

Investigating the effects of IL-36 γ on
signal transduction in human dermal
fibroblasts

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Abstract

Psoriasis is a chronic inflammatory skin condition affecting around 2% of the population. IL-36 γ is a member of the IL-1 family of cytokines and has emerged as a pivotal cytokine in psoriasis pathophysiology. The mTOR pathway serves as a key integrator of a wide range of environmental cues, and functions as a central regulator of cellular processes involved with growth and repair.

Inhibitors of mTOR have been successfully used in the treatment of several immune mediated conditions, however have been relatively unsuccessful in psoriasis. It has not been studied how mTOR inhibition affects the contribution of stromal cells to psoriasis.

We assessed signal transduction downstream of IL-36 γ , in primary dermal fibroblasts, with a particular focus on the mTOR axis, and identified a negative role in AKT activation, which could be attributed to feedback loops involved in mTOR signalling. It was demonstrated that NF κ B is necessary for cytokine induction by IL-36 γ .

We established that mTORC1 negatively regulated cytokine induction, through treatment with rapamycin. However, we were unable to demonstrate a clear reciprocal effect from the upregulation of mTOR via RNA interference.

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Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

1. Introduction

1.1 Psoriasis Background

1.1.1 Clinical Presentations of Psoriasis

Psoriasis is an immune-mediated, chronic inflammatory skin condition affecting around 2% of the population (Feldman & Pearce 2008). Psoriasis vulgaris (a.k.a. chronic plaque psoriasis) is the most common form of psoriasis, representing around 90% of psoriasis patients, and is understandably the best-researched. Clinically, it typically presents as well demarcated, erythematous plaques with silvery-white dry scaling, and classically has a distribution forming predominantly on extensor surfaces such as elbows and knees, however, scalp, ears, and the lower back are also commonly involved. (Kim et al. 2017)

Psoriasis is however a varied disease, and multiple other patterns of psoriasis exist. Examples include flexural psoriasis, which mainly affects axillary, perineal and inframammary regions, and although well demarcated, the erythematous lesions lack the scale that is associated with psoriasis vulgaris (Syed & Khachemoune 2011). Guttate psoriasis is classically post-infective, occurring after group B streptococcus infection of the upper respiratory tract and results in many droplet shaped lesions in centripetal distribution (Kim et al. 2017). Although usually a relatively benign condition, when psoriatic lesions have total or near-total involvement, it becomes known as erythrodermic psoriasis, which can profoundly disrupt the homeostatic capabilities of the skin, leading to hypothermia, metabolic dysregulation, and high output cardiac failure (Singh et al. 2016). Generalised pustular psoriasis is a rare, potentially life-threatening disease, in which the patient is systemically unwell and covered in sterile pustules which often coalesce, forming sheets. (Langley et al. 2005)

1.1.2 Systemic effects of Psoriasis

Psoriasis is a systemic illness and has non-cutaneous manifestations, with around 30% of patients developing psoriatic arthritis (Boehncke & Schön 2015). Like psoriasis, psoriatic arthritis is a largely heterogeneous disease which commonly presents as an asymmetrical oligoarthritis of smaller joints, however there are many other distributions, affecting both peripheral joints, and the spine. Arthritis mutilans is a severe deforming arthritis and predominantly occurs in individuals with psoriatic arthritis. Psoriatic arthritis and psoriasis have a significant degree of overlap in pathways driving disease pathology, specifically the central roles of tumour necrosis factor α (TNF α) and the interleukin-23/17 (IL-23/IL-17) axis, which is reflected in the shared therapeutic arsenal targeting these pathways (Kim et al. 2017).

Extracutaneous effects extend beyond arthropathies, with psoriatic disease being linked to increased cardiovascular risk, obesity, hypertension, and impaired insulin sensitivity (Hu & Lan 2017). Extensive psychosocial impacts accompany psoriatic disease and it has been shown to result in stigmatisation, poor self-esteem, increased stress, reduced social functioning, impaired relationships and increased incidence of depression (Fried et al. 1995)

1.1.3 Genetic and environmental risk factors for psoriasis

As a multifactorial disease, psoriasis is caused by interplay between both heritable and environmental risks. Multiple susceptibility loci have been described for psoriasis. Accounting for around 50% of disease heritability is Psoriasis Susceptibility Gene 1 (PSORS1), a 200 kb segment of the major histocompatibility complex on chromosome 6p21.3. HLA-Cw6 is most likely the primary risk allele (Trembath et al. 1997). Variations in multiple other loci have been identified, predominantly with roles in both innate and adaptive immunity, specifically nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), interferon signalling and the IL-23/Th17 axis (Ghoreschi 2013). Well documented triggers for psoriasis include mechanical trauma to the skin (as described by the Koebner Phenomenon), stress, infection and certain classes of drugs such as lithium and β-blockers (Mahajan & Handa 2013).

1.2 Pathophysiology of Psoriasis

1.2.1 Hallmarks of Psoriasis

There are several well-categorised histological hallmarks in psoriatic disease, which can be used to describe the disease pathology. The most striking feature of psoriasis is the marked increase in keratinocyte proliferation, which is accompanied by increased angiogenesis, and immune cell infiltrate.

Differentiation of keratinocytes normally takes around 5 weeks, however in psoriatic tissues this is greatly accelerated, taking only 3-4 days. This leads to thickening of the skin (acanthosis) and incomplete terminal differentiation that normally occurs in granular keratinocytes, leading to squamous keratinocytes retaining their nuclei (parakeratosis). Extracellular lipids that are normally involved in cementing together the stratum corneum are underproduced, leading to the flaking scale seen in psoriatic skin (De Rosa & Mignogna 2007).

Psoriatic lesions show marked vascular changes. Dermal papillary capillaries demonstrate morphological alterations before epidermal hyperplasia becomes evident (Kulka 1964). Capillaries are dilated, tortuous and demonstrate increased permeability, facilitating increased leukocyte migration to lesions (Heidenreich et al. 2009). The morphological vascular changes in psoriatic lesions are eloquently demonstrated in a clinical setting via eliciting Auspitz sign, which describes pinpoint bleeding when lesions are mechanically descaled. Papillary micro-vessels also demonstrate upregulation of inflammation associated adhesion molecules such as intracellular adhesion molecule (ICAM), vascular cell adhesion protein (VCAM) and E-Selectin, facilitating leukocyte adhesion to the endothelium (Springer 1994). Pro-angiogenic mediators including vascular endothelial growth factor, hypoxia inducible factors, angiopoietin, as well as cytokines like TNF α , IL-8, and IL-17 are all upregulated in psoriasis (Heidenreich et al. 2009).

The neovascularization and hyperproliferation observed in psoriatic disease can be explained by the institution of an inflammatory cytokine network by resident epithelial, stromal, immune cells as well as infiltrating T lymphocytes and cells of the innate immune system.

1.2.2 Role of Keratinocytes and Innate immunity

Keratinocytes and the innate immune system provide a non-specific, rapid response to damage and infection, which is dysregulated in psoriasis. Besides forming the barrier of human skin, keratinocytes can be a source of antimicrobial peptides such as b-defensins, psoriasin (S100A7) and cathelicidin (LL-37). Generally, the antimicrobial peptides have chemotactic functions and can influence dendritic cells and T lymphocytes (Büchau & Gallo 2007). Besides producing antimicrobial peptides, keratinocytes can respond to interferon γ (IFN- γ), TNF α and IL-17, and are sources of IL-1 family cytokines, IL-8, C-X-C motif chemokine 10 (CXCL10) and C-C motif ligand 20 (CCL20). IL-8 production by keratinocytes has been shown to drive recruitment of neutrophils to the epidermis, where they form micro-abscesses. (Balato et al. 2012)

Dendritic cells are antigen presenting cells and function as sentinels, bridging the gap between innate and adaptive immunity. When activated, dendritic cells migrate from the skin to draining lymph nodes, where they present antigens and activate a T cell response. Both dermal myeloid dendritic cells, and plasmacytoid dendritic cells (pDC) have repeatedly been shown to be significantly upregulated in psoriatic skin. Using a xenograft mouse model, a role for pDCs in the initiation of psoriasis was established, as although pDCs are usually tolerant to self RNA and DNA, LL-37 has the potential to complex self or pathogen derived DNA and stimulate Toll-like Receptor 9 (TLR-9) on plasmacytoid dendritic cells. This results in host DNA

functioning as a proinflammatory stimulus in psoriasis (Kim et al. 2014). LL-37 can stimulate cytokines IL-1 family cytokines, including IL-36 γ . Dendritic cells act the main source of IL-23 in psoriatic skin, which is a key cytokine in the IL-23/Th17 axis. It has been established that both TNF α and IL-36 stimulate IL-23 production by DCs, and that DCs are a critical source of TNF α and inducible Nitric Oxide Synthase (iNOS) in the psoriatic dermis. (Lowes et al. 2005)

1.2.3 Role of T cells

Although psoriasis involves both the innate and adaptive immune system, the importance of T cells was initially demonstrated by accident in when cyclosporine was successfully used in the treatment of psoriatic arthritis (Olivieri et al. 1997). More recently, T cell targeting biologic therapies, such as alefacept and efalizumab, have been very successful in the treatment of psoriatic disease (Rønholt & Iversen 2017).

One of the best-studied pathways in psoriasis is the IL-23/Th17 axis. T helper 17 (Th17) cells represent a specific subset of T-Lymphocytes characterised by their expression of IL-17, and are distinct from classical Type 1 T helper (Th1) lymphocytes (Steinman 2007). Like Th1 lymphocytes, Th17 cells play a significant role in the pathogenesis of psoriasis, amongst other chronic inflammatory diseases (Lowes et al. 2008; Neurath 2007). Predominantly, expansion and survival of this Th-17 subset is driven by IL-23, a cytokine produced by myeloid cells, acting on memory T cells, since naive T cells lack an IL-23 receptor (Bettelli et al. 2007.) Other cytokines such as IL-9 have been shown to enhance Th17 associated inflammation in the context of psoriasis (T. P. Singh et al. 2013). Once activated Th17 cells produce IL-17, amongst other proinflammatory cytokines, inducing some of the hallmark changes associated with psoriasis such as keratinocyte hyperproliferation, and angiogenesis. The significance of this pathway is demonstrated by the success

of clinical trials of biologic therapies targeting the IL-23/IL-17 - axis (Fotiadou et al. 2018).

1.2.4 Fibroblasts in Immunity

Fibroblasts are a heterogeneous population of stromal cells. Morphologically they are spindle shaped, with oval nuclei. There is no universal fibroblast marker and they lack markers associated with epithelial, vascular or leukocytic lineage. Traditionally recognised for their critical role in producing and remodelling extracellular matrix, it has also been demonstrated that they have the ability to maintain inflammatory cell infiltrates by responding to and producing relevant cytokines and chemokines (Jordana et al. 1994). Fibroblasts differ depending on their anatomical derivation, pathologies affecting tissues they are taken from, and even within tissues, and these differences persist even after in vitro culture (Chang et al. 2002).

Fibroblasts produce the structural proteins, adhesive protein and ground substance that make up extracellular matrix, as well as being responsible for its maintenance and reabsorption. They play an active role in wound healing, angiogenesis, inflammation, fibrosis as well as supporting neoplastic progression. With regards to inflammation, fibroblasts are responsive to many inflammatory cytokines such as $\text{TNF}\alpha$, $\text{IL-1}\beta$ and IL-6 , and in response can produce a variety of cytokines and chemokines (Van Linthout et al. 2014). The ability to respond to and produce growth factors and cytokines in a reciprocal fashion, allows fibroblasts to maintain homeostasis of local cells such as keratinocytes, endothelial cells or local immune cells. Fibroblasts support the generation of Th17 cells by enhancing release of IL-23 by dendritic cells (Schirmer et al. 2010)

Fibroblasts demonstrate the ability to alter duration, intensity and cellular proportions of inflammatory responses, by providing a “stromal address code” (Parsonage et al. 2005), and there is evidence of crosstalk between fibroblasts and leukocytes. Fibroblasts are also critical in angiogenesis, where they produce vascular endothelial growth factor (VEGF), matrix proteins and cytokines which are indispensable for endothelial cell mediated lumen function (Newman et al. 2011).

1.2.5 Fibroblasts in Psoriasis

Because of their role in immune reactions, fibroblasts contribute to the inflammatory microenvironment in psoriasis. Fibroblasts respond to proinflammatory cytokines such as TNF α by induction of psoriasis associated messengers such as IL-6 and IL-8, and this induction is significantly higher in lesional fibroblasts compared to non-involved skin (Zalewska et al. 2006). However, it has been demonstrated that lesional fibroblasts alone cannot induce keratinocyte proliferation by supernatant or co-culture assay, nor do normal fibroblasts inhibit keratinocyte proliferation (Priestley & Lord 1990). It has been demonstrated that IL-17A/TNF- α stimulation causes IL-8 release by fibroblasts (Yin et al. 2017). Modulation of neutrophil cytokine output has been demonstrated as a result of fibroblast-neutrophil interactions in the context of psoriasis (Glowacka et al. 2010).

1.3 MicroRNA

Normal immune function can be regulated on a post-transcriptional basis by microRNA (miRNA), which are small, single-stranded non-coding RNAs around 22 nucleotides long. They have been demonstrated to regulate a broad range of developmental and cellular processes, through binding to targets in the 3' untranslated region (UTR) of target messenger RNA (mRNA) sequences, which ultimately represses protein expression through a variety of mechanisms. miRNAs represent only around 3% of the human genome, however have the ability to regulate around 90% of genes (Pauley et al. 2009). The majority of miRNAs are encoded by genes in the introns of protein coding genes, however they can also be found in either untranslated gene regions or intergenic regions. (Lee et al. 2004)

In the context of immune function, miRNA genes have been shown to be controlled by activation of various mitogen Activated Protein Kinase (MAPK) pathways (Misawa et al. 2010), and NF κ B (Gatto et al. 2008). Following their induction, microRNAs have the potential to regulate multiple steps in immune responses, not limited to cytokine and chemokine expression, regulation of adhesion molecules, and cell polarisation (Zhou et al. 2011).

The biogenesis of microRNA is a process which involves multiple processing steps both inside and outside the nucleus. A long primary miRNA (pri-miRNA) transcript is generated in the nucleus by RNA polymerase II, and has a hairpin structure, within which the miRNA resides. This pri-miRNA is processed by a complex called Microprocessor, consisting of a nuclear RNase III Drosha, and its essential co-factor DiGeorge Syndrome Critical Region 8 (DGCR8), resulting in a miRNA precursor (pre-miRNA) of ~70 nucleotides. Following nuclear processing, pre-miRNAs are exported into the cytoplasm by Exportin 5 (XPO5). In the cytoplasm the pre-miRNA is cleaved by an RNase III Dicer which interacts with two cofactors, the HIV-1 TAR RNA Binding

Protein (TRBP) and protein activator of PKR (PACT), resulting in a small RNA duplex. An RNA-induced silencing complex is formed when the RNA duplex is loaded onto an Argonaut protein (AGO). One strand of the duplex (the passenger) is removed, leading to a mature RNA-Induced Silencing Complex (RISC) (Krol et al. 2010).

RNA silencing can then occur via translational repression, mRNA deadenylation, or decay, facilitated by AGO recruitment of active factors. The miRNA functions as a guide, with a 6-8 nucleotide stretch, known as the seed region being critical in binding with target mRNA, and the degree of complementarity determining the mechanism of silencing (Kehl et al. 2017). miRNA demonstrate both redundancy and pleiotropy, meaning complex regulatory networks can form between miRNA and their target mRNAs.

Since microRNA act to regulate normal immune function, alterations in their expression can influence disease pathologies. Several miRNAs have been identified as being dysregulated generally in inflammatory disease, with specific miRNA profiles exhibited in psoriatic inflammation (R. P. Singh et al. 2013; Joyce et al. 2011). Certain miRNAs can be used prognostically, and correlate with Psoriasis Severity Index (PASI) (Ichihara et al. 2012), and miRNA profiles alter following different systemic treatments (Hawkes et al. 2016).

1.4 IL-36

Recently a pivotal cytokine for psoriasis has been identified in IL-36. IL-36 cytokines are novel members of the IL-1 cytokine family, and include agonists IL-36 α , IL-36 β , and IL-36 γ (previously identified as IL-1F6, IL-1F8, and IL-F9) as well as a natural antagonist IL-36Ra (previously known as IL-1F5) (Sims & Smith 2010). IL-36 cytokines are much more tissue-specific than IL-1, generally its receptors are involved in the regulation of tissue-specific inflammation and expression is generally limited to tissues such as skin, lung, and gut (Ding et al. 2018).

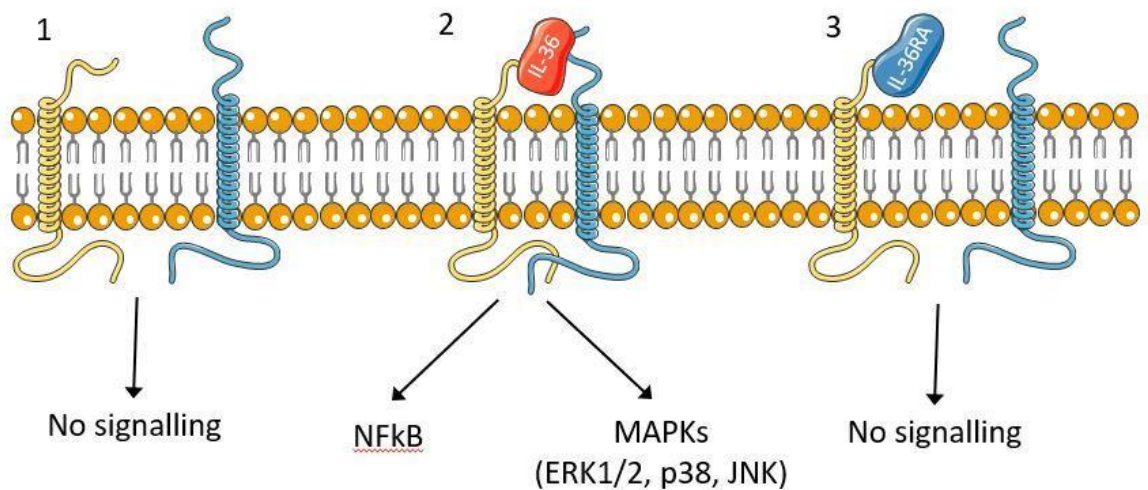


Figure 1. IL-36 Receptor Signalling

The IL-36 receptor requires two components for signalling, IL-36R (IL-1Rr12) and a co receptor, IL-1 Accessory Protein (IL-1AcP) which is shared with the IL-1 Receptor. IL-1AcP has no capacity for signalling on its own. Following binding between an IL-36 cytokine and IL-36R, IL-1AcP is recruited, leading to interactions of the TIR domains of the two proteins, and subsequent activation of MAPK and NF κ B pathways. (Towne et al. 2004) Downregulation occurs via IL-36RA which can successfully bind IL-36R, however does not result in recruitment of IL-1AcP, nor any downstream signalling (Gabay & Towne 2015). IL-36g is not released in an activated form and required processing by cathepsin S leading to a dramatic increase in its activity (Ainscough et al. 2017)

1.4.2 IL-36 in psoriasis

Much of the research surrounding IL-36 has taken place in the context of psoriatic disease, where it has been shown to be highly upregulated, and function as a biomarker. IL-36 has been shown to be upregulated in gene expression studies (Blumberg et al. 2007) as well as the functioning protein (D'Erme et al. 2015). IL-36 could be valuable in delineating between different clinically similar skin diseases such as atopic dermatitis and psoriasis. Strong expression of IL-36 γ is seen in the upper epidermal layer of psoriatic skin, compared to other inflammatory skin diseases where it is only weakly expressed (D'Erme et al. 2015). Serum levels of IL-36 are enhanced in psoriasis compared to other inflammatory skin conditions, and importantly correlate well with disease severity (as measured by PASI), not only in untreated individuals, but also in patients who underwent anti-TNF therapy where IL-36 in serum decreased with symptoms of disease (D'Erme et al. 2015).

IL-36 is thought to be mainly produced by keratinocytes and other epithelial cells in response to inflammatory mediators such as TNF- α and IL-1 (Carrier et al. 2011). IL-36 has effects on a range of cell types including epithelial cells, fibroblasts and immune cells. IL-36 contributes to psoriatic inflammation via its central position in the interplay between immune cells and keratinocytes. Over-expression of IL-36 in mouse skin results in a phenotype that is similar to psoriasis, with regards to acanthosis, hyperkeratosis and inflammatory infiltrate. Genes associated with psoriasis such as IL-17, IL-22, IL-23, antimicrobial peptides and S100 proteins are also upregulated (Towne & Sims 2012).

There is evidence of multiple mechanistic links between IL-36 and the IL-23/17 axes. IL-17 can induce IL-36 from human keratinocytes, and the degree of this induction is significantly stronger in non-lesional skin from psoriasis patients, than it is from unaffected individuals (Muhr et al. 2011). IL-36 γ induces its own expression as well as that of TNF α , IL-6 and IL-8 in keratinocytes (Carrier et al. 2011). Stimulation with IL-36 leads to the production of IL-23 by macrophages, and this induction is enhanced in macrophages of psoriasis patients (Bridgwood et al. 2018). IL-36 augments IL-17's role in the production of antimicrobial peptide production (Carrier et al. 2011). This describes a feedback loop for IL-36, in which it is not only regulated by Th-17 cytokines, but also enhances their activity.

Interestingly, in certain individuals affected by generalised pustular psoriasis, a loss of function mutations of IL-36RA has been identified (Marrakchi et al. 2011). Due to loss of control of IL-36 antagonist activity by IL-36RA, IL-36 stimulation of cells from patients with mutated IL-36RA (keratinocytes or peripheral blood mononuclear cells) induced cytokines (IL-1 α , IL-6, IL-8, TNF α) more strongly than it did in cells from control individuals. (Onoufriadis et al. 2011) (Marrakchi et al. 2011)

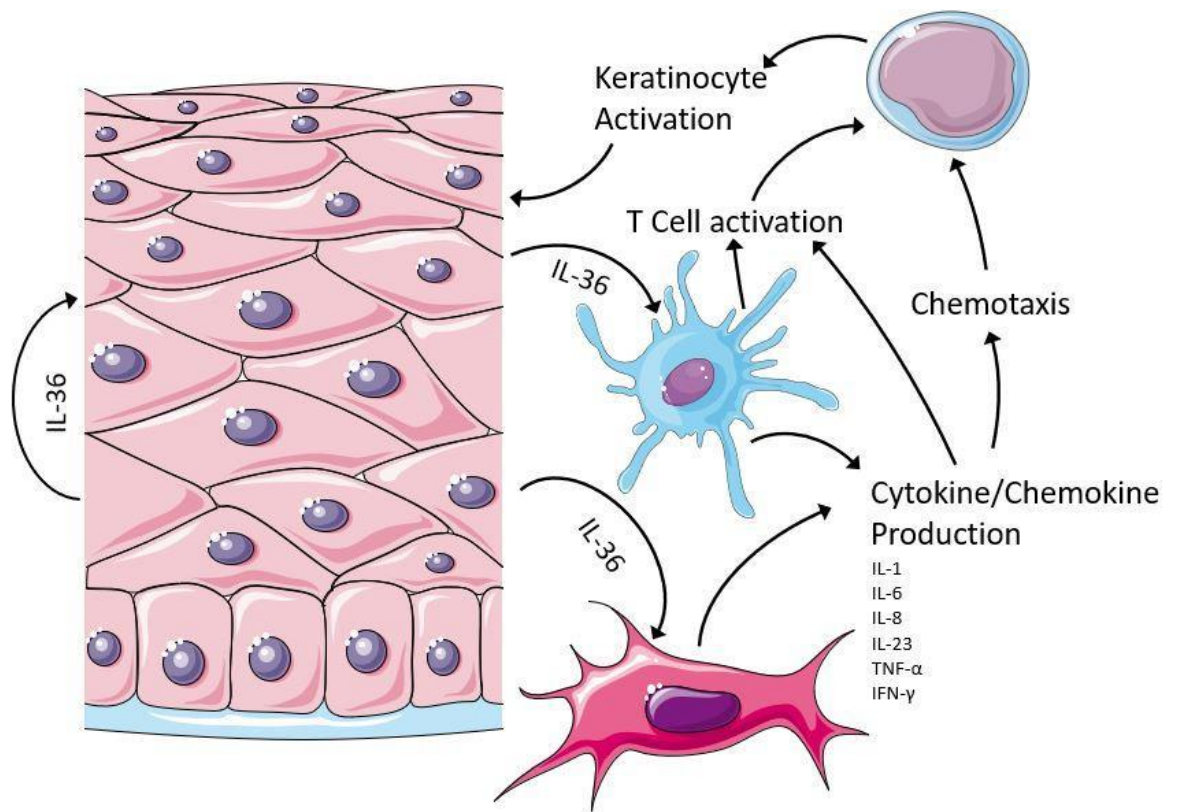


Figure 2. Role of IL-36 in Psoriasis Pathology

IL-36 is released by epithelial cells, following inflammatory stimulation. IL-36 can act on keratinocytes in both autocrine and paracrine fashion, leading to increased proliferation, as well as inducing itself and other proinflammatory cytokines. IL-36 has actions on fibroblasts and local immune cells such as dendritic cells, through which a Th1/17 response is driven. This T cell driven response creates further inflammatory stimulus for keratinocytes and contributes to the establishment of a proinflammatory cytokine and chemokine network.

1.5.1 mTOR

Mechanistic Target Of Rapamycin (mTOR) is a serine/threonine kinase that is a central regulator of metabolic control, and is highly conserved from yeast to humans. mTOR is sensitive to a wide range of extracellular signals, such as growth factors, hormones, cytokines as well as ligation of TLRs and T-cell Receptors (TCR). mTOR has also developed to respond to intracellular cues, and is responsive to nutrient availability (including amino acids and glucose), and cellular energy change (AMP: ATP ratio). In response to signals mTOR regulates cell growth and proliferation via a variety of targets (Laplante & Sabatini 2012).

1.5.2 mTOR complexes

mTOR exists in two discrete, interacting complexes that differ both structurally and functionally. mTORC1 is the better characterised of the mTOR complexes. It contains mTOR; regulatory protein associated with mTOR (Raptor), and mammalian lethal with Sec13 protein 8 (mLST8, aka GβL) (Kim et al. 2003). Furthermore, mTORC1 is also comprised of the two inhibitory subunits Proline-Rich AKT substrate of 40 kDa (PRAS40) and DEP domain containing mTOR interacting protein (Deptor) (Sancak et al. 2007).

mTORC2 shares mTOR and mLST8 (Jacinto et al. 2004), however Rapamycin insensitive companion of mTOR (Rictor) replaces raptor (Sarbasov et al. 2004). mTORC2 also contains the regulatory proteins mSin1 and Protor1/2 (Pearce et al. 2011; Frias et al. 2006).

1.5.3 Upstream of mTOR

A key upstream regulator of mTOR is the heterodimeric Tuberous Sclerosis Complex (TSC), which is comprised of TSC 1 (hamartin) and TSC 2 (tuberin). TSC is named after a multi-systemic disease, that is due to mutations in the genes encoding for either of the components of the heterodimer. TSC is characterised by malformation and multiple benign tumours, illustrating both the proliferative role of mTOR and TSC1/2's place as a key negative regulator (Lam et al. 2017).

TSC1/2 exerts its effects on mTORC1 by acting as a GTP-activating protein for Ras Homolog Enriched in the Brain (Rheb). Rheb interacts with mTORC1 directly to stimulate activity, and is rendered inactive when guanosine diphosphate (GDP) bound (Inoki et al. 2003). Many signals converge on TSC1/2, and canonically it is downstream of growth factors, via the phosphoinositide 3-kinase (PI3K) and Ras pathways. Kinases such as protein kinase B (AKT), extracellular signal related kinases 1/2 (ERK1/2), and ribosomal protein S6 kinase 1 (S6K1) phosphorylate TSC1/2, removing its opposition to mTORC1 activity (Laplante & Sabatini 2012).

Much like growth factors, proinflammatory cytokines such as TNF α have been demonstrated to relieve TSC1/2 control of mTORC1 via I κ B Kinase beta (Lee et al. 2007). Signals can also converge on mTOR via TSC independent mechanisms, for instance AKT, which can directly phosphorylate PRAS40, an mTORC1 inhibitor (Sancak et al. 2007).

1.5.4 Downstream of mTOR

The best characterised function of mTORC1 is increased protein output, via targets such as S6K1 and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). As the rate limiting step in protein synthesis is translation initiation, it makes sense that 4E-BP1 is an output of mTOR. Following phosphorylation, 4E-BP1 cannot bind to the cap-binding protein eukaryotic translation initiation factor 4 (eIF4E), facilitating the formation of the eIF4F complex, promoting cap-dependent translation (Ma & Blenis 2009).

S6K1 positively controls protein synthesis through a variety of mechanisms including enhancing translation initiation and increasing ribosome biogenesis (Sabatini 2017). mTORC1 also has a role in positively regulating metabolism and adenosine triphosphate (ATP) production, and in the presence of favourable growth signals mTORC1 acts as a strong negative regulator of autophagy, through phosphorylation and inactivation of Unc-51 Like Autophagy Activating Kinase 1 (ULK1) (Nazio et al. 2013).

Like mTORC1, mTORC2 is sensitive to growth factors in a PI3K dependent fashion, however amino acids do not seem to influence its activation (Laplante & Sabatini 2012). By direct phosphorylation of AKT on Ser473, mTORC2 enhances some of its functions, such as shifting towards glycolytic metabolism, and a Forkhead Box protein O1 (FOXO1) mediated transition towards growth (Sarbasov et al. 2005).

1.5.5 Regulatory loops affecting mTOR

Due to the broad activity of mTOR and its wide range of inputs, it is understandable that it is a tightly regulated system involving multiple feedback loops. In order to avoid unbridled mTOR activation, PI3K is inhibited by mTORC1 at multiple levels. S6K1 inhibits Insulin Receptor Substrate 1 (IRS1) through phosphorylation and relocalisation, resulting in impaired signalling (Takano et al. 2001). Via an analogous mechanism, S6K1 also inhibits PI3K activation from Platelet-Derived Growth Factor Receptors (PDGFR) (Zhang et al. 2007). It has been demonstrated that S6K1 also inhibits ERK/MAPKs (Carracedo et al. 2008).

There are also feedback loops between the mTOR complexes. Phosphorylation of AKT on Ser473 by mTORC2 is necessary for its maximum activation when phosphorylated by Phosphoinositide-Dependent Kinase 1 (PDK1) on Thr308. This means that mTOR functions both up and downstream of AKT (Sarbasov et al. 2005). Independent of its action on mTORC1, TSC seems to be necessary for mTORC2 activity (Huang et al. 2008). S6K1 leads to phosphorylation of Rictor, which does not impact most mTORC2 targets, but does reduce phosphorylation of AKT Ser473 (Dibble et al. 2009).

Inhibition of mTORC1 via rapamycin results in upregulation of mTORC2 activity, as measured by phosphorylation of AKT on Ser473 due to the previously mentioned feedback pathways. These overlapping mechanisms have been cited as mechanisms for the failure of rapalogs in a variety of disease states, including both inflammatory disease and cancer (Faes et al. 2017; Raychaudhuri & Raychaudhuri 2014).

1.5.6 mTOR in immune function

Appropriate degrees of mTOR activation have been demonstrated to be essential for shaping immune responses from a wide range of cells in both the innate and adaptive immune systems. Particular effects directly attributable to mTOR include regulating metabolism, cytokine response, antigen presentation, the polarisation of both macrophages and T cells, and the migratory potential of immune cells (Weichhart et al. 2015; Powell et al. 2012).

T cells appear to be extremely sensitive to mTOR inhibition, as demonstrated by the fact that rapamycin is highly effective in inhibiting antigen-induced T cell proliferation (Sehgal & Bansbach 1993). This has led to relatively widespread use clinically where it is effective in preventing organ rejection (Calne et al. 1989). Rapamycin has also proven to be effective in treatment of immune mediated disorders such as Systemic Lupus Erythematosus (SLE) (Fernandez et al. 2006). With respect to the polarisation of T cells, mTORC1 seems to be essential for the differentiation of Th1 and Th17 cells, and the development of Th2 cells depends on mTORC2 (Delgoffe et al. 2011).

In cells of the innate immune system, however, it would appear that mTOR has different roles depending on cell type, with mTORC1 inhibition resulting in increased inflammatory output in Peripheral Blood Mononuclear Cells (PBMCs) as measured by their capacity to produce proinflammatory cytokines (Weichhart et al. 2008; Byles et al. 2013). Whereas in neutrophils, mTOR inhibition results in decreased migration and activity (Gomez-Cambronero 2003). Modulation of mTOR activity can affect the way non-immune cells respond to inflammatory stimuli. It has been demonstrated in Fibroblast-Like-Synoviocytes (FLS) that, in a nutrient rich environment, mTOR signalling significantly decreases NFκB gene expression (Karonitsch et al. 2018).

1.5.6 mTOR in Psoriasis

Enhanced activation of mTOR and its downstream effectors has been observed in lesional psoriatic skin, and the degree of its activation has been linked to disease severity (Buerger et al. 2013; Raychaudhuri & Raychaudhuri 2014). It has been suggested that the inflammatory environment in psoriasis activates mTOR inappropriately, and that mTOR regulates both the proliferation of keratinocytes and induction of relevant cytokines (Raychaudhuri and Raychaudhuri 2014). Deregulation mTORC1 signalling is not limited to skin and was identified in PBMCs of psoriasis patients (Huang et al. 2014)

Based on the activation of mTOR in psoriatic skin, and the use of mTOR inhibition in successful immunosuppression regimes, mTOR inhibition seemed like a promising therapy for relieving psoriasis symptoms. However, although mTOR inhibition seems to be able to ameliorate imiquimod induced psoriasis in mice (Bürger et al. 2017), trials of topical sirolimus in a double blind study, revealed only a mild anti-psoriatic effect of mTOR inhibition in affected humans. Although there was a reduction in clinical score, as well as a histological reduction in CD4+ and proliferating cells, the effects were only modest, and there was no measurable alteration in plaque thickness or erythema (Ormerod et al. 2005).

1.5.7 mTOR in Fibroblasts

The effects of mTOR inhibition on fibroblasts have also been studied to some degree. It has been shown in dermal fibroblasts that mTOR enhances collagen production, and rapamycin treatment leads to some improvements in systemic sclerosis, a fibroblast mediated disease (Tamaki et al. 2014). The effects of mTOR inhibition in fibroblasts also extend to cytokine signalling. In human orbital fibroblasts, rapamycin treatment has been demonstrated to enhance TNF α production of proinflammatory cytokines (Lee et al. 2013).

Although the effect of mTOR inhibition has been well studied in a variety of immune cell types, how it affects stromal cell contribution to the psoriatic environment remains to be elucidated.

1.6 Project aims

The aims of this project were to:

- 1) determine the effect of IL-36 γ on psoriasis-associated proinflammatory mediators and adhesion molecules in HFFs;
- 2) explore the effect of IL-36 γ on signal transduction with a focus on the mTOR pathway
- 3) test whether mTOR modulation alters IL-36 γ induced cytokine signalling in fibroblasts.

2 Materials and methods

2.1 Cell culture

Human foreskin fibroblasts (HFFs) were obtained from frozen stocks in Lagos Lab, and were initially purchased from ATCC (PCS-201-012). HFFs were grown in Dulbecos Modified Eagle Medium (DMEM) (Gibco), supplemented with 10% foetal calf serum (FCS) (Hyclone), 1% L-Glutamine (Gibco), and 1% penicillin/streptomycin (Gibco) in 10cm dishes (BD Falcon). All experiments were seeded at 5×10^4 cells per well of 6 well plates (BD Falcon) and grown for 16h-24h prior to any treatment or starvation. Where indicated, serum starvation took place in DMEM for 16 hours.

2.2 siRNA knockdown

A transfection mix comprised of Opti-MEM (Invitrogen), TransIT-siQuest (Mirus), and siTSC (Dharmacon) or siNTC (Dharmacon) according to the manufacturer's recommended instructions. SiRNA were used at a final concentration of 50nM, and volume of 800 μ l. After 6 hours, transfection mix was supplemented by 10% FCS DMEM. Media/transfection mix was removed and washed with phosphate buffered saline (PBS) following 16 hours, and fresh media was added. Assays were performed 24 hours later.

2.3 RNA Extraction

Prior to RNA extraction, work surfaces and pipettes were treated with RNAase ZAP (ThermoFisher). For adherent cells, lysis was performed directly on 6 well plates (initially seeded with at 5×10^4 cells per well) using 700 μ l of Qiazol (Qiagen). For

non-adherent cells, lysis was performed with 700µl of Qiazol (Qiagen) on a centrifuged pellet of 1×10^5 cells.

Lysates were frozen at -80°C until RNA extraction. From this point RNase-free tubes (Appleton) were used for all elements of RNA work. RNA extraction was performed using mMiRNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA was eluted using 30µl of RNase free H_2O , and immediately analysed using a Nanodrop (2000 ThermoFisher) with 1µl of sample. Absorbance at 260/280 was used to determine RNA purity, and A260/230 to assess for contaminants such as remaining phenol.

2.4cDNA synthesis

cDNA synthesis for mRNA was performed using random hexamers. At least 1µl of RNA sample was incubated with 1µl random Hexamers (50ng/µl), 1µl of dNTP mix (10µM) and made up to 12.5µl with Nuclease free H_2O . Samples were then incubated at 65°C for 5 mins, and kept on ice, until the addition of 4µl of First strand buffer (Invitrogen), 2µl of 0.1M DTT (Invitrogen), 1µl of RNaseOUT (Invitrogen) and 1µl of superscript III (Invitrogen). Sample and reverse transcription mix were incubated at 25°C for 10 mins, 50°C for 50 minutes, and 5 minutes at 85°C to terminate reaction. cDNA was stored at -20°C or used for qPCR immediately. For miRNA, prior to reverse transcription, RNA was diluted to give $3\text{ng } \mu\text{l}^{-1}$. miRNA cDNA synthesis using the TaqMan® miRNA reverse transcription Kit (Applied Biosystems), following the manufacturer's instructions. Samples were stored at -20°C or used immediately for q-RT PCR.

2.5 Quantitative Reverse Transcription PCR (qRT-PCR)

Primers used for target sequences using either SYBR Green (Applied Biosystems) or TaqMan primers are detailed in Table 1. Each reaction contains 1 μ l of cDNA, 10 μ l of SYBR Green mix (Applied Biosystems), 0.6 μ l of forward primer, 0.6 μ l of reverse primer, and 7.8 μ l of nuclease-free water. MicroAmp Fast Optical 96 well reaction plates (Applied Biosystems) were sealed using MicroAmp Optical adhesive film (Applied Biosystems) and centrifuged at 1200g for 90 seconds. StepOnePlus Real Time PCR systems (Applied Biosystems) were used for 40 cycles, and thresholds determined using StepOne software (Applied Biosystems). Generation of a single amplicon was confirmed through melt curve analysis, also using StepOne software (Applied Biosystems).

GAPDH and U6 were used as loading controls for mRNA and miRNA respectively, and where appropriate (i.e. target was readily detectable) the $\Delta\Delta$ CT method was used to determine the relative expression of targets.

Target	Primer Sequence
GAPDH (F)	5'-GGAGTCAACGGATTTGGTCGTA-3'
GAPDH (R)	5'-GGCAACAATATCCACTTTACCAGA-3'
IL-1RAcp (F)	5'-GACCCTCCGTGGAGTTTTGGAGAA-3'
IL-1RAcp (R)	5'-TAGAACAACCAGGAGGCGTC -3'
IL-36 Receptor (F)	5'-GCTGGAGTGCCACAGCATA-3'
IL-36Receptor (R)	5'-GCGATAAGCCCTCCTATCAA-3'
IL-1 β (F)	5'-AGGATGACTTGTTCTTTGAAGCTGA -3'
IL-1 β (R)	5'-TGCCTGAAGCCCTTGCTG -3'
IL-8 (F)	5'-ATGACTTCCAAGCTGGCCGTGGCT -3'
IL-8 (R)	5'-TCTCAGCCCTCTCAAAAATTCTC-3'
VCAM (F)	5'-CGTCTTGGTCAGCCCTTCCT -3'
VCAM (R)	5'-ACATTCATATACTCCCGCATCCTTC -3'

ICAM (F)	5'-AGGCCACCCCAGAGGACAAC -3'
ICAM (R)	5'-CCCATTATGACTGCGGCTGCTA -3'

2.6 Protein Extraction and Quantification

Protein extraction was performed using radioimmunoprecipitation assay buffer RIPA buffer (see appendix), supplemented using Protease Inhibitor Cocktail (containing AEBSF, Aprotinin, Bestatin, E-64, Leupeptin, and Pepstatin A), Phosphatase Inhibitor Cocktail 2 (containing sodium orthovanadate, sodium molybdate, sodium tartrate, and Imidazole) and 3 (containing cantharidin, Bromolevamisole oxalate and Calyculin A) all from Sigma. Following removal of supernatant, plates were washed with cold PBS, which was carefully removed from the tilted plate. Between 30 μ l and 45 μ l of RIPA was then used, depending on confluence, due to increased viscosity of lysate with higher cell numbers. RIPA was then spread and lysate collected using a cell scraper, before being pipetted into Eppendorfs and placed on ice. Lysates were spun at 10,000g for 15mins at 4°C and the supernatant removed and stored at -20°C.

Quantification of proteins was performed using Pierce BCA Protein Assay Kit (Thermofisher Scientific). 1000 μ g ml⁻¹ Albumin provided with the Kit was serially diluted 6 times and used to generate a standard curve. Samples were diluted 1:5, with 5 μ l added to 95 μ l BCA reagent mix. Samples were prepared in duplicate and incubated in 96 well tissue culture plates at 37°C for 30 mins. Absorbance was measured at 562nm and concentrations calculated relative to a standard curve.

2.7 Western Blotting

Protein lysates were prepared to give 10µg of protein per sample where possible, however in circumstances where limited protein was available in some samples this was scaled to be as low as 6µg per well. Lysates were prepared using 5µl of 4x SDS reducing sample buffer (see appendix).

Protein denaturation was performed at 95°C for 10mins, then samples were placed on ice prior to loading. 18µl of denatured sample, and 8µl of Page Ruler Plus (ThermoFisher Scientific) were loaded into appropriate lanes, and 4.5µl of 4x SDS sample loading buffer into remaining empty lanes. Protein separation was performed on a 1.5mm thick 12% polyacrylamide gel (recipe below) in SDS-Page buffer (National Diagnostics) using the mini-PROTEAN tetra system (BioRad) for 90mins at 120V.

Transfer was performed at 0.2A, 25V for 65 minutes using semi-dry transfer machine (BioRad) and Transfer Buffer (National Diagnostics) with 10% methanol. Proteins were transferred to a polyvinyl fluoride (PVDF) membrane (Immobilon), which was activated for 1 minute in methanol and 1 minute in ddH₂O, before being stored in transfer buffer. Following transfer, membranes were blocked in 2% (w/v) BSA-TBS/T for 1 hour at room temperature on rollers in a 50ml Falcon Tube.

After blocking, membranes were incubated with 5-10ml of primary antibody (Table 2), at 4°C overnight, again in a 50ml Falcon Tube on a roller. Primary antibodies were then removed, and membranes washed 3 times for 5 mins in TBS/T on a rocking platform. Membranes then underwent a 1-hour room temperature incubation in an appropriate horseradish peroxidase (HRP) conjugated secondary antibody in another 50ml Falcon tube on a roller.

Membranes then underwent another 3 washes, before visualisation which was performed using Enhanced Chemiluminescent (ECL) western blotting reagent (GE

Healthcare). Signal was detected using either Hyperfilm ECL (GE Healthcare) using dark room facilities or using a Chemidot™ (BioRad).

Following detection, membranes were washed and re-probed for new targets and, when necessary, blots were stripped for 15 minutes in Bright Clear Stripping Buffer (Thermo) at room temperature, on a shaking platform, before being blocked as above.

Films were scanned at 600 DPI, and chemiluminescence images were saved as TIFs, with the signal from each band being quantified by densitometric analysis using ImageJ. Band signal was normalised against β actin, for each lane as a loading control.

Antibody	Manufacturer	Source	Dilution
pAKTthr308 (C31E5)	CST	Rabbit	1:1000 4% BSA
pAKTser473 (D9E)	CST	Rabbit	1:1000 4% BSA
AKT (C67E7)	CST	Rabbit	1:1000 4% BSA
P-S6 (#2215)	CST	Rabbit	1:1000 4% BSA
S6 (5G10)	CST	Rabbit	1:1000 4% BSA
β -actin (ab6276)	Abcam	Mouse	1:5000 5% Milk
Polyclonal Goat Anti-Mouse Immunoglobulins/HRP	DAKO	Goat	1:5000, 5% milk
Polyclonal Goat Anti-Rabbit Immunoglobulins/HRP	DAKO	Goat	1:5000, 5% milk

2.8 Enzyme-linked Immunosorbent Assay

HFFs were seeded at 5×10^4 cells per well, in 6 well plates, and 24 hours later, underwent relevant pre-treatments, transfection or stimulations. Wells were washed in PBS before having 650 μ l of media and stimulant added. Media was collected and frozen at -80°C until use. Media was spun for 5 mins at 300g 4°C and supernatant collected into fresh Eppendorf tubes. Nunc plates (Thermo Scientific) were incubated overnight, 4°C with 50 μ l of 1:200 capture antibody in commercially obtained carbonate coating buffer pH 9.5 (BioLegend).

Plates were then washed four times in TBS/T (see Appendix) before blocking for 1 hour in 5% FCS TBS/T, on a gently rocking platform at room temperature. After washing another four times, samples and standards were added and incubated for 2 hours at room temp. Standards were prepared in 5% FCS TBS/T using serial dilution. Where appropriate, based on preliminary experiments, samples were diluted 1:5, 1:10 or 1:20 in 5% FCS TBS/T.

Following washing, plates were incubated with 50 μ l of detection antibody solution (1:200), in 5% FCS for 1 hour. After washing a further four times, plates were incubated with 50 μ l of avidin-HRP for 30 minutes at room temperature. After a final wash involving repeated 30 second soaking steps, 100 μ l of TMB solution (1:1 A+B BioLegend) was added until an appropriate colour changes evolved; approximately 25 minutes. To stop the reaction, 100 μ l of 1M sulphuric acid was added. Plates were read at 450nm and 570nm, using Molecular Devices Spectromax Monochromatic Reader, then analysis was performed in SoftMax Pro.

2.9 Statistics

Results are presented as mean \pm standard error of the mean (SEM). All statistical tests and graphs were generated in GraphPad Prism 6 (GraphPad Software). One-way ANOVA and paired T tests were used where appropriate and is indicated in figure legends.

3 Results

3.1 HFFs express IL-36R and respond to IL-36 γ

Since both IL-36R and IL-1AcP are required for signalling due to IL-36 γ , and IL-36R is expressed in a cell type restricted fashion, confirmation that primary human foreskin fibroblasts express IL-36R and its co-receptor IL-1AcP was necessary. HFFs represent a ready source of dermal fibroblasts that are taken from healthy donors without local pathology. They are easily obtainable, well categorised, and commonly used as model fibroblasts in the study of skin pathologies and psoriasis (Yin et al. 2017).

Confirmation of receptor components in HFFs was performed using qRT-PCR, both of which were demonstrated to be present through comparison between the raw Ct values obtained from HFF cDNA and the NTC ($p < 0.0001$). IL-36R mRNA was not readily detectable in JURKATs, an immortalised T lymphoma cell line (Figure 3).

In HFFs, FCS was shown to result in a statistically insignificant trend towards upregulation of both the receptor and IL-1AcP mRNA compared to unstimulated samples (Figure 4). IL-8 was measured to assess for a functional response to IL-36 γ , and stimulation caused an upregulation of IL-8 mRNA ($p = 0.0016$) (Figure 5A). This effect was also observed by ELISA ($p = 0.0015$) (Figure 5B). It is noteworthy that the degree of this induction is more pronounced on a protein level than mRNA.

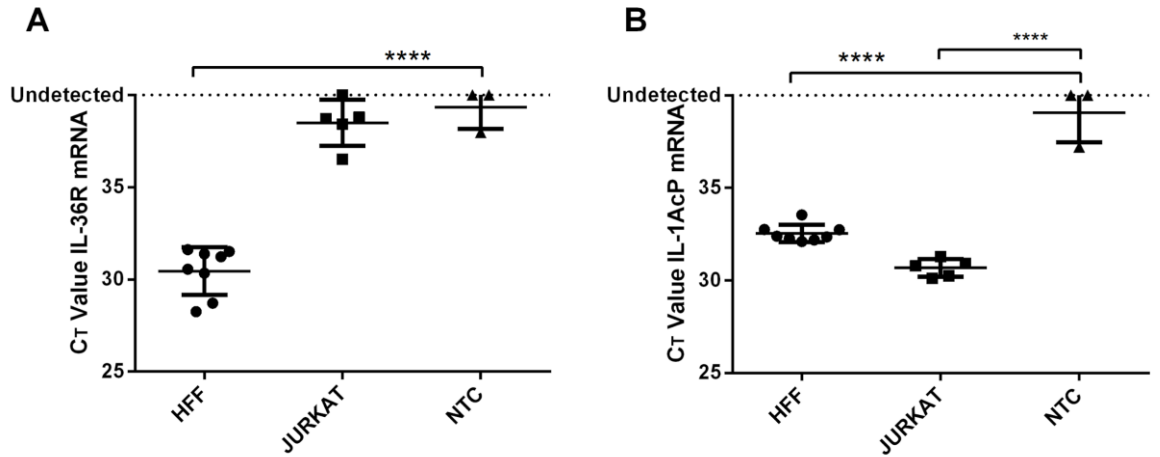


Figure 3. qRT-PCR Analysis of IL-36R and IL-1AcP in HFFs and JURKATs. Untreated HFFs and JURKATs were analysed for (A) IL-36R and (B) IL-1AcP. In samples where target was undetectable, CT was set to 40 cycles. For HFFs and JURKATs, $n \geq 5$. (A) IL-36R was detectable in HFFs, however there was no significant difference between JURKAT and NTC. (B) IL-1AcP was readily detected in both HFFs and JURKATs. Significance was determined via one-way ANOVA.

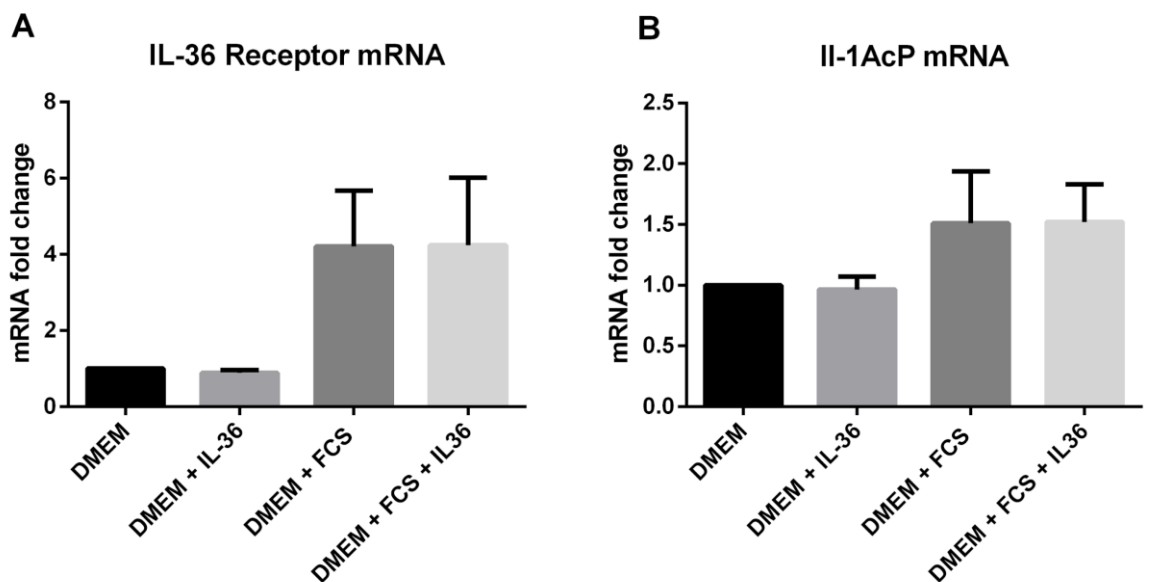


Figure 4. FCS upregulates IL-36R and IL-1AcP mRNA in HFFs. HFFs were serum starved for 24 hours, before 6 hours stimulation with 10% FCS and 100ng/ml IL-36. Sample mRNA is normalised to GAPDH. qRT-PCR analysis demonstrates that FCS upregulates both (A) IL-36R and (B) IL-1AcP, however IL-36 stimulation does not alter expression of the receptor. $n = 4$. Significance was determined via one-way ANOVA.

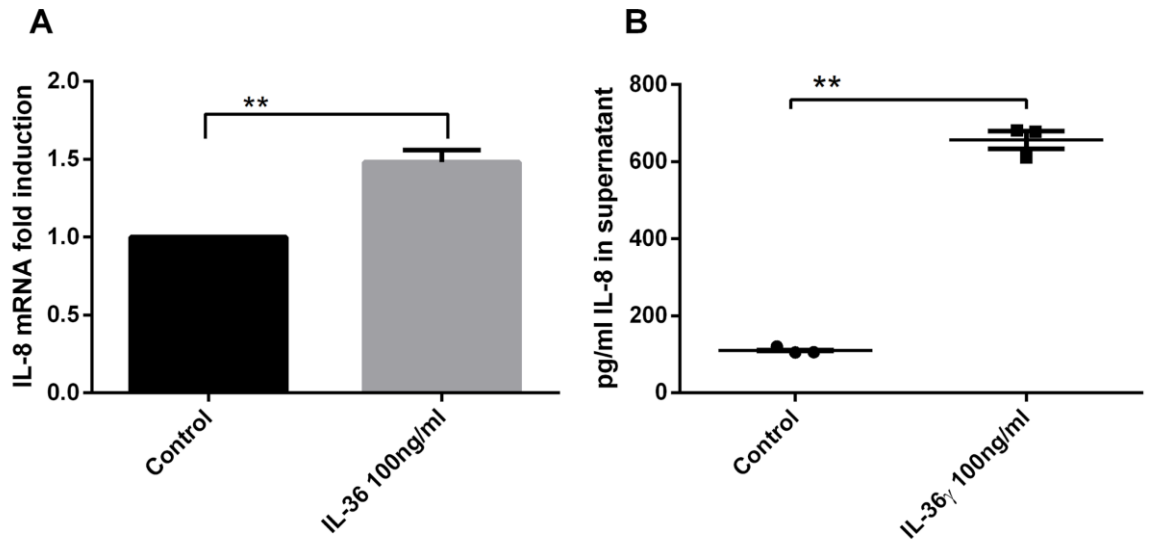


Figure 5. IL-36 Induced IL-8 in HFFs. HFFs were treated with 100ng/ml IL-36 γ and analysed after (A) 6 hours and (B) 24 hours by qRT-PCR and ELISA respectively. Sample mRNA is normalised to GAPDH. (A) n = 6. (B) n = 3. IL-8 upregulation was on both a mRNA and protein level. Significance was determined via paired T test.

3.2 IL-36 γ induces proinflammatory cytokines and adhesion molecules

To investigate the proinflammatory effects of IL-36 on HFFs, archetypal proinflammatory cytokine IL-1 β , chemokine IL-8, and adhesion molecules ICAM and VCAM were measured. Serum starvation was performed since there are many unidentified proteins in FCS that may interfere with IL-36. Additionally, FCS induces its own changes in cells due to its high concentration of growth factors, proteins and cytokines, so subtle effects may not be observable behind its stimulation. Treatment with FCS does, however, give an indication of the general effect of growth factors on induction of cytokines and adhesion molecules.

In serum-starved conditions, there was a trend towards upregulation of IL-1 β and IL-8 mRNA, however this was not statistically significant for either (Figure 6 A+B). When treated with FCS and IL-36, IL-1 induction was observed ($p=0.0038$) however the same effect was not statistically significant for IL-8 mRNA (Figure 6 A+B).

To determine protein production of chemokines, ELISA for IL-8 was performed. Any induction of IL-8 by IL-36 in serum-starved conditions was not statistically significant, however with FCS, IL-36 does induce IL-8 ($p=0.0004$) (Figure 7).

With regards to adhesion molecules, no statistically significant effects were apparent. However there was a trend towards upregulation of ICAM mRNA when treated with FCS ($p=0.0604$)(Figure 6C).

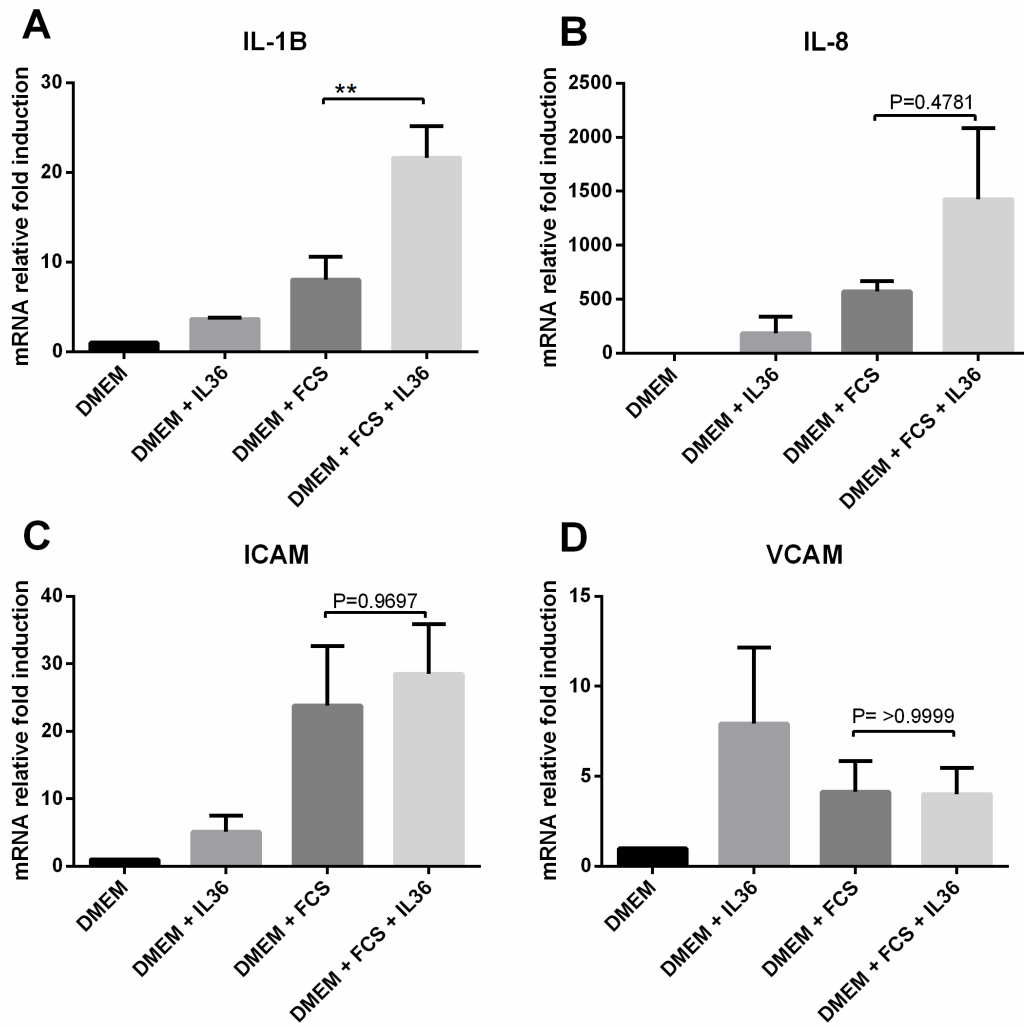


Figure 6. FCS enhances induction of proinflammatory molecules by IL-36. HFFs were serum starved overnight, then treated with either 10% FCS or 100ng/ml IL-36 γ . Sample mRNA is normalised to GAPDH. qRT-PCR was performed for (A) IL-1, (B) IL-8, (C) ICAM and (D) VCAM at 6 hours, and (E) ELISA for IL-8 was performed at 24hours. $n \geq 4$. Targets were normalised to unstimulated samples. Significance was determined via one-way ANOVA.

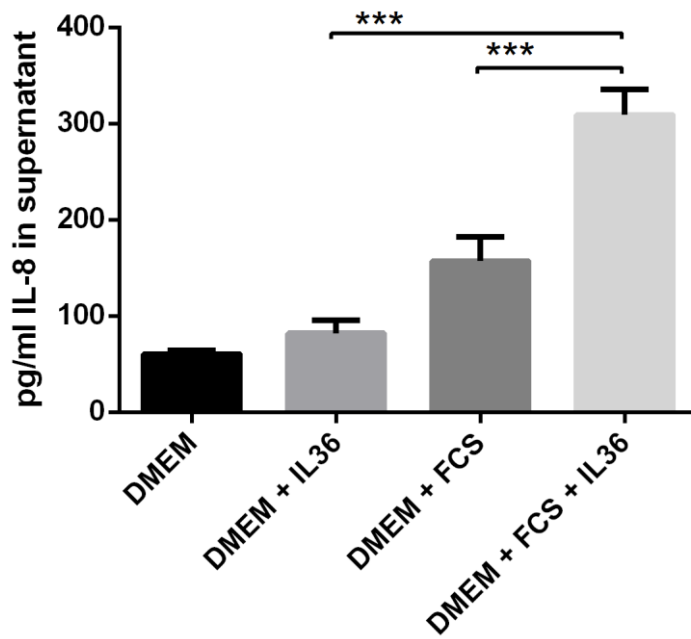


Figure 7. FCS increases IL-8 induction by IL-36. HFFs were serum starved overnight, then treated with either 10% FCS or 100ng/ml IL-36 γ . ELISA for IL-8 was performed at 24hours. n = 4. Significance was determined via one-way ANOVA.

3.3 IL-36 γ upregulates miR-21

To determine if microRNA are induced because of IL-36 stimulation in HFFs, we probed for five microRNA targets through qRT-PCR. MicroRNA to be examined were selected for being associated with both mTOR and psoriasis, with evidence suggesting their expression is altered in lesional tissues. miR-21 is a well-studied oncoMiR, elevated in psoriatic skin, and its expression has a strong correlation with TNF α mRNA (Guinea-Viniegra et al. 2014). It is associated with altered cytokine expression through STAT3 activation (Guinea-Viniegra et al. 2014) and matrix metalloproteinase production (Gabriely et al. 2008). miR-146a is a negative regulator of the innate immune response, via direct targeting of tumour necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) and interleukin-1 receptor-associated kinase 1 (IRAK1), both of which are important components in signal transduction for proinflammatory cytokines (O'Connell et al. 2012). It has been found to be highly upregulated in psoriatic skin in a number of studies looking at different compartments and infiltrating immune cells (Løvendorf et al. 2015; Xia et al. 2012; Sonkoly et al. 2007). miR-155 is found to be elevated in psoriatic skin in some of the same studies (Løvendorf et al. 2015; Ichihara et al. 2011). miR-210 upregulated in psoriasis (Lerman et al. 2011) Like miR-21, miR-221 is associated with the production of matrix metalloproteinases albeit in a negative fashion (Zibert et al. 2010).

We identified that miR-21 is upregulated by IL-36 γ stimulation in HFFs at 6 hours ($p=0.0420$) and there is possibly a non-significant trend towards upregulation miR-221 (and $p=0.1757$) (Figure 8A + E). There does not appear to be any consistent modulation of miR-146a, miR-155 or miR-210 (Figure 8B-D).

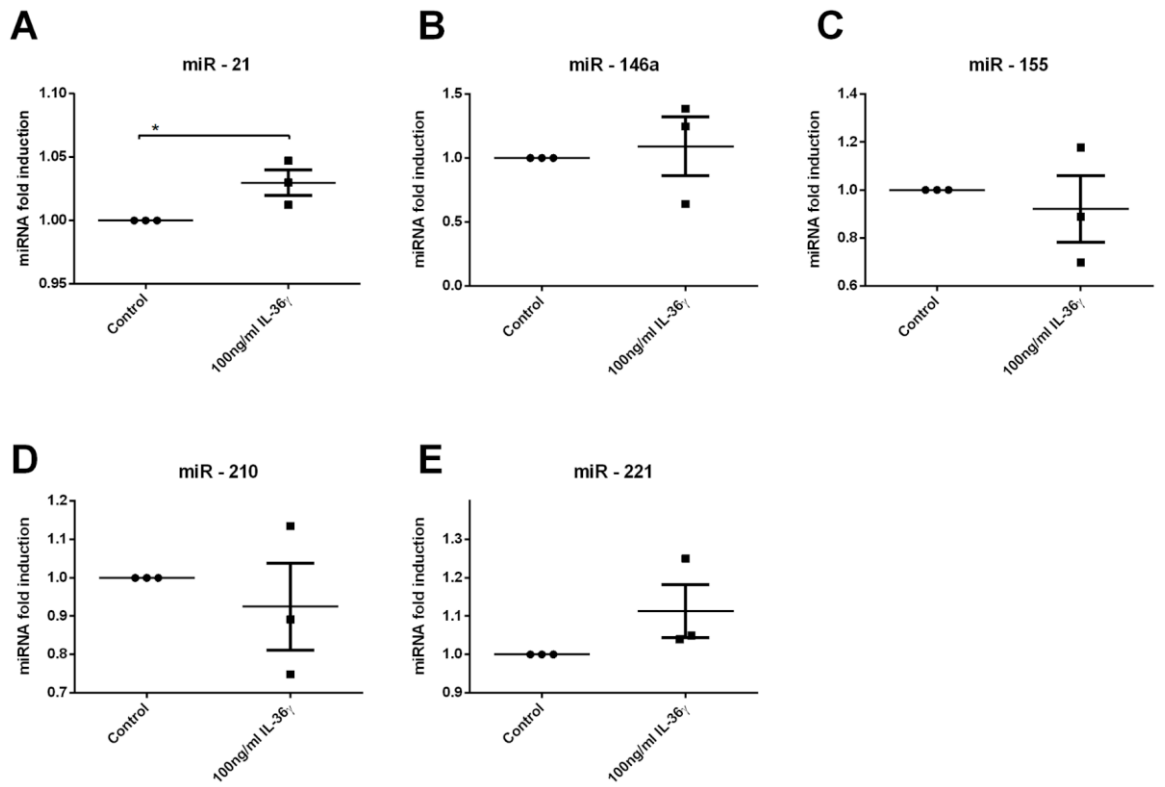


Figure 8. IL-36 upregulates miR-21 HFFs were treated with 100ng/ml IL-36 γ and analysed after 6 hours by qRT-PCR for miR-21 (A), miR-146a (B), miR-155 (C), miR-210 (D) and miR-221. Sample miRNA is normalised to U6. Significance was determined via paired T test.

3.4 IL-36 γ induces IL-8 via NF κ B

To investigate IL-36 induced signal transduction, a pharmaceutical inhibitor of NF κ B, BAY 11-7082, was utilised. In an unstimulated state, NF κ B is maintained in an inactive form in the cytosol by association with regulatory proteins, termed inhibitors of κ B (I κ B). In response to inflammatory cytokines or cellular stresses, I κ B is phosphorylated by I κ B Kinase (IKK), which, together with the ubiquitination and proteolytic degradation of I κ B, leads to the activation and nuclear translocation of NF κ B. BAY 11-7082 exerts its effects by inhibiting the activity of IKK, thereby maintaining NF κ B in its inactive complex with I κ B (Pierce et al. 1997).

BAY-11 treatment initially results in a reduction of basal IL-8 mRNA ($p < 0.0001$) (Figure 9A), and although protein measured by ELISA does demonstrate a downregulation, the effect is not significant ($p = 0.0705$) (see Figure 9B). BAY-11 also inhibited IL-36 γ induced IL-8 mRNA ($p < 0.0001$) (Figure 9A) and protein (< 0.0001) (Figure 9B), indicating the cytokine inducing effects of IL-36 γ are NF κ B dependent.

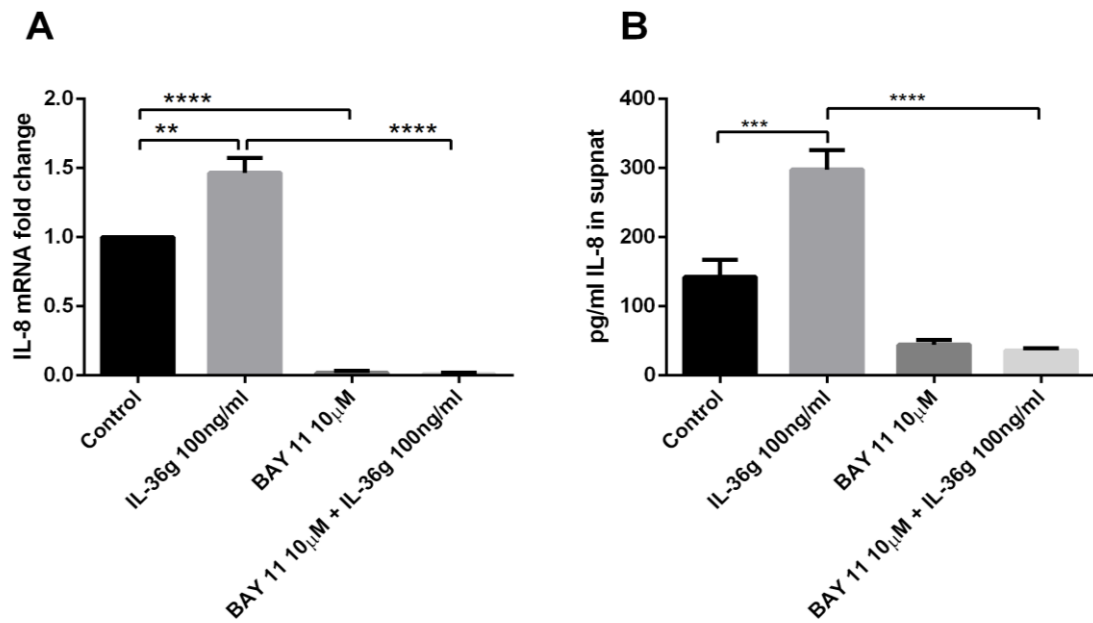


Figure 9. NF κ B inhibition eliminates basal and IL-36 induced IL-8 expression. HFFs were pre-treated with 10 μ M BAY 11-7082 for 1 hour, prior to stimulation with 100ng/ml IL-36 γ . Reduction in both basal and IL-36 induced IL-8 expression was observed by both (A) qRT-PCR and (B) ELISA. n=3. Sample mRNA is normalised to GAPDH. Significance was determined via one-way ANOVA.

3.5 IL-36 γ modulates AKT and mTOR activity

The key serine/threonine kinase AKT is activated following two phosphorylation events. AKT is phosphorylated on thr308 by PDK1 and on ser473 by mTORC2 (Laplante and Sabatini 2012). Expectedly, no phosphorylation of AKT was observable in serum starved conditions, this was regardless of the administration of IL-36 γ (Figure 10). FCS caused a significant upregulation of pAKTthr308 ($p < 0.0001$), there was also an upregulation when treated with FCS alongside IL-36 ($p = 0.0026$), however to a lesser degree than FCS alone ($p = 0.0007$) (see Figure 11C). Similarly, unobservable while starved, pAKTser473 was strongly induced by FCS ($p = 0.0285$). Again, this was ameliorated by concurrent IL-36 γ treatment ($p = 0.2905$) (Figure 11D). This indicates that IL-36 does not activate AKT, and may have a role in its inhibition.

pS6 is commonly used as a read out of mTOR signalling (Andreoli et al. 2015). In serum-starved cells pS6 signalling is only observable 30 minutes following DMEM replenishment (Figure 10), probably reflecting increased amino acid and sugar availability. Replenishment of DMEM alongside IL-36 γ stimulation does not lead to a statistically significant change, although a downward trend may be apparent ($p = 0.0857$) (Figure 11A). As expected FCS stimulation causes upregulation of pS6, observable at 30 minutes and continuing up to 24 hours (Figure 11A). However, unlike in serum-starved conditions, when treated with FCS and IL-36 γ there is a statistically insignificant initial increase in pS6 at 30 minutes. At 24 hours there is a downregulation of pS6 signalling ($p = 0.026$) (Figure 11A, B).

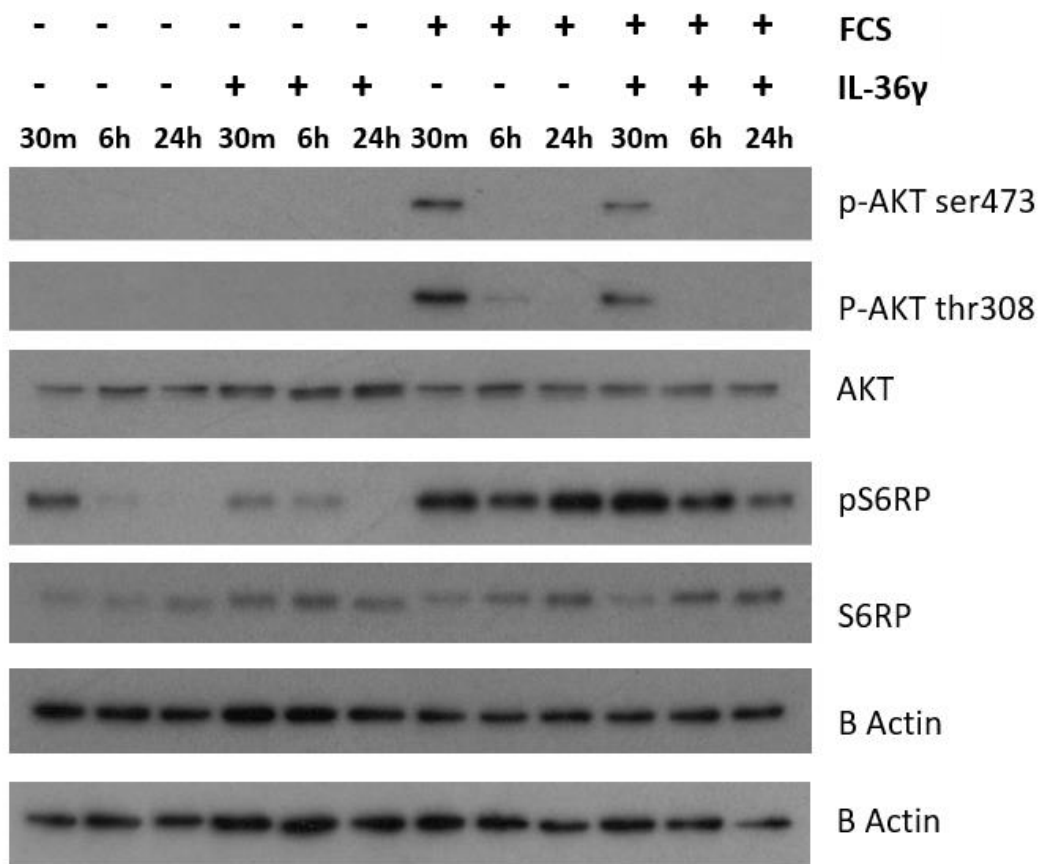


Figure 10. IL-36 reduces AKT phosphorylation and alters pS6 signalling. HFFs were serum starved for 16 hours, before media replacement, or treatment with 10% FCS or 100ng/ml IL-36 γ . A representative western blot showing pAKTser473, pAKTthr308 and pS6RP signalling at 30min, 6 and 24-hour timepoints.

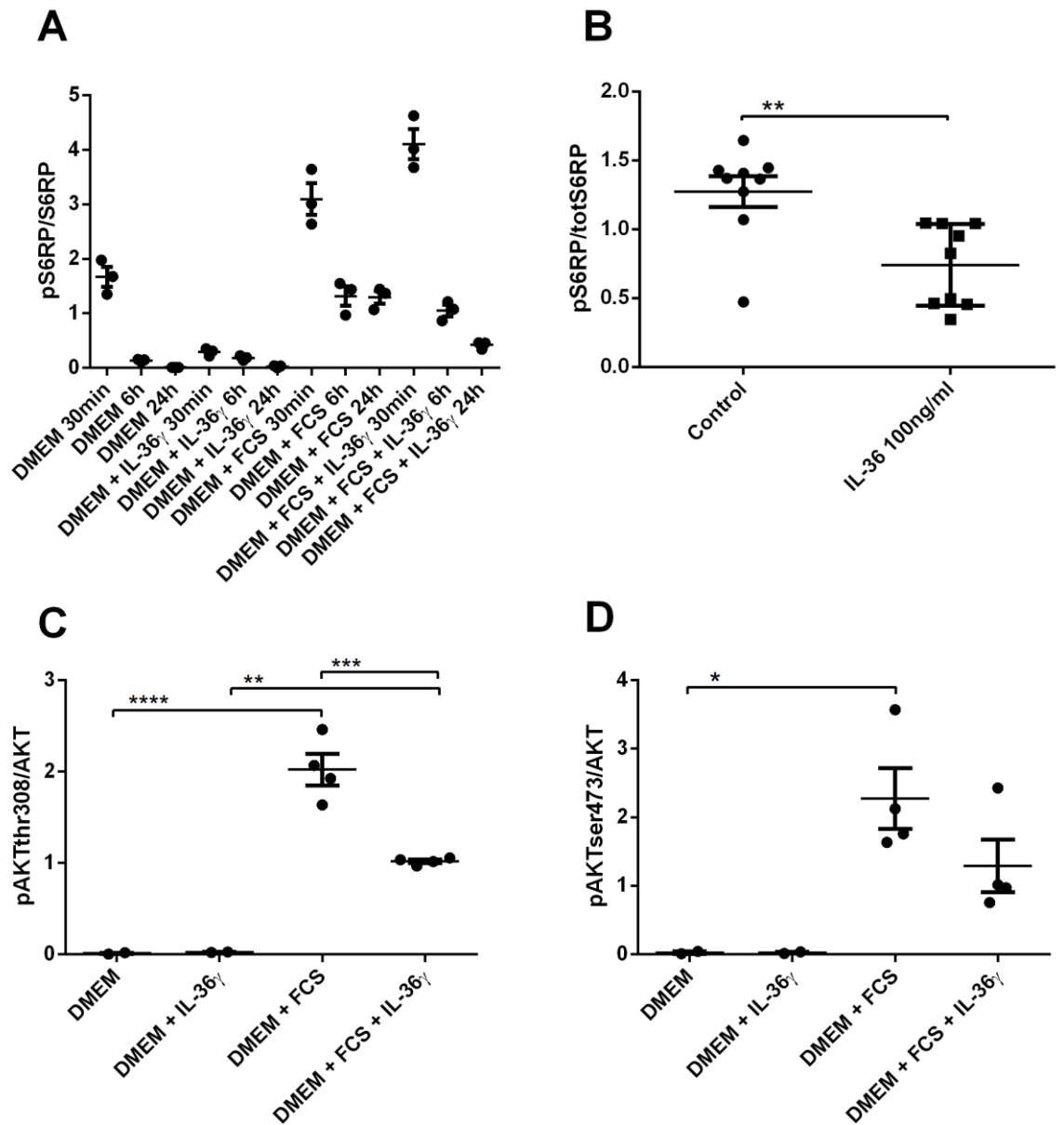


Figure 11. IL-36 reduces AKT phosphorylation and alters pS6 signalling. HFFs were serum starved for 16 hours, before media replacement, or treatment with 10% FCS or 100ng/ml IL-36 γ . Densitometric analysis was performed on western blots for (A + B) pS6RP/S6RP, (C) pAKTthr308/AKT, and (D) pAKTser473/AKT. Panel B represents 24 hours following stimulation. Panels (C + D) represent 30 mins following stimulation. Serum starved conditions for pAKT have two biological repeats, and all other samples have between three and nine.

3.6 mTOR inhibition increases IL-8 induction by IL-36 γ

Rapamycin is (at 50nM) a specific inhibitor of mTOR1 activity, via an indirect mechanism (Foster & Toschi 2009). Rapamycin was used to demonstrate whether mTOR modulation results in alteration of inflammatory cytokine signalling from IL-36. This was measured by qRT-PCR of IL-1 β and IL-8 mRNA, and IL-8 was measured on a protein level by ELISA. Again, western blot for S6 was performed in order to measure mTORC1 activity.

Diminution of detectable pS6 signalling occurs following treatment with rapamycin ($p=0.0387$) (Figure 12). With regards to IL-8 mRNA, an induction is observable with rapamycin treatment ($p<0.0001$), and a larger induction when treated with IL-36 γ and rapamycin, compared to IL-36 alone ($p<0.0001$) (Figure 13A). Upregulation of IL-8 by rapamycin was also apparent when measured by ELISA ($p=0.0028$), as well as enhanced IL-8 induction by IL-36 γ during mTOR inhibition ($p<0.0001$) (Figure 13B).

A non-significant trend towards upregulation was observed for IL-1 β ($p=0.1759$), and it does appear there is an increase in variance in IL-36 γ and rapamycin-treated cells (see Figure 13C).

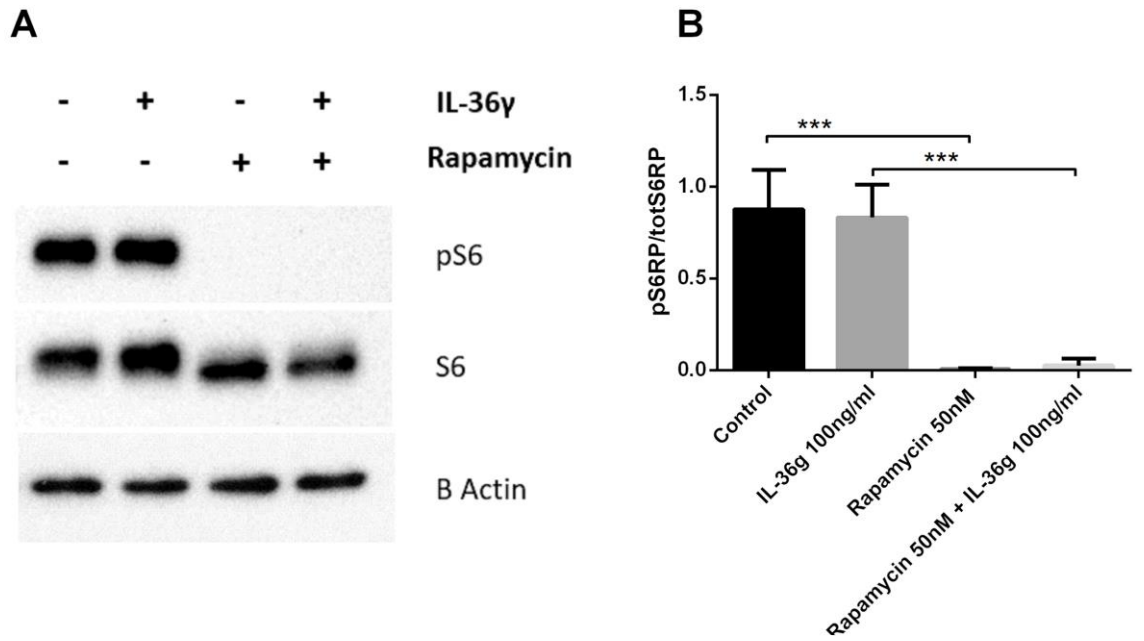


Figure 12. Rapamycin reduces mTOR signalling. HFFs were serum starved overnight, pre-treated with 50nM rapamycin, then stimulated with 10% FCS and 100ng/ml IL-36 γ . n = 3. Representative western blot (A) and densitometric analysis (B) show complete reduction in pS6/S6.

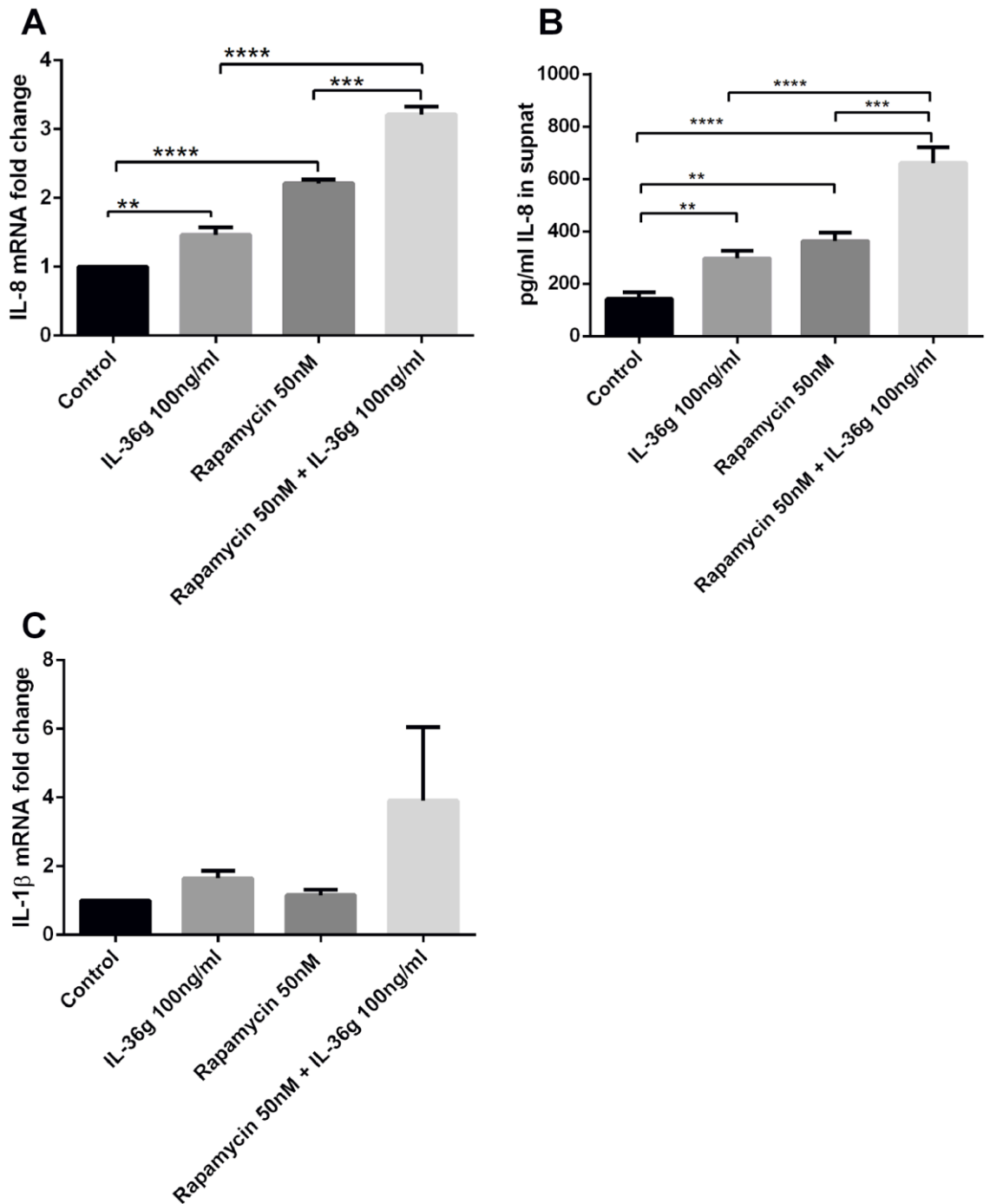


Figure 13. mTOR inhibition enhances effects of IL-36. HFFs were serum starved overnight, pre-treated with 50nM rapamycin, then stimulated with 10% FCS and 100ng/ml IL-36 γ . n = 3. Rapamycin upregulates IL-8 and increases IL-36 induced upregulation of IL-8 when measured by qRT-PCR (A) and ELISA (B). Rapamycin also increases upregulation of IL-1 β on a mRNA level (C). Sample mRNA is normalised to GAPDH. Significance was determined via one-way ANOVA

3.7 TSC knockdown activates mTOR but does not alter IL-8 induction by IL-36 γ

To determine if a downregulation of proinflammatory cytokine signalling would be observed with mTOR upregulation, reciprocal to rapamycin treatment, TSC knockdown was performed. The TSC complex is a negative regulator of mTOR activity, TSC1 knockdown was used to induce mTOR signalling in serum depleted conditions (Laplante and Sabatini 2012). Western blot for S6 was performed to measure mTORC1 activity, and this was followed by ELISA for IL-8 to determine a functional effect on cytokine induction by IL-36, following TSC1 knockdown. Serum starvation was used in this experiment so that pS6 would be observable, and not obscured by FCS induced mTORC1 activation.

Indeed, TSC1 knockdown did lead to increased pS6 signalling ($p=0.0300$) (Figure 14A + B). However, this did not translate to any observable alteration in IL-8 induction by IL-36 γ . Although generally IL-8 was detected at much lower levels in samples that had undergone transfection with either siNTC or siTSC (Figure 15), this level of induction is comparable to IL-8 induction in serum starved cells, where it was also found to be insignificant.

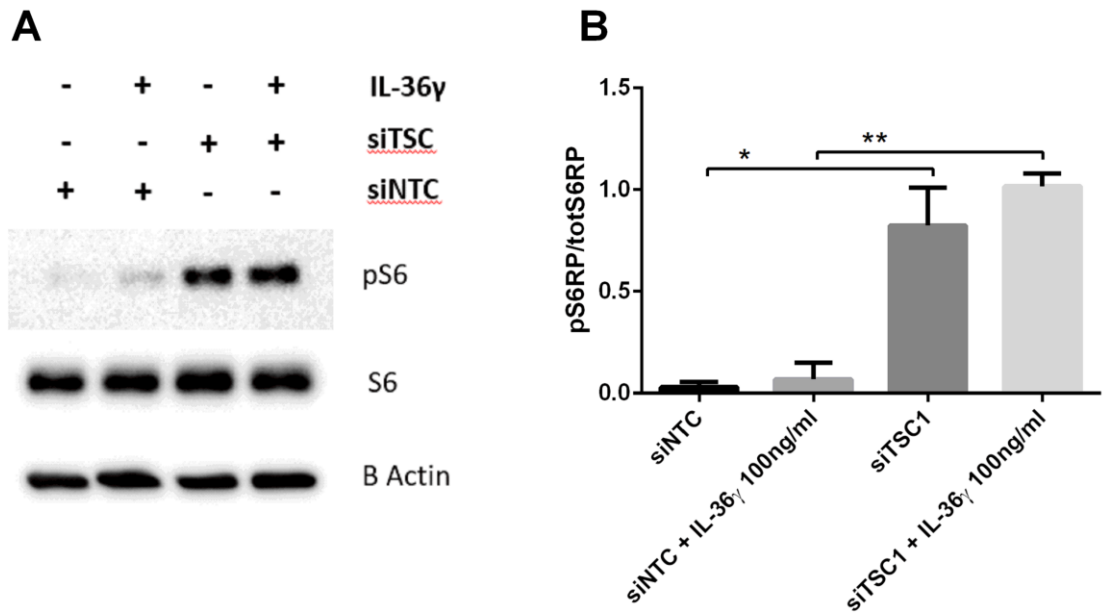


Figure 14. TSC knockdown induces mTOR activity. HFFs were transfected with 50nM siNTC or siTSC, and following 24h placed in FCS free conditions, and stimulated with 100ng/ml IL-36 γ . n=3. Western blot (A) and densitometric analysis (B) demonstrate TSC knockdown results in upregulation of pS6/S6. Significance was determined via one-way ANOVA

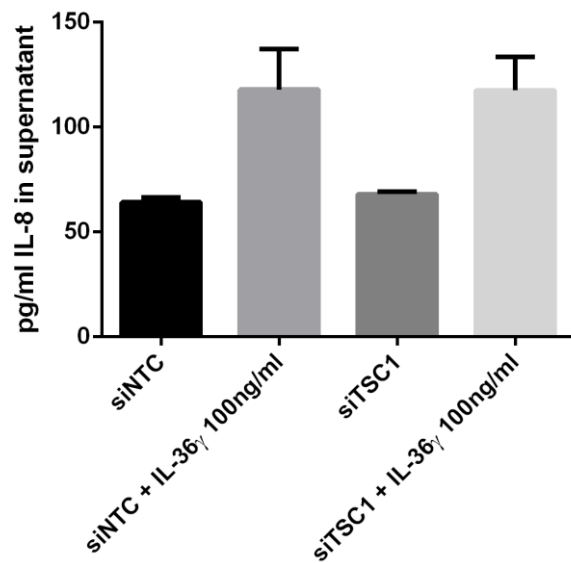


Figure 15. TSC knockdown does not alter IL-8 induction by IL-36. HFFs were transfected with 50nM siNTC or siTSC, and following 24h placed in FCS free conditions, and stimulated with 100ng/ml IL-36 γ . n=3. ELISA demonstrates that transfection with siTSC does not alter IL-8 production, nor does it alter IL-36 induced IL-8 production. Significance was determined via one-way ANOVA

4. Discussion

4.1 Summary

This project aimed to determine the proinflammatory effects of IL-36 γ on stromal cells, before exploring the effects of IL-36 γ on signal transduction, and testing if mTOR modulation affects IL-36 induced cytokine production. To achieve this, we assessed signal transduction downstream of IL-36 and identified a negative role for IL-36 γ in AKT activity, possibly due to mTOR induction via an independent mechanism. We identified that NF κ B is necessary for cytokine induction in dermal fibroblasts by IL-36 γ . Finally, it was demonstrated that mTORC1 negatively regulates IL-8 induction in fibroblasts, however we were unable to demonstrate an effect from upregulation of mTOR using RNAi.

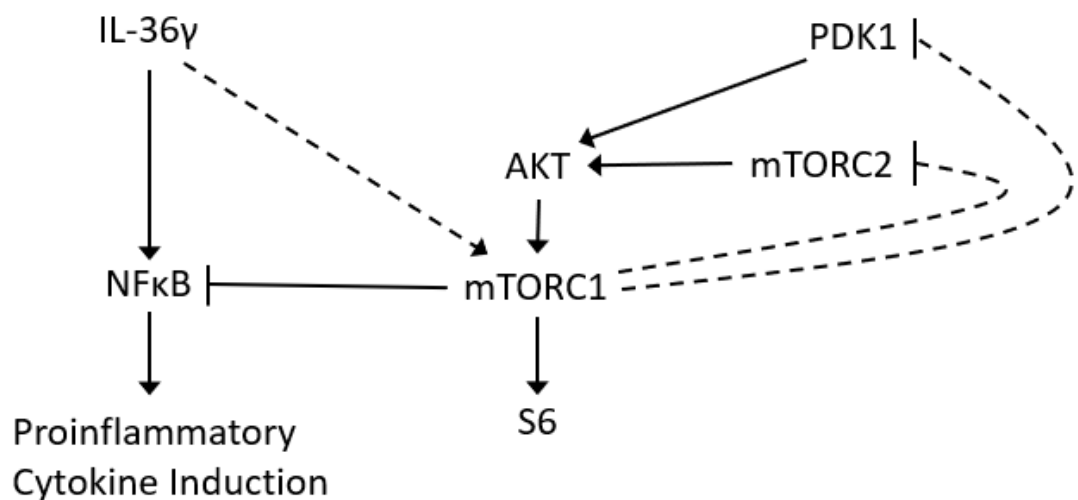


Figure 16. Synopsis of crosstalk between IL-36 signalling and mTOR in HFFs

4.2 The effects of IL-36 γ on signal transduction

There are multiple explanations for the downregulation in AKT activity observable at 30 minutes. It could be suggested that there is brief phosphorylation of AKT followed by a compensatory downregulation, as in human lung fibroblasts (HLFs) AKT phosphorylation due to IL-36 γ occurs as quickly as 5 minutes following stimulation. However, this effect was shown to be sustained for at least an hour (Zhang et al. 2017). In mouse embryonic fibroblasts (MEFs) TNF α induced AKT activation was at its maximum at 30 minutes post stimulation (Ghosh et al. 2006). Additionally, no induction of AKT was seen in serum starved conditions.

Activation of AKT by inflammatory signalling has been demonstrated to be a cell type specific phenomenon (Ozes et al. 2001; Delhase et al. 2000). In the context of human fibroblasts, multiple kinases such as ERK, p38 and c-Jun N-Terminal kinase (JNK) have been demonstrated to modulate mTOR through an AKT independent mechanisms (Li et al. 2003; Ma et al. 2005). In multiple breast cancer lines IKK beta (a major upstream regulator of NF κ B) has been shown to activate mTOR via the TSC complex, which has a IKK β target site at Ser511 (Lee et al. 2007).

Therefore, a better explanation for the changes in AKT would be that they are due to mTOR activation via an AKT independent mechanism, because of the well described compensatory downregulation mechanisms of AKT and mTORC2 following mTORC1 activation (Laplante & Sabatini 2012).

A clear limitation to the interpretation of signalling events in IL-36 γ stimulation, is the lack of statistical significance in the quantification of western blot data for S6 at 30 minutes. Although this was not deemed significant by ANOVA, because of variation in degrees of induction, an upregulation was observable in all three

repeats of this experiment. Due to time constraints it was not possible to perform more repeats of this experiment, and this should be done before a firm association between mTOR activation and IL-36 signalling is made. Conditional on this, AKT could be either pharmaceutically inhibited or through RNAi to see if the effects of IL-36 on mTOR are preserved. Inhibition of other targets such as ERK, p38, JNK and IKK β could be performed to elucidate which kinases are necessary for mTOR activation. Knockdown of TSC1 or 2 would reveal if this is a TSC dependant mechanism, as found in other cell types (Lee et al. 2007). Unfortunately, due to time constraints assessing knockdown of TSC1 on mTOR activation 30 minutes post stimulation with IL-36 γ was not performed.

BAY-11 treatment eliminated basal IL-8 and its induction by IL-36 γ suggesting this is mediated by NF κ B. Although it has also been shown that BAY-11 has effects independent of NF κ B inhibition with inhibitory effects on inflammasome activity (Juliana et al. 2010) as well as indirect suppression of MAPK activation pathways (Lee et al. 2012). Verification that IL-36 γ signals through NF κ B would be supported by blotting for phospho-NF κ B, however we unable to successfully do this. These findings agree with research performed on synovial fibroblasts, where NF κ B activation is necessary for IL-36 γ transduction (Frey et al. 2013). However, our findings contrast to those of Zhang et al, where it was demonstrated that BAY-11 treatment does not alter IL-8 release by any of the IL-36 cytokines in human lung fibroblasts, or human bronchial epithelial cells. (Zhang et al. 2017)

4.3 Upregulation of miR-21

miR-21 was shown to be induced at 6 hours following IL-36 stimulation. In fibroblasts miR-21 alters fibroblast expression of matrix metalloproteinases production, an enzyme with pathologic significance in psoriasis (Gabriely et al. 2008). In macrophages miR-21 interferes with TNF α production through a mechanism involving Phosphatase and tensin homolog (PTEN), a phosphatase which acts as a key negative regulator of the PI3K/AKT/mTOR axis (Das et al. 2014). Elsewhere, lipopolysaccharide (LPS) has been demonstrated to upregulate miR-21 in fibroblasts at 4 hours, with no substantial increase between 4 and 8 hours (Sheedy et al. 2010). Although the magnitude of effect on miR-21 was small, this is a very highly expressed microRNA, and stimulation with Angiopoietin 1 (ANG1), a strong mTOR inducer, only results in a 1.2-1.5 fold induction. (Warner et al. 2016). The role of miR-21 in dermal psoriatic fibroblasts should be investigated more deeply. Due to time constraints later time points were not performed, but may have revealed changes in the other miRNA that were probed for.

4.4 Effects of mTOR modulation on cytokine induction

To assess if modulation of mTOR impacts on cytokine expression, cells were treated in several ways. Firstly, it was shown that FCS upregulates mTOR activity. Induction of IL-8 by IL-36 was increased in the presence of FCS, this was demonstrated by both qRT-PCR and ELISA, with FCS also leading to some induction in the absence of IL-36 γ and similar effects were observed in IL-1 β mRNA.

There are a number of significant limitations when using FCS to induce mTOR activity, as it is a variable natural product containing many undefined proteins,

including multiple growth factors, it has potential to variably alter cytokine profiles of treated cells, and its effects are broad with effects far beyond that of mTOR activation, as although mTOR is downstream of many growth factor receptors, it does not exist in isolation as their only effector (Hayashi et al. 1978; Saxton and Sabatini 2017). When assessing subtle signalling dynamics or cytokine changes, and where serum starvation is inappropriate, it would be beneficial to use a chemically defined media.

Furthermore, although phenotypically similar, in human foreskin fibroblasts, mTORC1 inhibition and serum starvation leads to divergent and unique transcriptomic profiles (Gillespie et al. 2015). Further dissection reveals that following rapamycin treatment, cytokine related pathways were amongst the most highly altered genes in the array, especially IL-8 which was confirmed by both qRT-PCR and ELISA (Gillespie et al. 2015). This suggests that using serum is perhaps not the best tool for assessing the contribution of mTOR to inflammatory signalling.

In the dose range used, rapamycin is a selective inhibitor of mTORC1 (Foster & Toschi 2009). Rapamycin treatment leads to an increase in IL-8 expression, as well as augmented induction by IL-36 γ , on both a transcriptional and a protein level. It is possible that upregulation of inflammatory cytokines by mTORC1 inhibition is not limited to IL-8, as although alone rapamycin does not induce IL-1 β , there was a non-statistically significant trend towards the upregulation IL-36 γ mediated expression. Due to time constraints this was unable to be repeated, and for the same reason IL-1 β was not measured on a protein level, however this may be a valuable avenue in future work.

This suggests that mTORC1 has a negative role in the regulation of NF κ B. The negative role of mTOR in proinflammatory cytokine expression by fibroblasts has been demonstrated elsewhere where rapamycin has been shown to enhance TNF α

stimulated production of both IL-6 and IL-8, in human orbital fibroblasts (Lee et al. 2013) However, it cannot be said explicitly if this is directly due to mTORC1, as it is well documented that rapamycin treatment upregulates both mTORC2 activity and components of pathway above mTORC1 such as AKT and effects seen in rapamycin treatment may reflect the upregulation of other pathways. As S6K1 also inhibits MAPKs (Carracedo et al. 2008), the dynamics of other signalling pathways, such as ERK, p38 or JNK, in rapamycin treated HFFs needs to be elicited. Indeed activator protein 1 (AP-1), the putative target of JNK is activated in IL-36 signalling and has been demonstrated to be altered in some instances of rapamycin treatment (Weichhart et al. 2008).

The clinical implications of this must be interpreted with caution because prolonged exposure to rapamycin has been shown to result in impaired mTORC2 signalling in some cell lines, through a mechanism where free mTOR becomes bound and unable to form functional mTORC2 complexes (Sarbasov et al. 2006). This is significant as in its clinical context patients are often exposed to long treatment regimes. In cell culture, this is something that could be investigated by utilising prolonged treatment with rapamycin or by use of mTORC1/mTORC2 dual inhibitors.

Additionally, rapamycin does not inhibit all of the functions of mTORC1, as although phosphorylation of S6 is reduced, inhibition of 4E-BP1 may be spared (Choo et al. 2008). It could be explored further if complete mTOR inhibition results in a different effect on inflammatory signalling.

RNAi of TSC1 was successful with regards to the fact mTOR signalling was induced, despite a background of serum-starvation, however this did not translate into any measurable effect on IL-8 induction by IL-36 γ . In contrast, it has been reported elsewhere that activation of mTOR by loss of the inhibitory TSC1/TSC2 function

resulted in decreased NF κ B activity in murine embryonic fibroblasts (Ghosh et al. 2006).

This may either reflect a difference between murine and human fibroblasts or may reflect altered signalling dynamics in stable knockout. Additionally, we utilised serum starvation, so phosphorylation of S6 would be readily observable, and to avoid some of the limitations of FCS already discussed. This may possibly indicate that some degree of growth factor signalling may be necessary for mTOR to regulate NF κ B. Another interpretation could be that since most of the experiments were done on a background of stable TSC2 knockout, the regulation of NF κ B is specific to TSC2 rather than the functioning of the heterodimeric complex. Alternatively, a difference in IL-8 induction may simply have been very small, since in serum starved conditions, induction of IL-8 by IL-36 γ is less substantial compared to in serum replete conditions, indeed in previous experiments the induction of IL-8 by IL-36 γ during serum starvation has not been statistically significant.

4.5 Other implications of findings

mTORC1 is sensitive to modulation by amino acids particularly glutamine, leucine, and arginine (Jewell et al. 2013; Rebsamen & Superti-Furga 2016). This is significant for future investigations as the effect of rapamycin treatment may reflect a role for dermal fibroblasts in altering/coordinating the local inflammatory microenvironment to nutrient availability and could be relatively easily be studied through the restriction and introduction of amino acids. Indeed, this has been performed in fibroblast-like-synoviocytes, an effector cell in rheumatoid arthritis, where it was revealed that amino acid restriction leads to upregulation of IL-8, through a mTOR dependant mechanism (Karonitsch et al. 2018).

Pharmaceutical inhibition of mTOR through rapamycin has been shown to have rather limited clinical efficacy in psoriatic disease, despite its promise as potent immunosuppressive agents (Ormerod et al. 2005). Using an imiquimod induced psoriasis mouse model, rapamycin successfully reduced disease severity. With reduction in skin thickness and a marked reduction in neovascularization in the skin. It was speculated that these anti-psoriatic effects were driven by mTORC1 inhibition enhancing differentiation of keratinocytes, and possibly reducing their proinflammatory cytokine output (Bürger et al. 2017). This is accompanied by the well documented effects of rapamycin on T cells (Sehgal & Bansbach 1993). However, it has been shown elsewhere that in innate immune cells such as monocytes and dendritic cells, that mTOR inhibition results in an excessively proinflammatory phenotype (Weichhart et al. 2008), it has been shown here that the induction of inflammatory associated cytokines by IL-36 is enhanced by rapamycin treatment in primary dermal fibroblasts, which may somewhat explain residual psoriatic symptoms following mTOR inhibition.

The complexities of mTOR having a pleiotropic role in inflammatory responses are illustrated by events such as interstitial pneumonitis, occurring in patients receiving rapamycin as part of a post-transplant immunosuppression regimen (Singer et al. 2000). This work could suggest that some portion of this effect may be attributable to enhancement induction of proinflammatory cytokines in stromal cells of affected individuals.

4.6 Further limitations

Regulation of the IL-36 receptor by either FCS or IL-36 γ was not statistically significant and was only assessed on a mRNA level. However, the dynamics of receptor expression on a functional level would need to be assessed over a greater time frame, and through a modality which can appreciate changes in protein, and cell surface expression, since regulation may occur on a non-transcriptional level.

The degree of induction as measured by qRT-PCR and ELISA differed. Induction of cytokines occurs at multiple levels, both transcriptionally and post transcriptionally via mechanisms such as affecting the stability of mRNA, or the proteins may require post translational processing for full functional activity as is the case with both IL-1 β and IL-36 γ (Rubartelli et al. 1993; Ainscough et al. 2017). Furthermore secreted cytokines may interact with in vitro cultured cells, resulting in their removal from supernatant either by degradation or consumption. Treatments such as FCS, rapamycin and siTSC may alter this uptake (Verfaillie et al. 2001). Additionally IL-8 is not the only readout of fibroblast activation, as activation is more nuanced than a global activation.

Due to the functional diversity observable in fibroblasts, HFFs may not be entirely representative of fibroblasts found in the psoriatic environment, because of both the location of their derivation and disease state. This is exemplified by the abolishment of IL-8 induction in our HFFs treated with BAY-11, and IL-36 γ , when in HLFs this was shown to have no effect (Zhang et al. 2017). as well as increased IL-8 induction by TNF in lesional fibroblasts compared to non-involved skin (Zalewska et al. 2006).

4.7 Future Work

These findings help explain the failure of rapalogs, which were initially seen as promising therapeutic agents, in the treatment of psoriatic disease. Future work could investigate more complete mTORC1 inhibition, or dual inhibition of both mTOR compounds.

In order to better define a mechanism for the alteration of AKT/mTOR signalling observed here, we suggest the use of inhibitors for MAPKs, PI3K, and NF- κ B signalling components. It would also be worthwhile investigating the effects of mTOR modulation on other cytokines, as well as alterations in ECM production, which have been shown to alter both angiogenesis and immune cell function in the psoriatic environment

To investigate whether fibroblasts have the potential to function as nutrient-sensing sentinel cells we propose modulating mTOR activity through amino acid availability. This could be relatively easily achieved through the restriction and introduction of amino acids associated with mTORC1 activation.

Finally, due to the limitations of using HFFs as a model for fibroblasts found in the psoriatic environment, future work should confirm whether IL-36 initiates a change in AKT/mTOR signalling in lesional fibroblasts. This should be followed by a direct comparison between lesional and non-lesional samples of dermal fibroblasts.

5 Conclusions

This project aimed to investigate signal transduction downstream of IL-36 γ , in primary dermal fibroblasts, with a focus on the mTOR axis. In addition to the upregulation of proinflammatory cytokines, we revealed a negative role for IL-36 γ in AKT activation, which could be attributed to feedback loops following AKT independent activation of mTOR signalling.

We demonstrated that NF κ B is necessary for cytokine induction by IL-36 γ .

Furthermore, we established that mTOR modulation effects induction of proinflammatory cytokines by IL-36 γ . Inhibition of mTORC1, through treatment with rapamycin, resulted in an upregulation of IL-8, however, we were unable to demonstrate a clear reciprocal effect resulting from the upregulation of mTOR via RNAi.

Appendices

Solution	Reagents
RIPA buffer	150mM NaCl 10mM Tris HCl 0.1% (w/v) SDS 0.1% (v/v) Triton X1000 1% (w/v) Sodium Deoxychlorate 5mM EDTA
Sample Buffer	250mM Tris HCL pH 6.8 8% (w/v) SDS 10% (w/v) Glycerol 5% (v/v) β ME 0.05% (w/v) Bromophenol Blue
Stacking Gel	ddH ₂ O 4.1ml 30% Acrylamide 1mL 1.5M Tris (pH 6.8) 750 μ l 10% SDS 60 μ l 10% APS 60 μ l TEMED 6 μ L
Resolving Gel	ddH ₂ O 6ml 30% Acrylamide 8mL 1.5M Tris (pH 8.8) 5mL 10% SDS 200 μ l 10% APS 200 μ l TEMED 8 μ L
10X TBS	12.2g Tris HCl 87.65 NaCl 1L ddH ₂ O pH 8 Western Blot; pH 7.4 ELISA

Western Blot TBS/T (1%)	100ml 10X TBS pH 8.0 900ml ddH2O 1ml Tween-20
ELISA TBS/T (0.5%)	100ml 10X TBS pH 7.4 900ml ddH2O 0.5ml Tween-20

Abbreviations

4E-BP1: Eukaryotic translation initiation factor 4E-binding protein 1

AGO: Argonaute protein

AKT: Protein kinase B

ANOVA: Analysis of variance

ATP: Adenosine Triphosphate

BCA: bicinchoninic acid

BSA: bovine serum albumin

CT: cycle threshold

CXC10: C-X-C motif chemokine 10

DGCR8: DiGeorge syndrome critical region 8

DMEM: Dulbecco's modified Eagle's Medium

DNA: Deoxyribose nucleic acid

ECL: Enhanced chemiluminescence

ECM: extracellular matrix

ECM: Extracellular matrix

ELISA: enzyme-linked immunosorbent assay

ERK: extracellular signal-regulated kinase

FCS: Foetal calf serum

FLS: Fibroblast-like synoviocyte

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GDP: Guanosine diphosphate

GTP: Guanosine triphosphate

HFF: Human Foreskin Fibroblasts

HIV: Human immunodeficiency virus

HRP: Horseradish peroxidase

ICAM: Intercellular Adhesion Molecule

IFN- γ : Interferon gamma

IKK: I κ B Kinase

IL: Interleukin

IL-1RI2: Interleukin 1 Receptor like 2

IL-36R: Interleukin 36 Receptor

IRAK1: Interleukin-1 receptor-associated kinase 1

JNK: c-Jun N-Terminal kinase

LL-37: cathelicidin

MAPK: Mitogen-activated protein kinases

MEF: Mouse embryonic fibroblasts

mRNA: messenger RNA

mTOR: mechanistic Target of Rapamycin

NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cell

NTC: non-targeting control

PACT: Protein Activator of PKR

PACT: Protein activator of PKR

PASI: Psoriasis associated severity index

PBS: Phosphate buffered saline

PDGFR: Platelet-derived growth factor receptor

PTEN: Phosphatase and tensin homolog

PVDF: Polyvinylidene fluoride

qRT-PCR: Reverse transcription polymerase chain reaction

RBD: double-stranded RNA-binding domains

RIPA: Radioimmunoprecipitation assay buffer

RISC: RNA induced silencing complex

RNA: double-stranded RNA

RNAi: RNA interference

S100A7: psoriasin

SDS: Sodium dodecyl sulphate

SEM: Standard error of the mean

SLE: Systemic lupus erythematosus

STAT3: Signal transducer and activator of transcription 3

TBS: Tris Buffered Saline

TCR: T Cell Receptor

TLR: Toll like receptor

TNF α : Tumour necrosis factor

TRAF6: Tumour necrosis factor receptor (TNFR)-associated factor 6

TRBP: HIV-1 TAR RNA binding protein

TSC: Tuberos Sclerosis Complex

TSC: Tuberos Sclerosis complex

UTR: Untranslated Region

VCAM: Vascular cell adhesion protein

VEGF: Vascular endothelial growth factor

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