IL-36 member activation and secretion, and their role in immune defence

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Submitted in accordance with the requirements for the degree of Doctor of Philosophy

The University of Leeds

Faculty of Medicine and Health

November 2016

The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Chapter 4 uses data published in Macleod, T., Doble, R., McGonagle, D., Wasson, C.W., Alase, A., Stacey, M., Wittmann, M., Neutrophil Elastase-mediated proteolysis activates the anti-inflammatory cytokine IL-36 Receptor antagonist. *Scientific Reports* **6**, 24880 (2016). Conceived and designed experiments; M.S., M.W., R.D., T.M. Conducted experiments; T.M., R.D., A.A. Contributed to cell culture; A.A., C.W.W. Wrote manuscript; T.M., M.S., M.W.

Acknowledgements

I would firstly like to thank everyone in the lab that has helped me through the years; particularly Rosie for showing me the ropes in the first year, Joe in the last year for experimental guidance, and Becky throughout for making it such a pleasant place to work. I would also like to thank my supervisors Martin Stacey and Miriam Wittmann for their undying respective skepticism and optimism (and of course their continual support and guidance) and Delyth Reid for passing on some of her excellent skills in hybridoma production and housing me for a night. I'd also like to give thanks to Chris, for his never-ending persistence in maintaining a functional tissue culture room, Chi for making sure I have some form of a social life, and frankly anyone reading this – particularly if you manage to get through to the end. Lastly, I'd like to thank my family for their continual support (financial and otherwise!) throughout the PhD and writing process, Will for the thesis soundtrack contributions, and Cliff for just always being there. And of course huge thanks goes to Lucy for putting up with me and providing a constant welcome source of distraction.

Abstract

The IL-36 cytokines are a recently identified subset of the IL-1 superfamily that are becoming firmly established as instigators of pathogenic inflammation in a number of inflammatory conditions. IL-36γ, in particular, has been strongly linked to psoriatic inflammation of the skin. As with many IL-1 proteins, the IL-36 cytokines must undergo post-translational truncation in order to become biologically active, however the manner in which this occurs and their subsequent release from cells is poorly understood. Furthermore, research into these questions is greatly hindered by the lack of available IL-36 specific antibodies.

This work has therefore focused on 1) producing IL-36γ specific monoclonal antibodies to facilitate further IL-36 research, 2) the post-translational processing by endogenous and exogenous proteases, 3) the physiological role of IL-36 in epithelial barrier homeostasis.

The results of this work include successful production and characterisation of 4 anti-IL-36γ monoclonal antibodies and subsequent development of an IL-36γ ELISA that has been used to identify IL-36γ as a reliable psoriasis-specific disease marker. Secondly, an epithelial protease, cathepsin S, has been identified as able to cleave and activate IL-36γ and has been shown to be widely expressed within the skin compartment. Furthermore, it has identified that exogenous proteases produced by the opportunistic pathogen *Aspergillus fumigatus* are capable of cleaving and activating IL-36γ to initiate IL-36-mediated inflammation. Finally, IL-36γ was shown to promote epithelial barrier function by enhancing epithelial proliferation and providing resistance against apoptosis, which may be indicative of a role in wound healing. Overall from these findings we propose that the IL-36 cytokines may act as sensors of epithelial damage in response to protease-dependant activation that play a prominent role in the first line of defence against invading pathogens.

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Abbreviations

AcP – accessory proteins

AD – atopic dermatitis

AEBSF - 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride

AIM2 - absent in melanoma 2

AMPs – antimicrobial peptides

ANOVA – analysis of variance

APC – antigen presentation cells

ASC - apoptosis-associated speck-like protein containing a CARD

ATP - adenosine 5'-triphosphate

Bcl-2 - B-cell lymphoma 2

BSA – bovine serum albumin

CAPS - cryopyrin-associated periodic syndromes

CARD - caspase activation and recruitment domain

CASPAR - classification criteria for psoriatic arthritis

Caspase - Cysteine-dependent aspartate-directed proteases

CATSi – cathepsin S inhibitor

CCL - C-C motif ligand

CCR - C-C chemokine receptor type

CFA - Complete Freund’s Adjuvant

CFU – colony forming unit

CG – cathepsin G

CLA - cutaneous lymphocyte associated-antigen

CLR – C-type Lectin Receptor

COPD - chronic obstructive pulmonary disease

DAMPs - damage associated molecular patterns

DAPI - 4',6-diamidino-2-phenylindole

DC – dendritic cell

DIRA - deficiency of IL-1 receptor antagonist

DITRA - deficiency of IL-36 receptor antagonist

DMEM- Dulbecco’s modified eagle media

DMSO - Dimethyl sulfoxide

DNA – deoxyribonucleic acid

DSS - dextran sodium sulphate

DTT - Dithiothreitol

E – elastase

EDTA - Ethylenediaminetetraacetic acid

EGF – epidermal growth factor

ELISA – enzyme-linked immunosorbent assay

FDA – food and drug administration

FCS – fetal calf serum

FITC - fluorescein isothiocyanate

G-CSF - granulocyte colony stimulation factor

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GAS - group A *Streptococcus*

GFP – green fluorescent protein

GPP - Generalised pustular psoriasis

HAT - hypoxanthine-aminopterin-thymidine

HB-EGF - heparin binding EGF-like growth factor

hBD – human beta defensin

HEK – human embryonic kidney

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPK – human primary keratinocytes

HRP – horse radish peroxidase

IAA – iodoacetic acid

IAP - inhibitor of apoptosis protein

IFA - Incomplete Freund's Adjuvant

IFN – interferon

Ig - Immunoglobulin

IL- interleukin

IL-1Ra - IL-1 receptor antagonist

IL-36Ra – IL-36 receptor antagonist

ILCs – innate lymphoid cells

IPTG - isopropyl β-D-1-thiogalactopyranoside

KGM – keratinocyte growth media

KLK5 - kallikrein-related peptidase 5

LAMP1 - Lysosomal-associated membrane protein 1

LDH - Lactate dehydrogenase

LEKTI - lympho-epithelial Kazal type inhibitor

LoB – limit of blank

LoD – limit of detection

LoQ – limit of quantification

LPS - lipopolysaccharide

MAPK - mitogen-activated protein kinases

MCP - monocyte chemoattractant protein

MDDCs - monocyte derived dendritic cell

MHC II - major histocompatibility complex type 2

MIP1α – macrophage inflammatory protein 1 α

MMPs - Matrix metalloproteinases

NETs - neutrophil extracellular traps

NF-κB - nuclear factor-kappa B

NHS-biotin - *N*-hydroxysuccinimidobiotin

NK – natural killer

NLR - nod-like receptors

NLRP3 - NOD like receptor protein 3

NLS - nuclear localisation sequence

NOD - nucleotide-binding domain

NOMID – neonatal-onset multisystem inflammatory disease

NS – non-stimulated

PAGE – polyacrylamide gel electrophoresis

PAMPs - pathogen-associated molecular patterns

PAR-2 - protease-activated receptor 2

PARP - Poly (ADP-ribose) polymerase

PASI - psoriasis activity and severity score

PBMCs – peripheral blood mononuclear cells

PBS – phosphate buffered saline

pcAb – polyclonal antibody

PCR – polymerase chain reaction

pDCs - plasmacytoid dendritic cells

PEG – Polyethylene glycol

PI – propidium iodide

PI – protease inhibitor

PMA - phorbol 12-myristate 13-acetate

PMN – polymorphonuclear

PMSF - phenylmethylsulfonyl fluoride

Poly(I:C) - polyinosinic:polycytidylic acid

PR3 – proteinase 3

PRRs - pattern recognition receptors

PsA – psoriatic arthritis

PSORS1 - Psoriasis susceptibility region 1

qRT-PCR – quantitative real time PCR

Ra – receptor antagonist

RANTES - Regulated on Activation, Normal T Cell Expressed and Secreted

RNA – ribonucleic acid

ROC - receiver operating characteristic

RPMI - Roswell Park Memorial Institute

SALT - skin-associated lymphoid tissue

SDS - sodium dodecyl sulphate

SEC – size exclusion chromatography

SFM – serum free media

siRNA – small interfering RNA

SIS – skin immune system

SpeB - streptococcal cysteine protease, streptococcal exotoxin B

SUMO - Small Ubiquitin-like Modifier

TGF-β - transforming growth factor β

T_H1 – T helper cell 1

T_H17 – T helper cell 17

T_H2 – T helper cell 2

TIR - Toll-IL-1-receptor

TLRs - toll like receptors

TMB – Tetramethylbenzidine

TNBS - trinitrobenzene sulfonic acid

TNFα - tumour necrosis factor α

VEGF – vascular endothelial growth factor

XIAP - X-linked inhibitor of apoptosis protein

Chapter 1 - Introduction

1.1 Structure and cellular population of the skin

The skin can be separated into two distinct layers; the epidermis and dermis. The epidermis is the upper-most layer and sits a-top the dermis, separated by a basement membrane as shown in Figure 1-1. The epidermis consists primarily of tightly packed keratinocytes that progressively differentiate as they progress outward through the epidermis consequently forming a highly organized structure. As such, the epidermis is separated into four distinct layers, each consisting of keratinocytes at distinct stages of differentiation. The first layer of the epidermis, termed the stratum basale, comprises of undifferentiated proliferating basal keratinocytes attached to the basement membrane via hemidesmosomes which provide the epidermis with a continual turnover of cells. The next layer, the stratum spinosum, consists of keratinocytes that have begun to differentiate, attached to one another via desmosomes. At the third layer, the stratum granulosum, intermediately differentiated keratinocytes have begun to lose their nuclei and take on a granular appearance. Finally, the stratum corneum consists of terminally differentiated keratinocytes (corneocytes) that are anuclear, encased in cornified envelope proteins and tightly attached to one another via corneodesmosomes forming a physical barrier to the outside world [1]. As the keratinocytes differentiate they express different proteins (such as keratin), and secrete polar lipids that are converted to non-polar lipids that contribute to the epidermal barrier function [2]. Through identification of these differentially-expressed proteins the differentiation state of keratinocytes can be defined. Although the vast majority of the epidermis is made up of keratinocytes, other cells can also be found. Langerhans cells are often found in the stratum spinosum whilst melanocytes and sensory merkel cells are found in the stratum basale and a low number of $CD8^+$ T cells can be found in both [3, 4].

The dermis can be divided into two defined layers; the inner stratum reticulare and outer stratum pappilare, beyond which lies the basement membrane and epidermis. The dermis has a more complex anatomy than the epidermis consisting of several cell types and numerous integrated

tissues, but the predominant resident cells are fibroblasts. The fibroblasts secrete fibrous proteins such as collagen and elastin that form an extracellular matrix in which numerous nerve type cells and immune cells reside. The dermis is populated by a much more diverse set of specialized immune cells than the epidermis, including antigen presenting cells, conventional and non-conventional T cells. The dermis also contains both a capillary and lymphatic network providing the dermis with blood and allowing the influx and efflux of migratory immune cells in response to inflammatory mediators produced by both the dermis and epidermis [3, 5].

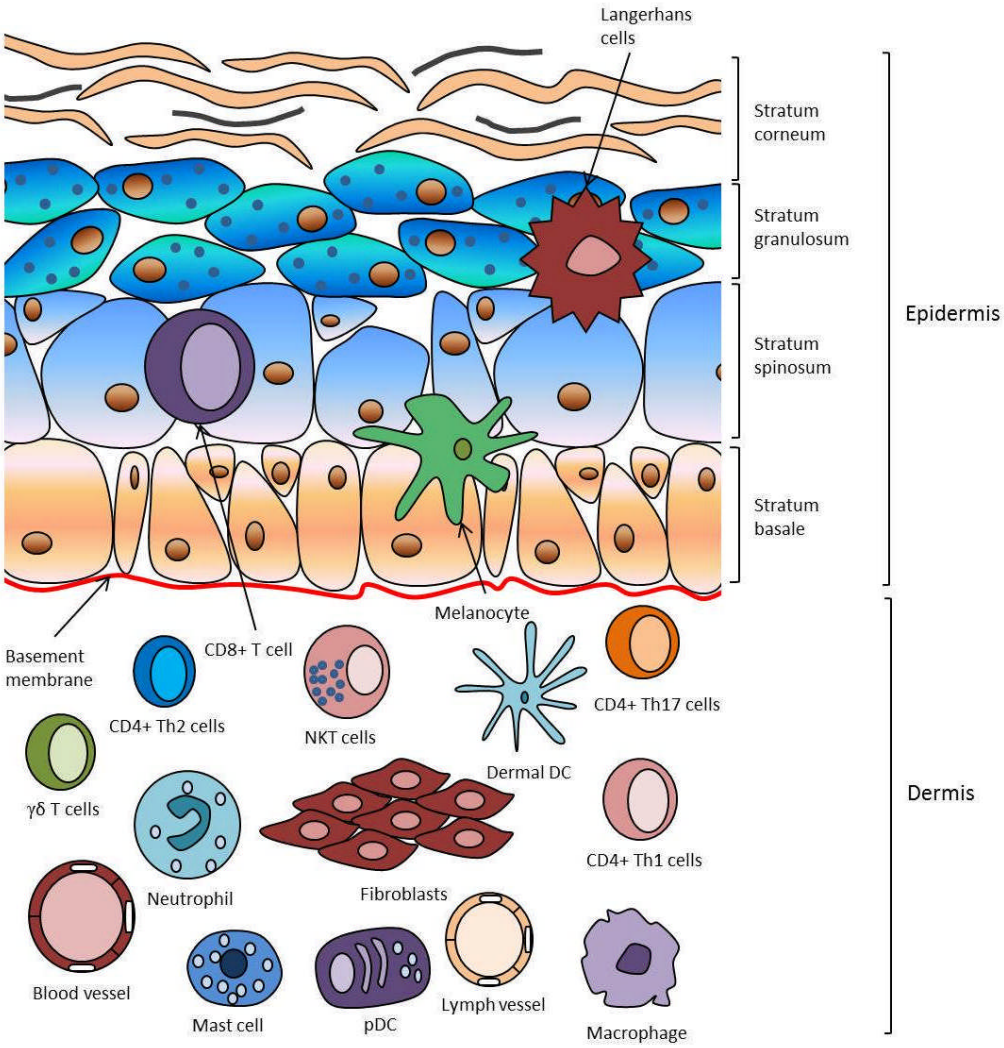


Figure 1-1 Diagram illustrating the cellular structure of the skin

This diagram illustrates the stratified epidermis formed by keratinocyte differentiation and the cells found within both the dermis and epidermis. The red line represents the basement membrane that separates the two [3].

1.2 Skin function in immunity, inflammation and disease (Immune response of the skin)

As the largest organ of the body that separates the vulnerable mesenchymal tissues and internal organs from the hazardous external environment, the skin (and other epithelial surfaces) is the first line of defense against invading pathogens and other harmful factors. The ability of the skin to ward off invading pathogens doesn't stop at the physical barrier formed by the structured epidermis and the antimicrobial environment it creates. This was recognized in 1983 by Streilein who introduced the concept of skin-associated lymphoid tissue (SALT) and subsequently the 'skin immune system (SIS)', identifying the importance of the blood and lymphatic system and immune cell trafficking to and from the skin [6, 7].

It has since been established that the skin is capable of mounting a strong immune response to prevent microbial invasion, acting immediately through innate measures and by effectively mobilizing an adaptive immune response. However, skin inflammation must be tightly controlled ultimately culminating in wound healing and bringing a resolution to the induced inflammation. Indeed, dysregulation of these tightly controlled immune processes are often linked to inflammatory skin disease.

1.2.1 Initiation of an immune response

In addition to creating a physical barrier against external pathogens, epidermal keratinocytes are capable of directly sensing and responding to an infection. Keratinocytes can detect a microbial presence through the expression of receptors termed pattern recognition receptors (PRRs). PRRs recognise evolutionarily conserved microbial components that are indispensable to the microbes' existence, such as bacterial flagellin or viral RNA, and were conceptualised by Janeway as pathogen-associated molecular patterns (PAMPs) [8]. The PRRs are made up of a number of distinct families, but the most abundant in the skin are the toll-like receptors (TLRs). The TLR family consists of 9 trans-membrane receptors, each

recognising distinct PAMPs. TLR 1, 2, 4, 5 and 6 are expressed on the cell surface and primarily recognise PAMPs of bacterial and fungal cell wall or membrane origin. TLR 3, 7, 8 and 9 recognise nucleic acids of viral and bacterial origin and are expressed within intracellular endosomes. Ligation of a PAMP to its respective TLR will activate the host cell, leading to activation of nuclear factor κB (NF-κB) and subsequent expression of a host of innate and adaptive immune mediators [9]. Through the expression of TLRs 1-6, and 9, epidermal keratinocytes are capable of responding to a wide variety of pathogens and hence initiating an immune response [10]. Whilst TLRs are responsible for recognition of a wide variety of pathogens, another class of PRR termed C-type lectin receptors (CLRs) are of great importance in the recognition of fungal carbohydrate PAMPs. Keratinocytes express a range of CLRs including Dectin-1 and Mannose Receptor C type 1 that recognise fungal pathogens via cell-wall β1,3-glucan and mannose respectively [11], prevalent on fungal pathogens such as *Candida albicans* and *Aspergillus fumigatus*. Ligation of PAMPs to CLRs also activates the host cell, transducing signalling via SYK activity, similarly activating NF-κB and Mitogen-activate protein kinases (MAPKs), resulting in a subsequent cytokine response [12]. In addition to sensing pathogens via PAMPs, keratinocytes can also recognise and respond to danger signals in the form of damage associated molecular patterns (DAMPs). DAMPs are molecules such as irritants, ultraviolet light, or host-cell derived molecules termed ‘alarmins’ including IL-1α and the S100 proteins, that are released upon damage caused by necrosis or trauma and as such notify neighbouring cells of danger [13-16]. As with PAMPs, they are primarily recognised via detection by PRRs. One such example is the detection of extracellular ATP released upon cell damage by nucleotide-binding domain, leucine-rich repeat-containing (NOD) like receptors (NLR). The NLRs are another family of PRRs highly expressed in the skin. Activation of NLRs leads to inflammasome activation and the subsequent release of pro-inflammatory mediators [17].

In response to TLR or CLR ligation, keratinocytes will express and secrete a range of cytokines and chemokines with which they can direct an immune

response, attracting both innate and adaptive immune cells and polarising them to the phenotype required to combat the pathogens detected. However, even before mobilisation of the immune system, keratinocytes can directly fight invading pathogens through the expression of anti-microbial peptides (AMPs). AMPs are evolutionarily conserved small cationic proteins that directly kill microbes by binding to and forming pores in their cell membranes [18]. Keratinocytes produce a number of AMPs, the most studied of which are the cathelicidin LL37 and human β defensins (hBDs), which are active against bacterial and fungal microbes. Of particular importance is hBD2, the expression and release of which is inducible in keratinocytes upon activation of TLR2 and TLR4 as well as in response to other inflammatory mediators such as IL-1 and tumour necrosis factor α (TNFα) [19, 20]. hBDs have not only been demonstrated to have potent bactericidal activity, but also have chemotactic properties, capable of binding and signalling though the chemokine receptor CCR6 expressed on DC, IL-17 producing type 3 innate lymphocyte subset (ILC3) and T_H17 cells. As such, AMPs both directly fight invading pathogens and contribute to the mobilisation of the immune system [21, 22].

Upon detection of a pathogenic intrusion via PRRs, keratinocytes will secrete a range of inflammatory mediators and thus begin to influence the surrounding cutaneous tissue and initiate an influx of leukocytes. Of great significance in skin immunity is the release of IL-1 family cytokines (discussed in section 1.3). Members of the IL-1 family have a broad range of effects on multiple cell types, ranging from activation of DC and $CD4^+$ T cells to maturation and clonal expansion of B cells, and will also act in an autocrine fashion to further activate surrounding keratinocytes [23, 24]. Keratinocytes are significant producers of chemokines and as mentioned above can attract different leukocyte subtypes into the skin compartment. For example, an influx of neutrophils is mediated – among others - through the secretion of IL-8 (CXCL8). $CCR6^+$ effector T cells (T_H17 and T_H22 cells) will migrate in response to local secretion of chemokine CCL20 [25, 26].

However, a specific immune response is dependent on professional antigen presenting cells. Langerhans cells and dermal DCs are activated by

keratinocyte produced mediators and in the presence of pathogens, following uptake and processing, will undergo maturation and migrate to the local draining lymph nodes where they present their antigens to naïve T cells [27, 28]. Depending on the antigens presented and the accompanying co-stimulation, Langerhans cells and dermal DCs initiate clonal expansion of specific T helper cells which then follow a chemokine gradient back to the site of infection. Once at the site of infection activated T helper cells secrete various immune mediators to enhance and strengthen the immune response. Of those that infiltrate into the skin, a number differentiate into memory T helper cells and remain there. These skin-resident T helper cells, identifiable by a cutaneous lymphocyte-associated antigen (CLA), are re-activated in the context of secondary antigen challenge and are therefore important skin-based effector cells [29, 30].

As previously mentioned, inflammation initiated after skin damage and microbial offense will result in an influx of neutrophils following an IL-8 chemokine gradient. Indeed it is well established that neutrophils are amongst the first immune infiltrate, present within minutes of tissue damage [31]. Neutrophils are crucial in the first steps of clearing a wound and mounting a strong response. In addition to their traditional phagocytic role, neutrophils secrete a host of antimicrobial molecules such as enzymes, superoxide radicals and neutrophil extracellular traps (NETs) and are extremely proficient at killing microbes [32, 33]. Furthermore they can also react to a wide range of stimuli and release a great amount of inflammatory mediators to further direct an immune response. An influx of neutrophils is shortly followed by monocytes and macrophages.

An effective cutaneous immune response results from the coordinated action of skin resident and infiltrating leukocytes. However, in order to properly repair a compromised skin barrier the inflammation must be brought to a resolution in order for wound healing to occur.

1.2.2 Resolution of inflammation and wound healing

Neutrophils are known to be short lived. Once they have undertaken their phagocytic role at the site of inflammation they then become apoptotic and

die. As outlined above, following neutrophil influx, monocytes and macrophages begin to traffic to the site of inflammation following a chemokine gradient. In addition to reinforcing the inflammatory immune response these monocytes and macrophages also clear the site of the apoptotic neutrophils. It is in this process, driven by macrophages and neutrophil apoptosis, that resolution of inflammation begins and skin barrier homeostasis can be regained.

As macrophages begin to enter the site of inflammation a depletion of neutrophil chemotactic chemokines begins, halting the influx of neutrophils and allowing resolution to progress. This is achieved in a number of ways. Macrophage-specific metalloproteinases such as MMP12 cleave neutrophil chemokines such as IL-8 to inactive forms, preventing them binding to chemokine receptors and hence preventing neutrophil chemotaxis [34]. Some chemokines are cleaved to produce receptor antagonists, allowing binding but preventing signal transduction [35]. Furthermore chemokines are sequestered by apoptotic neutrophils. As neutrophils undergo apoptosis they increase expression of chemokine receptor CCR5 which depletes surrounding pro-inflammatory chemokines such as macrophage inflammatory protein-1α and -1β (MIP-1α, MIP-1β) [36].

Once neutrophil influx is abrogated neutrophil apoptosis becomes abundant. An increase in macrophage presence promotes this through the expression of high amounts of death receptor ligands such as Fas-ligand and TNF [37, 38]. As neutrophils become apoptotic they secrete mediators that further inhibit neutrophil influx such as annexin A1, and start expressing molecules that mark them for clearance by macrophages termed ‘eat me’ signals, such as phosphatidylserine [39-41]. Upon phagocytosing apoptotic neutrophils, macrophages undergo a phenotypic change from pro-inflammatory cells and begin to act in an anti-inflammatory capacity. They secrete IL-10, IL-1 receptor antagonist (IL-1Ra) and transforming growth factor-β (TGF-β), important suppressors of inflammation and promoters of cell proliferation, as well as a host of lipid mediators such as protectins and resolvins which promote inflammation resolution by reducing neutrophil activity and promoting monocyte migration [42, 43].

In repair processes, alternatively activated macrophages (M2 macrophages) have been identified as critical, acting to further supress inflammation and induce reparative cell growth through secretion of growth factors [44]. Secretion of vascular endothelial growth factor (VEGF) induces angiogenesis providing a supply of oxygen to regenerating tissue, whilst TGF-β induces fibroblast differentiation into myofibroblasts which contribute to wound repair through production of interstitial fibrillar collagens [45, 46]. At the same time, secretion of MMPs by M2 macrophages allows for keratinocyte migration to occur and secretion of epidermal growth factor (EGF) and TGF-β induce epidermal proliferation [47]. Finally, basement membrane repair is promoted through the production of laminin, and the normal stratified epidermal structure is restored through keratinocyte differentiation and keratin production [48, 49].

The importance of immune response control becomes evident when stages in the initiation or resolution of inflammation become dysregulated.

1.2.3 Inflammatory disease in the skin

Examples of dysregulated inflammatory responses in the skin often manifest in inflammatory skin disease. Atopic dermatitis (AD) and psoriasis are amongst the most common and most extensively studied cutaneous inflammatory diseases and are both examples of highly heterogeneous multifactorial conditions involving environmental and genetic factors. AD and psoriasis have in common that leukocytes infiltrate into the epidermal compartment and thus keratinocyte lymphocyte interaction occurs.

In brief, AD is a common skin disease affecting up to 20% of children of school entry age in the UK [50]. Features of the disease are predominance of type 2 immune activation involving IL-4 and IL-13 driven cellular responses, IgE class type switch and high susceptibility to *Staphylococcus aureus* skin infection. The dominant clinical sign is pruritus and associated impairment of life quality, commonly due to loss of sleep [51].

Psoriasis is considered by many to be an autoinflammatory condition with possible associated autoimmune reactions that effects approximately 3% of

the population [52]. The condition has a significant impact on quality of life. It affects the skin and joints, and the more severe cases are being found to be associated with metabolic conditions, such as coronary heart disease and atherosclerosis. There are now up to 16 loci that have been associated with psoriasis susceptibility, emphasising the heterogeneous genetic nature of the condition [53]. Psoriasis is also phenotypically heterogeneous, and can be separated into 3 distinct clinical subtypes; vulgaris, guttate, and pustular. Psoriasis vulgaris, characterised by the dry red scaly plaques most commonly associated with psoriasis, is the most common form of psoriasis affecting 85-90% of sufferers [54]. Guttate psoriasis is characterised by small plaques of approximately 1 cm in diameter that often presents in an acute onset and is strongly associated with *Streptococcal* infection in young children and adolescents [55]. Pustular psoriasis has both generalised and localised manifestations, but generally features pustular lesions with marked infiltration of neutrophils into the dermis and epidermis [56]. Generalised pustular psoriasis (GPP) is a rare but potentially life threatening condition in which a subset of patients have been strongly associated with loss-of-function mutations in the gene encoding IL-36 receptor antagonist (IL-36Ra) [57].

Psoriasis can be triggered by a number of environmental factors that initiates an overactive self-perpetuating inflammatory loop often driven by multiple genetic factors that gives rise to the psoriatic lesions associated with the condition. Dysregulated inflammation leads to an abnormal influx of numerous inflammatory cell types into both the dermis and epidermis. These cells secrete pro-inflammatory cytokines and factors that recruit and activate T cell populations, the culmination of which results in increased epidermal proliferation and undifferentiated keratinocytes, ultimately leading to a compromised skin barrier and the associated scaly or flaky skin [58]. Psoriasis has primarily been thought of as a T cell driven disease, with T cell-associated cytokines such as TNFα, IL-22 and IL-17 playing critical roles in its development, and myeloid cytokines such as IL-23 inducing T cell differentiation [59, 60]. More recently, however, pro-inflammatory cytokines of the innate immune system have been recognised as playing pivotal roles

in psoriatic inflammation. The IL-36 family became of particular interest once noted by Blumberg *et al.* to be highly up-regulated in psoriatic skin, and are amongst the most highly up-regulated genes when comparing lesional to non-lesional skin [61]. The role of IL-36 cytokines in psoriasis and pathological inflammation is explored in greater detail in section 1.4.

1.3 The IL-1 family

The IL-1 family is very strongly associated with inflammation and the innate immune response. They are comprised of several distinct cytokine members that signal via their respective IL-1 family receptors and as a result have a wide-reaching influence on multiple cell types. Though typically associated with inflammation, the IL-1 family also contains members that work to regulate both general and specific IL-1 mediated inflammation. Given their importance in both inducing and regulating inflammation, and their far-reaching influence, the IL-1 family is of great interest in the context of inflammatory disease.

Their existence was first observed in the 1940s after it was noted that a factor in rabbit neutrophil-conditioned supernatant, then termed Leukocytic Pyrogen, could induce a rapid onset fever in rabbits [62]. Later it was identified that monocyte-conditioned media contained a factor capable of augmenting lymphocyte proliferation following antigen contact [63]. Eventually, in the late 1970s, these factors were purified and identified as the same protein, given the interleukin nomenclature of IL-1 [64]. Shortly afterwards, cDNA isolation revealed there were in fact two distinct forms of IL-1; IL-1α and IL-1β [65, 66]. Since then, the IL-1 family has grown to include another 9 family members, identified by protein purification and cloned from cDNA of various cells types or by *in silico* identification from gene data banks.

The IL-1 family consists of 11 cytokines. These were originally given an IL-1F identity from 1-11, but are now known by unique interleukin nomenclature and have been grouped into three sub families; the IL-1 subfamily, the IL-18 subfamily and the IL-36 subfamily (detailed in Table 1-1). All members of the IL-1 cytokines, with the exception of IL-1Ra, lack a signal peptide and are produced as immature intracellular proteins that must be processed into mature proteins. This processing removes a portion of the N-terminus, and it is by the size of the pro-piece removed that the IL-1 family is divided into subfamilies. The IL-1 subfamily, consisting of IL-1α, IL-1β and IL-33, has the largest pro-piece. The IL-18 subfamily has a comparatively smaller pro-piece

and consists of IL-18 and IL-37. The smallest pro-piece belongs to the IL-36 subfamily which is made up of 5 proteins; IL-36α, IL-36β, IL-36γ, IL-36 receptor antagonist (IL-36Ra) and IL-38 [67]. The IL-1 cytokines are thought to have arisen from a common ancestral gene as they have a highly conserved genetic structure, resulting in each protein folding into a 12-stranded β-barrel [68].

Sub-family	Cytokine agonist/antagonist	Receptor complex	Cleavage required for activity
IL-1	IL-1α	IL-1R1/IL-1RAcP	×
	IL-1β		✓
	IL-33	ST2/IL-1RAcP	×
	IL-1Ra	IL-1R1	×
IL-18	IL-18	IL-18Rα/IL-18Rβ	✓
	IL-37	IL-18Rα	×
IL-36	IL-36α	IL-36R/IL-1RAcP	✓
	IL-36β		✓
	IL-36γ		✓
	(IL-38)		?
	IL-36Ra	IL-36R	✓

Table 1-1 IL-1 family cytokines

Table shows IL-1 family cytokines, their associated signalling complexes and whether proteolytic processing is necessary for cytokine activation [69-71].

The IL-1 family cytokines signal through IL-1 receptors which belong to a large receptor superfamily called Toll-IL-1-receptor (TIR) domain-containing receptors. The IL-1 receptors consist of extracellular Ig-like domains to which the cytokines bind, and a cytosolic TIR domain that mediates receptor signalling. Binding of agonist to receptor causes recruitment of an accessory protein, creating a receptor complex capable of transducing a signal via

MyD88 and MAP kinases, often culminating in activation of NF-κB. As shown in Figure 1-2 the IL-1 cytokines bind distinct IL-1 receptors but all require recruitment of the IL-1R accessory protein (IL-1RAcP) for signal transduction (with the exception of IL-18 which has a unique accessory protein IL-18Rβ) [72]. In addition to the IL-1 family receptors through which IL-1 members transduce signals there are IL-1 family receptors that act in a regulatory capacity. IL-1R2 is a decoy receptor which binds IL-1 but lacks a TIR domain and as such cannot transduce a signal. It therefore acts to sequester IL-1α and IL-1β without producing pro-inflammatory signals [73]. IL-1R8 is another regulatory receptor which is recruited to IL-1 receptors similarly to IL-1RAcP but is incapable of transducing signals due to mutations in its TIR domain [74].

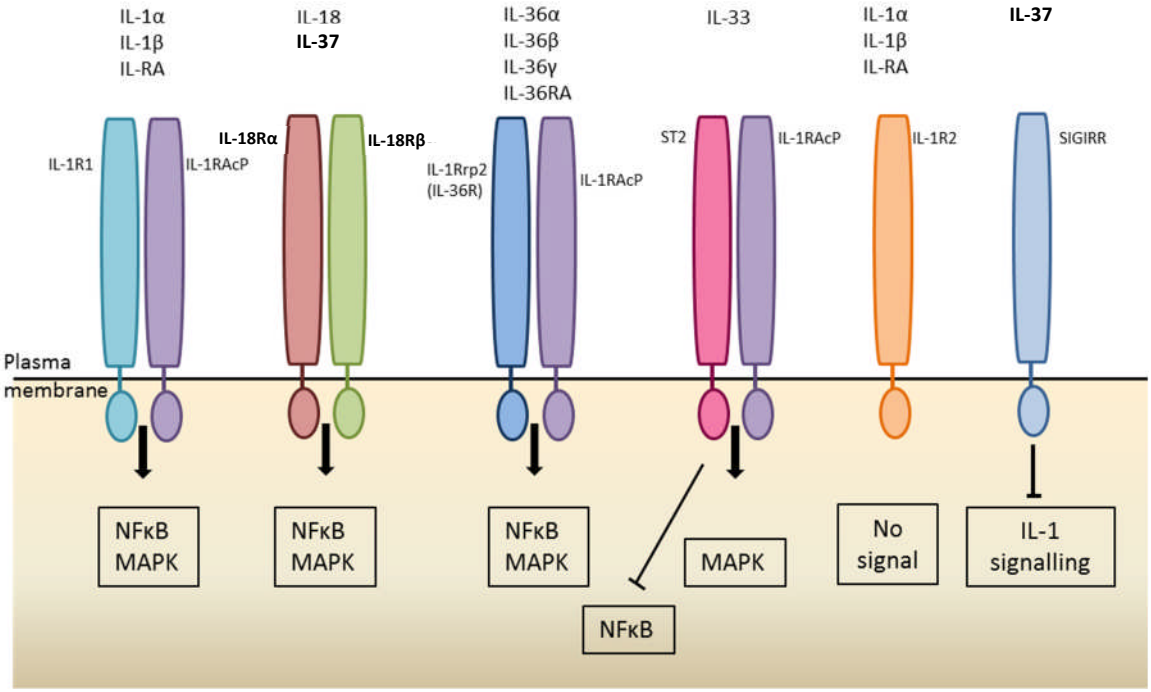


Figure 1-2: IL-1 family receptors

Diagram illustrating the IL-1 family receptors, their respective ligands, and the downstream signalling pathways [69].

1.3.1 Activation and function of IL-1 cytokines

IL-1 family cytokines influence all aspects of an immune response, ranging from polarization of T helper cells to T_H1, T_H2, or T_H17 phenotypes to the activation and regulation innate immune cells such as neutrophils, monocytes, macrophages and dendritic cells.

As previously mentioned, several IL-1 family cytokines require post-translational processing in order to become biologically active. Each IL-1 cytokine contains a consensus sequence motif of A-X-D, where A is an aliphatic residue, X is any residue and D is aspartic acid, within its 1st IL-1 beta-sheet. In order for full activity of an IL-1 cytokine to manifest, some IL-1 members require N-terminal truncation to within 9 amino acids of the A-X-D motif [71]. However, the manner in which the IL-1 proteins are truncated varies, and for many this process is poorly understood.

IL-1β and IL-18 activation is dependent upon caspase-1 cleavage that occurs at a caspase-1 consensus cleavage site 9 amino acids upstream of the A-X-D motif [75]. Activation of caspase-1 involves the formation of a cytosolic signalling complex termed the inflammasome that is present within most cells. An inflammasome is made up of a number of protein elements including a cytoplasmic PRR (primarily one of the NLR family), the adaptor molecule apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD) (ASC), and caspase-1 [76]. Inflammasomes are typically formed by NLR members such as NLR pyrin domain-containing 3 (NLRP3), and are named accordingly, but can also be initiated by other cytoplasmic sensors such as absent in melanoma 2 (AIM2) and pyrin [77]. Upon PRR activation, the cytoplasmic sensor will recruit ASC which in turn recruits caspase-1 and facilitates its activation [78, 79]. Once caspase-1 is active, cleavage of the caspase-1 sensitive IL-1 proteins can occur, mediating their activation and subsequent secretion from the cell.

Of the IL-1 family cytokines, IL-1β and IL-18 require inflammasome activation and subsequent caspase-1 processing to produce mature forms, and this is known to occur in a two-step process. Firstly cells must be primed, often through TLR stimulation, to initiate NF-κB dependent synthesis of pro-IL-1β,

pro-IL-18 and the sensor components of the inflammasome [80]. A second signal, activating the inflammasome sensor component, is then required to initiate the formation of the inflammasome and activation of caspase-1, which in turn processes the immature IL-1β and IL-18 proteins allowing for their secretion [81]. IL-1β is expressed by hematopoietic cells in response to PRR activation or stimulation by various inflammatory cytokines including IL-1 itself. In addition to its traditional fever-inducing function IL-1β influences the ILC3s to produce IL-17 and IL-22 and promotes T_H17 differentiation [69, 82]. Its dysregulated production is highly linked to autoinflammatory conditions such as neonatal onset multisystem inflammatory disease (NOMID) characterized by fever, rash and arthritis. Whilst the production of IL-1β requires an inflammatory stimulus, pro-IL-18 is constitutively expressed by most cell types. The primary producers of IL-18 are activated macrophages and monocytes, although keratinocytes are known to contribute to the production of active IL-18 in inflamed skin. Active IL-18, in the presence of IL-12 and IL-15, is considered as a T_H1 and ILC1 associated cytokine promoting proliferation of T_H1 cells and production of interferon-γ (IFN-γ). In the absence of IL-12 and IL-15, IL-18 can also induce a T_H2 phenotype [83]. IL-18 is therefore important in a number of inflammatory skin conditions, including atopic eczema and psoriasis [84].

IL-1α, unlike IL-1β, does not require N-terminal truncation as it is biologically active in its pro-form. Although both IL-1α and IL-1β signal through the same receptor and as such have very similar biological functions, IL-1α has some very distinct characteristics that set it apart from IL-1β. Whereas IL-1β is secreted upon maturation and can circulate systemically, IL-1α typically associates with the plasma membrane of the producing cell and so acts in a local capacity [85]. IL-1β is primarily produced by hematopoietic cells, such as macrophages and monocytes, but IL-1α is more widely expressed with notably high levels in epithelial tissues, including keratinocytes [86]. With activity in its immature form it has been postulated that IL-1α acts as a DAMP in the event of cellular damage causing its release into the extracellular matrix [87]. Furthermore, the immature form of IL-1α contains a nuclear localisation sequence (NLS) within its pro-piece which binds DNA, indicating

transcription factor activity [88]. Indeed some studies have identified oncogene functionality of IL-1α [89].

IL-1α is not the only IL-1 family member to have dual nuclear factor and cytokine activity. IL-33 and IL-37 both contain a NLS and can both regulate gene expression. IL-33 is expressed by a wide variety of cells, including keratinocytes, both constitutively and in response to stimuli [90]. Similarly to IL-1α, IL-33 is able to bind its receptor in its pro-form and is released primarily through cell death, functioning as a DAMP [87, 91]. As a soluble protein, IL-33 is known as a T_H2 cytokine that drives the polarization of ILC2s and T_H2 cells and is important in allergic inflammatory disease such as asthma [69, 90]. Whilst as a cytokine IL-33 acts in an inflammatory capacity, its nuclear localisation appears to have regulatory consequences. IL-33's N terminal NLS mediates its trafficking to the nucleus where IL-33 is thought to act as a transcriptional repressor by inducing chromatin compaction [92, 93]. IL-37 is also known to have anti-inflammatory activity, but to a much greater extent than IL-33. On both the extracellular and intracellular level IL-37 has been identified to regulate inflammation. IL-37 is produced in five isoforms by alternative splicing (IL-37a-e), each with their own specific expression profiles. Its expression is inducible by TGF-β in addition to TLR activation and IL-1β stimulation [94]. Upon stimulation with LPS, IL-37 has been demonstrated to traffic to the nucleus in a caspase-1 dependent manner where it exerts potent anti-inflammatory mechanisms (as yet not dissected) resulting in a decrease in both kinase phosphorylation and inflammatory cytokine production [95, 96]. IL-37 is structurally very similar to IL-18 and is able to bind the IL-18 receptor α chain. By doing so IL-37 appears to recruit the regulatory IL-1 receptor IL-1R8 (SIGIRR) and further exert anti-inflammatory activity [97].

IL-1-mediated inflammation is also controlled through the action of IL-1 members IL-1 receptor antagonist (IL-1Ra) and IL-36 receptor antagonist (IL-36Ra). Whereas IL-37 is known to be a potent regulator of general inflammation, IL-1Ra and IL-36Ra very specifically regulate IL-1 and IL-36 mediated inflammation by antagonising their specific receptors.

1.3.2 The IL-36 subfamily

The IL-36 subfamily consists of 4 members; IL-36α, IL-36β, IL-36γ and IL-36Ra, grouped together as they share the same receptor complex and have similar biological function [70]. The IL-36 agonists (IL-36α, IL-36β and IL-36γ) are able to transduce a signal upon binding of their receptor (IL-36R) as they facilitate recruitment of the IL-1R accessory protein (IL-1RAcP) thus enabling signal transduction. IL-36Ra however is able to bind to the IL-36R but cannot recruit IL-1RAcP and hence acts as a receptor antagonist, preventing signal transduction [98, 99]. IL-38 is very homologous to IL-36Ra and may also share the same receptor, so might be thought of as an IL-36 cytokine [100]. Unlike IL-1 and IL-18 members, their expression is quite restricted, with highest levels being expressed by epithelial cells of skin, joint and lung tissue. As with other IL-1 family members, the IL-36 cytokines require N-terminal cleavage for maturation. Unlike IL-1β and IL-18 the IL-36 cytokines contain no N-terminal consensus cleavage site, which for a while mislead the research field engendering the theory that, like IL-1α and IL-33, the IL-36 cytokines did not require N-terminal truncation to transduce a signal. This theory was dispelled when it was demonstrated, by progressively removing N-terminal amino acids of each protein, truncation to 9 amino acids upstream of the A-X-D motif yielded increases in activity of up to 10 000 fold [99]. Indeed recent crystallographic data suggests truncation of the N-terminus 9 amino acids upstream of the A-X-D motif is necessary for the IL-36 proteins to engage their receptor [101]. The post-translational processing of IL-36 members is a subject of on-going investigation that is explored in greater depth later.

With regards to the expression of the IL-36 cytokines, each member is slightly different. IL-36Ra is constitutively expressed by keratinocytes, whereas expression of the agonists requires a stimulus. All IL-36 members are expressed in epithelial tissue of the skin and lung, IL-36β mRNA is detectable in synovial tissue and IL-36α expression has been identified in human adipose tissue. IL-36 members are also thought to be expressed at low levels in joint, gut and brain tissue [67, 102-105]. Although the IL-36

members are predominantly expressed by epithelial tissue, their expression has also been identified in macrophages, monocytes and dendritic cells. The expression of IL-36 agonists is inducible by stimulation with inflammatory cytokines including TNFα, IL-17 and in an autocrine fashion by the IL-36 agonists themselves, and may be induced after activation of a number of TLRs [102, 106]. Their release from the cell is poorly understood and is an ongoing subject of investigation. As yet the only release of IL-36γ was observed in keratinocytes after TLR3 stimulation by polyinosinic:polycytidylic acid (poly(I:C)), and the mechanism of release was not clearly identified [107].

Given their abundant expression in the skin, the IL-36 members play a significant role in skin-based inflammation. As they all signal through the same receptor complex they influence their cellular environment in the same way, so any differences between each cytokine will arise through their differential expression. The IL-36R is widely expressed, so many cell types respond to IL-36 stimulation. In the skin both keratinocytes and fibroblasts are IL-36 responsive, secreting a range of pro-inflammatory mediators in response to stimulation, notably including chemokines IL-8 and CCL20 [106, 108]. IL-36 cytokines have been shown to induce maturation of monocyte-derived dendritic cells (MDDCs), causing an up-regulation in MHCII antigens, CD83 and CD86 thus promoting T cell differentiation, and induce LCs to produce a range of inflammatory cytokines including TNFα and IL-6 [102, 109]. IL-36 members therefore induce the production of T_H1 and T_H17 polarising cytokines, promote chemotaxis of activated leukocytes and maturation of dendritic cells to ultimately drive T_H1 cell and T_H17 cell proliferation. The IL-36 cytokines are greatly overexpressed in psoriatic skin, in which a T_H1 and T_H17 response is prominent, so it is of no surprise that the IL-36 cytokines are of great interest in the context of psoriatic disease [110].

1.4 IL-36 in disease

The IL-36 cytokines are strong inducers of inflammation and are emerging as being influential in a number of inflammatory conditions. Indeed it is in the

context of psoriatic inflammation that the IL-36 family is primarily studied. However, their involvement is notable in a number of other pathologies and they have also been indicated to have a role in immune defense.

1.4.1 IL-36 in psoriasis and inflammatory disease

The observation that IL-36 members are overexpressed in psoriatic skin first initiated interest in IL-36 and psoriatic inflammation [108, 110]. Furthermore it was observed that IL-36γ is one of the top 30 up-regulated genes when comparing non-lesional and lesional psoriatic skin, and that IL-36 expression is far more inducible in psoriatic keratinocytes than healthy keratinocytes [61, 111]. Further evidence that IL-36 plays a significant central role in the pathogenesis of psoriatic inflammation has been demonstrated through the development of a number of mouse models. Mice with forced transgenic overexpression of IL-36α presented with a phenotype very comparable to human psoriasis, exhibiting acanthosis, hyperkeratosis and an inflammatory infiltrate [110]. Although these psoriasis-like plaques spontaneously resolved after 2-3 weeks, subsequent treatment with phorbol ester bought a return of plaques within a few days that even more closely resembled human psoriasis. Furthermore the phorbol ester treated mice had an increased expression of a variety of psoriasis-associated inflammatory mediators including IL-23, IL-17 and IL-22 [112]. These cytokines are considered important in the pathogenesis of psoriasis, stimulating keratinocytes to produce growth factors and other inflammatory mediators, driving both epidermal and T_H17 cell proliferation and survival. Indeed blocking these pathways in the phorbol ester treated mice by targeting TNFα, IL-17 and IL-23 was very effective at preventing the skin lesions. Mouse studies have been conducted whereby psoriasis-like lesions are induced through the addition of imiquimod, a TLR7 agonist that has been demonstrated to strongly induce IL-36. Treatment with imiquimod causes skin thickening, expression of IL-17 and a notable infiltrate of inflammatory cells into the skin. These imiquimod induced effects, however, are completely ablated when performed on IL-36R-deficient mice, implicating a central role for IL-36 [113].

IL-36 cytokines induce the expression of a number of proteins associated with psoriasis, including T_H17-polarising cytokines. In turn T_H17 cytokines have been demonstrated to increase expression of IL-36 members, indicating the presence of a T_H17-inducing feedback loop [106, 108]. It has also been shown that IL-36 stimulation of keratinocytes induces secretion CCL20, promoting influx of T_H17 cells and γδ T cells that will further amplify the psoriatic inflammation through the secretion of IL-17 and IL-22. Furthermore IL-36 has been demonstrated to induce secretion of AMPs, the overexpression of which is a hallmark in psoriasis. Interestingly IL-36 has been suggested to increase expression of LL37, which has itself been implicated in the pathogenesis of psoriasis through binding self-RNA and promoting activation of dendritic cells upon stimulation of TLR7 and TLR8 [108, 114]. The importance of IL-36 in psoriasis was further demonstrated after identifying nonsense and missense mutations in the gene encoding IL-36Ra (*IL36RN*), illustrating the consequences of dysregulated IL-36-mediated inflammation. With a complete lack of IL-36 regulation, patients developed a severe systemic life-threatening form of psoriasis called general pustular psoriasis (GPP). The loss-of-function IL-36Ra mutations have been termed deficiency of IL-36 receptor antagonist (DITRA) due to the similarity with deficiency of IL-1 receptor antagonist (DIRA), and have been identified in a number of different populations. DITRA is the first case of a single gene mutation giving rise to a psoriatic condition, providing strong evidence of the importance IL-36-mediated inflammation plays in psoriasis [57, 115, 116].

In addition to psoriasis, the IL-36 cytokines have been implicated in rheumatoid arthritis and atopic dermatitis, and there is growing interest around their involvement in pulmonary disease and inflammatory bowel disease. IL-36α expression is overexpressed by synoviocytes in both rheumatoid and psoriatic arthritis, and stimulation of cultured synoviocytes with IL-36β is seen to induce secretion of IL-8 and IL-6 [117, 118]. Recent RNA trascriptome analysis comparing lesional and non-lesional skin of AD patients has identified increased expression of IL-36 cytokines in lesional skin, albeit to a lesser extent than in psoriasis [119]. IL-36α and IL-36γ expression has recently been identified to be enhanced in inflamed mucosa

of patients with inflammatory bowel disease, in particular ulcerative colitis. This same study showed IL-36 stimulation of intestinal epithelial cell lines induced expression of a host of inflammatory chemokines and enhanced activation of NF-κB, highlighting their propensity for promoting pathogenic gut inflammation [120].

There is a lot of evidence for a role of IL-36 in inflammatory pulmonary disease such as asthma and chronic obstructive pulmonary disease (COPD). Human bronchial epithelial cells have been shown to strongly produce IL-36γ in response to a number of inflammatory stimuli, including IL-17, IL-1β, TNFα and challenge with cigarette smoke (the causative agent in COPD) [121]. Rhinovirus has also been shown to induce IL-36γ expression, and in analogy to psoriatic keratinocytes is more inducible in bronchial epithelial cells from asthmatic donors than healthy cells [122]. Furthermore bronchial epithelial cells respond to IL-36 through NF-κB and MAPK activation producing neutrophil and T_H17 chemokines IL-8, CXCL3 and CCL20 [103]. The effect of IL-36 on lung inflammation has been studied more in depth in mice. Intranasal administration of IL-36γ results in rapid infiltration of neutrophils and other immune cells and an increase in mucus production [123]. Neutrophil infiltration has been implicated in the pathogenesis of a number of pulmonary diseases including asthma, acute respiratory distress syndrome and COPD, so as an inducer of neutrophil chemotaxis IL-36 is thought to contribute to the development of these conditions.

1.4.2 IL-36 in immune defence and wound healing

The majority of research on the IL-36 subfamily has been conducted in the context of pathogenic inflammation and as yet there is very little understanding with regards to their function in the immune system under normal conditions. With this said, there is emerging evidence suggesting that IL-36 might play a role in immune defence and wound healing at epidermal barriers. It has been demonstrated that both live and heat-killed *Aspergillus fumigatus* conidia and hyphae strongly induce expression of IL-36γ through TLR4 and dectin-1 pathways. Furthermore specifically inhibiting IL-36 signalling by IL-36Ra reduced *A. fumigatus*-induced expression of IL-17 and

IFNγ, suggesting IL-36 may be important in driving T_H17 and T_H1 responses in response to *Aspergillus* infection [124]. Given the IL-36 proteins give rise to a psoriatic phenotype, and that a psoriatic phenotype is very similar to that seen when fighting a fungal infection, it is perhaps logical that IL-36 proteins are important in fungal immunity.

It has also been postulated that IL-36 cytokines may play a role in wound healing. Although IL-36 has been implicated in the pathogenesis of inflammatory bowel disease, there is evidence to suggest the IL-36/IL-36R axis has a role in resolution and repair of damaged epithelial mucosa. This was demonstrated through inducing colitis by dextran sodium sulphate (DSS) in mice that lacked IL-36R. It was observed that IL-36R knock out mice initially had less severe colitis than wild type mice, indicating an inflammatory role for IL-36R signalling. However, wild type mice eventually recovered after stopping DSS treatment, whereas colitis in the IL-36R knock out mice progressed to the point the animals had to be euthanized. It was subsequently noted that IL-36R was crucial for the continued expression of IL-22 in colons of wild type mice, an important pro-resolving cytokine that promotes epithelial growth, restitution and protects from intestinal inflammation [125].

IL-36 is well established as a pro-inflammatory cytokine family that has a pathogenic role in a number of inflammatory conditions, but it is poorly understood in its normal function and may have a long term function in inflammation resolution. This is an area of IL-36 research that should be increased as it may yield insight into ways of better regulating IL-36-mediated inflammation and promoting inflammation resolution.

1.5 Post-translational processing of inflammatory mediators

As mentioned above, many IL-1 members are synthesized as immature proteins that require post-translational proteolysis in order to gain biological activity. Traditionally, as is the case with IL-1β and IL-18, this occurs in a caspase-1 dependent fashion involving formation of an inflammasome

proceeded by secretion of the mature cytokines. However, many IL-1 family proteins are processed by a range of different proteases, in both intracellular and extracellular environments. Post-translational proteolytic processing of inflammatory mediators is not limited to the IL-1 family. It is a method utilized by the immune system to regulate numerous inflammatory processes with both pro-inflammatory and regulatory consequences.

1.5.1 IL-1 post-translational processing

As mentioned above, IL-1β and IL-18 are both processed to their mature forms in a caspase-1 dependent manner after initiation of an inflammasome complex [75, 126]. Other members, however, do not contain a caspase-1 consensus cleavage site and thus are processed by other means. Furthermore, whilst many IL-1 family cytokines require proteolytic processing to become biologically active, some members are active in their immature form [91]. Yet, these have still been demonstrated to be susceptible to proteolytic cleavage having both activating and inactivating effects. Additionally, whilst cleavage of IL-1 family members often occurs within the cell, and in the case of IL-1β and IL-18 is a prerequisite of protein secretion, many IL-1 members are released in their immature forms and processed by extracellular proteases.

IL-1α is biologically active in its immature form and is released from cells upon mechanical tissue damage, acting as a DAMP to alert neighboring cells to danger [15, 127]. Whilst IL-1α is released in its immature form, it also undergoes intracellular truncation in activated cells by the calcium-dependent cysteine protease calpain, following which it is released into the extracellular space [128]. Comparisons between the unprocessed and calpain-processed IL-1α show that truncation causes up to a 5-fold increase in IL-1α activity by increasing its affinity for IL-1R, indicating activation of cells and calpain-mediated IL-1α processing may produce a stronger immune response than tissue damage alone [129]. Extracellular IL-1α is also processed to its mature form by neutrophil-derived proteases such as neutrophil elastase that are present in the extracellular environment, increasing its activity to a similar extent as calpain [129].

IL-33, like IL-1α, is active in its immature form and released primarily upon cell damage but can undergo post-translational processing to affect its biological activity. Processing of immature IL-33 can both increase and decrease its activity. Apoptotic caspases-3 and -7 have been demonstrated to inactivate IL-33, perhaps as a method of controlling inflammation during apoptosis, whilst similarly to IL-1α, processing of extracellular IL-33 by neutrophil-derived proteases increases its activity by up to 10-fold [130, 131].

Extracellular processing of IL-1 members is also observed with IL-1β and IL-18. Neutrophil and mast cell derived proteases have been demonstrated to process and activate pro-IL-1β and pro-IL-18 in the event of pre-cursor release into the extracellular environment after tissue damage or pyroptosis [132, 133]. These findings all show post-processing is an integral part of IL-1 biology; not only intracellular activation and secretion, but in immune modulation in the extracellular environment.

1.5.2 IL-36 post-translational processing

The IL-36 members are unique in the sense that all other IL-1 family members are either activated by caspase-1 or have biological activity in their full length immature form. Unlike others of the IL-1 family, the IL-36 proteins contain no caspase-1 cleavage motif, yet, as identified by Towne *et al.*, require precise truncation 9 amino acids upstream of the A-X-D motif. However, as illustrated in Figure 1-3, the amino acid sequence surrounding the cleavage sites bear little homology to one another. As a result, despite understanding where cleavage must occur, the responsible physiological proteases have remained elusive. It has been postulated that as the sequences surrounding the cleavage sites are markedly different, a number of different proteases may be responsible for their cleavage. There has been very little evidence to suggest the IL-36 cytokines undergo intracellular processing, and the method of release from cells is equally poorly understood. However, analogous to other IL-1 family cytokines, there is evidence to suggest that extracellular IL-36 cytokines are susceptible to proteolytic processing by neutrophil-derived proteases, which may act to enhance cytokine activity [134]. Work conducted by Henry *et al.* identified

neutrophil elastase, cathepsin G and proteinase 3 as capable of processing IL-36 cytokines resulting in an increase in their biological activity despite not producing the active forms of IL-36α or IL-36γ, and furthermore identified cathepsin G to produce the active truncation of IL-36β, highlighting the importance of neutrophils in processing extracellular IL-36. However, as of yet, the active forms of IL-36 cytokines identified by Towne *et al.* have not been isolated in vivo, and only extracellular activation of the cytokines has been shown in vitro.

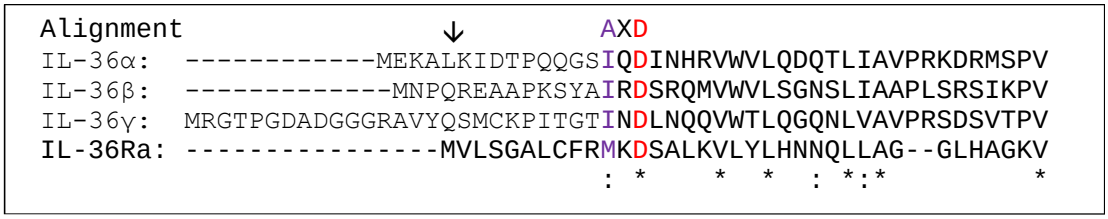


Figure 1-3 Protein sequence alignment of IL-36 cytokines

Alignment of the IL-36 cytokines' N termini shows amino acid homology between cytokine members. The A-X-D motif (where A = aliphatic residue) marks the beginning of the IL-1 domain shared by all IL-36 members. The arrow 9 amino acids upstream of the AXD motif indicates where cleavage must occur to generate biologically active cytokines, as identified by Towne *et al* [99]. ':' indicates shared R group type, '*' indicates conserved amino acid.

1.5.3 Post-translational processing of inflammatory mediators

Protease-mediated modulation of protein activity is not unique to the IL-1 family. Proteolysis is employed throughout biology as a means of regulation and there are numerous examples of protease activity both enhancing and dampening an inflammatory response through proteolytic processing of inflammatory mediators.

CC and CXC chemokines are an example of inflammatory mediators whose activity is also modulated by post-translational processing. CXCL8 (IL-8) is biologically active in its full length form and secreted through the secretory

pathway, however can be processed by a number of proteases, such as metalloproteinases, increasing its activity after N-terminal truncation [135, 136]. Similarly to the IL-1 family members, some CXC chemokines contain a motif in their N-terminus (ELR motif), and it has been shown that N-terminal truncation of these CXC chemokines up to the ELR motif enhances their biological activity [137, 138]. However, cleavage within the ELR motif deactivates chemokine activity. MMP12 cleavage of CXC chemokines in their ELR motif is recognized as an important step in inflammation resolution by halting inflammatory cell influx and favoring a wound healing phenotype [34].

Proteolytic processing of both CC chemokines and CXC chemokines that lack an ELR motif has been demonstrated to decrease their biological activity, imparting a regulatory effect on inflammation. Indeed it has been shown that N-terminal truncation of the monocyte chemoattractant proteins (MCPs) of the CC family causes a loss in signal transduction whilst retaining receptor binding capacity, generating chemokine antagonists from previously chemotactic proteins [35, 139].

Proteases are thus critical regulators of the immune response, capable of both enhancing and dampening an immune response, depending on the cocktail of proteases and the balance of protease inhibitors present in the inflammatory environment.

1.6 Project aims

IL-36 cytokines have become well established as important cytokines in the development of pathological inflammation, with a particularly significant role in psoriasis. However, despite this, a lot remains unclear with regards to their mechanisms of action and the processes that regulate their activity. As IL-1 family proteins, it is of vital importance to understand the factors that regulate their activity in order to fully appreciate the dynamics of IL-36-mediated inflammation. Furthermore, with such an emphasis in the literature on elucidating their pathological role, for many years research into their physiological function has been somewhat neglected. Yet, recent findings

are implicating IL-36 cytokines may have an active role in immune defence. For a comprehensive understanding of the IL-36 cytokines, it is necessary to understand not only how they are involved in pathological inflammation, but also how they contribute to an effective immune response under normal conditions.

The primary aims of this research are as follows:

- To elucidate the mechanisms of IL-36 cytokine activation within the skin compartment and gain an appreciation of how physiological IL-36-mediated inflammation contributes to immune defence.

Additional aims were:

- To generate and characterize IL-36-specific monoclonal antibodies to facilitate further research into IL-36 protein biology, with a particular focus on developing an IL-36γ-specific ELISA with which endogenous IL-36γ can be measured.

Chapter 2 - Materials and Methods

2.1. Materials

Unless otherwise stated all reagents were analytical grade and obtained from Sigma-Aldrich, UK.

2.1.1. Buffers used

Tape stripping lysis buffer: 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM DTT, 5 mM EDTA, 1 mM PMSF, 1% Triton X-100

Bacterial lysis buffer: 20 mM Tris pH 8, 10% Glycerol, 5 mM $MgSO_4$

2TY bacterial media: 3.3% Tryptone soya broth, 1% Yeast extract (Oxoid, Thermo Scientific)

Ni^{2+} column buffer: 500 mM NaCl, 20 mM Tris pH 7.4, 10% Glycerol, 20-500 mM Imidazole

Size exclusion buffer: 150 mM NaCl, 20 mM TRIS pH 7.4

Hybridoma freezing media: Foetal Calf Serum, 10% DMSO

Western blot transfer buffer: 90 mM glycine, 25 mM Tris 20% Methanol

Western blot blocking buffer: Tween-20 0.1% in PBS + 5% milk

SDS-PAGE running buffer: 25 mM Tris, 90 mM glycine, 0.1% SDS

SDS-PAGE loading buffer: 62.5 mM Tris pH 6.8, 10% Glycerol, 2% SDS, 0.001% bromophenol blue +/- 5% β2-mercaptoethanol

Coomassie stain: 40% Methanol, 10% Acetic Acid, 0.05% Brilliant Blue R-250, in dH2O

Coomassie de-stain: 10% Acetic Acid, 40% Methanol, in dH2O

TBE: 0.09 M Tris, 0.09 M Boric acid, 2 mM EDTA

6x DNA loading dye: 0.05% Orange G, 30% glycerol

Nuclear isolation lysis buffer: 320 mM sucrose, 5 mM $MgCl_2$, 10 mM HEPES, 1% Triton X-100 at pH 7.4

Nuclear isolation wash buffer: 320 mM sucrose, 5 mM $MgCl_2$, 10 mM HEPES at pH 7.4

Nuclear isolation staining buffer: 320 mM sucrose, 5 mM $MgCl_2$, 10 mM HEPES, 1% BSA, 0.1% NaN_3 at pH 7.4

NP-40 lysis buffer: 1% NP-40 (or equivalent), 0.15 M NaCl, 10 mM EDTA, 10 mM Tris-HCl pH 8

Cell extract buffer: 20 mM Hepes, pH 7.5, 10 mM KCl, 1 mM EDTA, 1mM DTT

Cell fractionation buffer: Tris HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM DTT

ELISA blocking buffer: 2% BSA, 0.1% Tween-20 in PBS

2.1.2. Cell lines and bacterial strains used

2.1.2.1. Cell lines

Human Embryonic Kidney 293 T (HEK293T) are adherent derivatives of the human embryonic kidney 293 cell line that was established by transformation with sheared adenovirus type 5 DNA [140]

HaCaT cells are an adherent spontaneously transformed cell line derived from adult human keratinocytes [141]

A549 cells are adenocarcinomic human alveolar basal epithelial cells [142]

2.1.2.2 Bacterial strains

BL21(DE3) and Shuffle T7 Xpress *Escherichia coli* were used for cloning and protein expression respectively:

BL21(DE3) genotype: F– *omp*T *hsd*SB(rB–, mB–) *gal dcm* (DE3)

Shuffle T7 Xpress genotype: F´ *lac*, *pro*, *lacIQ* / *Δ(ara-leu)7697 araD139 fhuA2 lacZ::T7 gene1 Δ(phoA)PvuII phoR ahpC* galE (or U) galK λatt*::pNEB3-r1-*cDsbC* (Spec^R, *lacI^q*) *ΔtrxB rpsL150*(Str^R) *Δgor Δ(malF)3*

2.2 General Methods

2.2.1 Obtaining primary cells

All human samples were taken in accordance with the Declaration of Helsinki and participants gave their written informed consent (REC number: 11/YH/0368; 14/NE/1199). Healthy volunteers were also recruited from the university and written consent was sought (BIOSCI09-001).

2.2.2 Peripheral blood collection

30-60 ml of peripheral blood was collected from healthy donors and psoriatic patients in sterile Vacutainer® lithium heparin (BD Biosciences, Oxford, UK) for isolation of neutrophils and peripheral blood mononuclear cells (PBMCs). Ethical approval was sought from the University of Leeds for healthy donors and national ethical approval was gained for patient material. Consent of donors was sought and anonymity 70 was strictly adhered to. Blood was kept at room temperature after donation and used immediately.

2.2.3 PMN isolation and culture

Whole blood was separated by using density gradient by layering Histopaque 1077 (Sigma- Aldrich, UK) in equal amounts on top of Histopaque 1119 (Sigma-Aldrich, UK). 1:2 PBS-diluted whole blood was then layered on top of the Histopaque 1077 and centrifuged at 400 g for 30 minutes with no brake. The polymorphonuclear cell (PMN) layer was then removed using a 10 ml serological pipette and cells were washed with PBS. Red blood cell lysis was conducted to remove contamination of erythrocytes by resuspended the pellet in 0.2% NaCl and repeatedly inverting the falcon tube 10x. An equal volume of 1.6% NaCl was then added and inverted once. Cells were centrifuged at 350 g for 4 minutes and re-suspended in RPMI-1640

supplemented with 5% FCS (PromoCell, Heidelberg, Germany), 0.5% L-Glutamine (Sigma-Aldrich, UK) and 0.05 mg/ml 72 streptomycin and 50 U/ml penicillin (Sigma-Aldrich, UK). Cells were stimulated on day of separation and were not cultured for longer than an hour.

2.2.4 Cell Culture

Human primary fibroblasts (HPFs) and all adherent cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Slough, UK) supplemented with 10% foetal calf serum (FCS), 0.5% L-Glutamine (Sigma-Aldrich, UK), 0.05 mg/ml streptomycin and 50 U/ml penicillin (Sigma-Aldrich, UK). Cells grown in suspension were cultured in Roswell Park Memorial Institute (RPMI) 1640 media supplemented in the same way. Human primary keratinocytes (HPKs) were grown in Keratinocyte Growth Media Kit II (KGM-2) (PromoCell, Heidelberg, Germany). Prior to keratinocyte stimulation, media was changed to KGM-2 minus hydrocortisone and human epidermal growth factor.

HPKs were grown to 70% confluence before passaging, whilst HPF and cell lines were grown to 90% confluence. Adherent cells were washed with sterile PBS and dissociated by incubation with trypsin (170 U/ml)-EDTA (200 mg/ml) (Lonza, Slough, UK) for two to ten minutes. Equal quantity of serum-containing DMEM was added to HPFs and other cell lines to neutralise the trypsin before washing and re-seeding. Trypsinized HPKs were neutralised by addition of trypsin neutralising solution (Lonza, Slough, UK) before washing and re-seeding. HPFs and cell lines were frozen by suspension in FCS and 10% DMSO and placed in a Mr. Frosty^{TM} (Thermo Scientific) at -80˚C for 24 hours before moving to liquid nitrogen for long term storage. HPKs were similarly frozen after suspension in PromoCell freezing media (PromoCell, Heidelberg, Germany).

Activity assays and transfections were performed in OptiMEM (Life technologies) supplemented with 0.05 mg/ml streptomycin and 50 U/ml penicillin (Sigma-Aldrich, UK).

2.2.5 Cell transfection and siRNA knockdown

Cells were cultured in supplemented growth media to a confluency of 80-90% in 6-well plates. Prior to transfection, growth media was substituted for OptiMEM (Gibco, Life Technologies, UK). 1 µg of DNA was diluted in 400 µl of OptiMEM and then mixed with 6 µl of TurboFect transfection regent (Thermo Scientific, UK). The solution was vortexed and incubated for 15-20 minutes at room temperature before administering to cells in a drop-wise fashion. After 8 hours the media was removed and replaced with 2 ml of fresh OptiMEM**.**

For siRNA knock down, cells were grown as above. For each well, 5 pM siRNA was diluted in 200 µl of OptiMEM and incubated at room temperature for 5 minutes. 8 µl of Lipofectamine (Thermo Scientific, Waltham, MA) was diluted in 200 µl of OptiMEM and incubated at room temperature for 5 minutes. The lipofectamine and siRNA preparations were then mixed for 20 minutes at room temperature. 1.6 ml of culture media (without penicillin/streptomycin) was added to the lipofectamine/siRNA mixture. The culture media was removed from each well and the siRNA preparations added. Cells were incubated for 24 hours at 37°C. The culture media was then replaced with OptiMEM and cells incubated for a further 48 hours. Following incubation, cell supernatants were removed and frozen at -80°C.

2.2.6 Growth assays

Cells were seeded in 24 well plates at a confluence of 10% with or without stimulation and grown in an IncuCyte® ZOOM system. Cells were scanned hourly over a period of growth ranging from 72-96 hours and confluence was analysed by the IncuCyte ZOOM system once all data were collected.

2.2.7 Cell lysis

Whole cell extracts were prepared by lysing cells in a NP40 lysis buffer supplemented with protease inhibitors (Complete EDTA-free protease inhibitors, Roche). Cells were removed from the tissue culture plate in lysis buffer and left on ice for 20 minutes before centrifugation for 10 minutes at

15,000 g to remove cell debris. Supernatant from the lysed cells was removed and samples were stored at -80°C or used immediately.

Cells to be used for activity assays were lysed in a hypotonic cell extract buffer. Cells were re-suspended in cell extract buffer and incubated on ice for 20 minutes, before being transferred to a Dounce-type homogeniser and undergoing homogenisation with 15 strokes of a B-type pestle. Centrifugation of the lysates at 15,000 g for 10 minutes removed cellular debris before storage at -80°C.

2.2.8 Cell fractionation

Cells were resuspended in cell fractionation buffer and incubated on ice for 20 minutes, before being transferred to a Dounce-type homogeniser and undergoing homogenisation with 15 strokes of a B-type pestle. The cell lysates were centrifuged at 1000 g for 10 minutes to remove nuclei and cell debris. Supernatant was collected then centrifuged at 15,000 g for 10 minutes to separate the lysosomes from the cytosol. After removing and storing the cytosol at -80°C, the lysosomes were resuspended in cell extract buffer and incubated on ice for 20 minutes. The lysosome fractions were subject to 3 serial freeze-thaw cycles before centrifugation at 15,000 g for 30 minutes to remove debris. Supernatants were stored at -80°C until use.

2.2.9 Activity assays

HaCaTs were plated at 10^5 cells/well (24-well plate) in supplemented DMEM (as described in section 2.2.4) and grown to approximately 70% confluency before the media was replaced with OptiMEM. Treatments detailed in results were added in conjunction with OptiMEM and cells were incubated for 24 hours at 37°C. Lysosome fractions were pre-incubated with protease inhibitors for 3 hours at 4°C before being added to HaCaTs when performing inhibition studies. For cell supernatant activity assays, A549 cells, primary keratinocytes or primary fibroblasts were plated at $0.5x10^5$ cells/well (6-well plate) in supplemented DMEM. Once 100% confluent, the media was replaced with OptiMEM and cells incubated for a further 24 hours. Following incubation, the conditioned media was removed and frozen at -80°C. Prior to

activity assays, the conditioned media was supplemented with 0.1 mM DTT. The conditioned media was added to HaCaTs with or without the proteins being tested, as detailed in the results. Following incubation, cell supernatants were removed and frozen at -80°C.

2.2.10 Apoptosis induction

HaCaTs were grown in supplemented DMEM to a confluency of 70% before initiating serum starvation. Cells were then washed 3 times with PBS to remove residual serum and media was replaced with serum free DMEM with or without proteins tested. Cells were serum starved for 24 hours before treatment with staurosporin (48 hours for cells to be analysed for caspase-3 activity). 500 ng/ml staurosporin was added to cells for 4 hours to induce apoptosis. Staurosporin-containing media was removed and cells were washed in PBS to remove residual staurosporin. Cells were then either immediately analysed for caspase-3 activity or incubated for a further 24 hours in serum free DMEM with or without stimulation before analysis of PARP cleavage or nuclear isolation and analysis of DNA degradation by flow cytometry.

2.2.11 Annexin V PI assays

Apoptosis assays were carried out using an annexin V PI staining kit obtained from BioLegend in accordance to the provided protocol and analysed by flow cytometry. Adherent cells were dissociated using EDTA to minimise cell membrane damage.

2.2.12 CellEvent Caspase-3/7 activity assay

CellEvent Caspase-3/7 green detection reagent sourced from ThermoFischer Scientific was used to label apoptotic cells. After induction of apoptosis (detailed in section 2.2.10), cells were incubated with CellEvent at 5 µM for 30 minutes. Fluorescent cells were imaged by an IncuCyte using an excitation wavelength of 460 nm and an absorption wavelength of 524 nm, and by phase contrast. Data was analysed by the IncuCyte Zoom software.

2.2.13 Nuclear isolation and staining

Cells were trypsinized and washed 2x in cold PBS before re-suspension in nuclear isolation lysis buffer at 10^6 cells/ml. Cells were gently vortexed and left on ice to lyse for 30 minutes. The lysed cells were centrifuged at 1000 g for 5 minutes at 4˚C to pellet the nuclei. The cytoplasmic fraction was removed and stored at -80˚C for future analysis. The nuclear pellet was washed 2x in nuclear isolation wash buffer, then 10^5 nuclei were re-suspended in 100 µl nuclear isolation staining buffer and stained with propidium iodide at 50 µg/ml for 15 minutes. The samples were made up to 500 µl in nuclear isolation staining buffer and analysed by flow cytometry.

2.2.14 Flow cytometry

Flow cytometry was performed on a BD LSRFortessa machine and data collected using BD FACSDiva software. 5000 – 10,000 events were collected and data was analysed with FlowJo software.

2.2.15 Fixing and staining cells for immunofluorescence

Following culture of cells on a 13mm glass cover slip in a 24 well plate cells were washed 3 times with PBS. 4% paraformaldehyde was then added to cells and incubated at room temperature for 15 minutes. Cells were washed with PBS 3 times then permiabilized with 0.1% saponin for 10 minutes. Following another wash cells were blocked with 2% BSA in PBS for 30 minutes at room temperature with agitation. Cells were incubated with 1 µg/ml of primary antibodies diluted in PBS containing 2% BSA and 0.05% saponin for 1 hour at room temperature with agitation. After 4 washes in PBS, cells were incubated in the dark with 1 µg/ml of secondary antibody diluted in PBS containing 2% BSA and 0.05% saponin for 1 hour. The cells were thoroughly washed with 4 x 10 minute washes in PBS before being mounted onto microscope slides using ProLong® Gold antifade containing 4',6-diamidino-2- phenylindole (DAPI) (Life Technologies). Once mounted, slides were kept at 4°C before imaging using an inverted LSM700 confocal microscope coupled to a LSM Image Browser.

2.2.16 Mouse inoculation and antibody titre analysis

Mouse inoculation was performed by Dr Delyth Reid at the University of Aberdeen. Mice were subcutaneously immunised with 200 µl PBS containing 50 µg of either recombinant IL-36α K6, IL-36γ S18, IL-36Ra V2 and IL-38 in combination with Complete Freund's Adjuvant (CFA). A second subcutaneous immunisation was performed 3 weeks later with 200 µl containing 25 µg of the same proteins in combination with Incomplete Freund's Adjuvant (IFA). Tail bleeds were performed 7 days after the second subcutaneous immunisation by cutting off 0.5 cm of the mice tails and collecting 100 µl blood. Blood was allowed to clot and serum was removed. Antibody titre was estimated by serial dilution of serum over a 96 well plate coated with 200 ng/well of antigen and detecting bound antibody by addition of HRP-conjugated anti-mouse antibody. A final intra-peritoneal immunisation was performed 4 days prior to the fusion without any adjuvant.

2.2.17 Hybridoma fusion and positive selection

Hybridoma fusion was performed with the help of Dr Delyth Reid. Immunised mice were humanely killed by authorised personnel. Mice were rinsed with 70% ethanol before removal of the spleen via an abdominal incision. The spleen was rinsed with serum free RPMI before homogenisation. Frosted microscope slides were rinsed with 70% ethanol and allowed to air dry before wetting with serum free RPMI. The spleen was homogenised by grinding between the frosted microscope slides to release cells. Homogenised spleen was washed with serum free RPMI to collect cells and filtered through a BD falcon strainer into a falcon tube.

Mouse splenocytes were fused with SP2 myeloma cell line obtained from ECACC. SP2 cells were washed in HBSS before mixing with mouse splenocytes at a ratio of 5:1. After washing in RPMI, cells were resuspended by gentle tapping. 1 ml PEG1500 was added drop-wise over 30 seconds whilst simultaneously gently agitating the SP2/splenocyte mix in a 37°C water bath. After a further 30 seconds, 1 ml of RPMI was similarly administered over 90 seconds, then 4 ml over 2 minutes, followed by 5 ml over 2 minute. The cells were rested for 2-3 minutes before washing with

RPMI. The pellet was resuspended in 100 ml RPMI supplemented with 20% Foetal Calf Serum and hypoxanthine-aminopterin-thymidine (HAT) then plated out into 10 x 96 well plates. Spleen cells and SP2 cells were added separately to 2 wells for controls to ensure HAT selection was working. Media was refreshed after 3 days by replacing 100 µl of media with fresh HAT selection media. After 5 days HAT media was replaced with HT medium to remove the selective pressure and colonies were allowed to grow.

2.2.18 Monoclonal hybridoma screening and selection

Immunosorbent 96 well plates were coated with avidin at 5 µg/ml overnight then washed and coated with biotinylated target proteins at 2 µg/ml. Once fusion colonies reached a size visible to the naked eye, 100 µl of neat hybridoma supernatant was removed and tested by ELISA. 1:10000 diluted HRP-conjugated goat anti-mouse antibody (SIGMA) was used as a detection antibody, and TMB solution used for colorimetric analysis. Positive colonies were grown up to be subject to a limiting dilution for monoclonal hybridoma selection.

Bulked up positive polyclonal hybridomas were diluted to 10 cells per ml and plated out into 2 x 96 well plates per clone, 100 µl per well, to give 1 cell per well. These were grown until colonies became visible to the naked eye before screening for positive colonies as above. Isolated positive monoclonal hybridomas were bulked up for antibody production.

2.2.19 Tape stripping

Tape stripping was performed by Dr Anna Keszegpal. Samples were collected with written informed consent in place of the patients, taken in accordance with the Declaration of Helsinki. Written consent was also sought for collecting samples from healthy volunteers recruited from the University of Leeds. The severity of lesions was clinically assessed before sample collection using D-squame adhesive discs of 3.8 cm^2 (Cuderm; Dallas TX). Non-lesional skin samples were taken preferably from the ventral lower arm area whereas location of lesional samples was guided by lesion appearance. Only non-erosive, non-oozing lesions were tape stripped. The

tapes were administered to the skin using gentle pressure for 5 seconds. The first tape was discarded and 10 subsequent tapes collected were put in an empty container and immediately stored on dry ice for transportation, or storage at -80°C until processing for protein extraction. For extraction tapes were placed in tape stripping lysis buffer for 30 min at 4 °C. Samples were sonicated for 20 seconds 3 times with a 20 second interval on ice between each sonication. Samples were centrifuged for 10 min at 15,000g before removal of supernatants and subsequent storage at -80°C.

2.2.20 Candida skin equivalent experiments

Skin equivalents and *Candida albicans* skin equivalent experiments were cultured and conducted by Dr Jeanette Wagener from the University of Aberdeen.

2.3 Molecular biology

2.3.1 Agarose gel electrophoresis

Gels were prepared with electrophoresis grade agarose dissolved in 1x TBE containing 0.2 µg/ml Ethidium Bromide. Samples were mixed with 6x DNA loading dye and run at 100 V alongside 1 kb or 10 kb DNA ladders depending on size of the sample. DNA in agarose was observed and photographed under short-wave UV light for observation or long-wave UV light when further cloning was required.

2.3.2 SDS-Polyacrylamide gel electrophoresis (PAGE)

Samples were mixed with loading buffer and boiled for 5 minutes. Following brief centrifugation, samples were loaded onto a 4% polyacrylamide stacking gel (pH 6.8) and resolved on a 10-15% acrylamide gel (pH 8.8) alongside molecular weight markers (New England Biolabs). 1.5 mm gels were cast and run using a Mini PROTEAN III gel system (Biorad). Gels were run in SDS-PAGE running buffer at 0.03-0.04 Amps per gel until good separation was achieved. Gels were either analysed by Coomassie staining followed by de-stain or were used in Western blots.

2.3.3 Western and dot blot

For western blotting, electrophoresis resolved polypeptides were transferred to HybondC+ nitrocellulose (Amersham biosciences) membranes in transfer buffer by applying 100V for 1 hour. In dot blotting samples were dotted directly onto HybondC+ nitrocellulose membranes applied through a dot blot apparatus via suction. Dot blot and transferred western membranes were blocked for 1 hour in blocking buffer before addition of primary antibodies diluted to the concentration advised by the manufacturer in blocking buffer. Membranes were incubated with agitation for 1 hour at room temperature and then washed with PBS 0.1% Tween-20 3 times each for 5 minutes at room temperature with agitation. Secondary horse radish peroxidase (HRP) antibodies (Sigma-Aldrich, UK) were diluted to a concentration advised by the manufacturer in blocking buffer and membranes were incubated for 1 hour with agitation at room temperature. Membranes were then washed as before. The ECL was used as per manufacturer's instructions (GE Healthcare) to detect specific antibody binding to membranes and emitted light was detected on X-ray film (GE Healthcare) following incubation of varying incubation times from 10 seconds to 5 minutes.

2.3.4 PCR for cloning

The proteins of interests' sequences were amplified from template DNA using sequence-specific primers (see Appendix) in conventional PCR using KOD hot start DNA polymerase (Novagen). Samples of the amplified PCR products were run on 1% agarose gels and compared to a DNA marker to verify the product size. PCR products to be ligated into TA overhang constructs (Champion pET SUMO expression system, Invitrogen, USA) were incubated with TAQ polymerase (Promega, USA) for 20 minutes at 72˚C to generate an adenosine overhang. After incubation, the products were run on a 1% agarose gel for purification, excised carefully with the aid of short wave UV for DNA visualisation, and extracted by QIAquick gel extraction kit (Qiagen) in accordance to the manufacturer's instruction.

2.3.5 Restriction digest

PCR products and vectors were incubated with relevant restriction enzymes with 1% BSA in optimal buffers (New England Biolabs) at 37˚C for 3 hours. Alkaline phosphatase was added to the vector for the final hour of incubation to prevent vector re-ligation. The digest products were run on a 1% agarose gel then excised and purified using QIAquick gel extraction kit (Qiagen) following manufacturer's instructions.

2.3.6 Ligation

Digested PCR products and vectors were ligated at a molar ratio of 1:3, with 1 ng of insert and 3 ng of vector. T4 ligase was added to the manufacturer's instruction (Invitrogen, USA), and the reaction was incubated at 16˚C overnight.

2.3.7 Transformation

The ligation mix was added to thawed competent bacteria on ice for 30 minutes. The bacteria/DNA mix was then heat shocked at 42˚C for 30 seconds and returned to ice for a further 2 minutes, before addition of SOCS medium (Invitrogen, USA) and incubation at 37˚C for 1 hour. The transformed bacteria were then plated out on 1% agar plates containing relevant selective antibiotics and incubated overnight at 37˚C.

A colony PCR using TAQ polymerase (Promega, USA) was performed on colonies that had formed overnight to identify successful ligation and transformation. Positive colonies were grown up in 2YT media and plasmid DNA was extracted by QIAprep Spin Miniprep Kit (Qiagen) before being sent to GATC-Biotech, Germany for Sanger-sequencing to confirm insert DNA sequence.

2.3.8 Expression of proteins in *E. coli*

Sequence-verified constructs were transformed into the expression strain of *E. coli* BL21-CodonPlus(DE3)-RIPL (E. coli B F– ompT hsdS(rB – mB–) dcm+ Tetr gal λ(DE3) endA Hte [argU proL Camr] [argU ileY leuW Strep/Specr]) that contain a plasmid encoding a Lac inducible T7 polymerase

(DE3) and a plasmid encoding rare tRNAs required for the expression of mammalian proteins (RIPL). Starter cultures 1/50th the volume of the desired expression culture were incubated overnight at 37˚C in 33 µg/ml kanamycin and 33 µg/ml chloramphenicol in order to inoculate the larger cultures containing the same antibiotics. The expression cultures were grown at 37˚C until an optical density of 0.6-0.8 (at 600 nm), indicative of an exponential growth phase, was reached. Expression was then induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0.8 mM and cultures were incubated at 25˚C overnight.

Cultures were centrifuged at 4000 g for 10 minutes to pellet the bacteria. Supernatant broth was discarded and the cells were re-suspended in bacterial lysis buffer with additional lysozyme (0.35 mg/ml) and DNase (20 µg/ml). The cells were sonicated on ice over 10 cycles of 15 seconds on 45 seconds off. The soluble and insoluble bacterial fractions were separated by centrifugation at 15,000 g for 50 minutes. The soluble fractions containing the expressed recombinant proteins were filtered through 0.45 nm syringe filters prior to purification by Ni^{2+} affinity chromatography.

2.3.9 Protein purification

Recombinant proteins were purified from filtered bacterial soluble fractions by Ni^{2+}-affinity chromatography with a GE Healthcare HisTrap column as the protein constructs had N-terminal His-tags incorporated into their N-terminal SUMO-tags. The column was equilibrated in 20 mM imidazole Ni^{2+} column buffer and the samples were made up to 20 mM imidazole by addition of 500 mM imidazole Ni^{2+} column buffer before running through the HisTrap column. The column was then washed with 5 column volumes of 20 mM Ni^{2+} column buffer followed by 5 column volumes of 50 mM Ni^{2+} column buffer. The recombinant proteins were eluted in 5 column volumes of 500 mM Ni^{2+} column buffer.

Ni^{2+}-affinity purified proteins were further purified by Size Exclusion Chromatography (SEC). Proteins were passed through a GE Healthcare Superdex 75 or Superdex 200 column depending on the size of the protein. Columns were washed through with 1 column volume of de-ionized, filtered

and degassed water then equilibrated in SEC buffer. Before purification, samples were filtered and concentrated to a suitable volume.

2.3.10 SUMO cleavage

SUMO protease ulp1 was added to SUMO proteins as a ratio of 1:50 protease to protein for 24-48 hours at 4°C. Cleavage was assessed by gel electrophoresis and then Coomassie staining. SUMO and ulp1 are both His tagged so were removed from the incubated samples by Ni^{2+} affinity chromatography leaving the pure untagged protein behind. For all constructs that were to be used for cell stimulation the Ni^{2+} column was thoroughly washed with 0.5 M NaOH before calibration with 20 mM imidazole Ni^{2+} column buffer. The sample was then added and the column eluent containing pure untagged protein collected. This was further purified by size exclusion chromatography to ensure protein purity. All glassware used to make buffers and the column itself was thoroughly washed with 0.5 M NaOH.

2.3.11 Antibody production and purification

Isolated monoclonal hyridomas were bulked up and frozen down in hybridoma freezing media for stocks. Monoclonal hybridomas were weaned onto hybridoma serum free media (H-SFM) over a period of 7 days and then grown up in 1 litre of H-SFM. Once at 1 litre, monoclonal hybridomas were allowed to grow until cell viability dropped below 40%, (approximately 4-5 days). Hybridoma media was then centrifuged to remove cells prior to antibody purification. Purification was carried out using 1ml Pierce™ Protein G agarose beads (ThermoFischer Scientific) packed in a column. The column was equilibrated in PBS before adding the antibody-containing media. The flow-through was collected and run through the column twice more to maximise antibody capture. After a PBS wash, the purified antibody was eluted in 5 ml pH 2.6 Glycine buffer neutralised by 500 µl pH 9 TRIS buffer. Finally, the antibody was dialyzed into PBS.

2.3.12 Biotinylation

Biotinylation of proteins and antibodies was achieved by *N*-hydroxysuccinimidobiotin (NHS-Biotin) from ThermoFisher Scientific, in accordance to the manufacturer's protocols. Proteins were dialysed into PBS prior to biotinylation.

2.3.13 Dialysis

Proteins dialysed were contained in 10 kDa cut-off dialysis membrane (Pierce) and suspended in 500x volume of the desired buffer with stirring for 5 hours. Dialysis was then repeated using fresh dialysis buffer.

2.3.14 Mass Spectrometry

Liquid chromatography mass spectrometry was performed in-house by the Mass Spectrometry Facility of the Faculty of Biological Sciences, University of Leeds.

2.3.15 Protease cleavage assays

Recombinant proteases and inhibitors were used at the concentration recommended by the manufacturer. Proteins of interest were incubated with cell supernatant or recombinant proteases at 37˚C for periods ranging from 2 minutes to 24 hours. Inhibitors were pre-incubated with the recombinant protease or cell supernatant for 30 minutes at 37˚C prior to addition of the protein of interest. Results were analysed by SDS-PAGE or western blot.

2.3.16 ELISA

Nunc-Immuno^{TM} MicroWell^{TM} 96 well plates (Scientific Laboratory Supplies LTD) were coated with capture antibody and ELISA proceeded as detailed in manufacturer instructions when using DuoSet human ELISA kits (RnD Systems, Abingdon, UK or BioLegend, Hatfield, UK). When using our antibodies, antibodies were diluted in PBS and 100 µl added to each well overnight at 4°C. After washing 5x in PBS 0.1% Tween-20 coated plates were blocked for 1 hour using 200 µl per well of ELISA blocking buffer at room temperature with agitation. After removal of blocking buffer 100 µl per

well of samples were added for 1 hour with agitation. Samples, if necessary, were diluted in ELISA blocking buffer. Plates were washed as before, then biotinylated detection antibody diluted in ELISA blocking buffer was added for 1 hour with agitation. After washing, 100 µl per well of Avidin-HRP (BioLegend) diluted 1/1000 in ELISA blocking buffer was added for 30 minutes. Plates were washed 6 times in PBS 0.1% Tween-20 before addition of 100 µl per well of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution. Reaction was stopped by addition of 100 µl per well of 2M H_2SO_4 and then absorbance was read using a Biotek PowerWave XS2 machine.

2.3.17 Immunoprecipitation

Samples were pre-cleared by addition of a quantity of Pierce™ Protein G agarose beads equal to that used in the pull down step. Samples were mixed with beads for 3 hours at 4°C whilst applying rotatory mixing. Clearing beads were removed by centrifugation, and immunoprecipitating antibodies were added to the samples at 5x concentration of target protein. Samples and antibodies were allowed to mix overnight at 4°C by applying rotatory mixing. Pierce™ Protein G agarose beads were added and mixed by rotatory action for 3 hours before beads and antibody complexes were removed by centrifugation at 8000 g for 2 minutes. Supernatant was removed and frozen at -20°C for later analysis. Beads were washed in 5 times in 100 µl PBS 0.1% Tween-20. Immunoprecipitated protein was either eluted for further analysis by ELISA, or denatured by addition of gel electrophoresis buffer for analysis by gel electrophoresis. Elution was performed using 1.5 M NaCl solution. Beads were incubated in 1.5 M NaCl for 15 minutes on ice with gentle agitation every 5 minutes. Beads were removed by centrifugation at 8000 g for 2 minutes and eluted protein was removed and stored at -20°C for further analysis.

2.3.18 Quantitative PCR

Quantitative real-time PCR was carried out by a RotorGen (Qiagen, Hilton, Germany) and a ΔΔCT-analysis formed from the generation of standard curves for the house keeping genes and genes of interest. RNA isolation

was carried out using the Qiagen RNeasy Mini Kit. cDNA was generated by a first strand cDNA synthesis kit (Thermo Fischer Scientific). Qiagen QuantiFast SYBR green PCR was used to carry out the qRT-PCR according to the manufacturer's protocol.

Chapter 3 - Generation and characterisation of anti-IL-36γ antibodies

3.1 Introduction

Research on the IL-36 family has been greatly hindered by the lack of effective commercially available antibodies. Without such antibodies, most significant research has been conducted on mice, via the examination of mRNA expression, recombinant proteins, or through the use of biological reporter assays utilising IL-36R transfected cell lines. In order to dissect the role of endogenous IL-36 cytokines that lack epitope tags and quantify IL-36 cytokine concentrations from patients or *in vitro* systems, it was therefore necessary to generate specific IL-36 antibodies.

IL-36γ is of particular interest as it is the most highly expressed IL-36 cytokine present in the skin compartment and is highly associated with psoriatic inflammation. Indeed, there is strong evidence implicating IL-36γ as a psoriasis-specific disease marker [143]. The ability to measure endogenous IL-36γ from patient samples may help differentiate what are otherwise phenotypically very similar conditions, as can be the case with psoriasis and atopic dermatitis. Furthermore the ability to extract endogenous IL-36γ from such patients by immunoprecipitation may provide further information regarding the dynamics of IL-36γ activity. In addition to the clinical aspect, IL-36γ specific antibodies will greatly facilitate research. There is a lot of uncertainty surrounding the expression, secretion and processing of IL-36γ which is in part compounded by the lack of biological tools necessary to dissect these questions. Therefore, anti-human IL-36γ monoclonal antibodies were produced in mice in the hope that they improve molecular analysis of IL-36γ and provide a means of diagnosis in skin-based inflammatory disease.

3.2 Generating recombinant active IL-36γ for immunisation

Recombinant IL-36γ was engineered with an N-terminal SUMO tag directly upstream of the IL-36γ start codon. In addition to facilitating purification via the SUMO His-tag, the very precise removal of the SUMO tag by SUMO protease (Ulp1) maintains the integrity of the recombinant protein N-terminus

by avoiding adding or removing any extra amino acids. This is of particular importance with the IL-36 proteins given the effect of N-terminal truncation on their activation, as discussed in the introduction.

Truncated IL-36γ cDNA encoding Ser18-D169 was cloned into the Champion pET SUMO expression system and transformed into DH5α. Verified clones were subsequently expressed by the *E. coli* strain BL21-Codon plus (DE3)-RIPL which are deficient in proteases Lon and OmpT, possess rare tRNAs and for mammalian codon bias and λDE3 lysogen inducible T7 driven expression.

Once expressed, the recombinant IL-36γ was initially purified by Ni^{2+} affinity chromatography, then by size exclusion chromatography, before removal of the SUMO tag by incubation of the recombinant protein with Ulp1. The cleaved tag and protease were purified away from the truncated recombinant protein by further Ni^{2+} affinity chromatography as both protease and tag contain His-tags that the truncated protein does not. A final purification by size exclusion was conducted to ensure a clean recombinant protein. The pure active recombinant IL-36γ was then tested on IL-36 responsive cells to validate activity. All instruments used in the purification process had been cleaned by 0.5 M NaOH in order to reduce LPS contamination that could interfere with subsequent assays. This process is outlined in Figure 3-1.

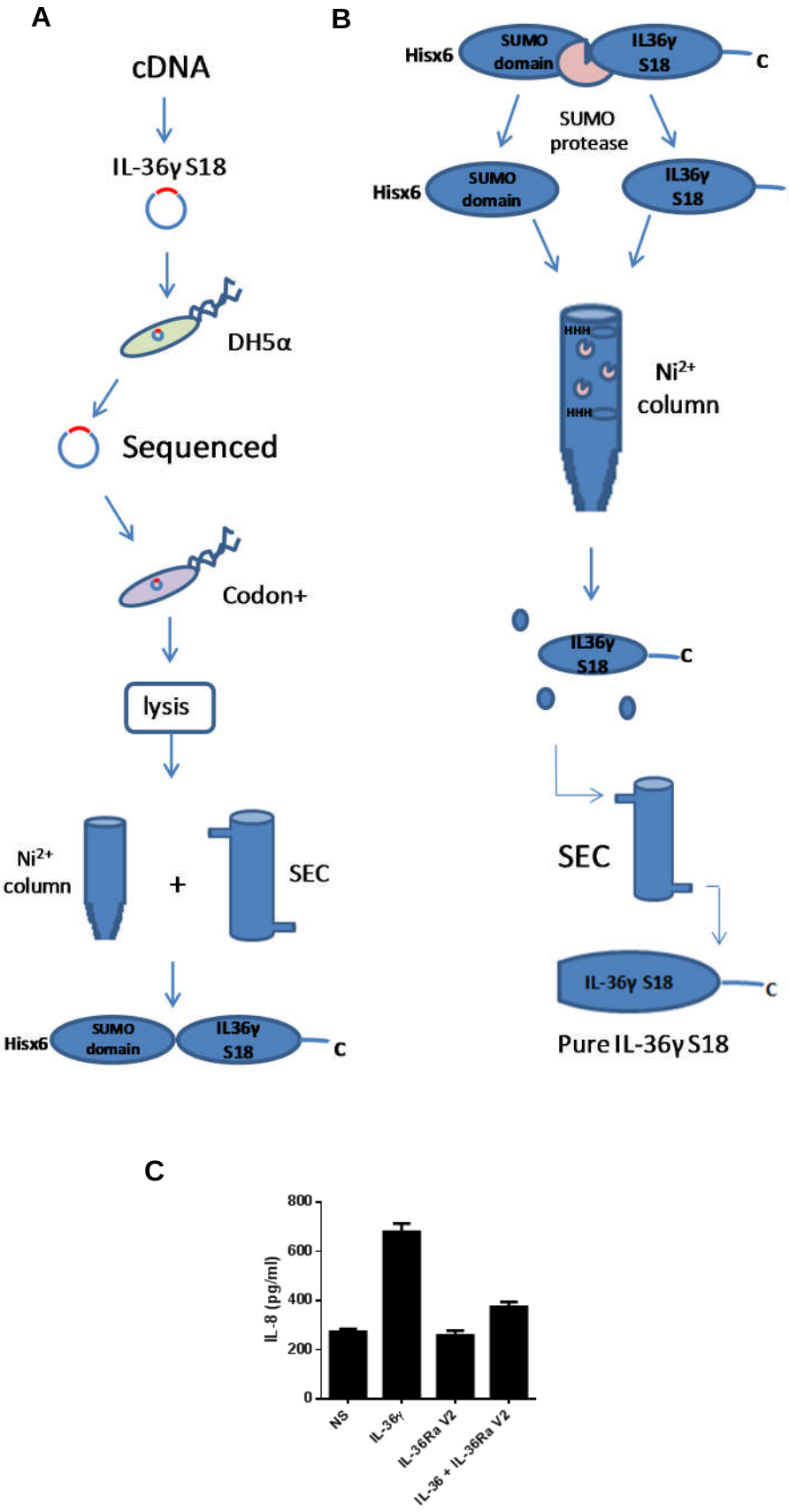

A
cDNA
IL-36γ S18
DH5α
Sequenced
Codon+
lysis
Ni2+ column
+
SEC
Hisx6
SUMO domain
IL36γ S18
c
B
Hisx6
SUMO domain
IL36γ S18
c
SUMO protease
Hisx6
SUMO domain
IL36γ S18
c
HHH
HHH
Ni2+ column
IL36γ S18
c
SEC
IL-36γ S18
c
Pure IL-36γ S18
C
IL-8 (pg/ml)
800
600
400
200
0
NS
IL-36γ
IL-36Ra V2
IL-36 + IL-36Ra V2

Figure 3-1: Production and purification of recombinant IL-36γ S18

(A) Diagram illustrates the process of cloning IL-36γ S18 and producing recombinant protein. (B) Diagram illustrates the process of removing N-terminal SUMO tag and purifying recombinant protein. (C) Purified recombinant IL-36γ S18 was tested on primary human keratinocytes to confirm activity of the produced protein. Keratinocytes were grown to 70% confluence before addition of recombinant IL-36γ S18 at 50 nM with or without 500 nM IL-36Ra. IL-36 activity was assessed by measuring secreted IL-8 via ELISA. n=2

3.3 Generation and production of human IL-36γ specific monoclonal hybridomas

Mice were immunised with recombinant IL-36γ at Aberdeen as described in methods section 2.2.16. Analysis of mouse serum from tail bleeds 7 days after their second immunisation showed IL-36γ-immunised mice had an antibody titre of <1/100 which was too low to proceed with the fusion, however no cross-reactivity against other IL-36 family members was detected (Figure 3-2). Therefore the mice were boosted again, as described in section 2.2.16, and re-tested the following week. The tail bleed showed an antibody titre of >1/1000 (data not shown – performed in Aberdeen), which was deemed adequate to proceed with spleen harvesting and hybridoma fusion.

Following the fusion, successfully fused hybridomas were selected by growth in hypoxanthine-aminopterin-thymidine (HAT) supplemented media. The selected hybridomas were then screened for identification of clones producing IL-36γ-specific antibodies as described below. At this point positive colonies were potentially polyclonal, containing hybridomas derived from multiple different B-cells. Therefore, positive colonies were amplified and further subjected to a limiting dilution resulting in a single cell per well of a 96 well plate thus generating monoclonal colonies (process detailed in methods section 2.2.18). Another round of screening was performed to identify monoclonal hybridomas generating IL-36γ-specific antibodies. These colonies were amplified and their expressed antibodies were purified as outlined in methods section 2.3.11. This process is outlined in Figure 3-2.

Screening was performed in an ELISA-like assay. Initially 200 ng/well recombinant IL-36γ was plated directly onto immunosorbent plates and then

probed with hybridoma supernatants. IL-36 binding antibodies were detected using anti-mouse-HRP conjugate and subsequent incubation and colour change of the TMB substrate (measured at 450 nm) (as detailed in methods section 2.2.18).

Intriguingly, it was later observed that direct adsorption of recombinant IL-36γ to the plate surface altered the protein's structure to the point of selecting antibodies unable to bind native protein. Therefore, in order to present IL-36γ to the antibodies in a soluble form, recombinant IL-36γ was conjugated to a biotinylated linker (EZ-Link NHS-Biotin, Pierce, UK) and added to avidin-coated immunosorbent plates. Screening using directly adsorbed protein generated a single monoclonal hybridoma producing IL-36γ specific antibodies, named 4A9. After modification of the screening process, an additional three were identified named 6A11, B5A2 and B5B2. Initially approximately 20 positive "clones" were identified by screening, however after isolation and expansion of each clone, subsequent screens revealed many of the hybridomas had stopped producing IL-36γ-specific antibodies, leaving only the four denoted above. This is likely due to chromosomal instability and non-clonal populations.

This process was also performed for IL-36α and IL-36Ra. Whereas the IL-36α fusion did not yield and IL-36α-specific antibody-producing hybridomas, one monoclonal hybridoma was isolated which generated IL-36Ra-specific antibodies which function in western blot.

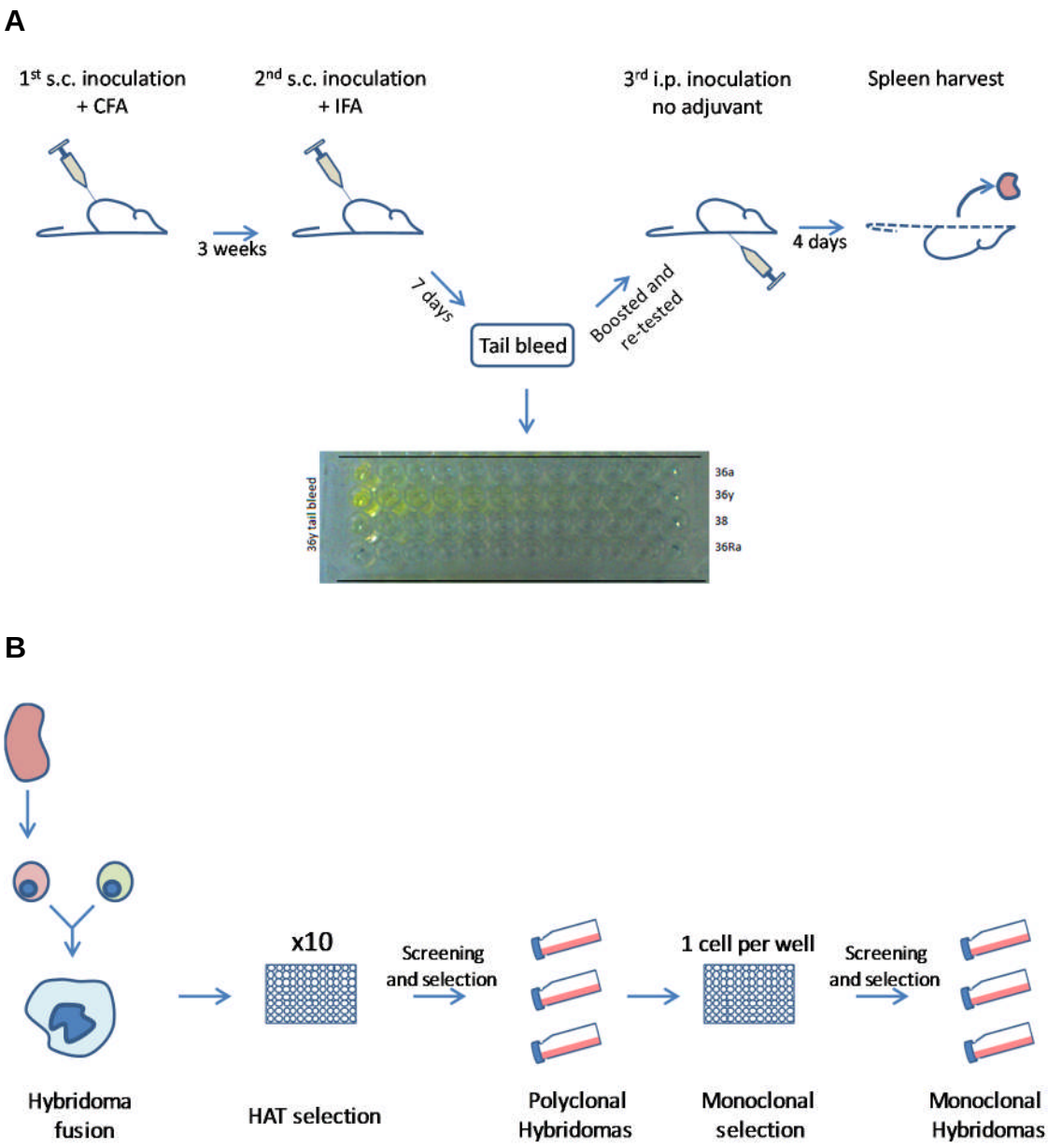


Figure 3-2: Diagram illustrating generation and selection of hybridomas

(A) Mice were inoculated with a subcutaneous (s.c.) injection of 50 µg IL-36γ with Complete Freund's Adjuvant (CFA). After 3 weeks mice received a s.c., booster of 25 µg IL-36γ with Incomplete Freund's adjuvant (IFA). Tail bleeds were then analysed to assess antibody titre. Serum was isolated and diluted 1:1 in PBS before addition to immunosorbent plates coated with 200 ng/well IL-36α, IL-36γ, IL-38 and IL-36Ra to check both antibody titre and cross-reactivity. Tail bleed serum was then subject to 1:10 serial dilution in PBS from left to right. Mice were boosted again by intraperitoneal (i.p.) injection of 25 µg IL-36γ without adjuvant and antibody titre was retested. After another 4 days mice were killed and spleen harvested. (B) Isolated splenocytes were fused with mouse myeloma SP2 cells and successful fusions were selected by culturing in hypoxanthine-aminopterin-thymidine supplemented RPMI. Hybridomas were grown until colonies were visible by the naked eye then screened for positive colonies. Positive colonies were isolated and expanded before being subject to a

limiting dilution and allowed to grow until colonies were visible and screened again. Monoclonal hybridomas were then isolated and expanded for antibody purification.

3.4 Antibody characterisation

Once positive hybridomas were observed by screening, these were grown up and stocks were frozen down (10% DMSO 90% FCS). The positive monoclonal hybridomas were then grown up in 1 litre 10% IgG depleted FCS RPMI and weaned onto serum free media over five days. Antibodies were purified via Protein G (ThermoFischer Scientific) as described in methods section 2.3.11 which generated approximately 1-2 mg/litre of conditioned hybridoma media. Antibodies were analysed by Coomassie stained SDS-PAGE to examine their purity, and were isotyped by Bio-Rad mouse isotyping test kit (shown in Table 3-1). They were tested on their ability to specifically detect IL-36γ in both native and denatured conformations through dot blot and western blot (Figure 3-3).

All antibodies specifically recognise IL-36γ when immobilised by dot blot, though B5A2 and 4A9 appear to generate the strongest signal. When analysed by western blot analysis, IL-36γ is only recognised by antibodies B5B2 and 4A9. Again, 4A9 appears to generate the strongest clear signal. Furthermore, no cross-reactivity is observed against other IL-36 family member IL-36α and IL-36Ra (Figure 3-3B and 3-3C).

Having established 4A9 is able to specifically detect recombinant IL-36γ by western blot, it remained to test whether this antibody would recognise native IL-36γ expressed by a cellular source. HaCaTs, a keratinocyte cell line known to express IL-36γ, were stimulated for 48 hours with IL-17 and poly(I:C) at 50 ng/ml and 10 µg/ml respectively, both of which have been described to induce expression of IL-36γ. Analysis of HaCaT cell lysates and supernatants by western blot using 4A9 as the primary antibody showed the presence of a single band present at 17 kDa in lysates of the stimulated HaCaTs that matched that of recombinant IL-36γ run in parallel (Figure 3-3). These results indicate the IL-36γ-specific antibody 4A9 functions as a primary western blot antibody capable of detecting IL-36γ expressed by a cellular source.

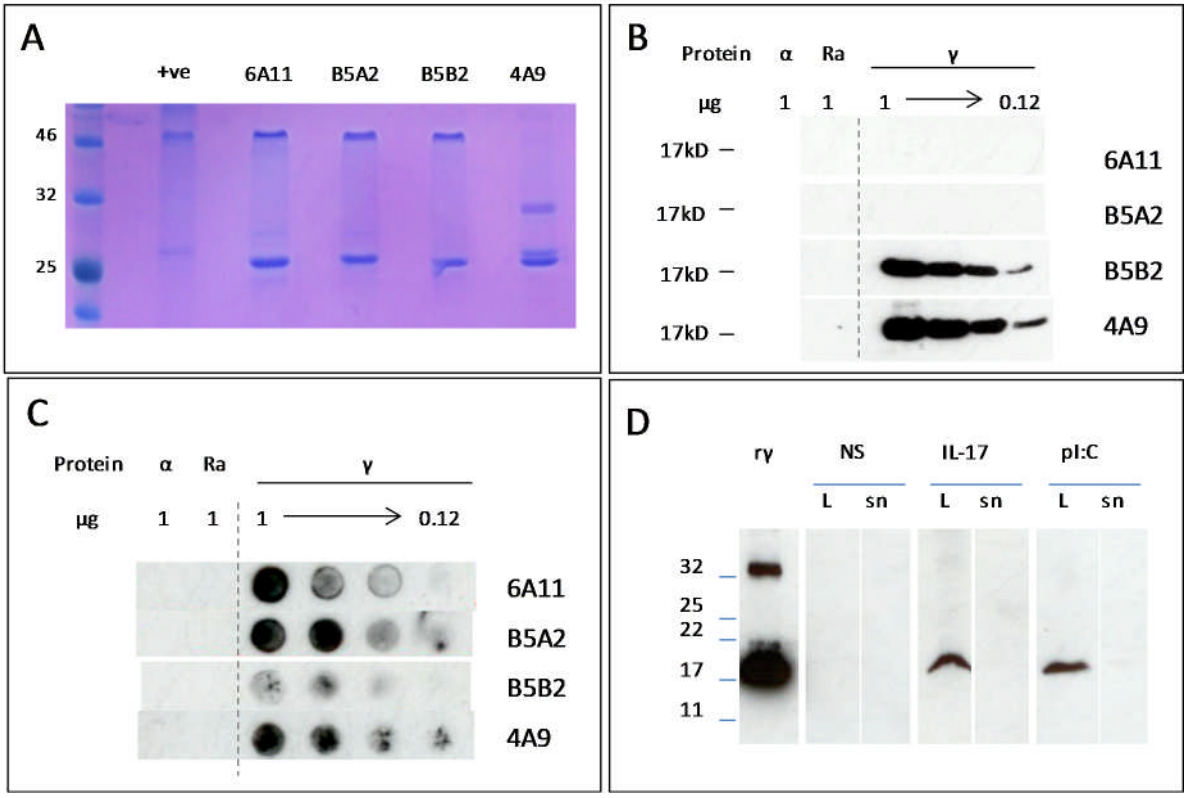


Figure 3-3: Characterisation of purified antibodies

(A) 2 µg of each purified antibodies were subject to SDS PAGE under reducing conditions and analysed by Coomassie staining. Purified antibodies were run alongside 2 µg of a random mouse IgG as a positive control (+ve). Antibodies were also tested for their ability to specifically detect recombinant IL-36γ by both western blot (B) and dot blot (C). 1 µg of recombinant IL-36α (α) and IL-36Ra (Ra) were used to determine occurrence of cross-reactivity whilst 1 µg of IL-36γ (γ) was serially diluted by 1:2 down to 0.12 µg. Proteins were either resolved by SDS PAGE and transferred to nitrocellulose (B) or immobilised on nitrocellulose by dot blotting (C). Membranes were then probed with 6A11, B5A1, B5B2, or 4A9 and detected by an anti-mouse secondary antibody. Antibody 4A9 was tested on its ability to detect endogenous IL-36γ expressed by HaCaTs (D). HaCaTs were cultured to a confluence of 70% in 6 well plates before addition of 50 ng/ml IL-17 or 10 µg/ml poly(I:C). Lysates and supernatants were collected and analysed alongside recombinant IL-36γ (rγ) by western blot using 4A9 as a primary antibody and HRP-conjugated anti-mouse secondary (Sigma).

Antibody	Isotype
6A11	κIgG1
B5A2	κIgG1
B5B2	κIgG1
4A9	κIgG2b

Table 3-1: Antibody isotypes

Table denoting purified antibody isotypes as characterised by Bio-Rad mouse isotyping test kit.

3.5 ELISA development

One of the greatest obstructions in IL-36 research is the absence of an antibody pair that functions in ELISA. Without this, it is very difficult to accurately measure low levels of protein and much research is limited to the mRNA level. As such, a primary objective has been to produce a pair of monoclonal antibodies that function as an ELISA pair.

3.5.1 Determining capture and detection ability

The antibodies were first tested on their ability to bind and retain soluble IL-36γ once adsorbed onto the surface of an immunosorbent plate. Antibodies unable to do so would not be suitable as a capture antibody in an ELISA pair. Similarly, the antibodies were also tested on their ability to detect IL-36γ immobilised and presented off the surface of an immunosorbent plate, mimicking the capture antibody-presented antigen.

In order to test their ability to function as capture and detection antibodies ELISA-like tests were developed. To test their capturing ability, immunosorbent plates were coated with the antibodies to be tested at 2 μg/ml and then probed with biotinylated IL-36γ. The plates were then probed with HRP-conjugated avidin which would bind to any biotinylated IL-36γ captured by the antibodies adsorbed to the plate. The addition of TMB

solution would then produce a quantifiable colour change to detect the captured IL-36γ. In order to generate a signal against which the antibodies could be compared, wells were coated with biotinylated mouse antibodies at 2 µg/ml and detected with HRP-conjugated avidin. To test the detection ability of the antibodies, biotinylated IL-36γ (biotinylated as described in methods section 2.3.12) was added to avidin-coated plates (2 µg/ml) to present the antigen away from the surface of the plate. The presented IL-36γ was then probed with the antibodies being tested. Antibodies that had bound the immobilised IL-36γ were detected by the addition of HRP-conjugated anti-mouse antibodies and subsequent addition of TMB solution. As a positive control plates were coated with 2 µg/ml of mouse antibodies and detected by HRP-conjugated anti-mouse antibody.

Tested in this manner, as shown in Figure 3-4, the antibodies 6A11 and B5A2 produced a strong signal when acting as both capture and detection antibodies. B5B2 and 4A9, however, in both tests produced a signal that was not significantly greater than that produced by the negative control, and as such would not be suitable as either capture or detection antibodies.

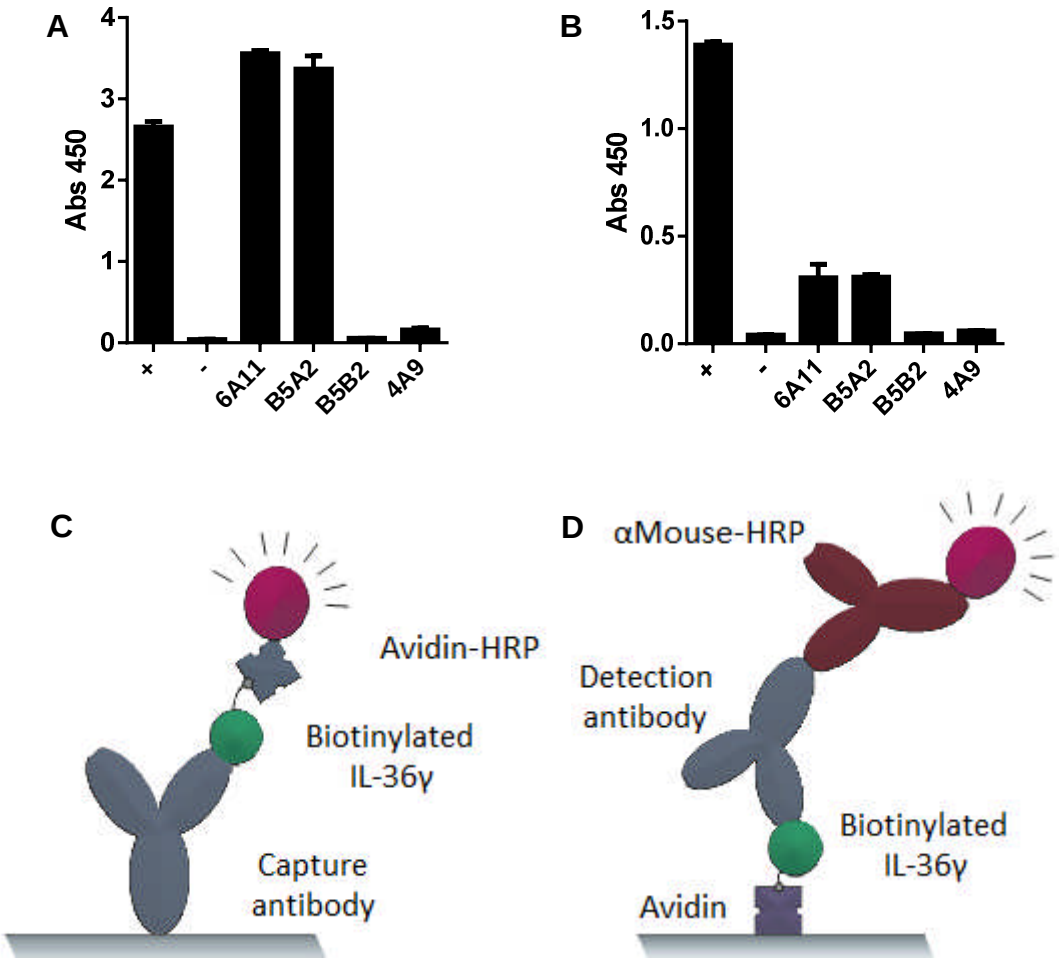


Figure 3-4: Capture and detection capability of purified antibodies

Purified antibodies were examined on their ability to both capture (A) and detect (B) recombinant IL-36γ. To assess capturing capability, immunosorbent plates were coated with 2 µg/ml capture antibody. Biotinylated IL-36γ was added at 1 µg/ml and detected with avidin-HRP (A,C). As a positive control (+) 2 µg/ml biotinylated antibody was added and directly detected with avidin-HRP. For assessing detection capability, immunosorbent plates were coated with 2 µg/ml avidin followed by biotinylated IL-36γ at 1 µg/ml. Detection antibodies were added at 2 µg/ml and detected HRP-conjugated anti-mouse secondary (Sigma) (B,D). As a positive control, plates were coated with 2 µg/ml mouse antibody and detected with anti-mouse HRP. In both experiments blocking buffer was added in place of biotinylated IL-36γ as a negative control (-) Data shown are mean ± S.E. (n = 3)

3.5.2 Antibody compatibility in sandwich ELISA

Having established both 6A11 and B5A2 function independently as capture and detection antibodies, it was necessary to determine whether they work as an ELISA pair. As both B5B2 and 4A9 tested very poorly for both capture and detection antibodies, these were not tested.

In order to test whether 6A11 and B5A2 were compatible with one another, a standard ELISA was performed. Immunosorbent plates were coated with either 6A11 or B5A2, washed with recombinant IL-36γ and probed with the other antibody. The detection antibodies were biotinylated to enable recognition by HRP-conjugated avidin and amplify the signal produced upon addition of TMB solution.

Initially, both capture and detection antibodies were used at 2 µg/ml as this is a standard concentration suggested for use in monoclonal antibody ELISAs. IL-36γ was added across a broad range of concentrations (1 µg/ml, 10 ng/ml, and 100 pg/ml). As shown in Figure 3-5, a strong signal was produced with both 6A11 and B5A2 as capture antibodies at 1 µg/ml of IL-36γ and a signal was also detectable at 10 ng/ml, albeit to a lesser degree. However, with the reaction allowed to progress for 5 minutes, no signal was detected at 100 pg/ml. The signal produced with B5A2 capturing and 6A11 detecting was slightly stronger than with 6A11 capturing and B5A2 detecting, suggesting this orientation would be more effective and sensitive for the detection of IL-36γ.

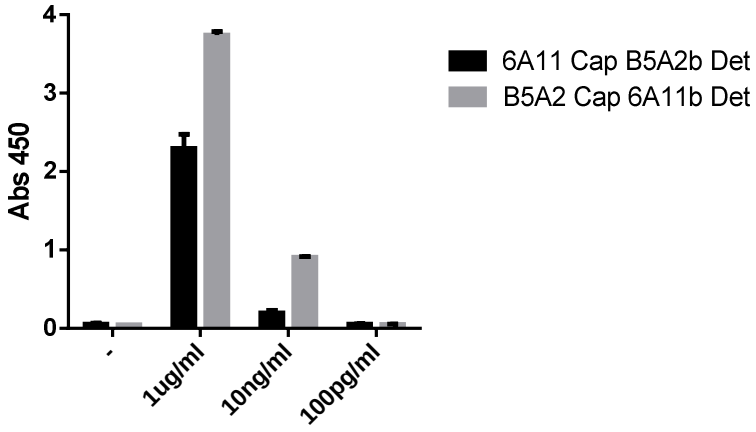


Figure 3-5: Functionality of B5A2 and 6A11 in sandwich ELISA

Immunosorbent plates were coated with 2 µg/ml of either 6A11 or B5A2 before addition of recombinant IL-36γ at 1 µg/ml, 10 ng/ml, and 100 pg/ml. IL-36γ was detected with 2 µg/ml of biotinylated B5A2 or 6A11. Reaction was allowed to progress for 5 minutes before reaction was stopped and absorption read at 450 nm. For a negative control, blocking buffer was added in place of recombinant IL-36γ. Data shown are mean ± S.E. (n = 3)

3.5.3 Optimization

B5A2 and 6A11 have been shown to function together as an ELISA pair, capable of detecting IL-36γ at 10 ng/ml. However, as most commercial ELISAs work in the range of 2 ng/ml to 30 pg/ml, the sensitivity of this ELISA must be improved in order to detect and quantify protein from cellular sources. As such, a number of optimisations were performed by modulating the concentration of capture and detection antibodies.

Immunosorbent plates were coated with B5A2 capture antibody at 10 µg/ml and subject to a serial dilution from left to right. IL-36γ was added to the coated plate at a concentration of 2 ng/ml so that optimisation was being performed at a relevant concentration of antigen. The plates were then probed with biotinylated 6A11 added at 10 µg/ml and serially diluted from top to bottom. Negative controls were included to identify any background signal The colour change after addition of TMB solution was allowed to progress for

30 minutes in order to allow the reaction to produce as much signal as possible.

This optimisation greatly increased the amount of signal produced by the ELISA when compared to the absorbance produced with 10 ng/ml of IL-36γ. The results shown in Figure 3-6 indicated 2.5 µg/ml of capture B5A2 used with 5 µg/ml of detection 6A11 produced the highest absorbance reading with the lowest acceptable level of background signal.

Det 6A11	Cap B5A2							
	10	5.000	2.500	1.250	0.625	0.313	0.156	0.000
10.000	0.091	0.419	0.605	0.643	0.472	0.317	0.184	0.055
5.000	0.071	0.363	0.544	0.607	0.47	0.301	0.18	0.065
2.500	0.055	0.317	0.479	0.569	0.442	0.298	0.188	0.059
1.250	0.048	0.262	0.419	0.508	0.424	0.274	0.162	0.053
0.625	0.051	0.22	0.355	0.451	0.373	0.248	0.152	0.05
0.313	0.049	0.168	0.292	0.385	0.323	0.211	0.133	0.051
0.156	0.042	0.125	0.23	0.323	0.267	0.189	0.127	0.043
0	0.049	0.05	0.042	0.077	0.046	0.046	0.041	0.034

Figure 3-6: Optimisation of B5A2:6A11 IL-36γ ELISA

Immunosorbent plates were coated with capture B5A2 antibody at a starting concentration of 10 µg/ml and serially diluted 1:2 from left to right to establish columns of varying capture antibody concentration. The last column was coated with blocking buffer. IL-36γ was added to all wells excluding the 1st column at 2 ng/ml. Blocking buffer was added to the first column in place of IL-36γ to act as a blank. Biotinylated 6A11 detection antibody was added at a starting concentration of 10 µg/ml and serially diluted 1:2 from top to bottom to establish rows of varying detection antibody concentration. The last row was detected using blocking buffer alone. Detection antibody was detected using a constant concentration of avidin-HRP. Values in white boxes denote the concentration of capture or detection antibody in µg/ml. Values in coloured boxes are the absorption value detected at 450 nm.

Despite the improvement, the absorbance generated by the optimised concentrations of capture and detection antibody was significantly less than that produced by commercial ELISA kits. A standard curve generated by serially diluting IL-36γ from 2 ng/ml to 31.25 pg/ml IL-36γ indicated the ELISA pair was still not sensitive enough to accurately quantitate low levels of

protein, despite producing absorbance readings at a concentration of 125 pg/ml.

To further increase the sensitivity of the ELISA, a commercial biotinylated polyclonal detection antibody obtained from R&D Systems was tested against both B5A2 and 6A11 capture antibodies. The polyclonal detection antibody was first optimised in a similar fashion as with B5A2 and biotinylated 6A11. Both capture and detection antibodies were serially diluted against one another in the manner shown in Figure 3-6. The polyclonal detection antibody was added at a starting concentration of 1 µg/ml as opposed to 10 µg/ml as non-specific binding is more common from polyclonal antibodies, and as such background may be more pronounced.

After having established the optimal concentrations of capture and detection antibody, standard curves were produced from 2 ng/ml of IL-36γ using both 6A11 and B5A2. The standard curves were then compared against those produced by the B5A2 and 6A11b monoclonal pair and a commercial IL-8 ELISA (Figure 3-7). The results from this comparison show that whilst the monoclonal pair is capable of detecting low levels of IL-36γ it is not as sensitive as when using the biotinylated polyclonal detection antibody. They also show that whilst the B5A2 capture and polyclonal detection antibodies are more sensitive than the monoclonal pair, the signal produced is not as strong as that produced by the commercial IL-8 ELISA.

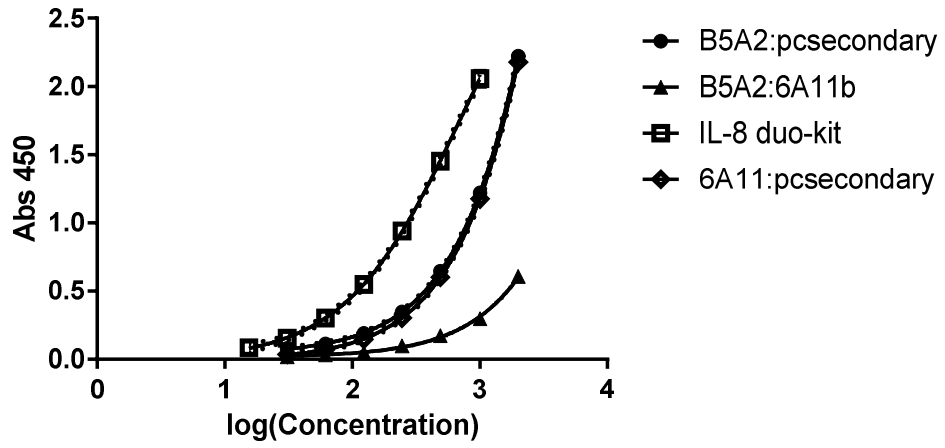


Figure 3-7: Comparison of IL-36γ ELISA standard curves

ELISA was performed with recombinant IL-36γ serially diluted from 2 ng/ml to 31.25 pg/ml using ELISA pairs B5A2:6A11b, B5A2:pcsecondary, and 6A11:pcsecondary. Standard curves were generated and interpolated from absorbance readings in GraphPad Prizm. Standard curve generated by a commercial IL-8 ELISA (BioLegend) from 1 ng/ml to 15.625 pg/ml was also included for comparison.

3.5.4 Establishing LoB, LoD, LoQ

In the development of a commercial ELISA it must be established to what level an ELISA pair can both reliably detect and quantify their antigen. This is done by establishing a Limit of Blank (LoB), Limit of Detection (LoD) and Limit of Quantification (LoQ). The LoQ can often be circumstantial, dictated by the purpose the ELISA serves and to what level of confidence that purpose requires, but is never less than the LoD. The LoD, however, is defined by the Clinical and Laboratory Standards Institute (CLSI) as the lowest concentration at which the measured analyte can be reliably distinguished from the LoB [144]. LoB can be defined as the highest apparent concentration of analyte found by measuring replicate samples containing no analyte. In order to establish how sensitive the B5A2 and 6A11 IL-36γ ELISAs are, the LoB and LoD for each were identified. For the purposes of these studies, the LoQ was defined as the LoD as the ELISAs need only be reliable enough to identify increases or decreases in IL-36γ

expression. Were it vital that precise IL-36γ concentrations were quantified with a high confidence, a more stringent LoQ would have been established.

To define the LoB and LoD of each ELISA, four replicate standard serial dilutions were performed for each antibody from 2 ng/ml with a lowest concentration standard at 31.3 pg/ml. Replicate blanks consisting of the assay diluent were also included to establish the LoB. LoB was estimated by using the mean absorbance and the standard deviation of the blank replicates, according to the formula shown below:

$$LoB = mean_{blank} + 1.645(SD_{blank})$$

LoD was then estimated using the LoB and the standard deviation of the lowest concentration replicate samples in the serial dilutions, utilising the formula outlines below:

$$LoD = LoB + 1.645(SD_{Low\ concentration\ sample})$$

The serial dilutions of IL-36γ for each ELISA were plotted to generate standard curves. From this, the LoD for each antibody was plotted on the standard curves and extrapolated to generate a concentration value, as shown in Figure 3-8. The B5A2 ELISA has an estimated LoD of 26 pg/ml, whilst the 6A11 ELISA has an LoD of 48 pg/ml. From this, it is clear the B5A2 ELISA is more sensitive, yet both ELISAs are capable of detecting concentrations of a level often secreted from cellular sources. As such, they both shall be viable to use for the quantification of IL-36γ protein, and shall prove invaluable for future use in IL-36γ research.

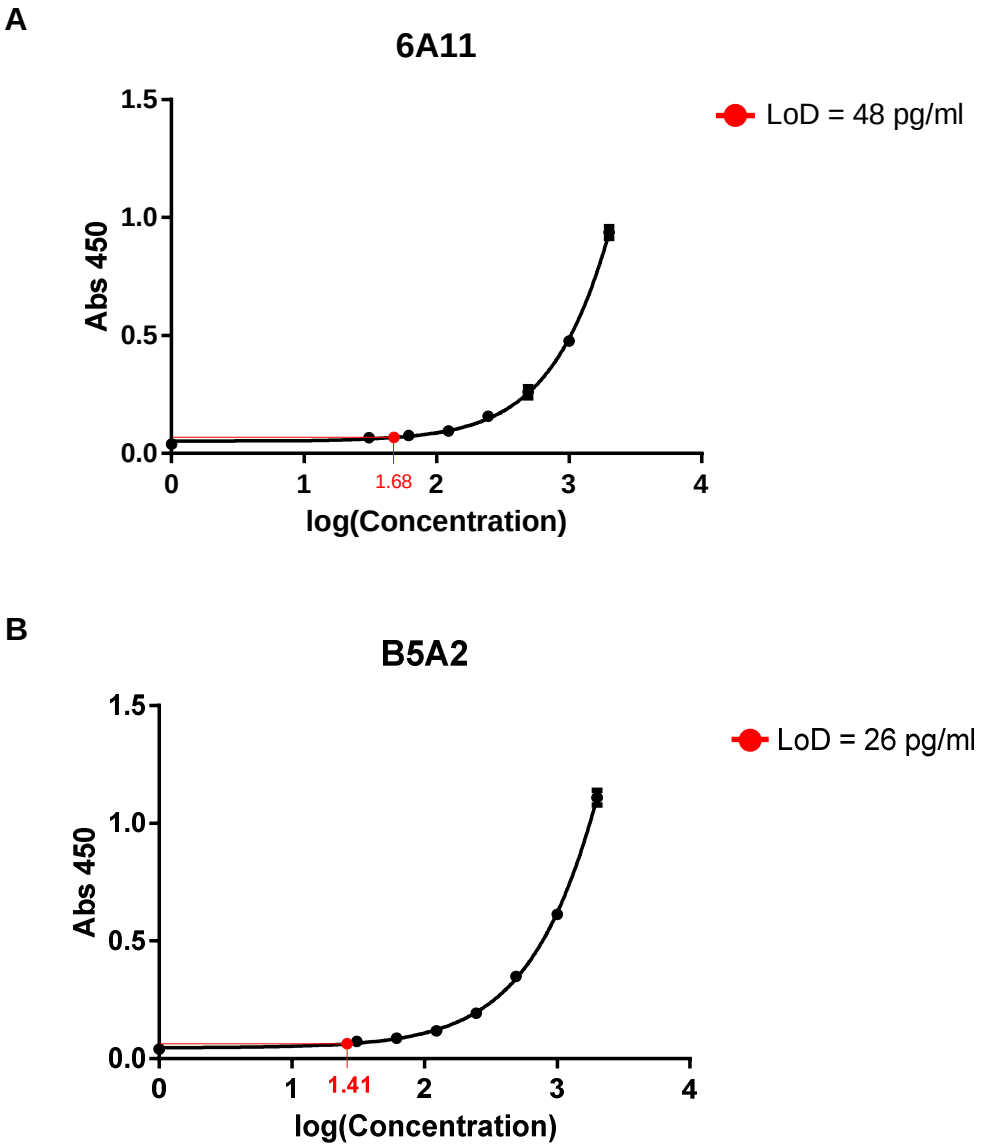


Figure 3-8: Limit of Detection for B5A2 and 6A11 ELISAs

An ELISA was performed using 6A11 (A) and B5A2 (B) capture antibodies with polyclonal detection antibody. A standard curve was generated for each antibody pair with 5 repeats of serially diluted recombinant IL-36γ from 2 ng/ml to 31.25 pg/ml and a blank sample. Limit of detection was calculated and interpolated against a standard curve generated by GraphPad Prizm

3.5.5 Measurement of endogenous IL-36γ by B5A2 ELISA

The results above have shown that the B5A2 ELISA is capable of reliably detecting recombinant IL-36γ from a concentration of 26 pg/ml. It was therefore necessary to test whether this can be applied to actual research, measuring endogenous IL-36γ from a cellular source. Keratinocytes were stimulated for 48 hours with 50 ng/ml of IL-17, TNFα and IFNγ; all of which are thought to induce expression of IL-36γ. Both cell supernatants and cell lysates were measured by B5A2 ELISA. Figure 3-9 shows the ELISA successfully detected IL-36γ in both supernatants and lysates, showing stimulation by both IL-17 and TNFα to greatly increase expression of IL-36γ whereas IFNγ induced no increase in expression. These results indicate the B5A2 ELISA is capable of detecting endogenous IL-36γ and as such would be appropriate to use in a research environment.

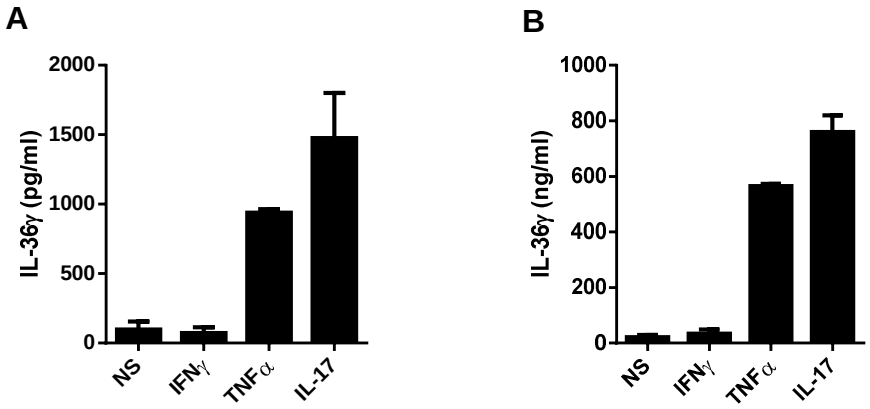


Figure 3-9: B5A2 ELISA detects endogenous IL-36γ

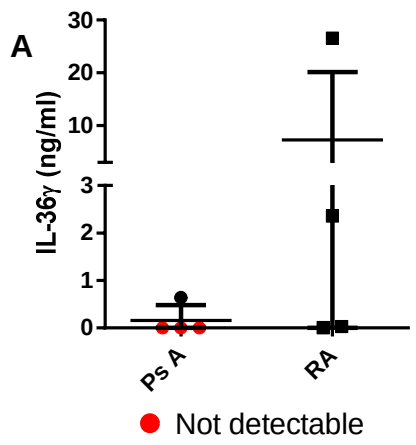
Primary keratinocytes were grown to a confluence of 70% before stimulation with 50 ng/ml IFNγ, TNFα or IL-17 for 48 hours. Keratinocyte supernatants were removed and cells lysed. Supernatants (A) and lysates (B) were then measured by B5A2:pcsecondary IL-36γ ELISA. Data shown are mean ± S.E. (n = 3).

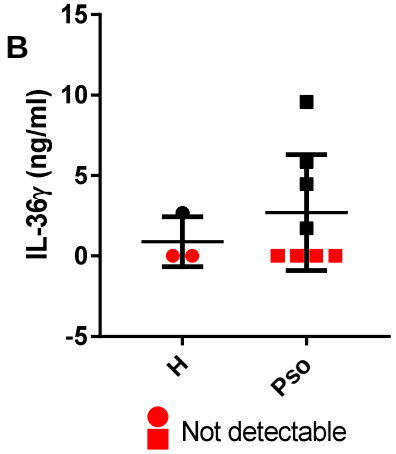
The ability to detect endogenous IL-36γ may serve to not only greatly help in a research setting, but may also prove to be an invaluable tool in a medical one. IL-36γ has been postulated to be a disease marker for psoriasis owing

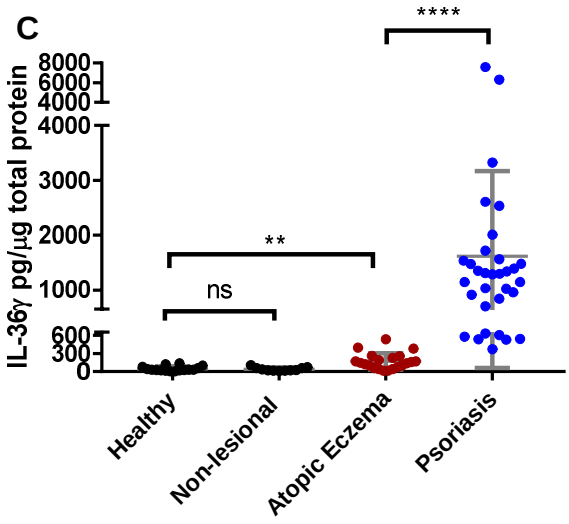
to its largely increased expression in psoriatic tissue. Whilst RNA analysis is a useful indicator of protein expression it does not always equate to the actual protein levels expressed. The ability to measure actual IL-36γ levels from diseased tissue may provide vital diagnostic information that may enable differentiation between otherwise phenotypically similar inflammatory conditions.

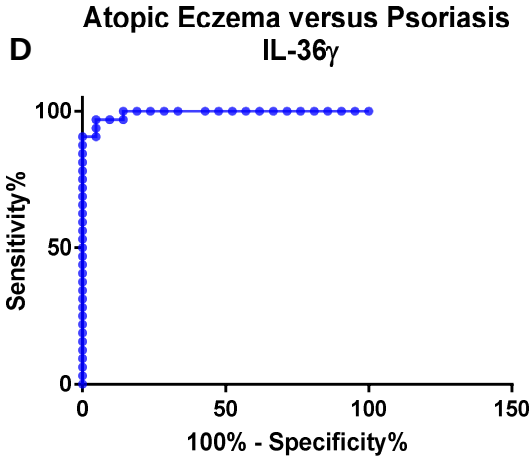
With this in mind, the B5A2 ELISA was tested on skin tape-strip biopsy, synovial and serum samples from both healthy donors and patients with atopic dermatitis (atopic eczema), psoriasis, psoriatic arthritis (PsA) and rheumatoid arthritis to assess the ELISAs capability of detecting IL-36γ in each biopsy method. Tape stripping was performed by Anna Keszegpal as outlined in methods section 2.2.19. As shown in Figure 3-10 IL-36γ can be measured in samples of all biopsy methods.

Indeed, with regard to the tape-stripping biopsies it appears the high level of IL-36γ expression in psoriatic skin is emulated at the protein level, lending credit to IL-36γ being a psoriasis-specific disease marker. An ROC curve generated from the data obtained from the tape-strip samples demonstrated that psoriasis can be differentiated from atopic dermatitis to a confidence of 95% when a lesional epidermal concentration of IL-36γ is in excess of 540 pg/µg of total protein (Figure 3-10). This therefore shows great promise for developing the IL-36γ ELISA into future diagnostic tests.









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Cut-off level	Sensitivity %	95% CI	Specificity %	95% CI	Likelihood ratio
> 316.1	100	89.11% to 100%	85.71	63.66% to 96.95%	7
> 376	96.88	83.78% to 99.92%	85.71	63.66% to 96.95%	6.781
> 387.9	96.88	83.78% to 99.92%	90.48	69.62% to 98.83%	10.17
> 461.8	96.88	83.78% to 99.92%	95.24	76.18% to 99.88%	20.34
> 532.7	93.75	79.19% to 99.23%	95.24	76.18% to 99.88%	19.69
> 538.6	90.63	74.98% to 98.02%	95.24	76.18% to 99.88%	19.03
> 540.5	90.63	74.98% to 98.02%	100	83.89% to 100%	
> 560.3	87.5	71.01% to 96.49%	100	83.89% to 100%	
> 592	84.38	67.21% to 94.72%	100	83.89% to 100%	

Figure 3-10: IL-36γ is detectable in blood serum, synovial fluid and by tape stripping

IL-36γ levels were measured by B5A2 ELISA in synovial fluid of patients with either psoriatic arthritis (PsA; n = 4) or rheumatoid arthritis (RA; n = 4) (A) and in blood serum from healthy volunteers (H; n = 3) or patients with psoriasis (Pso; n = 8) (B). Red points denote samples that had an undetectable level of IL-36γ. Epidermal IL-36γ was also measured by tape stripping (C). 10 CuDerm tapes were taken from healthy volunteers (n = 16) and lesional sites of patients with atopic dermatitis (n = 21) and psoriasis (n = 32). As there was no difference between non-lesional epidermal IL-36γ these samples were combined from patients with atopic dermatitis and psoriasis. Only samples from patients with a confirmed diagnosis were included. Data shown are mean ± S.D. Data from (C) were used to generate an ROC curve of atopic dermatitis versus psoriasis to determine the diagnostic potential of epidermal IL-36γ concentration in differentiating psoriasis and atopic dermatitis (D). The tabulated data from the ROC curve (E) indicate the cut-off for specific detection of psoriasis is at 540 pg/µg total protein.

3.6 Immunoprecipitation development

The ability to detect IL-36γ at the protein level would prove instrumental in further IL-36 research and have great potential as a diagnostic tool in a clinical setting. Whilst ELISA can quantitate the IL-36γ levels, it cannot elucidate any information on the activity of the protein. Knowledge of whether IL-36γ is present as processed active protein or the inactive precursor would prove to be extremely useful information, potentially giving more insight into disease phenotype. Whilst there is highly convincing evidence for IL-36γ S18 being the active form of IL-36γ, neither this truncated form nor indeed any form has been isolated from human tissue or a primary source. This would be made possible through immunoprecipitation followed by mass spectrometry interrogation. Therefore, the IL-36γ specific antibodies were tested on their ability to precipitate and purify IL-36γ.

6A11, B5A2, B5B2 and 4A9 antibodies were added to a solution of recombinant IL-36γ with bovine serum albumin. The precipitated antibody complexes were then analysed by western blot using an IL-36γ-specific primary antibody (as detailed in methods section 2.3.17). As shown in Figure 3-11 antibodies 6A11 and B5A2 appear to successfully precipitate IL-36γ as IL-36γ is strongly detected in the precipitation fraction with both antibodies

and is absent from the immunoprecipitation supernatant. In contrast, after immunoprecipitation by B5B2 and 4A9 IL-36γ is strongly detected in the supernatant and absent from the precipitated fraction, indicating neither B5B2 nor 4A9 are suitable for immunoprecipitation.

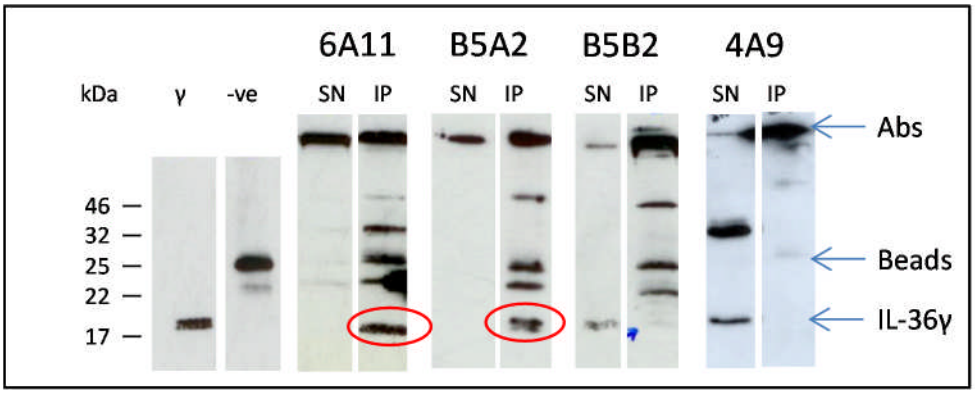


Figure 3-11: Immunoprecipitation of IL-36γ by purified antibodies

500 ng recombinant IL-36γ was mixed with 5 µg of either 6A11, B5A2, B5B2 or 4A9 overnight at 4°C. Antibodies were precipitated by addition of protein G Agarose beads for 3 hours at 4°C with rotatory mixing. Precipitated antibodies were collected by centrifugation and supernatant (SN) and immunoprecipitation (IP) fractions were analysed by western blotting. 500 ng recombinant IL-36γ was included (γ) to facilitate identification of successful immunoprecipitation. Immunoprecipitation was repeated substituting precipitating antibodies with PBS as a negative control (-ve). IL-36γ was detected using 4A9 primary antibody and HRP-conjugated anti-mouse secondary (Sigma). Arrows indicate proteins corresponding to bands. Red circles indicate successful immunoprecipitation.

After establishing 6A11 and B5A2 can precipitate recombinant IL-36γ under controlled conditions, it was examined whether endogenous IL-36γ expressed by a cellular source could be precipitated. As shown in Figure 3-9, keratinocytes express high levels of IL-36γ upon stimulation with IL-17. Therefore, primary keratinocytes were grown to a confluence of 70% and stimulated with IL-17 for 48 hours and immunoprecipitation was performed on cell lysates.

By measuring the concentration of IL-36γ in the keratinocyte lysates before and after immunoprecipitation an idea can be gauged of the success of the experiment. Figure 3-12 shows that a significant amount of IL-36γ (82% of the total lysate IL-36γ) was removed from the keratinocyte lysates after immunoprecipitation with B5A2, suggesting this antibody successfully precipitated IL-36γ. Furthermore, after elution of IL-36γ from B5A2 antibody by incubation in 1.5 M NaCl, purified IL-36γ was detected (albeit significantly less than was removed from the lysate after immunoprecipitation). These results indicate that B5A2 is capable of precipitating endogenous IL-36γ.

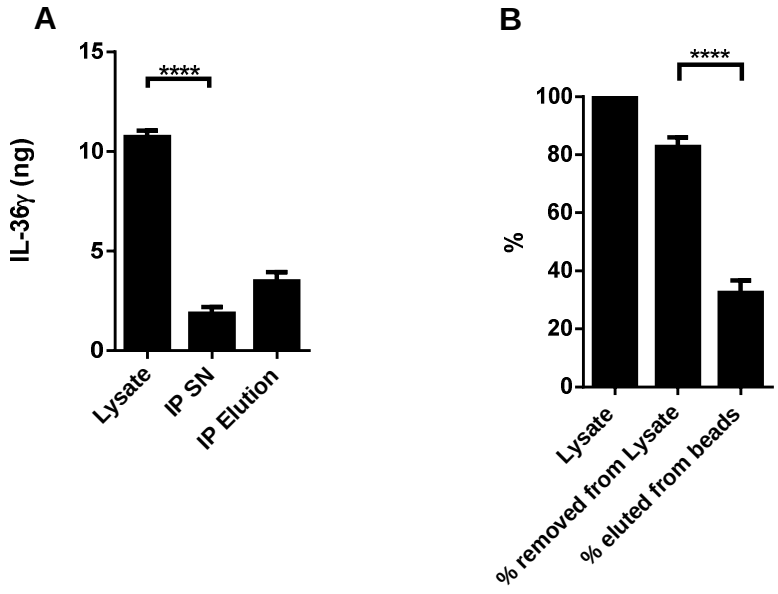


Figure 3-12: Immunoprecipitation of endogenous IL-36γ

Primary keratinocytes were grown to 90% confluence in 6 well plates before stimulation with 50 ng/ml IL-17 for 48 hours. Cells were lysed and IL-36γ immunoprecipitated with B5A2. IL-36γ was eluted by a high salt solution. Concentration of IL-36γ present in lysates before and after immunoprecipitation (Lysate; IP SN), and eluted fraction (IP Elution) was assessed by B5A2 ELISA (A). The percentage of total IL-36γ removed from the lysate and eluted is shown in B. Data shown are mean ± S.E. ****, $p < 0.0001$. ($n = 2$).

3.7 Blocking function

Antibodies are not only invaluable in a research setting but are being used as "biologics" in an increasing number of diseases, especial against inflammatory mediators [145]. Anti-IL-17 monoclonal antibodies such as secukinumab and the anti-IL-23p19 monoclonal antibody ustekinumab have both been shown to be very effective when used to treat psoriasis [146, 147]. Infliximab, an anti-TNFα antibody, is now widely used in the treatment of various inflammatory conditions and is well documented to be an effective treatment [148]. These antibodies typically function by chelating their inflammatory target peptide, preventing them from binding and activating their respective receptors. Given the importance IL-36 plays in psoriatic inflammation, particularly IL-36γ in the skin, anti-IL-36γ treatment may be a very attractive option to complement and use in conjunction with the existing monoclonal antibody biological treatments. Therefore the purified anti-IL-36γ antibodies were tested for their ability to prevent IL-36γ mediated signalling.

Antibodies were mixed with IL-36γ at various molar ratios and incubated for 20 minutes at room temperature prior to administering to HaCaTs for 48 hours. IL-8 secretion was measured as a read-out of IL-36 activity. As shown by Figure 3-13 neither 4A9 nor B5B2 showed any sign of blocking IL-36 activity at any of the molar ratios tested. In contrast, both 6A11 and B5A2 successfully reduced IL-36-dependent secretion of IL-8. The addition of 6A11 and IL-36γ in combination exhibited a significant reduction in IL-8 secretion at all the molar ratios tested when compared to IL-36γ alone. B5A2 appeared to have only a slight effect on IL-8 secretion at an equimolar concentration, however when used at higher molar ratios the antibody reduced the secretion of IL-8 to a level seen when cells are pre-treated with IL-36Ra prior to IL-36γ treatment. Furthermore this reduction of IL-8 secretion appears to be dose dependent, with IL-8 decreasing further as the molar ratio of B5A2 to IL-36γ is increased. These observations indicate both 6A11 and B5A2 are capable of blocking IL-36 signalling through binding of IL-36γ and sterically preventing receptor binding. The ability to block agonist signalling

indicates the antibodies may be useful in future blocking studies, with perhaps a long term aim to consider use in therapeutics.

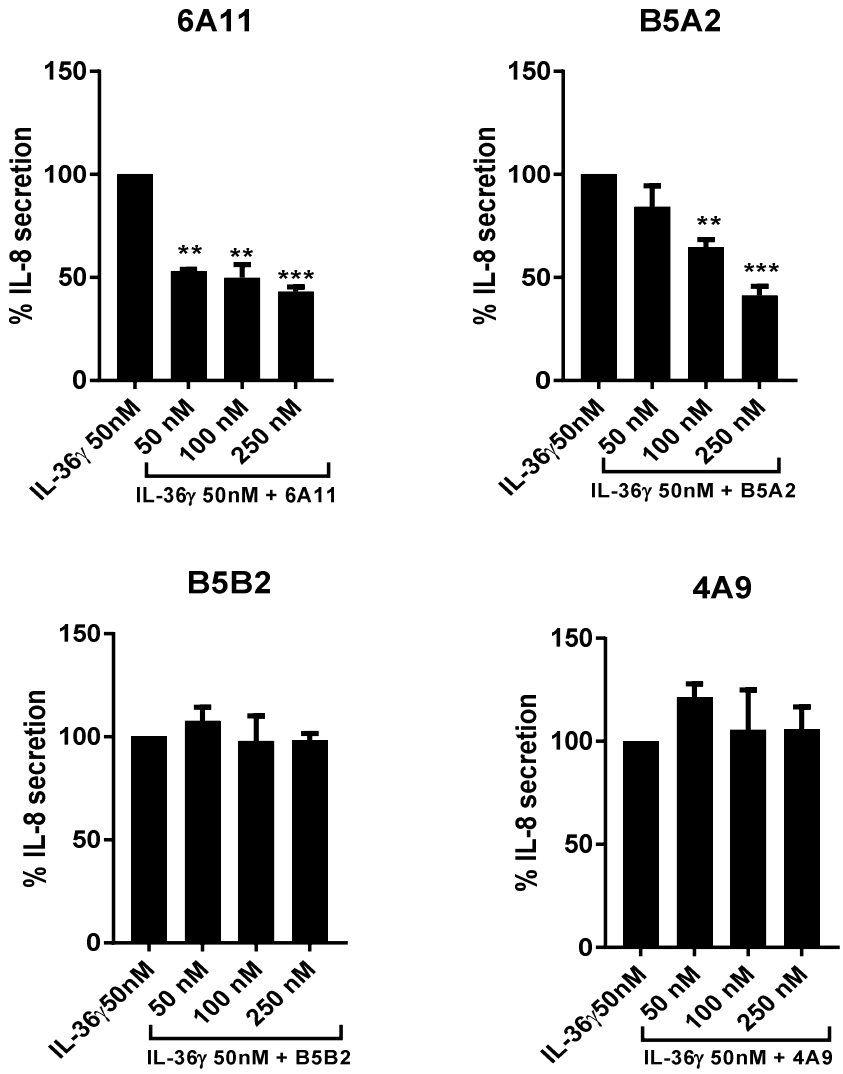


Figure 3-13: Blocking capabilities of purified antibodies

HaCaTs were grown to a confluence of 70% prior to stimulation. HaCaTs were stimulated with 50 nM IL-36γ alone or in combination with 6A11, B5A2, B5B2, or 4A9 at 1x, 2x, or 5x molar concentration for 48 hours. Antibodies were incubated with IL-36γ for 30 minutes at 4°C prior to addition to cells. IL-36γ activity was assessed by measuring IL-8 secretion via ELISA. Blocking ability of antibodies is expressed as percentage reduction in IL-36γ-induced IL-8 secretion. Data shown are mean ± S.E. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$. (n = 3).

3.8 Immunofluorescence

Little is known about the intracellular localisation and tracking of IL-36γ. As with many IL-1 family proteins the IL-36 cytokines lack a signal peptide and as such are not thought to enter the normal secretory pathway. The alternative pathways for protein secretion are not well described and quite difficult to interrogate, and as such research into the secretory pathway taken by IL-36 proteins has been quite sparse and often inconclusive. The ability to visualise intracellular IL-36 would prove to be very useful in further investigating the intracellular trafficking of IL-36, enabling visualisation of protein interactions and intracellular localisation. Therefore the anti-IL-36γ proteins were examined for their ability to function in immunofluorescent staining of fixed cells.

Prior to examining their ability to detect native IL-36γ, a positive control was established against which the efficacy of the anti-IL-36γ antibodies was tested. HEK293T cells were transfected with IL-36γ constructs containing C-terminal FLAG tags. The transfected HEK293T cells were fixed and stained with either mouse M2 anti-FLAG, 4A9, B5B2, B5A2 or 6A11 primary antibody as described in methods section 2.2.15. The mouse M2 anti-FLAG clone is well established to function well in immunofluorescence so should serve as a good positive control against which to compare the other antibodies. As all the primary antibodies used are mouse antibodies the same anti-mouse alexa-fluor 488 secondary was used on all samples. A negative control was also produced by staining FLAG-tagged IL-36γ expressing cells with the secondary antibody alone (not shown). All cells were also counter-stained with DAPI.

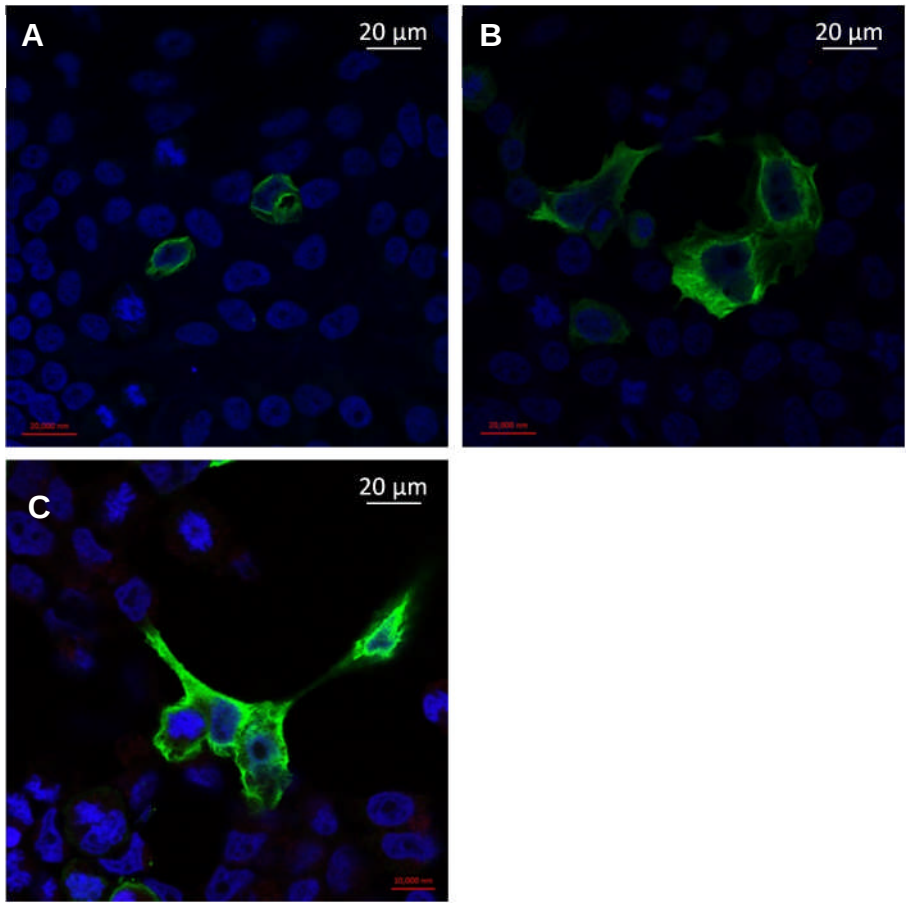


Figure 3-14: Detection of IL-36γ in IL-36γ-transfected HEK293T cells

HEK293T cells were cultured to 90% confluence before transfection with C-terminal FLAG tagged IL-36γ. Transfected cells were cultured for an additional 24 hours before fixation in 4% paraformaldehyde and staining with either 6A11 (A), B5A2 (B), or M3 anti-FLAG (C) and an alexa-fluor 488 conjugated anti-mouse secondary (ThermoFischer Scientific). Cells were counterstained with DAPI and slides were imaged by an inverted LSM700 confocal microscope coupled to a LSM Image Browser.

As shown in Figure 3-14 expression of FLAG-tagged IL-36γ was visualised by anti-FLAG staining indicating both the transfection and immunofluorescent staining were successful. Furthermore Figure 3-14 shows the expression of FLAG-tagged IL-36γ could also be visualised after staining with the anti-IL-36γ antibodies 6A11 and B5A2, albeit to a lesser degree than the anti-FLAG stained samples. However no IL-36γ-expressing cells could be identified when staining with 4A9 and B5B2 antibodies. These observations suggest

6A11 and B5A2 anti-IL-36γ antibodies may be suitable primary antibodies with which to investigate the intracellular expression and location of endogenous IL-36γ in primary cells.

Although the transfection of HEK293T cells with FLAG-tagged IL-36γ is seen above to be a good method of validating whether these antibodies are capable of staining IL-36γ under different fixation conditions, it is not necessarily an apt method for determining their ability to visualise endogenous protein. Therefore primary keratinocytes were stimulated with IL-17 to induce a high level of IL-36γ expression and were stained with both 6A11 and B5A2 anti-IL-36γ antibodies. However, no significant IL-36γ staining was observed despite IL-36γ ELISA data demonstrating a significant up-regulation in IL-36γ by IL-17 stimulation, suggesting perhaps either these anti-IL-36γ antibodies are not suitable for the visualisation of endogenous IL-36γ expressed by primary cells, or the cells are not expressing enough IL-36γ to be detected.

3.9 Discussion and future work

As illustrated in the introduction, the IL-36 cytokines are of great importance in psoriatic inflammation, and are becoming established as important mediators of inflammation in various epithelial barriers. IL-36γ in particular has been identified to be of significant importance in both the skin and lungs, as a proposed psoriasis-specific disease marker in the skin and with strong up-regulation noted in lung tissue in response to smoke condensate [103, 143]. However, much of the research conducted on IL-36 cytokines has been performed in mice models, or through RNA analysis. There are few human IL-36 specific antibodies available on the market, and those that are available have been found to be inadequate. This chapter, therefore, aimed to produce and characterise human IL-36γ specific monoclonal antibodies with which IL-36γ-specific research could be conducted, with a particular focus on developing an IL-36γ-specific ELISA.

By collaborating with Dr Delyth Reid at the University of Aberdeen, mice were inoculated with active IL-36γ and mouse hybridomas were generated from

the immunised mice splenocytes. After a series of selection and screening processes, four IL-36γ-specific monoclonal hybridomas were isolated and grown, from which monoclonal antibodies were purified. These were characterised and tested for their ability to function in a number of antibody-mediated protein assays, including ELISA, immunoprecipitation, immunofluorescence and activity blocking studies. An overview of each antibody and their characterised functions are listed in Table 3-2 below.

Antibody	Isotype	Western	Dot blot	ELISA	IP	IF	Blocking function
6A11	κIgG1	×	✓	✓	✓	✓×	✓
B5A2	κIgG1	×	✓	✓	✓	✓×	✓
B5B2	κIgG1	✓	✓	×	×	×	×
4A9	κIgG2b	✓	✓	×	×	×	×

Table 3-2: Characterised antibodies

Table to summarise purified antibodies and their optimised functions. '✓×' indicates antibodies could detect transfected IL-36γ but not endogenous IL-36γ

When screening the hybridomas for colonies that were expressing IL-36γ-specific antibodies, numerous positive hits were identified and cultured for antibody production. However a significant proportion of these stopped producing IL-36γ antibodies after an extended period of growth. As a result only 4 distinct clones were obtained from approximately 25 positive hits. This is likely due to chromosomal instability after cell fusion resulting in the loss of chromosome 12 or 6, containing the genes encoding IL-36γ-specific heavy and light immunoglobulin chains [149]. It has been observed that within clones of the same origin, those with fewer chromosomes proliferate at a greater rate [150]. It is therefore possible, over a period of prolonged growth, for an unstable monoclonal hybridoma population to become repopulated

with a clone of the same origin that has lost a chromosome encoding IL-36γ-specific antibody, and thus stop producing IL-36γ-specific antibody.

As shown in Table 3-2 the antibodies 4A9 and B5B2 functioned quite differently to 6A11 and B5A2 when examined in different antibody-mediated protein assays. Whereas both 6A11 and B5A2 functioned quite similarly and both performed relatively well in many of the assays tested, 4A9 and B5B2 only performed well in western and dot blot. This may, in part, be due to the different screening techniques utilised. As mentioned above, screening was initially performed by coating antigen directly onto the surface of immunosorbent plates to which hybridoma supernatants were added. It was noted, however, that this may have been altering the structure of the adsorbed protein to the point where the antibodies selected would not bind native soluble IL-36γ. The adsorption of proteins to plastic polymers is often mediated through hydrophobic interactions, and this has been demonstrated to cause a change in protein structure. This has been illustrated through the adsorption of both human growth hormone and lysozyme onto various solid surfaces, which have been shown to result in a less compact protein structure [151]. It may be possible that similar structural changes take place upon adsorption of IL-36γ to the point that adsorbed protein epitopes are distinct from soluble protein epitopes. Therefore, as the 4A9 hybridoma was selected by screening against directly adsorbed IL-36γ it is possible 4A9 antibodies do not bind soluble IL-36γ. Indeed, this seems to be supported when noting that 4A9 did not function as a capture antibody or a detection antibody, but recognised denatured IL-36γ when resolved by SDS-PAGE.

The antibodies 6A11, B5A2, and B5B2 were selected after screening against biotinylated IL-36γ presented off the surface of the plate by avidin. Presented in this way, IL-36γ should retain its soluble form, and as such the antibodies selected should bind native IL-36γ. Indeed, both 6A11 and B5A2 were shown to function as capture and detection antibodies, and could function in immunoprecipitation, indicating they bind native soluble IL-36γ. Furthermore, neither 6A11 nor B5A2 could detect IL-36γ by western blotting after resolution by SDS PAGE but were both capable of detecting native IL-36γ after immobilisation on nitrocellulose membrane by dot blotting,

indicating the epitope recognised by these antibodies is dependent on the 4 dimensional structure of the native protein. Intriguingly, despite being selected using avidin-presented IL-36γ, B5B2, like 4A9, did not function in immunoprecipitation or ELISA, but could detect IL-36γ immobilised on nitrocellulose and after resolution by SDS-PAGE in western blot,

The primary objective after production of IL-36γ-specific monoclonal antibodies was the development of an ELISA. During characterisation of the purified antibodies, 6A11 and B5A2 were identified as capable of both capturing soluble recombinant IL-36γ after adsorption onto immunosorbent plates and detecting biotinylated IL-36γ immobilised on avidin-coated immunosorbent plates. Furthermore they were shown to be compatible with each other in a sandwich conformation. However, further analysis revealed the sensitivity achieved by a B5A2 to 6A11 sandwich ELISA was not adequate for reliable quantification of low levels of recombinant IL-36γ. When compared to a commercial IL-8 sandwich ELISA, 1 ng/ml of IL-8 produced a 450 nm absorbance reading of 2 after only 10 minutes of incubation of the HRP substrate where 1 ng/ml of recombinant IL-36γ detected by B5A2 and 6A11 produced a 450 nm absorbance reading of 0.25 after allowing the colorimetric reaction to proceed for 30 minutes. Therefore commercial biotinylated polyclonal IL-36γ antibodies (RnD pcAb) were used to detect recombinant IL-36γ captured by either B5A2 or 6A11. This was shown to be an effective combination, with 1 ng/ml of IL-36γ detected by B5A2 and RnD pcAb generating a 450 nm absorbance reading of 1.2 after 30 minutes of incubation with HRP substrate. Further analysis revealed the B5A2 RnD pcAb ELISA could reliably detect recombinant IL-36γ down to 26 pg/ml, providing evidence of a sensitive ELISA. Finally the ELISA was demonstrated to detect endogenous IL-36γ in both cell lysates and cell supernatants of stimulated and non-stimulated primary keratinocytes, and thus was considered suitable to analyse the expression of IL-36γ protein.

Psoriasis and eczema are phenotypically similar conditions and often require an experienced dermatologist to differentiate between the two. In many cases, particularly when differentiating hand eczema and palmoplantar psoriasis, the only definitive method of differentiation is by punch biopsy and

histological examination; both a time-consuming diagnosis and an unpleasant painful procedure [152]. However, examination of mRNA expression from inflammatory skin lesions indicate increased IL-36γ expression is specific to psoriasis [143]. Using the ELISA generated in this chapter and tape-stripping biopsy methods the level of IL-36γ protein present in the epidermis of atopic eczema and psoriasis patients was evaluated to see whether the expression of IL-36γ could be used to differentiate the two conditions. In order to test whether a high level of IL-36γ correlates to a psoriatic disease phenotype, it was necessary to test patients that have a clearly defined diagnosis. In this fashion it has been demonstrated that high IL-36γ concentration strongly correlates with psoriatic inflammation. Indeed the ROC curve generated from this tape-strip data determined that skin-based IL-36γ in excess of 540 ng/μg is indicative of psoriasis to a confidence of 95%. It would therefore be interesting to determine whether measuring IL-36γ levels in skin lesions from patients not yet diagnosed by an experienced dermatologist would be able to correctly and reliably diagnose psoriasis, and furthermore distinguish phenotypically ambiguous psoriatic patients from eczema patients.

During the development of the ELISA it was observed that the monoclonal detection antibody was not sensitive enough to detect low levels of IL-36γ, yet that by using a polyclonal detection antibody with the same monoclonal capture antibody, the sensitivity of the ELISA was improved significantly. Intriguingly the polyclonal detection antibody significantly improved the sensitivity of the ELISA when used with either B5A2 or 6A11 as capture allowing for a LoD of 26 pg/ml and 48 pg/ml respectively, implicating that both B5A2 and 6A11 are capable of efficiently capturing IL-36γ and thus are to effectively bind IL-36γ. Yet once captured by one antibody, the other is not able to sensitively detect IL-36γ, as illustrated by the difference in signal produced by the monoclonal and polyclonal antibodies. This could be explained by a number of different scenarios. Firstly, as antibodies are large proteins and IL-36γ is relatively small, it is possible the capture antibody is partially masking the epitope of the detection antibody, causing fewer detection antibodies to successfully bind their target. Secondly, it has been

demonstrated that antibody binding can cause long-range conformational changes in the target antigen that can allosterically inhibit binding of a second antibody [153, 154]. It may therefore be possible that capture antibody binding is causing conformational changes in IL-36γ that reduces the affinity of the monoclonal detection antibody for its epitope. In both these cases the polyclonal detection antibody would maintain a high signal as it contains multiple clones of antibodies that recognise distinct epitopes, thus can compensate if an antibody is sterically or allosterically inhibited by the capture antibody. Finally there is the possibility the monoclonal detection antibodies were simply not adequately biotinylated, and were therefore not producing a strong signal after addition of avidin-conjugated HRP. This possibility should be explored in future experiments.

Ideally the ELISA developed in this study should undergo further development to establish a functioning pair of monoclonal antibodies. Whilst the monoclonal B5A2 capture to RnD polyclonal detection antibody pair have been shown here to function well, polyclonal antibodies are generated on an individual basis and as a result are subject to batch-to-batch variation, thus background and sensitivity in the ELISA can also vary. Indeed this is evident in Figures 3-7 and 3-8 when comparing the standard curves generated for comparison of monoclonal to monoclonal antibody, monoclonal to polyclonal antibody, and a commercial IL-8 ELISA (Figure 3-7) and for the determination of LoD (Figure 3-8). A different batch of polyclonal detection antibody was used for each figure whilst all other variables were maintained the same, yet batch 1 in Figure 3-7 generated a peak absorbance of 2.2 at 450 nm whilst batch 2 in Figure 3-8 generated a peak absorbance of 1.1 at 450 nm. As this ELISA may eventually be utilised in the diagnosis of psoriasis consistency is of vital importance. It is therefore necessary to develop an effective monoclonal detection antibody against IL-36γ that is compatible with B5A2 capture antibody. This should be relatively achievable by generating new hybridomas in a different species, such as rat or rabbit, and selecting for monoclonal hybridomas that produce compatible antibodies by screening against B5A2-presented IL-36γ. By using a different species for the detection antibody it is possible to differentiate positive hits from the capture

antibody by using species-specific antibodies. Screening for a monoclonal detection antibody in this manner will also select for antibodies that are not affected by steric and allosteric interference.

The antibodies generated in this chapter were also tested on their ability to block IL-36γ-mediated signalling. Both 6A11 and B5A2 appeared to have blocking capabilities, with B5A2 reducing IL-36-mediated IL-8 secretion in a dose dependent manner. Antibody therapy is a rapidly expanding field, and has been shown to be very effective in a number of inflammatory and rheumatologic conditions. In particular, biologics targeting IL-23 and IL-17 have demonstrated great efficacy in the treatment of psoriasis. Ustekinumab, approved for use in the USA and Europe in 2009, targets the p40 subunit shared by IL-23 and IL-12 and has been shown to be effective with 67.5% of patients achieving PASI 75 (a 75% reduction in psoriasis assessment and severity index (PASI) score) after 12 weeks of treatment [146]. In 2015 secukinumab, which targets IL-17A, was approved for use in the USA and Europe as a first-line of treatment of moderate to severe plaque psoriasis. Secukinumab has shown excellent efficacy, with 79% of patients achieving PASI 90 (a 90% reduction in PASI score) in 16 weeks [147]. IL-23 and IL-17 are both pivotal cytokines in the development of psoriatic plaques. IL-23 promotes the differentiation of T_H17 cells when in the presence of IL-6 and TGFβ, and subsequently promotes their survival [155]. The differentiated T_H17 cells produce high levels of IL-17, which promotes the development of psoriatic plaques by inducing secretion of neutrophil chemokines, AMPs and growth factors [156-158]. IL-36 has been proposed to be an attractive target for biologic treatment in psoriasis as it is an upstream cytokine that has been shown to initiate and regulate IL-17, IL-22 and IL-23 mediated inflammation [113]. As its expression is mostly limited to epithelial barriers, unlike IL-17, IL-22, IL-23 or TNFα, targeting IL-36 signalling is less likely to have adverse systemic side effects. Whilst it might be argued that targeting IL-36R would be more effective than simply targeting IL-36γ alone, given that IL-36R may have a role in wound healing completely blocking IL-36-mediated signalling may have detrimental long-term effects [125].

The treatment of conditions such as psoriasis, which often has localised areas of inflammation amongst uninvolved tissue, with systemic biologics can have disadvantageous side effects since the molecules targeted, such as IL-17, IL-23 and TNFα, are important in many aspects of immune function, not just the pathogenesis of psoriasis. In brodalumab clinical trial AMAGINE-3 (targeting IL-17 receptor A) it was identified that 56.8% of patients experienced adverse treatment-related effects with 1.4% developing serious adverse events after 12 weeks of treatment [159]. This included neutropenia and susceptibility to *C. albicans* infections, which is observed in patients that have IL-17 and IL-17RA deficiencies [160]. However, it may be possible to target biologic treatment specifically to a desired tissue or cell type through engineering recombinant bi-specific antibodies, thus limiting systemic side effects. It has been demonstrated that by linking a macrophage-specific single-chain antibody to a TNFα-specific high-affinity single domain antibody TNF inhibition can be specifically restricted to macrophages. By doing so the efficacy of the anti-TNF treatment is significantly enhanced [161]. Whilst IL-36 signalling is emerging as important in a number of different tissues, with implications in lung-based fungal defence and a wound healing protective role in the gut, psoriasis is primarily skin-based [124, 125]. Using a similar method as described by Efimov *et al.* it would be possible to specifically target skin-based IL-36-mediated inflammation through engineering bi-specific IL-36γ antibodies.

In addition to their blocking function and the development of an IL-36γ ELISA, the antibodies generated in this chapter were tested in immunoprecipitation and immunofluorescence. It was demonstrated that both 6A11 and B5A2 could immunoprecipitate recombinant IL-36γ and that B5A2 was capable of immunoprecipitating endogenous IL-36γ expressed by primary keratinocytes. As yet the endogenous active form of IL-36γ has not been isolated. With a functioning immunoprecipitating antibody this may be possible. Given that IL-36γ is measurable by ELISA from patient tape strips it seems logical that it should be possible to immunoprecipitate IL-36γ from these samples, and that provided enough is immunoprecipitated this could be analysed by mass spectrometry to provide information on the forms of IL-36γ present in the

sample. Furthermore, this may have a diagnostic application by providing information on how much of the present IL-36γ is in its active form. In immunofluorescence both B5A2 and 6A11 could detect IL-36γ expressed by HEK293Ts after transient transfection, yet neither could detect endogenous IL-36γ expressed by primary keratinocytes after stimulation with IL-17. This may be an issue with lower levels of expression compared to the transfected HEK293Ts as opposed to recognition of the antigen, however further optimisation is required to discern this. It may be worth trying different fixation conditions in case epitope masking is reducing the binding capabilities of B5A2 and 6A11 to IL-36γ and attempting antigen retrieval to reverse any epitope masking that might occur.

To conclude, the work undertaken in this chapter has produced 4 IL-36γ-specific antibodies which between them facilitate detection of IL-36γ in western blot, dot blot, ELISA and immunofluorescence, and immunoprecipitation of endogenous IL-36γ. Furthermore it has produced a reliable IL-36γ ELISA that has shown great potential for use as a diagnostic tool with which to differentiate psoriasis from eczema, and immunoprecipitating antibodies that should facilitate the isolation of endogenous active IL-36γ.

Chapter 4 - Proteolysis of IL-36 family members

4.1 Introduction

Like many IL-1 family cytokines, the IL-36 proteins require N-terminal truncation in order to become biologically active [99]. As mentioned previously, it has been demonstrated that this cleavage must be very precise, occurring exactly 9 amino acids upstream of the conserved A-X-D motif residing in the IL-1-like domain (illustrated in Figure 1-3). Inaccurate cleavage even by one amino acid upstream or downstream of this cleavage site will severely reduce the proteins' activity [99]. However, unlike other IL-1 family proteins the IL-36 members have no consensus cleavage site at their N-terminus, and the amino acid sequence surrounding the truncation sites bear little homology to one another (Figure 1-3). It has therefore proven difficult to predict any proteases that digest the IL-36 proteins and at the start of this research no IL-36 cleaving protease had been identified.

In order for cleavage to occur, the IL-36 proteins must come into contact with a protease. Given that IL-36 expression is predominantly restricted to lung, skin and joint tissue it is logical to assume that any endogenous activating proteases are present in these same tissues. The overabundance of IL-36 proteins in psoriatic lesions and their role in aggravating skin-based inflammation implicate the skin as a good candidate tissue from which to identify an IL-36 activating protease. The proteolytic cleavage and activation of IL-36 members was therefore investigated through the use of inflammatory and skin resident cells.

4.2 Neutrophil proteases truncate IL-36 members

Activation of the IL-36 members only requires removal of a small number of amino acids from the N-terminus. As such, cleavage would not be easily identified by SDS-PAGE or western blot. Therefore, incubations were performed using recombinant N-terminal SUMO-tagged IL-36 fusion proteins (illustrated in Figure 4-1). These were generated in the same fashion as detailed in chapter 3. By adding an N-terminal tag, the size of the peptide removed through cleavage significantly alters the size of the protein allowing

detection in both SDS-PAGE and western blot. Due to problems producing recombinant IL-36β, no experiments were conducted on this IL-36 cytokine.

SUMO-tagged full length IL-36α (IL-36α FL), IL-36γ (IL-36γ FL) and IL-36Ra (IL-36Ra FL) were incubated with media conditioned by keratinocytes, PMA-activated polymorphonuclear cells (PMNs) and fibroblasts for 24 hours (Figure 4-1). Analysis by SDS-PAGE showed that whilst no cleavage was seen in any of the proteins by keratinocyte or fibroblast conditioned media, PMN conditioned media cleaved all SUMO-tagged IL-36 cytokines. Furthermore, the cleaved products were approximately 17 kDa in mass which approximates to the size of the active IL-36 members. This suggests neutrophils may cleave IL-36 members in the region of their truncation site.

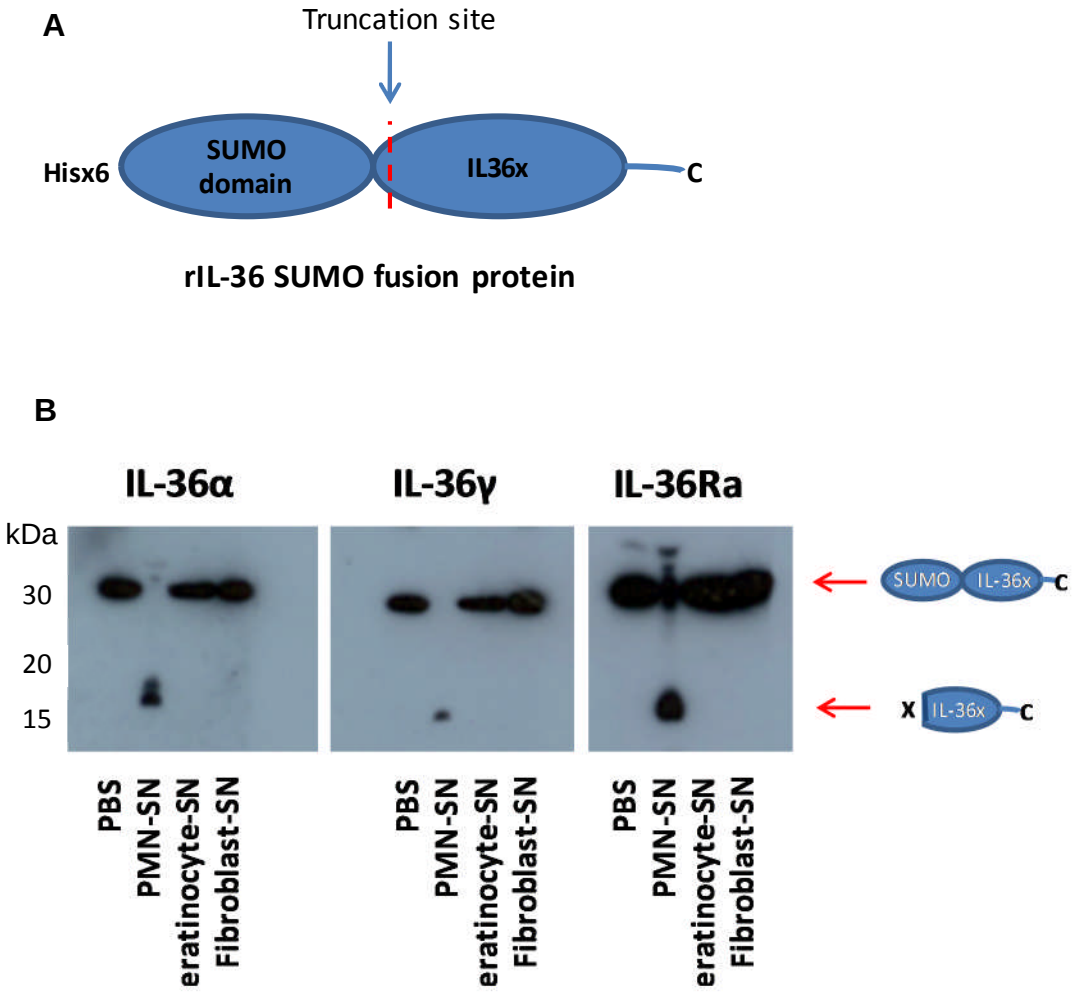


Figure 4-1: Polymorphonuclear cells truncate recombinant SUMO-IL-36 proteins

(A) Diagram depicts the SUMO-IL-36 fusion proteins used in the cleavage assays. The dashed red line depicts the approximate truncation site required for activation of the IL-36 cytokine. (B) SUMO-IL-36 recombinant proteins were incubated with supernatants of activated PMNs, keratinocytes and fibroblasts. PMNs isolated from whole blood of healthy donors were stimulated with 100 ng/ml of PMA for 1 hour at 37 °C. Human primary keratinocytes and fibroblasts were stimulated with 100 ng/ml of PMA for 24 hours at 37 °C. Supernatants were removed and incubated with SUMO-IL-36 recombinant proteins for 1 hour at 37 °C alongside a PBS control before analysis by western blot using mouse monoclonal anti-IL-36α, anti-IL-36γ and anti-IL-36Ra primary antibodies (R&D Systems and Sigma-Aldrich) and HRP-conjugated anti-mouse secondary (SouthernBiotech).

4.1 IL-36 proteolysis is mediated by neutrophil serine proteases

Neutrophils produce a vast number of proteases involved in inflammation, wound healing and tissue remodelling. In order to identify candidate proteases from those expressed by neutrophils, further refinement is required.

The proteins were again incubated with activated neutrophil conditioned media, but in the presence of molecular inhibitors that target specific protease families. The addition of iodoacetic acid (IAA), phenylmethylsulfonyl fluoride (PMSF), α1-antitrypsin and EDTA specifically inhibited cysteine, serine and metalloproteinases respectively to enable identification of a protease family actively cleaving IL-36. Western blot analysis revealed all SUMO-IL-36 proteins underwent cleavage when in the presence of IAA and EDTA, indicating the cleaving proteases were neither cysteine proteases nor metalloproteinases (Figure 4-2). Incubation with PMSF and α1-antitrypsin, however, prevented the cleavage of all SUMO-IL-36 members. This result suggests neutrophil-expressed serine proteases cleave IL-36 cytokines.

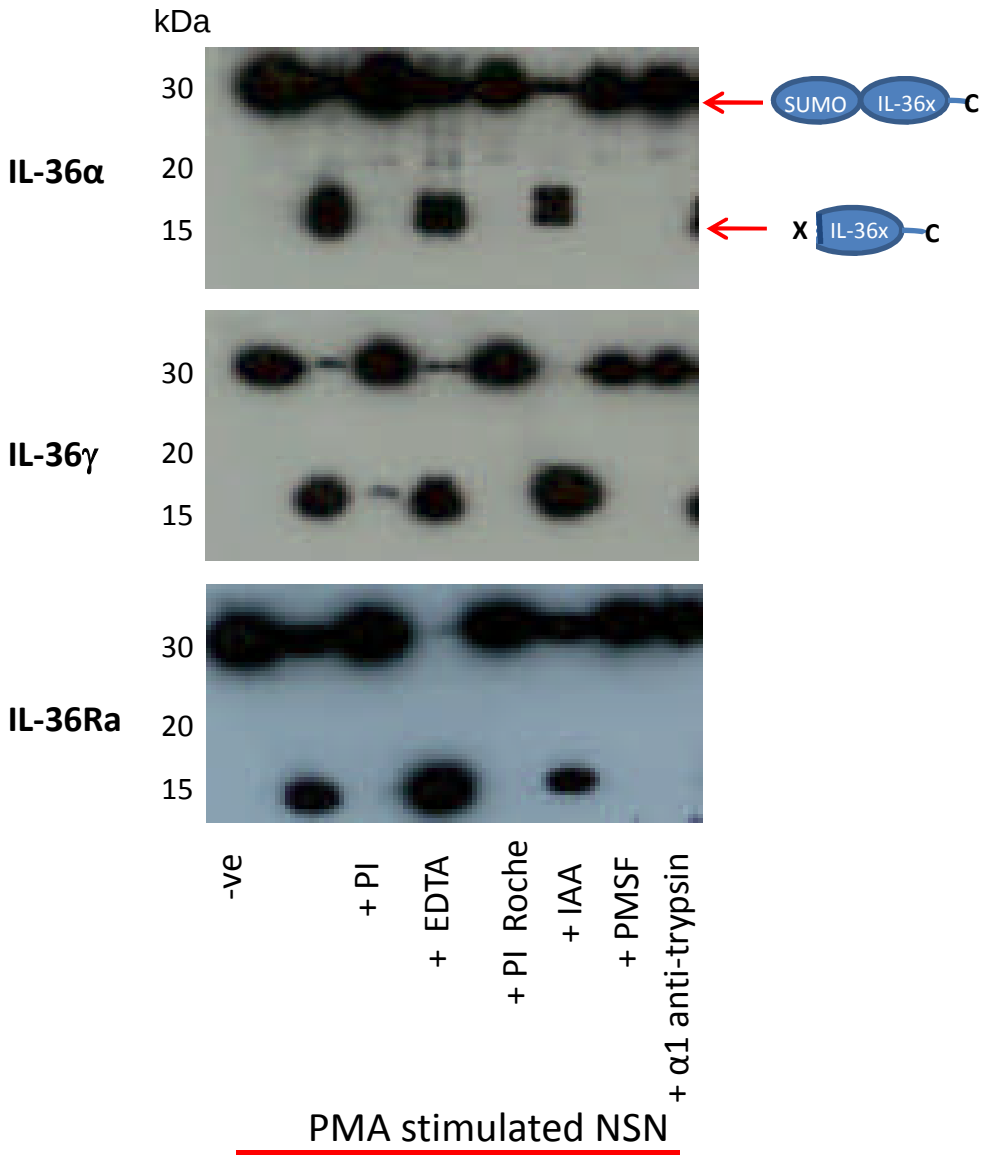


Figure 4-2: Neutrophil serine proteases truncate recombinant SUMO-IL-36 proteins

Recombinant SUMO-IL-36 proteins were incubated with PMA-stimulated PMN supernatants for 1 hour at 37°C in the presence or absence of a number of protease inhibitors; complete protease inhibitor cocktail (PI) (Roche), EDTA for the inhibition of metalloproteinases, complete ultra-protease inhibitor cocktail including aspartic protease inhibitors (PI Roche) (Roche), IAA for inhibition of cysteine proteases, PMSF and α1-antitrypsin for inhibition of serine proteases. Proteolysis was analysed by western blot using mouse monoclonal anti-IL-36α, anti-IL-36γ and anti-IL-36Ra primary antibodies (R&D Systems and Sigma-Aldrich) and HRP-conjugated anti-mouse secondary (SouthernBiotech).

4.2 Neutrophil elastase cleaves IL-36Ra to produce to produce both active and inactive IL-36Ra

Identifying neutrophil serine proteases as responsible for IL-36 cleavage refined the pool of candidate proteases. Therefore, SUMO-IL-36 proteins were incubated with a panel of recombinant serine proteases commonly expressed by neutrophils. SDS-PAGE analysis of the incubations revealed cathepsin G, neutrophil elastase and proteinase 3 all truncated IL-36Ra (Figures 4-3). Mass spectrometry analysis identified 2 major truncation products. IL-36Ra was cleaved by both cathepsin G and proteinase 3 between the third and fourth amino acid, leaving a serine at its N-terminus (IL-36Ra S4). Neutrophil elastase, however, generated an abundant truncated protein cleaved between the first and second amino acid leaving an N-terminal valine (IL-36Ra V2) (Figure 4-3). To ensure the cleavage generated by each protease was not influenced by the N-terminal SUMO tag, cleavages were repeated using IL-36Ra FL without an N-terminal SUMO tag. Mass spectrometry analysis showed the same results.

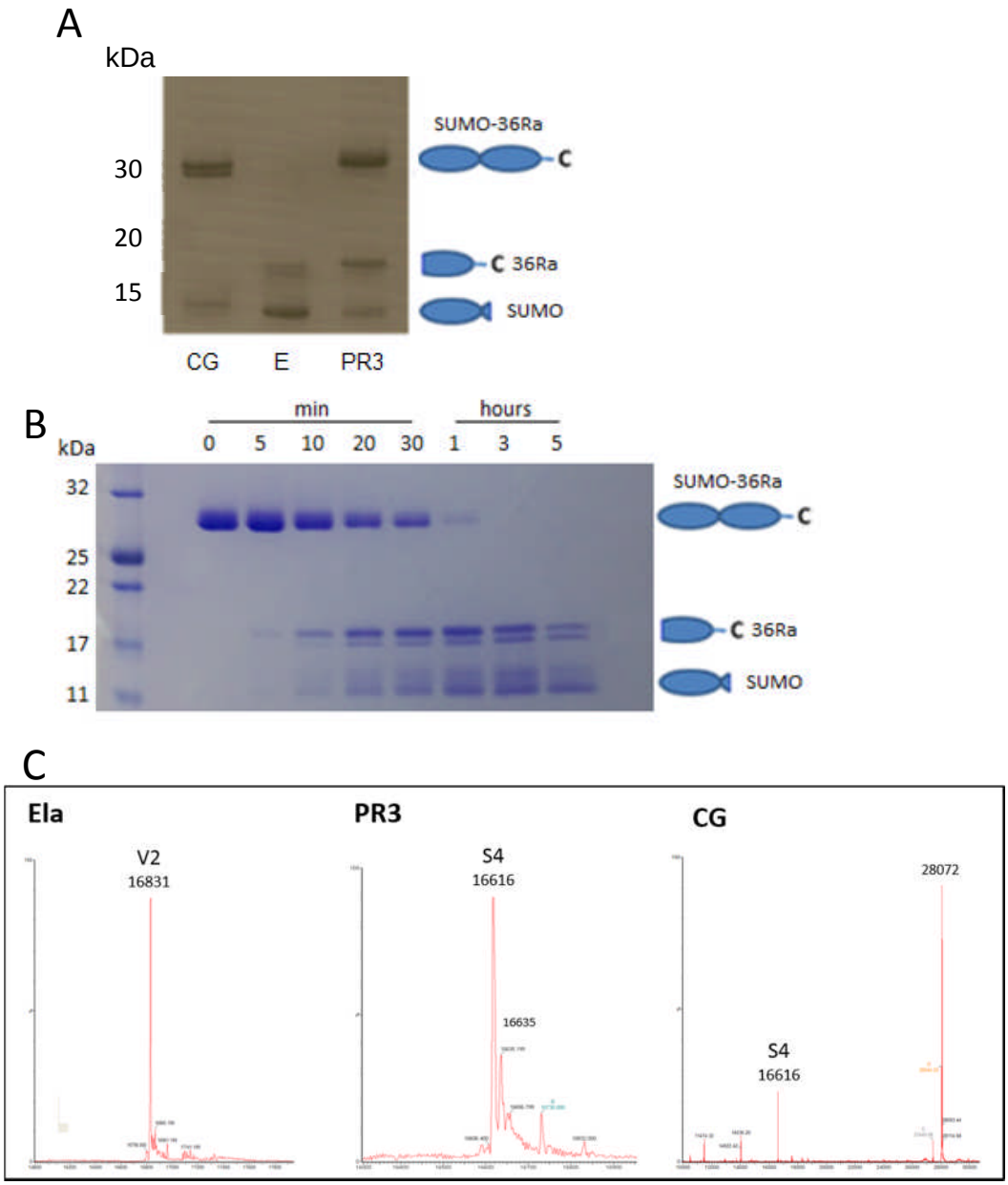


Figure 4-3: Neutrophil serine proteases efficiently truncate IL-36Ra

(A) Recombinant SUMO-IL-36Ra was incubated with recombinant neutrophil serine proteases cathepsin G (CG), elastase (E), and proteinase 3 (PR3) at a ratio of 1:100 protease:SUMO-IL-36Ra and incubated at 37°C for 1 hour. Proteolysis was analysed by Coomassie stained gel. (B) SUMO-IL-36Ra was incubated with elastase at a ratio of 1:100 protease:SUMO-IL-36Ra over a period of 5 hours at 37°C with various time points taken throughout the incubation. Proteolysis was analysed by Coomassie stained SDS-PAGE. (C) Liquid chromatography mass spectrometry result of neutrophil protease-incubated IL-36Ra showing truncation products produced by elastase (Ela), proteinase 3 (PR3) and cathepsin G (CG). Numbers denote mass of truncations whilst letters indicate the truncations' starting amino acids.

The IL-36Ra S4 truncation has an N-terminus that is 7 amino acids upstream from the A-X-D motif and therefore would not be predicted to be active. The IL-36Ra V2 truncation, however, produces an N-terminus precisely 9 amino acids upstream of the A-X-D motif generating a protein that would be predicted to have antagonistic activity. In order to test the biological activity of each IL-36Ra truncation compared to IL-36Ra FL, recombinant IL-36Ra V2 and IL-36Ra S4 were generated and used to stimulate IL-36-responsive cells. Primary keratinocytes were stimulated with active IL-36 agonists with or without IL-36Ra FL, IL-36Ra V2 and IL-36Ra S4 for 48 hours. Cell supernatant IL-8 concentration tested by ELISA was used as a biological read-out of IL-36 activity. As predicted, neither IL-36Ra FL nor IL-36Ra S4 showed any antagonistic activity. IL-36Ra V2, however, significantly reduced IL-36-mediated secretion of IL-8 (Figure 4-4). This strongly suggests neutrophils may be capable of activating IL-36Ra through the secretion of neutrophil elastase.

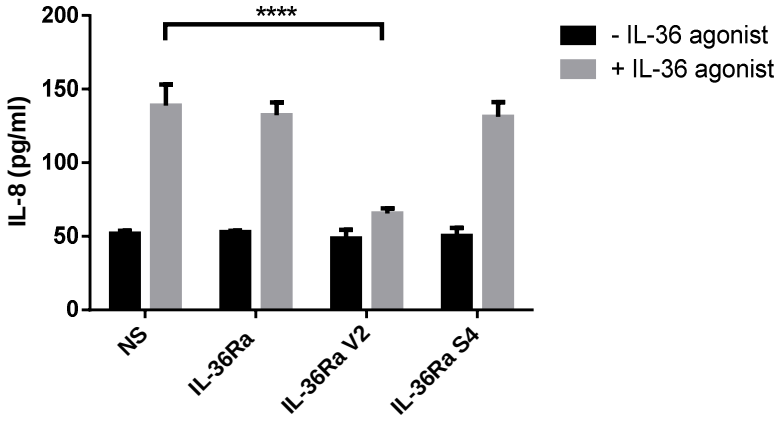
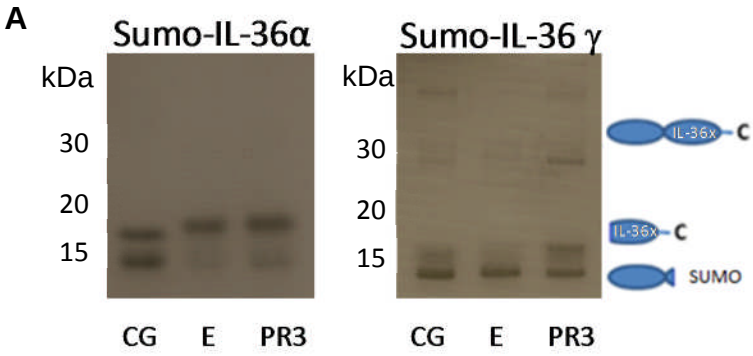


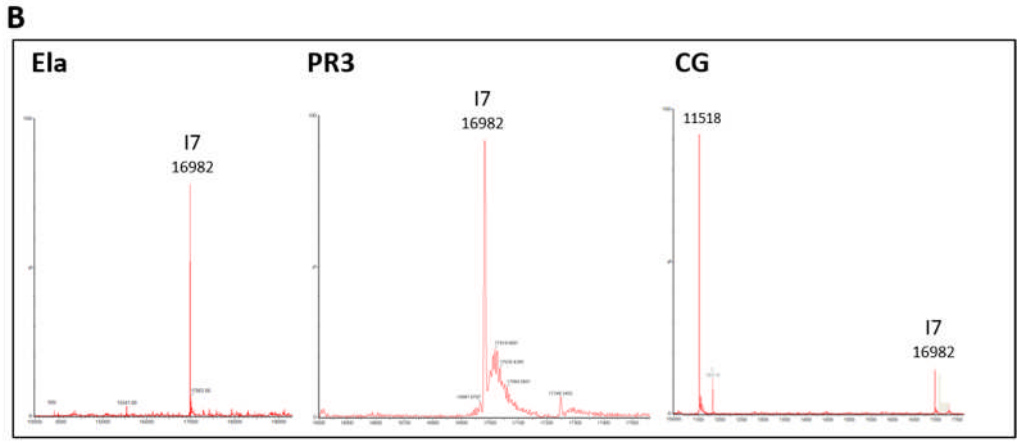
Figure 4-4: Antagonistic activity of neutrophil serine protease IL-36Ra truncations

Human primary keratinocytes were grown to a confluence of 70% before stimulation with IL-36Ra FL, IL-36Ra V2 or IL-36Ra S4 at 100 nM in the presence or absence of 10 nM active IL-36 agonist. IL-36 activity was assessed after 48 hours by measuring supernatant IL-8 concentration by ELISA. A one-way ANOVA was used to determine statistical significance of differences between treatment groups. ****, $p < 0.0001$. Data shown are mean ± S.E. ($n = 3$)

4.3 Neutrophil-mediated proteolysis of IL-36 agonists produces inactive truncations

As shown in Figure 4-5, IL-36α and IL-36γ are also truncated by cathepsin G, neutrophil elastase and proteinase 3. Mass spectrometry analysis of the truncations showed cathepsin G, neutrophil elastase and proteinase 3 all cleaved IL-36α between the sixth and seventh amino acid leaving an N-terminal isoleucine (IL-36α I7). Proteinase 3 and cathepsin G truncated IL-36γ between 15^{th} and 16^{th} and the 16^{th} and 17^{th} amino acids respectively, producing IL-36γ Y16 and IL-36γ Q17, whilst neutrophil elastase generated both truncations (Figure 4-5).


A
Sumo-IL-36α
Sumo-IL-36 γ
kDa
30
20
15
CG
E
PR3
IL-36x
C
SUMO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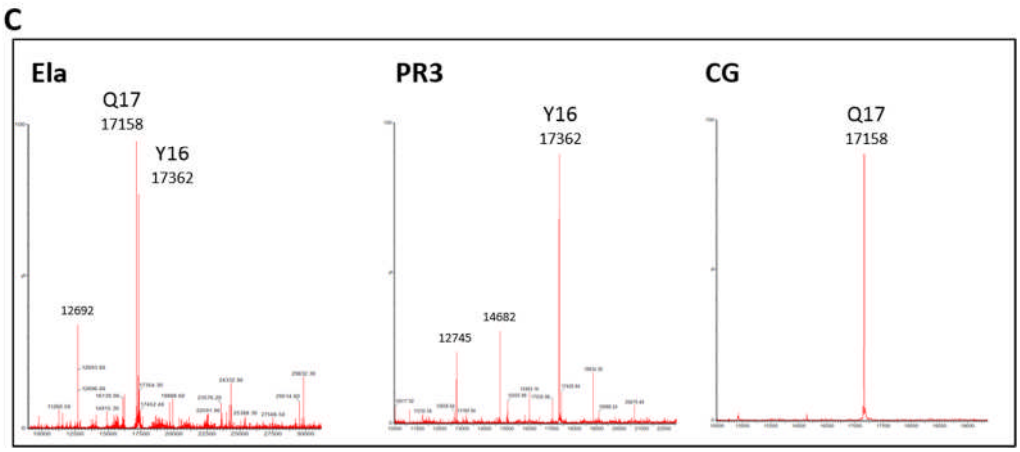
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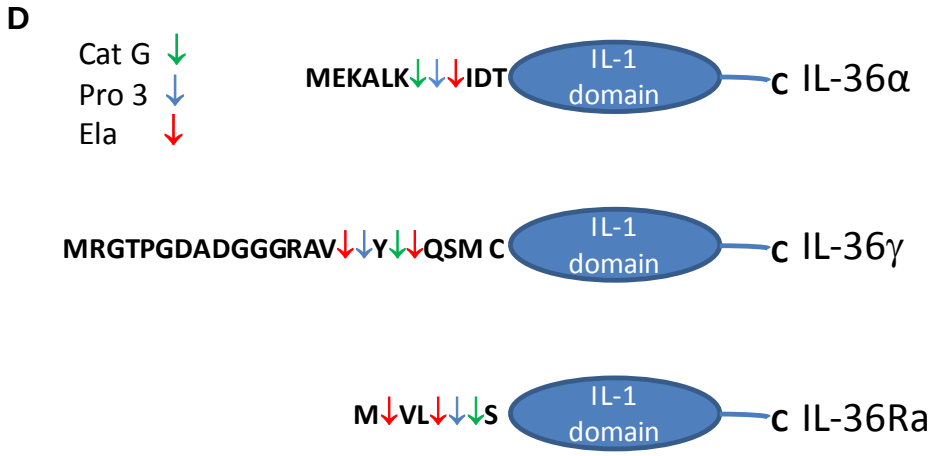
D
Cat G
Pro 3
Ela
MEKALKIDT
IL-1 domain
C IL-36α
MRGTPGDADGGGRAVYQSM C
C IL-36γ
MVLS
C IL-36Ra

Figure 4-5: Proteolytic processing of IL-36 cytokines by neutrophil serine proteases

(A) Recombinant SUMO-IL-36α and SUMO-IL-36γ were incubated with recombinant neutrophil serine proteases cathepsin G (CG), elastase (E), and proteinase 3 (PR3) at a ratio of 1:100 protease:SUMO-IL-36 and incubated at 37°C for 1 hour. Proteolysis was analysed by Coomassie stained gel. Mass spectrometry results of IL-36α (B) and IL-36γ (C) after 1 hour incubation with neutrophil proteases. Numbers denote mass of truncations whilst letters indicate the truncations' starting amino acids. (D) Diagram to illustrate the sites at which each neutrophil serine protease truncates the IL-36 cytokines as identified by liquid chromatography mass spectrometry. Arrows show cleavage position whilst colour denotes the cleaving protease, as shown in the key.

The truncations produced by these neutrophil serine proteases would not be predicted to be biologically active. As mentioned above, in order to produce biologically active IL-36 cytokines cleavage must occur precisely 9 amino acids upstream of the A-X-D motif [99]. For IL-36α this means cleavage between amino acid five and six to produce IL-36α K6, yet the proteases tested here produce the truncation IL-36α I7. For IL-36γ cleavage must occur between amino acids 17 and 18 to produce IL-36γ S18, yet the truncations produced by the neutrophil serine proteases are IL-36γ Y16 and IL-36γ Q17. By producing recombinant IL-36α I7, IL-36γ Y16 and IL-36γ Q17 and comparing them against full length agonists and the active IL-36α K6 and IL-36γ S18, it is shown that the truncations produced by these neutrophil serine proteases indeed have little or no agonist activity (Figure 4-6). These results show that whilst neutrophils are capable of cleaving IL-36 cytokines, they may not mediate their activation.

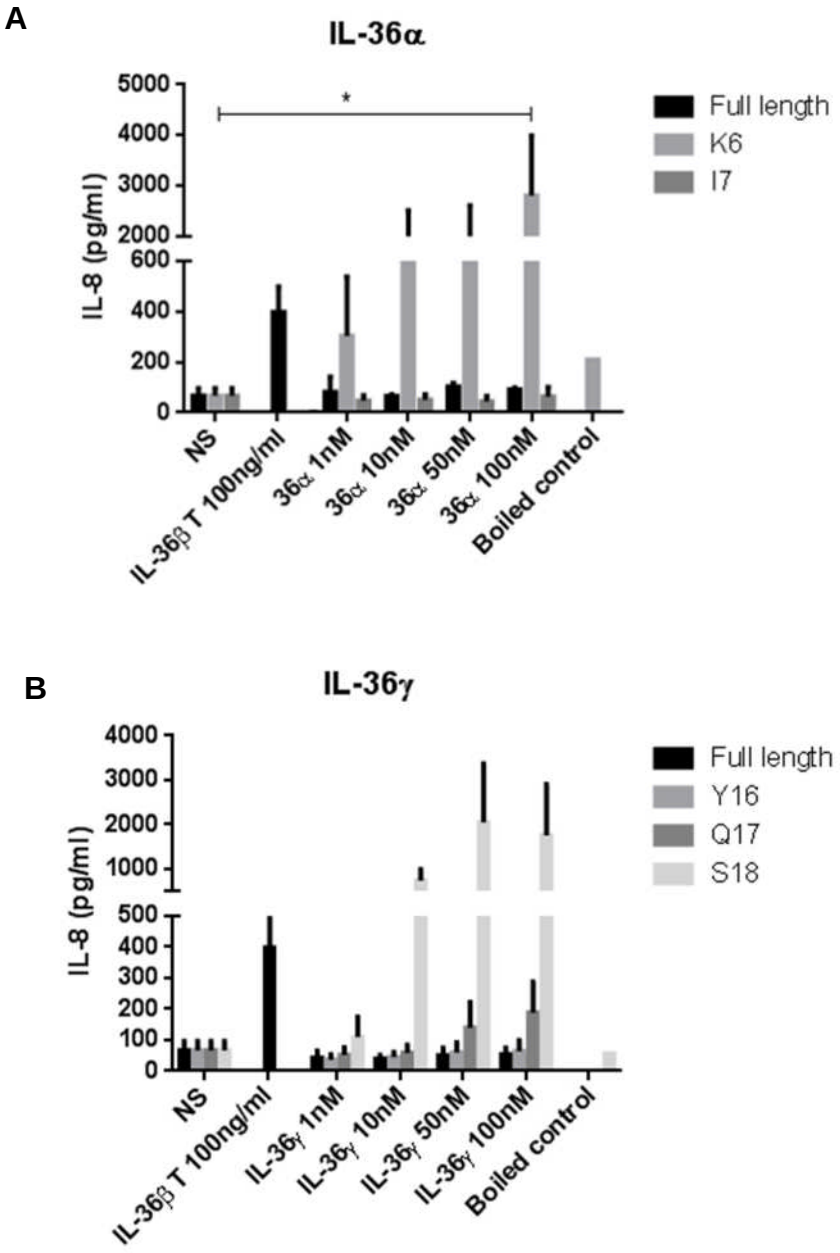

Figure 4-6: Biological activity of IL-36 agonist truncations produced by neutrophil serine proteases

Human primary fibroblasts were grown to a confluency of 70% before stimulation with the truncated IL-36 agonists generated by neutrophil serine proteases. (A) The biological activity of IL-36α I7, IL-36α FL, and the active IL-36α K6 were compared. (B) The biological activity of IL-36γ Y16, IL-36γ Q17, IL-36γ FL, and active IL-36γ S18 were compared. 100 ng/ml of active recombinant IL-36β was used as a positive control. Boiled controls were included to determine samples were free of contaminating LPS. IL-36 activity was assessed after 48 hours by measuring supernatant IL-8 concentration by ELISA. A one-way ANOVA was used to determine statistical significance of differences between treatment groups. *, $p < 0.05$. Data shown are mean ± S.E. (n = 3)

4.4 IL-36γ-activating protease is expressed within skin-resident cells

As previously mentioned, proteases are not only secreted but are also present intracellularly. Certainly, much IL-1 family proteolysis takes place within the cell prior to secretion of the processed protein. The IL-36 agonist-activating proteases may have been missed in previous experiments as only secreted proteases were interrogated.

Indeed, during the course of this research Henry *et al.* demonstrated that IL-36 agonists can be activated by incubation with neutrophil lysates [134]. Despite not identifying IL-36 cleavage products, a sensitive biological activity assay was employed to demonstrate IL-36 activity and subsequently identify the proteases involved in activation. As the cleavage via neutrophil proteases was not particularly efficient we hypothesised that the primary activator of IL-36 agonists would be present within the epithelial-type cells that express IL-36 agonist rather than infiltrating neutrophils. To test this, full length agonists were incubated with A549 cell lysates and IL-36 activity was examined by a biological reporter assay.

A549 cells were lysed in a hypotonic buffer, and lysates were added to the HaCaTs with or without IL-36α FL or IL-36γ FL, and IL-8 was used as a read out of IL-36 activity. When either A549 lysates or full length IL-36 agonists were added independently to HaCaTs, no increase was seen in IL-8 secretion. The same was true when IL-36α FL was added with A549 lysate, however when IL-36γ FL and A549 lysates were added together a modest yet significant increase in IL-8 secretion was observed (Figure 4-7). This is suggestive of an IL-36γ-activating protease present within A549 cells. Indeed, this increase in IL-8 secretion is ablated by the addition of IL-36Ra, indicating the observed IL-8 increase is dependent on IL-36 signalling.

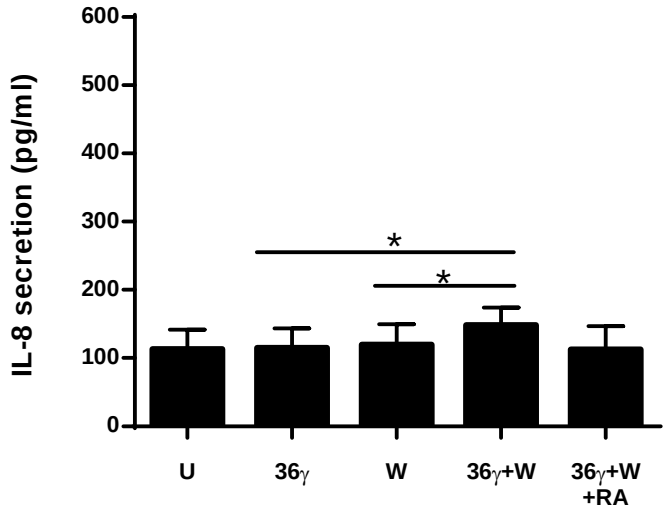

Figure 4-7: A546 whole cell lysates activate IL-36γ

HaCaTs were grown to a confluence of 90% before adding 50 nM IL-36γ FL (36γ), A549 whole cell lysates (W) or a combination of whole cell lysates and IL-36γ FL with and without 500 nM IL-36Ra (RA) for 24 hours. IL-36 activity was assessed by measuring supernatant IL-8. A one-way ANOVA was used to determine statistical significance of differences between treatment groups. *, $p < 0.05$. Data shown are mean ± S.E. (n = 3)

In order to further define the A549 lysate-mediated activation of IL-36γ, lysates were separated into their lysosomal and cytosolic fractions (as detailed in methods section 2.2.8) and activity assays were repeated with each fraction. Successful fractionation of cell lysates was confirmed through LAMP1 (lysosome) and GAPDH (cytosol) staining. The addition of cytosolic A549 fraction and IL-36γ FL produced no increase in IL-8 secretion when compared to the addition of each independently. However, as shown in Figure 4-8, addition of the lysosomal fraction with IL-36γ FL generated a large significant secretion of IL-8 relative to each independently. Again, this increase can be greatly reduced by addition of IL-36Ra indicating an IL-36-dependent IL-8 increase.

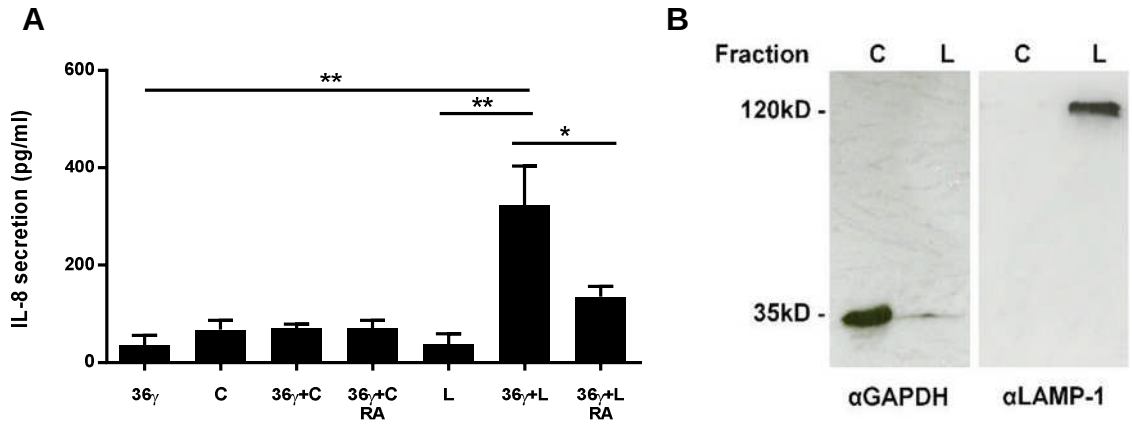

Figure 4-8: A549 lysosomal protease activates IL-36γ

(A) HaCaTs were grown to a confluence of 90% before addition of 50 nM IL-36γ FL (36γ), A549 cytosolic fraction (C), IL-36γ FL and cytosolic fraction in combination, with or without 500 nM IL-36Ra, A549 lysosomal fraction (L) and lysosomal fraction in combination with IL-36γ FL with and without IL-36Ra (RA) for 24 hours. IL-36 activity was assessed by measuring supernatant IL-8. (B) A549 cytosol and lysosome fractions were analysed by western blot using anti-LAMP-1 (abcam) and anti-GAPDH (Gene Tex) primary antibodies for lysosomal and cytosolic markers respectively. A one-way ANOVA was used to determine statistical significance of differences between treatment groups. *, $p < 0.05$, **, $P<0.01$. Data shown are mean ± S.E. (n = 3)

A549 cells are a cell line derived from alveolar epithelia and have been used for convenience as they grow rapidly and are easy to work with. Whilst they are a relevant cell line to work with, as lung tissue is known to express IL-36, it is important to test whether skin-derived cells also possess intracellular proteases that activate IL-36γ. Therefore lysosomal fractionations were performed on HaCaTs, primary keratinocytes and primary fibroblasts and activity assays were repeated using these skin cell fractionations. As shown in Figure 4-9, analogous to A549 lysosome fractions a strong IL-36-dependent induction of IL-8 was observed when each skin-cell lysosomal fraction was added with IL-36γ FL to HaCaTs. These results seem to suggest an IL-36γ activating proteases are commonly expressed by skin-resident cells.

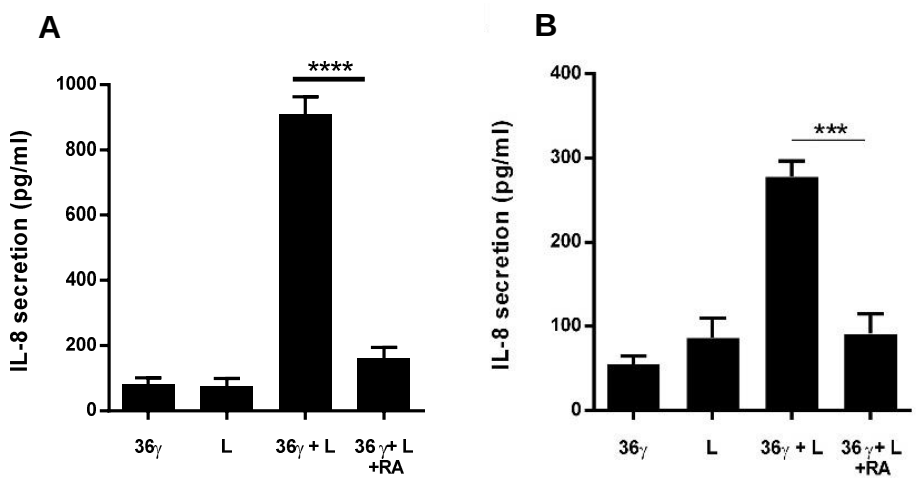

Figure 4-9: Keratinocyte and Fibroblast lysosomes contain IL-36γ-activating protease

HaCaTs were grown to a confluence of 90% before addition of 50 nM IL-36γ FL (36γ), lysosomal fraction (L) of either primary human fibroblasts (A) or primary human keratinocytes (B) alone and in combination with IL-36γ FL with and without 500 nM IL-36Ra (RA) for 24 hours. IL-36 activity was assessed by measuring supernatant IL-8. A one-way ANOVA was used to determine statistical significance of differences between treatment groups. ***, $p < 0.001$, ****, p, 0.0001. Data shown are mean ± S.E. (n = 3).

4.5 Activation of IL-36γ dependent upon lysosomal cathepsin S

As previously mentioned, a broad spectrum of proteases is expressed within the lysosome. In order to identify the protease responsible for IL-36γ activation, activity assays were repeated in the presence of the serine and cysteine protease molecular inhibitors 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) and E64 respectively. The addition of the serine protease inhibitor AEBSF had no effect on the activation of IL-36γ, however IL-8 secretion was completely ablated after addition of E64 indicating a cysteine protease is responsible for the activation of IL-36γ (Figure 4-10).

Lysosomal cysteine proteases are typically active at pH 4-5, yet the activity assays were performed at a buffered physiological pH (pH 6.8). This in itself rules out a large number of the candidate proteases and strongly implicates the cysteine protease cathepsin S as this protease has been well documented to function at a neutral pH [162, 163]. The activity assay was therefore repeated again in the presence of the specific cathepsin S inhibitor CATSi. Similarly, Figure 4-10 shows the addition of CATSi completely ablated the secretion of IL-8 observed when A549 lysosomal fractions and IL-36γ FL are added to HaCaTs.

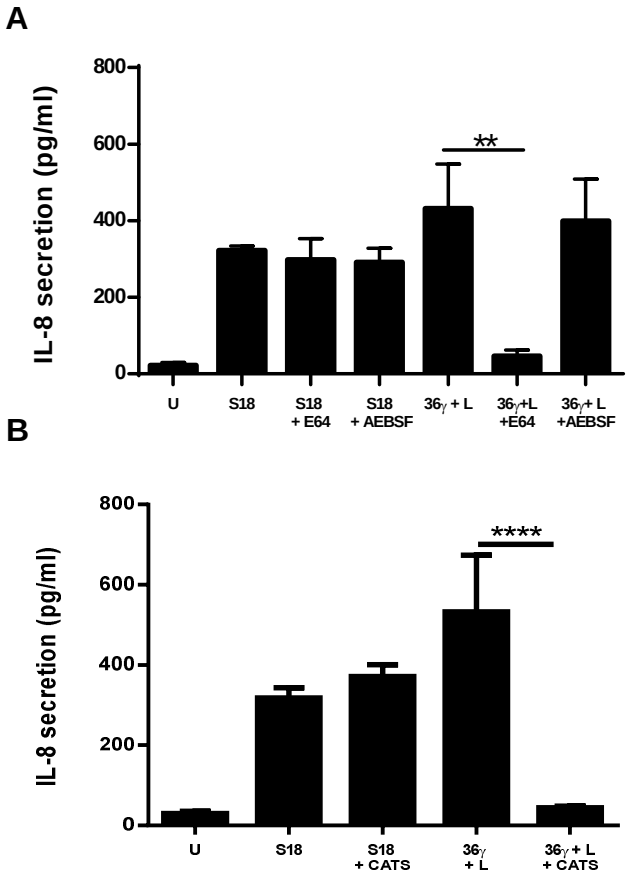

Figure 4-10: Lysosomal cysteine protease cathepsin S activates IL-36γ

(A) HaCaTs were grown to a confluence of 90% before addition of either 50 nM IL-36γ S18 (S18) or a combination of 500 nM IL-36γ FL (36γ) and A549 lysosomal fraction in the presence or absence of cysteine protease inhibitor (E64) or serine protease inhibitor (AEBSF) for 24 hours. (B) Experiment was repeated with the addition of a cathepsin S specific inhibitor (CAT S). The inhibitors were incubated with the proteins and lysosomal fraction for 2 hours at 4°C at 10 µM (E64), 100 µM (AEBSF) and 10 mM (CAT S). IL-36 activity was assessed by measuring supernatant IL-8. A one-way ANOVA was used to determine statistical significance of differences between treatment groups. **, $p < 0.01$, ****, $p < 0.0001$. Data shown are mean ± S.E. (n = 3).

The cleavage of IL-36γ FL to its active form IL-36γ S18 was further confirmed by incubating recombinant SUMO-IL-36γ FL with recombinant cathepsin S at 37°C for 24 hours. SDS-PAGE showed cathepsin S rapidly cleaved IL-36γ producing a truncated form of approximately 17 kDa. Mass spectrometry

confirmed a protein of 17031 Da, corresponding precisely to IL-36γ S18. To rule out the possibility of the N-terminal SUMO tag affecting the cleavage, this was repeated with un-tagged IL-36γ FL (Figure 4-11). The same product was identified and confirmed by N-terminal sequencing.

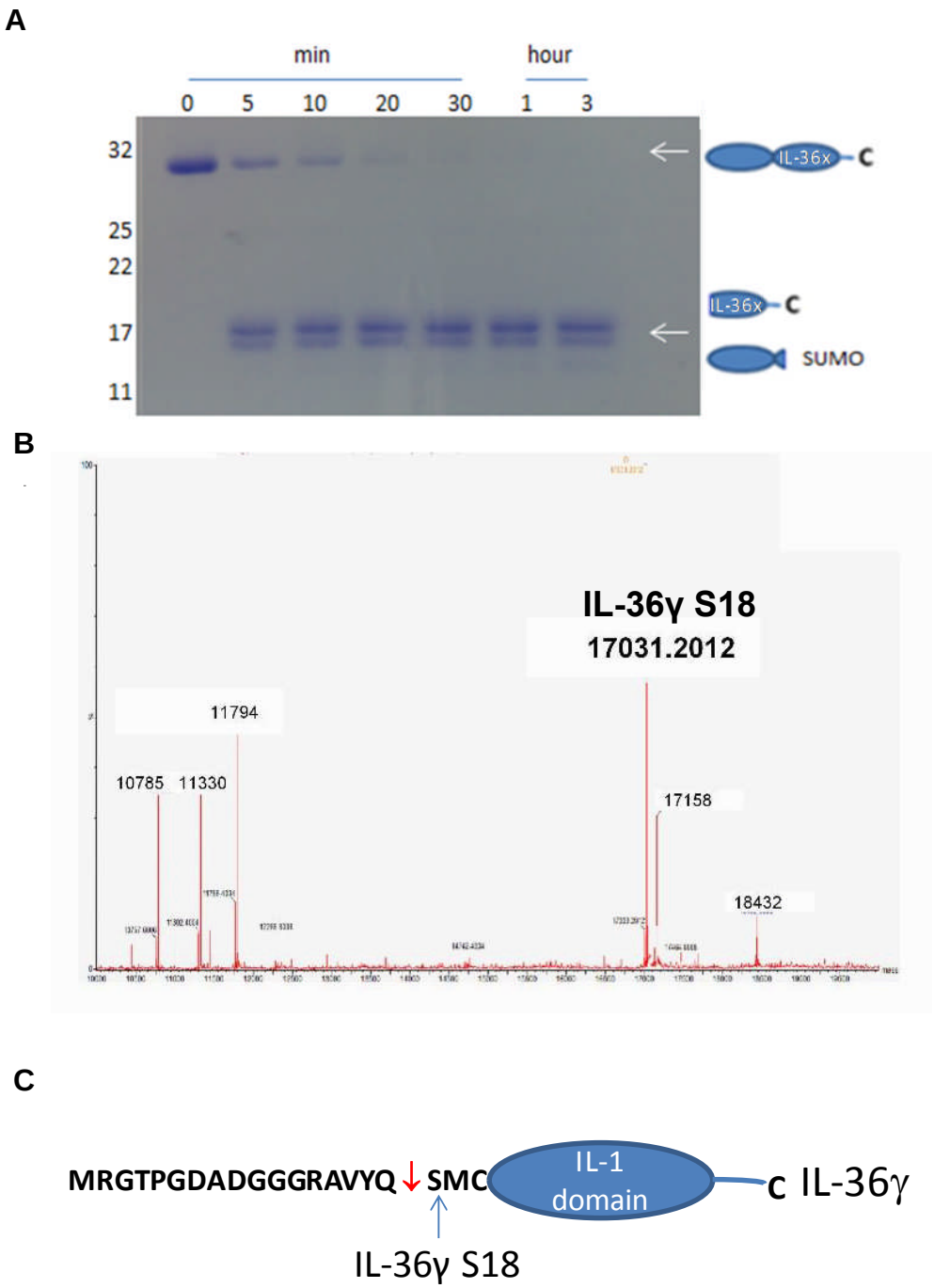

Figure 4-11: Cathepsin S truncates IL-36γ to produce active IL-36γ S18

(A) 2 µg recombinant SUMO-IL-36γ was incubated with recombinant cathepsin S at a ratio of 1:100 protease to protein at 37°C for 3 hours. Various time points were throughout the incubation and analysed by Coomassie stained gel electrophoresis. (B) Cleavage products were analysed by liquid chromatography mass spectrometry, which identified the predominant truncation as IL-36γ S18, illustrated in (C). Red arrow demarks cleavage site of cathepsin S.

4.6 Endogenous secretion of cathepsin S activates IL-36γ

Although lysosomal cathepsin S was demonstrated to activate IL-36γ, this may not necessarily be physiologically relevant as IL-36γ may not come into contact with cathepsin S within the cell. However, cathepsin S is known to be readily secreted by many cell types and thus may come into contact with IL-36γ in the extracellular environment. Therefore it was investigated whether secreted cathepsin S was capable of activating IL-36γ.

Cathepsin S was shown to be present in the conditioned media of A549 cells, primary keratinocytes and primary fibroblasts after 24 hours of culture at 100% confluence in 6 well plates. HaCaTs were then incubated with DTT-supplemented conditioned media from each cell type with 10 nM IL-36γ FL, in the presence or absence of 50 nM IL-36Ra. IL-8 secretion was significantly increased by the addition of both IL-36γ FL and conditioned media from all cell types (Figure 4-12).

To determine whether the observed IL-36γ FL processing by conditioned media was mediated by cathepsin S, siRNA was utilised to specifically down-regulate the expression of cathepsin S in A549 cells. Cathepsin S secretion was significantly reduced after transfection with cathepsin S siRNA when compared to non-transfected and scrambled siRNA transfected cells (Figure 4-12). Repeating the activity assays showed that the decrease in cathepsin S secretion seen by cathepsin S siRNA transfected cells coincided with a significant decrease in IL-36γ FL activity, showing a dependency on cathepsin S for IL-36γ FL processing. These results indicate secreted cathepsin S is capable of activating IL-36γ.

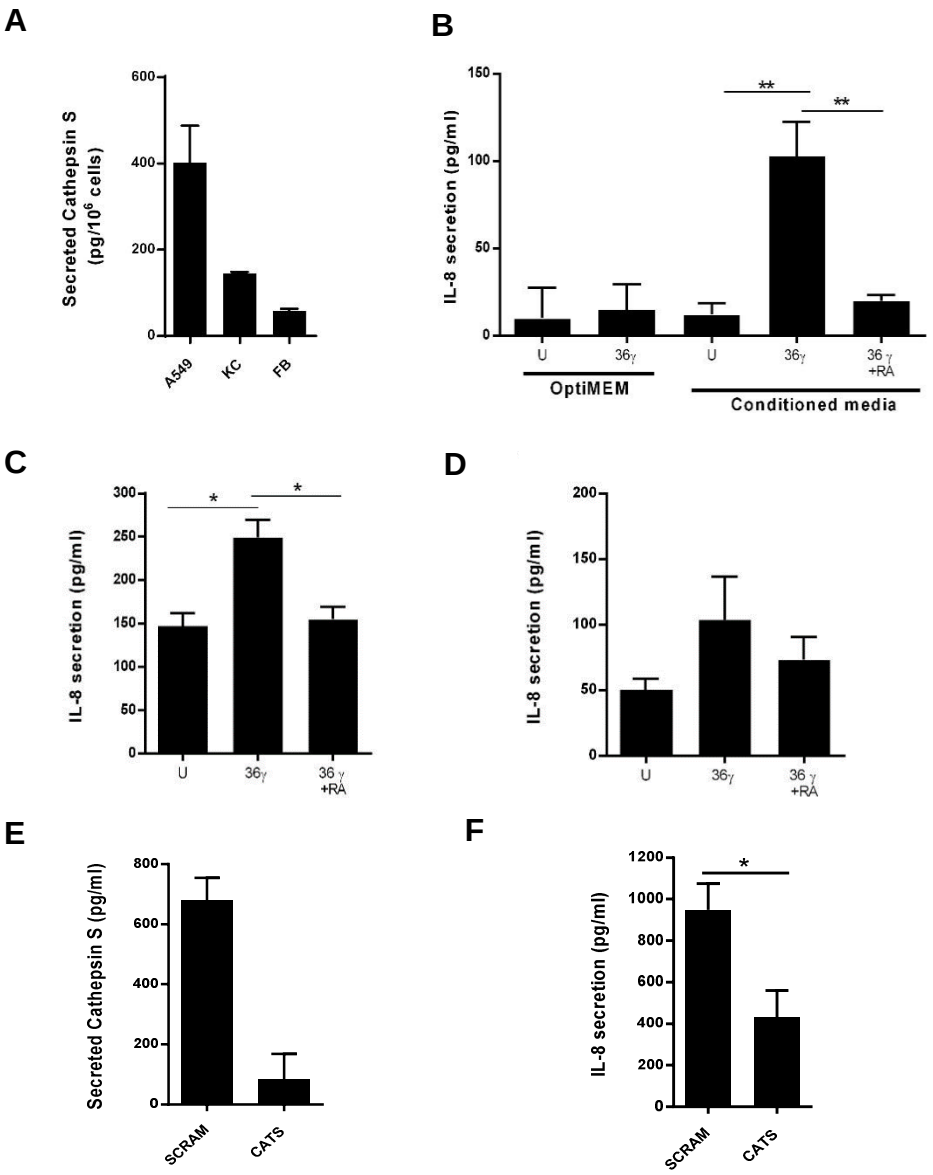

Figure 4-12: Cathepsin S secreted by epithelial cells activates IL-36γ

(A) A549 cells, primary keratinocytes, and primary fibroblasts were grown to confluence in 6 well plates and cultured for 24 hours before testing supernatants for secreted cathepsin S by ELISA. HaCaTs were grown to 90% confluency before addition of OptiMEM alone (U) or in combination with 500 nM IL-36γ FL (36γ), or the conditioned media of A549s (B), primary keratinocytes (C) or primary fibroblasts (D). Conditioned media was added alone (U) or in combination with 500 nM IL-36γ FL (36γ) with and without 500 nM IL-36Ra (RA). A549 cells were transfected with 25 nM cathepsin S siRNA (CATS) to knock down cathepsin S expression, or 25 nM scrambled siRNA (SCRAM) for a negative control, and cultured for 24 hours before removal of conditioned media. Cathepsin S level of transfected cell conditioned media was tested by ELISA (E) before being added to 90% confluent HaCaTs in combination with 500 nM IL-36γ FL (F). HaCaTs were incubated for 24 hours after addition of proteins. IL-36 activity was assessed by measuring supernatant IL-8. A one-way ANOVA was used to determine statistical significance of differences between treatment groups. *, $P < 0.05$. **, $p < 0.01$. Data shown are mean ± S.E. (n = 3).

4.7 Neutrophil proteases have a regulatory effect on IL-36γ activity

The results generated in this chapter have demonstrated that cathepsin S released by skin-based cells cleaves and activates IL-36γ, and that whilst neutrophil serine proteases are also capable of processing IL-36γ the truncations generated are predominantly inactive. However there is evidence in the literature to suggest neutrophil serine proteases are capable of activating IL-36γ to induce IL-36-mediated inflammation [134]. It therefore seemed appropriate to evaluate and compare the activating ability of neutrophil serine proteases and cathepsin S.

IL-36γ FL was incubated with either cathepsin S alone or cathepsin S with neutrophil serine proteases at a number of different molar ratios at 37˚C for 1 hour and then added to HaCaTs for 24 hours. IL-36-mediated signalling was then assessed by measuring IL-8 secretion. In doing so it was observed that at a ratio of 1 ng of protease to 100 ng IL-36γ FL the addition of neutrophil serine proteases to cathepsin S and IL-36γ FL reduces the subsequent IL-36-dependent secretion of IL-8 (Figure 4-13). This suggests neutrophil serine proteases have a more regulatory impact than inflammatory when involved in the proteolysis of IL-36γ.

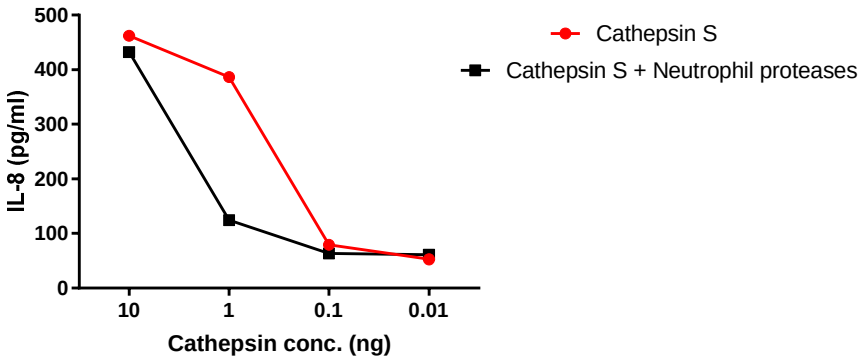

Figure 4-13: Neutrophil serine proteases reduce the IL-36γ activating potential of cathepsin S

1 µg IL-36γ FL was incubated with 0.01 – 10 ng of cathepsin S alone, or in combination with the molar equivalent neutrophil elastase, cathepsin G, and proteinase 3. IL-36γ FL was incubated with proteases for 1 hour at 37°C before addition to 90% confluent HaCaTs for 24 hours. IL-36 activity was assessed by measuring IL-8 secretion.

4.8 Discussion and Future work

The IL-1 family, consisting of 11 cytokines with a multitude of distinct functions, has been well established as of huge importance in innate immunity and inflammatory conditions [164]. As the activity of IL-1 cytokines is heavily reliant upon proteolytic processing, the IL-1 regulating proteases are therefore of equal importance. To have an in depth understanding of the role IL-1 family cytokines play in inflammation it is imperative to consider their proteolytic regulation. This is illustrated in cases where the function of IL-1-regulating proteases are affected, such as a dysregulated activation of caspase-1 leading to increased amounts of active IL-1β secretion that can result in cryopyrin-associated periodic syndromes [165]. The correct balance of inflammation and regulation needs to be maintained for an effective immune response and resolution to occur. In cases where the regulatory balance is lost, such as in DIRA and DITRA, severe systemic life-threatening inflammation can result [166]. It is therefore vital to understand the dynamics of how IL-1 cytokine activity is affected by proteolytic processing.

While proteolytic activation and regulation has been identified in many members of the IL-1 family, few advances have been made regarding the proteolytic processing of the IL-36 cytokines. It has been demonstrated that precise cleavage must occur for their activation, but until now there has been little convincing evidence implicating the proteases responsible for their activation. Given the importance IL-36 signalling plays in the pathological inflammation of psoriasis and its emerging role in wound healing and resolution of inflammation, it is imperative to understand the dynamics of their regulation and activation to properly comprehend the role IL-36 cytokines play in innate immunity. It was thus the objective of this chapter to investigate the proteolytic processing of IL-36 cytokine members and the effects this had on IL-36-mediated inflammation.

IL-36 cytokines were initially incubated with conditioned media derived from keratinocytes, fibroblasts, and neutrophils. Thus, activated neutrophil supernatant was demonstrated to cleave IL-36 members α, γ, and Ra in the region of their N-terminal IL-1-like domains. By a series of cleavage inhibition assays it was identified that neutrophil serine proteases cathepsin G, proteinase 3 and neutrophil elastase were responsible for cleavage, yet only neutrophil elastase cleavage of IL-36Ra produced a biologically active cytokine. The truncation products of the IL-36 agonists IL-36α and IL-36γ produced by neutrophil cleavage showed no – or very limited – biological activity. However, through the use of a biological activity assay it was demonstrated that lysosomal cathepsin S contained within epithelial cells was capable of activating IL-36γ and that, furthermore, cathepsin S secreted by primary keratinocytes, fibroblasts and A549 cells was able to activate IL-36γ and induce IL-36-mediated signalling.

Cathepsin S is a lysosomal cysteine protease that is primarily expressed by antigen presenting cells with a central role in antigen presentation. However, its expression is also present in human keratinocytes and can be strongly induced by stimulation with interferon-γ [167]. Unlike many lysosomal cysteine proteases, which require an acidic pH and reducing environment for optimal activity, cathepsin S is stable at a neutral pH [162]. Indeed whilst most lysosomal cysteine proteases become irreversibly deactivated after

release from the lysosome due to an increase in pH, cathepsin S retains its activity [163]. Intriguingly, when considering the expression of IL-36γ, cathepsin S is found expressed at much higher levels in psoriatic keratinocytes than in healthy. Furthermore the expression of cathepsin S in psoriatic keratinocytes is inducible by stimulation with TNFα, which also leads to cathepsin S secretion [168]. This is likely significant with regards to IL-36-mediated inflammation in psoriasis, given that the work in this chapter identified cathepsin S as a primary activator of IL-36γ. Due to an abundance of inflammatory cytokines in the epidermis of psoriatic tissue, it seems likely there should be increased levels of extracellular cathepsin S and thus increased activation of IL-36γ (which was demonstrated in chapter 3 to be present at significantly high levels in psoriatic epidermal tissue). Therefore cathepsin S is likely a central mediator of IL-36γ-driven inflammation in psoriatic tissue.

The release of IL-1 cytokines into the extracellular environment is a complex and poorly understood process. As the IL-1 family cytokines lack a signal peptide they are not channelled into the secretory pathway and thus must be released via non-classical means. Many mechanisms of release have been proposed, the most well established being release of IL-1β and IL-18 after activation of the inflammasome with intracellular protein processing a prerequisite for secretion [169]. However, for those members that are not internally processed the mechanisms involved in cytokine release are ill-defined and difficult to dissect. Despite this, it is well established that IL-1 members can act as DAMPs following release into the extracellular environment after necrotic cell death [87]. Regarding the release of IL-36 cytokines, there is very little evidence of active secretion and no evidence of intracellular processing, but it has been identified that IL-36γ is released upon necrotic cell death [170]. When considering that keratinocytes constitutively express IL-36γ and that this expression in strongly enhanced upon microbial insult, this may lend weight to the hypothesis that IL-36γ acts as a DAMP released in its inactive form that requires the presence of extracellular cathepsin S to initiate an inflammatory reaction.

In addition to an increased expression in psoriatic keratinocytes, cathepsin S expression has also been identified as elevated in dextran sulphate sodium-induced colitis [171]. Furthermore, through mouse knockouts, cathepsin S has been demonstrated to have an active role in the development of inflammation in trinitrobenzene sulfonic acid (TNBS) -induced colitis [172]. Given that IL-36γ expression is also increased in inflammatory bowel disease it seems logical that cathepsin S might contribute to intestinal inflammation through activation of IL-36γ. Additionally, like IL-36γ, expression of cathepsin S has been shown to increase upon stimulation of mice with smoke condensate and respiratory syncytial virus in a model of chronic obstructive pulmonary disease [173]. The mirrored expression of both IL-36γ and cathepsin S in a number of distinct inflammatory conditions lend weight to the hypothesis that cathepsin S is a primary activator of IL-36γ.

With the notion that IL-36γ-mediated inflammation is subject to cathepsin S activity, it becomes important to consider the regulation of cathepsin S. Indeed the balance of proteases involved in the processing of inflammatory mediators and the inhibitors that regulate them is crucial to maintain a controlled inflammatory response. This is illustrated in the case of Netherton syndrome; a disorder in which loss-of-function mutations in the gene encoding lymphoepithelial Kazal-type-5 serine protease inhibitor (LEKTI) results in development of a life-threatening form of ichthyosis with associated chronic skin inflammation [174]. Without the balance of LEKTI, unchecked serine protease activity compromises stratum corneum cohesion through elevated proteolytic activity. Subsequent stratum corneum thinning occurs with associated hypernatremic dehydration which can prove fatal in severely affected infants [175, 176]. LEKTI is important in the inhibition of kallikrein-related peptidase 5 (KLK5) which can induce the production of pro-inflammatory mediators by activating protease activated receptor-2 (PAR-2). Without functioning LEKTI, over-activation of PAR-2 induces expression of T_H2 polarising cytokines and inflammatory mediators such as TNFα, which results in an inflammatory phenotype similar to atopic dermatitis [177].

It is therefore important to understand the balance between cathepsin S and IL-36γ in order to properly assess IL-36γ-mediated inflammation. A good

understanding of the regulation and activity of cathepsin S might lead to the development of new treatment strategies to control IL-36γ-mediated inflammation. Cathepsin S is regulated on a number of different levels. Firstly, through its differential expression induced upon inflammatory stimuli as mentioned above. Secondly, cathepsin S is produced as an inactive zymogen thus itself requires activation through proteolytic processing. Finally, cathepsin S is irreversibly inhibited by cystatin C [178]. So if cathepsin S is a primary activator of IL-36γ, it stands to reason that IL-36γ-mediated inflammation can be controlled through the modulation of cathepsin S regulators.

It has previously been reported that neutrophil serine proteases are responsible for contributing towards IL-36 cytokine activation, yet the results of this chapter seem to contradict this observation. It has been identified that incubation of IL-36α and IL-36γ with cathepsin G and neutrophil elastase respectively produced active truncations that induced IL-36-mediated signalling in an activity assay [134]. However, the results obtained in this chapter identified the major IL-36 agonist truncation products produced by cathepsin G and neutrophil elastase to have little or no biological activity when used to stimulate IL-36-responsive HaCaTs. This was hypothesised to be due to cleavage not occurring 9 amino acids upstream of the A-X-D motif that has been shown to be crucial for IL-36 activation by Towne *et al.* [99]. Indeed, the truncations identified for neutrophil elastase-cleaved IL-36γ by Henry *et al.* were identical to those identified in this chapter, thus would also be predicted to be inactive. Furthermore, it was demonstrated here that the addition of neutrophil serine proteases to cathepsin S and IL-36γ FL reduced IL-36γ-mediated IL-8 secretion, suggesting neutrophil serine proteases may have a regulatory effect on IL-36γ-mediated inflammation. This experiment therefore suggests an influx of neutrophils into an environment with cathepsin S and IL-36γ present would dampen IL-36γ-mediated inflammation rather than compound it. The exposure of neutrophil proteases prevented cathepsin S dependent activation of IL-36, so it is therefore conceivable that under conditions of excess neutrophil activity the inflammatory effect of IL-36 might be regulated through cleavage and degradation. Such a phenomenon

has been observed in the lungs of cystic fibrosis patients in which the presence of neutrophils degrades inflammatory mediators such as IL-6 [179]. Furthermore, neutrophil serine proteases can deactivate a number of inflammatory chemokines including macrophage inflammatory protein 1α (MIP-1α) and RANTES through proteolytic processing, so the concept of neutrophil-mediated regulation through the degradation of inflammatory mediators is already well established [180, 181]. The regulation of IL-36-mediated inflammation is further supported when considering that neutrophil elastase was shown here to activate IL-36Ra.

Furthermore, when considering the concept of IL-36 cytokines functioning as DAMPs it seems more logical that IL-36-activating proteases are constantly present, or immediately inducible, so that a reaction can be immediately mounted in response to tissue damage. Indeed, in line with this hypothesis, cathepsin S is expressed in keratinocyte lysosomes and as reported here is readily released into the surrounding medium and likely to be released upon tissue damage, thus is able to immediately activate released IL-36γ and initiate an inflammatory response [167]. Yet activation by neutrophil serine proteases would require a pre-existing inflammatory response and a chemokine gradient to facilitate an influx of neutrophils, as neutrophils are not present in the epidermis under normal healthy conditions. Furthermore, activation of the IL-36 cytokines would then require neutrophil stimulation to mediate release of serine proteases. In light of this, the hypothesis of neutrophils activating IL-36 cytokines and IL-36 cytokines acting as DAMPs do not necessarily corroborate. It might, however, be hypothesised that IL-36γ alone in the skin acts as a DAMP, whilst IL-36α and IL-36β act as traditionally pro-inflammatory cytokines. In the skin, whilst IL-36α and IL-36β are induced upon microbial insult and inflammatory stimulation, only IL-36γ appears to be constitutively expressed [108]. In analogy to IL-1α, this indicates there are cellular stores of IL-36γ, but not IL-36α or IL-36β, available for activation in the event of tissue damage. Furthermore, whereas the work here has demonstrated that neutrophil serine proteases might regulate IL-36γ-mediated signalling, it is possible they may enhance activation of the other IL-36 cytokines. Indeed, the findings reported by

Henry *et al.* did identify the predicted active form of IL-36β (IL-36β R5) after incubation with cathepsin G and proteinase 3 [134]. It is clear, either way, that neutrophils are involved IL-36 biology, but further work is required to clarify their role in IL-36-mediated inflammation. Future work should endeavour to identify IL-36α-activating proteases so that a broader picture of IL-36 activation and regulation can be established.

To conclude, the work conducted in this chapter has identified that cathepsin S is a primary activator of IL-36γ in the skin and perhaps lung tissue. Furthermore, this work demonstrated that though neutrophils are involved in the post-translational processing of IL-36 cytokines, they may act in a more regulatory fashion. Not only do they produce inactive IL-36 agonist truncations, but they also activate IL-36Ra and have been shown to reduce the signalling capacity of cathepsin S-incubated IL-36γ FL. With the culmination of this work and that conducted by others it is now possible to start building a comprehensive idea of the proteolytic regulation of the IL-36 cytokines. Proteases have now been identified that activate IL-36Ra, IL-36β, and IL-36γ, and that also regulate IL-36 signalling. Through studying these proteases a better understanding of IL-36-mediated inflammation should be attainable, and potentially further down the line manipulated for a favourable disease outcome. However, further work is still required. An activating protease is yet to be identified for IL-36α, and the dynamics between neutrophil proteases and local epithelial proteases in the regulation of IL-36 cytokines requires clarification.

Chapter 5 - Role of IL-36γ in Immune Defence

5.1 Introduction

As discussed in the introduction, whilst a lot of research is centred on the role of IL-36 cytokines in pathological inflammation, there is a limited understanding of the physiological roles of IL-36 cytokines. It has been postulated that like other IL-1 members IL-36 cytokines act as DAMPs released by mechanical damage to epithelial tissue, but evidence exists to suggest a more involved role, with implications in both wound healing and protection against invading pathogens at epithelial barriers [87, 125, 182]. Psoriatic sufferers exhibit a skin phenotype that closely resembles fighting a skin-based fungal infection, with an increased expression of AMPs, thickening of the epidermis and polarisation of a T_H1 and T_H17 phenotype [54]. The overexpression of IL-36 cytokines, a hallmark in psoriatic inflammation, produces very similar phenotypes when induced in mice [112, 143]. A recent publication has shown that IL-36 expression is induced by exposure to *Aspergillus fumigatus*, a fungal pathogen that is constantly challenging our pulmonary epithelial barrier, and suggests IL-36 may have a role in combating *A. fumigatus* infection [124]. Additionally IL-36 cytokines are inducible upon TLR stimulation. Given this information and the effect IL-36 has in the skin compartment it seemed plausible that IL-36 may also function to combat skin-based fungal infection.

5.2 IL-36α and IL-36γ offer resistance against Candida in a skin epidermis model

To further investigate the relationship between IL-36 members and skin-based fungal infection, a skin equivalence model was utilised to model a skin-based *C. albicans* infection. Collaborators in Aberdeen grew epidermal skin equivalent models in the presence or absence of IL-36α and IL-36γ for 5 days before introducing live *C. albicans* to the surface of the epidermis for 12 hours, according to the model employed by Eyerich *et al.* [183]. Models grown with active IL-36 agonists suffered less epidermal damage, as measured by LDH release and histological observation, than models grown with inactive agonists or PBS control (Figure 5-1). In addition, the colony forming units (CFU) from *C. albicans*-infected epidermal raft were reduced in the presence of active IL-36 agonists. This resistance to *C. albicans* infection demonstrated an IL-36 dependent trend as the addition of IL-36Ra ablated the effects seen in the skin models grown with active IL-36 agonists. Furthermore, skin models grown with IL-36Ra alone showed more severe epidermal damage and higher *C. albicans* CFUs than the non-treated control skin models suggesting that endogenous IL-36γ and IL-36α may have a protective effect (although IL-36 levels were not measured at the time of experiments). IL-36 appears to increase the resilience of epidermal cells, reducing the amount of cell death, and reducing *C. albicans* viability.

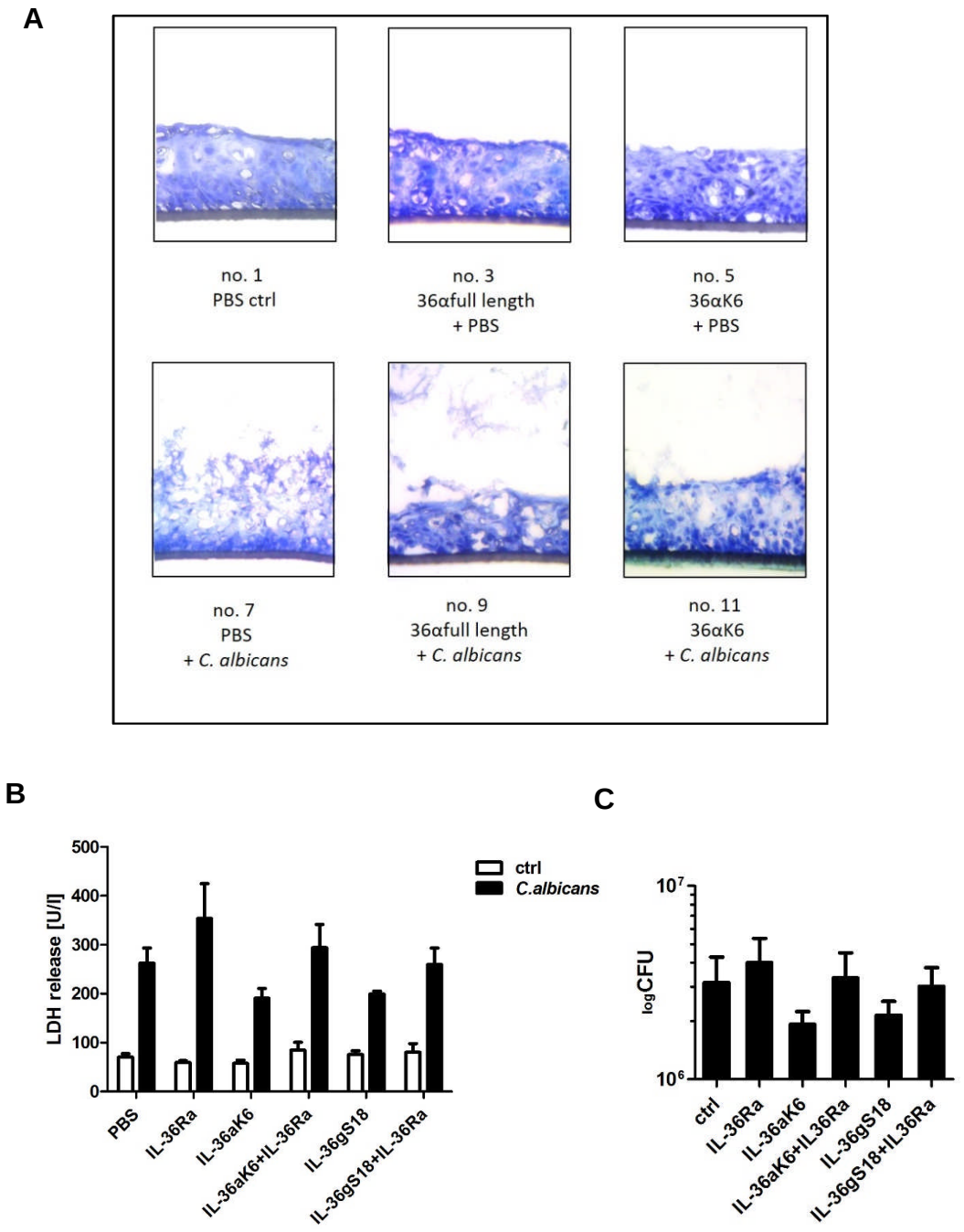

Figure 5-1: IL-36 agonists promote resistance to epidermal Candida infection

All work in this figure was conducted by collaborators at the University of Aberdeen. Epidermal skin equivalents were grown with and without active and inactive IL-36α or IL-36γ for 5 days and infected with *C. albicans* at day 5 for 12 hours (A) (IL-36α-treated models shown only). Epidermal cell death was analysed by LDH release (B) and viable *C. albicans* assessed by measuring the colony forming units (CFU) from each infected model at the end of the infection period (C).

5.3 Antimicrobial peptide expression is induced by IL-36 stimulation

Keratinocytes play a key role in the immune defence of the skin as the first point of contact to microbial intrusion. Not only do they help initiate the immune response through the release of pro-inflammatory mediators such as IL-8 and IL-1 cytokines, but they are capable of directly combating infection by secreting numerous AMPs. In psoriatic plaques, several AMPs are known to be up-regulated. Amongst them is hBD2, which has been demonstrated to have antimicrobial activity against *C. albicans* [184]. Given that the viability of *C. albicans* is reduced in skin models grown with IL-36 agonists, it could be hypothesised that IL-36 stimulation is causing secretion of AMPs that are directly killing the *C. albicans*. As such, the effect of IL-36 stimulation on secretion of hBD2 was examined.

Human keratinocytes were stimulated with IL-36γ at 50 nM with and without IL-36Ra at 500 nM for 48 hours. The concentration of hBD2 was then tested by ELISA. hBD2 levels were increased upon addition of IL-36γ agonists and reducible by the addition of IL-36Ra, indicating the increase was IL-36γ dependent (Figure 5-2).

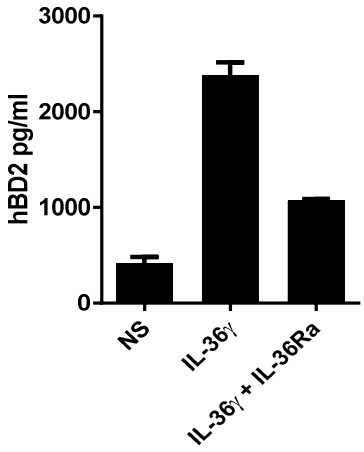

Figure 5-2: IL-36 induces hBD2 expression

Primary keratinocytes were stimulated with 50 nM IL-36γ S18 with and without 250 nM IL-36Ra V2 for 48 hours. Supernatants were removed and tested for hBD2 concentration by ELISA. Data shown are mean ± S.E. (n=2)

5.4 IL-36 promotes epidermal/keratinocyte growth

Maintaining barrier integrity is an important aspect of defence against skin-based infections. Once invading pathogens breach the epidermis they are more capable of establishing infection and causing greater tissue damage. The skin barrier function can be maintained by an increase in epidermal keratinocyte proliferation, increasing the buffer between infecting pathogens and the epidermal basement membrane. The skin models grown with IL-36 agonists appeared to suffer less damage and showed greater integrity compared to those that had either not been treated or were grown with both IL-36 agonists and antagonist. IL-36 may be helping maintain skin integrity by promoting keratinocyte growth in addition to inducing *C. albicans* killing via AMP induction. Therefore, the effect of IL-36 on keratinocyte proliferation was further investigated.

HaCaTs and human keratinocytes were grown in media with and without IL-36 agonists for 3 days. As shown in Figure 5-3, by monitoring and plotting their confluence throughout this period of growth it was noted that cells grown in the presence of IL-36α and IL-36γ grew at a faster rate than those without. Cells grown with both IL-36 agonists and antagonist grew at a rate elevated from the non-stimulated control, but at a lesser rate than those incubated with agonists alone, indicating an IL-36-dependent increase in proliferation.

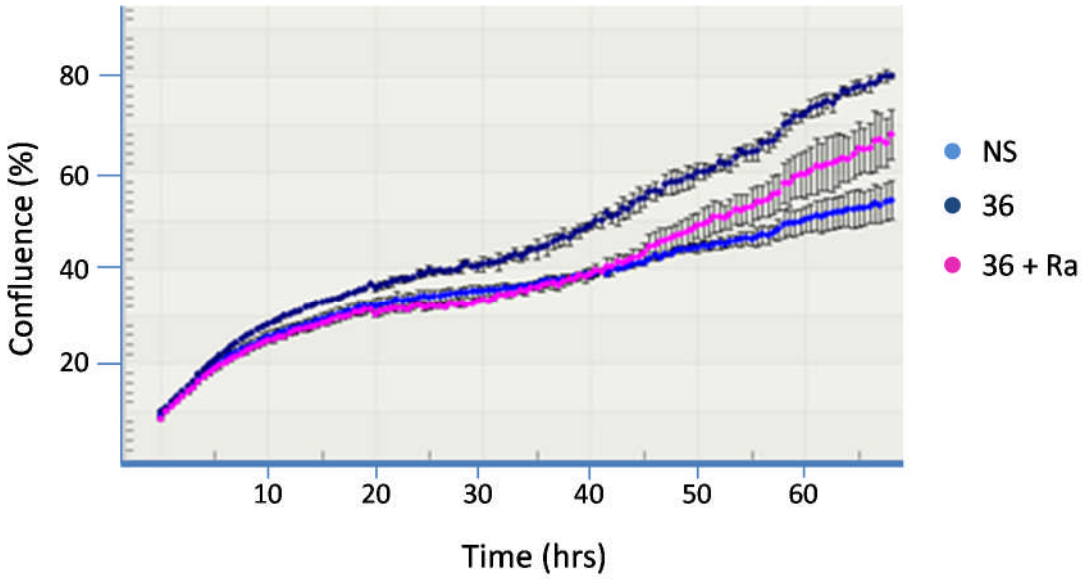

Figure 5-3: IL-36 stimulation promotes epidermal proliferation

HaCaTs were plated at 10% confluence and grown for 67 hours in the presence of 50 nM IL-36γ S18 (dark blue 36) with and without 250 nM IL-36Ra V2 (pink 36 + Ra) and compared to an untreated control (light blue NS). Confluence was measured throughout the 67 hour period by an IncuCyte and analysed by the IncuCyte Zoom software.

5.5 IL-36 increases resistance to apoptosis

As a breakdown in barrier integrity is primarily instigated through cell death, barrier integrity may also be maintained by preventing or delaying apoptosis. The *C. albicans*-infected skin models grown with IL-36 agonists suffered less cell death than those grown with both IL-36 agonists and antagonist. Whilst this may be due to the IL-36-mediated increase in epidermal proliferation and killing of the infecting *C. albicans*, IL-36 may also be influencing the survival capabilities of epidermal keratinocytes. In such a way, IL-36 may help maintain skin barrier function by delaying and resisting cell death. As such, the effect of IL-36 on apoptosis was further investigated.

Apoptosis is a highly controlled and complex programmed cell death that can be initiated in a variety of ways, but all culminate in the initiation of a caspase

cascade after activation of caspase-3. This results in DNA fragmentation, degradation of cellular and nuclear proteins, and ultimately the formation of apoptotic bodies expressing ligands that mediate uptake and clearing by phagocytic cells [185]. Given the complexity of this process, apoptosis was measured by a number of different methods at varying stages of the cascade. Typically, apoptosis is measured by an annexin V assay, however, as shown in Figure 5-4, this proved unsuitable with adherent keratinocyte cell lines as the processes required to dissociate the cells from their wells caused membrane damage that resulted in false positives. Instead, caspase-3 activation was measured via a caspase-3 activity assay and through the detection of PARP cleavage. In addition to this, apoptosis was measured by examining cellular degradation of DNA. By measuring multiple stages of apoptosis, a comprehensive picture was established.

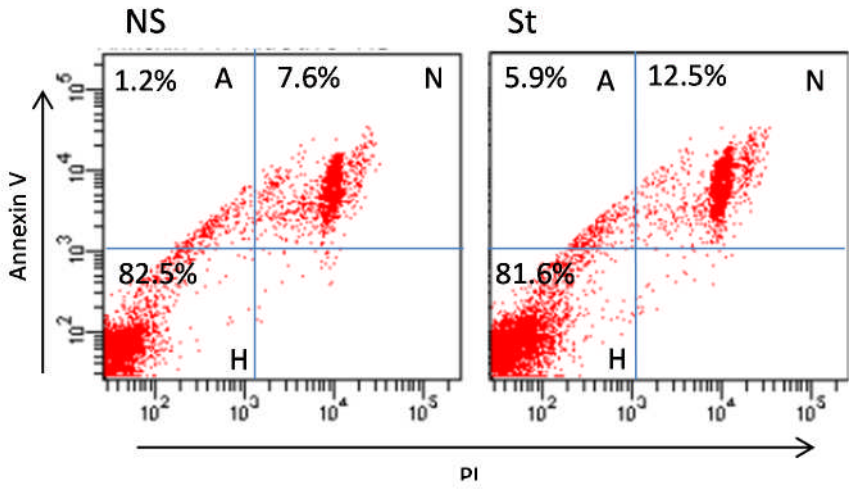

Figure 5-4: Annexin V analysis not viable for measuring apoptosis in HaCaTs

HaCaTs were grown to a confluence of 70% prior to treatment with 500 ng/ml staurosporin for 3 hours. Apoptotic and necrotic cells from non-stimulated (NS) and staurosporin treated (St) HaCaTs were analysed by flow cytometry by Annexin V expression and PI counter staining. 'H' indicates viable population, 'A' indicates apoptotic population, 'N' indicates necrotic population. Percentages of each population are displayed in their respective quadrants.

Initially, apoptosis was induced by 48 hours of serum starvation to produce a mild apoptotic state that might be rescuable, however serum starvation alone proved insufficient to induce measurable apoptosis. Therefore, HaCaTs were serum starved for 48 hours and treated with staurosporin to induce a strong measurable apoptotic response in which IL-36 activity could be assessed (detailed in methods section 2.2.10). HaCaTs were grown to a confluence of approximately 70% before addition of IL-36 proteins and initiation of serum starvation for 48 hours. Staurosporin was added at 500 ng/ml for 4 hours, with or without IL-36 proteins, immediately prior to measuring caspase-3 activity or 24 hours prior to measuring DNA degradation and PARP cleavage. As shown in Figure 5-5, the addition of staurosporin to serum starved cells produced a strong induction of caspase-3 activity, noticeable PARP cleavage, and a significant amount of DNA degradation. Figure 5-5 also shows HaCaTs starved in the presence of IL-36γ suffered approximately 30% less DNA degradation than those without IL-36γ after the induction of apoptosis. Examining PARP cleavage showed whilst cleavage was evident in both IL-36γ stimulated and non-stimulated cells, less cleaved PARP was present in those grown with active IL-36γ. Finally, 12 hours after induction of apoptosis, 15% of cells grown with IL-36γ showed caspase-3 activity compared to 20% of cells grown either without stimulation or stimulated with both agonist and antagonist. These results suggest IL-36γ may act to increase epidermal resistance to apoptosis, which in turn may help to maintain skin barrier integrity.

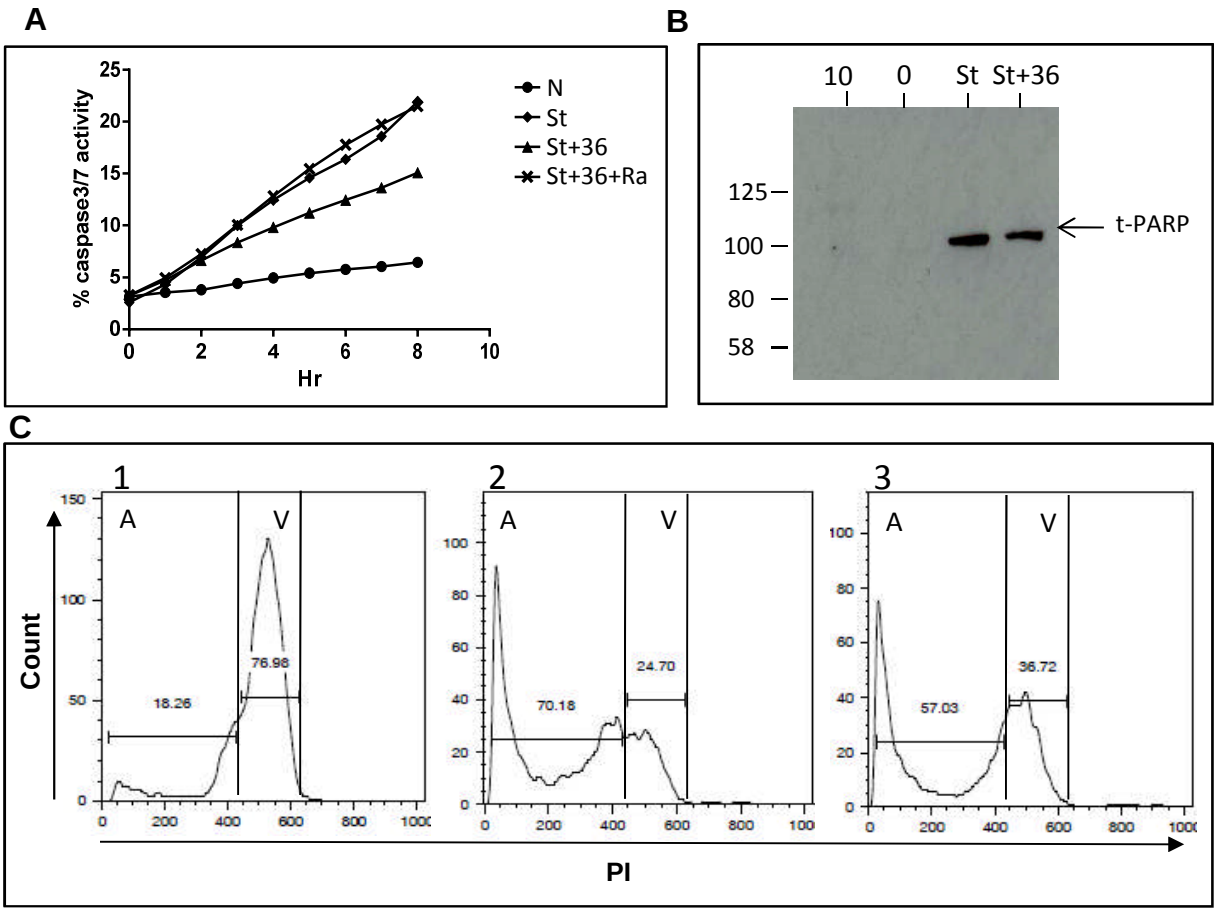

Figure 5-5: IL-36 provides resistance to staurosporin-induced apoptosis

HaCaTs were grown to 70% confluence in media containing 10% serum before undergoing 48 hours serum starvation in the presence or absence of 50 nM IL-36γ S18 with or without 250 nM IL-36Ra V2 as indicated. Cells were treated with 500 ng/ml staurosporin for 3 hours, 24 hours into the starvation period. (A) Caspase3/7 activity was analysed with CellEvent caspase-3/7 activity assay. Fluorescence was detected in real time using an IncuCyte and analysed with IncuCyte Zoom software expressed as fluorescent percentage of cell confluence. (B) Cleavage of PARP was examined by western blot analysis of HaCaT lysates using anti-cleaved PARP (Asp214) clone19F4 Mouse mAb (Human Specific) from Cell Signaling Technology. 'PARP' indicates full length PARP size, 't-PARP' indicates truncated PARP. (C) Apoptosis was analysed by visualising DNA degradation. HaCaTs were grown to 70% confluence in media containing 10% serum before undergoing 48 hours serum starvation (1). Cells were treated with 500 ng/ml staurosporin for 3 hours, 24 hours into the starvation period in the absence (2) or presence (3) of 50 nM IL-36γ S18. Nuclei were isolated from lysed cells and stained with PI before analysis by flow cytometry. After doublet exclusion, populations were gated to identify apoptotic 'A' and viable 'V' cells according to DNA degradation.

5.6 TLR stimulation induces expression of IL-36γ

In order to respond to and fight off invading pathogens, they must be recognised by the immune system. This is achieved through detection of pathogen associated molecular patters (PAMPs) by pattern recognition receptors (PRRs) [8]. As epithelial cell types are often the first point of contact for invading pathogens it is of no surprise that epithelial cell types express a broad range of PRRs [10, 186]. The most prominent group of PRRs are the toll-like receptors (TLRs) which respond to a wide range of PAMPs. As different types of pathogens require different immune responses, the PRRs will initiate a tailored response specific to the type of pathogen detected. Since it has been demonstrated that IL-36 cytokines appear to play a role in the skin-based immune defence, it seemed pertinent to see how TLR stimulation would affect IL-36 expression. Additionally, expression favoured by stimulation of bacterial, fungal or viral associated PRRs may allude to a function in immune defence.

Keratinocytes were stimulated with LPS to initiate the TLR4 pathway associated with a bacterial response, zymosan to initiate the TLR2/6 and dectin-1 pathway associated with a fungal response, and poly(I:C) to initiate a viral immune response through activation of TLR3. After incubation with the PAMPs for 48 hours, the conditioned cell media and lysates were tested for IL-36γ by ELISA. As shown in Figure 5-6, LPS stimulation appeared to have no effect on IL-36γ expression by keratinocytes. Stimulation with zymosan, however, caused a significant increase in IL-36γ expression lending supporting evidence to the hypothesis that IL-36γ is involved in fungal defence. Surprisingly, stimulation with poly(I:C) also strongly induced expression of IL-36γ suggesting that IL-36 may perhaps also be of importance in a viral immune response.

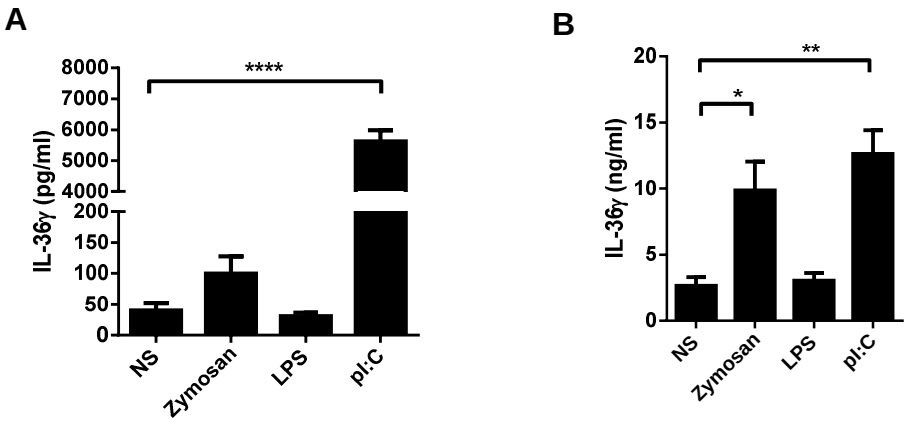

Figure 5-6: Zymosan and poly(I:C) induce IL-36γ expression in primary keratinocytes

Primary keratinocytes were cultured to 70% confluence and stimulated with 50 µg/ml zymosan, 100 ng/ml LPS and 10 µg/ml poly(I:C) for 48 hours. Keratinocyte supernatants (A) and lysates (B) were tested for IL-36γ concentration by B5A2 ELISA. A one-way ANOVA was used to determine statistically significant differences between treatment groups. *, $p < 0.05$, **, $p < 0.01$, ****, $p < 0.0001$. Data shown are mean ± S.E. (n = 3).

As demonstrated in chapter 4, cathepsin S is a protease responsible for activation IL-36γ. Whilst an up-regulation of IL-36γ may be indicative of IL-36γ-mediated inflammation, in order for inflammation to manifest the expressed IL-36γ must be activated. Thus, it is also necessary to measure the expression of IL-36γ-activating protease cathepsin S. Therefore, the same cell supernatants and lysates were also tested for cathepsin S concentration to elucidate whether the increase in IL-36γ concentration might also correlate with an increase in cathepsin S and therefore the capacity to activate the expressed IL-36γ. As shown in Figure 5-7, analogous to IL-36γ expression, stimulation with LPS caused no significant increase in cathepsin S concentration yet zymosan induced a modest increase. Interestingly again, poly(I:C) stimulation also generated a very strong production of cathepsin, with keratinocytes producing almost 10-fold more cathepsin S than in response to zymosan.

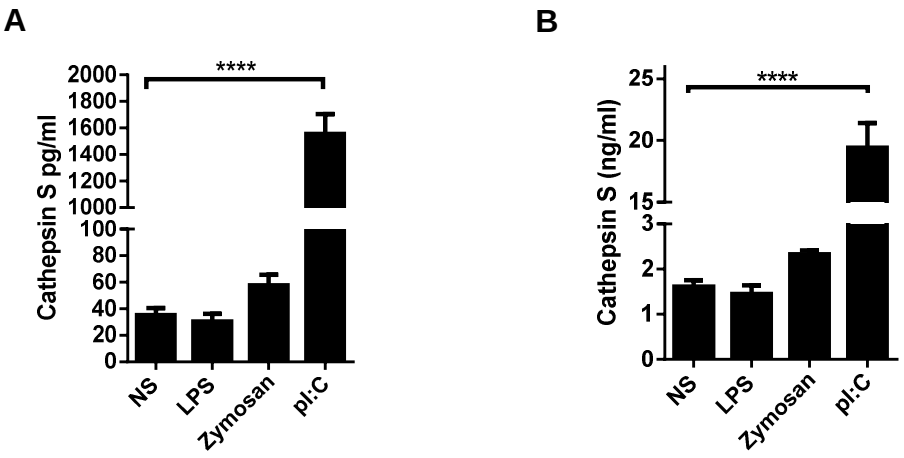

Figure 5-7: Zymosan and poly(I:C) induce cathepsin S secretion by primary keratinocytes

A – Primary keratinocytes were cultured to 70% confluence and stimulated with 100 ng/ml LPS, 50 µg/ml zymosan and 10 µg/ml poly(I:C) for 48 hours. Keratinocyte supernatants (A) and lysates (B) were tested for cathepsin S concentration by ELISA. A one-way ANOVA was used to determine statistically significant differences between treatment groups. ****, $p < 0.0001$. Data shown are mean ± S.E. (n = 3).

These observations lend weight to the hypothesis that IL-36 cytokines may be involved in fungal defence as stimulation of cells with fungal-associated PAMPs induced an increase in expression of IL-36γ and a proportional increase its activating protease cathepsin S that was not seen after stimulation with bacterial PAMPs. However, the surprising up-regulation of both IL-36γ and cathepsin S after stimulation with poly(I:C) may indicate a role in viral immune defence and the interferon response and requires further investigation.

Finally, the release of IL-36γ was examined in response to TLR activation. As, like all IL-1 members, the IL-36 cytokines lack a signal peptide they are not recruited into the secretory system and so must be subject to alternative secretory methods. For many IL-1 members secretion occurs after intracellular truncation of the immature protein, however the mechanisms of release are often ambiguous. Indeed, there is no evidence for intracellular

truncation of IL-36 cytokines and little evidence of release in response to stimulation. Therefore, the effect of TLR stimulation on release of IL-36γ was examined.

Primary keratinocytes were stimulated with zymosan, LPS and poly(I:C) for 48 hours. As the release of many IL-1 family cytokines is dependent upon inflammasome activation a second stimulus of ATP or nigericin was also administered. Supernatants and cell lysates were then collected and analysed for IL-36γ concentration and the released IL-36γ was expressed as the percentage of total IL-36γ contained within the supernatant. As shown in Figure 5-8, treatment with poly(I:C), but not zymosan or LPS, caused a significant release of IL-36γ. Furthermore, treatment with ATP and nigericin had no significant effect on secretion. These results indicate that whilst zymosan does induce expression of IL-36γ it does not cause a significant increase in its release. Poly(I:C), however, appeared to both strongly induce IL-36γ and result in its release. However, as poly(I:C) induces necrosis it is possible this release is due to cell death and further experiments are required to dissect this.

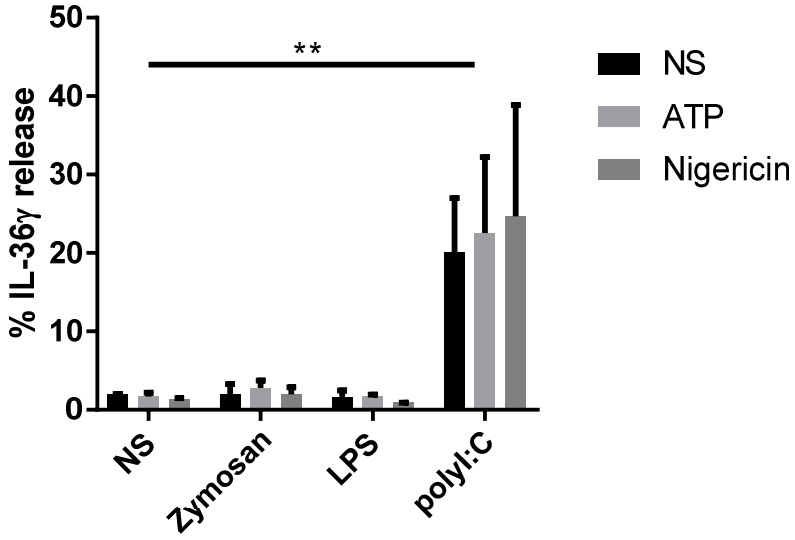

Figure 5-8: poly(I:C) stimulation, but not zymosan or LPS, causes release of IL-36γ

Primary keratinocytes were cultured to 70% confluence before stimulation with 100 ng/ml LPS, 50 µg/ml zymosan and 10 µg/ml poly(I:C) for 48 hours and then treated with either PBS, 50 µM ATP or 10 µM nigericin for 5 hours. Lysates and supernatants were tested by ELISA for IL-36γ concentration and released IL-36γ was expressed as a percentage of the total produced IL-36γ present in the supernatant. A two-way ANOVA was used to determine statistically significant differences between treatment groups. **, $p < 0.01$. Data shown are mean ± S.E. (n = 2).

5.7 *Aspergillus fumigatus* protease(s) cleave IL-36γ to its active form

It has previously been demonstrated in the literature that IL-36γ expression is increased after exposure to both live and dead *A. fumigatus*, and is therefore suggested that IL-36γ has a role to play in the immune defence against *A. fumigatus* infection [124]. As the IL-36 cytokines require post-translational processing for activation, and *A. fumigatus* produce and secrete a range of proteases, it seemed interesting to investigate the effect *A. fumigatus* proteases might have on IL-36γ activation [187].

It was first examined whether *A. fumigatus* proteases cleaved IL-36γ. SUMO tagged full length IL-36γ (IL-36γ FL) was incubated with *A. fumigatus* conditioned media (CEA10) (provided by Dr Sarah Herrick from the University of Manchester) and cleavage was analysed at various time points by electrophoresis. The results show that incubation with CEA10 cleaves IL-36γ producing products of ~17 kDa (Figure 5-9). Indeed, mass spectrometry analysis identified a cleavage product of 17029 Da, matching the mass of the active truncation IL-36γ S18 (±2 Da) (Figure 5-9).

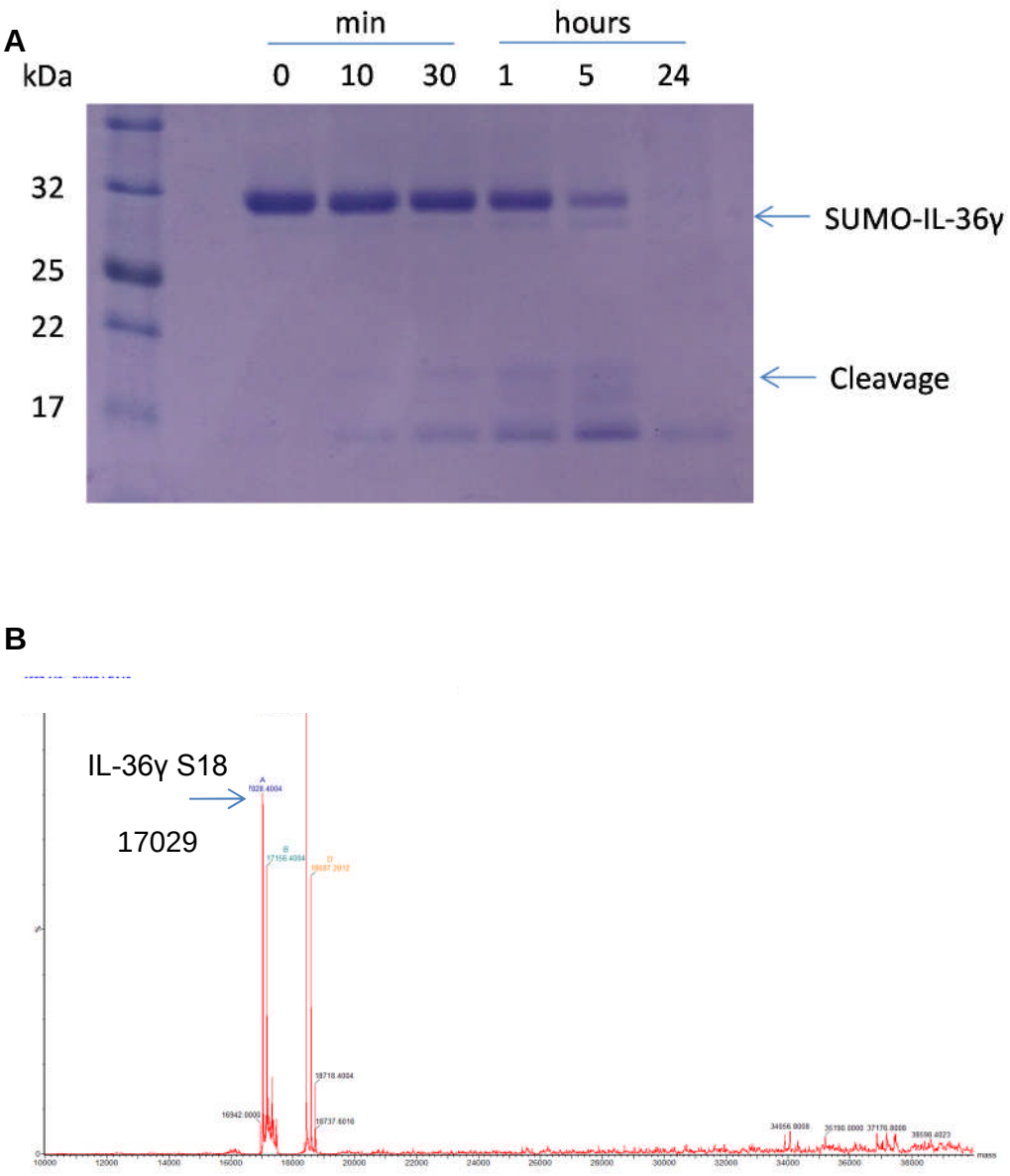

Figure 5-9: *A. fumigatus* conditioned media cleaves SUMO-tagged IL-36γ

2 µg of SUMO-tagged IL-36γ FL was incubated with 20 ng total protein from *A. fumigatus* conditioned media and incubated at 37˚C. Various time points were taken and analysed by Coomassie-stained SDS-PAGE. Arrows indicate full length fusion protein and cleaved product (A). Cleavage was repeated using untagged IL-36γ FL and cleavage products analysed by liquid chromatography mass spectrometry (B). Arrow indicates active IL-36γ S18 truncation.

Given that the active IL-36γ S18 truncation was identified by mass spectrometry after incubation with *A. fumigatus* proteases, an activity assay was performed to determine whether CEA10-mediated activation of IL-36γ could be reproduced in a bioassay and generate a measurable IL-36γ-dependent response. CEA10-incubated untagged IL-36γ FL was added to HaCaTs with or without IL-36Ra alongside non-incubated IL-36γ FL. To determine whether the *A. fumigatus* proteases might further digest and deactivate active IL-36γ, the incubation was also performed on IL-36γ S18. The proteins were incubated with CEA10 for 3 hours at 37°C before being stopped by addition of Roche total protease inhibitor and added to HaCaTs for 24 hours. The HaCaTs were then examined for IL-36γ activity by measuring IL-8 secretion. As shown in Figure 5-10, whilst non-incubated IL-36γ FL had a negligible effect on IL-8 secretion, after incubation with CEA10 there is a significant increase in IL-8. Furthermore, this increase in IL-8 secretion is completely ablated by the addition of IL-36Ra indicating the response is IL-36-dependent. When comparing CEA10-incubated and non-incubated IL-36γ S18 it is evident CEA10 does not reduce IL-36γ activity. On the contrary, a significant increase in IL-36γ activity was observed. This may be due to a priming effect from fungal PAMPs that might be contained within the conditioned media. In an attempt to dissect whether the CEA10 associated enhancement of IL-36γ S18 activity was conferred via PAMP priming, HaCaTs were primed with boiled or un-treated CEA10 for 3 hours before stimulation with IL-36γ S18. As shown in Figure 5-10, there was no noticeable difference in IL-8 secretion between primed or non-primed IL-36γ S18 stimulated cells, however this may be due to an inadequate period of contact with the cells required for the priming effect.

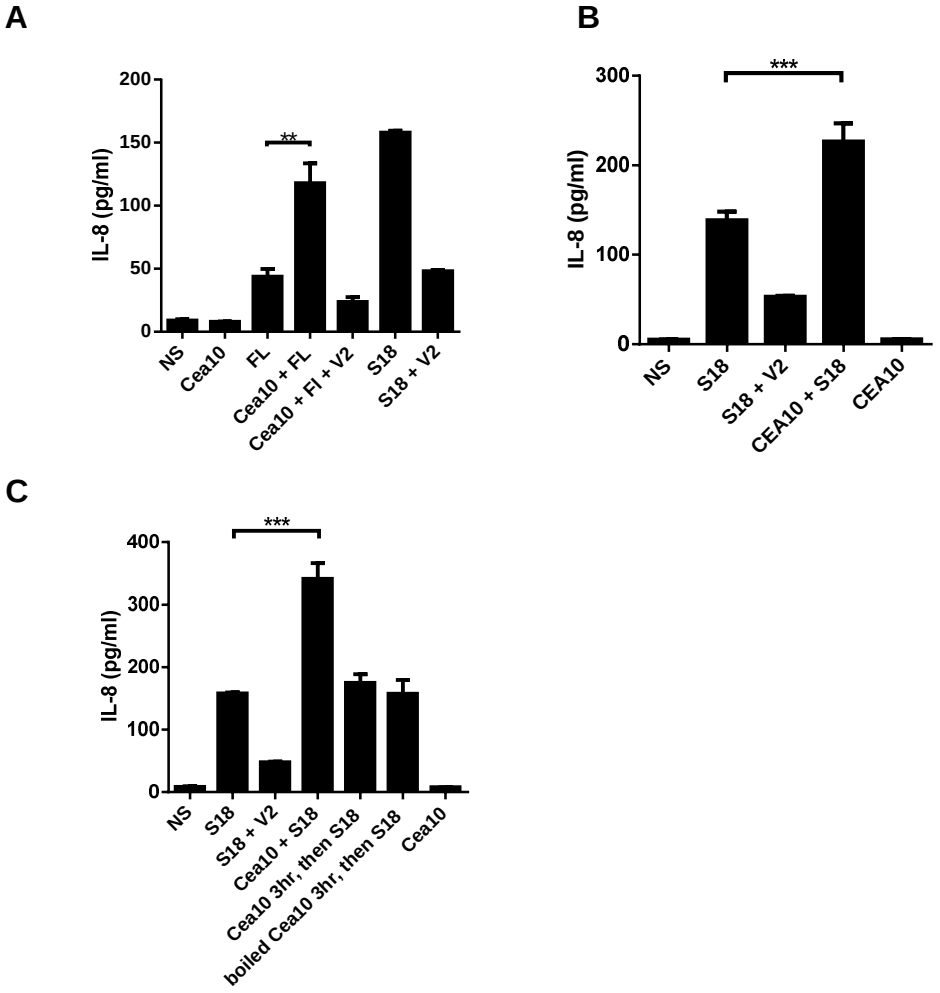

Figure 5-10: *A. fumigatus* conditioned media has an activating effect on IL-36γ FL and may enhance IL-36γ S18 activity

50 nM IL-36γ FL (A) and IL-36γ S18 (B) was incubated with CEA10 at 1:100 ratio of CEA10 to IL-36γ for 1 hour at 37˚C and then stopped by addition of Roche total protease inhibitor and incubated for a further 30 minutes. Incubated IL-36γ proteins were added to 90% confluent HaCaTs in the presence or absence or IL-36Ra V2 as indicated for 24 hours. IL-36 activity was assessed by analysing IL-36-induced IL-8 expression. To examine the priming effect of CEA10, HaCaTs were primed with boiled or non-treated CEA10 for 3 hours, washed with PBS and then treated with 50 nM IL-36γ S18 (C). HaCaTs were stimulated with non-incubated 50 nM IL-36γ FL, IL-36γ S18 and CEA10 alone for positive and negative controls. A one-way ANOVA was used to determine statistical significance of differences between treatment groups. **, $P < 0.01$, ***, $p < 0.001$. Data shown are mean ± S.E. (n = 2)

5.8 Discussion and future work

IL-36 has primarily been studied in the context of pathological inflammation due to its overexpression and pathological role in psoriasis. As a result, the physiological function of IL-36 cytokines in non-pathological inflammation has not yet been clearly elucidated. It has been proposed that IL-36 cytokines, like many other IL-1 family cytokines, act as alarmins, alerting surrounding cells to tissue damage [87]. Whilst this is probable, increased interest and research into the endogenous function of IL-36 cytokines is producing evidence to suggest they also may be involved in microbial defence and wound healing. It has been identified that infection of macrophages with *Mycobacterium tuberculosis* results in an increase in IL-36γ expression, and that *M. tuberculosis* growth is restricted in an IL-36R-dependant manner [188]. Recently published work has postulated IL-36γ plays a critical role in immune defence in the human reproductive tract (FRT) after identifying expression of IL-36γ in FRT epithelial cells increased in response to microbial insult [182]. In particular, it seems likely the IL-36 cytokines play an active role in the defence against fungal infection. It has been demonstrated that the IL-36 cytokines enhance production of T_H17 polarising cytokines, which are of great importance in fungal defence [106]. Furthermore challenging human peripheral blood mononuclear cells with *Aspergillus fumigatus* strongly induces expression of IL-36γ, further indicating a role in fungal defence [124]. In light of these observations, the work in this chapter sought to investigate the physiological role of IL-36 cytokines in the context of microbial infection and presents data implicating IL-36γ as significantly involved in maintaining epithelial integrity against pathogenic invasion, as summarised in Figure 5-11.

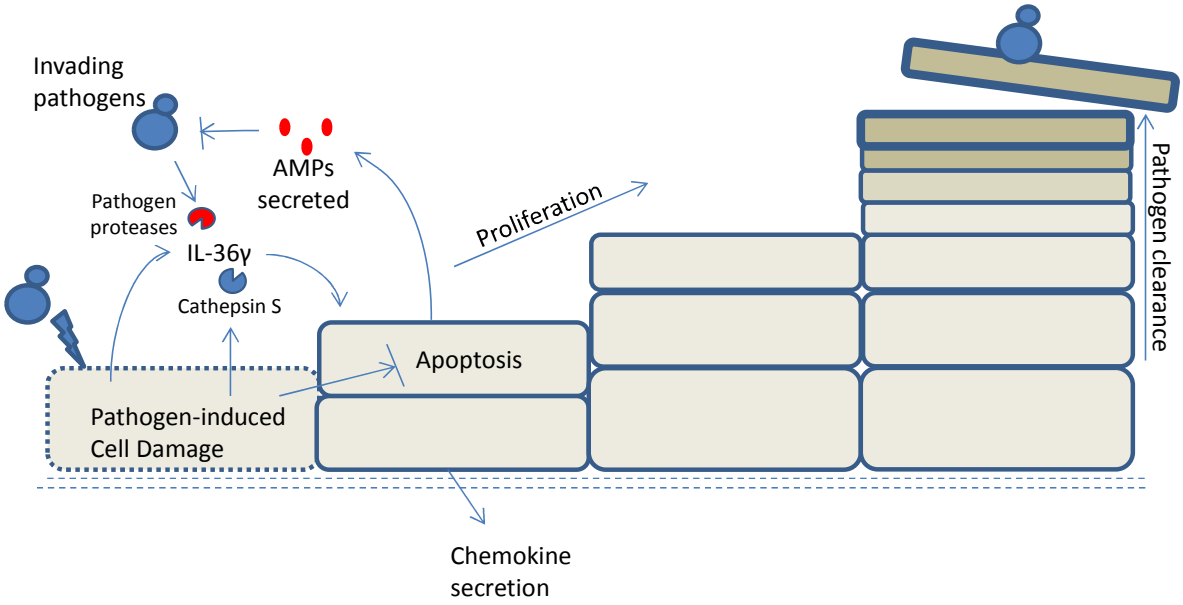

Figure 5-11: Physiological role of IL-36γ at epithelial barriers

Diagram to summarise the physiological activity of IL-36γ at epithelial barriers as implicated by the results of this thesis. Pathogen-induced cell damage induces release of IL-36γ and cathepsin S. Exogenous pathogen proteases and released cathepsin S cleave and activate IL-36γ leading to resistance of apoptosis, promotion of proliferation, secretion of chemotactic factors and antimicrobial proteins, all of which promote epithelial integrity and pathogen clearance.

By infecting epidermal skin models with *C. albicans* and treating with active IL-36 agonists, collaborators from the University of Aberdeen demonstrated that IL-36 agonists promoted a favourable outcome with less tissue damage and increased *C. albicans* killing. By investigating the mechanisms behind the IL-36-dependent effects observed, it was demonstrated that epidermal IL-36γ increases secretion of hBD2, increases resistance to apoptosis, and promotes epidermal growth; all of which would promote resistance to invading pathogens and maintain the integrity of the epidermal barrier. In addition, it was shown that IL-36γ is preferentially expressed in response to fungal PAMPs than to LPS, and that the cathepsin S expression profile matched that of IL-36γ suggesting a corresponding increase in IL-36γ activation. Finally, whilst there was no evidence for secretion of IL-36γ

occurring in response to fungal challenge, it was demonstrated that fungal proteases are capable of activating IL-36γ and initiating IL-36-mediated signalling, providing a direct link between fungal infection and IL-36-mediated inflammation. It may be hypothesised that a fungal presence would both increase expression of IL-36γ and result in its release through inducing damage and cell death. The released IL-36γ would then be susceptible to cleavage by both host and pathogen proteases and initiate an inflammatory response.

The resistance against skin-based *C. albicans* infection generated by IL-36 cytokines appeared to work at three levels; induction of AMP secretion, resistance to cell death, and promotion of epidermal growth. Importantly all of these mechanism act entirely on the epidermis and do not depend of the adaptive arm of the immune system, implicating a first line defence against microbial insult. The induction of hDB2 by IL-36γ may in itself be indicative of an anti-fungal role as hBD2 has been demonstrated to have fungicidal activity against *Candida* species in the skin, and may also act against *Aspergillus* species in the lungs [184, 189, 190]. However, in the experiments performed in this chapter only hBD2 expression was examined. It would be interesting to analyse the effect of IL-36γ stimulation on the expression of a broader range of AMPs as this may provide more information on the antimicrobial effect IL-36 cytokines have. Whilst hBD2 is known to be fungicidal, hBD3 has been demonstrated to have more potent fungicidal activity and so an IL-36-dependent increase in hBD3 may be more supportive of an antifungal role than increased expression of hBD2 alone [184]. Furthermore, in light of the pathological links of IL-36 cytokines to psoriasis, it may prove interesting to examine the effect IL-36 cytokine stimulation has on the expression of LL37; an AMP that is associated with a break-down in tolerance in the development of psoriasis [191]. It might also be interesting to investigate whether the IL-36 cytokines have any direct antimicrobial activity as have been shown with other cytokines such as IL-26 [192]. Immunohistochemistry micrographs of skin biopsies from patients with various forms of inflammatory conditions show that expression of IL-36γ, when detectable, is most concentrated in the upper levels of the epidermis,

which is characteristic of many AMPs [143]. Given that at as the keratinocytes differentiate and progress to the upper layers of the epidermis they become unresponsive, it seems unlikely that these keratinocytes would respond to the present IL-36γ. It seems plausible then, since IL-36γ appears most concentrated in the upper-most layers, that IL-36γ might have another role independent of keratinocyte stimulation.

The results in this chapter demonstrated that IL-36γ might promote epidermal integrity by promoting keratinocyte proliferation. Whilst the data demonstrating IL-36 cytokines increased proliferation of HaCaTs is quite preliminary, and there was no investigation into the mechanisms of how this enhanced proliferation, the notion that IL-36 cytokines might promote proliferation is not new. It has been shown in the literature that IL-36 stimulation of keratinocytes induces secretion of the keratinocyte mitogens transforming growth factor β (TGF-β) and granulocyte colony stimulation factor (G-CSF), and growth factors such as heparin binding EGF-like growth factor (HB-EGF) [109, 113]. These observations all support the idea that IL-36 cytokines promote epidermal growth. Furthermore, they also implicate a role in wound healing. HB-EGF is an important growth factor for epithelialization after cutaneous damage, demonstrated to promote wound healing by accelerating keratinocyte migration and fibroblast proliferation [193, 194]. Given the IL-36-mediated increase in expression of factors associated with wound healing, it would be interesting to further investigate the wound healing and inflammation-resolving capacity of IL-36 activity in the skin. Indeed, as previously mentioned, IL-36R has been shown in mice to be crucial for the resolution of dextran sodium sulphate-induced colitis [125]. In the wound healing process, keratinocyte and fibroblast migration is crucial for re-epithelialization and wound closure to occur, and angiogenesis is necessary to deliver oxygen to the repairing tissue [186]. Given that IL-36γ has been demonstrated to induce the expression of a number of growth factors, it would therefore be interesting to examine the effects IL-36 cytokines have on cell migration and the induction of angiogenesis.

The work conducted in this chapter identified that IL-36γ-stimulated HaCaTs exhibited resistance to apoptosis, however the mechanisms behind this

resistance were not elucidated and thus require further investigation. Apoptosis is a complex multi-step process that can be initiated through three distinct pathways, termed the extrinsic, intrinsic, and perforin/granzyme pathways, and can be controlled at several points. The extrinsic pathway is initiated through external stimulation of death receptors whereas the intrinsic pathway is initiated internally by the release of pro-apoptotic proteins from the mitochondrial intermembrane space. Each apoptotic pathway converges on the same execution pathway upon activation of caspase-3, resulting in DNA fragmentation, degradation of nuclear and cytoskeletal proteins, and the formation of apoptotic bodies [185]. Mitochondrial membrane disruption is a feature of both the intrinsic and extrinsic apoptosis pathways and as such, factors regulating the permeability of mitochondrial membranes have a regulatory effect on apoptosis. This is largely instigated by the Bcl-2 family proteins, which can be both pro- and anti-apoptotic depending upon whether they increase or decrease mitochondrial membrane permeability [195]. Apoptosis can also be controlled by the expression of inhibitor of apoptosis proteins (IAPs) such as X-linked inhibitor of apoptosis protein (XIAP) and survivin, which bind to and prevent activation of initiator and execution caspases [196]. The experiments carried out in this chapter induced apoptosis by staurosporin treatment, which, though its mechanisms are not completely understood, results in the activation of caspase-3 and caspase-7 [197]. As these are terminal caspases that mediate the final stages of apoptosis, any resistance observed after staurosporin treatment is most likely due to an increase in inhibitors of these caspases. In light of this, it seems likely IL-36γ-stimulation may increase expression of IAPs. Further work assessing the effect of IL-36γ stimulation on the expression of anti-apoptotic Bcl-2 proteins and IAPs are necessary to provide a better understanding of the mechanisms behind IL-36γ-mediated resistance to apoptosis.

The findings discussed above provide evidence of a cytokine that acts directly on the epidermis to maintain epidermal integrity and barrier function in the face of a destructive fungal infection. However, due to time constraints, the physiological role of IL-36γ in the epidermis was only evaluated through its effect on apoptosis and epidermal proliferation.

Epidermal integrity can be influenced by a variety of additional factors, most notably keratinocyte differentiation. Indeed, one of the most significant characteristics of psoriatic inflammation is the abnormally undifferentiated epidermis. Given that IL-36 cytokines – in particular IL-36γ – are strongly associated with psoriasis in the epidermis it would be intriguing to investigate the effect IL-36 cytokines have on keratinocyte differentiation. Furthermore, given that IL-36γ appears to increase the integrity of the epidermis, it might be interesting to examine its effect on keratinocyte adhesion molecules such as tight junctions and cadherins. This might provide more insight into the increase in epidermal integrity observed upon IL-36γ treatment.

The work in this chapter demonstrated that not only did fungal insult in the skin induce expression of both IL-36γ and its activating protease cathepsin S, thus indirectly resulting in IL-36-mediated inflammation, but that *Aspergillus fumigatus* conditioned media was able to directly activate IL-36γ and therefore directly induce IL-36-mediated inflammation. This is significant as it provides a direct link between fungal infection and IL-36 cytokine signalling. The IL-36 cytokines have previously been implicated in the defence against *Aspergillus* infection in the lung. It has been observed that IL-36γ expression by peripheral blood mononuclear cells (PBMCs) is strongly increased upon challenge with both live *A. fumigatus* conidia and heat-killed hyphae. Furthermore, it has been shown that the expression of IL-17 and IFNγ in response to *A. fumigatus* challenge is influenced by IL-36 signalling, thus IL-36 is important for driving the T_H1 and T_H17 responses in *A. fumigatus* infection [124]. In light of this, the observation that *A. fumigatus* can directly initiate IL-36γ-mediated signalling strongly implicates a role for IL-36γ in the defence against *A. fumigatus* infection in the lung. *A. fumigatus* are capable of expressing a wide variety of proteases, including serine proteases, aspartic proteases and metalloproteinases [187]. Whilst these are not considered as virulence factors for invasive *A. fumigatus* infections, they have been demonstrated to impact on the host response. Several proteases secreted by *A. fumigatus* have been identified as allergens that can induce an allergic response in atopic individuals [198]. Intriguingly, the metalloproteinase Asp f 5 and serine protease Asp f 13 have been shown to

induce inflammation and inflammatory cell infiltration in mice infected with *A. fumigatus,* contributing to a T_H2 response and an asthmatic pathology [199]. Whereas the proteases expressed by *A. fumigatus* have primarily been considered as allergenic in their activity by inducing allergic inflammatory responses, the initiation of IL-36γ signalling and subsequent T_H1 and T_H17 responses suggests the immune system may also utilize these proteases to mount an anti-fungal immune response. Indeed, it has recently been posited that IL-1β may act as a sensor of pathogen-associated proteolysis that occurs in group A *Streptococcus* (GAS) infection after identifying that GAS-expressed protease SpeB cleaved and activated IL-1β [200]. The activation of IL-36γ by *A. fumigatus* proteases appears to be analogous to this, suggesting IL-36 cytokines may potentially act as sensors of fungal proteolytic activity. *Candida* species are also known to secrete a range of proteases that can contribute to its pathogenicity [187]. If IL-36 cytokines act as sensors of fungal protease activity it would be interesting to examine whether a mechanism analogous to *A. fumigatus*-mediated IL-36γ activation exists with *Candida* species in the skin.

In addition to examining the endogenous effects of IL-36γ on the epidermis, this chapter also investigated the effect various PAMPs had on both the expression and secretion of IL-36γ. The secretion of IL-1 family members is a subject of much interest, as the IL-1 family members lack an N-terminal signal peptide and thus must be secreted via a non-classical mechanism. The best-characterised mechanism of IL-1 secretion occurs as a two-step process and is dependent upon formation and activation of the inflammasome. An initial stimulus induces expression of IL-1 pro-forms and inflammasome components [80, 81]. Upon a secondary stimulation, formation of the inflammasome occurs, which recruits and activates caspase-1 [201]. Subsequently, pro-IL-1 cytokines are truncated to their active forms and secreted from the cell [202]. However, this mechanism of secretion has only been described for the caspase-1 susceptible IL-1 family members IL-1β and IL-18. Indeed, in the experiments conducted in this chapter, whilst IL-36γ expression increased in response to an initial PAMP challenge, activation of the inflammasome by secondary stimulus of either ATP or

nigericin had no effect on the release of IL-36γ from the cell. This demonstrates the secretion of IL-36γ is not influenced by activation of the inflammasome.

For IL-1 members that are not influenced by inflammasome activation of caspase-1, it is difficult to elucidate a mechanism of release from the cell, and this is often shown to occur passively, either as a result of mechanical cell damage or via necrosis. However, a number of alternative mechanisms of IL-1 family cytokine release have been proposed. There have been many conflicting studies implicating the involvement of vesicular transport for release of IL-1 cytokines in the form of secretory lysosomes, multivesicular bodies and exosomes [203]. Indeed it has been reported that IL-36γ can be secreted by mice lung macrophages in microparticles and exosomes in an ATP-dependent manner [204]. Another proposed method of secretion is via secretory autophagy. It has been shown in the case of IL-1β that pro-IL-1β can be sequestered into autophagosomes upon TLR stimulation and that subsequent autophagy-based secretion can result in its release by serum-starved cells [205, 206]. With regards to IL-36γ and fungal infection, this is potentially interesting as fungal challenge has been shown to induce autophagy and subsequent unconventional protein secretion in human macrophages in a dectin-1 dependent manner [207]. Furthermore as cathepsin S is present in lysosomal fractions, the formation of autophagolysosomes may bring both cathepsin S and IL-36γ together, thus subsequent autophagy-based secretion may release active IL-36γ and initiate IL-36-mediated inflammation. However, the results obtained in this chapter indicate this may not be the case with keratinocytes. Whilst treating keratinocytes with zymosan, an agonist of dectin-1, did induce an increase in IL-36γ expression, no significant increase in secretion was observed.

Whilst with several IL-1 family cytokines it has been demonstrated that cytokine cleavage can occur internally, this has not been shown with IL-36γ. However, although cathepsin S is contained within a separate compartment to IL-36γ, as they are both expressed within the same cells it seems possible activation could happen internally. As mentioned above, cellular processes such as autophagy might bring cathepsin S and IL-36γ together, which could

facilitate its activation. Furthermore, the lysosome is in some cases susceptible to lysosomal disruption, resulting in of lysosomal contents into the cytosol. Indeed, it has been shown that lysosomal disruption can lead to the activation of the NLRP3 inflammasome via cathepsin B activity, so it stands to reason that cathepsin S released in the same manner might also activate IL-36γ [201]. Lysosomal disruption has been shown to occur in response to crystals of monosodium urate (MSU), a prevalent pathological characteristic of the inflammatory condition gout [208, 209].

Whilst there was no observed release of IL-36γ in response to zymosan or LPS stimulation of keratinocytes, there was a strong increase in both expression and release in response to the TLR3 agonist poly(I:C). Whereas the increase in IL-36γ release may be attributed to cell necrosis, as poly(I:C) is well characterised as an inducer of necrotic cell death, the significant increase in IL-36γ production is perhaps an intriguing finding. TLR3 is involved in the recognition of viral pathogens, yet IL-36-mediated inflammation generally results in the production of T_H17 polarising cytokines not typically associated with anti-viral immunity. However, the poly(I:C)-induced release of IL-36γ by keratinocytes has also been previously reported in the literature. It has been demonstrated that poly(I:C) induces pyroptosis in keratinocytes and the subsequent release of IL-36γ, supporting the notion that IL-36 cytokines act as alarmins passively released upon pyroptotic cell death [170]. However, another publication has shown that low concentration stimulation of keratinocytes with poly(I:C) is capable of inducing IL-36γ secretion in the absence of pyroptosis via extracellular vesicles, suggesting there does exist an active and controlled mechanism of IL-36γ release in response to viral pathogens [107].

The observation that TLR3 stimulation results in IL-36γ expression and possibly secretion is suggestive of IL-36γ having a role in viral immunity. This is perhaps supported by the observation that the IL-36γ activating protease cathepsin S is also up-regulated and released in response to poly(I:C) stimulation. Whilst IL-36-mediated inflammation is typically associated with a T_H17 response not typical of viral defence, there is evidence of IL-36 cytokines being involved in viral infections. Soon after its

discovery, IL-36γ was found to be up-regulated in response to herpes simplex virus-1 infection in mice [210]. IL-36γ was also found to be amongst the most consistently overexpressed genes identified by microarray analysis from recurrent respiratory papillomas caused by human papilloma virus infection [211]. Rhinovirus challenge of primary bronchial epithelial cells strongly induces expression of IL-36γ [122]. Additionally, in mouse models of chronic obstructive pulmonary disease, a condition in which IL-36γ-mediated inflammation is thought to be pathological, infection with respiratory syncytial virus has been shown to exacerbate inflammation and increase expression of cathepsin S [121, 173]. In light of this, and the large amount of viral challenge that must occur within lung epithelial tissue, it seems likely that IL-36 cytokines may be somehow involved in viral immunity. Were IL-36 cytokines to have an active role in the defence against respiratory viral infections, it may be interesting to investigate the effect on viral infection on the activation and secretion of IL-36 cytokines. Many respiratory viruses, including rhinoviruses, encode viral proteases and induce significant changes in cellular structure during reproduction, including autophagy, so it would be interesting to examine the effect this might have on IL-36-mediated signalling and inflammation.

To conclude, the culmination of the results obtained in this chapter paint an image of a cytokine that acts directly on the epidermis in response to invading pathogens to promote the epidermal barrier function by maintaining its integrity. The observation that IL-36γ is active in the defence against *C. albicans* in the skin, up-regulated in response to fungal PAMPs and activated by *A. fumigatus* proteases in lung tissue strongly supports the notion that IL-36γ is important in fungal defence. However, in light of the strong up-regulation of both cathepsin S and IL-36γ in response to TLR3 activation, and the correlation noted in the literature of IL-36γ expression and viral infections, it seems possible the IL-36 cytokines also have a role in antiviral immunity. Furthermore, with the observation that inflammasome activation had no effect on IL-36γ release, yet induction of necrosis resulted in a strong release, it seems logical that the primary mechanism of IL-36 cytokine release is through cell damage and necrosis. In light of the recent finding that IL-1β

acts as a sensor of pathogen-associated protease activity, it should prove an interesting subject to explore whether a similar mechanism exists for the IL-36 family of cytokines in response to fungal pathogens.

Chapter 6 - Discussion

The IL-36 cytokines have become well established as important mediators of inflammation in a number of inflammatory conditions, including psoriasis and chronic obstructive pulmonary disease. However, as relatively newly discovered members of the IL-1 family, our understanding of the IL-36 cytokines is by no means comprehensive. Whilst they have been implicated in numerous inflammatory conditions at epithelial surfaces, and have been shown to orchestrate pathological inflammation, a lot remains unclear regarding the mechanisms of their activation and role they play under physiological conditions. The work conducted in this thesis endeavored to clarify these mechanisms and explore how IL-36 cytokines might influence their environment, to build a more comprehensive understanding of the role IL-36 cytokines play in both pathological and physiological inflammation.

6.1 Diagnostic and research potential of IL-36γ antibodies

IL-36 cytokines first piqued the interest of researchers after recognizing the abundance of IL-36 cytokine expression in psoriatic plaques [108, 110]. A number of subsequent transgenic mouse studies further demonstrated that IL-36 overexpression is not only a characteristic of psoriasis, but that IL-36 signalling plays a pathological role in psoriatic skin inflammation [112, 113]. It was later identified through analysis of RNA extracted from patients and by immunohistology that psoriatic tissue specifically had highly elevated levels of IL-36γ expression when compared to a range of other inflammatory skin diseases [143].

There is a significant clinical need for advanced alternative diagnostic approaches. Firstly, with the lack of available specialists and restrictions to access of secondary care, GPs are in urgent need for non-invasive diagnostic tests to inform on inflammatory skin reactions. Uncertainty and inability to diagnose a skin rash leads to considerable overprescribing of systemic antibiotics and failure to initiate appropriate treatment pathways. Secondly, descriptive phenotyping and conventional diagnostic dermato-histopathology – which depends on invasive and expensive biopsy

procedures – insufficiently inform on stratified medicine approaches. As pure phenotype descriptions have failed to inform on the most suitable treatment approach for a given patient, current “stratified medicine” consortia and research initiatives in the area of inflammatory skin disease now focus on “molecular” subtypes or “endotypes” [212, 213].

Diagnostic challenges are also frequently encountered in specialist dermatology settings with regard to atopic dermatitis (AD) and psoriasis where special body locations (e.g. outer ear, palm, sole, scalp, skin fold area) are involved or phenotypic features of both diseases are present (often referred to as psoriasiform eczema or eczematised psoriasis) [152]. AD and psoriasis can appear very similar despite the distinct underlying mechanisms of disease. Whilst AD is typically driven by T_H2 responses and has strong links to allergy, psoriasis has a predominantly T_H1 and T_H17 profile [51, 59, 60]. There are therefore different inflammatory pathways at play, which require different forms of treatment. In this work, specific anti-IL-36γ monoclonal antibodies were generated and optimized to work in ELISA. These were used to measure the level of IL-36γ present in the skin of both AD patients and psoriasis patients, which identified that, as predicted by RNA data, IL-36γ is present at much higher levels in skin of patients with psoriasis than with AD. The results suggest that this difference in IL-36γ expression may, in the future, be used to differentiate patients with ambiguous symptoms that may be either AD or psoriasis. One example for a clinical setting were this is of specific value refers to the diagnostic criteria of psoriatic arthritis, where psoriatic skin symptoms are part of the classification criteria for psoriatic arthritis (CASPAR) [214]. Here, patients are attributed points according to specific symptoms (including psoriatic skin), and must have a score of ≥ 3 combined with inflammatory articular disease in order for a condition to be classified as psoriatic arthritis (PsA). However, in suspected early PsA, the symptoms exhibited by patients’ skin can often be minimal or very limited, complicating and delaying diagnosis.

This ELISA therefore has great potential as a diagnostic tool when used in combination with non-invasive sample collection by skin surface tape stripping. Furthermore, IL-36γ can also be detected in synovial fluid and

serum. The ELISA may thus be useful in further diagnostic methods for conditions involving systemic circulating IL-36γ or joint involvement such as rheumatoid arthritis or psoriatic arthritis, as well as to investigate lung fluid from patients with viral or fungal infections.

The potential for clinical use of the antibodies developed in this work is not limited to the ELISA. As shown in chapter 3, two of the antibodies generated are capable of blocking the signalling ability of active IL-36γ. Whilst the results are very preliminary these antibodies may have a therapeutic value. As discussed in chapter 3, IL-36 may be a desirable target in the treatment of psoriasis as, though still significantly involved in orchestration of inflammation, its expression is much more restricted than that of other targets such as TNF, IL-17 or IL-12/IL-23 and thus is less likely to have severe off-target consequences.

In addition to the demonstrated and potential value for clinical diagnostics and therapy, these antibodies have proved invaluable throughout the research conducted in this work. Previously, it has been very difficult to study the IL-36 cytokines at the protein level as the antibodies available on the market were inadequate. This made it impossible to measure IL-36 protein expression by cells. However, as demonstrated in chapter 5, the developed ELISA made it possible to examine the cellular expression of IL-36γ at the protein level and facilitated the investigation of IL-36γ secretion; an aspect of IL-36 cytokine biology that has not been studied in great detail, due – in part – to the lack of reagents. Furthermore, though not extensively utilized in this work, the antibodies were also optimized to function in immunoprecipitation and immunofluorescence, which shall prove of great use in the future. With the ELISA and immunoprecipitating antibodies it should be possible to not only determine under which conditions IL-36γ might be released from the cell, but whether IL-36γ is released in its active or inactive form. Furthermore, with functioning immunofluorescence antibodies it should also be possible to examine the intracellular trafficking events that occur upon IL-36γ activation and secretion.

6.2 Regulation of IL-36 activity by proteolysis and microbial insult

As repeatedly mentioned, the IL-1 family cytokines are predominantly produced as inactive precursors that require truncation to become fully active [215]. Their activity is therefore heavily dependent on the activity and expression of the proteases that truncate and activate them. This is evident in cases where the activity of IL-1 regulating proteases become affected through genetic mutations; particularly in cryopyrin associated periodic syndromes [216].

Whilst with some members of the IL-1 family, such as IL-1β, proteolysis can occur in a general region of the molecule to enhance its activity, the IL-36 cytokines require precise truncation (as has been demonstrated by Towne *et al.* and reiterated by the work conducted in this thesis) [99]. This is a significant factor to consider with the IL-36 cytokines, as has been illustrated in the work conducted in chapter 4. Whilst neutrophils are capable of truncating all IL-36 cytokines in the region of their truncation site, of the agonists only IL-36β is cleaved precisely 9 amino acids upstream of its A-X-D motif to activation [134]. Furthermore, whilst they generate inactive truncations of IL-36α and IL-36γ, they produce active IL-36Ra, which would suggest - contrary to some findings reported in the literature - that neutrophils may act in a more regulatory fashion with respect to IL-36-mediated inflammation. This is further supported by the finding presented in chapter 4 that by including neutrophil serine proteases in an incubation of full length IL-36γ and cathepsin S, the resultant IL-36γ-mediated signalling is reduced.

In contrast to truncation by neutrophil serine proteases, it was shown that IL-36γ is efficiently activated by cathepsin S upon truncation at precisely 9 amino acids upstream of its A-X-D motif. This is significant as cathepsin S is constitutively expressed by many epithelial cells as shown in chapter 4, including primary fibroblasts and keratinocytes of the skin, and thus the environment in which IL-36γ is expressed has the capacity to activate and induce IL-36γ-mediated inflammation without the need for an influx of inflammatory cells. Given the physiological role of IL-36γ in the epidermis

discussed in chapter 5, this would be crucial for IL-36γ to mount an effective defence against microbial insult.

With the observation that cathepsin S is a primary activator of IL-36γ, it has become important to also consider the expression and activation of cathepsin S in relation to IL-36 mediated inflammation. Indeed, in keeping with the pathological role attributed to IL-36-mediated inflammation in psoriasis, it is established that cathepsin S is also overexpressed in psoriatic tissue [167, 168]. This would suggest that in addition to elevated expression of IL-36γ, psoriatic lesions are also likely to have elevated IL-36γ activity when compared to healthy tissue. The identification of cathepsin S as an activator of IL-36γ may provide a genetic candidate to screen in psoriasis cohorts to facilitate a stratified treatment approach. As patients with increased cathepsin S are likely to have increased IL-36-mediated inflammation, treatment tailored towards targeting cathepsin S and IL-36 signalling might prove more efficacious than generalized treatments. Furthermore, identification of cathepsin S-mediated activation may provide new avenues of research in mouse models that could help to elucidate the importance of cathepsin S in IL-36-mediated development of psoriasis in a complex mammalian system.

Despite several identified methods of non-classical secretion, given the findings of this work it seems likely that IL-36γ, like IL-1α and IL-33, is primarily released upon necrosis and functions as an alarmin [87]. This seems logical when considering that IL-36γ directly influences the epidermis to increase epidermal integrity and wards off invading pathogens without requiring input from adaptive immune cells. Furthermore, with both IL-36γ and its activating protease cathepsin S expressed by the same cells, it seems likely that necrosis would cause a release of both cathepsin S and IL-36γ, thus facilitating its activation and the subsequent alarmin function of IL-36γ.

6.3 Endogenous role of IL-36 cytokines

IL-36 has been primarily studied in the context of pathological inflammation. However research into its physiological role suggests that the IL-36 cytokines play a role in microbial defence, and that whilst they are in many cases involved in the orchestration of pathological inflammation, they may also have a function in the resolution of inflammation and a progression to wound healing [124, 125].

Recent research has implicated IL-36 cytokines in the defence against invading pathogenic infection, with several publications demonstrating an increase in IL-36γ expression upon microbial insult, and even an active role in preventing microbial growth in the case of infection with *M. tuberculosis* [188]. Indeed, work in collaboration with the University of Aberdeen, presented in this thesis, demonstrate another example of where IL-36 cytokines actively protect against microbial insult, by promoting resistance to epidermal invasion by *C. albicans*. Furthermore, it was shown that IL-36γ directly influences neighboring keratinocytes and functions to increase the integrity of the epidermis by three separate mechanisms; promoting growth, resisting cell death, and promoting secretion of AMPs. When considering that IL-36γ is constitutively expressed in the skin, has been shown to be strongly up-regulated upon challenge by microbial PAMPs (along with its activating protease cathepsin S), and its primary mechanism of release appears to be through necrosis and mechanical cell damage, the above results paint the picture of an alarmin cytokine capable of orchestrating an immediate defence against invading pathogens by acting directly upon the epidermal cells. Furthermore, published information points to the fact that IL-36 cytokines can mobilize both the innate and adaptive immune system by inducing the production of chemokine gradients and promoting maturation of infiltrating dendritic cells and macrophages [102, 109, 217].

As discussed in chapter 5, a recent publication has proposed a novel function for IL-1β as a sensor of group A *Streptococcal* (GAS) infection, as the cytokine can be activated by the GAS-produced protease SpeB and the subsequent IL-1β-mediated inflammation is important for clearance of the

infection [200]. The results presented in this thesis have identified a similar mechanism of function for IL-36γ in relation to *Aspergillus* infection in the lung, demonstrating that proteases secreted by *Aspergillus fumigatus* activate and facilitate IL-36-mediated signalling. Whilst it was not shown that IL-36 signalling is crucial for resolution of an *A. fumigatus* infection, IL-36 signalling is known to promote T_H17 responses important in fungal immunity and it has been previously reported that blockade of IL-36 signalling reduces IL-17 and IFNγ secretion by *A. fumigatus*-challenged peripheral blood mononuclear cells [124]. This would imply that in addition to functioning to orchestrate the first line of defence against invading pathogens, IL-36γ may, analogous to IL-1β, also be able to sense the presence of certain fungal pathogens itself and initiate an immediate response without the need for up-regulation or secretion of a host activating protease.

6.4 Conclusion

To conclude, the work conducted in this thesis has produced four anti-IL-36γ specific monoclonal antibodies, which function in a number of assays. Most notably, the generation of these antibodies has facilitated the production and optimization of an IL-36γ-specific ELISA which has been used to detect and quantitate IL-36γ extracted from patient epidermal tissue. In doing so, it has demonstrated that psoriatic epidermis has significantly more IL-36γ than the epidermis of AD lesions, providing a diagnostic tool to differentiate the two conditions.

This work has identified cathepsin S as a primary activator of IL-36γ expressed at epithelial sites, and demonstrated that whilst neutrophils are capable of processing IL-36 cytokines, their truncations are predominantly inactive and thus act in a more regulatory capacity. Furthermore this work has demonstrated a role for IL-36γ in the epidermis to maintain and promote barrier integrity in the face of microbial infection – particularly fungal insult – that may even act as sensors of pathogenic protease activity. The sum of all the results presented in this thesis provides evidence of a versatile cytokine family closely controlled through its proteolytic processing that can not only orchestrate and mobilise a robust immune response, but can itself sense

microbial invasion and act immediately and directly upon the epithelial compartment to resist microbial invasion. Future experiments determining whether there is cross species conservation of these mechanisms will help pave the way to more insightful mouse models and further investigate the roles of IL-36 cytokines in pathological inflammation and the immune response.

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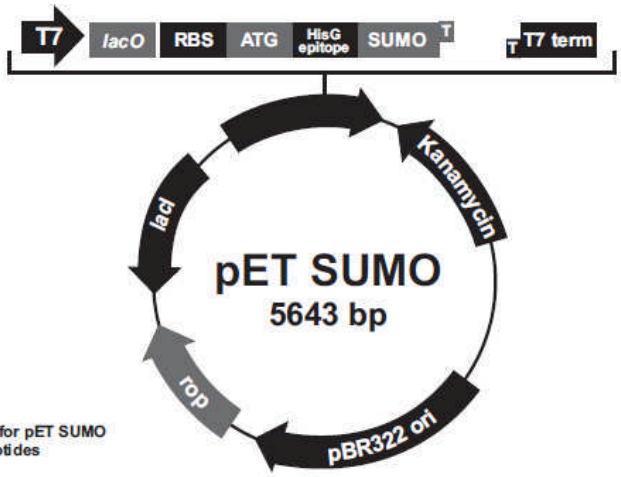
Appendix



Comments for pET SUMO
5643 nucleotides

T7 promoter: bases 209-225
lac operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-288
Initiation ATG: bases 297-299
HisG epitope: bases 309-329
SUMO ORF: bases 360-653
SUMO forward priming site: bases 549-571
TA Cloning site: bases 653-654
T7 reverse priming site: bases 783-802 (C)
T7 terminator: bases 744-872
Kanamycin resistance gene: bases 1431-2246 (C)
pBR322 origin: bases 2342-3015
ROP ORF: bases 3383-3574
lacI ORF: bases 4383-5474 (C)

(C) = complementary strand

```
121  ATAGGCGCCA GCAACCGCAC CTGTGGCGCC GGTGATGCCG GCCACGATGC GTCCGGCGTA GAGGATCGAG ATCTCGATCC

                    T7 promoter                    lac operator
201  CGCGAAATTA ATACGACTCA CTATAGGGGA ATTGTGAGCG GATAACAATT CCCCTCTAGA AATAATTTTG TTTAACTTTA

                                                        HisG epitope
                RBS                                Polyhistidine region
281  AGAAGGAGAT ATACAT ATG GGC AGC AGC CAT CAT CAT CAT CAC GGC AGC GGC CTG GTG CCG CGC GGC AGC
                  Met Gly Ser Ser His His His His His His Gly Ser Gly Leu Val Pro Arg Gly Ser

                                                SUMO fusion protein
354  GCT AGC ATG TCG GAC TCA GAA GTC AAT CAA GAA GCT AAG CCA GAG GTC AAG CCA GAA GTC AAG CCT GAG ACT
     Ala Ser Met Ser Asp Ser Glu Val Asn Gln Glu Ala Lys Pro Glu Val Lys Pro Glu Val Lys Pro Glu Thr

426  CAC ATC AAT TTA AAG GTG TCC GAT GGA TCT TCA GAG ATC TTC TTC AAG ATC AAA AAG ACC ACT CCT TTA AGA
     His Ile Asn Leu Lys Val Ser Asp Gly Ser Ser Glu Ile Phe Phe Lys Ile Lys Lys Thr Thr Pro Leu Arg

                                                                    SUMO forward priming site
498  AGG CTG ATG GAA GCG TTC GCT AAA AGA CAG GGT AAG GAA ATG GAC TCC TTA AGA TTC TTG TAC GAC GGT ATT
     Arg Leu Met Glu Ala Phe Ala Lys Arg Gln Gly Lys Glu Met Asp Ser Leu Arg Phe Leu Tyr Asp Gly Ile

570  AGA ATT CAA GCT GAT CAG ACC CCT GAA GAT TTG GAC ATG GAG GAT AAC GAT ATT ATT GAG GCT CAC AGA GAA
     Arg Ile Gln Ala Asp Gln Thr Pro Glu Asp Leu Asp Met Glu Asp Asn Asp Ile Ile Glu Ala His Arg Glu

642  CAG ATT GGT GGT GAC GAC AAG CTT AGG TAT T TAT TCG GGC GC AAA GTG CGT C GGG TGA TGC T
     GTC TAA CCA CCA PCR product TCT GTT C GAA TCC ATA A
     Gln Ile Gly Gly
                 SUMO cleavage site
701  GCCAACTTAG TCGAGCACCA CCACCACCAC CACTGAGATC CGGCTGCTAA CAAAGCCCGA AAGGAAGCTG AGTTGGCTGC

                T7 reverse priming site
781  TGCCACCGCT GAGCAATAAC TAGCATAACC
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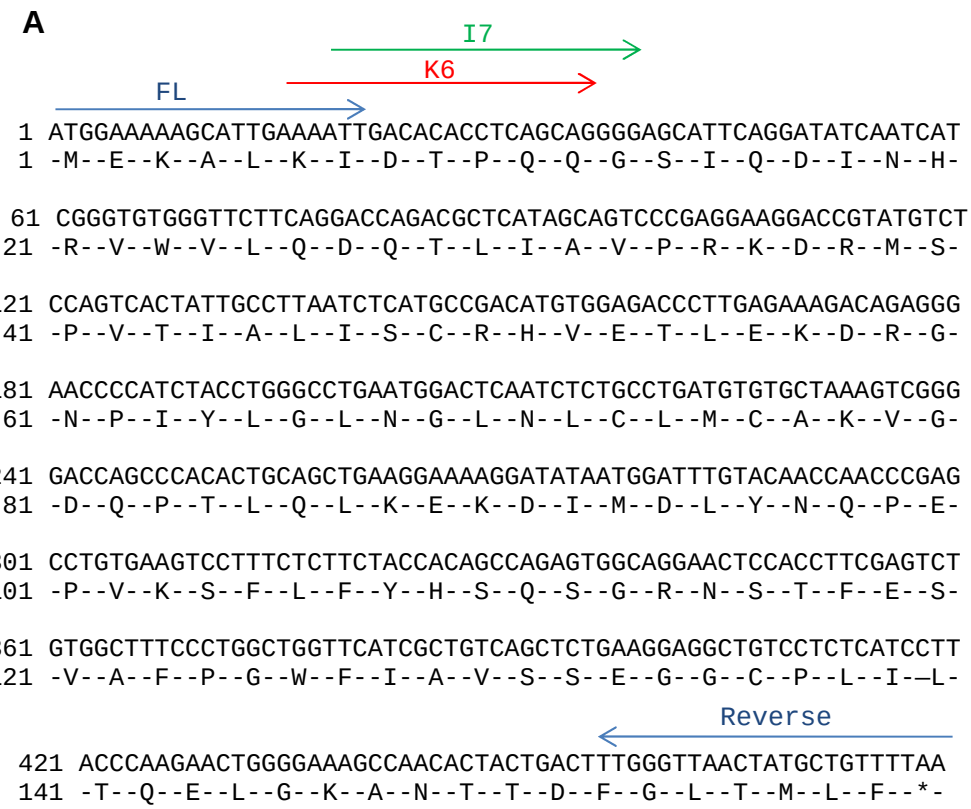
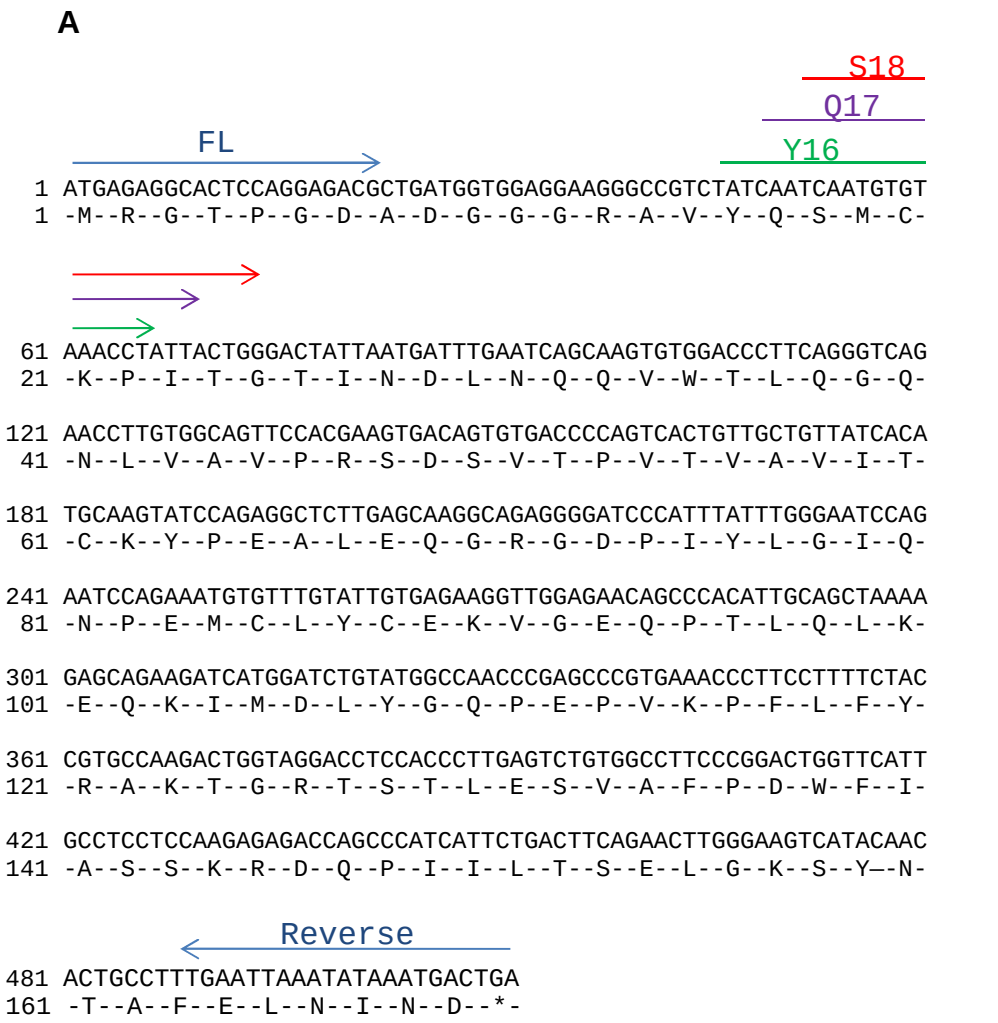
Appendix Figure 1: Champion pET SUMO bacterial expression

Vector used for bacterial expression of IL-36 members



Appendix Figure 2: IL-36α sequences

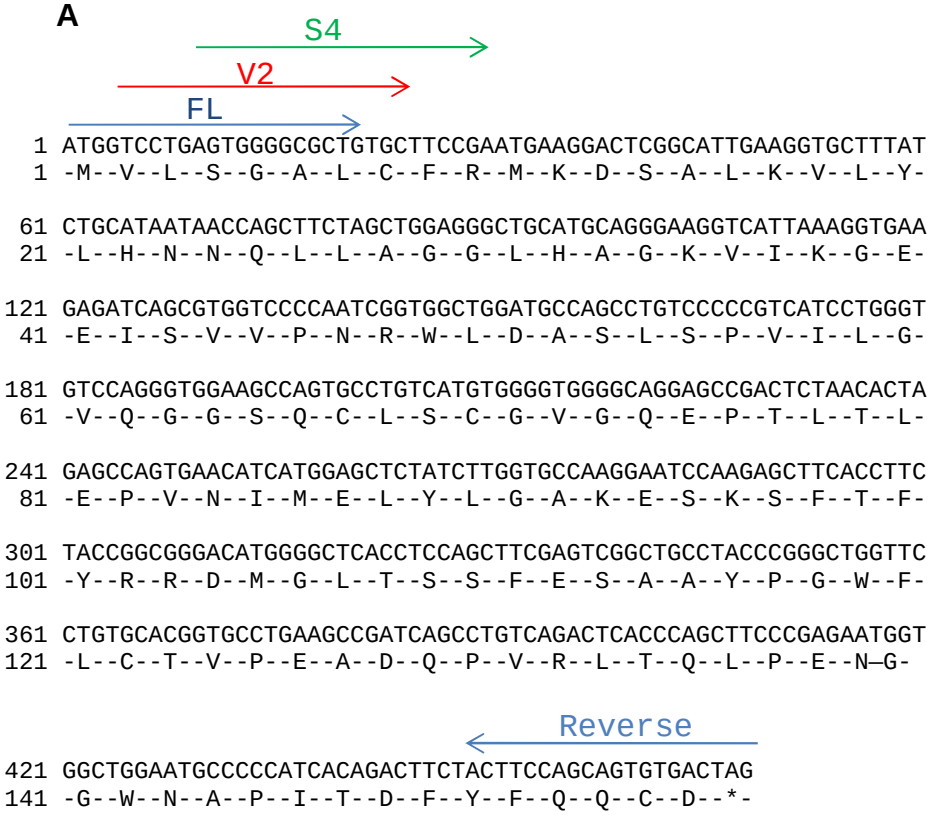
(A) Nucleotide sequence of IL-36α and the primer sequences used to amplify each form of IL-36α. (B) Peptide sequence of IL-36α with various truncated N termini coloured according to the corresponding primers shown in A.



Appendix Figure 3: IL-36γ sequences

(A) Nucleotide sequence of IL-36γ and the primer sequences used to amplify each form of IL-36γ. (B) Peptide sequence of IL-36γ with various truncated N termini coloured according to the corresponding primers shown in A.

A



B

MVLSGALCFRMK**D**SALKVLYLHNNQLLAGGLHAGKVIKGEEISVVPNRWLDASLSPVIL
GVQGGSQCLSCGVGQEPTLTLEPVNIMELYLGAKESKSFTFYRRDMGLTSSFESAAYPG
WFLCTVPEADQPVRLTQLPENGGWNAPITDFYFQQCD

Appendix Figure 4: IL-36Ra sequences

(A) Nucleotide sequence of IL-36Ra and the primer sequences used to amplify each form of IL-36Ra. (B) Peptide sequence of IL-36Ra with various truncated N termini coloured according to the corresponding primers shown in A.