The IL-1 family in relation to psoriasis

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

The University of Leeds

Faculty of Biological Sciences

July 2014

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 3 – based on Wittmann, M., Doble R., Bachmann M., Pfeilschifter J., Werfel T., Mühl H., IL-27 Regulates IL-18 binding protein in skin resident cells. PLoS One, 2012. 7(6): p. e38751. Conceived and designed the experiments: MW HM RD. Performed the experiments: RD MB. Analysed the data: RD MW HM MB. Contributed reagents/materials/analysis tools: JP TW HM MW. Wrote the paper: RD MW HM TW.

Chapter 4 – based on Doble, R., McDermott MF., Cesur O., Stonehouse NJ., Wittmann M., IL-17A RNA aptamer: possible therapeutic potential in some cells, more than we bargained for in others? J Invest Dermatol, 2014. 134(3): p. 852-5. Conceived and designed experiments: RD, NS, MW. Performed the experiments: RD, OC. Analysed the data: RD. Contributed reagents/materials/analysis tools: MW NS MD. Wrote the paper: RD, MW, NS.

Acknowledgements

I would firstly like to say a massive thank you to my supervisors, Dr Miriam Wittmann and Dr Martin Stacey for their huge amounts of support and guidance over the last 4 years, their input has allowed me to establish a wide range of techniques with confidence. I would also like to thank Professor Dennis McGonagle and Dr Neil Turner for their critique and input of ideas during the last 4 years. I am also hugely grateful to Professor Nicola Stonehouse and Dr Andrew Macdonald for their collaborative projects for which their guidance has been invaluable. I would also like to thank Chris Wasson for his patience and helpful guidance in construction of skin equivalents, as well as Özlem Cesur and Sophie Forrest for their help with my aptamer work. I would like to thank members of the Wittmann and Stacey group, Ade Alase and Tom Macleod for making the lab a pleasant place to work. I would also like to thank everyone who has been in the lab over the last 4 years especially those who have kept me sane and made the last 4 years enjoyable. I would like to thank Laura Knight, Hannah Kyle, Chris Wasson, Emma Prescott, James Findlay and Laura White for the regular tea/gossip breaks and who have all during the 4 years kept my spirits up. I am very grateful for all the support from my friends and family who despite not knowing what I am talking about still share in the excitement and frustration of research. I would lastly like to thank Mark for his unwavering support in listening and understanding the ups and downs of doing a PhD, no matter how grumpy I was.

Abstract

Psoriasis is a common chronic inflammatory skin disease which can also affect the joints. Its pathogenesis is still to be fully elucidated and involves a wide range of inflammatory mediators, tissue and immune cells. At present, there is no treatment available to cure psoriasis. Although biologics have considerably improved the treatment of the most severe cases there is still a pressing clinical need to improve therapy for specific disease subtypes (e.g. pustular psoriasis) and the vast majority of patients suffering from psoriasis classified as mild to moderate. In particular, efficient and well tolerated topical approaches are lacking.

This work has focused 1) on advancing our understanding of IL-36 cytokines which are recognised for their significance in pustular psoriasis, 2) on identifying endogenous disease limiting mediators such as IL-18 binding protein and how they could be manipulated in a therapeutic approach and 3) on IL-17 neutralising RNA aptamers as tools for topical therapy.

Main results include the identification of biologic activity of processed and non-processed IL-36 members. N-terminal cleavage is required to increase activity of all IL-36 members. The protease responsible for IL-36RA processing was elucidated. Neutrophil proteases as well as kallikrein 7 can cleave pro-inflammatory IL-36 members. However, a second processing step seems necessary for full activation and the potentially responsible aminopeptidase remains to be identified. Secondly, it was found that human primary fibroblasts produce significant levels of IL-18BP, which controls pro-inflammatory function of IL-18. Endogenous IL-18BP can be induced by IL-27 which, when given in combination with hydrocortisone does not induce pro-inflammatory responses. Thirdly, an IL-17 specific aptamer was verified to block IL-17A activity in fibroblast and fibroblast Th17 co-cultures but not in keratinocyte cultures. Significant uptake of the RNA aptamer by keratinocytes was identified as potentially responsible for the lack of neutralising capacity.

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Abbreviations

3-D – 3-dimensional

ACH - acrodermatitis continua of Hallopeau

AcP – accessory proteins

AD – atopic dermatitis

ADAM17 - a disintegrin and metalloprotease 17

AGEP – acute generalised exanthematous pustulosus

AMPs – antimicrobial peptides

APC – antigen presentation cells

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

ATP - adenosine 5'-triphosphate

BSA – bovine serum albumin

BP – binding protein

c/EBP - CCAAT-enhancer-binding protein

CAPS - cryopyrin-associated periodic syndromes

CARD - caspase activation and recruitment domain

Caspase - Cysteine-dependent aspartate-directed proteases

CCL - C-C motif ligand

CCR - C-C chemokine receptor type

CD – cluster of differentiation

c/EBP – CCAAT-enhancer-binding protein

CG – cathepsin G

CK10 – cytokeratin 10

CLA - cutaneous lymphocyte associated-antigen

CLE – cutaneous lupus erythematosus

CLF-1 – cytokine like-factor 1

CMV - human cytomegalovirus

COX - cyclooxygenase

CREB - cAMP response element-binding

CTLA-4 - cytotoxic T-cell antigen 4

CXCL10 - C-X-C motif chemokine 10

CXCR - C-X-C chemokine receptor

DAB - Di-AminoBenzidine

DAMPs - danger associated molecular patterns

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

DC – dendritic cell

Der p - *Dermatophagoides pteronyssinus*

DIRA - deficiency of IL-1 receptor antagonist

DITRA - deficiency of IL-36 receptor antagonist

DMEM- Dulbecco's modified eagle media

DNA – deoxyribonucleic acid

E - elastase

EBI3 - Epstein-Barr virus-induced 3

EDTA - Ethylenediaminetetraacetic acid

EGF – epidermal growth factor

EGFR - epidermal growth factor receptor

ELISA – enzyme-linked immunosorbent assay

ERK - extracellular signal-related kinases

FACS – fluorescence-activated cell sorting

FDA – food and drug administration

FCS – fetal calf serum

FGFR – fibroblast growth factor receptor

FITC - fluorescein isothiocyanate

GAG - galactosaminogalactan

GAS – gamma-activated sequence

GFP – green fluorescent protein

gp130 – glycoprotein130

GPP - Generalised pustular psoriasis

GWAS – genome wide associated studies

HBD – human beta defensin

H&E – haematoxylin & eosin

HDM – house dust mite

HEK – human embryonic kidney

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

HLA – human leukocyte antigen

HPK – human primary keratinocytes

HPV – human papillomavirus

HRP – horse radish peroxidase

IAA – iodoacetic acid

ICAM – intracellular adhesion molecule

IDECs - inflammatory dendritic epidermal cells

IFN - interferon

IL- interleukin

IL-18BP – IL-18 binding protein

IL-1RA - IL-1 receptor antagonist

IL-1Rrp2 – IL-1R related protein 2

IL-36RA – IL-36 receptor antagonist

IL-6R – IL-6 receptor

ILCs – innate lymphoid cells

IPTG - isopropyl β-D-1-thiogalactopyranoside

IRF – interferon regulatory factor

JAK – janus kinase

JNK - jun N terminal kinases

KGM – keratinocyte growth media

KLK – kallikrein related proteases

LAL - limulus amebocyte lysate

LEAF™ - low endotoxin, azide free

LEKTI - lympho-epithelial Kazal type inhibitor

LPS - lipopolysaccharide

MAPK - mitogen-activated protein kinases

MCP-1 - monocyte chemoattractant protein 1

M-CSF - macrophage colony stimulating factor

MDA5 - melanoma differentiation-associated protein 5

MDDCs - monocyte derived dendritic cell

MHC class II - major histocompatibility complex type 2

MIP1α – macrophage inflammatory protein 1 α

MMPs - Matrix metalloproteinases

MT3 - 3,3′,5-Triiodo-L-thyronine sodium salt

MS – multiple sclerosis

NETs - neutrophil extracellular traps

NF-κB - nuclear factor-kappa B

NK – natural killer

NLR - nod-like receptors

NLRP3 - NOD like receptor protein 3

NLS - nuclear localisation sequence

NOD - nucleotide-binding domain

NOMID – neonatel-onset multisystem inflammatory disease

NSP4 – neutrophil serine protease 4

NS – non-stimulated

OA – osteoarthritis

PAGE – polyacrylamide gel electrophoresis

PAMPs - pathogen-associated molecular patterns

PAR-2 - protease-activated receptor 2

PASI - psoriasis activity and severity score

PBMCs – peripheral blood mononuclear cells

PBS – phosphate buffered saline

PCR – polymerase chain reaction

pDCs - plasmacytoid dendritic cells

PE - phycoerythrin

PEG - polyethylene glycol

PI3K - phosphoinositide-3-kinase

PMA - phorbol 12-myristate 13-acetate

PMN – polymorphonuclear

PMSF - phenylmethylsulfonyl fluoride

Poly dA:dT - poly(deoxyadenylic-deoxythymidylic) acid

Poly I:C - polyinosinic:polycytidylic acid

PPP - palmoplanter pustular psoriasis

PR3 – proteinase 3

PRRs - pattern recognition receptors

PsA – psoriatic arthritis

PSORS1 - Psoriasis susceptibility region 1

qRT-PCR – quantitative real time PCR

RA – receptor antagonist

RIG-I - retinoic acid-inducible gene 1

RNA – ribonucleic acid

RNase - ribonuclease

ROI - reactive oxygen intermediates

ROS – reactive oxygen species

RPMI - Roswell Park Memorial Institute

SALT - skin-associated lymphoid tissue

Saps - secreted aspartic proteases

SDS - sodium dodecyl sulphate

SEFIR - SEF/IL-17R

SELEX - systemic evolution of ligands by exponential enrichment

SIS – skin immune system

SNPs – single nucleotide polymorphisms

SOCS – suppressor of cytokine signalling

SPINK5 - serine protease inhibitor Kazal-type 5

SPMs - specialised proresolving mediators

STAT - signal transducer and activator of transcription

T regs - regulatory T cells

TACE - TNF-converting enzyme

T-bet – T box expressed in T cells

TCR - T cell receptor

TGF-β - transforming growth factor β

Th1 – T helper cell 1

Th17 – T helper cell 17

Th2 – T helper cell 2

TIPE2 - TNF-α-induced protein-2

TLRs - toll like receptors

TNFR – TNF receptor

TNFα - tumour necrosis factor α

TRAF6 - TNFR-associated factor 6

UV – ultraviolet

VEGF – vascular endothelial growth factor

Chapter 1 - Introduction

1.1 Introduction

Psoriasis is a chronic inflammatory disease which predominantly affects the skin but can also involve the joints, leading to psoriatic arthritis. Recently co-morbidities such as cardiovascular disease and psychological disorders have been linked to the disease. Psoriasis is one of the most common chronic inflammatory skin diseases affecting around 2-3% of the population [1]. It is often ineffectively treated and considering the psychological burden and impact on the patient's quality of life it is important that the immunological basis of all disease subtype manifestations is more thoroughly understood. This will then provide a more personalised, stratified approach to treatment.

It is well recognised that there are many cell types and inflammatory mediators involved in psoriasis, including tumour necrosis factor α (TNFα), interleukin (IL)-1 and IL-17 [2]. The disease initiation is not well understood but a genetic component with an environmental trigger such as mechanical damage to the involved tissue (sometimes termed the Koebner phenomenon), may be involved. However, infiltrating immune cells reaching the skin as the result of chemokine gradients and other molecules released from skin resident cells play a critical role in disease development and chronicity [3].

The chronicity of psoriasis means that the inflammation is difficult to treat and the co-morbidities associated with this, such as psoriatic arthritis cause significant joint damage [4]. Therefore, it seems important that the psoriatic inflammation is treated as quickly as possible to prevent debilitating joint damage and other co-morbidities. At present the treatment for moderate-severe psoriasis insufficiently controlled by topical treatment relies on systemic immunosuppressant therapy (e.g. ciclosporin A, methotrexate) that may produce toxicities and the risk of infections. For patients that fail these conventional agents anti-cytokine biological therapies can be used. It is crucial that further understanding of the pathogenesis of the disease is achieved in order to develop novel therapeutics to induce sustained drug free remission or to define novel topical therapy strategies which are generally less likely to produce significant side effects.

There is good evidence that chronic inflammation in psoriasis is related to a disturbed balance between inflammatory cascade agonists and endogenous antagonists. This has

been demonstrated in the identification of a loss-of-function mutation in the IL-36 receptor antagonist which causes generalised pustular psoriasis (GPP), a life-threatening subtype of psoriasis [5]. The balance of these pro-inflammatory versus anti-inflammatory mediators may be a key to understanding disease pathogenesis and correcting this balance may be an achievable and effective therapeutic target. This has already been utilised with regard to IL-1 which can be neutralised by Anakinra®, which is a recombinant IL-1 receptor antagonist. Anakinra® is a highly effective treatment in a number of auto-inflammatory psoriasiform disease phenotypes such as the deficiency of IL-1 receptor antagonist (DIRA) syndrome [6, 7]. Understanding the complex immune dysfunction in psoriasis is crucial to provide patients with more effective therapy with fewer side effects.

1.2 Anatomy of the skin

The skin is divided into two distinct layers, the epidermis and the dermis separated by a basement membrane. Both layers contain a complex variety of cell types (see Figure 1-1). The epidermis is divided again into 4 layers, most inferiorly lies the stratum basale then moving outwards is the stratum spinosum, stratum granulosum and the stratum corneum. The outermost layer of the skin, stratum corneum consists of terminally differentiated keratinocytes (corneocytes) which form a crucial part of the physical barrier of the skin. Keratinocytes undergoing differentiation make up the majority of the epidermis. Differentiation-associated proteins are expressed by keratinocytes differentially throughout these layers. Keratins make up a large proportion of the intermediate filament cytoskeleton in epithelial cells and differentiation state of the keratinocytes can be ascertained by expression of certain keratins [8]. Apart from keratinocytes the epidermal layers also contain cells related to the hair follicle (including an epidermal stem cell compartment), Langerhans cells, pigment producing melanocytes and merkel cells which are sensory in nature. The physical barrier formed by the corneocytes and the acidic, hydrolipidic nature of the skin provide functional protection from invading pathogens. However, changes in keratinocyte differentiation or composition of the hydrolipidic layer undermine the barrier and play a substantial role in the pathogenesis of many skin diseases.

The dermal layer is composed of 2 layers, most inferiorly the stratum reticulare and superiorly the stratum papillare. The dermis is anatomically more complex, as well as resident immune cells there are fibroblasts and nerve related cells surrounded by a structural framework of collagen fibres. The dermis also has an extensive blood supply and lymphatic drainage which are crucial for migration of immune cells into the skin compartment [9]. Some immune cells are resident in both the dermis and epidermis. However, many more will migrate into the epidermis and dermis in an inflammatory state when tissue resident cells produce chemokines, such as C-X-C motif chemokine 10 (CXCL10) and IL-8. The infiltrating immune cells are also depicted in Figure 1-1.

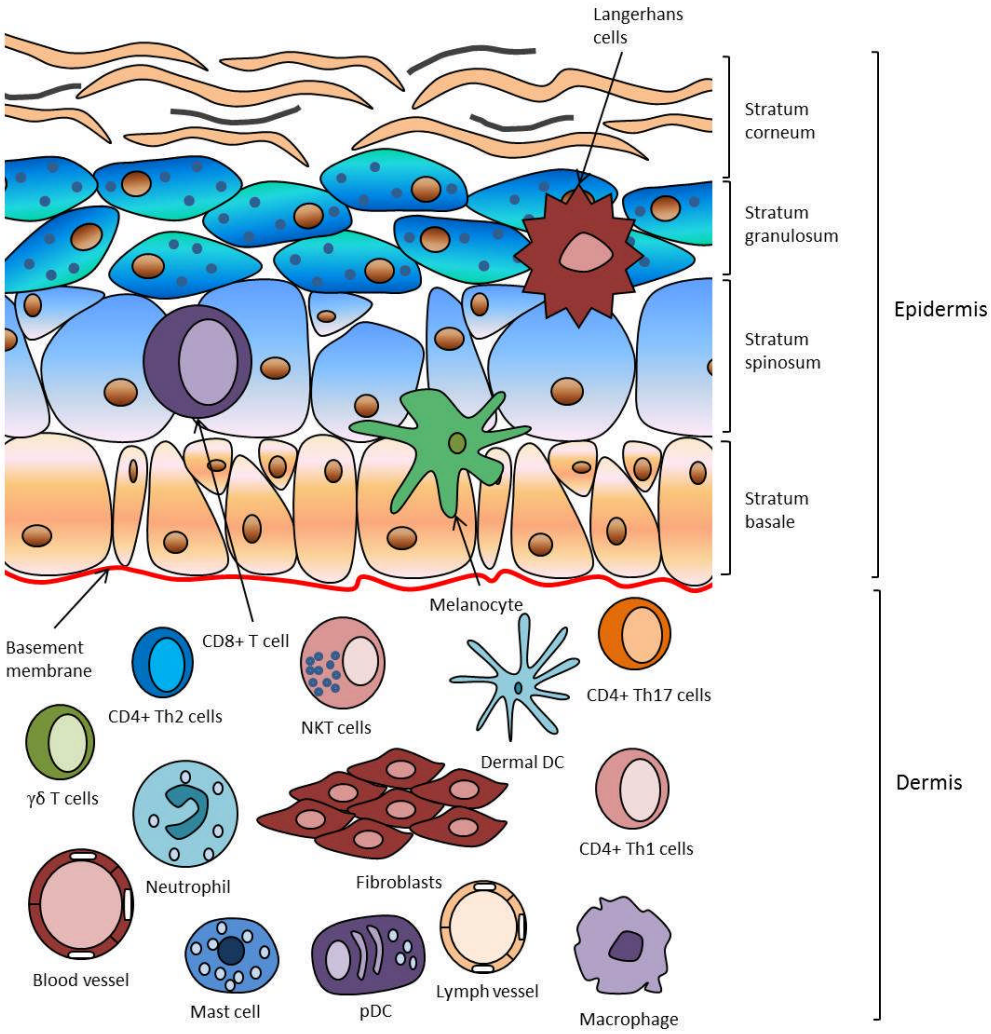


Figure 1-1. Diagrammatic representation of the layers of the skin.

The diagram clearly shows the 4 distinct layers of keratinocytes within the epidermis. The red layer at the base of the epidermis is the basement membrane. Resident immune cells are also depicted in the epidermis. Specialist cells are shown in the dermis however it is also rich in extracellular matrix and contains collagen, elastic fibres and reticular fibres [10]

1.3 Skin inflammation

The skin has a crucial function as a barrier, protecting the body from environmental challenges such as ultraviolet light, mechanical or chemical stress. Forming one of the largest organs in the body the skin is not just a traditional barrier but has roles as a sensory-receptive organ, a biochemical barrier to maintain hydration and also for synthesis of vitamins and hormones. The skin also plays an active role in mounting an

immune response against invading pathogens with barrier function disruption being associated with an increased risk of infection. The skin immune system is in a continued state of readiness to fend off microbial and other potentially detrimental agents so a dysregulated immune response may predispose to chronic, possibly auto-reactive, inflammation [10].

1.3.1 Initiation of skin inflammation

The skin immune function was first described in 1983 by Streilein who devised the term skin-associated lymphoid tissue (SALT) [11] which was later referred to as 'The skin immune system (SIS)' [12]. Streilein recognised that the skin had more than just a physical barrier function but was also important for local inflammatory response to trauma or pathogens and for the trafficking of immune cells from circulating blood and lymph to the skin. One of the important functions of the skin immune system in is wound healing. Moments after damage to the skin a well-orchestrated immune response occurs, this is the first step on the pathway to healing of the skin barrier.

The initiation of inflammation in the skin is importantly controlled by release of cytokines from skin resident cells and both resident and infiltrating immune cells. The specific cytokine profile indicates different physiological outcomes such as wound repair or immune activation, these different processes will involve different cell types. Keratinocytes are the cells that provide the 'first line of defence' against any invading pathogens. In a similar manner to other epithelial cells in mucosal linings keratinocytes can 'distinguish' between harmless resident organisms and harmful pathogens. The skin microbiome is extensive (10^{12} resident bacteria/m^2) and in normal conditions does not present a threat [13]. Keratinocytes express receptors named pattern recognition receptors (PRRs) that recognise evolutionary conserved microbial components proposed by Janeway as pathogen-associated molecular patterns (PAMPs) such as flagellin, nucleic acids and lipopolysaccharide (LPS) [14]. The most abundantly studied PRRs in the skin are toll like receptors (TLRs) with keratinocytes expressing a substantial number of these [15]. TLR 1, 2, 4, 5 and 6 recognise components of microbial cell wall and membranes unique to pathogens and are present on the cell surface. Whereas, TLR 3, 7, 8 and 9 recognise single and double stranded RNA from RNA viruses and DNA.

TLR 3, 7 and 9 are present in endosomes [16]. TLR activation leads to activation of nuclear factor-kappa B (NF-κB), mitogen-activated protein kinases (MAPK) and interferon regulatory factor (IRF) [17]. All of which are involved in the amplification of the immune response by increasing pro-inflammatory cytokines.

Keratinocytes also react to danger associated molecular patterns (DAMPs), which are non-infectious molecules that can initiate an immune response. This term was coined by Matzinger and revolutionised the way we explain recognition of danger by the immune system [18]. DAMPs involve molecules such as irritants, toxins, ultraviolet (UV) light and also cellular proteins or constituents that alert surrounding cells to danger following cell necrosis or mechanical damage to cells i.e. scratching or physical trauma (which is thought to be related to psoriatic lesion onset). It was more recently described that the nucleotide-binding domain, leucine-rich repeat-containing (NOD) like receptors (NLR) gene family can recognise both DAMPs and PAMPs and are highly expressed in the skin. Activation of these receptors which consequently up-regulates pro-inflammatory molecules is crucial in pathogen clearance and wound healing. LL-37 produced by keratinocytes in combination with RNA or DNA following cell damage can activate TLR7/8 on DCs which consequently causes release of cytokines such as IL-23. This is important for polarisation of IL-17 producing T cells and aberrant activation of pathways such as these lead to chronic inflammation such as psoriasis [19].

Pro-inflammatory cytokine release often occurs through activation of the inflammasome following TLR activation [20]. The inflammasome (described in more detail later in 1.4.1) is a large multi-protein oligomer that is present in the cytoplasm of most cells. Upon activation of the inflammasome, caspase-1 is activated from its pro-form. Active caspase-1 cleaves pro-inflammatory inactive cytokines such as IL-1β and IL-18 into their active forms [21]. These pro-cytokines are stored inside the cell and released upon activation of e.g. keratinocytes [22, 23]. The release of these IL-1 family cytokines, not only plays a role in amplifying the immune response it also has effects on the keratinocytes themselves inducing proliferation or differentiation therefore altering the barrier function of the skin. This is relevant in diseases, such as atopic dermatitis, which is linked to impaired barrier function [24]. The pro-inflammatory cytokines released

following inflammasome activation are controlled by binding proteins and receptor antagonists, such as IL-18 binding protein and IL-36 receptor antagonist. On the other hand, up-regulation of inflammatory cytokines such as IL-1, interferon (IFN)γ and IL-17 plays a vital role in removing invading pathogens by up-regulating antimicrobial peptides (AMPs), such as LL-37 and defensins [25]. These molecules are cationic and kill bacteria by creating holes in the anionic membranes of the bacteria or by removing iron required for bacterial growth [26]. AMPs such as LL-37 also have chemotactic roles, causing infiltration of immune cells into the skin.

There is evidence that points to the idea that activation of keratinocytes initiate inflammation before infiltration of any immune cells [27]. Although we understand that the skin resident cells play a crucial role in inflammation it is also known that without immune cell infiltration inflammation cannot occur to its full extent (See Figure 1-2). The skin contains several populations of dendritic cells (DCs) the best known of which is Langerhans cells. These are resident in the epidermis so are one of the first subsets of immune cells to come in to contact with any pathogen or insult to the skin. This subset of cells is a specialist DC that after migrating to draining lymph nodes effectively presents antigens to naïve T cells in order to cause clonal expansion of specific T helper cell subsets [28]. In health the DCs are likely to be more tolerogenic, however in pathogenic circumstances DCs may lose their tolerance and induce inflammation [29]. Depending on the type of antigen and the co-stimulatory signals presented by the Langerhans cells, or dermal dendritic cells, gives rise to proliferation of a specific subset of T helper cells. Following inflammation where a specific set of T helper cells have been involved, some remain in the skin on resolution of inflammation. These are then known as tissue-resident memory T cells and provide a faster immune response if infected with the same pathogen again. Twice as many of these memory cells have been suggested to be present in healthy skin compared to peripheral blood and 98% of these are cutaneous lymphocyte associated-antigen (CLA) + [30].

It is known that within minutes of damage to the skin neutrophils are present, recruited in to the skin by release of IL-8 from skin resident cells [31]. These cells play an invaluable role in immediate response to invading pathogens by release of, among

others, antimicrobial molecules, proteases and neutrophil extracellular traps (NETs) [32, 33]. Contrary to early research into the role of neutrophils as purely early inflammatory response cells it is now understood that neutrophils have a diverse role in both innate and adaptive immunity. Neutrophils express a wide range of receptors such as TLRs [34], C-type lectin receptor Dectin-1 [35], retinoic acid-inducible gene (RIG)-1 and melanoma differentiation-associated protein (MDA) 5 [36]. Therefore under specific circumstances when they migrate into the skin they can respond to damage or pathogens by release of a diverse range of cytokines [37].

Macrophages and DCs resident in the dermis are also crucial cells both for antigen presentation and phagocytosis of pathogens and apoptotic cells. Monocytes are rapidly recruited from the blood in response to keratinocytes chemokines such as monocyte chemoattractant protein (MCP)-1 [38]. Both macrophages and neutrophils release proteases and reactive oxygen species therefore providing potent antimicrobial properties and further inflammatory cascade amplification [39].

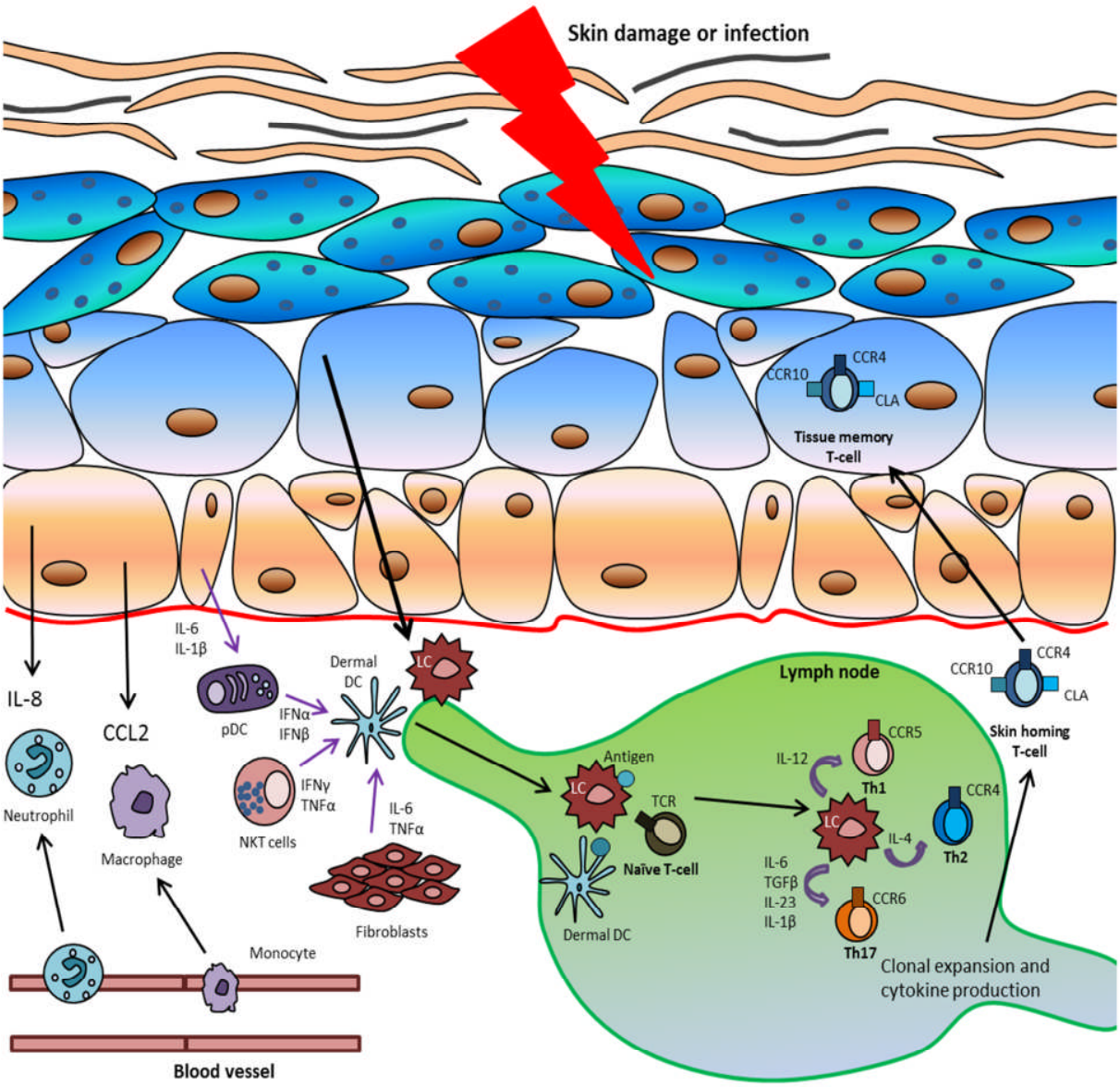


Figure 1-2. Diagrammatic representation of initiation of skin inflammation.

When skin is damaged or irritated both skin resident cells and infiltrating immune cells mount a well-orchestrated response to maintain skin homeostasis. Keratinocytes sense damage or pathogenic invaders and respond by secretion of chemokines and cytokines that attract infiltrating immune cells and activate fibroblasts. Fibroblasts in turn will produce further cytokines. Langerhans cells and dermal dendritic cells play a crucial role in antigen presentation of 'new' pathogens to naïve T cells which consequently produces clonal expansion and skin homing T cells of the relevant subset. Macrophages and neutrophils

also play a critical role in skin inflammation being recruited by chemokines produced predominantly by keratinocytes [40].

The initiation of inflammation requires a concerted effort from all cell types involved to deliver a well-orchestrated response to clear any pathogens or damage that may be compromising the skin barrier (as depicted in Figure1-2).

1.3.2 Maintenance and resolution of skin inflammation

In health a tightly controlled immune response orchestrates inflammation until the pathogen or damage has been cleared or repaired with subsequent resolution of inflammation and restoration of normal skin homeostasis. However, a recent publication has suggested that there is in fact a post-resolution phase which bridges the gap between innate and adaptive immunity [41]. A state of 'adaptive homeostasis' in the tissue allows for an appropriate response of repeat stimuli, of note this has been established in a murine model [41]. The regulation of inflammation is controlled by a number of mediators including cytokines, such as receptor antagonists and binding proteins as well as transcription factors, many of these are involved in prevention of spontaneous inflammation without microbial provocation or autoimmunity [42]. These molecules form a coordinated response that not only prevents spontaneous inflammation but also controls and aids resolution of acute inflammation [43].

Once the acute inflammatory 'trigger' has been resolved, leukocytes must be cleared from the skin in order to maintain normal skin homeostasis. This process is not a passive process as previously thought [44] but a metabolically active biochemical process. Neutrophils also play a role in the resolution of inflammation. At the final stages of acute inflammation when neutrophils undergo apoptosis a switch from production of lipid mediators (such as eicosanoids) to specialised proresolving mediators (SPMs) occurs [45]. The specialised SPMs include resolvins and lipoxins which prevent further leukocyte infiltration and encourage clearance of debris and apoptotic cells by macrophages in a non-phlogistic manner [46]. Soluble proresolving mediators reduce leukocyte infiltration by down-regulation of integrins such as CD11b which prevents diapedesis [47]. It has recently become evident that neutrophil production of lipid mediators is dependent on the environment they are in and the cells they interact with.

Other cell types in the skin such as fibroblasts can also produce SPMs on contact with neutrophils, as well as macrophages in a specific cytokine micro milieu [48]. A novel function of these SPMs has also been shown to regulate microRNAs that are involved in resolution of inflammation [49]. Neutrophils are not only responsible for release of SPMs but the increase of these mediators up-regulates receptors such as C-C chemokine receptor type 5 (CCR5) by apoptotic neutrophils. These receptors act as decoy receptors for chemokines that would otherwise attract further infiltration of leukocytes [50].

In a proresolving environment cytokines released by macrophages also act on neutrophils to actively release IL-1 receptor antagonist (IL-1RA) which will reduce the inflammatory actions of IL-1 [51]. Lipoxins also act upon epithelial cells to reduce IL-8 release in response to TNFα [52]. Of note, lipoxins also block CD3-specific antibody binding restricting NF-κB signalling in T cells, therefore reducing release of pro-inflammatory cytokines [53]. SPMs also regulate macrophages. It is generally regarded that M1 differentiated macrophages are more pro-inflammatory whereas M2 exert more anti-inflammatory functions. The differentiation fate of macrophages is determined by micro milieu of cells and cytokines [54, 55]. SPMs have been shown to cause differentiation of macrophages into an M2 lineage, favouring a more anti-inflammatory role [47, 56]. Macrophages are also crucial to resolution of inflammation, being the most prevalent phagocytic cells, and on ingestion of dead and dying cells they release anti-inflammatory molecules such as transforming growth factor (TGF)-β and IL-10 [57, 58]. However, this anti-inflammatory signature cytokine release only occurs on phagocytosis of apoptotic cells and not necrotic cells.

As with initiation of inflammation in the skin, the resident cells are also important in controlling inflammation. As discussed further in chapter 3 we have shown that on following IL-27 stimulation skin fibroblasts and keratinocytes will initially respond by releasing CXCL10, which initiates infiltration of IFNγ producing lymphocytes. *In vitro,* after 24 hours pro-inflammatory mediators will no longer be released and a switch to anti-inflammatory molecules such as binding proteins (BP) and receptor antagonists (RA) is seen [59]. Up-regulation of RA can be detected along with its pro-inflammatory

agonist and this seems to be an important control mechanism to prevent an uncontrolled inflammation [60].

To maintain homeostasis the skin has many, not yet fully elucidated mechanisms by which to control inflammation and resolve it in a timely manner. If these mechanisms fail, chronic inflammation may occur.

1.3.3 Chronic inflammation

Chronic inflammation can occur for various reasons including the persistence of an inflammatory trigger, genetically determined autoinflammation and lack of inflammatory resolution. An example of chronic inflammation caused by persistence of an inflammatory trigger is the presence of non-degradable particles of asbestos or silica which consistently activate the inflammasome therefore causing aberrant release of pro-inflammatory cytokines, such as IL-1β and IL-18 [61]. This is particular relevant in chronic inflammation of the lung epithelium on inhalation of particulates which often leads to neoplastic disease [62] or in chronic joint inflammation seen in gout [63]. Chronic inflammation seen in atopic dermatitis (AD) involves breakdown of the barrier, super infection and of course allergen exposure. Therefore, a persistent inflammatory trigger is present. However, AD also has a strong genetic component and the most widely replicated risk factor is a loss-of-function mutation in the gene encoding filaggrin, which is involved in epidermal barrier maintenance [64, 65]. Chronic inflammation in AD may be related to barrier function breakdown with persistent antigen access and risk of lesional skin super infection [66].

Another cause underlying the development of chronic inflammation is the presence of an abnormal, overactive inflammatory response related to a genetically determined autoinflammation. In Crohn’s disease a subset of patients have a NOD2 mutation which results in an aberrant response to inflammatory stimuli due to increased inflammasome activity [67]. Neonatal-onset multisystem inflammatory disease (NOMID) is an example of a disease where aberrant IL-1 release results in systemic inflammation including significant skin pathologies [68]. This genetic disease is due to a gain-of-function

mutation in the inflammasome complex which activates caspase-1 and consequently activates IL-1β, leading to excessive IL-1β release.

Chronic inflammation can also be related to lack of inflammatory resolution and infiltrating immune cells remaining in the tissue, chronic inflammation has been regarded as lymphocyte, macrophage and DC mediated until more recently [69]. Neutrophils, for example, have been shown to play a role in the resolution of inflammation, however if excessive or prolonged neutrophil infiltration into the skin occurs then this can lead to release of enzymatic granule contents during incomplete phagocytosis [31]. This consequently leads to tissue damage which is prolonged beyond clearance of the pathogen and may lead to a pattern of chronic inflammation [31]. Excessive or prolonged neutrophil infiltration is seen in chronic inflammatory skin diseases such as psoriasis (discussed in detail in 1.3). However, more recently it has been proposed that fibroblasts play an important role in transition from acute to chronic inflammation. The cytokine micro milieu produced by skin resident cells defines the inflammatory infiltrate, therefore if there is aberrant production of pro-inflammatory mediators by fibroblasts this prevents resolution of inflammation [70].

1.4 Psoriasis and psoriatic arthritis

Psoriasis is a common inflammatory skin disease. It has, however, recently become evident that psoriasis can no longer be considered as solely a skin disease but potentially a systemic disease that can affect the joints and the cardiovascular system [71]. Psoriatic disease of the skin is present in around 2-3% of the European and North American population and affects more than 25 million people in Europe and North America [72]. Psoriasis can be divided into 3 main subtypes: vulgaris (which simply means common psoriasis), guttate and pustular. Psoriasis vulgaris is most extensively researched due to the fact that it affects approximately 85-90% of psoriasis sufferers [73]. It is characterised by ‘scaly’ plaques that involve an increased capillary network in the upper dermis, and inflammatory infiltrate including mononuclear cells, T cells and neutrophils into both the dermal and epidermal compartment. The epidermis is greatly enlarged (acanthosis) with keratinocytes moving through the epidermis every 4-5 days, a tenfold acceleration compared to healthy skin. Parakeratosis (maintenance of nuclei into the

cornified layer) and loss of stratum granulosum is present, with aberrant expression of keratins in the epidermis namely cytokeratin 16 [19].

1.4.1 Clinical characterisation of psoriasis

Clinical severity of psoriasis is determined by using the psoriasis activity and severity score (PASI) which assesses; erythema (redness), induration (thickness), and desquamation (scale) of the plaques in different body sections, with 72 as the maximal score [19].

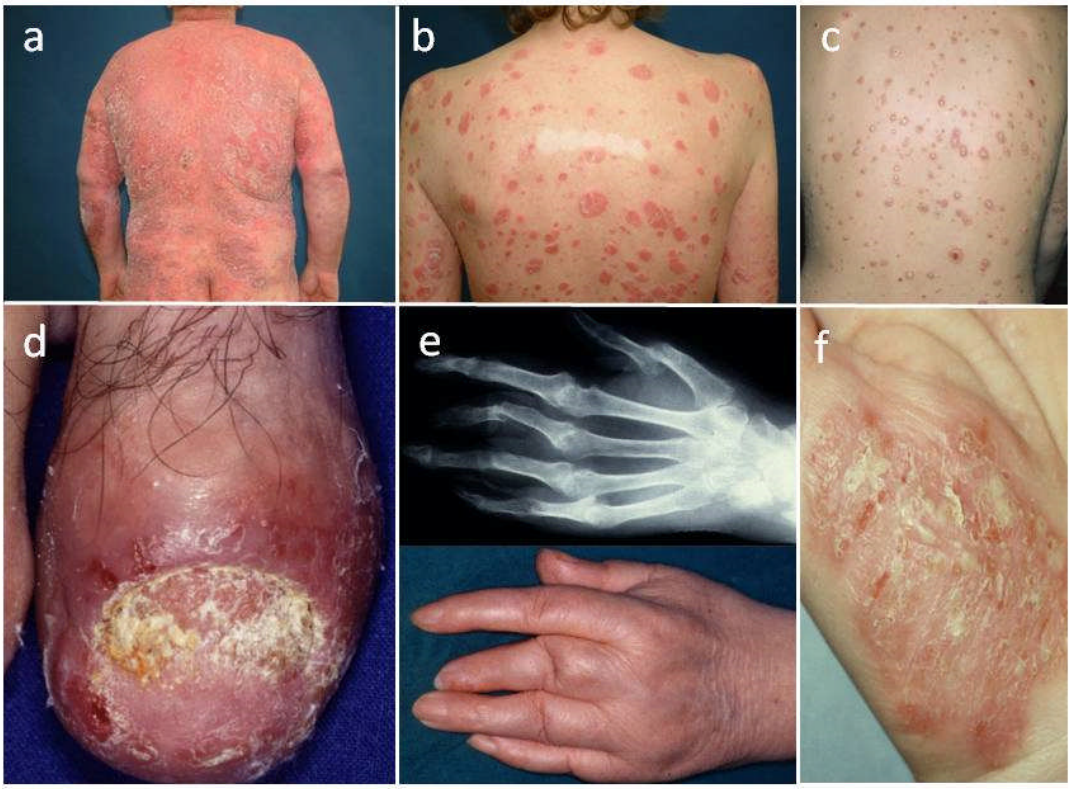


Figure 1-3. Clinical characteristics of the psoriatic subtypes.

Clinical photos showing a typical phenotype for each psoriatic subtype. a – Erythrodermic psoriasis. b – Psoriasis vulgaris. c – Guttate psoriasis. d - Acrodermatitis continua of Hallopeau (a pustular psoriasis subtype). e – Psoriatic arthritis. f – Palmoplanter pustular psoriasis (a pustular psoriasis subtype). Photos kindly provided by Dr Miriam Wittmann.

1.4.1.1 Psoriasis vulgaris

Psoriasis vulgaris is characterised by demarcated dry, red plaques with silvery white loosely adherent scales. A typical symptom is the Auspitz phenomenon where mild disruption to the superficial layer of the lesion results in pin point bleeding. These plaques characteristically form on the extensor region of the elbows and knees and lumbosacral region and also commonly effect the scalp [3]. Moderate to severe psoriasis effects 3-10% of psoriasis vulgaris patients and is termed as such if more than 10% of the body is covered [19]. Moderate psoriasis effects 25% and mild psoriasis effects 65% of patients. The development of psoriasis vulgaris is complex and is likely to be different for different subgroups of patients. The Koebner phenomenon, which is defined by mechanical stress causing development of a lesion, suggests that lesions often occur after mechanical trauma to the skin.

1.4.1.2 Guttate psoriasis

Guttate psoriasis in contrast to psoriasis vulgaris is characterised by erythematous forming small droplet (‘gutta’) shaped plaques about 1cm in diameter. Guttate psoriasis often presents with an acute onset of the disease so all plaques are at the same developmental stage. It is common in children and adolescents following a *Streptococcal* infection or upper respiratory tract infection [74]. Unlike psoriasis vulgaris the disease is often localised to the trunk. Guttate psoriasis is most often self-limiting and will spontaneously resolve, however following an outbreak of guttate psoriasis the risk of developing chronic psoriasis vulgaris is increased [75].

1.4.1.3 Pustular psoriasis

Pustular psoriasis can either be localised or generalised. Lesions are characteristically tender sterile pustules which have underlying erythematous. Histologically diffuse neutrophil infiltration is detectable in the dermis and epidermis [76]. Localised pustular psoriasis can present as palmoplanter pustular psoriasis (PPP) or acrodermatitis continua of Hallopeau (ACH). PPP is characterised by sterile pustules and hyperkeratosis of the palmar and plantar surfaces and it is commonly associated with psoriatic nail involvement [77]. ACH affects predominately the fingers and toes, however the pustules

can become confluent and spread to the dorsal aspects of the hands and feet. Generalised pustular psoriasis (GPP) is a rare subtype of psoriasis, however it is a severe form and affected patients may need intensive care treatment. A subset of GPP has recently been associated with a loss-of-function mutation in the IL-36 receptor antagonist (IL-36RA) gene [5]. Treatment can be difficult but success has been described using conventional drugs such as Acitretin or methotrexate but also biologics involving both TNF and IL-1 inhibitors [78].

1.4.1.4 Erythrodermic psoriasis

This sub type of psoriasis is characterised by widespread erythrema involving most of the body surface, with some nail involvement. It is often due to poorly managed psoriasis vulgaris or sudden halt of systemic medication. It can also be related to a drug reaction, such as lithium, or systemic inflammation [79]. As for GPP, this subtype can be life threatening if left untreated.

1.4.1.5 Psoriatic arthritis (PsA)

PsA is thought to affect 20-30% of people suffering from psoriasis vulgaris [80-82]. PsA was originally described by Wright as having 5 clinical characteristics; distal predominant pattern, oligoarticular asymmetrical, polyarticular rheumatoid arthritis-like, spondylitis, and arthritis mutilans [82]. It has now been identified that PsA is often associated with enthesitis [83], for which diagnosis has progressed with use of MRI imaging [84]. It has been suggested that 20% of PsA patients will develop an aggressive debilitating disease as seen in rheumatoid arthritis if not treated early and effectively [85]. As a general rule patients with PsA have a more severe skin disease and are at a higher risk of developing other co-morbidities associated with severe psoriasis [86].

1.4.2 Pathogenesis of psoriasis

It is widely accepted that psoriasis pathogenesis involves a complex interplay between genetic and environmental factors. It has been described that genetic factors predispose to an overreaction to environmental stimuli such as mechanical stress [10]. Plaque initiation is thought to involve pDCs and myeloid DCs which respond to release of DAMPs and self DNA by damaged keratinocytes [19]. This view is supported by a

murine model where imiquimod (a TLR7 agonist) initiates a psoriasis-like plaque [87], however this does fail to fully represent human psoriasis. Psoriasis is characterised by a distinctive micro milieu of cytokines that interact in a network manner (see Figure 1-4). It has been shown that in a co-culture of keratinocytes and activated CD4+ T cells large amounts of IL-17 can be detected [88]. This response was firstly dependent on IL-1β and secondly on IL-23 [88]. It is known that IL-1β and IL-17 can induce each other which indicates a possible role for these cytokines in a positive loop mechanism within psoriatic skin. IL-17 is predominantly produced by Th17 cells [89] and these cells are regulated and polarised by IL-23, IL-1 and IL-6 [90] in humans. In psoriatic plaques it has been described that the CCR6+ Th17 cells are attracted into the skin by a chemokine, C-C motif ligand (CCL)20, which can be released from keratinocytes in response to TNFα [91]. On binding of IL-17 to its receptor further induction of pro-inflammatory cytokines, such as TNFα, IL-8 and IL-36 (see 1.4.3), occur which leads to neutrophil attraction [90]. These increased levels of neutrophils can be linked to psoriasis due to the accumulation of neutrophils in the form of Munro's micro-abscesses [92], suggesting that IL-8 is important in the pathogenesis of psoriasis. IL-22 is also released from activated Th17/Th22 cells and is recognised to cause the phenotypic epidermal symptoms by primarily acting on keratinocytes [93]. As well as Th17 cells Th1 cells are also present in lesional psoriatic skin and the inflammatory environment contains Th1 related cytokines such as IFNγ [94]. IL-12 release from DCs is crucial for Th1 polarisation [19]. IL-12 and IL-23 are heterodimeric cytokines and share the p40 subunit, due to the known role of these cytokines in psoriatic pathogenesis this p40 subunit has been targeted for antibody therapy (Ustekinumab) [95]. Ustekinumab prevent binding to of these cytokines to their receptors therefore preventing polarisation of Th1 and Th17 cells and it has also been shown to inhibit activity of neutrophils and monocytes [95]. This therapy shows good clinical outcome however more therapeutics targeting small molecules within this pathway are also being developed such as those targeting tyrosine kinase 2 and JAK1 which prevents IL-23 signalling [96].

Members of the IL-1 family have also been implicated in the pathogenesis of psoriasis. IL-36γ has been shown to be increased in psoriatic plaques and is thought to increase expression of AMPs [97]. It has also been shown to be up-regulated at the RNA level in

psoriatic keratinocytes compared to healthy keratinocytes upon stimulation with IL-17 [60] which points to an intrinsic difference in IL-36γ regulation in psoriasis. AMPs such as human beta defensin (HBD) 2 and 3, are known to be up-regulated in psoriatic skin and are thought to be responsible for the low infection rate of psoriatic plaques. An increase in copy number for HBD2 has also been described contributing to the genetic background of the disease [98]. The mentioned mediators often have synergistic effects, this has been shown for TNFα and IL-17 [2]. The combination of IL-1α with EGFR ligands such as TGFα induces much higher up-regulation of AMPs, such as HBD-2 and S100A7, than any of the mediators alone [97].

The innate immune system may play a more substantial role in psoriasis pathogenesis than first thought [99]. Of interest, the recently identified type 3 innate lymphoid cells (ILCs) have been identified as a significant source of IL-17 and IL-22 [100]. There have been 3 subsets of ILCs identified in humans and these are grouped in relation to their dependence on specific transcription factors and the cytokines released. The ILC subset which produces IL-17 and IL-22 [101] is NKp44 + and has been shown to be enriched in peripheral blood and skin from psoriasis patients [102, 103]. Following treatment with biologics such as anti-TNFα this enrichment reduces back down to baseline [104]. This further implicates the role of not only the adaptive immune system but also innate cells in the complex pathogenesis of psoriasis.

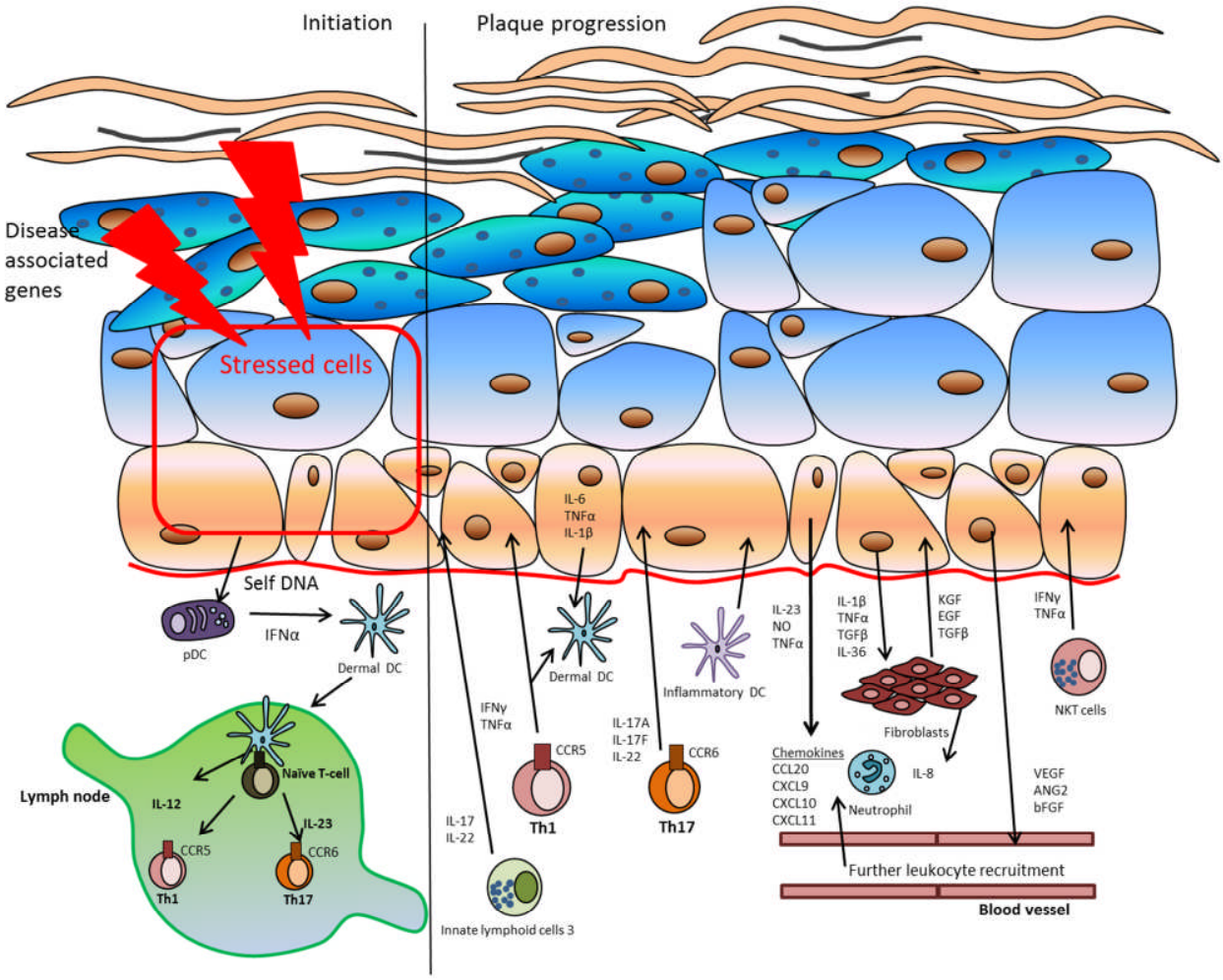


Figure 1-4. Diagrammatic representation of psoriatic inflammation.

The immunological basis for initiation of psoriatic plaques is not fully understood and the many information are derived from murine models. However, it is thought that in human disease DCs are important in the initiation of disease following keratinocyte damage. Infiltrating immune cells and the cytokines released by resident and infiltrating immune cells drive chronicity of the disease.

The pathogenesis of psoriasis is very complex. However, with further understanding of both the pathogenesis and the genetic background for different subtypes of disease treatment will be more effective following a more 'personalised' medicine approach.

1.4.3 Associated co-morbidities in psoriasis

There has been a long-standing appreciation for the fact that there were co-morbidities present in connection with psoriasis. Nail dystrophy, psoriatic arthritis and depression

are well known co-morbidities [3]. Recent studies have shown that psoriasis has a severe psychological burden on patients [105] which may explain the links to depression and other psychological co-morbidities such as smoking and alcoholism [86]. More recently cardiovascular co-morbidities such as atherosclerosis [106] as well as obesity, diabetes and metabolic syndrome have all been associated with psoriasis with a prevalence ranging from 7-50% [80, 107, 108]. Taken together this reinforces the concept that psoriasis is an extremely complex disease of the immune system not only in the skin but also systemically which indicates need for multidisciplinary management and targeted therapy of these patients.

1.4.4 Genetic implications in psoriasis

The notion that genetic changes play a role in the disease is supported by 70% concordance rate in monozygotic twins and 20% in dizygotic twins [109]. There are multiple genetic variants that contribute to a psoriatic phenotype. Psoriasis susceptibility region 1 (PSORS1) is the strongest susceptibility locus located in the major histocompatibility complex and human leukocyte antigen (HLA)-Cw6 is the suggested primary allele. Patients who are heterogeneous for this mutation are more susceptible to develop early onset psoriasis vulgaris or guttate psoriasis [110].

The increase in genome wide association studies (GWAS) has led to 36 single nucleotide polymorphisms (SNPs) being identified in the European population [99]. Most of these SNPs are related to immune function or skin barrier function (see Table 1-1). However, 2 mutations identified can cause disease independently, *IL-36RN* and *CARD14.* The loss-of-function mutation of IL-36RA identified in 2011 [5, 111] leads to un-controlled activity of the IL-36 cytokines which cause downstream activation of NF-κB leading to a multitude of inflammatory mediator release (discussed in more detail in 1.4.3.2). The *CARD14* mutation is a gain-of-function mutation. CARD14 is involved in activation of NF-κB and this mutation causes an increase in activity therefore again causing an increase in inflammatory mediators [112, 113]. These poignant mutations give us insight into psoriatic disease pathogenesis due to the lack of regulation of inflammatory mediators leading to this inflammatory disease.

Known susceptibility loci	Notable genes	Amino-acid substitution	Function
rs9988642	*IL23R*	p.Arg381Gln (P)	IL-23 signalling
rs27432	*ERAP1*	p.Gln730Glu	Encodes for endoplasmic reticulum aminopeptidase 1 – important in immune function due to cleavage of receptors and inflammatory cytokines [114].
rs1295685	*IL13*	p.Arg144Gln	Th2 cytokine
rs33980500	*TRAF3IP2*	p.Asp19Asn (S,P)	A TNF associated receptor factor that encodes Act1. Mediates NF-κB signalling [115].
rs2066819	*STAT2*	p.Met594Ile	Transcription factor involved in type 1 IFN signalling [116].
rs12445568	*PRSS53*	p.Pro406Ala	Encodes polyserases, serine proteases [117].
rs11652075	*CARD14*	p.Arg820Trp (S)	Encodes a family of caspase recruitment domain-containing scaffold proteins (CARD) and membrane associated guanylate kinase-like domain-containing protein (CARMA), mediates recruitment and activation of NF-κB [118].
rs34536443	*TYK2*	p.Pro110Ala (S,P)	Encodes tyrosine kinase 2 and a member of the Janus kinase family and involved in downstream signalling of many pro-inflammatory cytokines [119].
rs12720356	*TYK2*	p.Ile684Ser (S,P)	
rs4821124	*YDJC*	p.Ala263Thr	Function not well described, however may play a role in cleavage of cellobiose-phosphate [120].

Table 1-1. Potential causal SNPs following meta-analysis of Immunochip and GWAS data to identify psoriasis susceptibility loci.

A summary table to show SNPs that have potential causal effects in development of psoriasis in relation to a recent publication analysing 3 GWAS studies and 2 independent studies genotyped using an Immunochip. The notable genes, the predicted amino-acid change and the function are also shown. High-confidence damaging effect (amino-acid change), amino-acid substitution, predicted by SIFT (S) or Polyphen (P). This meta-analysis was performed using 3 genome wide association studies (GWAS) identified 15 new susceptibility loci and confirmed other SNPs that had been previously associated to psoriasis **[98]**.

Table 1-1 shows potentially causal SNPs for the disease and gives us an insight into the importance of the innate immunity in psoriasis, it is worth noting that this is not all the SNPs identified but those identified as potentially causal in a recent publication [99]. However it is well regarded that psoriasis is not simply a genetic disease but other factors are involved for this disease to develop.

1.5 The IL-1 family

The IL-1 family of cytokines play an important role in immune regulation and can control inflammation at both the receptor and nuclear level. These cytokines act on nearly all cells and therefore all organs of the body and are the classical endogenous pyrogens (fever inducing), which consequently leads to their role as pathogenic mediators in many diseases [121]. IL-1 was identified when researchers were looking for the cause of fever; it was observed in the 1940's by Beeston and Menkin that supernatant from rabbit neutrophils induced fever and the cause of this was shown to be small proteins [122]. We now know these as IL-1α and IL-1β and these were identified in the 1970's. These members along with IL-1 receptor antagonist (RA) and IL-18 were identified following purification from cells, whereas other members were identified using *in silico* methods from gene banks.

Some IL-1 family cytokines have potent pro-inflammatory effects which are tightly regulated on the pre- and post-translational level which includes soluble receptors and receptor antagonists. IL-1 family cytokines impact on all aspects of the immune response, including polarisation and regulation of Th1, Th2 and Th17 lymphocytes as well as mast cells, neutrophils and tissue resident cells. The pro-inflammatory IL-1

cytokines are crucial to protect cells from infection by activation of systemic and local responses. However if this tight regulation is disturbed at any level it can be detrimental, as seen in septic shock [123].

There are 11 members of the IL-1 family and they were originally given the nomenclature IL-1F1-11, however with elucidation of their functional properties, most have been given individual IL designations [124] (See Table 1-2). All of these members are located in a cluster on chromosome 2 suggesting they arose from a common ancestral gene [125].

Original Name	**Alternative Name**	**Property**	**Receptor**	**N-terminal cleavage required for activity**
IL-1F1	IL-1α	Agonist Nuclear transcription factor	IL-1RI, IL-1RAcP IL-1R11	No
IL-1F2	IL-1β	Agonist	IL-1RI, IL-1RAcP IL-1R11	Yes
IL-1F3	IL-1Ra	Receptor antagonist	IL-1RI	No
IL-1F4	IL-18/IFN-γ inducing factor	Agonist	IL-18Rα, IL-18Rβ	Yes
IL-1F5	IL-36Ra	Receptor antagonist	IL-1Rrp2/IL-36R	Yes
IL-1F6	IL-36α	Agonist	IL-1Rrp2/IL-36R, IL-1RAcP	Yes
IL-1F7	IL-37	Anti-inflammatory	IL-18Rα	?
IL-1F8	IL-36β	Agonist	IL-1Rrp2/IL-36R, IL-1RAcP	Yes
IL-1F9	IL-36γ	Agonist	IL-1Rrp2/IL-36R, IL-1RAcP	Yes
IL-1F10	IL-38	Possible antagonist [126]	IL-1Rrp2/IL-36R?	?
IL-1F11	IL-33	Agonist Transcriptional repressor	ST2, IL-1RAcP	No

Table 1-2. The IL-1 family nomenclature and associated receptors.
This includes newly designated names that relate to the functions of the cytokines [127] [128] [129] [122].

The IL-1 family can be categorised into functional sub groups based on the length of the precursor and taking into account the members that have antagonistic properties on the

others. One functional group includes IL-1α, IL-1β and the antagonist IL-1RA, another group includes IL-18 and its antagonist IL-18 binding protein (described in further detail in 1.4.4). The third group includes IL-36α, IL-36β, IL-36γ and the antagonist IL-36RA. Apart from IL-1RA all of the IL-1 family members do not contain a signal peptide and are primarily intracellular precursors. This means that release of these cytokines from the cell has to be via a non-conventional pathway or following cell death. All IL-1 members have a conserved IL-1 domain that consists predominantly of beta strands [118]. The receptor antagonists and binding proteins that block the activity of the pro-inflammatory members of the IL-1 family represent potential therapeutic molecules for diseases where dysregulation of IL-1 production occurs, such as in auto-inflammatory disorders [119]. Unlike other cytokines, IL-1 members can control inflammation at both the protein level and transcriptional level, both IL-1α and IL-33 have been described to relocate to the nucleus and act as transcription factors [122].

1.5.1 Activity and processing of the IL-1 family members

A common feature of some members of the IL-1 family is that cleavage at the N-terminus is required in order to increase binding affinity to the receptor resulting in increased activity (see Table 1-2).

The activation of pro-IL-1β and pro-IL-18 is dependent on cleavage by caspase-1 [129]. Caspase-1 is activated by the inflammasome, which is a macromolecular protein complex present in the cytoplasm of most cells. The classical inflammasome is made up of a cytosolic PRR which is often the NLR, the adapter molecule apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD) (ASC) domain, and caspase-1 [130]. These cytosolic PRRs that make up the inflammasome can either be in the NLR family of receptors or pyrin and HIN domain containing receptors [131]. There are three proposed mechanisms for activation of the inflammasome. One is that extracellular adenosine 5'-triphosphate (ATP) stimulates the purogenic P2X7 ATP-gated ion channel triggering a K+ efflux which is thought to cause formation of pores by pannexin-1 delivering the specific ligand to the inflammasome [132]. A second proposed mechanism is that DAMPs are engulfed by phagocytes which can result in lysosomal damage and release of lysosomal enzymes, such as cathepsin B,

which activate NOD like receptor protein 3 (NLRP3) [133]. The third mechanism is that NLRP3 inflammasome ligands cause production of reactive oxygen species (ROS) which will then activate the NLRP3 inflammasome [61]. The activation mechanism of the inflammasome may differ depending on cell type as it has been shown that UV initiates an increase in intracellular Ca^{2+} which activates the NLRP3 inflammasome in keratinocytes [134].

At present four inflammasome complexes have been described that will assemble in the presence of different stimuli which are given in brackets; NLRP1 (Anthrax lethal toxin), NLRP3 (Cell stress), NLRC4 (Flagellin), AIM2 (double stranded DNA). The best studied is the NLRP3 inflammasome which is known to be activated by various stimuli including bacteria, bacterial RNA and danger associated molecular patterns (DAMPs) [135]. It is well regarded that the processing of caspase-1 dependent IL-1 members is a two-step process. Signal 1 is required to prime the cells and increase transcription of pro-IL-18 or pro-IL-1β and NLRP3 expression via NF-κB, this is often due to microbial products such as LPS via TLR activation [136, 137]. The second signal is required to initiate assembly of the inflammasome therefore leading to active caspase-1 and consequently active IL-18 or IL-1β, this is often a stimulus such as ATP or a crystal (e.g. urate) [138, 139].

It is thought that caspase-1 may also play a role in release of mature IL-1β, either in a secretory lysosomal manner or by microvesicles, where the complex of mature IL-1β and caspase-1 causes formation of the microvesicles [140, 141]. It is thought that ATP stimulation then results in release of the microvesicle contents [142]. This microvesicle release has been proposed as a 'protected' form of release. IL-1β has a short half-life in plasma so when it is protected by a microvesicle it suggests it is destined for an area away from the local inflammation [143]. IL-1β release has also been proposed to be via exosomes, which are small vesicles secreted from late endosomes. This is release believed to be caspase-1 independent but dependent on NLRP3 activation [144]. Lastly, both mature and proforms of IL-1 molecules will be released into the extracellular space in the context of necrotic cell death. The NLRP3 inflammasome has been shown to be present in human skin [145] as well as synovial fibroblasts [146], this suggests pro-IL-

18 and pro-IL-1β can be activated in the areas affected by psoriatic inflammation.

In contrast to IL-1β, which requires cleavage in order to be active IL-1α is active in its full length form. It is most widely accepted that IL-1α is an intracellular DAMP only released following necrotic cell death [147, 148]. Animal models have suggested that both IL-1α and IL-1β are important in clearance of fungal infection [149] and the inflammatory response to nanoparticles [150]. This is of interest due to the fact that these two models show both members to be important but both will activate the NLRP3 inflammasome. Recent studies have shown more clearly that activity or release of IL-1α may involve assembly of the inflammasome [151, 152]. Two papers in the late 1980's [153, 154] did suggest that IL-1α was cleaved by calpain to increase activity, and more recently granzyme B has been proposed [155]. Calpain has shown to be active only during loss of plasma membrane integrity so this could be a trigger for necrotic release of IL-1α [156]. However, the biological consequences of calpain cleavage are still unclear and no mechanisms of action have been confirmed. In contrast to IL-1β, IL-1α is present not only in the cytosol but also the nucleus. The proform of IL-1α contains a nuclear localisation sequence (NLS) which allows for binding of IL-1α to DNA, this pro-IL-1α shuttles rapidly between the cytosol and nucleus [121]. Release of pro-IL-1α has been shown to only occur during cell necrosis because during normal apoptosis IL-1α remains tightly bound to chromatin [157]. Pro-IL-1α will remain in the cytosol when a signal such as hypoxia marks the cell for necrotic death [158]. However, it has also been shown that even during necrotic cell death IL-1α remains in the nucleus in order to dampen down sterile inflammation [159]. The specific role of IL-1α in inflammation is still not clear, especially in human cells. This is due to the lack of information regarding the release and activation of IL-1α. However, more recently caspase-1 has been implicated in this. It is well regarded that IL-1α is active in its full length form, however if IL-1α is cleaved by caspase-1 it may be released from the cell and this mature form may also be active, in a similar manner to the proposed mechanisms for release of IL-1β [160]. It has also been shown that caspase-1 may play a role in the secretion of various leaderless proteins, IL-1α in its proform has been shown to be released in a caspase-1 dependent manner [161]. Further studies will elucidate the specific role of IL-1α and its role as an extracellular cytokine as well as a nuclear transcription factor.

IL-33 has some similar properties to its family member IL-1α due to its possible indications as a transcription factor. However it is predominantly involved in type 2 immune responses due to its ability to activate mast cells and its capability to polarise type 2 innate lymphocytes. It is a driver for allergic inflammatory disease such as asthma [162], however it has also been implicated in non Th2 driven diseases such as cardiovascular disease [163] and arthritis [164]. It has more recently been shown to increase antimicrobial activities of the skin in *Staphylococcus aureus* infection [165] and induce neutrophilic infiltration [166]. IL-33 is expressed by many stromal and haematopoietic cell types and acts on leukocytes, in particular macrophages, mast cells and keratinocytes [167], stromal and endothelial cells [121]. Similarly to IL-1α IL-33 is not cleaved into activation by caspase-1 and is active in its proform. More recently it has been suggested that IL-33 is inactivated by caspase-1 [168]. As described with other IL-1 members such as IL-1β, IL-33 has been shown to be cleaved into more active mature forms by extracellular proteases such as cathepsin G and elastase [169]. Interestingly the IL-33 receptor, ST2 has been implicated as a plasma biomarker in the prediction of treatment resistant graft-versus-host disease which has significant skin implications, with increased levels indicating therapy resistance [170].

IL-37 and IL-38 are newly recognised members of the IL-1 family. IL-37 has five splice variants, IL-37b being the most complete. An outstanding feature of IL-37 is its structural similarity to IL-18 [171]. IL-37 has an instability sequence so the mRNA has a limited half-life [172]. Many cell types can express IL-37, however it is highly regulated at the transcriptional level due to the instability of its mRNA, so it requires a signal such as TLR activation in order to induce expression [173]. It has been suggested that caspase-1 is also involved in cleavage of IL-37 into its active form and allows for more efficient nuclear translocation [174, 175]. Similar to IL-33 and IL-1α, IL-37 can be found in the nucleus where it is thought to have suppressive functions on cytokine transcription [175]. This has been confirmed in keratinocyte cultures both with and without a psoriatic inflammatory induced phenotype [176]. It has now emerged that IL-37 like other IL-1 members may be a dual function cytokine with functions both at the intracellular and extracellular level. It has been shown that IL-37 can suppress immune response by reducing pro-inflammatory cytokine release. This is thought to be reduction

of DC activation on the cellular level and by interaction with kinases in inflammatory signalling pathways on the molecular level [177]. Release of full length and mature IL-37 has been detected in transfected human cells following activation with LPS indicating a role for caspase-1 dependent and independent release [178]. Murine models have suggested that IL-37 over-expression provides protection against inflammation [179], which has indicated IL-37 as an inhibitor of adaptive immunity. Due to the seemingly outstanding roles of IL-37 in calming inflammation, there is no doubt further work is required to assess this cytokine as a possible therapeutic.

IL-38 is the least well defined member of the IL-1 family and was identified *in silico*. Using immunohistochemistry IL-38 expression has been identified in the skin [121]. It shares homology with IL-1RA and IL-36RA suggesting that it may have a receptor antagonist role. As well as its homology to the receptor antagonists IL-38 also has been shown to bind to IL-1R1, however the binding affinity is a lot lower than IL-1RA or IL-1β. This has also been shown for the IL-36R, however the binding affinity is again a lot lower than IL-36RA or other IL-36 members [126]. At present these binding affinity studies have only been shown with the full length form of the protein and in order to increase binding affinity to the IL-36R N-terminal cleavage may be required as with other IL-36 members (see 1.4.3 for further discussion). GWAS studies have linked IL-38 polymorphisms to various inflammatory diseases such as ankylosing spondylitis [180] and PsA [181], which may point to a role for IL-38 in the pathogenesis of these diseases.

1.5.2 The IL-1 receptors and negative regulators

IL-1Rs belong to a phylogenetically conserved superfamily of proteins, which also includes the TLR family that are involved in innate immunity and inflammation. The majority of the IL-1 receptors are comprised of two Ig domains and a cytoplasmic signalling domain. The conserved domain in this family is the cytoplasmic Toll/IL-1R (TIR) domain [182], this is involved in the activation of protein kinases such as p38, c-jun N terminal kinases (JNK), extracellular signal-related kinases (ERKs) and mitogen-MAPKs. Downstream signalling of the cytosolic TIR domain also allows for translocation of NF-κB into the nucleus [183]. Within this cytoplasmic TIR domain

there are different functional motifs, some more important than others for downstream signalling [184]. The IL-1 receptor family contains receptors and accessory proteins (AcP) as well as inhibitory receptors, such as SIGIRR (See Figure 1-5). The AcP component of the IL-1 receptor is recruited on ligand binding (not RA binding), and this is common to all IL-1 members apart from IL-18 which has its own AcP. When the accessory protein is recruited this allows downstream signalling to occur [124, 185, 186]. IL-1R1 is widely expressed in tissues and signals through the NF-κB pathway. ST2 is expressed in a constitutive and inducible manner in many cell types including stromal and haematopoietic cells. Signalling is MyD88-dependent and negative regulation is via the soluble ST2 and soluble IL-1AcP, which forms a complex and competes for IL-33 binding with the membrane bound signalling receptor complex [121]. The receptors and accessory proteins and the ligands of the IL-1 family are depicted in Figure 1-5.

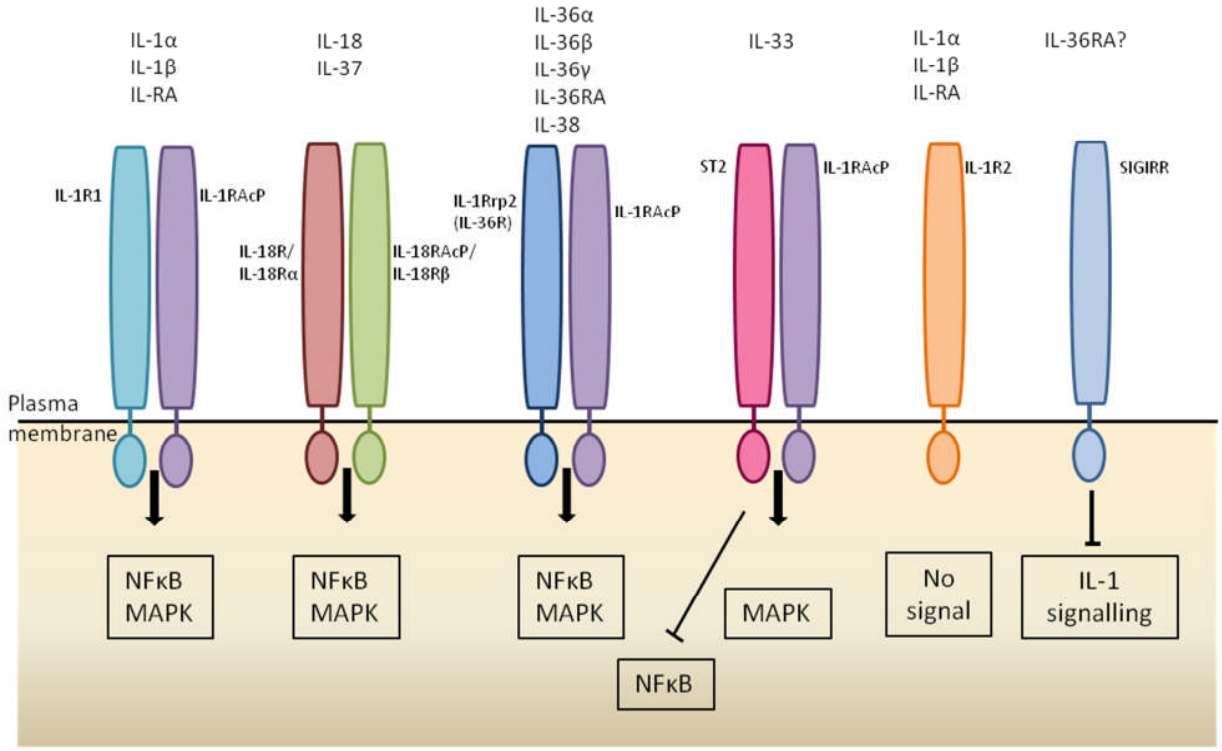


Figure 1-5. A diagrammatic representation of the IL-1 receptor family.

This diagram identifies known IL-1 receptors and there known ligands as well as the proposed downstream signalling pathways [121].

The negative regulators within the IL-1 family are also crucially important. These consist of the receptor antagonists, IL-1RA and IL-36RA (discussed further in 1.4.3), which compete with IL-1α, IL-1β and IL-36α, IL-36β, IL-36γ (respectively) to bind to IL-1R1 and IL-36R (respectively). IL-1RA binds to IL-1R1 with greater affinity compared to its agonists, however the IL-1RAcP is not recruited thereby preventing downstream signalling. In addition to the soluble extracellular IL-1RA there are also intracellular forms which are thought to be a reserve of IL-1RA which is released upon cell death in order to dampen down the inflammatory response [121]. The IL-1 family also has two decoy receptors, IL-1R2 and IL-18 binding protein (discussed in 1.4.4.2). A negative regulatory receptor SIGIRR also exists as well other orphan receptors that are thought to play a role in IL-1 signalling namely TIGIRR and IL-1RAPL2, however their ligands and actions are still to be elucidated [187].

IL-1R2 was first described in 1999 [188] as a typical decoy receptor and more recently a decoy receptor has been identified for IL-18 as well [189]. IL-1R2 lacks a signalling domain (TIR) due to its small cytoplasmic domain, therefore on IL-1 binding no downstream signalling occurs, therefore not only is it acting as a decoy receptor but also as a 'scavenger' for excess IL-1. This decoy receptor is more than just a sequestering tool for IL-1 it is an active regulator because it binds IL-1RA 100 times less than the agonists and in a complex with IL-1RAcP it binds pro-IL-1β preventing cleavage into its active form [190]. However, caspase-1 will dissociate IL-1R2 from IL-1α therefore restoring the biological activity of IL-1α [191]. IL-1R2 is expressed on a relatively limited variety of cell types compared to IL-1R1, of note expression of IL-1R2 is high on fibroblasts. Its expression is enhanced by anti-inflammatory signals such as IL-4 and IL-13 [192]. It has been described that IL-1R2 can also be cleaved and released in a soluble form by a disintegrin and metalloprotease 17 (ADAM17) [193], thereby acting almost as a binding protein. It is thought that IL-1R2 may be a mechanism by which steroids such as prednisone may be effective, patients treated orally with this showed increased levels of IL-1R2 on monocytes and levels of IL-1R2 related to response to the drug [194].

SIGIRR, also known as TIR8, is unique in the IL-1R family due to its ability to inhibit not only cytokine signals but also TLR signalling [195]. It is comprised of a single extracellular Ig domain and a cytoplasmic TIR domain, which has two amino acid substitutions compared to the other IL-1Rs. These structural differences in SIGIRR suggest a potential differential signalling pathway [121]. SIGIRR is expressed in a variety of tissues including the lung, liver, kidney, lymph tissues and digestive tract [196], however due to the extensive expression of IL-1 this negative regulation may be limited to these tissues that express SIGIRR. The negative regulatory role of this receptor is due to its ability to inhibit NF-κB and JNK activation upon agonist binding to IL-1R or TLRs by interfering with the recruitment of TIR-containing adapter molecules [195]. In murine models deficient in SIGIRR it has been shown that following infection with *Candida albicans* or *Aspergillus fumigatus* mice are more prone to lung infection and have increased mortality and fungal burden. These symptoms following fungal infection are attributed to increased IL-1 and Th17 cytokine release [197]. The ligand that binds to SIGIRR is a contentious issue as it is thought that the small extracellular Ig domain prevents a ligand binding [198]. However, it has been shown that in glial cells interaction of IL-36RA with SIGIRR attenuates the inflammatory role of IL-36 [199]. The importance of SIGIRR is demonstrated due to the fact that a deficiency is associated with an increased risk of chronic inflammation, such as psoriasis, in murine models [200]. This murine model indicates that SIGIRR dampens down IL-1 mediated Th17 differentiation and therefore reducing release of IL-17A [200]. SIGIRR has also been proposed to play a role in Th2 mediated immunity, it has been shown that SIGIRR can form a complex with ST2 upon IL-33 binding to inhibit downstream signalling [201]. It is thought that the intracellular TIR domains of TIGIRR/IL-1RAPL are similar to that of SIGIRR and do not induce NF-κB activation [202], suggesting they may play a similar negative regulatory role in inflammation.

1.5.3 IL-36, a subgroup of the IL-1 family

The IL-36 family has four members IL-36α, IL-36β, IL-36γ and IL-36RA which all bind to IL-36R (formerly known as IL-1Rrp2), and the accessory protein IL-1RAcP apart from IL-36RA (see Figure 1-5) [203, 204]. IL-1RAcP is the signalling element of the

receptor which when recruited by the IL-36R activates the NF-κB and MAPK pathways. This signalling element of the receptor is not recruited when the IL-36RA binds to the IL-36R, despite its increased binding affinity, therefore no downstream signalling occurs [185]. IL-36RA (which binds to IL-36R) acts in the same way as IL-1RA (which binds to IL-1RI).

N-terminal truncation increases the biological activity of all members of the IL-36 family by 1000-10000 fold compared to the full length proteins. Each member of the family requires a different truncation of the N-terminus to increase activity [128]. IL-36RA requires only removal of the methionine to drastically increase its activity. Whereas IL-36α (K6), IL-36β (R5) and IL-36γ (S18) require cleavage 9 amino acids away from the IL-1 domain conserved region (A-X-Asp – A = aliphatic amino acid, X = any amino acid, Asp – Aspartic acid) at the N-terminus (N-terminal amino acid and the amino acid number from N-terminus are given in brackets) [128]. However, unlike other IL-1 family members caspase-1 is not likely to be the protease responsible for this cleavage due to its very specific consensus sequence for cleavage, this consensus sequence is not present around the IL-36 cleavage site [205]. This has been confirmed *in vitro* where LPS/ATP stimulated macrophages with confirmed caspase-1 activity could not cleave IL-36α [206].

1.5.3.1 Expression and role of IL-36 cytokines

The expression patterns of these molecules are slightly different when looking at the four family members. IL-36RA is constitutively expressed in keratinocytes whereas the agonists often require induction. IL-36β mRNA levels have been detected in human synovial tissue [207] and IL-36α shown to be increased in chronic kidney disease [208]. IL-36γ has recently been shown to be released from keratinocytes expressing human papillomavirus (HPV) 11 interestingly at lower levels than normal keratinocytes [209]. In the same study on HPV11 human monocyte derived immature Langerhans cells were shown to respond to IL-36γ by release of further pro-inflammatory cytokines [209]. IL-36γ has also been implicated in lung pathology and is known to be expressed by lung epithelial cells in mice where it can induce neutrophil infiltration independently of IL-1α and IL-1β [210]. Interestingly this study showed that induction of inflammation was

present when the full length form of IL-36α was administered, however the truncated form or possible *in vivo* cleavage/activation was not analysed in this study. All of the IL-36 cytokines are expressed in the skin and most of the work on IL-36 is focused around psoriasis. In keratinocytes IL-36 expression is induced by TNFα, IL-17 and IL-1α [60, 97, 211, 212]. Human lung fibroblasts produce IL-8 and CCL20 in response to IL-36γ [213]. IL-36β and IL-36γ have also been implicated in monocyte derived DCs (MDDCs) maturation with IL-36β inducing release of IL-18 and IL-12p70 along with increased expression of CD40 and CD80 resulting in Th1 cell activation [214]. IL-36γ has also been implicated in human Th1 cell lineage and subsequent Th1 type immunoactivation by activation of T-box expressed in T cells (T-bet) [215]. T-bet is a transcription factor involved in Th1 lineage polarisation and activation and consequent release of IFNγ [216, 217]. This same study also showed that IL-36γ from both myeloid cells and keratinocytes may promote a Th1 driven inflammation and also enhance levels of chemokines such as CCL20, which plays a role in Th17 infiltration. This has also been confirmed in murine models where IL-36 members have been shown to be expressed by innate immune cells and lymphocytes. This consequently leads to increased release of pro-inflammatory cytokines and chemokines. This role of IL-36 leads to Th1 and Th17 cell proliferation and polarisation and DC activation [218]. Interestingly it was also noted that active forms of IL-36γ induce further release of IL-36γ from primary human keratinocytes [215]. This was also shown for IL-36β [212] and suggests that the IL-36 cytokines show a similar pattern to that of IL-1, where an autocrine effect has been indicated [219]. This work along with others indicates a pivotal role for IL-36γ in innate and adaptive immunity. Due to the fact that in humans IL-36 expression seems largely limited to epithelial cells, IL-36 activity blockade could be of interest for therapy in inflammatory skin diseases such as psoriasis.

Release of IL-36 from the cell is at present not well understood. TLR activation has been shown to induce transcription of IL-36γ in primary human keratinocytes, however the TLR3 agonist polyinosinic:polycytidylic acid (Poly I:C) was the only stimulus that allowed visualisation of IL-36γ outside the cell [211]. However, this was consequently shown to be due to induce caspase-3/7 and pyroptosis, programmed cell death, which was indicated to be a caspase-1 dependent mechanism. Interestingly it was also shown

that IL-36γ transcription is caspase-1 dependent, suggesting expression is switched on before the cell goes into a state of pyroptosis [211]. Release of IL-36α in bone marrow derived macrophages has been shown to only occur following a stimulus coupled mechanism i.e. two stimuli such as LPS and ATP, in a similar manner to IL-1β. It was assumed that IL-36α did not undergo proteolytic cleavage during release due to its appeared similar motility on an sodium dodecyl sulphate (SDS) page gel in the supernatant and lysate [206]. However, we now know that the mature form of IL-36α only requires cleavage of 5 amino acids from the N terminus and the difference would not be detectable on an SDS page gel.

It has to be highlighted that current knowledge on IL-36 regulation is mainly based on mRNA and the lack of protein expression data or *in vivo* detection of IL-36 members suggests there is still a plethora of knowledge still to be gained regarding these elusive IL-1 members.

1.5.3.2 The relevance of IL-36 in psoriasis

Psoriatic inflammation is complex involving many factors of the skin cytokine network. IL-36 appears to be another important mediator as highlighted by the severe clinical phenotype seen in patients with unleashed IL-36 activity (loss-of-function mutation in the RA).

The recent interest in the role of IL-36 in psoriasis is due to 1) findings related to the loss-of-function mutations [5, 111] and 2) data showing that IL-36 is highly up-regulated in psoriatic skin [60, 97, 220]. It was shown that IL-36 expression could be induced in human primary keratinocytes by treatment with IL-17 and this induction was much more prominent in keratinocytes derived from psoriatic patients [60]. A multitude of genome studies and microarrays have shown that IL-36γ frequently appears as one of the top 30 up-regulated genes when comparing non-lesional and lesional psoriatic skin [221]. Most recently IL-36γ has appeared in the top 20 when comparing psoriatic skin to skin from cutaneous lupus erythematosus [222]. These findings were strengthened when it was shown that mice over-expressing IL-36α developed a psoriasis-like skin phenotype including epidermal thickening and immune cell infiltration, this phenotype

was worsened by a deficiency in IL-36RA [220]. This supports the view that the balance between IL-36 agonists and antagonists plays a crucial role in psoriatic inflammation. Of interest, mice over expressing IL-36α also showed over-expression of the psoriasis relevant mediators IL-17A, IL-23 and TNFα. Inhibition of IL-36R prevented psoriatic like changes in the skin [223]. In murine models of imiquimod induced psoriasiform dermatitis increased IL-36 expression in DCs was noted. Mice deficient in IL-36R are protected from developing the imiquimod induced psoriasiform dermatitis, and accordingly mice deficient in IL-36RA had exacerbated disease [224].

IL-36 members can induce AMPs, such as HBD, expression. AMPs are highly up-regulated in psoriasis and the IL-36 may play a role as an important amplifier [97]. Along with the induction of AMPs IL-36 has also been shown to not only be up-regulated in response to Th17 cytokines but also induce Th17 cytokine release in psoriatic skin lesions [212]. A study has also suggested a link between IL-36 and rheumatoid arthritis and PsA, IL-36α was shown to be up-regulated in the synovium which consequently induced IL-8 and IL-6 release from the synovial fibroblasts [207, 225]. IL-36β mRNA has also been detected in synovial fibroblasts [207].

The link between IL-36 and psoriasis has been further strengthened by the discovery of a loss-of-function mutation in IL-36RA which consequently causes unleashed activity of the IL-36 agonists. This mutation causes a rare, potentially life threatening form of psoriasis termed generalised pustular psoriasis (GPP). This disease phenotype related to the IL-36RA mutation has been given the name deficiency of IL-36 receptor antagonist (DITRA) as a comparison to the IL-1 related disease, deficiency of IL-1 receptor antagonist (DIRA) [5, 111, 226]. DITRA has now been identified in a variety of populations, however the mutation is only present in a subset of GPP patients. Mutations in IL-36RA have now also been identified in patients suffering from severe adverse drug reactions which manifests as acute generalised exanthematous pustulosus (AGEP) [227]. This is suggestive of a role for IL-36 in a pustular phenotype of disease indicative of neutrophil inflammation and innate immunity activation.

In light of recent publications IL-36 is evidently an important mediator in psoriasis and PsA along with other pro-inflammatory cytokines such as IL-17, IL-22, TNFα and IL-8.

1.5.4 Interleukin-18

As previously mentioned IL-18 is a member of the IL-1 family. It was first described as 'IFNγ inducing factor' in 1989 because following intraperitoneal injection of endotoxin into mice IL-18 was identified in the serum. It was then first cloned in 1995 [228]. IL-18 as with other IL-1 family members contains no signal peptide, so is therefore secreted from the cell via an unconventional pathway. IL-1β and IL-18 are similar in the fact that they are produced in a pro-form and are then cleaved into activity. However, pro-IL-18 is constitutively expressed in a wide range of cells, the best described being in blood monocytes that express pro-IL-18 but not pro-IL-1β [229]. The expression pattern of IL-18 is indeed similar to IL-1α and IL-33, all of which are present in keratinocytes and a large proportion of epithelial cells. However, pro-IL-18 is structurally similar to IL-37 suggesting a close association between the two [230].

1.5.4.1 Processing and function of IL-18

Cleavage of pro-IL-18 is very similar to that of IL-1β in that assembly of the NLRP3 inflammasome is required in order to cleave pro-caspase-1 into its active form which then consequently cleaves pro-IL-18 into its active form. As mentioned previously assembly of the NLRP3 inflammasome requires a two-step stimulation and this often occurs following cellular stress. In humans LPS appears to be a strong stimulus for IL-18 production which is detectable in the peripheral blood from sepsis patients [231]. This confirms that idea that stimulation with a TLR agonist followed by a cellular stress signal is required in order to see release of mature IL-18 [232]. Activation of the NLRP3 inflammasome in keratinocytes has also been shown to occur by *Dermatophagoides pteronyssinus* (house dust mite) in a cysteine protease dependent manner, suggesting that such allergens can be triggers for skin inflammation [233]. Following activation of IL-18 by caspase-1 IL-18 is secreted, however up to 80% of the pro-IL-18 remains unprocessed and intracellular. The importance of caspase-1 in activation of IL-18 has been shown using caspase-1 deficient mice where very little circulating IFNγ is detectable following endotoxin challenge [234]. Conversely, in caspase-1-deficient murine macrophages stimulated with Fas ligand biologically active IL-18 was detected [235]. In a similar manner to IL-1α and IL-33 pro-IL-18 is also released following cell

death, which could be Fas signalling mediated. However it is yet to be elucidated whether pro-IL-18 is then cleaved in the extracellular space by neutrophilic proteases [236]. Mature active IL-18 binds to IL-18Rα with low affinity unless in the presence of the accessory receptor, IL-18Rβ. On binding of mature IL-18 to the IL-18Rα and IL-18Rβ a complex is formed which allows for downstream signalling via the TIR domain and subsequently activates NF-κB [237]. The IL-18Rα is widely expressed on cells whereas the expression of IL-18Rβ is more limited and found predominantly on T cells, ILC1 and DCs. This limits the cell types that respond to IL-18, however cytokines such as IL-2 can induce expression of IL-18Rβ [238]. IL-18 is also present as a membrane protein on around 30-40% of macrophages stimulated with macrophage colony stimulating factor (MCSF) [239].

The main cellular source for IL-18 is activated macrophages and monocytes [240]. However, keratinocytes have also been shown to produce bioactive IL-18 in the inflammatory state [241]. In the presence of IL-12 or IL-15 IL-18 is a potent inducer of IFNγ and Th1 polarisation [242], the co-stimulation is required in order to up-regulate IL-18Rβ, as mentioned previously. IL-18 as a polariser along the Th1 and ILC1 pathway [243] plays a role in particular in chronic inflammatory conditions including chronic atopic eczema, lupus erythematosus and psoriasis [244] [245].

Interestingly IL-18 can also enhance a Th2 response [246] in the appropriate microenvironment. It can enhance IL-13 production and when combined with IL-2 it activates T cells and NK cells [247]. Keratinocytes also respond to IL-18, following a dual signal in order to up-regulate the receptor, by production of CXCL10 which is a chemokine that attracts C-X-C chemokine receptor (CXCR)3 IFNγ producing T cells (Th1 subset) [245].

1.5.4.2 IL-18 binding protein (IL-18BP)

IL-18 is tightly regulated at the transcriptional level, the protein level by controlling cleavage as well as at the receptor level, where IL-18BP prevents binding of IL-18. IL-18BP was first identified when screening potential soluble receptors for IL-18 [248]. It has now been shown that it is constitutively expressed and binds with high affinity to

IL-18, [249] preventing binding to its receptor. In fact IL-18BP binds IL-18 at a much higher affinity than it binds to IL-18Rα. There have been four isoforms of IL-18BP described in humans a, b, c and d. IL-18BPa and IL-18BPc neutralise IL-18 to >95% at a molar excess of 2. However, IL-18BPb and IL-18BPd lack complete Ig domains on the C-terminus so have therefore lost any neutralising capacity [250]. IL-18BP is not categorised as a traditional soluble receptor because it does not contain the extracellular ligand binding domain of the IL-18R. IL-37b has also been suggested to bind to IL-18BP following which a complex is formed with IL-18Rβ preventing the formation of a functional receptor complex and therefore inhibiting downstream signalling. Of note, IL-37b binds the IL-18Rα and prevents recruitment of the IL-18Rβ [171].

In healthy human serum IL-18BP is present at a 20 molar excess compared to IL-18 [251]. IL-18 and IL-18BP are often up-regulated together especially in the context of inflammatory conditions. IL-18BP has been shown to be robustly induced by IFNγ in a diverse number of non-leukocyte cell types [252, 253]. This is in line with the concept that on induction of pro-inflammatory molecules, the antagonist is also up-regulated to control inflammation. In disorders such as familial hemophagocytosis IFNγ is unable to up-regulate IL-18BP therefore the activity of IL-18 is uncontrolled [254]. It is thought that the imbalance between IL-18 and IL-18BP may play a role in the pathogenesis of IFNγ or IL-18 mediated diseases. In systemic lupus erythematosus IL-18 and IL-18BP are hugely increased over healthy controls, however IL-18BP is not sufficiently up-regulated to neutralise IL-18 effectively [255]. This insufficient up-regulation in IL-18BP in the plasma has also been shown for patients suffering from chronic liver disease [256]. Over-expression of IL-18BP in murine models has also been shown to suppress antigen presentation cells (APC)-derived Th17 polarising cytokines, suggesting that if IL-18BP was used as a possible therapeutic it may have effects on not only Th1 derived cytokines but Th17 derived cytokines [257]. IL-18BP has been trialled (Phase I) as a subcutaneous therapeutic in patients suffering from RA and psoriasis vulgaris, the drug was well tolerated and it was shown that the drug displayed dose dependent pharmacokinetics [258].

1.5.4.2.1 Utilisation of IL-18BP by viruses

Some viruses have developed a strategy to avoid immune surveillance by either up-regulating host IL-18BP or producing their own homologue. This allows the virus to prevent IL-18 mediated immune responses. Poxviruses produce a homologue of IL-18BP which shares 20-40% amino acid sequence with the human IL-18BP [259]. Interestingly, this is thought to have been acquired from the host and then modified by natural selection. Molluscum contagiosum virus has an IL-18BP homologue that contains a long C-terminal tail which has been shown to provide increased binding properties [260]. Furthermore, specific human papillomavirus (HPV) proteins have been shown to up-regulate the endogenous form of IL-18BP in the host which may support viral immune evasion [261].

1.6 Interleukin-27

IL-27 is a heterodimeric cytokine and member of the IL-12 family which was first described as a cytokine thought to act on T cells [262]. Since the discovery of IL-27 many studies have tried to fully elucidate its functional roles within the immune system and both pro-inflammatory and anti-inflammatory roles have been highlighted. However many roles of this cytokine still remain enigmatic [263]. IL-27 is comprised of two subunits, p28 and Epstein-Barr virus-induced 3 (EBI3) (see Figure 1-6). The IL-27 subunits also have other binding partners which consequently form different cytokines, such as IL-12 and IL-35. Due to the weak binding of the subunits it is thought they may act independently as natural antagonists of the heterodimeric IL-27 [264]. IL-27 is produced by APCs early in the immune response and increases sensitivity of target cells to IL-12 [265].

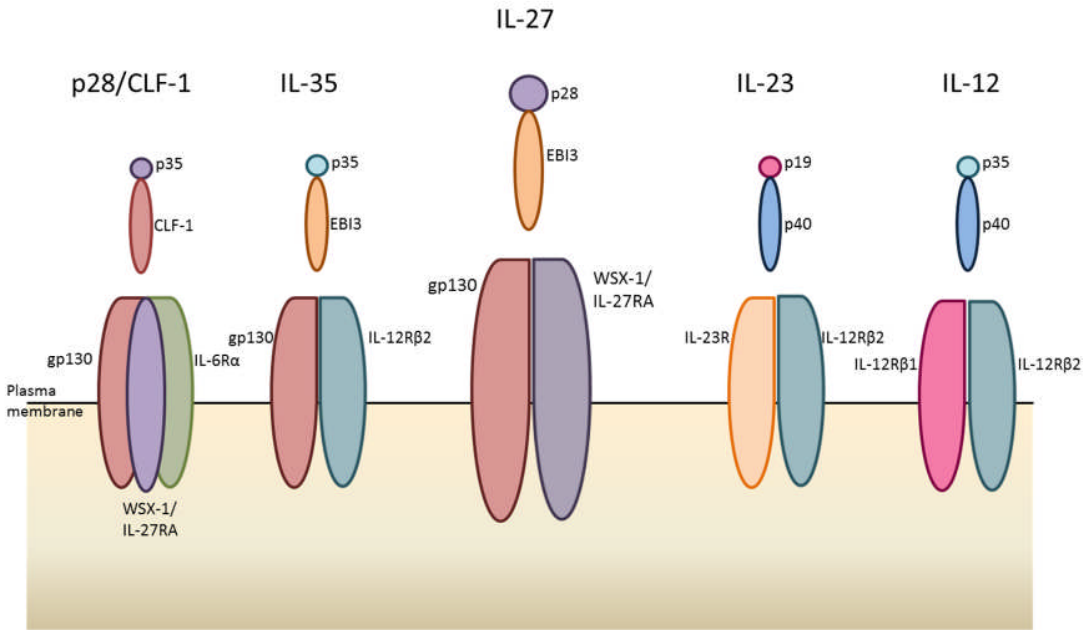


Figure 1-6. Diagrammatic representation of the IL-12 family of cytokines and receptors.

This demonstrates the significant homology of IL-27 with other IL-12 family members. EBI3 is shared with IL-35 and p28 is shared with cytokine like factor-1 (CLF-1) and may also signal on its own. The IL-27 receptor complex also has homology with the other IL-12 family receptors, sharing glycoprotein 130 (gp130) with the IL-35 receptor and 2 receptor subunits with CLF-1 [266]. Of note, the IL-6R also contains a gp130 subunit [267].

1.6.1 IL-27 receptor and signalling

The IL-27 receptor is a heterodimeric receptor comprised of WSX-1/IL-27RA and gp130 (see Figure 1-6). WSX-1/IL-27RA contains a cytoplasmic domain which contains box 1 binding motifs for janus kinase (JAK)1/2 which contributes to the downstream signalling of the WSX-1/gp130 complex [268]. Downstream signalling of IL-27 leads to activation of both STAT1 and 3 pathways [269]. A STAT3-responsive molecule, suppressor of cytokine signalling (SOCS) 3, has been shown to inhibit STAT3 signalling by binding directly to the gp130 receptor chain preventing JAK phosphorylation [270]. SOCS3 also cross-regulates STAT1 activation. Interestingly keratinocytes fail to up-regulate SOCS3 efficiently when compared to autologous macrophages and as a consequence fail to regulate/stop the production of pro-inflammatory cytokines such as CXCL10 in the context of repeated stimulation with IL-

27 [271]. The two receptor subunits of IL-27 are differentially expressed in tissues as with many heterodimeric receptors. Gp130 is expressed relatively widely whereas WSX-1 is expressed predominately on immune cells such as B and T cells [272]. A soluble WSX-1/IL-27RA has also been identified released from a range of cell lines and primary cells, metalloproteases have been implicated in production of this soluble receptor [273].

1.6.2 Pro-inflammatory roles of IL-27

IL-27 was initially described to provide a rapid clonal differentiation and expansion of Th1 cells, consequently causing production of IFNγ [262, 274]. Antigen-stimulated WSX-1-deficient cells have significantly reduced IFNγ levels, suggesting a role of IL-27 in increasing responsiveness of T cells to IL-12 leading to increased Th1 polarisation [262]. WSX-1 deficient mice with proteoglycan-induced arthritis had delayed onset of disease and reduced severity, this was associated with fewer Th1 and Th17 cells [275]. However, the role of IL-27 in arthritis is not entirely clear as local addition of IL-27 into the joints of mice with collagen induced arthritis show disease amelioration [276].

With regard to human pathologies, in the serum of psoriatic patients IL-27 is significantly higher compared to healthy controls. IL-27-secreting cells were identified in the dermal layer of psoriatic lesions and these cells were not present in healthy controls or atopic dermatitis lesions, suggesting that IL-27 may be involved in the development of psoriatic lesions [277]. However, further studies have also shown that IL-27 is expressed in chronic eczematous skin lesions. IL-27 can prime keratinocytes to be more responsive to pro-inflammatory signals [278]. Increased serum levels of IL-27 have also been identified in patients suffering from ankylosing spondylitis which was indicated to correlate with disease severity [279]. Monocytes, mast cells [272, 280], primary human keratinocytes, fibroblasts and human antigen presenting cells all react to IL-27 [59, 278]. In inflammatory dendritic epidermal cells (IDECs) and keratinocytes, IL-27 acts as a priming signal for IL-23 and CXCL10 production respectively. This effect is specific to IL-27 and was not shown for any other IL-12 family members [281].

1.6.3 Anti-inflammatory roles of IL-27

As previously discussed it has been suggested that IL-27 plays a pro-inflammatory role in initiation or early stages of inflammation, however in the later stages of inflammation a more anti-inflammatory role has been proposed. This suggests that IL-27 may be an important mediator in the prevention of chronicity of inflammation. The suggestion that IL-27 played a role in Th1 differentiation was shattered, at least in murine models, when it was shown that WSX-1 knockout mice only had a reduction in IFNγ briefly following infection with *Leishmania major*, subsequently which normal IFNγ levels were detected [282]. However, these mice did develop organ damage thought to be due to exacerbated action of T cells, possibly indicating a role for IL-27 as an anti-inflammatory mediator. Mice deficient in WSX-1 infected with *Toxoplasma gondii* are still capable of mounting a sufficient inflammatory response but the inflammation is not resolved and consequently developed a lethal T cell mediated inflammation [283]. The ability of IL-27 to dampen down wide ranging inflammation opened up questions as to the mechanisms involved. It has been shown that IL-27 can antagonise the activity of IL-2 and has also been shown to reduce the expression of both Gata3 and RORγt, which may explain its role in dampening down T cell mediated inflammation [284]. Another anti-inflammatory role of recombinant IL-27 has been shown by its ability to reduce ROS released from activated macrophages and neutrophils [285].

A number of the regulatory roles of IL-27 have been attributed to the up-regulation of IL-10. It is thought that IL-27 acts on all T helper subtypes to enhance IL-10 production [286-288]. This was initially shown in 2007 using murine models and has now been attributed to human IL-27 as well in the context of visceral Leishmaniasis [289]. During respiratory viral infections, such as influenza CD8+ T cells respond to IL-27 with increased expression of IL-10. However memory CD8+ T cells in the same viral infection lose the gp130 receptor which consequently deems them unable to respond to IL-27 on repeat infection [290] [291]. Interestingly, patients suffering from multiple sclerosis (MS) are often treated with IFNβ and it has been shown that this causes increases in IL-10 and a decrease in IL-23 and IL-1β. It is thought that response to IFNβ therapy in MS is mediated by IL-27 because responders to therapy show significantly

more IL-27 release from PBMCs compared to non-responders. This is supported by murine models of experimental autoimmune encephalomyelitis where treating with IFNβ increased IL-27 and attenuated signs of disease [292]. *In vitro* experiments have shown that stimulation of fibroblast-like synoviocytes derived from rheumatoid arthritis patients with IL-27 showed a significant reduction in release of pro-inflammatory mediators such as IL-6 and CCL20 [293]. Of note, many of the studies looking at the anti-inflammatory or regulatory roles of IL-27 are in mice therefore the role of IL-27 in humans remains to be fully accounted for. However, a recent publication in human PBMCs have indicated a complex mechanism between IL-27 and IL-17A, where the inflammatory state may determine responses and the actions between the two are by no means unidirectional. This implies a possible anti-inflammatory role in surrounding resting cells to prevent over-activation but also boosting inflammatory responses by their reciprocal actions [294]. This current finding is of interest in IL-17A mediated diseases such as psoriasis.

1.7 Proteolytic cleavage of inflammatory mediators in the skin

It is well known that both IL-1β and IL-18 require cleavage in order to gain biological activity as described previously. However, proteolytic cleavage is not just limited to that of the IL-1 family members but extends to other cytokines such as IL-6, IL-2 and TNFα and chemokines as well as cytokine receptors. This proteolytic cleavage may be intracellular such as via the inflammasome, or by a protease present in the extracellular space. Proteolytic cleavage discussed in this section will be inflammasome independent as cleavage dependant on the inflammasome has been discussed in 1.4.1.

1.7.1 Proteases in the skin

On the basis of the catalytic domain proteases are grouped as follows; aspartate, cysteine, glutamate, metallo-, serine and threonine with the prime aim to break peptide bonds. The prominent proteases present in the skin compartment are metallo-, serine, cysteine and aspartic proteases [295]. Of note, aminopeptidases are also likely to be present in the skin due to their widespread expression. Aminopeptidases are often membrane bound and will remove a single amino acid from the N-terminus. The most

well studied of these is aminopeptidase N (AMN) or CD13 which is Zn^{2+} dependent ectopeptidase [296]. Within the skin compartment serine proteases appear to play a role in barrier homeostasis. Proteases will also be present in the skin as fungal or bacterial derivatives as well as proteases produced by infiltrating immune cells such as polymorphonuclear (PMN) cells. Proteases themselves require cleavage to gain activity often by removal of an N-terminal amino acid which then initiates a downstream cascade of activation and is important to prevent constant activation of inflammatory mediators [295]. The balance of proteases and protease inhibitors is crucial in maintaining healthy skin homeostasis and there are also many protease inhibitors expressed in the skin that have wide ranging inhibition often to a specific catalytic domain protease. This is evident in the rare genetic disorder, Netherton syndrome which leads to widespread break down of the epidermal barrier. It is caused by mutations in the serine protease inhibitor Kazal-type 5 (SPINK5) gene [297]. There are now an increasing number of disease states especially within the skin that are being attributed to the imbalance of protease and protease inhibitor.

1.7.1.1 Metalloproteinases in the skin

Metalloproteinases are proteases whose catalytic activity is dependent on the presence of a metal ion. Matrix metalloproteinases (MMPs) are Zn^{2+} dependent and are important in remodelling of tissue by cleavage of collagens. MMPs expressed in mammals primarily cleave specific collagens as well as some growth factors. Aberrant expression of MMPs has been attributed to many disease states where tissue degradation is present such as arthritis and psoriasis [298]. ADAMs are a family of metalloproteinases of which ADAM10 and ADAM17 are expressed in the skin. This family of proteases are important in the cleavage of receptors from the plasma membrane to produce soluble receptors [299] which are crucial in the control of inflammation. Loss of function of ADAM17 causes chronic inflammation [295].

1.7.1.2 Cysteine and aspartic proteases in the skin

Cysteine proteases can be classed into two categories in mammals based on the structural organisation of the active site, one group being related to papains and one

which includes the caspases and legumains [300]. It is thought that these cysteine and aspartic proteases in the skin are essential for desquamation and thereby are important in the maintenance of the skin barrier.

Cysteine-dependent aspartate-directed proteases (caspases) are a family of 14 endoproteases that rely on a catalytic cysteine residue in the caspase active site and only cleave after aspartic amino acids in the substrate [301]. Caspases are widely expressed and as discussed previously caspase-1 plays a crucial role in intracellular cleavage of IL-18 and IL-1β. An important epidermal enzyme is caspase-14 which is required for the breakdown of filaggrin into natural moisturising factors in order to maintain barrier homeostasis [302] [303]. Like other caspases it is produced in a pro-form and thought to be cleaved into activation by a serine protease with elastase-like properties in the cornified layer of the epidermis [304]. Another cysteine protease expressed in the skin is cathepsin C which is a lysosomal protease in the papain family. Mutations in the cathepsin C gene are responsible for two genetic disorders, Papillon-Lefevre [305] and Haim-Munk [306], both of which have clinical phenotypes that indicate a role for cathepsin C in the organisation of the epidermis and barrier function. However, cathepsin C deficient mice do not display the phenotype of the human disease but do have deficiencies in processing and activation of granzyme A and B which are essential for T cell mediated killing [307].

Cathepsin D is an aspartic protease and is the most abundant protease in the endolysosomes. It is active at the pH of healthy skin, around 5.5. Cathepsin D is thought to be crucial in the final stages of desquamation [308] and therefore deficiency of cathepsin D results in down-regulation of the proteins such as involucrin. Clinically this produces symptoms such as dry skin with hyperkeratosis [309].

1.7.1.3 Serine proteases in the skin

Serine proteases are large family of proteases that use a catalytic triad in the substrate binding pocket (Ser, His, Asp). It is thought that as with the cysteine and aspartic proteases, the serine proteases produced by keratinocytes are important in terminal differentiation and desquamation of keratinocytes therefore maintaining barrier

homeostasis. Serine proteases can be divided in to three distinct groups; trypsin-like enzymes (cleaves C-terminally at Arg or Lys), chymotrypic enzymes (cleaves after aromatic or bulky hydrophobic amino acids) and elastase-like enzymes (cleaves after small or medium non-polar amino acids) [300].

Matriptase and prostasin are important serine proteases produced by keratinocytes in the interphase between the stratum granulosum and stratum corneum. These proteases are transmembrane anchored proteases that crucially cleave profilaggrin in to its active form [310]. This is an extremely important protein in barrier homeostasis and mutations in the *filaggrin* gene have been linked to atopic dermatitis and asthma, possibly due to the disturbed barrier allowing penetration of allergens as well as infectious agents [311]. Matriptase is thought to be activated by exposure to acidic pH in the skin and this in turn may activate prostasin [312]. However, activation of these proteases is tightly controlled due to the complex mechanism by which activation occurs, therefore a small window is present where they are both active and have the ability to cleave the substrate [313]. The importance of these proteases is not just limited to activation of filaggrin but it has also been proposed that matriptase may activate the largest family of serine proteases in the skin, kallikrein-related proteases [314], suggestive of a role for these proteases in mediation of inflammatory skin diseases.

Kallikrein-related proteases (KLKs) are tryptic or chymotryptic serine proteases. These proteases are produced by keratinocytes in the stratum granulosum where they are released from lamellar bodies [300]. At present eight members have been described in healthy skin, the most important described as KLK5, KLK7, KLK8 and KLK14 in skin homeostasis. KLK 5 and KLK7 have been isolated in an active form in the stratum corneum and KLK14 has been predominantly identified in the sweat glands [315]. The specific functions of the KLKs in the skin remains to be fully elucidated, however with the use of murine models and *in vitro* studies some light has been shed on their possible roles in the skin. Kallikreins have been shown to cleave corneodesmosomes, which as with other proteases produced by keratinocytes suggests crucial involvement in desquamation and barrier homeostasis [316]. There has also been suggested roles in regulating antimicrobial activity in the skin [317] which may contribute to the

pathogenesis of rosacea [318]. KLK5 and KLK14 have been shown to cleave and consequently activate protease-activated receptor 2 (PAR-2) [319]. These receptors are involved in maintenance of the barrier, however they also play a role in inflammation, pruritis and scar formation [315]. KLK7 over-expression in murine models displays an itchy, hyperproliferative, inflamed phenotype with reduced barrier integrity [320]. This suggests that KLK7 may be involved in the pathogenesis of skin diseases that develop this phenotype such as atopic dermatitis. The only kallikrein knockout mouse that has been published is KLK8. This murine model has apparently normal skin apart from when inflammation is induced using UV light and resolution of inflammation is delayed [321]. Due to the unique environment of the skin and the necessity for control of desquamation, the activity of KLKs are pH and water dependent [322].

Apart from the serine proteases produced by keratinocytes, those produced by infiltrating cells also contribute to inflammatory pathologies. The serine proteases produced by these cells may play an important role in the pathogenesis of inflammatory disorders. The serine proteases produced by neutrophils are present in the azurophils and are thought to be responsible for intracellular degradation of microorganisms [323]. These proteases also have action in the extracellular space and it is thought that avoidance of the numerous circulating serine proteases inhibitors may be via the serine proteases remaining on the cell membrane but maintaining activity [324].

Three structurally similar serine proteases are present in neutrophils; cathepsin G (CG), proteinase 3 (PR3) and neutrophil elastase (E). Murine models deficient in these specific proteases has indicated an important role in bacterial clearance [325]. It is also worth noting that a fourth serine protease is also present in neutrophil granules, neutrophil serine protease 4 (NSP4). This protease shares <40% structural homology with the other neutrophil serine proteases and is unique in that it has a preference for cleavage with an Arg at the P1 position [326]. However, mice deficient in NSP4 show no changes in phenotype that seems of clinical relevance [327]. Neutrophil serine proteases require processing for enzymatic activity, in a murine model cathepsin C, which is a lysosomal cysteine protease, has been identified for processing and activation of PMN proteases [328]. C-terminal cleavage is also required for normal trafficking within the cell [329].

1.7.2 Inactivation of inflammatory mediators by proteolytic cleavage

As previously described proteases are prevalent in the skin and provide important extracellular proteolytic activities especially at the sites of inflammation. TNFα was the first pro-inflammatory cytokine to be identified as rapidly inactivated following incubation with activated PMNs [330] and also recombinant E and CG. The cleavage of TNFα into at least two fragments results in loss of cytotoxic activity [331]. This has also been shown for the inflammatory cytokine IL-6 at the sites of inflammation. Recombinant PMN serine proteases cleave IL-6 at different sites; however, all lead to inactivation. IL-6 is known to activate neutrophils so cleavage may be a way to provide negative regulation [332, 333]. IL-2, the potent T cell activator has also been shown to be susceptible to cleavage by E and these cleaved products actually have a negative regulatory role in preventing the pro-adhesive properties of IL-2 thereby preventing further immune cell infiltration [334]. Chemokines including CXCL12 and CCL3 have also been shown to be cleaved by serine proteases released by neutrophils and recombinant E, CG and PR3 resulting in loss of chemotactic activity [335, 336].

Another important modulatory role of proteases within the skin is the shedding of cytokine receptors. Membrane bound metalloproteases as well as PMN serine proteases have been implicated in this role. PMN serine proteases cleave the IL-2 receptor, TNF and IL-6 receptor (IL-6R) close to the plasma membrane and form soluble receptors [337]. However, ADAM17 is the predominate sheddase that cleaves both TNF receptor (TNFR) I and TNFRII as well the IL-6R and IL-15R. ADAM10 also cleaves IL-6R, and both ADAM10 and ADAM17 are involved in cleavage of adhesion molecules to alters cellular adhesion and consequently cellular infiltration [338, 339]. This not only provides soluble receptors to act as decoys but is also important in regulation of cell adhesion controlling cellular infiltration into inflamed areas.

1.7.3 Activation of inflammatory mediators by proteolytic cleavage

Pro-inflammatory cytokines often require cleavage before activation occurs. Proteases released from immune cells only present in inflammation are common mechanisms by which pro-inflammatory cytokines are activated. However, other proteases present in the

skin may also play a crucial role in the cleavage of cytokines into bioactive forms. As previously discussed IL-1β is cleaved in to activity by caspase-1 within the cell. However if pro-IL-1β is present in the extracellular space due to cell necrosis alternative cleavage of IL-1β can occur to enable activity. This has been confirmed by turpentine-induced inflammation in murine models; those deficient in IL-1β were protected from inflammation whereas those deficient in caspase-1 were not [340, 341]. Serine proteases released from neutrophils can cleave IL-1β into its active form, specifically PR3 [342]. This is not the same cleavage site as caspase-1 but between the valine-arginine adjacent to this caspase-1 site [337]. However, MMPs have also been shown to cleave pro-IL-1β such as stromelysin 1 and gelatinases A and B [343]. There has also been some suggestion that PR3 released from neutrophils can cleave pro-IL-18, however it has not been confirmed as to whether this cleavage produces the bioactive product [236] [344].

Chemokines are crucial in the inflammatory response and enable infiltration of immune cells to the site of inflammation. Chemokines however often require cleavage. CXCL8 or IL-8 is a potent chemokine that attracts neutrophils. IL-8 is cleaved to increase its chemotactic functions by PR3 [345]. This is a protease produced predominantly by neutrophils suggesting that on infiltration of neutrophils release of proteases encourages further infiltration. There are various isoforms of IL-8, clustering into 3 main categories one of which has no chemotactic ability while the other 2 have significant biological activity. This needs to be taken into account when assessing levels of IL-8 and the balance between active and inactive forms following cleavage [346]. CG cleaves CXCL5 into activation to increase its neutrophil chemotactic ability [347]. The chemotactic ability of CCL15 (MIP3δ) to attract monocytes can be increased 1000 times following N-terminal cleavage by CG [348].

TNF is produced as a pro-form that is membrane bound and is proteolytically released from the membrane. A metalloprotease was identified that cleaves TNF into its active from and this was named TNF-converting enzyme (TACE) [349], which is now known as ADAM17. This has been confirmed by using murine models which express an inactive form of ADAM17. T cells from these mice have significantly reduced TNF levels. [350]. As well as ADAM17 it has also been shown that PR3 can cleave TNF

into its active form [351]. Interestingly the two other prominent serine proteases released from neutrophils, E and CG, have been shown to inactivate mature TNF [352]. The cleavage sites of PR3 and ADAM17 in order to activate TNF are not identical. PR3 cleaves between a valine-arginine in the immediate vicinity to the site of ADAM17 [337].

1.8 RNA aptamers

RNA aptamers are single stranded oligonucleotides selected *in vitro* to bind a target with high specificity and affinity. Single stranded RNA is conformationally very flexible and can adopt a range of 3-dimensional (3-D) structures, some of which will bind to the target. Of note, ssDNA aptamers can also be generated and are being developed in parallel with RNA aptamers. RNA aptamers can function in a similar way to antibodies and small molecule inhibitors that are currently used in therapy. However, in comparison to antibodies they provide a non-immunogenic alternative and have advantages due to their small size and also their ability to target epitopes that antibodies cannot. The structural flexibility of aptamers also means that RNA aptamers can often bind epitopes that are hidden from antibody targeting. Due to these unique features of RNA aptamers they present as interesting potential therapeutics [353]. RNA aptamers are also being developed to act as delivery systems that can be loaded with toxins, enzymes and chemotherapy agents to target specific cells [354]. Fusion of siRNA and shRNA with RNA aptamers has been shown to effectively deliver these interfering RNAs to specific cells [355].

1.8.1 Production of RNA aptamers

RNA aptamers are traditionally generated through the use of systemic evolution of ligands by exponential enrichment (SELEX). This can either be performed *in vitro* or using whole cells, such as cancer cells which may provide an array of cellular targets for establishing healthy cell from cancerous cell (Cell-SELEX) [356]. *In vitro* SELEX is performed using a library of RNA molecules which is then incubated with the receptor or ligand of interest. This is heated and cooled to encourage formation of stable structures, followed by washing to remove unbound molecules. The RNA structures that

bound to the target are eluted and incubated with a control sample, this provides negative selection. The molecules that bound in the positive round and did not bind in the negative round are then isolated, reverse transcribed and then amplified using polymerase chain reaction (PCR) and then the process is repeated until the pool of RNA molecules are specific for the required target. This pool is then cloned and sequenced to obtain the individual sequences (see Figure 1-7). Due to the *in vitro* nature of this process it can be altered depending on the properties required for the end product, such as different temperatures and different forms of negative selection, which can be repeated more than once [357]. Cell-SELEX is performed in a similar manner, for example using live cancer cells for the positive selection round and healthy cells for the negative selection. The library of single stranded oligonucleotide segments incubated with the cells can be fluorescently labelled, allowing the whole process to be monitored using flow cytometry [356].

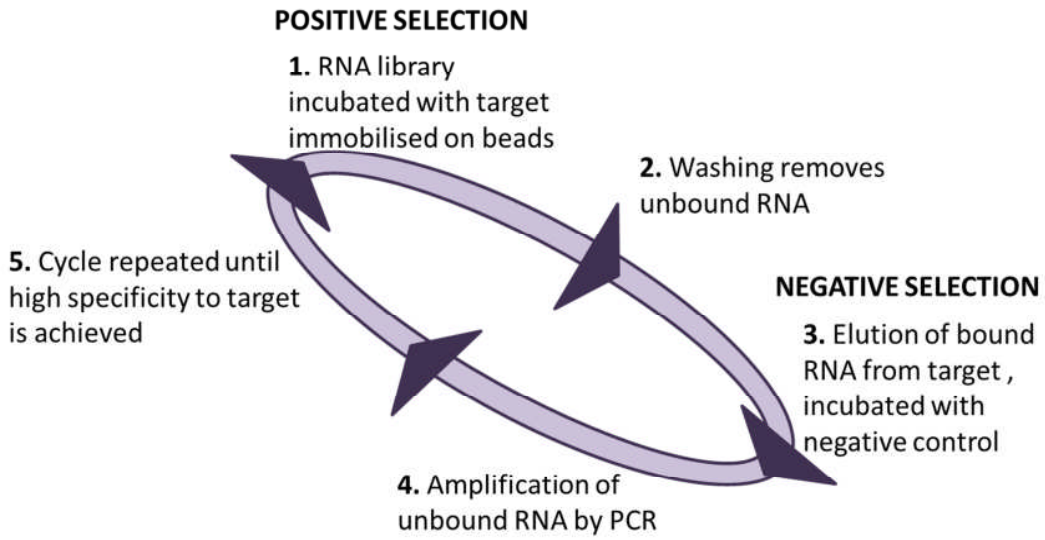


Figure 1-7. Summary of the SELEX process.

Steps 1 – 4 are performed before the cycle is repeated until a few RNA molecules are bound with high specificity and affinity. These are then cloned and sequenced and then chemical modification can be performed on the specific RNA molecules [358].

This method is undoubtedly faster than producing monoclonal antibodies and more specific due to the multiple rounds of selection and screening. However, RNA molecules are inherently unstable, so without chemical modification these molecules would be of no use as a therapeutic or research tool [359]. Chemical modification such modifying the 2'OH position of the ribose of pyrimidines to incorporate a fluorine reduces RNase degradation and maintains hair-pin loops and single stranded structures that are thought to be important for the binding of the aptamer to its target. Other common modifications used are the addition of O-methyl or amino group to the 2' position of the ribose [360]. However, fluorine is the most common modification due to its small size compared to the other chemical modifications available. These chemical modifications have been shown to allow stability of aptamers for 2 hours in human serum [361]. These chemical modifications also increase thermodynamic stability therefore maintaining structure of the RNA molecule. Aptamers can also be easily labelled with fluorophores in order to track the aptamers, this has been utilised in an experimental setting however it can also be used in diagnostics to identify diseased cells

[357]. Aptamers can also be used as a diagnostic due to the binding affinity of RNA aptamers, detecting cancerous cells at extremely low levels [357].

1.8.2 Potential clinical use of RNA aptamers

The use of aptamers as therapeutics is relatively novel and aptamers have shown promising results so far. A number of aptamers are in pre-clinical trials and at least nine aptamers are in various stages of clinical testing [362]. Apart from the above mentioned, another advantage of aptamers over antibodies is their small size which allows increased transport and tissue penetration. However aptamers are not likely to be utilised as a systemic therapy so antibodies will be advantageous when systemic therapy is required [353]. Small molecule inhibitors which are increasing in popularity especially for treatment of inflammatory diseases are a similar size to aptamers and therefore the systemic clearance will be comparable. However, aptamers have the advantage of being potentially more specific avoiding off target side effects [363]. The majority of aptamers that have been produced as a potential therapeutics bind a target to modulate downstream signalling, they are most commonly used as neutralisers rather than stimulators. This typically occurs by the binding of the target and preventing structural changes that are required for downstream signalling [364] or prevent binding to the receptor [365]. However, a few aptamers have been produced as agonists, namely aptamers against the extracellular domain of the EGFR [366] and isoleucyl tRNA synthetase [367].

RNA aptamers have been produced most commonly as a cancer therapeutic. Aptamers produced using Cell-SELEX can provide a tool to distinguish between healthy and cancerous cells [368]. This is essential for cancer therapy due to the need to reduce healthy tissue death and side effects. RNA aptamers have been produced to target various aspects of the disease including adhesion molecules such as E-selectin which prevents metastases [369]. Modulation of the immune system has also been targeted including cytotoxic T-cell antigen 4 (CTLA-4) [370]. An aptamer has been produced against CXCL12, which is involved in aiding tumour metastases and angiogenesis [371]. Immunological targeting by aptamers has also been demonstrated as an alternative to antibody neutralising therapy, prevention of pro-inflammatory cytokines

binding to the receptor is an invaluable tool to control dysregulated immune responses. Pro-inflammatory cytokines such as IL-17A have been targeted for aptamer therapy which is prominent in many inflammatory diseases such as psoriasis [365].

1.8.3 Potential mechanisms of internalisation of oligonucleotides

In order for RNA aptamers to function inside the cell, targeting signalling pathways or nuclear proteins directly, aptamers need to cross the cell membrane. This would either have to be achieved using chemical introduction into the cells or by utilising physiological mechanisms. If an aptamer was to be used therapeutically then it would be preferable to utilise cellular mechanisms without the need for chemical treatment of the cells which could damage the cell membrane and may cause unwanted immune responses. A DNA aptamer, AS1411, has been described as an effective anti-neoplastic aptamer which targets nucleolin, which is often over-expressed in cancer cells and binding of the aptamer consequently interferes with DNA replication and leads to cell death [372]. In contrast to initial studies it has now been shown that this DNA aptamer is in fact internalised by healthy cells and cancer cells rather than cancer cells alone. However in cancer cells the aptamer is thought to be taken up by macropinocytosis and in healthy cells it is thought that the uptake is via an alternative mechanisms which may lead to lysosomal degradation [373]. Interestingly the AS1411 uptake mechanism appears to play a role in the efficacy of the aptamer, with the aptamer only having an effect in cancer cells [373]. Macropinosomes have been shown to be leaky, therefore possibly allowing delivery of the aptamer in to the cell.

Another mechanism that has been suggested for aptamer uptake is via specific receptors. The uptake of AS1411 was shown in a fibroblastic cell line, however plasmid DNA uptake has also been identified in human primary keratinocytes. This uptake was shown to be receptor-dependent and indicated a possible role for DNA-binding proteins. It was shown that ezrin and moesin, which are DNA-binding membrane-cytoskeleton proteins, are involved in the uptake and trafficking of DNA however, the specific transmembrane DNA receptor is yet to elucidated [374]. Receptor mediated uptake has also been shown utilising the IL-6R, the RNA aptamer specific to this receptor could carry cargo (toxins or siRNA) 10x its molecular weight and deliver this into the cell [375]. Therefore

possible mechanisms by which aptamer uptake in to cells occurs has been shown to be via both macropinocytosis [373] and receptor-dependent [374, 375]. These mechanisms deliver the oligonucleotide molecules into the cells in a form by which they still maintain neutralising effects. However, mechanisms have also been suggested by which uptake occurs and the oligonucleotides internalised are targeted for degradation and are therefore not functional [373].

More extensive research has been performed in the delivery of siRNA into cells and various mechanisms are utilised to increase efficacy. It is thought that siRNA is internalised predominantly via endocytotic mechanisms, and delivery mechanisms can be improved by association with other molecules such as aptamers or antibodies that target a specific ligand to initiate receptor-dependent endocytosis [376]. An important feature of delivering siRNA into the cell via endocytosis is the endosomal escape to avoid degradation and enter the cytosol to enable their silencing effects [377]. This is often overcome by promoting fusion with the endosomal membrane or causing disruption of the membrane and this would be important in the formulation of siRNA to ensure escape of endosomal degradation [377]. However, as with the aptamers macropinocytosis has also been described as an effective delivery mechanism for siRNA [378].

1.8.4 The challenges of using RNA aptamers for therapeutics

Following the development of the aptamer technology it took 15 years for an RNA aptamer to be available on the market as a therapeutic. Currently there is only one food and drug administration (FDA) approved therapeutic aptamer on the market, Macugen® (Pegaptanib) which is in use for treatment of age-related macular degeneration as well a diabetic retinal disease [379]. There are many challenges that present when establishing oligonucleotide therapy. Aptamers have been administered intravenously, subcutaneously and intravitreally. However nuclease degradation, renal clearance and rapid biodistribition need to be overcome [380]. Nuclease degradation is overcome by the chemical modifications made to oligonucleotides either before or after SELEX. Following SELEX further modifications can be made including capping of oligonucleotide termini protecting from exonuclease activity. Protection from nucleases

can also be achieved by the use of Spiegelmers. This is the replacement of the $_{D}$-ribose with an $_{L}$-ribose which yields unnatural $_{L}$-nucleotides [381, 382]. Renal clearance of aptamers has been overcome by the addition of high molecular weight polyethylene glycol (PEG). This is a commonly used strategy for therapeutics and has been shown to slow renal clearance and increase half-life therefore increasing bioavailability [383]. Toxicity of aptamer therapy has not been extensively studied however in trials Macugen® and AS1411 have shown minimal toxicity [380]. The immune response to oligonucleotides has been studied in relation to the activation of TLRs, which are known to respond to both RNA and DNA and produce an IFN type immune response. However this response is primarily following sensing of extensive regions of double stranded RNA or DNA [384]. This would have to be a consideration when assessing an aptamer for therapeutic use.

1.9 Interleukin-17

In 2005 it was demonstrated that with regard to Th cells, there were more than the three lineages, Th1, Th2 and T reg. This new lineage was described to produce IL-17 and consequently this new subset of Th cells was named Th17 [89, 385]. IL-17A was first cloned and described in 1993 and was previously known as CTLA8 [386]. IL-17A and IL-17F are the best characterised of the IL-17 family and are the only members known to be produced by Th17 cells. However, genomic sequencing has also identified IL-17B, IL-17C, IL-17D and IL-17E (also known as IL-25) [387]. All of these are covalent homodimers although a heterodimer of IL-17A and IL-17F has also been described [388] (see Figure 1-8). There are diverse cellular sources and expression of the IL-17 family members which all are thought to have pro-inflammatory roles [389].

1.9.1 IL-17 receptors and signalling pathways

The IL-17 receptor family comprises of five distinct subunits, IL-17RA-IL-17RE (see Figure 1-8). All of these subunits contain a single transmembrane protein domain, with a conserved extracellular fibronectin III-like domain and a cytoplasmic SEF/IL-17R (SEFIR) domain. It is still to be fully elucidated how these receptor subunits form a functional receptor [387].

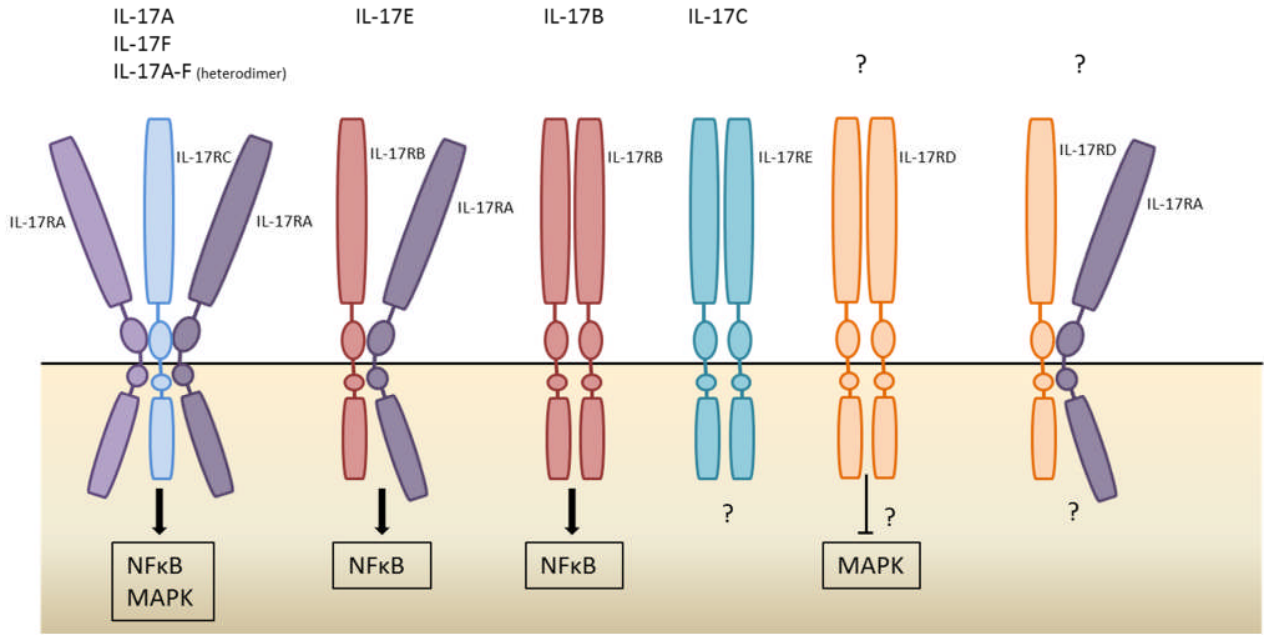


Figure 1-8. Diagrammatic representation of the IL-17 receptor subunits and the pairings formed to produce functional receptors.

IL-17RA-IL-17RC has three known ligands whereas IL-17RB-IL-17RA and IL-17RE have only one know ligand. IL-17RD-IL-17RA and IL-17RD have no known ligands. Downstream signalling pathways have been established for IL-17RA, IL-17RD-IL-17RA, IL17RB and IL-17RD.

IL-17RA is the largest IL-17R complex [390] and was initially identified as the receptor for IL-17A. It has now been shown to bind IL-17F and the heterodimer of IL-17A and IL-17F. Initial studies with human IL-17 suggested that binding of IL-17A to IL-17RA was not sufficient to elicit a response [391], it was not until much later that it was described that IL-17RC was required to have a fully functional receptor complex [392]. However, it is not fully understood what number of each of these subunits is present in this receptor complex and many theories have been proposed as to how these subunits form the functional receptor [387]. At present it is thought that a trimeric receptor is likely, as shown in figure 1-8. IL-17RA expression is widespread including epithelial cells, fibroblasts and macrophages [390] and also can be induced in T cells by IL-21 and down-regulated by phosphoinositide-3-kinase (PI3K) [393, 394]. Unlike other cytokine receptors the IL-17R is required at high levels to elicit a response and it has been suggested that IL-17RA may act in a regulatory role by internalising IL-17A upon

binding and removing it from the inflammatory environment [394]. The other IL-17 receptor subunits are poorly described and the pairing shown in figure 1-8 is the proposed mechanism by which these subunits form their functional receptors [395].

The downstream signalling from the IL-17 receptors is complex and many pathways have been reported to be activated, such as the JAK pathway which was implicated following inhibition of JAK which consequently reduced IL-17A expression [396]. However, these results using small molecule inhibitors should be interpreted with caution due to the possible non-specific effects and possible transcription factor activation due to cytokines up-regulated down stream of IL-17 [397]. Due to the highly pro-inflammatory role of IL-17A it was originally proposed that the downstream signalling of its receptor may well compare to the IL-1R and the TLRs. Downstream of the IL-17RA activation of p65 and p50 has been shown which suggests that the canonical NF-κB pathway may play a role in the inflammatory signature of IL-17A [398]. Act1 has been shown to be recruited to the IL-17RA intracellular domain, which is a known adapter molecule for NF-κB activation [399]. MAPKs are also activated downstream of IL-17RA, the most strongly activated of these are ERKs [400]. Unlike IL-17RA, IL-17RB contains a TNFR-associated factor 6 (TRAF6) binding motif in its cytoplasmic component of the receptor, this is a well-known mechanism by which NF-κB is activated [401]. The downstream signalling of IL-17RD is still to be fully elucidated although activation of MAPKs have been proposed due to the evolutionary origins of IL-17RD and its role in non-primate development [402].

1.9.2 Functional roles of IL-17

As previously mentioned IL-17A and IL-17F are thought to be predominantly leukocyte derived cytokines, T cells and innate lymphocytes such as ILCs have been described to produce IL-17 [403, 404]. IL-17 producing cells express the transcription factor associated with IL-17 production, RORγt. Most studies have also shown that IL-6 and IL-1β is required for Th17 development [405]. However IL-21, IL-23 and TGFβ signalling has also been implicated [406-408]. The role of IL-23 in the development of the Th17 subset in an inflammatory context has now been extensively researched [409, 410]. A well-known biological role of IL-17 is the induction of pro-inflammatory

cytokines, chemokines and MMPs from tissue resident cells leading to tissue remodelling and, among others, recruitment of neutrophils to site of inflammation [411].

IL-17 and the often co-expressed IL-22 play a prominent role in induction of AMPs and mice deficient in IL-17 are highly susceptible to both fungal and bacterial infections. This has also been confirmed in humans with genetic mutations leading to reduced Th17 numbers, or upon treatment with IL-17 neutralising antibodies [412]. Dominant-negative mutations in STAT3 lead to a hyper-IgE syndrome which prevents generation of Th17 cells and consequently these patients suffer from mucocutaneous candidiasis and pulmonary infections, predominantly due to *Staphyloccus aureus* [413]. STAT3 is essential for Th17 development downstream of IL-6 [414]. These observations indicate a crucial role for IL-17 in the defence against invading pathogens. Together with the environmental stimuli, levels of IL-21, IL-23 and TGFβ control the magnitude of the IL-17 mediated response by stimulating surrounding cells to produce chemokines such as IL-8 to further recruit inflammatory cells such as neutrophils [405]. IL-17 has been shown to promote contact-dependent cytotoxicity by IFNγ-induced up-regulation of intracellular adhesion molecule (ICAM)-1 on keratinocytes [415]. The role of IL-17 in Th1 differentiation is controversial with some studies suggesting it plays a role in Th1 generation [416] and some that suggest a negative feedback loop by RORγt on Th1 differentiation [417]. IL-17 has also been shown to induce other pro-inflammatory mediators such as the IL-36 family members [212].

1.9.3 The role of IL-17 in disease

As previously discussed IL-17 is highly pro-inflammatory and is an effective mediator in host defence. However if IL-17 expression is dysregulated this can lead to uncontrolled and chronic inflammation and tissue damage. The role of IL-17 in autoimmunity was established when it was shown in murine models that Th17 cells were crucial in the initial stages of experiment autoimmune encephalitis [418]. IL-17A and IL-17F are also highly expressed in rheumatoid arthritis [419]. It is thought that in rheumatoid arthritis IL-17 causes a induction of cytokines (e.g. TNFα and IL-6) and chemokines (e.g. IL-8) as well as destructive enzymes such as MMP1 from

synoviocytes [420]. IL-17 has also been shown to inhibit apoptosis of synoviocytes causing tissue hyperplasia [421].

IL-17 has extensively been described as playing a pivotal role in the pathogenesis of psoriasis. After the identification of the Th17 subset of cells, it was shown that these are highly prevalent in lesional psoriatic skin [3]. Specifically keratinocytes have been identified as targets for IL-17 and IL-22 activity. In these cells, IL-17 acts synergistically with TNFα on inflammatory mediators known to be critical in psoriatic inflammation [2]. IL-17 and IL-22 play a role in the change of barrier homeostasis typical for psoriatic plaque [422]. Interestingly, in a relatively small cohort of psoriasis patients Th17 cells were not shown to be the most prominent producers of IL-17A but mast cells and neutrophils [423]. It has also been shown that the CD8+ T cells in psoriatic skin contain significantly more IL-17 producing CD8+ cells compared to healthy skin [423]. Due to the apparent importance of IL-17 in psoriasis it is an important target for antibody therapies. Data on the extremely high efficacy of secukinumab (blocks IL-17A) holds the promise to not only control but clear psoriasis with this treatment [424]. There is also a monoclonal antibody that blocks the p40 subunit of IL-12 and IL-23 (ustekinumab), two cytokines crucial for Th1 and Th17 differentiation. Ustekinumab has shown good clinical responses in skin psoriasis and data for PsA seem promising [387].

1.10 Project aims

Although the pathogenesis of psoriasis is extensively studied there are still shortfalls in our understanding as to the role of all aspects of the disease pathogenesis. There is no doubt that this is a highly inflammatory disease [425] which is predominantly mediated by an influx of immune cells [3]. However, the role of the skin resident cells particularly the largely overlooked fibroblasts have not been well described in the chronic inflammation seen in psoriasis. The inflammatory response is a highly regulated process that is tightly controlled in order to prevent tissue damage and chronic inflammatory states. This control occurs at various levels, including transcriptional, translation and receptor regulation. At the receptor level the balance of agonists, antagonists and

binding proteins is crucial. This balance is not well understood, especially in disease states.

Therapeutic approaches for psoriasis patients varies greatly depending on severity of disease. The most severe patients are most commonly treated with systemic anti-inflammatories that are not always effective and frequently show side effects. However, for these patients recently developed biologics hold the promise for long-term, efficient control of the disease. Due to the very high costs of biologics this approach is not available for the majority of patients. There is a great need in particular for the mild to moderate disease group, to develop more effective and well tolerated topical therapies.

The main aim of this investigation on psoriatic inflammation was:

- To understanding the role of the novel group of IL-1 family cytokines, IL-36 in psoriasis and the importance of proteolytic cleavage of these proteins.

Additional aims were as follows:

- To identify how endogenous IL-18BP production can be enhanced in the skin compartment. The focus is on IL-27, which has previously indicated as a possible inducer of IL-18BP. To investigate the therapeutic value of an IL-17 targeting RNA aptamers in relation to psoriatic skin inflammation.

Chapter 2 – Materials and methods

2.1 Materials

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

Table 2-1. Primary antibodies used

Antibody	Specificity	Species and isotype	Source
162601	Human IL-36β	Mouse IgG_1	R&D systems
162122	Human IL-36α	Mouse IgG_{2B}	R&D systems
2A8	Human IL-36γ	Mouse $IgG_{1\kappa}$	Sigma-Aldrich
GTX108466	Human IL-36γ	Rabbit IgG	GeneTex
278706	Human IL-36γ	Rat IgG_{2A}	R&D systems
190524	Human IL-36RA	Mouse IgG_{2B}	R&D systems
ab171844	Human IL-1RL2	Rabbit IgG	Abcam
GT239	Human GAPDH	Mouse IgG_{2B}	GeneTex
CD28.2	Human CD28 (LEAF™)	Mouse $IgG_{1\kappa}$	BioLegend
OKT3	Human CD3 (LEAF™)	Mouse $IgG_{2A\kappa}$	BioLegend
A-5598	Myc tag (HRP)	Rabbit IgG	Sigma-Aldrich
R6H1	Human CCR6 (PE)	Mouse IgG_1	eBioscience
SC-23877	Human cytokeratin 10	Mouse IgG_1	Santa Cruz
D1.3	Hen egg lysozyme (Isotype ctrl)	Mouse IgG_1	Produced in-house

Table 2-2. Secondary antibodies used

Antibody	Specificity	Species	Label	Source
A-0168	Mouse IgG	Goat	HRP	Sigma-Aldrich
A-9542	Rat IgG	Rabbit	HRP	Sigma-Aldrich
STAR124P	Rabbit IgG	Goat	HRP	AbD Serotec
STAR120F	Mouse IgG	Goat	FITC	AbD Serotec

2.1.1 Buffers used

Loading dye for SDS-PAGE gels – 62.5 mM Tris pH 6.8, 10% Glycerol, 2% SDS, 0.001% bromophenol blue +/- 5% β2-mercaptoethanol

Gel electrophoresis running buffer – 25 mM Tris, 90mM glycine, 0.1% SDS

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

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

PBS Tween (for washing Western blot membranes) – PBS, 0.1% Tween-20

NP-40 lysis buffer – 1% NP-40 (or equivalent), 0.15M NaCl, 10mM EDTA, 10Mm NaN_3, 10mM Tris-HCl pH8

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

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

Bacterial lysis buffer – 20mM Tris pH8, 10% Glycerol, 5mM $MgSO_4$

Nickel column buffer – 500mM NaCl, 20mM Tris pH7.4, 10% Glycerol, 20-500mM Imidazole

Size exclusion column buffer – 20mM Tris pH7.4, 300mM NaCl, 5% Glycerol

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

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

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

Complete E-media - E-media contained per litre; 600ml DMEM HEPES (Sigma-Aldrich, UK), 320ml Hams F-12 (Gibco Invitrogen), 0.10 mg/ml streptomycin and 100U/ml penicillin (Gibco Invitrogen), 5% FCS, 10ug Cholera Toxin (Sigma-Aldrich, UK), 5mg/ml hydrocortisone (Sigma-Aldrich, UK), 5ng/ml epidermal growth factor (EGF) (BD Biosciences), 10ml 100x cocktail mix containing; 0.18M Adenine (Sigma-Aldrich, UK), 5mg/ml Insulin (Gibco), 5mg/ml Transferrin (Sigma-Aldrich, UK), 2x10-8µg MT3 (Sigma-Aldrich, UK).

2.1.2 Cell lines used

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 [426].

Human Embryonic Kidney 293 TT (HEK293TT) cell lines are adherent derivatives of 293 cells with a sheared adenovirus type 5 DNA and simian virus-40 [427]

Human Embryonic Kidney 293 FT (HEK293FT) cell lines are also adherent derivatives of 293 cells which stably expresses the SV40 large T antigen from the pCMVSPORT6TAg.neo plasmid. Expression of the SV40 large T antigen is controlled by the human cytomegalo-virus (CMV) promoter.

COS-7 are an adherent fibroblast-like cell line established from SV40 T antigen transformed CV-1 simian cells from African Green Monkey kidney cells [428].

HaCat cells are an adherent spontaneously transformed cell line derived from adult human keratinocytes [429].

2.2 General methods

Unless otherwise stated all reagents are from Sigma-Aldrich, UK. Unless otherwise stated cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Slough, UK) supplemented with 5% fetal calf serum (FCS) (PromoCell, Heidelberg,

Germany), 0.5% L-Glutamine (Sigma-Aldrich, UK) and 0.05 mg/ml streptomycin and 50U/ml penicillin (Sigma-Aldrich, UK) at 37°C, 5% CO_2 and 95% humidity.

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 NHS patients – see Appendix figure 25). Healthy volunteers were also recruited from the university and written consent was also sought (BIOSCI09-001).

2.2.2 Culture of primary cells and cell lines

Human primary keratinocytes (HPK) were cultured in Keratinocyte Growth Media (KGM) Kit II (PromoCell, Heidelberg, Germany). Culture medium was changed every second to third day. On stimulation keratinocytes were placed in Keratinocyte Growth Media with all supplements apart from epidermal growth factor and hydrocortisone. When the fibroblasts reached 90% confluency and HPK 60-70% confluency the cells were passaged. For primary fibroblasts and all adherent cell lines, cells were washed with sterile phosphate buffered saline (PBS) (Gibco, Life technologies, UK) and then trypsin (170.000U/L)-EDTA (200mg/ml) (Lonza, Slough, UK) was added and placed in the incubator for 2 minutes at 37°C, 5% CO_2. An equal amount of serum containing DMEM was added to the trypsinised cells in order to neutralise the enzyme. The cells were then centrifuged at 230xg and then re-seeded. For HPK, cells were also washed with sterile PBS and then EDTA (Versen) 1% (Pan Biotech, Germany) was added for 2 minutes at 37°C, 5% CO_2. Trypsin was added in the same manner as for the fibroblasts however neutralisation was achieved with trypsin neutralising solution (Lonza, Slough, UK). Cell lines were cultured in the same manner as human primary fibroblasts. In order to freeze cells they were re-suspended in freezing media (PromoCell, Heidelberg, Germany) and placed in a Mr. Frosty™ (Thermo Scientific) at -80°C for 24 hours before long-term storage in liquid nitrogen.

2.2.3 Outgrowth of fibroblasts from healthy and patient derived skin and tendon biopsies

Biopsies were taken from healthy controls or patients following consent (NHS patient donors - REC number: 11/YH/0368) and transported in DMEM supplemented with 5% FCS (PromoCell, Heidelberg, Germany), 0.5% L-Glutamine (Sigma-Aldrich, UK), 0.05 mg/ml streptomycin and 50U/ml penicillin (Sigma-Aldrich, UK) and 0.5% of 250µg/ml amphotericin B (Sigma-Aldrich, UK). Skin biopsies were then cut into small sections using a scalpel. The sections were carefully placed into a six well plate and left to air dry in the cell culture hood for approximately 15 minutes. The same media that the biopsies were transported in was then carefully added to the wells ensuring that the tissue stays adhered to the plate. Biopsies were then cultured at 37°C, 5% CO_2 for 1-2 weeks before cells were sufficiently confluent to passage and consequent culture was in media with out amphotericin B.

2.2.4Transfection of primary cells and cell lines

Cells were cultured in normal growth media until confluency of 80-90% was reached in a 6-well plate. On the day of transfection normal media was substituted for reduced serum Opti-MEM (Gibco, Life Technologies, UK). 1µg of DNA was then diluted in 400µl of Opti-MEM and then 6µl of TurboFect transfection regent (Thermo Scientific, UK) was added. The solution was vortexed and incubated for 15-20 minutes at room temperature. This solution was then added dropwise into the 6 well plate containing 4ml of Opti-MEM in each well. After 8 hours the media was removed and replaced with 2ml of fresh Opti-MEM**.**

2.2.5 Peripheral blood collection

In order to isolate neutrophils and peripheral blood mononuclear cells (PBMCs) 30-60ml of peripheral blood was collected from healthy donors and psoriatic patients in sterile Vacutainer® lithium heparin (BD Biosciences, Oxford, UK). 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

was strictly adhered to. Blood was kept at room temperature after donation and used immediately.

2.2.6 Isolation of PBMCs from whole blood

PBMCs were isolated from peripheral whole blood using density gradient. Peripheral blood was diluted 1:1 with sterile PBS (Gibco, Life technologies, UK). An equal amount of diluted blood was then carefully layered onto Ficoll-Paque plus (GE Healthcare, Buckinghamshire, UK). This was then centrifuged for 40 minutes at 400xg with no brake. PBMCs were then removed and diluted 1:1 with sterile PBS. Cells were then centrifuged at 350xg for 4 minutes and supernatant removed. PBMCs were either used immediately or cultured in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 5% FCS (PromoCell, Heidelberg, Germany), 0.5% L-Glutamine (Sigma-Aldrich, UK) and 0.05 mg/ml streptomycin and 50U/ml penicillin (Sigma-Aldrich, UK).

2.2.7 CD4+ T cell isolation from PBMCs – using MACS® cell separation

PBMCs were centrifuged at 350xg for 4 minutes. The supernatant was removed before washing with sterile PBS. Using the CD4+ T cell isolation kit II (Miltenyi Biotec, Surrey, UK) cells were then stained with magnetic labels as per manufacturer's instructions. After magnetic labelling the cells were then put through LS columns (Miltenyi Biotec, Surrey, UK) as per manufacturer's instructions. The method used allows for collection of untouched CD4+ cells so there is no magnetic tags which could potentially interfere with further experiments. After separation, cells were centrifuged at 350xg for 4 minutes and either used immediately or cultured in RPMI-1640 supplemented with 5% FCS (PromoCell, Heidelberg, Germany), 0.5% L-Glutamine (Sigma-Aldrich, UK) and 0.05 mg/ml streptomycin and 50U/ml penicillin (Sigma-Aldrich, UK).

2.2.8 Culture of primary CD4+ T cells

Freshly isolated T cells were cultured in RPMI-1640 media (Lonza, Slough, UK) supplemented with 5% FCS (PromoCell, Heidelberg, Germany), 0.5% L-Glutamine (Sigma-Aldrich, UK) and 0.05 mg/ml streptomycin and 50U/ml penicillin (Sigma-

Aldrich, UK). If CD4+ T cells were cultured for longer than 2 days the media was supplemented with 5ng/ml of IL-2 (Immunotools, Friesoythe, Germany).

2.2.9 Enrichment of CCR6+ T cells from CD4+ T cells

Following magnetic separation of CD4+ population cells were centrifuged at 300xg for 10 minutes and then re-suspended in 40µl of a PBS based buffer, pH 7.2, containing 0.5% bovine serum albumin (BSA) and 2mM EDTA. Cells these were then stained with 10µl of PE labelled CCR6 antibody (eBioscience, Hatfield UK. Clone: R6H1) for 10 minutes at 4°C. In order to remove unbound primary antibody 1-2ml of buffer was then added before centrifugation at 300xg for 10 minutes. Supernatant was then removed and 80µl of buffer used to re-suspend the cells, 20µl of Anti-PE microbeads (Miltenyi Biotec, Surrey, UK) was then added and incubated for 15 minutes at 4°C. The cells were washed again with 1-2ml of buffer and then re-suspended in 500µl of buffer before magnetic separation was performed with LS columns (Miltenyi Biotec, Surrey, UK) as per manufacturer's instructions. Cells were either used immediately or cultured in RPMI-1640 supplemented with 5% FCS (PromoCell, Heidelberg, Germany), 0.5% L-Glutamine (Sigma-Aldrich, UK) and 0.05 mg/ml streptomycin and 50U/ml penicillin (Sigma-Aldrich, UK).

2.2.10 Isolation of PMNs from whole blood

Whole blood was separated by using density gradient in a similar manner to that used for isolation of PBMCs however instead of using Ficoll, Histopaque 1077 (Sigma-Aldrich, UK) was layered in equal amounts on top of Histopaque 1119 (Sigma-Aldrich, UK). The diluted whole blood was then layered on top of this and centrifuged at 400xg for 30 minutes with no brake. The PMN layer, which is above the red blood cell pellet, was then removed using a 10ml sereological pipette and cells were washed with PBS. In order to remove contamination of erythrocytes the pellet was re-suspended in 0.2% NaCl and the tube was inverted 10x. An equal volume of 1.6% NaCl was then added and inverted once. Cells were then centrifuged at 350xg for 4 minutes and the cell pellet was re-suspended with RPMI-1640 supplemented with 5% FCS (PromoCell, Heidelberg, Germany), 0.5% L-Glutamine (Sigma-Aldrich, UK) and 0.05 mg/ml

streptomycin and 50U/ml penicillin (Sigma-Aldrich, UK). Cells were stimulated on day of separation and were not cultured for longer than an hour.

2.2.11 Construction of skin equivalents

Human primary fibroblasts from either healthy controls or psoriatic patients were cultured as described previously. The first step for skin equivalent construction involves making a dermal scaffold to support the human primary keratinocytes. For this 1 – 2 x 10^6 of human primary fibroblasts were used for each collagen scaffold. In order to make the collagen plug all solutions and plastic ware were kept cold and then following harvesting and pelleting of fibroblasts (as previously described) the pellet was re-suspended in 0.3ml of 10xDMEM + 0.3ml of 10x reconstitution buffer (2.2g $NaHCO_3$, 4.77g HEPES, 100ml 0.05M NaOH). Then 2.4ml of Collagen G (Type 1 and 4 – Biochrom AG, Merck Millipore) was added and 3mls of fibroblast/collagen mix was added to a 35mm Petri dish. The gels were allowed to solidify at 37°C 5% CO_2 for 30 minutes before adding 1.5ml of E-medium (with 5ng/ml EGF). These were then cultured for 2 days. 1 – 2 x 10^6 of primary keratinocytes was then seeded on the top of each collagen scaffold and cells were allowed to grow to confluence, the media was changed daily (E-medium + EGF). After 2 days skin equivalents were ready for the air/liquid interface stage. The support grids (Sigma, UK) were placed in 10cm Petri dishes and then using sterile forceps the skin equivalent was carefully placed on the support grid. E-medium (no EGF) was then added, ensuring that media did not come over the edge of the support grid and only sat underneath it touching the underside of the skin equivalent. Media was changed every other day for 14 days and if stimulation was required this was added into the culture media 96 hours before fixing rafts with 4% paraformaldehyde. Skin equivalents were sectioned, mounted onto slides and H&E stained by Propath Ltd UK (Hereford, UK).

2.2.12 Enzyme-linked immunosorbent assay (ELISA)

Cell-free supernatant was collected, stored at -20 (short term) or -80°C and analysed for the content of protein using a DuoSet human ELISA kit (RnD Systems, Abingdon, UK or BioLegend, Hatfield, UK) following the manufacturer's instructions.

2.2.13 Flourescence assisted cell sorting (FACS)

In order to stain for surface receptors cells were detached, if adherent, using EDTA (Versen) 1% (Pan Biotech, Germany) and then washed with PBS. Cells were then incubated with blocking buffer (5% serum in PBS) for 1 hour on ice before spinning down and washing twice with PBS. Cells were then incubated with the primary antibody at the concentration recommended by supplier for 1 hour on ice following which they were washed twice with PBS. Cells were then incubated with secondary antibody at the concentration suggested by supplier for 1 hour on ice following which cells were washed 3 times with PBS. Cells were re-suspended in PBS and kept on ice before analysis.

2.2.14 Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was performed on a RotorGen (Qiagen, Hilden, Germany) using a ΔΔCT-analysis based on the generation of standard curves for both the housekeeping gene (U6snRNA – Forward ctcgcttcggcagcaca, Reverse gcaaattcgtgaagcgtt) and the target gene (QuantiTect Primer Assay, Qiagen unless otherwise stated). For RNA isolation Quick-RNA MiniPrep (Zymo Research, Cambridge Bioscience, Cambridge, UK) was used. First strand cDNA synthesis kit (Fermentas / Thermo Fisher Scientific, Loughborough, UK) was used for reverse transcription. QuantiFast SYBR green PCR (Qiagen) was used to carry out the RT-PCR following manufacturer's instructions.

2.2.15 Fixing and staining cells for fluorescence imaging

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. For experiments detecting fluorescent molecules within the cells, following fixation antibody staining was not required. Cells were washed with PBS 3 times, the last wash containing 1µg/ml of 4',6-diamidino-2-phenylindole (DAPI) to stain the nucleus. Before mounting, slides were washed with dH_2O. Coverslips were mounted onto Poly-lysine coated slides using Fluoromount-G™ (eBioscience, Hatfield, UK) and sealed around the edges. Once mounted, slides were

kept at 4°C before imaging using an inverted LSM510 confocal microscope coupled to a LSM Image Browser.

2.2.16 Di-AminoBenzidine (DAB) staining of paraffin embedded skin equivalents

Skin equivalents were sectioned and mounted onto microscope slides by Propath UK. Mounted microscope slides were rehydrated and deparaffinised by submerging in Xylene (VWR chemicals) for 5 minutes, this was repeated twice. Slides were then sequentially submerged in 100%, 90% and 70% ethanol for 1 minute before being submerged for 5 minutes in water. Water was changed after 2 minutes of incubation. Slides were then submerged in 10mM sodium citrate + 0.05% Tween (pH 6) and boiled for 10 minutes in order to unmask antigens from paraffin embedding before antibody staining. Slides were again washed with water 3 times for 5 minutes before incubating with 3% hydrogen peroxide for 10 minutes. Slides were washed twice in water for 5 minutes each before being washed in PBS for 5 minutes. Slides were then placed in a humidity chamber and blocking buffer added (10% FCS in PBS) for 1 hour or overnight at 4°C. Following incubation sections were washed for 5 minutes in PBS, this was repeated 5 times. Slides were then incubated in humidity chamber with primary antibody diluted in 1.5% blocking buffer (1.5% FCS in PBS) for an hour at room temperature. Washing in PBS was performed again 5 times before addition of secondary antibody diluted in 1.5% blocking buffer (1.5% FCS in PBS) and incubated in humidity chamber for 1 hour at room temperature. PBS washing was repeated again 5 times. DAB staining was performed using a Vector Laboratories (Peterborough, UK) substrate kit following manufacturer's instructions. A counterstain was performed using haematoxylin (Sigma-Aldrich) for 20 seconds before washing repeatedly with water. Sections were then dehydrated by incubation of slides in 95% ethanol twice for 10 seconds. Sections were then incubated in 100% ethanol twice for 10 seconds, then in xylene twice for 10 seconds. Slides were then air-dried and mounted with coverslips using DPX mounting media (VWR chemicals). Slides were then imaged using a Leiss light microscope.

2.3 Molecular biology

2.3.1 SDS-Polyacrylamide gel electrophoresis (PAGE)

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 15-20 minutes before being centrifuged to remove cell debris. Supernatant from the lysed cells was removed and samples were stored at -20°C or used immediately. Samples were mixed with 4 x loading buffer and boiled for 5 minutes. Following brief centrifugation of the samples they were loaded onto a 4% polyacrylamide stacking gel (pH6.8) and resolved on a 12-17% acrylamide gel (pH 8.8) alongside molecular weight markers (New England Biolabs). 1.5mm gels were cast and run using a Mini PROTEAN III gel system (Biorad). Gels were run in running buffer at 0.03-0.04 Amps per gel as long as required for good separation. Gels were either analysed by Coomassie staining followed by de-stain or were used in Western blots.

2.3.2 Western blotting

Following SDS electrophoresis, resolved polypeptides were transferred to HybondC+ nitrocellulose (Amersham biosciences) or Immobilon-P (Millipore) membranes in transfer buffer by applying 100V for 1-2 hours. Membranes were then blocked for 1 hour in blocking buffer. Primary antibodies were diluted to the concentration advised by the manufacturer in blocking buffer and incubated with agitation for 1 hour at room temperature. Membranes were 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 20 seconds to 5 minutes.

2.3.3 Agarose gel electrophoresis

Gels were prepared using electrophoresis grade agarose in 1xTBE containing 0.2µg/ml Ethidium Bromide. DNA samples were mixed with 6x DNA loading dye and run at 100V alongside 1 kb DNA ladders. DNA in agarose was observed and photographed under short-wave UV light for observation or long-wave UV light when further cloning was required.

2.3.4 Conventional PCR for cloning

Primers were designed (see Appendix for all primers, figures 3-23) in order to amplify the sequence for the protein of interest using conventional PCR (KOD hot start DNA polymerase, Novagen). A sample was then taken from the PCR product and run on a 1% agarose gel alongside a DNA marker (New England Biolabs) and UV light used to assess whether a band was present at the correct size. Extra adenine nucleotides were added on to the end of the product using TAQ polymerase (Promega, USA) in preparation for insertion into a TA overhang vector (Champion pET SUMO expression system, Invitrogen, USA). Following addition of nucleotides the product was run on a 1% agarose gel and the band carefully cut out with a scalpel. DNA was extracted from the gel using QIAquick gel extraction kit (Qiagen) following manufacturer's instructions.

2.3.5 Restriction digest

PCR products and vectors were cut with relevant restriction enzymes in optimal buffers with 1% BSA (New England Biolabs). Reactions were carried out at 37°C for 3 hours. For the vectors after 2 hours of incubation alkaline phosphatase was added to prevent re-ligation of the vector. Following digest products were run on a 1% agarose gel and then bands cut out and purified using QIAquick gel extraction kit (Qiagen) following manufacturer's instructions.

2.3.6 Ligation

The ligation of the insert into the vector was carried out at a molar ratio of 1:3 vector to insert and 1ng of insert was added. T4 ligase was used following manufacturer's instructions (Invitrogen, USA). The reaction was carried out overnight at 16°C.

2.3.7 Transformation

Following ligation the vector was transformed into DH5α cells (Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1) in order to allow enough DNA proliferation to sequence. The ligation reaction was added to the thawed cells for 30 minutes on ice. The cells were then heat shocked at 42°C for 30 seconds, then placed back on ice for 2 minutes before adding SOCS medium (Invitrogen, USA) and then placed at 37°C with shaking for 1 hour. Cells were then plated on 2% agar plates containing relevant antibiotics.

Following overnight incubation at 37°C colonies were picked and a colony PCR performed using TAQ polymerase (Promega, USA). Positive colonies were then grown up in 2YT media containing relevant antibiotics overnight at 37°C. DNA was extracted from the cells using QIAprep Spin Miniprep kit (Qiagen) following manufacturer's instructions. Sequencing was then confirmed using Sanger sequencing (GATC- Biotech, Germany). Sequence profiles were analysed using a BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) programme for comparison with the predicted sequence (see Appendix for all sequences).

2.3.8 Expression of IL-1 family members in *E. coli*

Following successful sequencing of the constructs 1ng DNA was then transformed in to expression strains of *E. coli* – BL-21 (DE3) (*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdSλ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin)* and 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]) in the same manner as with the DH5α cells. (DE3) encodes an Lac inducible T7 polymerase. The BL21-CodonPlus (DE3)-RIPL cells also contain a plasmid encoding rare tRNA that are needed for expression of mammalian proteins. Cells were then grown overnight in either

33µg/ml of kanamycin for BL21 (DE3) or both kanamycin and 33µg/ml chloramphenicol for the BL21-CodonPlus cells. Larger cultures containing relevant antibiotics and 5% filtered glucose, to prevent leaky expression were then inoculated with 1 50^{th} of the overnight culture. Initially cells were grown at 37°C until they reached an optical density (at 600 nm) of 0.6 and then cells were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0.8mM and then grown over night at 16°C, 25°C and 37°C. The optimal temperature for expression was assessed by running the bacterial lysates before and after induction on an SDS page gel and then stained over night with Coomassie and de stained the following day. Following optimisation larger volumes of culture were grown up at the ideal temperature in order to get the required amount of protein. After overnight growth of cultures they were spun down at 4000g for 5 minutes. The bacterial pellet was then re-suspended in bacterial lysis buffer. Lysozyme (0.35mg/ml) and DNase (20µg/ml) were added and the bacteria were sonicated on ice for 8 cycles of 20 seconds on 30 seconds off. The lysed bacteria were then spun at 30,000g for 30 minutes and the supernatant removed and filtered. The constructs produced contained a His tag so protein was purified using a nickel-affinity chromatography. The column was equilibrated with 20mM imidazole nickel column buffer and then the filtered bacterial supernatant was added containing 20mM imidazole to prevent non-specific binding to the column. Following addition of bacterial supernatant the nickel column was washed with 5 column volumes of 20mM imidazole and then 50mM imidazole. Protein was eluted from the column in 5 column volumes of 100mM imidazole and then 500mM and 1ml fractions collected to run on a SDS page gel and stain with Coomassie to analyse when the protein comes off the nickel column. Fractions that contained the protein were then pooled.

2.3.9 Purification of protein using size exclusion column

Pooled fractions from nickel-affinity chromatography purification were concentrated to 5ml and then sterile filtered at 0.45µm. The size exclusion column was prepared by running 1 column volume of degassed sterile filtered water and then the same for column buffer. The protein was loaded and fractions were collected when a 280nm peak was present, excluding any peaks that were present in the void volume or any that were

suggestive of a protein dimer. Fractions were pooled and concentrated before being run on an SDS page gel and stained with Coomassie to check that the purity of the protein. Western blots were also carried out to check the protein expressed and purified was correct.

2.3.10 Removing SUMO from relevant constructs

Following size exclusion protein purification when it was necessary the SUMO tag was removed from the constructs. Optimisation of the amount of SUMO protease, His-ULP-1, was carried out and this differed depending what the preceding amino acid was. Therefore, between 1:50 and 1:200 ratio protease to protein was used for between 24-48 hours at 4°C. Cleavage was assessed by running on an SDS page and then Coomassie stained. Due to the SUMO and SUMO protease containing His tags these constructs could be removed using a nickel column to purify protein away from the SUMO and SUMO protease. For all constructs that were to be used in cell culture the nickel column was thoroughly washed with 0.5M NaOH before calibration with 20 mM imidazole buffer. The sample was then added and the column elute collected which would represent the pure cut protein. This was then re-run on the size exclusion column to ensure purity and only monomers of the protein were present. Again, all glassware used to make buffers and the column itself was thoroughly washed with 0.5M NaOH.

2.3.11 RNA aptamer chemical synthesis

Chemical syntheses of the RNA aptamers used were produced in house by the University of Leeds. The aptamers were produced with a 5’ monophosphate and a fluorine modification at the 2’ on pyrimidine bases. The sequenced produced without flourophores was: 5' GGU CUA GCC GGA GGA GUC AGU AAU CGG UAG ACC 3'. Addition of a 5’ Cy3 flourophore: 5' cy3-GGU CUA GCC GGA GGA GUC AGU AAU CGG UAG ACC 3'. Addition of a 5’ Cy3 and 3’ Cy5: 5' cy3-GGU CUA GCC GGA GGA GUC AGU AAU CGG UAG ACC-cy5 3'.

Chapter 3 – IL-18 binding protein is up-regulated by IL-27

3.1 Introduction

The production and release of pro-inflammatory cytokines by skin resident cells during an inflammatory response is crucial to aid in eradication of invading pathogens and maintain skin homeostasis (see 1.2.2 for in depth discussion). However, resolution of inflammation once the environmental threat has been removed is crucial to avoid permanent tissue damage (e.g. scarring) or chronic inflammatory conditions (see 1.2.3 for in depth discussion). The skin resident cells possess a control mechanism in that release of pro-inflammatory mediators is followed by release of anti-inflammatory mediators to antagonise their activity. These mechanisms are tightly regulated and are essential for the prevention of chronic inflammation (see 1.2.4 for in depth discussion). The focus of this chapter is to describe a novel anti-inflammatory mechanism of IL-27 which is its ability to up-regulate IL-18 binding protein (BP) which antagonises IL-18.

IL-27 is a member of the IL-12 family of cytokines that has been shown to have pleiotropic roles in inflammation and both anti-inflammatory and pro-inflammatory actions have been identified. Interestingly, IL-27 appears to possess unique features that are not shared with other IL-12 family members. IL-27 has been shown to up-regulate the IL-12R which displays high binding affinity with its agonists and is crucial in Th1 polarisation [265]. IL-27 has also been shown to induce IL-23 release from APCs, which plays a role in Th17 polarisation but also IFNγ release from memory Th1 cells [281]. In human macrophages, monocytes and keratinocytes IL-27 has been described to play an inflammatory role by induction of CXCL10. Due to the property of CXCL10 to attract CXCR3+, IFNγ producing T cells to the area of inflammation, IL-27 has been proposed as an important mediator in inflammatory skin diseases such as eczema and psoriasis [277, 278]. However, in contrast work carried out in murine models has suggested a role for IL-27 as an anti-inflammatory mediator in the later stages of infection. IL-27 knock-out mice have been shown to be more susceptible to experimental autoimmune encephalitis [430]. Mice over-expressing WSX-1 displayed reduced lupus erythematosus like symptoms in a disease model [431]. Conversely to what has been shown in human cells, IL-27 has also been shown to inhibit Th17 differentiation in mice in a STAT1-dependent but IFNγ-independent manner [430]. IL-

27 has also been shown to stimulate murine cells to produce IL-10, a well-known anti-inflammatory mediator [432].

IL-18 is a member of the IL-1 family of cytokines and has been described to have a highly pro-inflammatory function [186, 433, 434]. Within the skin IL-18 is expressed by resident skin DCs and also keratinocytes. However, expression of IL-18 is not seen by fibroblasts in the skin compartment [435, 436]. IL-18 supports both Th1 and Th2 differentiation depending on the surrounding cytokine milieu and is important in regulation of innate and adaptive immunity [433, 437]. Murine models deficient in IL-18 have a significantly reduced ability to produce IFNγ as well a reduction in NK cell activity [438]. In murine asthma models deficiency in IL-18 reduced airway remodelling and chronic inflammation [439]. IL-18 and its receptor have also been found to be highly expressed in chronic inflammatory skin lesions, such as psoriatic plaques [440, 441] and cutaneous lupus erythematosus (CLE) [442]. It is thought that IL-18 plays a role in the chronification of inflammatory diseases and its role in inflammatory fibrosis has been shown in lung [443], kidney [444] and cardiac pathologies [445]. Skin epithelial cells isolated from patients with CLE were shown to be more responsive to IL-18 with regard to production of TNFα compared to healthy controls. TNFα induced apoptosis in response to IL-18 was also noted in this study, which is crucially important in the disease pathogenesis of CLE [446]. Healthy keratinocytes respond to IL-18 stimulation by producing CXCR3 ligands such as CXCL10 [245] and increased surface expression of MHC I and II [442]. IL-18 has recently been found to activate IFNγ producing innate lymphoid cells 1 (ILC1). The recently described ILCs seem to be closely linked to tissue responses at epithelial surfaces [447].

Due to the evident pro-inflammatory effects of IL-18, it is important as with any pro-inflammatory cytokine that it is controlled. IL-18 is controlled by its natural antagonist, IL-18BP [248]. IL-18BP prevents IL-18 binding to its cell surface receptor and exhibits a high neutralising capacity [249]. The balance between IL-18 and IL-18BP is crucial in understanding pathologies related to over-expression of IL-18 or IFNγ. However, in inflammatory disorders both IL-18 and IL-18BP are up-regulated and current methods have limitations in quantitative determination of cytokine activity in the tissue (in

particular as IL-18 exists as a pro- and active form) [444, 448, 449]. At the start of this project IFNγ was the only known inducer of IL-18BP. However, IFNγ not only induces IL-18BP but also the chemokine CXCL10 which attracts further Th1 cells into the area of inflammation. This has been shown in a variety of non-leukocytic cell types [252]. It has been identified that in DLD-1 colon carcinoma cells IL-18BP expression is dependent on STAT1 binding to the GAS element in the IL-18BP promoter [450]. The importance of IL-18BP can be demonstrated by the fact that viruses such as human papillomavirus (HPV) and pox viruses either produce a IL-18BP homologue or up-regulate the endogenous form leading to increased viral virulence [260, 261, 451, 452].

It is of importance to understand potential alterations in the balance between anti-inflammatory and pro-inflammatory mediators, particularly in the development of chronic inflammatory skin diseases. Experimental work presented in this chapter aimed to identify and describe novel mediators that have the ability to up-regulate endogenous IL-18BP in human skin resident cells.

3.2 IL-18BP expression following IL-27 stimulation

IL-27 was established as an interesting molecule for IL-18BP production when all the IL-12 family cytokines were used to treat human primary keratinocytes to assess their functional responses. IL-27 has also been previously described to induce CXCL10 which suggests STAT1 activation occurs downstream of the IL-27 receptor, which supposedly is also required for IL-18BP expression [278].

Human primary keratinocytes were treated with 50 ng/ml of IL-12 family cytokines including IL-12, IL-27 and IL-23. IFNγ was used as a positive control at 10ng/ml and 50ng/ml to assess cell responsiveness (Figure 3-1 a). After 48 hours of treatment cell free supernatant was removed and IL-18BP levels were detected using ELISA. A significant difference was detected between the non-stimulated control and IL-27 treatment. No significant expression of IL-18BP was detected with the other two IL-12 family members. IFNγ at a concentration of 50 ng/ml showed approximately a 100 fold stronger response compared to IL-27 at the same concentration in human primary keratinocytes. Human primary keratinocytes were treated with increasing concentrations

of IL-27 ranging from 5 ng/ml to 100 ng/ml for 48 hours before IL-18BP protein concentrations were analysed by ELISA (Figure 3-1 b). A significant difference was shown between the non-stimulated control and treatment with IL-27 at 50 ng/ml and 100 ng/ml. IL-18BP protein release showed no further increase after 100 ng/ml (data not shown). This experiment was also performed in HaCat cells. IL-27 treatment using HaCat cells ranged from 1 ng/ml to 100 ng/ml and again significance between non-stimulated control and IL-27 treatment at 50 ng/ml and 100 ng/ml was detected (Figure 3-1 c). At 10ng/ml HaCat cells also produced significant levels of IL-18BP compared to non-stimulated control. HaCat cells displayed a similar expression of IL-18BP in response to IL-27 as primary cells. Interestingly, human primary keratinocytes were the only skin resident cells to have a basal expression of IL-18BP (Figure 3-1 and Figure 3-3).

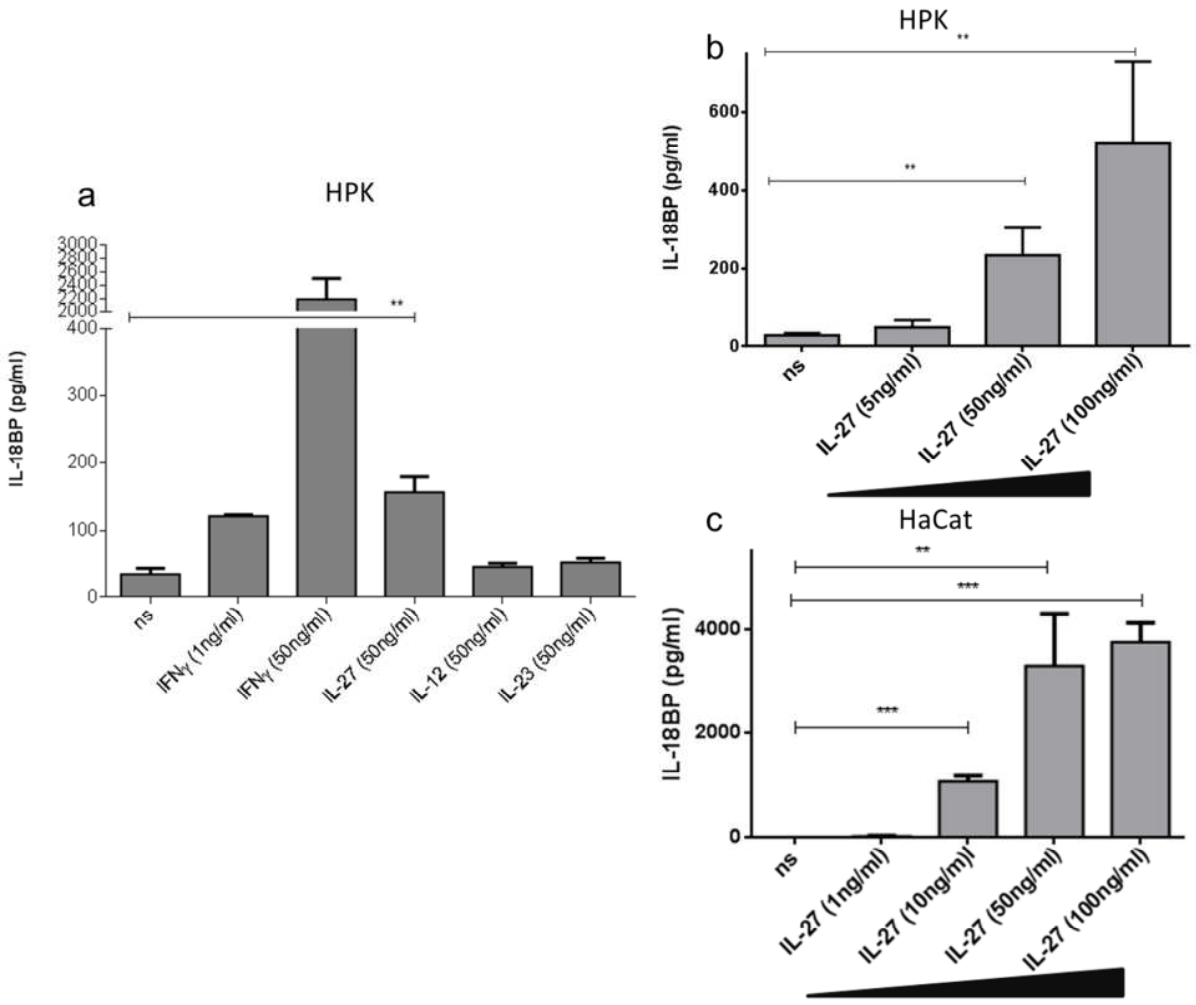


Figure 3 - 1. IL-27 dose dependently up-regulates IL-18 binding protein (BP) in human keratinocytes.

Human keratinocytes and HaCat cells were plated at 20,000 cells per well and then stimulated for 48 hours following which supernatants were removed and IL-18BP protein concentration analysed by ELISA. **a** - Human primary keratinocytes (HPK) were stimulated with varying concentrations of IFNγ and IL-12 family member cytokines. n = 7, independent experiments and different donors. **b** - Human primary keratinocytes were stimulated with IL-27 (5 ng/ml-100 ng/ml). n = 3, independent experiments and different donors. **c** – Human keratinocyte cell line, HaCat cells were also stimulated with IL-27 (1 ng/ml-100 ng/ml). n = 3. Mean +/- Standard error of the mean (SEM) is depicted on all graphs. ns = Non-stimulated. Raw data was analysed using an un-paired student's t-test (GraphPad Prism 5.03, GraphPad Software, San Diego, CA). ** = $p \leq 0.01$ *** = $p \leq 0.001$. Data in this figure was collected in collaboration with Division of Immunodermatology and Allergy Research, Department of Dermatology, Hannover Medical School, Hannover, Germany [59]

In order to study whether primary human dermal fibroblasts also produced IL-18BP in response to IL-27 cells were treated with increasing concentrations of IL-27 (1ng/ml to 100 ng/ml). Following 48 hours of treatment IL-18BP concentrations analysed by ELISA (Figure 3-2 a). Human primary fibroblasts produced unexpectedly high amounts of IL-18BP and significant differences in IL-18BP levels were seen compared to non-stimulated control at IL-27 concentrations of 10 ng/ml, 50 ng/ml and 100 ng/ml. As IFNγ is presently the only known inducer of IL-18BP, cells were also treated with equivalent concentrations (50 ng/ml) of IL-27 and IFNγ for 48 hours prior to IL-18BP measurement (Figure 3-2 b). In contrast to human primary keratinocytes, IL-27 (50ng/ml) stimulated fibroblasts produced IL-18BP in the same range as after stimulation with the equivalent concentration of IFNγ at 50 ng/ml. It is also worth noting that human primary fibroblasts produced higher concentrations of IL-18BP compared to human primary keratinocytes. Human primary fibroblasts are responsive to extremely low levels of IL-27, around 70% of donors responded to 1 ng/ml of IL-27.

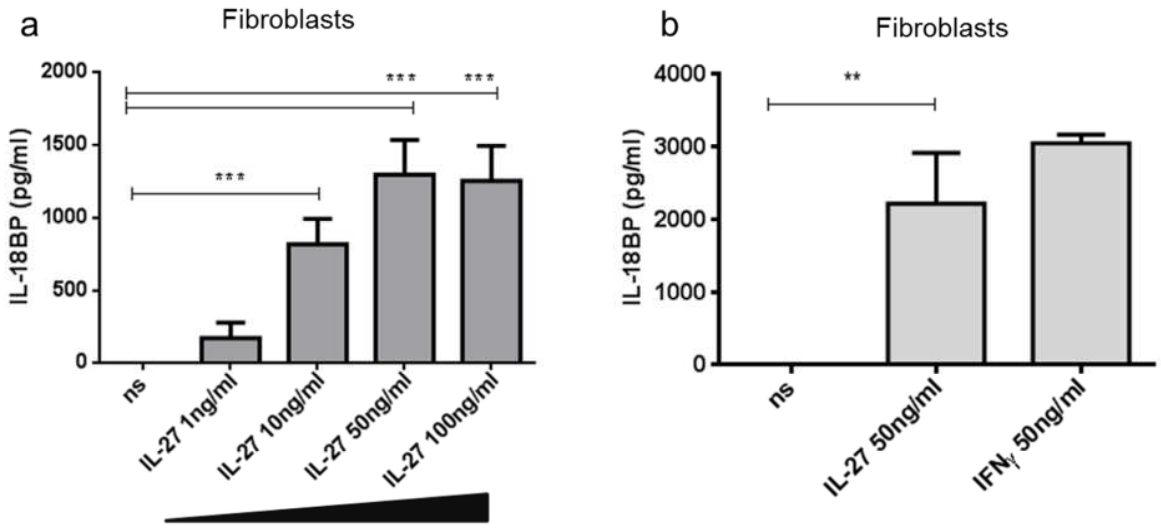


Figure 3 – 2. IL-27 dose dependently increases levels of IL-18BP in human primary fibroblasts.

Human primary fibroblasts were plated at 20,000 cells per well and then stimulated for 48 hours following which supernatants were removed and IL-18BP protein concentration was analysed by ELISA. **a** – Human primary fibroblasts were stimulated with IL-27 (1 ng/ml-100 ng/ml) for 48 hours. n = 7, independent experiments and different donors. **b** – Human primary fibroblasts were stimulated with 50 ng/ml of IL-27 and IFNγ for 48 hours. n = 4, independent experiments and different donors. Mean +/- SEM is depicted on all graphs. ns = Non-stimulated. Raw data was analysed using an un-paired student's t-test (GraphPad Prism 5.03, GraphPad Software, San Diego, CA). ** = $p \leq 0.01$ *** = $p \leq 0.001$ [59]

These experiments indicated that 50 ng/ml of IL-27 was sufficient to induce significant levels of IL-18BP and this concentration was used for all future experiments.

3.3 Time dependent expression of IL-18BP and CXCL10 in response to IL-27

As a known inducer of IL-18BP IFNγ was used a comparator in this experimental setup. Human primary fibroblasts and human primary keratinocytes were treated with 50 ng/ml of IL-27 for 5 hours and 16 hours before mRNA was isolated and qRT-PCR was performed to determine mRNA levels of IL-18BP (Figure 3-3). In primary human fibroblasts there was a significant increase in mRNA levels at 16 hours compared to non-stimulated control (Figure 3-3 a). No significant differences were seen in human

primary keratinocytes, however a slight increase in IL-18BP mRNA levels was present (Figure 3-3 b). The mRNA levels of IL-18BP were hugely increased (10 fold to 100 fold) in human primary fibroblasts compared to human primary keratinocytes, which is in line with what has been shown on protein expression levels.

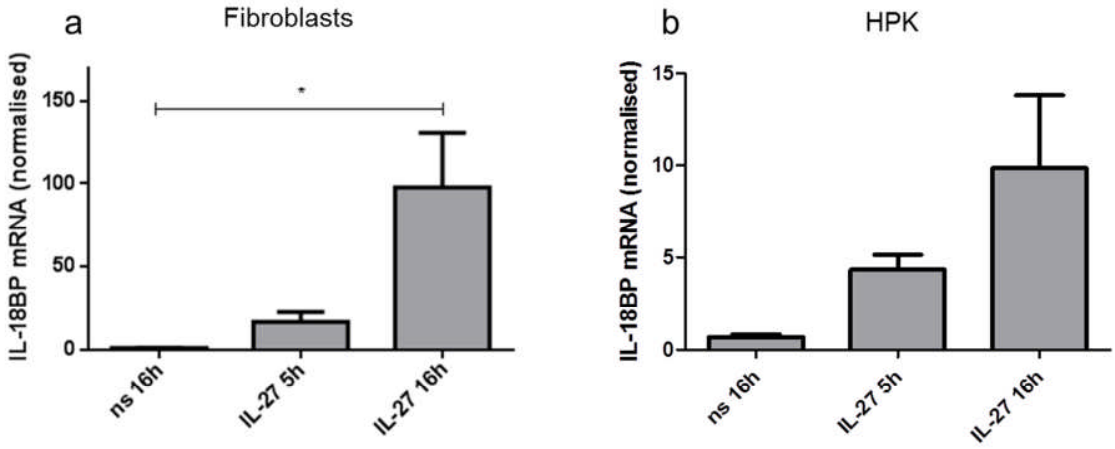


Figure 3 – 3. mRNA levels of IL-18BP are up-regulated in IL-27 stimulated human primary fibroblasts.

Primary human cells were plated at 20,000 cells per well and then stimulated with 50 ng/ml IL-27 and mRNA expression of IL-18BP was determined by quantitative PCR (qPCR), the values were normalised using a housekeeping gene, U6 and then normalised to the ns control to depict fold increase. **a** - Human primary fibroblasts were stimulated with 50 ng/ml IL-27 for 5 and 16 hours. n = 2, independent experiments and different donors. **b** – Human primary keratinocytes (HPK) were stimulated with 50 ng/ml of IL-27 for 5 and 16 hours. n = 2, independent experiments and different donors. Mean +/- SEM is depicted on all graphs. ns = Non-stimulated. Raw data was analysed using an un-paired student's t-test (GraphPad Prism 5.03, GraphPad Software, San Diego, CA). * = $p \leq 0.05$ [59]

In order to establish whether protein expression of IL-18BP was also increased over time human primary fibroblasts and HaCat cells were stimulated with 50 ng/ml of IL-27 for 24, 48, 72 and 96 hours before cell-free supernatants were removed and IL-18BP protein expression was analysed using ELISA (Figure 3-4). A significant increase of IL-18BP expression was detected in the human primary fibroblasts after 48, 72 and 96 hours; whereas HaCat cells only showed a statistically significant difference after 72 and

96 hours. In human primary fibroblasts the production of IL-18BP was maintained until 96 hours and the same time kinetic was also seen in human primary keratinocytes (data not shown). *In vitro* experiments are difficult to perform for longer than 96 hours, however experiments performed at 120 hours suggest that IL-18BP production and consequent accumulation plateaus at 96 hours.

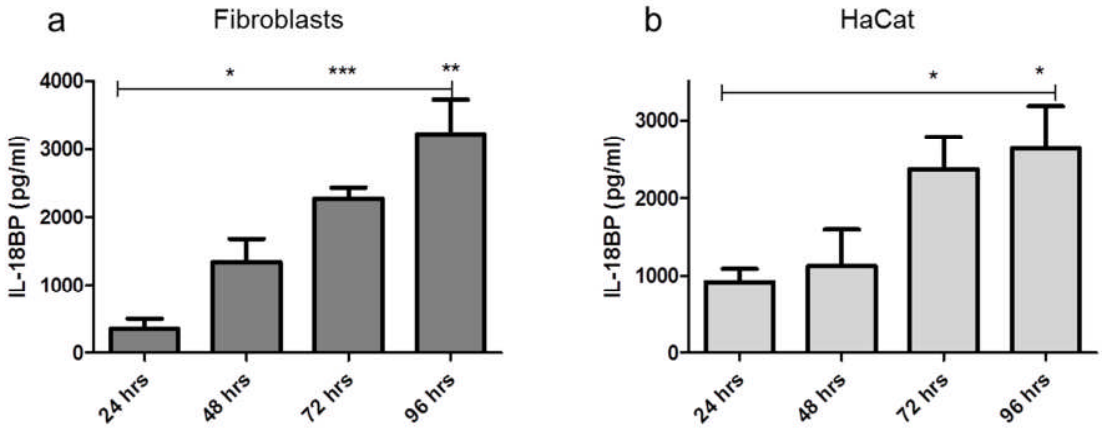


Figure 3 – 4. IL-18BP is released in a time dependent manner in response to IL-27.

Human primary fibroblasts and HaCat cells were plated at 20,000 cells per well and then stimulated with 50 ng/ml of IL-27 for varying amounts of time before supernatants were collected and IL-18BP protein concentration assessed by ELISA. **a** – Human primary fibroblasts were stimulated with 50 ng/ml of IL-27 for varying time points (24, 48, 72, 96). n = 4, independent experiments and different donors. **b** – HaCat cells were stimulated with 50 ng/ml of IL-27 for varying time points (24, 48, 72, 96). n = 4. Mean +/- SEM is depicted on all graphs. Raw data was analysed using an un-paired student's t-test (GraphPad Prism 5.03, GraphPad Software, San Diego, CA). * = $p \leq 0.05$ ** = $p \leq 0.01$ *** = $p \leq 0.001$[59]

It is known that both IFNγ [252] and IL-27 [278] induce CXCL10 therefore levels of CXCL10 in response to both IL-27 and IFNγ were studied in human primary fibroblasts in order to compare the pro-inflammatory ability of these mediators. Cells were treated with 50 ng/ml of IL-27 or with 10 ng/ml of IFNγ, which is within the physiological range for these cytokines (Figure 3-5). After 24, 48, 72 and 96 hours cell-free supernatants were removed and analysed by ELISA. Results from these experiments showed that CXCL10 is released along with IL-18BP at 24 hours however IL-18BP expression is maintained for longer. This is consistent with observation from other

mediators where upon stimulation expression of the anti-inflammatory molecule is seen at a later time point than the pro-inflammatory one. IL-27 induced lower levels of CXCL10 compared to IFNγ. In the cells treated with IFNγ CXCL10 levels peaked at 24 hours whereas IL-27 treated cells peaked at a later time point maintaining the expression for longer, however at a lower level overall. Taken results from Figure 3-2 b into account these results may suggest that IL-27, compared to IFNγ, promotes the production of the anti-inflammatory IL-18BP rather than the pro-inflammatory CXCL10.

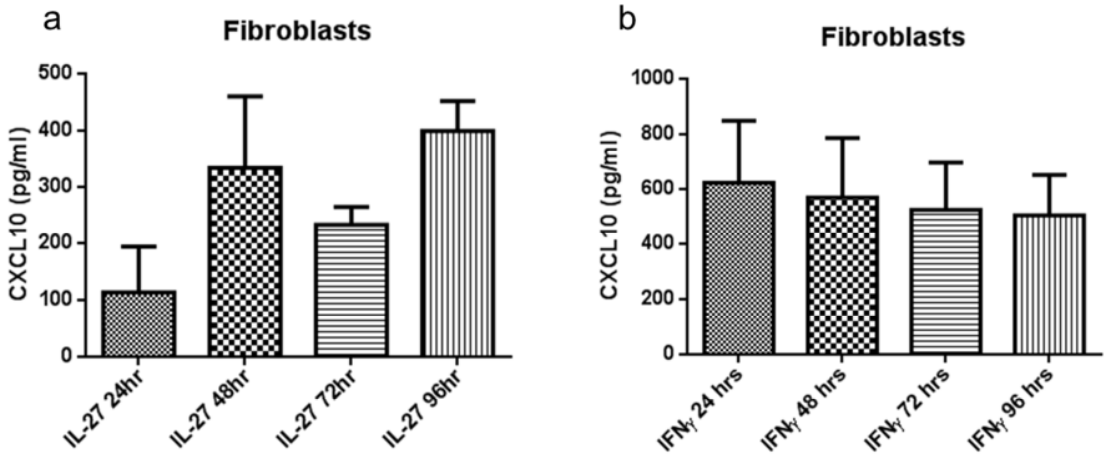


Figure 3 – 5. CXCL10 is differentially expressed compared to IL-18BP in response to IL-27 or IFNγ.

Human primary fibroblasts were plated at 20,000 cells per well and then stimulated with 50 ng/ml of cytokine for varying amounts of time before supernatants were collected and CXCL10 protein concentration assessed by ELISA. **a** – Human primary fibroblasts were stimulated with 50ng/ml of IL-27 for varying time points (24, 48, 72, 96). n = 4, independent experiments and different donors. **b** – Human primary fibroblasts were stimulated with 10 ng/ml of IFNγ for varying time points (24, 48, 72, 96). n = 4. Mean +/- SEM is depicted on all graphs.

3.4 IL-27 activates STAT1 downstream of its receptor

It has previously been shown that STAT1 activation is crucial for IL-18BP expression as well as expression of CXCL10 [450]. In order to decipher the signalling pathways involved in the ability of IL-27 to up-regulate IL-18BP experiments were performed in collaboration Prof. Heiko Muhl at Pharmazentrum Frankfurt/ZAFES. This research

group had previously performed experiments regarding STAT1 activation and IL-18BP promoter in IFNγ stimulated DLD-1 colon carcinoma cells [450]. Here, HaCat cells were treated with 100 ng/ml of IL-27 or 20 ng/ml of IFNγ for 30 minutes before cell lysis. Cell lysates were run on an SDS page gel before western blotting for both STAT1 and phosphorylated STAT1 (Figure 3-6 a). IL-27 initiated STAT1 phosphorylation and thus activation after 30 minutes however this was to a lesser degree than seen for IFNγ. mRNA levels of IL-18BP in HaCat cells were also confirmed using qRT-PCR. Significant levels of IL-18BP mRNA were detected following treatment with IL-27 as seen in human primary keratinocytes (Figure 3-6 b); however IFNγ did up-regulate IL-18BP mRNA to a greater extent. Luciferase reporter assays were performed to analyse the IL-18BP promoter activity in response to IL-27 (Figure 3-6 c). Wild type promoter (pGL3-BPwt) indicates induction of IL-18BP which was significantly inhibited when the proximal GAS site (pGL3-BPmt/prox) was mutated. In contrast, if the distal GAS site (pGL3-BPmt/dist) was mutated there was no significant difference in mRNA levels of IL-18BP. A double mutation in both the distal and proximal GAS site (pGL3-BPmt/prox/dist) resulted in a similar inhibition of the gene promoter compared to a single mutation in the proximal GAS site. These results indicate a crucial role for the proximal GAS site in the IL-18BP promoter.

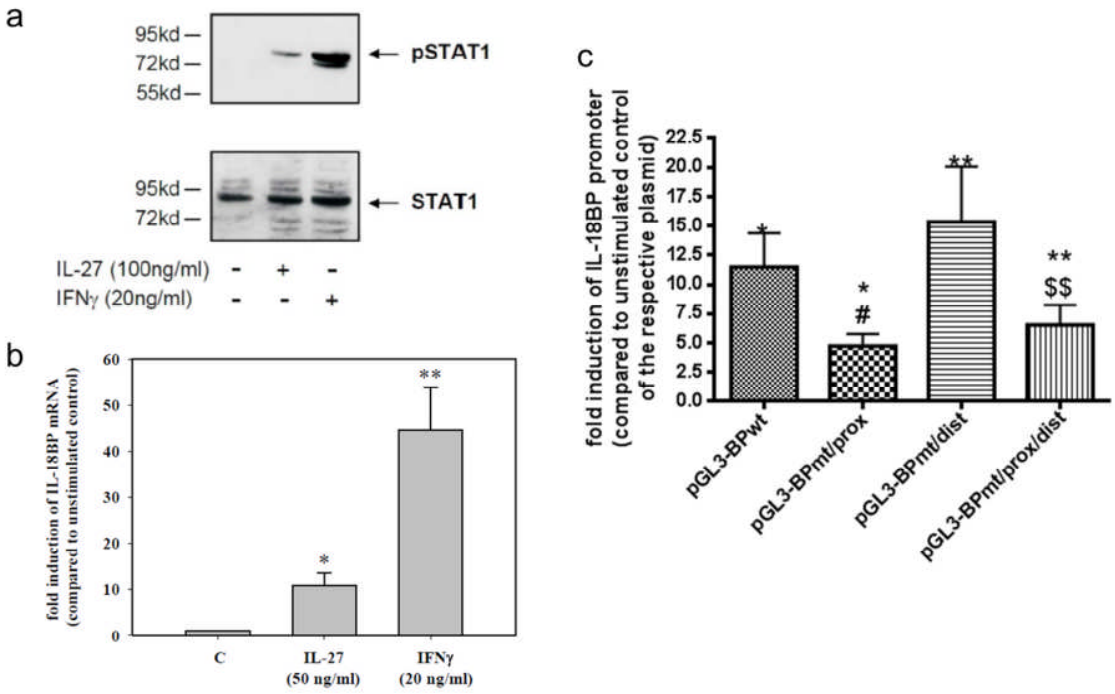


Figure 3 – 6. IL-27 induces IL-18BP via a STAT1 pathway and the proximal GAS site in the IL-18BP promoter is crucial for gene activation.

HaCat cells were stimulated with IL-27 and IFNγ and analysis performed in order to elucidate downstream signalling for release of IL-18BP. **a** – HaCat cells were stimulated for 30 min with 100 ng/ml of IL-27, 20 ng/ml of IFNγ or used as a non-stimulated control. Cells were lysed and the obtained nuclear extract analysed by Western blot using antibodies specific for total STAT1 and pSTAT1-Y701. n=3, one representative experiment shown. **b** – HaCat cells were stimulated for 24 h with 50 ng/ml of IL-27, 20 ng/ml of IFNγ or used as un-stimulated control and mRNA expression of IL-18BP was determined by qRT-PCR. IL-18BP mRNA was normalised to that of GAPDH and is shown as mean fold induction compared to un-stimulated control. n = 6. **c** – HaCat cells were transfected with the indicated IL-18BP promoter constructs. After 24 h, cells were kept as non-stimulated control or stimulated with 100 ng/ml of IL-27. After another 24 h, cells were harvested and luciferase assays were performed. Data are expressed as mean fold-luciferase induction (compared to the non-stimulated control transfected with the same plasmid). n = 4, independent experiments. * = $p \leq 0.05$ and ** = $p \leq 0.01$ compared to non-stimulated control of the respective plasmid; #p,0.05 compared pGL3-BPwt under the influence of IL-27, $$p,0.01 compared to pGL3-BPmt/dist under the influence of IL-27. Experiments carried out in collaboration with Prof. Heiko Muhl (Pharmazentrum Frankfurt/ZAFES, University Hospital Goethe-University Frankfurt, Frankfurt am Main, Germany). [59]

3.5 Understanding the different expression levels of IL-18BP and CXCL10 at different time points

The results shown in figures 3-4 and 3-5 indicate that CXCL10 and IL-18BP are released at different kinetics following activation by IL-27 or IFNγ. One possibility for prolonged protein expression is increased stability of the IL-18BP mRNA. Therefore in order to study this fibroblasts were stimulated with 50 ng/ml of IL-27 for 4 hours and then actinomycin D was added, which stops all transcription. mRNA was isolated at the point of actinomycin D addition and then 1, 2, 3 and 4 hours after actinomycin D addition (Figure 3-7 a). Once the mRNA was isolated qRT-PCR was performed for IL-18BP and IL-8. IL-8 mRNA is known to be relatively unstable, therefore this provided a good comparator to assess whether IL-18BP mRNA was stabilised following IL-27 treatment. IL-18BP mRNA showed a kinetic very similar to IL-8 mRNA in this experiment so we concluded that mRNA stability was not a key factor in prolonged IL-18BP production. Another hypothesis was that IL-27 may cause constant activation of STAT1 and the consequent maintenance of IL-18BP expression that has been shown in previous experiments. In order to study this hypothesis human primary fibroblasts were treated with 50 ng/ml of IL-27. The media was then removed and fresh media added after washing cells 1, 4, 12, 24 hours after IL-27 treatment. The cells were then cultured for 48 hours (from the point of initial stimulation) and supernatants removed, IL-18BP levels were analysed by ELISA. Interestingly, removal of the stimulus from the supernatant after an hour did not have a marked reducing effect on IL-18BP production, possibly suggesting that constant receptor activation is not required to have a continued expression of IL-18BP.

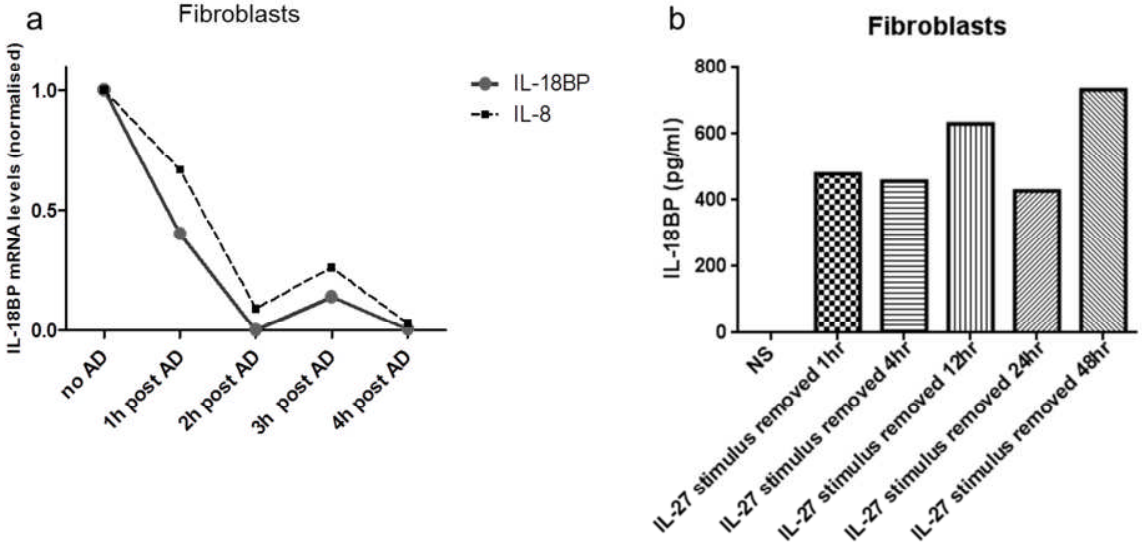


Figure 3 – 7. IL-18BP RNA does not show increased stability and constant IL-27 receptor activation is not required for prolonged production of IL-18BP.

Human primary fibroblasts were plated at 20,000 cells per well and then stimulated with 50 ng IL-27. **a** – Human primary fibroblasts were stimulated and then 4 hours later treated with actinomycin D (AD), cells were lysed at 1, 2, 3 and 4 hours following treatment. qPCR was performed using primers for IL-18BP and IL-8, a known low stability RNA. n = 1 **b** – Human primary fibroblasts were stimulated with IL-27 and then media removed and cells washed at 1, 12, 24 hours and fresh media added. Following addition of fresh media cells were cultured for 48 hours from point of initial stimulation for levels of IL-18BP protein using ELISA. n = 1. NS = Non-stimulated. [59]

If IL-27 was to be used therapeutically as a topical inducer of the anti-inflammatory protein IL-18BP in IL-18 mediated diseases then the release of CXCL10 would need to be completely inhibited. This was attempted *in vitro* by treating human primary fibroblasts with 50 ng/ml of IL-27 and then adding 10 µg/ml hydrocortisone at the same time or 1, 4, 8, 12 and 24 hours either before or after IL-27 treatment (Figure 3-8). Treatment of human primary fibroblasts with 50 ng/ml of IL-27 induced significantly less CXCL10 compared to IL-18BP. However, addition of hydrocortisone 1 hour before IL-27 treatment (Figure 3-8 a) or hydrocortisone 1 hour after IL-27 treatment (Figure 3-8 b) completely inhibited CXCL10 expression while maintaining IL-18BP levels and this was maintained for up to a 12 hour gap between treatments. It is worth noting that

addition of IL-27 and hydrocortisone at the same time did not reduce CXCL10 expression.

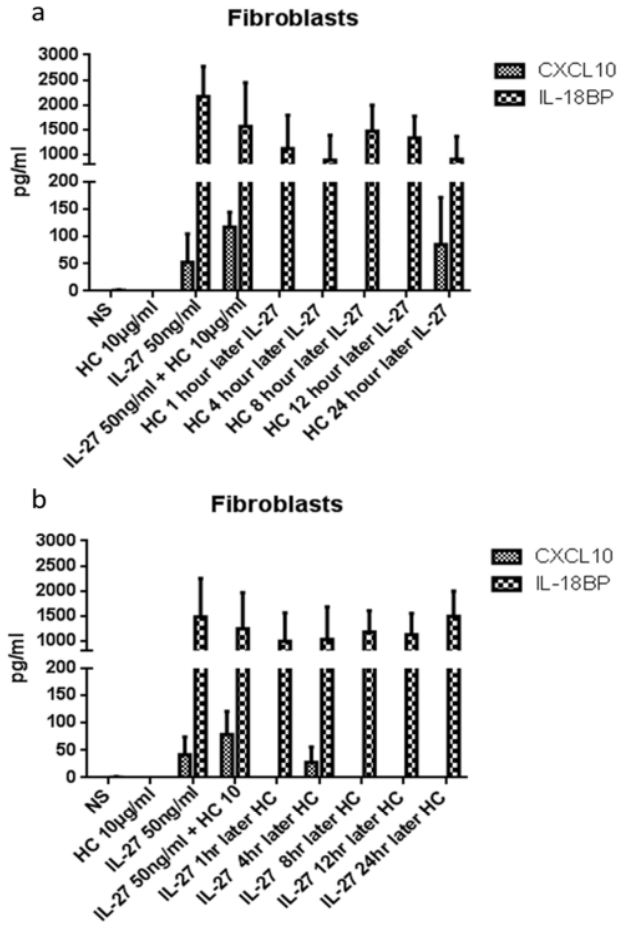


Figure 3 – 8. CXL10, but not IL-18BP release is prevented by hydrocortisone.

Primary human fibroblasts were plated at 20,000 cells per well and then stimulated with 50 ng/ml of IL-27 in the presence and absence of 10µg/ml of hydrocortisone (HC) and IL-18BP and CXCL10 ELISAs were carried out to establish the level of protein released. **a** – Human primary fibroblasts were stimulated with 10µg/ml of HC and then at time points ranging from 1 – 24 hours later 50 ng/ml of IL-27 was added. After 48 hours IL-18BP and CXCL10 were analysed via ELISA.. n = 3. **b** – Human primary fibroblasts stimulated with 50 ng/ml of IL-27 and then at time points ranging from 1 – 24 hours 10 µg/ml of HC was added. After 48 hours IL-18BP and CXCL10 were analysed via ELISA. n = 3. Mean +/- SEM is depicted on all graphs. NS = Non-stimulated.

3.6 Discussion and future work

Skin resident cells play a crucial role in the pathogenesis of many chronic inflammatory skin diseases such as psoriasis and CLE. These diseases often have uncontrolled levels of pro-inflammatory cytokines and it is thought that an imbalance between the pro-inflammatory and the anti-inflammatory mediators may play a role in the development of chronic disease. In this chapter a novel anti-inflammatory mechanism of human IL-27 has been defined in skin resident cells. As previously described, IL-18 is thought to play a pathogenic role in many inflammatory skin diseases [453]. IL-18 may well be important in the maintenance of inflammation due to its ability to induce TNFα and initiate IFNγ release from Th1 lymphocytes and ILC1. In addition, its ability to induce MMPs may be important in chronic inflammation. As well as its involvement in chronic inflammation IL-18 has also been described to play a role in allergic contact dermatitis [454], which is a common, difficult to treat skin manifestation of delayed type hypersensitivity.

At present, in depth knowledge about the expression levels of IL-18 and its natural antagonist IL-18BP is lacking in chronic inflammatory skin diseases. It is has been identified that the amount of free IL-18 and the amount bound to IL-18BP needs to fully understood in relation to skin disease [455]. For other diseases, levels of IL-18 and IL-18BP have been determined and up-regulation of both has been shown in chronic liver disease. However, in advanced cirrhotic liver disease Ludwiczek *et al.*, suggests that IL-18BP is not sufficiently up-regulated to control IL-18 mediated immune responses [256]. Heart failure patients have also been described to have increased IL-18 but decreased IL-18BP, again suggestive of an uncontrolled inflammation due to imbalance of pro- and anti-inflammatory mediators [456]. In contrast, a recent study has shown that IL-18 and IL-18BP are both highly up-regulated in the blood of patients with atherosclerosis [449]. However, some imbalance may be present as blocking of IL-18 has been shown to reduce the size and inflammatory state of the atherosclerotic plaque [457]. IL-18 has also been implicated in adult-onset Still's disease and the analysis of free IL-18 has been used as an important factor in the assessment of disease remission [458].

IL-18BP is clearly a crucial mediator that is important for controlling the highly inflammatory functions of IL-18 and possibly important in the resolution of inflammation. It is thought that there are basal levels of IL-18BP present in the circulation and also data in this chapter has highlighted the ability of keratinocytes to produce basal levels of IL-18BP. It is worth noting that data presented here suggest that dermal fibroblasts are significant producers of IL-18BP. These cells do no produce IL-18, which points to a possible role for dermal resident cells as 'controllers' of inflammation. Thus dermal fibroblasts which produced significantly higher levels of IL-18BP compared to the keratinocytes, seem to play an important role in the complex network of cell types and mediators that provide the fine tune control of inflammatory responses.

As described previously IL-27 is a pleiotropic cytokine that has been described to have both pro-inflammatory and anti-inflammatory roles. It has been shown to prime keratinocytes, macrophages and IDECs for IL-23, CXCL10 and TNFα production and has therefore been described to play a role in many inflammatory diseases [277, 278, 281, 432]. On the contrary other studies have suggested a role for IL-27 as an anti-inflammatory mediator in the later stages of infection. In one study IL-27 was shown to down-regulate cytokines involved in Th1, Th2 and Th17 subset development [459]. This study and others support the notion that IL-27 may have pro-inflammatory functions to produce a robust inflammatory response, following which at the later stages of infection IL-27 plays a more regulatory role. The complex mechanism by which IL-27 acts may be due to its downstream signalling, both STAT1 and STAT3 are known to be activated during IL-27 receptor activation. Of note, STAT3 does not play a role in the expression of IL-18BP in colon carcinoma cells [460]. The anti-inflammatory roles of IL-27 have predominantly been described in murine models however, in this chapter a novel anti-inflammatory mechanism of IL-27 has been described. This may be crucial in understanding the role of IL-27 in the pathogenesis of inflammatory diseases.

The anti-inflammatory roles of IL-27 have predominantly been described in murine models however, data presented in this chapter add a novel anti-inflammatory mechanism of IL-27 relevant for human tissues. Data presented indicate that the

proximal GAS site on the IL-18BP promoter is crucial for IL-18BP expression. However, a previous study has shown that in cardiac myocytes the β2-adrenergic receptor triggers a signalling cascade that leads to cAMP response element-binding (CREB) and CCAAT-enhancer-binding protein (c/EBP) β dependent IL-18BP release [461]. This was also shown in another study where c/EBP bound to the proximal GAS site on the IL-18BP promoter in HepG2 cells [462]. Taken together this suggests that there may be differential transcription factors involved in IL-18BP expression in different cell types, the studies shown here are in transfected cell lines and murine cell which may differ greatly to human primary cells. Therefore this is an important avenue for further work. However, due to the emerging interest in the IL-18/IL-18BP axis in cardiomyopathy it would be interesting to further elucidate a role for c/EBP in the induction of IL-18BP in human cardiac myocytes.

It would be of therapeutic interest to manipulate the IL-18/IL-18BP axis. There has been one Phase I clinical trial in patients with rheumatoid arthritis and plaque psoriasis where subcutaneous doses of IL-18BP were well tolerated and levels were maintained in the serum if injections were repeated every two days [258]. However, efficacy was not fully determined in that Phase I study and further work is required to establish whether treatment with IL-18BP would reduce chronic inflammation in different organs. It was however noted that IL-18BP at intermediate doses did in fact improve some clinical symptoms, interestingly at high doses this disease altering effect and reduced cell activation was not seen [258]. This is consistent with murine models of collagen-induced arthritis where IL-18BP at high doses increased disease severity [463]. It is a possibility that high levels IL-18BP may be bound by the IL-1 family member, IL-37 depriving IL-18BP of its antagonist properties [464]. In some immune responses it would not be beneficial to reduce IL-18. For example in viral infection where virus virulence is increased by the ability of the virus to increase either endogenous IL-18BP or homologues of this molecule [261]. In this situation the antiviral properties of IL-18 are crucial to reduce viral infection. However, in myocardial infarction and murine models of heart failure IL-18BP seems to play a protective role against tissue damage and may therefore in some situations be a viable therapeutic [465]. Due to the issues

demonstrated using recombinant IL-18BP as a therapeutic, up-regulation of endogenous IL-18BP may present a promising alternative option as a therapeutic.

Interestingly, data in this chapter has shown that the inflammatory mediator CXCL10 that is also induced following IL-27 stimulation and consequent STAT1 activation can be inhibited in skin resident cells with the simple addition of commonly used anti-inflammatory, hydrocortisone. However, this effect was not seen if given at the same time as IL-27, hydrocortisone only inhibited CXCL10 if given an hour before or after IL-27. Hydrocortisone and hydrocortisone acetate are often used topically in skin inflammation. These drugs exert their action by binding to the glucocorticoid receptor in the cytoplasm and this consequently leads to reduction in pro-inflammatory transcription factors. This has been shown to involve reduction in NF-κB pathway activation but it has been suggested that there may be other pathways targeted [466]. Interestingly, treatment of cells with hydrocortisone had no effect on levels of IL-18BP. This suggests that the downstream signalling of IL-27 may be more complex than currently reported and these cytokines may not only be STAT1 dependent, as previously discussed c/EBP may also play a role [462].

Taken together, the presented data suggest that IL-27 could be used as a therapeutic. However, having an hour between treatments is not a practical solution and more work is needed make use of the anti-inflammatory properties of IL-27. IL-27 could be a novel way to induce endogenous IL-18BP in the skin. This could be achieved by using a combination of IL-27 and a commonly used immune suppressive topically for the treatment of inflammatory skin diseases. However, IL-27 would need to be assessed more thoroughly for its ability to induce any other pro-inflammatory mediators. In conclusion, the results presented describe a novel anti-inflammatory mechanism for the pleiotropic cytokine, IL-27. IL-27 has the ability to preferentially up-regulate IL-18BP and may control IL-18 mediated inflammation. The data show that IL-18BP expression following treatment with IL-27 is dependent on STAT1 activation and indicates the importance of the proximal GAS site on the IL-18BP promoter. Following stimulation of skin resident cells with IL-27 IL-18BP seems to have a delayed production, peaking at around 48 hours but with a prolonged ability to release protein, up to 120 hours. This

has been proposed as accumulation of stabilised protein rather than stabilisation at the transcriptional level as we failed to see high mRNA stability. A previous study has suggested that IL-18BP accumulates in the supernatant of cultured cells [462]. Constant receptor activation was not shown to impact IL-18BP protein levels, again suggestive of an accumulation of protein that is stable for a prolonged period. However, washing the cells with PBS may not have sufficiently removed the IL-27, therefore more work is required to analyse activation of downstream transcription factors over a range of time periods. Data in this chapter also suggests that the pro-inflammatory cytokine CXCL10, which is also up-regulated to a lesser degree than IL-18BP by IL-27, could be fully inhibited by the use of well know immune suppressive drugs in combination with IL-27. However, further work is needed to fully elucidate the downstream signalling pathway following IL-27 receptor activation before this can be used in pilot studies as a therapeutic molecule.

Chapter 4 – Establishing the efficacy of the anti-IL-17A RNA aptamer, Apt21-2 in skin resident cells

4.1 Introduction

IL-17A is a pro-inflammatory cytokine that has been implicated in the pathogenesis of many inflammatory diseases. This cytokine is predominantly produced by Th17 lymphocytes and is a key mediator in chronic tissue inflammation [467]. However, Th17 cells do not exclusively produce IL-17 but also produce IL-21 and IL-22 [468], the latter of which has also been implicated in psoriasis [469]. IL-17A was initially described as a player in autoimmunity, being identified in human rheumatoid arthritis as an up-regulated mediator. More recently, the IL-17 family has been identified as having six members; IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. IL-17A and IL-17F have been most well described and are thought to be involved in IL-17 mediated disease [387]. Apt21-2 has been shown to be a specific neutraliser of IL-17A. Lack of crossreactivity is not surprising considering that IL-17F and IL-17A only share 55% homology [365].

Murine models and use of neutralising antibodies against IL-17 have provided useful insights into the pathogenic role of IL-17. This has led to the development of neutralising anti-IL-17 therapeutic antibodies for treatment of both multiple sclerosis and ankylosing spondylitis [470]. However, IL-17 has also been linked directly to disease in humans. Th17 cells were identified in the lesional skin of psoriasis which initially linked IL-17 to a possible role in disease pathogenesis [3]. IL-23, which has been shown to be crucial in Th17 development, is also up-regulated in psoriasis [471]. The effector cytokines released from Th17 cells such as IL-17A cause the pathogenic skin changes seen in psoriasis as well as increased inflammatory mediators that further enhance Th17 activity. This positive feedback loop maintains the psoriatic lesion and contributes to the chronicity of the disease. These observations along with extensive publications suggesting a pathogenic link between psoriasis and IL-17 has led to the development of novel biologics targeting this pathway [472]. Humanised antibodies targeting IL-17A (secukinumab and ixekizumab) and blocking its pro-inflammatory action by preventing binding to its receptor have been developed, as well as antibodies targeting the IL-17 receptor and preventing agonist binding (brodalumab) [424, 473]. At

present these biologics are showing promising results in clinical trials, affirming the concept that IL-17 is a critical cytokine in the pathogenesis of psoriasis [474].

These highly pro-inflammatory and pathogenic roles of IL-17A make this molecule an obvious target for therapeutic intervention. Th17 cells co-express IL-22 which is described to play a role in psoriatic skin symptoms but it has also been shown to be protective in colitis [469]. This suggests that the pathogenic role of Th17 cytokines may be relevant to the tissue context and surrounding cytokine milieu and this needs to be taken into account when designing therapeutics to target this pathway.

The importance of IL-17 in disease is evident. However, at present humanised monoclonal antibodies and limited small molecule inhibitors are the only therapies specifically targeting this pathway. Antibodies have disadvantages in that they can be immunogenic and anti-drug antibodies can be detected in a substantial number of patients [475]. This can lead to symptoms ranging from mild skin reactions to anaphylaxis and often significant reduction of the therapeutic efficacy [475]. Therapeutic antibodies are also costly to produce and they have systemic immunosuppressive properties. Systemic therapy is however not necessarily required in a skin limited disease. Treatment of systematic diseases, such as multiple sclerosis and severe psoriasis, may be suited to an antibody based systemic therapy but these biologics do not work in all patients. For this reason and the fact that these biologic therapies are often associated with side effects an effective, topical strategy is required for treatment of psoriasis and other chronic inflammatory skin diseases [476].

RNA aptamers are single stranded oligonucleotides segments that bind targets specifically and with great affinity [379] (See 1.7 for more detailed discussion). These molecules are non-immunogenic due to the lack of a 5’ triphosphate and limited regions of double stranded RNA which prevents activation of intracellular retinoic acid-inducible gene 1 (RIG-I) or TLR3 [477, 478]. RNA aptamers are easily produced *in vitro* keeping costs down. Multiple rounds of selection allows for highly specific binding. The small size and structural flexibility of these RNA molecules also allows for binding to epitopes on the target molecules that would not be detected by antibodies [380]. The first RNA aptamer was FDA approved in 2004 against VEGF for treatment

of age-related macular degeneration (Macugen®) [479], many more are currently in various stages of clinical trials for treatment ranging from cancer to inflammatory diseases [353]. An anti-human IL-17A RNA aptamer was produced in 2011 by Ishiguro *et al.* [365], who showed that this aptamer prevented IL-17A binding to its receptor consequently preventing downstream signalling. Murine models were used to assess efficacy of the aptamer and it was shown to inhibit IL-17 action in the Th17 mediated autoimmune models. This chemically produced RNA aptamer was named Apt21-2 and will be referred to as this from here on. A RNA aptamer has also been produced that specifically binds and antagonises the heterodimeric form of IL-17A/IL-17F, however this aptamer is still at its early stages and requires optimisation to increase its neutralisation capacity [480]. Of note, this aptamer is not being designed for therapeutic use but for a tool to overcome the hurdles of specifically identifying this heterodimeric form of IL-17.

This chapter will aim to assess the efficacy of Apt21-2 in human skin resident cells with the view to further elucidate the potential of this aptamer as a topical therapy for the treatment of Th17 mediated skin diseases such as psoriasis. This will focus on both primary human keratinocytes and dermal fibroblasts and also whether Apt21-2 can neutralise the effects of endogenous human IL-17A.

4.2 Structure of Apt 21-2

Apt21-2 was produced synthetically in house at the University of Leeds according to the sequence of the previously published functional anti-IL-17A RNA aptamer [365]. Apt21-2 is 33 nucleotides long and includes pyrimidines modified with the addition of 2’ fluorine to increase stability of the RNA molecule. Apt21-2 has very limited sections of double-stranded RNA which is advantageous for reducing activation of the intracellular TLR3 and RIG-I receptors (Figure 4-1). Apt21-2 was also produced with fluorophores added on either exclusively the 3’ end (Cy3) or both the 3’ (Cy3) and 5’ (Cy5) end (for sequences see 2.2.18). This was to allow monitoring of the aptamer using fluorescence microscopy and the double-labelled aptamer allowed for visualising whether the RNA molecule was still intact.

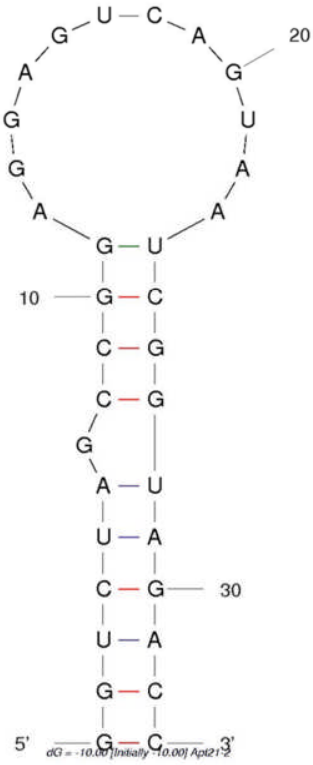


Figure 4 – 1. IL-17A aptamer - Apt21-2

An mfold (Michael Zuker & Nick Markham, the mfold web server) diagram of the potential structure Apt21-2 is depicted. It is worth noting that this is only a prediction and Apt21-2 may form a different structure and oscillate between many. This aptamer was chemically synthesised due to its known ability to antagonise IL-17A activity. It is 33 nucleotides long and has a small portion of double stranded RNA, which is advantageous in reducing TLR and RIG-I responses The pyrimidines have been modified by the addition of fluorine to increase stability of the RNA.

4.3 Efficacy of Apt21-2 in human primary fibroblasts

The previously published study describes the efficacy of Apt21-2 in murine models and human cells. However in order for this to be a potential therapeutic to treat skin-related disease the ability of Apt21-2 to neutralise the effects of physiologic IL-17A on skin resident cells need to be assessed. The focus of this chapter is on skin resident cells due to the known relevance of IL-17A in the pathogenesis of psoriasis. In order for Apt21-2 to be developed as a topical therapy it needs to be confirmed that it has human IL-17A neutralising capacity with regard to both human primary keratinocytes (results depicted

in 4.4) and fibroblasts activation. In order to address these questions human primary fibroblasts were treated with 10 ng/ml of recombinant IL-17A and then increasing concentrations of Apt21-2 (5 nM-100 nM). After 24 hours cell-free supernatant was removed and IL-6 protein concentrations analysed using ELISA. In the previously published study [365] IL-6 was used as the pro-inflammatory readout and levels were reduced in the presence of Apt21-2, hence why it has been used in this study. A size-matched control aptamer was also used in these experiments. This aptamer (47tr) was synthesised against polymerase from foot-and-mouth disease virus [481].

Human primary fibroblasts treated with increasing doses of Apt21-2 in the presence of 10ng/ml of recombinant IL-17A showed significant reduction of IL-6 release, the significant reduction in IL-6 correlated with increasing aptamer concentrations (Figure 4-2 a). This data set was normalised to the IL-17A treated cells without Apt21-2 and compared to this control in the presence of 80 nM of Apt21-2 more than a 50% reduction in IL-6 was seen. No significant reduction of IL-6 production was seen when cells were treated with increasing concentrations of 47tr in the presence of IL-17A, suggestive of the fact that IL-17A cannot be neutralised by a size matched control RNA aptamer. Recombinant TNFα was also used in combination with increasing concentrations of Apt21-2 to assess whether this aptamer was specific to IL-17A (Figure 4-2 b). Human primary fibroblasts were treated with 10ng/ml of IL-17A and TNFα in the presence and absence of increasing concentrations of Apt21-2 (5 nM – 100 nM). This data set was also normalised to IL-17A or TNFα alone and a reduction in IL-6 release was evident in the presence of Apt21-2 but not in the presence of the control aptamer, 47tr. This indicates that Apt21-2 is a specific neutralising RNA aptamer against IL-17A and does not have an effect on other pro-inflammatory cytokines. These experiments were carried out in healthy human primary fibroblasts as well as fibroblasts isolated from psoriatic patients (Figure 4-2 c). Both healthy and psoriatic fibroblasts in the presence of 10ng/ml of IL-17A and increasing concentrations of Apt21-2 (5 nM – 100 nM) showed reductions in IL-6 levels. This indicated that Apt21-2 also had an effective neutralising capacity in fibroblasts derived from psoriatic patients.

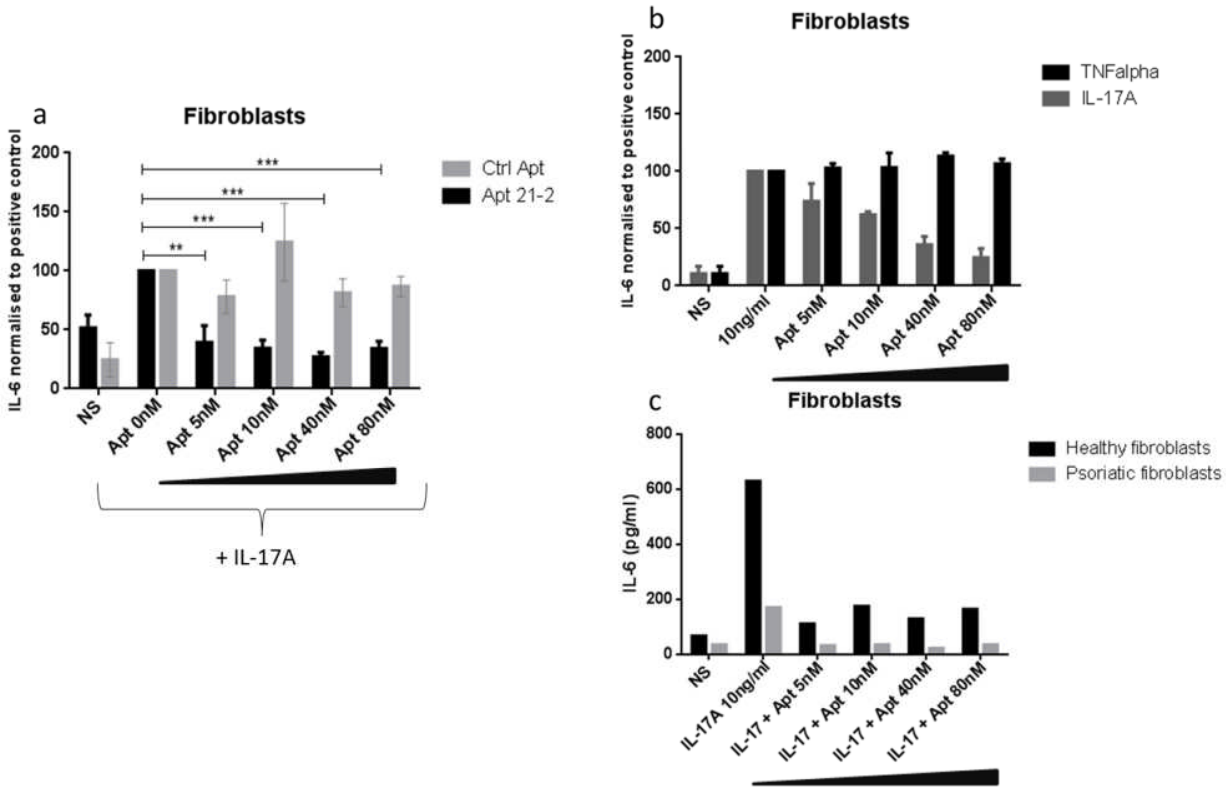


Figure 4 – 2. Apt21-2 effectively neutralises recombinant IL-17A in healthy and psoriatic human primary fibroblast culture.

Human primary fibroblasts were stimulated with 10 ng/ml of IL-17A in the presence or absence of increasing concentrations of aptamer. After 24 hours IL-6 levels were analysed by ELISA. **a** – Human primary fibroblasts were stimulated with 10 ng/ml of IL-17A in the presence or absence of chemically synthesised control aptamer (47tr) (n = 3) and Apt21-2 (n = 4). IL-6 levels shown represent normalised values to the control stimulated with IL-17A only. **b** – Human primary fibroblasts were stimulated with 10 ng/ml of IL-17A or 10ng/ml of TNFα in the presence and absence of Apt21-2. IL-6 levels shown represent normalised values to the IL-17A only control. n = 2. **c** – Human primary fibroblasts isolated from both healthy control and psoriatic patients were stimulated with 10ng/ml of IL-17A in the presence and absence of Apt21-2. n = 1. Mean +/- Standard error of the mean (SEM) is depicted on all graphs. NS = Non-stimulated. Raw data was analysed using a one way ANOVA with Bonferroni post-test (GraphPad Prism 5.03, GraphPad Software, San Diego, CA). ** = $p \leq 0.01$ *** = $p \leq 0.001$. [476]

Human primary fibroblasts were also co-cultured with human CD4+ CCR6+ IL-17 producing T cells to assess the functionality of Apt21-2 on endogenous rather than recombinant IL-17A (Figure 4-3). In order to do this CD4+ CCR6+ T cells were isolated

from PBMCs using MACS® separation from both healthy and psoriatic donors. Following isolation CD4+ CCR6+ T cells were cultured with healthy human primary fibroblasts in the presence of antiCD3/antiCD28 to activate the T cells and increasing concentrations of Apt21-2 (5 nM – 100 nM). As shown with the recombinant IL-17A there was a substantial decrease in IL-6 release following treatment with the aptamer. Interestingly the CD4+ CCR6+ T cells derived from psoriatic patients required a higher concentration of Apt21-2 in order to detect neutralisation of IL-6, suggestive of the fact that more IL-17A was produced by these cells.

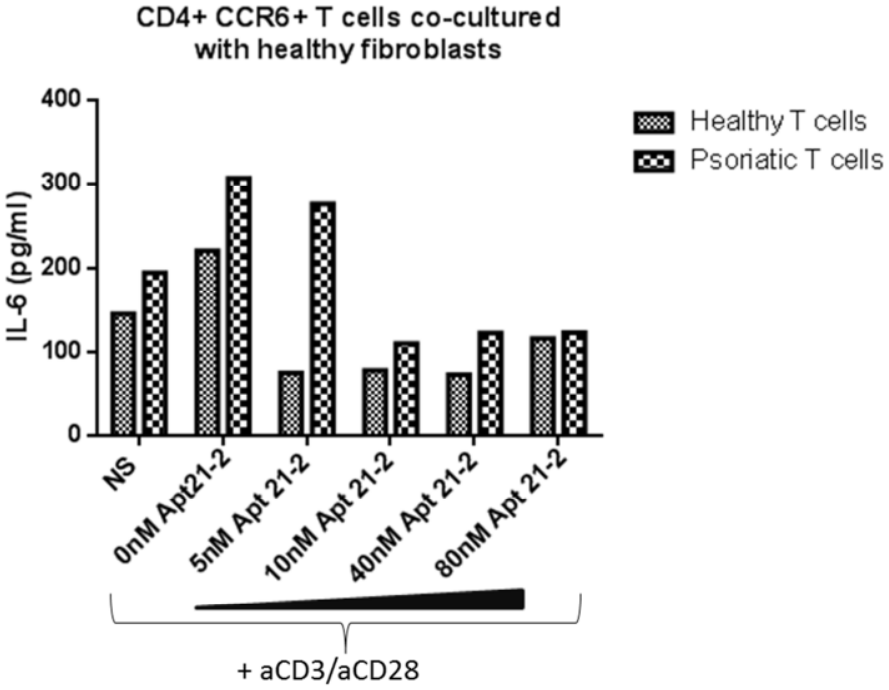


Figure 4 – 3. Apt 21-2 effectively neutralises physiologic IL-17A in a T cell – fibroblast co-culture.

Human primary fibroblasts were cultured in the presence or absence of CD4+ CCR6+ (Th17 cells) isolated from either healthy donors or psoriatic donors. Th17 cells were stimulated with 450 ng/ml and 200 ng/ml of aCD3/aCD28 antibodies respectively. Increasing concentrations (5 nM – 80 nM) of Apt 21-2 was added to the co-culture. After 24 hours IL-6 levels were analysed by ELISA. n = 1. NS = Non-stimulated. [476]

4.4 Efficacy of Apt21-2 in human primary keratinocytes

Apt21-2 was confirmed to have neutralising effects in human primary fibroblasts therefore experiments were also carried out in human primary keratinocytes. Keratinocytes were treated with 10 ng/ml of recombinant IL-17A in the presence and absence of increasing concentrations of Apt 21-2 and control aptamer, 47tr (5 nM – 100 nM). After 24 hours of stimulation cell free supernatants were removed and both IL-6 (Figure 4-4 a) and IL-8 protein (Figure 4-4 b) concentrations were analysed by ELISA. IL-8 was used as an additional pro-inflammatory mediator that is produced by keratinocytes in response to various inflammatory mediators [482] and in these experiments the human primary keratinocytes were producing low levels of IL-6. Unexpectedly no significant neutralisation of IL-8 or IL-6 release was detected in the human primary keratinocytes, in contrast to the human primary fibroblasts where neutralisation could be seen with the addition of only 5 nM of Apt21-2. High concentrations of Apt21-2 (80 nM) were also added to the cells alone to assess for possible activation of the keratinocytes. However levels were not significantly different to the non-stimulated control. No difference in the effects of Apt21-2 and the size matched control, 47tr could be detected.

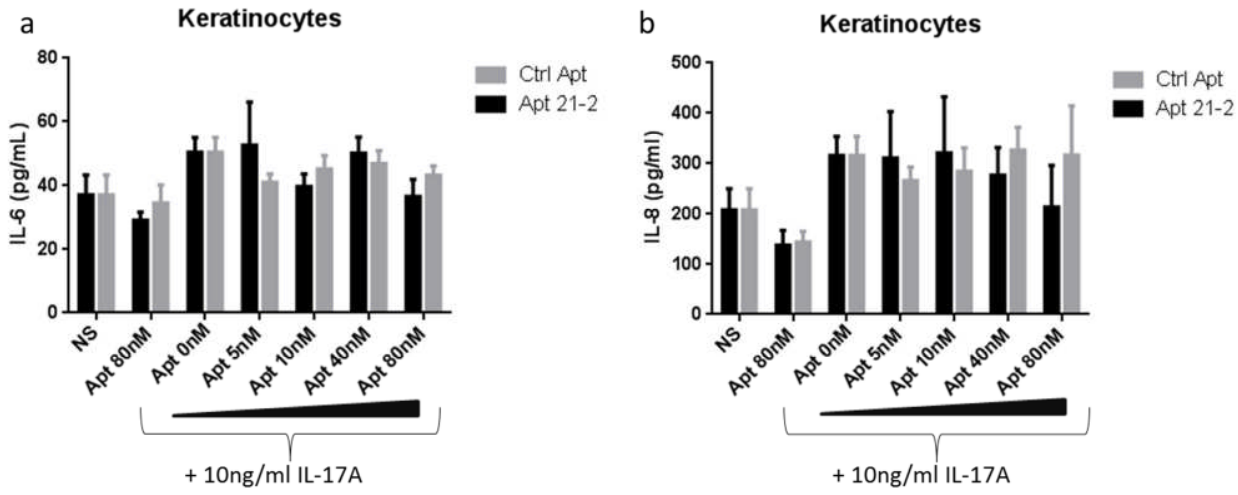


Figure 4 – 4. Apt 21-2 does not neutralise IL-17A in human primary keratinocyte culture.

Human primary keratinocytes were stimulated with 10ng/ml of IL-17A in the presence or absence of increasing concentrations of aptamer (5 nM-80 nM) and 80 nM of Apt21-2 alone. Cell supernatants were then removed after 24 hours and protein levels analysed by ELISA. **a** – Human primary keratinocytes were stimulated with 10 ng/ml of IL-17A in the presence or absence of Apt 21-2. Cell supernatants were collected after 24 hours and IL-6 levels analysed using ELISA. n = 3. **b** - Human primary keratinocytes were stimulated with 10 ng/ml of IL-17A in the presence or absence of Apt 21-2. Cell supernatants were collected after 24 hours and IL-8 levels analysed using ELISA. Mean +/- SEM is depicted on all graphs. NS = Non-stimulated. [476]

4.5 Unexpected uptake of Apt21-2

Results depicted in 4.3 and 4.4 indicate a huge contrast in efficacy of Apt21-2 in the two different skin resident cells. Therefore, this led to some exploratory experiments and due to the availability of fluorescently labelled aptamers this allowed for tracking of the aptamer following its addition to cells. It had previously been suggested that relatively small DNA molecules were internalised by cells [373], therefore this led to investigate the possibility of RNA molecules being taken up in skin resident cells. Human primary keratinocytes were treated with 3' Cy3 labelled Apt21-2 in serum containing conditions and imaged using live cell imaging at 3 hours and 20 hours (Figure 4-5 a + b). Interestingly, even after the relatively short time period of 3 hours the images suggested that the aptamer was being internalised by the human primary keratinocytes and this uptake was further clarified at 20 hours. This was also confirmed in fixed cells using

fluorescent microscopy. Primary human keratinocytes were treated with 3' and 5' (Cy3 and Cy5 respectively) labelled Apt21-2, as well as a 3' Cy3 labelled 47tr size matched control aptamer. After 4 hours of treatment with the aptamer cells were fixed with 4% paraformaldehyde and counterstained with DAPI to distinguish the nuclei, following which cells were mounted and imaged. After 4 hours both control aptamer (Figure 4-5 c) and Apt21-2 (Figure 4-5 d-f) were convincingly internalised by the human primary keratinocytes. It appears that the keratinocytes have the ability to internalise any small RNA molecules due to the control aptamer being internalised to the same degree. Cy3 and Cy5 were co-localised in images where cells had been treated with the double-labelled aptamer (Figure 4-5 d-f), suggestive of the fact that the RNA aptamer had been internalised and remained intact within the cells.

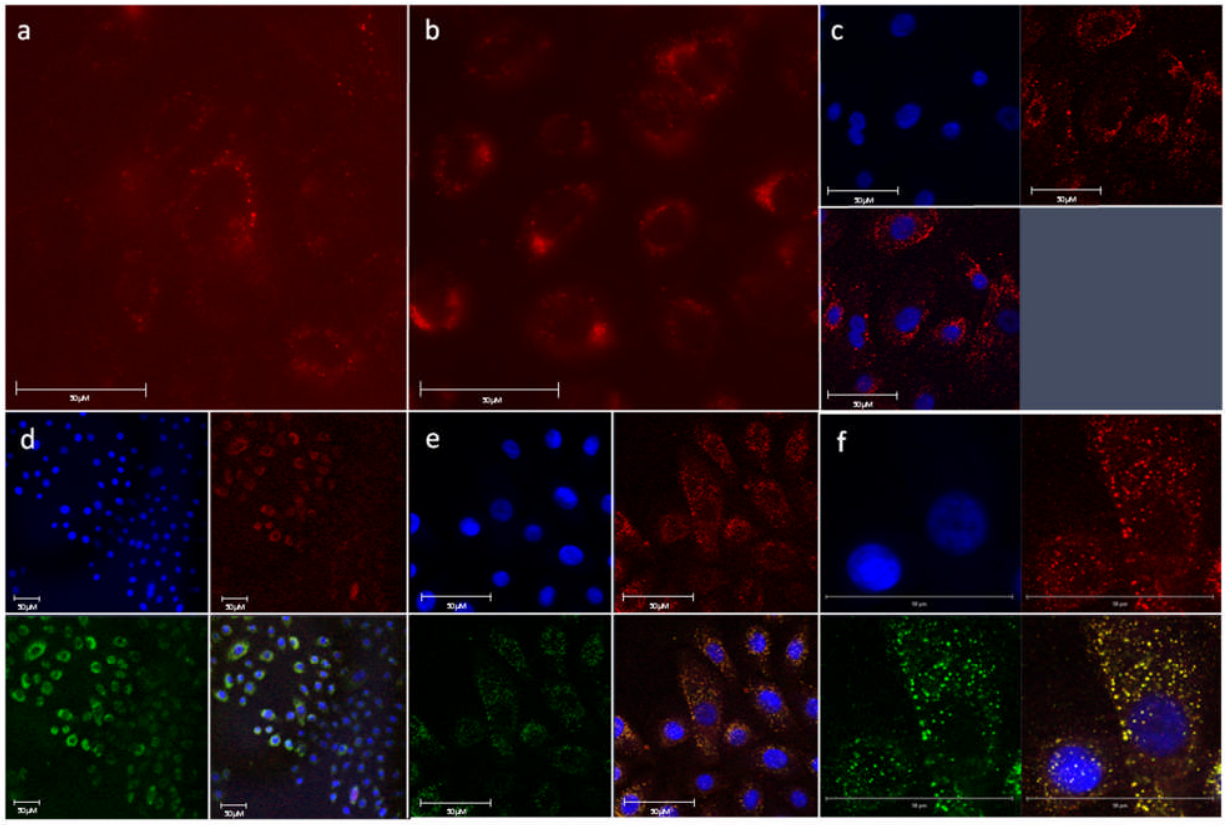


Figure 4 – 5. Small RNA molecules are rapidly internalised by human primary keratinocytes.

Human primary keratinocytes were treated with 80 nM of labelled Apt21-2 at various time points in order to track uptake of the RNA. **a, b** - Primary human keratinocytes treated with Cy3-labelled Apt21-2 at 3 hours (**a**) and 20 hours (**b**), using live-cell imaging. **c** - Human primary keratinocytes were also treated with a Cy3-labelled control aptamer (47tr) for 4 hours. DAPI (diamidino-2-phenylindole, top left), Cy3 (top right hand), and merged (lower left). **d–f** Human primary keratinocytes were also treated with a 3'Cy3 and 5'Cy5 double-labelled aptamer for 4 hours. Images were taken at increasing magnification from **d** through to **f**. DAPI (top left), Cy3 (top right hand), Cy5 (lower left), and merged (lower right). Following incubation cells were fixed with 4% paraformaldehyde and stained for DAPI. Slides were imaged using an inverted LSM510 confocal microscope coupled to a LSM Image Browser. Bars = 50 µM. [476]

Previous publications have suggested that aptamer uptake may be receptor mediated [375], therefore uptake may only occur in the presence of the target. In order to assess whether this was true for Apt21-2 the human primary keratinocytes were treated with 80

nM 3’ Cy3 labelled Apt21-2 alone (Figure 4-6 a-c) or with 10 ng/ml of recombinant IL-17A in the presence of 3’ Cy3 labelled Apt21-2 (Figure 4-6 d-f). No difference could be seen in the uptake ability whether IL-17A was present or not which is not surprising because Apt21-2 should prevent IL-17A binding to its receptor. However, it does indicate that Apt21-2 bound to IL-17A does not prevent internalisation.

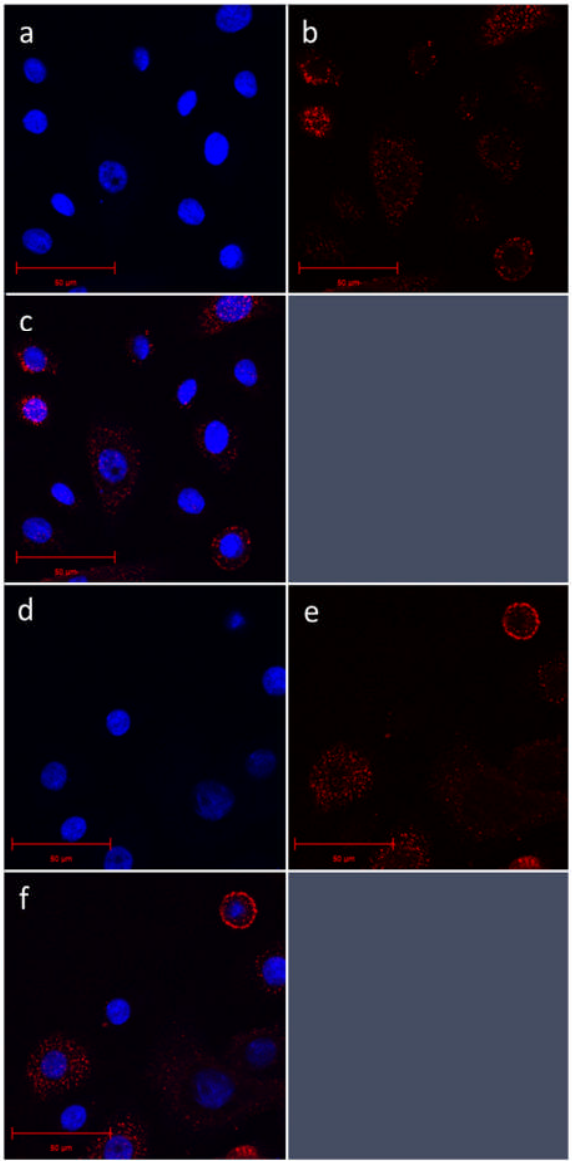


Figure 4 – 6. Apt21-2 is not dependent on IL-17A in order for internalisation to occur.

Human primary keratinocytes were treated with 80 nM of 3' cy3 labelled Apt21-2 for 4 hours in the presence and absence of 10 ng/ml of recombinant IL-17A in order to track uptake of the RNA. **a – c** Human primary keratinocytes were incubated for 4 hours with 80 nM of 3'Cy3 labelled aptamer. **a** – Nuclei stained with DAPI. **b** – Cy3 fluorescence. **c** – Merge of Cy3 fluorescence and DAPI. **d - f** Human primary keratinocytes were incubated for 4 hours with 80nM of 3'Cy3 labelled aptamer in the presence of 10 ng/ml of recombinant IL-17A. **d** – Nuclei stained with DAPI. **e** – Cy3 fluorescence. **f** – Merge of Cy3 fluorescence and DAPI. Following incubation cells were fixed with 4% paraformaldehyde and stained for DAPI. Slides were imaged using an inverted LSM510 confocal microscope coupled to a LSM Image Browser. Images representative of two experiments. Bars = 50 µM.

Due to the unexpected finding in human primary keratinocytes human primary fibroblasts were also analysed for double-labelled 3’ and 5’ (Cy3 and Cy5 respectively) labelled Apt21-2 uptake. Dermal fibroblasts were treated with Apt21-2 and then incubated for 6 hours before fixation with 4% paraformaldehyde and then counterstained with DAPI to distinguish the nuclei (Figure 4-7). Slides were then mounted and imaged using fluorescent microscopy. Dermal fibroblasts were treated for 6 hours due to the fact that human primary fibroblasts have not been described to have the same uptake ability as keratinocytes. After the 6 hour incubation there is some uptake of Apt21-2 in to the cells and co-localisation is present, suggestive of an intact RNA molecule. However, due to the higher level of background seen in the fibroblasts compared to the keratinocytes it is suggestive that not as much Apt21-2 is taken up or a longer time period is required for uptake.

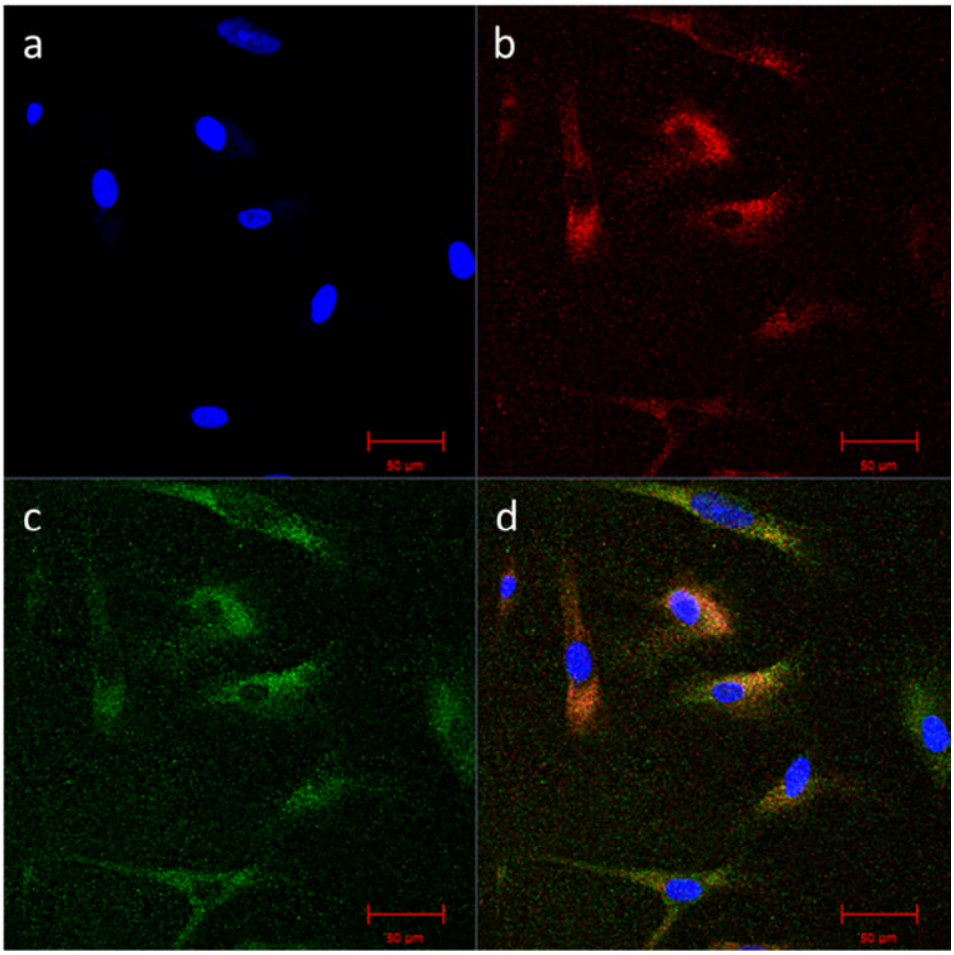


Figure 4 – 7. Small RNA molecules are also taken up by human primary fibroblasts.

Human primary fibroblasts were treated with 80 nM of 3'Cy3 and 5'Cy5 labelled Apt21-2 for 6 hours. **a** – Nuclei stained with DAPI. **b** – 3'Cy3 fluorescence. **c** – 5'Cy5 fluorescence. **d** – Merge of Cy3 and Cy5. Following incubation cells were fixed with 4% paraformaldehyde and stained for DAPI. Slides were imaged using an inverted LSM510 confocal microscope coupled to a LSM Image Browser. Bars = 50 µM.

4.6 Immune activation by Apt21-2

Results depicted in 4.4 and 4.5 indicate that human primary keratinocytes and fibroblasts effectively internalise RNA molecules however this may cause a RIG-I response by the cell, which is a cytosolic sensor of intracellular PAMPs, particularly RNA [483]. Endosomal TLRs such as TLR3, TLR7, TLR8 and TLR9 are also important

in sensing intracellular pathogen derived nucleic acids [484]. Activation of these intracellular receptors leads to release of type-I IFNs and pro-inflammatory cytokines. However, these receptors importantly sense double stranded RNA and a 5' triphosphate therefore Apt21-2 should not be detectable by these intracellular receptors. In order to investigate whether uptake of Apt21-2 was in fact eliciting response from these receptors, primary human keratinocytes and fibroblasts were treated with either 80nM Apt21-2, 100ng/ml poly (deoxyadenylic-deoxythymidylic) acid (Poly dA:dT) or 1µg/ml Poly I:C. Poly dA:dT is a synthetic double stranded DNA and Poly I:C is a synthetic double stranded RNA and both were used as positive controls that would activate the intracellular pathogen sensors. Cells were treated for 24 hours before RNA was isolated and qRT-PCR was performed to analyse known downstream genes of the intracellular TLRs and RIG-I-like receptors as well as IFN induced genes such as IFNλ (Figure 4-8). MX1 and Gbp-1 were analysed and these are normally up-regulated following activation of the cell by IFNs. IFNβ was analysed due to the fact that it is a known downstream mediator of TLR3. Both the human primary keratinocytes and human primary fibroblasts did not show any increased expression of the panel of IFN and intracellular receptor response genes, in comparison to the positive controls which did induce these IFN response genes in different patterns depending on cell type. These results are suggestive of the fact that internalisation of Apt21-2 is not eliciting an immune response by either human primary keratinocytes or fibroblasts.

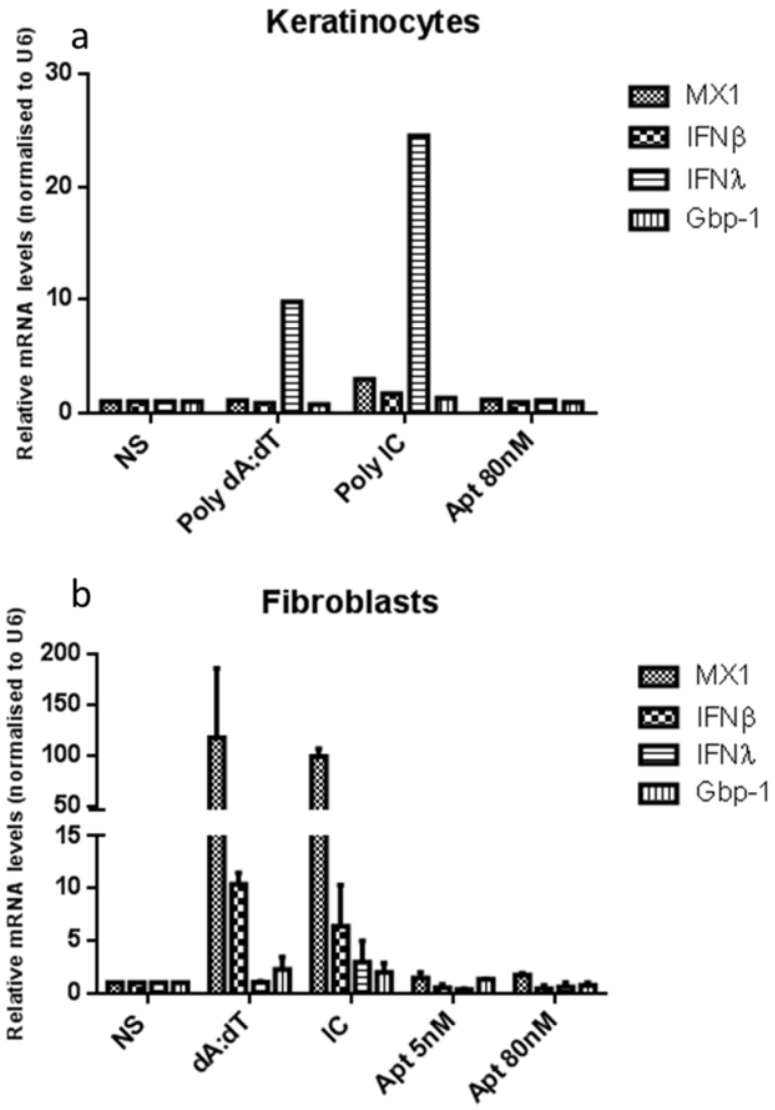


Figure 4 – 8. Human primary cells were treated with small RNA molecules and no up-regulation of interferon response genes could be detected.

Human primary cells were stimulated with either 1µg/ml Poly I:C, 100ng/ml Poly dA:dT or 80nM of Apt21-2 for 24 hours before mRNA was isolated and qRT-PCR performed. **a** – Human primary keratinocytes were stimulated with Poly I:C, Poly dA:dT and 80nM of Apt21-2 for 24 hours. qRT-PCR was performed to assess levels of MX1, IFNβ, IL-29 and Gbp-1. **b** - Human primary fibroblasts were stimulated with Poly I:C, Poly dA:dT and 80nM of Apt21-2 for 24 hours. qRT-PCR was performed to assess levels of MX1, IFNβ, IL-29 and Gbp-1. All qRT-PCR data was normalised to the housekeeping gene U6. Mean +/- SEM is depicted on all graphs. NS = Non-stimulated.

4.7 Discussion and future work

The role of IL-17 in chronic inflammatory skin diseases such as psoriasis is well described. IL-17 has been identified as a key mediator involved in driving of the inflammatory cascade seen in psoriatic lesions [424]. For this reason it has been highlighted as a possible therapeutic target in the treatment of psoriasis. Humanised monoclonal antibodies are currently in the latter stages of clinical trials or already licensed for treatment and efficacy is promising in psoriasis showing potent and rapid reduction of disease symptoms [474]. However this is a systemic therapy which if effective in blocking the pathogenic IL-17 will also lead to side effects such as fungal infections, due to the important role of IL-17 in anti-fungal immune responses [414]. There are also questions over the long term safety of these drugs and the extremely high costs mean that alternatives are also necessary [485]. Therefore, it is crucial that for chronic inflammatory skin diseases limited to the skin a topical therapy is developed to avoid systemic side effects.

Developments have been made in moving the treatment of psoriasis to a more topical rather than systemic approach. A recent study in mice has shown that the newly developed skin penetrating methotrexate ameliorated imiquimod induced psoriasiform inflammation using clinical criteria [486]. Methotrexate is used extensively in dermatology as an inexpensive effective systemic therapy, however it is toxic to various cell types including liver and bone marrow. These serious side effects limit the use of this drug, however this recent study has established an effective novel way to use this drug topically [486]. These up and coming topical therapies in psoriasis will be crucial to increase effective treatments for patients where conventional therapy has failed. RNA aptamers present as an ideal molecule to use for topical therapy. They are cost effective due to the *in vitro* production and non-immunogenic. Due to the unique structure of these molecules they can also bind epitopes that antibodies cannot so therefore maybe an invaluable therapeutic tool when biologic therapy has failed. RNA aptamers are currently being produced against a huge range of targets ranging from anti-cancer therapies to anti-viral therapies [487]. One RNA aptamer is at present FDA approved

and is used topically to treat age-related macular degeneration [379], therefore a topical therapy for skin using this technology is within reach.

The experiments in this chapter aimed to establish whether Apt21-2 could be a potential therapeutic for the topical treatment of IL-17 mediated skin diseases. Apt21-2 was successful at neutralising the effects of recombinant IL-17A and endogenous IL-17 in primary human fibroblasts however this same positive effect was not seen in the human primary keratinocyte culture. This was likely due to the rapid uptake of Apt21-2 in to the cells. The specific site by which Apt21-2 binds IL-17A was not established in this study, however Ishiguro *et al.*, [365] who designed the aptamer did establish binding affinity (K_d - 48.5 pM) and specificity using surface plasmon resonance. This in conjunction with our data showing reduction in IL-6 levels following addition of IL-17A in combination with Apt21-2 indicates that Apt21-2 is preventing binding of IL-17A to its receptor. Ishiguro *et al.*, [365] identified that Apt21-2 showed neutralising effects for both murine and human IL-17A, for which there is 63% homology suggesting binding is occurring in this conserved region. However, in order to confirm the region of Apt21-2 binding that prevents IL-17A binding to its receptor co-crystallisation of IL-17A in combination with the aptamer would be required. Alternatively, mutational analysis of the aptamer or recombinant IL-17A could be performed to assess which regions are required for binding.

Our previous work has shown that dermal fibroblasts are undoubtedly a significant source of pro-inflammatory cytokines, so therefore reducing IL-17 mediated effects may be beneficial for modulating disease. However, for a topical therapy to work keratinocytes need to be taken into account because they form the barrier of the skin and therefore are the first cells to come into contact with anything placed on the skin. If Apt21-2 was to be used as a topical therapeutic in treating chronic inflammatory skin diseases then keratinocytes would need to be bypassed. At present there are various methods by which drugs can be administered directly into the dermis [488]. Results depicted in this chapter indicate that despite internalisation of Apt21-2 in keratinocytes and to a lesser degree fibroblasts there is not a type-1 IFN response. This is crucial when designing therapeutics and despite the non-immunogenic features of small RNA

aptamers, siRNA has been shown to activate a RIG-I response [489]. Therefore the lack of a type-1 IFN response in human primary fibroblasts is an encouraging result for developing Apt21-2 as a therapeutic.

There is an increasing demand for delivery of drugs or vaccines directly into the dermis bypassing the difficult to penetrate stratum corneum, therefore technologies are being developed. These include jet injectors, which involve a high powered stream of liquid that pierces the skin and delivers the drug into the dermis [488]. Hollow microneedles containing aqueous drugs [490] and biodegradable drug incorporated needles [491] have both been developed as dermal delivery systems. A patch system has also been developed where drug coated microneedles are attached to an adhesive patch and can be placed on the skin piercing the epidermis and reaching the dermis [492]. These newly developed technologies allow for therapeutic potential of Apt21-2 to prevent IL-17 mediated inflammation in the dermis. However, further studies are needed to establish the penetrative capabilities of RNA aptamers once in the skin because at present this has not been investigated. This needs to be established due to the abundant number of RNases that are present in the skin. Ribonuclease (RNase) 7 and 5 are particularly well described within the skin compartment due to their role in cutaneous defence [493]. The effects of these RNAses on Apt21-2 integrity need to be established before the concept of using RNA aptamers as a topical skin therapy can be taken further.

Data presented in this chapter indicates that Apt21-2 effectively neutralises both recombinant and endogenous IL-17A in a human primary fibroblast culture. However, the ineffective neutralising capacity in the human primary keratinocytes and the finding that this small RNA aptamer was passively taken up into the cell was not an expected finding. This may however explain the lack of neutralising ability because Apt21-2 was not available in the extracellular space. Of note, the keratinocytes were inefficiently stimulated by IL-17A and this may also account for lack of neutralisation, the low levels of activation that were seen were however not altered. Data presented in this chapter indicate that Apt21-2 neutralises endogenous IL-17 however it is worth noting that this experiment was only performed once and the experimental setup has limitations. CD4+ CCR6+ enrichment of PBMCs can yield small numbers of cells which is likely to be the

reason for the low levels of IL-6 seen following stimulation. This experiment need repeating to confirm that this effect with the inclusion of an IL-17 neutralising antibody control as well as FACS staining in conjunction to indicate efficient enrichment and presence of IL-17, with these controls the neutralising effect of Apt21-2 could be confirmed as IL-17 mediated.

The uptake of RNA molecules by keratinocytes is a novel finding as uptake of RNA aptamers in to primary skin resident cells has not previously been described. However, human primary keratinocytes do have tendencies to take up substances from the environment and uptake of melanosomes is crucial for keratinocyte survival and protection from UV. The mechanisms by which melanosome transfer into keratinocytes occurs are not all together clear [494]. The rapid uptake of Apt21-2 may well explain the lack of IL-17A neutralising capacity in human primary keratinocytes, however the human primary fibroblasts also appeared to internalise the aptamer but still indicated significant IL-17A neutralisation. The amount of background that is evident despite the longer incubation compared to the keratinocytes is suggestive that the fibroblasts do not internalise the RNA molecules as effectively. In order to establish this conclusively a time resolve would be required comparing human primary keratinocytes and fibroblasts which could be carried out using an *in vitro* assay on an IncuCyte™. Quantification of uptake could also be analysed using FACS and again human primary keratinocytes and fibroblasts could be compared. Uptake of oligonucleotides by human primary fibroblasts has not been described in the literature, however uptake of the DNA aptamer AS1411 has been described in a human fibroblastic cell line, Hs27 [373]. This uptake was described to be by a mechanism which led to endosomal entrapment or lysosomal degradation in healthy cells [373]. Healthy human primary keratinocytes have been described to passively take up plasmid DNA [374]. Interestingly in comparison to what has been shown in Hs27 cells uptake of plasmid DNA led to a small amount of plasmid transcription suggestive of the fact that not all DNA was degraded in lysosomes or endosomes [374]. In fact in this study it was suggested that macropinocytosis was the mechanism by which uptake of plasmid DNA occurred due to inhibition of the uptake by phosphoinositide 3-kinase [495]. The DNA aptamer AS1411 was taken up by macropinocytosis only in the cancer cells tested, however this did effectively deliver the

aptamer to its intracellular target due to the leaky nature of macropinosomes. This is consistent with the uptake of DNA plasmid where low levels of plasmid transcription occurred. It is thought that unlike Gram-positive bacteria which express specific receptors for uptake of DNA, mammalian cells are more likely to take up DNA by utilising existing endocytic pathways [374]. It has been shown in muscle cells that plasmid DNA is taken up by potocytosis, which is receptor mediated endocytosis [496]. AS1411 uptake has previously been described as being nucleolin dependent, however it also appears to be taken up by alternative mechanisms which are not receptor dependent [373]. RNA aptamer uptake has been described in a receptor mediated manner in a haematopoietic cell line and in this study the RNA uptake was dependent on IL-6 receptor expression. Interestingly this RNA aptamer was used to carry cargo into the cell and by this receptor mediated endocytosis cargo could be delivered into the cell 10 times the size of the 19 nucleotide RNA aptamer [375].

Although in human primary keratinocytes Apt21-2 is not a viable option for neutralisation of IL-17A, it does present as a possible novel way to target intracellular mediators with the use of RNA aptamers without the need for chemical introduction. There is undoubtedly a need for cell penetrating molecules that can deliver therapeutics or act inside the cell [497]. The fact that Apt21-2 was effectively internalised in the presence of its target suggests that there could be scope for the use of Apt21-2 as an RNA aptamer to take cargo therapeutics into the cell. In order for new RNA aptamers to be developed for use inside the cell the exact mechanisms by which uptake occurs need to be established. It is likely due to published literature that this is an active mechanism, however this needs to be confirmed in the human primary cells by assessing uptake ability when the cells are at 4°C [374]. Lack of activity suggests an endocytic uptake whereas internalisation at 4°C suggests a passive mechanism.

Following an initial temperature dependency experiment, more detailed experiments would need to be performed in order to confirm what had been shown. As described in Reyes-Reyes *et al*., [373] inhibition of various uptake mechanisms can be utilised to establish the mechanism by which RNA aptamers are being taken up by the primary skin resident cells. Macropinocytosis could be a mechanism by which uptake occurs as

well as endocytosis, which is generally regarded as the mechanism by which siRNA is internalised [376]. Endocytosis can be split into clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin and caveolae-independent endocytosis [498]. Specific inhibitors would need to be used to establish if endocytosis was responsible for uptake. Treatment of cells with cytocholasin D has been shown to inhibit endocytosis therefore this could be used to assess this as an internalisation mechanism [373]. Dynasore could be used to inhibit both clathrin and caveolae dependent endocytosis due to the requirement for GTPase dynamin in these forms of endocytosis [499]. Fluorescent staining of markers for endosomes such as EEA1, Rab5, and Rab11 would also be necessary to establish where the intact RNA aptamer was within the cell [500]. If Apt21-2 was shown to be internalised by endocytosis, the development of the formulation by which delivery was performed would have to carefully designed in order to escape the endocytotic pathway by disrupting the endosomal membrane to deliver the RNA aptamer into the cytosol [377]. However, this is a well-regarded essential mechanism for siRNA delivery therefore many technologies are available to escape endosomal degradation [377].

Macropinocytes is another possibility that may be responsible for internalisation of Apt21-2 if endocytosis was ruled out. However it is worth noting that previous studies have identified different internalisation pathways in different cell types, therefore a number of relevant cell types would need to be tested. Following uptake of the aptamer in to the cells co-staining with dextran would indicate whether macropinocytosis was responsible for uptake, as well as chemical inhibition of this process using amiloride [373]. Of note, hyperstimulation of macropinocytosis has been indicated to cause a novel mechanism of cell death which was not seen in this study [501, 502].

Before Apt21-2 could be taken forward as a therapeutic more in depth studies would need to be carried out regarding activation of the cells. In this chapter it was shown that a RIG-I or TLR response was not evident in the panel of mediators analysed following uptake of Apt21-2, which is to be expected due to the 5’ monophosphate group on RNA aptamers. The intracellular pathogen sensors predominantly detect 5’ triphosphate groups and double stranded RNA [477, 478] which are absent or limited in the RNA

molecules used. Cell viability and cell death would also need to be assessed following uptake of RNA aptamers using longer time points compared to what has been currently carried out. Cell death is commonly regarded as a positive outcome in aptamer development targeting cancer cells using specific cancer cell markers, therefore RNA aptamers for intracellular targets would need to be carefully designed to avoid this.

In conclusion, this chapter has confirmed the neutralising efficacy of Apt21-2 on both recombinant and endogenous IL-17 in human primary fibroblast culture. Unexpectedly, it has also described a novel feature of human primary keratinocytes in their ability to take up small RNA molecules. More work is required to fully elucidate the pathways involved in this uptake. RNA aptamers have been shown to modulate protein-protein interactions therefore aptamers could be designed to utilise this function inside the cell both in an experimental and therapeutic setting [503]. This unique function of keratinocytes could present with an invaluable tool to target pro-inflammatory signalling pathways without the need for chemical introduction in to the cell. This could be utilised in the topical treatment of chronic inflammatory skin diseases predominantly affecting the epidermis which would not lead to the widespread side effects seen in systemic therapies.

Chapter 5 – Elucidating the role of IL-36 cleavage

5.1 Introduction

IL-36 is a relatively newly described subfamily in the IL-1 family of cytokines. The family includes four members; IL-36α, IL-36β, IL-36γ and IL-36 receptor antagonist (RA) that bind to the receptor IL-1Rrp2 (IL-36R). All apart from IL-36RA also bind to the common accessory protein to all IL-1 members, IL-1RAcP [203, 204]. The IL-36 members constitute an independent system from IL-1, however the IL-36R does also signal via NF-κB [185]. In 2011 it was identified that, similar to other IL-1 family members the IL-36 family members also require N-terminal cleavage to gain biological activity [128]. In line with other IL-1 family members the IL-36RA plays a crucial role in controlling the pro-inflammatory activity of IL-36α, IL-36β and IL-36γ. As for other IL-1 members the balance between the agonists and antagonists is important in maintaining control over inflammatory responses. It has also been proposed that another cytokine, IL-38 may also have antagonistic properties on IL-36 by binding in a similar manner as IL-36RA to the IL-36R [126]. However, there is at present limited literature on IL-38.

The predominant source of the IL-36 cytokines is keratinocytes. IL-36RA is constitutively expressed, whereas IL-36γ is effectively induced following treatment with phorbol 12-myristate 13-acetate (PMA) and pro-inflammatory cytokines [60, 211]. IL-36 expression has also been identified in other epithelial tissues such as the lung, however lack of available reagents has hampered the investigation at the protein level [207, 208, 210]. Most recently it has been shown that IL-36 stimulates maturation of DCs therefore driving T cell proliferation and actively playing a role in skin inflammation [504]. Due to IL-36 being mainly expressed by epithelial cells the majority of the research in this area is focused on the skin (and bronchial epithelial cells). Therefore, emerging evidence in both human cells and in murine models indicates that IL-36 may play a crucial role in skin inflammation.

IL-36 has particularly been implicated in the pathogenesis of psoriasis and different studies have identified IL-36 mRNA and protein in psoriatic plaques [505]. IL-36γ is consistently one of the most up-regulated genes in these arrays and our unpublished work suggests that it may be a biomarker to distinguish psoriasis from other inflammatory skin conditions [97, 221, 222]. The link to psoriasis pathogenesis has been further strengthened by the identification of a disease causing missense

mutation in the IL-36RA gene that leads to dysfunctional IL-36RA. This mutation has been identified in a subset of GPP patients in a number of populations [5, 111].

Biophysical analysis showing N-terminal cleavage results in >30000 fold increase in receptor affinity [128]. However, the bioactive, cleaved form of IL-36 has not yet been identified in physiological conditions therefore it is crucial to understand how and when these pro-inflammatory cytokines are being activated. In addition to this the protease responsible for IL-36 cleavage is still to be elucidated. As previously discussed some IL-1 family members such as IL-1β and IL-18 are cleaved in to their active form inside the cell by inflammasome dependent caspase-1 [129]. It is however unlikely that IL-36 is cleaved by caspase-1 due to its requirement for a specific consensus sequence [205]. Both IL-1β and IL-18 if released from the cell in their inactive full length forms can be cleaved by a number of different proteases [344] including the neutrophil serine protease, PR3 [342], however the activity of IL-18 following cleavage by PR3 is yet to be fully elucidated [236]. Given the lack of data on IL-36 bioactivity *in vivo*, a greater understanding of the biology of IL-36 and the protease/s responsible for cleavage and the mechanisms by which IL-36 is released is needed.

The aims of this work were to (1) elucidate the role of the predicted active forms of IL-36 *in vitro* using primary skin resident cells derived from both healthy and psoriatic donors, and (2) to elucidate which protease could be responsible for this cleavage. Due to paucity of knowledge about the release of these cytokines from the cell this will also be investigated using *in vitro* techniques. In order to establish potential functional roles of IL-36 on skin phenotype 3-D skin equivalents as well as single layer cultures will be treated with different forms of IL-36.

5.2 Response of primary skin resident cells to IL-36

Activated skin resident cells can produce significant levels of pro-inflammatory cytokines, such as IL-8 which is a potent chemoattractant that will recruit neutrophils in to the skin, this along with other cytokines aid in the mounting an inflammatory response [506]. IL-36 has been shown to induce pro-inflammatory cytokines [218] and due to the relevance of IL-36 in psoriasis and the importance of neutrophils in this disease [3] IL-8 was used as a marker cytokine for IL-36 bioactivity. Biophysical analysis suggested that cleavage of IL-36 at the N-terminus increased

activity [128]. Cleaved IL-36 was commercially available therefore these were tested using human skin resident cells.

5.2.1 IL-36α

In order to study this, human primary keratinocytes and fibroblasts derived from both healthy and psoriatic donors were stimulated with 100 ng/ml of both IL-36α in its full length form and its cleaved form (5 amino acids removed from the N-terminus IL-36α K6), these were both commercially available (Figure 5-1). After 48 hours of stimulation levels of IL-8 were analysed by ELISA. In the healthy human primary fibroblasts a significant increase of IL-8 production was detected between the non-stimulated control and the truncated IL-36α K6 that was not seen following treatment with the full length IL-36α (Figure 5-1 a). A significant increase of IL-8 was also seen between the non-stimulated control and IL-36α K6 in the healthy human primary keratinocytes and no significant difference was seen following treatment with the full length IL-36α (Figure 5-1 b). This suggests that in healthy human primary fibroblasts and keratinocytes IL-36α requires N-terminal cleavage to have a significant pro-inflammatory effect. However, although no statistically significant differences were seen in the psoriatic human primary fibroblasts there was a defined increase in IL-8 following treatment with IL-36α truncated (Figure 5-1 a). The psoriatic human primary keratinocytes were un-responsive (Figure 5-1 b). This was possibly due to high passage number and due to limited availability of psoriatic keratinocytes no alternative donors were available for testing. No other IL-36 members were tested using this donor due to the un-responsiveness.

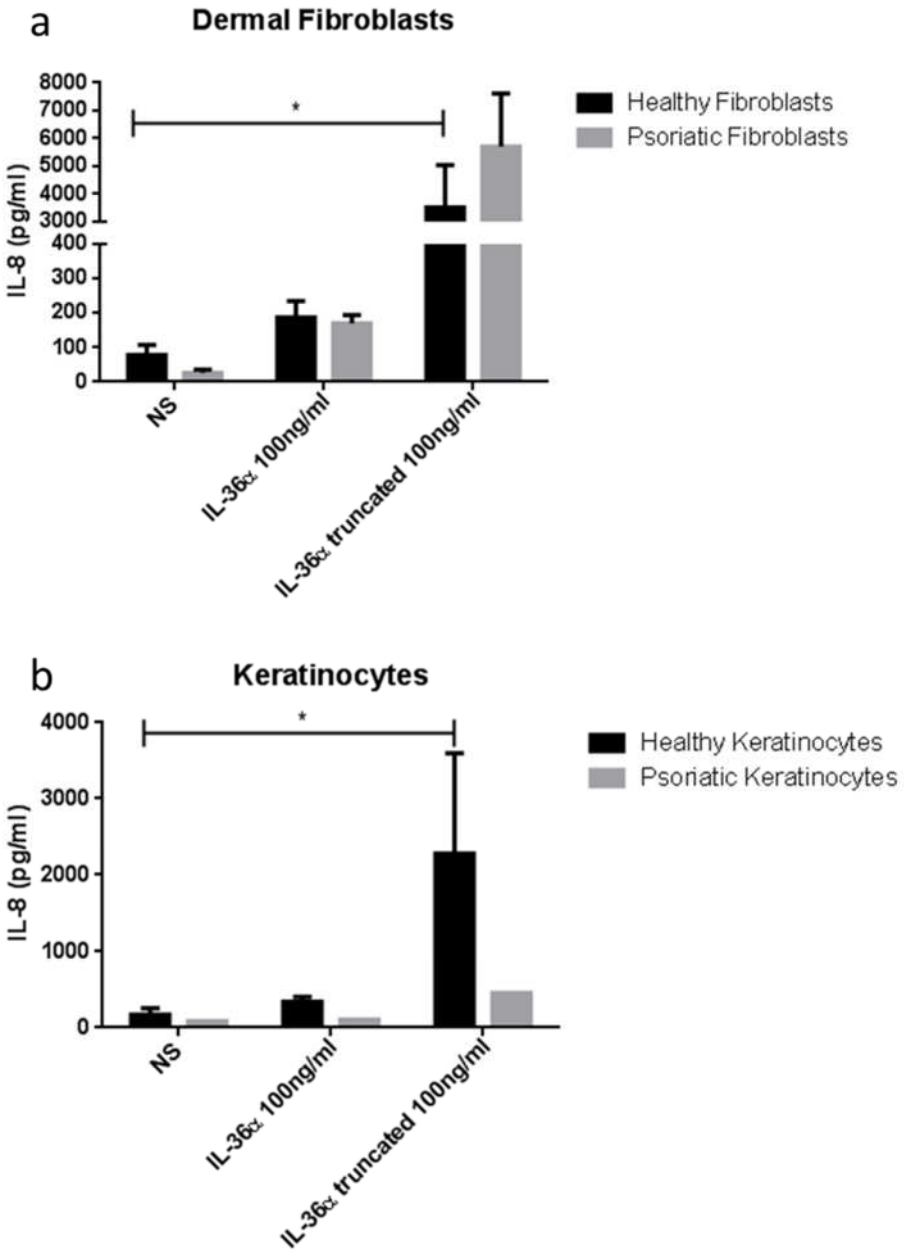


Figure 5 – 1. IL-36α requires cleavage for activity in human primary fibroblasts and keratinocytes.

Human primary cells were plated at 20,000 cells per well and stimulated for 48 hours following which supernatants were removed and IL-8 levels analysed by ELISA. **a** – Human primary fibroblasts from both healthy and psoriatic donors were stimulated with 100 ng/ml of both IL-36α full length and truncated forms. n = 3, independent experiments and different donors. **b** – Human primary keratinocytes from both healthy and psoriatic donors were stimulated with 100 ng/ml of both IL-36α full length and truncated forms. Healthy donors n = 3, Psoriatic donors n = 1, independent experiments and different donors. Mean +/- Standard error of the mean (SEM) is depicted on all graphs. NS = Non-stimulated. Raw data was analysed using a Kruskal-Wallis test (GraphPad Prism 5.03, GraphPad Software, San Diego, CA). * = $p \leq 0.05$

5.2.2 IL-36β

Human primary skin resident cells were also treated with IL-36β in both its full length and truncated form. Human primary fibroblasts derived from both healthy and psoriatic donors and healthy human primary keratinocytes were stimulated with 100 ng/ml of full length IL-36β and truncated IL-36β (4 amino acids removed from the N-terminus) (Figure 5-2). After 48 hours cell free supernatant was removed and IL-8 concentrations were analysed using ELISA. Although, with the limited available sample number, no statistically significant differences were detected in either the healthy or psoriatic human primary fibroblasts there is no doubt that following treatment with IL-36β truncated compared to full length there is an increased production of IL-8 (Figure 5-2 a). This was also seen in the healthy human primary keratinocytes (Figure 5-2 b). This data although lacking in statistical significance does suggest that IL-36β in line with the other IL-36 family members requires N-terminal cleavage to gain significant pro-inflammatory activity.

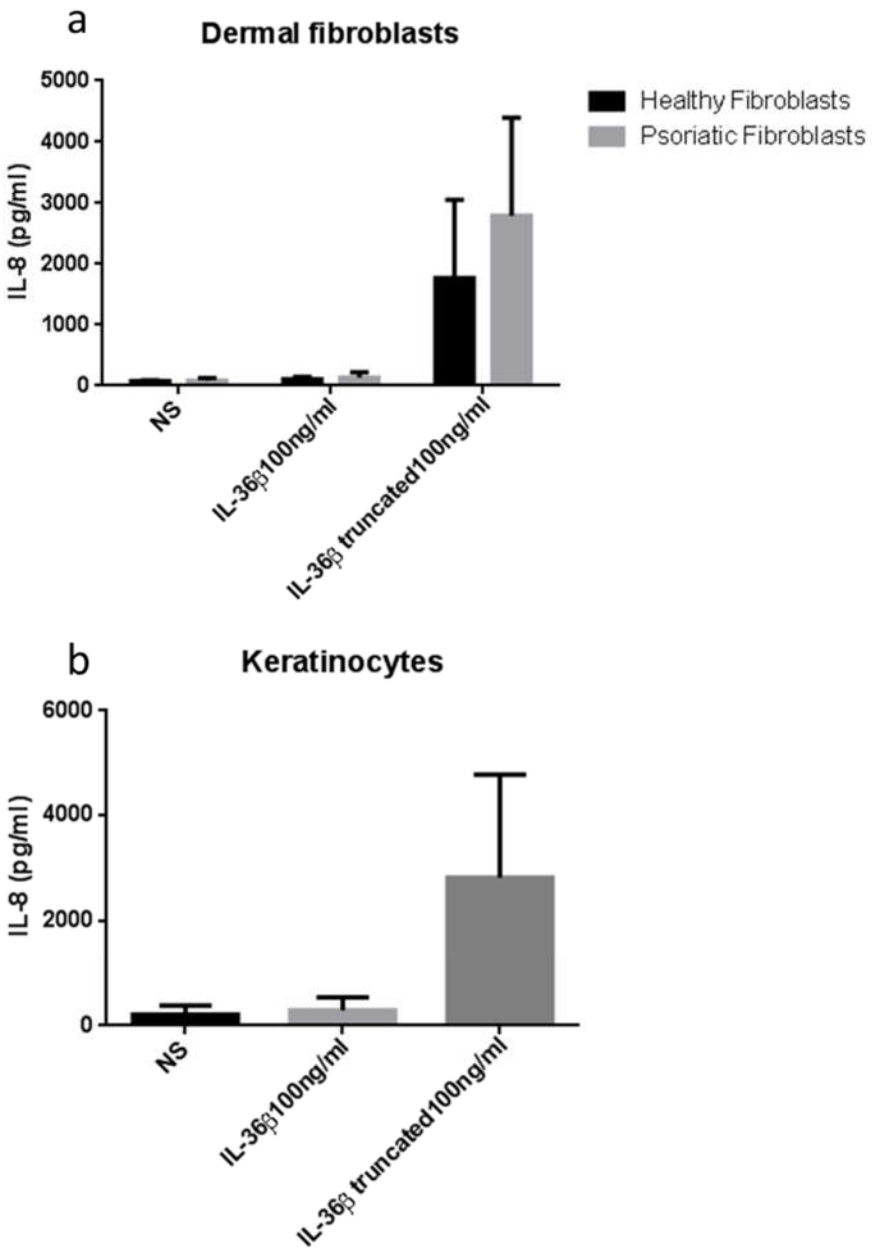


Figure 5 – 2. IL-36β requires cleavage for activity in human primary fibroblasts and keratinocytes.

Human primary cells were plated at 20,000 cells per well and stimulated for 48 hours following which supernatants were removed and an IL-8 ELISA performed. **a** – Human primary fibroblasts from both healthy and psoriatic donors were stimulated with 100 ng/ml of both IL-36β full length and truncated forms. n = 3, independent experiments and different donors. **b** – Human primary keratinocytes from healthy donors were stimulated with 100 ng/ml of both IL-36β full length and truncated forms. n = 3, independent experiments and different donors. Mean +/- SEM is depicted on all graphs. NS = Non-stimulated. Raw data was analysed using a Kruskal-Wallis test (GraphPad Prism 5.03, GraphPad Software, San Diego, CA).

5.2.3 IL-36γ

Human primary skin resident cells were also treated with IL-36γ in its full length form and also its truncated form. Human primary fibroblasts derived from both healthy and psoriatic donors as well as healthy human primary fibroblasts were stimulated with 100 ng/ml of full length and truncated IL-36γ (17 amino acids removed from the N-terminus – S18). After 48 hours of stimulation IL-8 protein concentrations were analysed by ELISA (Figure 5-3). In human primary fibroblasts a significant increase in IL-8 release was detected following treatment with truncated IL-36γ (S18) compared to full length IL-36γ and this was seen in both fibroblasts derived from healthy and psoriatic donors (Figure 5-3 a). Interestingly, a significant increase in IL-8 production was also detected between the healthy and psoriatic human primary fibroblasts treated with 100 ng/ml IL-36γ truncated (S18) (Figure 5-3 a). Preliminary data suggests that this difference in response is not related to differential expression of the IL-36 receptor on the cells, as confirmed by FACS. Although no statistical difference was detected in IL-8 production following treatment with truncated IL-36γ (S18) in healthy human primary keratinocytes there was a defined increase in IL-8 production that was not seen upon treatment with full length IL-36γ (Figure 5-3 b). This again is suggestive that IL-36γ requires N-terminal cleavage in order to gain significant pro-inflammatory activity.

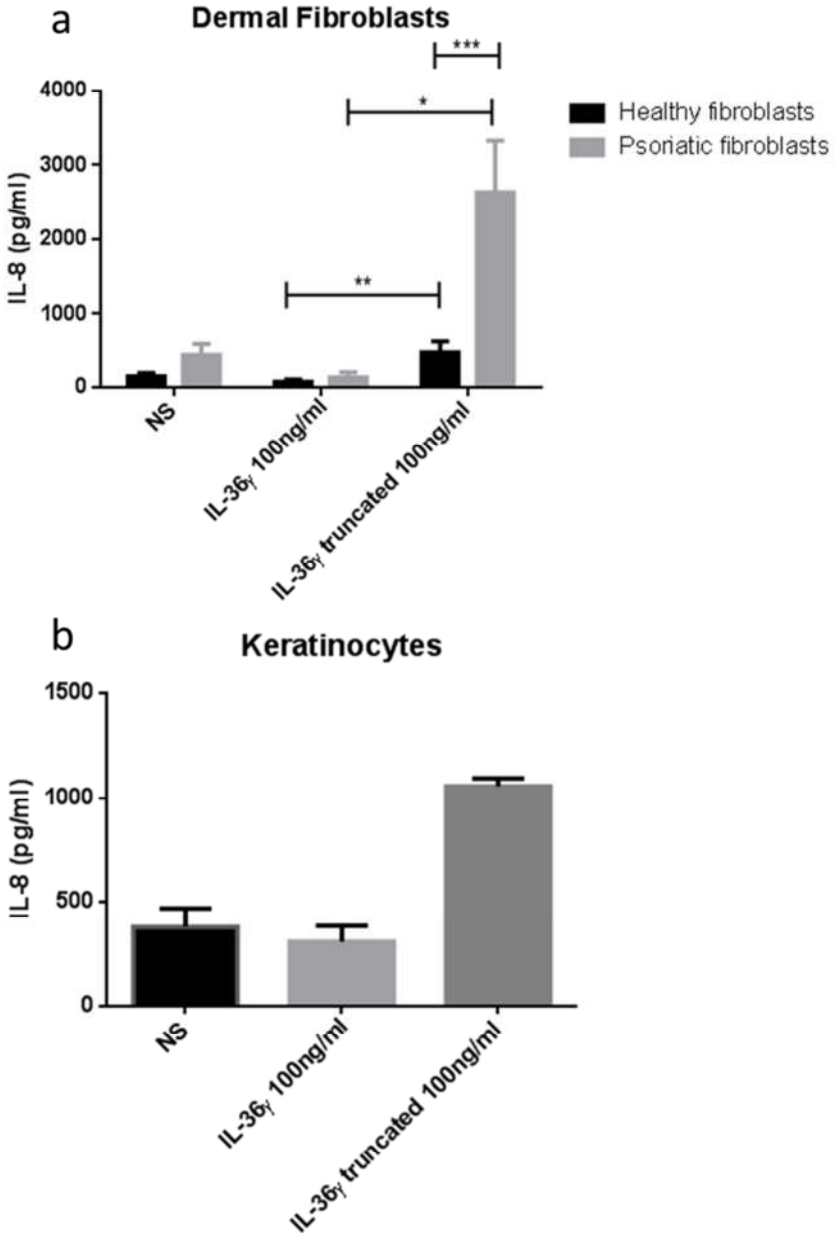


Figure 5 – 3. IL-36γ requires cleavage for activity in human primary fibroblasts and keratinocytes.

Human primary cells were plated at 20,000 cells per well and stimulated for 48 hours before analysis by IL-8 ELISA was performed. **a** – Human primary fibroblasts from both healthy and psoriatic donors were stimulated with 100 ng/ml of both IL-36γ full length and truncated forms (S18). Healthy donors n = 6, psoriatic donors n = 4, independent experiments and different donors. **b** – Human primary keratinocytes from healthy donors were stimulated with 100 ng/ml of both IL-36γ full length and truncated forms. Healthy donors n = 3, independent experiments and different donors. Mean +/- SEM is depicted on all graphs. NS = Non-stimulated. Raw data was analysed using a Kruskal-Wallis test (GraphPad Prism 5.03, GraphPad Software, San Diego, CA). * = $p \leq 0.05$ ** = $p \leq 0.01$ *** = $p \leq 0.001$.

5.3.4 Effects of IL-36 on PMN and T cell activation

The truncated cleaved forms of IL-36 were also tested to assess their direct activity on PMNs and PBMCs. Standard transwell chemotaxis assays were performed to assess the effect of IL-36 on the migratory capacity of the PMNs. However, no chemokinetic or chemotactic effects were observed. No increase in PMN activation following IL-36 treatment was identified, as assessed by CD11b expression, generation of reactive oxygen species from the PMNs or CD62L shedding. PBMCs were also treated with both the full length and active cleaved forms of IL-36. Levels of activation were established by looking at CD69 levels, however no T cell activation was identified.

5.3 Cleavage of full length IL-36

As shown in the previous figures (5.1 to 5.3), IL-36 family members require N-terminal cleavage to increase their pro-inflammatory functions. As previously mentioned a distinctive feature of psoriasis is the accumulation of neutrophils in epidermal-dermal junctions, known as Munro's abscesses [3]. Therefore it was logical to assume that with an increased level of neutrophils in a disease where increased levels of IL-36 were also present that these highly protease rich cells may be involved in the cleavage of IL-36. In order to study this hypothesis PMNs were isolated from whole blood of healthy donors and immediately after isolation PMNs were stimulated with 100 ng/ml of PMA for 1 hour at 37°C. After stimulation cells were removed and supernatant was incubated with full length IL-36γ. IL-36γ was used for these experiments as it was found to be the most up-regulated IL-36 member in psoriasis. As well as the fact that the truncated form has 17 amino acids removed from the N-terminus which means difference in mobility of the protein on an SDS PAGE gel was detectable. This is not the case for other IL-36 members. IL-36γ was incubated with the PMN supernatant for 10 minutes and 30 minutes at 37°C (Figure 5-4).

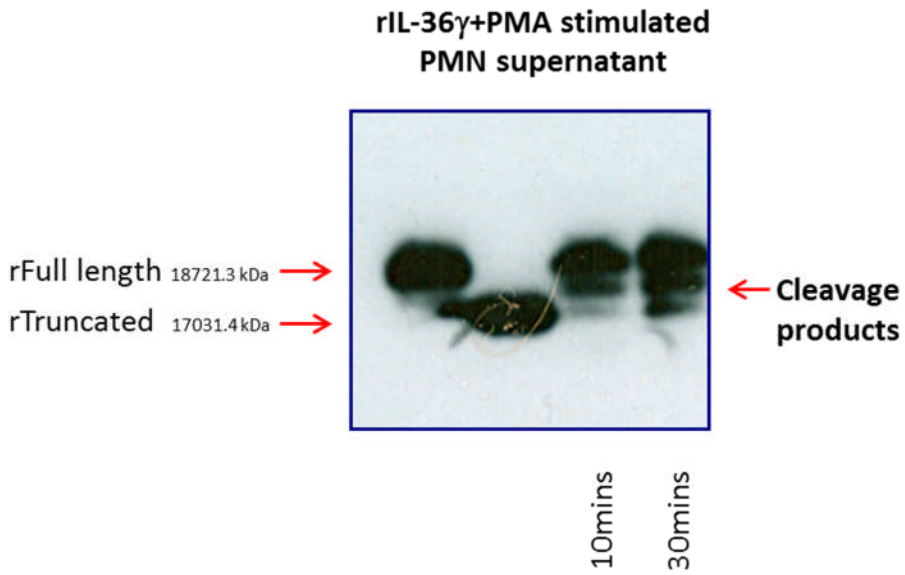


Figure 5 – 4. Supernatant from stimulated polymorphonuclear (PMNs) cells can cleave IL-36γ.

PMNs were isolated from peripheral blood of healthy donors and stimulated with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) for 1 hour at 37°C. Following this incubation cells were removed and supernatant either used immediately or frozen at -80°C. Supernatant was then incubated with full length IL-36γ for 10 minutes and 30 minutes. Western blot analysis including a full length and cleaved control is shown. Western blot is representative of 3 independent experiments.

Following incubation samples were analysed by Western blotting. It was shown that after even after 10 minutes of incubation with PMN supernatant cleavage of protein could be detected which was more prominent after 30 minutes. The lower molecular weight bands on the gel were suggestive of the full length protein being cleaved by a protease derived from activated PMNs.

5.4 Production of recombinant tagged IL-36

Due to the exciting results depicted in Figure 5-4 numerous attempts were performed to pull down the cleaved products seen in the lower bands on the Western blot using immunoprecipitation. However, it was concluded that the commercially available antibodies did not bind with great enough affinity for immunoprecipitation to retrieve enough protein to analyse. Also, the experiments depicted in 5.3 could only be performed on IL-36γ due to the small number of amino acids removed from the N-terminus of the other proteins. Therefore, in order to elucidate the accurate products following protease cleavage without suitable reagents recombinant proteins were produced containing an N-terminal SUMO tag. This allowed for identification of N-terminal cleavage due to the size difference being evident on an SDS PAGE

gel. Recombinant IL-36 proteins were also produced with an N-terminal SUMO tag and a C-terminal His tag using a pET 28 (+) vector (see Appendix figure 2 for vector map). This would allow for nickel affinity chromatography following digestion if further analysis was required and needed to be isolated from the proteases that had cleaved IL-36.

All figures depicted in this section are representative images of the production and purification of N-terminally SUMO tagged IL-36γ, however the same method was utilised for all of the IL-36 proteins and for both the N-terminally tagged and N and C-terminally tagged proteins. A diagrammatic representation of the constructs produced is depicted in figure 5-5 a. The IL-36 members were all cloned into a Champion pET SUMO expression system (see Appendix figure 1 for vector map) and then transformed into DH5α cells to check accuracy of cloned sequence (See Appendix figure 4 - 23 for primers and sequences of all IL-36 members). The IL-36 members were expressed in BL21-CodonPlus (DE3)-RIPL strain of *E. coli* and the overnight growth temperature following induction with IPTG was optimised (Figure 5-5 b). Following overnight culture samples were taken and run on an SDS PAGE gel and stained with Coomassie. Compared to the pre-induction samples a band relating to IL-36 can clearly be seen (indicated by the red arrow) at all temperatures, however the strongest band is present at 25°C.

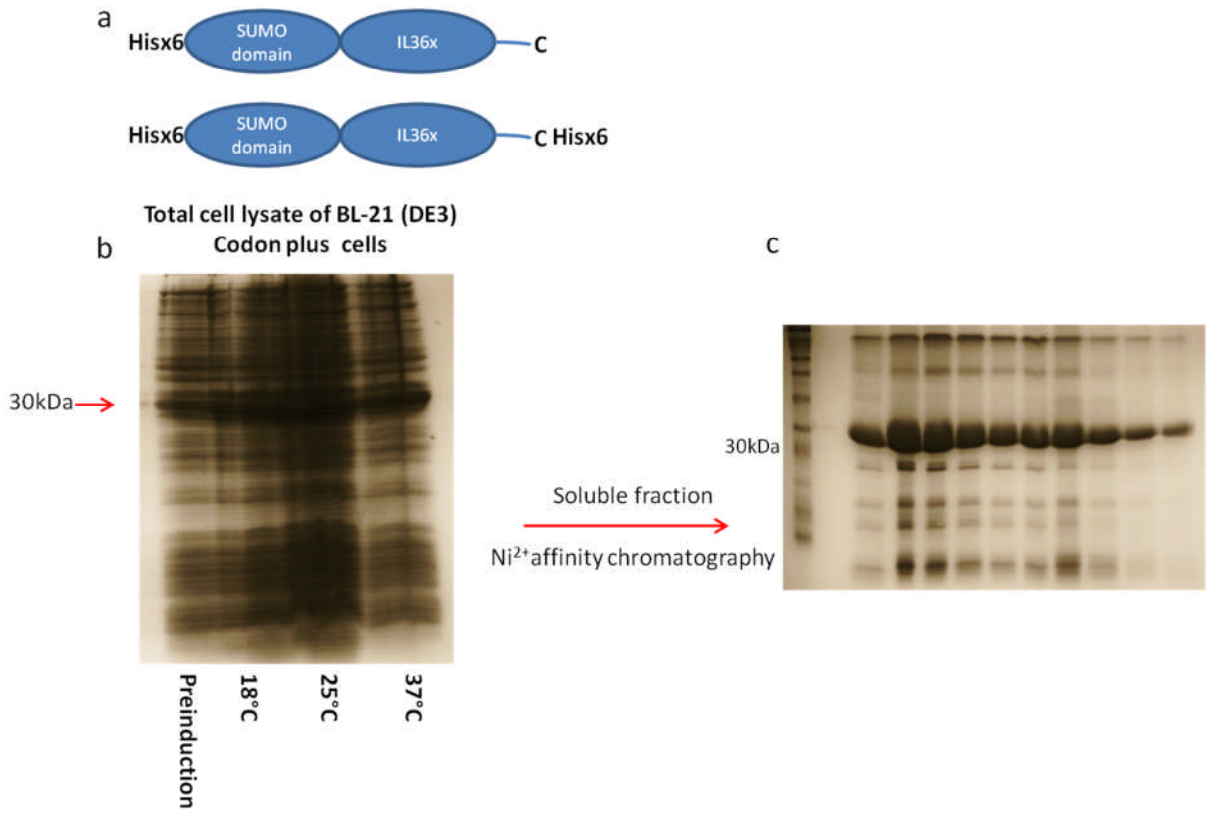


Figure 5 – 5. Optimisation of IL-36 expression in *E. coli*

IL-36 members (IL-36α, IL-36γ, IL-36RA) were cloned using a pET expression system including a SUMO-his tag in order to purify the protein and aid solubility. **a** - Diagrammatic representation of the N-terminal SUMO-His tag as well as constructs produced including a C-terminal His tag. **b** – IL-36s were cloned and then transformed into expression strains of *E. coli.* A sample was taken prior to induction and then compared to overnight growth following induction at 18°C, 25°C and 37°C. Coomassie stained gel shows samples at different temperatures following overnight incubation. **c** – Coomassie stained gel of nickel affinity chromatography soluble fractions. These figures are representative experiments for one construct however this was carried out for all constructs.

All further larger scale expression cultures were induced using 0.8mM IPTG and then grown overnight at 25°C due to optimisation results depicted in Figure 5-5 b. Following overnight growth and cell lysis (see 2.3.8) the protein present in the soluble fraction of the cells was then purified using nickel affinity chromatography. Protein was eluted from the nickel column using increasing concentrations of imidazole, 1ml fractions were taken and a sample of each run on an SDS PAGE gel and stained using Coomassie. In Figure 5-5 c the prominent band is the purified IL-36 protein. This method was used for all IL-36 recombinant proteins produced.

Following nickel affinity chromatography IL-36 proteins were further purified using a size exclusion column which then allowed for differentiation between monomers, dimers and aggregates therefore allowing monomers to be purified and used for further experiments (Figure 5-6 a). The fractions that represent the monomer peak were run on an SDS PAGE gel and stained with Coomassie to assess purity. As shown in Figure 5-6 b following this purification method the sample is extremely pure.

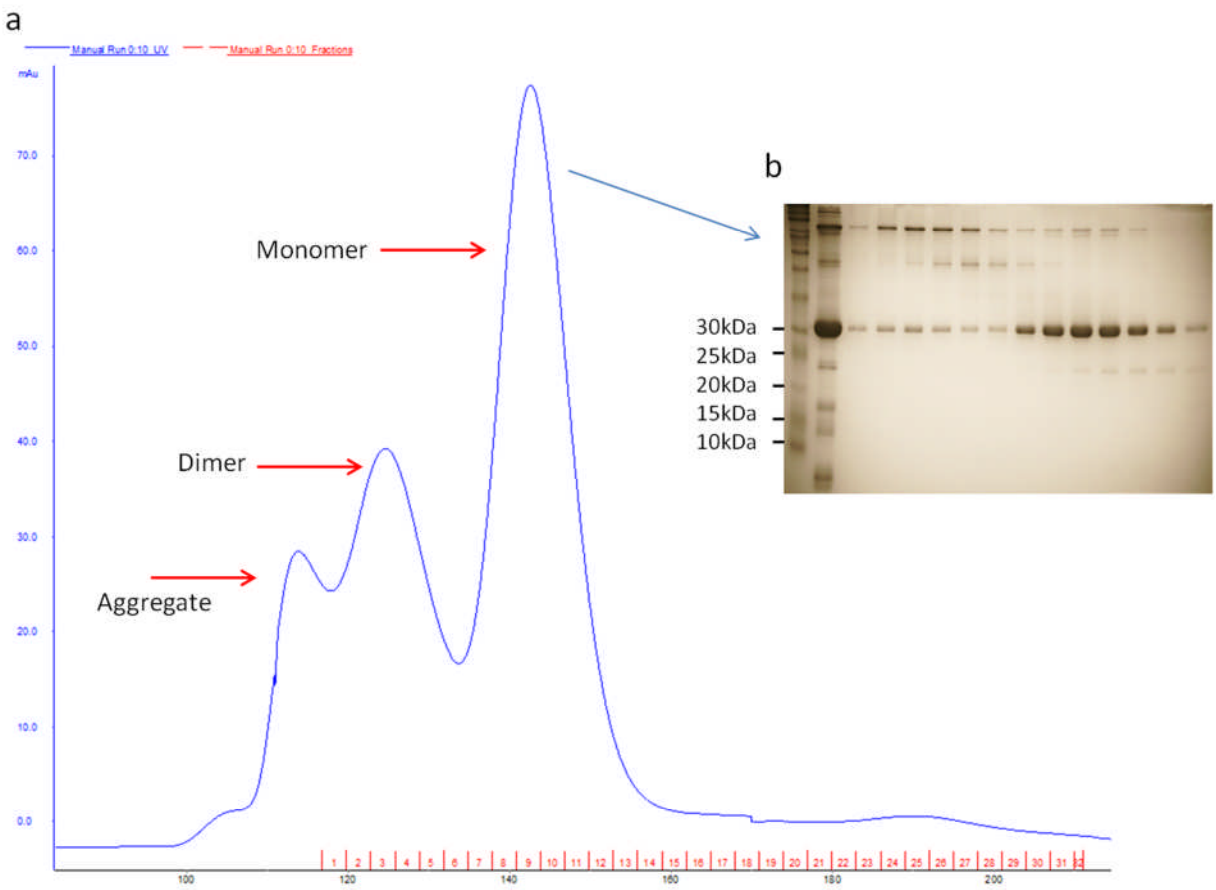


Figure 5 – 6. Further purification of IL-36 proteins using size exclusion.

IL-36 members were purified using nickel column chromatography following which they were further purified using size exclusion. **a** – A representative 280nm UV graph taken from the software used to analyse the fractions removed from the size exclusion column. Fractions from the peak that represented the monomer fraction was collected and concentrated. **b** – Coomassie stained gel of size exclusion fractions. The depicted experiments are representative of the experiments carried out for all constructs.

Following numerous attempts to express IL-36β it was concluded that expression was not viable. Various optimisation methods were performed including using different strains of expression *E. coli* – Shuffle® competent cells, Rosetta™ 2 (DE3)

competent cells and ArticExpress competent cells. The induction stage of the *E. coli* was also optimised with the use of auto-induction and alterations of glucose and lactose concentrations. However, none of these techniques led to any IL-36β expression so therefore from here on IL-36β was not used in any of the experiments (See Appendix figure 10 – 13 for primers used and sequences). The lack of expression could be due to its reduced phylogenetic relationship and therefore less structural similarity to the other IL-36 members [507].

5.5 Identifying putative proteases responsible for IL-36 cleavage

In order to identify specific proteases that cleave IL-36 all IL-36 proteins were produced as described in 5.4 with an N-terminal SUMO tag. The tag allowed for visualisation of N-terminal cleavage via SDS PAGE analysis. Therefore different cellular supernatants could be tested in order to assess their cleavage activity. In order to study this, PMN supernatant was obtained as described in 5.3 and human primary fibroblasts and keratinocytes were also stimulated with 100ng/ml of PMA for 24 hours at 37°C before supernatant was removed. Supernatant from the three different cell types was then incubated with IL-36α (Figure 5-7 a), IL-36γ (Figure 5-7 b) and IL-36RA (Figure 5-7 c) for 1 hour at 37°C. Following incubation samples were then analysed by Western blot. N-terminal cleavage of the IL-36 members was only present when incubated with supernatant from activated PMN. This is in line with what was shown using the commercially available IL-36γ in 5.3 and it is interesting to note that all of the IL-36 members are cleaved.

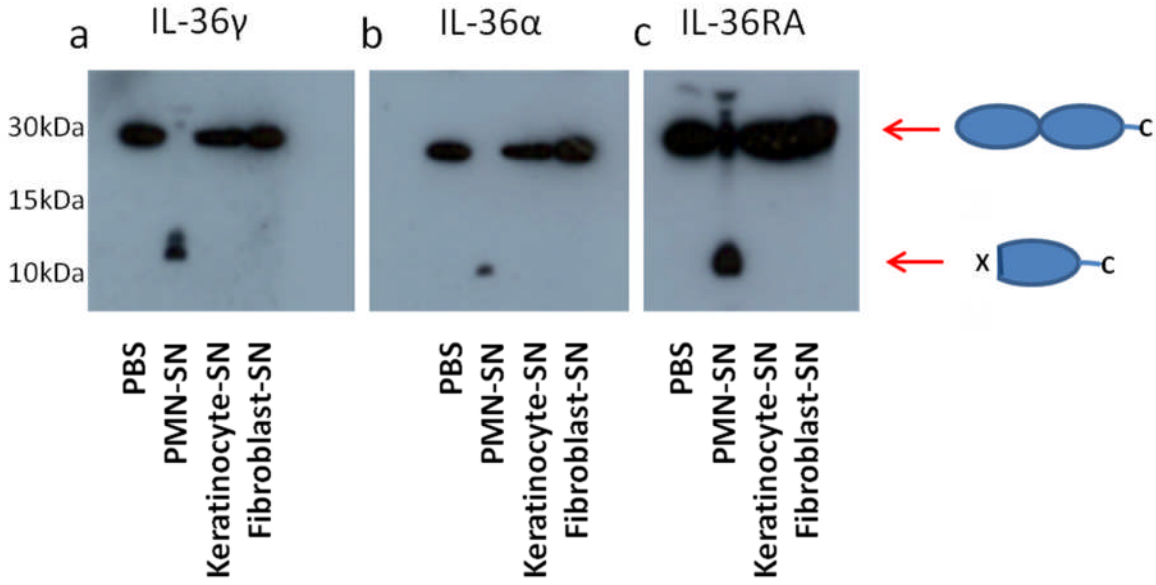


Figure 5-7. IL-36 proteins with a SUMO tag incubated with activated cellular supernatants are cleaved by supernatant from PMNs.

SUMO tagged IL-36s were incubated with a range of supernatants from activated cells. PMNs were isolated from whole blood of healthy donors and then 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. Supernatant was then removed from the cells and incubated with IL-36α (**a**), IL-36γ (**b**) or IL-36RA (**c**) for 1 hour at 37°C, along with a PBS control which was also incubated for 1 hour at 37°C. Samples were then analysed by Western blotting.

In order to further elucidate the protease that appears to be responsible for the cleavage of IL-36 all proteins were cleaved using supernatant from activated PMNs in the presence and absence of various protease inhibitors. IL-36α (Figure 5-8 a), IL-36γ (Figure 5-8 b) and IL-36RA (Figure 5-8 c) were incubated with activated PMN supernatant for 1 hour at 37°C. This was carried out in the presence of pan serine, cysteine and aspartic protease inhibitors (PI, PI Roche), MMP inhibitors (EDTA), pan cysteine protease inhibitors (IAA) or pan serine proteases inhibitors (PMSF, α1 anti-trypsin). Following incubation, samples were analysed via Western blot analysis and run alongside a positive control and a sample that was incubated with activated PMN supernatant and did not contain any protease inhibitors. As expected both pan protease inhibitors prevented cleavage in all IL-36 members tested. EDTA and IAA did not prevent cleavage which suggests that an MMP or cysteine protease was not responsible for IL-36 cleavage. However, both serine protease inhibitors (PMSF, α1

anti-trypsin) prevented cleavage which is suggestive of a PMN derived serine protease that is responsible for IL-36 cleavage.

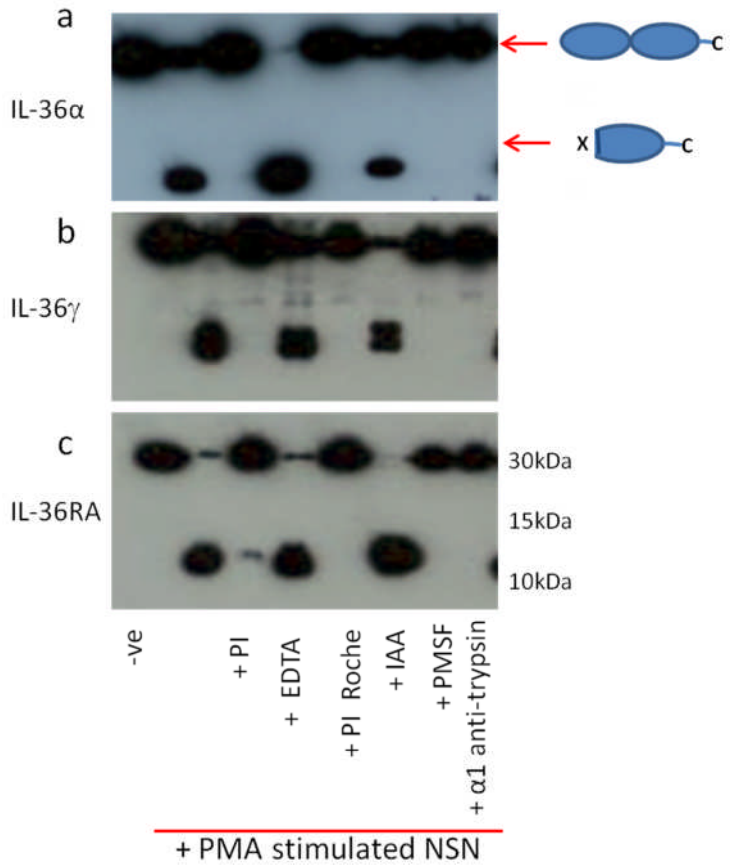


Figure 5 – 8. IL-36 cleavage by activated PMN supernatant can be prevented by serine protease inhibitors.

Sumo tagged IL-36s were incubated with supernatant from PMNs stimulated with PMA for 1 hour at 37°C. Incubation was performed in the presence or absence of a range of protease inhibitors. PI = complete protease inhibitor cocktail (Roche), EDTA = Ethylenediaminetetraacetic acid, inhibitor of proteases where a metal ion is required for cleavage, PI Roche = complete ultra-protease inhibitor cocktail which includes aspartic proteases (Roche), IAA = iodoacetic acid a pan cysteine protease inhibitor, PMSF = phenylmethylsulfonyl fluoride a pan serine protease inhibitor, α1 anti-trypsin is another pan serine protease inhibitor. **a** – IL-36α was incubated with activated PMN supernatant in the presence and absence of the panel of protease inhibitors. **b** - IL-36γ was incubated with activated PMN supernatant in the presence and absence of the panel of protease inhibitors. **c** - IL-36RA was incubated with activated PMN supernatant in the presence and absence of the panel of protease inhibitors. Samples were then analysed using Western blotting.

Although Figures 5-7 and 5-8 were suggestive of PMN serine proteases being responsible for cleavage other proteases were analysed for completeness. Due to the expression of the IL-36 members being largely limited to human epithelium and the

known relevance of IL-36 in inflammatory skin diseases we focused on cleavage in the skin compartment. Therefore in collaboration with Dr Ulf Meyer-Hoffert at the University of Kiel, Germany the SUMO tagged IL-36 members were also subjected to cleavage by recombinant kallikreins. These are serine proteases that are produced predominantly by keratinocytes [300]. In order to study this, IL-36α was incubated with kallikrein 5 (Figure 5-9 a) and kallikrein 7 (Figure 5-9 b) for increasing periods of time. There was no cleavage of IL-36α following even prolonged incubation with kallikrein 7, however after 3 hours of incubation with kallikrein 5 N-terminal cleavage could be detected. IL-36γ was also incubated with kallikrein 5 (Figure 5-9 c) and kallikrein 7 (Figure 5-9 d) for increasing periods of time. Minimal cleavage of IL-36γ was detected following 24 hours of incubation with kallikrein 5, however substantial cleavage of IL-36γ was detected after only 5 minutes of incubation with kallikrein 7 and after 24 hours nearly all protein was cleaved. IL-36RA was also incubated with kallikrein 5 (Figure 5-9 e) and kallikrein 7 (Figure 5-9 f) for increasing periods of time. No remarkable cleavage could be detected when IL-36RA was incubated with kallikrein 5 or 7 at even 24 hours of incubation.

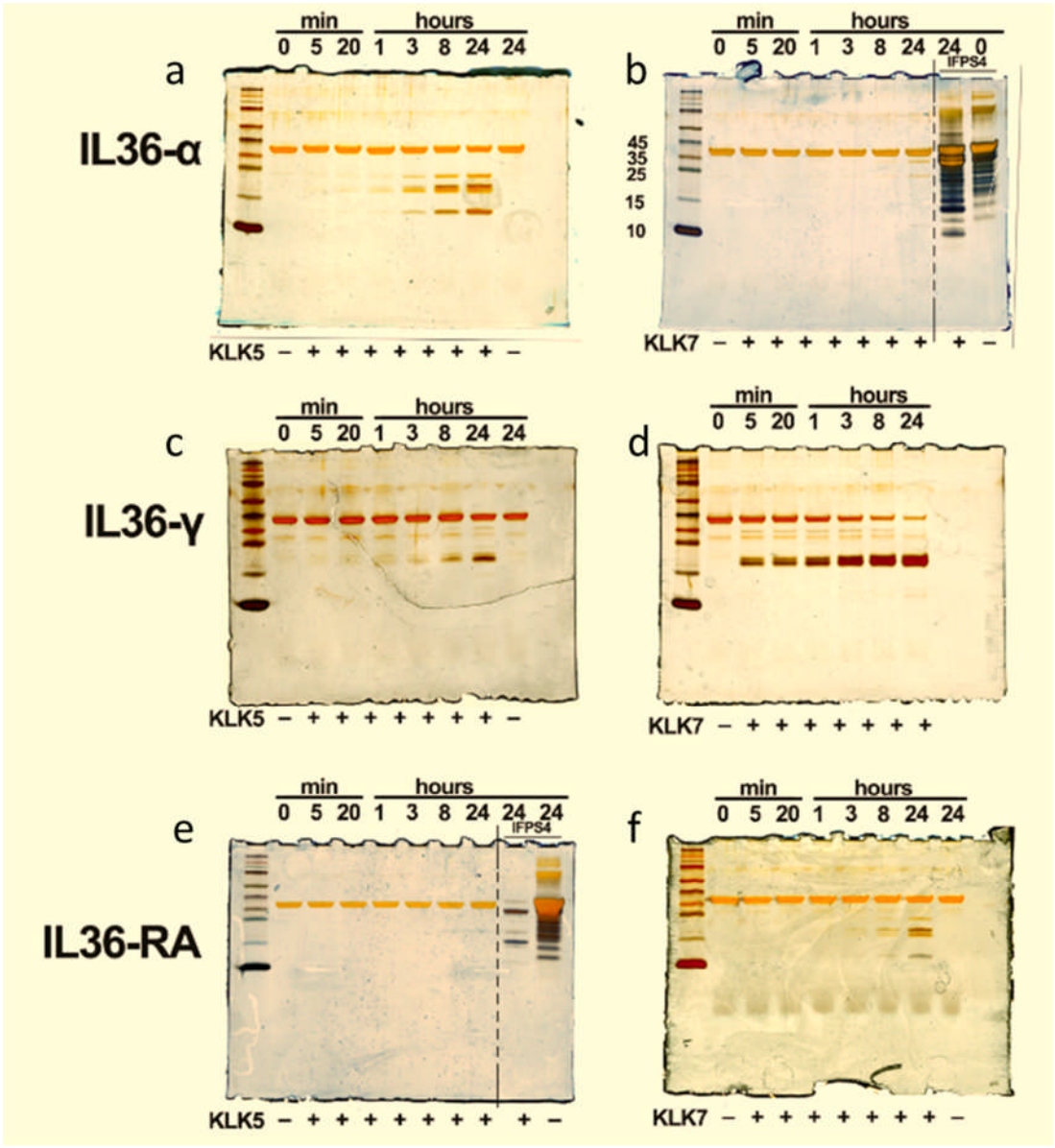


Figure 5-9. IL-36 proteins with a SUMO tag can be cleaved by recombinant kallikreins.

SUMO tagged IL-36 proteins were incubated with recombinant kallikreins for a range of time periods. **a** – IL-36α was incubated with recombinant kallikrein 5 (KLK5). **b** - IL-36α was incubated with recombinant kallikrein 7 (KLK7). **c** – IL-36γ was incubated with recombinant kallikrein 5. **d** - IL-36γ was incubated with recombinant kallikrein 7 (KLK7). **e** - IL-36RA was incubated with recombinant kallikrein 5 (KLK5). **f** - IL-36RA was incubated with recombinant kallikrein 7 (KLK7). Silver stained gels show protein bands following incubation with the kallikreins. Cleaved samples were then analysed by mass spectrometry. The data displayed in this figure was carried out by Dr Ulf Meyer-Hoffert and Jan Fischer, Department of Dermatology, University Hospital Schleswig-Holstein, Kiel, Germany.

The cleavage products of IL-36γ identified in Figure 5-9 d were analysed by mass spectrometry and the results will be discussed along with all other mass spectrometry data in 5.6 (see Table 5-1).

5.6 IL-36 cleavage products identified

Figure 5-8 suggested that a serine proteases derived from PMNs were responsible for IL-36 cleavage. In order to identify the precise cleavage products by mass spectrometry recombinant proteases were used in order to maintain a relatively pure sample suitable for mass spectrometry. The three most prominent neutrophil serine proteases, cathepsin G (CG), elastase (E) and proteinase 3 (PR3) were tested. In order to study this, recombinant CG, E and PR3 were incubated with SUMO tagged IL-36α (Figure 5-10 a), IL-36γ (Figure 5-10 b) and IL-36RA (Figure 5-10 c) for 10 minutes at 37°C. Following incubation samples were run on an SDS PAGE gel and protein bands determined by Coomassie stain. Incubation with all of the recombinant PMN serine proteases results in complete cleavage of IL-36α and IL-36γ, however cleavage of IL-36RA was detectable but not all IL-36RA was cleaved. The red arrows indicate the N-terminally cleaved IL-36 and the SUMO tag, which is depicted as the higher band on the Coomassie stained gels.

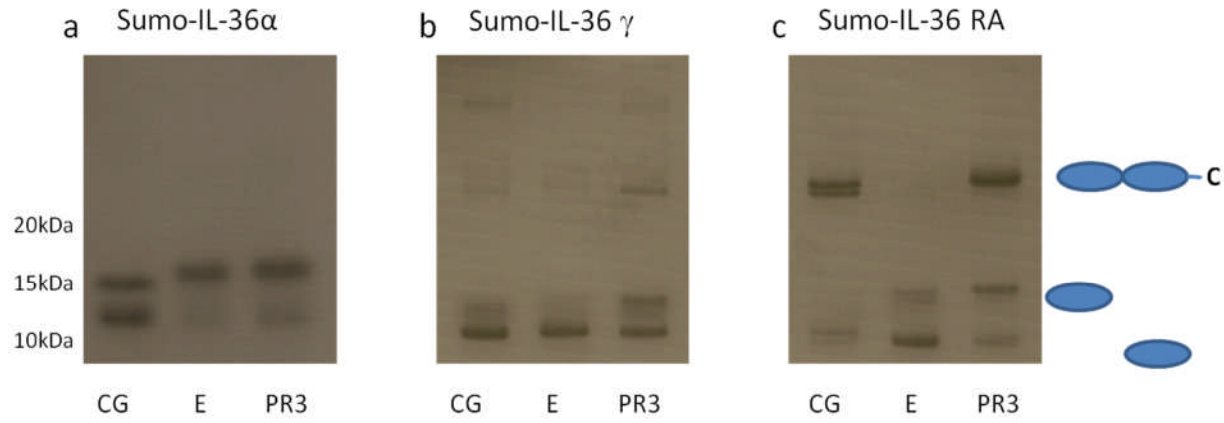


Figure 5 – 10. IL-36 proteins can be efficiently cleaved using recombinant neutrophil serine proteases.

Sumo tagged IL-36 proteins were incubated with the three most common neutrophil serine proteases, cathepsin G (CG), elastase (E) and proteinase 3 (PR3) at 1:20th of IL-36 added for 10 minutes at 37°C. **a** - IL-36α. **b** – IL-36γ. **c** – IL-36RA. Coomassie stained gel shows samples following incubation.

This incubation was exclusively carried out with recombinant proteins therefore the samples were relatively pure, as depicted on the Coomassie stained gels on Figure 5-10 a-c. This allowed for further analysis by mass spectrometry and N-terminal sequencing. The N-terminal cleavage products identified are diagrammatically depicted in Figure 5-11 a-c. From here on the N-terminal cleavage products are referred to as the N-terminal amino acid and the number of amino acids from the N-terminus.

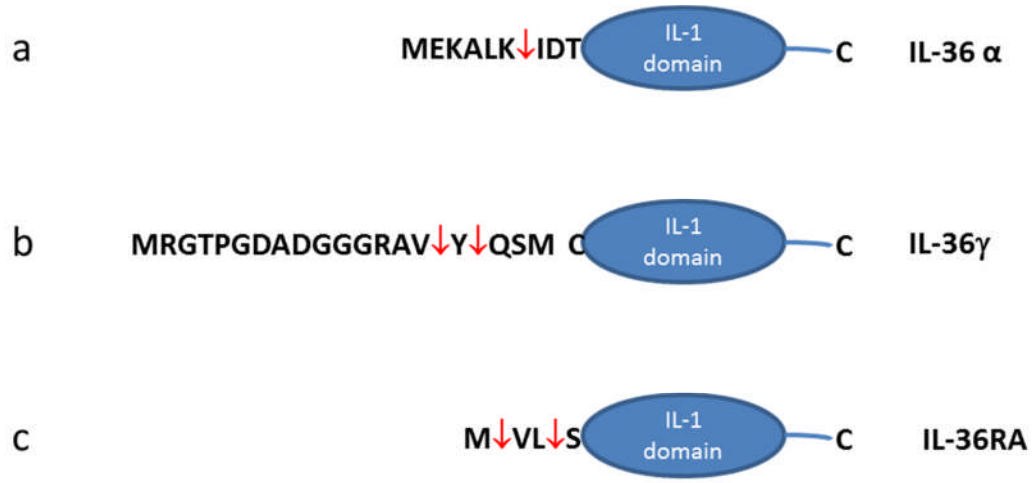


Figure 5-11. Diagrammatic representation of cleavage products identified following incubation with recombinant neutrophil serine proteases.

Following incubation with recombinant serine proteases samples were analysed by mass spectrometry and N-terminal sequencing. Diagrammatic representation of the N-terminal cleavage products detected for IL-36α (**a**), IL-36γ (**b**) and IL-36RA (**c**), depicted by the red arrows.

The cleavage product of IL-36α identified was I7 following incubation with recombinant neutrophil serine proteases. Two IL-36γ cleavage products were identified following incubation with recombinant serine proteases, these were Y16 and Q17. Two cleavage products were also identified for IL-36RA, S4 and V2. The biophysical prediction of cleavage products identified IL-36RA V2 as the most active form of the receptor antagonist [128]. As depicted in Figure 5-8 IL-36γ following cleavage with kallikrein 7 was also analysed by mass spectrometry and the same products were identified as with the recombinant serine proteases, which were Y16 and Q17.

IL-36α	Elastase	Proteinase 3	Cathepsin G	PMN SN	PMN SN + AMN
K6					
I7			x		
L5					x

N-terminal sequencing of product cleaved with all 3 proteases confirm I7

IL-36γ	Elastase	Proteinase 3	Cathepsin G	KLK7	PMN SN	PMN SN + AMN
Y16	x	x			x	x
Q17	x		x	x		x
S18						

N-terminal sequencing of product cleaved with all 3 proteases confirm Y16 and Q17

IL-36RA	Elastase	Proteinase 3	Cathepsin G	PMN SN	PMN SN + AMN
V2	x				
L3				x	
S4	x	x	x	x	x

Table 5-1. Summary table of cleavage products identified by mass spectrometry and N-terminal sequencing.

Depicted are all the cleavage products identified by mass spectrometry and N-terminal sequencing following incubation with recombinant serine proteases (elastase, proteinase 3 and cathepsin G) as well as with activated PMN supernatant (PMN SN). IL-36 proteins were also incubated with activated PMN supernatant following which aminopeptidase N (AMN) was added and incubated further. As described in Figure 5-9 KLK7 cleavage of IL-36γ was also analysed using mass spectrometry. The IL-36 cleavage products are depicted as the amino acid and the number from the N-terminus indicating the amount of amino acids removed from the N-terminus.

The cleavage products identified for IL-36α and IL-36γ were not products that had been predicted as active forms by Towne *et al*., [128] (see Table 5-1). In order to assess whether these active products could be identified other proteases were incubated with IL-36. Owing to the fact that the products identified for IL-36α and IL-36γ only had one extra amino acid compared to the active form, aminopeptidases were a logical target due to their known cleavage functions. Aminopeptidase N (AMN), also known as CD13, is a type II metalloprotease that cleaves single amino acids from the N-terminus of proteins [508]. In order to assess the role of AMN it was incubated at 1:20th and 1:100th of the IL-36 protein, this was added to the IL-36 proteins following cleavage by recombinant serine proteases or in combination with recombinant serine proteases. However, following cleavage with AMN the same products were identified by mass spectrometry to those following incubation with recombinant serine proteases alone. However, this may have been due to the AMN not working. In order to test whether the N-terminal SUMO could possibly affect cleavage the SUMO tag was removed prior to cleavage. This did not affect the cleavage products detected. All cleavage products are summarised in table 5-1.

In order to compare the cleavage products following incubation with recombinant neutrophil serine proteases and the proteases responsible for the cleavage in the activated PMN supernatant, the IL-36 proteins that had N- and C-terminal tags to allow for purification following cleavage were used. Using these tagged IL-36 proteins cleavage was carried out with supernatants and cells relevant to inflammatory skin diseases, including activated PMN supernatant, activated PMNs, detached and attached activated keratinocytes and a punch skin biopsy from healthy and psoriatic donors. This was carried out with a range of stimulations to activate the cells known to be relevant in psoriatic inflammation (IL-17 and TNFα; also added as stimulus: PMA). However, following purification and analysis by mass spectrometry the biophysical predictions of the active IL-36 forms were not identified and results were inconclusive due to apparent complete digestion of the protein, therefore experiments need to be performed using a more diverse range of time points.

5.7 Production of identified cleaved products

In order to test the activity of the identified IL-36 cleavage products in mammalian cell culture all the identified cleavage products shown in 5.6 were produced as recombinant proteins. These proteins were generated in the same manner as described in 5.4 with the specific cleaved sequence cloned into the Champion pET SUMO expression system. However once the SUMO tagged proteins were purified the SUMO tag was removed using a specific protease (His-ULP-1) that cleaves only the SUMO tag (leaving the exact N-terminus). Both His-ULP-1 protease and the SUMO tag following cleavage contain His tags, allowing for purification of the IL-36 proteins away from the protease and SUMO tag (Figure 5-12 a). Following purification, the IL-36 proteins were verified using mass spectrometry. The predicted active products [128] for IL-36α (K6) and IL-36γ (S18) were also produced to act as positive controls. A summary gel of all constructs produced with SUMO tags removed is depicted in Figure 5-12 b.

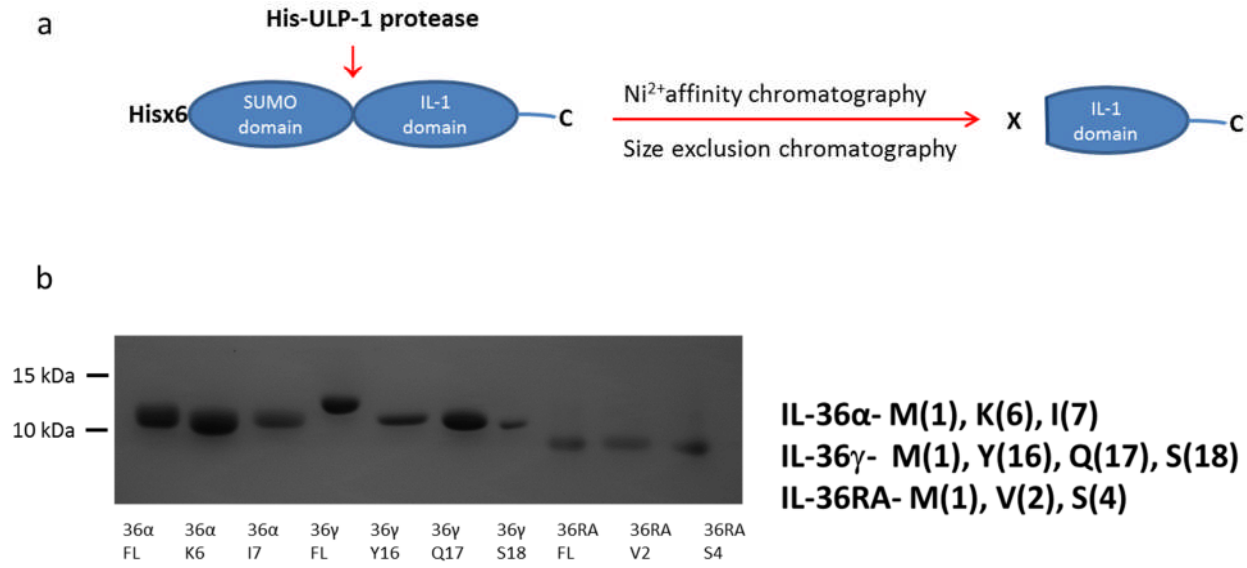


Figure 5 - 12. Cleavage products identified following serine protease cleavage are produced in *E. coli.*

The cleavage products identified following recombinant neutrophil serine protease cleavage depicted in Figure 5 – 10 were produced in *E. coli*. **a** – The IL-36 cleavage products were produced in the same manner as the full length IL-36 proteins using a Champion pET SUMO expression system and then purifying the protein using nickel affinity chromatography followed by size exclusion chromatography. After purification the SUMO tag was removed using a highly specific protease, His-

ULP-1 which also contains a His tag following which the His-ULP-1 and the SUMO tag were purified by nickel affinity chromatography. Mass spectrometry analysis was performed to ascertain that the exact protein sequence had been produced. **b** – Coomassie stained gel of all IL-36 cleaved proteins produced including positive controls, 36γ S18 and 36α K6 that were not identified following serine protease cleavage.

5.8 Response of human primary fibroblasts to the cleaved IL-36 proteins

In order to determine the activity of the truncated IL-36 proteins produced as described in 5.7 primary human skin resident cells were treated with these proteins. Human primary fibroblasts were stimulated with increasing concentrations of IL-36 proteins and after 48 hours concentrations of IL-8 were analysed using ELISA. Healthy human primary fibroblasts were treated with 100 ng/ml of commercially available IL-36β truncated (T), which was used as a positive control to confirm cell responsiveness. Increasing concentrations (1 nM – 100 nM) of IL-36α full length, K6 and I7 were used to stimulate the fibroblasts (Figure 5-13 a). A statistically significant increase in release of IL-8 was detected following treatment with 100 nM IL-36α K6 compared to non-stimulated control. A steady increase in IL-8 was evident with increasing concentrations of IL-36α K6, however the full length and I7 IL-36α did not induce any substantial release of IL-8. In order to control for LPS contamination a control IL-36 sample which was boiled for 20 minutes prior to addition to cells confirms that the increase in IL-8 is not related to LPS contamination. A limulus amebocyte lysate (LAL) test was also performed following manufacturer's instructions in order to assess endotoxin levels, levels were deemed at a low enough level for tissue culture experiments (<1 EU/μg).

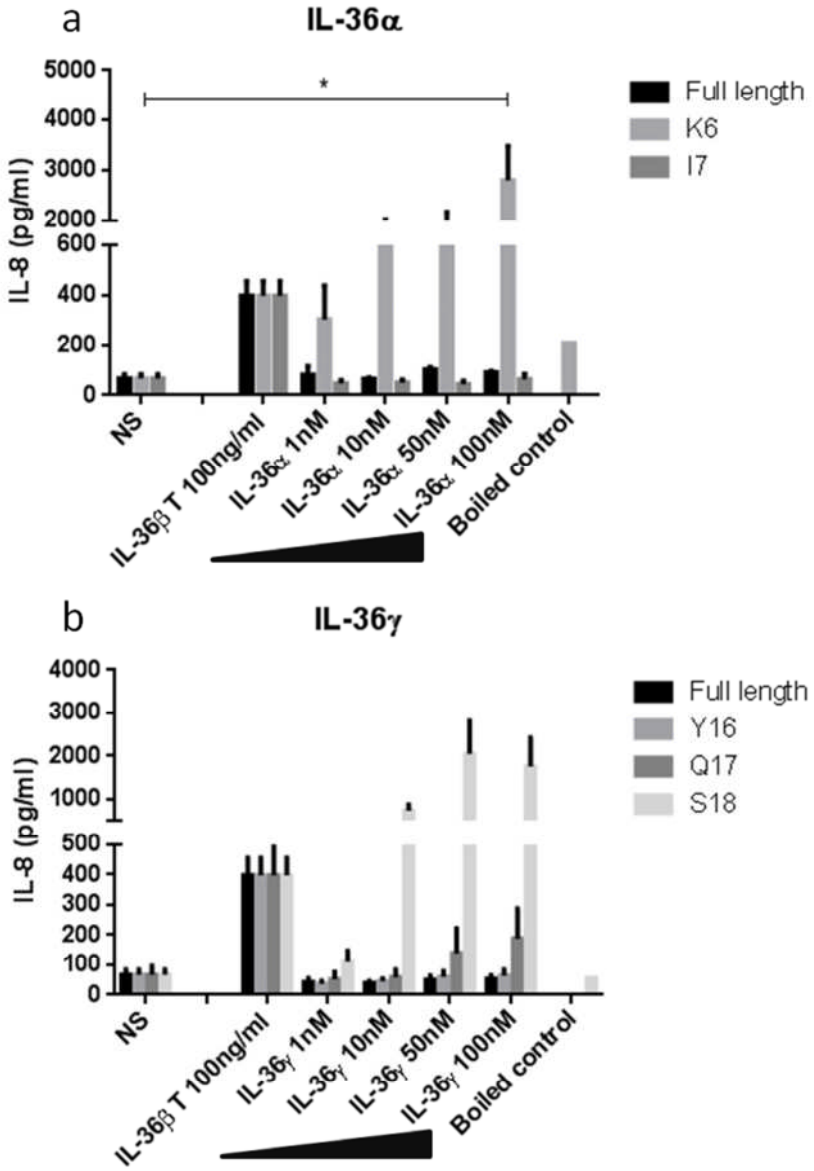


Figure 5 – 13. IL-36 proteins require specific N-terminal cleavage in order to increase activity in healthy human primary fibroblasts.

Healthy human primary fibroblasts were plated at 20,000 cells per well and stimulated with the IL-36 cleavage products identified previously for 48 hours before levels of IL-8 were analysed by ELISA. **a** – Human primary fibroblasts were stimulated with IL-36α cleavage products (K6 and I7) as well as the full length control. n = 3, independent experiments and different donors. **b** - Human primary fibroblasts were stimulated with IL-36γ cleavage products (Y16, Q17 and S18) as well as the full length control. n = 3, independent experiments and different donors. IL-36β truncated (T) at 100 ng/ml (5.4nM) was used as positive control in both experiments, which is a commercially available IL-36 which has previously been shown to be active. Mean +/- SEM is depicted on all graphs. NS = Non-stimulated. Raw data was analysed using a Kruskal-Wallis test (GraphPad Prism 5.03, GraphPad Software, San Diego, CA). * = $p \leq 0.05$

Healthy human primary fibroblasts were also treated with increasing concentrations (1 nM – 100 nM) of IL-36γ in its full length and cleaved forms, Y16, Q17 and S18 (Figure 5-13 b). Although no statistical significance was shown in the IL-36γ S18 treated samples, it is evident that a dose dependent increase of IL-8 release can be seen following increasing concentrations of IL-36γ S18. A boiled control for IL-36γ S18 was also performed and this confirms the increase in IL-8 is not related to LPS contamination. There was no substantial IL-8 release following treatment with full length or Y16 IL-36γ however a small induction of IL-8 could be detected following treatment with 100 nM of IL-36γ Q17.

In order to establish differences in response to IL-36 in cells derived from psoriatic patients compared to healthy cells psoriatic human primary fibroblasts were also stimulated with IL-36. This was performed in the same manner as previously described with the cleavage products of IL-36α and IL-36γ and then after 48 hours IL-8 concentrations analysed by ELISA. Psoriatic human primary fibroblasts were treated with increasing concentrations (1 nM- 100 nM) of full length and cleavage products (K6 and I7) of IL-36α as well as commercially available IL-36β truncated (T) as a positive control (Figure 5-14 a). Statistically significant increases of IL-8 release were detected following treatment with 50 nM and 100 nM of IL-36α K6. No significant increases in IL-8 were detected following treatment with the full length protein or IL-36α I7. Interestingly, IL-8 expression in response to 50 nM and 100 nM of IL-36α K6 is significantly higher in psoriatic fibroblasts (~3000 pg/ml to ~7000 pg/ml) compared to healthy fibroblasts (Figure 5-14 c). Psoriatic human primary fibroblasts were also treated with full length and cleavage products (Y16, Q17 and S18) of IL-36γ alongside IL-36β truncated (T) as a positive control (Figure 5-14 b). A statistically significant increase in IL-8 release was detected following treatment with both 50 nM and 100 nM of IL-36γ S18. As in the healthy human primary fibroblasts there was little IL-8 release following treatment with IL-36γ full length or Y16. However IL-36γ Q17 did induce some IL-8 which was significantly increased (~200 pg/ml to ~600 pg/ml) compared to the concentration that was detected in healthy human primary fibroblasts. This may be physiologically significant however it is much lower than the IL-8 levels seen with IL-36γ S18. In the same manner as to with the IL-36α K6 the levels of IL-8 released in the psoriatic

compared to healthy human primary fibroblasts was significantly higher (~2000 pg/ml to ~6000 pg/ml) (Figure 5-14 d).

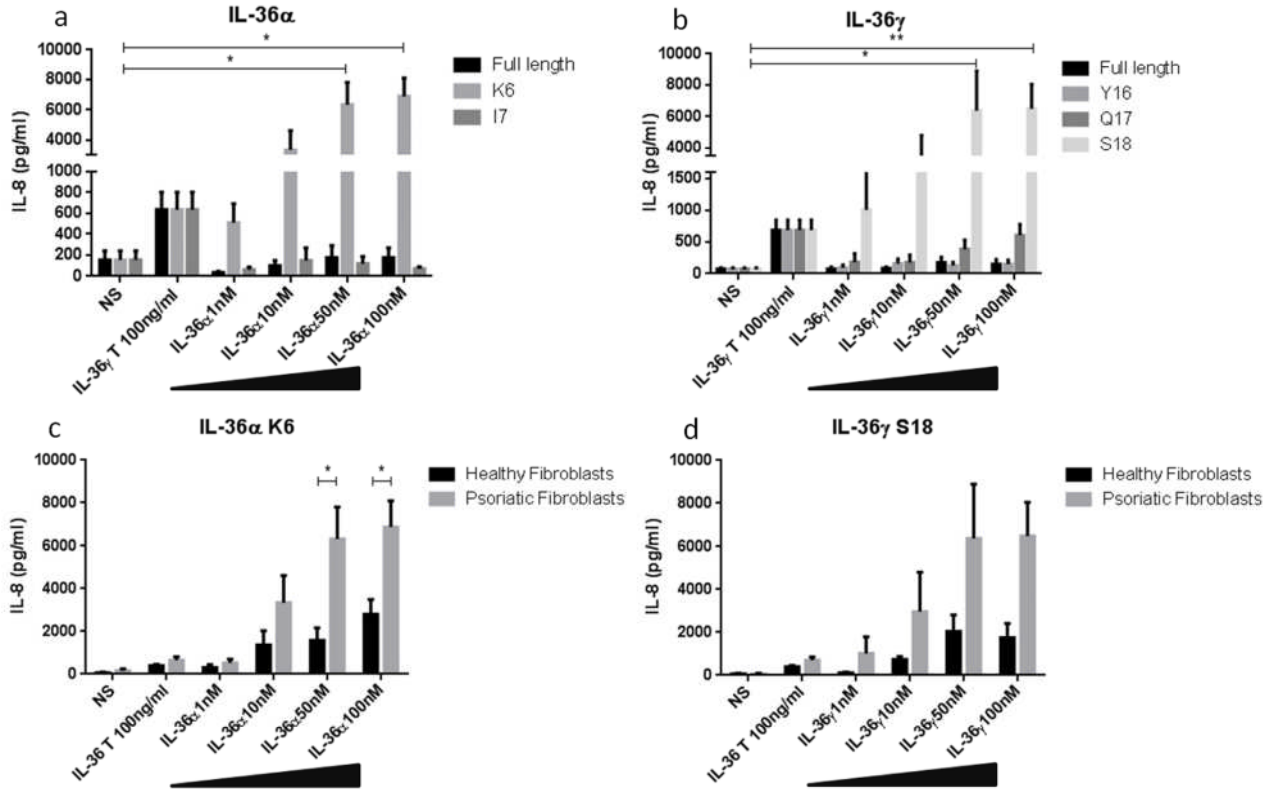


Figure 5 – 14. IL-36 proteins require specific N-terminal cleavage in order to increase activity in psoriatic human primary fibroblasts.

Psoriatic human primary fibroblasts were plated at 20,000 cells per well and stimulated with the IL-36 cleavage products identified previously for 48 hours and levels of IL-8 analysed by ELISA. **a** – Human primary fibroblasts were treated with IL-36α cleavage products (K6 and I7) as well as the full length control. n = 4, independent experiments and different donors. **b** - Human primary fibroblasts were stimulated with IL-36γ cleavage products (Y16, Q17 and S18) as well as the full length control. n = 3, independent experiments and different donors. **c** – Comparison of human primary fibroblasts from both healthy (Figure 5-13 a) and psoriatic donors stimulated with IL-36α K6 (Figure 5-14 a). **d** – Comparison of human primary fibroblasts from both healthy (Figure 5-13 b) and psoriatic (Figure 5-14 b) donors stimulated with IL-36γ S18. IL-36γ truncated (T) at 100 ng/ml (5.4nM) was used as positive control in both experiments (commercially available IL-36). Mean +/- SEM is depicted on all graphs. NS = Non-stimulated. Raw data was analysed using a Kruskal-Wallis test (a+b) and multiple student's T test with Holm-Sidak post-test (GraphPad Prism 5.03, GraphPad Software, San Diego, CA). * = $p \leq 0.05$ ** = $p \leq 0.01$

The activity of the IL-36 proteins appeared to be determined by the removal of a precise number of amino acids from the N-terminus (see Figure 5-14). In order to study this further mutational analysis was performed for both IL-36α K6 and IL-36γ

S18 to assess whether the specific amino acid or the length of the N-terminus was crucial for activity. The N-terminal amino acid was therefore substituted for an alanine. Following amino acid substitution from a lysine (IL-36α K6) or a serine (IL-36γ S18) to an alanine IL-36γ showed no reduction in activity (Figure 5-15) suggestive of the importance of the N-terminal length rather than a specific amino acid. Preliminary data suggests that the same is true for IL-36α (data not shown).

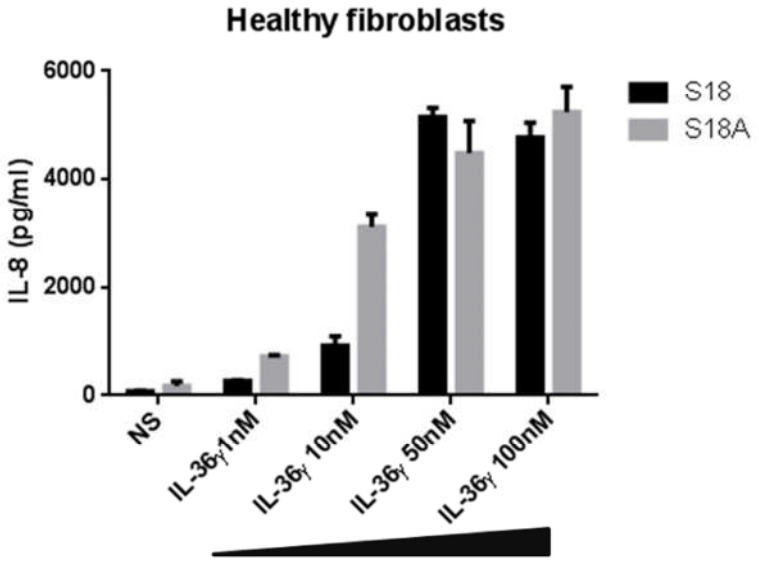


Figure 5-15. IL-36γ requires a specific N-terminal length in order to maintain activity.

Healthy human primary fibroblasts were treated with both IL-36γ S18 and IL-36γ S18A, where the N-terminal serine has been substituted for an alanine. Following 48 hours of stimulation IL-8 levels were detected using ELISA. n = 3, independent experiments and different donors. Mean +/- SEM is depicted on all graphs. NS = Non-stimulated. Preliminary results suggest that these results also reflect what is seen for IL-36α K6.

As mentioned previously RAs are crucial in order to control inflammation. Following cleavage of IL-36RA by the recombinant neutrophil serine protease, elastase, a cleavage product was detected where the methionine was removed from the N-terminus. Also following cleavage by recombinant neutrophil serine proteases and activated PMNs another product was detected with three amino acids removed from the N-terminus (IL-36RA S4). In order to test the efficacy of these differentially cleaved receptor antagonists both healthy and psoriatic human primary fibroblasts were treated of IL-36RA V2 in the presence and absence of commercially available IL-36γ truncated (T) at 100 ng/ml (5.4nM) or the active IL-36γ S18 at 10 nM. In both the healthy and psoriatic human primary fibroblasts the IL-36RA

effectively reduced the IL-36γ T or IL-36γ S18 induced IL-8 release on increasing concentrations of IL-36 V2 (Figure 5-16 a + b). In the healthy human primary fibroblasts complete reduction of IL-36γ T induced IL-8 to non-stimulated levels was seen at 50 nM of IL-36RA V2 (Figure 5-16 a). However, in the psoriatic human primary fibroblasts higher concentrations (100 nM) of IL-36RA V2 were required to reduce IL-8 levels to non-stimulated control, this is likely to be due to the increased responsiveness of these cells (Figure 5-16 b). In contrast to IL-36RA V2 which alone showed no activation of the cells and in combination with IL-36γ truncated (S18) showed antagonistic properties IL-36RA S4 indicated no antagonistic properties (Figure 5-16 c).

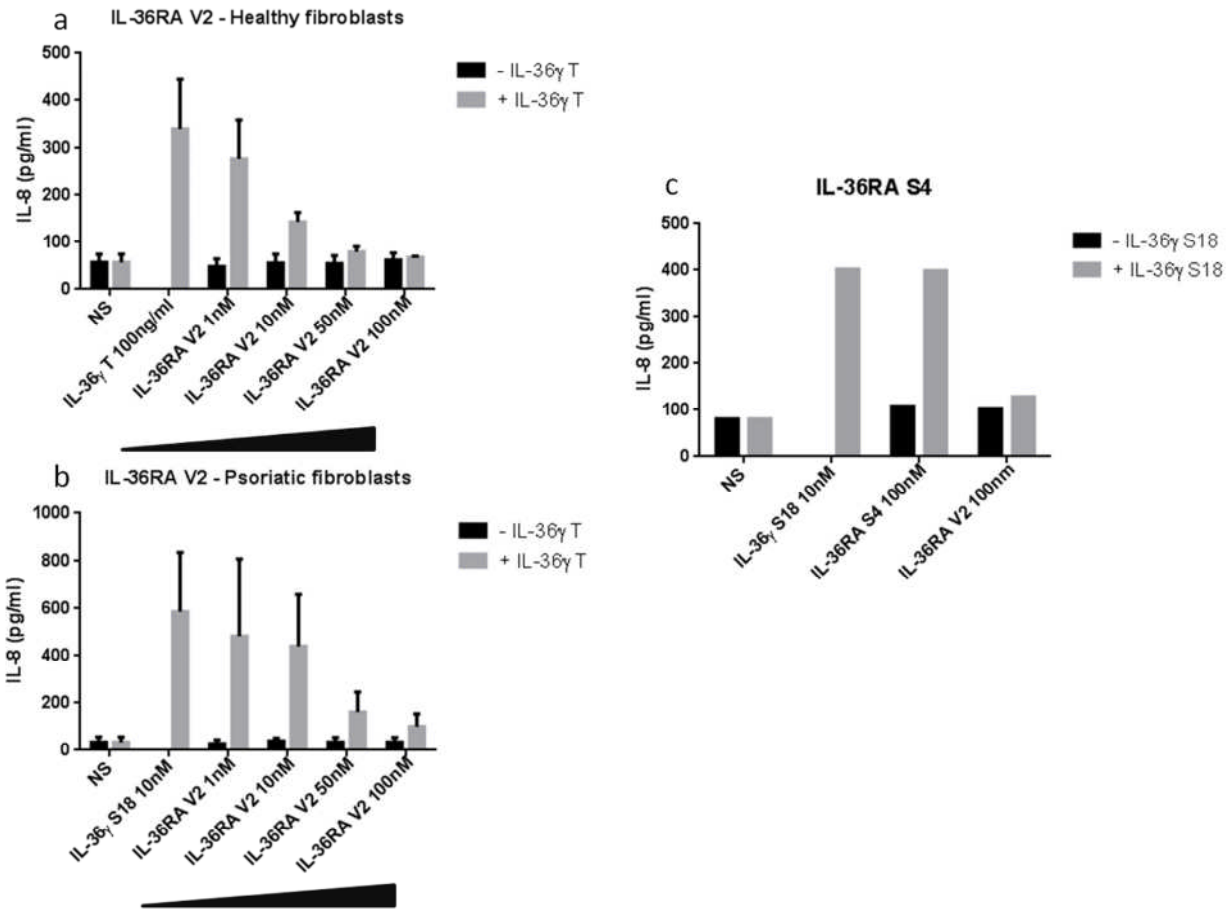


Figure 5 – 16. IL-36 receptor antagonist requires removal of the N-terminal methionine to enable antagonistic properties.

Healthy and psoriatic human primary fibroblasts were plated at 20,000 cells per well and treated with IL-36RA cleavage products (V2 and S4) in the presence and absence of commercially available IL-36γ truncated (T) or IL-36γ S18 at 100ng/ml (5.4nM) or 10nM, which has previously shown to be active. After 48 hours of treatment IL-8 concentrations analysed by ELISA. **a** – Healthy human primary fibroblasts were stimulated with IL-36RA V2 in the presence and absence of commercially available IL-36γ T. n = 3, independent experiments and different donors. **b** - Psoriatic human primary fibroblasts were stimulated with IL-36RA V2 in the presence and absence of active IL-36γ S18. n = 3, independent experiments and different donors. Boiled controls were performed for IL-36RA V2 to rule out LPS contamination (data not shown) **c** – Healthy human primary fibroblasts were treated with 100nM of IL-36RA S4 in the presence and absence of IL-36γ S18. n = 1. Mean +/- SEM is depicted on all graphs. NS = Non-stimulated.

5.9 Response of skin equivalent models to stimulation by IL-36

In 5.8 it was shown that the cleaved IL-36α K6 and IL-36γ S18 induce release of high levels of IL-8 in human primary fibroblasts. However, due to the significance of the IL-36 members in chronic inflammatory skin diseases such as psoriasis it was

presumed that IL-36 may play a more complex role than simply increasing IL-8 release. Therefore in order to study the potential interplay between fibroblasts and keratinocytes in a more physiological setting, 3-D skin equivalents were produced using human primary keratinocytes and fibroblasts derived from both healthy and psoriatic donors.

3-D skin equivalent models were produced using healthy human primary keratinocytes and fibroblasts. These models were cultured for 10 days at the air-liquid interface before addition of 20 ng/ml of IL-22, 100 nM IL-36α full length and IL-36γ full length or 100 nM IL-36α K6 and IL-36γ S18 for a further 96 hours. Following 14 days of culture at the air-liquid interface skin equivalents were fixed with 4% formaldehyde, sectioned and mounted onto microscope slides. Sections were then stained using H&E and stained for the presence of cytokeratin 10 (CK10). Figure 5-17 identifies active IL-36 compared to untreated control and IL-22, which is regarded as an inducer of psoriatic phenotype in skin equivalents [509], IL-36α K6 and IL-36γ S18 indicate a marked change in phenotype (Figure 5-17 a-c). An enlarged epidermis is clearly present however, the CK10 which is a differentiation marker, staining does not suggest that this is related to aberrant differentiation because the CK10 levels look relatively consistent in untreated, IL-22 and IL-36 treated skin equivalent sections.

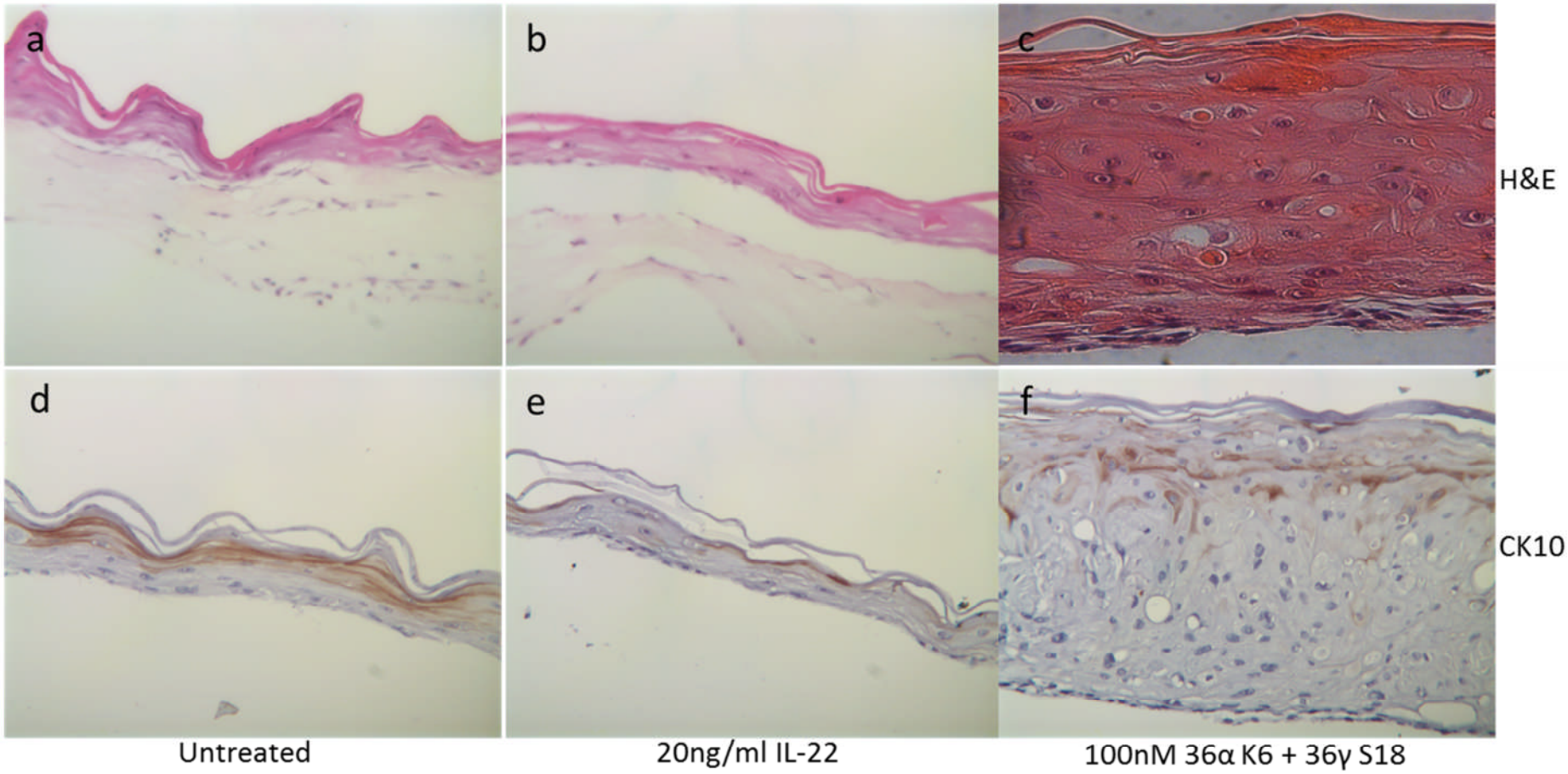

a
b
c
H&E
d
e
f
CK10
Untreated
20ng/ml IL-22
100nM 36α K6 + 36γ S18

Figure 5 – 17. Treatment of skin equivalents constructed with healthy fibroblasts and keratinocytes with IL-36 shows a defined phenotype.

Skin equivalents produced from healthy human primary keratinocytes and fibroblasts were treated for 96 hours with either IL-22 or IL-36α K6 and IL-36γ S18. Following which skin equivalents were fixed in 4% paraformaldehyde, sectioned and stained with H&E or cytokeratin 10 (CK10) using a DAB detection system and counterstained with haematoxylin then imaged using a Leiss light microscope at a 16x objective. Multiple images were taken and a representative image is displayed. Untreated skin equivalent sections were stained using H&E (**a**) and CK10 (**d**). n = 1 experiment. IL-22 (20 ng/ml) treated skin equivalent sections were stained using H&E (**b**) and CK10 (**e**). n = 1 experiment. IL-36α K6 and IL-36γ S18 (100 nM) treated skin equivalent sections were stained using H&E (**c**) and CK10 (**f**) n = 1 experiment.

The images taken of the CK10 stained sections were counterstained with haematoxylin enabling nuclei counting to be performed for each image frame and an average nuclei count to be analysed (Figure 5-18). A statistically significant increase in the number of nuclei was detected following treatment with 100 nM of IL-36α K6 and IL-36γ S18, however an increase in the number of nuclei was also seen in the skin equivalents treated with 100nM of full length IL-36α and IL-36γ.

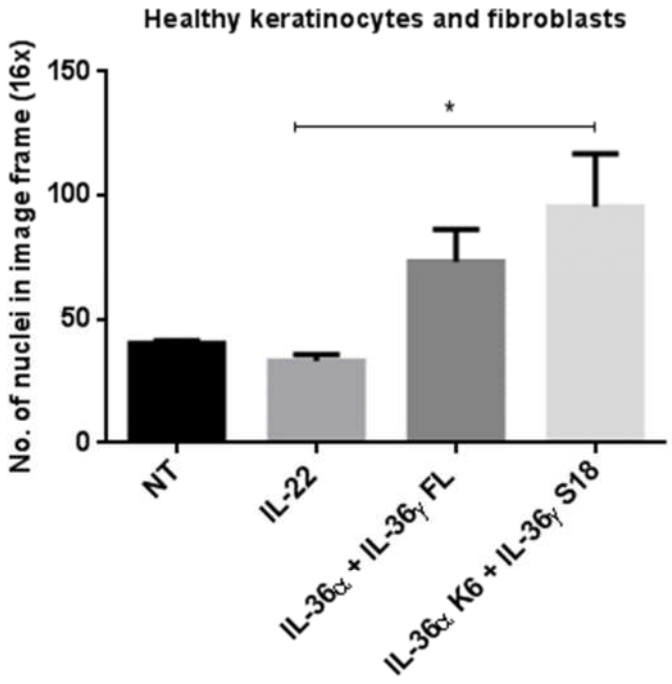


Figure 5 – 18. IL-36 treatment of skin equivalents derived from healthy keratinocytes and fibroblasts induces proliferation in the epidermal layer.

Skin equivalents produced from healthy fibroblasts and keratinocytes were stained with CK10 and counterstained with haematoxylin which allowed quantification of nuclei. Nuclei were counted in each x16 image frame using Image J software. 4 representative images of each skin equivalent were counted and the IL-36α K6 and IL-36γ S18 represented 2 experiments. NT = non-treated. Mean +/- SEM is depicted on the graph. Raw data was analysed using a Kruskal-Wallis test (GraphPad Prism 5.03, GraphPad Software, San Diego, CA). * = $p \leq 0.05$

3-D skin equivalent models were also produced using keratinocytes and fibroblasts from healthy and psoriatic patients to attempt to elucidate the intrinsic roles of skin cells derived from psoriatic patients. However, the results were not conclusive therefore data is not shown.

5.10 IL-38 as a possible IL-36 antagonist in human fibroblasts

IL-38 has been described to have antagonistic properties on IL-36 acting in a similar manner as the IL-36RA [126]. IL-38 proteins were produced in the Champion pET SUMO expression system in the same manner as the IL-36 proteins described in 5.4 and 5.7. Four different forms of IL-38 were produced, one full length and 3 others with amino acids removed from the N-terminus (IL-38 C2, IL-36 S3, IL-38 L4). These forms were produced due to the alignment with other IL-1 members in the same manner as was used for prediction of IL-36 cleavage (see Appendix figure 24 for alignment). The SUMO tag was removed using His-ULP-1 protease before the IL-38 proteins were used to treat human primary cells. In order to test this in a culture system in which the IL-36RA V2 had been shown to be an effective antagonist on IL-36 members (see 5.8), healthy human primary fibroblasts were stimulated with increasing concentrations (1 nM – 100 nM) of full length and cleaved IL-38. This was performed in the presence and absence of 100ng/ml of the commercially available IL-36γ truncated (T) (Figure 5-19). After 48 hours of stimulation IL-8 concentrations analysed by ELISA.

Following treatment with increasing concentrations of full length IL-38 in the absence of IL-36γ T increasing levels of IL-8 were detected and a further increase of IL-8 was seen when IL-38 in combination with IL-36γ T was used (Figure 5-19 a). This suggests that full length IL-38 binds to a receptor on the cells causing downstream signalling and therefore displays an agonist and not antagonistic role. Full length IL-38 was also boiled for 20 minutes before being added to the cells and this confirmed that LPS was not responsible for the increase in IL-8 release (data not shown). When human primary fibroblasts were treated with increasing concentrations of IL-38 C2 in the absence of IL-36γ an increase in IL-8 release was also seen and this was increased further when IL-38 C2 was used in combination with IL-36γ T (Figure 5-19 b). This shows that removing one amino acid from the N-terminus does not enable the antagonistic properties in this experimental set-up. In contrast both IL-38 S3 and IL-38 L4 even at 100 nM in the absence of IL-36γ T did not induce IL-8 (Figure 5-19 c + d). In combination with IL-36γ T IL-38 S3 does still not display any antagonistic properties, however IL-38 L4 in combination with IL-36γ T showed a slight tendency to reduce IL-36γ T induced IL-8 release in some

donors. However, the overall trend is that neither the full length IL-38 nor the cleavage products produced here have an antagonistic role on IL-36.

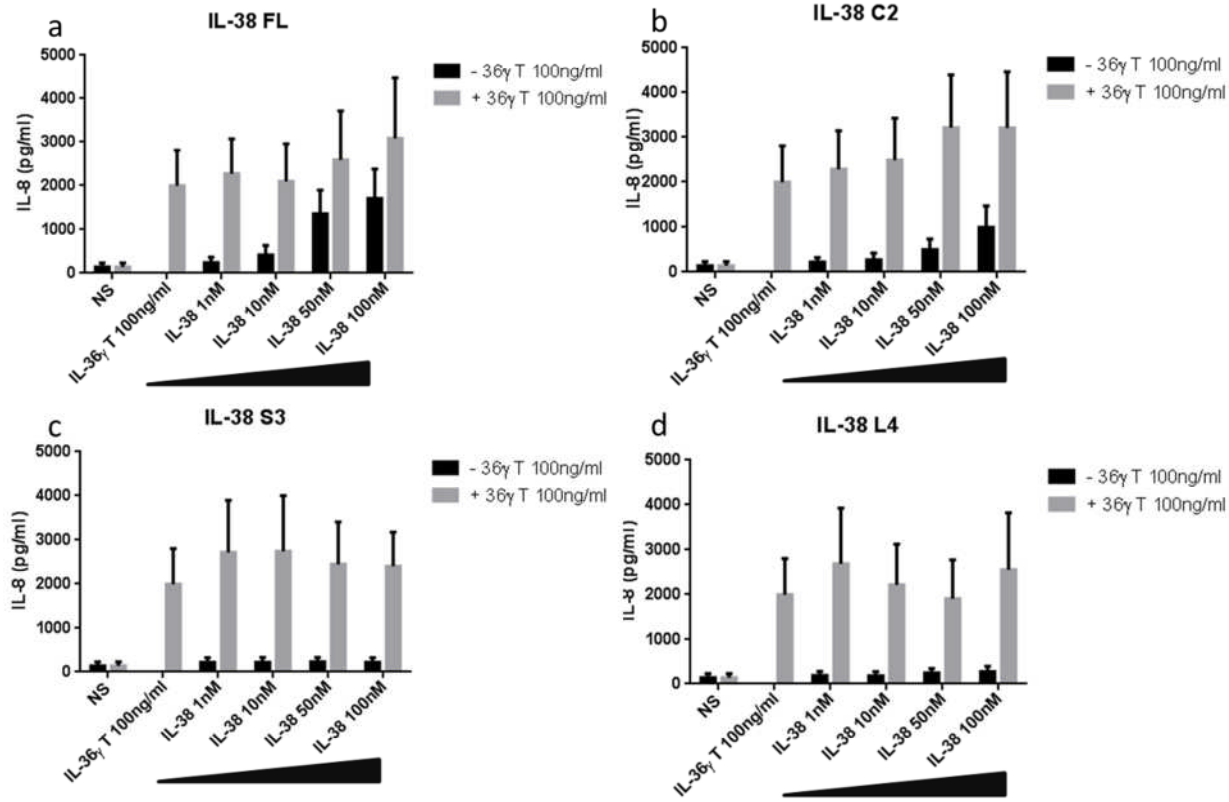


Figure 5 – 19. Both full length IL-38 and cleavage products fail to antagonise IL-36.

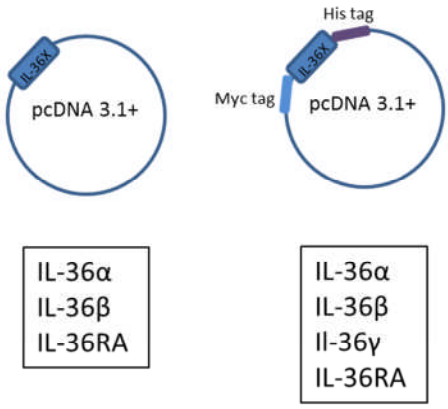
Human primary fibroblasts were plated at 20,000 cells per well treated with cleavage products (C2, S3 and L4) of IL-38 in the presence or absence of a commercially available IL-36γ truncated (T) that is known to be active. 48 hours after stimulation supernatants were removed and IL-8 protein concentrations were assessed by ELISA. **a** – Human primary fibroblasts were stimulated with full length (FL) IL-38 in the presence and absence of IL-36γ T. n = 4, independent experiments and different donors. **b** - Human primary fibroblasts were stimulated with IL-38 C2 in the presence and absence of IL-36γ T. n = 4, independent experiments and different donors. **c** - Human primary fibroblasts were stimulated with IL-38 S3 in the presence and absence of IL-36γ T. n = 4, independent experiments and different donors. **d** - Human primary fibroblasts were stimulated IL-38 L4 in the presence and absence of IL-36γ T. n = 4, independent experiments and different donors. NS = non-stimulated. Mean +/- SEM is depicted on all graphs.

5.11 Mammalian expression of IL-36

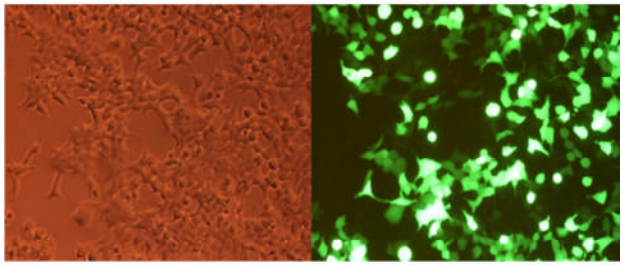
As mentioned previously, the majority of the IL-1 family proteins do not contain a leader sequence, therefore release from the cell has to be via a non-conventional pathway. Caspase-1 is thought to play a role in the release of some IL-1 members

(shown for IL-1α and IL-1β, discussed in 1.4.1) from the cell. Therefore, the cleavage and consequent increased activity of the IL-36 members may occur inside the cell prior to release or it may be released and cleaved into activation outside the cell. In order to study this, a vector, pcDNA 3.1+ (see Appendix figure 3 for vector map), over-expressing the IL-36 members with and without N- (myc) and C-(His) terminal tags was transfected in to a range of mammalian cells (depicted in Figure 5-20 a). The figures depicted in 5-20 are representative but have been performed in all conditions and different cell types. The transfection efficiency was confirmed using a green fluorescent protein (GFP) vector, which identified a good percentage of cells were successfully transfected (Figure 5-20 b). After 24 hours of transfection mRNA was isolated and levels of IL-36 mRNA established using qRT-PCR which confirmed that the plasmid was being successfully transcribed (Figure 5-20 c). However, no IL-36 protein could be detected using the plasmid containing IL-36 alone or in combination with the myc and His tags. This was attempted in various cell lines including 293T, 293TT, 293FT, Cos7 cells as well as primary cells. Optimisation was extensively carried out altering transfection time, transfection reagents and cell confluency. The transfection was also carried out 12 hours prior to the addition of the proteasome (MG132) or lysosome (chloroquine) inhibitors to prevent protein degradation before cells were harvested for protein analysis. Unfortunately, due to the lack of protein seemingly being produced the trafficking and cleavage of IL-36 could not be monitored in a cellular system. Of note, available commercial antibodies also fail to detect intracellular IL-36.

a



b



c

IL-36a	GOI Ct	U6 Ct	Fold change
GFP control - 293T	17.32	16.61	1
IL-36a transfection - 293T	8.84	16.23	275.017
GFP control - Cos7	24.61	15.3	1
IL-36a transfection - Cos7	8.96	15.31	51958.544
IL-36RA			
GFP control - 293T	16.07	16.61	1
IL-36RA transfection - 293T	8.83	15.97	96.539
GFP control - Cos7	25.12	15.3	1
IL-36RA transfection - Cos7	10	15.27	35106.19

Figure 5 – 20. IL-36 is potentially post-translationally regulated.

Various cell lines (293T, 293TT, 293FT and Cos7) and human primary cells were transfected with pcDNA 3.1+ containing IL-36 constructs and mRNA levels and transfection efficiency analysed. **a** – Diagrammatic representation of the pcDNA 3.1+ plasmid constructs used. IL-36α, IL-36β and IL-36RA were cloned into the plasmid without N- and C-terminal tags. All IL-36 products were also cloned into the pcDNA 3.1+ plasmid containing a C-terminal His tag and N-terminal myc tag. **b** – A GFP control plasmid was also transfected in conjunction with the experimental plasmids to assess transfection efficiency. These images represent the transfection efficiency in all cell lines transfected. **c** – 24 hours after transfection mRNA was isolated from the cells and qRT-PCR performed for IL-36α and IL-36RA, Ct values are shown following ΔΔCT-analysis and fold increase is depicted compared to GFP transfection control. U6 – housekeeping gene, GOI = gene of interest. This table represents results for 293T and Cos7 cells, however similar results were identified for all other cell types used.

5.12 Discussion and future work

The IL-36 family of cytokines have only been described relatively recently and there is currently a significant lack of suitable reagents e.g. for protein detection. As a consequence the full IL-36 functional activity, intra- and extracellular trafficking and regulation still needs to be elucidated. This is of clinical relevance due to clear link between subtypes of GPP and an uncontrolled IL-36 activity due to a loss-of-

function mutation in the RA [5]. Numerous studies have also identified that IL-36 is highly up-regulated in lesional psoriatic skin compared to the non-lesional skin in the same patients [222]. Keratinocytes derived from psoriatic patients intrinsically produce more IL-36 in response to IL-17 and TNFα [60]. These clear links with psoriasis suggests that the IL-36 family may be of interest as a therapeutic target. It is consequently crucial to gain insight in mechanisms and pathways leading to IL-36 activation and inactivation.

In this chapter the previously predicted cleavage products that were thought to be the most active using biophysical analysis [128] were tested in human primary skin resident cells. It was confirmed that compared to the full length proteins the cleaved IL-36α, IL-36β and IL-36γ were all significantly more active in their ability to increase IL-8 release (>10 fold increase, see Figure 5-1 - 3) in both keratinocytes and fibroblasts, which is in line with the biophysical predictions. IL-8 is a chemokine that attracts neutrophils, and the significant levels released from skin resident cells may drive the neutrophilic inflammation seen in psoriasis. This effect has also been shown in lung fibroblasts, where treatment of IL-36 induces significant levels of IL-8 [213]. However it was shown that IL-36 had to be used at high concentrations to see an effect, suggestive of the fact that full length proteins were used in these experiments [213]. Results depicted in this chapter demonstrate that the truncated IL-36α caused significantly more IL-8 release compared to truncated IL-36γ. This is in line with finding from Towne *et al*., [128] who analysed the binding affinity of the cleaved products. All IL-36 cleavage products displayed a higher binding affinity compared to the full length protein, however the cleaved IL-36α had a significantly higher binding affinity compared to the cleaved IL-36β and IL-36γ [128]. In this chapter, data was presented in support of human primary fibroblasts being more efficient responders to IL-36 in their production of IL-8 compared to human primary keratinocytes. This is in line with the suggestion that human primary fibroblasts are the predominant responders to IL-36 in the skin whereas the human primary keratinocytes are the significant producers of IL-36 [60].

Of note, in response to the cleaved IL-36 proteins human primary fibroblasts derived from psoriatic patients produced significantly higher levels of IL-8. This is suggestive of the fact that psoriatic cells are intrinsically more responsive to IL-36. This may well be due to increased IL-36R levels on these cells, however preliminary

experiments carried out alongside the work shown in this chapter did not indicate any changes in receptor levels compared to healthy human primary fibroblasts. Due to these findings it is unlikely that differential expression of IL-36 receptor is the cause for increased responsiveness. As previously discussed the IL-36 receptor signals via NF-κB [185]. It is well regarded that in some psoriatic patients the NF-κB pathway is dysregulated due to common genetic mutations (see Table 1-1). These mutations effect negative regulators in this pathway causing potential over activity and consequent increase in pro-inflammatory mediator release [99]. It would be an interesting avenue for further work to assess responsiveness of psoriatic fibroblasts to IL-36 according to the patient's genotype.

The results depicted in this chapter suggest that all the IL-36 members require cleavage to increase binding affinity to the IL-36 receptor in order to see an increase in pro-inflammatory cytokine release downstream of the NF-κB pathway. This work has been focused on elucidating the cleavage of IL-36 proteins into their most bioactive forms. Of note, a newly described neutrophil serine protease, NSP4, was not tested in the panel of recombinant neutrophil serine proteases. This was due to its known low abundance in neutrophil azurophils, NSP4 is released at levels representing only 5% of the cathepsin G released [510]. However, if this neutrophil protease was responsible for cleavage then the active IL-36 products would have been identified following incubation with activated PMN supernatant.

IL-36RA is cleaved in to its active and highly antagonistic form by the recombinant serine protease, elastase. This is an important finding due to the crucial role of receptor antagonists in controlling inflammation and preventing the possibility of chronic inflammation. In contrast the proteases released from activated PMNs and recombinant neutrophil serine proteases cleave the IL-36 agonists (IL-36α and IL-36γ) into a form in which they are inactive. However, the cleavage site identified in this work is in close proximity to the site in which cleavage would lead to the highly active form [128]. This is not consistent with the N-terminal cleavage of IL-1β which can be cleaved at different points within a small range on the N-terminus with no effect to activity [511]. This specific cleavage site required for IL-36 is likely to be related to activation of the active site within the receptor. In this chapter the length of the N-terminus rather than the specific amino acid has been shown to play a crucial in biological activity. In order to establish the specific receptor binding sites

for IL-36 the crystal structure should be solved in complex with its receptors, this would be an important area for further work. A recent publication has reported the crystal structure of IL-36γ [512] and previously the structure of murine IL-36RA had been reported [513]. This crystal structure despite not being co-crystallised with the receptors does allow modelling of IL-36γ with the previously published IL-1 receptor complexes [512]. This is in line with what was shown in Figure 5-15 that the length and not the amino acid on the N-terminus is important for receptor binding. When IL-36γ is modelled in the IL-1 receptors the backbone of the serine at the N-terminus forms a hydrogen bond with an aspartate in the receptor. Therefore, confirming the length of the amino acid is important to form the hydrogen bond and the backbone of any amino acid could form this bond. Any extra amino acids would cause steric hindrance for receptor binding. The structural analysis of IL-36γ and consequent *in vitro* techniques indicated a mutation at amino acid 162 from an alanine to aspartic acid increased binding affinity [512]. This is of interest due to the possibility of a gain-of-function mutation in a subset of psoriatic patients.

The identified IL-36γ cleavage product Q17 did initiate some IL-8 release which was more pronounced in the psoriatic fibroblasts, at a local concentration this could be physiologically relevant. It is known that neutrophils, the large proportion of cells in PMNs, are present at a high density in psoriatic lesions [3]. The data presented in this chapter favours a regulatory role of PMNs in a psoriatic lesion regarding IL-36-mediated inflammation. This is not all together surprising considering the substantial role neutrophils play in the resolution of inflammation (see 1.2.3). The IL-36α and IL-36γ cleavage products, I7 and Y16, Q17 respectively, detected were highly reproducible with the neutrophil proteases and kallikreins tested. This could suggest that these cleavage products are highly physiologically relevant and there is a possibility that another cleavage step is required to cleave IL-36α and IL-36γ into their active form, however this remains to be elucidated. This could however be due to a susceptible free region at the N-terminus present in all IL-36 members as shown by previously published crystal structures [512] [513]. This structural analysis suggests that the N-termini of the IL-36 proteins contain exposed flexible regions that are discrete from the IL-1 domain that could simply make the N-terminus more susceptible to proteolytic processing.

The results depicted in this chapter do however leave the protease responsible for cleavage of the agonist IL-36 members in to their active forms undetermined. The data suggest that proteases released from skin resident cells or from activated PMNs are not, on their own, responsible for processing IL-36 into the fully active form. The lack of detection of the active forms could also potentially indicate that the IL-36 members are in fact not released from the cell in their full length form, which would be in line with other IL-1 family members such as IL-1β and IL-18 (see 1.4.1 for in depth discussion on other IL-1 members) [140]. IL-36 may either be released from the cell in its active form, a cleaved inactive form or its full length form. If it was released in an inactive but cleaved form then cleavage by proteases present in the skin compartment need to act on IL-36 proteins to process it in to its active form outside the cell. However, it may the case, as with IL-1β that following necrotic cell death full length inactive IL-1β is present in the extracellular space and neutrophil serine proteases then cleave it into its active form (see 1.6.3) [342].

There is evidence in murine models to suggest that the full length IL-36 is cleaved outside of the cell rather than prior to release from the cell. Intratracheal administration of full length IL-36α [514] or IL-36γ [210] in to mice induces a significant influx of neutrophils in an IL-1α/IL-1β independent manner and also increases expression of other pro-inflammatory cytokines. In this *in vivo* model it is likely that IL-36 is cleaved by an extracellular protease in order to exert its pro-inflammatory effects. This theory could be tested by producing mutants that are un-cleavable and injecting this protein to compare the pro-inflammatory effects exerted by the mutant and physiologic full length IL-36. Therefore, despite having tested inflammatory cells and skin resident cells for their ability to cleave IL-36 the conditions or microenvironment to enable IL-36 processing may have been overlooked. Psoriasis shows altered protease activity and serine proteases mutations have been identified which are linked with disease susceptibility (see Table 1-1) [99, 515]. Therefore increased protease activity in psoriasis may actually lead to more active IL-36 and play a significant role in disease pathogenesis.

There are many proteases in the skin including MMPs, cysteine and serine proteases (discussed in more detail in 1.6.1) as well as proteases derived from the skin microflora. Experiments presented in this chapter included skin biopsies from both healthy and psoriatic patients that were incubated with IL-36 before the protein was

purified and analysed. However, results from these experiments failed to demonstrate biological active IL-36 forms. This may be due to the incubation period being too long, if the tissue was available then numerous shorter time points could be performed to rule this out and this is a crucial area for further work. One healthy skin biopsy was stimulated with cytokines thought to be important in psoriasis, IL-17 and TNFα, and the other biopsies left untreated including one from a psoriatic donor. Although cleavage was detected following incubation with both treated and untreated skin biopsies, the correct cytokine micro milieu may not have been present in order to produce proteases that could cleave IL-36 into its active form. This reiterates the need for sensitive reagents to detect IL-36 and its active cleaved forms *in vivo* to fully understand the role it plays in disease.

Another possibility for the lack of active form identified is that the cleavage could be occurring by a protease derived from an exogenous source. Skin biopsies were removed from healthy and psoriatic donors and washed extensively with antibiotics to prevent bacterial contamination in culture. However, this means that the potential influence of skin microbiota derived proteases may have been missed. The skin has a diverse microbial flora and in its healthy state contains 10^{12} resident bacteria/m_2, therefore the proteases produced by bacteria must be taken into consideration. Skin relevant bacteria such as *Staphylococci aureus* and *S. epidermidis*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa* produce extracellular proteases [300]. These include serine-, cysteine- and metallo-proteases. Fungi also present a source for proteases in the skin, *Candida albicans* and *Malazessia* are commensal yeasts present on healthy skin without any symptoms. When fungal colonisation becomes pathogenic, proteases are produced as a virulence factor to aid the infective process. For *Candida albicans* these proteases include secreted aspartic proteases (Saps) and may disturb the endogenous balance of protease and protease inhibitor [516]. House dust mite (HDM) presents as a common allergen in many allergic disease, it has been shown that the main allergen from HDM displays protease activity. There are 4 known proteases; *Dermatophagoides pteronyssinus* (Der p), Der p1, Der p3, Der p6, Der p9 and they all require cleavage to facilitate activity. These proteases have been shown to play a role in cleavage of PAR-2 and other membrane associated receptors [517]. Der p1 does have a preference for a serine at its P1' site (MEROPS database), therefore this may well be a target for cleavage of IL-36γ in to its active form. Of

note, it has also been shown in murine models that IL-36γ is up-regulated in response to HDM challenge [518]. All of these possible exogenous proteases may well shift the balance of the endogenous protease – protease inhibitor system and may be related to pathogenesis of inflammatory skin diseases, this could potentially also involve increasing activity of possible mediators of disease such as IL-36. It has been shown that IL-36 is up-regulated in response to live scabies mites in a skin equivalent model [519] and also in various bacterial [520, 521] and fungal infection [126, 522]. It is well known that scabies mites produce significant levels of proteases in order to aid their life cycle [523] and the fungal and bacterial proteases have been discussed previously. Therefore it is possible that these exogenous proteases may play a role in IL-36 cleavage following up-regulation of IL-36 upon infection. Of note, there is rarely infection seen in psoriatic skin lesions and there is reduced microbiome diversity [524]. However in comparison to other chronic inflammatory skin disease there is a unique microbiome which may be relevant to disease pathogenesis [524]. As previously discussed there are many emerging roles of IL-36 in the inflammatory response. From data depicted in this chapter there is no doubt that it plays a role in neutrophil infiltration in to the skin and there is certainly much more to elucidate about this group of cytokines.

A type of tissue immune cells not discussed with regard to IL-36 cleavage are mast cells which are abundant in tissues such as the skin [525]. These cells would have undoubtedly been present in a skin biopsy however they may not have been activated sufficiently in order to degranulate and release their plethora of proteases. It has been shown that in response to LPS mast cells will only release cytokines and no obvious degranulation was detected [526]. Therefore this may be an area for further investigation in the pursuit of the protease responsible for IL-36 cleavage.

IL-36 release from the cell remains to be fully elucidated due to the lack of a leader peptide which is a common feature of all IL-1 family members [527]. There are many studies identifying increases in IL-36 mRNA in various epithelial cell types however there is a significant lack of data with regard to IL-36 protein and in particular IL-36 in the extracellular space. At present the literature suggests that IL-36 is released from the cell following loss of membrane integrity such as pyroptosis [206, 211]. A double activation signal of cells has also been suggested as a mechanism for release of IL-36 in a similar manner to IL-1β [405]. It seems that IL-

36 protein is often detected in the extracellular space following treatment of cells with dsRNA [213] which as observed by Lian *et al*., [211] causing loss of cellular membrane integrity.

In this chapter it was demonstrated that attempts were made to over express IL-36 members in mammalian cell culture in order to try and elucidate the point at which IL-36 cleavage is likely to occur. In order to assess whether the active form was released from the cell an *in vitro* set up with N- and C-terminal tags was required, as described in 5.11. N- and C-terminal tags were required due to the minimal difference in molecular weight it would be difficult to assess the difference between active and inactive forms simply by the mobility on an SDS PAGE gel. The problems encountered whilst attempting to over express IL-36 may be due to the presence of mRNA instability elements. This has been described for other IL-1 family members, IL-18 and IL-37, in which the rapid degradation of mRNA is thought to be a tightly maintained control mechanism utilised to control release of pro-inflammatory cytokines [172]. Another option for detection of cleaved and full length forms would be to produce antibodies specifically against the active cleaved IL-36 forms. This may be difficult to achieve however it would an invaluable tool to identify the different forms of IL-36 found *in vivo*. There is minimal data identifying IL-36 *in vivo*, however IL-36α has been identified in the synovium of patients suffering from rheumatoid arthritis and PsA [225]. At present the commercially available antibodies appear to lack sensitivity and this may therefore be the reason for the paucity in IL-36 protein data. A crucial area for further work will be to identify the cleaved active forms *in vivo* and produce more sensitive reagents to study IL-36.

IL-36 has been shown to be up-regulated in psoriasis as described previously (see 1.4.3.2). However, at present, IL-36 has not been implicated in causing morphological changes in psoriatic plaque. In this chapter a novel function of the active form of IL-36 has been described regarding the hyperproliferation of the epidermal compartment in a 3-D skin equivalent model following 96 hours of cleaved active IL-36 treatment. The induction of a psoriatic like phenotype using pro-inflammatory cytokines has been previously demonstrated in a skin equivalent model using a de-epidermised dermis to seed keratinocytes [509]. IL-22 treatment was used as a control due to its known role in epidermal thickening and psoriatic

pathogenesis [528]. However IL-36 was a much more potent inducer of epidermal enlargement and keratinocyte proliferation. In comparison to the full length IL-36 active cleaved IL-36 induced significantly more epidermal proliferation. However this has also been alluded to in murine models. In a fibroblast growth factor receptor (FGFR) deficient murine model severe barrier defects are seen as well as activation of keratinocytes and T cells, with an increased expression of IL-36β in the epidermis [529]. The relevance of IL-36 in the barrier defects seen was confirmed by injecting recombinant IL-36β intradermally, this showed increased keratinocyte proliferation after 24 hours [529]. Another study has shown that ADAM17, which is downstream of FGFR2, deficient mice show a similar phenotype and at 10 days old mRNA levels of IL-36α and IL-36β are significantly increased [530]. Data shown in this chapter as well as previously published murine models indicates a potential role for IL-36 in the epidermal changes that are characteristically identified in psoriasis. An interesting avenue for further work would be to include IL-36RA along with the active cleaved IL-36 to assess how much of the hyperproliferation could be ameliorated in the skin equivalent cultures. This would then establish whether the hyperproliferation seen was purely IL-36 mediated. It may well be that IL-36 is inducing other mediators, therefore it would be interesting to analyse the supernatants from these skin equivalents and assess levels of pro-inflammatory mediators using a multiple analyte detection method, such as FlowCytomix™. The IL-36 effect may well have been overridden by mediators released from the psoriatic skin resident cells that were not present in the healthy cells, which may be the reason for the limited changes in morphology seen when psoriatic cells were used.

This experimental set-up used both healthy keratinocytes and fibroblasts, however interestingly when psoriatic keratinocytes were used in combination with healthy fibroblasts an increased epidermal compartment was observed. This was regardless of the treatment conditions suggestive of an intrinsic proliferative role of psoriatic keratinocytes. However, it is worth noting these experiments are preliminary and need to be repeated with different donors due to the donor variability that is often seen in primary cultures. In addition the fact that the psoriatic keratinocytes used were of high passage number and therefore in an altered ‘differentiation’ state needs to be taken into consideration. Proliferation was determined in this chapter using nuclei counting, however further work is required to stain for proliferation markers

such as Ki67. Further work is also required to expand the panel of markers used to stain the skin equivalent sections such as cytokeratin 16 which is known to be dysregulated in psoriasis [531], this is required to substantiate the preliminary findings shown in this chapter. However the treatment of the healthy skin equivalent models with active IL-36 was reproduced using cells from two different donors suggestive of this being a reproducible finding. In order to fully replicate psoriatic skin the skin equivalent model would ideally be populated with autologous immune cells that are known to be relevant in psoriasis in a similar manner as described by van den Bogaard *et al*., [532]. It would be of great interest to assess the role of IL-36 in an immune cell populated 3-D skin equivalent model.

IL-38 was also investigated in this chapter due to its potential role as an antagonist of the IL-36 family [126]. The aforementioned study was performed using full length IL-38 and PBMCs, this suggested antagonistic properties on IL-36 however this was shown to be much weaker than IL-36RA. Preliminary work is depicted in this chapter which indicates that IL-38 is not an antagonist for the IL-36 family in human primary fibroblasts. Cleavage products were produced for IL-38 using the same basis that appears to hugely increase the activity of IL-36 (alignment shown in Appendix figure 24). Using the method of counting from the conserved IL-1 domain at the N-terminus correctly identified the active IL-36 products, this method would suggest that IL-38 S3 should be active. However, this could not be verified in the preliminary work depicted here. This may be because IL-8 is not the ideal downstream marker to analyse, therefore a range of pro-inflammatory mediators should be assessed. It is also possible that IL-38 is binding to a different receptor that is not necessarily expressed on human primary fibroblasts but exerts inhibitory effects on the IL-36 downstream signalling in other cells such as SIGIRR, which functions at present are poorly understood.

In conclusion, the truncated active form of IL-36 stimulates significant IL-8 release from skin resident cells which consequently attracts neutrophils into the skin and this IL-8 release is increased further in fibroblasts derived from psoriatic patients. This points to an intrinsic difference of these cells regarding responsiveness to IL-36 and indicates a role for IL-36 in the neutrophilic inflammation seen in psoriasis. The activated neutrophils present in psoriatic skin fail to fully activate the pro-inflammatory IL-36 members and activate the IL-36RA which suggests that

neutrophils could play a regulatory role in the IL-36-mediated inflammation. IL-36 also potentially plays a role in the psoriatic phenotype in which the epidermis is enlarged and keratinocyte proliferation is evident. Therefore, IL-36 may be a pivotal cytokine in psoriatic inflammation and may be an invaluable target for psoriatic therapeutics as well as other chronic inflammatory disorders. However, more work is required to fully elucidate the release, activation and control of this newly described family of cytokines.

Chapter 6 – Discussion

A key problem for treating diseases such as psoriasis and eczema is their tendency to become chronic and the complex disease pathogenesis including different subtypes of disease. It is crucially important to understand processes leading to chronic inflammation and subtypes of disease in order to develop therapeutics that effectively reduce inflammation without severe side effects. There are many suggestions of how chronic inflammation occurs and a complex network of immune cells, surrounding stromal cells as well as the cytokine micro milieu all contribute to the process.

6.1 The balance between pro- and anti-inflammatory mediators

As discussed throughout this work pro-inflammatory cytokine release is always accompanied by expression of regulatory molecules that inhibits the pro-inflammatory function. These control mechanisms are present at various levels including the receptor level. Agonists are prevented from binding to their receptor by receptor antagonists, decoy receptors, binding proteins [187] as well as altering binding capacity of the mediators by proteolytic cleavage. Control mechanisms are also present downstream from the receptor at transcription and translational levels.

There are numerous examples of the importance of regulators within the IL-1 family and much has been learned from monogenetic diseases affecting the inflammasome in which excessive activation of IL-1β leads to episodic fever syndromes such as Muckle Wells [533]. These syndromes are often successfully treated with Anakinra® which is a recombinant IL-1RA which neutralises IL-1 activity. This illustrates the consequences and severe symptoms that ensue when the imbalance between pro- and anti-inflammatory mediators are disturbed. The up-regulation of IL-1RA by a component of *Aspergillus fumigatus* cell wall (galactosaminogalactan) has also been shown to be a virulence factor in *Aspergillus fumigatus* infection [534]. This illustrates the powerful immune modulatory effects these ‘controllers’ of the immune system play.

The newly described subset of IL-1 family members, IL-36, are also crucially controlled by the IL-36RA and loss-of-function mutations in this protein lead to a severe, life-threatening subset of psoriasis, GPP [5, 111]. This, as well as data depicted in chapter 5 indicating the importance of IL-36RA in controlling the pro-inflammatory IL-36 members suggests that a recombinant IL-36RA therapeutic may

be a valid option for treatment of psoriasis. As has been already described, IL-36γ specifically is highly up-regulated in psoriasis therefore controlling these IL-36 members, could be of potential therapeutic benefit.

The IL-1 family consists of one binding protein, IL-18BP. This BP importantly controls the highly pro-inflammatory IL-18. Previously IFNγ was the only known inducer of IL-18BP [450]. In chapter 3 a novel mechanism by which IL-18BP can be up-regulated was described. IL-27 was described to efficiently up-regulate IL-18BP in human primary skin resident cells. Interestingly, levels of IL-18BP were significantly higher in human primary fibroblasts which indicates a potentially more regulatory role of the dermal layer of the skin compared to the highly reactive and pro-inflammatory keratinocytes. One could speculate that some tissue cells are programmed to be more regulatory than pro-inflammatory and work on mesenchymal stromal cells which used dermal fibroblasts as control cells has already delivered hints for the generally more regulatory properties of fibroblasts [535]. The importance of the IL-18/IL-18BP balance is currently of great interest in disease states where IL-18 has been long regarded as highly up-regulated. Recent literature is focused on the importance of this IL-18/IL-18BP balance in cardiac dysfunction [465, 536]. Inefficient up-regulation of IL-18BP to control the increase of IL-18 in diseases such as systemic lupus erythematosus is also thought to be crucial to the chronicity of this disease [537]. IL-18 currently also re-gains increased interest as a key factor in ILC1 polarisation [121, 243]. In chapter 3, IL-27 in addition to hydrocortisone was shown to up-regulate IL-18BP without the induction of the pro-inflammatory chemokine CXCL10. Therefore endogenous induction of IL-18BP using IL-27 in combination with hydrocortisone could be a therapeutic tool to re-address this balance.

6.2 Control of pro-inflammatory mediators by proteases

Pro-inflammatory mediators are also controlled at the transcriptional level and changes in mRNA stability is a well described mechanism by which this occurs. This has been described for both IL-37 and IL-18 within the IL-1 family [172]. Another mechanism by which pro-inflammatory mediators are regulated is by their proteolytic activation. As extensively discussed in this thesis, proteolytic cleavage is important in the control of IL-1 family members. In chapter 5 the importance of

proteolytic cleavage of IL-36 is demonstrated. The results demonstrate that the activity of IL-36 changes dramatically between removal of only one extra amino acid from the N-terminus. This is however different for the related family member IL-1β which can be cleaved into activation at different sites in close proximity on the N-terminus [511].

As previously mentioned, over-activation of caspase-1 seen in cryopyrin-associated periodic fever syndromes leads to excessive IL-1 release and consequently autoinflammation [68, 538]. This may be of relevance in psoriasis in which IL-36 may be the driver of disease for a subset of patients and over-activation of the protease cleaving IL-36 or diminished IL-36RA activity may cause the chronic skin inflammation seen. Given the recent description of DITRA as an autoinflammatory psoriasis subtype and our current knowledge on the IL-36 system, it can be postulated that psoriatic inflammation (even in the absence of the IL-36RA loss-of-function mutation) shows features of autoinflammation and that IL-36 may promote this [538]. Probably due to the limited expression of IL-36 mostly in epithelial tissues and its specific activation requirements it may be unlikely to cause systemic inflammation. Results presented in chapter 5 highlight release of IL-8 from skin resident cells as a functional property of active IL-36. The chemokine IL-8 leads to neutrophilic infiltration into the skin and this is a defined feature of psoriatic histology. Over-activation of a protease and consequent over-activation of IL-36 could be a driver of psoriatic disease however, it is clear that T cell activation and recruitment into the skin is also key to the psoriatic phenotype.

In health there is a balance not only between pro- and anti-inflammatory mediators but also between proteases and protease inhibitors. A reduced activity of protease inhibitors can lead to uncontrolled protease activity, as is seen in Netherton syndrome. A genetic mutation in the serine protease inhibitor Kazal-type 5 (SPINK5) which encodes lympho-epithelial Kazal type inhibitor (LEKTI) 5 leads to chronic skin inflammation and loss of epidermal integrity due to uncontrolled serine protease activity [539]. *In vitro* using SPINK5 knockdown skin equivalents the epidermal thickness and increased tendency for the epidermis to detach from the dermis was reduced when KLK5 and KLK7 were also knocked down. This indicates that kallikrein inhibitors may of therapeutic benefit in this disease [539].

6.3 Possible novel therapeutics in psoriasis

Due to the importance of proteases in the activation of IL-36, protease inhibitors could be of therapeutic value in psoriasis, the benefits of these being that they are more cost effective than biological therapy. Phase I trials have indeed been carried out for cathepsin S inhibitors for treatment of psoriasis. This was performed due to the up-regulation of cathepsin S by psoriatic keratinocytes [540]. At present, protease inhibitors are used extensively in anti-viral therapies. However, in diseases such as asthma where altered levels of MMPs have been reported [541] broad range MMP inhibitors such as tetracycline and doxycycline have been investigated as potential treatment strategies [542]. Once the protease/s that cleaves IL-36 is determined therapeutic approaches could be developed aiming to inhibit IL-36 activation. Doxycycline has also been shown to indirectly inhibit the proteolytic activity of kallikrein-related peptidases [543] as well as other more specific trypsin-like serine protease inhibitors (ε-aminocaproic acid) [544]. These have been suggested for use in rosacea where increased activity of KLK5 has been shown [544]. α1 Antitrypsin deficiency leads to a lack of serine protease inhibition and predisposes to chronic obstructive pulmonary disease and liver disease, treatment of this disease is achieved by readdressing the balance of proteases/protease inhibitors [545]. This could be of relevance if a dysregulation of proteases is identified in relation to IL-36 activity in psoriasis.

Psoriasis is undoubtedly related to uncontrolled pro-inflammatory mediators. Genetic mutations in the NF-κB pathway, namely *A20* and *CARD14* have been linked to the most common form of psoriasis [99], psoriasis vulgaris. This points to a loss of control at the signalling level which leads to high levels of pro-inflammatory mediator expression. Chronic inflammation as seen in rheumatoid arthritis and lupus erythematosus but also skin psoriasis has been shown to have a negative impact on life expectancy, thought to be related to the increased risk of cardiovascular disease. As with rheumatoid arthritis severe psoriasis also presents with co-morbidities [86]. Cardiovascular disease is also thought to be a life limiting factor in severe psoriasis, this is likely due to the uncontrolled systemic inflammation [546]. Importantly, on reducing inflammation this risk is also reduced [547, 548] strongly supporting the view that any state of chronic inflammation should be treated early and efficiently. Rheumatoid arthritis, as other chronic inflammatory diseases such as inflammatory

bowel diseases and psoriasis can often be successfully treated and even cleared with biological therapies. Currently the most widely used biologics block the action of TNFα thereby reducing the uncontrolled inflammation [549]. In most health systems only a minority of severely affected patients will receive biologics due to the enormous costs of this therapy. However, given the emerging view that chronic inflammation may drastically increase the risk of cardiovascular events, health economists may have to re-calculate the long term benefit of efficient and early treatment.

There are many options for treatment of psoriasis ranging from topical anti-inflammatories to systemic biological therapy for more severe disease. However, traditional systemic therapy such as ciclosporin A, methotrexate, acitretin and fumaric acid can cause significant side effects, slow onset or suboptimal efficacy. On the other hand, topical therapy which is often used for localised disease is often not sufficient to clear the skin of psoriatic plaques and has low patient compliance [550]. Therefore, more targeted therapies are required and over the past decade biologic therapy targeting specific cytokine pathways have been developed. Small molecule inhibitors have more recently been developed which target pro-inflammatory signalling pathways and neutralise action of transcription factors such as JAKs [550]. At present, there is an increasing array of FDA approved anti-TNFα humanised monoclonal antibodies (etanercept, adalimumab, infliximab, golimumab) used to successfully treat psoriasis in some patients [551]. The IL-17 pathway is also being targeted for biologic therapies and various neutralising antibodies have been produced against one IL-17 family member, IL-17A (secukinumab, ixekizumab), [552]. However, despite the promising results achieved for patients following treatment with these biologic therapies there have also been severe side effects in some patients. The most prominent issue with blocking cytokine function is the increased risk of serious infection. IL-17A and IL-17F are both crucial for mucocutaneous fungal defence and genetic mutations in the IL-17RA subunit have been shown to lead to recurrent *Candida albicans* infection [553]. There are other risks including immunogenicity of the antibodies and also infusion reactions can occur which has recently been extensively reviewed by Carrascosa *et al*., [554]. There is no doubt that for severely affected patients these biologic therapies have revolutionised psoriasis treatment and there is an ongoing discussion if they should

be utilised early to prevent the ‘psoriatic march’ of co-morbidities [1]. However, for some patients these therapies are not an option due to unavailability (e.g. when disease activity is scored mild or moderate by PASI), side effects or lack of efficacy and therefore other avenues must be developed. It is also well recognised that there is paucity in topical treatment for limited and/or localised disease that would prevent unnecessary side effects in these patients.

In chapter 4 the use of RNA aptamers for topical therapy of inflammatory skin diseases was explored using an RNA aptamer to neutralise IL-17A. Topical therapy is advantageous because it is not associated with the infection risks and other side effects seen in systemic therapy. RNA aptamers are still at the early stages of clinical use, however one RNA aptamer is FDA approved (Macugen®) and many are in clinical trials. In chapter 4 the concept of RNA aptamers as a topical skin therapy was described due to the novel discovery that small RNA molecules can be taken up by keratinocytes without the need for chemical permeabilisation of the cell. This would allow for a new generation of topical therapy that could target specific pro-inflammatory signalling pathways in a similar way to the small molecule inhibitors but without the widespread immune system suppression. However, more work is needed to show whether this approach to target intracellular molecules with RNA apatmers is indeed feasible.

6.4 Conclusion

In conclusion, understanding the mechanisms by which regulatory mechanisms are controlled is crucial in order to fully elucidate chronic inflammation and produce effective specific therapeutics. The focus of this thesis has been on psoriasis which was originally portrayed as simply a skin disease, however recently the disease has been further investigated and in its severe form is now regarded as a systemic disease which may have symptoms in the joints and cardiovascular system as well as some psychological associations. It is therefore important that therapies to treat this life-altering and probably life-expectancy reducing disease are constantly evolving mirroring the progressive treatment options available in rheumatology clinics. In the future, the aim for treatment of psoriatic disease would be to screen for genetic mutations in order to subcategorise patients and assess the best pathway to target. Such stratified medicine approaches are currently being explored in large national

and international initiatives. However, novel ways to target these pathways need to established and this work is continuing, as described in chapter 4. This would prevent the ‘trial and error’ method of treatment and hopefully reduce side effects and increase treatment efficacy for patients.

There also needs to be further work into the concept of biomarkers to specifically distinguish subtypes of psoriasis further than what is currently performed by simply categorising based on clinical phenotype. Currently progress is being made in distinguishing psoriasis from other chronic inflammatory skin diseases using molecular signatures, of which IL-36 has been identified as a marker for psoriasis [555]. However biomarkers for subtypes of psoriasis are still be identified. This would hopefully identify those that require early biologic intervention to prevent psoriatic arthritic joint damage as well as distinguish between other similar chronic inflammatory skin diseases and treat more specifically. As described in this thesis, targeting IL-36, IL-18 or IL-17 pathways topically or systemically may all be of significant clinical benefit if the most suitable patient subgroup for these approaches can be identified.

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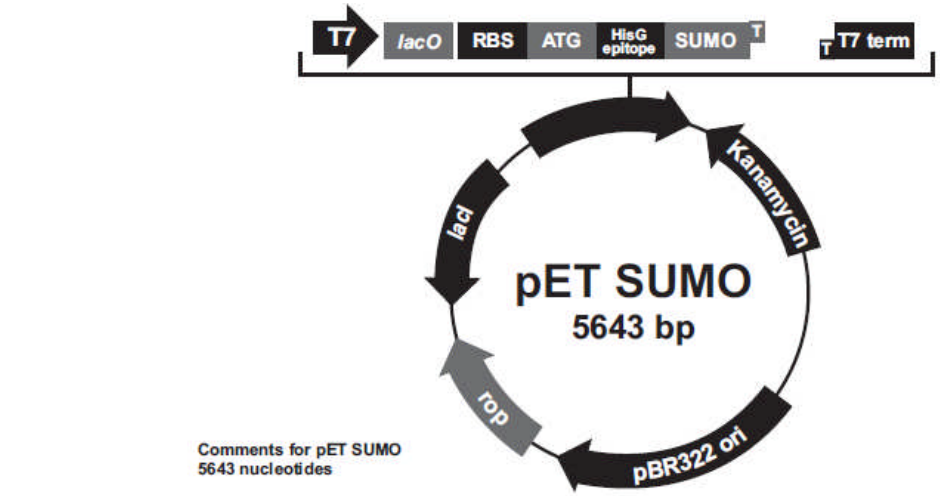
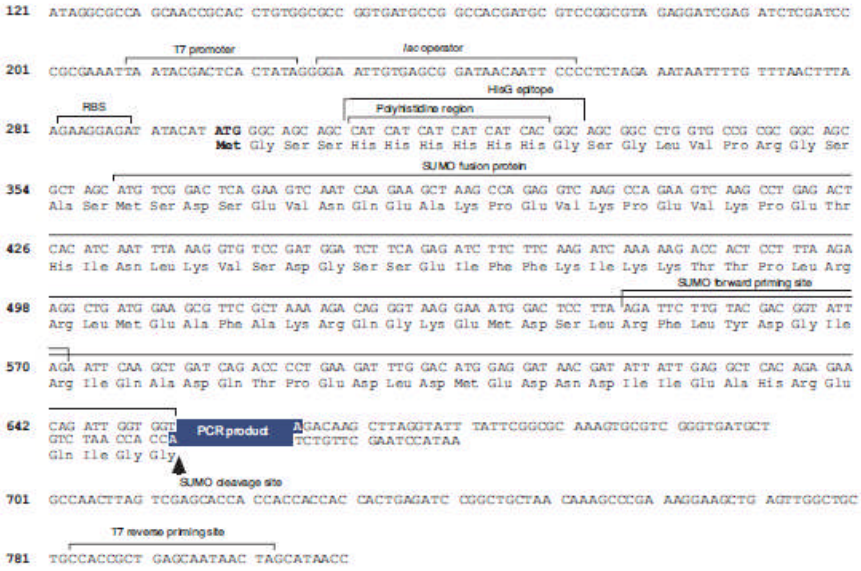
Appendix



Comments for pET SUMO
5643 nucleotides

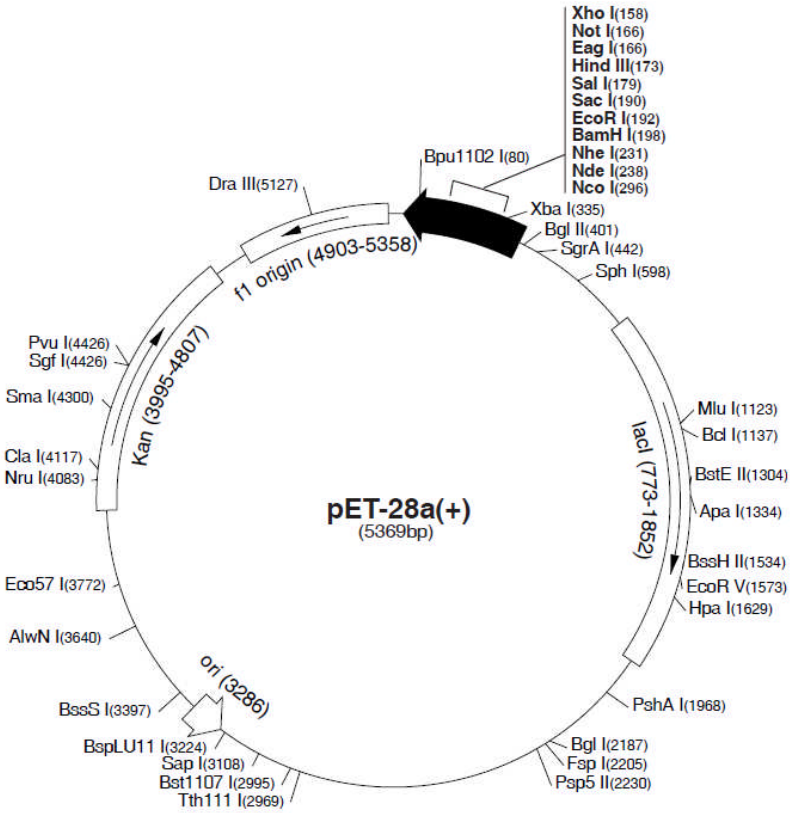
T7 promoter: bases 209-225
lac operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-288
Initiation ATG: bases 297-299
HisG epitope: bases 309-329
SUMO ORF: bases 360-653
SUMO forward priming site: bases 549-571
TA Cloning site: bases 653-654
T7 reverse priming site: bases 783-802 (C)
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)

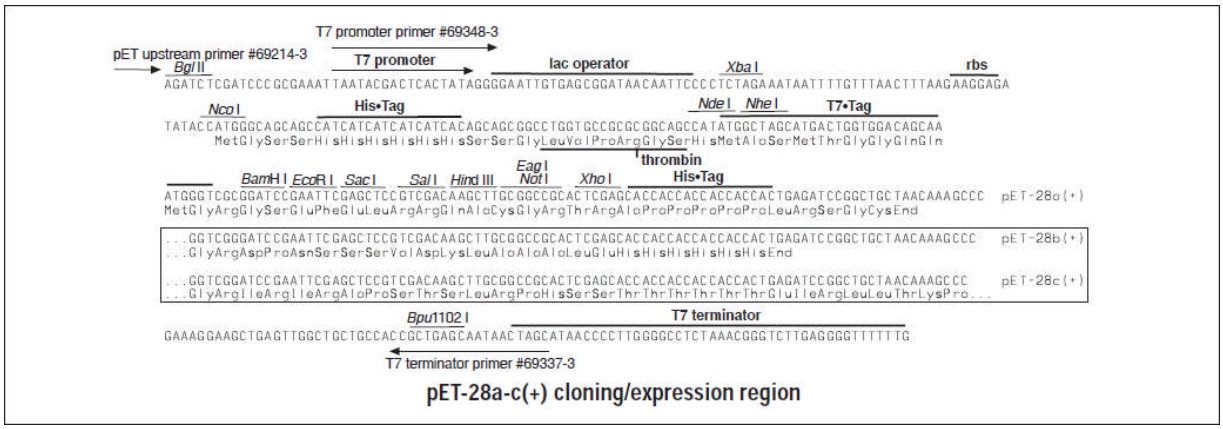
(C) = complementary strand



Appendix Figure 1 - Champion pET SUMO bacterial expression (Invitrogen).

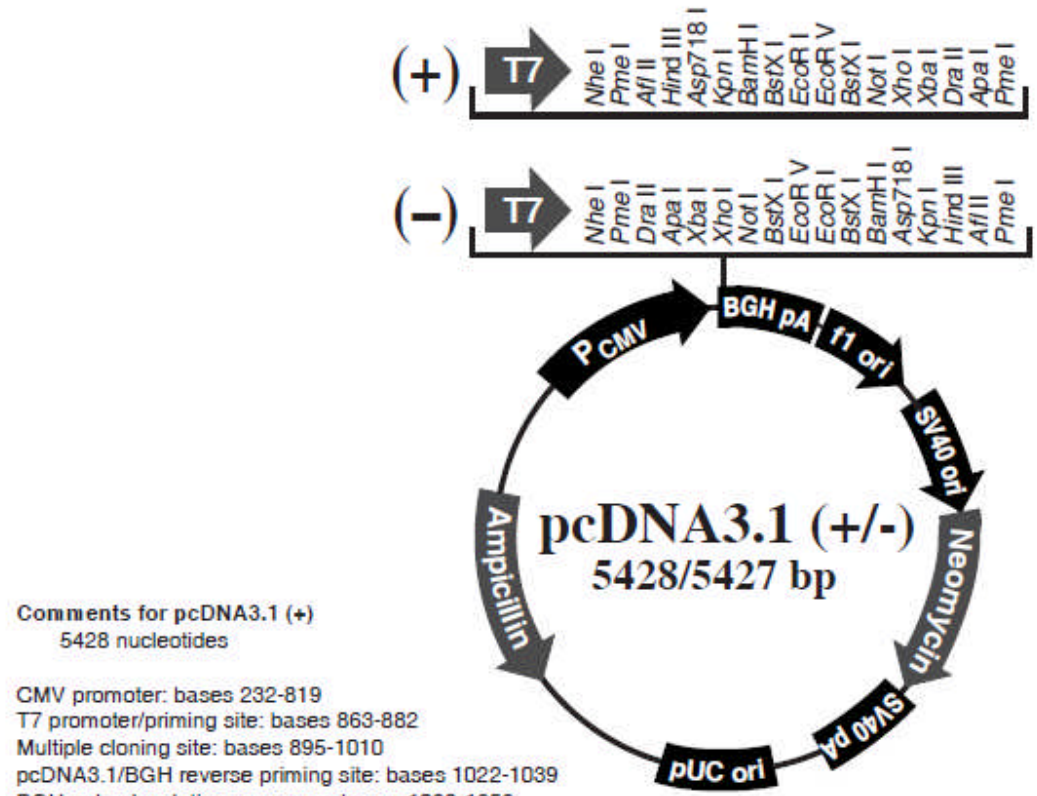
Schematic representation of the vector used for the bacterial expression of IL-36.





Appendix Figure 2 - pET-28a(+) used for bacterial expression (Novagen).

Schematic diagram of the vector used for bacterial expression of IL-36 with N-terminal SUMO (from another vector) and C terminal His.



Comments for pcDNA3.1 (+)
5428 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
pcDNA3.1/BGH reverse priming site: bases 1022-1039
BGH polyadenylation sequence: bases 1028-1252
f1 origin: bases 1298-1726
SV40 early promoter and origin: bases 1731-2074
Neomycin resistance gene (ORF): bases 2136-2930
SV40 early polyadenylation signal: bases 3104-3234
pUC origin: bases 3617-4287 (complementary strand)
Ampicillin resistance gene (*bla*): bases 4432-5428 (complementary strand)
ORF: bases 4432-5292 (complementary strand)
Ribosome binding site: bases 5300-5304 (complementary strand)
bla promoter (P3): bases 5327-5333 (complementary strand)

Appendix Figure 3 - pcDNA3.1+ used for mammalian expression (Invitrogen).

Schematic diagram of vector used for mammalian expression of IL-36 for both untagged and tagged proteins, with tags being incorporated with the primers.

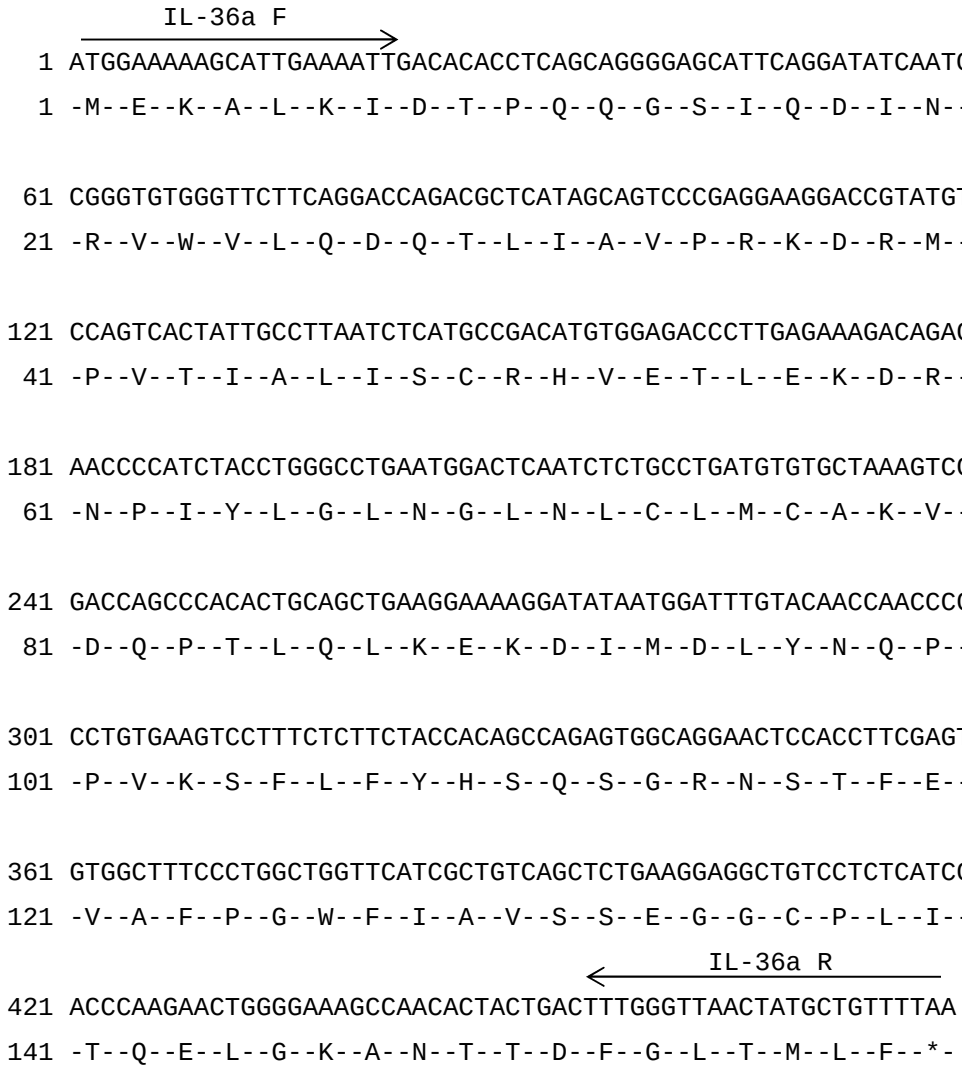
MEKALK IDTPQQGSIQDINHRVWVLQDQTLIAVPRKDRMSPVTIALISCRHVETLEKDRG

NPIYLGLNGLNLCLMCAKVGDQPTLQLKEKDIMDLYNQPEPVKSFLFYHSQSGRNSTFES

VAFPGWFIAVSSEGGCPLILTQELGKANTTDFGLTMLF

Appendix Figure 4 – IL-36α sequence.

IL-36α sequence depicting predicted IL-1 domain (Blue), active form [128] (Red), PMN cleavage products (space).



Appendix Figure 5 - IL-36α – primers for Champion pET SUMO vector.

IL-36α sequence containing primers depicted by arrows – F = forward primer, R = reverse primer. Following successful cloning and transformation into DH5α cells sequences were confirmed by Sanger sequencing.

A

36a F no tag

HindIII

TAGAGAAAGCTTCCACCATGGAAAAAGCATTGAAAATTGACAC

36a R no tag

Not1

TAGAGGCGGCCGCTTAAAACAGCATAGTTAACCCAAAG

B

```
aagcttccacccatggaaaaagcattgaaaattgacacacctcagcag
             M  E  K  A  L  K  I  D  T  P  Q  Q
gggagcattcaggatatcaatcatcgggtgtgggttcttcaggaccagacgctcatagca
 G  S  I  Q  D  I  N  H  R  V  W  V  L  Q  D  Q  T  L  I  A
gtcccgaggaaggaccgtatgtctccagtcactattgccttaatctcatgccgacatgtg
 V  P  R  K  D  R  M  S  P  V  T  I  A  L  I  S  C  R  H  V
gagacccttgagaaagacagagggaaccccatctacctgggcctgaatggactcaatctc
 E  T  L  E  K  D  R  G  N  P  I  Y  L  G  L  N  G  L  N  L
tgcctgatgtgtgctaaagtcggggaccagcccacactgcagctgaaggaaaaggatata
 C  L  M  C  A  K  V  G  D  Q  P  T  L  Q  L  K  E  K  D  I
atggatttgtacaaccaacccgagcctgtgaagtcctttctcttctaccacagccagagt
 M  D  L  Y  N  Q  P  E  P  V  K  S  F  L  F  Y  H  S  Q  S
ggcaggaactccaccttcgagtctgtggctttccctggctggttcatcgctgtcagctct
 G  R  N  S  T  F  E  S  V  A  F  P  G  W  F  I  A  V  S  S
gaaggaggctgtcctctcatccttacccaagaactggggaaagccaacactactgacttt
 E  G  G  C  P  L  I  L  T  Q  E  L  G  K  A  N  T  T  D  F
gggttaactatgctgttttaagcggccgctcgagtctagagggcccgtttaaacccgctg
 G  L  T  M  L  F  -  A  A  A  R  V
```

Appendix Figure 6 – IL-36α primers for mammalian expression with no tags.

A - Primers for pcDNA3.1 (+) vector – no tags. Highlighted red sequence depicts restriction sites used. F = forward and contains a HindIII restriction site and R = reverse and contains a Not1 restriction site. B – Following cloning and transformation into DH5α cells the sequence was verified by Sanger sequencing, sequence depicted. Red highlighted section depicts Kozak sequence and the restriction sites are also highlighted (green – HindIII, blue – Not1).

A

36a F N and C tag

HindIII Myc Kpn1

TAGAGAAGCTTCCACCATGGAGCAGAAACTCATCTCTGAAGAGGATCTGGGTACCATGGAAAAAGCATTGA
AAATTGACAC

36a R N and C tag

Not1 His EcoR1

CTCTAGCGGCCGCTCAGTGATGGTGATGGTGATGGAATTCAAACAGCATAGTTAACCCAAA

B

```
   1 - ACGTGCTGGTTTACTTTAGCTTCACCATGGAGCAGAAACTCATCTCTGAAGAGGATCTGG - 60
   1 -   V  L  V  Y  F  S  F  T  M  E  Q  K  L  I  S  E  E  D  L  G   - 20

  61 - GTACCATGGAAAAAGCATTGAAAATTGACACACCTCAGCAGGGGAGCATTCAGGATATCA - 120
  21 -   T  M  E  K  A  L  K  I  D  T  P  Q  Q  G  S  I  Q  D  I  N   - 40

 121 - ATCATCGGGTGTGGGTTCTTCAGGACCAGACGCTCATAGCAGTCCCGAGGAAGGACCGTA - 180
  41 -   H  R  V  W  V  L  Q  D  Q  T  L  I  A  V  P  R  K  D  R  M   - 60

 181 - TGTCTCCAGTCACTATTGCCTTAATCTCATGCCGACATGTGGAGACCCTTGAGAAAGACA - 240
  61 -   S  P  V  T  I  A  L  I  S  C  R  H  V  E  T  L  E  K  D  R   - 80

 241 - GAGGGAACCCCATCTACCTGGGCCTGAATGGACTCAATCTCTGCCTGATGTGTGCTAAAG - 300
  81 -   G  N  P  I  Y  L  G  L  N  G  L  N  L  C  L  M  C  A  K  V   - 100

 301 - TCGGGGACCAGCCCACACTGCAGCTGAAGGAAAAGGATATAATGGATTTGTACAACCAAC - 360
 101 -   G  D  Q  P  T  L  Q  L  K  E  K  D  I  M  D  L  Y  N  Q  P   - 120

 361 - CCGAGCCTGTGAAGTCCTTTCTCTTCTACCACAGCCAGAGTGGCAGGAACTCCACCTTCG - 420
 121 -   E  P  V  K  S  F  L  F  Y  H  S  Q  S  G  R  N  S  T  F  E   - 140

 421 - AGTCTGTGGCTTTCCCTGGCTGGTTCATCGCTGTCAGCTCTGAAGGAGGCTGTCCTCTCA - 480
 141 -   S  V  A  F  P  G  W  F  I  A  V  S  S  E  G  G  C  P  L  I   - 160

 481 - TCCTTACCCAAGAACTGGGGAAAGCCAACACTACTGACTTTGGGTTAACTATGCTGTTTG - 540
 161 -   L  T  Q  E  L  G  K  A  N  T  T  D  F  G  L  T  M  L  F  E   - 180

 541 - AATTCCATCACCATCACCATCACTGAGCGGCCGCTCGAGTCTAGAGGGCCCGTTTAAACC - 600
 181 -    F  H  H  H  H  H  H  *
```

Appendix Figure 7 – IL-36α primers for mammalian expression with N and C-terminal tags.

A - Primers for pcDNA3.1 (+) vector – N and C-terminal tags. Highlighted red sequence depicts restriction sites used. F = forward and contains a HindIII and Kpn1 restriction site as well as a myc tag (blue). R = reverse and contains a Not1 and EcoR1 restriction site (red) and a His tag (purple). B – Following cloning and transformation into DH5α cells the sequence was verified by Sanger sequencing, sequence depicted. Red highlighted section depicts the restriction sites (Kpn1 and EcoR1) and tags are also highlighted (blue – myc, purple – His).

A

F no his SUMO Nco1

Nco1 SUMO

TAGTAGCCATGGATGTCGGACTCAGAAGTCAATCAAG

B

```
      ATGTCGGACTCAGAAGTCAATCAAGAAGCTAAGCCAGAGGTCAAGCCAGAAGTCAAGCCT - 60
  1 - M  S  D  S  E  V  N  Q  E  A  K  P  E  V  K  P  E  V  K  P   - 20

 61 - GAGACTCACATCAATTTAAAGGTGTCCGATGGATCTTCAGAGATCTTCTTCAAGATCAAA - 120
 21 - E  T  H  I  N  L  K  V  S  D  G  S  S  E  I  F  F  K  I  K   - 40

121 - AAGACCACTCCTTTAAGAAGGCTGATGGAAGCGTTCGCTAAAAGACAGGGTAAGGAAATG - 180
 41 - K  T  T  P  L  R  R  L  M  E  A  F  A  K  R  Q  G  K  E  M   - 60

181 - GACTCCTTAAGATTCTTGTACGACGGTATTAGAATTCAAGCTGATCAGACCCCTGAAGAT - 240
 61 - D  S  L  R  F  L  Y  D  G  I  R  I  Q  A  D  Q  T  P  E  D   - 80

241 - TTGGACATGGAGGATAACGAT
 81 - L  D  M  E  D  N  D

      ATTATTGAGGCTCACAGAGAACAGATTGGTGGTATGGAAAAAGCATTGAAAATTGACAC - 300
 81 -  I  I  E  A  H  R  E  Q  I  G  G  M  E  K  A  L  K  I  D  T   - 100

301 - ACCTCAGCAGGGGAGCATTCAGGATATCAATCATCGGGTGTGGGTTCTTCAGGACCAGAC - 360
101 -  P  Q  Q  G  S  I  Q  D  I  N  H  R  V  W  V  L  Q  D  Q  T   - 120

361 - GCTCATAGCAGTCCCGAGGAAGGACCGTATGTCTCCAGTCACTATTGCCTTAATCTCATG - 420
121 -  L  I  A  V  P  R  K  D  R  M  S  P  V  T  I  A  L  I  S  C   - 140

421 - CCGACATGTGGAGACCCTTGAGAAAGACAGAGGGAACCCCATCTACCTGGGCCTGAATGG - 480
141 -  R  H  V  E  T  L  E  K  D  R  G  N  P  I  Y  L  G  L  N  G   - 160

481 - ACTCAATCTCTGCCTGATGTGTGCTAAAGTCGGGGACCAGCCCACACTGCAGCTGAAGGA - 540
161 -  L  N  L  C  L  M  C  A  K  V  G  D  Q  P  T  L  Q  L  K  E   - 180

541 - AAAGGATATAATGGATTTGTACAACCAACCCGAGCCTGTGAAGTCCTTTCTCTTCTACCA - 600
181 -  K  D  I  M  D  L  Y  N  Q  P  E  P  V  K  S  F  L  F  Y  H   - 200
```

```
601 - CAGCCAGAGTGGCAGGAACTCCACCTTCGAGTCTGTGGCTTTCCCTGGCTGGTTCATCGC - 660
201 -  S  Q  S  G  R  N  S  T  F  E  S  V  A  F  P  G  W  F  I  A   - 220

661 - TGTCAGCTCTGAAGGAGGCTGTCCTCTCATCCTTACCCAAGAACTGGGGAAAGCCAACAC - 720
221 -  V  S  S  E  G  G  C  P  L  I  L  T  Q  E  L  G  K  A  N  T   - 240

721 - TACTGACTTTGGGTTAACTATGCTGTTTGAATTCCATCACCATCACCATCACTGAGCGGC - 780
241 -  T  D  F  G  L  T  M  L  F  E  F  H  H  H  H  H  H  *  A  A   - 260

781 - CGCACTCGAGCACCACCACCACCACCACTGA
```

Appendix Figure 8 – IL-36α primers for N and C-terminal tagged protein bacterial expression.

A - Primers for pET 28 (+) vector using IL-36α in pET expression system as template. Highlighted red sequence depicts restriction sites (Nco1) used. Reverse primers as depicted in Appendix Figure 7. B – Following cloning and transformation into DH5α cells the sequence was verified by Sanger sequencing, sequence depicted. N-terminal Sumo tag is depicted in blue, EcoR1 restriction site (red) and His tag is depicted in purple.

MNPQ**R** EAAPKSYAIR**D**SRQMVWVLSGNSLIAAPLSRSIKPVTLHLIACRDTEFSDKEKGN
MVYLGIKGKDLCLFCAEIQGKPTLQLKLQGSQDNIGKDTCWKLVGIHTCINLDVRESCFM
GTLDQWGIGVGRKKWKSSFQHHHLRKKDKDFSSMRTNIGMPGRM

Appendix Figure 9 – IL-36β sequence.

IL-36β sequence depicting predicted IL-1 domain (Blue), active form [128] (Red), PMN cleavage products (space).

Appendix Figure 10 - IL-36β – primers for Champion pET SUMO vector.

IL-36β sequence containing primers depicted by arrows – F = forward primer, R = reverse primer. Following successful cloning and transformation into DH5α cells sequences were confirmed by Sanger sequencing.

A

```
36b F no tags
       BamH1
TAGAGAGGATCCCCACCATGAACCCACAACGGGAGGCAGCACCC
36b R no tags
        Not1
TCTCTAGCGGCCGCCTACATCCTTCCTGGCATTCCTATGTTG
```

B

```
ggatccccaccatgaacccacaacgg
           M  N  P  Q  R
gaggcagcacccaaatcctatgctattcgtgattctcgacagatggtgtgggtcctgagt
 E  A  A  P  K  S  Y  A  I  R  D  S  R  Q  M  V  W  V  L  S
ggaaattctttaatagcagctcctcttagccgcagcattaagcctgtcactcttcattta
 G  N  S  L  I  A  A  P  L  S  R  S  I  K  P  V  T  L  H  L
atagcctgtagagacacagaattcagtgacaaggaaaagggtaatatggtttacctggga
 I  A  C  R  D  T  E  F  S  D  K  E  K  G  N  M  V  Y  L  G
atcaagggaaaagatctctgtctcttctgtgcagaaattcagggcaagcctactttgcag
 I  K  G  K  D  L  C  L  F  C  A  E  I  Q  G  K  P  T  L  Q
cttaagcttcagggctcccaagataacatagggaaggacacttgctggaaactagttgga
 L  K  L  Q  G  S  Q  D  N  I  G  K  D  T  C  W  K  L  V  G
attcacacatgcataaacctggatgtgagagagagctgcttcatgggaacccttgaccaa
 I  H  T  C  I  N  L  D  V  R  E  S  C  F  M  G  T  L  D  Q
tggggaataggagtgggtagaaagaagtggaagagttcctttcaacatcaccatctcagg
 W  G  I  G  V  G  R  K  K  W  K  S  S  F  Q  H  H  H  L  R
aagaaggacaaagatttctcatccatgcggaccaacataggaatgccaggaaggatgtag
 K  K  D  K  D  F  S  S  M  R  T  N  I  G  M  P  G  R  M  -
gcggccgctcgagtctagagggcccgtttaaacccgctgatcagcctcgactgtgccttc
 A  A  A  R  V
```

Appendix Figure 11 – IL-36β primers for mammalian expression with no tags.

A - Primers for pcDNA3.1 (+) vector – no tags. Highlighted red sequence depicts restriction sites used. F = forward and contains a BamH1 restriction site and R = reverse and contains a Not1 restriction site. B – Following cloning and transformation into DH5α cells the sequence was verified by Sanger sequencing, sequence depicted. Red highlighted section depicts Kozak sequence and the restriction sites are also highlighted (green – BamH1, blue – Not1).

A

36b F N + C

BamH1 Myc Kpn1

TAGAGGGATCCCCACCATGGAGCAGAAACTCATCTCTGAAGAGGATCTGGGTACCATGAACCCACAACGGG
AGGCA

36b R N + C

Not1 His EcoRV

CTCTAGCGGCCGCTCAGTGATGGTGATGGTGATGGATATCCATCCTTCCTGGCATTCC

B

```
   1 - CCATGTACGTTTACTTAGCTTGGTACCGAGCTCGGATCCCCACCATGGAGCAGAAACTCA - 60
   1-   M  Y  V  Y  L  A  W  Y  R  A  R  I  P  T  M  E  Q  K  L  I   - 20

  61 - TCTCTGAAGAGGATCTGGGTACCATGAACCCACAACGGGAGGCAGCACCCAAATCCTATG - 120
  21 -   S  E  E  D  L  G  T  M  N  P  Q  R  E  A  A  P  K  S  Y  A   - 40

 121 - CTATTCGTGATTCTCGACAGATGGTGTGGGTCCTGAGTGGAAATTCTTTAATAGCAGCTC - 180
  41 -   I  R  D  S  R  Q  M  V  W  V  L  S  G  N  S  L  I  A  A  P   - 60

 181 - CTCTTAGCCGCAGCATTAAGCCTGTCACTCTTCATTTAATAGCCTGTAGAGACACAGAAT - 240
  61 -   L  S  R  S  I  K  P  V  T  L  H  L  I  A  C  R  D  T  E  F   - 80

 241 - TCAGTGACAAGGAAAAGGGTAATATGGTTTACCTGGGAATCAAGGGAAAAGATCTCTGTC - 300
  81 -   S  D  K  E  K  G  N  M  V  Y  L  G  I  K  G  K  D  L  C  L   - 100

 301 - TCTTCTGTGCAGAAATTCAGGGCAAGCCTACTTTGCAGCTTAAGCTTCAGGGCTCCCAAG - 360
 101 -   F  C  A  E  I  Q  G  K  P  T  L  Q  L  K  L  Q  G  S  Q  D   - 120

 361 - ATAACATAGGGAAGGACACTTGCTGGAAACTAGTTGGAATTCACACATGCATAAACCTGG - 420
 121 -   N  I  G  K  D  T  C  W  K  L  V  G  I  H  T  C  I  N  L  D   - 140

 421 - ATGTGAGAGAGAGCTGCTTCATGGGAACCCTTGACCAATGGGGAATAGGAGTGGGTAGAA - 480
 141 -   V  R  E  S  C  F  M  G  T  L  D  Q  W  G  I  G  V  G  R  K   - 160

 481 - AGAAGTGGAAGAGTTCCTTTCAACATCACCATCTCAGGAAGAAGGACAAAGATTTCTCAT - 540
 161 -   K  W  K  S  S  F  Q  H  H  H  L  R  K  K  D  K  D  F  S  S   - 180

 541 - CCATGCGGACCAACATAGGAATGCCAAGGATGGATATCCATCACCATCACCATCACTGAG - 600
 181 -   M  R  T  N  I  G  M  P  R  M  D  I  H  H  H  H  H  H  *  A   - 200
```

Appendix Figure 12 – IL-36β primers for mammalian expression with N and C-terminal tags.

A - Primers for pcDNA3.1 (+) vector – N and C-terminal tags. Highlighted red sequence depicts restriction sites used. F = forward and contains a BamH1 and Kpn1 restriction site as well as a myc tag (blue). R = reverse and contains a Not1 and EcoRV restriction site (red) and a His tag (purple). B – Following cloning and transformation into DH5α cells the sequence was verified by Sanger sequencing, sequence depicted. Red highlighted section depicts restriction sites (Kpn1 and EcoRV) tags are also highlighted (blue - myc, purple – His).

A

F no his SUMO Nco1

Nco1 SUMO

TAGTAGCCATGGATGTCGGACTCAGAAGTCAATCAAG

B

```
   1 - ATGTCGGACTCAGAAGTCAATCAAGAAGCTAAGCCAGAGGTCAAGCCAGAAGTCAAGCCT - 60
   1 - M  S  D  S  E  V  N  Q  E  A  K  P  E  V  K  P  E  V  K  P  - 20

  61 - GAGACTCACATCAATTTAAAGGTGTCCGATGGATCTTCAGAGATCTTCTTCAAGATCAAA - 120
  21 - E  T  H  I  N  L  K  V  S  D  G  S  S  E  I  F  F  K  I  K  - 40

 121 - AAGACCACTCCTTTAAGAAGGCTGATGGAAGCGTTCGCTAAAAGACAGGGTAAGGAAATG - 180
  41 - K  T  T  P  L  R  R  L  M  E  A  F  A  K  R  Q  G  K  E  M  - 60

 181 - GACTCCTTAAGATTCTTGTACGACGGTATTAGAATTCAAGCTGATCAGACCCCTGAAGAT - 240
  61 - D  S  L  R  F  L  Y  D  G  I  R  I  Q  A  D  Q  T  P  E  D  - 80

 241 - TTGGACATGGAGGATAACGATATTATTGAGGCTCACAGAGAACAGATTGGTGGTATGAAC - 300
  81 - L  D  M  E  D  N  D  I  I  E  A  H  R  E  Q  I  G  G  M  N  - 100

 301 - CCACAACGGGAGGCAGCACCCAAATCCTATGCTATTCGTGATTCTCGACAGATGGTGTGG - 360
 101 - P  Q  R  E  A  A  P  K  S  Y  A  I  R  D  S  R  Q  M  V  W  - 120

 361 - GTCCTGAGTGGAAATTCTTTAATAGCAGCTCCTCTTAGCCGCAGCATTAAGCCTGTCACT - 420
 121 - V  L  S  G  N  S  L  I  A  A  P  L  S  R  S  I  K  P  V  T  - 140

 421 - CTTCATTTAATAGCCTGTAGAGACACAGAATTCAGTGACAAGGAAAAGGGTAATATGGTT - 480
 141 - L  H  L  I  A  C  R  D  T  E  F  S  D  K  E  K  G  N  M  V  - 160

 481 - TACCTGGGAATCAAGGGAAAAGATCTCTGTCTCTTCTGTGCAGAAATTCAGGGCAAGCCT - 540
 161 - Y  L  G  I  K  G  K  D  L  C  L  F  C  A  E  I  Q  G  K  P  - 180

 541 - ACTTTGCAGCTTAAGCTTCAGGGCTCCCAAGATAACATAGGGAAGGACACTTGCTGGAAA - 600
 181 - T  L  Q  L  K  L  Q  G  S  Q  D  N  I  G  K  D  T  C  W  K  - 200

 601 - CTAGTTGGAATTCACACATGCATAAACCTGGATGTGAGAGAGAGCTGCTTCATGGGAACC - 660
 201 - L  V  G  I  H  T  C  I  N  L  D  V  R  E  S  C  F  M  G  T  - 220

 661 - CTTGACCAATGGGGAATAGGAGTGGGTAGAAAGAAGTGGAAGAGTTCCTTTCAACATCAC - 720
```

```
221 - L  D  Q  W  G  I  G  V  G  R  K  K  W  K  S  S  F  Q  H  H   - 240

721 - CATCTCAGGAAGAAGGACAAAGATTTCTCATCCATGCGGACCAACATAGGAATGCCAGGA - 780
241 - H  L  R  K  K  D  K  D  F  S  S  M  R  T  N  I  G  M  P  G   - 260

781 - AGGATGGATATCCATCACCATCACCATCACTGAGCGGCCGCACTCGAGCACCACCACCAC - 840
261 - R  M  D  I  H  H  H  H  H  H  *  A  A  A
```

Appendix Figure 13 – IL-36β primers for N and C-terminal tagged protein bacterial expression.

A - Primers for pET 28 (+) vector using IL-36α in pET expression system as template. Highlighted red sequence depicts restriction sites (Nco1) used. Reverse primers as depicted in Appendix Figure 7. B – Following cloning and transformation into DH5α cells the sequence was verified by Sanger sequencing, sequence depicted. N-terminal Sumo tag is depicted in blue, EcoR1 restriction site (red) and His tag is depicted in purple.

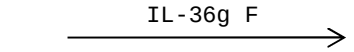
MRGTPGDADGGGRAV Y Q**S**MCKPITGTIN**D**LNQQVWTLQGQNLVAVPRSDSVTPVTVAVIT

CKYPEALEQGRGDPIYLGIQNPEMCLYCEKVGEQPTLQLKEQKIMDLYGQPEPVKPFLFY

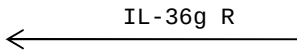
RAKTGRTSTLESVAFPDWFIASSKRDQPIILTSELGKSYNTAFELNIND

Appendix Figure 14 – IL-36γ sequence.

IL-36γ sequence depicting predicted IL-1 domain (Blue), active form [128] (Red), PMN cleavage products (space).




```
  1 ATGAGAGGCACTCCAGGAGACGCTGATGGTGGAGGAAGGGCCGTCTATCAATCAATGTGT
  1 -M--R--G--T--P--G--D--A--D--G--G--G--R--A--V--Y--Q--S--M--C-

 61 AAACCTATTACTGGGACTATTAATGATTTGAATCAGCAAGTGTGGACCCTTCAGGGTCAG
 21 -K--P--I--T--G--T--I--N--D--L--N--Q--Q--V--W--T--L--Q--G--Q-

121 AACCTTGTGGCAGTTCCACGAAGTGACAGTGTGACCCCAGTCACTGTTGCTGTTATCACA
 41 -N--L--V--A--V--P--R--S--D--S--V--T--P--V--T--V--A--V--I--T-

181 TGCAAGTATCCAGAGGCTCTTGAGCAAGGCAGAGGGGATCCCATTTATTTGGGAATCCAG
 61 -C--K--Y--P--E--A--L--E--Q--G--R--G--D--P--I--Y--L--G--I--Q-

241 AATCCAGAAATGTGTTTGTATTGTGAGAAGGTTGGAGAACAGCCCACATTGCAGCTAAAA
 81 -N--P--E--M--C--L--Y--C--E--K--V--G--E--Q--P--T--L--Q--L--K-

301 GAGCAGAAGATCATGGATCTGTATGGCCAACCCGAGCCCGTGAAACCCTTCCTTTTCTAC
101 -E--Q--K--I--M--D--L--Y--G--Q--P--E--P--V--K--P--F--L--F--Y-

361 CGTGCCAAGACTGGTAGGACCTCCACCCTTGAGTCTGTGGCCTTCCCGGACTGGTTCATT
121 -R--A--K--T--G--R--T--S--T--L--E--S--V--A--F--P--D--W--F--I-

421 GCCTCCTCCAAGAGAGACCAGCCCATCATTCTGACTTCAGAACTTGGGAAGTCATACAAC
141 -A--S--S--K--R--D--Q--P--I--I--L--T--S--E--L--G--K--S--Y--N-
```




```
481 ACTGCCTTTGAATTAAATATAAATGACTGA
161 -T--A--F--E--L--N--I--N--D--*-
```

Appendix Figure 15 - IL-36γ – primers for Champion pET SUMO vector.

IL-36γ sequence containing primers depicted by arrows – F = forward primer, R = reverse primer. Following successful cloning and transformation into DH5α cells sequences were confirmed by Sanger sequencing.

A **36g F no tag**

HindIII

TAGAGAAAGCTTCCACCATGAGAGGCACTCCAGGAGACGCTGATG

36g R no tag

Not1

CTCTCAGCGGCCGCTCAGTCATTTATATTTAATTCAAAGGCAGTGTTGTATG

B aagcttccaccatgagaggcactccaggagacgctgatggtggaggaagggccgtctatcaatcaatgtgt

M R G T P G D A D G G G R A V Y Q S M C

aaacctattactgggactattaatgatttgaatcagcaagtgtggacccttcagggtcag

K P I T G T I N D L N Q Q V W T L Q G Q

aaccttgtggcagttccacgaagtgacagtgtgaccccagtcactgttgctgttatcaca

N L V A V P R S D S V T P V T V A V I T

tgcaagtatccagaggctcttgagcaaggcagaggggatcccatttatttgggaatccag

C K Y P E A L E Q G R G D P I Y L G I Q

aatccagaaatgtgtttgtattgtgagaaggttggagaacagcccacattgcagctaaaa

N P E M C L Y C E K V G E Q P T L Q L K

gagcagaagatcatggatctgtatggccaacccgagcccgtgaaacccttccttttctac

E Q K I M D L Y G Q P E P V K P F L F Y

cgtgccaagactggtaggacctccacccttgagtctgtggccttcccggactggttcatt

R A K T G R T S T L E S V A F P D W F I

gcctcctccaagagagaccagcccatcattctgacttcagaacttgggaagtcatacaac

A S S K R D Q P I I L T S E L G K S Y N

actgcctttgaattaaatataaatgactgagcggccgc

T A F E L N I N D -

Appendix Figure 16 – IL-36γ primers for mammalian expression with no tags.

A - Primers for pcDNA3.1 (+) vector – no tags. Highlighted red sequence depicts restriction sites used. F = forward and contains a HindIII restriction site and R = reverse and contains a Not1 restriction site. B – Following cloning and transformation into DH5α cells the sequence was verified by Sanger sequencing, sequence depicted. Red highlighted section depicts Kozak sequence and the restriction sites are also highlighted (green – HindIII, blue – Not1).

A

36g F N + C tag

HindIII Myc Kpn1

TAGAGAAGCTTCCACCATGGAGCAGAAACTCATCTCTGAAGAGGATCTGGGTACCATGAGAGGCACTCCAG
GAGAC

36g R N + C tag

Not1 His EcoR1

TCTCAGCGGCCGCTCAGTGATGGTGATGGTGATGGAATTCGTCATTTATATTTAATTCAAA

B

```
   1 - CCCGGGGTGCGTTTACTTAGCTTCACCATGGAGCAGAAACTCATCTCTGAAGAGGATCTG - 60
   1 - P  G  V  R  L  L  S  F  T  M  E  Q  K  L  I  S  E  E  D  L  - 20

  61 - GGTACCATGAGAGGCACTCCAGGAGACGCTGATGGTGGAGGAAGGGCCGTCTATCAATCA - 120
  21 - G  T  M  R  G  T  P  G  D  A  D  G  G  G  R  A  V  Y  Q  S  - 40

 121 - ATGTGTAAACCTATTACTGGGACTATTAATGATTTGAATCAGCAAGTGTGGACCCTTCAG - 180
  41 - M  C  K  P  I  T  G  T  I  N  D  L  N  Q  Q  V  W  T  L  Q  - 60

 181 - GGTCAGAACCTTGTGGCAGTTCCACGAAGTGACAGTGTGACCCCAGTCACTGTTGCTGTT - 240
  61 - G  Q  N  L  V  A  V  P  R  S  D  S  V  T  P  V  T  V  A  V  - 80

 241 - ATCACATGCAAGTATCCAGAGGCTCTTGAGCAAGGCAGAGGGGATCCCATTTATTTGGGA - 300
  81 - I  T  C  K  Y  P  E  A  L  E  Q  G  R  G  D  P  I  Y  L  G  - 100

 301 - ATCCAGAATCCAGAAATGTGTTTGTATTGTGAGAAGGTTGGAGAACAGCCCACATTGCAG - 360
 101 - I  Q  N  P  E  M  C  L  Y  C  E  K  V  G  E  Q  P  T  L  Q  - 120

 361 - CTAAAAGAGCAGAAGATCATGGATCTGTATGGCCAACCCGAGCCCGTGAAACCCTTCCTT - 420
 121 - L  K  E  Q  K  I  M  D  L  Y  G  Q  P  E  P  V  K  P  F  L  - 140

 421 - TTCTACCGTGCCAAGACTGGTAGGACCTCCACCCTTGAGTCTGTGGCCTTCCCGGACTGG - 480
 141 - F  Y  R  A  K  T  G  R  T  S  T  L  E  S  V  A  F  P  D  W  - 160

 481 - TTCATTGCCTCCTCCAAGAGAGACCAGCCCATCATTCTGACTTCAGAACTTGGGAAGTCA - 540
 161 - F  I  A  S  S  K  R  D  Q  P  I  I  L  T  S  E  L  G  K  S  - 180

 541 - TACAACACTGCCTTTGAATTAAATATAAATGACGAATTCCATCATCATCATCATCATTGA - 600
 181 - Y  N  T  A  F  E  L  N  I  N  D  E  F  H  H  H  H  H  H  *  - 200
```

Appendix Figure 17 – IL-36γ primers for mammalian expression with N and C-terminal tags.

A - Primers for pcDNA3.1 (+) vector – N and C-terminal tags. Highlighted red sequence depicts restriction sites used. F = forward and contains a HindIII and Kpn1 restriction site as well as a myc tag (blue). R = reverse and contains a Not1 and EcoR1 restriction site (red) and a His tag (purple). B – Following cloning and transformation into DH5α cells the sequence was verified by Sanger sequencing, sequence depicted. Red highlighted section depicts restriction sites (Kpn1 and EcoR1) tags are also highlighted (blue - myc, purple – His).

A

F no his SUMO Nco1

Nco1 SUMO

TAGTAGCCATGGATGTCGGACTCAGAAGTCAATCAAG

B

```
   1 - GGTGACGAATTCCCTCTAAATAATTTTGTTTACTTTAAGAAGGAGATATACCATGGATGT - 60
   1 -   *  R  I  P  S  K  *  F  C  L  L  *  E  G  D  I  P  W  M  S  - 20

  61 - CGGACTCAGAAGTCAATCAAGAAGCTAAGCCAGAGGTCAAGCCAGAAGTCAAGCCTGAGA - 120
  21 -   D  S  E  V  N  Q  E  A  K  P  E  V  K  P  E  V  K  P  E  T  - 40

 121 - CTCACATCAATTTAAAGGTGTCCGATGGATCTTCAGAGATCTTCTTCAAGATCAAAAAGA - 180
  41 -   H  I  N  L  K  V  S  D  G  S  S  E  I  F  F  K  I  K  K  T  - 60

 181 - CCACTCCTTTAAGAAGGCTGATGGAAGCGTTCGCTAAAAGACAGGGTAAGGAAATGGACT - 240
  61 -   T  P  L  R  R  L  M  E  A  F  A  K  R  Q  G  K  E  M  D  S  - 80

 241 - CCTTAAGATTCTTGTACGACGGTATTAGAATTCAAGCTGATCAGACCCCTGAAGATTTGG - 300
  81 -   L  R  F  L  Y  D  G  I  R  I  Q  A  D  Q  T  P  E  D  L  D  - 100

 301 - ACATGGAGGATAACGATATTATTGAGGCTCACAGAGAACAGATTGGTGGTATGAGAGGCA - 360
 101 -   M  E  D  N  D  I  I  E  A  H  R  E  Q  I  G  G  M  R  G  T  - 120

 361 - CTCCAGGAGACGCTGATGGTGGAGGAAGGGCCGTCTATCAATCAATGTGTAAACCTATTA - 420
 121 -   P  G  D  A  D  G  G  G  R  A  V  Y  Q  S  M  C  K  P  I  T  - 140

 421 - CTGGGACTATTAATGATTTGAATCAGCAAGTGTGGACCCTTCAGGGTCAGAACCTTGTGG - 480
 141 -   G  T  I  N  D  L  N  Q  Q  V  W  T  L  Q  G  Q  N  L  V  A  - 160

 481 - CAGTTCCACGAAGTGACAGTGTGACCCCAGTCACTGTTGCTGTTATCACATGCAAGTATC - 540
 161 -   V  P  R  S  D  S  V  T  P  V  T  V  A  V  I  T  C  K  Y  P  - 180

 541 - CAGAGGCTCTTGAGCAAGGCAGAGGGGATCCCATTTATTTGGGAATCCAGAATCCAGAAA - 600
 181 -   E  A  L  E  Q  G  R  G  D  P  I  Y  L  G  I  Q  N  P  E  M  - 200

 601 - TGTGTTTGTATTGTGAGAAGGTTGGAGAACAGCCCACATTGCAGCTAAAAGAGCAGAAGA - 660
 201 -   C  L  Y  C  E  K  V  G  E  Q  P  T  L  Q  L  K  E  Q  K  I  - 220

 661 - TCATGGATCTGTATGGCCAACCCGAGCCCGTGAAACCCTTCCTTTTCTACCGTGCCAAGA - 720
```

```
221 -   M  D  L  Y  G  Q  P  E  P  V  K  P  F  L  F  Y  R  A  K  T   - 240

721 - CTGGTAGGACCTCCACCCTTGAGTCTGTGGCCTTCCCGGACTGGTTCATTGCCTCCTCCA - 780
241 -   G  R  T  S  T  L  E  S  V  A  F  P  D  W  F  I  A  S  S  K   - 260

781 - AGAGAGACCAGCCCATCATTCTGACTTCAGAACTTGGGAAGTCATACAACACTGCCTTTG - 840
261 -   R  D  Q  P  I  I  L  T  S  E  L  G  K  S  Y  N  T  A  F  E   - 280

841 - AATTAAATATAAATGACGAATTCCATCATCATCATCATCATTGAGCGGCCGCACTCGAGC - 900
281 -   L  N  I  N  D  E  F  H  H  H  H  H  H  *  A  A  A  L  E  H   - 300
```

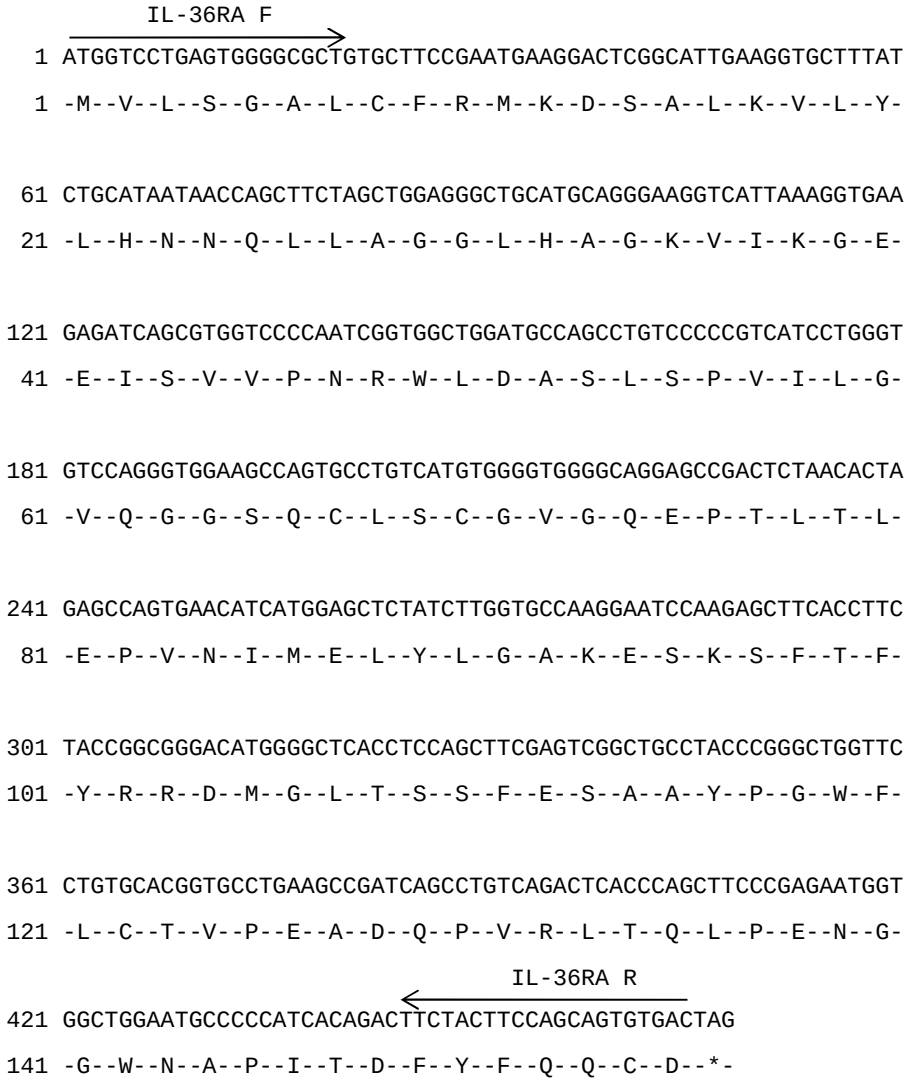
Appendix Figure 18 – IL-36γ primers for N and C-terminal tagged protein bacterial expression.

A - Primers for pET 28 (+) vector using IL-36α in pET expression system as template. Highlighted red sequence depicts restriction sites (Nco1) used. Reverse primers as depicted in Appendix Figure 7. B – Following cloning and transformation into DH5α cells the sequence was verified by Sanger sequencing, sequence depicted. N-terminal Sumo tag is depicted in blue, EcoR1 restriction site (red) and His tag is depicted in purple.

M **V**L SGALCFRMK**D**SALKVLYLHNNQLLAGGLHAGKVIKGEEISVVPNRWLDASLSPVILG
VQGGSQCLSCGVGQEPTLTLEPVNIMELYLGAKESKSFTFYRRDMGLTSSFESAAYPGWF
LCTVPEADQPVRLTQLPENGGWNAPITDFYFQQCD

Appendix Figure 19 – IL-36RA sequence.

IL-36RA sequence depicting predicted IL-1 domain (Blue), active form [128] (Red), PMN cleavage products (space).



Appendix Figure 20 - IL-36RA – primers for Champion pET SUMO vector.

IL-36RA sequence containing primers depicted by arrows – F = forward primer, R = reverse primer. Following successful cloning and transformation into DH5α cells sequences were confirmed by Sanger sequencing.

A

36RA F no tags

HindIII

TAGAGAAAGCTTCCACCATGGTCCTGAGTGGGGCGCTGTGC

36RA R no tags

Not1

TCTCTAGCGGCCGCCTAGTCACACTGCTGGAAGTAGAAGTC

B

```
aagcttcaccatggtcctgagtggggcgctgtgcttccgaatgaag
          M  V  L  S  G  A  L  C  F  R  M  K
gactcggcattgaaggtgctttatctgcataataaccagcttctagctggagggctgcat
 D  S  A  L  K  V  L  Y  L  H  N  N  Q  L  L  A  G  G  L  H
gcagggaaggtcattaaaggtgaagagatcagcgtggtccccaatcggtggctggatgcc
 A  G  K  V  I  K  G  E  E  I  S  V  V  P  N  R  W  L  D  A
agcctgtcccccgtcatcctgggtgtccagggtggaagccagtgcctgtcatgtggggtg
 S  L  S  P  V  I  L  G  V  Q  G  G  S  Q  C  L  S  C  G  V
gggcaggagccgactctaacactagagccagtgaacatcatggagctctatcttggtgcc
 G  Q  E  P  T  L  T  L  E  P  V  N  I  M  E  L  Y  L  G  A
aaggaatccaagagcttcaccttctaccggcgggacatggggctcacctccagcttcgag
 K  E  S  K  S  F  T  F  Y  R  R  D  M  G  L  T  S  S  F  E
tcggctgcctacccgggctggttcctgtgcacggtgcctgaagccgatcagcctgtcaga
 S  A  A  Y  P  G  W  F  L  C  T  V  P  E  A  D  Q  P  V  R
ctcacccagcttcccgagaatggtggctggaatgcccccatcacagacttctacttccag
 L  T  Q  L  P  E  N  G  G  W  N  A  P  I  T  D  F  Y  F  Q
cagtgtgactaggcggccgctcgagtctagagggcccgtttaaacccgctgatcagcctc
 Q  C  D  -  A  A  A  R  V
```

Appendix Figure 21 – IL-36RA primers for mammalian expression with no tags.

A - Primers for pcDNA3.1 (+) vector – no tags. Highlighted red sequence depicts restriction sites used. F = forward and contains a BamH1 restriction site and R = reverse and contains a Not1 restriction site. B – Following cloning and transformation into DH5α cells the sequence was verified by Sanger sequencing, sequence depicted. Red highlighted section depicts Kozak sequence and the restriction sites are also highlighted (green – HindIII, blue – Not1).

A

36RA F N and C tags

HindIII Myc Kpn1

TAGAGAAGCTTCCACCATGGAGCAGAAACTCATCTCTGAAGAGGATCTGGGTACCATGGTCCTGAGTGGGG
CGCTG

36RA R N and C tags

Not1 His EcoR1

TCTCAGCGGCCGCTCAGTGATGGTGATGGTGATGGAATTCGTCACACTGCTGGAAGTAGAA

B

```
   1 - CCGTGTAGGTTTACTTAGCTTCCACCATGGAGCAGAAACTCATCTCTGAAGAGGATCTGG - 60
   1 -   V  *  V  Y  L  A  S  T  M  E  Q  K  L  I  S  E  E  D  L  G   - 20

  61 - GTACCATGGTCCTGAGTGGGGCGCTGTGCTTCCGAATGAAGGACTCGGCATTGAAGGTGC - 120
  21 -   T  M  V  L  S  G  A  L  C  F  R  M  K  D  S  A  L  K  V  L   - 40

 121 - TTTATCTGCATAATAACCAGCTTCTAGCTGGAGGGCTGCATGCAGGGAAGGTCATTAAAG - 180
  41 -   Y  L  H  N  N  Q  L  L  A  G  G  L  H  A  G  K  V  I  K  G   - 60

 181 - GTGAAGAGATCAGCGTGGTCCCCAATCGGTGGCTGGATGCCAGCCTGTCCCCCGTCATCC - 240
  61 -   E  E  I  S  V  V  P  N  R  W  L  D  A  S  L  S  P  V  I  L   - 80

 241 - TGGGTGTCCAGGGTGGAAGCCAGTGCCTGTCATGTGGGGTGGGGCAGGAGCCGACTCTAA - 300
  81 -   G  V  Q  G  G  S  Q  C  L  S  C  G  V  G  Q  E  P  T  L  T   - 100

 301 - CACTAGAGCCAGTGAACATCATGGAGCTCTATCTTGGTGCCAAGGAATCCAAGAGCTTCA - 360
 101 -   L  E  P  V  N  I  M  E  L  Y  L  G  A  K  E  S  K  S  F  T   - 120

 361 - CCTTCTACCGGCGGGACATGGGGCTCACCTCCAGCTTCGAGTCGGCTGCCTACCCGGGCT - 420
 121 -   F  Y  R  R  D  M  G  L  T  S  S  F  E  S  A  A  Y  P  G  W   - 140

 421 - GGTTCCTGTGCACGGTGCCTGAAGCCGATCAGCCTGTCAGACTCACCCAGCTTCCCGAGA - 480
 141 -   F  L  C  T  V  P  E  A  D  Q  P  V  R  L  T  Q  L  P  E  N   - 160

 481 - ATGGTGGCTGGAATGCCCCCATCACAGACTTCTACTTCCAGCAGTGTGACGAATTCCATC - 540
 161 -   G  G  W  N  A  P  I  T  D  F  Y  F  Q  Q  C  D  E  F  H  H   - 180

 541 - ACCATCACCATCACTGAGCGGCCGCTCGAGTCTAGAGGGCCCGTTTAAACCCGCTGATCA - 600
 181 -   H  H  H  H  *
```

Appendix Figure 22 – IL-36RA primers for mammalian expression with N and C-terminal tags.

A - Primers for pcDNA3.1 (+) vector – N and C-terminal tags. Highlighted red sequence depicts restriction sites used. F = forward and contains a HindIII and Kpn1 restriction site as well as a myc tag (blue). R = reverse and contains a Not1 and EcoR1 restriction site (red) and a His tag (purple). B – Following cloning and transformation into DH5α cells the sequence was verified by Sanger sequencing, sequence depicted. Red highlighted section depicts restriction sites (Kpn1 and EcoR1) tags are also highlighted (blue - myc, purple – His).

A

F no his SUMO Nco1

Nco1 SUMO

TAGTAGCCATGGATGTCGGACTCAGAAGTCAATCAAG

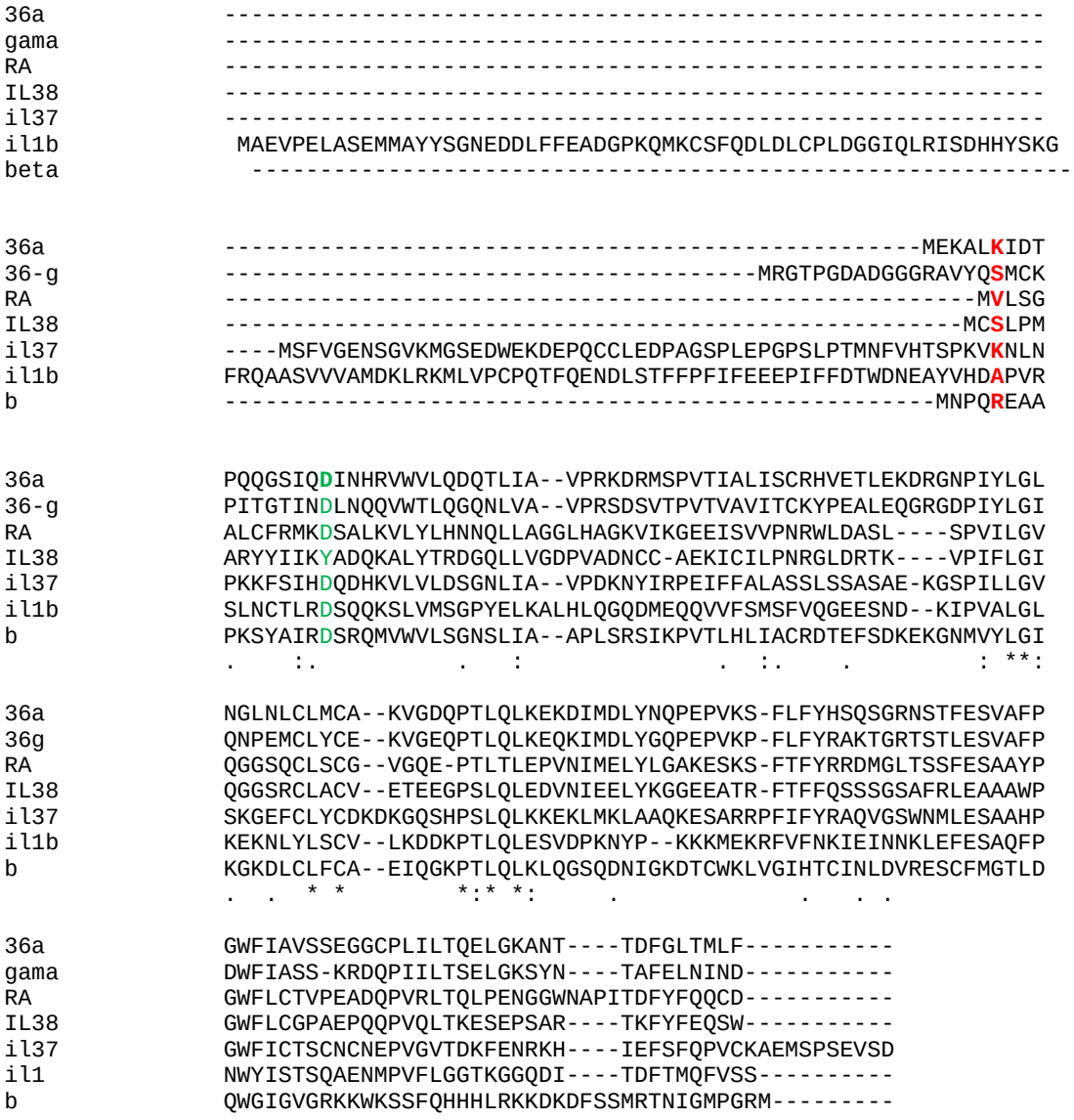
B

```
   1 - CCTGGACGGGTAAATTCCCTCTAGAAATATTTTGTTTAACTTTAAGAAGGAGATATACCA - 60
   1 - P  G  R  V  N  S  L  *  K  Y  F  V  *  L  *  E  G  D  I  P  - 20

  61 - TGGATGTCGGACTCAGAAGTCAATCAAGAAGCTAAGCCAGAGGTCAAGCCAGAAGTCAAG - 120
  21 - W  M  S  D  S  E  V  N  Q  E  A  K  P  E  V  K  P  E  V  K  - 40

 121 - CCTGAGACTCACATCAATTTAAAGGTGTCCGATGGATCTTCAGAGATCTTCTTCAAGATC - 180
  41 - P  E  T  H  I  N  L  K  V  S  D  G  S  S  E  I  F  F  K  I  - 60

 181 - AAAAAGACCACTCCTTTAAGAAGGCTGATGGAAGCGTTCGCTAAAAGACAGGGTAAGGAA - 240
  61 - K  K  T  T  P  L  R  R  L  M  E  A  F  A  K  R  Q  G  K  E  - 80

 241 - ATGGACTCCTTAAGATTCTTGTACGACGGTATTAGAATTCAAGCTGATCAGACCCCTGAA - 300
  81 - M  D  S  L  R  F  L  Y  D  G  I  R  I  Q  A  D  Q  T  P  E  - 100

 301 - GATTTGGACATGGAGGATAACGATATTATTGAGGCTCACAGAGAACAGATTGGTGGTATG - 360
 101 - D  L  D  M  E  D  N  D  I  I  E  A  H  R  E  Q  I  G  G  M  - 120

 361 - GTCCTGAGTGGGGCGCTGTGCTTCCGAATGAAGGACTCGGCATTGAAGGTGCTTTATCTG - 420
 121 - V  L  S  G  A  L  C  F  R  M  K  D  S  A  L  K  V  L  Y  L  - 140

 421 - CATAATAACCAGCTTCTAGCTGGAGGGCTGCATGCAGGGAAGGTCATTAAAGGTGAAGAG - 480
 141 - H  N  N  Q  L  L  A  G  G  L  H  A  G  K  V  I  K  G  E  E  - 160

 481 - ATCAGCGTGGTCCCCAATCGGTGGCTGGATGCCAGCCTGTCCCCCGTCATCCTGGGTGTC - 540
 161 - I  S  V  V  P  N  R  W  L  D  A  S  L  S  P  V  I  L  G  V  - 180

 541 - CAGGGTGGAAGCCAGTGCCTGTCATGTGGGGTGGGGCAGGAGCCGACTCTAACACTAGAG - 600
 181 - Q  G  G  S  Q  C  L  S  C  G  V  G  Q  E  P  T  L  T  L  E  - 200

 601 - CCAGTGAACATCATGGAGCTCTATCTTGGTGCCAAGGAATCCAAGAGCTTCACCTTCTAC - 660
 201 - P  V  N  I  M  E  L  Y  L  G  A  K  E  S  K  S  F  T  F  Y  - 220
```

```
661 - CGGCGGGACATGGGGCTCACCTCCAGCTTCGAGTCGGCTGCCTACCCGGGCTGGTTCCTG - 720
221 - R  R  D  M  G  L  T  S  S  F  E  S  A  A  Y  P  G  W  F  L   - 240

721 - TGCACGGTGCCTGAAGCCGATCAGCCTGTCAGACTCACCCAGCTTCCCGAGAATGGTGGC - 780
241 - C  T  V  P  E  A  D  Q  P  V  R  L  T  Q  L  P  E  N  G  G   - 260

781 - TGGAATGCCCCCATCACAGACTTCTACTTCCAGCAGTGTGACGAATTCCATCACCATCAC - 840
261 - W  N  A  P  I  T  D  F  Y  F  Q  Q  C  D  E  F  H  H  H  H   - 280

841 - CATCACTGAGCGGCCGCACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAAC - 900
281 - H  H  *  A  A  A  L  E  H  H  H  H  H  H  *  D  P  A  A  N   - 300
```

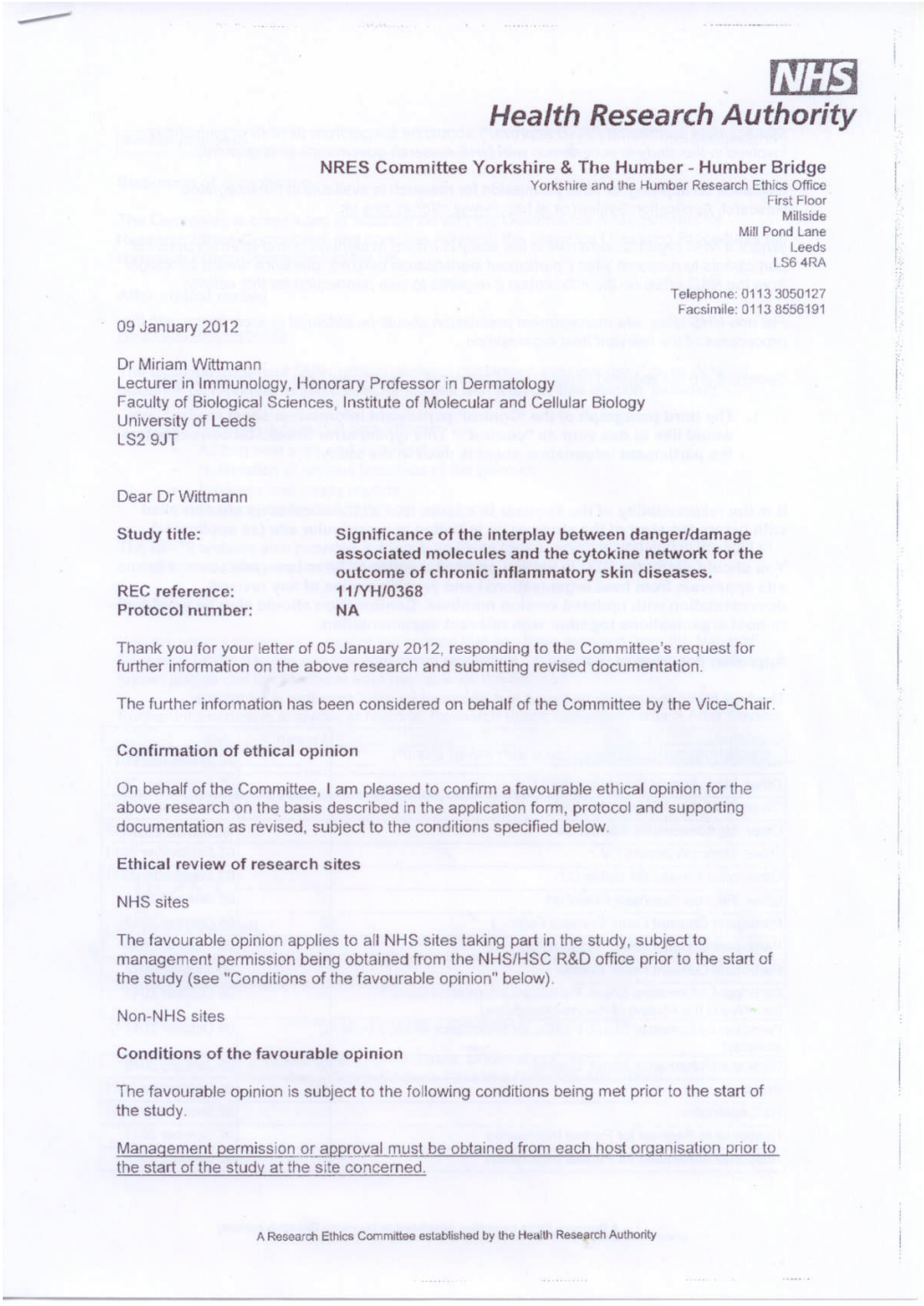
Appendix Figure 23 – IL-36RA primers for N and C-terminal tagged protein bacterial expression.

A - Primers for pET 28 (+) vector using IL-36α in pET expression system as template. Highlighted red sequence depicts restriction sites (Nco1) used. Reverse primers as depicted in Appendix Figure 7. B – Following cloning and transformation into DH5α cells the sequence was verified by Sanger sequencing, sequence depicted. N-terminal Sumo tag is depicted in blue, EcoR1 restriction site (red) and His tag is depicted in purple.



Appendix Figure 24 – Alignment of IL-36s, IL-38, IL-37 and IL-1β.

Alignment of all of these IL-1 members allows for comparison of the site of cleavage in order to increase activity. The red highlighted amino acid indicates the point of cleavage for IL-36 and IL-1β to increase activity, however of note IL-1β can also be cleaved into activity around this point. IL-37 and IL-38 are speculated to have increased activity if cleaved at this point. The green highlighted amino acids are the conserved aspartic acids 9 amino acids away from the cleavage point that have been used in order to predict active forms of IL-36.



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09 January 2012

Dr Miriam Wittmann
Lecturer in Immunology, Honorary Professor in Dermatology
Faculty of Biological Sciences, Institute of Molecular and Cellular Biology
University of Leeds
LS2 9JT

Dear Dr Wittmann

Study title: **Significance of the interplay between danger/damage associated molecules and the cytokine network for the outcome of chronic inflammatory skin diseases.**
REC reference: **11/YH/0368**
Protocol number: **NA**

Thank you for your letter of 05 January 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Vice-Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

A Research Ethics Committee established by the Health Research Authority

Appendix figure 25 – Ethical approval letter

Letter confirming ethical approval had been granted in order to take biopsies or collect excess skin from NHS patients.