatants was measured by ELISA.

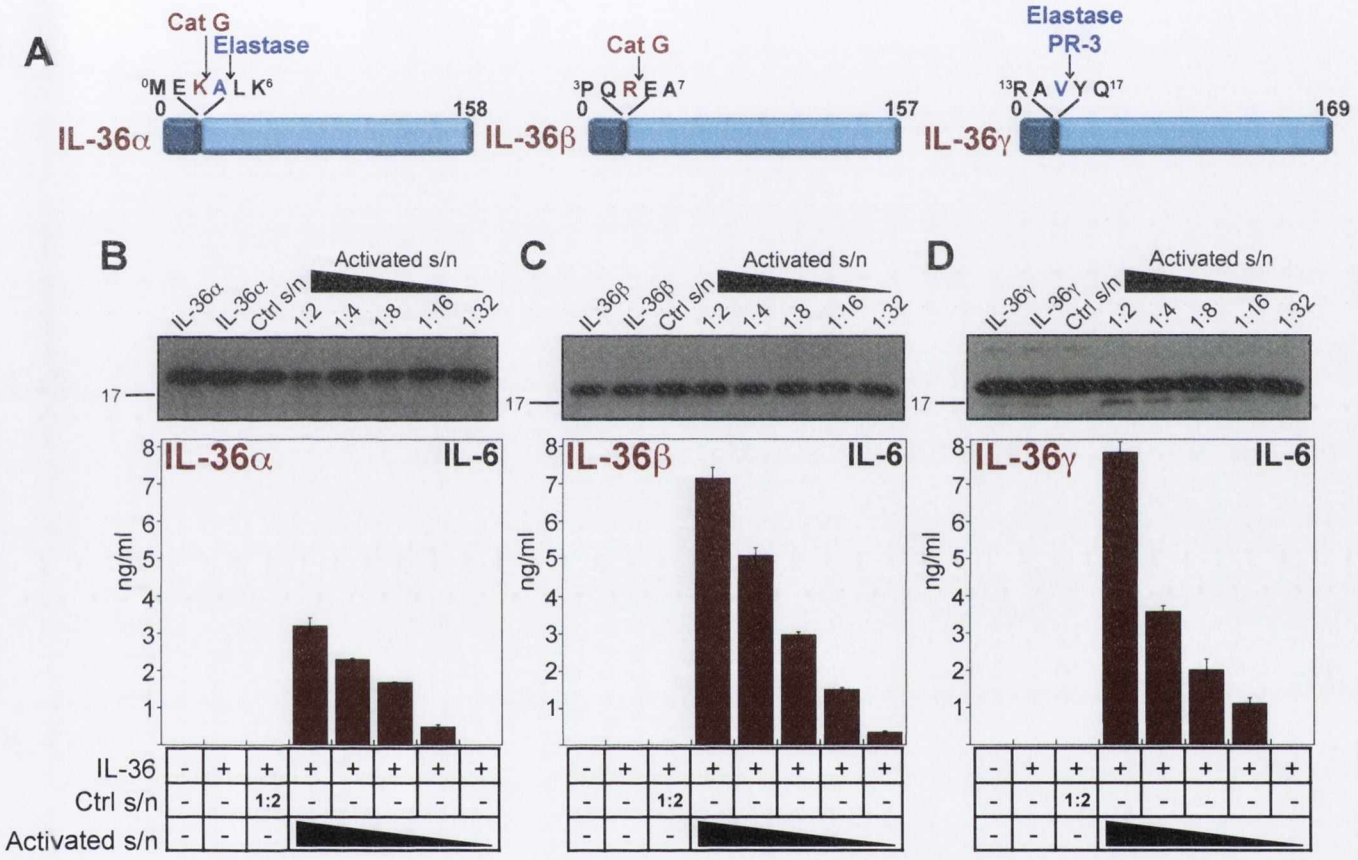

Figure 4.18 IL-36 cytokines are processed and activated by neutrophil proteases
A. Schematic of IL-36α, IL-36β and IL-36γ proteins indicating processing sites by neutrophil proteases. **B-D.** Recombinant **B.** IL-36α, **C.** IL-36β and **D.** IL-36γ (50 nM) was incubated with control or PMA-activated neutrophil degranulate for 2 h at 37 °C. Processing of IL-36 cytokines by neutrophil degranulates was analysed by immunoblot. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-36α, IL-36β or IL-36γ (1 nM), as indicated. After 18 h, IL-6 levels in cell culture supernatants was measured by ELISA.

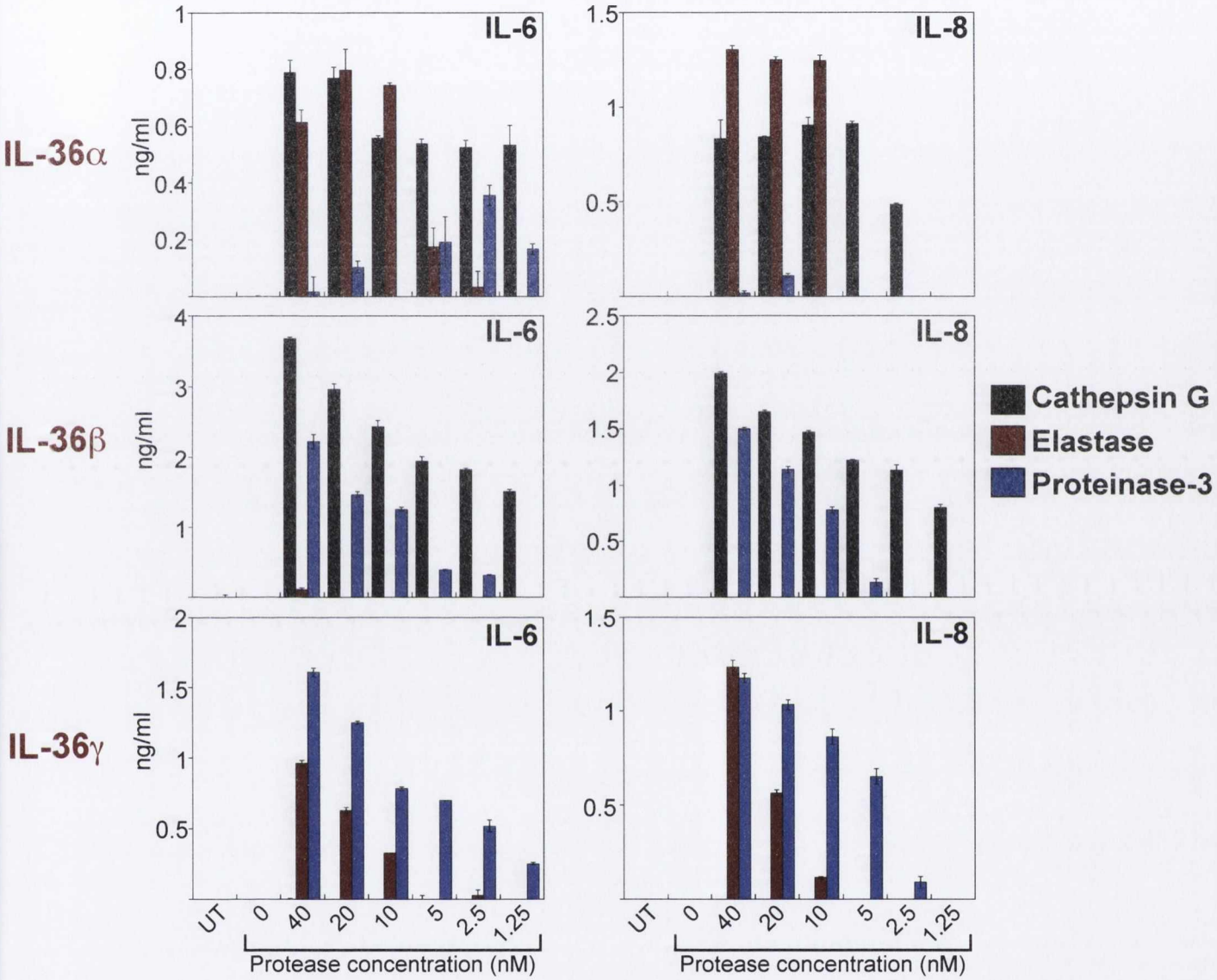

Figure 4.19 Purified neutrophil proteases robustly activate IL-36 cytokines
Recombinant IL-36α, IL-36β and IL-36γ (50 nM) were incubated with cathepsin G, elastase or proteinase-3, as indicated for 2 h, 37 °C. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were stimulated with IL-36α, IL-36β or IL-36γ (1 nM), as indicated. After 18 h, IL-6 and IL-8 concentrations in culture supernatants was measured by ELISA. This data was contributed by Graeme Sullivan.

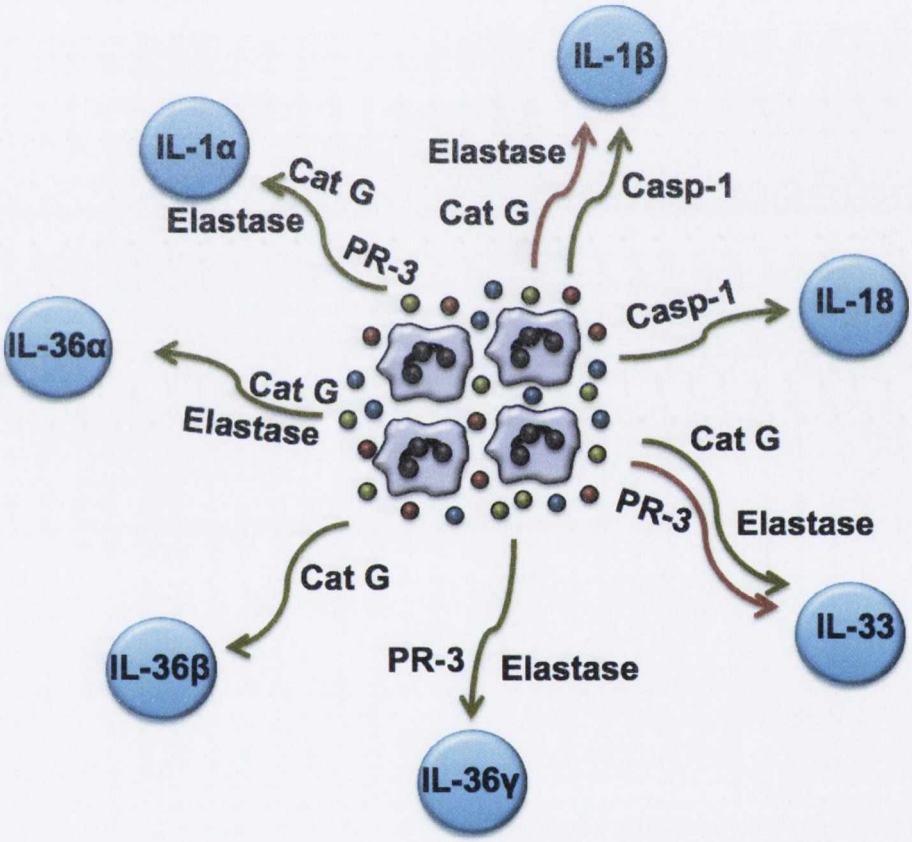

Figure 4.20 Neutrophil proteases modulate the activity of multiple IL-1 family cytokines Neutrophil granule proteases play a major role as modulators (both positively and negatively) of the activation state of multiple pro-inflammatory IL-1 family cytokines. Green arrows denote activation of IL-1 family cytokine by neutrophil proteases, while red arrows denote inactivation or no activation.

Overall, the results presented here point to neutrophil serine proteases as key regulators of the activity of most IL-1 family cytokines. These proteases serve to either activate or inactivate these cytokines to shape inflammatory responses and modulate immunity.

4.3.1 Neutrophil proteases as mediators of inflammation: Lessons from knock-out mice

Neutrophil serine proteases are important effector enzymes stored in neutrophil azurophilic granules, and are rapidly deployed to eliminate invading microbes. Upon injury or infection, neutrophils are recruited from the blood stream by chemotactic signals to the injured site. Once these cells reach their target damaged tissue, they are fully activated and mobilise their granules to either fuse to the phagosome to kill intracellular bacteria, or fuse to the plasma membrane to release anti-microbial effectors into the extracellular space (Amulic et al., 2012)..

Neutrophil granule proteases have been shown to play an important role in the elimination and clearance of microbial insults. Neutrophil serine proteases are synthesized as inactive zymogens, requiring processing by dipeptidyl peptidase I (DPPI) to achieve their fully active state (Adkison et al., 2002). Studies utilizing mice deficient in these proteases underline their importance as antimicrobial enzymes, with these animals displaying increased susceptibility and mortality to bacterial and fungal infections, despite normal neutrophil maturation and function (Belaaouaj et al., 1998; Reeves et al., 2002; Tkalcevic et al., 2000; Weinrauch et al., 2002). While the importance of neutrophil-derived proteases as antibacterial enzymes has been widely studied, their contribution to inflammation is also becoming increasingly apparent.

Neutrophil derived proteases were first identified as destructive enzymes that participate in the breakdown of ECM at sites of injury or infection, leading to host tissue damage (Korkmaz et al., 2008). Thus, the current view of neutrophil enzymes in inflammatory conditions tends to be restricted to non-specific proteolysis that damages host tissue. However, increasing evidence suggests that neutrophil proteases are actively involved in the innate inflammatory response and act as important mediators of local inflammation. Massive neutrophil infiltration is associated with a range of inflammatory diseases including cystic fibrosis, COPD, acute lung injury, rheumatoid arthritis and psoriasis (Korkmaz et al., 2010).

In line with this, mice deficient in multiple neutrophil proteases display dramatically reduced inflammatory responses, underscoring the importance of these enzymes as inflammatory mediators. Mice deficient in DPPI, required to process and activate all three neutrophil serine proteases, are dramatically protected against collagen-induced arthritis compared to wild type mice, with no neutrophil accumulation detected in joints of $DPPI^{-/-}$ mice. These mice have defective protease expression and loss of enzymatic activity, but generally have normal neutrophil recruitment and function in response to thioglycollate injection or the neutrophil chemokine IL-8, suggesting that local cytokines and chemokines are required for neutrophil influx to joints, rather than an overall defect in neutrophil migration. Similar results were seen with single knockout mice deficient in elastase or cathepsin G alone. $DPPI^{-/-}$ mice are also resistant to inflammation in response to OVA immune complexes, with a local reduction of pro-inflammatory cytokines IL-1β and TNFα (Adkison et al., 2002).

Additionally, mice doubly deficient in elastase and cathepsin G are profoundly protected against LPS-induced endotoxic shock. In this model, neutrophil recruitment to the peritoneal cavity or migration to the liver were unaffected in the absence of elastase and cathepsin G, and TNFα production was also unaffected, but mice deficient in these proteases survived longer than wild type mice. The effect on IL-1 cytokines in this model was not examined (Tkalcevic et al., 2000). Similarly, elastase deficient mice are protected from challenge with zymosan, due, in part, to impaired generation of pro-inflammatory mediators including IL-1β, KC and MIP-1α (Young et al., 2004). Taken together, these studies suggest that neutrophil-derived proteases play an important role in amplifying inflammatory responses and act as regulators of local inflammation by inducing IL-1 and promoting production of other cytokines and chemokines.

4.3.2 Neutrophil proteases act as key regulators of IL-1 family cytokines to amplify inflammation

Accumulating evidence suggests that neutrophil granule proteases can process extracellular cytokines and chemokines to promote inflammation. For example, previous studies have suggested that proteinase-3 can process IL-8 to generate a more potent chemotactic fragment and cathepsin G has been reported to similarly modulate the activity of CCL15 (Pham, 2006). While processing of extracellular chemokines may contribute to further neutrophil recruitment to damaged sites, it does not explain the dramatic reduction in inflammatory responses seen in granule protease deficient mice.

IL-1 cytokines are potent pro-inflammatory cytokines and act at the apex of cytokine cascades to trigger inflammation. As discussed in the introduction, intermittent reports

over the last 20 years have linked neutrophil-derived proteases in the processing and activation of IL-1 family members. However, these studies fail to directly investigate the ability of neutrophil proteases to activate IL-1 cytokines to enhance their biological activity. The data presented here comprehensively shows that neutrophil-derived proteases act as key regulators of IL-1 family cytokines to promote and potentiate inflammation. Using PMA-activated neutrophil degranulate supernatant, which contains potent protease activity, as well as individual purified neutrophil proteases, we have shown that neutrophil-derived proteases can act as both positive and negative regulators of IL-1 family members, in a system that mirrors a physiologically relevant scenario.

Neutrophils may regulate IL-1 cytokine activity to promote and shape the inflammatory response. Upon tissue damage and subsequent necrotic cell death, IL-1 cytokines are released as DAMPs and stimulate surrounding cells to produce cytokines and chemokines. These signals recruit neutrophils to the damaged site where they release their granule proteases which, in turn, regulate IL-1 cytokines to modulate their biological activity and amplify inflammation. Data presented here argue that neutrophil-derived proteases act as specific regulators of IL-1 family members to enhance and alter immune responses, challenging the view of these proteases as merely antimicrobial enzymes.

4.3.3 Neutrophil-derived proteases may spatially and temporally regulate IL-1 cytokines

Neutrophil proteases released at sites of injury or infection may spatially and/or temporally regulate the activation of IL-1 cytokines to shape the local inflammatory environment and skew towards the appropriate adaptive immune responses. We have found that neutrophil proteases can act as both positive and negative regulators of IL-1 cytokine activation states. Because these proteases can both enhance and suppress the activity of IL-1 family members, it may seem that these processing events are random and non-specific outcomes of aberrant release of cathepsin G, elastase and proteinase-3 at inflammatory sites. However, when the expression patterns and downstream effects of each IL-1 cytokine is taken into account, it becomes apparent that processing of these cytokines by neutrophil proteases may represent precise mechanisms to shape inflammatory responses. Not all IL-1 cytokines are expressed or released in the same tissues so the availability of these cytokines for neutrophil proteases will vary depending on the inflammatory site.

IL-1α is highly expressed across a wide range of cell types including keratinocytes, epithelial cells and endothelial cells and is constitutively active in its full-length form. IL-1α is a key alarmin released upon necrotic cell death and can promote inflammation in sterile wounds (Chen et al., 2007; Rider et al., 2011). Because IL-1α activity is potently enhanced by neutrophil proteases (Fig. 4.4 and 4.5), this cytokine may act as a general indicator of tissue damage which can be enhanced by cathepsin G, elastase and proteinase-3.

Similarly, IL-36 cytokines are highly expressed in the skin and have been heavily implicated in the pathogenesis of psoriasis. Psoriasis is characterized by massive neutrophil influx and disease progression can be exacerbated by tissue damage in a phenomenon known as the Koebner effect (Nestle et al., 2009; Towne and Sims, 2012; Weiss et al., 2002). Thus, the tissue-specific expression of IL-36 in the skin makes it an important mediator of this disease. IL-36γ is highly expressed in inflammatory bowel disorders and recent work has implicated IL-36 signalling as an important mediator of wound healing in mouse models of colitis. IL-36 receptor deficient mice failed to recover from DSS-induced bowel damage and have profound defects in wound healing, failing to close colonic wounds. This correlated with impaired neutrophil accumulation in the wound and reduced IL-22 expression from neutrophils, resulting in increased mortality of IL-36R$^{-/-}$ mice. This may suggest a dual function of IL-36 activity whereby at early timepoints, IL-36 cytokines are highly pro-inflammatory and contributes to the colitis progression, whereas later, IL-36 signalling is required to promote neutrophil accumulation and subsequent IL-22 production to promote wound closure (Medina-Contreras et al., 2016).

Conversely, the expression of IL-1β and IL-18 is more restricted, primarily limited to hematopoietic cells such as monocytes and macrophages. Neutrophil proteases do not activate IL-1β or IL-18. The activation of these cytokines is under tight regulation by the inflammasome complex and intracellular caspase-1-mediated processing (Latz et al., 2013). Indeed, a recent study from our laboratory has demonstrated that extracellular IL-1β is not processed by caspase-1 after inflammasome activation, suggesting that IL-1β activation by caspase-1 does not occur extracellularly (Cullen et al., 2015). Neutrophil proteases do, however, potently suppress the activity of mature IL-1β generated by caspase-1 cleavage. This inactivation may occur temporally to limit inflammation, with inactivation occurring only when high concentrations of neutrophil proteases are present. This idea is reflected in results presented here, as low concentrations of cathepsin G and elastase have no effect on p17 IL-1β activity, with inactivation only occurring at higher concentrations of proteases (Fig. 4.9). This may

act as a negative feedback loop to control IL-1β activity and prevent the development of a chronic inflammatory environment. Similar negative regulation of IL-1 activity is mediated by IL-1Ra and soluble decoy receptors, both of which are upregulated by IL-1β itself (Garlanda et al., 2013). Combined effects of neutrophil proteases result in suppression of IL-33 activity, again, perhaps as mechanism of limiting inflammation.

4.3.4 Neutrophil proteases modulate IL-1 cytokine activation states to skew toward appropriate adaptive immune responses

IL-1 family cytokines are potent mediators of innate immune responses but also act as important determinants of the type of T cell responses activated during adaptive immunity after tissue injury or infection. IL-1α and IL-1β skew towards Th17 responses, while IL-18 promotes predominantly Th1 cell responses through induction of IFNγ (Chung et al., 2009; Dinarello, 1999; Garlanda et al., 2013). IL-33 potently activates Th2 immunity and induces Th2-associated cytokines including IL-5 and IL-13 (Schmitz et al., 2005). Interestingly, previous studies have shown that IL-33 administration can protect against LPS-induced sepsis by promoting neutrophil influx and subsequent bacterial clearance. IL-33-treated mice had higher neutrophil recruitment and a reduction in systemic cytokine levels, but importantly, Th2-associated cytokines were not induced (Alves-Filho et al., 2010; Verri et al., 2010). This suggests that while IL-33 can induce neutrophil migration, the expected Th2 response was not induced, perhaps because neutrophil proteases inactivate IL-33 activity upon arrival at the inflammatory site.

Interestingly, a recent study has also implicated IL-1 cytokines in the resolution of inflammation by activating regulatory T cells to promote lung tissue repair. Treg cells suppress T cell responses and blunt pro-inflammatory gene activation by disrupting positive feedback loops on activated T cells. Thus, these cells promote a switch from an inflammatory environment to a wound healing program to mediate tissue repair. Arpaia and colleagues report that Treg cells are a major source of the EGF-R ligand amphiregulin during influenza virus infection (Arpaia et al., 2015). Amphiregulin activity regulates cell survival, proliferation and motility and plays an important role in the development of numerous tissues (Berasain and Avila, 2014). Conditional knockout of amphiregulin in Treg cells results in severe tissue damage in influenza infected lungs, however this does not disrupt Treg suppressor function. Induction of amphiregulin in Treg cells is independent of TCR stimulation, but instead is mediated by IL-33 and IL-18 activity released from damaged cells (Arpaia et al., 2015). Thus, by differentially activating or inactivating IL-1 family members, neutrophil proteases can selectively skew innate and adaptive inflammatory responses.

Chapter 5

Neutrophil extracellular traps can serve as platforms for activation of IL-1 family cytokines

5.1 Introduction

Studies over the past 10 years have demonstrated that activated neutrophils undergo an unusual form of cell death, termed NETosis, which culminates in the expulsion of chromatin into the extracellular space in the form of neutrophil extracellular traps (NETs). While the precise mechanism of this mode of cell death remains under investigation, it appears that NETosis may be an additional anti-microbial strategy employed by neutrophils to entrap bacteria, fungi, yeast and other micro-organisms in a mesh-like structure made up of DNA, histones, anti-microbial proteins and neutrophil proteases such as elastase and cathepsin G (Brinkmann et al., 2004; Fuchs et al., 2007; Papayannopoulos and Zychlinsky, 2009). Indeed, after histones, elastase was found to be the most abundant protein constituent of NETs, although a specific role for this protease in this context has not been demonstrated (Urban et al., 2009).

As shown in chapter 4, neutrophil proteases are involved in the processing and activation of multiple IL-1 family cytokines, thus, we wondered whether neutrophil serine proteases found on NETs were capable of activating members of the IL-36 cytokine family. High concentrations of NETs have been detected in a range of autoinflammatory and autoimmune diseases including systemic lupus erthymatosus (SLE), cystic fibrosis, necrotising vasculitis and psoriasis, and so may serve as platforms for cytokine activation by exposing proteases in the extracellular space to potentiate inflammation (Branzk and Papayannopoulos, 2013; Brinkmann and Zychlinsky, 2012). Here we show that neutrophils release NETs in response to PMA and that these NETs are decorated with neutrophil serine proteases cathepsin G, elastase and proteinase-3. NET-associated protease activity robustly activated IL-36α, IL-36β and IL-36γ and potently enhanced their biological activity. Activation of IL-36 cytokines was confirmed to be specifically mediated by NET-bound neutrophil serine proteases using specific inhibitors of these proteases, as well as mutated forms of IL-36 in which neutrophil protease cleavage sites are mutated. Degradation of NETs using DNase I also suppressed activation of IL-36 cytokines. Thus, NETs may serve as platforms for cytokine activation during infection or injury or as part of autoinflammatory disorders to enhance inflammation and the immune response.

5.2 Results

5.2.1 Activated neutrophil supernatants contain soluble protease activity

Upon treatment with activating stimuli such as PMA, neutrophils undergo an oxidative burst that leads to degranulation and release of their granule contents. Neutrophils were activated with PMA to induce degranulation and release of granule proteases into cell supernatants (Fig. 5.1 A, B), however here, cells were treated with PMA for 4 hours to promote NET formation, as described previously (Brinkmann et al., 2010). As expected, neutrophils stimulated with PMA released proteases that could be detected by FLF-sBzL and AAPV-AMC hydrolysis assays (Fig. 5.1 B).

As well as measuring protease enzymatic activity by fluorimetric methods, we also analysed neutrophil cell and supernatant fractions by immunoblot. As shown in Fig. 5.2, neutrophils released cathepsin G, elastase and proteinase-3 into cell culture supernatants in response to PMA, whereas these proteins could not be detected in untreated neutrophil supernatants. Interestingly, activated neutrophils also retained some cathepsin G, elastase and proteinase-3 in the cellular fraction, suggesting that some proteases remain cell-bound, possibly associated on NET structures being extruded from cells (Fig. 5.2). Activated neutrophils appeared to undergo actin rearrangments in response to PMA. Actin polymerisation and cytoskeletal reorganization occurs after activation and may be important for effective neutrophil adhesion, chemotaxis, and phagocytosis (Nathan, 2006).

5.2.2 Activated neutrophils release neutrophil extracellular traps

Activated neutrophils undergo degranulation to release their proteases and anti-microbial effectors, however, a seminal study by Zychlinsky and colleagues identified NETs, an additional weapon utilized by neutrophils during infection or trauma. NETs are mesh-like structures consisting of smooth filaments decorated with granular proteins including neutrophil serine proteases (Brinkmann et al., 2004; Urban et al., 2009). During NET formation, neutrophils undergo striking morphological changes, particularly in their nuclei, ultimately resulting in nuclear breakdown into the cytosol and expulsion into the extracellular space (Fuchs et al., 2007; Papayannopoulos et al., 2010).

To induce and visualize NETs, neutrophils were stimulated with PMA for 4 hours, followed by fixation of cells and associated NETs. DNA was visualised using the cell-permeable fluorescent dye Hoechst 33258 and NETs were analysed by confocal microscopy. NET formation was counted by classing stained cells according to four distinct morphologies as described previously: lobulated or delobulated nuclei or diffuse or spread NETs (Hakkim et al., 2011). Untreated, resting neutrophils had classical

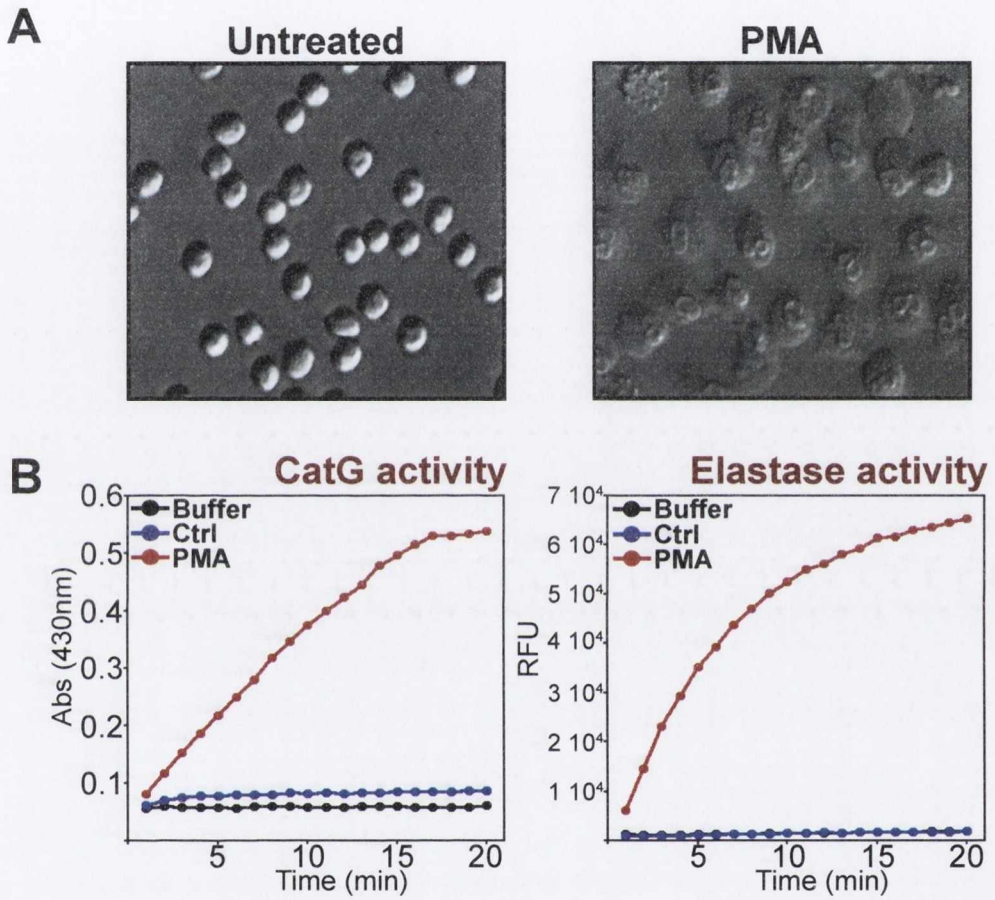

Figure 5.1 Activated neutrophils release granule proteases and degranulate supernatants contain soluble protease activity

A. Primary human neutrophils isolated from peripheral blood were stimulated with PMA (100 nM) for 4 h. Cells were visualised by phase-contrast microscopy. **B.** Primary human neutrophils (1 x 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, respectively, over 20 minutes.

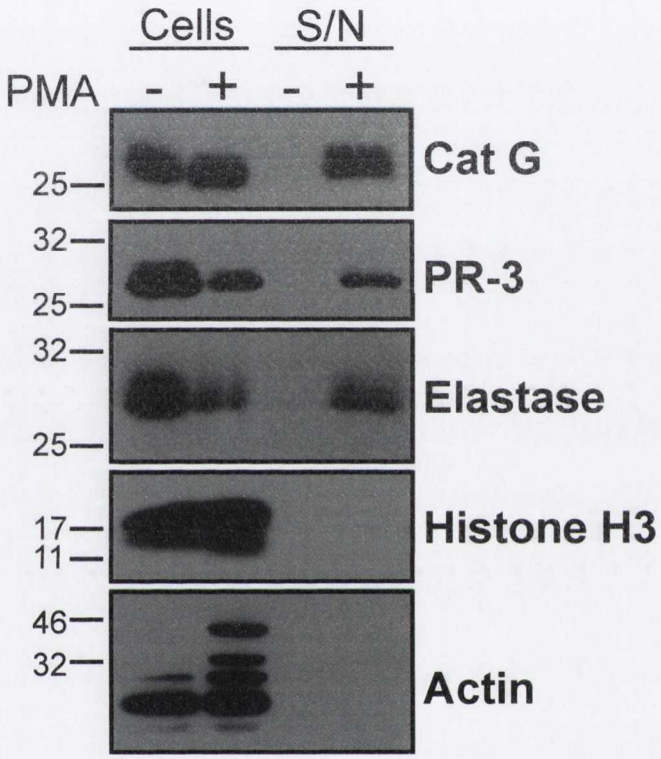

Figure 5.2 Activated neutrophils retain some granule proteases after degranulation
Neutrophils (1 x 10^7 cells/ml) were stimulated, or not, with 100 nM PMA for 4 h, 37 °C in eppendorfs. After 4 h, cells were centrifuged at 500 g for 5 minutes to separate supernatant and cellular fractions. Cell lysates versus supernatants were electrophoresed and immunoblotted using specific antibodies, as indicated.

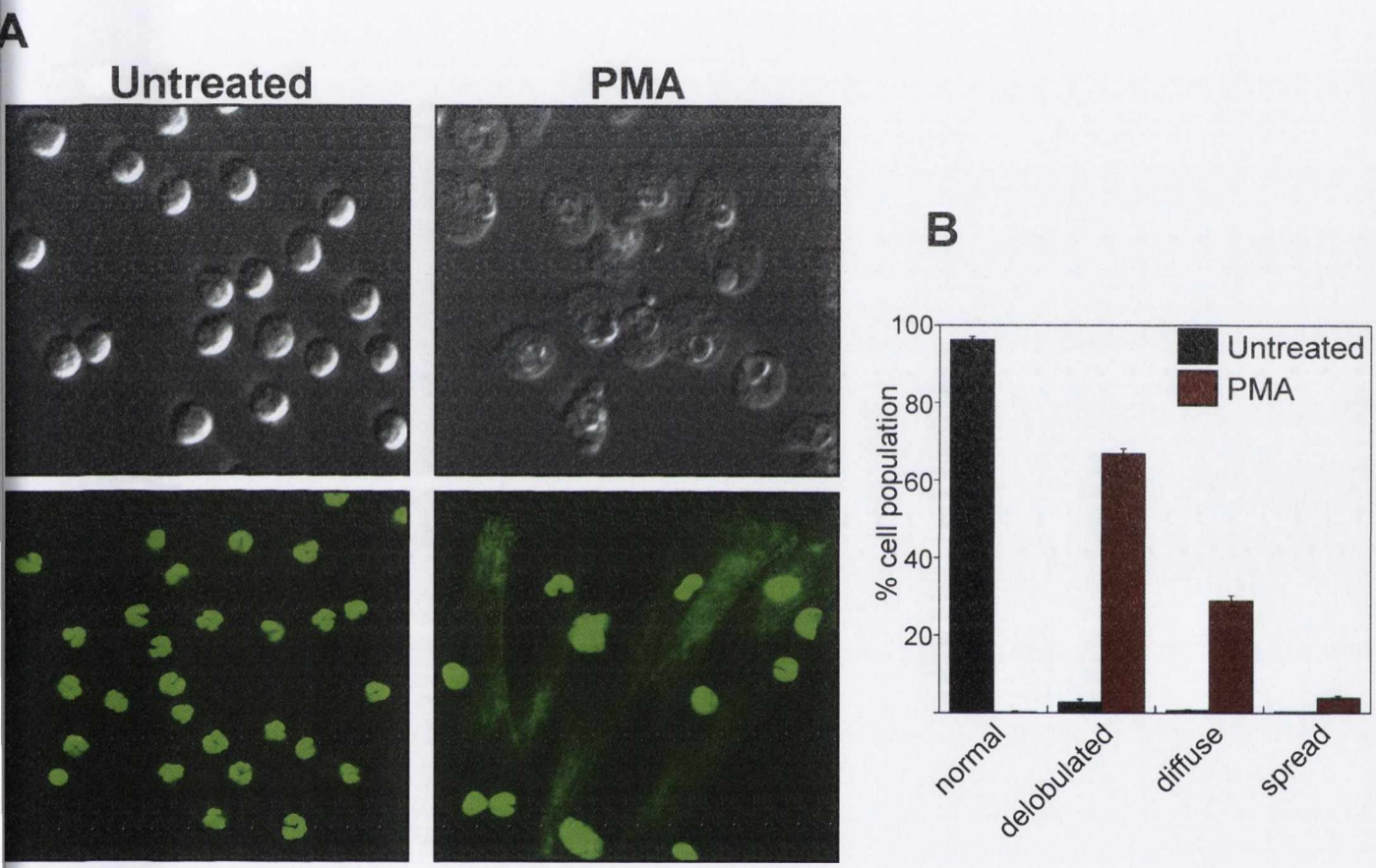

Figure 5.3 Activated neutrophils release neutrophil extracellular traps
A. Neutrophils (2 x 10^6 cells/well) were stimulated, or not, with 100 nM PMA for 4 h, 37 °C. After fixing and permeabilisation, cells were stained with Hoechst 33258 to visualise DNA. Neutrophil extracellular traps were visualised by confocal microscopy. DNA staining was pseudocoloured to green using Adobe Photoshop CS6. **B.** NETs were assessed and counted by confocal microscopy. Error bars represent SD of triplicate counts of a minimum of 100 cells.

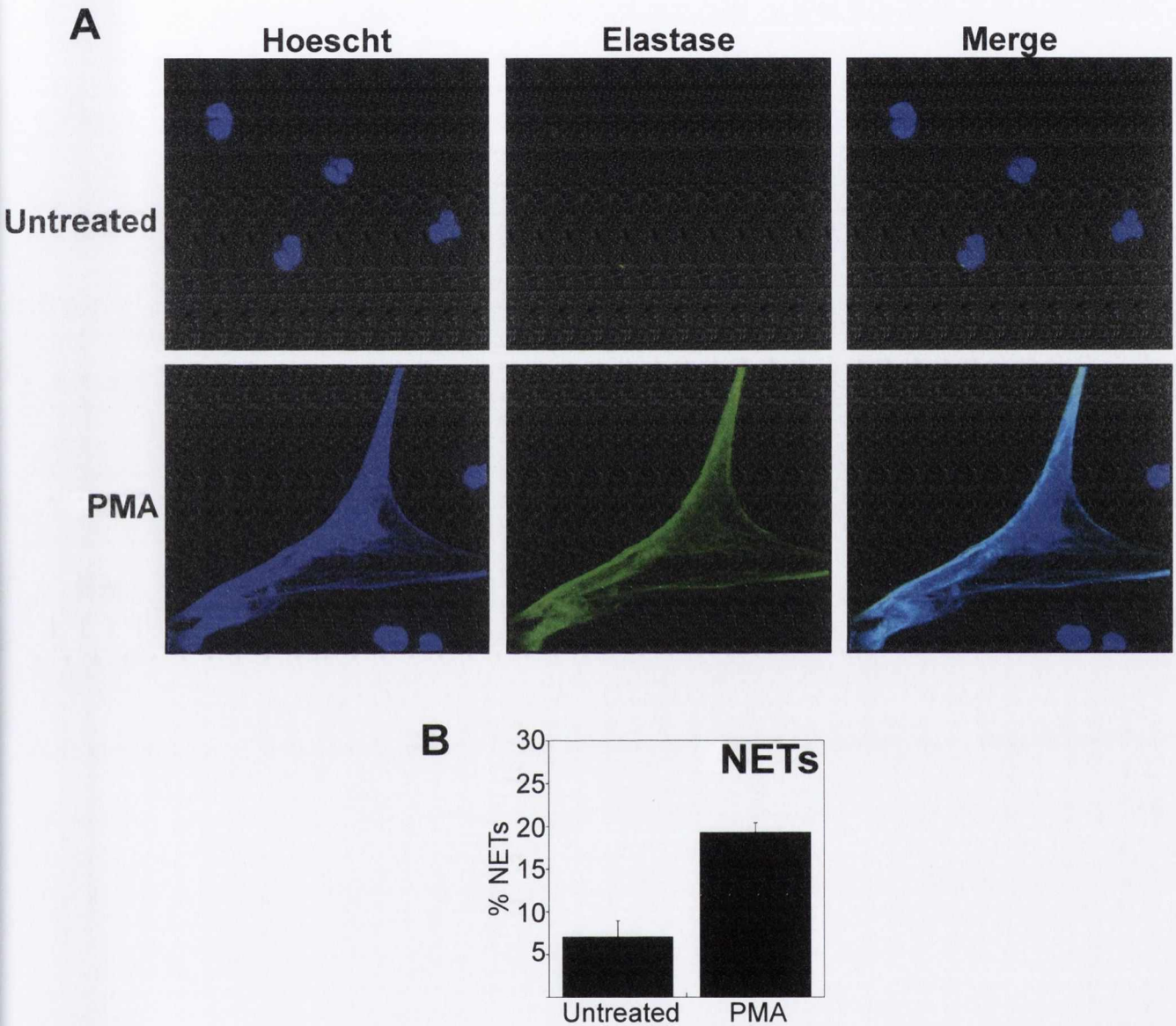

Figure 5.4 Neutrophil extracellular traps contain elastase that co-localises with NET chromatin structures

A. Neutrophils (1 x 10^6 cells/well) were stimulated, or not, with 100 nM PMA for 4 h, 37 °C. Cells were immunostained with elastase (green) and DNA was stained with Hoechst (blue) and visualised by confocal microscopy. **B.** NETs were assessed and counted by confocal microscopy.

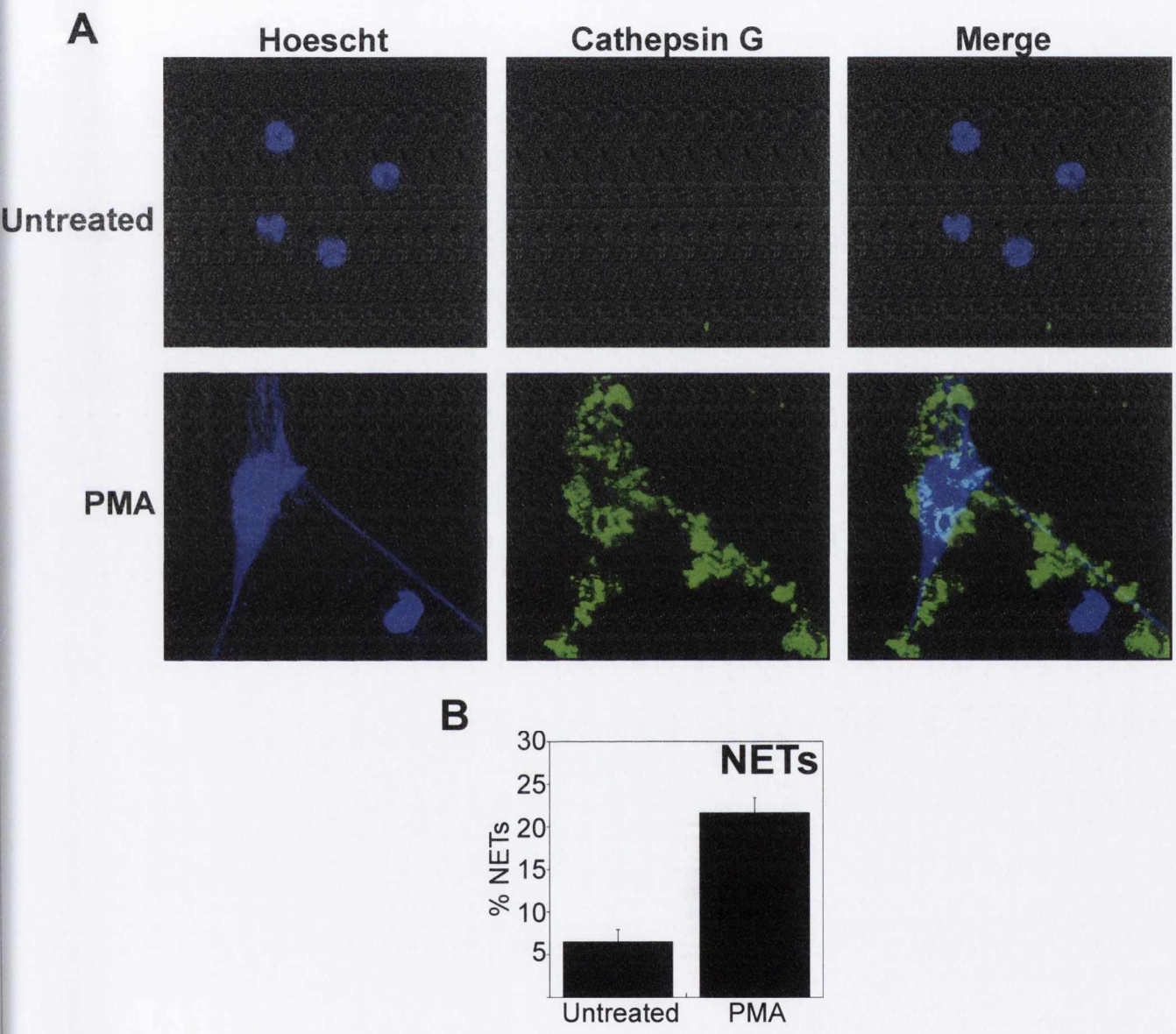

Figure 5.5 Neutrophil extracellular traps contain cathepsin G
A. Neutrophils (1 x 10^6 cells/well) were stimulated, or not, with 100 nM PMA for 4 h, 37 °C. Cells were immunostained with cathepsin G (green) and DNA was stained with Hoechst (blue) and visualised by confocal microscopy. **B.** NETs were assessed and counted by confocal microscopy.

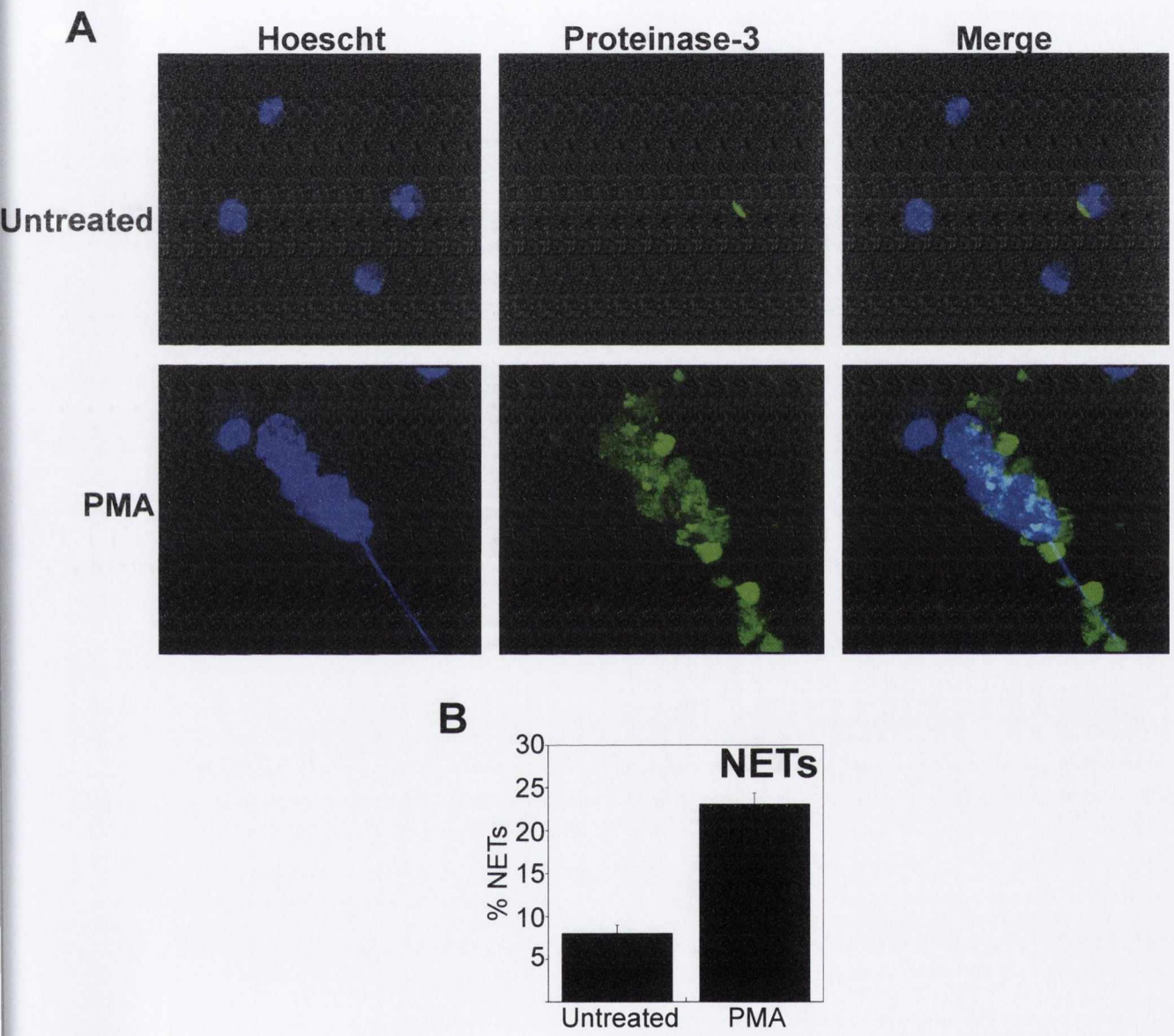

Figure 5.6 Neutrophil extracellular traps contain proteinase-3
A. Neutrophils (1 x 10^6 cells/well) were stimulated, or not, with 100 nM PMA for 4 h, 37 °C. Cells were immunostained with proteinase-3 (green) and DNA was stained with Hoechst (blue) and visualised by confocal microscopy. **B.** NETs were assessed and counted by confocal microscopy.

lobulated nuclei, whereas PMA-treated neutrophils had diffuse cloud-like NETs and long spread NETs mirroring the long extracellular chromatin fibres described in previous studies (Fig. 5.3 A, B) (Brinkmann et al., 2010). These data clearly demonstrate that NETs are formed in response to PMA stimulation and that NETs can be visualised and quantified by staining with a DNA-specific stain.

5.2.3 Neutrophil extracellular traps contain granule serine proteases

Previous studies have implicated neutrophil serine proteases as components of NETs (Papayannopoulos et al., 2010; Urban et al., 2009). We sought to confirm the presence of neutrophil granular proteins on NETs using immunofluorescence.

Neutrophils were stimulated with PMA, as before, and cells were stained with specific antibodies against cathepsin G, elastase and proteinase-3 as well as Hoechst staining of DNA to determine if these proteases co-localised with NET structures. It is important to note here that neutrophils were not permeabilised before staining, so any protease-specific staining represents extracellular cathepsin G, elastase or proteinase-3.

As shown in Fig. 5.4, activated neutrophils released NETs which were highly decorated with elastase. Elastase staining co-localised with extracellular DNA associating this protease with NET structures, while delobulated neutrophils that had not yet undergone NETosis failed to externalize elastase. Similar results were seen with cathepsin G and proteinase-3 (Fig 5.5; Fig 5.6). This observation confirms the presence of neutrophil serine proteases cathepsin G, elastase and proteinase-3 on NETs and implicates NETosis as a method of releasing neutrophil granule proteases into the extracellular space where they can modulate and amplify immune responses (Pham, 2008).

5.2.4 Neutrophil extracellular traps contain cell-associated protease activity

NETs constitute a source of neutrophil proteases bound to long chromatin fibres that may be more concentrated than proteases released during degranulation which may be dispersed rapidly in the extracellular space. Thus, we hypothesized that NETs may act as a platform for cytokine activation by exposing and retaining neutrophil proteases at the site of infection or inflammation. We sought to investigate the processing and activation of IL-36 cytokines by NET-bound proteases using an *in vitro* assay. Briefly, freshly isolated neutrophils were stimulated with PMA to induce NETosis, as before. Activated neutrophils were washed to removed soluble proteases released during degranulation, leaving only NET-bound protease activity on the remaining neutrophil population. The remaining neutrophils and the associated NETs were then treated with

inactive, full-length IL-36 cytokines. Activation was measured by titrating NET-treated IL-36 on HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells to check for enhanced IL-36 activity (Fig. 5.7).

To ensure that any activation of IL-36 cytokines could be specifically attributed to NET-bound protease activity, and not activity from residual soluble proteases, we measured soluble and cell-associated protease activity after washing of the NETs. As observed previously, supernatants from activated neutrophils contained potent elastase activity, as measured by AAPV-AMC hydrolysis assay (Fig. 5.8, A). Strikingly, after washing, no soluble protease activity could be measured in activated neutrophil supernatants, even up to one hour after washing (Fig. 5.8, B). Importantly, however, elastase enzymatic activity could be detected associated with the neutrophil/NET population (Fig. 5.8, C). These data suggest that neutrophils do not continue to degranulate after removal of the activating stimulus, PMA, and washing of the cell population. However, activated neutrophils do contain cell-associated elastase activity, suggesting that any enhanced activation of IL-36 cytokines after incubation with these cells is mediated by cell-associated or NET-bound protease activity.

5.2.6 Neutrophil extracellular traps activate IL-36 cytokines

Using the *in vitro* assay described previously (Fig. 5.7), we investigated the ability of NETs to activate IL-36 cytokines. All recombinant cytokines used in these assays were expressed and purified using ClearColi BL-21 *E. coli* to eliminate endotoxin contamination of protein preparations which may interfere with these assays as neutrophils are highly sensitive to LPS stimulation (Malcolm et al., 2003; Mamat et al., 2013). As previously dissected in chapter 3 and 4, IL-36 cytokines are inactive as full-length proteins and require N-terminal processing to release their pro-inflammatory activity. We incubated IL-36α, IL-36β and IL-36γ with neutrophils and their associated NETs and checked for enhanced bioactivity. As seen in Fig. 5.9, while untreated neutrophils had no effect on the activity of full-length IL-36α, incubation of IL-36α with NETs robustly enhanced the activity of this cytokine, reflected in high levels of IL-6 and IL-8 secretion from HeLa$^{IL\text{-}36R}$ cells. Similar results were seen with IL-36β (Fig. 5.10) and IL-36γ (Fig. 5.11), with untreated neutrophils having little or no effect on cytokine activity, whereas IL-36 activity was strongly potentiated by NETs.

These results suggest that neutrophil extracellular traps contain granule serine proteases with measurable enzymatic activity that can process and enhance IL-36 cytokine activity in the extracellular space.

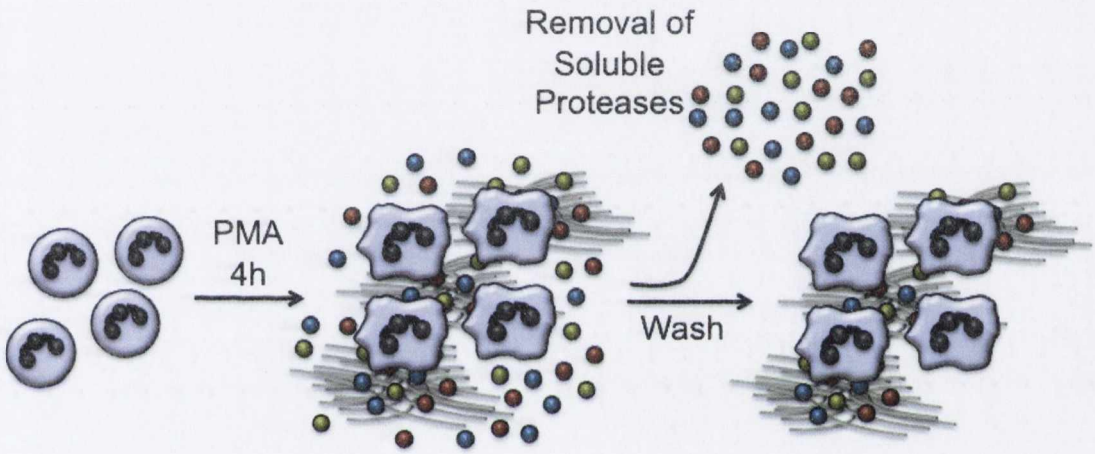

Figure 5.7 In vitro bioassay to investigate the activation of IL-36 cytokines by NETs
Schematic showing assay procedure to investigate if IL-36 cytokines can be activated by NETs. Neutrophils ($5 \times 10^6 - 1 \times 10^7$ cells/well) were stimulated by PMA for 4 h to induce degranulation and NET release. Neutrophils were washed with PBS to remove soluble proteases released during degranulation, leaving only NET-bound protease activity on the remaining neutrophil population. Remaining neutrophils and their associated NETs were treated with IL-36 cytokines for 30 minutes to allow cytokine activation, followed by a titration of NET-activated IL-36 cytokines on $HeLa^{IL\text{-}36R\text{-}SEAP}$ to check for enhanced IL-36 activity.

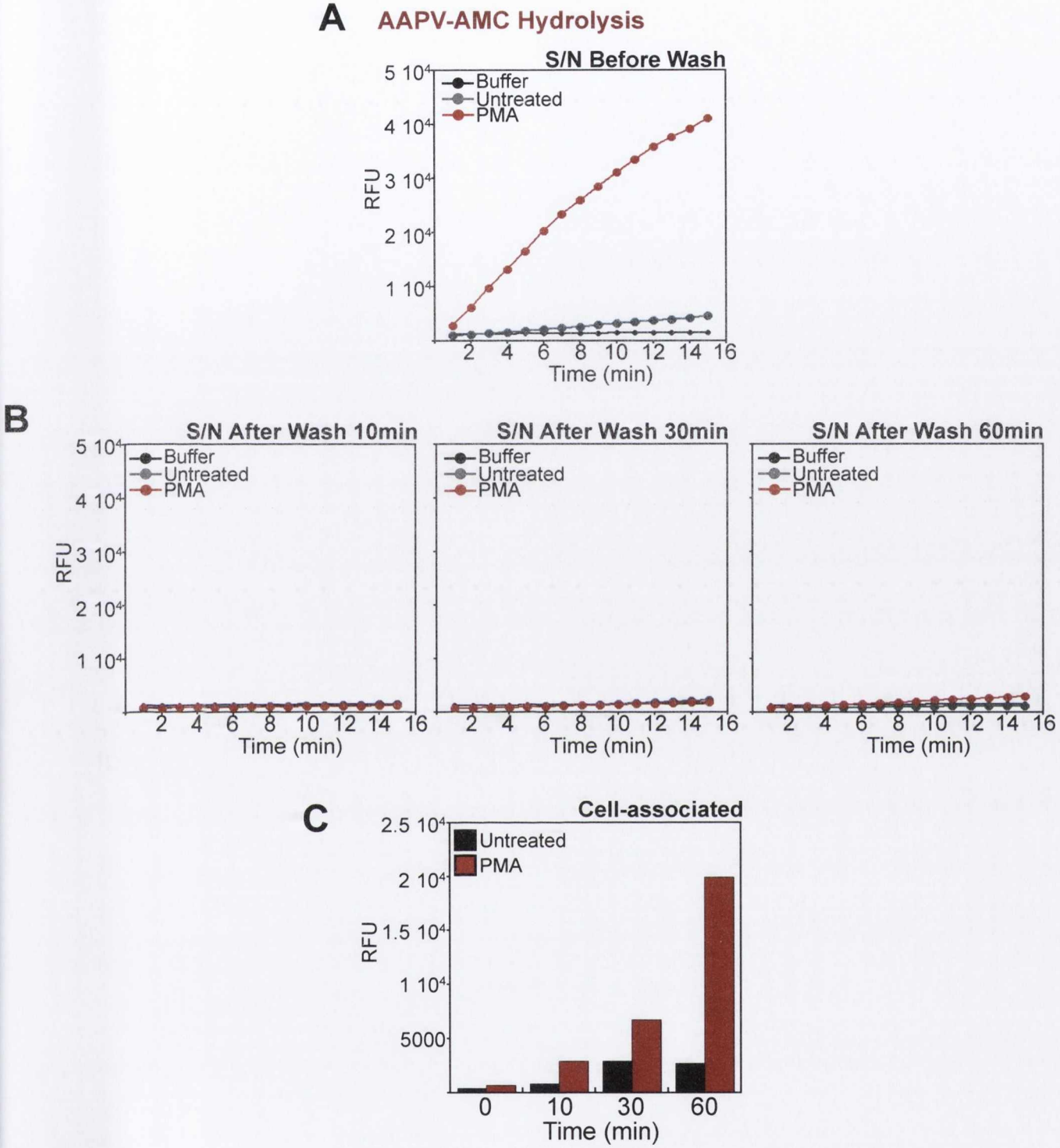

Figure 5.8 Neutrophils retain cell-associated protease activity after activation
A. Neutrophils (1 x 10^7 cells/ml) were stimulated with PMA (100 nM) for 4 h at 37 °C. After 4 h, neutrophil supernatants were harvested and elastase enzymatic activity in the supernatants was measured by AAPV-AMC hydrolysis assay. **B.** Cells were treated as in **A.** After 4 h, cells were washed 3 times with 2 ml of PBS followed by incubation with fresh HBSS/0.25% BSA. After washing, samples of neutrophil supernatant were taken at indicated timepoints and elastase enzymatic activity was measured by AAPV-AMC hydrolysis assay. **C.** Neutrophils were treated as before, washed and incubated with AAPV-AMC (50 μM) to determine cell-associated elastase enzymatic activity. Samples were taken at indicated timepoints after addition of AAPV-AMC.

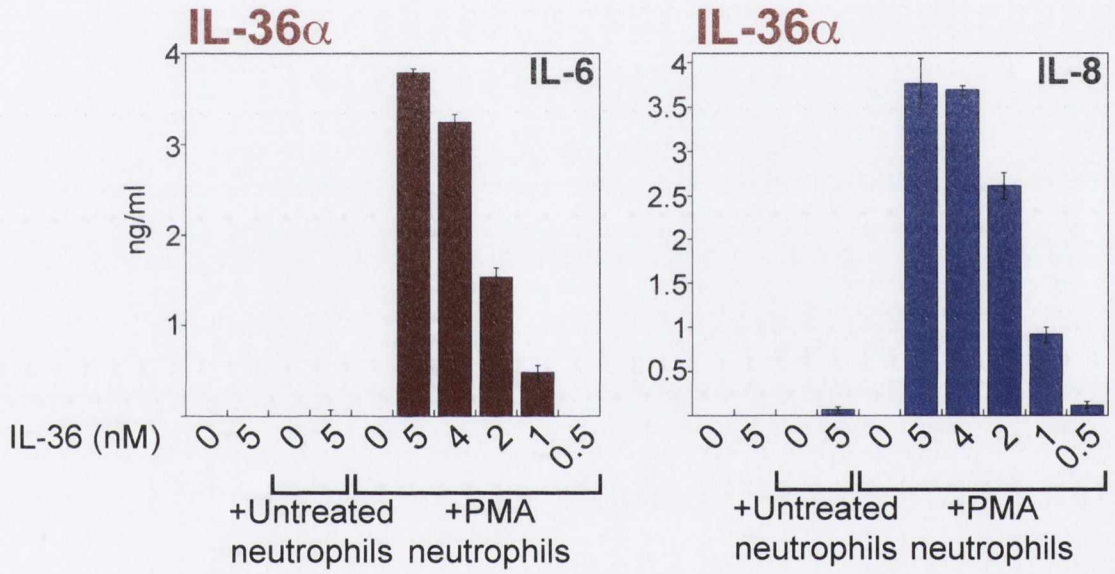

Figure 5.9 Neutrophil extracellular traps activate IL-36α
Neutrophils (1 x 10^7 cells/well) were stimulated, or not, with PMA (100 nM) for 4 h at 37 °C. Neutrophil degranulate supernatant was removed and cells were washed 3 times with 2 ml PBS to remove any residual soluble proteases. Cells were incubated with 50 nM IL-36α for 30 minutes. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were treated with a titration of IL-36α, as indicated. IL-6 and IL-8 concentration in cell culture supernatants was measured by ELISA after 18 h.

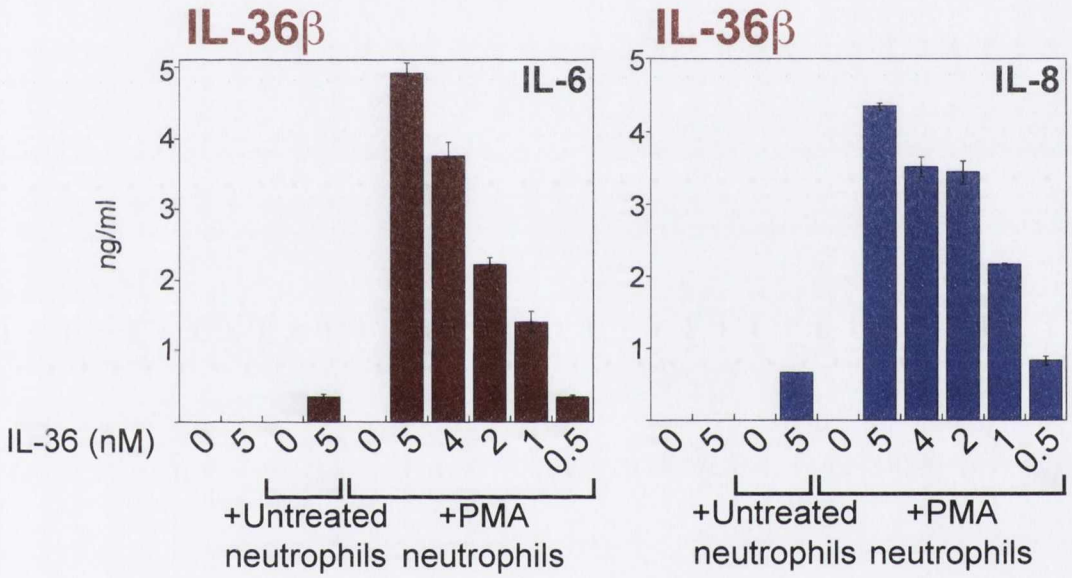

Figure 5.10 Neutrophil extracellular traps activate IL-36β
Neutrophils (1 x 10^7 cells/well) were stimulated, or not, with PMA (100 nM) for 4 h at 37 °C. Neutrophil degranulate supernatant was removed and cells were washed 3 times with 2 ml PBS to remove any residual soluble proteases. Cells were incubated with 50 nM IL-36β for 30 minutes. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were treated with a titration of IL-36β, as indicated. IL-6 and IL-8 concentration in cell culture supernatants was measured by ELISA after 18 h.

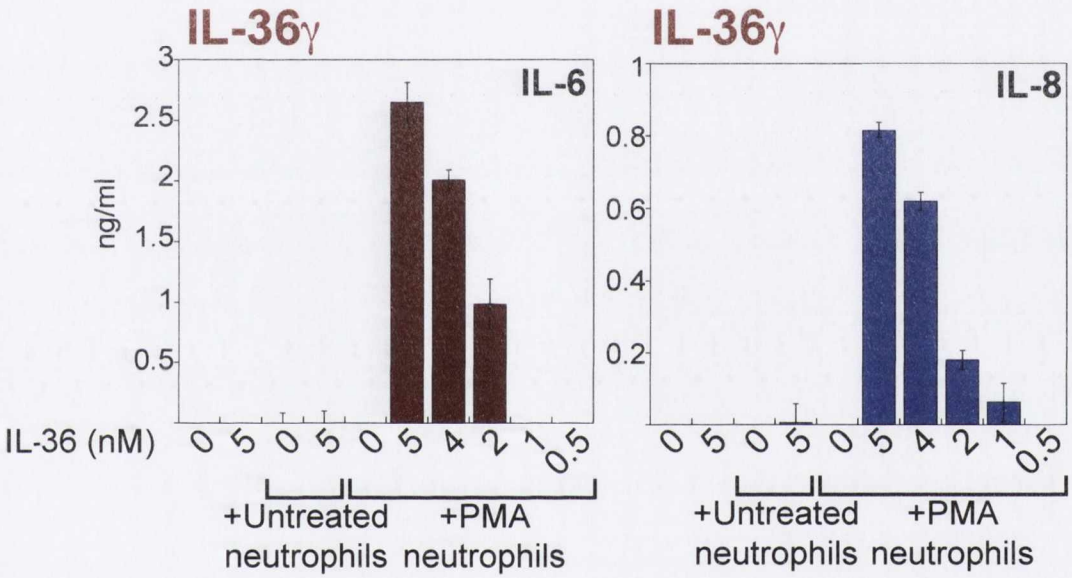

Figure 5.11 Neutrophil extracellular traps activate IL-36γ
Neutrophils (1 x 10^7 cells/well) were stimulated, or not, with PMA (100 nM) for 4 h at 37 °C. Neutrophil degranulate supernatant was removed and cells were washed 3 times with 2 ml PBS to remove any residual soluble proteases. Cells were incubated with 50 nM IL-36γ for 30 minutes. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were treated with a titration of IL-36γ, as indicated. IL-6 and IL-8 concentration in cell culture supernatants was measured by ELISA after 18 h.

5.2.7 NET-mediated activation of IL-36 cytokines is specific to neutrophil-derived granule proteases

We have shown that neutrophil extracellular traps contain cathepsin G, elastase and proteinase-3 (Fig. 5.4 – 5.6), however, NETs are comprised of many more components. While neutrophil serine proteases are found in high abundance in NETs, histones, anti-bacterial peptides and many other proteins can be found on these structures (Urban et al., 2009). Thus, the possibility arose that neutrophil proteases alone may not be the source of IL-36 cytokine processing and activation.

We have previously identified the cleavage sites in IL-36β and IL-36γ targeted by cathepsin G and elastase, respectively (Henry et al., 2016). Using N-terminal Edman degradation sequencing of cathepsin G-treated IL-36β preparations, we identified a candidate processing site at Arg5. Therefore, we generated a point mutation (R5A) at this site to assess its role in cathepsin G-mediated activation of IL-36β (Fig. 5.12, A). Mutation of this site eliminated the 17 kDa cleavage fragment generated by cathepsin G processing and more importantly, completely eliminated activation of IL-36β by cathepsin G (Henry et al., 2016). To investigate the contribution of cathepsin G in the activation of IL-36β by NETs, we treated NETs as before with wild-type IL-36β or the IL-36β^{R5A} point mutant. As shown in Fig. 5.12, B, wild-type IL-36β was robustly activated by NETs, as before. Strikingly, however, NETs were completely unable to activate the IL-36β^{R5A} mutant, suggesting that cathepsin G bound to NETs is mediating the activation of IL-36β (Fig 5.12, B).

Using a similar approach, we identified Val15 in IL-36γ as the residue that is cleaved by elastase to promote activation of this cytokine. Mutation of this residue (V15G) dramatically attenuated activation of IL-36γ by elastase and proteinase-3, as well as by PMA-activated neutrophil supernatants (Fig. 5.13, A) (Henry et al., 2016). As before, we compared the activation of wild-type IL-36γ and mutated IL-36γ^{V15G} by NETs. As expected, exposure of wild-type IL-36γ to NETs resulted in enhanced bioactivity, while mutated IL-36γ^{V15G} was not activated (Fig. 5.13, B). These data suggest that neutrophil-derived proteases bound to NETs selectively and effectively process and activate IL-36 cytokines to enhance their bioactivity.

5.2.8 Inhibition of proteases on NETs block IL-36 cytokine activation

To further confirm that NET-bound serine proteases are indeed the activators of IL-36 cytokines, we used chemical inhibitors of cathepsin G and elastase to block their enzymatic activity. As before, neutrophils were stimulated with PMA to induce NET

formation, followed by washing NETs to remove residual soluble protease activity. NETs were treated with cathepsin G or elastase inhibitors, or a combination of both, for 30 minutes to block protease activity, followed by treatment with IL-36 cytokines (Fig. 5.14).

As shown in Fig. 5.15, blocking cathepsin G activity potently suppressed activation of IL-36α and IL-36β, while inhibition of elastase had little effect on NET-mediated activation of these cytokines. This correlates with previous results in chapter 4 showing cathepsin G is the primary mediator of IL-36α and IL-36β activation. Both cathepsin G and elastase inhibition attenuated IL-36γ activation by NETs (Fig. 5.15). The contribution of cathepsin G to IL-36γ activity is surprising here, as we previously implicated elastase as the main activator of IL-36γ. NET-bound proteases are poor activators of IL-36γ as soluble proteases consistently give better activation and more potent enhancement of activity of this cytokine.

These observations, coupled with results using non-cleavable IL-36 mutants, underline that NET-induced activation of IL-36 cytokines is mediated by extracellular neutrophil granule proteases bound to chromatin strands.

5.2.9 DNase I treatment of NETs partially abrogates IL-36β activation

NETs can be dismantled by targeting the DNA backbone of these structures using DNase I, an endonuclease that non-specifically processes DNA (Yousefi et al., 2009). As mentioned previously, expression of DNases by bacteria promote the degradation of NETs and facilitate their escape from these traps (Beiter et al., 2006; Buchanan et al., 2006). To further confirm that NET-bound proteases mediate activation of IL-36β, we used a similar set up as before where washed NETs were incubated with DNase I for 30 minutes to promote their degradation. After DNase I treatment, the cell population was washed again to remove any proteases that may have been liberated from dismantled NETs. Then, as before, IL-36β was incubated on the cell population to check for enhanced bioactivity (Fig. 5.14).

Treatment with DNase I lead to a partial, albeit not complete, removal of NETs from PMA-activated neutrophils. As seen in Fig. 5.16 A, untreated neutrophils stained for DNA (blue) display the characteristic lobulated nuclei. After PMA treatment, neutrophil nuclei decondense and long NET structures are formed that co-stain for elastase (green). After DNA treatment, long NET structures are lost, but some elastase staining remains on diffuse, decondensed cells (Fig. 5.16, A). In the activity assay, again, untreated neutrophils do not modulate IL-36β, while PMA-activated neutrophils potently

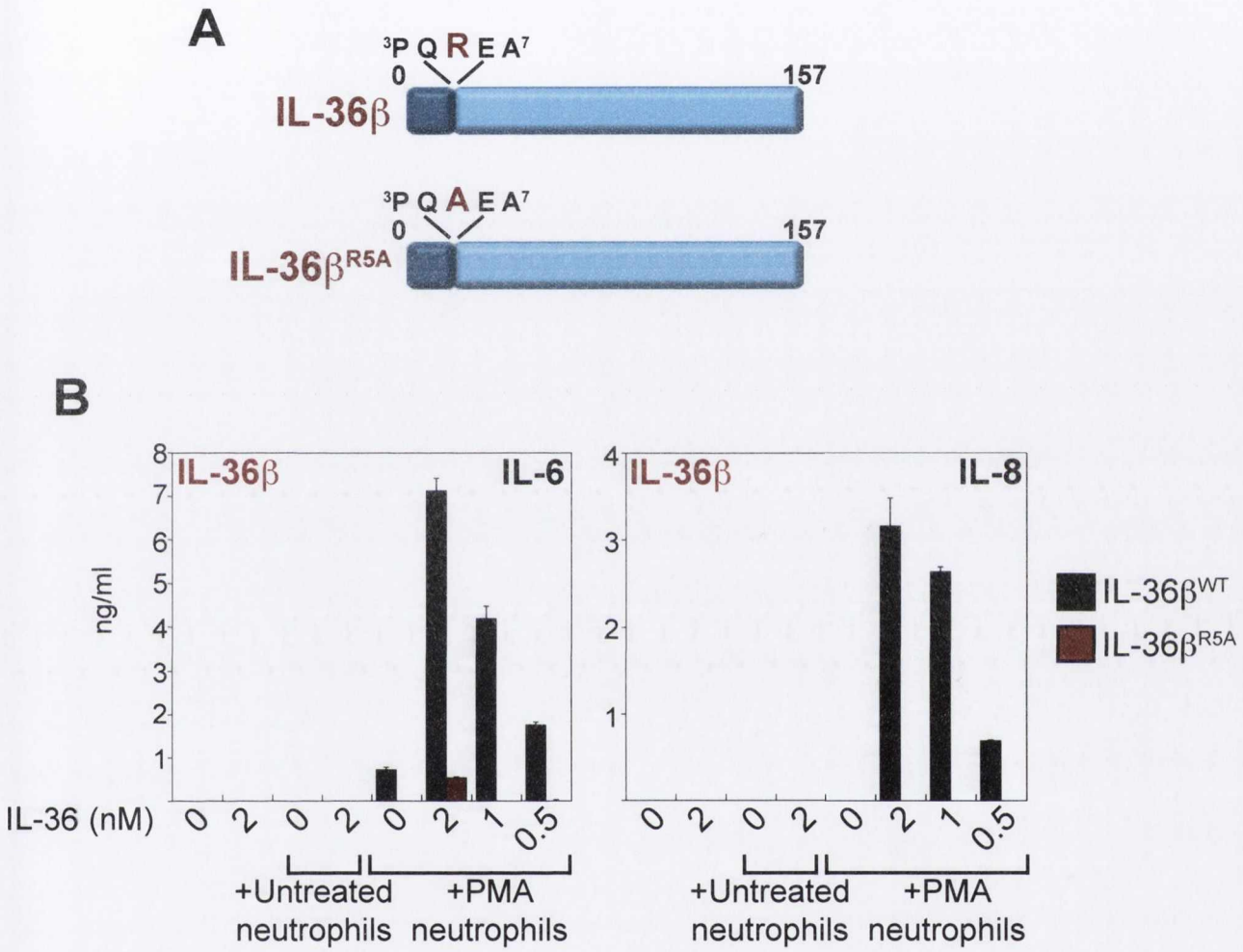

Figure 5.12 Neutrophil extracellular traps activate IL-36β but not the non-cleavable mutant IL-36β^{R5A}

A. Schematic of wild-type (WT) IL-36β indicating the PQR^{5} cleavage motif targeted by cathepsin G and the non-cleavable mutant IL-36β^{R5A}. **B.** Neutrophils (5 x 10^{6} cells/well) were stimulated, or not with PMA (100 nM) for 4 h at 37 °C. After 4 h, cell supernatants were removed and cells were washed 3 times with PBS. Cells were treated with IL-36β^{WT} or IL-36β^{R5A} (10 nM) for 30 minutes. HeLa$^{IL-36R-SEAP}$ cells were treated with a titration of IL-36β^{WT} or IL-36β^{R5A}, as indicated. IL-6 and IL-8 concentration in cell culture supernatants was measured by ELISA after 18 h.

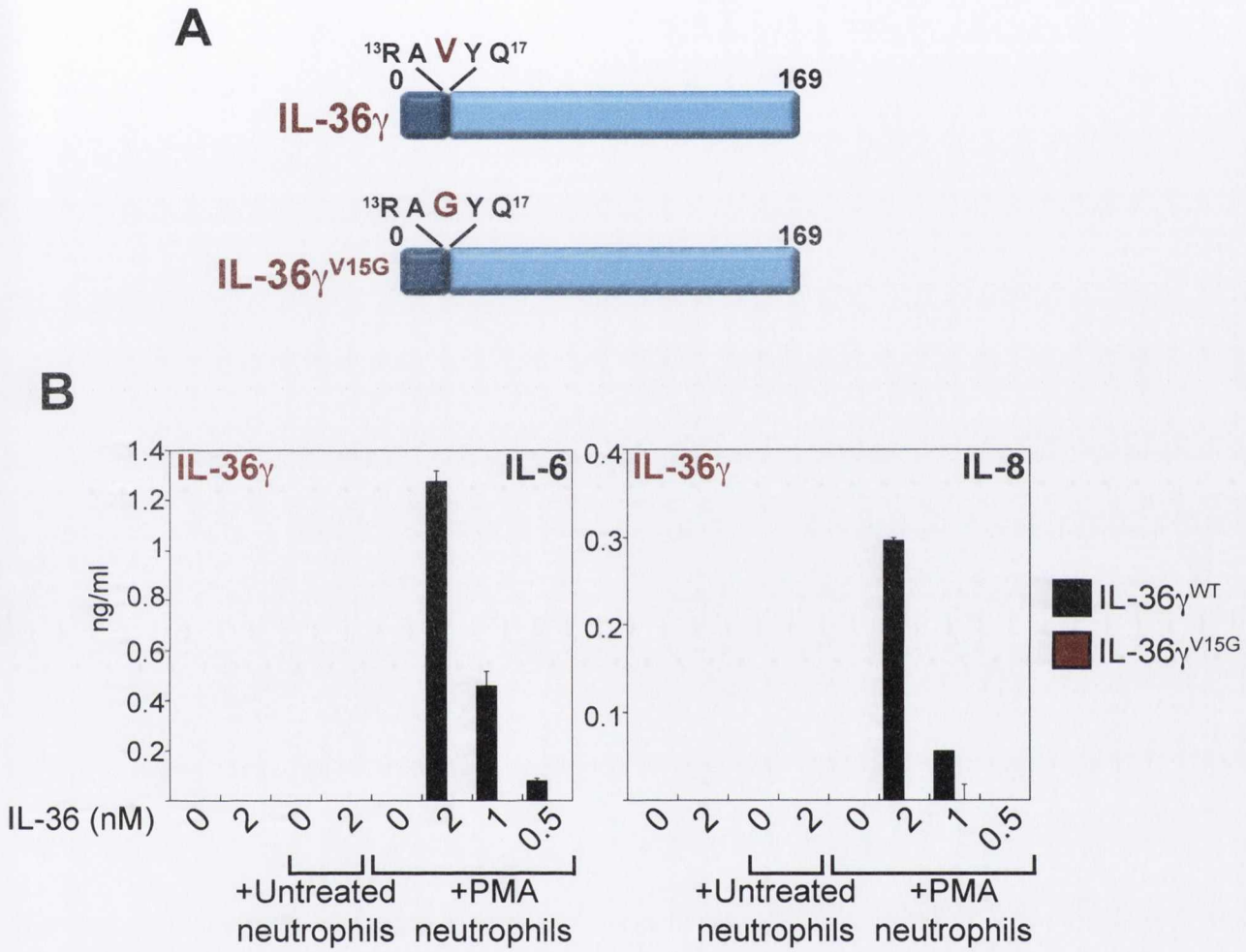

Figure 5.13 Neutrophil extracellular traps activate IL-36γ but not the non-cleavable mutant IL-36γ^{V15G}

A. Schematic of wild-type (WT) IL-36γ indicating the RAV^{15} cleavage motif targeted by cathepsin G and the non-cleavable mutant IL-36γ^{V15G}. B. Neutrophils (5 x 10^6 cells/well) were stimulated, or not, with PMA (100 nM) for 4 h at 37 °C. After 4 h, cell supernatants were removed and cells were washed 3 times with PBS. Cells were treated with IL-36γ^{WT} or IL-36γ^{V15G} (10 nM) for 30 minutes. HeLa$^{IL-36R-SEAP}$ cells were treated with a titration of IL-36γ^{WT} or IL-36γ^{V15G}, as indicated. IL-6 and IL-8 concentration in cell culture supernatants was measured by ELISA after 18 h.

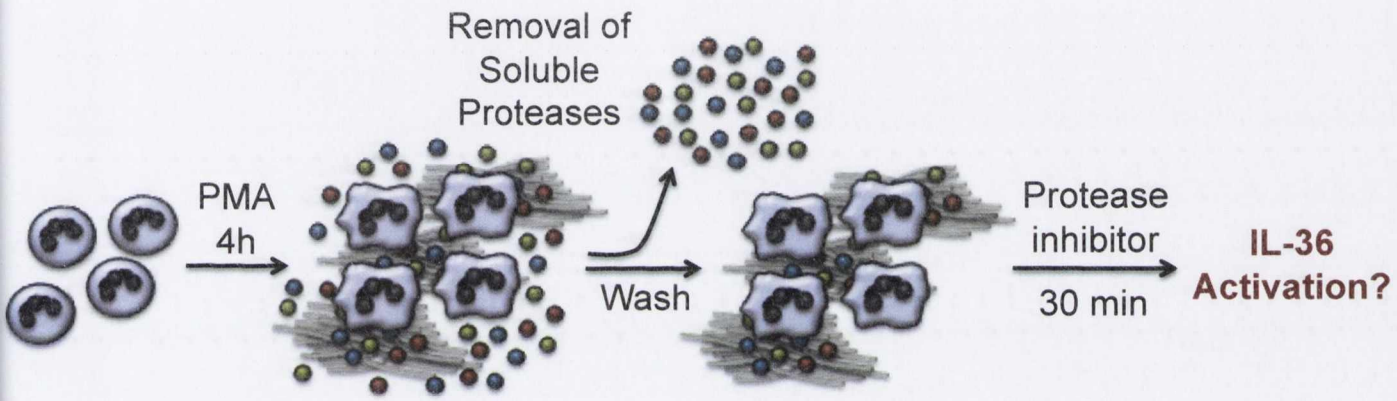

Figure 5.14 Blocking activation of IL-36 cytokines by NETs using protease inhibitors
Schematic showing assay layout of activation of IL-36 cytokines by NETs, with, or without the addition of protease inhibitors. Neutrophils ($5 \times 10^6 - 1 \times 10^7$ cells/well) were stimulated, as before, by PMA for 4 h to induce degranulation and NET release. Neutrophils were washed with PBS to remove soluble proteases released during degranulation, leaving only NET-bound protease activity on the remaining neutrophil population. Cells were incubated with cathepsin G or elastase chemical inhibitors for 30 minutes, followed by incubation with IL-36 cytokines.

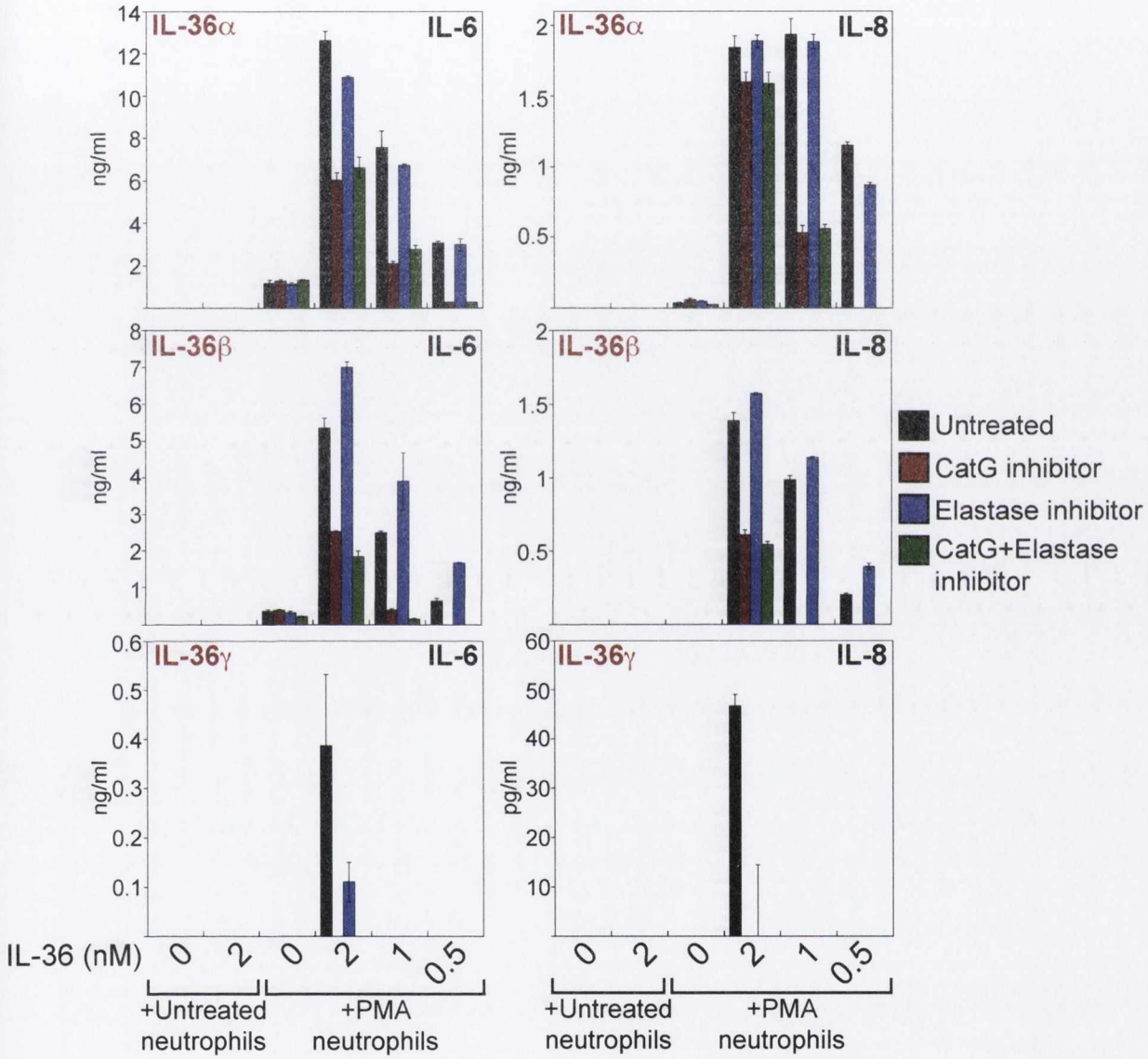

Figure 5.15 NET-bound cathepsin G and elastase inhibition blocks IL-36 cytokine activation

Neutrophils (5 x 10^6 cells/well) were stimulated with PMA (100 nM) for 4 h. Cells were washed 3 times with PBS followed by incubation with 5 μM cathespin G inhibitor, elastase inhibitor, or both for 30 min. Neutrophils were then treated with IL-36α, IL-36β or IL-36γ (10 nM) for 30 minutes. HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were treated with a titration of IL-36 cytokines, as indicated, and IL-6 and IL-8 concentrations in cell culture supernatants was measured by ELISA after 18 h.

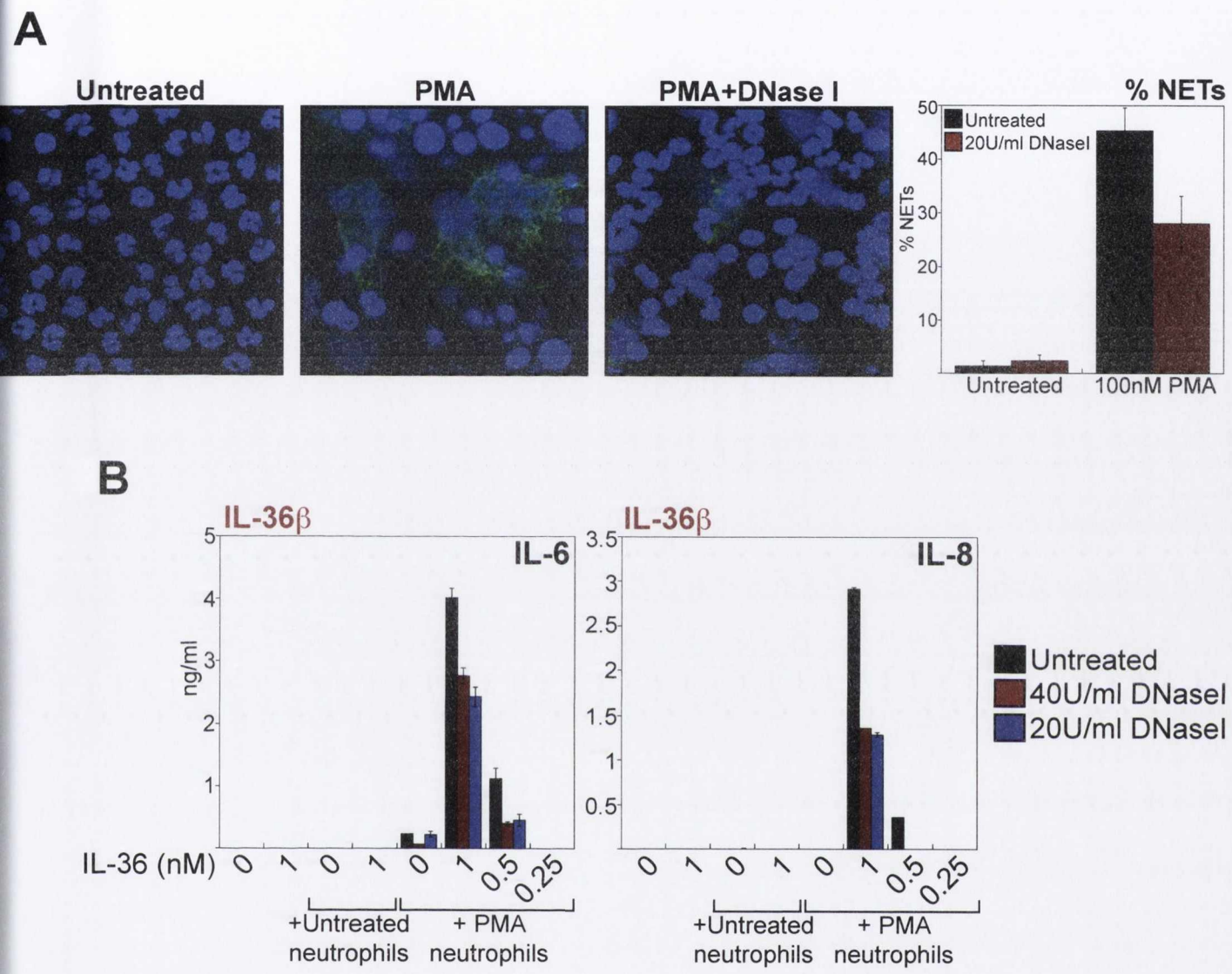

Figure 5.16 DNase I treatment partially ablates IL-36β activation by NETs
A. Neutrophils (1 x 10^7 cells/well) were stimulated with PMA for 4 h. Neutrophils were washed 3 times with PBS, followed by treatment with DNase I (20 U/ml) for 30 minutes. Cells were immunostained with elastase (green) and DNA was stained with Hoechst (blue). NETs were visualised and counted by confocal microscopy. **B.** Neutrophils were treated as in **A.** After DNase I treatment, cells were washed again with PBS and treated with IL-36β (5 nM). HeLa$^{IL\text{-}36R\text{-}SEAP}$ cells were treated with a titration of IL-36β, as indicated and IL-6 and IL-8 in cell culture supernatants was measured by ELISA after 18 h.

enhance IL-36β activity. Treatment of neutrophils with DNase I partially ablates IL-36β activation and reduces IL-6 and IL-8 secretion from HeLa cells (Fig. 5.16, B). This mirrors the results obtained with confocal microscopy, whereby DNase I partially removes NETs from the cell population.

5.2.10 Activated neutrophils produce cytokines and chemokines

Neutrophils recruited to sites of infection or injury undergo rapid activation by microbial and endogenous factors to trigger phagocytosis, degranulation and NETosis. Additionally, neutrophils initiate *de novo* production and secretion of a range of cytokines and chemokines that can further modulate immune responses. Neutrophils exposed to TNFα, IFNγ and PAMPs including LPS and fMLP rapidly induce the expression of cytokines and chemokines, however the full range of stimuli able to promote neutrophil cytokines has yet to be illustrated (Tecchio et al., 2014).

Previous studies have identified TNFα and LPS as potent inducers of cytokine expression, particularly IL-8, from neutrophils (Fujishima et al., 1993; Malcolm et al., 2003; Zhang et al., 2004). To confirm these findings, we incubated freshly isolated peripheral blood neutrophils with physiologically relevant concentrations of TNFα or LPS and measured cytokine secretion by ELISA after 24 and 48 hours. TNFα induced high levels of IL-8, IL-1β and RANTES from neutrophils, even after 24 hours, with higher concentrations of these cytokines detected after 48 hours. TNF-treated neutrophils also secreted VEGF and CXCL1 but not GM-CSF (Fig. 5.17 A and B). TNFα treatment resulted in marginally enhanced neutrophil cell death after 24 hours (Fig. 5.18). Similarly, treatment with LPS induced a wide range of cytokines and chemokines from neutrophils. LPS-treated neutrophils secreted high levels of IL-8, IL-1β, VEGF, CXCL1, GM-CSF and RANTES, suggesting that LPS is a more potent inducer of cytokine expression in neutrophils (Fig. 5.19 A and B). Akin to TNFα, LPS treatment also resulted in slightly enhanced cell death after 24 hour incubation (Fig. 5.20).

We also investigated the ability of IL-1 cytokines to promote cytokine expression and secretion from neutrophils. IL-1 cytokines were expressed and purified in ClearColi BL-21 *E. coli* to exclude endotoxin contamination as neutrophils are exquisitely sensitive to LPS and any contamination of the protein preparation may lead to false positive results (Tecchio et al., 2014). Neutrophils were incubated with full-length or cathepsin G-activated IL-36β and cytokine production was measured by ELISA. Neutrophils failed to make any cytokines or chemokines in response to IL-36β, even at longer timepoints (Fig. 5.21). IL-36β treatment also had no effect on neutrophil viability (Fig. 5.22). We

also examined the ability of other IL-1 cytokines to induce cytokines from neutrophils. IL-1α induced robust secretion of IL-8, VEGF and RANTES and IL-1β, albeit to a lesser extent. Little to no induction of CXCL1 or GM-CSF was seen with IL-1α treatment (Fig. 5.23). Conversely, only very low concentrations of cytokines were induced after treatment with IL-1β (Fig. 5.24). These results suggest that neutrophils can induce robust expression and secretion of a variety of cytokines, but the cytokine signature induced can vary depending on the stimulus used.

5.3 Discussion

We have shown that NET-bound serine proteases process and activate IL-36 cytokines to release their biological activity. PMA-treated neutrophils undergo NETosis and project long DNA fibres into the extracellular space. We have demonstrated that NETs contain neutrophil-derived granule proteases cathepsin G, elastase and proteinase-3 that co-localise with the chromatin backbone. Using an *in vitro* assay, we have shown that NET-bound proteases have enzymatic activity and activate IL-36α, IL-36β and IL-36γ to enhance their pro-inflammatory potential. The requirement for protease activity in NET-mediated enhancement of IL-36 activity was confirmed using specific inhibitors of cathepsin G and elastase activity, as well as IL-36 cleavage mutants.

5.3.1 Neutrophil extracellular traps in inflammatory and autoimmune diseases

Neutrophils undergo NETosis and release extracellular DNA traps in response to a variety of activating stimuli including PMA, IL-8 and LPS. NETs are an important arm of neutrophil defence during infectious disease, however abnormal or dysfunctional NET activity can result in chronic inflammation and autoimmunity. Excessive NET formation or defective NET clearance can contribute to inflammation and tissue damage and promote autoimmunity by externalizing autoantigens. NETs have been implicated in the pathogenesis of a number of inflammatory and autoimmune diseases including rheumatoid arthritis, cystic fibrosis, SLE and psoriasis (Branzk and Papayannopoulos, 2013).

Neutrophils and NETs are found at high abundance in synovial fluid from arthritic joints in rheumatoid arthritis patients. Neutrophils from individuals suffering from rheumatoid arthritis have increased NET formation when compared to neutrophils from healthy controls. Citrullinated histones, a post-translationally modified form of histone found particularly on NETs, induces the production of autoantibodies. These autoantibodies, in conjunction with increased pro-inflammatory cytokine levels can trigger NET release and potentiate inflammation in rheumatoid arthritis (Barnado et al., 2016; Khandpur et

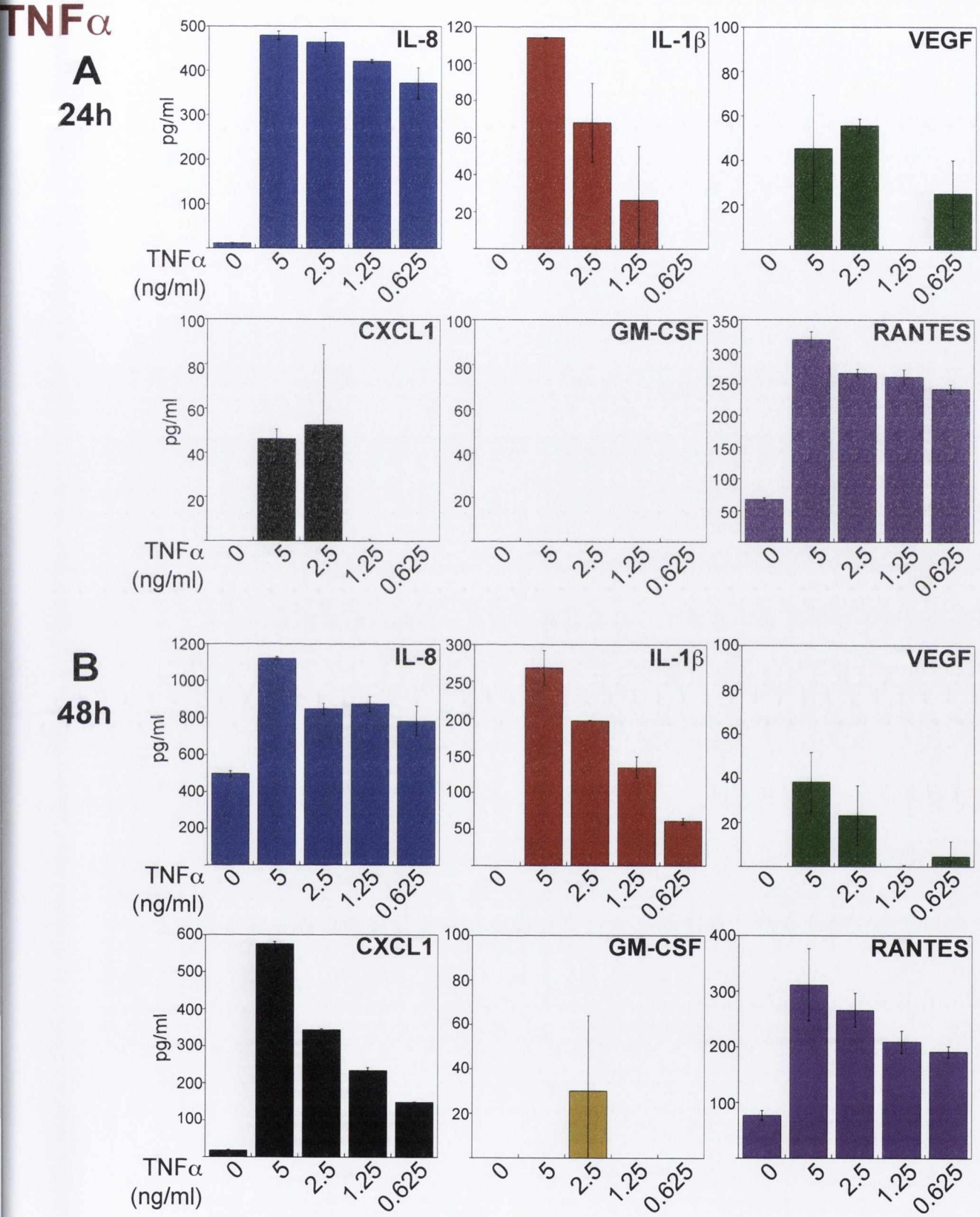

Figure 5.17 TNFα induces cytokine and chemokine secretion from neutrophils
Neutrophils (1 x 10^7 cells/ml) were treated with a titration of TNFα, as indicated. Cytokine concentrations in cell culture supernatants were measured after **A.** 24 h and **B.** 48 h.

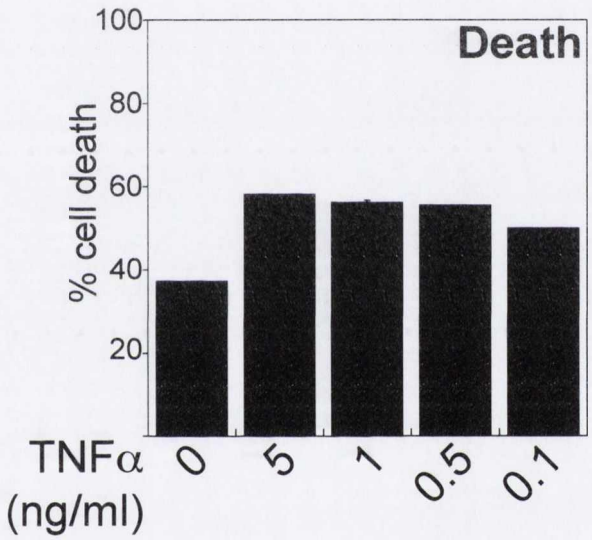

Figure 5.18 Neutrophil viability after TNFα treatment
Neutrophils (1 x 10^7 cells/ml) were treated with a titration of TNFα, as indicated. Cell death was measured by FACs analysis using annexin V/PI staining after 24 h.

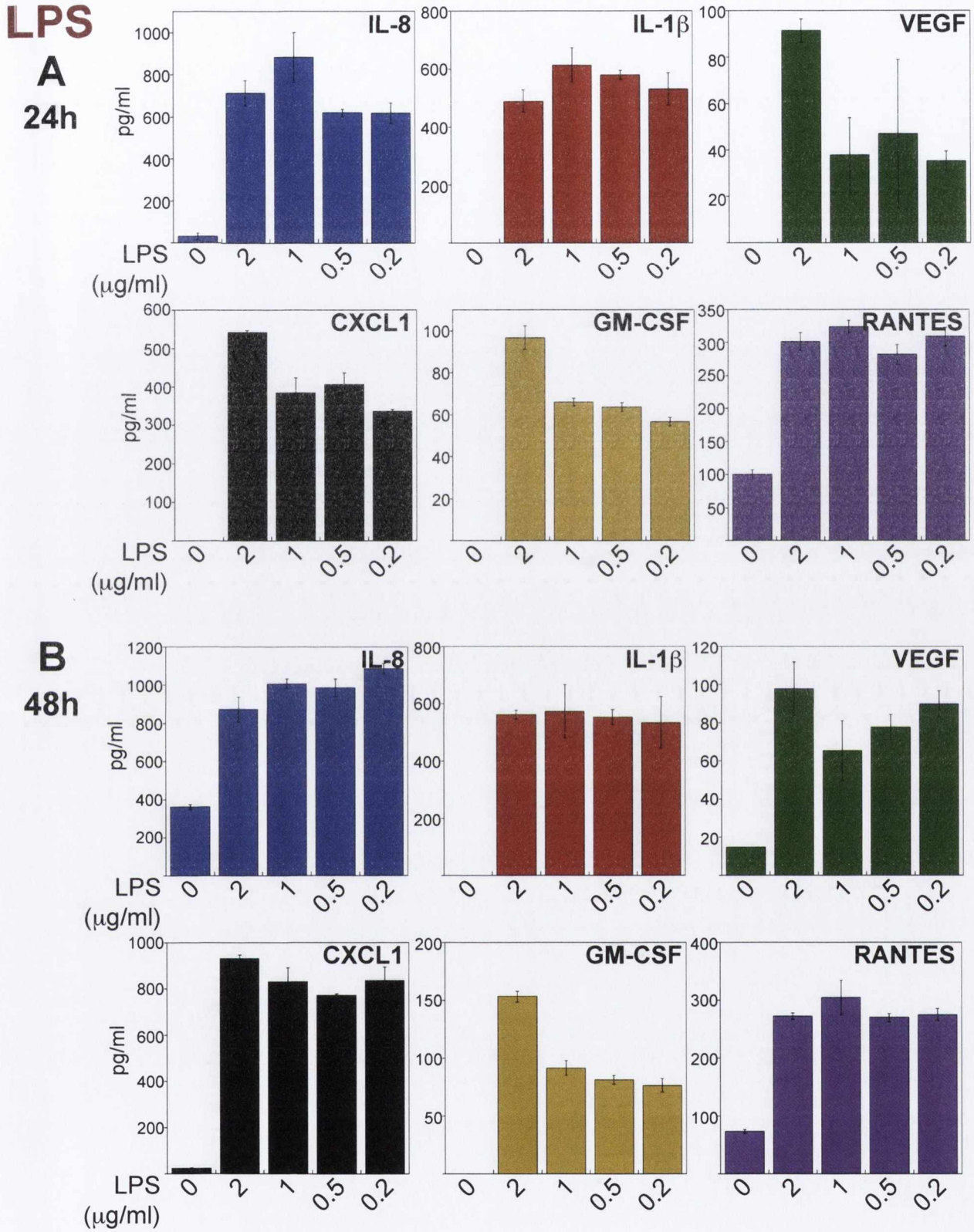

Figure 5.19 LPS induces cytokine secretion from neutrophils
Neutrophils (1 x 10^7 cells/ml) were treated with a titration of LPS, as indicated. Cytokine concentrations in cell culture supernatants were measured after **A.** 24 h and **B.** 48 h.

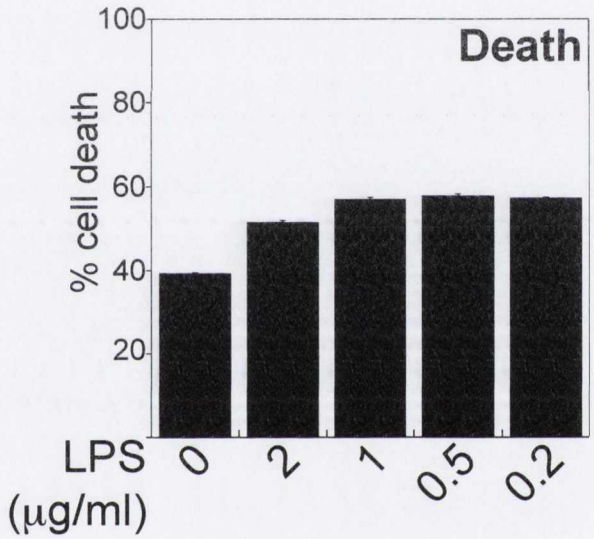

Figure 5.20 Neutrophil viability after LPS treatment
Neutrophils (1 x 10^7 cells/ml) were treated with a titration of LPS, as indicated. Cell death was measured by FACs analysis using annexin V/PI staining after 24 h.

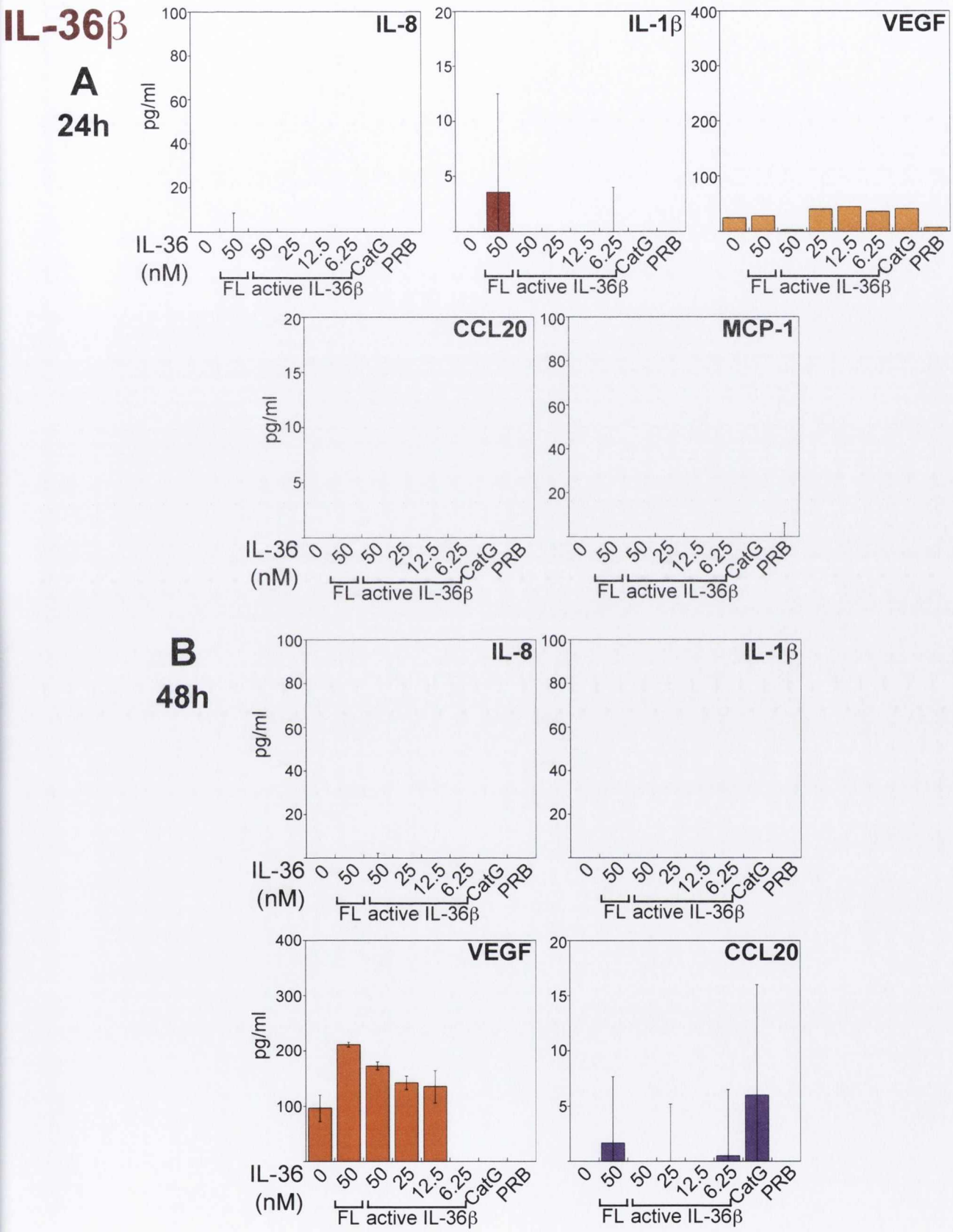

Figure 5.21 Neutrophils do not respond to active IL-36β
Neutrophils (1 x 10^7 cells/ml) were treated with a titration of full-length or cathepsin G-activated IL-36β, as indicated. Cathepsin G (CatG) or protease reaction buffer (PRB) alone were used as negative controls. Cytokine concentrations in neutrophil supernatants were measured after **A.** 24 h and **B.** 48 h.

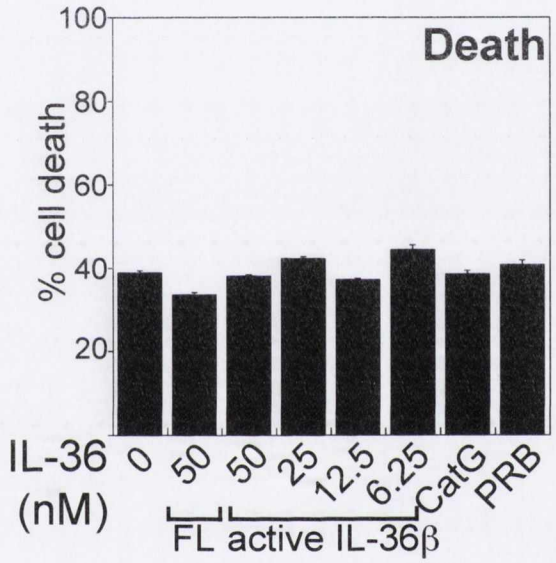

Figure 5.22 Neutrophil viability after IL-36β treatment
Neutrophils (1 x 10^7 cells/ml) were treated with a titration of full-length or cathepsin G-activated IL-36β, as indicated. Cell death was measured by FACs analysis using annexin V/PI staining after 24 h.

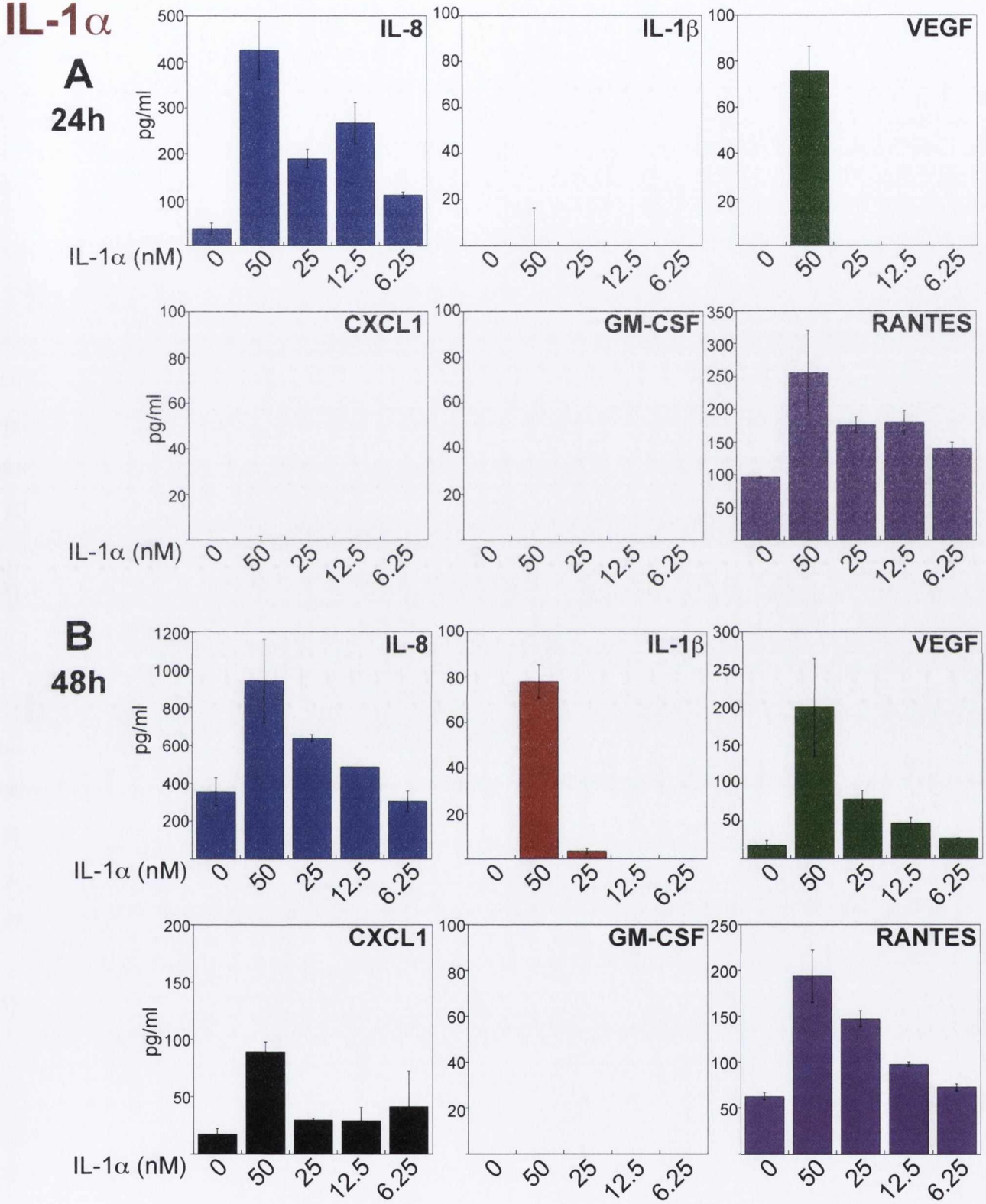

Figure 5.23 IL-1α induces cytokine and chemokine secretion from neutrophils
Neutrophils (1 x 10^7 cells/ml) were treated with a titration of IL-1α, as indicated. Cytokine concentrations in cell culture supernatants were measured after **A.** 24 h and **B.** 48 h.

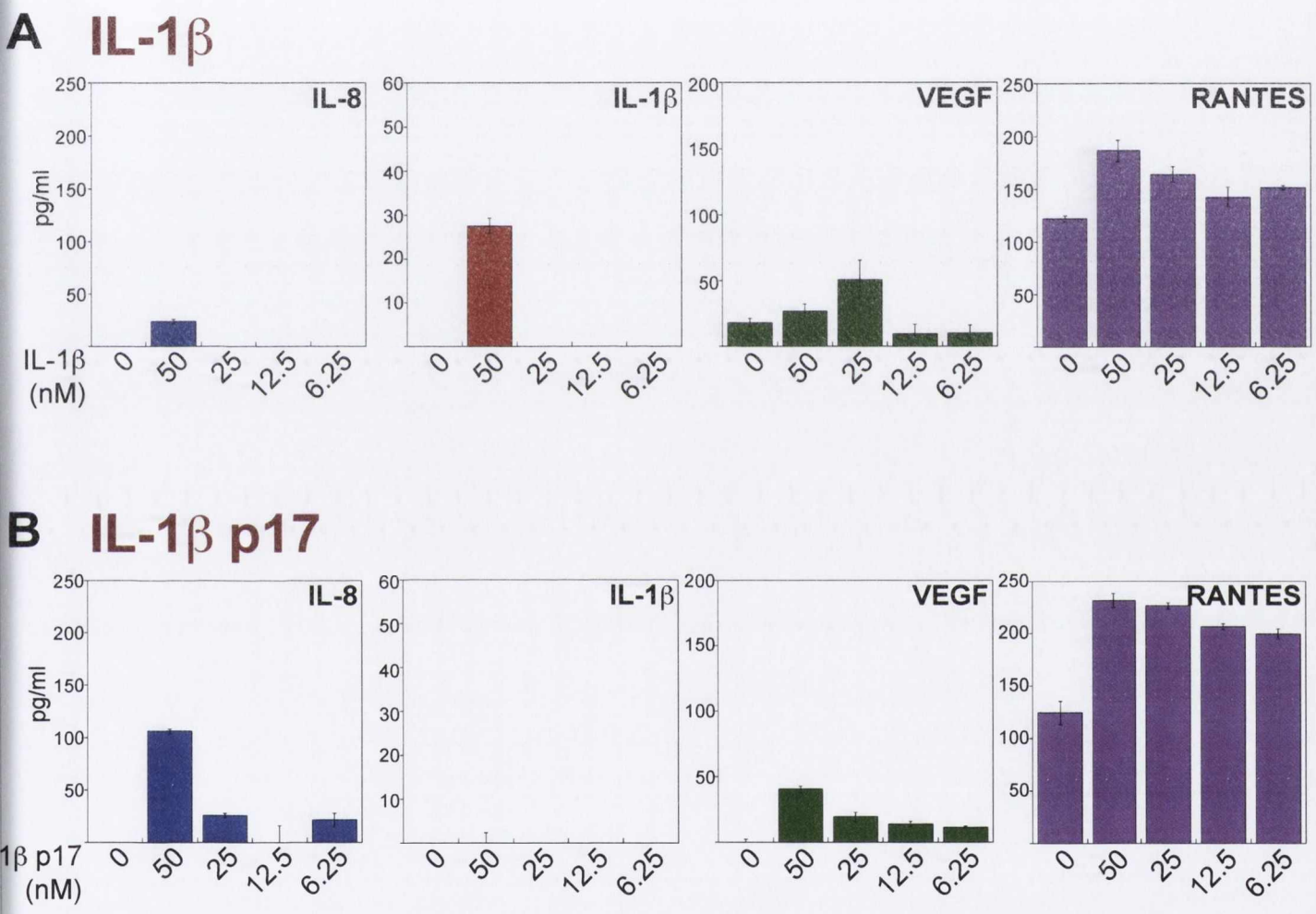

Figure 5.24 Neutrophils respond poorly to IL-1β

A. Neutrophils (1 x 10^7 cells/ml) were treated with a titration of full-length IL-1β, as indicated. Cytokine concentrations in cell culture supernatants were measured after 24 h. **B.** Neutrophils (1 x 10^7 cells/ml) were treated with a titration of active p17 IL-1β, as indicated. Cytokine concentrations in cell culture supernatants were measured by ELISA after 24 h.

al., 2013). NETs also amplify inflammation and disease severity in cystic fibrosis. Cystic fibrosis is a genetic lung disorder characterized by high viscosity sputum that blocks airways and allows bacterial colonization in the lung. Sputum viscosity is caused by increased levels of extracellular DNA from NETs. Granule proteins including elastase and MPO are also found in high levels in sputum and elastase activity may mediate lung damage (Branzk and Papayannopoulos, 2013). Cystic fibrosis patients undergo DNase I treatment to solubilise viscous sputum. Elastase activity enhances sputum solubilisation by DNase I, however this treatment can also have detrimental effects as DNA-bound elastase is released and can contribute to further lung injury (Brinkmann and Zychlinsky, 2012; Papayannopoulos et al., 2011).

Deregulated NET formation can also break self-tolerance and lead to the development of autoimmunity. SLE is an autoimmune disease characterized by skin rashes and internal organ damage. NETs have been found in biopsies from SLE patients, along with elevated levels of circulating DNA, ANCAs and autoantibodies against histones and DNA (Amulic et al., 2012; Branzk and Papayannopoulos, 2013). Defects in NET clearance have been attributed to SLE progression. Sera from SLE patients fail to degrade NETs and NET levels correlated with disease severity. These patients may have decreased DNase I activity or express DNase I inhibitors and are more likely to develop lupus nephritis (Hakkim et al., 2010). SLE NETs may also activate complement to further exacerbate inflammation and disease severity (Kaplan and Radic, 2012).

Psoriasis is characterized by massive neutrophil influx in skin plaques and major roles for pro-inflammatory cytokines including IL-36, IL-17, IL-22 and TNFα in this disease have been identified. NETs can initiate and maintain the chronic inflammatory phenotype associated with this disease. Neutrophil-derived LL-37, in complex with DNA can potently activate skin pDCs to produce high levels of IFNα. Elastase in complex with the inhibitor SLP1 and DNA has similar activating effects on pDCs and can contribute to the production of type I IFNs and pro-inflammatory cytokines (Lande et al., 2007; Pinegin et al., 2015). IL-17 is central to the pathology of psoriasis and drugs targeting this cytokine have had great success in the treatment of this disease. These treatments were thought to target and block T cells producing IL-17, however a recent study has challenged this view and identified neutrophils and mast cells as the predominant IL-17-producing cells in psoriatic skin. This study demonstrated that psoriatic neutrophils had a higher propensity to undergo NETosis and IL-17 could be detected on extracellular NET structures (Lin et al., 2011).

Abnormal NET formation and function intensifies sterile immune responses and can result in chronic inflammation and autoimmune diseases. However, the direct mechanism of how NETs mediate many of these inflammatory diseases remains to be elucidated. For example, in psoriasis, LL-37 can propagate inflammation by activating pDCs but it is not considered a major player in the pathogenesis of psoriasis compared to pro-inflammatory cytokines like IL-36, IL-17, TNFα and IL-23. Similarly, while IL-17 has been observed on NETs, it is unclear whether neutrophils themselves express this cytokine as no IL-17 mRNA could be detected from psoriatic neutrophils. It may be that circulating IL-17, found at high levels in psoriatic plaques, can stick non-specifically to NETs allowing for their detection on these structures. NETosis exposes endogenous cellular proteins that can act as alarmins and autoantigens to stimulate immunity and NET-bound proteins have cytotoxic activity, however the role of NETs in modulating extracellular cytokines and chemokines has not been considered.

5.3.2 NETs act as platforms for extracellular cytokine activation to promote inflammation

NETs have been implicated in the pathogenesis of a number of inflammatory disorders and can be found in high abundance at inflammatory sites. A recent study has postulated that NETs may enhance inflammation in atherosclerosis by inducing IL-1β. Due to its potent activity, IL-1β release is tightly regulated and requires two signals for inflammasome activation, however the priming signal needed to induce IL-1 transcription during sterile inflammation is unknown. Warnatsch and colleagues have shown that cholesterol crystals induce NET formation, which can go on to prime monocytes to upregulate IL-1β. Exposure to NETs, in combination with cholesterol, promotes the release of mature, active IL-1β from monocytes, which in turn exacerbates atherosclerosis (Warnatsch et al., 2015). This study suggests that NETs can modulate inflammation by modulating pro-inflammatory cytokine release, however the direct effect of NETs on cytokine and chemokine activity has not been investigated.

As previously discussed in detail, neutrophil serine proteases can modulate the activity of extracellular cytokines and chemokines by proteolytic processing. Elastase and proteinase-3 have previously been found to be associated with NETs (Kessenbrock et al., 2009; Papayannopoulos et al., 2010), therefore, we hypothesized that NET-bound proteases may have similar effects. Data presented here convincingly shows that NET-bound cathepsin G, elastase and proteinase-3 can potently activate IL-36 cytokines to enhance their biological activity. This activation was dependent on protease activity on NETs, as inhibition of the enzymatic activity of these proteases ablated IL-36 activation. Previous reports have suggested that binding of elastase to DNA diminishes its

enzymatic activity, however this suppression of activity was only partial (Duranton et al., 2000). We have demonstrated here that NET-bound serine proteases are sufficiently active to promote IL-36 processing and enhance their pro-inflammatory activity. DNase I treatment only partly suppressed NET-mediated IL-36 activation, however, previous studies have shown that activated neutrophils display granule proteases on the cell membrane (Csernok et al., 1994; Owen et al., 1995). This remaining protease activity on neutrophil cell surfaces after NET degradation may promote IL-36 activation. Furthermore, association of neutrophil proteases with NETs or the neutrophil membrane may protect them from inactivation by endogenous circulating protease inhibitors (Zawrotniak and Rapala-Kozik, 2013).

The modulation of cytokine activity by NET-bound granule proteases may be an important mediator of a range of inflammatory diseases, particularly psoriasis. All three IL-36 cytokines have been detected in psoriatic lesional skin and these cytokines are seen as key mediators of the pathogenesis of psoriasis (Towne and Sims, 2012). As discussed above, NETs have been detected in psoriatic lesions and correlate with disease severity as scored by the psoriasis area severity index (PASI). NET-mediated activation of IL-36 cytokines may also be important in psoriasis due to the high abundance of NETs in this environment.

Similar regulation of the activity of other cytokines and chemokines may also be mediated by NET-bound proteases. As shown in chapter 4, neutrophil granule proteases robustly enhance IL-1α activity and can differentially regulate IL-33. Following on from this, NET-bound proteases should also potently activate IL-1α to increase immune responses. Similarly, NETs may also enhance IL-33 activity, however this will be dependent on the ratio of proteinase-3 to elastase and cathepsin G present in NETs, as proteinase-3 promotes inactivation of IL-33. As well as activating IL-1 cytokines, NET-bound granule proteases may also regulate the action of other cytokines and chemokines including IL-8 and TNFα and ECM components. Neutrophil proteases are released extracellularly during degranulation however these soluble proteases may diffuse rapidly. Extrusion of these proteases as part of NETs prevents their dispersion into the surrounding tissue and increases the local concentration of enzymatic activity to allow effective cytokine activation.

NETs may also have an anti-inflammatory role to limit immune responses. In gout, MSU crystals promote the recruitment and activation of neutrophils. At low densities, neutrophils undergo NETosis and cytokine release in response to MSU crystals. However, later in the inflammatory response, increased numbers of neutrophils

accumulate and at high densities, NETs aggregate and degrade pro-inflammatory cytokines including IL-1β, TNF and MIP-1α. In this model, neutrophil recruitment and activation at the inflammatory site proceeds until a critical mass of neutrophils is reached. At this stage, NETs aggregate and diminish cytokine activity to resolve inflammation (Schauer et al., 2014).

Along with the capacity to impede microbial colonization and infection, we have shown that NETs have another important function as immune modulators. NET-bound proteases specifically and effectively regulate extracellular IL-36 cytokine activity to fine-tune inflammatory responses.

5.3.3 Neutrophil-derived cytokines and chemokines modulate immune responses

Activated neutrophils recruited to damaged or diseased tissue release a panoply of cytokines and chemokines as well as deploying antimicrobial mechanisms. Previously, it was speculated that neutrophils were bereft of transcriptional activity and only released pre-made effectors stored in their granule compartments. It is now apparent that neutrophils are capable of initiating a vigorous transcriptional response, resulting in the *de novo* expression of a large array of cytokines. Neutrophils express up to 20 times less RNA than other immune cells but due to the high densities reached by these cells in inflammatory sites, neutrophil-derived cytokines play a huge role in inflammatory responses. Due to the broad variety of cytokines produced by these cells, neutrophils may participate in physiological processes, such as angiogenesis and wound healing or inflammatory responses and autoimmunity (Cassatella, 1995; Tecchio et al., 2014).

We have shown that neutrophils produce an assortment of pro-inflammatory cytokines and chemokines in response to TNFα, LPS and IL-1α. Activated neutrophils secrete high levels of chemokines, including IL-8 and RANTES, and IL-1β to promote further recruitment of immune cells and to promote inflammation. TNFα, LPS and IL-1α-treated neutrophils also released considerable levels of VEGF, a potent angiogenic factor required during wound healing responses. Interestingly, neutrophils do not respond to IL-36 treatment, with no cytokines or chemokines detected by ELISA and no effect on cell viability. Previous reports have suggested that neutrophils do not express the IL-36 receptor, rendering them incapable of responding to IL-36 (Bozoyan et al., 2015; Foster et al., 2014). Surprisingly, stimulation with IL-1β did not result in robust cytokine secretion from neutrophils. The ability of IL-18 and IL-33 to induce cytokine expression from neutrophils requires further investigation.

Here, we have measured just a small subset of neutrophil-derived cytokines and chemokines. Activated neutrophils can upregulate a plethora of soluble factors that influence immune responses, resolution of inflammation and wound healing (Malcolm et al., 2003; Theilgaard-Monch et al., 2004). Cytokine expression from neutrophils can be stimulated by microbial components such as LPS, fMLP or intact bacteria as well as endogenous factors including IL-8, TNFα, and as demonstrated here, IL-1α (Martinez et al., 2004; Zhang et al., 2004). Stimulation of neutrophils by IL-1 cytokines, and the resulting cytokine signature may act as a positive feedback loop to recruit further leukocytes to the damaged site and further potentiate inflammation.

Chapter 6

General Discussion

6.1 Introduction

Throughout this thesis, the contribution of IL-1 family members to inflammation and their regulation by neutrophil-derived proteases has been investigated. Elucidation of the complex crosstalk between IL-1 cytokines and immune cells, particularly neutrophils, during sterile immune responses will lead to a better understanding of how the body responds to damage during acute and chronic inflammation and may result in the development of novel therapeutics to resolve inflammatory diseases.

The IL-36 cytokine subfamily is made up of three distinct cytokines, IL-36α, IL-36β and IL-36γ and have been heavily implicated in skin inflammation. Akin to other pro-inflammatory IL-1 cytokines, IL-36 cytokines require proteolytic processing to become fully active but until recently, the proteases mediating IL-36 activation were unknown (Towne et al., 2011). In chapter 3, we describe the development of a novel method to generate active IL-36 cytokines through insertion of a caspase cleavage motif, DEVD, in the N-termini of these cytokines. Processing with caspase-3 specifically truncates these proteins after the DEVD motif, resulting in active IL-36 cytokines, which we then used to elucidate IL-36 receptor signalling pathways. We found that the IL-36R signalling pathway is closely related to IL-1R and shares common adaptor molecules and kinases including MyD88, IRAK4 and TRAF6. We also identified the deubiquitinase OTULIN as a novel regulator of IL-36R signalling that restrains excessive IL-36 activity by targeting and degrading linear ubiquitin chains required for optimal signal transduction.

Over the last 20 years, isolated studies have linked neutrophil proteases in the regulation of IL-1 cytokine activity. Neutrophil granule proteases, in particular proteinase-3, are widely cited as alternative, inflammasome-independent mediators of IL-1β and IL-18 activation (Dinarello, 2011; Netea et al., 2010). Similarly, neutrophil proteases are also implicated in the processing and activation of other IL-1 family members such as IL-1α and IL-33 (Afonina et al., 2011; Lefrancais et al., 2012). However, direct evidence for enhanced IL-1 activation in these previous studies was lacking. Early studies showed that neutrophil elastase and cathepsin G could efficiently process IL-1α and IL-1β, however the functional outcome of this processing was not rigorously investigated. Studies with mice deficient in caspase-1 have further added to the notion of neutrophil proteases as regulators of IL-1β, but these studies often fail to comprehensively identify the alternative proteases responsible for IL-1 activation (Dinarello, 2011). In the course of this work, we sought to determine the effect of neutrophil proteases on IL-1 family cytokine activation in a comprehensive study using

all pro-inflammatory members of this family. To reflect physiological conditions found at inflammatory sites, we used neutrophil degranulate supernatants containing a mixture of proteases, as well as purified neutrophil proteases at typical concentrations found extracellularly during inflammation. We found that neutrophil serine proteases can potently enhance the activity of IL-1α and IL-36 cytokines, but rapidly inactivate mature IL-1β. Interestingly, neutrophil proteases differentially regulate IL-33 activity, with combined action of these proteases resulting in inactivation of IL-33. Furthermore, NET-bound proteases can also robustly activate IL-36 cytokines, suggesting a novel function of NETs as platforms for extracellular cytokine activation during inflammatory responses.

Taken together, the data presented in this thesis identifies neutrophils as dynamic immune cells that fine-tune inflammatory responses by modulating cytokine activity at a damaged or infected site. As shown here, neutrophil-derived proteases released during degranulation and NETosis, are potent regulators of IL-1 family cytokine activity. By regulating the activity of these cytokines, neutrophils function to modulate inflammatory responses and activate appropriate adaptive immune responses. This data suggests that neutrophils play an important role in coordinating specific immune responses and challenges the previously held view that these cells are primarily phagocytic microbial killers.

6.2 IL-1 family cytokines represent model DAMPs

Polly Matzinger proposed the 'danger hypothesis' in 1994 and suggested that rather than sensing 'self' versus 'non-self', the immune system recognizes and responds to host damage through damage-associated molecular patterns, or DAMPs (Matzinger, 1994). DAMPs are endogenous nuclear or cytosolic proteins released from damaged, dying cells during necrosis or tissue injury. Once released, DAMPs can activate receptors on surrounding cells to initiate sterile inflammation, host defence or repair (Kim et al., 2013).

Several candidate DAMPs have been proposed since the introduction of Matzinger's original danger hypothesis including ATP, genomic DNA, and HMGB1, among many others. Intracellular proteins released by necrosis have been postulated to initiate inflammation by activating specific receptors on surrounding cells such as TLR and RAGE receptors. For example, it has been claimed that HMGB1 promotes inflammation by binding to TLR2, TLR4 or RAGE to activate NFκB. However, studies by Rock and colleagues have explored the inflammatory response to necrotic cells and have excluded TLR signalling as a requirement for inflammatory responses in response

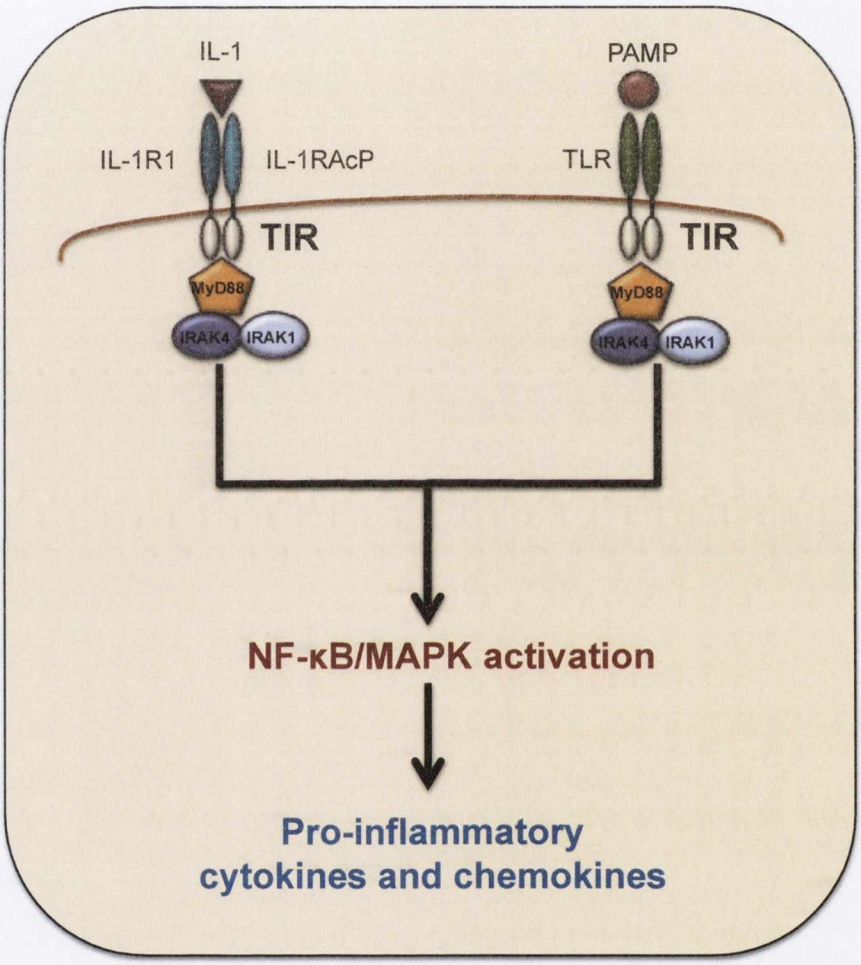

Figure 6.1 IL-1 receptor signalling closely resembles TLR signalling due to the shared TIR domain

IL-1 and TLR receptor signalling are closely related due to the highly conserved Toll/IL-1 receptor (TIR) domain shared by both receptor complexes. The TIR domain is essential for signalling after activation of these receptors and proximally recruits MyD88 to transduce downstream signals. Due to the conservation of the TIR domain between these receptors, many of the same adaptor proteins and kinases are involved in their downstream signalling pathways and both converge on NFκB and MAP kinase activation for the production of pro-inflammatory cytokines and chemokines.

to necrotic cells (Chen et al., 2007; Chen et al., 2006; Rock et al., 2010; Kono et al., 2010). Strikingly, necrotic HMGB1$^{-/-}$ cells elicited an immune response similar to wild-type cells, suggesting that HMGB1 has minimal pro-inflammatory activity and does not represent an essential DAMP. In fact, this study identified IL-1α and IL-1R signalling as key determinants of the inflammatory response in response to dying cells and identifies IL-1α as a major DAMP (Chen et al., 2007).

IL-1 family cytokines are ideal candidates for danger signals as they are profoundly pro-inflammatory and capable of stimulating a broad range of cells and tissues throughout the body (Martin, 2016). IL-1 family cytokines are constitutively expressed in barrier tissues such as the skin, lung, and intestinal and urinary tract and can be further upregulated by stress and noxious stimuli (Garlanda et al., 2013). IL-1 cytokines are not conventionally secreted through the ER/Golgi pathway. The majority of IL-1 family members are expressed as leaderless cytokines that lack a signal peptide for conventional secretion. In fact, the only known mode of release of these cytokines to date is from necrotic cells, another key feature of DAMPs (Martin, 2016).

Similar to PAMPs encountered during microbial infection, DAMPs must activate receptors on adjacent cells to initiate and amplify inflammation. PAMPs trigger inflammatory responses by activating PRRs. The TLR family is one of the largest and most extensively studied of the PRR families and can recognise a broad range of bacteria, viruses and fungi (O'Neill, 2008). TLR and IL-1 receptor signalling is closely related due to the Toll/IL-1 receptor (TIR) domain shared by both receptor complexes. Although the extracellular portions of TLRs and IL-1 family receptors are divergent, the intracellular cytosolic TIR domain is highly conserved and has been found in vertebrates, invertebrates and plants. IL-1R and TLR downstream signalling is broadly analogous due to the conservation of the TIR domain, and both function similarly to activate NFκB and MAP kinases (Fig 6.1) (Martin and Wesche, 2002; Verstrepen et al., 2008). Thus, TLRs mediate the response to microbial infection by recognising and responding to PAMPs, while IL-1 family receptors regulate sterile inflammation through the activity of IL-1 family cytokines released as DAMPs during tissue damage (Martin, 2016).

IL-1α and IL-33 have previously been identified as potential DAMPs as they are constitutively expressed and released during necrosis to promote inflammation. IL-1α has been shown to be involved in the initiation of sterile inflammation by promoting neutrophil recruitment, while IL-33 is an alarmin abundantly expressed in blood vessels (Chen et al., 2007; Moussion et al., 2008; Rider et al., 2011). Interestingly,

mechanisms have evolved to prevent deregulated IL-1 cytokine release during normal tissue homeostasis. IL-1α is normally found in the cytosol however, during apoptosis, IL-1α translocates to the nucleus through the action of its NLS domain, blocking its extracellular release and subsequent pro-inflammatory activity. Similarly, IL-33 activity is attenuated during apoptosis through cleavage and inactivation of this cytokine by caspase-3 and -7 (Luthi et al., 2009). Thus, these cytokines are differentially regulated during apoptotic or necrotic cell death to control their pro-inflammatory effects. During necrosis, unrestrained IL-1α and IL-33 are released from dying cells, resulting in enhanced inflammation (Cohen et al., 2010; Cayrol and Girard, 2009; Martin, 2016).

IL-1β activity is regulated at the transcriptional level and through the requirement for proteolytic processing by caspase-1 to release its bioactivity. A recent study from our laboratory showed that several NLRP3 inflammasome-inducing stimuli were also cytotoxic. This study showed that IL-1β is released by necrosis after inflammasome activation and suggests that IL-1β may represent an inducible danger signal (Cullen et al., 2015). Remarkably, in one of the first papers describing the cDNA of IL-1β published over 30 years ago, the authors speculate that due to its lack of signal peptide, IL-1β release from monocytes may occur through cell 'leakage' after damage (Auron et al., 1984). This mirrors our recent findings and suggests that IL-1β, along with the wider IL-1 cytokine family, are potent DAMPs released during necrosis that mediate sterile inflammatory responses.

6.3 How are neutrophils recruited to sites of sterile inflammation?

During infection, neutrophils are recruited to inflammatory sites by microbial-derived factors such as LPS and fMLP. In the absence of microbial infection, neutrophils are mobilized to areas of sterile inflammation by endogenous factors including DAMPs, particularly IL-1. Neutrophil recruitment is a hallmark of sterile inflammation and is associated with trauma, myocardial infarction, ischemia and stroke (Rock et al., 2010).

IL-1 cytokines, particularly IL-1α and IL-33, escape from necrotic cells during tissue damage and act on resident stromal cells and local immune cells to promote the production of cytokines and chemokines including IL-1β. These pro-inflammatory signals induce the recruitment of further immune cells such as neutrophils, macrophages and dendritic cells to the damaged area. Cytokines such as IL-6 are released into circulation and can stimulate the liver to produce acute phase proteins to further enhance the innate immune response (Kim et al., 2013). Neutrophils recruited to a sterile inflammatory site degranulate and undergo NETosis, exposing granule proteins including cathepsin G, elastase and proteinase-3 into the extracellular space.

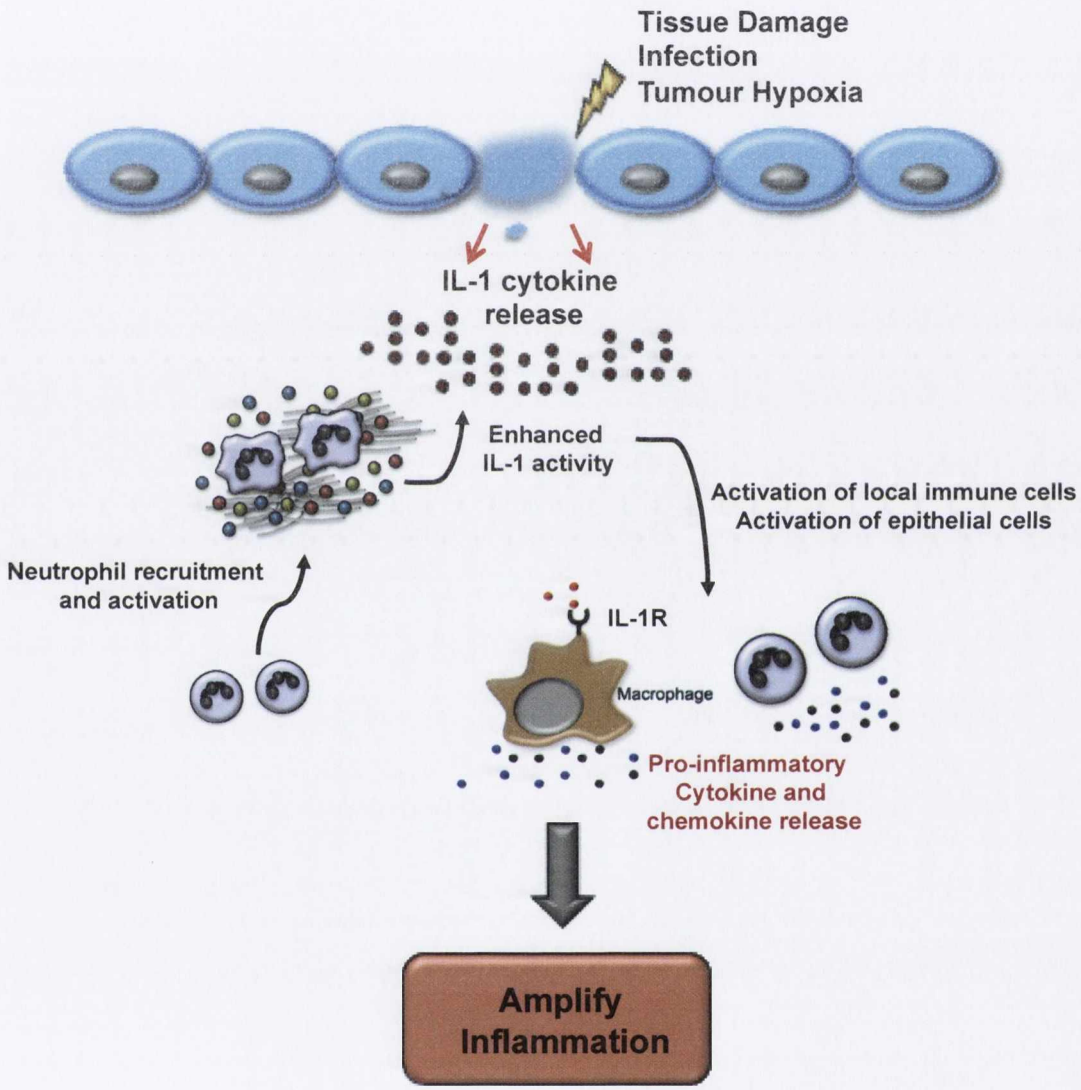

Figure 6.2 IL-1 cytokines released from necrotic cells as DAMPs are activated by neutrophil granule proteases to amplify inflammation

Tissue damage or infection induces necrotic cells death and the release of IL-1 family cytokines as DAMPs. IL-1 cytokines released from damaged tissues stimulate adjacent epithelial cells, endothelial cells and immune cells to induce neutrophil recruitment and activation. Proteases released from activated neutrophils during degranulation or NETosis modulate IL-1 activity to promote release of pro-inflammatory cytokines and chemokines from surrounding cells to further amplify inflammatory responses.

These proteases can subsequently process and regulate the activity of IL-1 cytokines released during necrosis to further augment inflammation (Fig. 6.2).

DAMP-mediated neutrophil recruitment during sterile inflammation may be important for the resolution of inflammation and to initiate a wound healing response. Neutrophils engulf and clear cell debris at damaged sites and play an important role in wound debridement during healing (Shaw and Martin, 2009). Apoptotic neutrophils prevent the recruitment of further immune cells and engulfment of dying neutrophils by macrophages reprograms these cells to an anti-inflammatory phenotype (Pittman and Kubes, 2013). Therefore, neutrophil recruitment by DAMPs during sterile inflammation may be beneficial for the resolution of inflammation but unbalanced, excessive neutrophil activity may lead to chronic inflammation and autoimmunity.

6.4 Neutrophils are key regulators of sterile inflammatory responses

As discussed in detail in chapter 5, neutrophils and their associated NETs have been implicated in the pathogenesis of a broad spectrum of inflammatory and autoimmune diseases including psoriasis, SLE, rheumatoid arthritis and acute lung diseases. The view of neutrophils solely as antimicrobial effectors is becoming obsolete, with more and more evidence confirming the role of these cells in inflammation. Recent studies have suggested that distinct subsets of neutrophils exist, indicating that neutrophil cell populations are plastic and heterogeneous based on their receptor expression profiles and function (Kruger et al., 2015).

One such neutrophil subset is known as low density granulocytes (LDGs), a subpopulation of neutrophils first described in SLE but also found in ANCA vasculitis and psoriasis. LDGs were identified in the PMBC preparations from SLE patients but were found to express high levels of neutrophil-specific genes. These cells are phenotypically similar to immature neutrophils and contain non-segmented nuclei and higher expression of MPO, elastase and defensins (Amulic et al., 2012; Carmona-Rivera and Kaplan, 2013). LDGs make up a division of pro-inflammatory neutrophils that have enhanced pro-inflammatory cytokine production and IFN synthesis, with a reduced capacity for phagocytosis. Moreover, LDGs from psoriasis and SLE patients have a higher propensity to undergo NETosis and release NETs (Carmona-Rivera and Kaplan, 2013; Grayson and Kaplan, 2016; Lin et al., 2011; Villanueva et al., 2011). LDGs and NET-bound histones also participate in tissue damage and are cytotoxic towards endothelial and epithelial cells (Denny et al., 2010; Saffarzadeh et al., 2012). This suggests that neutrophils may participate in an amplification loop to promote inflammation. Activated neutrophils release proteases and NETs that can activate

cytokines, particularly IL-1 family members at the site of inflammation. Concomitantly, pro-inflammatory LDGs and NETs can induce further tissue damage and cell death, releasing additional IL-1 cytokines as DAMPs which can be activated by exposed neutrophil proteases to amplify inflammation. Breaking this positive feedback loop between neutrophils and IL-1 cytokine activation may be beneficial for the treatment of a range of inflammatory diseases, particularly psoriasis.

6.5 Neutrophil proteases represent promising therapeutic targets for the treatment of inflammatory disorders

Results shown throughout this thesis identify neutrophil granule proteases as key regulators of IL-1 family cytokines. As a consequence, these proteases may represent highly promising therapeutic targets for the treatment of inflammatory diseases mediated by IL-1 activity and neutrophil infiltration including psoriasis, rheumatoid arthritis, gout, neurodegenerative diseases and pulmonary diseases (Lukens et al., 2012).

Currently, only one elastase inhibitor is approved for clinical use. Sivelestat is available in Japan as a treatment for acute lung injury associated with sepsis and systemic inflammation, however the use of this inhibitor in USA was terminated in 2003, due to the failure to show improved survival in clinical trials (Zeiher et al., 2004; Zeiher et al., 2002). Another elastase inhibitor developed by AstraZeneca, AZD9668, is currently undergoing clinical trials for treatment of chronic inflammatory lung disease (Stevens et al., 2011). Recombinant elafin, an endogenous inhibitor of elastase and proteinase-3, has also been tested in clinical trials to investigate its efficacy on post-operative inflammation following major surgery (Shaw and Wiedow, 2011). There are currently no inhibitors of cathepsin G or proteinase-3 in clinical use or undergoing clinical trials.

Although the contribution of neutrophil proteases to the pathology of a range of inflammatory diseases has long been recognized, there has been little progress in the development of effective inhibitors to target these enzymes. Systemic administration of these inhibitors may fail to reach the site of infection at concentrations required to effectively block the activity of these proteases to resolve inflammation. Furthermore, elafin, SLP-1 and other endogenous inhibitors are susceptible to proteolysis and inactivation by ROS and neutrophil proteases at high concentrations found in chronic inflammatory conditions. Elafin variants containing mutations that render them resistant to proteolysis, as well as peptide fragments of SLP-1 that resist cleavage but retain inhibitory activity are now being tested as more effective inhibitors that may have use in a clinical setting (Henriksen, 2014; Small et al., 2015).

Inhibition of neutrophil protease activity may have potential as anti-inflammatory agents through antagonizing the activation of IL-1 family cytokines, especially IL-36 in psoriasis. Currently, multiple therapeutics directed against cytokines such as TNFα and IL-17 have been approved for the treatment of psoriasis. However, while this approach is effective, systemic administration of cytokine neutralizing antibodies can have detrimental effects including increased susceptibility to opportunistic infections (Ali et al., 2013). Neutrophil protease inhibitors have not yet been considered as a treatment for psoriasis, however a previous study has highlighted the potential success of this approach. Targeted depletion of neutrophils in GPP patients lead to a dramatic reduction in disease severity, suggesting that targeting neutrophils or their proteases may be highly beneficial for the treatment of psoriasis, even in mild-to-moderate cases. Due to problems associated with systemic administration of inhibitors, it may be more desirable to neutralize protease activity only in affected skin lesions. Topical application of small molecule inhibitors of these proteases directly to affected areas of skin may represent attractive therapeutics for skin inflammatory diseases such as psoriasis. Targeting neutrophil proteases in this way may diminish IL-1 cytokine activation at inflammatory sites and help to reduce and resolve inflammation in chronic inflammatory disorders.

6.6 Crosstalk between neutrophils and IL-1 family cytokines in cancer

The link between inflammation and cancer has long been recognized. Chronic inflammation and the persistence of immune cells at an inflamed site has been strongly associated with the initiation and progression of many types of cancers. Furthermore, established tumours maintain an inflammatory microenvironment to assist in their growth and eventual metastasis (Lin and Karin, 2007). Immune cells, in particular macrophages and neutrophils, are harnessed by tumours to promote their own development and progression. Neutrophils are found in many different types of cancer including glioblastoma, melanoma, hepatocellular carcinoma, colorectal cancer, renal cell carcinoma and head and neck cancer. The presence of neutrophils in a tumour correlates with poor patient prognosis, however it is not clear how neutrophil function promote tumour progression (Powell and Huttenlocher, 2016). IL-1 cytokine activity and neutrophil infiltration are hallmarks of sterile inflammation so it is feasible to think that similar crosstalk between these inflammatory mediators may occur in cancer.

Neutrophils may promote tumour development through ROS, cytokine secretion and the release of MMPs. It was proposed that neutrophil-derived ROS may initiate tumour formation by causing genomic instability, however *in vivo* evidence for this is still

lacking. Neutrophils release a plethora of inflammatory mediators that can promote angiogenesis, further leukocyte recruitment and ECM remodeling through the action of MMPs and other granule enzymes (Amulic et al., 2012; Powell and Huttenlocher, 2016). Neutrophils also promote cancer progression by enhancing early tumour metastasis. In mouse models of breast cancer, signals from primary cancers promoted pro-tumorigenic neutrophil accumulation in distinct organs, namely the lung, that was closely followed by tumour metastasis to the lung (Coffelt et al., 2015; Tuting and Visser, 2016; Wculek and Malanchi, 2015). The presence of intratumoral NETs also correlates with worse outcomes, potentially by trapping circulating tumour cells to promote metastasis (Cools-Lartigue et al., 2013; Powell and Huttenlocher, 2016). Furthermore, a previous study has suggested that distinct neutrophil subsets may exist within the tumour microenvironment. These anti- (N1) or pro-tumorigenic (N2) are polarized by TGFβ and, similar to tumour-associated macrophages (TAMs), may modulate cancer progression. Anti-tumorigenic N1 neutrophils release pro-inflammatory cytokines and are cytotoxic to tumour cells, while N2 neutrophils are immunosuppressive and release high levels of arginase to inhibit T cell function to benefit tumour survival and progression (Fridlender et al., 2009).

Neutrophil recruitment to tumours closely resembles neutrophil recruitment to sterile wounds or sites of infection (Fig. 6.2). Cytokines and chemokines in the inflammatory microenvironment promote leukocyte infiltration to the tumour. In particular, IL-1 cytokines are abundant at tumour sites. As solid tumours develop, they quickly outgrow their blood supply, leading to areas of hypoxia and damage within the tumour. IL-1 family cytokines released from hypoxic, necrotic tumour cells enter the tumour microenvironment where they stimulate resident macrophages and T cells to secrete cytokines and chemokines. This promotes neutrophil infiltration and degranulation, further activation of IL-1 cytokines by neutrophil proteases and stimulation of surrounding cells and tissues to promote angiogenesis, tumour growth and metastasis. In line with this, a recent study showed that IL-1β generates neutrophil recruitment, increased elastase and cathepsin G activity which results in thrombospondin-1 cleavage and increased levels of pro-inflammatory mediators, resulting in enhanced tumour metastasis in mouse lungs (El Rayes et al., 2015). Similarly, a human monoclonal antibody specific for IL-1α, MABp1, has been tested in phase I clinical trials for the treatment of advanced cancers by reducing inflammation that is beneficial for tumour growth. IL-1α released from hypoxic tumour areas may mediate tumour progression, especially in epithelial cell tumours that express constitutively high levels of IL-1α, such as breast and lung cancers (Hong et al., 2014).

6.7 Conclusion

The overarching goal of this thesis has been to investigate the proteolytic regulation of IL-1 family cytokine activity by neutrophil granule proteases. First, we have shown that IL-36 cytokines require processing to achieve their fully active state and that IL-36 cytokine signalling closely resembles IL-1 receptor signalling, with an important role for linear ubiquitination in the regulation of this pathway. The elucidation of downstream mediators of IL-36 signalling may represent attractive therapeutic targets, especially in psoriasis. Secondly, we have identified neutrophil serine proteases cathepsin G, elastase and proteinase-3 as key modulators of pro-inflammatory members of the IL-1 cytokine family. Furthermore, we also identified that NET-bound neutrophil serine proteases can potently activate IL-36 cytokines to further intensify inflammatory responses.

IL-1 cytokines released from damaged cells synergise with neutrophil proteases to amplify inflammation. This suggests that in addition to their role as sentinel cells in host defence, neutrophils are potent immunomodulatory cells working at the forefront of innate immunity. Thus, therapeutic targeting of neutrophil granule proteases released during degranulation and NETs represents an attractive novel mechanism to effectively treat inflammatory diseases by suppressing IL-1 family cytokine activity.

Chapter 7

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