Investigating the Role of Epigenetics in the Regulation of Inflammatory Skin Disease

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Abstract

Psoriasis represents a complex interplay between genetic predisposition, the environment and inflammatory responses, and increasing evidence suggests alterations in the epigenome, including histone acetylation, plays a role. Bromodomain-containing proteins regulate gene expression by binding to acetyl-lysine residues on histones and recruiting transcription factors to gene regulatory regions. The development of small molecule inhibitors of bromodomain extraterminal (BET) proteins has enabled interrogation of this pathway. Interestingly BET inhibitors demonstrate anti-proliferative and anti-inflammatory effects in *in vitro* and *in vivo* models of cancer and inflammation. An established *in-vitro* keratinocyte model of cutaneous inflammation was further developed to characterise IL-6 and IL-8 (mRNA and protein) responses to TNFα + IL-17 stimulation. Chromatin immunoprecipitation (ChIP) studies showed psoriasis-relevant stimuli induced dynamic, gene-specific alterations of the epigenome, including histone hyperacetylation, with co-ordinated recruitment of BET proteins (Brd2, Brd3 and Brd4) and RNA polymerase II, to the promoter region of IL-6 and IL-8. The effects of TNFα + IL-17 stimulation in keratinocytes were validated through global gene expression array studies which showed stimulation modulated expression of keratinocyte genes known to be differentially expressed in psoriasis and involved in its pathogenesis. The hypothesis that BET proteins are involved in regulating inflammatory responses in keratinocytes was tested using a specific BET inhibitor, I-BET151; this blocked pathogenic inflammatory responses. In particular, IL-6 and IL-8 responses to TNFα + IL-17 stimulation demonstrated potent sensitivity to I-BET151 treatment; this could be accounted for by the decreased binding of BET proteins and RNA polymerase II to IL-6 and IL-8 gene promoter regions in the presence of the BET inhibitor. Global gene expression array studies showed genes sensitive to BET inhibition were primarily involved in the cell cycle and inflammation, with many relevant to the pathogenesis of psoriasis. In addition, ~20% of genes identified in a previously published meta-analysis of five psoriasis transcriptomic studies were differentially modulated by I-BET151 treatment in TNFα + IL-17 stimulated NHEKs. Furthermore, acetate, a principle metabolite of ethanol, a factor implicated in the development and exacerbation of psoriasis, enhanced IL-6, but not IL-8, responses to TNFα + IL-17 stimulation through gene-specific epigenetic modifications at the promoter region. IFNγ also enhanced IL-6, but not IL-8, response to TNFα + IL-17 stimulation, suggesting i) IL-6 is more sensitive to enhancement by additional disease relevant stimuli and ii) IL-6 and IL-8 are differentially regulated at the transcriptional level; ChIP studies showed increased enrichment of Brd4/p65 at the IL-6 promoter compared to the IL-8 promoter.

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Abbreviations

- ACSS Acetyl coenzymeA synthetase
- ADH Alcohol dehydrogenase
- ALDH Aldehyde dehydrogenase
- BET Bromodomain extraterminal
- Brd Bromodomain
- BSA Bovine serum albumin
- ChIP Chromatin immunoprecipitation
- Ct Cycle threshold
- DEGs Differentially expressed genes
- DMEM Dulbecco's Modified Eagle Medium
- DMSO Dimethyl Sulfoxide
- DNA Deoxyribonucleic acid
- ELISA Enzyme-linked immunosorbent assay
- FCH Fold change
- FCS Fetal Calf Serum
- FDR False discovery rate
- FSL-1 $Pam_2CGDPKHPKSF$
- H3 Histone-H3
- H3K9ac Histone H3, acetylated on lysine 9 residue
- H3K27ac Histone H3, acetylated on lysine 27 residue
- H3K4me1 Histone H3, mono-methylated on lysine 4 residue
- H3K4me3 Histone H3, tri-methylated on lysine 4 residue
- HAT Histone acetyl transferase
- HDAC Histone deacetylase
- HKGS Human Keratinocyte Growth Supplement
- HKSA Heat-killed Staphylococcus *aureus*
- I-BET151 GSK1210151A, BET inhibitor
- IC_{50} Half-maximum inhibitory concentration
- IFN Interferon
- IL Interleukin
- IL-17 Interleukin-17A
- IPA Ingenuity® Pathway Analysis

- LPS — Lipopolysaccharide derived from Escherichia *coli* K235
- LTA — Lipoteichoic acid
- mg — milligram
- ml — millilitre
- mM — millimolar
- MTT — Thiazolyl blue betrazolium bromide
- NFAT — Nuclear factor of activated T cells
- NF-κB — Nuclear factor kappa-light-chain-enhancer of activated B cells
- NHEKs — Normal human epidermal keratinocytes
- nM — nanomolar
- Pam3Cys — Pam_3Cys-Ser-$(Lys)_4$ trihydrochloride
- PASI — Psoriasis area severity index
- PBS — Phosphate Buffered Saline
- PCR — Polymerase chain reaction
- pg — picogram
- PGN — Peptidoglycan derived from Staphylococcus *aureus*
- pol II (S2P) — RNA polymerase II, phosphorylated at its serine 2 residue
- Poly:IC — Polyinosinic:polycytidylic acid
- PSA — Penicillin Streptomycin Amphotericin B
- qPCR — Real-time quantitative polymerase chain reaction
- qRT-PCR — Quantitative reverse transcriptase polymerase chain reaction
- RNA — Ribonucleic acid
- S100 — S100 calcium binding protein
- SED — Standard erythema dose
- SEM — Standard error of the mean
- SRB — Sulphorhodamine B
- S/W — stimulation window
- TE — Trypsin ethylene diamine tetraacetic acid
- TNFα — Tumour necrosis factor alpha
- µg — microgram
- µl — microliter
- µM — micromolar

Chapter 1.

Introduction

1. Introduction

1.1. Structure and function of the skin

The skin covers over $2m^2$ in adult humans and is divided into two layers (Figure 1-1A); the epidermis which is generally 75-150nm thick, but may be up to 600nm on palms and soles; and the dermis which is usually up to 2mm thick, but may be up to 4.5mm on the back; below these two layers is the subcutaneous tissue (Tobin, 2006). Keratinocytes are the major cell type of the epidermis (approximately 80%), other cell types include; T-cells, melanocytes, Langerhans' cells and monocytes. The dermis is composed mainly of collagen-based connective tissue interspersed with blood vessels but also hair follicles, sweat glands, and sebaceous glands. Below the dermis is subcutaneous tissue composed mainly of adipose tissue. The skin has important barrier functions, with protective functions against ultraviolet radiation, infection and excess fluid loss; the majority of these functions are provided by the stratum corneum of the epidermis.

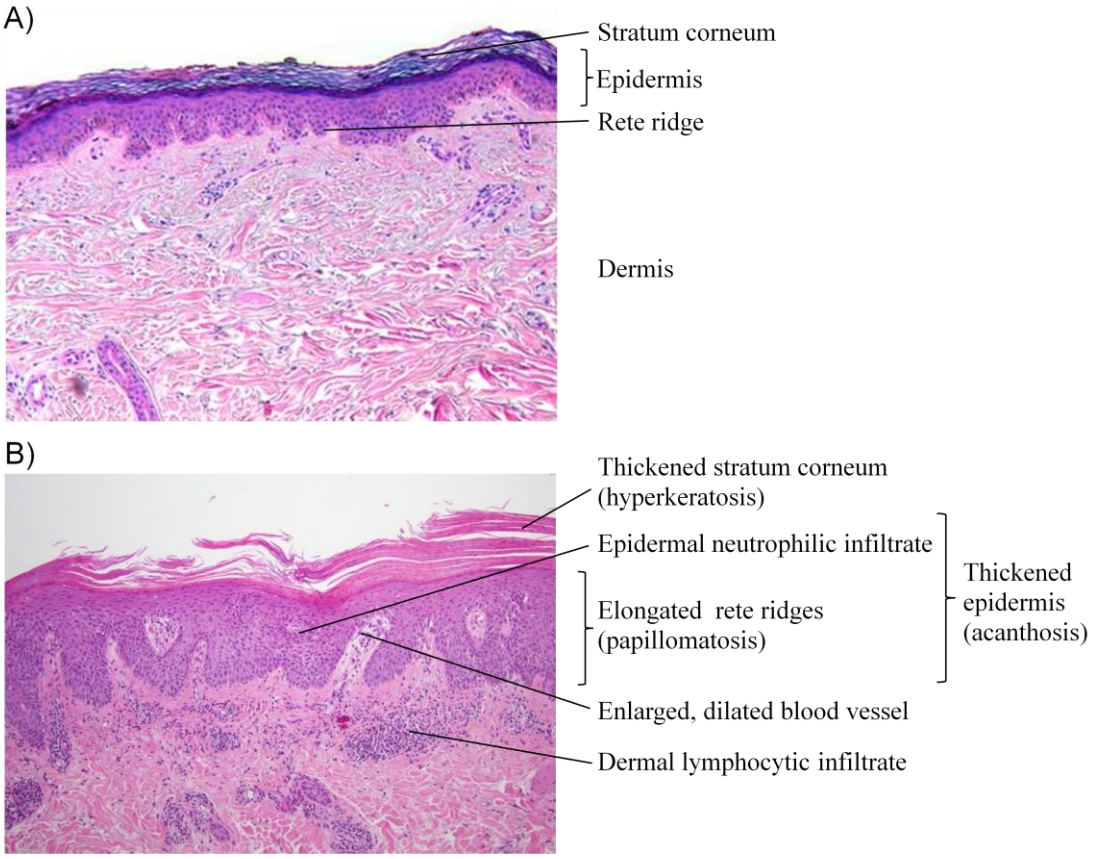


Figure 1-1: Haematoxylin and eosin stain of normal and psoriatic skin (40x objective)

A) Normal skin. Keratinocytes make up 80% of the epidermis which undergo progressive differentiate within the 4 layers of the epidermis. The basal layer contains keratinocyte stem cells; the stratum spinosum contains proliferating cells committed to differentiation (transit amplifying cells); the stratum granulosum contains the majority of differentiating cells and; the stratum corneum consists of anucleated differentiated squames that are eventually shed. Corneocytes within the stratum corneum have a lipid-embedded, crossed-linked protein membrane (termed a "cornified envelope"), which effectively forms a physical barrier to prevent *e.g.* outward water loss and inward infection. The dermis is composed mainly of collagen-based connective tissue interspersed with blood vessels **B**) In psoriasis the epidermis is thickened (acanthosis), with elongation of the rete ridges (papillomatosis), thickening of the stratum corneum (hyperkeratotic) and of the stratum spinosum. The stratum granulosom is thinned or absent (hypogranulosis) due to the loss of differentiation which normally occurs in this layer. Excess keratinocyte proliferation and abnormal differentiation, results in retention of nuclei within the stratum corneum (parakeratosis). In psoriasis, erythema is due to an increased vasculature which reaches into the tips of the dermal papillae and becomes dilated. Epidermal neutrophilic infiltration is also a feature and may localise to form microabscesses. Additional inflammatory infiltrate is composed mainly of myeloid dendritic cells and CD4+ T cells within the dermis and CD8+ cells in the epidermis. (Adapted from www.skinpathology.org)

1.2. Introduction to psoriasis

1.2.1. Epidemiology and characteristics of psoriasis

Psoriasis is a chronic inflammatory dermatosis, which is a category of disease encompassing a broad spectrum of dermatological conditions including atopic eczema, psoriasis, acne and lichen planus; common features include skin barrier disruption and inflammation. These conditions have high prevalence and severity can range from mild to life-threatening. Psoriasis is a chronic relapsing and remitting inflammatory skin disease which typically affects 2-3% of the Western population, although this figure can vary between studies (Stern *et al.*, 2004; Gelfand *et al.*, 2005; Parisi *et al.*, 2015). Psoriasis affects both sexes equally, although some report that men suffer more severe disease than women (Hagg *et al.*, 2013). In patients with psoriasis, approximately 90% will have chronic plaque psoriasis (Figure 1-2) which demonstrates characteristic histological features (Figure 1-1B). Other subtypes of psoriasis include inverse/flexural psoriasis, pustular psoriasis, guttate psoriasis, erythrodermic psoriasis and palmo-plantar psoriasis. There may also be nail and joint involvement with each subtype of psoriasis.

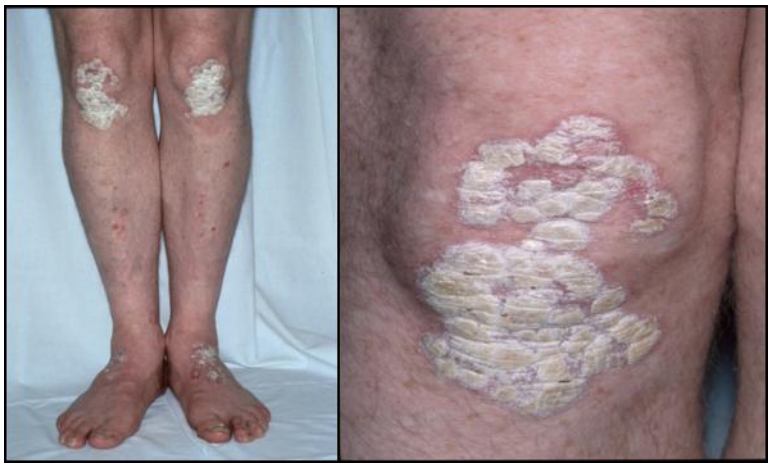


Figure 1-2: Chronic plaque psoriasis

Chronic plaque psoriasis is characterised clinically by well-defined plaques of erythema with thick silver scale often on extensor surfaces, including elbows and knees.
(Source: Prof. N.J. Reynolds, Newcastle Upon Tyne Hospitals, NHS Foundation Trust)

Chronic plaque psoriasis can itself be subdivided into type I, in which onset is <40 years old, there is a strong family history and accounts for approximately 75% of patients with chronic plaque psoriasis; and type II, in which onset is ≥40 years old and accounts for approximately 25% of patients with chronic plaque psoriasis. Recently, the concept of disease endotype has been applied to complex multi-factorial diseases; an endotype can be defined by a genetic signature (*e.g.* IL-36RN in pustular psoriasis or HLA-Cw6 in plaque psoriasis (Gudjonsson *et al.*, 2003; Onoufriadis *et al.*, 2011) or by a molecular signature (*e.g.* high IFNα in systemic lupus erythematosus (Niewold *et al.*, 2010) (section 1.2.4). There is currently great interest in the association of disease endotypes with clinical presentation and course of disease, as well as their potential to inform therapeutic stratification.

Clinical severity of psoriasis can be assessed using the psoriasis activity and severity index (PASI), which includes assessment of erythema (redness), desquamation (scale), induration (thickness) and body sites affected, with a maximum total score of 72. In clinical trials, a baseline PASI score (measured prior to treatment) is usually compared to repeated PASI scores undertaken during treatment. Most clinical trials consider an intervention to be effective if it leads to a 75% improvement in PASI compared baseline (PASI75). A major criticism of this is that patients with very severe disease could still have a high PASI score/disease severity even after PASI75 is achieved. In addition to PASI or as an alternative, disease severity can be measured using Physician Global Assessment (PGA) and impact of disease on a patient's quality of life can be measured by Dermatology Life Quality Index **(**DLQI).

1.2.2. The financial burden of psoriasis and impact on quality of life

Psoriasis can have a significant impact on a patient's quality of life, with some patients rating the impact of skin disease as highly as some major medical diseases, including cancer and heart disease (Rapp *et al.*, 1999). In addition, it is reported that some patients with psoriasis consume more alcohol following diagnosis to cope with the impact of disease (Braathen *et al.*, 1989), reflecting the negative impact that chronic inflammatory dermatoses can have on quality of life (Kirby *et al.*, 2008). Patient with severe psoriasis are also more likely to suffer from depression with an adjusted hazard ratio (HR) for depression of 1.72 in those with severe psoriasis compared to an HR of 1.38 in those with mild disease. In addition to effects on patients, psoriasis can be a considerable financial burden for the wider society; a European study showed the

economic cost of patients with moderately severe psoriasis to be ~£4500 per patient per year, while the economic cost of patients with severe disease was ~£10000 per patient per year (Colombo *et al.*, 2008), with time out of work accounting for ~40% of these costs (Fowler *et al.*, 2008). A more recent 2015 study in the US showed the cost of treating moderate to severe psoriasis was ~£15000 per patient per year (Schaefer *et al.*, 2015).

1.2.3. Current therapies and unmet needs in psoriasis

Current available treatments for psoriasis tends to progress in a step-wise fashion (Figure 1-3). Approximately 70-80% of patient with psoriasis will have mild disease which may respond to topical treatment alone (Schon and Boehncke, 2005). However topical treatments are often time consuming to apply or cosmetically unacceptable (for example being greasy, smelly or stain clothing/bedding) and may not lead to complete resolution of disease; resulting in poor compliance (Jabbar-Lopez *et al.*, 2014). Systemic therapy, for moderate to severe disease, often carries the risk of significant side effects, including renal and hepato-toxicity, increased risk of life-threatening infections and cancer; and not all patients will respond to treatment (Jabbar-Lopez *et al.*, 2014). Interestingly the field of pharmacogenetics, with respect to psoriasis, is expanding with some groups reporting, in a small preliminary study, genetic polymorphisms associated with response to biologic therapy (Tejasvi *et al.*, 2012). This may have important implications for the selection of the most appropriate treatments for patients. However, despite these advances there is a significant unmet therapeutic need, not all patients will respond to currently available treatments which may have many side-effects and there are no predictive biomarkers of response or failure. In addition, even in patients who do show an initial response to biologic therapy, loss of efficacy over time remains a challenge. Furthermore, none of the current therapies offer a potential cure or very long-term remission.

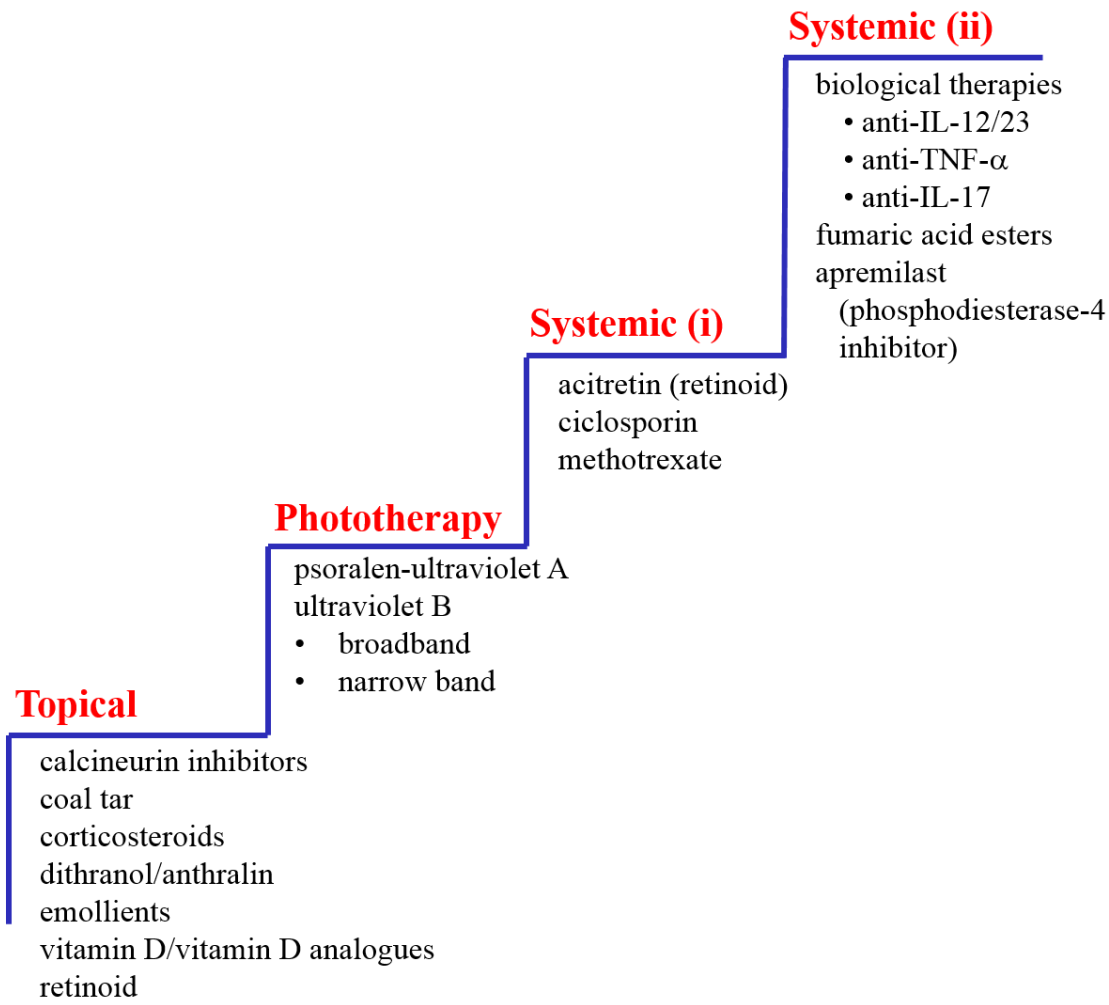


Figure 1-3: Psoriasis treatment ladder

The choice of therapy will be determined by a variety of factors including severity and site of psoriasis. Generally, the greater the severity of disease, the further up the treatment ladder, but patients may receive treatment from several rungs of the ladder and do not necessarily have to transition through each step, *e.g.* if a patient presents with very severe disease for the first time, then topical treatment alone may be inappropriate. (Adapted from Jabbar-Lopez *et al*., 2014)

1.2.4. Genetic studies in psoriasis

It is unknown what regulates the pattern of psoriasis seen clinically; for example, the boundary between lesional and non-lesional skin, the typical sites of chronic plaque psoriasis or the relapsing/remitting nature of the disease, although it is known that there is a genetic component. Evidence for genetic involvement in psoriasis initially came from twin studies demonstrating higher concordance between monozygotic twins (65–72% concordance) compared to dizygotic twins (15–30% concordance) (Bowcock and Cookson, 2004).

More recently our knowledge of the genetic susceptibility of psoriasis has increased and genome wide association studies have increased the number of psoriasis susceptibility loci in individuals of European ancestry to 45 (Tsoi *et al.*, 2012; Tsoi *et al.*, 2015). Each susceptibility loci may contain sequences for several candidate genes and, while it is currently unknown what all these genes are and which might contribute to disease, deep sequencing in the future may identify these genes. However, some loci are known to include candidate genes involved in, for example, i) T-cell regulation, *e.g.* STAT3 which is involved in downstream signalling of numerous cytokines implicated in psoriasis including IL-6, IL-22 and IL-23; ii) innate immune response, *e.g.* SOCS1 which regulates STAT3 transcriptional activity and CARD14 which is involved in activation of the NF-kB pathway; and iii) cell signalling, *e.g.* KLF4 which is involved in macrophage activation (Tsoi *et al.*, 2012; Tsoi *et al.*, 2015). 36 of the previously discovered psoriasis associated genetic regions were predicted to account for only approximately 14% of the total variance of psoriasis risk or approximately 22% of its estimated heritability (Tsoi *et al.*, 2012), which together with the observation that monozygotic twins do not show complete concordance for psoriasis, suggests that non-genetic factors must also contribute to disease.

Interestingly different genetic associations have been made with particular subtypes of psoriasis. For example, type I chronic plaque psoriasis (section 1.2.1) is most strongly associated with the PSORS1 locus, a 220-kb region in the major histocompatibility complex (MHC) on chromosome 6 which is known to contain at least 10 genes (Trembath *et al.*, 1997). Within the PSORS1 locus there is consensus that HLA-C is the most likely gene involved in psoriasis pathogenesis (Elder, 2006). Specifically, the HLA-Cw0602 allele is believed to confer the greatest risk of developing psoriasis; up to 60% of patients with psoriasis carry the HLA-Cw0602 allele and homozygosity confers a relative risk of developing psoriasis of approximately 20 and a relative risk of approximately 10 in heterozygotes (Gudjonsson *et al.*, 2003). Despite strong association with psoriasis, the role of HLA-Cw0602 in the pathogenesis of psoriasis remains unknown. Interestingly HLA-Cw0602 is known to interact genetically with the endoplasmic reticulum aminopeptidase-1 (ERAP1) such that variants of ERAP1 are only associated with psoriasis in those also carrying the HLA-Cw6 risk allele (Strange *et al.*, 2010). This is of particular biological interest as ERAP is involved in antigen binding to MHC class I proteins including HLA-C. Type II plaque psoriasis (section 1.2.1), on the other hand is not associated with HLA-Cw0602, different disease

pathways may therefore feature more prominently in particular disease subtypes. Furthermore, different ethnic groups may show unique genetic susceptibility factors (Sun *et al.*, 2010).

With the observation that i) there is discordance in psoriasis between monozygotic twins and ii) in patients with chronic plaque psoriasis, there are both affected and unaffected areas, despite all cells generally harbouring the same underlying genetic sequences and mutations, it is clear that psoriasis cannot be a purely genetic disease. In this respect it is known that environmental factors can also contribute to disease; alcohol, streptococcal throat infection, stress and drugs, such as beta-blockers and lithium, are associated with psoriasis (section 1.4). It is, however, unknown how the environment interacts with genetic components and adaptive and innate immune systems to produce the disease phenotype in psoriasis, although epigenetics may play a role (section 1.3.5).

1.2.5. Pathophysiology of psoriasis

The pathogenesis of psoriasis is not fully understood but the most widely accepted model of psoriasis is that proliferation of keratinocytes, which themselves may be dysfunctional or hypersensitive to stimuli (section 1.2.6), is driven by over-active adaptive and innate immune systems, involving T cells, dendritic cells, neutrophils, mast cells, natural killer (NK) cells, and macrophages, with at least 3 key cytokine networks/axes having prominent roles:

i. IFNγ/TNFα/T helper 1 cells (T_H1 cells)
ii. IL-23/IL-17/T helper 17 cells (T_H17 cells)
iii. IL-22/T helper 22 cells (T_H22)

In addition to key roles for TNFα, IL-17, IL-23 and IFNγ in disease pathogenesis, IL-6, IL-7, IL-8, IL-12, IL-18 and vascular endothelial growth factor (VEGF) also have important roles and their serum levels are elevated in patients with psoriasis compared to healthy controls (Takahashi *et al.*, 2010). Although cytokine networks/axes will be discussed individually, for simplicity, psoriasis represents a complex interplay between these networks with multiple positive feedback loops between networks and different cell types driving disease.

Psoriasis can be triggered by a variety of stimuli including injury/trauma (known as Koebner phenomenon), drugs, alcohol, and infections. Exactly how these stimuli may

influence psoriasis is unknown but it is thought that environmental insults to keratinocytes cause cell death and release of epidermal products including anti-microbial peptide LL37 (cathelicidin)/nucleic acid complexes which bind to toll-like receptors on plasmacytoid dendritic cells (Gilliet and Lande, 2008). This is thought to lead to dendritic cell activation and production of type I interferons IFNα and β, TNFα and IL-2, which skews the maturation of naïve T cells into T_H1 cells which are known to produce IFNγ, TNFα, IL-1 and IL-18 (Gilliet and Lande, 2008). TNFα is a pro-inflammatory cytokine that acts as a chemoattractant for immune cells and is an activator of T-cells and dendritic cells; thus a positive feedback loop between keratinocytes and dendritic cells is created. TNFα is also produced by a wide variety of other cells in psoriasis, including keratinocytes, macrophages, lymphocytes and endothelial cells. IFNγ is a type II interferon and drives further recruitment of lymphocytes as well as further stimulating dendritic cells to produce IL-23 and IL-1. The IFNγ/TNFα/T_H1 cytokine axis is considered one of the main divers of psoriasis pathogenesis (Perera *et al.*, 2012).

Dendritic cells are also known to be the main producers of IL-23 in psoriatic skin (Lee *et al.*, 2004). IL-23, together with IL-6 and IL-12, triggers activation and differentiation of T cells into IL-17 producing T_H17 cells. IL-6-dependent activation of STAT3 leads to T_H17 differentiation, by activating transcription of *IL-23R*, *IL-17* family members and *RORC* (retinoic acid receptor-related orphan receptor C), and subsequent T_H17 proliferation (Hirahara *et al.*, 2010). Once activated, T_H17 cells produce key cytokines including IL-17A (IL-17), IL-17F and IL-22 which are increased in psoriatic skin and known to drive keratinocyte proliferation (Johansen *et al.*, 2009). IL-17 is also produced by CD4+/8+ T cells, neutrophils, mast cells, NK cells and macrophages (Baliwag *et al.*, 2015). This second cytokine axis, IL-23/IL-17/T_H17, is also considered key to disease pathology (Perera *et al.*, 2012). TNFα and IL-17 act on many cells types, although keratinocytes are the main cell type to express the IL-17 receptor, causing them to produce antimicrobial peptides (*e.g.* β-defensins and S100 proteins) chemokines (*e.g.* CXCL-5, CXCL-6, CXCL10 and CCL-20) and cytokines (*e.g.* IL-6, IL-8, IL-17, IL-23) which act as chemoattractants for immune cell infiltration into the skin (Nograles *et al.*, 2008; Chiricozzi *et al.*, 2011). The positive feedback loop between IL-17-induced production of IL-6 from non-immune cells (*e.g.* keratinocytes and fibroblasts) and IL-6-induced differentiation/activation of naïve T-cells into T_H17 cells, resulting in further IL-17 release, plays a key role in linking immune and non-immune

tissues (Saggini *et al.*, 2014). IL-17 is also known to link the innate and adaptive immune systems, through recruitment and activation of neutrophils (Weaver *et al.*, 2007). Neutrophil recruitment and activation is further enhanced by IL-6, IL-8 and CXCL-5 release from keratinocytes in response to IL-17 (and TNFα) stimulation (Saggini *et al.*, 2014), thus demonstrating complex interplay between non-immune and immune (adaptive and innate) tissues as well as key roles for both IL-6 and IL-8.

In addition to T_H17 cells secreting IL-22, more recently a subset of T-cells were found to secrete IL-22 and TNFα, but not IFNγ, IL-4 or IL-17; these were termed T_H22 cells and are known to infiltrate the epidermis in patients with psoriasis (Eyerich *et al.*, 2009) and differentiation of naive T cells to T_H22 cells was previously found to be dependent on both dendritic cells as well as IL-6 and TNFα (Duhen *et al.*, 2009). NK cells also secret IL-22, as well as TNFα and IFNγ (Lowes *et al.*, 2014). Interestingly, the IL-22 receptor is expressed only in non-immune cells, including epidermal keratinocytes, which could explain why IL-22 stimulation previously was shown to have no effect on T-cells (Wolk *et al.*, 2010). IL-22 can induce a variety of responses in keratinocytes which are known to be important in psoriasis. For example, IL-22 can stimulate production of anti-microbial peptides in keratinocytes, including S100 proteins, β-defensins and LL-37, which are over-expressed in psoriasis and have roles in recruiting immune cells and potentially initiating psoriasis in response to keratinocyte injury, as discussed above (Wolk *et al.*, 2004; Gilliet and Lande, 2008). Furthermore, IL-22 can also induce keratinocyte proliferation and inhibit differentiation, two key features of psoriatic epidermis (Boniface *et al.*, 2005). IL-17 secretion from T_H17 cells may also drive epidermal hyperplasia (Rizzo *et al.*, 2011)

Cytokine signalling plays a clear role in linking complex networks and regulating different cell types. This signalling is tightly regulated and relies on multiple positive feedback loops, thus inhibiting key cytokine pathways has the potential to disrupt pathogenesis leading to disease remission. This is supported by the finding that monoclonal antibodies to individual key cytokines are effective treatments for psoriasis. Monoclonal antibodies to both TNFα and IL-23/12 are both widely used clinically and the central role of IL-17 in psoriasis is supported by the observation that IL-17 receptor antagonism reverses the psoriatic phenotype (Hueber *et al.*, 2010) and a monoclonal antibody against IL-17 has recently been licensed for treating moderate-severe psoriasis (section 1.2.6). In addition to targeting TNFα, IL-23/12 and IL-17, IL-6 has also been

evaluated as a potential target for treatment (Saggini *et al.*, 2014). In addition to antibody-targeting of specific cytokines, more recently small molecule inhibitors have been developed which either inhibit production of cytokines or inhibit downstream signalling pathways. For example, a phosphodiesterase 4 inhibitor (apremilast) has recently been licensed for moderate-severe psoriasis and is known to downregulate production of TNFα, IFNγ and IL-23 in inflammatory cells, while a Janus kinase 3 inhibitor (tofacitinib, currently in phase III clinical trials) blocks signal transduction of IL-2/-7/-15 and -21 which are involved in T-cell development (Mansouri and Goldenberg, 2015).

1.2.6. *In vitro* keratinocyte models/studies of psoriasis

Although there is strong evidence for the role of both the adaptive and innate immune system in the pathogenesis of psoriasis, there is also good evidence to support the role of defects or alterations in keratinocyte functions in psoriasis (Al-Daraji *et al.*, 2002). For example, psoriasis can present in distinct linear patterns often with a sharp, midline cut off; this is known as linear psoriasis and is characterised by a linear distribution of psoriatic lesions along Blaschko's lines (Brinca *et al.*, 2011). Blaschko's lines represent the routes of embryological keratinocyte migration and do not follow any known nervous, vascular or lymphatic structures in the skin (Jackson, 1976). Linear psoriasis is hypothesised to arise from either somatic mutations or crossing over in embryonic keratinocyte stem cells (resulting in exchange of genetic material between homologous chromosomes and therefore recombination of genes), which may therefore harbour a predisposing gene in daughter cells within a Blaschkoid migratory segment (Happle, 1991). In addition to predisposing genes, as with chronic plaque psoriasis, environmental factors are also thought to be necessary for the linear psoriasis phenotype to develop, this could explain why disease presents later in life and not at birth (Happle, 1991). In addition to potential keratinocyte defects in linear psoriasis, these may also exist in keratinocytes from chronic plaque psoriasis. For example, keratinocytes from psoriatic patients have been shown to undergo abnormally rapid proliferation; injection of radiolabelled thymidine into skin 1h prior to skin biopsy showed that the ratio of mitotic count to number of labelled cells was higher in involved psoriatic skin compared to normal healthy skin, with uninvolved psoriatic skin having an intermediate number (Goodwin *et al.*, 1973). Furthermore, keratinocytes derived from psoriasis patients are also more resistant to apoptosis compared to ones derived from normal skin and could contribute to the excess keratinocyte number observed in psoriasis (Wrone-Smith *et al.*,

1997). Finally, keratinocytes have also been shown to have altered intracellular signalling, including activated STAT3, which is known to have roles in cell survival and proliferation (Sano *et al.*, 2005). Interestingly, Sano *et al*. demonstrated that transgenic mice with constitutively active STAT3 developed a psoriatic phenotype in the presence of activated T-cells (Sano *et al.*, 2005).

In addition to the above, psoriasis therapies targeting keratinocytes can lead to disease remission which further suggests that keratinocytes have an important role in the pathogenesis of psoriasis. For example, it is known that treatment with narrow band ultraviolet B (UVB) can induce remission (Kirke *et al.*, 2007; Chen *et al.*, 2013). Although phototherapy has a complex mechanism of action, targeting keratinocytes and skin immune cells, it has been shown that keratinocyte apoptosis is an important mechanism of psoriatic plaque remodelling and clearance in response to UVB (Weatherhead *et al.*, 2011; Zhang *et al.*, 2015). Together these findings suggest keratinocytes are not innocent/passive bystanders but may be genetically predisposed to be hyper-responsive to endogenous or exogenous inflammatory stimuli. Furthermore, correction of keratinocyte dysfunction could lead to long-term clearance of psoriasis. In order to explore the role of keratinocytes in psoriasis further, this thesis will focus on keratinocyte models/studies of psoriasis.

Although a variety of cytokines, chemokines and growth factors are upregulated in psoriasis, it remains unclear which are the key mediators of disease and therefore which of these potential stimuli are the most important for reproducing an *in vitro* keratinocyte model of psoriasis to further understand the pathogenesis. Perhaps the most relevant factors are those which correlate with disease activity and also respond to therapy. In one of the largest studies of its kind, involving 122 patients, serum cytokine and growth factors levels in patients with psoriasis were compared to healthy controls and also correlated to disease activity, measured by PASI, both before and after psoriasis treatment (Takahashi *et al.*, 2010). Psoriasis patients showed increased serum levels of TNFα, IFNγ, IL-6, IL-7, IL-8, IL-12, IL-17, IL-18 and vascular endothelial growth factor (VEGF) compared to healthy controls but decreased IL-10 compared to healthy controls (Takahashi *et al.*, 2010). Furthermore, apart from IL-6, IL-7 and IL-8, all factors correlated with PASI and were downregulated with psoriasis treatment, except IL-10 which was inversely correlated, suggesting that increased levels of TNFα, IFNγ, IL-12, IL-17, IL-18 and VEGF may be most relevant to disease activity.

Interestingly monoclonal antibodies to a number of these cytokines (TNFα, IL-17 and IL-12/23) are currently licensed and are effective for the treatment of psoriasis, reinforcing the hypothesis that these particular stimuli are key mediators of disease and therefore potentially most relevant to a model of psoriasis. For example, currently in widespread use for the treatment of psoriasis are monoclonal antibodies against TNFα (*e.g.* etanercept, adalimumab and infliximab) and IL-23/12 (ustekinumab). Furthermore, an anti-IL17, secukinumab, antibody has recently received approval for the treatment of psoriasis. A further monoclonal antibody against IL-17 (ixekizumab) and one against IL-17 receptor A (IL-17RA) (brodalumab), are both currently in phase III clinical trials with promising results to date (Leonardi *et al.*, 2012; Papp *et al.*, 2012). As we will focus on keratinocyte-based studies/models of psoriasis, and IL-23/12 act through stimulation of T cells, further discussion of *in vitro* keratinocyte models of psoriasis will be around the effects of TNFα and IL-17.

IL-17 belongs to the IL-17 family, composed of 6 isoforms: IL-17A (IL-17), IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. The isoforms share 20-50% sequence homology, with IL-17F being the most homologous to IL-17 (Johansen *et al.*, 2009). However only expression of IL-17, IL-17C and IL-17F is increased in psoriatic skin (Martin *et al.*, 2013). As expression of IL-17B, IL-17D and IL-17E are not increased in lesional psoriatic skin compared to non-lesional skin, these members of the IL-17 family are less likely to play a key role in psoriasis, compared to IL-17, IL-17C and IL-17F (Johansen *et al.*, 2009).

It has previously been reported that stimulation of primary keratinocytes with a combination of TNFα (10ng/ml) and IL-17 (200ng/ml), both key cytokines in the pathogenesis of psoriasis as previously discussed (section 1.2.5), had a synergistic effect on the upregulation of 160 genes and an additive effect on the upregulation of 196 genes, compared to stimulation with either cytokine on their own (Chiricozzi *et al.*, 2011). Interestingly synergistically upregulated genes included some of the most highly over expressed genes in psoriasis including IL-8, IL-23, S100 calcium binding proteins, human β-defensin and serpin peptidase inhibitors. Furthermore, synergy between TNFα and IL-17 may have therapeutic relevance, as analysis of data from psoriasis patients treated with etanercept (a monoclonal antibody against TNFα) showed that there was a statistically significant greater reduction in expression of genes

synergistically regulated by TNFα and IL-17 compared to genes regulated by TNFα alone (Zaba *et al.*, 2009; Chiricozzi *et al.*, 2011).

Underlying the synergy between TNFα and IL-17, it has been shown that TNFα stabilised IL-17 induced transcripts thereby potentiating its effects (Hartupee *et al.*, 2007). There is also potential synergy in downstream signalling pathways. TNFα is known to signal via a number of pathways, including NF-κB. In unstimulated cells, NF-κB is localised to the cytoplasm and is inhibited by its binding to inhibitory κB (IκB). Binding of TNFα to its receptor, for example on keratinocytes, leads to a signalling cascade which ultimately results in nuclear translocation of NF-κB and subsequent binding to DNA and transcriptional activation. NF-κB can be activated by many other stimuli, plays a major role in psoriasis and mutations in genes whose protein products are involved in signalling NF-κB have been identified as psoriasis susceptibility loci *e.g. CARD14* and *CARM1* (Tsoi *et al.*, 2012). *CARD14* is of particular interest as it is known to be upregulated by >2.5 fold in the psoriasis transcriptome and a *do novo CARD14* mutation has been identified in a child with severe psoriasis, without a family history of the disease (Jordan *et al.*, 2012). *CARD14* encodes caspase recruitment domain-containing protein 14, a scaffolding protein which interacts with and activates Bcl10, which in turn activates NF-κB. *CARD14* is expressed in keratinocytes and psoriasis-associated CARD14 mutations lead to enhanced NF-κB activation and increased expression of inflammatory cytokines and chemokines (Jordan *et al.*, 2012; Harden *et al.*, 2014). Mutations in *CARD14* are also implicated in pityriasis rubra pilaris, another type of chronic inflammatory dermatoses which is less common than psoriasis (Fuchs-Telem *et al.*, 2012).

IL-17 signalling pathways are not fully resolved, but it is known that IL-17 can activate NF-κB and mitogen-activated protein kinase (MAPK) pathways; IL-17 binding to its receptor results in recruitment of the signalling adaptor protein ACT1, also known as TRAF3 interacting protein 2, which is encoded by *TRAF3IP2*, and is involved in IL-17-mediated NF-κB activation (Gaffen *et al.*, 2014; Harden *et al.*, 2015). ACT1 mediates ubiquitylation of TRAF6 (TNF receptor-associated factor 6) which activates the NF-κB canonical pathway (through TAK1 activation) as well as MAPK pathway (including ERK, extra-cellular signal-regulated kinase; p38; and JNK, JUN N-terminal kinase) (Gaffen *et al.*, 2014), thus synergy between TNFα and IL-17 could be mediated in part through NF-κB activation and interestingly variant in *TRAF3IP2* are associated with

psoriasis (Huffmeier *et al.*, 2010). In addition to signalling through NF-κB, TNFα can also activate MAPK kinase (*e.g.* JNK) pathways, through TRAF (Gaur and Aggarwal, 2003), therefore providing further potential interactions between TNFα and IL-17 signalling, which could underlie their synergistic effects.

Interestingly although anti-IL-17 therapy is very effective for the treatment of psoriasis and has recently been licensed for this indication, surprisingly stimulation of monolayer keratinocytes with IL-17 alone altered the expression of only 47 genes, compared to 459 genes with TNFα stimulation (Chiricozzi *et al.*, 2011). In addition to signalling through NF-κB and MAPK pathways, IL-17 acts through ERK and GSK-3 β (glycogen synthase kinase 3β) to phosphorylate the transcription factor C/EBPβ (C/CAAT-enhancer-binding proteins β) (Gaffen *et al.*, 2014). Therefore IL-17 mediates some of its effects through C/EBPβ, which is upregulated in psoriasis. TNFα may also play a role in its regulation, thus providing a further node of interaction between TNFα and IL-17. In this respect, keratinocytes grown in monolayer demonstrate relatively low expression of C/EBPβ, which may explain the limited effect of IL-17 stimulation on keratinocytes grown in monolayer (Chiricozzi *et al.*, 2014). Stimulation of monolayer keratinocytes with TNFα and IL-17 enhanced C/EBPα expression, which is known to form heterodimers with C/EBPβ (Chiricozzi *et al.*, 2011). This may partly explain the synergistic effects of stimulation with a combination of TNFα and IL-17 on keratinocytes grown in monolayer. Of note, immuno-staining of C/EBPβ in psoriatic skin (lesional and non-lesional) showed expression was considerably higher compared to expression in normal health skin, where C/EBPβ was only slightly detectable (Chiricozzi *et al.*, 2014).

In summary TNFα and IL-17 could mediate its synergistic effects through mRNA stabilisation and interactions of signalling pathways involving NF-κB and MAPK as well as upregulation of downstream transcription factors which mediate their effects on target genes.

Although there was an impressive overlap between genes which were synergistically or additively regulated by TNFα and IL-17 stimulation and the psoriasis gene signature, as well as being seemingly therapeutically relevant, this data may need to be interpreted with caution. Firstly, experiments were performed in primary keratinocytes from only healthy 4 donors and it is unclear what the variation between donors was and whether

similar results would be observed in keratinocytes derived from patients with psoriasis. Secondly, keratinocytes were harvested for mRNA only at a single timepoint (24h) after stimulation, therefore immediate and early gene responses may have been missed. Finally, Chiricozzi *et el*. reported all genes with a false discovery rate (FDR) <0.3, therefore some of these observations may be due to chance. Of note, the authors refer only to genes which were upregulated by TNFα and IL-17 stimulation, thus there may be a further set of genes which were synergistically or additively downregulated by this combination of stimuli.

Interestingly stimulation of keratinocytes with TNFα and IL-17 resulted in >50 fold increase in IL-17C mRNA expression (Chiricozzi *et al.*, 2011) and the potential role for IL-17C in psoriasis has been recently investigated. IL-17C shares 23% sequence homology to IL-17, although they are located on different chromosomes (Li *et al.*, 2000). Both IL-17C and IL-17 mRNA expression were found to be significantly increased in lesional psoriatic skin, compared to non-lesional, whereas expression of IL-17B and IL-17D were both decreased in lesional psoriasis skin (Johnston *et al.*, 2013), in keeping with previous findings (Johansen *et al.*, 2009). Johnston *et al*. showed that while IL-17 transcript levels were approximately 10-fold more abundant than IL-17C transcript levels, in contrast IL-17C protein was ~125 higher than IL-17 protein in lesional psoriasis skin. Similarly in normal healthy skin IL-17C protein levels were ~100 fold higher compared to IL-17 protein levels. Therefore, overall, upregulation of both IL-17 and IL-17C protein was similar each demonstrating ~2-2.5 fold elevation in lesional psoriatic skin compared to normal healthy skin (or non-lesional psoriatic skin) (Johnston *et al.*, 2013). Stimulation of primary human keratinocytes with IL-17C (200ng/ml) had little effect on pro-inflammatory gene expression but in combination with TNFα (2ng/ml) increased expression of some genes previously shown to be additively or synergistically regulated by TNFα and IL-17 stimulation, including TNFα, IL-8, IL-1β, S100 calcium binding proteins and human β-defensin genes, amongst others (Johnston *et al.*, 2013). Johnston *et al*. also demonstrated that over-expression of IL-17C in mice resulted in a psoriasiform phenotype. Although these findings are interesting, there were few direct comparisons between IL-17C and IL-17 within the same system. For example, in comparing effects of IL-17 and IL-17C in keratinocytes, the effects of IL-17 stimulation were inferred from a different laboratory using keratinocytes derived from different donors and the IL-17 *versus* IL-17C protein expression data reported by Johansen *et al*. was not consistent with previously published

data (Johansen *et al.*, 2009). Johansen demonstrated that IL-17C protein expression was only ~4 fold higher compared to IL-17 in lesional psoriatic skin; but, there was actually greater upregulation of IL-17 protein (~10 fold) compared to IL-17C (~4 fold) in lesional psoriatic skin compared to non-lesional psoriatic skin. There are several possible explanations for these discrepancies. Patient numbers were low in both studies; 3 (Johnston *et al.*, 2013) and 5 (Johansen *et al.*, 2009); differences could represent patient variability. From the methods in the Johnston *et al*. study it was unclear how some of the data was derived. For example the protein concentrations of IL-17 in skin (<10pg/ml for lesional psoriasis and <5pg/ml for non-lesional psoriasis and normal skin) were below the sensitivity for the ELISA kit (~15pg/ml according to Manufacturer's, R&D Systems, protocol) quoted to be used in their methods (Johnston *et al.*, 2013) and levels were lower than the Johansen *et al*. study. In a separate study, stimulation of keratinocytes with IL-17C produced a concentration dependent increase in human β-defensin 2 and granulocyte colony-stimulating factor, but again no direct comparison was made with IL-17 (Ramirez-Carrozzi *et al.*, 2011). The relative expression of IL-17 and IL-17C therefore remains unclear as does the biological relevance of increased IL-17C expression, in psoriasis.

Although IL-17F shows 50% sequence homology to IL- 17, the effect of IL-17F in keratinocytes is less studied (Johansen *et al.*, 2009). However, IL-17F has previously been shown to upregulate expression of both IL-6 and IL-8 protein to a greater degree, than an equivalent concentration of IL-17 in human keratinocytes, ~4 fold and ~ 8 fold differences respectively. (Watanabe *et al.*, 2009; Fujishima *et al.*, 2010) Although the same concentrations of IL-17 and IL-17F were used in these experiments, expression of IL-17F protein is ~6 fold higher in lesional psoriatic skin compared to IL-17 protein expression (Johnston *et al.*, 2013).

Although the precise roles of elevated IL-17, IL-17C and IL-17F in psoriasis remains to be elucidated, perhaps most tellingly, blockade of IL-17 alone compared to combined blockade of IL-17, IL-17C and IL-17F have similar effects on psoriasis endpoints in phase II clinical trials (Leonardi *et al.*, 2012; Papp *et al.*, 2012). Ixekizumab is a humanized IgG4 monoclonal antibody that is known neutralise IL-17 and in a randomised, double-blinded placebo controlled trial of 142 patients with moderate-to-severe plaque psoriasis, PASI75 was achieved in ~83% of patients and a 90% reduction in PASI (PASI90) was achieved by ~71% of patients, both at week 12 (Leonardi *et al.*, 2012). In comparison, brodalumab is a human monoclonal antibody against the IL-17

receptor A (IL-17RA). IL-17RA is known to bind IL-17, IL-17C and IL-17F of the IL-17 family (Chang *et al.*, 2011; Song *et al.*, 2011; Papp *et al.*, 2012). In a brodalumab randomised, double-blinded placebo controlled trial of 198 patients with moderate-to-severe plaque psoriasis, PASI75 was achieved in ~82% of patients and PASI90 was achieved by ~75% of patients, both at week 12 (Papp *et al.*, 2012). Although these trials were not direct head-to-head comparisons, the finding that blockade of IL-17 alone vs. blockade of IL-17, IL-17C and IL-17F binding had similar functional consequences in psoriasis clinical trial endpoints, could suggest that IL-17 is the main mediator (of IL-17 family members) of psoriasis and that IL-17C and IL-17F have redundant roles. Alternatively, blockade of a single member of the IL-17 family had a maximal effect and further blockade of additional family members has no additional effect.

1.2.7. Gene array studies in psoriasis

Psoriasis represents a complex interaction between genetic predisposition, environmental factors and an altered immune profile. While the most widely accepted model of psoriasis suggest that proliferation of keratinocytes, which themselves may be dysfunctional or hypersensitive to stimuli, is driven by an over-active immune system, with T cells, dendritic cells, macrophages and inflammatory cytokines playing key roles (section 1.2.5), the underlying changes in signalling pathways are not understood. With improvements and increased validation of microarray technology, transcriptomic data has become a valuable tool to study disease. Using this data, many groups have compared lesional psoriatic skin (PP) to non-lesional psoriatic skin (PN) to define a list of differentially expressed genes (DEG), which can be used to help understand psoriasis pathogenesis, treatment response and identify novel targets for therapy. Although global gene expression array studies have been performed in psoriatic tissues samples other than skin, *e.g.* peripheral blood mononuclear cells (Palau *et al.*, 2013), this discussion will focus on array studies performed in skin as these are most relevant to our array study (chapter 6).

Although a number of psoriasis microarray studies have been reported, there is often little overlap in DEG between studies. An explanation for this could be due to the heterogeneity in methodologies adopted by different groups. For example, skin biopsies were taken from different anatomical sites *e.g.* buttock area (Swindell *et al.*, 2012), arm (Kulski *et al.*, 2005) and in many studies the body site or specific area of the plaque (edge or centre) was not defined (Yao *et al.*, 2008), which could affect results due to the

differences in relative thickness of different layers of the skin and therefore contributing cell types. In theory this may have little effect provided that PP and PN biopsies are taken from similar sites. In practice, however, in some studies PN samples were taken adjacent to psoriatic lesions (Kulski *et al.*, 2005) and in other studies PN samples were taken further away (Swindell *et al.*, 2012). Laser capture micro-dissection is a technique which can be used to isolate subpopulations of cells from tissue, thus avoiding the pitfalls associated with using mixed populations of cells (Mitsui *et al.*, 2012). Furthermore, the location within psoriatic lesions that the PP sample was taken also varied between studies; in some studies samples being taken from the edge (Reischl *et al.*, 2007) and in other studies the sample was taken from the centre of the lesion (Swindell *et al.*, 2012). This could affect results as there is evidence that psoriatic plaques have a "leading edge" which behaves differently to the centre, with potentially differential responses to treatment (Goodfield *et al.*, 1994). For some studies it was unclear whether samples were taken from patients with chronic plaque psoriasis or another subtype of psoriasis, furthermore the severity of disease was often not reported. Finally sample preparation also varied with some investigators placing samples in "RNA later" (Reischl *et al.*, 2007) and others "snap freezing" samples in liquid nitrogen (Suarez-Farinas *et al.*, 2012) prior to RNA extraction. Although most studies utilised Qiagen RNAesy kits for RNA extraction, not all documented assessment of RNA quality prior to analysis. One study commented that some of the RNA samples obtained were unsuitable for microarray analysis, although it was unclear how these samples were prepared and whether the same RNA extraction methods were used for all samples (Yao *et al.*, 2008). Finally in addition to the biological and experimental differences discussed, there were also technical differences including array platforms and both computational and statistical analyses also varied between studies. In order to improve the comparability between transcriptomic studies, standardisation of methodologies has previously been suggested (Cahan *et al.*, 2007).

To take into account these differences in methodologies, various approaches have been used to compare transcriptomic data sets, the underlying premise being that key pathways will be conserved across different donors despite different sampling and analytical techniques. The main advantage of comparing data sets from different studies is the improved statistical power to detect significant and reproducible changes; heterogeneity between samples means larger sample numbers are required to detect significant changes.

As previously discussed, taking a traditional Venn diagram to understand overlapping DEG sets, often yields few results (Suarez-Farinas *et al.*, 2010), partly due to the variability in sample preparation as described above. Instead other approaches have been taken. The first is Gene Set Enrichment Analysis (GSEA), where a reference gene list from one study is used to compare against DEG lists from other studies. Instead of comparing the expression of individual genes across transcriptomes, GSEA compares differential expression patterns of groups of genes which have similar functions or pathways, thus GSEA demonstrates which cellular pathways are altered in psoriasis (Suarez-Farinas *et al.*, 2010). A disadvantage of the GSEA approach is that it relies on the reference gene list to be complete and it becomes difficult to compare datasets where there are less probes in the reference dataset compared to the data sets to be compared. Furthermore, GSEA does not provide weighting according to the size of the study; some studies had as few as 4 psoriatic patients (Kulski *et al.*, 2005). To overcome these disadvantages in GSEA analysis, more recently a "meta-analysis" approach to comparing transcriptomic studies has been used (Tian *et al.*, 2012). Essentially this method compared DEGs across similar transcriptomic studies (*e.g.* studies of chronic plaque psoriasis using particular microarray platforms), but weighted these studies according to the sample sizes of the individual studies. The main disadvantage to the meta-analysis approach is that small changes in gene expression within a smaller dataset may not be significant in other larger studies, with the potential that this information could be lost/ignored in comparative studies. Using this approach, with a false-discovery rate (FDR) of <0.05 and fold change (FCH) >2, Tian *et al*. reported that across 5 transcriptomic studies analysed, there were 677 upregulated and 433 downregulated genes, which the authors termed the meta-analysis derived ("MAD-5") transcriptome. Within the 5 studies analysed, there were 3 studies which used a microarray platform (Affymetrix HGU133aPlus) with twice the number of probes as the microarray platform (Affymetrix HGU133a and HGU133a2) used in the other 2 studies. Interestingly when meta-analysis of these 3 studies were compared to meta-analysis of the 5 studies, there was good correlation: the top 10 up-regulated genes in each meta-analysis had 8 genes in common (SERPINB4, S100A12, TCN1, SPRR2C, DEFB4A, AKR1B10, PI3 and IL-8); and the top 10 down-regulated genes in each meta-analysis had 6 genes in common (WIF1, HSD11B1, CCL27, ZBTB16, MSMB and GAL) suggesting this method provided reproducible concordance across studies (Tian *et al.*, 2012). Using Ingenuity Pathway Analysis, Tian *et al*. were able to identify pathways which were over-represented in the DEG data set, these pathways included interferon

signalling, role of IL-17 in psoriasis as expected but also not-immediately obvious pathways including atherosclerosis signalling, which is of interest as psoriasis is increasingly recognised as an independent risk factor for cardiovascular disease (Armstrong *et al.*, 2013). To date the MAD-5 data set represents the best comparative transcriptomic study.

It should be noted that microarray studies in general can have limitations. For example, the "snap-shot" approach of investigating gene expression at a single timepoint could make it difficult to understand the evolution and resolution of disease, although different approaches to predict either upstream regulators or downstream responses are possible. It is also important to understand that microarrays can have poor sensitivity for low abundance transcripts resulting in under estimation of fold-changes which could account for why key cytokines including IL-6, IL-17, IL-22 and IFNγ were not included as DEGs in the MAD-5 transcriptome (Suarez-Farinas *et al.*, 2010; Tian *et al.*, 2012). However if other related genes are also differentially regulated then pathway analysis (*e.g.* using IPA) should conclude that a particular pathway is disrupted even if not all genes within that pathway demonstrate significant change. Although studies of global gene expression have traditionally been array based, more recently direct RNA-sequencing has been used, which may show increased sensitivity for low abundance transcripts (Li *et al.*, 2014) as well as greater ability to detect transcript isoforms and greater dynamic range of detection. However the relatively high cost of RNA-sequencing compared to arrays as well as the more challenging analysis required, are current factors in preventing more wide-spread adoption of this technology (Zhao *et al.*, 2014).

1.3. Introduction to epigenetics

1.3.1. Overview

Although there is disagreement over the precise definition of epigenetics, it is generally accepted that this describes heritable changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence (Bird, 2007). The principle epigenetic mechanisms are histone modifications and DNA methylation and recent evidence suggests epigenetics playing a role in psoriasis and disease resolution (section 1.3.5).

Nucleosomes are the fundamental units of chromatin structure, comprising a core of 8 histones which DNA is wrapped around. Chromatin functions include: packaging DNA into nuclei, there is over 2m of DNA in each nucleus; strengthening DNA during mitosis/meiosis; providing mechanisms to control gene expression and DNA replication, through post-translational histone modifications.

Chromatin exists in two states; heterochromatin ("compacted/closed") and euchromatin ("extended/open"). In its inactive state, chromatin that is not being actively transcribed is found tightly associated with structural proteins and highly compacted into tertiary structures (heterochromatin). In the heterochromatin state DNA is relatively inaccessible to polymerases, transcription factors and polymerases although the N-terminus of histone tails protrudes from these structures and are subject to covalent modifications (section 1.3.2). During gene activation, chromatin undergoes conformational changes from its compacted/closed heterochromatin state to a more loosely packaged extended/open, euchromatin state in which the DNA template is more accessible to transcription factors and polymerases. Although the underlying mechanisms which account for these changes are not fully understood, the effects of lysine acetylation on chromatin structure is the most studied and understood (section 1.3.2). DNA methylation is also known to contribute to these changes (section 1.3.3).

In addition to histone modifications and DNA methylation, some investigators also consider microRNAs a form of epigenetic change and these are often included as an epigenetic mechanisms in reviews (Zhang *et al.*, 2011a). MicroRNAs are non-coding RNAs approximately 22 nucleotides in length which target mRNAs in a sequence specific manner and normally results in gene silencing by translational repression or target degradation. Other investigators, make a distinction between mircoRNAs from histone modifications and DNA methylation (Chuang and Jones, 2007). Detailed investigation and discussion about microRNAs are beyond the scope of this thesis but interestingly there appears to be considerable interaction between microRNAs and more widely accepted epigenetic changes; expression of microRNAs can be affected by DNA methylation and histone modifications and microRNAs can themselves target mRNA transcripts coding proteins involved in regulating traditional epigenetic modifications *e.g.* DNA methylating enzymes and histone deacetylases (Chuang and Jones, 2007). In addition, microRNAs are also implicated in the pathogenesis of psoriasis, with some microRNAs being under expressed and others being over expressed. For example, miR-

125b is abnormally decreased in psoriasis; miR-125b normally targets TNFα for post-translational repression, hence downregulation of miR-125b may be lead to over-production of TNFα in psoriasis (Zhang *et al.*, 2011a). On the other hand miR-203 is over expressed in psoriatic lesional skin; miR-203 targets cytokine signalling-3 (SOC-3) which is a known to negatively regulate STAT3 (Sonkoly *et al.*, 2007). Over expression of miR-203 would therefore lead to excess STAT3 signalling (a feature of psoriasis), via decreased SOC-3 levels.

1.3.2. Histone modifications: writers, erasers and readers

Core histones are predominantly globular in structure except for their N-terminus (sometimes referred to as the "histone tail"), which is flexible and protrudes from the nucleosome surface. Histones are subject to at least 16 different enzyme-catalysed covalent modifications at specific amino acid residues mostly in the histone tail *e.g.* acetylation of lysines; methylation of lysines and arginines; phoshorylation of serines, threonines and tyrosines; citrullination of lysines and arginines; sumoylation of lysines; succinylation of lysines; formylation of lysines; ubiquitylation of lysines; butyrylation of lysines; propionylation of lysines; isomerisation of prolines; ADP ribosylation of glutamines; and deamination of arginines *etc* (Kouzarides, 2007; Sanchez and Zhou, 2009; Tessarz and Kouzarides, 2014). These covalent modifications are dynamic, occur post-replication and are generated, removed and recognised by distinct families of enzymes; "writers", "erasers" and "readers" respectively (Table 1-1) (Kouzarides, 2007). In humans there are over 10^8 histone tails which can all potentially be modified, but the functional consequences of histone modifications or how they are integrated is not well understood (Rakyan *et al.*, 2011).

Modification	Target amino acid	Writer	Eraser	Reader
Acetylation	Lysine	Acetylase	Deacetylase	Bromodomain
Methylation	Lysine, Arginine	Methylase	Demethylase	Chromodomain
Phosphorylation	Serine, Threonine Tyrosine	Kinase	Phosphorylase	PHD finger, WD40 repeat

Table 1-1: Examples of common histone modifications, their regulators and binding partners

Amino acid residues on histones tails are subject to covalent modifications. These-enzyme-catalysed modifications are regulated by the balance between specific but opposing "writers" and "erasers". Specific histone modifications function as binding sites for specific chromatin "readers", which in turn orchestrate the recruitment of other transcription factors and co-activators. For example, lysine acetylation is catalysed by histone acetyltransferases, the epitope is recognised by bromodomain-containing proteins and removed by histone deacetylases.

Histone acetylation of lysine residues is the most widely studied histone modification and was first reported in 1964 (Allfrey *et al.*, 1964). Since then, it is recognised that lysine acetylation is regulated by the balance of opposing histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity (Figure 1-4). Although all core histones are acetylated, acetylation of histone-H3 and histone-H4 has been more extensively studied than acetylation of histone-H2A and histone-H2b. HATs are a family of enzymes which transfer the acetyl group from acetyl-coA onto lysine residues. The negatively charged acetyl group counteracts the positive charge on the histones, thereby decreasing the affinity of histones with the negatively charged phosphate groups of DNA (Bannister and Kouzarides, 2011). As a result, tightly condensed heterochromatin undergoes a conformational change into the more relaxed structure of euchromatin, thereby increasing the accessibility of transcriptional regulators and RNA polymerase to the chromatin template, thus facilitating gene transcription (Struhl, 1998). In the reverse, deacetylation, reaction, HDACs remove acetyl groups from lysine residues so that chromatin resumes back to its condensed heterochromatin resting state; increased HDAC activity is usually associated with repression of transcription (Kimura *et al.*, 2005). Individual members of both the HAT and HDAC families can demonstrate distinct specificities for particular lysine residues and different histones. For example, CBP and P300 of the HAT family are known to acetylate the lysine 14

and 18 residues on histone H3 (H3K14 and H3K18), whereas TIP60 is known to acetylate the lysine 5, 8, 12 and 16 residues on histone H4 (H4K5, H4K8, H4K12, H4K16) (Kouzarides, 2007). This may reflect particular biological functions of different family members but the mechanisms regulating these specificities are not well understood (Turner, 2000).

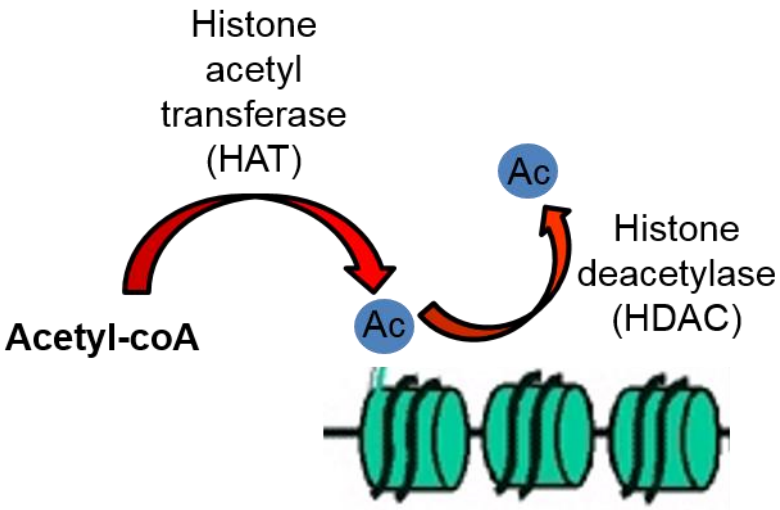


Figure 1-4: Histone acetylation is regulated by the balance between histone acetyl transferase and histone deacetylase activity

Interestingly a particular amino acid position can be subject to a variety of different covalent modification. For example, the lysine 27 residue of histone-H3 can be mono-, di- or tri-methylated (H3K27me1, H3K27me2 and H3K27me3 respectively) as well as acetylated (H3K27ac), although methylation and acetylation are mutually exclusive epitopes *i.e.* the same lysine 27 residue cannot be both acetylated and methylated at the same time (Tie *et al.*, 2009). In fact, the change from H3K27 tri-methylation (H3K27me3) to acetylation (H3K27ac) has been shown to act as an important switch from gene repression to transcriptional activation (Pasini *et al.*, 2010). Within a single nucleus, there may be both tri-methylation and acetylation of the lysine 27 residue on different H3 histones; in hepatocellular carcinoma, it has been shown that H3K27me3 localised to the heterochromatin-rich peripheral region of nuclei whereas H3K27ac localized to the euchromatin-rich central region (Hayashi *et al.*, 2014).

In addition to writers and erasers having specificity for histone modification on particular amino acid residues, histone marks on specific amino acid residues are also known to have specific effects. These effects can depend on whether the histone modification is associated with the promoter or enhancer region of a gene (section

1.3.4). Furthermore although lysine acetylation is considered a permissive histone modification, which generally signifies actively transcribed genes, the effects of other histone modifications are less well studied and it is unknown how cell-type specific these modifications are. In the most comprehensive study of its kind, the Bernstein group analysed 9 chromatin marks, across 9 different human cell types (including normal epidermal keratinocytes), in different chromatin states *e.g.* repressed/poised/active promoter or strong/weak enhancers (section 1.3.4) and revealed some intriguing findings (Ernst *et al.*, 2011). For example, some epitopes demonstrated very defined effects *e.g.* acetylation of the lysine 9 or lysine 27 residue on histone-H3 (H3K9ac and H3K27ac) was associated with an active promoter in >95% of cases and a strong enhancer in 97% of cases for H3K27ac and 86% of cases for H3K9ac. Ernst *et al*. showed that the level of methylation at a particular residue affected the function: mono-methylation of the lysine 4 of histone-H3 (H3K4me1) was associated with an active promoter in only 17% of cases, whereas di-methylation (H3K4me2) was associated with an active promoter in 93% of cases and tri-methylation (H3K4me3) was associated with an active promoter in 99% of cases. Interestingly, the effect of a particular histone modification was dependent on the regulatory region it was associated with: although H3K4me1 was associated with an active promoter in only 17% of cases, the same epitope was associated with a strong enhancer in 96% of cases (Ernst *et al.*, 2011).

Although, some epigenetic epitopes are known to have co-operative effects (Figure 1-5), it is unknown exactly how multiple histone modifications interact to regulate gene expression although there are several theories including; charge neutralization (Dion *et al.*, 2005) and the histone code (Jenuwein and Allis, 2001). There is on-going debate of exactly how multiple histone modifications are integrated to regulate gene expression, with constantly evolving viewpoints. For example, previously one group wrote "the recruitment of proteins and complexes with specific enzymatic activities is now accepted dogma of how (histone) modifications mediate their function" (Bannister and Kouzarides, 2011) but more recently the same group suggested the role of histone modifications in regulating chromatin structure may be equally important (Tessarz and Kouzarides, 2014). Histone modification might, indeed, mediate effects through several mechanisms and these might not be mutually exclusive. For example, it has already been discussed that histone acetylation can lead to recruitment of bromodomain-containing proteins but can also lead to changes in chromatin conformation from

heterochromatin to euchromatin, potentially through "charge neutralisation", allowing greater accessibility of the DNA template, thus acetylation can have multiple, co-operative functions to regulate gene expression.

1.3.3. DNA methylation

DNA methylation involves covalent transfer of a methyl group from S-adenosyl-methionine onto the 5'-carbon position of a cytosine ring (5mC), almost exclusively within cytosine/guanine dinucleotide pairs (CpG), and is traditionally considered to be a repressive epigenetic mark when present near gene regulatory regions (Bestor and Coxon, 1993). DNA methylation is involved in regulating imprinted genes and X-chromosome inactivation (Jones and Takai, 2001). In human somatic cells, approximately 70-80% of CpG dinucleotide are methylated and primordial germ cells show lower levels of DNA methylation (Bird, 2002). Unmethylated cytosine residues are often grouped in cytosine/guanine repeats/clusters, termed CpG islands, which are resistant to methylation; approximately 70% of mammalian gene promoters are linked to CpG islands and the remaining 30% contain a low CpG content (Saxonov *et al.*, 2006). Approximately 50% of CpG islands are associated with gene transcription start sites (Bird, 2002) and methylation of these sites are typically inversely correlated to the level of expression of the associated gene (Bell *et al.*, 2011). The mechanism by which DNA methylation silences gene expression is unknown but two possible hypotheses exist (Bird, 2002). The first proposes that the methyl group of 5mC interferes with binding of proteins onto CpG sequences, in this respect it is known that binding of the CTCF protein onto DNA can be blocked by methylation. The second hypothesis proposes that, rather than blocking binding, the methyl group of 5mC increases affinity of binding of particular repressive proteins, for example methyl CpG binding protein 2 (MECP2) (Figure 1-5).

Cytosine methylation is catalysed by DNA methyltransferase (DNMT) enzymes (Figure 1-6) and in order to preserve DNA methylation, maintenance methylation activity is necessary after each DNA replication cycle; as the replication machinery itself is unable to methylate DNA, resultant daughter strands are unmethylated. In humans, 3 DNMTs together are responsible for maintaining and establishing DNA methylation: DNMT1 is thought to be responsible for maintaining DNA methylation by preferentially binding hemi-methylated DNA and copying DNA methylation patterns to the daughter strands

during DNA replication, Figure 1-6, whereas DNMT3a and DNMT3b are thought to be responsible for *de novo* DNA methylation patterns early in development (Bird, 2002).

More recently there has been some focus on 5-hydroxymethylcytosine (5hmC) which results from the oxidation of 5mC to 5hmC, catalysed by ten eleven translocation proteins. However, in contrast to the gene silencing effects associated with 5mC, 5hmC has generally been associated with increased gene expression. Unlike 5mC, mapping of 5hmC has not yet been performed in psoriasis, for this reason this epitope will not be discussed further.

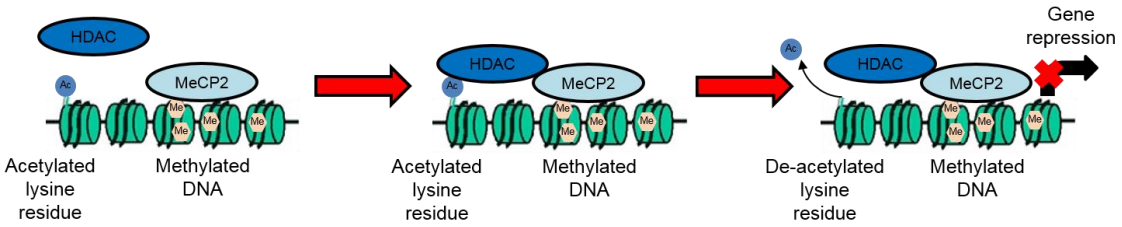


Figure 1-5: Epigenetic epitopes can have co-operative effects

Methyl CpG binding protein 2 (MECP2) binds methylated DNA, recruiting a histone deacetalyse enzyme (HDAC) which deacetylates a histone lysine residue. Methylated DNA and de-acetylation of lysines residues on histones is associated with gene silencing.

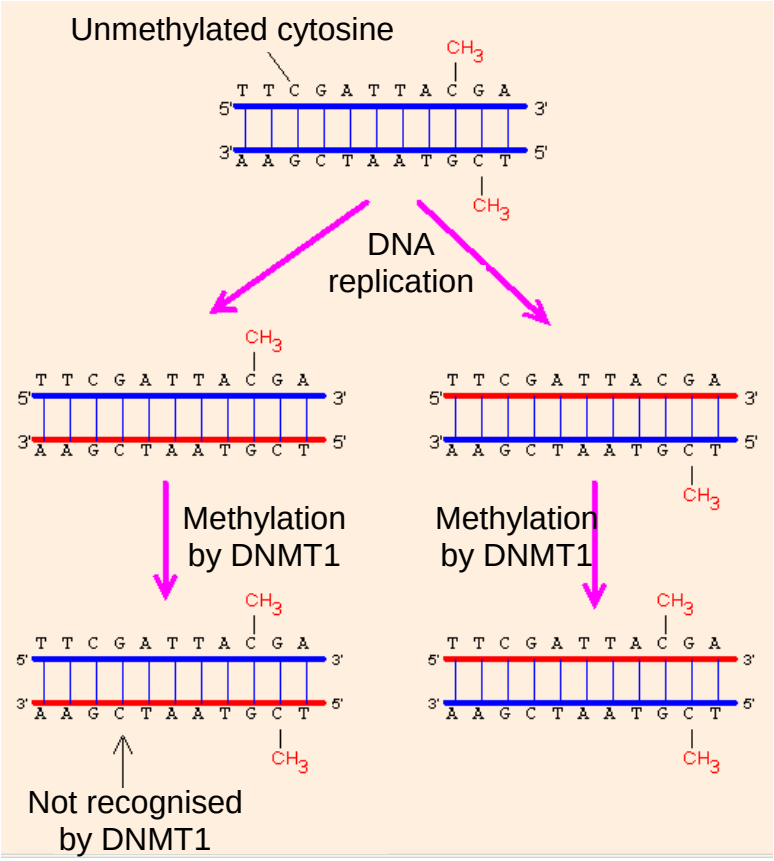


Figure 1-6: DNA methylation in maintained by DNA methyltransferase 1

The semi-conserved nature of DNA replication results in initially hemi-methylated daughter strands of DNA. DNA methyltransferase 1 (DNMT1) preferentially binds hemi-methylated DNA, thus methylation only occurs on a CG sequence paired with a methylated CG. A CG sequence not paired with methylated CG will not be methylated. Hence, the original pattern can be maintained after DNA replication. (Adapted from www.web-books.com)

1.3.4. Epigenetic modifications at gene regulatory regions

In humans, gene expression is a highly regulated process which determines the fate and function of a cell. A key early event is transcription initiation which involves the assembly of transcriptional machinery including RNA polymerase II at the gene promoter region, at or near the transcriptional start site (TSS). In addition, gene expression can be further regulated by enhancer regions, potentially located far from the TSS.

A promoter is a region of DNA which initiates transcription of a gene and is typically 100-1000bp long, although the "core promoter" is defined as the minimum DNA sequence to that directs initiation of transcription (Danino *et al.*, 2015). Promoters tend to be located at or just upstream of the TSS. Distal promoters are DNA sequences further upstream of the gene that may contain additional regulatory elements, with

generally weaker effects on gene expression than more proximal promoters. Active promoters are associated with reduction or depletion of nucleosomes, DNAseI hypersensitivity, and enrichment of specific epigenetic marks including di- and tri-methylation of lysine 4 residue on histone-H3 (H3K4me2 and H3K4me3) and acetylation of lysine 4, 9 and 27 on histone-H3 (H3K4ac, H3K9ac, H3K27ac) (Ernst *et al.*, 2011; Danino *et al.*, 2015).

In contrast to promoters which tend to be at or near the TSS, enhancers are regulatory regions of DNA which are capable of regulating/activating expression of genes that are potentially hundreds of bases and even megabases away (Heinz *et al.*, 2015). In their active state, enhancers are bound by RNA polymerase II, transcription factors and co-activators important in the regulating expression of the target gene (Calo and Wysocka, 2013). The exact mechanisms by which enhancers regulate expression of genes long-distances away are unknown but there are 2 main theories: "tracking" and "looping" (Bulger and Groudine, 2011). The "tracking" model proposes that RNA polymerase II, transcription factors and co-activators track/move along the length of DNA to the target gene, whereas the "looping" model proposes that conformational changes in chromatin and "looping out" of intervening DNA, allows the close association of an enhancer region and its target gene so that proteins can move directly between the two regions (Bulger and Groudine, 2011). Of the two the "looping" hypothesis is more widely accepted and more easily explains how enhancer regions can be megabases away from target genes (Calo and Wysocka, 2013). Interestingly, enhancer regions can be characterised by a number of epigenetic characteristics including presence of high levels H3K27ac and H3K4me1 (with a high ratio of H3K4me1 to H3K4me3; this ratio tends to be reversed at promoter regions) as well as DNAseI hypersensitivity, binding of co-activators, transcription factors and RNA polymerase II and relative depletion of nucleosomes when enhancers are in their active state (Calo and Wysocka, 2013). Although the human genome contains millions of potential enhancers, it is said that only a subset are active in a particular type of cell and that cell-specific enhancers, through regulation of gene expression, may themselves define the cell type; a major challenge is to determine which cell-type defining enhancers connect to which genes (Heintzman *et al.*, 2009; Heinz *et al.*, 2015).

On the basis of the characteristics used to define enhancer regions, a given cell type is said to have between 10,000 – 150,000 enhancers, suggesting that there are potentially more enhancers in a given cell compared to the number of expressed genes (Pott and

Lieb, 2015). However, although a single enhancer can potentiate the expression of a target gene, the concept of super-enhancers had been suggested; these are typically large regions of DNA (often tens of kilobases in length) which are enriched with a high density of multiple "typical" enhancer loci. Super-enhancer can be further characterised by H3K27 acetylation, binding of Mediator (a multi-protein coactivator that may facilitate interactions between transcription factors and RNA polymerase II) and, interestingly, binding of bromodomain containing protein Brd4 (Brown *et al.*, 2014; Heinz *et al.*, 2015; Pott and Lieb, 2015). Most cell types contain 300-500 super-enhancer regions, which can regulate expression of multiple genes and the combined function of "typical" enhancers within these regions, are associated with genes encoding cell type-specific transcription factors and co-activators, which are central to defining cell lineage (Hnisz *et al.*, 2013; Heinz *et al.*, 2015). Interestingly, DNA sequence variations in cancer have been shown to occur within super-enhancer regions or lead to the acquisition of super-enhancer regions known to regulate multiple cancer-related genes, within specific cell types relevant to disease (Hnisz *et al.*, 2013). Defining enhancer and super-enhancer regions within individual cell types is of considerable interest as is the observation that super-enhancers may mediate their effects partly through Brd4; inhibiting Brd4 binding to this region may represent a strategy for cancer treatment (section 1.3.7).

1.3.5. Epigenetic studies in psoriasis

Traditionally the study of human disease has focused on determining genetic contributions to disease and in some cases, environmental and age-related factors. However, it is now increasingly understood that epigenetics could provide the link to explain how environmental factors and age interact with the genome to precipitate disease (Grolleau-Julius *et al.*, 2010). Furthermore, while genetic susceptibility may be unavoidable and potentially irreversible, epigenetic abnormalities are potentially dynamic and reversible through the use of pharmacological inhibitors to target regulators of the epigenome (Egger *et al.*, 2004), for example through the use of inhibitors to target writers, erasers or readers of the epigenome, Table 1-1.

In addition to the rapidly expanding evidence that epigenetics plays a role in many diseases including autoimmune diseases and cancer, there is increasing evidence that epigenetics plays a role in psoriasis and therapies that are currently used in the treatment of psoriasis have been shown to have effects on the epigenome (reviewed below).

Furthermore, early evidence suggests that pharmacological agents targeting regulators of the epigenome may lead to promising treatments for psoriasis.

There have been a limited number of studies examining histone modifications in psoriasis and these suggest histone modifications are aberrant in psoriasis. Zhang *et al*. reported global histone H4 hypoacetylation in peripheral blood mononuclear cells (PBMCs) from patients with psoriasis compared to unaffected controls, with negative correlation between degree of histone H4 acetylation and psoriasis disease activity (Zhang *et al.*, 2011a; Trowbridge and Pittelkow, 2014). Zhang *et al*. also reported that expression of specific HATs (*e.g.* p300 and CBP) were decreased in PBMCs from patients with psoriasis, which also showed increased expression of some HDACs (*e.g.* HDAC-1); this could account for the hypoacetylation observed in psoriatic PBMCs (Zhang *et al.*, 2011a; Zhang *et al.*, 2011b). Interestingly, downregulated of the HDAC Sirtuin-1 (SIRT1) was reported, which has previously been shown to inhibit keratinocyte proliferation and promote differentiation, and it is suggested that decreased expression of SIRT1 could account for phenotypic changes in psoriatic keratinocytes (Blander *et al.*, 2009; Zhang *et al.*, 2011b). Recently, a SIRT1 activator has been evaluated in a clinical trial for the treatment of moderate to severe psoriasis, although the results are not yet available (ClinicalTrials.gov Identifier: NCT01154101).

Most studies investigating epigenetic factors in psoriasis have focused on DNA methylation, initially at the promoter region of specific genes relevant to psoriasis. Psoriasis is characterised by excess keratinocyte proliferation which may arise as a result of resistance to apoptosis of keratinocytes derived from patients with psoriasis, compared with healthy patients (Wrone-Smith *et al.*, 1997). In keeping with this hypothesis, DNA methylation of the $p16^{INK4a}$ promoter was found in the epidermis of 30% of patient with psoriasis (Chen *et al.*, 2008). $p16^{INK4a}$ is a tumour suppressor protein with anti-proliferative effects, whose down-regulation has previously been associated with increased promoter methylation in squamous cell carcinoma and enhanced cellular self-renewal (Brown *et al.*, 2004). Patients with methylation at the $p16^{INK4a}$ promoter showed significantly lower expression of $p16^{INK4a}$ mRNA and greater psoriasis severity, compared to psoriasis patients with an unmethylated $p16^{INK4a}$ promoter. Interestingly the promoter of $p14^{ARF}$, a gene homologous to $p16^{INK4a}$, is also hypermethylated, with corresponding decreased expression, in keratinocytes derived from patients with psoriasis (Zhang *et al.*, 2010). Together these findings suggest that aberrant methylation of specific genes, including the promoter region of both $p16^{INK4a}$

and $p14^{ARF}$, has a potential role in the pathogenesis of psoriasis. However, in addition to aberrant hypermethylation of these genes, hypomethylation of other genes has been observed in psoriasis. For example, SHP-1 is a regulator of cell growth and proliferation, with 2 functional promoters and psoriatic keratinocytes show demethylation of the SHP-1 promoter 2 compared to keratinocytes from normal skin or from eczema (Ruchusatsawat *et al.*, 2006). Ruchusatsawat *et al*. also demonstrated that psoriatic skin had higher expression of SHP-1 compared to normal skin, suggesting that aberrant hypomethylation is at least partly involved in regulating SHP-1 in psoriasis.

In addition to investigation of DNA methylation changes of single genes, global DNA methylation changes in psoriasis have more recently been investigated. Roberson *et al*., were the first to investigate global DNA methylation changes in psoriatic skin and found that psoriatic skin had a greater number of hypermethylated regions than hypomethylated regions and that DNA methylation states at ~1100 (out of a total of ~27500 CpG sites examined) differentiate psoriatic lesional skin from normal skin. Interestingly, 12 of these sites mapped to the epidermal differentiation complex, which contains a large number of genes involved in epidermal differentiation and function. All 12 sites showed decreased methylation in psoriatic lesional skin compared to normal skin and mapped near to genes which are known to be upregulated in psoriasis, *e.g.* S100 genes (Roberson *et al.*, 2012). The greatest differences in methylation were between psoriatic lesional skin and normal skin (*i.e.* from non-psoriatic individuals) and the top 50 differentially methylated sites (25 with increased methylation and 25 with decreased methylation) could be used to separate psoriatic lesional skin and normal skin, with non-lesional psoriatic skin showing intermediate levels of methylation. Furthermore, treatment of patients with anti-TNFα therapy resulted in a reversion of DNA methylation from the lesional state towards the non-lesional state even prior to the resolution of psoriatic plaques (Roberson *et al.*, 2012). This finding suggests restoration of aberrant DNA methylation to its normal state could drive the resolution of psoriasis, rather than being a consequence of resolution.

More recently methylated DNA immunoprecipitation followed by sequencing (MeDIP-Seq) has been used to compare involved and uninvolved psoriatic skin and it was reported that there was significantly higher hypermethylated than hypomethylated regions in affected skin (Zhang *et al.*, 2013), consistent with previously published data (Roberson *et al.*, 2012). Zhang *et al*. demonstrated that differentially methylated regions were enriched for genes involved in immunity, cell cycle and apoptosis *i.e.*

functions known to be dysregulated in psoriasis. Gene expression inversely correlated with levels of DNA methylation for a number of genes including TIMP2, a matrix metalloproteinase inhibitor, whose loci was hypermethylation and expression downregulated in lesional psoriatic skin, although the function of this gene has not yet been characterised in psoriasis (Zhang *et al.*, 2013). In addition to DNA methylation studies in skin, MeDIP-Seq has been used to investigate DNA methylation status of circulating immune cells (CD4+ T cells) in patients with psoriasis compared to healthy controls and, as with lesional skin, overall DNA methylation values were higher in psoriatic CD4+ T cells patients compared to those derived from healthy controls (Park *et al.*, 2014). In addition, hypermethylated regions were enriched at gene promoter sites, and this correlated with expression of associated genes (Park *et al.*, 2014) in CD4+ T cells. In the most recent study of DNA methylation in psoriatic skin, lesional psoriasis samples compared to normal healthy skin showed over 3600 differentially methylated regions, determined by bisulphite sequencing, with psoriatic samples demonstration overall hypomethylation (Gu *et al.*, 2015). Differentially methylated regions enriched for genes with functions relevant to the pathogenesis of psoriasis including inflammation, regulation of cell motion and regulation of programmed cell death (Gu *et al.*, 2015). However, the overall hypomethylation observed by Gu *et al.* was in contrast to the overall hypermethylation observed in other studies (Roberson *et al.*, 2012; Zhang *et al.*, 2013). This discrepancy may have arisen due to differences in sample preparation; in this latest study, DNA methylation of only the epidermal part of skin (by separation from the dermis using ammonium thiocyanate) was determined, (Gu *et al.*, 2015) whereas previous studies examined whole-skin biopsies *i.e.* epidermis, dermis and any underlying sub-cutaneous tissue (Roberson *et al.*, 2012; Zhang *et al.*, 2013). Whole-skin biopsies of psoriatic tissue vs. normal skin may have contained relatively more inflammatory infiltrate compared to epidermis-only samples, and the hypermethylation observed in psoriatic CD4+ T cells, discussed above (Park *et al.*, 2014), may have contributed to the overall hypermethylation pattern when whole-skin biopsies were sampled.

Interestingly, therapies that are currently used in the treatment of psoriasis have been shown to have effects on the epigenome. For example, methotrexate is a folate antagonist used in the treatment of psoriasis (Figure 2-1), and treatment lead to the reversal of global DNA hypomethylation observed in PBMCs derived from patients with psoriatic arthritis, indicating that DNA methylation changes in psoriasis are

dynamic (Kim *et al.*, 1996). As previously discussed, anti-TNFα psoriasis therapy resulted in reversion of DNA methylation from lesional skin towards the non-lesional state (Roberson *et al.*, 2012). Furthermore, narrow-band UVB phototherapy has also been shown to reverse aberrant DNA methylation changes in psoriasis (Gu *et al.*, 2015). In addition to current therapies in psoriasis reversing aberrant changes in the epigenome, compounds targeting readers or regulators of the epigenome may also have therapeutic value in the treatment of psoriasis (section 1.3.7). Collectively these findings suggest a role for epigenetics in the pathophysiology of psoriasis and also provide some evidence that underlying epigenetic processes may be potential therapeutic targets.

However the epigenetics studies described do have a number of caveats which could influence the interpretation or indeed the results of investigations. Firstly epigenetic studies utilising whole tissue, including skin, will be affected by the relative proportions of different cell types which will have differences in their epigenome, this becomes particularly complex when the state of disease itself influences the composition of cells within tissue. For example, in psoriasis it is known that there is an increased inflammatory infiltrate within the skin, therefore epigenetic differences between psoriatic skin biopsy samples and normal healthy samples could simply represent these differences in cell types. Secondly the state of differentiation of a cell will be governed by changes in the epigenome and in psoriasis, where for example keratinocytes demonstrated excess proliferation and abnormal proliferation, comparisons of the epigenome between involved and uninvolved epidermis may simply reflect these changes in cellular phenotype or proliferation. Thirdly, variations in the epigenome may cause disease or be a consequence of it and deciphering these differences could prove difficult, but functionally important to determine especially when considering potential therapeutic targets. Finally, although epigenetic epitopes are dynamic and potentially reversible, it is unknown how processing of samples affects these regulatory marks. Overcoming these challenges is fundamental for understanding the role of epigenetics in disease pathogenesis and resolution.

1.3.6. Bromodomains and the bromodomain extraterminal protein family

Bromodomain (BRD)-containing proteins are reader proteins known to bind to acetylated lysine residues, Table 1-1. The human genome encodes 61 distinct bromodomains (BRDs) each ~110 amino acids long, contained within 46 BRD-

containing proteins, with each protein containing between 1 – 6 BRDs (Basheer and Huntly, 2015). The BRD structure consists of 4 α helices separated by a variable loop regions; together these form a hydrophobic binding cavity and is the only domain known to specifically recognise acetylated lysine residues (Dhalluin *et al.*, 1999; Filippakopoulos and Knapp, 2012). BRD-containing proteins are grouped into families based on sequence homology and are known to have very diverse functions *e.g.* as histone acetyl transferases, methyltransferases, transcriptional co-activators and nuclear scaffolding proteins *etc* (Filippakopoulos *et al.*, 2012). The bromodomain extraterminal (BET) family of BRD-containing proteins (namely Brd2, Brd3, Brd4 and BrdT), contains two tandem amino-terminal BRDs (known as BD1 and BD2) and a conserved extraterminal domain (Figure 1-7). Brd2, 3, and 4 are ubiquitously expressed across multiple cell types and tissues, whereas BrdT has only been found in testes (Jones *et al.*, 1997; Basheer and Huntly, 2015).

BET proteins are known to have essential, non-redundant roles in growth and development. For example, homozygous Brd2 or Brd4 knockout mouse embryos fail to develop after implantation and Brd4 heterozygotes have pre- and post-natal defects including head malformations, absence of subcutaneous fat and cataracts showing that Brd4 heterozygosity demonstrates haploinsufficiency (Houzelstein *et al.*, 2002; Shang *et al.*, 2009).

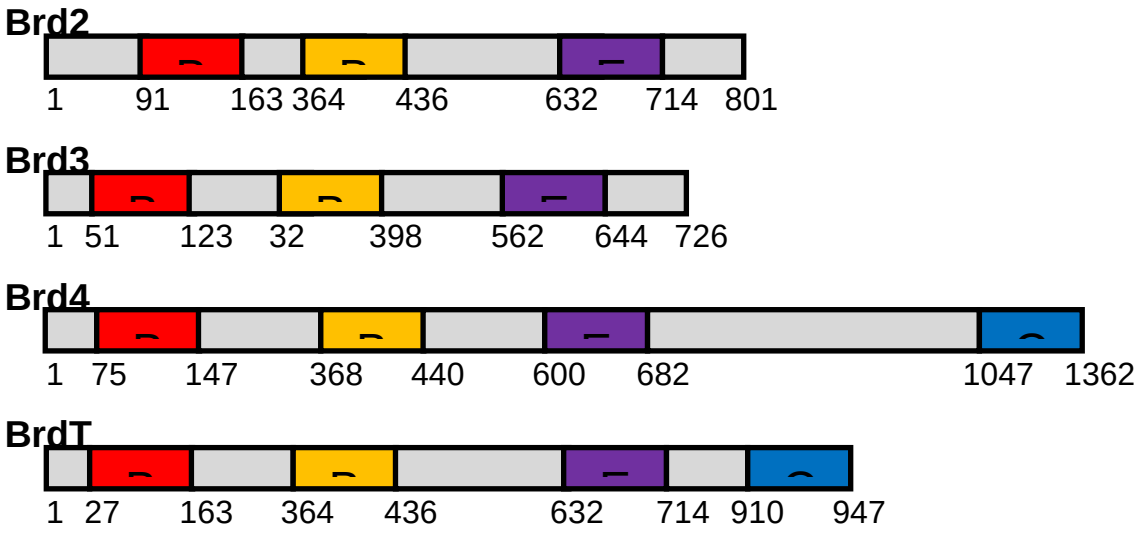


Figure 1-7: Bromodomain extraterminal (BET) protein structures

Numbers indicate the position of amino acids. BD = bromodomain, ET = extraterminal, CTM = C-terminal motif. (Adapted from Basheer *et al.*, 2015)

Interestingly BET proteins are also known to be involved in disease pathogenesis. For example, NUT midline carcinoma is a rare but aggressive form of squamous cell carcinoma in which tumours arise from midline anatomical structures, especially the upper aerodigestive tract and mediastinum. Most patients have a chromosomal translocation t(15;19) involving Brd4 (or less commonly Brd3) and NUT (nuclear protein in testes) resulting in a Brd4-NUT (or Brd3-NUT) fusion oncoprotein, which contain BET tandem BRDs, the extraterminal domain and virtually the whole of the NUT protein. The fusion protein is targeted to chromatin by BET BRDs and the NUT protein is known to recruit HAT enzymes (p300/CBD) leading to histone hyperacetylation which is known to drive gene expression (French, 2012). BET inhibitors are currently in clinical trials for the treatment of this life-threatening disease (section 1.3.7).

BET proteins have been shown to associate primarily with euchromatin, and rarely with heterochromatin, suggesting early on that these proteins play a role in regulating gene expression (Dey *et al.*, 2003). It is now recognised that, through their BRDs, BET proteins bind acetylated lysine residues commonly found on histones and non-histone targets and orchestrate recruitment of other proteins involved in transcriptional activation/inactivation, thus regulating gene transcription (Figure 1-8) (Sanchez and Zhou, 2009). It has previously been calculated that there are over 24,000 lysine acetylation sites in human cells (Filippakopoulos and Knapp, 2014), but it unknown how BRD-containing proteins achieve their binding specificity. Investigations into BET protein binding specificities have previously been undertaken and, although there are no systematic studies comparing specificities and affinities for individual BET proteins and particular acetyl-lysine residues, results across several studies suggest that binding of BET protein is highly regulated and complex and is currently poorly understood.

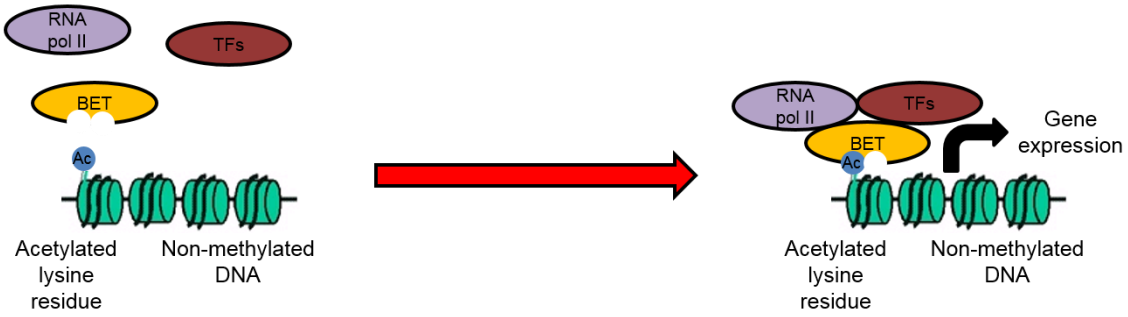


Figure 1-8: Bromodomain (BRD) containing proteins

These proteins bind acetylated lysine residues found on histones and stabilise and recruit transcription factors (TFs) to chromatin, thereby regulating gene transcription. In addition to histone targets, BRDs can also bind acetyl-lysine residues on non-histone targets.

Most studies investigate binding *in-vitro* to acetylated histone-tail peptides, and BET proteins are known to bind with greater affinity when exposed to multiple acetylated lysine residues on the same (cis) histone tail rather than single acetylated lysines, although these proteins can also bind acetylated lysine residues on different (trans) histone tails that are in close proximity to each other (Filippakopoulos and Knapp, 2012; Basheer and Huntly, 2015). Published substrate affinities are usually low and have been determined by a variety of methods, including pull-down assays and peptide arrays. Even when similar methods were used, conditions often varied *e.g.* different peptide length used in peptide binding assays; these factors make direct comparisons of specificity and affinity between studies difficult. Comparisons within a single study are more straight-forward; one study demonstrated that Brd4 did not bind to non-acetylated H3 or H4 and binding of Brd4 to mono-acetylated H3 was lysine residue specific; there was weak binding to H3K14ac but not to H3K9ac (Dey *et al.*, 2003). In certain circumstances, acetylation of multiple lysine residues within a short amino acid span can enhance BET protein binding. Dey *et al*. demonstrated that histone di-acetylation can enhance Brd4 binding in a lysine-residue specific manner; di-acetylated H3 (H3K9acK14ac) and di-acetyl H4 (H4K5acK12ac) enhanced Brd4 binding compared to mono-acetylation at each lysine residue alone but an alternative di-acetyl H4 (H4K8acK16ac) had no effect on Brd4 binding and tetra-acetyl H4 (H4K5acK8acK14acK16ac) resulted in enhanced binding of Brd4; compared to mono-acetylation at each lysine residue alone but less than binding to di-acetyl H4 (H4K5acK12ac). In addition to lysine acetylation affecting BET protein binding interestingly other, non-acetylation, histone modifications can also affect binding. For

example, in peptide binding studies, the association of Brd4 to di-acetyl H3 (H3K4acK9ac) was enhanced by the phosphorylation of the H3 threonine residue at position 3 (H3T3pK4acK9ac) (Filippakopoulos *et al.*, 2012). These findings suggest that although BET proteins can bind single acetyl-lysine residues on histones, binding is enhanced by specific patterns of histone modifications, suggesting a co-operative role of neighbouring sites for substrate binding. However it remains unknown how these multiple covalent histone modifications are integrated into a single output to active, suppress or pause gene expression. Furthermore, the role of each of the tandem BRDs (BD1 and BD2) remain unknown although they are said to have different substrate affinities (Filippakopoulos and Knapp, 2012), and deletion of BD1 in BrdT in mice caused sterility by inhibiting sperm maturation, suggesting BD1 at least has a non-redundant role (Shang *et al.*, 2007).

BRD-mediated interactions with acetylated residues on histones results in localisation of BET proteins to specific regions of chromatin where they are associated with other proteins involved in regulation of gene expression. This is accomplished through protein interactions with i) the other BRD if not bound to a second acetylated lysine residue, ii) the conserved extra-terminal domain of BET proteins or iii) the carboxy-terminal (C-terminal) domain, thus BET proteins exert their multiple functions through the association with other proteins (Basheer and Huntly, 2015). In this respect Brd4, through the BRDs, is known to bind p65/RelA in an acetylated lysine-310 dependent manner, leading to mutual co-activation and recruitment of Brd4/p65 to the promoters of NF-κB regulated genes (Huang *et al.*, 2009). Furthermore, Brd4 also recruits cyclin-dependent kinase 9 (CDK9), which phosphorylates the serine 2 residue of RNA polymerase II of its carboxy-terminal domain (pol II S2P) leading to its activation, thus facilitating the initiation of transcription of NF-κB dependent genes (Huang *et al.*, 2009). BET proteins play a further role in initiation of transcription through the interaction with Mediator, which Brd4 has been shown to bind; Mediator is a multi-protein coactivator that may facilitate interactions between transcription factors and RNA polymerase II (Wu and Chiang, 2007). Brd4/Mediator complexes stabilise each other's occupancy at regulatory regions and have been identified at the transcription start site and enhancers of many genes across the genome (Loven *et al.*, 2013).

The functions of BET proteins do not rest solely on their BRDs, BET proteins also interact with other proteins through the BET C-terminal domain, which is unique to Brd4 and BrdT and not present in Brd2 nor Brd3 (Basheer and Huntly, 2015). For

example, Brd4 is used as an adaptor by papillomaviruses and the C-terminal domain of Brd4 is known to interact with the E2 protein encoded by these viruses, this interaction tethers viral DNA to mitotic chromosomes of the host cell for segregation of the viral genome into daughter cells (You *et al.*, 2004; Wu and Chiang, 2007). Positive transcription elongation factor b (pTEFb, a complex of cyclin T1 and CDK9) is also known to interact with the C-terminal domain and BD2 of Brd4 (Schroder *et al.*, 2012). pTEFb (along with SEC, super elongation complex) promotes transcription elongation, by phosphorylation of the negative elongation factors DSIF (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole sensitivity-inducing-factor) and NELF (negative transcription elongation factor) (Bartholomeeusen *et al.*, 2012). After transcription initiation and promoter clearance, DSIF and NELF assemble on RNA polymerase II, blocking its movement; phosphorylation of DSIF and NELF inhibits their repressive function and also converts DSIF into an elongation promoting factor (Bartholomeeusen *et al.*, 2012). pTEFb itself exists in either a kinase-inactive form, when bound to its negative regulators (HEXIM1 and 7SKsnRNA complex), and an active form (free pTEFb) (Yang *et al.*, 2005). In this respect, Brd4 is known to displace the negative regulators from pTEFb, thus activating pTEFb which can then phosphorylate DSIF and NELF as well as RNA polymerase II (Yang *et al.*, 2005; Bartholomeeusen *et al.*, 2012). Therefore in addition to their role in initiation of transcription, BET proteins also play a role in transcriptional elongation.

The extraterminal domain of BET proteins is less studied and little is known about the functions of this domain compared to the BET bromo- and C-terminal domains. All BET proteins have been linked to transcriptional regulation. However, only Brd4 and BrdT possess the C-terminal domain which is known to recruit pTEFb, therefore BET proteins may possess transcriptional regulatory functions which are independent of the C-terminal domain. Using transfected tagged fragments of Brd4 regions of interest and a nuclear localisation signal, the extraterminal domain of Brd4 (and also Brd2/3) was shown to interact with NSD3, JMJD6, CHD4, GLTSCR1 and ATAD5. Furthermore NSD3, JMJD6 and CHD4 were found to be involved in transcriptional activation, as determined by the effect of siRNA knockdown on transcriptional activation in a luciferase reporter assay (Rahman *et al.*, 2011). Interestingly CHD4 (chromodomain helicase DNA binding protein 4) is known to be involved in chromatin remodelling suggesting that Brd4 could be involved in chromatin remodelling via its interaction with CHD4, although its downstream effects on gene expression are unknown.

1.3.7. Epigenetic modifications as therapeutic targets: bromodomain extraterminal (BET) protein inhibitors

In 2010, two separate groups reported the development of small molecules (GSK525762A (I-BET762), and JQ1) which specifically bind to the BRDs (BD1 and BD2) of the BET family of proteins, with high affinity over BRDs contained within other families of BRD-containing proteins (Filippakopoulos *et al.*, 2010; Nicodeme *et al.*, 2010). These compounds competitively bind within the acetyl-lysine binding pocket of the tandem BRDs of

Brd2, Brd3, Brd4 and BrdT and block association with acetylated proteins, including histone (Figure 1-9) and non-histone targets (Zou *et al.*); they represent a novel drug target class (Nicodeme *et al.*, 2010).

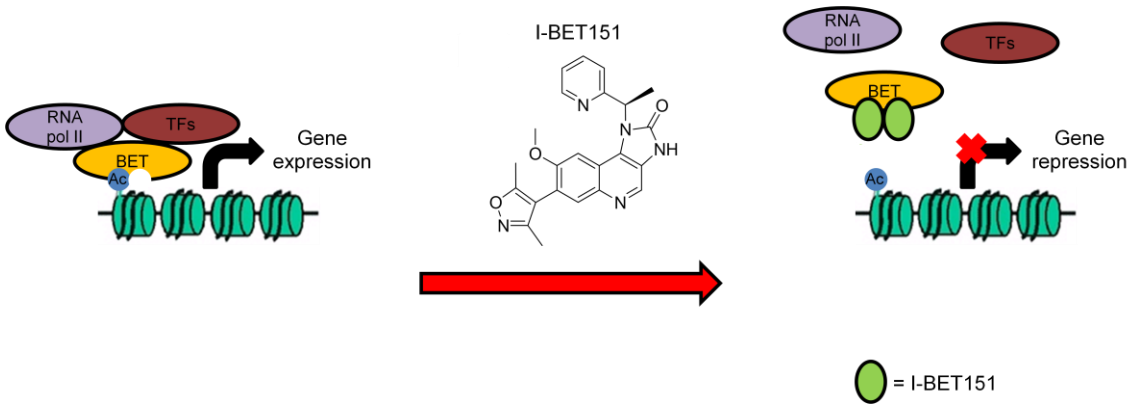


Figure 1-9: BET inhibitors

These inhibitors, *e.g.* I-BET151 target the acetyl-lysine binding pocket of BET proteins and block association with acetylated lysine containing proteins which are mainly involved in transcriptional complex formation thereby modulating gene expression (shown here are histone targets and the effect of gene silencing). (Source of I-BET151 structure: Epinova, GlaxoSmithKline)

I-BET762 was demonstrated to inhibit the secondary inflammatory response in lipopolysaccharide-stimulated mouse macrophages *in-vitro* and mouse models of sepsis *in-vivo* (Nicodeme *et al.*, 2010). I-BET762 treatment of lipopolysaccharide-stimulated

macrophages lead to the inhibition of expression of LPS-sensitive genes important in the inflammatory response, for example cytokines, *IL-6* and *IL-1b;* and chemokines, *Cxcl9* and *Ccl-12*; as well as transcription factors, *Rel* and *Irf4* (Nicodeme *et al.*, 2010); chromatin immunoprecipitation (ChIP) studies demonstrated that inhibition of BET binding to promoters of affected genes correlated with their decreased expression. However, in addition to I-BET762 reducing BET binding to promoter region of LPS-sensitive genes, I-BET762 treatment also resulted in reduction in histone acetylation associated with LPS-sensitive genes which could have been as a result of either i) increased accessibility of HDACs to acetyl-lysine residues no longer bound by BET proteins and/or ii) inability of coactivators (including HATs) to be recruited to acetyl-lysine residues to maintain acetylation, in the absence of BET binding (Nicodeme *et al.*, 2010). This shift in balance between HDAC and HAT activity could thus favour histone deacetylation. This study provided the first demonstration of efficacy for a BET inhibitor in models of inflammation and sepsis. The potential use of BET inhibitors in treating inflammatory diseases was further supported by the finding that JQ1 prevented differentiation of naïve CD4+ T cells into IL-17 producing T helper 17 cells (T_H17 cells) and inhibited activation of previously differentiated T_H17 cells (Mele *et al.*, 2013) with associated suppression of T_H17 cell-associated genes including IL-17 and IL-22, which are central to the pathogenesis of psoriasis, amongst other auto-inflammatory diseases (section 1.2.5). In addition to effects on immune cells, BET inhibitors have been shown to suppress the inflammatory response in other cell types. For example, I-BET151 suppressed IL-6 and IL-8 gene expression in response to either TNFα or IL-1β stimulation in patient-derived rheumatoid arthritis synovial fibroblasts (Klein *et al.*, 2014).

As well as inhibiting inflammatory responses, BET inhibitors have also been shown to have anti-proliferative effects in models of cancer. In the first of these cancer studies JQ1 demonstrated efficacy in a pre-clinical cancer model of NUT midline carcinoma which, as previously discussed, results from the formation of a Brd4-NUT or Brd3-NUT oncoprotein, due to chromosomal translocation, whose oncogenic function lies in the ability of Brd3/4 to bind acetylated chromatin thus providing a rationale for treatment of NUT-midline carcinoma with a BET inhibitor (section 1.3.6). *In vitro* investigations using NUT-midline carcinoma cell lines, demonstrated that JQ1 displaced Brd4-NUT from chromatin and resulted in terminal differentiation and apoptosis of malignant cells (Filippakopoulos *et al.*, 2010). Furthermore, in patient-

derived xenograft mouse models of NUT-midline carcinoma, *in vivo* administration of JQ1 lead to increased survival with minimal toxicity in normal tissue (Filippakopoulos *et al.*, 2010). Although these results clearly demonstrated a potentially exciting advancement for the treatment of NUT-midline carcinoma, and BET inhibitors are now in phase I/II clinical trials for this aggressive disease (Basheer and Huntly, 2015), the majority of other types of cancers do not display chromosomal translocations involving BET. Therefore, since this pivotal study, other investigations have evaluated the effect of BET inhibition in cancers that lack BET translocations. For example, the BET inhibitor GSK1210151A (I-BET151) has been shown to inhibit growth of human mixed lineage leukaemic-fusion cell lines *in-vitro* by inducing early cell cycle arrest and apoptosis and, in a mouse model of MLL-fusion leukaemia, *in-vivo* treatment with I-BET151 lead to increased survival (Dawson *et al.*, 2011). The inhibition of tumour growth by I-BET151 could be explained, at least in part, by the inhibitory effects on transcription of key oncogenes including *C-MYC* and *BCL2* as a result of I-BET151 inhibiting binding of BET proteins to these gene promoters (Dawson *et al.*, 2011). Of great interest was the observation that in models of other types of cancer, treatment with BET inhibitors also lead to inhibition of growth, again at least in part by the downregulation of *C-MYC* and/or *BCL2* in both haematological and solid organ tumours *e.g.* JQI had an anti-proliferative effect on both multiple myeloma cells lines and primary cells derived from patients and in an *in vivo* mouse model of disease, with accompanying decreases in expression of *C-MYC* expression *C-MYC* dependent genes (Delmore *et al.*, 2011); and I-BET762 inhibited tumour growth in *in vitro* and *in vivo* models of neuroblastoma, in which down-regulation on *BCL2* (and *MYCN*) was also observed (Wyce *et al.*, 2013). Over-expression of MYC or BCL2 led to reversal of tumour growth inhibition by JQ1 or I-BET151, respectively (Dawson *et al.*, 2011; Mertz *et al.*, 2011). Together these findings suggest that a wide variety of haematological and solid organ tumours may be sensitive to BET inhibition and these compounds may mediate their tumour suppressive effects, at least in part, through inhibition of expression of *MYC* and *BCL2*. BET inhibitors are currently in phase I/II clinical trials for the treatment for NUT-midline carcinoma and a number of other diseases, mainly haematological in origin, including Hodgkin's and non- Hodgkin's disease, myelodysplastic syndrome and myeloproliferative neoplasm, section 1.3.7 (Basheer and Huntly, 2015). BET inhibitors are also being trialled in non-cancer diseases, including type II diabetes and atherosclerosis (Filippakopoulos and Knapp, 2014).

Interestingly in the above studies, BET inhibitors decreased expression of only a subset of genes, not all genes were affected equally, even though BET proteins are found at the transcriptional start site of a wide variety of genes (Loven *et al.*, 2013). In addition BET inhibition appeared to have effects which were cell type specific; as discussed, JQ1 treatment of leukaemic cells lead to downregulation of *MYC*, but in contrast had little effect on *MYC* expression in fibroblasts (Zuber *et al.*, 2011). To account for this, it has previously been hypothesised that BET inhibitors might mediate many of their effects through displacement of BET proteins at enhancer regions; where there is often high levels of cell-type specific BET binding *e.g.* Brd4 binding at super-enhancers as previously discussed (section 1.3.4). Support for this hypothesis came from the finding that a particular multiple myeloma cell line (MM1.S) was found to have a translocation so that *MYC* expression became under the regulation of the IgH enhancer, which showed 10-fold greater enrichment of Brd4 compared to Brd4 binding at the *MYC* promoter (Delmore *et al.*, 2011). In this cell line, *MYC* expression was highly sensitive to BET inhibition by JQ1 (Delmore *et al.*, 2011), suggesting that BET binding to enhancer regions might be the main driver of BET-sensitive gene expression. Furthermore as enhancer and super-enhancer may be both cell-type specific and regulate the expression of a specific subset of genes (Heintzman *et al.*, 2009; Heinz *et al.*, 2015), displacement of BET proteins from enhancers and super-enhancers could explain the cell-type specific effects of BET inhibition and also why only a subset of gene are inhibited by BET inhibitors.

In addition to BET inhibitors disruption recruitment of transcriptional complexes to regulatory regions, either through inhibiting BET binding to acetylated histones or to transcription factors and coactivators, BET inhibitors were also found to have additional interesting mechanisms of action which could be used to clinical advantage. For example, the HIV transactivator Tat is known to compete with Brd4 for binding to pTEFb; Tat binding to pTEFb leads to transactivation of the HIV promoter and viral replication. On the other hand Brd4 binding to pTEFb, sequesters pTEFb thereby preventing the formation of Tat-pTEFb complexes, leading to HIV latency thus allowing the virus to evade clearance with anti-retroviral treatment (Bisgrove *et al.*, 2007). BET inhibition (by JQ1) was shown to release pTEFb from Brd4, thereby allowing free pTEFb to associate with Tat and reactive viral transcription which could increase HIV sensitivity to anti-retroviral therapies (Li *et al.*, 2013).

Given that BET inhibitors demonstrate both anti-inflammatory and anti-proliferative effects, as well as inhibiting differentiation of naïve CD4+ T cells into T_H17 cells, this provided rationale for investigating the role of BET proteins and evaluating the effects of BET inhibitors in psoriasis, a disease characterised by chronic inflammation and excessive proliferation (sections 1.2.1 and 1.2.5) which may be driven by alterations in the underlying epigenome (section 1.3.5). In this respect, *in vivo* topical application of JQ1 was very recently shown to reduce inflammation in an imiquimod-induced mouse model of psoriasiform inflammation (Nadeem *et al.*, 2015). In mice, topical application of imiquimod (IMQ, a TLR-7 agonist which leads to induction of NF-κB) is known to induce psoriasis-like cutaneous inflammation and Nadeem *et al*. demonstrated that topical treatment with a pan-BET inhibitor (JQ1) either before or after application of IMQ prevented the psoriasis-like phenotype including reduction in acanthosis, hyperkeratosis and elongation of the rete ridges (Figure 1-1B). There was a corresponding reduction in key cytokines relevant psoriasis including IL-17 and IL-22 (but not other cytokine including IL-23 and TNFα); this was mediated by JQ1 inhibiting epidermal expression of the retinoic acid receptor-related orphan receptor C (RORC) (mRNA and protein), a transcription factor known to regulate expression of IL-17 and IL-22 (Nadeem *et al.*, 2015).

The multitude of potentially useful but diverse effects of BET inhibitors, of course raises the question of whether these effects could be due to off-target effects. To address this question, the effects of pan-BET inhibitors I-BET151 and JQ1 were compared in rheumatoid arthritis synovial fibroblasts stimulated with IL-1β; it was found that patterns of inhibition were almost identical between the two chemically unrelated compounds (Klein *et al.*, 2014). Furthermore, screening with I-BET762 has previously not identified any non-BET protein binding partners (Nicodeme *et al.*, 2010). Together these findings suggest that the BET inhibitors discussed mediate their effects via specific binding and inhibition of BET proteins.

1.4. Environmental toxins implicated in the pathogenesis of psoriasis.

In addition to genetic components, environmental factors including alcohol, streptococcal throat infection, stress and prescription drugs, such as beta-blockers and lithium may also contribute to the pathogenesis of psoriasis (Peters *et al.*, 2000). Although there is strong evidence that these factors play a role in psoriasis, how

environment factors interact with genetic components and adaptive and innate immune systems to produce or exacerbate the psoriasis phenotype is poorly understood.

1.4.1. The role of alcohol and its metabolites in the pathogenesis of psoriasis

Although it is proposed that alcohol abuse is a risk factor for the subsequent development and also exacerbation of pre-existing psoriasis, the exact nature of this relationship has not always been clearly defined. Although data from larger population studies in Scandinavia suggested that there was increased alcohol consumption in patients with psoriasis (Braathen *et al.*, 1989), some investigators reported that patients consumed more alcohol following the diagnosis of psoriasis, reflecting the negative impact that chronic inflammatory dermatoses can have on quality of life (Kirby *et al.*, 2008). In this respect, increased alcohol consumption after the onset of psoriasis could act as a coping mechanism for chronic disease but not necessarily a cause for it. Although some early studies did not demonstrate alcohol abuse preceding the onset of psoriasis (Grunnet, 1974), there is increasing evidence from large epidemiological studies, and it is now generally accepted, that alcohol is a risk factor for development (and exacerbation) of psoriasis (Lindegard, 1986), as well as other skin diseases including discoid eczema, rosacea, post-adolescent acne, and porphyria cutanea tarda (Higgins and Du Vivier, 1994b). Interestingly some groups that initially reported no significant association between alcohol intake and the risk of developing psoriasis (Naldi *et al.*, 1992), have more recently reported that alcohol is a significant risk factor (Naldi, 1998).

A common problem with many of the epidemiological studies on this subject was the reliance on self-reporting of alcohol intake; an underestimation of alcohol intake was likely to occur. Many studies were retrospective in nature, with recall bias and potential under reporting being a problem. Other studies included cases of self-diagnosed psoriasis. In addition, the control population in studies varied; some had normal healthy controls without any skin disease and others had patients with skin diseases other than psoriasis (Poikolainen *et al.*, 1990). In one of the largest studies of its kind, a prospective study of nurses in the US sought to address some of these issues (Qureshi *et al.*, 2010). The study was based in a cohort of over 115,000 nurses in the US who undertook biennial questionnaires which included question regarding the amount of alcohol consumed and dermatological diseases, including physician-diagnosed psoriasis. Only participants without a diagnosis of psoriasis at the start of the study

were followed up over a 15 year period. This study found that the relative risk of developing psoriasis was 1.73 (95% confidence interval, 1.16–2.58) in those who consumed ≥ 2.3 alcoholic drinks/week compared with those who did not drink any alcohol (Qureshi *et al.*, 2010). The strength of the study was its prospective nature and the population size. Perhaps the greatest weakness was that only women were included in this study and it is unknown if the same conclusions could be made about male subjects, although it has previously been reported that alcohol may be a greater risk factor for psoriasis in males compared to females (Higgins, 2000).

In addition to alcohol consumption being a risk factor for the development of psoriasis, many investigators also reported that alcohol exacerbated pre-existing psoriasis (Higgins, 2000), is associated with poor treatment response (Gupta *et al.*, 1993) and that abstinence can induce remission (Vincenti and Blunden, 1987). Interestingly, alcohol may also affect psoriasis in the long term, beyond the acute phase of alcohol intake; it has previously been shown that in patients who abstain from alcohol during in-patient treatment for psoriasis, their previous alcohol intake can predict treatment outcome (Gupta *et al.*, 1993).

As the role of alcohol in the development and exacerbation of psoriasis becomes clearer, there is also evidence that patients with high alcohol intake have a tendency towards more severe, widespread, inflamed psoriasis (Monk and Neill, 1986; Higgins and Du Vivier, 1994b). They may also have distinct psoriatic phenotypes; the first type being a highly inflammatory form, with minimal scale, mainly confined to the flexures and face and the second type being characterised by intensely thick scale (hyperkeratosis) in an acral distribution *e.g.* hands and feet (Higgins and du Vivier, 1992).

In addition to alcohol being a risk factor for the development and exacerbation of psoriasis, excess alcohol consumption can also decrease the efficacy of systemic treatments for psoriasis, as well as being associated with poorer patient compliance (Gupta *et al.*, 1993; Higgins and du Vivier, 1994a). Furthermore, alcohol can increase the potential toxicity of systemic treatments. For example, alcohol can increase the esterification of acitretin, a systemic retinoid used to treat psoriasis, to etretinate which, as a result of its slow elimination and accumulation in adipose tissue, increases possible side effects of acitretin including its teratogenicity (Larsen *et al.*, 1993).

1.4.2. Ethanol metabolism

In humans, ingested alcohol is absorbed mainly in the duodenum, by passive diffusion. Following absorption, ethanol diffuses rapidly throughout water-based compartments of the body. For example, in human sweat ethanol concentration is ~80-95% of that found in blood (Buono, 1999) and in rats, skin ethanol levels reached 12–18% of blood values 2h after a single oral ingestion of ethanol (Brand *et al.*, 2006). During metabolism, ethanol is initially oxidised to acetaldehyde, mostly by cytoplasmic alcohol dehydrogenase (ADH), (Figure 1-10) which is then oxidised to acetate, mostly by mitochondrial aldehyde dehydrogenase (ALDH); conversion of ethanol to acetate occurs predominantly in the liver (Crabb *et al.*, 2004). Acetate itself can be oxidised to carbon dioxide and water, alternatively acetate can freely circulate in plasma to peripheral tissue where it can be metabolised to acetyl-coA, by acetyl coenzymeA synthetase (ACSS). Acetyl-coA is involved in many biochemical reactions, including the Krebs cycle, but can also be used as a substrate for histone acetyl transferase enzymes (*e.g.* for acetylation of lysine residues on histones) or be converted to acetone, through ketosis (Yamashita *et al.*, 2001).

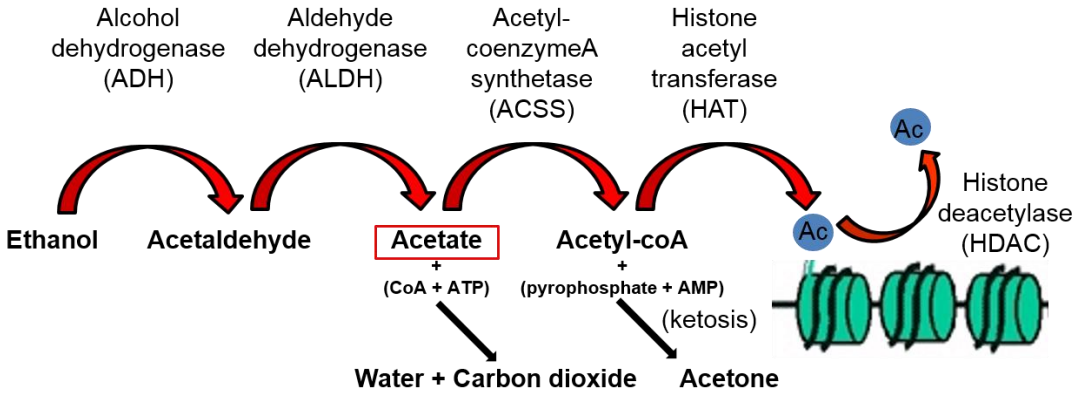


Figure 1-10: Ethanol metabolism

In humans there are six classes of ADH (1-6), each with different substrate specificities, although class I ADH (consisting of ADH1A, ADH1B and ADH1C) is the main ethanol-metabolising isoenzyme (Cheung *et al.*, 1999). In comparison, there are 3 classes of ALDH (1-3), each with different substrate specificities, but ALDH1 and ALDH2 are the main acetaldehyde-metabolising isoenzymes (Cheung *et al.*, 2003)

Although metabolism of acetate occurs in peripheral tissue, oxidation of ethanol to acetate occurs predominantly in hepatocytes where ADH and ALDH enzymes are principally expressed (Crabb *et al.*, 2004). In comparison, it has previously been shown that expression of ADH and ALDH in skin was ~12-30 fold lower than in liver (Cheung *et al.*, 1999; Cheung *et al.*, 2003). In addition to skin showing low expression of enzymes involved in ethanol metabolism, enzyme activities and affinity for ethanol has also been found to be low. For example, comparisons of substrate utilisation rates of ADH derived from human skin for 7 different primary alcohols (ethanol, propranolol, butanol, pentanol, 2-methylpropranolol, 3-methylbutanol and 2,2-demethyl-propranolol) showed that, apart from 2,2-demethyl-propranolol, ethanol showed the lowest rate of substrate utilisation (Wilkin and Stewart, 1987). In addition the specific enzyme activity of ADH from different tissues, using ethanol as a substrate, has previously shown that ADH activity in skin is approximately ~190 fold lower than in liver (Saleem *et al.*, 1984). Furthermore while it is clearly not possible to measure total ADH activity in the whole human body or in individual organs, total ADH activity in individual organs has been calculated using data from ADH activity/gram in organ-specific samples and the average total weight of these organs in the human body; these calculations showed that ~96 to 98.8% of total ADH activity in the human body is confined to the liver and only 0.05% occurs in the whole of the skin (Saleem *et al.*, 1984). Together these findings suggest that very little ethanol metabolism occurs in skin as a whole and even less in individual keratinocytes.

1.4.3. Potential, non-epigenetic, mechanisms of alcohol involvement in the pathogenesis of psoriasis

Although there is mounting evidence that alcohol is a causal factor in the development and exacerbation of psoriasis, the mechanism(s) by which alcohol might contribute to disease remains unknown. However, a number of studies support the hypothesis that ethanol, and a number of its metabolites (Figure 1-10), contributes to the psoriasis phenotype through a variety of potential mechanisms.

Firstly ethanol and its downstream metabolite, acetone (Figure 1-10), have both previously been shown to induce proliferation in HaCaT cells, an immortalised keratinocyte cell line (Farkas *et al.*, 2003). Excess keratinocyte proliferation is a key feature in psoriasis (Goodwin *et al.*, 1973) and ethanol and acetone were both shown to upregulate expression of genes characteristic of keratinocyte proliferation including α5

integrin, keratinocyte growth factor receptor and cyclin D1; which have all been shown to be upregulated in lesional psoriatic tissue (Farkas *et al.*, 2003).

Another mechanism proposes that metabolism of ethanol to acetylaldehyde leads to the generation of reactive oxygen species (ROS), which activates pro-inflammatory signalling pathways including mitogen-activated protein kinase/activator protein 1 (MAPK/AP1), NF-κB and Janus kinase signal transducers and activators of transcription (JAK-STAT) leading to upregulation of pro-inflammatory cytokines including TNFα, IL-1, IL-6, IL-8 and IFNγ, causing a shift towards a predominantly Th1 response seen in psoriasis (Farkas and Kemeny, 2010).

As well as potential effects on epidermal proliferation and the immune response, alcohol may also affect the skin's barrier function. In rats, oral ethanol intake can affect the skin's barrier function, as determined by transdermal absorption of topically applied agents; the authors proposed this may be due to enhanced solubility, physical alteration in the skin barrier and/or changes in the skin due to ethanol or its metabolites (Brand *et al.*, 2006). Impaired barrier function can also increase susceptibility to infection and have effects on the skin microbiome, which may contribute to inflammatory skin disease. As previously described, ethanol concentration in sweat is ~80-95% of that in blood (Buono, 1999) and skin ethanol levels are ~12–18% of blood (Brand *et al.*, 2006).

In addition to local cutaneous effects, alcohol can have more generalised effects which could have an impact on the development or exacerbation of psoriasis. For example, alcohol abuse can cause nutritional deficiencies either because of a primary deficiency due to lack of intake or a secondary deficiency due to, for example, malabsorption or inability to metabolise or store nutrients normally; this could result from pancreatic insufficiency or small bowel dysmotility as a result of alcohol abuse. Heavy abusers of alcohol may be deficient in protein and vitamins, including A,B, C and E as well as micronutrients including magnesium, zinc and selenium (Smith and Fenske, 2000). Many nutritional deficiencies are known to have cutaneous manifestations, for example; protein deficiency can lead to scaling of the skin and flexural fissures; vitamin B_3 deficiency can lead to dermatitis (as part of the pellagra); vitamin C deficiency can lead to peri-follicular haemorrhages and corkscrew hair and zinc deficiency can lead to impaired wound healing, angular stomatitis and pustular paronychia. Nutritional deficiencies, as a result of alcohol abuse, could therefore play a role in the pathogenesis of psoriasis.

1.4.4. Potential, epigenetic, mechanisms of alcohol involvement in the pathogenesis of psoriasis

As previously discussed, psoriasis is characterised by hyper-proliferative and abnormally differentiated keratinocytes, driven in part by intrinsically dysfunctional keratinocytes (Goodwin *et al.*, 1973) but also by an altered immune system with excess expression of growth factors and cytokines (Takahashi *et al.*, 2010). As epigenetic modifications, section 1.3.2, by definition, regulate gene expression (Bird, 2007) and there is increasing evidence that these are aberrant is psoriasis (Gudjonsson and Krueger, 2011; Zhang *et al.*, 2011b), section 1.3.5, it is plausible that alcohol interacts with the epigenome to influence the development, exacerbation and phenotype of psoriasis.

To date, there have been no publications investigating the potential epigenetic effects of alcohol, or its metabolites, in psoriasis. However, studies outside of the field of psoriasis suggest that alcohol or its metabolites can poise a cell into a pro-inflammatory state through alterations of the epigenome and its regulators.

Park *et al*. were one of the earliest investigators to demonstrate that ethanol treatment, in rat derived hepatocytes, resulted in histone hyperacetylation (Park *et al.*, 2003). The same group have since demonstrated that ethanol effects on the epigenome may be tissue specific; ingestion of alcohol in rats *in vivo* lead to enhanced histone acetylation in liver and spleen but not in brain or kidney (effects in skin were not investigated) (Kim and Shukla, 2006). The balance between opposing writer (*e.g.* HAT) and eraser (*e.g.* HDAC) activity governs the state of epigenetic marks and ethanol has previously been shown to enhance HAT activity and decrease HDAC activity *in vitro*, thus promoting a state of hyperacetylation (Choudhury and Shukla, 2008), and in keeping with evidence of ethanol-induced hyperacetylation of histones *in vivo* (Kim and Shukla, 2006). *In vivo*, ethanol undergoes zero-order metabolism and it has previously been shown that ethanol could mediate its (epigenetic) effects through one of its principle metabolites, acetate. In nuclear extracts prepared from rat derived hepatocytes, both ethanol and acetate exposure lead to the selective enhancement of acetylation of H3 at lysine 9 (H3K9ac) (Park *et al.*, 2005) and inhibition of enzymes involved in ethanol metabolism decreased ethanol-induced histone-H3 acetylation (Park *et al.*, 2003).

In addition to generalised effects on histone acetylation, ethanol has also been shown to enhance acetylation of histones associated with the promoter region of genes

upregulated in alcohol-associated diseases. For example in a macrophage model of acute alcoholic hepatitis, ethanol enhanced cytokine responses to LPS through hyperacetylation of histones at the promoter region of target response genes *e.g.* IL-6 and TNFα (Kendrick *et al.*, 2010). The hyperacetylation (greater for histone-H3 compared to histone-H4) could be explained by the observation that both ethanol and acetate increased HAT activity, decreased HDAC activity and up-regulated expression of ACSS (Kendrick *et al.*, 2010). In this system acetate had similar effects to ethanol, supporting the earlier hypothesis that ethanol's effects on the epigenome could be mediated through this metabolite (Park *et al.*, 2003; Park *et al.*, 2005).

In addition to ethanol driving increased acetylation of histone-H3 over histone-H4 (Kendrick *et al.*, 2010), ethanol has been shown to preferentially induce acetylation of particular lysine residues on histone-H3 over others. For example, in rat-derived hepatocytes, ethanol selectively enhanced acetylation of H3 at lysine 9 (H3K9ac) but had no significant effect on H3K14ac, H3K18ac nor H3K23ac, these results were also observed in the liver of rats treated with ethanol *in vivo* (Park *et al.*, 2005). Although it is known that histone acetylation is regulated by the family of HAT and HDAC enzymes, the specificity of particular family members for individual lysine residues and different histones is not fully understood. Therefore, whilst Park *et el*. demonstrated that ethanol modulated the activity of HAT(s), which demonstrated selectively for H3K9ac, they could not elucidate which member(s) of the HAT family were activated by ethanol or indeed whether specific HDACs were also involved (Park *et al.*, 2005).

More recently, ethanol has been shown to regulate recruitment of specific HATs. In a model of alcohol-induced cardiac hypertrophy, ethanol-exposed mice demonstrated an ethanol-induced increase in H3K9ac at the NKX2.5 promoter and increased expression of the NKX2.5 gene, which encodes a transcription factor involved in and upregulated in cardiac development and hypertrophy (Peng *et al.*, 2015). In contrast there was no increased H3K9ac acetylation at the *RPL13A* promoter, a gene for which the expression was not enhanced with ethanol treatment (Peng *et al.*, 2015). Furthermore Peng *et al*. demonstrated that specific members of the HAT family (p300, CBP, PCAF, SRC1 and GCB5) bound to the NKX2.5 promoter and that ethanol enhanced the binding of a specific subset of these (p300, CBP, PCAF and SRC1 but not GCB5) (Peng *et al.*, 2015). Although Peng *et al*. did not investigate the effects of ethanol on HDAC binding to the NKX2.5 promoter, it was demonstrated that the pan-HAT inhibitor, anacardic acid, reduced binding of p300 and PCAF with corresponding attenuation of H3K9ac.

Together these findings suggest ethanol can induce acetylation of individual lysine residues on histones associated with particular promoter regions of target genes through the recruitment of specific HATs, which potentially play a greater role than HDACs in ethanol-enhanced expression (of NKX2.5). Although this is a novel and intriguing finding, it is unknown if similar mechanisms regulate other ethanol-sensitive genes in this system or other alcohol-related diseases or whether HDACs play a greater role in different conditions.

Although the effects of ethanol on histone acetylation have been the most widely studied epigenetic modification, ethanol has also been shown to have effects on other types of histone modifications. For example, treatment of rat-derived hepatocytes has been shown to alter the methylation status of lysine residues on histones (Pal-Bhadra *et al.*, 2007). Interestingly, Pal-Bhadra *et al*. demonstrated that ethanol affected methylation patterns in a lysine-residue specific manner; ethanol induced an increase in global dimethylation on lysine 4 on histone-H3 (H3K4me2) but a decrease in global dimethylation on lysine 9 on histone-H3 (H3K9me2). Furthermore, in ChIP studies, H3K4me2 was enriched at the promoter region of genes which were upregulated with ethanol treatment, whereas H3K9me2 was enriched at the promoter region of genes which were downregulated with ethanol treatment (Pal-Bhadra *et al.*, 2007). These findings support the hypothesis that ethanol can regulate recruitment of specific epigenetic writers and potentially erasers to particular histone modifications associated with specific genes, but in addition to affecting recruitment of regulators of histone acetylation (Peng *et al.*, 2015), the findings by Pal-Bhadra *et al*. suggest that ethanol could also affect regulators of histone methylation (methylases and demethylases), although the exact mechanism remains unknown.

In summary, it is known that alcohol abuse is associated with increased risk of organ specific diseases especially inflammation and cirrhosis of liver with mounting evidence for a role in psoriasis pathogenesis, amongst other dermatological diseases. As described above, investigations of the epigenetic effects of ethanol in non-dermatological diseases/models suggest that ethanol can have organ specific epigenetic effects and has been shown to affect both histone acetylation and methylation. By regulating the recruitment of specific epigenetic writers (and potentially erasers), ethanol can modulate individual histone modifications at the promoter region of particular disease-related genes, thereby regulating their expression, potentially precipitating or exacerbating disease. Furthermore, it was shown that acetate often had

similar effects to ethanol suggesting that ethanol could mediate its effects through acetate, one of its principle metabolites.

1.4.5. Lithium and beta-blockers in the pathogenesis of psoriasis

In addition to alcohol, there is very strong evidence and it is widely accepted that non-recreational drugs including lithium and beta-blockers can also trigger and exacerbate pre-existing psoriasis (Peters *et al.*, 2000; Rongioletti *et al.*, 2009).

Lithium is a mood stabiliser and is licensed, as lithium carbonate and lithium citrate, for the treatment of bipolar disorder and depression (BNF, 2015). It is well recognised that lithium can both exacerbate pre-existing psoriasis and, less commonly, provoke it in patients with or without a previous personal or family history of psoriasis; although not all patients exposed to lithium will develop psoriasis (Sasaki *et al.*, 1989; Brauchli *et al.*, 2009). Incidence of exacerbation or induction of psoriasis ranges between 3.4 – 45% and is not necessarily dose related and latency periods of 20 weeks (mean) for exacerbation and 48 weeks (mean) for induction of psoriasis are reported (Rongioletti *et al.*, 2009; Hampton *et al.*, 2012). A case-control analysis of over 36,000 newly diagnosed cases of psoriasis using the UK-based General Practice Research Database, showed that patients who received 5 or more prescriptions of lithium had an odds ratio of 1.68 (95 confidence interval: 1.18-2.39) for developing psoriasis compared to those who did not receive lithium (Brauchli *et al.*, 2009). In addition to a link between lithium and plaque psoriasis, lithium has also been reported to precipitate pustular psoriasis in a patient with pre-existing plaque psoriasis and this occurred again on re-challenge with lithium (Lowe and Ridgway, 1978). Although the majority of case studies involved a limited number of patients, it has been previously reported that lithium provoked psoriasis was reversed after withdrawal of the drug and that re-challenging these patients with lithium resulted in a return of their psoriasis within a shorter period of time (Skoven and Thormann, 1979). Although this suggests a causal relationship between lithium and psoriasis, the underlying mechanism of lithium provoked or lithium exacerbated psoriasis remains uncertain.

Interestingly, previously published data has shown that lithium did not potentiate release of IL-6 or IL-8 protein in unstimulated keratinocytes derived from patients with psoriasis or healthy controls, although levels of transforming growth factor alpha (TGFα) were increased (Ockenfels *et al.*, 1996). Furthermore, lithium treatment in patients with bipolar disorder has previously been shown to down regulate expression of

key psoriasis inflammatory cytokines, including IL-6 and TNFα, in patient derived monocytes (Padmos *et al.*, 2008). This is in contrast with the potentiation effects of ethanol on IL-6, IL-8 and TNFα responses to LPS stimulation, as previously discussed. It has previously been reported that lithium can increase T-cell proliferation and, more recently, keratinocyte proliferation, key features of psoriasis, through inhibition of glycogen synthase kinase 3 (GSK-3) (Ohteki *et al.*, 2000; Hampton *et al.*, 2012). In keratinocytes, it was demonstrated that clinically relevant concentrations of lithium lead to increased keratinocyte proliferation and that this could be reproduced by either pharmacological inhibition of GSK-3 or retroviral transduction of GSK-binding protein, a known endogenous inhibitory binding partner of GSK-3, which resulted in both increased nuclear translocation of Nuclear factor of activated T cells 2 (NFAT2) and transcriptional activation of NFAT (Hampton *et al.*, 2012).

In addition to lithium being strongly implicated as a causal factor in the precipitation or exacerbation of psoriasis, beta-blockers (β-blockers) which are licensed to treat hypertension, amongst other indications (BNF, 2015) are also widely accepted to both provoke and exacerbate existing psoriasis and latency can be over 1 year (Rongioletti *et al.*, 2009). As with lithium, not all patients exposed to β-blockers will develop psoriasis and discontinued use of the offending drug will induce remission of β-blocker-induced psoriasis in the majority of patients (Arntzen *et al.*, 1984). A retrospective study of nearly 600 patients with psoriasis suggested that in those patients prescribed β-blockers, over 70% experienced exacerbations of their psoriasis over a 1 year period (Gold *et al.*, 1988).

Although the underlying mechanism for β-blocker-exacerbated or induced psoriasis is uncertain it has been suggested that this may be through decreased levels of cyclic adenosine monophosphate, as a direct result of beta-blockade, leading to decreased levels of intracellular calcium and therefore reduced cellular (keratinocyte) differentiation and increased proliferation, as observed in psoriasis (Wu *et al.*, 2014). Therefore, as potentially the case with lithium, β-blockers could influence the psoriasis phenotype through promoting keratinocyte proliferation, although through a different mechanism to lithium; rather than through enhanced effects on inflammation which may be the underlying mechanism, at least in part, for ethanol-exacerbated or induced psoriasis.

In summary psoriasis is a common, complex chronic inflammatory dermatosis, whose pathogenesis is not fully understood. While the most widely accepted model of psoriasis suggests hyperproliferation of keratinocytes (which themselves may be dysfunctional or hypersensitive to stimuli) is driven by an over-active immune system and environmental insults, the underlying changes which regulate intracellular signalling and inflammation are not fully understood, but increasing evidence suggests alterations in the epigenome, including histone acetylation, plays a role. Bromodomain containing proteins bind acetyl-lysine residues on histones and recruit transcription factors, thereby regulating gene expression. Small molecule inhibitors of bromodomain extraterminal (BET) proteins demonstrate anti-proliferative and anti-inflammatory effects in *in vitro* and *in vivo* models of cancer and inflammation. There is little documentation about the function of BET proteins or the effects of BET inhibition in keratinocytes. However, given that psoriasis is characterised by chronic inflammation and excessive keratinocyte proliferation, it is hypothesised that epigenetic modifications and BET proteins are involved in regulating cutaneous inflammatory responses and that BET inhibitors could block pathogenic inflammatory responses in keratinocytes.

1.5.Study aims

Psoriasis represents a complex interplay between genetic predisposition, the environment and inflammatory responses. The central hypothesis of this project is that epigenetics plays a role in the regulation of cutaneous inflammation and inflammatory skin disease. There is significant interest in utilising and further developing models of skin inflammation which can be used to increase understanding about the pathogenesis of inflammatory skin disease, including the role of epigenetics. Additionally, these studies aim to establish models amenable to pre-clinical testing of potential therapeutic compounds, including topical treatments.

Cytokine-stimuli relevant to the pathogenesis of psoriasis may alter gene-regulating epigenetic epitopes, resulting in a primed/pro-inflammatory state. In addition, it is known that exogenous factors can precipitate and exacerbate the psoriasis phenotype and we hypothesised that these may modify the epigenome to further promote expression of pro-inflammatory and psoriatic genes.

The main aims of this project were to develop a model of cutaneous inflammation in human keratinocytes using pro-inflammatory cytokines and various stimuli of the innate immune system that can be used for:

- Further understanding the pathogenesis of inflammatory skin disease and the role of epigenetics, including acetylation of lysine residues on histones and bromodomain-containing proteins that recognise these particular epitopes.
- Investigating how exogenous factors, including alcohol, might exacerbate responses to stimuli relevant to inflammatory skin disease; and to understand whether any potentiation of pro-inflammatory responses may be through an epigenetic mechanism.
- *In-vitro*, pre-clinical, evaluation of potential therapeutic compounds, including those targeting proteins known to bind to the epigenetic epitopes.

Chapter 2.

Materials and methods

2. Materials and Methods

2.1. General principles

2.1.1. Laboratory work

All experimental procedures were carried out according to the Control of Substances Hazardous to Health (COSHH) regulations. All laboratory work at Newcastle University was conducted in accordance with Newcastle University Health and Safety regulations and in a sterile containment level 2 safety hood, where appropriate. MesoScale Discovery® Platform cytokine analysis and chromatin immunoprecipitation (apart from stimulation and fixation of cells) were both carried out at GlaxoSmithKline Pharmaceutical Research and Development Facility, Stevenage, according to GlaxoSmithKline Health and Safety regulations and with relevant training.

2.1.2. Ethical approval

The study adhered to the code of ethics of the world medical association (Declaration of Helsinki) for medical research involving human subjects, was approved by Newcastle and North Tyneside Research Ethics Committee (Ref 08/H0906/95+5_Lovat) and was sponsored by The Newcastle upon Tyne Hospitals NHS Foundation Trust (trust approval for R&D project ref 4775). Written informed consent was obtained from all subjects and the research performed was in accord with the terms of the informed consents. All patient samples were obtained from Newcastle Upon Tyne Hospitals, NHS Foundation Trust.

2.1.3. Sources of materials

All chemical were supplied by Sigma-Aldrich, Gillingham, UK, unless otherwise stated.

2.2. Primary keratinocyte cell culture

2.2.1. Primary human keratinocytes isolation and culture

After obtaining informed consent from patients undergoing elective surgery, non-psoriatic human skin samples which would normally be discarded after surgery (*e.g.* circumcision, breast or abdominal reductions) were used to extract and culture normal human epidermal keratinocytes (NHEKs) (Todd and Reynolds, 1998). Immediately following surgical removal, skin samples were placed in 10ml Epilife® keratinocyte medium (Invitrogen, Paisley, UK) supplemented with 2% penicillin streptomycin

amphotericin B (PSA) (Lonza Biologics, Switzerland). Prior to transfer from theatre to the Newcastle University Dermatological Sciences Laboratories, samples were stored at 4°C. Upon receipt, samples were stored at 4°C and processed the same day. In a sterile containment level 2 safety hood, samples were washed in phosphate buffered saline (PBS) (Sigma-Aldrich, Gillingham, UK) with added PSA. Forceps and scissors were washed in 100% ethanol and flame sterilised and under clean conditions sub-cutaneous tissue (fat, connective tissue and blood vessels) was removed; the remaining tissue (epidermis and dermis) was scored with a scalpel then incubated in dispase II (1ml) (Roche Diagnostics, Burgess Hill, UK), PSA (1ml) and PBS (8ml) at 4°C, overnight. Dispase II is a protease, produced in *Bacillus polymyxa*, which hydrolyses the N-terminal peptide bonds of non-polar amino acid residues, which are found at a high frequency in collagen. Overnight incubation of the sample in dispase II, allows the separation of the epidermis from the dermis the following day, using flamed utensils. Isolated epidermis was placed in a universal tube containing 0.05% trypsin ethylene diamine tetraacetic acid (TE) (Sigma-Aldrich, Gillingham, UK) and incubated at 37°C for 5 min, shaking vigorously half way through, prior to TE neutralisation with fetal calf serum (FCS) (Sigma-Aldrich, Gillingham, UK). The sample was centrifuged at 3000g for 5mins at room temperature. The resulting pellet was resuspended in Epilife® medium with human keratinocyte growth supplement (HKGS) (Invitrogen, Paisley, UK) and 1% PSA then placed into a T175cm^2 tissue culture flask (T175) (Corning, New York, USA). Cells were cultured in a 37°C, 5% CO_2, 95% humidity incubator. Culture medium was changed every two days until cells reached approximately 80% confluency.

2.2.2. Cell passaging

At required confluency, typically 80%, cells were washed twice in PBS, then TE added and incubated at 37^{o}C for 5-7mins until NHEKs detached. Cells were centrifuged in FCS and PSA supplemented Dulbecco's Modified Eagle Medium (DMEM) (Lonza biologics, Switzerland) for 5mins at 3000g. After resuspending in Epilife®/HKGS media, cells were split 1 in 3 or 1 in 4, depending on proliferation efficiency of individual donors. Culture medium was changed every 2 days until cells reached 80% confluency. All cells used for experiments within this project were passage 1-4.

2.2.3. Ethanol, acetate, lithium and propranolol culture conditions

In experiments investigating the effects of ethanol, acetate, lithium or propranolol exposure on inflammatory responses, these were used at clinically relevant concentrations. The ethanol concentration (86mM, 0.5%, 400mg/dl) used was equivalent to five times the legal blood ethanol concentration for driving in the UK and equivalent to heavy alcohol consumption in humans (Jones, 1999). 1mM acetate concentration was equivalent to the corresponding serum concentration seen in individuals metabolising ethanol at the concentration used (86mM) (Mascord *et al.*, 1992) (Nuutinen *et al.*, 1985). The concentration of lithium (1mM) was equivalent to the therapeutic concentration used clinically (BNF, 2015) and the concentration of propranolol (150ng/ml) was equivalent to the concentration required to achieve physiological beta-receptor blockade (Duff *et al.*, 1986).

Ethanol (VWR, Poole, UK) was added to Epilife® culture media (to a final concentration of 0.5% v/v). Additionally as ethanol is a volatile substance, evaporation could lead to changes in ethanol concentration. To overcome this problem, several options were considered. Firstly sealing the culture flasks/plates, but this would have led to impaired gas exchange. Secondly to frequently replace the culture media but in experiments involving measurement of secreted cytokines over a 48h period, this would not be practical. The use of ethanol in an incubator's water reservoir at twice the concentration as added to the culture media has previously been described as a solution to this problem (Zhang *et al.*, 2001). The concept is that ethanol in the water reservoir evaporates, filling the incubator with ethanol vapour which equilibrates with the ethanol dissolved in the media; the vapour pressure of the ethanol in the incubator thus maintains the ethanol concentration in the media, provided the circuit remains closed. This method was adopted for the experiments involving ethanol, control or acetate treated cells were grown in a different incubator. A concentration of 0.5% ethanol was added to the media and a concentration of 1% ethanol was used in the reservoir in the incubator, which was replaced every 48h and each time the incubator door was opened (personal communication Dr. Stuart Kendrick, Newcastle University). In experiments investigating the effects of acetate exposure on inflammatory responses, 1mM sodium acetate (Sigma-Aldrich, Gillingham, UK) was added to culture media and replaced every 48h, similarly for experiments involving lithium and propranolol.

In experiments where cells were exposed to ethanol, acetate, lithium carbonate (Sigma-Aldrich, Gillingham, UK), lithium chloride (Sigma-Aldrich, Gillingham, UK) or propranolol hydrochloride chloride (Sigma-Aldrich, Gillingham, UK), the time indicated is the total time of exposure to these reagents prior to stimulation. As shown in Figure 2-1, cells were exposed to 1mM acetate while being cultured in T175 flask for 6 days, then seeded into 12 or 48-well plates and left for a further 1 day in 1mM acetate prior to stimulation (with TNFα + IL-17A (IL-17), again in 1mM acetate). A similar protocol was used for testing effects of ethanol, lithium carbonate, lithium chloride or propranolol hydrochloride and for testing different stimulation conditions. Control cells were treated in the exactly the same way, apart from excluding acetate/ethanol/lithium carbonate/lithium chloride/propranolol hydrochloride where relevant from Epilife® media, *i.e.* NHEKs were cultured in T175 flasks with Epilife® media for 6 days, prior to being re-seeded into 12 or 48-well plates and 1 day later, cells were stimulated (with treatments diluted in Epilife®).

The only exceptions to this were ChIP (chromatin immunoprecipitation) studies. Due to the large number of cells required for ChIP, cells were grown in T175 flasks for the total duration indicated (7 days) in the presence of media or media + 1mM acetate, before being stimulated (with TNFα + IL-17) in these T175 flasks (again treatments were diluted in media or media + 1mM acetate as relevant).

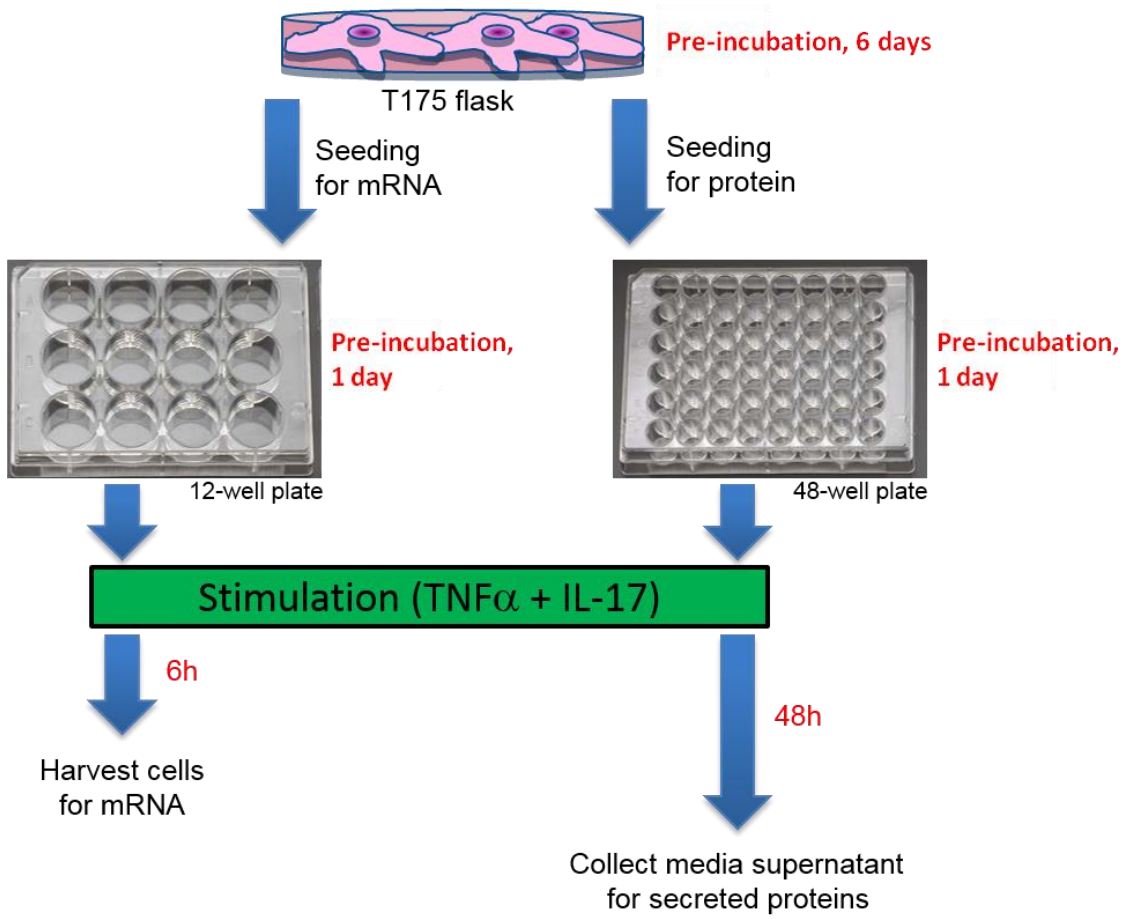


Figure 2-1: Experimental set-up to investigate the effects of pre-incubation with specified agents (*e.g.* acetate) for 7 days, on keratinocyte responses to pro-inflammatory stimuli

Cells were grown in media or media + specified agent (*e.g.* 0.2-1mM acetate, 86mM ethanol) in T175 flasks for 6 days. Cells were then seeded into 12-well or 48-well plates, for mRNA or protein studies respectively, in media or media + specified agent. 1 day later, *i.e.* after a total of 7 days' exposure to media or media + specified agent, cells were stimulated with TNFα + IL-17 (diluted in media or media + specified agent, as relevant). Cells were harvest at timepoints indicated in results chapters *e.g.* 6h post-stimulation for RNA. Media supernatants were collected at 48h post-stimulation for secreted proteins.

2.3. Treatment of keratinocytes

2.3.1. Stimulation of keratinocytes

Except where otherwise stated, for experiments investigating effects on secreted protein, NHEKs were seeded into 48-well plates at a density of 18,000 cells/well in 200µl Epilife® media. The following day, when cells were approximately 70% confluent, Epilife® media was removed and replaced with Epilife® media containing the following stimuli or pharmacological compounds, at concentrations as described in individual experiments (see results section).

- TNFα (Immunotools, Friesoythe, Germany)
- IL-17A (IL-17) (Immunotools, Friesoythe, Germany)
- IL-22 (Immunotools, Friesoythe, Germany)
- IFNγ (Immunotools, Friesoythe, Germany)
- Pam_3Cys-Ser-$(Lys)_4$ trihydrochloride (Pam3Cys) (MerckMillipore, Massachusetts, USA)
- Peptidoglycan derived from Staphylococcus *aureus* (PGN) (Sigma-Aldrich, Gillingham, UK)
- Pam_2CGDPKHPKSF (FSL-1) (Invivogen, California, USA)
- Heat-killed Staphylococcus *aureus* (HKSA) (Invivogen, California, USA)
- Lipoteichoic acid (LTA) (Sigma-Aldrich, Gillingham, UK)
- Polyinosinic:polycytidylic acid (Poly:IC) (Sigma-Aldrich, Gillingham, UK)
- Lipopolysaccharide derived from Escherichia *coli* K235 (LPS) (Sigma-Aldrich, Gillingham, UK)
- Ciclosporin (MerckMillipore, Massachusetts, USA)
- GSK1210151A (I-BET151, batch ID # P10633-080-PJ1) (GlaxoSmithKline, Stevenage, UK)
- Sodium acetate (Sigma-Aldrich, Gillingham, UK)
- Ethanol (VWR, Poole, UK)
- Dimethyl sulfoxide (DMSO, vehicle) (Sigma-Aldrich, Gillingham, UK)

For the "unstimulated" control, mock-stimulation was performed *i.e.* Epilife® media was replaced with fresh Epilife® media at the same time as treatment with stimuli or compounds in other wells. For the "vehicle" control, cells were stimulated with vehicle diluted in Epilife® media at the same concentration as the vehicle dilution for compound. For example, I-BET151 was initially dissolved in DMSO and then diluted in Epilife® media so that the final concentration of DMSO was consistently 1:1200, regardless of the final concentration of I-BET151. Therefore for I-BET151, the vehicle control was DMSO 1:1200 diluted in Epilife® media. For ciclosporin, the vehicle was ethanol (at a final dilution of 1:2000, 0.05%). Note, apart from ciclosporin and I-BET151, all other compounds were dissolvable in aqueous solution/media, abrogating the need for a separate vehicle control for these conditions. Unless otherwise stated, media supernatants were collected at 48h post-stimulation and stored at -20^{o}C, until analyte concentrations were measured. Quantitative measurements of secreted

cytokines were performed by Enzyme-linked immunosorbent assay (ELISA) or MesoScale Discovery® (MSD) Platform (section 2.4).

For mRNA analysis, NHEKs were seeded in 12-well plates at a density of 100,000 cells/well in 1ml Epilife® media. One day later, when cells were approximately 90% confluent, cells were treated as described above and in individual experiments. Unless otherwise stated, cells were harvested at 6h post stimulation and RNA was extracted using the ReliaPrep™ RNA Cell Miniprep System (Promega, Wisconsin, USA) according to the manufacturer's protocol (section 2.6).

In experiments comparing the effects of isolating and culturing NHEKs in Epilife® media vs. MCDB 153 media (Sigma-Aldrich, Gillingham, UK), keratinocytes were extracted from skin samples (section 2.2.1) and cultured separately in either Epilife® media or MCDB 153 media, both supplemented with HKGS, for 2 passages. Cells were seeded and stimulated with TNFα and/or IL-17 (diluted in Epilife® media or MCDB 153 media) at passage 3, as above, and media supernatants collected 48h post-stimulation and secreted protein concentrations measured by ELISA (section 2.4.1).

Where possible wells at the edges of culture plates were filled with PBS and not used to seed keratinocytes. This was to minimise potential evaporation effects, which could have altered the concentration of compounds.

2.3.2. Treatment of keratinocytes with compounds

In experiments investigating effects of I-BET151 concentrations on keratinocyte responses to TNFα + IL-17 stimulation, NHEKs were seeded (section 2.3.1) and I-BET151 (at concentrations indicated in results) or vehicle (DMSO, 1:1200) was added 1h prior to stimulation with TNFα + IL-17, to allow equilibration of the compound across cell membranes. In experiments investigating effects of ciclosporin on keratinocyte responses to TNFα + IL-17 stimulation, NHEKs were seeded (section 2.3.1) and ciclosporin (1μM) or vehicle (ethanol, 1:2000, 0.05%) was added 1h prior to stimulation with TNFα + IL-17. Cells were harvested at 6h post stimulation and RNA was extracted using the ReliaPrep™ RNA Cell Miniprep System (Promega, Wisconsin, USA) according to the manufacturer's protocol (section 2.6). Media supernatants were collected at 48h post-stimulation and secreted cytokines concentrations measured by Enzyme-linked immunosorbent assay (ELISA) (section 2.4.1).

In experiments investigating effects of timing of I-BET151 addition on keratinocyte responses to TNFα + IL-17 stimulation, NHEKs were seeded (section 2.3.1) and treated with I-BET151 before, during or after stimulation (Figure 2-2). Cells were harvest at 6h post stimulation for RNA (section 2.6). Media supernatants were collected at 48h post-stimulation and secreted cytokines concentrations measured (section 2.4.1).

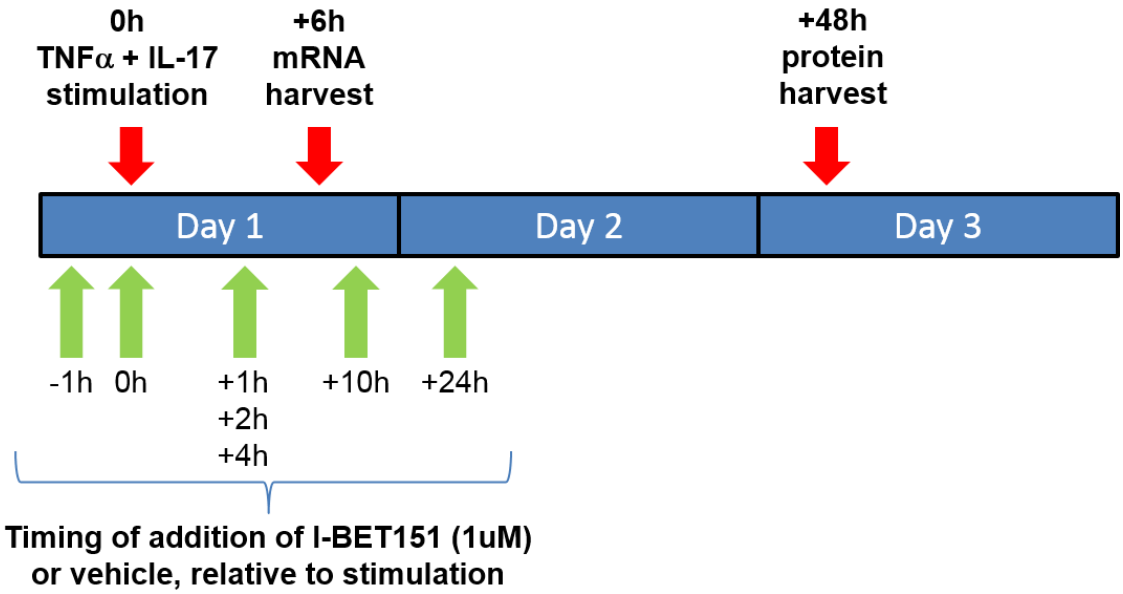


Figure 2-2: Experimental set-up to investigate of timing of I-BET151 addition on keratinocyte responses to TNFα + IL-17 stimulation

NHEKs were seeded and treated with I-BET151 (1μM) or vehicle (DMSO, 1:1200) either 1h before stimulation with TNFα + IL-17, at time of stimulation or at various times after (1, 2 or 4h after stimulation for mRNA studies and 1, 2, 4, 10 or 24h after stimulation for protein studies). Cells were harvested for mRNA 6h post-stimulation and media supernatants collected for secreted cytokines 48h post-stimulation.

2.4. Protein determination by immunoassays

Quantitative measurements of media supernatants (see section 2.3 for details of preparation) for secreted cytokines were performed using MesoScale Discovery® (MSD) Platform (at GlaxoSmithKline, Stevenage) or Enzyme-linked immunosorbent assay (ELISA) (at Newcastle University) as indicated in results sections.

2.4.1. Enzyme-linked immunosorbent assay

Measurement of secreted protein concentration was performed by ELISA using the DuoSet ELISA Development kit (R&D Systems®, Minnesota, USA) according to the manufacturer's protocol. Briefly, a "capture" antibody was coated onto a clear-flat

bottomed well and incubated at room temperature overnight, prior to washing 3 times (0.05% Tween®20 (Sigma-Aldrich, Gillingham, UK) in PBS was used for all washing steps). Non-specific protein binding was blocked using 1% bovine serum albumin (Sigma-Aldrich, Gillingham, UK) in PBS, incubating at room temperature for 2h, prior to washing 3 times. Sample media supernatant was incubated in the well at 4°C overnight, to allow binding of the protein of interest to the "capture" antibody, before the media was removed and the well washed 3 times. A biotinylated "detection" antibody was then added, which binds to a different epitope of the protein of interest (which is now bound to the "capture" antibody), incubating at 37°C for 2h before washing 3 times. Streptavidin-horseradish peroxidase (HRP) was added which binds the biotinylated "detection" antibody; the plate was sealed and incubated at room temperature for 20mins before washing 3 times. Substrate solution was added and incubated for 20mins at room temperature. Bound-HRP catalyses a colour change in the substrate (tetramethylbenzidine) solution and a blue colour develops in proportion to the amount of analyte present in the sample. Colour development was terminated using sulphuric acid (Sigma-Aldrich, Gillingham, UK), turning the colour in the wells from blue to yellow. Optical density was read at 450nm and a correction wavelength at 540nm using a SpectraMax M250 plate reader (Molecular Devices, UK). The correction wavelength reading at 540nm was subtracted from the 450nm reading and compared against a set of standard controls (run at the same time as test media supernatants) using a four parameter logistic (4-PL) curve-fit to determine cytokine concentrations. Wavelength-specific absorbance is proportional to the concentration of analyte of interest in each sample. The R&D Systems® ELISA Duoset range of detection was 9.38 - 600 pg/ml for IL-6 and 31.20 - 2,000 pg/ml for IL-8, according to the manufacturer. Where concentrations were above the upper limit of detection, further stored media supernatant samples were diluted before being assayed again.

2.4.2. MesoScale Discovery® Platform

During the initial phase of development of the cutaneous model of inflammation secreted protein concentrations were determined by MesoScale Discovery® Platform (MSD) (MesoScale, Rockville, Maryland, USA) at GlaxoSmithKline (Stevenage, UK). MSD is a proprietary technology, based on a similar principle to ELISA, and uses electrochemiluminescent labels (SULFO-TAG™) that are coupled to detection antibodies. It also facilitates the assay of multiple analytes in one sample by a multiplex format. In MSD platforms, the "capture" antibody is bound to a carbon electrode on the

bottom of the well and each well can contain several "capture" antibodies in distinct areas ("spots") of the well allowing for multiple analytes to be detected from the same media supernatant sample (Figure 2-3). "Detection" antibodies/SULFO-TAG™ bind to analytes bound to capture antibody and generate light when stimulated by electricity in the appropriate buffer environment. Electricity is applied to the plate electrodes at the bottom of the well, leading to light emission by SULFO-TAG™ labels, which is measured by a charge-coupled camera within an MSD analyser and, through comparison against a set of standard controls (run at the same time as test media supernatants), the concentration of an analyte can be determined.

Media supernatant samples were analysed by MSD, according to the manufacturer's protocol. Briefly, 25µl of test supernatant (or standards) was added to each well and incubated at room temperature on an orbital shaker for 2h. "Detection" antibodies coupled to SULFO-TAG™ were added and incubated for a further 2h at room temperature on an orbital shaker. After washing, read buffer was added and samples analysed on an MSD sector S 600 analyser (MesoScale, Rockville, Maryland, USA). Analyte concentrations were determined, by comparison against the standard set of controls, using MSD Workbench software (MesoScale, Rockville, Maryland, USA).

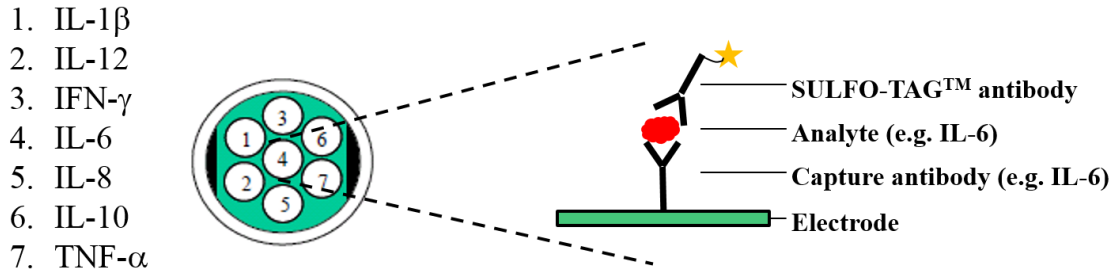


Figure 2-3: Cytokine analysis by MesoScale Discovery® Multi-Spot® Human ProInflammatory-7® platform

Each well contains seven spots, each pre-coated with a defined capture antibody. A specific cytokine binds to its corresponding capture antibody. The SULFO-TAG™ emits quantifiable light upon electrostimulation at the electrode surface of the plate. Typical lower limits of detection are 0.5-10pg/ml. (Adapted from www.mescoscale.com)

2.5. Measurement of cell viability

2.5.1. SRB assay

Total cellular protein content was determined using the Sulforhodamine B (SRB) assay which is based on the ability of SRB to bind to basic amino acids of cellular proteins (Voigt, 2005) and has been shown to be highly correlated with cell density (Skehan *et al.*, 1990). NHEKs were seeded into 48-well plates at a density of 18,000 cells/well in 200µl Epilife® media. The following day, when cells were approximately 70% confluent, Epilife® media was removed and replaced with Epilife® media containing stimuli or pharmacological compounds, at concentrations as described in individual experiments (see section 2.3 and results sections). 48h after treatment, media was removed and cells fixed with 200µl of 10% trichloroacetic acid in water for 1h at 4^{o}C. After fixing, plates were washed in water 5 times and allow to air-dry. 200µl of 0.4% (w/v) SRB (Sigma-Aldrich, Gillingham, UK) dissolved in 1% glacial acetic acid (Sigma-Aldrich, Gillingham, UK) was added to each well and incubated for 30mins at room temperature. Plates were washed five times in 1% glacial acetic acid, until residual dye was removed. Plates were dried at 60^{o}C for 1h, then 400µl 10mM Tris base pH10.0 (Sigma-Aldrich, Gillingham, UK) was added to each well and incubated for 15mins at room temperature on an orbital shaker. Absorbance was read at 490nm, using a SpectraMax M250 plate reader (Molecular Devices, UK).

2.5.2. MTT assay

Cellular metabolism was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay. Yellow MTT is reduced to purple formazan when mitochondrial reductase enzymes are active; therefore if the reduction takes place the cell is considered to be metabolically active and viable (van Meerloo *et al.*, 2011). Keratinocytes were seeded and treated as described previously for SRB assays (section 2.5.1). 48h after treatment, 40µl of 5mg/ml of MTT dissolved in PBS was added to each well and incubated at 37^{o}C for 3.5h. MTT solution was then removed and 300µl MTT solvent (4mM HCL, 0.1% Nonident P-40 in isopropanol) was added and incubated in the dark on an orbital shaker for 15mins. Absorbance was read at 590nm using a SpectraMax M250 plate reader (Molecular Devices, UK).

2.6. Gene expression determination by quantitative reverse transcriptase polymerase chain reaction (PCR)

Gene expression was assayed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). mRNA extracted from samples (section 2.6.1) was first converted to cDNA by reverse transcription (section 2.6.3). Polymerase chain reaction (PCR), of the newly synthesised cDNA, was then undertaken, using primers specifically designed to the region of interest (section 2.6.4). In comparison to standard PCR, where the product of the reaction is detected at the end *e.g.* by ethidium bromide agarose gel, real time PCR is made quantifiable by incorporating fluorescent markers into the reaction. As the reaction progresses a detectable signal, proportional to the number of amplicons formed, is generated; amplified DNA can therefore be detected as the reaction progresses in "real time". Samples which initially had a higher number of mRNA transcripts (higher gene expression), will produce more amplicons and therefore more detectable signal, after a given number of PCR cycles.

There are two main methods used in quantitative PCR. The first uses a dye (SYBR® green) which binds to dsDNA; SYBR® dye-dsDNA complexes emit quantifiable light. A caveat with this method is that SYBR® green will bind to any dsDNA present; it is therefore important to control for non-specific PCR products, double stranded DNA contamination in the PCR assay mix and primer dimers. Alternatively, amplicon-specific DNA probes consisting of an oligonucleotide sequence that is labelled with a fluorophore marker and quencher can be used (*e.g.* Taqman® probe). While the probe is intact, fluorescence emitted by the fluorophore (after laser excitation) is quenched by the quencher molecule, by fluorescence resonance energy transfer (FRET). However during PCR extension, when *Taq* polymerase copies the (cDNA) template from 3' to 5', the 5' exonuclease activity of *Taq* polymerase hydrolyses the sequence-bound probe. Degradation of the probe releases the fluorophore from the locality of the quencher, thus releasing the emission of a detectable fluorescence upon excitation. The fluorescence detected in quantitative PCR is proportional to the fluorophore released and the amount of cDNA template initially present in the PCR reaction.

In quantitative PCR studies, using either of the above methods, the Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal/PCR amplification curve to cross the threshold (*i.e.* exceed background level). Ct values are inversely proportional to the amount of target nucleic acid in the sample *i.e.* the greater the

amount of starting cDNA template (which reflects gene expression) the lower the Ct value.

2.6.1. RNA extraction

All water used in RNA work was nuclease-free (Integrated DNA Technologies, Coralville, Iowa, USA). Contamination from RNAses was minimised by careful technique, use of RNAse-free plasticware, regular changing of gloves and cleaning all equipment and work surfaces routinely with RNAseZap™ (Sigma-Aldrich, Gillingham, UK).

For mRNA analysis, NHEKs were seeded and treated as per section 2.3 and, unless otherwise stated, harvested at 6h post-stimulation for RNA. RNA extraction was undertaken using the ReliaPrep™ RNA Cell Miniprep System (Promega, Wisconsin, USA) according to the manufacturer's protocol. The standard protocol supplied with the ReliaPrep™ RNA Cell Miniprep System was followed as below:

i. To each well of a 12-well plate, 150µl BL+TG buffer (Promega, Wisconsin, USA) was added (or 250µl to each well of a 6-well plate) and pipetted up and down 5 times to lyse cells.
ii. The lysate was transferred to a ReliaPrep™ minicolumn and centrifuged at 14000*g* for 30secs at room temperature.
iii. 500µl of RNA Wash Solution (Promega, Wisconsin, USA) was added to the minicolumn and centrifuged at 14000*g* for 30secs.
iv. To each minicolumn membrane the following was added: 24µl of Yellow Core Buffer (Promega, Wisconsin, USA), 3µl 0.09M $MgCl_2$ and 3µl of DNase I enzyme and incubated for 15mins at room temperature.
v. 200µl of column wash solution (Promega, Wisconsin, USA) was added and centrifuged at 14000*g* for 15secs.
vi. 500µl of RNA wash solution was added and centrifuged at 14000*g* for 30secs.
vii. The minicolumn was placed into a new collection tube and 300µl of RNA wash solution added and centrifuged at 14000*g* for 2mins.
viii. The minicolumn was transferred to an elution tube, and nuclease-free water added directly onto the membrane (20µl for RNA from a 12-well plate, 30µl for RNA from a 6-well plate) and incubated for 1min.
ix. The minicolumn was centrifuged at 14000*g* for 1min to elute RNA.

2.6.2. Nucleic acid quantification

The concentration of nucleic acid solutions can be determined spectrophotometrically at 260nm, where 1 absorbance unit (A260) equals 40µg of single-stranded RNA/ml. Nucleic acid purity was estimated by spectrophotometry from the relative absorbances at 230, 260 and 280nm. Proteins have a peak absorbance at 280nm and an A_{260}/A_{280} ratio of 1.8-2.0 suggests that protein contamination is relatively low. Phenol, Ethylenediaminetetraacetic acid (EDTA) and carbohydrates and have peak absorbance near 230nm and an A_{260}/A_{230} ratio of 2.0-2.2 suggests that contamination from these is relatively low. After extraction, RNA yield was quantified and purity determined using a NanoDrop 2000 Spectrophotometer (ThermoScientific, Illinois USA) with 1µl sample and referenced to a blank sample of nuclease-free water, without nucleic acid.

2.6.3. cDNA preparation

Reverse transcription was performed using SuperScript™ III Reverse Transcriptase/First-strand synthesis system (Invitrogen, Paisley, UK) according to the manufacturer's protocol, using a standardised amount of RNA in each reaction (Table 2-1). Where possible "master cocktails" were made up to minimise variations in volumes of components. Reactions mixes were sealed and briefly centrifuged before being transferred to an Applied Biosystems GeneAmp® PCR System 9700 Machine (Invitrogen, Paisley, UK) using standard PCR cycle conditions as per Table 2-1:

Process	Reagents	Vol. per reaction (µl)	Temp (^{o}C)	Time (mins)
Denature	Total RNA (up to 500ng) 100µM random primers 10mM dNTP mix Water	10 (max) 1 1 to 12	65 4	5 1
Anneal	To RNA/primer mix add: 5X First-strand buffer 0.1M DTT RNaseOUT^{TM} (40 U/µl) M-MLV RT (200 U/µl) Water	 4 2 0.125 0.5 to 20	25	10
cDNA synthesis			37	50
Inactivation of reaction			70	15

Table 2-1: Summary of reverse transcription/cDNA preparation, prior to real time polymerase chain reaction

2.6.4. Real time PCR

All primer/probes sets used were TaqMan® Gene Expression Assay (Invitrogen, Paisley, UK); except IL-6, Brd2/3/4 and RPL13A primer/probes (sequences and optimal running concentrations kindly supplied by Dr N.R. Harker, GlaxoSmithKline, Stevenage, UK) which were synthesised by Integrated DNA Technologies (Coralville, Iowa, USA) (Table 2-2). Primers used span exon-exon junctions; complementary sequences to exon-spanning primers only appear in cDNA and not in (contaminating) genomic DNA, therefore only cDNA is amplified. RPL13A was selected as a reference/housekeeper gene for "loading control" as this gene has previously been identified as a suitable reference/housekeeper gene in human keratinocytes (Minner and Poumay, 2009). Furthermore, RPL13A demonstrated the least variation in cycle threshold values determined by qRT-PCR (section 2.6.5) in samples treated with TNFα + IL-17 stimulation as well as I-BET151, compared to other candidates assayed *e.g.*

18S, keratin14, and histone H4. Negative control wells (containing no DNA) were used for each primer/probe to ensure the system was free of DNA contamination

Real-time quantitative polymerase chain reaction (qPCR) was performed with GoTaq Probe qPCR Master Mix (Promega, Wisconsin, USA) according to the manufacturer's protocol, adjusted to a 10µl reaction with a standardised amount of cDNA in each reaction (typically ~200ng). "Master cocktails" were made up to minimise variations in volumes of PCR buffer, probe and primers in each well. Reaction were set up in optical 96-well plates (Invitrogen, Paisley, UK) on a pre-chilled rack, sealed and briefly centrifuged before being transferred to an Applied Biosystems Step One Plus Machine (Invitrogen, Paisley, UK) using standard PCR cycle conditions as follows:

1. 50^{o}C for 2mins
2. 95^{o}C for 10mins
3. 95^{o}C for 15secs
4. 60^{o}C for 60secs
5. Plate read
6. Repeat steps 3-5 x 40 times

In the conditions above, DNA polymerase was activated by incubation at 95^{o}C for 10mins, followed by 40 cycles of denaturation at 95^{o}C for 15secs and annealing/extension at 60^{o}C for 60secs. Heating DNA to 95^{o}C denatures the DNA allowing the two strands to separate and cooling to 60^{o}C allows primers to anneal to complementary strands. This process was repeated 40 times.

2.6.5. Analysis of real time PCR

A cycle threshold (Ct) value (section 2.6) was measured by qRT-PCR for each reaction, and RPL13A was used as a "housekeeper gene/loading control" to calculate a fold change correction for each sample. After correction of the "housekeeper gene/loading control", the Delta-Delta Ct method was used for analysis (*i.e.* the difference between the two Ct values) (Livak and Schmittgen, 2001). The difference was expressed as a fold change (equivalent to $2^{\text{Delta-Delta Ct}}$).

Target	Assay ID/sequence	Concentration used in qPCR
IL-8	Hs00174103_m1 (Applied Biosciences) (FAM/NFQ-MGB)	Primers: 900nM Probe: 100nM
ADH1A	Hs00605167_g1 (Applied Biosciences) (FAM/NFQ-MGB)	Primers: 900nM Probe: 100nM
ADH1B	Hs00605175_m1 (Applied Biosciences) (FAM/NFQ-MGB)	Primers: 900nM Probe: 100nM
ADH1C	Hs02383872_s1 (Applied Biosciences) (FAM/NFQ-MGB)	Primers: 900nM Probe: 100nM
ALDH1	Hs00946916_m1 (Applied Biosciences) (FAM/NFQ-MGB)	Primers: 900nM Probe: 100nM
ALDH2	Hs01007998_m1 (Applied Biosciences) (FAM/NFQ-MGB)	Primers: 900nM Probe: 100nM
ACSS1	Hs00287264_m1 (Applied Biosciences) (FAM/NFQ-MGB)	Primers: 900nM Probe: 100nM
ACSS2	Hs00218766_m1 (Applied Biosciences) (FAM/NFQ-MGB)	Primers: 900nM Probe: 100nM
IL-6	Forward: AATGGAAAGTGGCTATGCAG Reverse: GTTAGCCATTTATTTGAGGTAAGC Probe: TGTTTCAGAGCCAGATCATTTCTTGGA (FAM/TAMRA)	Forward: 900nM Reverse: 400nM Probe:100nM
Brd2	Forward: GAAGCTGGGCCGAGTTGT Reverse: CGTAGGCAGGAAAGGACATAG Probe: CCAAGCCAGGGAGCCCTCTTTACGTGA (FAM/TAMRA)	Forward: 400nM Reverse: 900nM Probe:100nM
Brd3	Forward: AGCAGCTCCTCCGAGTCTGG Reverse: TCCGAAGCCAGTTCATTCTGAG Probe: AGCGGGTCCAGCTCTGACAGCAGTGA (FAM/TAMRA)	Forward: 900nM Reverse: 900nM Probe:100nM
Brd4	Forward: GCAGCTGAGGATTGCAGAGC Reverse: GCTCGTAACAAGGCGTGTGC Probe: CTTACGGCCAGTCGGACAG (FAM/TAMRA)	Forward: 900nM Reverse: 900nM Probe:100nM
RPL13A	Forward: GGGACTGCAGGTGGTGAC Reverse: GGCCTCAGATGGTAGTGCAT Probe: AGTTTCCCGACCATGAGATG (FAM/TAMRA)	Forward: 900nM Reverse: 900nM Probe:100nM

Table 2-2: Primer/probe sets used in real time polymerase chain reactions

All primer/probe sets were TaqMan® Gene Expression Assay (Invitrogen, Paisley, UK); except IL-6, Brd2/3/4 and RPL13A (sequences and optimal running concentrations kindly supplied by Dr N.R. Harker, GlaxoSmithKline, Stevenage, UK) which were synthesised by Integrated DNA Technologies (Coralville, Iowa, USA). All probes contained a FAM reporter dye and a non-fluorescent quencher.

2.7. Dual-luciferase assays

NF-κB activity after stimulation with TNFα and/or IL-17 was measured using a NF-κB firefly luciferase reporter (gift from Dr D J McKean, Mayo foundation, Rochester, MIN, USA). Keratinocytes were seeded into a 48-well plate at a density of 16000 cells/well. 24h later, when ~60% confluent, cells were transfected with 0.2μg NF-κB firefly construct and 0.005μg renilla using TransIT-Keratinocyte specific transfection reagent (Geneflow, Litchfield, UK), according to manufacturer's instructions, with renilla as an

internal control vector (Flockhart *et al.*, 2008). The next day, cells were stimulated with TNFα (10ng/ml) and/or IL-17 (100ng/ml). At the times indicated for each experiment post-stimulation, cells were harvested using 40µl passive lysis buffer (Promega, Wisconsin, USA), incubating at room temperature on an orbital shaker for 15mins then stored at -80^{o}C. Luciferase activity was measured using Dual-luciferase reporter system (Promega, Wisconsin, USA) following the manufacturer's protocol. Briefly, 20µl of defrosted lysate was added to a blacked-out 96-well plate suitable for luminescence assays. Using a Glomax 96 Microplate reader (Promega, Wisconsin, USA), 60µl LAR and 60µl S+G was added to each well using two injectors built into the luminometer. Values were presented as a ratio of firefly to renilla, then normalised to mock/unstimulated control.

2.8. Gene array

2.8.1. Study design

The following conditions were considered to be the most valuable in order to understand the effects of i) TNFα + IL-17 stimulation in keratinocytes and ii) BET inhibition in this system:

1. Mock/unstimulated (*i.e.* media change)
2. TNFα (10ng/ml) + IL-17 (100ng/ml)
3. TNFα (10ng/ml) + IL-17 (100ng/ml) + I-BET151 vehicle (DMSO (1:1200))
4. TNFα (10ng/ml) + IL-17 (100ng/ml) + I-BET151 (1µM)

A number of valuable comparisons could be considered. For example, comparison of 1 vs. 2 would determine which genes were regulated by TNFα + IL-17 stimulation in the model. The set of differentially expressed genes (DEGs) could then be compared to a psoriasis transcriptome to determine the overlap and understand their relevance to psoriasis. 2 vs. 3 would determine which genes, in TNFα + IL-17 stimulated NHEKs, were affected by DMSO and 4 vs. 3 would determine which genes, in TNFα + IL-17 stimulated NHEKs, were affected by I-BET151. I-BET151 sensitive genes could in turn be compared to a psoriasis disease set of genes and a variety of psoriasis-treatment transcriptomes, respectively.

As we were interested in early/primary response genes and IL-6 and IL-8 mRNA responses to TNFα + IL-17 stimulation were maximal at 6h post-stimulation (section

3.3.2), it was decided to stimulate NHEKs with TNFα + IL-17 for 6h prior to harvesting cells for mRNA/microarray analysis. However, late response genes may not show changes as early as 6h. Furthermore, Chiricozzi *et al*. had chosen a timepoint of 24h after stimulation with TNFα + IL-17, in studying the effects of these stimuli on inflammatory responses in human keratinocytes (Chiricozzi *et al.*, 2011). For these reasons, it was decided that in addition to a 6h timepoint, NHEKs would also be stimulated under the above conditions for 24h in order to study effects on late gene response, to allow for easier comparison to published data set (Chiricozzi *et al.*, 2011) and also to understand dynamic gene expression changes in responses at 6h vs. 24h. Such data may also allow clustering of distinct response patterns. Thus there were 4 treatment conditions, each at 2 timepoints for each donor.

Power calculations were undertaken in collaboration with Dr. Erika Cule (GlaxoSmithKline, Stevenage, UK), to determine the number of donors required to detect statistically significant changes in gene expression in response to TNFα + IL-17 stimulation and also inhibition by I-BET151. Power calculations were based on pilot qRT-PCR data (N=8) for IL-6 and IL-8 in the TNFα (10ng/ml) + IL-17 (100ng/ml) stimulated human keratinocyte model, and also considering the effects of I-BET151 (1μM) on these endpoints. Based on this analysis, it was calculated that, for example, 5 donors would have 80% statistical power (with type-I error of 5%) to detect a ~6-fold change in gene expression in stimulated compared to unstimulated NHEKs and ~85% inhibition by I-BET151, based on variability in IL-6 mRNA responses across donors (IL-8 showed similar variability to IL-6). In comparison, 20 donors would have 80% power (with type-I error of 5%) to detect a ~2-fold change in gene expression in stimulated compared to unstimulated NHEKs and ~50% inhibition by I-BET151, based on variability in IL-6 mRNA responses across donors.

The final consideration was the microarray platform and provider. After cost effectiveness analysis, it was decided that the Illumina human HT-12 v4.0 microarray platform (Illumina, San Diego, California, USA), outsourced to Central Biotechnology Services, Cardiff University, Cardiff, UK (http://centralbioserv.co.uk/) would provide the most cost effective and reliable service. Each Illumina human HT-12 v4.0 BeadChip allowed 12 samples to be run; although empty runs are wasted.

In summary it was decided that the 4 treatment conditions, each at 2 timepoints would be run in 12 donors which, based on power calculation from pilot data, would have 80%

power (with type-I error of 5%) to detect a ~2.85-fold change in gene expression after stimulation with TNFα + IL-17 and ~60% inhibition by I-BET151, based on variability in IL-6 mRNA responses across donors. These power calculations were based solely on variability in IL-6 and IL-8 responses (which both demonstrated similar variability), with RPL13A as a reference/loading control. If other gene responses show greater variability, the power to detect significant changes will be reduced for these genes.

2.8.2. Sample preparation

For microarray studies, NHEKs were seeded in 6-well plates at a density of 150,000 cells/well in 2ml Epilife® media. 24h later, when NHEKs were approximately 80% confluent, cells were stimulated as follows:

1. Mock/unstimulated (*i.e.* media change), 6h stimulation
2. TNFα (10ng/ml) + IL-17 (100ng/ml), 6h stimulation
3. TNFα (10ng/ml) + IL-17 (100ng/ml) + I-BET151 vehicle (DMSO (1:1200)), 6h stimulation
4. TNFα (10ng/ml) + IL-17 (100ng/ml) + I-BET151 (1μM), 6h stimulation
5. Mock/unstimulated (*i.e.* media change), 24h stimulation
6. TNFα (10ng/ml) + IL-17 (100ng/ml), 24h stimulation
7. TNFα (10ng/ml) + IL-17 (100ng/ml) + I-BET151 vehicle (DMSO (1:1200)), 24h stimulation
8. TNFα (10ng/ml) + IL-17 (100ng/ml) + I-BET151 (1μM), 24h stimulation

Note that stimulation times were staggered so that conditions 5-8 were set up first and 18h later conditions 1-4 were set up. Cells from all conditions were therefore harvested at the end of the experiment, at a single timepoint. This was to ensure a similar number of cells were harvested for each condition and to minimise any differences in harvesting of cells. Harvested cells from each donor were "snap frozen" in liquid nitrogen and stored at -80^{o}C. Once all donors were stimulated and cells harvested, RNA was extracted from all donors over a 2-day period (section 2.6, eluting into 30μl of nuclease-free water), in order to minimise variations in handling. In total NHEKs from 14 consecutive donors were stimulated (over a period of approximately 3 months, each at passage 3) and harvested for RNA. qRT-PCR for IL-6 and IL-8 were undertaken

(section 2.6) for each of the 14 donors and their 8 conditions. 12 of the 14 donors were selected for microarray analysis (section 6.3.1).

2.8.3. Array processing on Illumina human HT-12 v4.0 microarray platform

To avoid unnecessary freeze/thawing of samples, 2 aliquots of each sample of RNA (at a concentration of 100ng/μl, in nuclease free water) was transferred on dry ice by Biocair (Cambridge, UK) to Central Biotechnology Services (Cardiff University, UK). One aliquot (containing 2μl of RNA) was used for analysis RNA quality and verify quantity and the other aliquot (containing 5μl of RNA) was used for microarray analysis on an Illumina human HT-12 v4.0 microarray platform (Illumina, San Diego, California, USA). RNA quality and quantity was analysed using Agilent RNA 6000 Nano kit (Agilent, Santa Clara, California, USA) on an Agilent on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, California, USA), using 1μl of sample; 4 of the 96 samples had (RNA integrity number) RIN values between 8.6 - 8.9, all remaining samples had RIN values which were above 9. RIN values are a measure of RNA degradation and range between 1 - 10, where 10 indicates completely intact RNA. RIN values above 7 are recommended for microarrays, but values above 8 are preferable; therefore all 96 samples had RIN values suitable for microarray analysis. 100ng of RNA per sample was labelled using Ambion Illumina TotalPrep-96 RNA Amplification Kit (Invitrogen, Paisley, UK) and resulting cRNA hybridised onto one of eight Illumina human HT-12 v4.0 BeadChip microarray platforms. The Illumina human HT-12 v4.0 microarray platform contains over 47,000 probes/oligonucleotides, coated onto beads which are mapped onto an array surface and each probe is represented, typically on over 30 beads (http://www.illumina.com/products/humanht_12_expression_beadchip_kits_v4.html). After hybridisation, washing and staining steps, BeadChips were scanned using iScan system (Illumina, San Diego, California, USA).

Note samples were randomised at Newcastle University prior to shipment to Central Biotechnology Services, who were blinded to the randomisation. Samples from different conditions and different donors were therefore allocated at random to different BeadChips to minimise any sampling differences due to RNA labelling, BeadChip or date of BeadChip hybridisation/analysis (Central Biotechnology Services advise that this was performed on 2 separate days).

2.8.4. Array analysis

After BeadChips were scanned, data was imported by Central Biotechnology Services into GenomeStudio® (Illumina, San Diego, California, USA), background correction was applied and values for missing probes imputed. Output intensity data files (.idat files) were transferred to Newcastle University for further analysis. Microarray analysis was performed in collaboration with Dr. Simon Cockell (Bioinformatics Support Unit, Newcastle University, UK)

Expression values were derived from SampleProbeProfile.txt. files imported in R Software using the lumi package. Expression values were initially log transformed (log base 2) using a Variance Stabilising Transformation (Lin *et al.*, 2008) which takes advantage of the technical replication present in the Illumina human HT-12 v4.0 microarray platform. Data was then normalised using the Robust Spline Normalisation algorithm designed for BeadChip data (Du *et al.*, 2008). Three different methods were used for the quality assessment of arrays. Firstly, the mean absolute difference of each array from all other arrays was considered - a threshold for outlier detection was determined from the distribution of mean absolute differences. Secondly, the principal component analysis was considered for outlier detection - outliers being determined by visual inspection and expert judgement. Finally, the array intensity distribution was assessed - an array will be marked as an outlier by this method if the distribution tests are significantly different from the pooled intensity distribution of the other arrays (by the Kolmogorov-Smirnov statistic).

After quality control, a number of initial analyses were performed in R software, *e.g.* principle component analysis and hierarchical clustering. These analyses revealed variance between samples was primarily due to the effect of I-BET151, although effects of TNFα + IL-17 stimulation and donor were also apparent. Following these initially analyses and decisions on cut off values for fold change and p value (section 6.3.1), comparisons were made, in R software, between the following groups of conditions (section 6.3):

i. TNFα + IL-17 stimulation compared to mock stimulation at 6h
   i.e. condition 1 vs. 2 (section 2.8.2)
ii. TNFα + IL-17 stimulation compared to mock stimulation at 24h
   i.e. condition 5 vs. 6 (section 2.8.2)

iii. TNFα + IL-17 compared to TNFα + IL-17 + DMSO at 6h
i.e. condition 2 vs. 3 (section 2.8.2)

iv. TNFα + IL-17 compared to TNFα + IL-17 + DMSO at 24h
i.e. condition 6 vs. 7 (section 2.8.2)

v. TNFα + IL-17 + I-BET151 compared to TNFα + IL-17 + DMSO at 6h
i.e. condition 3 vs. 4 (section 2.8.2)

vi. TNFα + IL-17 + I-BET151 compared to TNFα + IL-17 + DMSO at 24h
i.e. condition 7 vs. 8 (section 2.8.2)

vii. Comparison between i. and ii

viii. Comparison between v. and vi.

ix. Comparison between vii. and viii

x. A previously published data set of the effects of TNFα + IL-17 stimulation in NHEKs (Chiricozzi *et al.*, 2011) was compared to ii.

xi. A previously published meta-analysis of psoriasis transcriptomic studies (Tian *et al.*, 2012) was also compared i., ii, v., v. and ix.

Lists of differentially expressed genes (DEGs) derived from the above analyses were further interrogated using Ingenuity® Pathway Analysis (IPA) Summer 2015 release software (Qiagen, Hilden, Germany). IPA enables analysis of gene-to-gene interactions by creating networks which link DEGs by identifying relationships and relevant connections between genes *e.g.* upstream signalling pathways, targets of kinases *etc*. This allows DEGs to be grouped into pathways, diseases or biological functions, for example, allowing the biological relevance of changes in gene expression to be better understood. IPA also enables predictions of upstream regulators or downstream effects of DEGs to be made. DEGs were also compared to a meta-analysis of psoriasis transcriptomic datasets (Tian *et al.*, 2012) and an IPA-defined psoriasis network. The information within IPA is derived from data in primary literature, in *e.g.* journal articles, review articles and textbooks, as well as publicly available databases. Furthermore as the information in IPA is manually curated, analysis in IPA can give reliable meaning to DEGs.

2.9. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) is a method used to study interactions between specific proteins of interest (*e.g.* particular transcription factors or modified histones) and genomic DNA. The method involves several steps:

i. Cross-linking of proteins to DNA (this step is excluded in "native" ChIP) (section 2.9.2).
ii. Lysis of cells and shearing of chromatin into smaller fragments (section 2.9.3).
iii. Selective enrichment of chromatin, using an antibody to a particular protein of interest (sections 2.9.4 and 2.9.5).
iv. Purification and identification of immunoprecipitated DNA fragments *e.g.* by sequencing or PCR using primers designed to particular DNA regions of interest. Enrichment of a particular region of DNA reflects the extent to which this region was associated with the protein of interest (sections 2.9.6 and 2.9.7).

The following ChIP protocol was kindly supplied by Dr N.R. Harker (GlaxoSmithKline, Stevenage, UK) then optimised for use in keratinocytes.

2.9.1. Stimulation of cells

A) For ChIP experiments investigating binding of BET proteins to gene regulatory regions, NHEKs were grown in T175 culture flasks and, when ~90% confluent, stimulated as follows, timepoints were chosen based on maximal IL-6 and IL-8 mRNA expression (section 3.3.2):
   1) TNFα (10ng/ml) + IL-17 (100ng/ml) + DMSO (1:1200) (stimulated for 3h)
   2) TNFα (10ng/ml) + IL-17 (100ng/ml) + I-BET151 (3μM) (stimulated for 3h)
   3) TNFα (10ng/ml) + IL-17 (100ng/ml) + DMSO (1:1200) (stimulated for 4.5h)
   4) TNFα (10ng/ml) + IL-17 (100ng/ml) + I-BET151 (3μM) (stimulated for 4.5h)
   5) TNFα (10ng/ml) + IL-17 (100ng/ml) + DMSO (1:1200) (stimulated for 6h)
   6) TNFα (10ng/ml) + IL-17 (100ng/ml) + I-BET151 (3μM) (stimulated for 6h)

   NB: As indicated, DMSO or I-BET151 was added to cells at the same time as stimulation with TNFα + IL-17.

B) For ChIP experiments investigating effects of TNFα + IL-17 stimulation and pre-treatment with acetate, NHEKs were grown in T175 culture flasks with or without 1mM acetate for 7 days (section 2.2.3), when ~90% confluent, before being stimulated as follows, timepoints were chosen based on maximal IL-6 and IL-8 mRNA expression (section 3.3.2):

1) Media, mock/unstimulated
2) Media + 1mM acetate, mock/unstimulated
3) Media + TNFα (100ng/ml) + IL-17 (100ng/ml) (stimulated for 1h)
4) Media + 1mM acetate + TNFα (100ng/ml) + IL-17 (100ng/ml) (stimulated for 1h)
5) Media + TNFα (100ng/ml) + IL-17 (100ng/ml) (stimulated for 4h)
6) Media + 1mM acetate + TNFα (100ng/ml) + IL-17 (100ng/ml) (stimulated for 4h)

NB: In conditions where NHEKs were cultured with 1mM acetate for 7 days prior to stimulation, subsequent stimulation was also performed in 1mM acetate/media (*i.e.* conditions B2, B4 and B6 above). See section 2.2.3 for further details.

Note that stimulations were performed in a total volume of 15ml and stimulation times were staggered so that conditions with the longest stimulation times were stimulated first (including the mock/unstimulated control) followed by conditions with shorter periods of stimulation. For each experiment, cells from all conditions were therefore fixed and harvested at the end of the experiment, at a single timepoint. This was to ensure a similar number of cells were harvested for each condition and to minimise any differences in fixation and harvesting of cells (section 2.9.2). $1x10^6$ keratinocytes was used in each immunoprecipitation (section 2.9.4). A T175 culture flask contains approximately $5x10^6$ keratinocytes, therefore the number of T175 flasks stimulated was increased accordingly, to accommodate for the number of immunoprecipitations.

2.9.2. Fixation

After stimulation for the indicated times, 1/10th volume (1.67ml) fix solution (10% formaldehyde, 0.1M NaCl, 1mM EDTA, 0.5mM EGTA, 10mM Tris-HCl pH8) was added drop wise to the flask and incubated for 10mins at room temperature. Fixation

was terminated by the addition of 875µl glycine (125mM final concentration) and incubated at room temperature for 5mins. Cells were scraped and pelleted by centrifuging at 1200rpm at 4°C for 5mins. The pellet was washed twice with ice cold PBS, centrifuging at 1200rpm at 4°C for 5 minutes after each wash. The cell pellet was "snap frozen" in liquid nitrogen and stored at -80°C. Cell pellets were transferred on dry ice by Biocair (Cambridge, UK) to GlaxoSmithKline, Stevenage, UK, where the remainder of the ChIP protocol and qPCR was performed.

2.9.3. Sonication

The cell pellets were defrosted on ice and resuspended in 100µl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8, protease inhibitor cocktail (Roche Diagnostics, Burgess Hill, UK) per $1x10^6$ NHEKs, incubating on ice for 10mins with intermittent mixing. Cell lysate were aliquoted into pre-chilled polystyrene sonication tubes (Active Motif, Carlsbad, California, USA) and sonicated for 15mins (30secs on and 30secs off, on high power) using Bioruptor®Pico waterbath sonicator (Diagenode, Seraing, Belgium). Cell debris was removed by centrifugation (8°C, 10mins at 13000rpm). As primary keratinocytes from different donors had previously shown variable responses to sonication, 25µl of test sample was assayed to ensure adequacy of sonication. This sample was eluted, cross-links reverse and DNA was purified (section 2.9.6) and run on a 2% agarose gel (Invitrogen, Paisley, UK) to ensure the majority of DNA was sonicated to 200-500bp. If sonication was inadequate, sonication was repeated on all conditions from that donor (further sonication time was dependent on the efficiency of initial sonication). A further 25µl of test sample was assayed to ensure adequacy of any further sonication.

2.9.4. Incubation with antibody

Chromatin was aliquoted into 100µl aliquots ($1x10^6$ cells) and diluted 1:10 with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH 8, 167mM NaCl, protease inhibitor cocktail). Samples were incubated at 4^oC with rotation overnight with 5µg antibody (Table 2-3 and Table 2-4). IgG was selected as a negative control and α-pan H3 a positive control. 25µl of chromatin (250,000 cells) was reserved as input and stored at -20°C.

Antibody	Company/Cat No.	Lot and conc^{n}	Vol for 5ug
Rabbit IgG	Abcam ab37415	GR164350-1 5mg/ml	1ul
Rabbit α-pan H3	Abcam ab1791	GR125634-1 1mg/ml	5ul
Rabbit α-Pol II S2P	Abcam Ab5095	GR154006-2 1mg/ml	5ul
Rabbit α-Brd2	Bethyl A302-583A	A302-583A-1 0.2mg/ml	25ul
Rabbit α-Brd3	Bethyl A302-368A	A302-368A-1 0.2mg/ml	25ul
Rabbit α-Brd4	Bethyl A301-985A	A301-985A-1 1mg/ml	5ul
Rabbit α-NF-κBp65	Abcam7970	GR150309-4 0.2mg/ml	25ul

Table 2-3: Antibodies used in chromatin immunoprecipitation studies, section 2.9.1A

Antibody	Company/Cat No.	Lot and conc^{n}	Vol for 5ug
Rabbit IgG	Abcam ab37415	GR164350-1 5mg/ml	1ul
Rabbit α-pan H3	Abcam ab1791	GR182539-1 1mg/ml	5ul
Rabbit α-pol II (S2P)	Abcam Ab5095	GR154006-2 1mg/ml	5ul
Rabbit α-Acetyl H3	Millipore 06-599	2430399 1mg/ml	5ul
Rabbit α-H3K9ac	Abcam Ab4441	GR196840-1 1mg/ml	5ul
Rabbit α-H3K27ac	Abcam Ab4729	GR184557-1 1mg/ml	5ul
Rabbit α-H3K4me3	Abcam Ab8580	GR188707-1 1mg/ml	5ul
Rabbit α-H3K4me1	Millipore ABE1353	Q2464806 1.5mg/ml	3.3ul
Rabbit α-H3K27me3	Abcam ab108245	161273-2 1mg/ml	5ul

Table 2-4: Antibodies used in chromatin immunoprecipitation studies, section 2.9.1B

2.9.5. Immunoprecipitation and wash

Protein A + G magnetic beads (Millipore, Massachusetts, USA and Invitrogen, Paisley, UK) were washed twice in dilution buffer (section 2.9.4) and resuspended in their original volume. 20µl Protein A + G magnetic beads were added to each sample and incubated with rotation for 5h at 4°C. Samples were washed once in low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150mM NaCl, 20 mM Tris-HCl pH 8), once in high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500mM NaCl, 20 mM Tris-HCl pH 8), once in lithium chloride buffer (0.25M LiCl, 1% NP40, 1% Na Deoxycholate, 1mM EDTA, 10mM Tris-HCl pH8) and twice in Tris-EDTA buffer (10mM Tris-HCl pH8, 1mM EDTA). All washes were performed at room temperature. After the final wash, samples were centrifuged at 13000rpm for 30secs and remaining traces of Tris-EDTA buffer removed prior to addition of elution buffer, below.

2.9.6. Elution, reversal of cross links and DNA purification

Chromatin bound to protein A + G magnetic beads were resuspended in 100µl elution buffer (1% SDS, 100mM $NaHCO_3$) and incubated at RT with rotation for 15mins. Eluted chromatin was removed, and the beads were incubated for 15mins with a further 100µl elution buffer, then 65°C for 5mins. The two eluates were then combined. 8µl 5M NaCl was added to the combined eluates and incubated at 65°C overnight to reverse protein-DNA cross-links. The samples were then incubated with 0.25µl 20mg/ml RNaseA for 1h at 37°C then with 4µl 0.5M EDTA, 8µl 1M Tris-HCl pH6.5 (MerckMillipore, Massachusetts, USA) and 0.4µl 20mg/ml Proteinase K (Invitrogen, Paisley, UK) for 1h at 60°C. The DNA was purified using Zymo ChIP DNA Clean & Concentrator Kit (Cambridge Bioscience, Cambridge, UK). Following the manufacturer's instructions, 1ml DNA binding buffer was added to each sample, mixed and the samples loaded onto the purification columns in 2 batches with a 13000 rpm 30secs centrifugation in between. The columns were washed twice with 200µl DNA wash buffer with a 13000 rpm 30secs centrifugation in between. 100µl of elution buffer was added directly onto the membrane of each column and after 1min incubation at room temperature, the column was centrifuged at 13000rpm for 1min to elute DNA. Samples were stored at -20°C.

2.9.7. Real time PCR

Real-time PCR (qPCR) was undertaken using specifically designed primers to the IL-6 promoter, IL-8 promoter and to a region previously described as an IL-6 enhancer in macrophages (Table 2-5). All primers were optimised for SYBR-based qPCR using genomic standards (1 – $1x10^5$ copies). qPCR was performed, in triplicate technical repeat, in 10µl reactions each containing the following: 3µl DNA template or human genomic standard (1 – $1x10^5$ copies) (Promega, Wisconsin, USA), 5µl SYBR green master mix (Invitrogen, Paisley, UK), 0.1µl forward primer, 0.1µl reverse primer and 1.8µl water, and run on an Applied Biosystems 9700HT Machine (Invitrogen, Paisley, UK) using standard PCR cycle conditions as follows:

1. 50°C for 2mins
2. 95°C for 10mins
3. 95°C for 15secs
4. 60°C for 60secs
5. Plate read
6. Repeat steps 3-5 x 40 times

Target	Assay ID/sequence	Concentrations
IL-6 Promoter (Qiao *et al.*, 2013)	Forward: ACCCTCACCCTCCAACAAAG Reverse: GCAGAATGAGCCTCAGACATC	Forward: 250nM Reverse: 250nM
IL-6 Enhancer (Qiao *et al.*, 2013)	Forward: GGACACACACAGGACTCTCACA Reverse: GAGGTAGATTTGGTTCTCCCATT	Forward: 250nM Reverse: 250nM
IL-8 Promoter (Costa *et al.*, 2013)	Forward: GGGCCATCAGTTGCAAATC Reverse: TTCCTTCCGGTGGTTTCTTC	Forward: 900nM Reverse: 900nM

Table 2-5: Sequences of real time primers used in chromatin immunoprecipitation qPCR studies

All primers were synthesised by Sigma-Aldrich (Gillingham, UK).

2.9.8. Analysis of chromatin immunoprecipitation real time PCR

Based on comparisons of Ct values from samples vs. genomic standards used to obtain a standard curve, the quantity of DNA following amplification was determined using Applied Biosystems SDS Software (Invitrogen, Paisley, UK) and a mean of the triplicate qPCR technical repeats was calculated, excluding any obvious outliers *e.g.* due to pipetting errors. Values from immunoprecipitated DNA samples were expressed as a percentage of values from non-immunoprecipitated input sample, which represents the amount of chromatin initially used in the ChIP, prior to immunoprecipitation with antibody. The calculated percentage input value from the negative control (IgG) was subtracted from the calculated percentage input values obtained using specific antibodies to proteins/histone modifications of interest. Enrichment of a particular region of DNA (*e.g.* IL-6 promoter) reflects the extent to which this region was associated with the protein or histone modification of interest.

2.10. Measurement of epigenetic writer and eraser activity

The balance between epigenetic writer and eraser activity regulates epigenetic histone modifications; in the case of lysine acetylation this balance is between histone acetyl transferase (HAT) and histone deacetylase (HDAC) activity. The effects of acetate on HAT and HDAC activity, in NHEKs nuclear extracts, were measured after exposure to 1mM acetate, or media alone, for 7 days.

2.10.1. Preparation and quantification of keratinocyte nuclear extracts

NHEKs were grown in T175 culture flasks with or without 1mM acetate for 7 days, reaching 90% confluency. Flasks were washed twice with ice-cold PBS, then scraped with a cell-scraper into of ice-cold PBS and transferred into 1.5ml microcentrifuge tube and pelleted by centrifugation at 500*g* for 3mins at 4^0C. Supernatant was removed and the cell pellet "snap-frozen" in liquid nitrogen and stored at -80^0C before nuclear extraction was performed using NE-PER® nuclear and cytoplasmic extraction reagents (CER) (ThermoScientific, Rockford, Illinois, USA) according to the manufacturer's instruction as follows:

1. 300µl ice-cold cytoplasmic extraction reagent (CER) I (ThermoScientific, Rockford, Illinois, USA) was added to the cell pellet.
2. The sample was vortexed for 15secs then incubated on ice for 10mins.

3. 16.5µl ice-cold CER II (ThermoScientific, Rockford, Illinois, USA) was added to the sample, then vortexed for 5secs, incubated on ice for 1min and vortexed again for 5secs before centrifugation for 5mins at 16000*g* at 4^0C.
4. The supernatant (cytoplasmic extract) was transferred to a clean pre-chilled tube, "snap-frozen" in liquid nitrogen and stored at -80^0C.
5. 150µl ice-cold nuclear extraction reagent (NER) (ThermoScientific, Rockford, Illinois, USA) was added to the cell pellet.
6. The sample was vortexed for 15secs then incubated on ice with further 15secs vortexing every 10mins for a total of 40mins before centrifugation for 10mins at 16000*g* at 4^0C.
7. The supernatant (nuclear extract) was transferred to a clean pre-chilled tube, "snap-frozen" in liquid nitrogen and stored at -80^0C.

Quantification of nuclear extracts was undertaken using Pierce® BCA protein assay kit (ThermoScientific, Rockford, Illinois, USA) according to the manufacturer's instructions, using 5µl of nuclear extract and incubating the samples and standards (each in triplicate repeat) at 37^0C for 30mins. Absorbance of samples were read at 562nm using a SpectraMax M250 plate reader (Molecular Devices, UK) and compared against the set of standard controls using a four parameter logistic (4-PL) curve-fit to determine nuclear extract concentrations.

2.10.2. Measurement of histone acetyl transferase enzyme activity

HAT activity was measured using HAT activity colorimetric assay kit (Sigma-Aldrich, Gillingham, UK) that detects acetylation of a substrate by HAT enzymes, using acetyl-coA as a substrate; which releases of co-A. Co-A acts as a co-enzyme for the production of NADH, which can be detected as a colour change after reaction with a tetrazolium dye. HAT activity, using 50µg of keratinocyte nuclear extract in 40µl water, was measured according to the manufacturer's protocol. Each sample also contained 50µl 2x HAT assay buffer, 5µl HAT substrate I, 5µl HAT substrate II and 8µl NADH generating enzyme and samples were incubated at 37^0C for 4h. Absorbance of samples was read at 440nm using a SpectraMax M250 plate reader (Molecular Devices, UK). Background absorbance reading (obtained by running the reaction with water but no nuclear extracts) was subtracted from samples readings.

2.10.3. Measurement of histone deacetylase enzyme activity

HDAC activity was measured using HDAC assay kit, colorimetric (Active Motif, Carlsbad, California USA) and HDAC assay kit, fluorescent (Active Motif, Carlsbad, California USA) that detects deacetylation of an acetylated lysine residue; in subsequent reactions the deacetylated lysine residue results in release of either i) a chromophore, which can be detected by a quantifiable colour change or ii) a fluorophore which can be detected by fluorescence, depending on the kit used. According to the manufacturer, the colorimetric kit measures total activity of HDAC 1,2,6,10,11, whereas the fluorescence kit measures total activity of HDAC 1,2,6,8,10,11.

For each of the protocols, 45µg of keratinocyte nuclear extract in 30µl water was used. Each reaction also contained 10µl HDAC assay buffer (for colorimetric or fluorescent kit) and 10µl HDAC substrate (for colorimetric or fluorescent kit). Samples were incubated at 37^0C for 1h, 50ul HDAC assay developing solution (for colorimetric or fluorescent kit) was then added and incubated at room temperature for 15mins. For the colorimetric assay, absorbance of samples was read at 440nm using a SpectraMax M250 plate reader (Molecular Devices, UK). For the fluorescent assay, fluorescence was read with an excitation wavelength of 360nm and emission wavelength of 460nm using a NanoDrop 2000 Spectrophotometer (ThermoScientific, Illinois USA). For both assays, background absorbance reading (obtained by running the reaction with water but no nuclear extracts) was subtracted from samples readings.

2.11. Statistical analysis

All initial analysis was carried out using Microsoft Excel (Microsoft Corporation, Redmond, Washington) and all statistical analysis was carried out using Prism 5.0 (GraphPad version 5.0 software, San Diego, CA).

Data is generally presented as the mean of data points ± SEM. N depicts the number of independent donors and n indicates the total number of independent data points (typically representing data obtained from independent wells). Thus N=3 indicates that the experiment was conducted in 3 independent donors. For all figures: *$p<0.05$, **$p<0.01$, ***$p<0.001$ and where results were analysed by two statistical methods (*e.g.* section 7.3.9), the second statistical anaylsis is shown as $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$; statisitical significance was taken at $p<0.05$, thus non-significant (ns) was $p>0.05$.

Data was tested for normal distribution before statistical analysis was performed. Where appropriate, results show pooled data from multiple donors presented as mean ± standard error of the mean (SEM). In experiments where there was large variability in absolute values between donors (*e.g.* of secreted cytokines), values were normalised to control (*e.g.* unstimulated) within each donor before data was pooled. This was done to prevent responses in some donors, which showed greater absolute values, masking the responses of other donors, which showed lower absolute values, if a mean of absolute values was calculated.

For I-BET151 concentration-response curves, results were expressed as % inhibition of (*e.g.* IL-6) calculated as follows:

$$\frac{\text{(IL-6 value for "TNF}\alpha\text{/IL-17 + vehicle") - (IL-6 value for "TNF}\alpha\text{/IL-17 + I-BET151 at given concentration")}}{\text{(IL-6 value for "TNF}\alpha\text{/IL-17 + vehicle") – (IL-6 value for "mock stimulated + vehicle")}} \text{ X 100}$$

"Best fit curve", IC_{50} and maximum inhibition were drawn/derived using 4-parameter logistic curve fit software, Prism 5.0 (GraphPad version 5.0 software, San Diego, CA). "Mean IC_{50}" was calculated as the mean of IC_{50} values from individual donors.

The effects of TNFα (10ng/ml) + IL-17 (100ng/ml) stimulation on the induction of IL-6 and IL-8, compared to stimulation with TNFα (10ng/ml) or IL-17 (100ng/ml) alone were classified by the following definitions (Chiricozzi *et al.*, 2011):

- **Synergistic effect:** the combined effect of interacting cytokines was greater than the sum of their individual effects.
- **Additive effect:** the combined effect of interacting cytokines was greater than the individual effect for each cytokine, but less than the sum of their individual effects.
- **Antagonistic effect:** the combined effect of interacting cytokines was less than the individual effect for either or both cytokines.

Using this method of analysis, synergy is defined as a greater than expected additive effect, and antagonism is defined as a less than expected additive effect.

Two-way ANOVAs were conducted when analysing the effect of two independent variables. An example within this project was the effect I-BET151 on BET protein recruitment at the IL-6 promoter at different timepoints (section 4.3.4). A two-way ANOVA is useful when one of the variables being compared is a repeated measure. The analysis therefore acknowledges that readings from different timepoints are not independent readings and therefore provides a more stringent statistical analysis between treatments, by taking into account measurements over several timepoints. A Bonferroni post-hoc test was then conducted to determine at which timepoint(s), during stimulation with TNFα + IL-17, I-BET151 treatment had a significant inhibitory effect on BET protein binding at the IL-6 promoter.

Chapter 3.

In vitro model of cutaneous inflammation

3. *In vitro* model of cutaneous inflammation

3.1. Introduction

There is considerable interest in developing *in vitro* models of cutaneous inflammation in order to further understand underlying mechanisms of inflammatory skin diseases such as psoriasis, and for pre-clinical evaluation of potential therapeutic compounds. This chapter focuses on the development of *in vitro* models of cutaneous inflammation, including the further development of an existing model in which normal human epidermal keratinocytes (NHEKs) were stimulated with a combination of TNFα + IL-17A (IL-17); this combination of cytokine stimulation was previously found to regulate expression of key keratinocyte genes central to the pathogenesis of psoriasis (Chiricozzi *et al.*, 2011).

Additionally, we investigated the effects of compounds used clinically to treat psoriasis in this *in vitro* model, in part to further validate the utility of the model for pre-clinical evaluation of potential therapeutic compounds.

3.2. Aims

- To develop *in vitro* model(s) of cutaneous inflammation for further understanding the pathogenesis of inflammatory skin disease and the role of epigenetics, including acetylation of lysine residues on histones, in the regulation of genes relevant to psoriasis.
- To evaluate the model(s) of cutaneous inflammation for *in-vitro*, pre-clinical, testing of potential therapeutic compounds, including those targeting epigenetic epitopes.

3.3. Results

3.3.1. Stimulation of keratinocytes with a combination of TNFα + IL-17 produced the most robust level of secreted IL-6 and IL-8, with no significant effect on cellular metabolism or total cellular protein

Initial data obtained during the MRes preceding this PhD project showed robust secretion of IL-6 and IL-8 by NHEKs in response to TNFα and/or IL-17 stimulation. Responses were dependent on TNFα concentrations up to 10ng/ml and the effect of stimulation with IL-17 was maximal at the lowest concentration tested of 100ng/ml. Maximal IL-6 and IL-8 responses were achieved with a combination of 10ng/ml TNFα +100ng/ml IL-17. The combination of TNFα + IL-17 was previously reported to have a synergistic effect on keratinocyte IL-6 and IL-8 mRNA expression, compared to stimulation with either cytokine on their own (Chiricozzi *et al.*, 2011). We extended this model by investigating the effects of TNFα + IL-17 stimulation on secreted IL-6 and IL-8 protein in NHEKs. We found that this combination of stimuli also exerted a synergistic effect on secreted IL-6 and IL-8 protein. In addition, the absolute level of secreted IL-8 protein was greater than that of secreted IL-6. Levels of secreted IL-10, IL-12, IL-1β and IFNγ protein were at or below the lowest limit of detection for the MSD® Multi-Spot® platform (typically 0.5-10ng/ml); therefore these cytokines were not explored in future studies. It was thus concluded that a combination of 10ng/ml TNFα +100ng/ml IL-17 would produce optimal stimulation of NHEKs and determined that data could only be reliably obtained from secreted IL-6 and IL-8 protein in this model.

To explore the specificity of the IL-6 and IL-8 responses and to develop further models of cutaneous inflammation, NHEKs were stimulated with a variety of cytokines and toll-like receptor (TLR) ligands, at concentrations previously described in the literature: IL-22, IFNγ, Pam_3Cys-Ser-$(Lys)_4$ trihydrochloride (Pam3Cys), peptidoglycan derived from Staphylococcus *aureus* (PGN), Pam2CGDPKHPKSF (FSL-1), heat-killed Staphylococcus *aureus* (HKSA), lipoteichoic acid (LTA), polyinosinic:polycytidylic acid (Poly:IC) or lipopolysaccharide derived from Escherichia *coli* (LPS) (Kollisch *et al.*, 2005; Begon *et al.*, 2007; Donnarumma *et al.*, 2011). Supernatants were collected 48h after stimulation and IL-6 and IL-8 concentrations measured by ELISA.

As shown in Figure 3-1, the combination of TNFα + IL-17 stimulation induced significant secreted IL-6 and IL-8 protein responses; approximately 27-fold and 17-fold

changes respectively compared to unstimulated cells ($p<0.001$ for both IL-6 and IL-8 induction). Interestingly the addition of IFNγ to TNFα + IL-17 stimulation enhanced the IL-6 response ($p<0.001$) but inhibited the IL-8 response ($p<0.05$) compared to stimulation with TNFα + IL-17 in NHEKs, suggesting IL-6 and IL-8 are differentially regulated. TNFα + IL-17 + IFNγ induced ~120-fold increase in IL-6 secreted protein and ~10-fold increase in IL-8 secreted protein compared to unstimulated ($p<0.001$ for both IL-6 and IL-8 induction). IL-17 stimulation of NHEKs resulted in a significant IL-8 response ($p<0.001$) but not IL-6. The remaining stimuli had no significant effect on IL-6 and IL-8 secreted cytokine production.

Having established that both TNFα (10ng/ml) + IL-17 (100ng/ml) and TNFα (10ng/ml) + IL-17 (100ng/ml) + IFNγ (10ng/ml) produced the most robust and reproducible IL-6 and IL-8 responses, the effects of these stimuli on cellular metabolism and total protein were investigated. NHEKs were stimulated with combinations of TNFα, IL-17 and IFNγ. The effects of cytokine stimulation on cellular metabolism and total protein were determined at 48h by thiazolyl blue betrazolium bromide (MTT) and Sulphorhodamine B (SRB) assays respectively.

As shown in Figure 3-2, NHEKs stimulation with TNFα, IL-17 or TNFα + IL-17 for 48h had no significant effects on cellular metabolism or total protein. IFNγ treatment inhibited cellular metabolism, by approximately 30% ($p<0.05$), and total protein, by approximately 25% ($p<0.01$). However, the combination of TNFα + IL-17 + IFNγ treatment had the greatest and most significant effect on cellular metabolism and total protein, with reduction of approximately 31% ($p<0.01$) and 42% ($p<0.001$) respectively. Although NHEKs stimulation with TNFα + IL-17 + IFNγ resulted in significant secretion of IL-6 and IL-8 protein, the interpretation of this was difficult due to the corresponding inhibition of both cellular metabolism and total protein; it was unclear if the IL-6 and IL-8 responses to TNFα + IL-17 + IFNγ stimulation were products of non-inflammatory pathways, *e.g.* cytotoxicity, rather than inflammatory responses *per se*. Furthermore it appeared that negative effects on cellular metabolism or total protein could be attributed to IFNγ, as both IFNγ and TNFα + IL-17 + IFNγ had similar effects in the MTT and SRB endpoints. For these reasons, only effects of TNFα, IL-17 or TNFα + IL-17 on inflammatory responses were subsequently investigated.

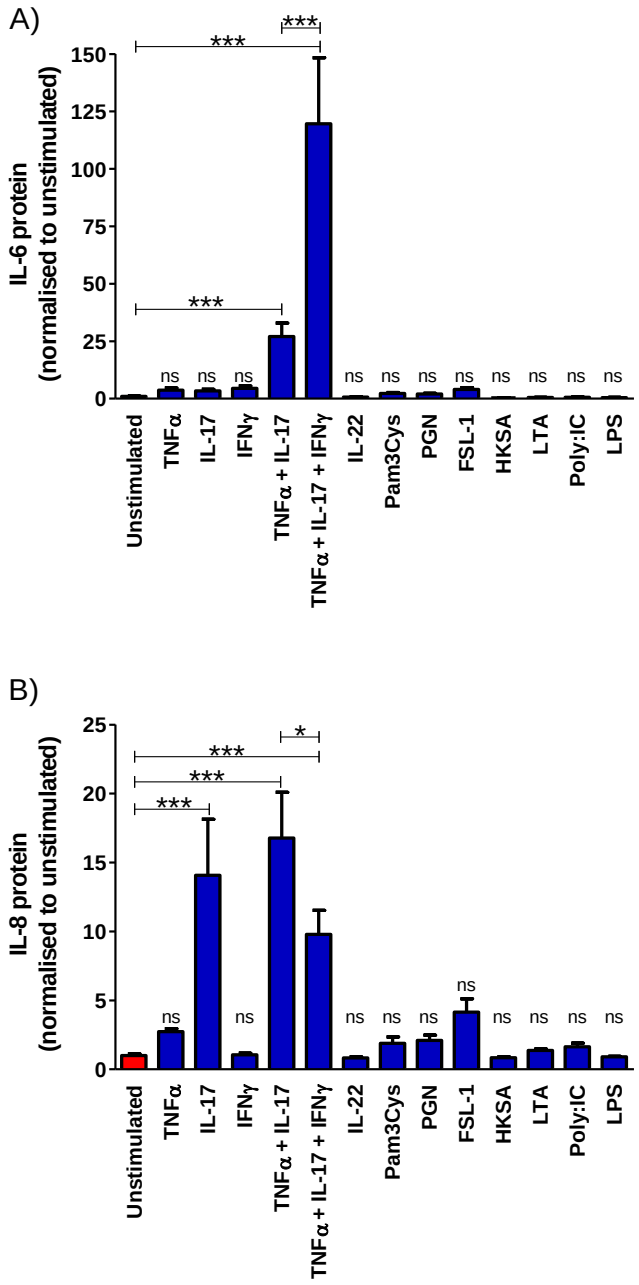


Figure 3-1: IL-6 and IL-8 secreted protein responses by NHEKs in response to a variety of cytokine and TLR agonists. Stimulation with TNFα + IL-17 and TNFα + IL-17 + IFNγ produced the greatest secreted IL-6 and IL-8 responses.

NHEKs were stimulated with TNFα (10ng/ml), IL-17 (100ng/ml), IFNγ (10ng/ml), TNFα (10ng/ml) + IL-17 (100ng/ml), TNFα (10ng/ml) + IL-17 (100ng/ml) + IFNγ (10ng/ml), IL-22 (100ng/ml), Pam3Cys (10μg/ml), PGN (20μg/ml), FSL-1 (100ng/ml), HKSA ($1x10^7$/ml), LTA (20μg/ml), Poly:IC (0.1μg/ml), LPS (1μg/ml) or mock stimulated. Compounds were soluble in aqueous solution/media; therefore vehicle controls were not necessary. Supernatants were harvested 48h post-stimulation and IL-6 (**A**) and IL-8 (**B**) protein concentration measured by ELISA. (N=3, with each donor performed in triplicate repeat) Analysis by two-way ANOVA with Bonferroni post-test; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points (normalised to unstimulated) ± SEM.

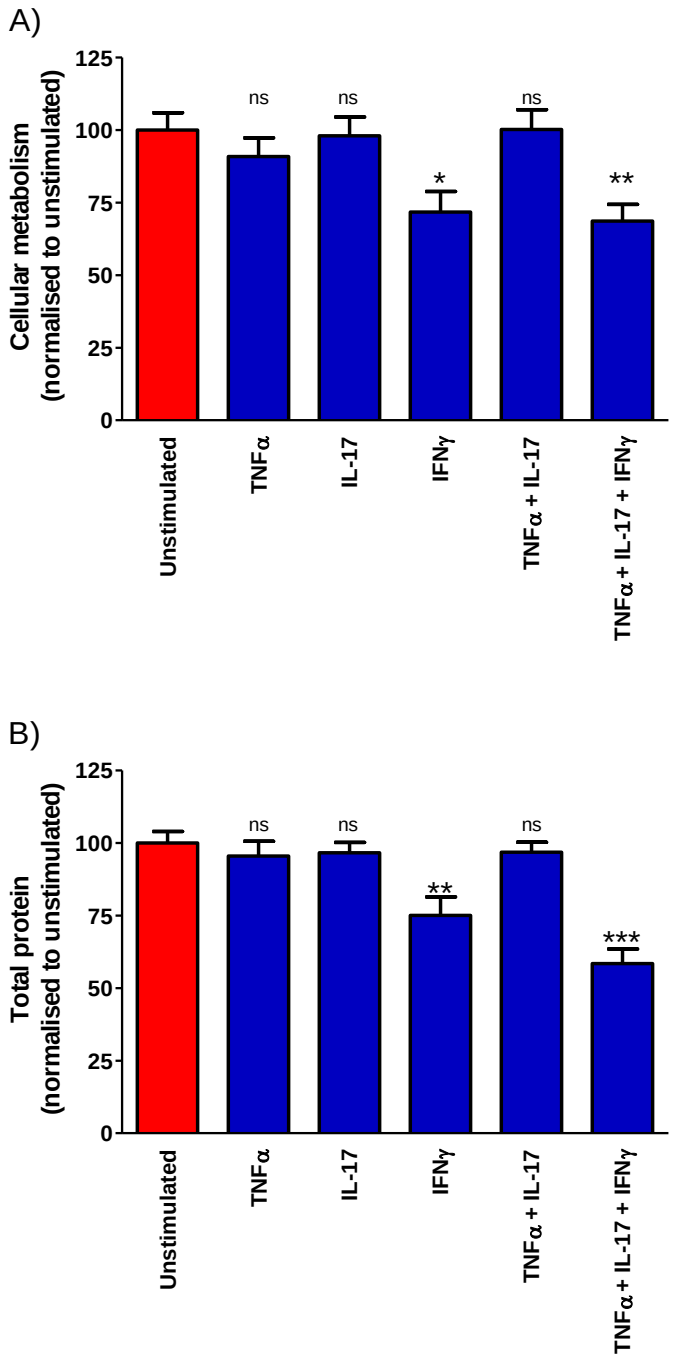


Figure 3-2: TNFα and/or IL-17 stimulation had no significant effect on NHEK metabolism or total protein, in contrast to addition of IFNγ alone or in combination which reduced NHEK metabolism and total protein.

NHEKs were stimulated with TNFα (10ng/ml), IL-17 (100ng/ml), IFNγ (10ng/ml), TNFα (10ng/ml) + IL-17 (100ng/ml), TNFα (10ng/ml) + IL-17 (100ng/ml) + IFNγ (10ng/ml) or mock stimulated. The effects of cytokine stimulation on cellular metabolism were measured at 48h by MTT (**A**) and total protein determined by SRB (**B**). (N=6, with each donor performed in triplicate repeat) Analysis by one-way ANOVA with Dunnett's post-test; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points (normalised to unstimulated control) ± SEM.

3.3.2. IL-6 and IL-8 mRNA expression was maximal at 6h after TNFα + IL-17 stimulation whereas secreted protein levels were cumulative up to 48h

From the data on cellular metabolism and total protein, and TNFα + IL-17's effects on IL-6 and IL-8 production, we concluded that the combination of TNFα (10ng/ml) + IL-17 (100ng/ml) produced the most robust inflammatory stimulus for use as an *in vitro* model of cutaneous inflammation in order to study the role of epigenetics in the regulation of inflammatory responses and for *in vitro* evaluation of potential therapeutic compounds. These two cytokines have central roles in psoriasis pathology, they induce expression of key genes central to psoriasis pathogenesis (Chiricozzi *et al.*, 2011) and anti-IL-17 and anti-TNFα biological therapies are licensed and effective for the treatment of psoriasis.

To characterise the kinetics of expression of IL-6 and IL-8 in the TNFα + IL-17 model of cutaneous inflammation, IL-6 and IL-8 mRNA levels were measured (by qRT-PCR) at 1, 3, 6, 10 and 24h after stimulation with TNFα, IL-17 or TNFα + IL-17 and secreted protein levels were measured (by ELISA) at 10, 24 and 48h after stimulation.

Both IL-6 and IL-8 mRNA expression was maximal at 6h post-TNFα + IL-17 stimulation (Figure 3-3), whereas secreted IL-6 and IL-8 protein was cumulative up to 48h (Figure 3-4). Measuring secreted protein production beyond 48h is difficult owing to the need to feed cells with fresh media, thereby altering the inflammatory milieu. Subsequent investigations, *e.g.* compound inhibition and chromatin immunoprecipitation (ChIP) studies were designed around these optimised time points *i.e.* 6h post- TNFα + IL-17 stimulation for studies concerning mRNA expression and 48h post-stimulation for protein studies.

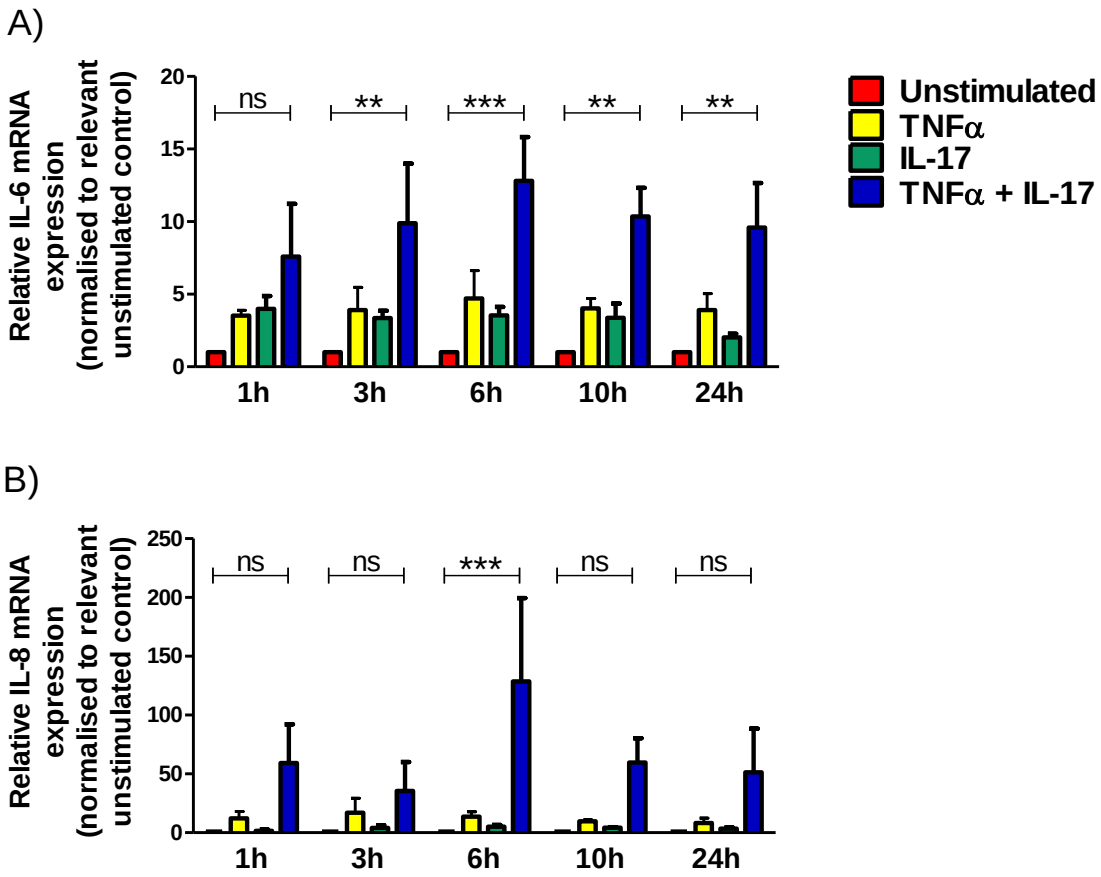


Figure 3-3: NHEK IL-6 and IL-8 mRNA expression was maximal at 6h after TNFα + IL-17 stimulation.

NHEKs were stimulated with TNFα (10ng/ml), IL-17 (100ng/ml), TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. Cells were harvested for mRNA at timepoints indicated and expression of IL-6 (**A**) and IL-8 (**B**) determined by qRT-PCR. (N=3, with each donor performed in duplicate repeat) Analysis by two-way ANOVA with Bonferroni post-test; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points (normalised to unstimulated control within individual timepoints) ± SEM.

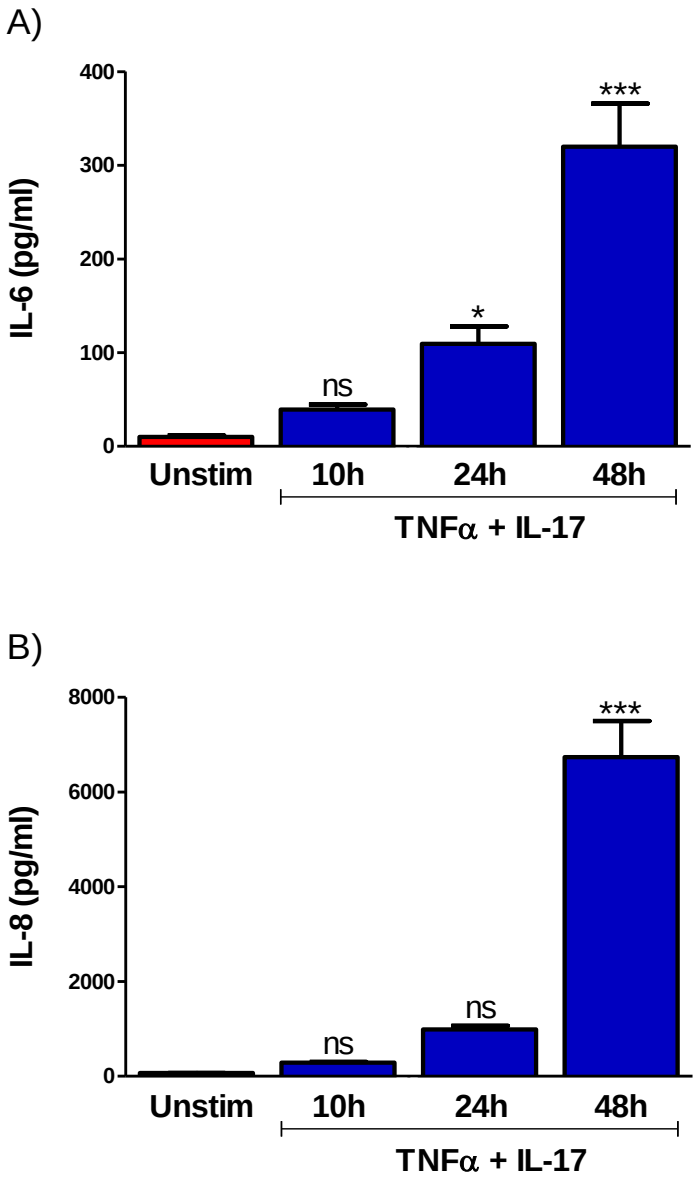


Figure 3-4: Secreted IL-6 and IL-8 protein by NHEKs was cumulative up to 48h after TNFα + IL-17 stimulation.

NHEKs were stimulated with TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. Supernatants were harvested at timepoints indicated post-stimulation and IL-6 (**A**) and IL-8 (**B**) protein concentrations measured by ELISA. (N=3, with each donor performed in triplicate repeat) Analysis by one-way ANOVA with Dunnett's post-test; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points ± SEM.

3.3.3. TNFα + IL-17 induced expression of IL-6 and IL-8 in keratinocytes, with a reproducible synergistic effect for IL-6 protein and a donor-dependent synergistic effect for IL-8 protein

As shown in Figure 3-1, TNFα + IL-17 exerted a synergistic effect on IL-6 secretion into the medium but not IL-8 secretion (N=3). Data obtained during the MRes preceding this PhD project, showed that for some donors TNFα + IL-17 also had a synergistic effect on IL-8 induction. To understand the potential effect of donor variability, we investigated TNFα and/or IL-17 stimulation on IL-6 and IL-8 secretion in further donors. Effects of TNFα + IL-17 stimulation compared to stimulation with TNFα or IL-17 alone were classified according to the following definitions (Chiricozzi *et al.*, 2011):

- **Synergistic effect:** the combined effect of interacting cytokines was greater than the sum of their individual effects.
- **Additive effect:** the combined effect of interacting cytokines was greater than the individual effect for each cytokine, but less than the sum of their individual effects.
- **Antagonistic effect:** the combined effect of interacting cytokines was less than the individual effect for either or both cytokines.

NHEKs were stimulated with TNFα (10ng/ml), IL-17 (100ng/ml) or TNFα (10ng/ml) + IL-17 (100ng/ml). For IL-6 and IL-8 mRNA expression studies, NHEKs were harvested for mRNA extraction at 6h post-stimulation and expression was measured by qRT-PCR. Supernatants were harvested 48h post-stimulation and IL-6 and IL-8 protein concentration measured by ELISA.

As shown in (Figure 3-5), there was considerable variability in IL-6/-8 mRNA and protein expression between donors. Basal levels of secreted IL-8 protein (Figure 3-5D) were higher than IL-6 protein (Figure 3-5B). The magnitude of IL-8 mRNA and protein responses (Figure 3-5C and Figure 3-5D) to stimulation were greater than IL-6 mRNA and protein responses (Figure 3-5A and Figure 3-5B). Stimulation with either TNFα or IL-17 alone induced IL-6/-8 (mRNA and protein) to a similar degree. TNFα + IL-17 stimulation had a robust effect on IL-6/-8 mRNA and protein induction, although there was considerable variability between donors in terms of both magnitude of responses

and to the combined effects of TNFα + IL-17 stimulation compared with stimulation with either cytokine alone (*i.e.* synergistic, additive or antagonistic).

As shown in Table 3-1, TNFα + IL-17 stimulation had a synergistic effect on IL-6 mRNA induction in 65% of donors and an additive effect in 35% of donors; whereas for IL-6 secreted protein, synergy between TNFα + IL-17 was observed in 94% of donors. On the other hand, TNFα + IL-17 stimulation had a synergistic effect on IL-8 mRNA induction in all donors, but a synergistic effect on IL-8 protein production in only 70% of donors, an additive effect in 17% and antagonistic effect in 13% of donors.

The effects of TNFα + IL-17 stimulation in NHEKs for individual donors are shown in Figure 3-6. In some donors stimulation with TNFα + IL-17 had similar effects on both IL-6 and IL-8 protein production. For example, in donor A, TNFα + IL-17 stimulation had a synergistic effect on both IL-6 and IL-8 expression, (Figure 3-6A and Figure 3-6B, respectively); whereas in donor B, TNFα + IL-17 stimulation had an antagonistic effect on IL-6 and IL-8 expression**,** (Figure 3-6C and Figure 3-6D, respectively). In other donors the IL-6 and IL-8 responses to TNFα + IL-17 stimulation differed. For example, in donor C, TNFα + IL-17 stimulation had a synergistic effect on IL-6 production but an additive effect on IL-8 production (Figure 3-6E and Figure 3-6F, respectively); whereas in donor D, TNFα + IL-17 had a synergistic effect on IL-6 production but an antagonistic effect on IL-8 production (Figure 3-6G and Figure 3-6H, respectively). Furthermore, the absolute level of secreted IL-6 or IL-8 protein in response to TNFα + IL-17 stimulation was variable across different donor. For example, IL-6 production in response to TNFα + IL-17 stimulation in donor D (Figure 3-6G) was ~ 10 fold higher than in donors A, B or C (Figure 3-6A, Figure 3-6C, Figure 3-6E, respectively) and IL-8 production in response to TNFα + IL-17 stimulation in donor A (Figure 3-6B) was ~ 5 fold higher than in donors B, C or D (Figure 3-6D, Figure 3-6F, Figure 3-6H, respectively). Furthermore, the absolute levels of secreted proteins were not dependent on whether TNFα and IL-17 had a synergistic effect or not. For example, TNFα + IL-17 stimulation induced similar levels of IL-6 protein in donor B (Figure 3-6C) and C (Figure 3-6E), despite TNFα and IL-17 having an antagonistic effect in donor B but a synergistic effect in donor E. Similarly, TNFα + IL-17 stimulation induced comparable levels of IL-8 protein in donor C (Figure 3-6F) and D (Figure 3-6H), despite TNFα and IL-17 having an additive effect in donor C but an antagonistic effect in donor D.

In some donors, the effects of TNFα + IL-17 on IL-6 and IL-8 protein were also observed at the mRNA level; in donor E (Figure 3-7A, B, C, D) TNFα + IL-17 had a synergistic effect on IL-6 and IL-8 protein which was also evident at the mRNA level. However in other donors this was not the case; in donor F (Figure 3-7E, F, G, H), TNFα + IL-17 had a synergistic effect on IL-6 and IL-8 secreted protein and IL-8 mRNA but an additive effect on the IL-6 mRNA response.

The different effects of TNFα + IL-17 stimulation on IL-6 and IL-8 mRNA expression suggest these two cytokines are differentially regulated at a transcriptional level and the differential effects of stimulation on IL-6/-8 mRNA vs. protein suggests both cytokines are subject to post-transcriptional regulation. In order to understand whether donor-dependent effects could be related to underlying disease, for example, clinical notes were extensively analysed; the donor dependent IL-6 and IL-8 responses to TNFα + IL-17 stimulation were not correlated with donor age, personal or family history of medical illness, or current medications.

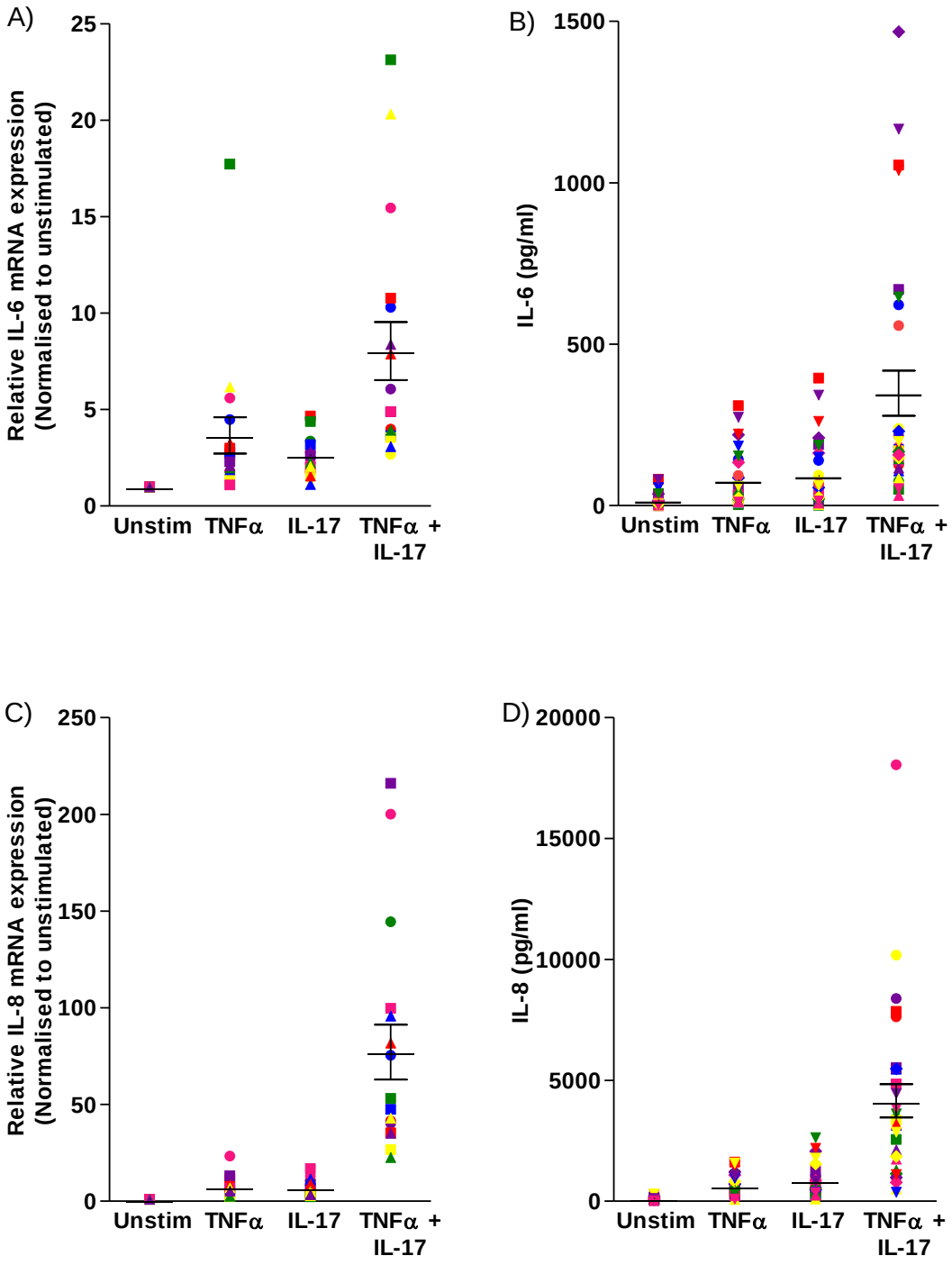


Figure 3-5: TNFα + IL-17 stimulation of NHEKs induced IL-6 and IL-8 responses, with considerable variability in magnitude of responses between donors

NHEKs were stimulated with TNFα (10ng/ml), IL-17 (100ng/ml), TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. Cells were harvested for mRNA at 6h post-stimulation and expression of IL-6 (**A**) and IL-8 (**C**) mRNA determined by qRT-PCR. Supernatants were harvested 48h post-stimulation and secreted IL-6 (**B**) and IL-8 (**D**) protein concentration measured by ELISA. Graphs show the effect TNFα and/or IL-17 stimulation in individual donors represented by different colours/symbols, black line shows the mean ± SEM. (for mRNA, N=17; for protein, N=30).

Effect of TNFα + IL-17 stimulation	IL-6 mRNA Donor N (%)	IL-6 protein Donor N (%)	IL-8 mRNA Donor N (%)	IL-8 protein Donor N (%)
Synergistic	11 (65%)	28 (94%)	17 (100%)	21 (70%)
Additive	6 (35%)	1 (3%)	0	5 (17%)
Antagonistic	0	1 (3%)	0	4 (13%)
Total	17	30	17	30

Table 3-1: TNFα + IL-17 had a reproducible synergistic effect on NHEK IL-6 protein and IL-8 mRNA induction and donor-dependent effects on IL-6 mRNA and IL-8 protein. TNFα + IL-17 effects on IL-6/-8 mRNA were not always comparable to effects on IL-6/-8 protein

Table shows number of donors and proportion of donors with synergistic, additive or antagonistic effects in response to TNFα + IL-17 stimulation, compared to stimulation with either TNFα or IL-17 individually. Data derived as described in Figure 3-5. (for mRNA, N=17, with each donor performed in duplicate repeat; for protein, N=30, with each donor performed in triplicate repeat).

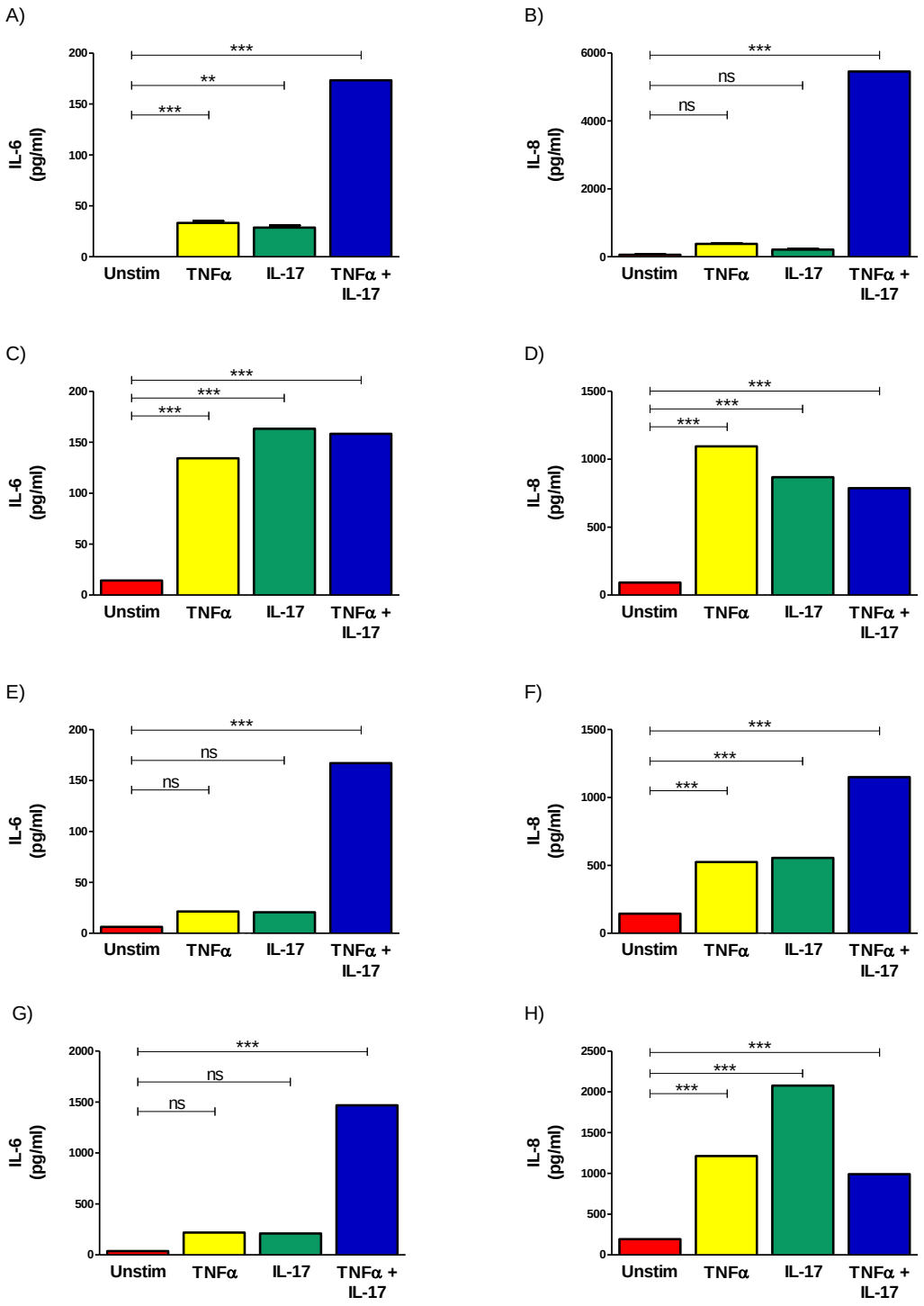


Figure 3-6: Donor dependent effects of TNFα + IL-17 stimulation on IL-6 and IL-8 secreted protein responses

NHEKs from donors A (graphs **A** and **B**), B (**C** and **D**), C (**E** and **F**) and D (**G** and **H**) were stimulated with TNFα (10ng/ml), IL-17 (100ng/ml), TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. Supernatants were harvested 48h post-stimulation and IL-6 (**A**, **C**, **E**, **G**) and IL-8 (**B**, **D**, **F**, **H**) protein concentration were measured by ELISA. (for each graph: N=1, with each donor performed in triplicate repeat) Analysis by one-way ANOVA with Dunnett's post-test; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points.

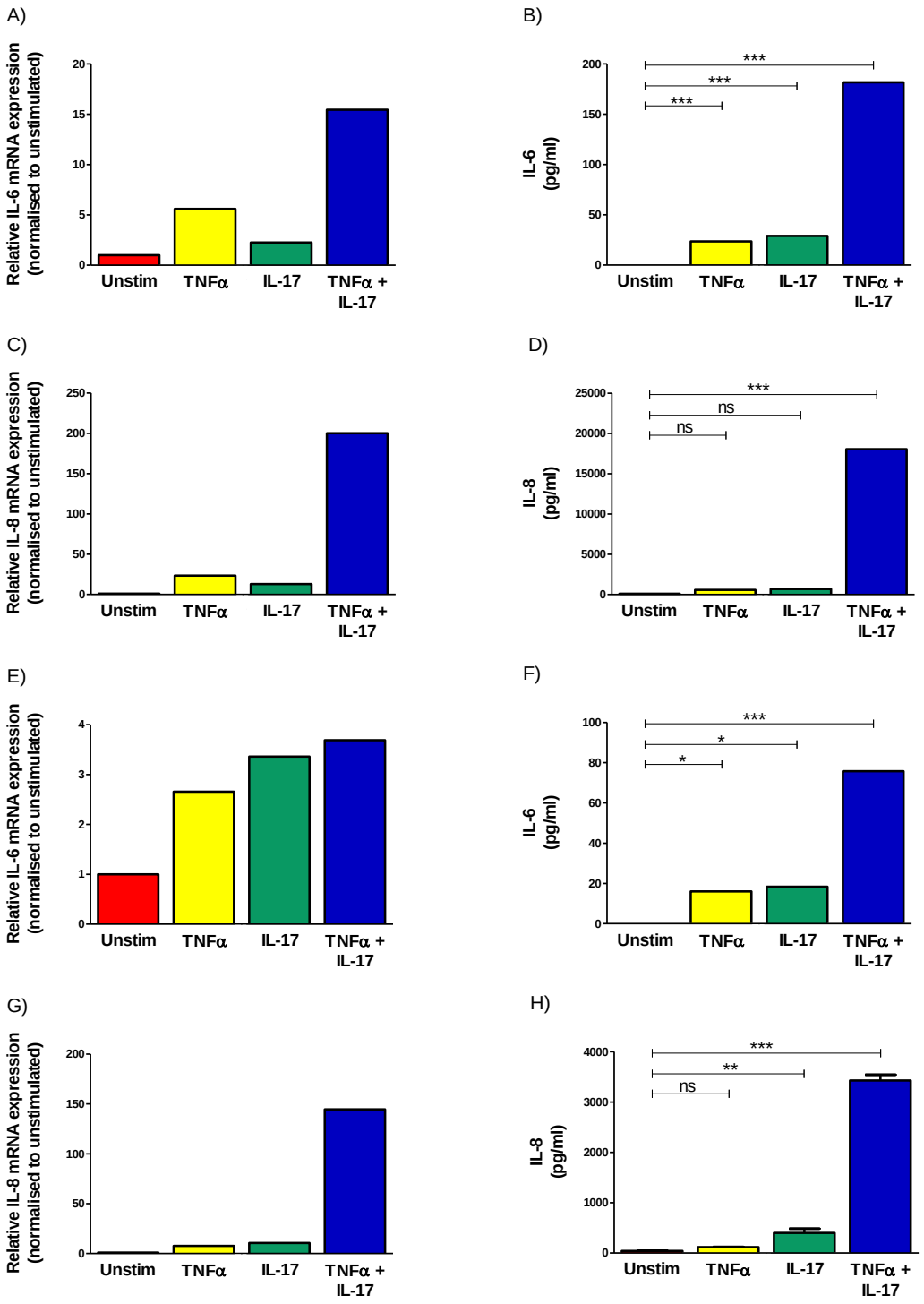


Figure 3-7: Donor dependent effects of TNFα + IL-17 stimulation on IL-6 and IL-8 mRNA and secreted protein responses

NHEKs from donor E (graphs **A**, **B**, **C**, **D**) and F (**E**, **F**, **G**, **H**) were stimulated with TNFα (10ng/ml), IL-17 (100ng/ml), TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. Cells were harvested for mRNA at 6h post-stimulation and expression of IL-6 (**A**, **E**) and IL-8 (**C**, **G**) determined by qRT-PCR. Supernatants were harvested 48h post-stimulation and IL-6 (**B**, **F**) and IL-8 (**D**, **H**) protein concentration were measured by ELISA. (for each graph: mRNA, N=1; protein, N=1, with each donor performed in triplicate repeat) Analysis by one-way ANOVA with Dunnett's post-test; *$p<0.05$, **$p<0.01$, ***$p<0.001$. Graphs show mean of data points (for mRNA, normalised to unstimulated).

3.3.4. The synergistic effect of TNFα + IL-17 on IL-6 and IL-8 induction were reproducible and not dependent on cell density, type of growth media or cell passage

Having observed that TNFα + IL-17 stimulation appeared to have donor dependent effects on IL-6 and IL-8 responses, the consistency of these findings over different cell passage numbers and potential impact of culture conditions were investigated.

Initially NHEKs density was investigated; NHEKs were seeded at a density of either 16,000 or 18,000 cells per well of a 48 well plate prior to stimulation with TNFα, IL-17 or TNFα + IL-17. Supernatants were harvested 48h post-stimulation and IL-6 and IL-8 protein concentration measured by ELISA. At the time of supernatant harvesting, wells originally seeded with 16,000 cells had reached ~70% confluency whereas those seeded with 18,000 cells had reached ~100% confluency. As shown in

Figure 3-8, the density of NHEKs did not affect the pattern of response to TNFα and/or IL-17 stimulation. However, as expected, absolute values of IL-6 and IL-8 were greater where there were higher densities of NHEKs, reflecting the greater number of cells at the end of the experiment.

Next the potential effect of cell passage on NHEKs responses to TNFα + IL-17 stimulation were investigated; NHEKs isolated from a single donor were stimulated at passage 3 with TNFα, IL-17 or TNFα + IL-17. Supernatants were harvested 48h post-stimulation and IL-6 and IL-8 protein concentration measured by ELISA. The same experiment was repeated on NHEKs from the same donor at passage 4, with a delay of 5 days between passage 3 and passage 4 growth/stimulation. As shown in Figure 3-9, there were no significant difference between passage 3 and passage 4 responses to TNFα, IL-17 or TNFα + IL-17 and effects were reproducible on different days of stimulation/supernatant harvest.

Finally, different growth media were investigated. NHEKs were cultured in either Epilife® or MCDB 153, both supplemented with human keratinocyte growth supplement, prior to stimulation with TNFα, IL-17 or TNFα + IL-17. Supernatants were harvested 48h post-stimulation and IL-6 and IL-8 protein concentrations measured by ELISA. Growth of NHEKs in Epilife® or MCDB 153 produced no significant differences in inflammatory responses to TNFα, IL-17 or TNFα + IL-17 (Figure 3-10).

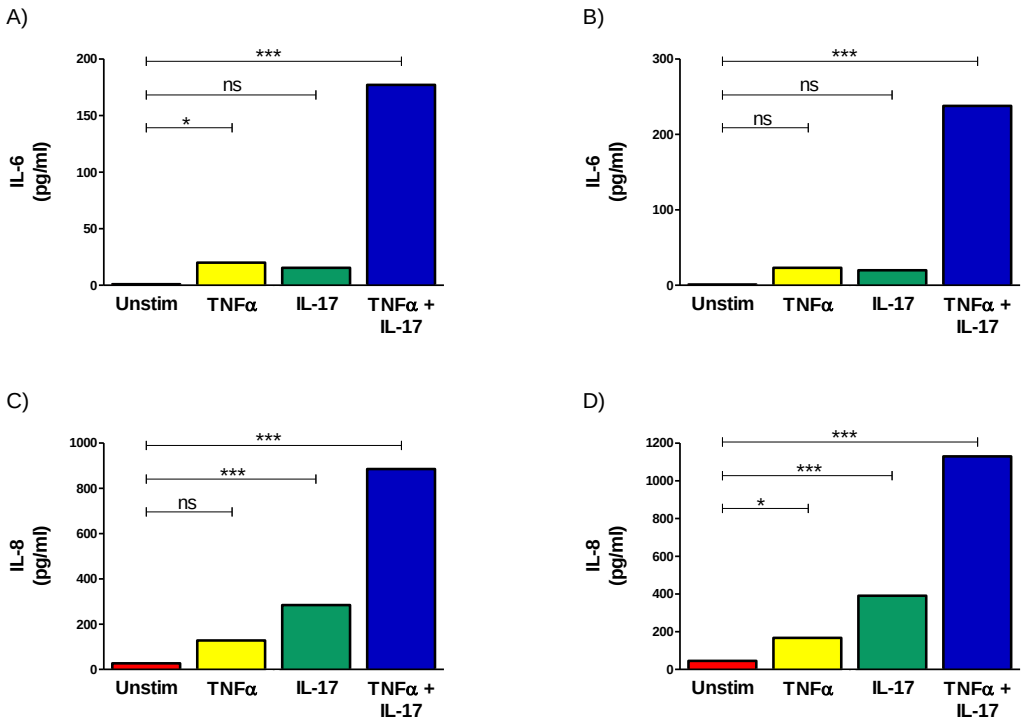


Figure 3-8: The synergistic effects of TNFα + IL-17 on IL-6 and IL-8 induction were independent of NHEK cell density

NHEKs from donor H were seeded at a density of either 16,000 NHEKs per well of a 48 well plate (**A**) and (**C**) or 18,000 NHEKs per well of a 48 well plate (**B**) and (**D**) then stimulated with TNFα (10ng/ml), IL-17 (100ng/ml), TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. Supernatants were harvested 48h post-stimulation and IL-6 (**A and B**) and IL-8 (**C and D**) and protein concentration were measured by ELISA. At the end of the experiment A and C, NHEKs had reached 70% confluency whereas NHEKs in experiment B and D had reached 100% confluency. (N=1, with each donor performed in triplicate repeat) Analysis by one-way ANOVA with Dunnett's post-test; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points.

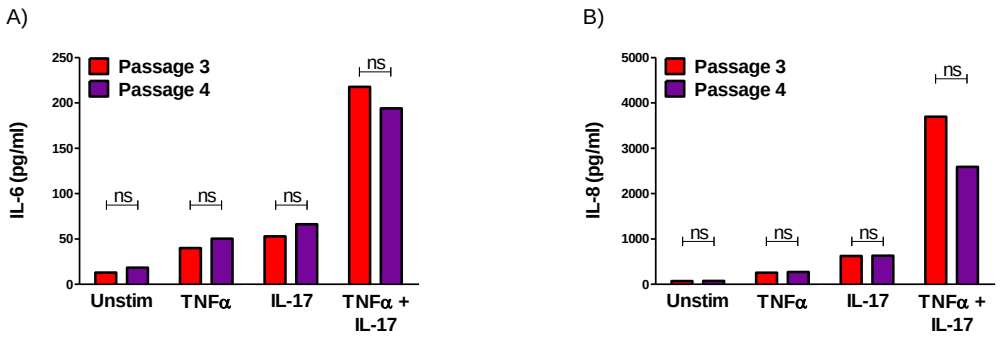


Figure 3-9: Synergistic effects of TNFα + IL-17 on IL-6 and Il-18 induction were independent of NHEK cell passage and reproducible on different days

NHEKs from donor I were cultured to passage 3 or 4 then stimulated with TNFα (10ng/ml), IL-17 (100ng/ml), TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated, the delay between passage 3 and 4 was 5 days. Supernatants were harvested 48h post-stimulation, for each of passage 3 and 4; IL-6 (**A**) and IL-8 (**B**) protein concentration were measured by ELISA. (N=1, with each donor performed in triplicate repeat) Analysis by two-way ANOVA with Bonferroni post-test. Graphs show mean of data points.

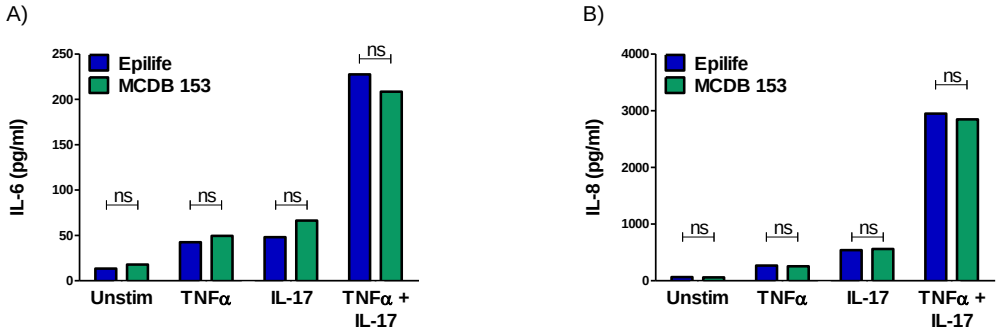


Figure 3-10: Induction of IL-8 by TNFα + IL-17 stimulation was not dependent on the type of growth media used

NHEKs from donor J were isolated and cultured for 2 passages in either Epilife® or MCDB 153, each supplemented with Human Keratinocyte Growth Supplement, prior to stimulation with TNFα (10ng/ml), IL-17 (100ng/ml), TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. Supernatants were harvested 48h post-stimulation and IL-6 (**A**) and IL-8 (**B**) protein concentration were measured by ELISA. (N=1, with each donor performed in triplicate repeat) Analysis by two-way ANOVA with Bonferroni post-test. Graphs show mean of data points.

3.3.5. TNFα + IL-17 stimulation had an additive effect on upstream NF-κB signalling

Having observed that TNFα and IL-17 could have a synergistic effect on IL-6 and IL-8 mRNA expression, it was hypothesised that this might be through synergistic effects on upstream signalling pathways. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is known to be a key regulator of inflammatory responses in psoriasis, including IL-6 and IL-8, and both TNFα and IL-17, have been shown to signal through NF-κB (Libermann and Baltimore, 1990; Hoffmann *et al.*, 2002; Gaffen *et al.*, 2014; Harden *et al.*, 2015). This raised the hypothesis that TNFα + IL-17 stimulation in NHEKs would have a synergistic effect on NF-κB activation. The effects of stimuli were therefore investigated in an NF-κB dual luciferase reporter system (Sherf *et al.*, 1996). NHEKs were transiently transfected with an NF-κB luciferase reporter and stimulated with TNFα, IL-17 or TNFα + IL-17. As expression of IL-6 and IL-8 mRNA were maximal at 6h after TNFα + IL-17 stimulation, we reasoned that activation of upstream signalling pathways would precede maximal transcription. Luciferase activity was therefore measured at 2 and 4h post-stimulation.

As shown in Figure 3-11, TNFα and IL-17 produced an at least additive effect on NF-κB signalling which was more evident at 4h post-stimulation. TNFα stimulation appeared to have a greater effect on NF-kB activation, compared to IL-17 stimulation, although this was not significant.

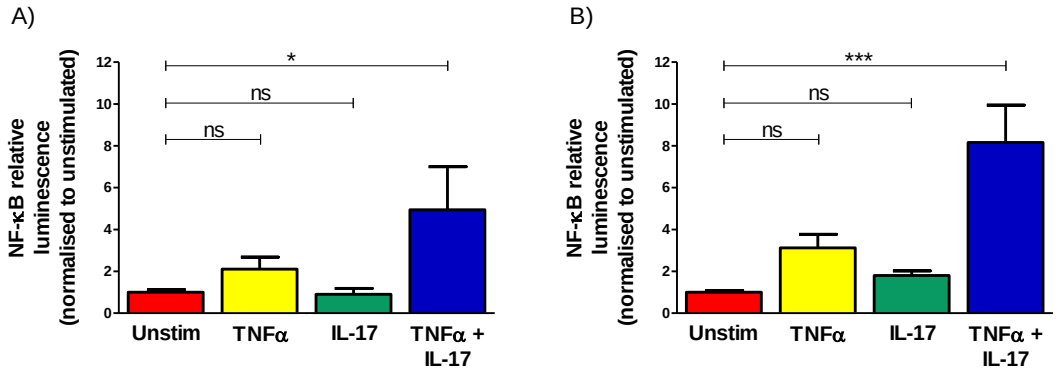


Figure 3-11: TNFα + IL-17 stimulation had an additive effect on NF-κB activation

NHEKs were transfected with NF-κB firefly and renilla luciferase constructs prior to stimulation with TNFα (10ng/ml), IL-17 (100ng/ml), TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. Cells were lysed at 2h (**A**) or 4h (**B**) post-stimulation and luciferase activity measured using a luminometer. (N=3, with each donor performed in triplicate repeat) Analysis by one-way ANOVA with Dunnett's post-test; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of relative luminescence (firefly/renilla ratio) ± SEM.

3.3.6. Induction of IL-6 and IL-8 by TNFα + IL-17 stimulation was inhibited by ciclosporin

To explore whether the TNFα + IL-17 model of cutaneous inflammation could be used for *in vitro* testing of potential therapeutic compounds, the effect of ciclosporin, a compound used clinically to treat psoriasis (Jabbar-Lopez *et al.*, 2014), on IL-6 and IL-8 responses to TNFα + IL-17 was investigated. NHEKs were treated with ciclosporin for 1h prior to stimulation with TNFα + IL-17. Forty eight hours after stimulation, supernatants were collected and secreted IL-6 and IL-8 concentrations were measured by ELISA.

Consistent with the effects of ciclosporin on keratinocyte calcineurin/NFAT signalling, ciclosporin significantly inhibited IL-6 and IL-8 responses to TNFα + IL-17 stimulation (Figure 3-12) compared to the vehicle control ($p<0.01$ and $p<0.001$ for IL-6 and IL-8, respectively), suggesting that the model could be used for *in vitro*, pre-clinical, evaluation of potential therapeutic compounds for the treatment of psoriasis. Ciclosporin did not significantly affect basal IL-6 or IL-8 levels in unstimulated NHEKs. In comparison DMSO, a commonly used drug vehicle, had no significant effects on basal IL-6/-8 levels or on IL-6/-8 responses to TNFα + IL-17 stimulation.

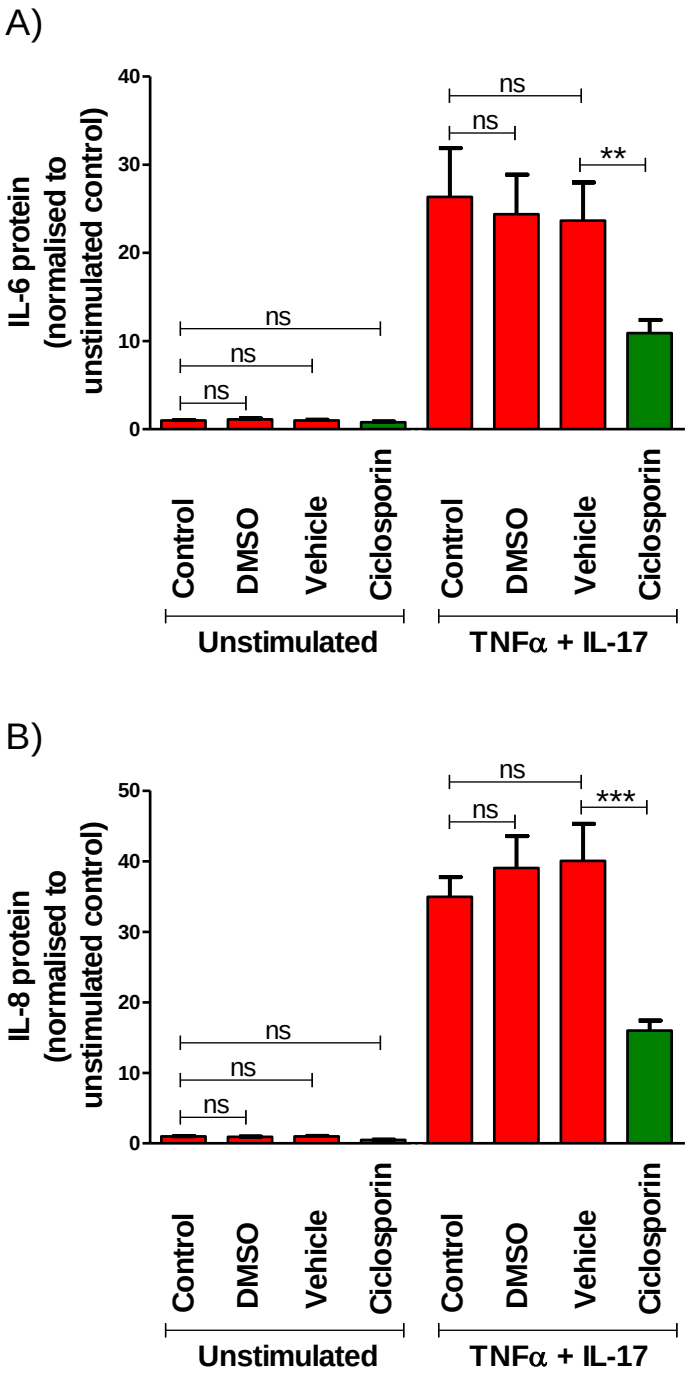


Figure 3-12: Ciclosporin inhibited IL-6 and IL-8 induction by TNFα + IL-17 stimulation

NHEKs were incubated with ciclosporin (1μM), vehicle (ethanol, 0.05%) or DMSO (1:1200) for 1h prior to stimulation with TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. Supernatants were harvested 48h after stimulation and IL-6 (**A**) and IL-8 (**B**) concentrations were determined by ELISA. (N=3, with each donor performed in triplicate repeat) Analysis by two-way ANOVA with Bonferroni post-test. Graphs show mean of data points (normalised to unstimulated control) ± SEM.

3.4. Discussion

An existing model of cutaneous inflammation, in which NEHKs were stimulated with TNFα + IL-17 (Chiricozzi *et al.*, 2011), was extended to show that secreted pro-inflammatory cytokines were significantly increased after TNFα + IL-17 stimulation. In addition it was demonstrated that inflammatory responses could be inhibited by ciclosporin, a compound used clinically to treat psoriasis. TNFα and IL-17 are key cytokines in the pathogenesis of psoriasis, whose serum levels correlate with disease severity and are known to regulate the expression of keratinocyte genes relevant to the pathogenesis of psoriasis (Takahashi *et al.*, 2010; Chiricozzi *et al.*, 2011). Together these findings suggest the *in vitro* TNFα + IL-17 model of cutaneous inflammation, relevant to psoriasis, can be used to i) investigate the regulation of inflammatory skin disease and ii) be used for pre-clinical evaluation of potential therapeutic compounds. Furthermore, in the first time using a model relevant to psoriasis, it was demonstrated that there were donor-dependent inflammatory responses to TNFα + IL-17 stimulation, in terms of both magnitude of responses and synergism, or lack of, between TNFα and IL-17 stimulation.

It was observed that IL-6 and IL-8 mRNA expression was maximal at 6h after TNFα + IL-17 stimulation, whereas secreted protein was cumulative up to 48h post-stimulation. The kinetics of expression were in keeping with previously published effects of TNFα + IL-17 stimulation in synovial fibroblasts on IL-6 and IL-8 mRNA induction (Katz *et al.*, 2001) and also the effects of oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (Ox-PAPC) stimulation in human endothelial cells on IL-8 mRNA and protein induction (Yeh *et al.*, 2001). The absolute concentrations of secreted IL-6 and IL-8 we observed in response to TNFα + IL-17 stimulation were approximately 5-10 fold higher than co-culture of keratinocytes with Staphylococci (Sasaki *et al.*, 2003).

Compared to stimulation with each cytokine individually, the combination of TNFα + IL-17 stimulation had a consistently synergistic effect on IL-8 mRNA induction in all donors but donor dependent effects (synergistic or additive) on IL-6 mRNA induction. Overall TNFα + IL-17 had a synergistic effect on IL-6 mRNA induction in only 65% of donors and an additive effect in 35% of donors (N=17) (Table 3-1). Donor effects did not correlate with donor age, medical history, family history of illness, or medications. In NHEKs, Chiricozzi *et al*. previously described TNFα + IL-17 having synergistic

effects on both IL-6 and IL-8 mRNA induction (Chiricozzi *et al.*, 2011). A number of differences in experimental set up exist and these were considered with respect to this discrepancy. Firstly the concentration of IL-17 used by Chiricozzi *et al*. was 200ng/ml, compared to 100ng/ml in these investigations (the same concentration of TNFα, 10ng/ml, was used in both studies). However, results obtained during the MRes preceding this PhD showed that NHEKs stimulation with TNFα (10ng/ml) plus IL-17 at either 100ng/ml, 200ng/ml or 400ng/ml had no significant difference in the magnitude of IL-6 and IL-8 protein responses (*i.e.* IL-6 and IL-8 responses were maximal with IL-17 100ng/ml). Furthermore, Katz *et al*. previously demonstrated that in primary synovial fibroblasts the synergistic effects of TNFα and IL-17, on IL-6 and IL-8 mRNA production, could be observed for all concentrations and combinations of TNFα and IL-17 assayed; up to 10ng/ml and 100ng/ml respectively (Katz *et al.*, 2001). Differences in concentration of IL-17 were therefore unlikely to account for the discrepancy in results. Secondly Chiricozzi *et al*. investigated effects on mRNA expression 24h after stimulation, whereas the experiments described above investigated effects at 6h, when expression of IL-6 and IL-8 was maximal (Figure 3-3). The kinetics of IL-6 and IL-8 mRNA expression in our model were in keeping with the effects of TNFα + IL-17 stimulation in synovial fibroblasts, which demonstrated maximal IL-6 and IL-8 mRNA responses at 4-18h after stimulation with a peak at around 7h (Katz *et al.*, 2001) and Figure 3-3, demonstrates that maximal synergistic response to TNFα + IL-17 stimulation occurred at 6h for both IL-6 and IL-8; differences in timepoints are therefore also unlikely to account for the discrepancy in results. Finally, primary keratinocytes were used in these experiments, therefore donor variability may account for some of these differences. As our dataset had over 4 times the number of donors it is likely to be more robust (N=4 in Chiricozzi *et al*.'s dataset and N=17 in our dataset); subtle effects of TNFα + IL-17 stimulation on IL-6 mRNA responses may have only been detectable with greater donor numbers.

To begin to investigate potential underlying mechanisms of synergy between TNFα and IL-17, we studied the effects of stimulation on NF-κB signalling, which TNFα and IL-17 have both been shown to signal through (Gaffen *et al.*, 2014; Harden *et al.*, 2015) (section 1.2.6). Using a NF-κB luciferase reporter assay, combined TNFα + IL-17 stimulation was found to have at least an additive effect on NF-κB signalling. This is consistent with the synergistic effects of TNFα + IL-17 on NF-κB nuclear translocation

observed in rheumatoid arthritis synoviocytes; similar synergistic effects on nuclear translocation of other transcription factors, including c-FOS and c-JUN, were also observed (Granet *et al.*, 2004). It would have been interesting to correlate the effects of TNFα and/or IL-17 stimulation on NF-κB signalling, with effects on IL-6 and IL-8 induction in individual donors. However, as NHEKs demonstrated differential effects of TNFα + IL-17 stimulation on IL-6 and IL-8 induction in some donors (for example in donor F, Figure 3-7, TNFα + IL-17 stimulation had an additive effect on IL-6, but a synergistic effect on IL-8 gene expression), the effect on NF-κB signalling alone, which is known to regulate both IL-6 and IL-8 (Libermann and Baltimore, 1990; Hoffmann *et al.*, 2002), is unlikely to account for the different responses of downstream effector genes entirely. In addition to signalling through NF-κB, both TNFα and IL-17 have been shown to activate mitogen-activated protein kinase (MAPK) pathways (*e.g.* JUN N-terminal kinase (JNK)), through TNF receptor-associated factor (TRAF) (Gaur and Aggarwal, 2003; Gaffen *et al.*, 2014) (section 1.2.6). It may therefore be informative to understand the effects of TNFα + IL-17 stimulation on MAPK signalling pathways on NHEKs.

It was observed that TNFα + IL-17 stimulation of NHEKs had a consistently synergistic effect on IL-8 mRNA induction, but a more variable effect on IL-6 mRNA, and the magnitude of IL-8 mRNA response to TNFα + IL-17 stimulation was greater than IL-6 mRNA response. In comparison, the addition of IFNγ enhanced IL-6, but inhibited IL-8, response to TNFα + IL-17 stimulation. Interestingly IFNγ has also previously been shown to enhance IL-6 mRNA response to histamine in keratinocytes, but had an inhibitory effect on histamine-induced IL-8 mRNA (Kohda *et al.*, 2002). Together these findings suggest IL-6 and IL-8 are differentially regulated at the transcriptional level. In support of this, in Jurkat T cells stimulated with phorbol 12-myristate 13-acetate (PMA) (an analogue of diacylglycerol and activator of protein kinase C, which activates nuclear factor of activated T cells (NFAT)) showed IL-8, but not IL-6, was closely associated with the activation pattern of NFAT (Khalaf *et al.*, 2013). Furthermore in a model of HIV-associated neurotoxicity, stimulation of astrocytes with methamphetamine and HIV-1 glysoproein120 had a synergistic effect on IL-6, but not IL-8, induction, and the synergistic effect was shown to be mediated through phosphatidylinositol 3'-kinase/ protein kinase B (PI3/Akt) pathways (Shah *et al.*, 2012). Interestingly, epigenetic changes are implicated in mechanisms underlying synergistic effects of combined stimulation; in macrophages, pre-treatment with IFNγ

synergistically upregulated subsequent LPS-induced responses of effector cytokines including IL-6 (Qiao *et al.,* 2013). Qiao *et al*. demonstrated IFNγ treatment resulted in increased acetylation of the lysine 27 residue on histone H3 (H3K27ac) at the promoter and enhancer region of IL-6 and TNFα, which was associated with increased recruitment of histone acetyl transferase (HAT) enzymes to these regions. IFNγ-induced priming of IL-6 regulatory regions did not activate IL-6 transcription but subsequent LPS stimulation led to enhanced and prolonged recruitment of transcription factors (including p65) and RNA polymerase II to IL-6 promoter and enhancer regions, which accounted for IFNγ-induced potentiation of IL-6 response to LPS stimulation (Qiao *et al.,* 2013). It would be very interesting to investigate whether similar epigenetic mechanisms underlie the synergy between TNFα + IL-17 stimulation in NHEKs, especially as IFNγ enhanced IL-6 response to TNFα + IL-17 stimulation (Figure 3-1A). It would also be interesting to understand if the magnitude of synergism, or lack of, between TNFα + IL-17 on IL-6 induction correlates with epigenetic changes at the IL-6 promoter in NHEKs and whether similar epigenetic changes occur at the IL-8 promoter.

Although Chiricozzi *et al*. did not investigate effects of TNFα + IL-17 stimulation on IL-6 and IL-8 protein responses (Chiricozzi *et al.*, 2011), importantly, we extended the model of NHEKs stimulation with TNFα + IL-17 by investigating effects of stimulation on secreted IL-6 and IL-8 protein. TNFα + IL-17 stimulation had a reproducible synergistic effect on secreted IL-6 protein production (94% of donors, N=30) (Table 3-1). In contrast, the effects on secreted IL-8 protein were indicative of TNFα + IL-17 stimulation having a synergistic effect on IL-8 protein production in only 70% of donors, an additive effect in 17% and antagonistic effect in 13% of donors (N=30) (Table 3-1). The donor dependent effects of TNFα + IL-17 stimulation (synergistic, additive or antagonistic) could not be accounted for by tissue culture conditions *e.g.* cell density, type of growth media or cell passage and effects were reproducible on different days of stimulation. As shown in Figure 3-7, the effect of TNFα + IL-17 stimulation on IL-6 mRNA expression did not necessarily translate to effects on secreted IL-6 protein, suggesting that there is post-transcriptional regulation for both IL-6 and IL-8. This suggestion is supported by findings by Katz *et al*. who showed that in primary synovial fibroblasts, TNFα and IL-17 had a greater synergistic effect on secreted protein responses compared to mRNA expression, for both IL-6 and IL-8 (Katz *et al.*, 2001). It

has previously been shown that stimulation with TNFα + IL-17 can have co-operative effects on target gene mRNA stability, in human colonic myofibroblasts (Hata *et al.*, 2002). Hata *et al*. showed that co-stimulation of myofibroblasts with TNFα + IL-17 enhanced IL-6 mRNA stability compared to stimulation with either TNFα or IL-17 alone and observed that enhancement of IL-6 protein responses to TNFα + IL-17 stimulation was dependent on IL-6 mRNA stability (Hata *et al.*, 2002). In contrast IL-8 mRNA was more stable than IL-6 mRNA and the stability of TNFα-induced IL-8 was not modulated by the addition of IL-17 (Hata *et al.*, 2002). Similar post-transcriptional mechanisms could exist in NHEKs and these may account for the differential effects of TNFα + IL-17 on mRNA vs. protein in response to stimulation.

With regard to the variability in magnitude of IL-6 and IL-8 responses to stimulation and the donor dependent effects of synergy between TNFα and IL-17, it has previously been demonstrated that primary cells from different donors can show considerable variability in the amount of proteins they secrete including cytokines, chemokines and growth factors in both basal/unstimulated cells and also in response to inflammatory stimuli, including TNFα and IL-1β (Zhukareva *et al.*, 2010). Interestingly Zhukareva *et al*, demonstrated donor dependent IL-8 responses (to IL-1β stimulation). Donor heterogeneity in response to TNFα, IL-17 or combined stimulation has also previously been reported, in fibroblast-like synoviocytes (FLS). Fischer *et al*. demonstrated that TNFα + IL-17 stimulation of FLS from different donors tended to have an additive effect on both IL-6 and IL-8 protein production in the majority of donors, but in some donors a synergistic effect was observed (Fischer *et al.*, 2015). Our understanding of individual donor responses to stimuli is increasing and a genetic component is likely to be involved in the underlying mechanism, this is perhaps unsurprising given that different culture or stimulation conditions could not account for the donor variability demonstrated in NHEKs. Dombroski *et at*. previously investigated B cell responses to endoplasmic reticulum (ER) stress and found that, in a sample of 60 donors, some genes (*e.g. RNASE4*, which encodes a protein that plays a role in mRNA cleavage) showed large variations in responses across donors, but other genes demonstrated less variation (*e.g. EIF3B*, which encodes a protein involved in initiation of protein synthesis) (Dombroski *et al.*, 2010). Although highly variable genes did not demonstrate enrichment for any particular functions, Dombroski *et al*. assessed whether there were genetic components underlying this variance by investigating ER stress responses in immortalised B cells derived from monozygotic (MZ) twins compared to unrelated

individuals. Immortalised B cells derived from MZ twins were found to be more similar in their responses to ER stress compared to unrelated individuals, suggesting a genetic component could account for donor variability to stimuli. Interestingly correlation between MZ twins varied for different responses induced by ER stress, and the lack of complete correlation between MZ twins suggested other, non-genetic factors, were also involved in mechanisms underlying donor variability (Dombroski *et al.*, 2010).

Recently the concept of expression quantitative trait loci (eQTLs) has emerged. Genome wide association studies (GWAS) of gene expression have identified genetic loci associated with the expression of specific genes, these loci have been termed eQTLs (Ding *et al.*, 2010). Most eQTLs in humans map to within a few hundred kilobases of the associated gene, theses have been termed cis-eQTLs, and eQTLs which map further away have been termed trans-eQTLs (Ding *et al.*, 2010). eQTLs have been identified for a wide variety of genes involved in disease pathogenesis (Cookson *et al.*, 2009) and interestingly donor heterogeneity of IL-6 responses to lipopolysaccharide (LPS) have been mapped to eQTLs (Fairfax *et al.*, 2010). Fairfax *et al*. showed that IL-6 mRNA expression correlated with single nucleotide polymorphisms (SNPs) in a number of genes including *LEP* (encoding leptin, an adipokine secreted mainly by adipocytes), in both unstimulated and LPS stimulated peripheral blood mononuclear cells (PMBCs). For example, heterozygous carriers of a thymine to cytosine mutation at position 3030 (3030T>C) of the *LEP* gene had 7-fold higher expression of basal IL-6 mRNA compared to those not carrying this mutation. In addition, after LPS stimulation donors with the 3030T>C mutation in *LEP* demonstrated 2.3-fold higher expression of IL-6 (Fairfax *et al.*, 2010). The 3030T>C mutation in *LEP* was associated with a 5.5-fold higher expression in *LEP* mRNA expression and basal expression of leptin correlated with the magnitude of IL-6 responses to LPS stimulation in PMBCs (Fairfax *et al.*, 2010). The mechanisms underlying *LEP* associated enhancement of IL-6 production are unknown, but does not appear to be through non-specific, pro-inflammatory responses, as expression of other cytokines (*e.g.* IL-12) are no affected (Fairfax *et al.*, 2010). Interestingly, leptin has been shown to increase secreted IL-6, IL-8 and TNFα in keratinocytes (Xue *et al.*, 2013). Of further note, a recent meta-analysis of 11 studies, comprising 773 patients with psoriasis and 579 controls, showed significantly elevated leptin levels in serum samples from psoriasis patients (weighted mean difference 7.24ng/ml, $p<0.0001$), although this was less significant when plasma

was analysed (weighted mean difference 12.47ng/ml, $p<0.02$) (Zhu *et al.*, 2013). It would be very interesting to understand if any eQTLs (including in *LEP*) correlate with increased IL-6 or IL-8 responses to TNFα + IL-17 stimulation in NHEKs or to synergistic effects of TNFα + IL-17 stimulation on IL-6 or IL-8 mRNA and protein induction and whether these are also associated with an increased risk of developing psoriasis, or indeed whether any of the currently identified psoriasis susceptibility loci (Tsoi *et al.*, 2012; Tsoi *et al.*, 2015) can predict IL-6 or IL-8 responses to TNFα + IL-17 stimulation.

In the final investigation of the TNFα + IL-17-stimulated NHEKs model of cutaneous inflammation, the model was evaluated for *in vitro* pre-clinical testing of potential therapeutic compounds. Ciclosporin is a an effective treatment for psoriasis (Ellis *et al.*, 1986) and is known to inhibit calcineurin and therefore block phosphorylation and subsequent translocation of NFAT (Flanagan *et al.*, 1991). In addition to inhibition of the NFAT pathway, ciclosporin has more recently been shown to have inhibitory effects on NF-κB signalling (Nishiyama *et al.*, 2005). In TNFα + IL-17 stimulated NHEKs, ciclosporin inhibited both IL-6 and IL-8 responses. This result is consistent with the *in vivo* inhibitory effects of ciclosporin on cytokine production in psoriatic patients treated with this drug (Elder *et al.*, 1993). More recent studies have shown ciclosporin can inhibit IL-6 and IL-8 production in keratinocytes *in vitro*, consistent with our findings (Kobayashi *et al.*, 2007; Takei-Taniguchi *et al.*, 2012). Other studies have shown that ciclosporin can have direct effects on skin independent of T-cells, for example ciclosporin has anti-proliferative effects on cultured keratinocytes (Fisher *et al.*, 1988). Furthermore, calcineurin/NFAT have been shown to be expressed in keratinocytes (Al-Daraji *et al.*, 2002). This, together with our results, suggests that ciclosporin effects in keratinocytes, in addition to its effects in T-cells, may account in part for its effectiveness in inflammatory skin disease. Furthermore, the results suggest that the TNFα + IL-17 model of cutaneous inflammation could be a valid assay for pre-clinical testing of potential therapeutic compounds for psoriasis treatment.

3.5. Future work

Our results, combined with Chiricozzi et al.'s data (Chiricozzi *et al.*, 2011) suggest the NHEKs model of cutaneous inflammation is a valid *in vitro* model which can be used to further understand the response of epidermal keratinocytes to inflammatory cytokines,

the pathogenesis of psoriasis and the role of epigenetics in regulating cutaneous inflammation. A number of key questions, however, remain:

- In the NHEKs model of cutaneous inflammation which factors govern donor dependent responses to TNFα + IL-17 stimulation? Although it has previously been shown that TNFα and IL-17 can have co-operative effects on several levels, *e.g.* receptor upregulation, upstream signalling pathways and mRNA stability of target, it is unknown how these co-operative effects vary between donors (Hartupee *et al.*, 2007; Gudjonsson *et al.*, 2012; Gaffen *et al.*, 2014; Harden *et al.*, 2015). Are changes in co-operative effects at different levels responsible for differences in magnitude of response and synergism (or lack of) between TNFα + IL-17 stimulation. We hypothesise that specific eQTLs (Fairfax *et al.*, 2010) are associated with donor variability in response to TNFα and/or IL-17 stimulation. Furthermore it would be interesting to investigate whether specific eQTLs (Fairfax *et al.*, 2010) associated with increased pro-inflammatory responses are also associated with specific endotypes of psoriasis and potentially to their response to treatment, including anti-TNFα/anti-IL-17 therapy.
- Combined with increased understanding of eQTLs, further mechanistic studies of epigenetic changes and signalling pathways engaged by TNFα and IL-17 in NHEKs and their potential role in regulating i) inter-individual differences in IL-6/IL-8 mRNA responses and ii) synergistic responses of IL-8 and IL-6 following stimulation with TNFα + IL-17 would also be of interest.

3.6. Conclusions

- NHEKs stimulation with TNFα + IL-17 induced significant IL-6 and IL-8 mRNA and protein responses, although there were inter-donor differences in the magnitude of responses.
- Combined stimulation with TNFα + IL-17, compared to stimulation with either cytokine alone, had a reproducible synergistic effect on IL-8 mRNA induction in all donors. On the other hand, TNFα + IL-17 stimulation had donor dependent responses on IL-6 mRNA expression, with only 65% of donors showing a synergistic effect of stimulation.

- In contrast to effects on IL-6/-8 mRNA responses; a synergistic effect between TNFα + IL-17 stimulation on IL-6 protein induction was observed in 94% of donors. On the other hand, TNFα + IL-17 stimulation had a synergistic effect on IL-8 protein production in only 70% of donors, an additive effect in 17% and antagonistic effect in 13% of donors.
- TNFα + IL-17 stimulation resulted in maximal IL-6 and IL-8 mRNA expression at 6h after stimulation, whereas protein was cumulative up to 48h post-stimulation.
- Ciclosporin, a compound used clinically to treat psoriasis, inhibited TNFα + IL-17 induction of both IL-6 and IL-8.
- Taken together, these data suggest the NHEKs model of cutaneous inflammation could be used to further understand the pathogenesis of psoriasis and also for *in intro* pre-clinical testing of potential therapeutic compounds.

Chapter 4.

The role of BET proteins in the *in vitro* model of cutaneous inflammation

4. The role of BET proteins in the *in vitro* model of cutaneous inflammation

4.1. Introduction

As discussed in section 1.3.5, differential DNA methylation and histone modifications have been associated with psoriasis (Zhang *et al.*, 2011a; Roberson *et al.*, 2012; Trowbridge and Pittelkow, 2014). Lysine acetylation is the most widely studied histone modification and is mostly associated with gene expression (Ernst *et al.*, 2011). Through their bromodomains, bromodomain extraterminal (BET) proteins (Brd2, Brd3, Brd4 and BrdT, although BrdT is only expressed in testes) are known to bind acetylated lysine residues on histones and act as adapter proteins to regulate gene expression through recruitment of other proteins involved in transcription initiation and also elongation (Basheer and Huntly, 2015).

Specific and potent inhibitors of BET-bromodomains have been developed (Filippakopoulos *et al.*, 2010; Nicodeme *et al.*, 2010) *e.g.* I-BET151 (Dawson *et al.*, 2011) (section 1.3.7). These compounds bind within the acetyl-lysine binding pocket of the tandem bromodomains of BET proteins and block association with acetylated proteins, including histones (Zou *et al.*). In *in vitro* and *in vivo* models of inflammation and cancer, BET inhibitors demonstrated anti-inflammatory and anti-proliferative effects (Filippakopoulos *et al.*, 2010; Nicodeme *et al.*, 2010) and have been shown to inhibit differentiation of naïve CD4+ T cells into T_H17 cells (Mele *et al.*, 2013).

There is little documentation about the function of BET proteins or the effects of BET inhibition in keratinocytes or skin. However, given that BET inhibitors have anti-proliferative and anti-inflammatory effects both *in vitro* and *in vivo* (Filippakopoulos *et al.*, 2010; Nicodeme *et al.*, 2010) and that psoriasis is characterised by chronic inflammation and excessive proliferation, with T_H17 cytokines playing key roles in driving pathogenesis, we hypothesised that BET proteins may be involved in regulating expression of pro-inflammatory cytokines relevant to psoriasis and that BET inhibition would block pro-inflammatory responses in keratinocytes. In support of this hypothesis, BET inhibition has recently been shown to inhibit psoriasis-like cutaneous inflammation in an imiquimod-induced mouse model of psoriasis (Nadeem *et al.*, 2015). To test these hypotheses the activity of a specific BET inhibitor, I-BET 151, (Dawson *et al.*, 2011) was evaluated in the TNFα + IL-17A (IL-17) *in vitro* model of cutaneous inflammation and the results are described in this chapter.

4.2. Aims

- To characterise the role of BET proteins in regulating expression of pro-inflammatory cytokines, using the TNFα + IL-17 in vitro model of cutaneous inflammation
- To test the activity of BET inhibitor I-BET151 in the TNFα + IL-17 *in vitro* model of cutaneous inflammation, as a potential therapeutic compound for the treatment of psoriasis.
- To use the specific BET inhibitor, I-BET151, as a tool for further understanding the role of BET proteins in regulating inflammatory responses in the TNFα + IL-17 *in vitro* model of cutaneous inflammation.

4.3. Results

4.3.1. Keratinocyte expression of BET proteins is not regulated by TNFα + IL-17 stimulation

To characterise the role of BET proteins in the TNFα + IL-17 *in vitro* model of cutaneous inflammation, BET expression in keratinocytes was investigated. Previously, expression of IL-6 and IL-8 mRNAs in TNFα + IL-17 stimulated normal human epidermal keratinocytes (NHEKs) was found to be maximal at 6h after stimulation (section 3.3.2) and it was hypothesised that this would involve recruitment of pre-formed BET proteins rather than through increased synthesis of BET proteins *de novo*. NHEKs were stimulated with TNFα, IL-17 or TNFα + IL-17 and Brd2/3/4 mRNA expression was measured by qRT-PCR at 1, 3, 6, 10 and 24h after stimulation.

As shown in Figure 4-1, unstimulated NHEKs expressed Brd2 Brd3 and Brd4, and stimulation with TNFα and/or IL-17 had no significant effect on their expression.

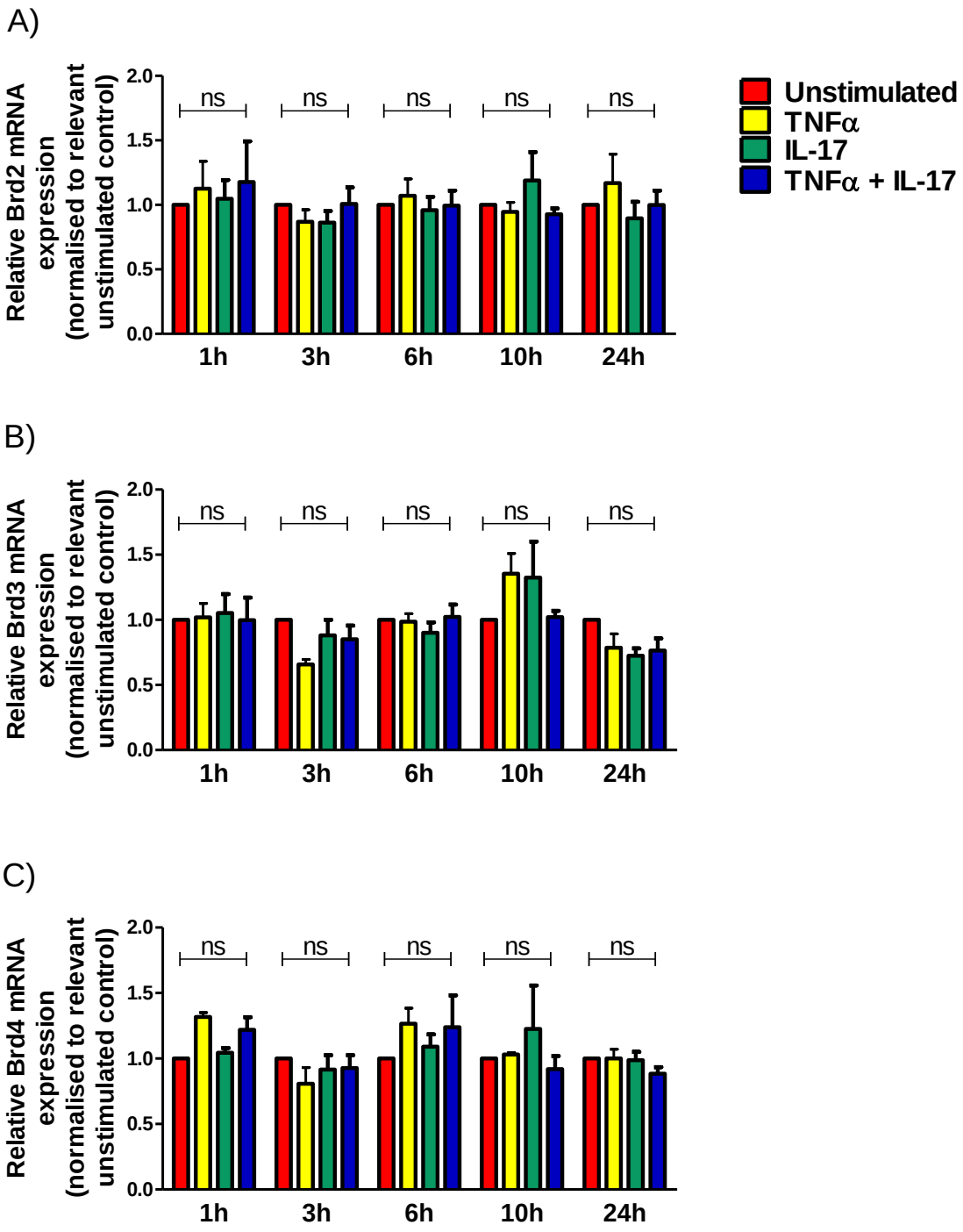


Figure 4-1: TNFα + IL-17 stimulation did not affect mRNA BET expression

NHEKs were stimulated with TNFα (10ng/ml), IL-17 (100ng/ml), TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. Cells were harvested after stimulation, at timepoints indicated, and expression of Brd2 (**A**), Brd3 (**B**) and Brd4 (**C**) determined by qRT-PCR/Taqman® gene expression assays. (N=3, with each donor performed in triplicate repeat) Analysis by two-way ANOVA with Bonferroni post-test, comparing against the relevant unstimulated control. Graphs show mean of data points (normalised to unstimulated control within the relevant timepoint) ± SEM.

4.3.2. I-BET151 treatment inhibited induction of IL-6 protein, reduced IL-6 mRNA expression, but had donor-dependent effects on IL-8 protein and mRNA expression in TNFα + IL-17 stimulated NHEKs.

To understand the effects of BET inhibition on keratinocyte inflammatory responses and to further investigate the pharmacology of I-BET151, NHEKs were treated with a range of increasing concentrations of I-BET151 for 1h prior to stimulation with TNFα + IL-17 *in-vitro*, to allow time for I-BET151 to equilibrate across cell membranes prior to stimulation; the effects of addition of compound either at time of or after stimulation was also studied (section 4.3.7). For IL-6 and IL-8 gene expression studies, NHEKs were harvested at 6h post-stimulation, when IL-6 and IL-8 mRNA expression was maximal (section 3.3.2), and IL-6 and IL-8 mRNA expression was measured by qRT-PCR. As IL-6 and IL-8 secreted protein was cumulative up to 48h post-stimulation (section 3.3.2), media supernatants were collected at this timepoint and secreted IL-6 and IL-8 concentrations were determined by ELISA,

I-BET151 inhibited IL-6 mRNA response to TNFα + IL-17 stimulation in NHEKs in a concentration dependent manner, although the activity of I-BET151 on IL-6 mRNA response was variable across the three donors; IC_{50} (half-maximum inhibitory concentration) range = 57.6 – 173.4nM and maximum inhibition of IL-6 mRNA range = 61.0 - 74.9% (Figure 4-2A, C and E). Across the 3 donors, the mean IC_{50} = 133.6nM (95% confidence interval (95% CI) = 86.5 – 180.7nM) and mean maximum inhibition = 69.5% (95% CI = 64.2 – 74.8%) (Figure 4-4A and E).

I-BET151 also inhibited IL-8 mRNA response to TNFα + IL-17 stimulation in NHEKs in a concentration dependent manner and, as with the effect on IL-6 mRNA response, the activity of I-BET151 on IL-8 mRNA response was variable across the three donors; IC_{50} (half-maximum inhibitory concentration) range = 18.4 – 117.8nM and maximum inhibition of IL-6 mRNA range = 44.1 - 76.0% (Figure 4-2B, D and F). Across the 3 donors, the mean IC_{50} = 55.5nM (95% confidence interval (95% CI) = 16.7 – 94.3nM) and mean maximum inhibition = 61.4% (95% CI = 49.9 – 72.9%) (Figure 4-4C and E).

Interestingly, donor 3 showed the most sensitive IL-6 and IL-8 mRNA responses to I-BET151, IC_{50} = 57.6% and 64.1%, respectively, (Figure 4-2E and F) and also displayed the widest stimulation window (*i.e.* difference between the positive and negative controls for stimulation) for both IL-6 and IL-8 mRNA (Figure 4-2E and F). In comparison, I-BET151 had the greatest maximum inhibitory effect in donor 1 for both

IL-6 and IL-8 mRNA responses, although this did not correlate with the stimulation window or basal/stimulated levels of IL-6/-8 mRNA (Figure 4-2A and B). These finding could suggest donors with the greatest stimulation window could be most sensitive to BET inhibition, although further investigation in additional donors would be required to confirm this.

I-BET151 inhibition of IL-6 protein response to TNFα + IL-17 stimulation in NHEKs was concentration dependent and had broadly similar activity across the seven donors; IC_{50} range = 21.1 – 80.2nM and maximum inhibition of IL-6 protein range = 85.2 - 98.6%) (Figure 4-3A, C, E, G, I, K and M). Across the 7 donors, the mean IC_{50} = 44.6nM (95% CI = 36.1 – 53.1nM) and mean maximum inhibition = 91.2% (95% CI = 88.9 – 93.5%) (Figure 4-4C and Figure 4-4E).

In contrast to the effect on IL-6 protein response, I-BET151 had a variable and weaker effect on IL-8 secreted protein response to TNFα + IL-17 stimulation in NHEKs (Figure 4-3B, D, F, H, J, L, N). Although all donors assayed demonstrated a degree of IL-8 protein inhibition in response to I-BET151 treatment, the range of maximum IL-8 inhibition was variable (42.0 – 89.5%). Furthermore, only some donors (4, 5 and 8) showed concentration-dependent sensitivity to I-BET151. In these donors, the activity of I-BET151 was more variable (IC_{50} range = 4.5 – 108.4 nM) compared to IL-6 protein sensitivity (IC_{50} range = 21.1 – 80.2 nM) and the compound exhibited only partial inhibition of IL-8 protein (Figure 4-4D). As a result of donor variability, the mean values of these data points for IL-8 protein, mean IC_{50} = 59.0nM (95% CI = 29.5 – 88.5nM) and mean maximum inhibition = 63.3% (95% CI = 49.7 – 76.9%) were not representative of any one of the contributing 3 donors (Figure 4-4D and Figure 4-4E). The stimulation window for IL-8 protein in different donors could not account for donor differences in sensitivity to I-BET151. For example while donor 4 and donor 10 had broadly similar basal and stimulated levels of IL-8 secreted protein, donor 4 demonstrated sensitivity to I-BET151 but donor 10 did not (Figure 4-3B and N). In addition the variable effect of I-BET151 on IL-8 protein responses was not dependent on the underlying histological diagnosis of donor samples (Table 4-1).

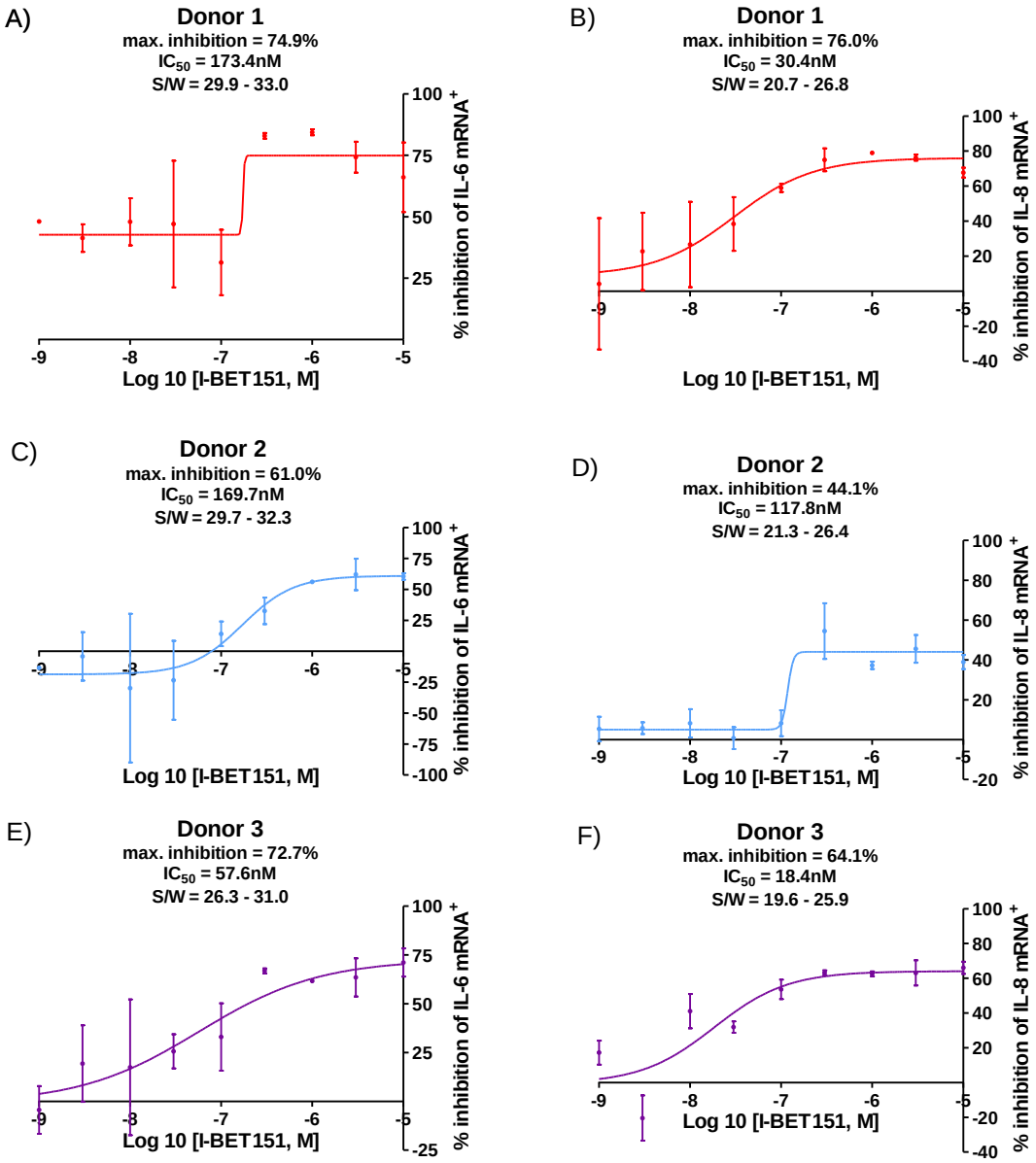
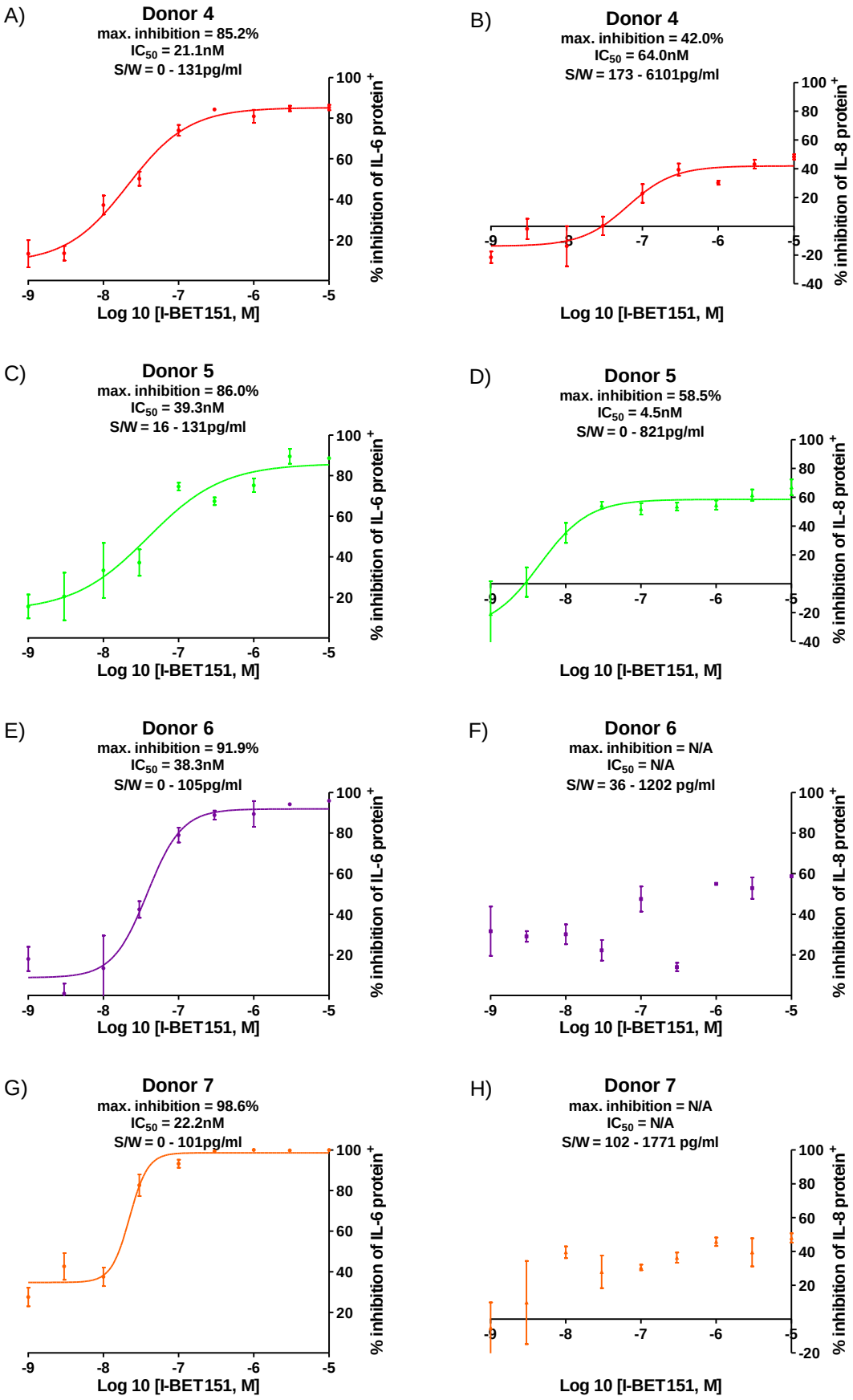


Figure 4-2: I-BET151 inhibition of IL-6 and IL-8 mRNA responses to TNFα + IL-17 stimulation in keratinocytes was concentration dependent

NHEKs were treated with increasing concentrations of I-BET151 for 1 h prior to stimulation with TNFα (10ng/ml) + IL-17 (100ng/ml). Stimulated and mock stimulated cells were also pre-treated for 1h with vehicle (DMSO, 1:1200) as positive and negative controls for stimulation, and to determine the stimulation window (S/W). Cells were harvested 6h post-stimulation and expression of IL-6 (**A**, **C**, **E**) and IL-8 (**B**, **D**, **F**) mRNA was determined by qRT-PCR. Coloured lines are mean data for individual donors (for each graph: N=1, with each donor performed in duplicate repeat). Best fit curve was fitted using 4-parameter logistic curve fit software (Graphpad Prism); maximum inhibition, IC_{50} and S/W (qRT-PCR cycle threshold (Ct) values for positive and negative controls for stimulation) are shown for each graph. [+]Results expressed as "% inhibition of IL-6", were calculated:

$$\frac{\text{(IL-6 value for "TNFα/IL-17 + vehicle") - (IL-6 value for "TNFα/IL-17 + I-BET151 at given concentration")}}{\text{(IL-6 value for "TNFα/IL-17 + vehicle") – (IL-6 value for "mock stimulated + vehicle")}} \text{ X 100}$$

"% inhibition of IL-8" calculated in a similar manner.


A)
Donor 4
max. inhibition = 85.2%
IC50 = 21.1nM
S/W = 0 - 131pg/ml
% inhibition of IL-6 protein
Log 10 [I-BET151, M]
B)
Donor 4
max. inhibition = 42.0%
IC50 = 64.0nM
S/W = 173 - 6101pg/ml
% inhibition of IL-8 protein
Log 10 [I-BET151, M]
C)
Donor 5
max. inhibition = 86.0%
IC50 = 39.3nM
S/W = 16 - 131pg/ml
% inhibition of IL-6 protein
Log 10 [I-BET151, M]
D)
Donor 5
max. inhibition = 58.5%
IC50 = 4.5nM
S/W = 0 - 821pg/ml
% inhibition of IL-8 protein
Log 10 [I-BET151, M]
E)
Donor 6
max. inhibition = 91.9%
IC50 = 38.3nM
S/W = 0 - 105pg/ml
% inhibition of IL-6 protein
Log 10 [I-BET151, M]
F)
Donor 6
max. inhibition = N/A
IC50 = N/A
S/W = 36 - 1202 pg/ml
% inhibition of IL-8 protein
Log 10 [I-BET151, M]
G)
Donor 7
max. inhibition = 98.6%
IC50 = 22.2nM
S/W = 0 - 101pg/ml
% inhibition of IL-6 protein
Log 10 [I-BET151, M]
H)
Donor 7
max. inhibition = N/A
IC50 = N/A
S/W = 102 - 1771 pg/ml
% inhibition of IL-8 protein
Log 10 [I-BET151, M]

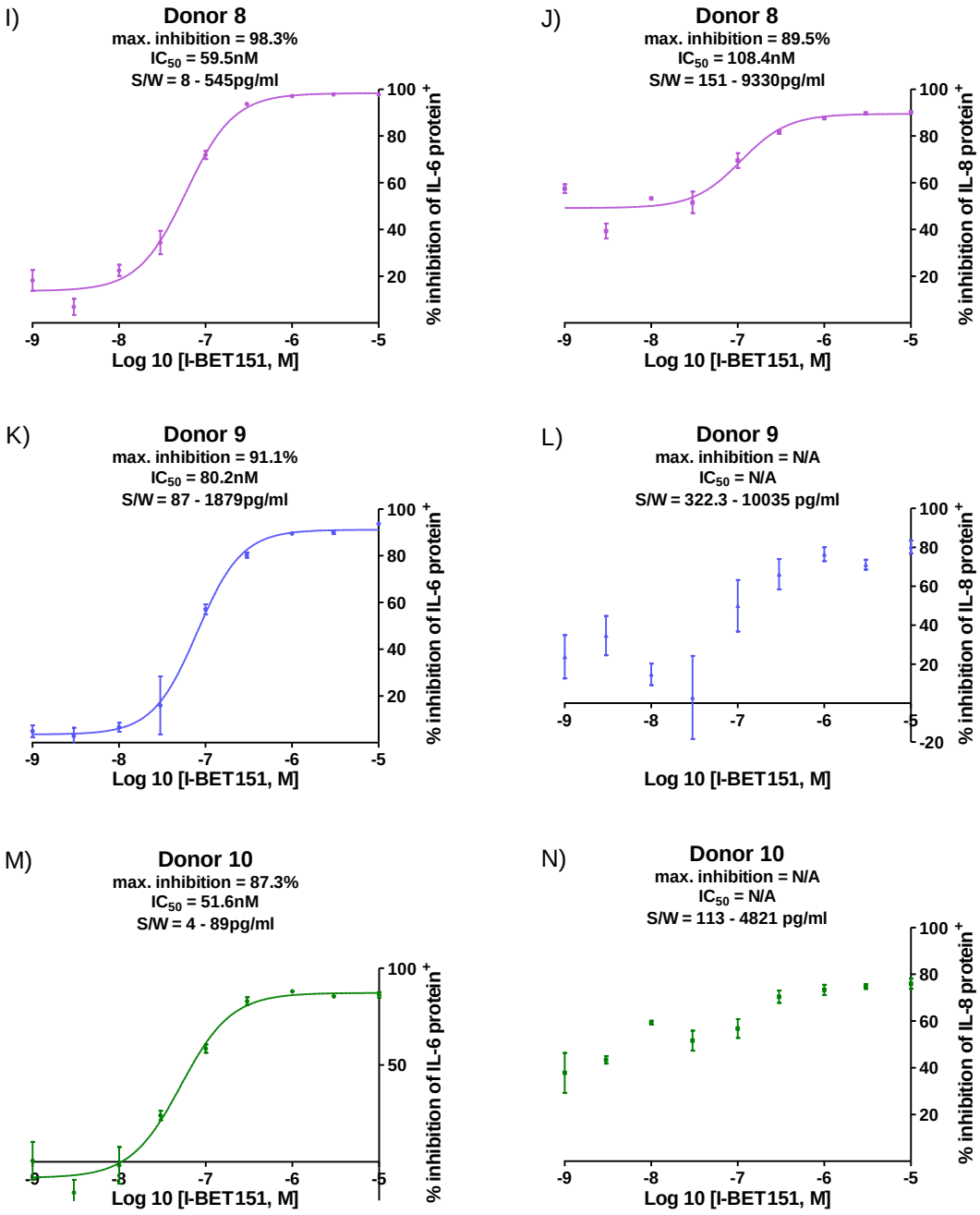
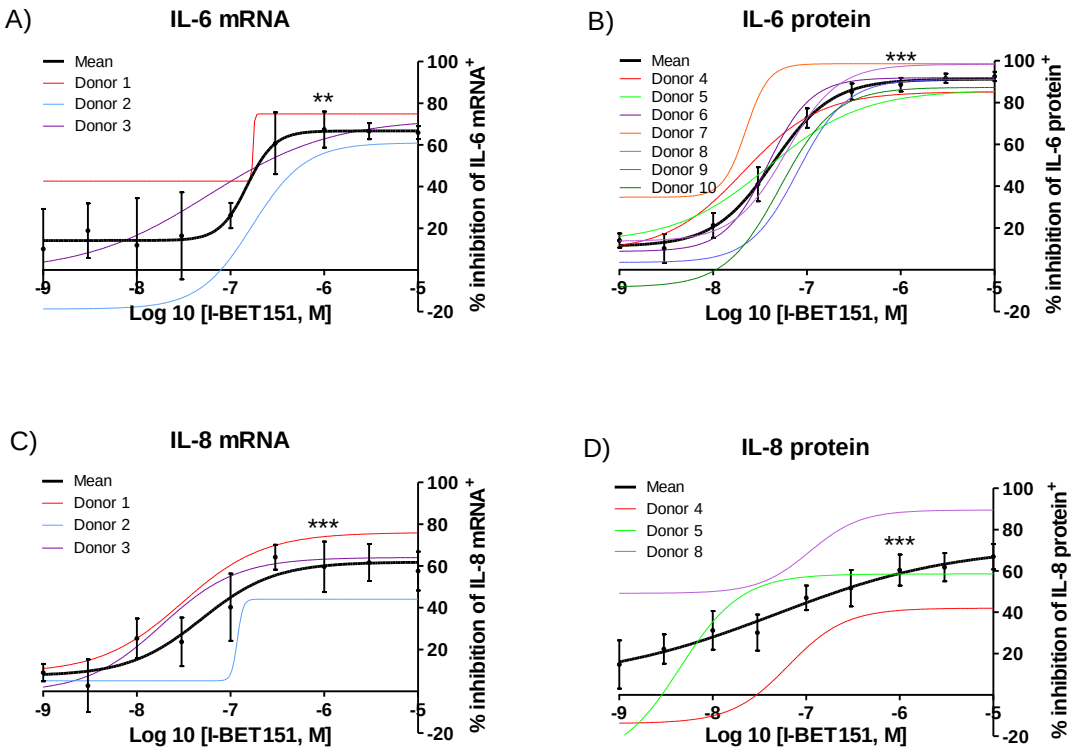


Figure 4-3: Across donors I-BET151 had a concentration-dependent inhibitory effect on IL-6 protein response to TNFα + IL-17 stimulation in keratinocytes, but donor-dependent effects on IL-8 protein response

See Figure 4-2 for experimental details. Media supernatants were collected 48h after stimulation and secreted IL-6 (**A**, **C**, **E**, **G**, **I**, **K**, **M**) and IL-8 (**B**, **D**, **F, H, J, L, N**) protein concentrations were measured by ELISA. Coloured lines are mean data for individual donors (for each graph: N=1, with each donor performed in triplicate repeat). Where possible, best fit curve was fitted using 4-parameter logistic curve fit software (Graphpad Prism); maximum inhibition, IC_{50} and stimulation window (S/W) (for negative and positive controls for stimulation, in pg/ml) are shown for each graph. $^{+}$Results expressed as "% inhibition of IL-6 (or IL-8)" calculated as per Figure 4-2.



E)

	Mean IC_{50}, nM (95% confidence interval)	Mean max. inhibition, % (95% confidence interval)
IL-6 mRNA	**133.6** (86.5, 180.7)	**69.5** (64.2, 74.8)
IL-6 protein	**44.6** (36.1, 53.1)	**91.2** (88.9, 93.5)
IL-8 mRNA	**55.5** (16.7, 94.3)	**61.4** (49.9, 72.9)
IL-8 protein	**59.0** (29.5, 88.5)	**63.3** (49.7, 76.9)

Figure 4-4: I-BET151 inhibited IL-6/-8 mRNA expression and IL-8 protein release, with a greater maximal inhibitory effect on IL-6 protein in TNFα + IL-17 stimulated keratinocytes

See Figure 4-2 for experimental details. Cells were harvested 6h post-stimulation and expression of IL-6 (**A**) and IL-8 (**C**) mRNA was determined by qRT-PCR. Media supernatants were collected 48h after stimulation and secreted IL-6 (**B**) and IL-8 (**D**) protein concentrations were measured by ELISA. Coloured lines are mean data for individual donors (Figure 4-2 and Figure 4-3), the black line is a composite curve derived from mean of normalised % inhibition data points across donors ± SEM; (for mRNA: N=3, with each donor performed in duplicate repeat; for protein: N=7, with each donor performed in triplicate repeat). Best fit curve was fitted using 4-parameter logistic curve fit software (Graphpad Prism). The mean IC_{50} was calculated as the mean of IC_{50} values from individual donor curves and the mean maximum inhibition was calculated in a similar manner, 95% confidence interval shown in brackets (**E**). $^{+}$Results expressed as "% inhibition of IL-6 (or IL-8)", calculated as per Figure 4-2.

Donor	Histological diagnosis
4	Balanitis xerotica obliterans
5	Balanitis xerotica obliterans
6	Balanitis xerotica obliterans
7	Non-specific balanitis
8	Lichenoid inflammation, consistent with early balanitis xerotica obliterans
9	Non-specific balanitis
10	Lichenoid inflammation, consistent with early balanitis xerotica obliterans

Table 4-1: Underlying histological diagnosis of donors

Table shows the underlying histological diagnosis of tissue samples from which keratinocytes were isolated, cultured and assayed in Figure 4-3.

4.3.3. I-BET151 inhibited total protein at high concentration, but had no significant effect on cellular metabolism

Having established that I-BET151 inhibited IL-6 and IL-8 responses to TNFα + IL-17 stimulation in NHEKs, the effects of BET inhibition on cell viability were investigated by determining the effects of I-BET151 on cellular metabolism and total cellular protein. NHEKs were incubated with I-BET151, vehicle (DMSO, 1:1200) or fresh media alone. 48h later, effects on cellular metabolism and total protein were measured, by MTT and SRB respectively.

I-BET151 had no significant effect on cellular metabolism (Figure 4-5A) and although there was significant inhibition of total protein at high I-BET151 concentrations (Figure 4-5B), the effects were relatively modest (~25% inhibition) and could not account for the reduction in IL-6 and IL-8 previously observed (Figure 4-4). For example, at 3μM I-BET151 had no effect on cellular metabolism (Figure 4-5A) and inhibited total protein by only approximately 25% ($p<0.05$, Figure 4-5B), but inhibited both the IL-6 secreted protein response to TNFα + IL-17 stimulation, by approximately 90% ($p<0.001$, Figure 4-4B), and the IL-8 secreted protein response, by approximately 60% ($p<0.001$, Figure 4-4D).

In subsequent chromatin immunoprecipitation-experiments, I-BET151 was used at a concentration of 3μM; this was towards the top/plateau of concentration-response curve (Figure 4-4). At this concentration I-BET151 had a maximal inhibitory effect on IL-6 and IL-8 mRNA and protein responses to TNFα + IL-17 stimulation (significance values $p<0.01$ for IL-6 mRNA, $p<0.001$ for IL-6 protein, $p<0.001$ for IL-8 mRNA and $p<0.001$ for IL-8 protein responses) (Figure 4-4), without any significant effect on cellular metabolism and only a small inhibitory effect on total protein at 48 hours (Figure 4-5).

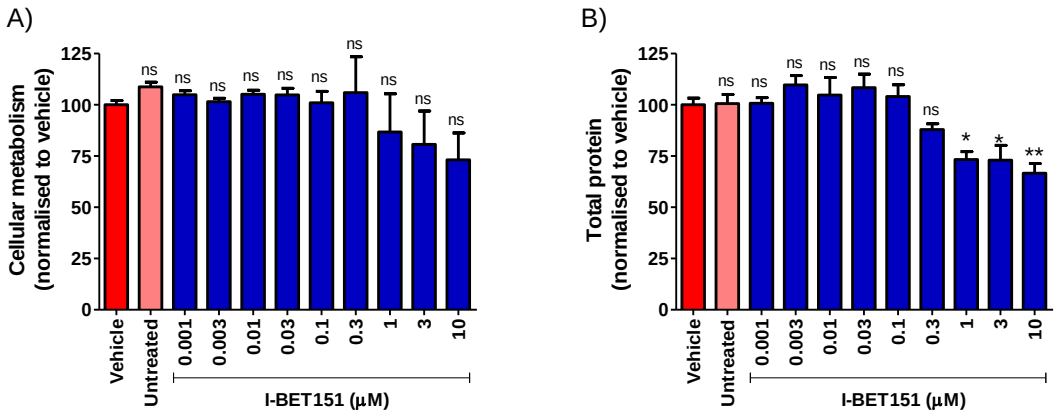


Figure 4-5: I-BET151 had no significant effect on keratinocyte cellular metabolism but at high concentration did have a small, although significant, inhibitory effect on total cellular protein

NHEKs were incubated with increasing concentrations of I-BET151, vehicle (DMSO, 1:1200) or media alone ("untreated"). 48h later, I-BET151 effects on cellular metabolism were measured by MTT (**A**) and total protein determined by SRB (**B**). (N=3, with each donor performed in triplicate repeat) Analysis was by one-way ANOVA with Dunnett's post-test comparing to vehicle; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points (normalised to vehicle) ± SEM.

4.3.4. I-BET151 blocked transient recruitment of Brd4 and RNA polymerase II to the IL-6 promoter

Having demonstrated that I-BET151 blocked TNFα + IL-17 induced IL-6 expression, we hypothesised that i) BET proteins would be involved in regulating IL-6 transcription and ii) TNFα + IL-17 stimulation would drive recruitment of BET proteins to the IL-6 promoter region. Furthermore, we hypothesised that I-BET151 would inhibit this recruitment, as well as the co-recruitment of RNA polymerase II (pol II (S2P)). To investigate these hypotheses, chromatin immunoprecipitation (ChIP) experiments were undertaken to evaluate the effects of TNFα + IL-17 stimulation on BET protein and pol II (S2P) recruitment to the promoter region of target genes, as well as the effects of I-BET151. TNFα + IL-17 was demonstrated to enhance NF-κB upstream signalling (section 3.3.5) and as Brd4 is known to bind p65 (Huang *et al.*, 2009), we investigated whether p65 would also be recruited to the promoter region of TNFα + IL-17 responsive genes such as IL-6 and tested whether I-BET151 would inhibit any p65 binding.

NHEKs were stimulated with TNFα + IL-17 in the presence of I-BET151 or vehicle. As maximum expression of IL-6 (and IL-8) occurred at 6h post-TNFα + IL-7 stimulation (section 3.3.2), we reasoned that peak recruitment of BET, pol II (S2P) and p65 would occur within 6h of stimulation. Cells were therefore harvested at 3h, 4.5h and 6h post-TNFα + IL-7 stimulation for ChIP studies using antibodies to RNA polymerase II phosphorylated (and therefore activated) at its serine 2 residue (pol II (S2P)), Brd2, Brd3, Brd4 and p65 for selective immunoprecipitation (antibodies to histone-H3 and IgG were used as positive and negative controls respectively). After reverse cross-linking, co-immunoprecipitated DNA was purified prior to being assayed by real-time PCR (qPCR) for enrichment of the IL-6 promoter region (using specifically designed primers directed at the IL-6 promoter region, (Qiao *et al.*, 2013). Enrichment of a particular region of DNA (*e.g.* IL-6 promoter) reflects the extent to which this region was associated with the protein of interest.

As shown in Figure 4-6, TNFα + IL-17 stimulation induced transient, recruitment of pol II (S2P), BET proteins and p65 to the IL-6 promoter, maximal at 4.5h post-stimulation. I-BET151 appeared to reduce binding of Brd2/3/4, although this inhibition was only significant for Brd4 ($p<0.05$ at 4.5h). I-BET151 also impaired recruitment of pol II

(S2P) to the IL-6 promoter ($p<0.05$ taking all timepoints into account), consistent with I-BET151's inhibition of IL-6 expression in response to TNFα + IL-17 stimulation (Figure 4-4). Interestingly, binding of Brd4 and p65 at the IL-6 promoter was ~35 fold and ~25 fold higher, respectively, than at the Il-8 promoter (Figure 4-7). Furthermore, p65 binding tracked with changes in Brd4 binding at the Il-6 promoter and in addition to I-BET151 blocking Brd4 binding to the Il-6 promoter, there was a trend for I-BET151 inhibiting p65 binding to the IL-6 promoter.

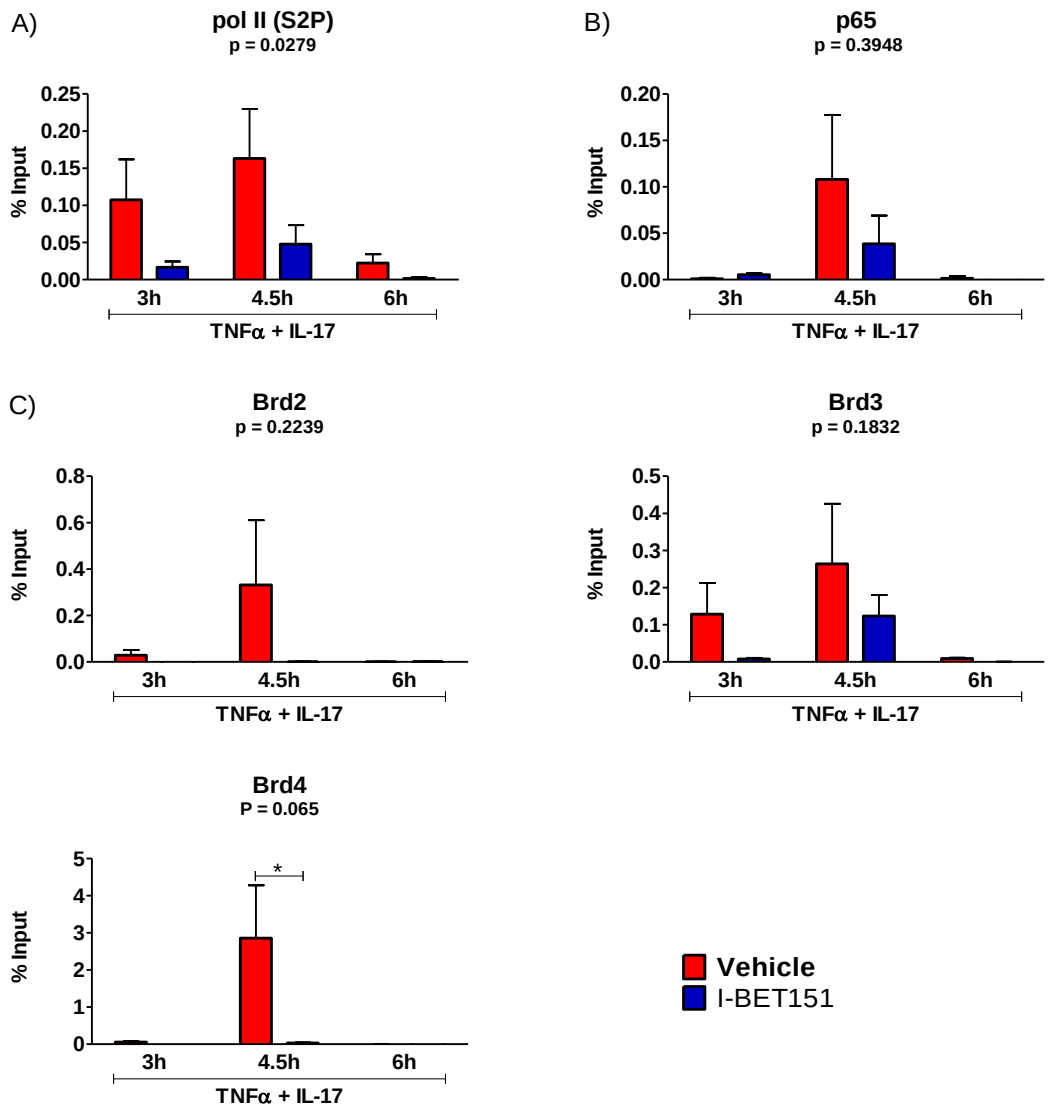


Figure 4-6: I-BET151 blocked transient, TNFα + IL-17-induced, recruitment of pol II (S2P) and Brd4 to the IL-6 promoter

NHEKs were stimulated with TNFα (10ng/ml) + IL-17 (100ng/ml) in the presence of I-BET151 (3μM) or vehicle (DMSO, 1:1200) and harvested at 3h, 4.5h and 6h post-stimulation for ChIP studies using antibodies to pol II (S2P) (**A**), p65 (**B**) and BET proteins (**C**). Quantification of co-immunoprecipitated DNA was performed by qPCR, using primers to the IL-6 promoter region; (N=3) p value above each graph shows the overall statistical significance of I-BET151 compared to vehicle, taking into account all timepoints, two-way ANOVA; Bonferroni post-test, comparing I-BET151 against vehicle; *p<0.05, **p<0.01, ***p<0.001. Data points show mean (after subtracting background/non-specific binding and normalised to an input control) ± SEM.

4.3.5. I-BET151 blocked transient recruitment of Brd3, Brd4 and RNA polymerase II to the IL-8 promoter

Having observed that I-BET151 inhibited TNFα + IL-17-induced recruitment of Brd4 and pol II (S2P) to the IL-6 promoter (Figure 4-6) and given that I-BET151 also inhibited IL-8 transcription induced by TNFα + IL-17 (Figure 4-4), we hypothesised that I-BET151 would also block recruitment of BET proteins and pol II (S2P) to the IL-8 promoter in TNFα + IL-17 stimulated keratinocytes. After reverse cross-linking, co-immunoprecipitated, purified DNA (samples prepared as per section 4.3.4) was assayed by qPCR for enrichment of the IL-8 promoter region (using specifically designed primers directed at the IL-8 promoter region, (Costa *et al.*, 2013) in I-BET151 treated NHEKs and compared to vehicle treated cells.

TNFα + IL-17 stimulation induced, transient, recruitment of pol II (S2P) and BET proteins to the IL-8 promoter, maximal at 4.5h post-stimulation for Brd2/3 proteins and cumulative up to 6h for pol II (S2P) (Figure 4-7). Binding of Brd4 and pol II (S2P) to the IL-8 promoter appeared to be more sustained, compared to binding at the IL-6 promoter. Although I-BET151 appeared to block binding of Brd2/3/4, this inhibition was only significant for Brd3 ($p<0.01$ taking all timepoints into account and Bonferroni post-test showed this was also significant at 4.5h, $p<0.01$) and Brd4 ($p<0.001$ taking all timepoints into account,). I-BET151 also inhibited recruitment of pol II (S2P) to the IL-8 promoter ($p<0.05$ taking all timepoints into account, Bonferroni post-test showed this was also significant at 6h, $p<0.01$). This was consistent with inhibition of IL-8 expression, in response to TNFα + IL-17 stimulation, by I-BET151. I-BET151 had no significant effect on p65 binding at the IL-8 promoter.

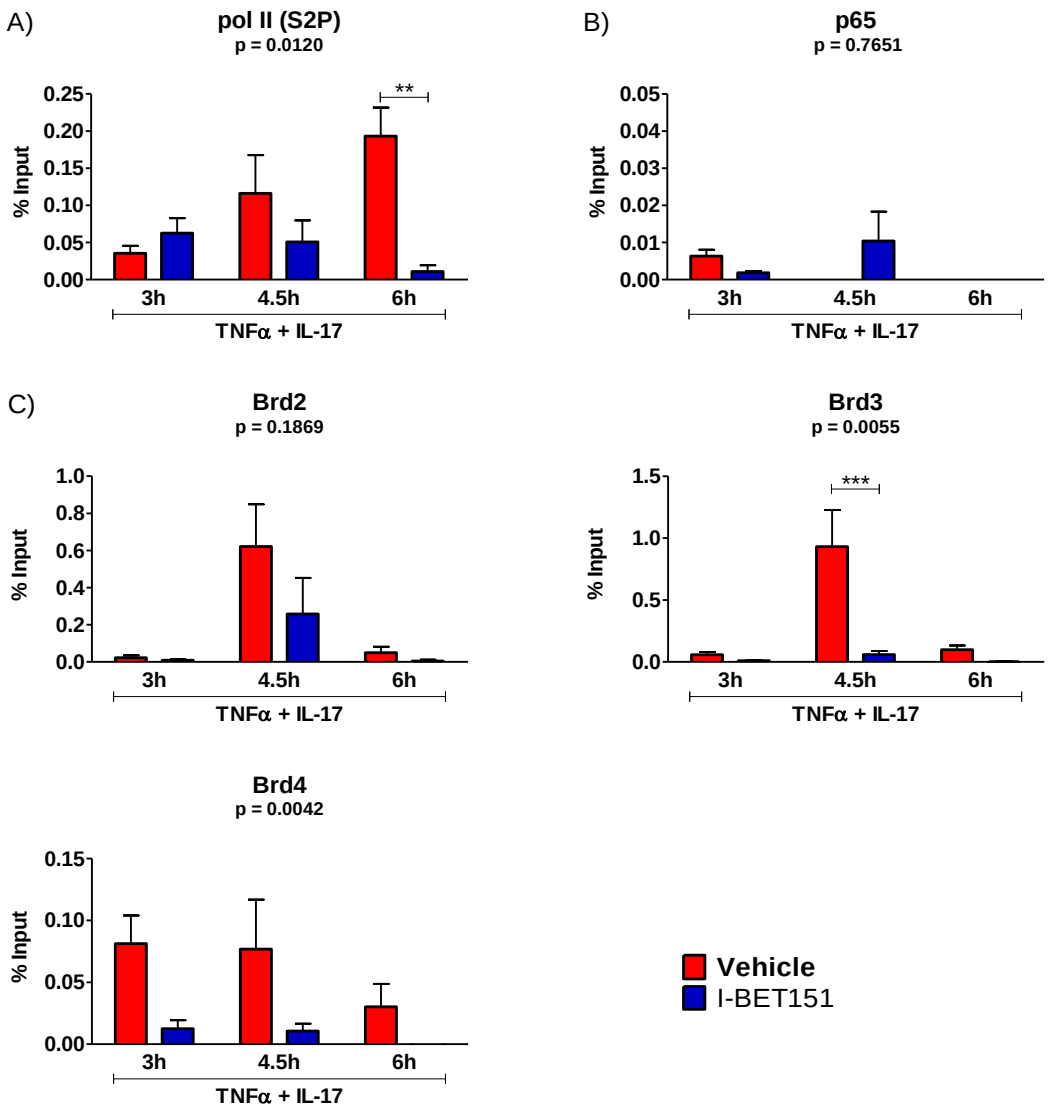


Figure 4-7: At the IL-8 promoter, I-BET151 blocked, TNFα + IL-17 induced, transient recruitment of pol II (S2P), Brd3 and Brd4

NHEKs were stimulated with TNFα (10ng/ml) + IL-17 (100ng/ml) in the presence of I-BET151 (3μM) or vehicle (DMSO, 1:1200) and harvested at 3h, 4.5h and 6h post-stimulation for ChIP studies using antibodies to pol II (S2P) (**A**), p65 (**B**) and BET proteins (**C**). Quantification of co-immunoprecipitated DNA was performed by qPCR, using primers to the IL-8 promoter region. (N=3); p value above each graph shows the overall statistical significance of I-BET151 compared to vehicle, taking into account all timepoints, two-way ANOVA; Bonferroni post-test, comparing I-BET151 against vehicle; *p<0.05, **p<0.01, ***p<0.001. Data points show mean (after subtracting background/non-specific binding and normalised to an input control) ± SEM.

4.3.6. I-BET151 blocked binding of BET proteins and RNA polymerase II to the potential IL-6 enhancer in keratinocytes

Having observed that I-BET151 inhibited IL-6 induction by TNFα + IL-17 and that I-BET151 significantly inhibited recruitment of Brd4 and pol II (S2P) to the IL-6 promoter, we hypothesised that I-BET151 would also block BET protein and pol ll (S2P) recruitment at the IL-6 enhancer. Previously prepared co-immunoprecipitated, purified DNA samples (section 4.3.4) were assayed by qPCR for enrichment of a region previously described as an IL-6 enhancer in macrophages (using specific primers directed at this sequence, (Qiao *et al.*, 2013)).

As shown in Figure 4-8, I-BET151 inhibited binding of Brd2 ($p<0.05$ at 3h), Brd3 ($p<0.05$ taking all timepoints into account), Brd4 ($p<0.05$ taking all timepoints into account, Bonferroni post-test showed this was significant at 4.5h, $p<0.05$) and pol II (S2P) ($p<0.01$ taking all timepoints into account, Bonferroni post-test showed this was significant at 3h, $p<0.05$). In contrast, I-BET151 had no significant effect on p65 binding at the potential IL-6 enhancer in keratinocytes.

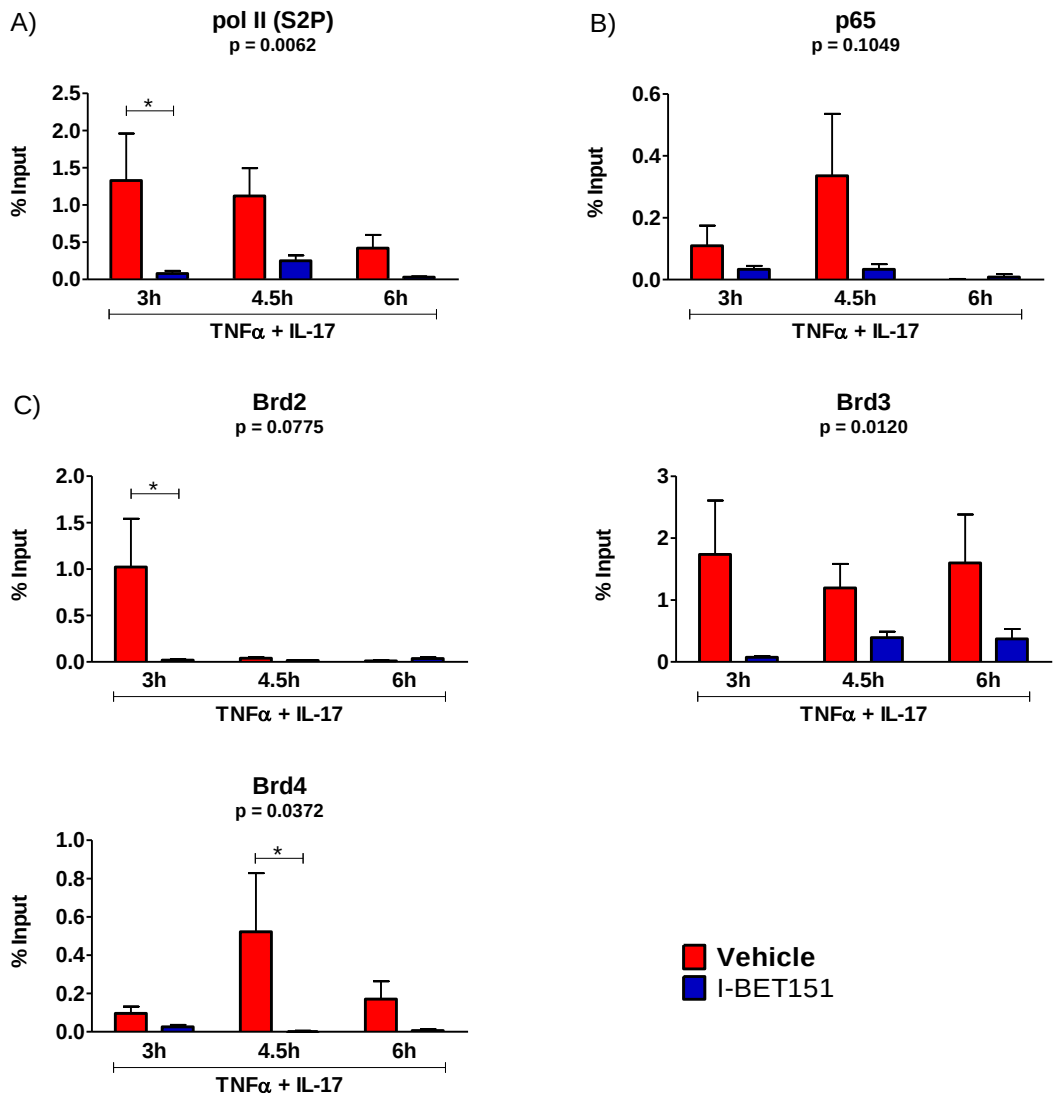


Figure 4-8: At the IL-6 enhancer, I-BET151 inhibited binding of pol II (S2P), Brd2/3/4

NHEKs were stimulated with TNFα (10ng/ml) + IL-17 (100ng/ml) in the presence of I-BET151 (3μM) or vehicle (DMSO, 1:1200) and harvested at 3h, 4.5h and 6h post-stimulation for ChIP studies using antibodies to pol II (S2P) (**A**), p65 (**B**) and BET proteins (**C**). Quantification of co-immunoprecipitated DNA was performed by qPCR, using primers to a region previously described as an IL-6 enhancer in macrophages. (N=3); p value above each graph shows the overall statistical significance of I-BET151 compared to vehicle, taking into account all timepoints, two-way ANOVA; Bonferroni post-test, comparing I-BET151 against vehicle; *p<0.05, **p<0.01, ***p<0.001. Data points show mean (after subtracting background/non-specific binding and normalised to an input control) ± SEM.

4.3.7. I-BET151 remains active if added after TNFα + IL-17 stimulation

Having observed that I-BET151 inhibited IL-6 and IL-8 transcription in response to TNFα + IL-17 stimulation (Figure 4-4) via reducing binding of BET proteins and pol II (S2P) to IL-6 and IL-8 gene regulatory regions in chromatin (Figure 4-6 and Figure 4-7), the dynamics of BET inhibition were further investigated.

As BET proteins and pol II (S2P) were recruited to IL-6 and IL-8 gene regulatory regions after stimulation with TNFα + IL-17, during the phase of active chromatin remodelling, it was hypothesised that I-BET151 would retain activity during this phase of remodelling, even when added after stimulation with TNFα + IL-17. In previous experiments I-BET151 had been added either 1h before (Figure 4-4) or at the same time as TNFα + IL-17 stimulation (Figure 4-6, Figure 4-7 and Figure 4-8). The effect of timing of I-BET151 treatment in relation to TNFα + IL-17 stimulation on IL-6 and IL-8 mRNA and protein expression was therefore explored. NHEKs were treated with I-BET151 (1μM) either 1h before stimulation with TNFα + IL-17, at time of stimulation or at various times after (1, 2 or 4h after stimulation for mRNA studies and 1, 2, 4, 10 or 24h after stimulation for protein studies). IL-6 and IL-8 mRNA were measured, by qRT-PCR, at 6h after stimulation, when maximal expression of these genes was previously observed (section 3.3.2). Media supernatants were collected for determination of secreted IL-6 and IL-8 protein concentrations, by ELISA, at 48h post-stimulation (section 3.3.2).

As shown in Figure 4-9, I-BET151 inhibited IL-6 mRNA production when added before, during or after stimulation with TNFα + IL-17, this was statistically significant up to 2h after stimulation ($p<0.01$) and, interestingly, I-BET151 appeared to have more effect on inhibition of IL6 mRNA when added during or after stimulation. The effect of time of I-BET151 treatment on IL-6 mRNA production translated to inhibitory effects on secreted IL-6 protein; where the compound still had an inhibitory effect even when added up to 24h after stimulation ($p<0.001$).

I-BET151 also inhibited IL-8 mRNA production when added up to 2h after TNFα + IL-17 stimulation ($p>0.01$) (Figure 4-10). As previously observed (Figure 4-3), the effects on secreted IL-8 protein were more variable; although there was a trend for inhibition when I-BET151 was added after stimulation, this was only significant for 10h

($p<0.001$). This was partly due to the variability in donor sensitivity, as shown by the larger error bars for effects on IL-8 protein compared to IL-6.

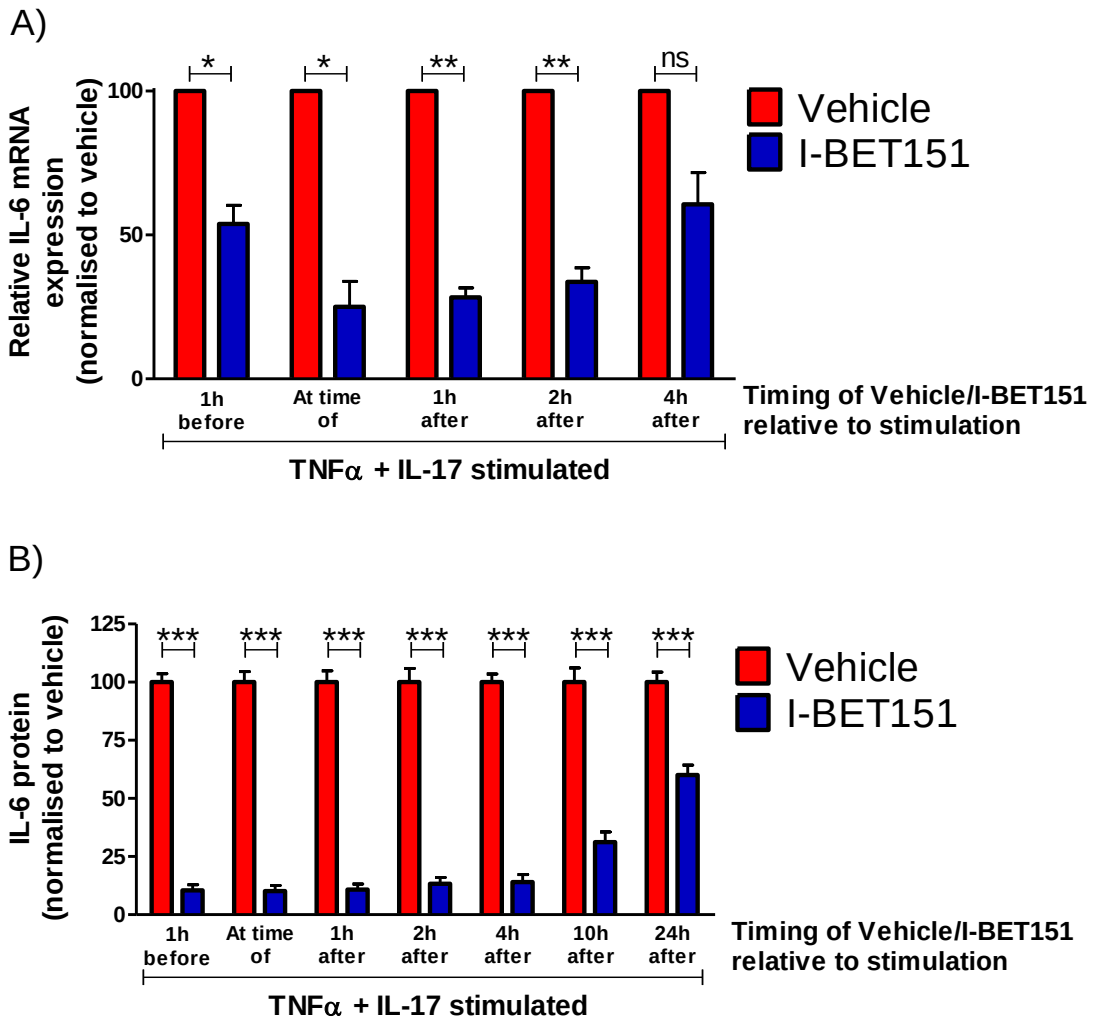


Figure 4-9: I-BET151 was pharmacologically active when added after TNFα + IL-17 stimulation

NHEKs were treated with I-BET151 (1μM) or vehicle (DMSO, 1:1200) either 1h prior to stimulation with TNFα (10ng/ml) + IL-17 (100ng/ml), at time of stimulation or afterwards (at timepoints indicated below graphs). Cells were harvested 6h post-stimulation and IL-6 mRNA expression (**A**) determined by qRT-PCR. Supernatants were collected 48h post-stimulation and secreted IL-6 protein (**B**) concentration measured by ELISA. (for mRNA: N=3; for protein: N=3, with each donor performed in triplicate repeat) Analysis by two-way ANOVA with Bonferroni post-test, comparing against the relevant DMSO (vehicle) control and time point; *$p<0.05$, **$p<0.01$, ***$p<0.001$. Graphs show mean of data points (normalised to vehicle within relevant timepoint) ± SEM.

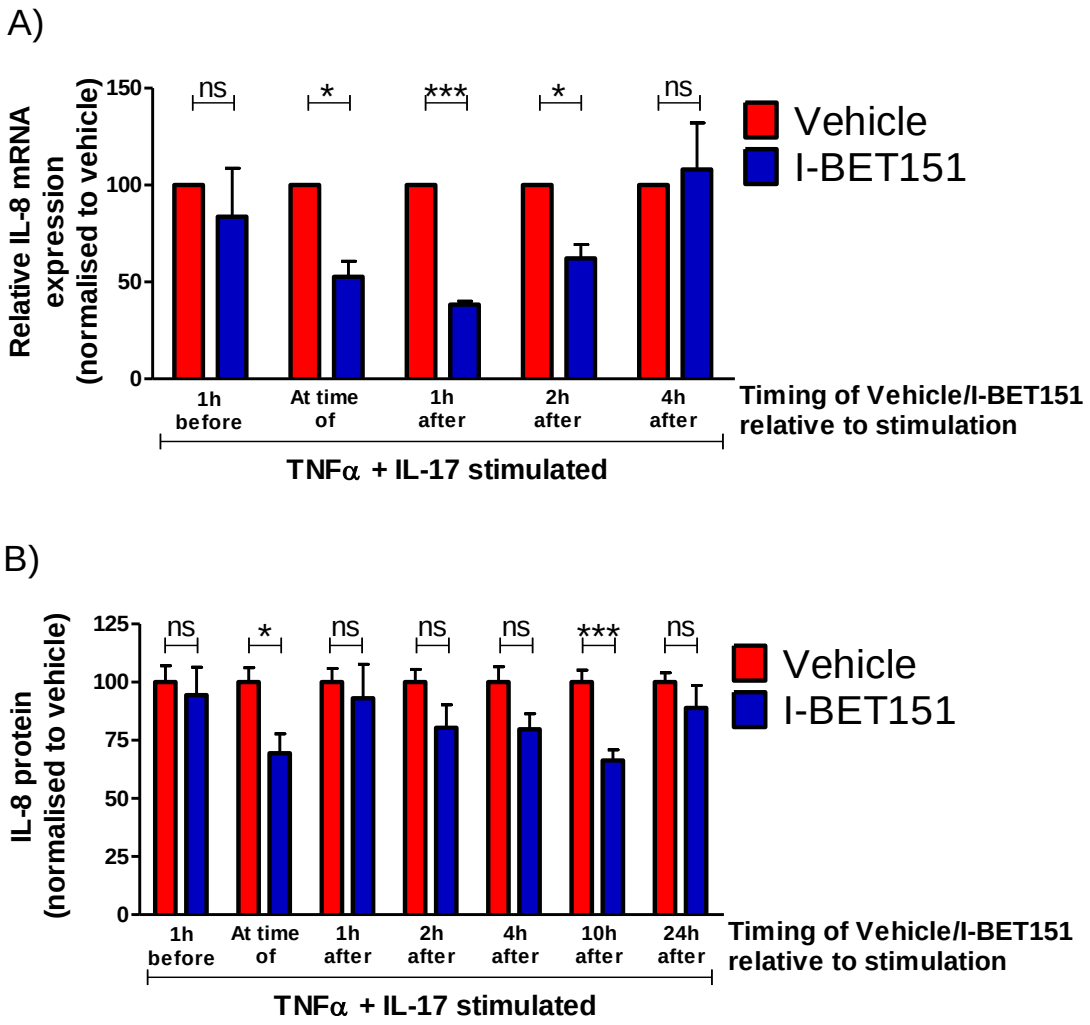


Figure 4-10: I-BET151 was pharmacologically active when added after TNFα + IL-17 stimulation

NHEKs were treated with I-BET151 (1μM) or vehicle (DMSO, 1:1200) either 1h prior to stimulation with TNFα (10ng/ml) + IL-17 (100ng/ml), at time of stimulation or afterwards (at timepoints indicated below graphs). Cells were harvested 6h post-stimulation and IL-8 mRNA expression (**A**) determined by qRT-PCR. Supernatants were collected 48h post-stimulation and secreted IL-8 protein (**B**) concentration measured by ELISA. (for mRNA: N=3; for protein: N=3, with each donor performed in triplicate repeat) Analysis by two-way ANOVA with Bonferroni post-test, comparing against the relevant DMSO (vehicle) control and time point; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points (normalised to vehicle within relevant timepoint) ± SEM.

4.4. Discussion

In this chapter the effects of a specific BET inhibitor, I-BET151, on keratinocyte pro-inflammatory responses were investigated. I-BET151 treatment inhibited expression of IL-6 and IL-8 genes as well as secretion of IL-6 and IL-8 protein in response to TNFα + IL-17 stimulation. These effects could not be attributed to relatively minor effects of I-BET151 on cellular metabolism or total protein. These data indicate for the first time that BET proteins are involved in regulating expression of pro-inflammatory genes in keratinocytes and notably, in an *in vitro* model of cutaneous inflammation relevant to psoriasis, we demonstrated that BET proteins were transiently recruited to the promoter region of pro-inflammatory genes in response to stimuli directly relevant to disease. Furthermore inhibition of IL-6 and IL-8 mRNA induction could be explained by the reduction in recruitment of BET proteins and pol II (S2P) to the promoter region of these genes in the presence of I-BET151. These novel findings suggest BET proteins are involved in the regulation of genes relevant to psoriasis in keratinocytes and that BET proteins could represent novel targets for psoriasis treatment.

I-BET151 inhibited both IL-6 and IL-8 mRNA expression in TNFα + IL-17 stimulated NHEKs with statistically similar IC_{50} and maximum effects. I-BET151 also inhibited IL-6 and IL-8 secreted protein responses with statistically similar potency, IC_{50} = 44.6nM (95% CI 36.1 – 53.1) and 59.0nM (95% CI 29.5 – 88.5) for IL-6 and IL-8, respectively, in I-BET151 sensitive-donors. Inhibition of IL-6 inflammatory response by I-BET151 has previously been described; in lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells, I-BET151 inhibited IL-6 secreted protein response, with an IC_{50} = 160nM (Dawson *et al.*, 2011). These findings demonstrate IL-6 secreted protein response to TNFα + IL-17 stimulation in NHEKs, was more sensitive to I-BET151 treatment than IL-6 secreted protein response to LPS stimulation in peripheral blood mononuclear cells or human whole blood. Differential sensitivity to I-BET151 could suggest that, while BET proteins are likely to be involved in the inflammatory response in a variety of cell types, they may play in different roles different cell types or in response to different stimuli.

TNFα + IL-17 stimulated transient recruitment of Brd2/3/4 proteins as well as pol II (S2P) to the promoter region of both IL-6 and IL-8; this is in keeping with the positive

effects of TNFα + IL-17 stimulation on IL-6 and IL-8 expression (section 3.3.2). This was also consistent with previously published data which showed that Brd2/3/4 proteins are recruited to the IL-6 gene promoter after LPS stimulation (Nicodeme *et al.*, 2010). In addition Brd4 was previously shown to be recruited to the IL-8 promoter in airway smooth muscle cells stimulated with fetal calf serum (FCS) + transforming growth factor-beta (TGF-β); recruitment of Brd2/3 was not investigated in this model (Perry *et al.*, 2015). In TNFα + IL-17 stimulated NHEKs, there appeared to be ~35 fold greater enrichment of Brd4 to the IL-6 promoter compared to the IL-8 promoter (~3% input and ~0.08% input respectively), whereas the IL-8 promoter demonstrated relatively greater enrichment of Brd3 (~1% input compared to ~0.03% input at the IL-6 promoter). Although it is difficult to make direct comparisons about enrichment of different epitopes, as enrichment will be influenced by antibody affinity to the target protein, these results suggest differential regulatory roles of Brd2/3/4 for different genes in keratinocytes. These findings are supported by findings in LPS-stimulated macrophages, where enrichment of Brd2 at the TNFα promoter was approximately twice that at the IL-6 promoter (~0.16% and ~0.09% respectively); in comparison enrichment of Brd3 at the TNFα promoter was 20-times that at the IL-6 promoter (~0.4% and ~7%, respectively) (Nicodeme *et al.*, 2010).

I-BET151 treatment of TNFα + IL-17 stimulated NHEKs, significantly inhibited the recruitment of Brd4 and pol II (S2P) to the IL-6 promoter and although there were trends for blocking Brd2/3 binding at all timepoints assayed these effects were not significant. At the IL-8 promoter, I-BET151 significantly inhibited binding of Brd3 and Brd4 as wells as pol II (S2P), with a non-significant trend for inhibiting Brd2 binding at all timepoints assayed. Similar to our findings in keratinocytes, in LPS stimulated macrophages, treatment with a BET inhibitor I-BET762 which is structurally distinct from I-BET151, significantly inhibited binding of Brd3, Brd4 and pol II (S2P) to the IL-6 promoter, but not Brd2 (Nicodeme *et al.*, 2010). In FCS + TGF-β stimulated ASM cells with JQ1 (a third BET inhibitor, widely used in research as a preclinical tool compound) blocked recruitment of Brd4 to the IL-8 promoter (Perry *et al.*, 2015). Although I-BET151 targets Brd2,3,4, the affinities of I-BET151 for Brd2, Brd3 and Brd4 differ as determined by ligand displacement assays (Dawson *et al.*, 2011), together these results support the hypothesis that BET proteins have different regulatory functions for different genes, which are likely to be context dependent (on both stimulus and cell type) and could suggest that in keratinocytes Brd4 plays a relatively more

important role in regulation of IL-6; whereas at the IL-8 promoter, both Brd3 and Brd4 play important roles. It would be interesting to understand if disease states may differentially affect recruitment of BET proteins in keratinocytes, as previously demonstrated in ASM cells derived from asthmatic and non-asthmatic donors, and therefore whether these might represent novel targets for therapy (Perry *et al.*, 2015).

Tracking with the greater enrichment of Brd4 at the IL-6 promoter, compared to the IL-8 promoter, there was also greater enrichment of p65 at the IL-6 promoter. Furthermore, changes in p65 binding tracked with changes in Brd4 binding to the IL-6 promoter and I-BET151 inhibited both Brd4 and p65 binding to the IL-6 promoter, although this was only a trend for p65. These findings are consistent with Brd4 being a co-factor for p65 binding (Huang *et al.*, 2009); the tandem bromodomains of Brd4 have previously been shown to have co-operative effects on binding to acetylalted-lysine-310 of p65, this in turn leads to the Brd4 induced activation of cyclin dependent kinase 9, leading to the phosphorylation and activation of RNA polymerase II which ultimately drives transcription (Huang *et al.*, 2009). As I-BET151 did not block total p65 binding to the IL-6 or IL-8 promoter, it may be informative to perform ChIP studies with a specific antibody against acetylalted-lysine-310 of p65, although this may be difficult to detect if levels are very low. The differential enrichment of Brd4/p65 and Brd3 at IL-6 and IL-8 promoters supports the suggestion that IL-6 and IL-8 are differentially regulated and the relatively greater enrichment of Brd4/p65 at the IL-6 promoter could suggest the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway plays a relatively greater role in regulating IL-6 expression compared to IL-8 expression. In support of this, as previously discussed (section 3.4) in Jurkat T cells IL-6 expression was closely associated with the activation pattern of NK-kB, whereas IL-8 expression was more closely associated with the activation pattern of nuclear factor of activated T cells (NFAT) (Khalaf *et al.*, 2013). Our finding that, in NHEKs stimulated with TNFα + IL-17, ciclosporin had a greater inhibitory effect on IL-8 induction compared to IL-6 (section 0), could be in keeping with Khalaf *et al.*'s findings. It would be interesting to understand, in further ChIP studies of TNFα + IL-18 NHEKs, whether NFAT shows greater enrichment at the IL-8 promoter than at the IL-6 promoter.

The second tandem bromodomain (BD2) of Brd4 is known to bind and direct recruitment of a kinase, positive transcription elongation factor b (*pTEFb*), to RNA polymerase II, thus regulating RNA polymerase II-dependent transcriptional elongation,

(Jang *et al.*, 2005; Schroder *et al.*, 2012), this may represent an additional mechanism through which BET proteins regulate IL-6 and IL-8 responses in keratinocytes. Therefore, in addition to I-BET151 inhibiting BET binding to regulatory regions of TNFα + IL-17-responsive genes in NHEKs, I-BET151 may also act through additional mechanisms including disrupting Brd4 binding to acetyl-p65 as well as *pTEFb*.

Interestingly, I-BET151 had a more potent and sensitive effect on IL-6 secreted protein compared to its effect on mRNA expression (maximal inhibition of IL-6 protein = 91.2% (95% CI = 88.9 – 93.5) and IC_{50} = 44.6nM (95% CI = 36.1 – 53.1) compared to maximal inhibition of IL-6 mRNA expression = 69.5% (95% CI = 64.2 – 74.8) and IC_{50} = 133.6nM (95% CI = 86.5 – 180.7), whereas I-BET151 had statistically similar effects on IL-8 mRNA and protein responses to TNFα + IL-17 stimulation, in I-BET151 sensitive donors (Figure 6-4E). For the first time, our results demonstrate differential effects of I-BET151 on IL-6 mRNA vs. secreted protein and suggest BET inhibition may have post-transcriptional effects. These effects could be through I-BET151 i) inhibiting the function of proteins/molecules with post-transcriptional roles *e.g.* mRNA translation or protein trafficking, or ii) inhibiting expression of these proteins/molecules (section 6.3.3). An increased inhibitory effect on protein compared to mRNA has previously been observed in compounds targeting regulators of the epigenome. In a model of rheumatoid arthritis trichostatin A (TSA), an histone deacetylase (HDAC) inhibitor (HDACi) blocked IL-6 induction by IL-1β in fibroblast-like synoviocytes (Grabiec *et al.*, 2012). Grabiec *et al*. demonstrated that 1μM TSA had a greater inhibitory effect on secreted IL-6 protein (over 80% inhibition) compared to the effect on IL-6 mRNA (70% inhibition) and it was demonstrated that treatment with TSA (or ITF2357, an HDACi) lead to accelerated decay in IL-6 mRNA (Grabiec *et al.*, 2012). Grabiec *et al*. also observed this effect in LPS stimulated monocyte-derived macrophages, suggesting accelerated IL-6 mRNA decay could be a common mechanism by which IL-6 translation is suppressed by HDAC inhibition and may explain the differential effects of HDAC inhibition (and potentially BET inhibition) observed on IL-6 mRNA vs. protein responses. Grabiec *et al*. hypothesised HDACi might modulate the expression of specific microRNAs, which are known to regulate mRNA stability, although no specific microRNAs for IL-6 have yet been identified. Additionally, Grabiec *et al*. hypothesised that HDACs might associate with proteins involved in selective regulation of IL-6 mRNA stability; Zc3h12 RNAse is known to both associate with HDACs and regulate IL-6 mRNA stability (Matsushita *et al.*, 2009).

Interestingly it has previously been shown that BET inhibitors and HDAC inhibitors both modulate similar genes, with similar overall biological effects and can have synergistic effects when assayed in combination (Bhadury *et al.*, 2014; Heinemann *et al.*, 2015), therefore there may be shared mechanisms between HDAC inhibitors and I-BET151 to explain the differential effects of I-BET151 on IL-6 mRNA *versus* protein.

Of note, Grabiec *at al*., reported that their preliminary data suggested HDAC inhibition had no effect on IL-8 mRNA stability (Grabiec *et al.*, 2012). Interestingly, although I-BET151 had donor-dependent effects on IL-8 protein responses to TNFα + IL-17 stimulation, in I-BET151-responsive donors the effects of I-BET151 on IL-8 mRNA vs. protein were more consistent, compared to effects on IL-6; maximal inhibition of IL-8 protein = 63.3% and IC_{50} = 59.0nM compared to maximal inhibition of IL-8 mRNA expression = 61.4% and IC_{50} = 55.5nM. This could suggest that, unlike the effects on IL-6, I-BET151 did not have post-transcriptional regulatory effects on IL-8 mRNA and further supports the suggestion that BET and HDAC inhibitors have similar biological effects (Grabiec *et al.*, 2012).

In NHEKs stimulated with TNFα + IL-17, it was demonstrated that BET inhibition had a consistent effect on IL-6 secreted protein responses across donors, but a more variable effect on secreted IL-8. Some donors showed little IL-8 sensitivity to low concentrations of I-BET151, and robust I-BET151 concentration vs. IL-8 protein responses could not be generated in these donors. Even in donors that demonstrated IL-8 sensitivity to I-BET151, their I-BET151 concentration vs. IL-8 protein responses showed considerable donor variability. A similar observation has been described in airway smooth muscle cells (ASM) stimulated with FCS + TGF-β in the presence of the BET inhibitor, JQ1, which showed donor-dependent effects on secreted IL-8 protein but more consistent effects on secreted IL-6 protein across donors (Perry *et al.*, 2015). For example, 10nM JQ1 consistently inhibited secreted IL-6 protein by ~10-15% across different groups of donors but inhibited secreted IL-8 protein by ~40% healthy controls, ~25% in the non-severe asthma group and appeared to enhance IL-8 release by ~15% in the severe asthma group (Perry *et al.*, 2015). In the severe asthma group a JQ1 concentration of 300nM was required to inhibit secreted IL-8 protein by ~40% (Perry *et al.*, 2015). Interestingly, ASM from asthmatics demonstrated greater basal expression of IL-6 and IL-8 compared to healthy controls and in keeping with this observation, Perry *et al*. reported greater enrichment of Brd4 at IL-6 and IL-8 promoters in ASM from asthmatic patients compared to healthy controls. Furthermore ASM from severe

asthmatics demonstrated greater IL-6 and IL-8 responses to stimulation with increased enrichment of Brd4 to the IL-8 (but not IL-6) promoter, compared to ASM from non-severe asthmatics, although this difference was a trend and not significant. Perry *et al*. hypothesised that increased basal recruitment of Brd4 to the IL-8 promoter in severe asthmatics could explain the reduced sensitivity and variability of IL-8 responses (but not IL-6 responses) to BET inhibition.

In order to understand if similar findings could explain donor-dependent IL-8 responses to I-BET151 treatment in NHEKs, the underlying histological diagnosis of donor samples were investigated, but no correlation between severity of underlying inflammation (as determined by histological analysis of donor samples) and NHEKs IL-8 sensitivity to I-BET151 could be demonstrated (Figure 4-3 and Table 4-1). However, the underlying histological diagnosis of donors may need to be interpreted with caution, as typically a macroscopically diseased portion of a sample would be sent for histological diagnosis and the remainder of the sample would be donated, with patient consent, for research. Therefore the histological diagnosis of the macroscopically diseased portion of a sample might not necessarily reflect pathology, if any, present in the portion donated for research and used in these experiments. It may have been informative to perform ChIP studies in NHEKs whose IL-8 response was relatively insensitive to I-BET151 treatment, to understand if TNFα + IL-17 stimulation lead to greater enrichment of BET proteins to the IL-8 promoter compared to NHEKs which were more sensitive I-BET151 treatment.

On the basis of our findings, it can be concluded that TNFα + IL-17 stimulation results in chromatin remodelling, facilitating histone hyperacetylation and thus transient recruitment of BET protein to the promoter region of response genes. We hypothesised that I-BET151 would demonstrate inhibition of IL-6 and IL-8 expression when added after stimulation with TNFα + IL-17, during the phase of active chromatin remodelling. Indeed I-BET151 inhibited IL-6 and IL-8 mRNA expression when added up to 4h after TNFα + IL-17 stimulation, consistent with the observation that maximal recruitment of BET proteins to IL-6 and IL-8 promoters occurred at ~4.5h post-stimulation. Interestingly, I-BET151 had a greater inhibitory effect on both IL-6 and IL-8 transcription when added at time of stimulation, compared to addition 1h before stimulation, and the most significant inhibition was observed when I-BET151 was added 1h after stimulation. It is uncertain why this phenomenon was observed but we

postulate that in unstimulated cells where there was low IL-6 and IL-8 expression, chromatin associated with these promoters would be in a "closed"/inactive state (heterochromatin) with little accessibility for BET protein binding. Therefore when I-BET151 is added before stimulation, the compound may be sequestered to promoters of other actively transcribed genes (where BET proteins are bound) which could limit the availability of compound when cells are later stimulated, with TNFα + IL-17. However if I-BET151 is added at time of or after stimulation, during the phase of chromatin remodelling, chromatin associated with IL-6 and IL-8 promoters will be expected to rapidly become "open"/active (euchromatin) facilitating binding of BET proteins, and I-BET151 might be expected to have most access to actively transcribed genes (*e.g.* IL-6 and IL-8). Therefore addition of compound at time of stimulation or shortly afterwards may be expected to have most effect on TNFα + IL-17-sensitive genes. As it was observed that BET recruitment was maximal 4.5h after TNFα + IL-17 stimulation, it is unlikely that inhibition of IL-6 and IL-8 protein responses, by I-BET151 added at 10h or 24h after stimulation, would be through inhibiting BET binding to IL-6 and IL-8 promoters. Potential post-transcriptional effects of BET inhibition are discussed in sections 6.3.3 and 6.4, and it is perhaps more likely that it is through these mechanisms predominate when I-BET151 was added 10h or 24h after stimulation. Interestingly addition of I-BET151 1h before TNFα + IL-17 stimulation had no significant effect on IL-8 mRNA response in these donors (Figure 4-10), in contrast to the significant effects in other donors (Figure 4-2) and the effects on IL-8 protein response were variable; these findings further confirm that I-BET151 has donor-dependent effects on IL-8 response to TNFα + IL-17 stimulation. As discussed in section 4.3.2, maximal effects and sensitivities to I-BET151 in different donors were not clearly related to the stimulation window or to the underlying histological diagnosis in donor tissue.

In NHEKs stimulated with TNFα + IL-17, I-BET15 blocked binding of Brd2/3/4 as well as pol II (S2P) to a region previously defined as an IL-6 enhancer in macrophages (Qiao *et al.*, 2013). However, as discussed in sections 5.3.3 and 5.4, the epigenetic modifications (induced by TNFα + IL-17 stimulation) at this region suggest it does not function as an IL-6 enhancer in keratinocytes. In addition to enhancer regions being characterised by high levels H3K27ac and H3K4me1 (with a high ratio of H3K4me1 to H3K4me3), DNAseI hypersensitivity, binding of co-activators, transcription factors and RNA polymerase II when enhancers are in their active state (Calo and Wysocka, 2013)

(section 1.3.4), a further characteristic of enhancers, especially "super enhancers", can be the relatively greater enrichment Brd4 (Brown *et al.*, 2014). Brown *et al*. demonstrated that in human endothelial cells activation via the NF-κB pathway by TNFα stimulation, resulted in increased co-localisation of p65 and Brd4 to inflammatory gene promoters as well as H3K27ac-enriched *de-novo* inflammatory enhancers (Brown *et al.*, 2014). However results in TNFα + IL-17 stimulated NHEKs demonstrated ~6 fold lower enrichment of Brd4 at the region previous described as an IL-6 enhancer in macrophages compared to the IL-6 promoter and p65 enrichment at these two regions was broadly comparable. The relatively poor enrichment of Brd4 and p65 together with the lack of enrichment of H3K27ac and H3K4me1 after TNFα + Il-17 stimulation (section 5.3.3), suggest that the region identified as an IL-6 enhancer in macrophages does not have the same function in keratinocytes and supports the view that enhancer regions are cell-type specific (Heinz *et al.*, 2015). As this region does not appear to function as an IL-6 enhancer in keratinocytes and its function has not been defined, it is unclear what the functional relevance of BET recruitment to this region is or what the consequences of inhibiting BET protein binding are.

4.5. Future work

In summary stimulation of NHEKs with TNFα + IL-17 demonstrated BET epigenetic reader proteins are recruited to the promoter region of pro-inflammatory genes in response to stimuli relevant to the pathogenesis of psoriasis. Furthermore, using BET inhibitor I-BET151 as a pharmacological tool to further understand the relevance of BET proteins in regulating expression of pro-inflammatory genes in keratinocytes, we showed that BET bromodomains are involved in mediating the inflammatory cytokine responses, confirming the suggestion that BET proteins could represent novel targets in the treatment of psoriasis (Nadeem *et al.*, 2015). Importantly we showed that inhibition of IL-6 and IL-8 responses by I-BET151 can be attributed to the blockade of BET protein and pol II (S2P) recruitment to hyperacetylated IL-6 and IL-8 promoters respectively, demonstrating the tractability of targeting this epigenetic process. Additionally I-BET151 profoundly blocked IL-6 protein release suggesting that the compound may also modulate post-transcriptional mechanisms to impair protein synthesis and or secretory functions.

A number of key questions remain:

- Which proteins/molecules are targeted by I-BET151 to account for the differential effects on IL-6 mRNA vs. protein response to TNFα + IL-17 stimulation and which other responses are affected? (section 6.3.3)
- What factors are responsible for donor-dependent IL-8 protein sensitivity to I-BET151, which other responses demonstrate this variability and how are these relevant to psoriasis?
- In addition to inhibiting recruitment of BET proteins to regulatory regions of I-BET151-senstitive genes, does I-BET151 mediate the effects through any additional mechanisms *e.g.* inhibiting Brd4 binding to acetyl-p65 or *pTEFb* for example?
- In the NHEKs model of cutaneous inflammation, apart from IL-6 and IL-8, which other genes are regulated by TNFα + IL-17 stimulation, how are these relevant to psoriasis, are they regulated by BET proteins and can their expression be modulated by BET inhibitors? (section 6.3)

4.6. Conclusions

- TNFα + IL-17 stimulated transient differential recruitment of BET proteins to IL-6 and IL-8 promoter regions with relatively greater enrichment of Brd4 to the IL-6 promoter and relatively greater enrichment of Brd3 to the IL-8 promoter.
- Inhibition of cytokine responses by I-BET151 could not be accounted for by its negative effects on cellular metabolism or total protein alone.
- ChIP studies demonstrated that I-BET151 blocked Brd4 and pol II (S2P) recruitment to the IL-6 promoter, and blocked recruitment of both Brd3/4 and pol II (S2P) to the IL-8 promoter. These findings link BET proteins to pol II recruitment in keratinocytes and support the hypothesis that BET inhibitors mediate their effects on gene transcription through inhibiting binding of BET proteins to gene regulatory regions. These data also support the hypothesis that distinct BET proteins regulate expression of IL-6 and IL-8 in TNFα + IL-17 stimulated NHEKs.
- There was greater enrichment of p65 at the IL-6 promoter, than at the IL-8 promoter, and p65 tracked with changes in Brd4 binding at the IL-6 promoter and, in addition to I-BET151 blocking Brd4 binding to the Il-6 promoter, there was a trend for I-BET151 inhibiting p65 binding to the IL-6 promoter. Together

these findings suggest Brd4/p65 make play a relatively greater role in regulating expression of IL-6 compared to IL-8.

- I-BET151 had donor dependent effects on secreted IL-8 responses to TNFα + IL-17 stimulation and a differential effect on IL-6 mRNA *versus* secreted protein responses to TNFα + IL-17 stimulation, suggesting that BET inhibition has post-transcriptional effects. This could be through functional inhibition of proteins involved in *e.g.* mRNA translation/stability or cytokine secretion or, alternatively, through inhibiting expression of these proteins. (section 6.3.3)
- I-BET151 was able to inhibit expression of IL-6 and IL-8 when added after TNFα + IL-17 stimulation, consistent with chromatin remodelling taking place after stimulation.
- The lack of enrichment of Brd4 and p65 to the IL-6 enhancer defined previously in macrophages (Qiao *et al.*, 2013) suggests this region does not perform the same function in keratinocytes, supporting the general consensus that enhancer functions are cell-type specific.

Chapter 5.

Epigenetic regulation in the *in vitro* model of cutaneous inflammation

5. Epigenetic regulation in the *in vitro* model of cutaneous inflammation

5.1. Introduction

It is increasingly evident that alterations in the epigenome play an important role in regulating inflammatory skin conditions. As discussed fully in section 1.3.5, differential DNA methylation and histone modifications have been associated with psoriasis and some epigenetic modifications were shown to correlate with disease severity and treatment response (Zhang *et al.*, 2011a; Roberson *et al.*, 2012; Trowbridge and Pittelkow, 2014).

In sections 4.3.3 and 4.3.4, it was demonstrated that stimulation of normal human epidermal keratinocytes (NHEKs) with TNFα + IL-17A (IL-17) resulted in transient recruitment of BET proteins and RNA polymerase II to IL-6 and IL-8 promoters, which corresponded to increased expression of these genes. Furthermore IL-6 and IL-8 mRNA responses to TNFα + IL-17 stimulation were sensitive to a BET inhibitor, I-BET151, and these findings could be accounted for by the observation that I-BET151 blocked recruitment of BET proteins and RNA polymerase II to IL-6 and IL-8 promoters. As BET proteins are known to bind acetylated lysine residues, this raised the hypothesis that histone modifications, including acetylation, plays a role in regulating inflammatory responses in keratinocytes.

Acetylation of lysine residues on histones is considered a permissive histone modification, which generally signifies actively transcribed genes; the change from H3K27 tri-methylation (H3K27me3) to acetylation (H3K27ac) is an important switch from gene repression to transcriptional activation (Pasini *et al.*, 2010). In general our understanding of the effects of different histone modifications is limited, although some epitopes have very well defined functions. For example, acetylation of the lysine 9 or lysine 27 residue on histone-H3 (H3K9ac and H3K27ac) is associated with an active promoter in >95% of cases (Ernst *et al.*, 2011). Interestingly the level of methylation at a particular residue affects its function: mono-methylation of the lysine 4 of histone-H3 (H3K4me1) is associated with an active promoter in only 17% of cases, whereas tri-methylation (H3K4me3) is associated with an active promoter in 99% of cases (Ernst *et al.*, 2011).

To further understand the role of histone modifications in the regulation of the psoriasis phenotype histone modifications, including lysine acetylation, at regulatory regions of key effector genes were evaluated in NHEKs stimulated with TNFα + IL-17.

5.2. Aims

- To use the *in vitro* keratinocyte model of cutaneous inflammation to understand the role of histone modifications, including acetylation of lysine residues on histones, in regulating expression of inflammatory genes relevant to psoriasis.

5.3. Results

5.3.1. TNFα + IL-17 stimulation induced RNA polymerase II recruitment, global histone acetylation and hyperacetylation of specific lysine residues associated with active gene expression at the IL-6 promoter

Having observed that stimulation of NHEKs with TNFα + IL-17 increased IL-6 mRNA expression and resulted in a transient increase in BET protein and RNA polymerase II recruitment to the IL-6 promoter (section 4.3.4), we hypothesised that stimulation would lead to chromatin remodelling. Specifically, we hypothesised a mechanism involving hyperacetylation of lysine residues on histones and recruitment of RNA polymerase II (RNA pol II); thus promoting IL-6 expression. To test this hypothesis chromatin immunoprecipitation (ChIP) studies were undertaken to investigate the effect of TNFα + IL-17 on defined histone modifications and RNA pol II recruitment. As expression of IL-6 mRNA was maximal at 6h after TNFα + IL-17 stimulation, we reasoned that any chromatin remodelling and epigenetic changes would precede maximal transcription. NHEKs were therefore harvested at 1h and 4h post-TNFα + IL-17 stimulation for the ChIP experiments. The following ChIP assays were conducted using antibodies directed to:

- RNA polymerase II, phosphorylated (and therefore activated) at its serine 2 residue (pol II (S2P))
- epigenetic epitopes known to positively regulate gene expression (global acetylation of histone H3, acetyl-H3; acetylation of the lysine 9 residue on histone H3, H3K9ac; acetylation of the lysine 27 residue on histone H3, H3K27ac; and tri-methylation of the lysine 4 residue on histone H3, H3K4me3)
- an epigenetic epitope known to have repressive effects on gene expression (mono-methylation of the lysine 4 residue on histone H3, H3K4me1)
- IgG was used as a negative control and anti-pan H3 a positive control
- ChIP studies were also performed using an antibody to tri-methylation of the lysine 27 residue on histone H3 but the data are not shown as the manufacturer (Abcam, Cambridge, UK) subsequently advised the antibody was not specific (with cross-reactivity to H3K4me3 and H3K9me3 as determined by western blot and ELISA), therefore the results were not interpretable.

Co-immunoprecipitated DNA was purified as described in methods section (section 2.9.6) prior to being assayed by qPCR for enrichment of the IL-6 promoter region (using specifically designed primers, (Qiao *et al.*, 2013) in TNFα + IL-17 stimulated NHEKs compared to unstimulated NHEKs. Enrichment of a particular region of DNA (*e.g.* IL-6 promoter) reflects the extent to which this region was associated with the histone modification or protein of interest.

As shown in Figure 5-1, TNFα + IL-17 stimulation resulted in a time-dependent increase in pol II (S2P) recruitment to the IL-6 promoter ($p<0.05$ at 4h). Furthermore, and consistent with the positive effects of TNFα + IL-17 on IL-6 expression, there was a corresponding increase in global H3 acetylation ($p<0.05$ at 4h), as well as hyperacetylation of specific lysine residues on histone H3 known to positively regulate gene expression; H3K9ac ($p<0.01$ at 4h) and H3K27ac ($p<0.05$ at 4h). Although there was a trend for an increase in H3K4me3, this was not significant. In contrast, there was no significant increase in the repressive mark H3K4me1 at the IL-6 promoter, again this was entirely in keeping with the positive effects of TNFα + IL-17 on IL-6 expression.

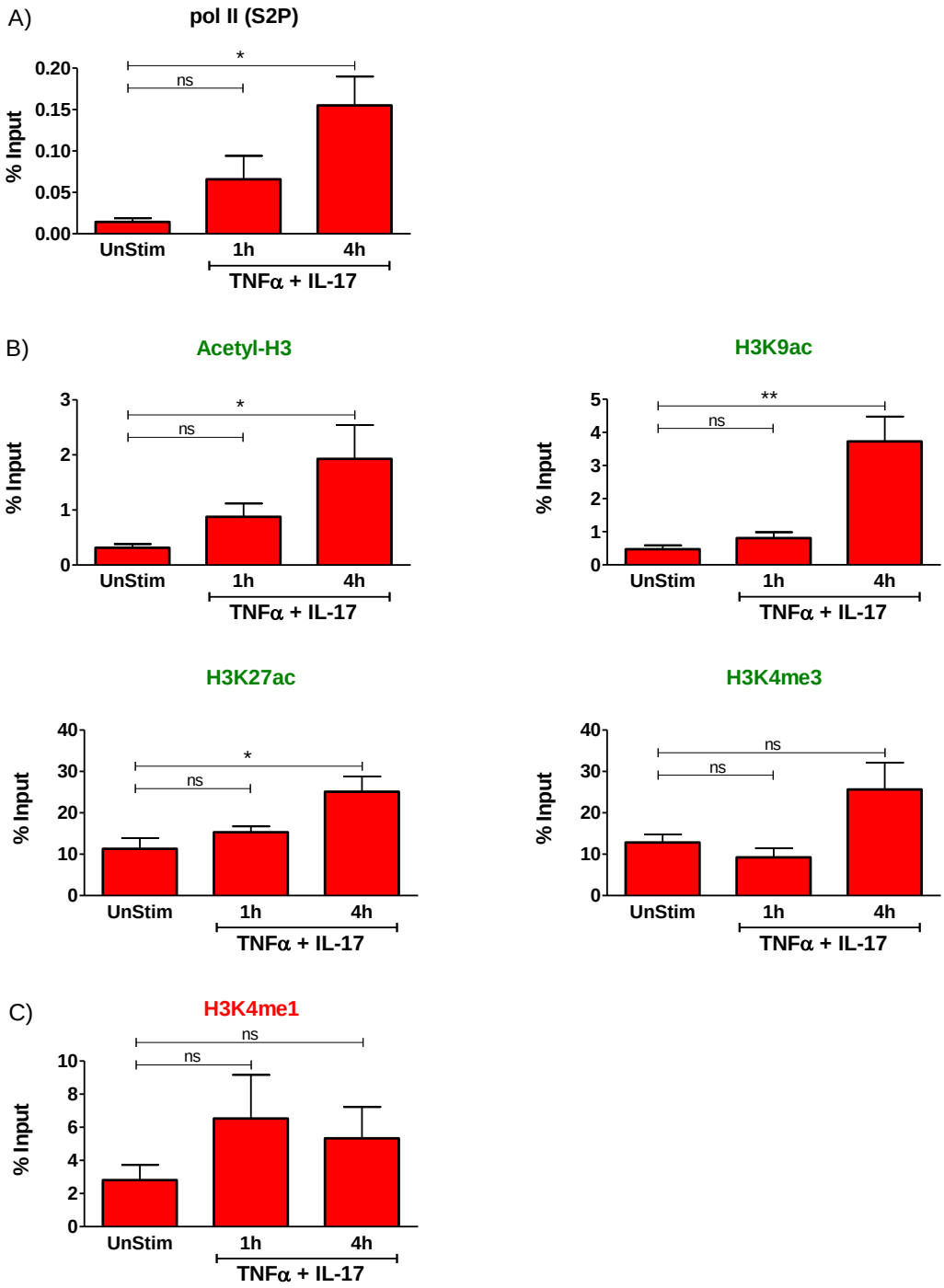


Figure 5-1: At the IL-6 promoter, TNFα + IL-17 stimulation increased active epigenetic epitopes and pol II (S2P) recruitment

NHEKs were stimulated with or without TNFα (10ng/ml) + IL-17 (100ng/ml) and harvested at 1h and 4h post-stimulation for ChIP studies using antibodies to pol II (S2P) (**A**), epigenetic epitopes that positively regulate gene expression, highlighted in green (**B**) and epigenetic epitopes with repressive effects on gene expression, highlighted in red (**C**). Quantification of co-immunoprecipitated DNA was performed by qPCR, using primers to the IL-6 promoter region. (N=3) Analysis by one-way ANOVA with Dunnett's post-test, comparing against the unstimulated media control; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Data points show mean (after subtracting background/non-specific binding and normalised to an input control) ± SEM.

5.3.2. At the IL-8 promoter, TNFα + IL-17 stimulation induced RNA polymerase II recruitment, global histone hyperacetylation and acetylation of specific lysine residues associated with active gene expression

As TNFα + IL-17 increased both IL-6 and IL-8 mRNA expression in NHEKs, we hypothesised that, similar to the effects at the IL-6 promoter, TNFα + IL-17 stimulation would also result in chromatin remodelling and enhance pol II (S2P) recruitment to the IL-8 promoter, thus promoting IL-8 expression. As with IL-6, maximal expression of IL-8 mRNA occurred at 6h post-TNFα + IL-17 stimulation, therefore NHEKs were harvested at 1 and 4h after stimulation with TNFα + IL-17 for ChIP experiments.

Co-immunoprecipitated, purified DNA (samples prepared as per section 5.3.1) were assayed by qPCR for enrichment of the IL-8 promoter region using specifically designed primers (Costa *et al.*, 2013) in TNFα + IL-17 stimulated NHEKs compared to unstimulated NHEKs.

As shown in Figure 5-2, TNFα + IL-17 stimulation resulted in a time-dependent and transient increase in pol II (S2P) recruitment to the IL-8 promoter ($p<0.05$ at 1h). Similar to the effects at the IL-6 promoter, at the IL-8 promoter, TNFα + IL-17 stimulation resulted in increased global H3 acetylation ($p<0.05$ at 1h) and H3K27ac ($p<0.01$ at 1h). There were also trends for increases in other active marks, H3K9ac and H3K4me3, although these were not significant. Post-test analysis for linear trend showed the increase in H3K9ac enrichment was not significant. Interestingly pol II (S2P) recruitment was maximal at 1h for IL-8 but cumulative up to 4h for IL-6. Furthermore the changes in pol II (S2P) recruitment tracked the general changes in active marks at the IL-6 and IL-8 promoter. There was also greater enrichment of pol II (S2P) at the IL-8 promoter than at the IL-6 promoter in both unstimulated and TNFα + IL-17 stimulated NHEKs. There were no significant changes in the repressive mark H3K4me1 at the IL-8 promoter with TNFα + IL-17 stimulation. These findings were consistent with the positive effects of TNFα + IL-17 on IL-8 expression. Of note the enrichment of acetyl-H3 and H3K9ac was approximately 5-10 fold lower at the IL-8 promoter than for the IL-6 promoter, suggesting these genes are differentially regulated.

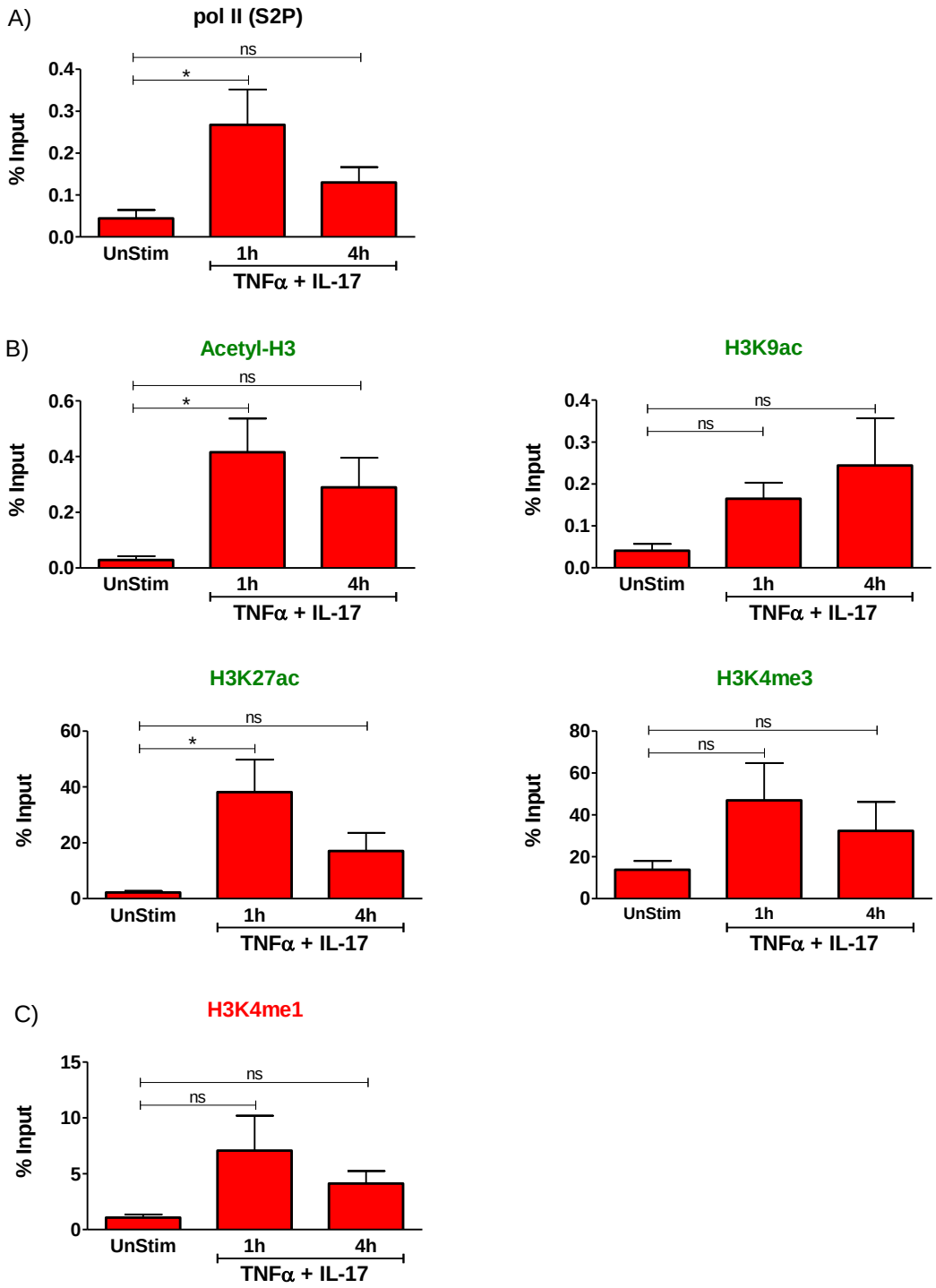


Figure 5-2: At the IL-8 promoter, TNFα + IL-17 stimulation increased active epigenetic epitopes and pol II (S2P) recruitment

NHEKs were stimulated with or without TNFα (10ng/ml) + IL-17 (100ng/ml) and harvested at 1h and 4h post-stimulation for ChIP studies using antibodies to pol II (S2P) (**A**), epigenetic epitopes that positively regulate gene expression, highlighted in green (**B**) and epigenetic epitopes with repressive effects on gene expression, highlighted in red (**C**). Quantification of co-immunoprecipitated DNA was performed by qPCR, using primers to the IL-8 promoter region. (N=3) Analysis by one-way ANOVA with Dunnett's post-test, comparing against the unstimulated media control; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Data points show mean (after subtracting background/non-specific binding and normalised to an input control) ± SEM.

5.3.3. At the potential IL-6 enhancer in keratinocytes, TNFα + IL-17 stimulation had no significant effect on histone modifications or RNA polymerase II binding

Having observed that in ChIP studies of the IL-6 promoter, TNFα + IL-17 enhanced recruitment of pol II (S2P) as well as an increase in permissive epigenetic epitopes, TNFα + IL-17 stimulation was hypothesised to have similar effects at a potential IL-6 enhancer region in NHEKs, thus potentiating IL-6 expression. Although an IL-6 enhancer region has not previously been described in NHEKs, one has previously been identified in primary human macrophages (Qiao *et al.*, 2013) 25kb upstream of the IL-6 transcriptional start site (TSS). This region was therefore evaluated as a potential IL-6 enhancer in NHEKs, by undertaking ChIP studies to investigate the enrichment of epitopes characteristic of enhancers (H3K27ac, H3K4me1and RNA polymerase II (Calo and Wysocka, 2013) to this region in NHEKs. Note, H3K4me1 is associated with active enhancer regions, compared to a generally repressive effect at promoter regions (Ernst *et al.*, 2011; Calo and Wysocka, 2013).

Co-immunoprecipitated, purified DNA samples (prepared as per section 5.3.1) were assayed by qPCR for enrichment of marks at the IL-6 enhancer region as defined in primary human macrophages (forward and reverse primer sequences were as defined in (Qiao *et al.*, 2013)). The ChIP experiment was conducted in TNFα + IL-17 stimulated NHEKs compared to unstimulated NHEKs.

As shown in Figure 5-3, TNFα + IL-17 had no significant effect on pol II (S2P) recruitment to the potential IL-6 enhancer in NHEKs or enrichment of epigenetic epitopes which normally characterise active enhancers, *e.g.* H3K27ac and H3K4me1. Additionally, there was no significant enrichment of acteyl-H3, H3K9ac or H3K4me3 at this region in response to TNFα + IL-17 stimulation. Furthermore, this region demonstrated relatively low enrichment of acetyl-H3, H3K9ac and H3K27ac compared to levels at IL-6 and IL-8 promoter regions.

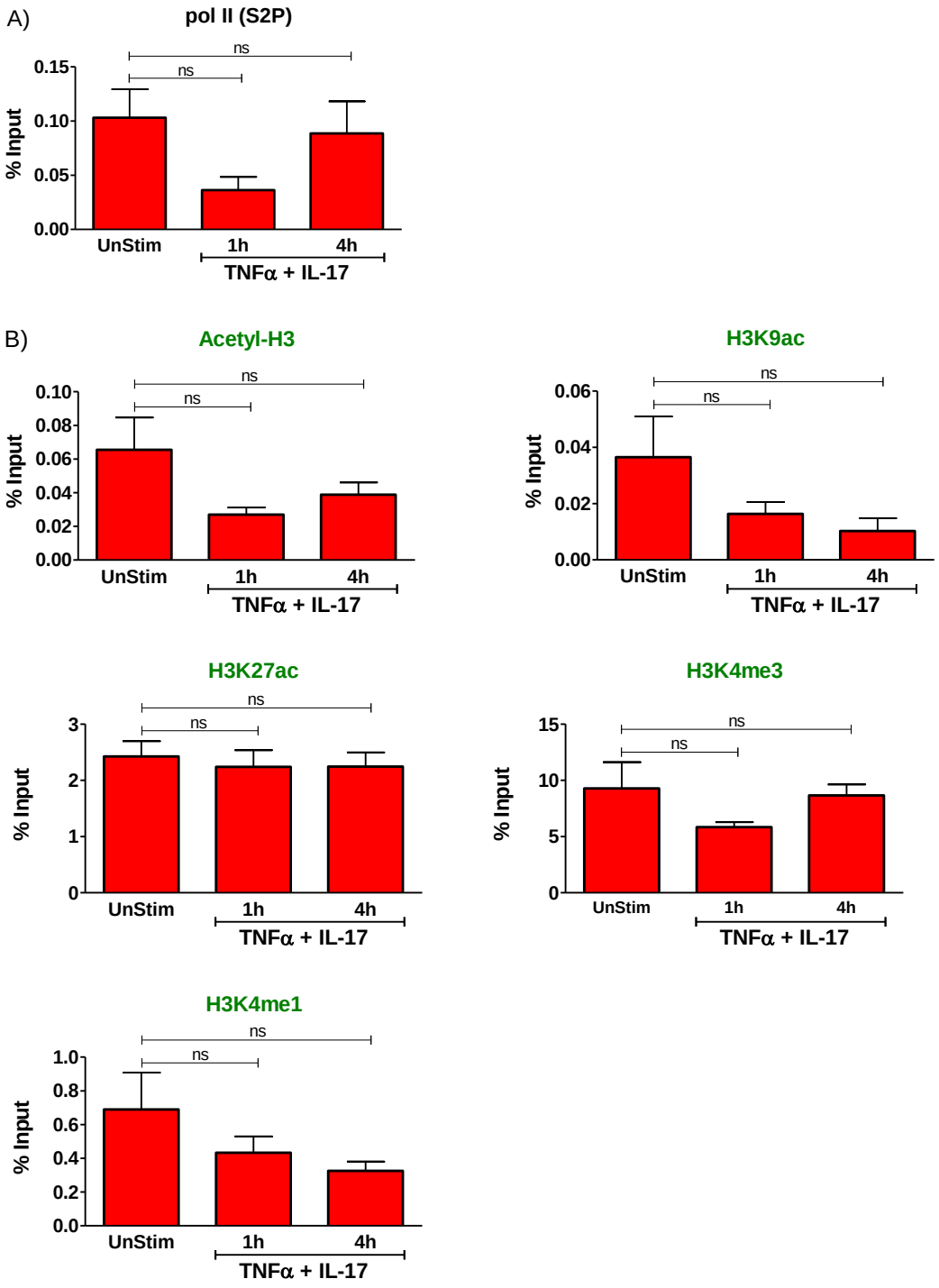


Figure 5-3: At the potential NHEK IL-6 enhancer, TNFα + IL-17 stimulation had no significant effect on active epigenetic epitopes and/or pol II (S2P) recruitment

NHEKs were stimulated with or without TNFα (10ng/ml) + IL-17 (100ng/ml) and harvested at 1h and 4h post-stimulation for ChIP studies using antibodies to pol II (S2P) (**A**) or epigenetic epitopes that positively regulate gene expression (**B**). Quantification of co-immunoprecipitated DNA was performed by qPCR, using primers to the potential IL-6 enhancer region. (N=3) Analysis by one-way ANOVA with Dunnett's post-test, comparing against the unstimulated media control; $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$. Data points show mean (after subtracting background/non-specific binding and normalised to an input control) ± SEM.

5.4. Discussion

The skin and serum of patients with psoriasis are both rich in TNFα and IL-17 (Takahashi *et al.*, 2010) and these cytokines are known to regulate the expression of genes relevant to the pathogenesis of psoriasis (Chiricozzi *et al.*, 2011). TNFα + IL-17 stimulation upregulated IL-6 and IL-8 expression in NHEKs, with maximal expression occurring at 6h post stimulation (section 3.3.2). For the first time in a model relevant to psoriasis, and consistent with the observations above, we demonstrated that TNFα + IL-17 stimulation resulted in chromatin remodelling facilitating histone hyperacetylation and pol II (S2P) binding at the promoter regions of IL-6 and IL-8. These changes preceded maximal transcription. There was greater enrichment of pol II (S2P) at the IL-8 promoter than at the IL-6 promoter, in both unstimulated and stimulated cells; this may explain higher expression of IL-8 compared to IL-6, in both the basal state and in inflammatory responses. These findings provide mechanistic insight into how stimuli relevant to psoriasis regulate inflammatory responses through changes in the epigenome. As histone acetylation is dynamic and the balance between histone acetyl transferase and histone deacetylase activity, which governs the acetylation state of histones, can be modulated with pharmacological treatments, these findings could lead to the identification of novel targets for psoriasis therapy.

Although the ChIP results supported the hypothesis that TNFα + IL-17 stimulation induced chromatin remodelling, the comparative studies also highlighted differences between the epigenome at the IL-6 and IL-8 promoters in response to TNFα and IL-17 stimulation. The results for IL-6 and IL-8 loci are discussed in turn.

At the IL-6 promoter, there were significant increases in permissive histone modifications (acetyl-H3, H3K9ac and H3K27ac, each cumulative up to 4h after stimulation), but no significant change in the inhibitory mark H3K4me1 in response to TNFα + IL-17 stimulation. Although there was a trend for increased enrichment of H3K4me3 at 4h post-TNFα + IL-17 stimulation, this was not significant. Of note, TNFα + IL-17 induced changes in the epigenome preceded maximal IL-6 expression (6h). Furthermore, pol II (S2P) recruitment to the IL-6 promoter tracked with changes in histone acetylation and was also cumulative up to 4h post-stimulation. Together, these findings suggest histone acetylation ultimately drives recruitment of pol II (S2P) to the IL-6 promoter, thus regulating IL-6 expression. Our findings were consistent with epigenetic regulation of IL-6 found in other systems. Sahar *et al*. demonstrated

that angiotensin II treatment of rat vascular smooth muscle cells led to increased H3K9ac enrichment at the IL-6 promoter, with a parallel increase in IL-6 expression (Sahar *et al.*, 2007). In neutrophils and monocytes, stimulation with R848 (a TLR7/TLR8 agonist) induced increases in H3K27ac, with a corresponding increase in IL-6 induction (Zimmermann *et al.*, 2015). Further in keeping with our results, Zimmermann *et al*. found no significant change in the inhibitory mark H3K4me1 early after stimulation (6h) of neutrophils with R848, although there was significant enrichment by 20h after stimulation. It would be interesting in future to understand if NHEKs show a similar delayed increase in H3K4me1 at the IL-6 promoter. Delayed enrichment of inhibitory marks, including H3K4me1, could account for the gradual decline in IL-6 (and IL-8) mRNA expression 6h after stimulation with TNFα + IL-17. It would also be informative to evaluate the effects of prolonged TNFα + IL-17 stimulation on permissive histone modifications (including lysine acetylation) at the IL-6 promoter to investigate the how the balance between permissive and repressive epigenetic marks shift with time, to understand the mechanisms responsible for switching from gene expression to silencing.

At the IL-8 promoter, TNFα + IL-17 stimulation resulted in significant enrichment of acetyl-H3 and H3K27ac, but not H3K9ac. Of interest, enrichment of permissive histone modifications (acetyl-H3 and H3K27ac) at the IL-8 promoter was transient and maximal at 1h after TNFα + IL-17 stimulation. Similar to findings at the IL-6 promoter, pol II (S2P) recruitment to the IL-8 promoter tracked with transient changes in histone acetylation and preceded maximal IL-8 expression (6h). Together, these findings suggest histone acetylation drives recruitment of pol II (S2P) to the IL-8 promoter, thus regulating IL-8 expression. Furthermore, the decline in permissive epigenetic marks and pol II (S2P) recruitment, by 4h post-TNFα + IL-17 stimulation, may explain the gradual decline in IL-8 mRNA expression after 6h stimulation. This data suggests that an increase and subsequent decline in permissive epigenetic marks (rather than changes in repressive epitopes) may be primarily responsible for switching on/off IL-8 expression in NHEKs. However before this conclusion can be drawn, it may be informative to undertake longer timecourse studies to understand if there is a delayed increase in H3K4me1 at the IL-8 promoter, as demonstrated at the IL-6 promoter in neutrophils, as discussed above (Zimmermann *et al.*, 2015). In addition further ChIP studies investigating additional repressive epitopes, *e.g.* H3K27me3, as originally undertaken prior to being contacted by the manufacturer about cross-

reactivity problems with this antibody (section 5.3.1) may aid additional understanding of the switch between gene expression and silencing. In this respect, the change from H3K27 tri-methylation (H3K27me3) to acetylation (H3K27ac) has been shown to act as an important switch from gene repression to transcriptional activation (Pasini *et al.*, 2010).

Our observation that stimulation of NHEKs (with TNFα + IL-17) resulted in histone hyperacetylation at the IL-8 promoter, which in turn drives gene expression, is consistent with previously published findings describing epigenetic regulation of IL-8 expression in keratinocytes. For example, stimulation of human keratinocytes with *Malassezia* furfur, a yeast which signals through TLR2 receptors, increased H3 acetylation with corresponding upregulation of pro-inflammatory cytokine responses including IL-8, as well as IL-1α, IL-6 and TNFα (Angrisano *et al.*, 2013). However, in contrast to the kinetics of IL-8 expression and histone acetylation observed in TNFα + IL-17 stimulated NHEKs, Angrisano *et al*. observed maximal IL-8 gene expression at 4h after stimulation with *Malassezia* furfur and a transient increase in acetyl-H3, which was also maximal at 4h after-stimulation and returned to baseline by 24h. These differences may be due to the different stimuli used, although Angrisano *et al*. did not perform ChIP studies prior to the 4h timepoint, when maximal expression of IL-8 was observed, and it is likely that chromatin would undergo remodelling prior to maximal IL-8 gene expression. For these reasons, direct comparisons of kinetics of histone modifications would be difficult.

As described in section 3.3.3, basal expression of IL-8 protein, in unstimulated NHEKs, was higher than expression of IL-6 protein and the magnitude of IL-8 mRNA and protein responses to TNFα + IL-17 stimulation were greater compared to IL-6 responses. It was therefore hypothesised that IL-6 and IL-8 genes were under differential epigenetic regulation. In support of this hypothesis, the enrichment of acetyl-H3 and H3K9ac was approximately 5-10 fold higher at the IL-6 promoter than at the IL-8 promoter. Furthermore, and consistent with this observation, there was approximately 35 fold greater enrichment of Brd4 at the IL-6 promoter compared to the IL-8 promoter (sections 4.3.4 and 4.3.5). Although enrichment of pol II (S2P) tracked with histone acetylation at the promoter region of IL-6 and IL-8, as discussed above, the increased enrichment of histone modifications (acetyl-H3 and H3K9ac) and Brd4 at the IL-6 promoter was not reflected by increased recruitment of pol II (S2P). The opposite

was actually observed *i.e.* enrichment of pol II (S2P) was greater at the IL-8 promoter compared to enrichment at the IL-6 promoter; approximately 2.9 fold higher at the IL-8 promoter compared to IL-6 promoter in unstimulated cells and approximately 1.6 fold higher at the IL-8 promoter compared to IL-6 promoter in TNFα + IL-17 stimulated cells (Figure 5-1 and Figure 5-2). Increased recruitment of pol II (S2P) at the IL-8, promoter than at the IL-6 promoter, may partly explain the greater expression of IL-8 *versus* IL-6 in both unstimulated and TNFα + IL-17 stimulated NHEKs. As differences in epigenetic modifications and Brd4 recruitment could not account for differences in pol II (S2P) recruitment to IL-6 and IL-8 promoters, it is likely that additional epigenetic marks/transcription factors and co-activators are also involved in regulating recruitment of pol II (S2P), thus IL-6/-8 expression, in keratinocytes. In this respect increased di-methylation of the lysine 4 of histone-H3 (H3K4me2) is associated with increased IL-8 expression in keratinocytes and hyperacetylation of H4 is associated with increased IL-6 expression in neutrophils (Angrisano *et al.*, 2013; Zimmermann *et al.*, 2015). In addition Brd4 is known to bind Mediator, a multi-protein co-activator that may facilitate interactions between transcription factors and RNA polymerase II (Wu and Chiang, 2007) and Brd4/Mediator complexes have been identified at the transcription start site of many genes across the genome (Loven *et al.*, 2013). It may therefore be informative to direct future investigations towards understanding how these additional factors may regulate cutaneous inflammation. Additional regulatory regions, *e.g.* enhancers, may also be involved in regulating cutaneous inflammatory responses, although these have not been previously defined for IL-6/-8 in keratinocytes. It was also noted that the differences in kinetics of histone modifications and pol II (S2) recruitment at IL-6 and IL-8 promoters (Figure 5-1 and Figure 5-2) were not reflected by differences in kinetics of IL-6 and IL-8 expression (section 3.3.2). This supports the hypothesis that additional factors, or regulatory regions, are also involved in regulating cutaneous inflammation, as discussed above.

It is known that BET proteins bind acetylated lysine residues on protein, including histones. It was shown that in NHEKs stimulation with TNFα + IL-17 resulted in chromatin remodelling and a significant increase in global acetylation of histone H3 at IL-6 and IL-8 promoters, as well as acetylation of specific lysine residues associated with active promoters (at the IL-6 promoter H3K9ac and H3K27ac, at the IL-8 promoter H3K27ac and a trend for increased H3K9ac). Furthermore, at the IL-8 promoter maximal enrichment of permissive epitopes and pol II (S2P) was at 1h post-stimulation,

but lagged at the IL-6 promoter where maximal enrichment was at 4h post-stimulation. These differences in kinetics of lysine acetylation at the IL-6 vs. IL-8 promoter did not appear to translate to differences in kinetics of BET recruitment to IL-6 and IL-8 promoters; BET proteins showed transient recruitment, which tended to be maximal at 4.5h post-stimulation, at both IL-6 and IL-8 promoters. As the differences in kinetics of lysine acetylation at IL-6 and IL-8 promoters was not reflected by differences in timing of BET recruitment to these regulatory regions, these findings could suggest that while H3K9ac and H3K27ac are both associated with active promoters, they may not be (the only) binding targets of BET proteins. However definitive conclusions may be difficult to draw as acetylation of other lysine residues were not investigated.

Furthermore, ChIP experiments in NHEKs investigating effects on epigenetic modification (sections 4.3.4 and 4.3.5) and BET recruitment were performed in different donors, which may have shown subtle differences in kinetics as suggested by the different binding profiles of pol II (S2P) to IL-6 and IL-8 promoters in these two experiments. Investigations into BET proteins binding targets suggest regulation of binding is highly complex and that BET proteins might recognise a pattern of histone modifications not simply single acetyl-lysine residue, and modifications other than acetylation might also play a role. For example, histone di-acetylation can enhance Brd4 binding in a lysine-residue specific manner; di-acetylated H3 (H3K9acK14ac) and di-acetyl H4 (H4K5acK12ac) enhanced Brd4 binding compared to mono-acetylation at each lysine residue alone but an alternative di-acetyl H4 (H4K8acK16ac) had no effect on Brd4 binding (Dey *et al.*, 2003) and binding of Brd4 to di-acetyl H3 (H3K4acK9ac) is enhanced by the phosphorylation of the H3 threonine residue at position 3 (H3T3pK4acK9ac) (Filippakopoulos and Knapp, 2012). Investigation of BET binding patterns through ChIP in primary donors is therefore unlikely to be a particularly efficient method of screening. Further investigations through ChIP-sequencing could be an informative method to discover where, for example, Brd4 (other BET proteins), p65 or acetyl marks map within the genome in order to further understand their regulatory roles. Advances in technology using low cell numbers could allow this to be performed in activated primary keratinocytes in the future (Mundade *et al.*, 2014; Jakobsen *et al.*, 2015)

In contrast to the observations and the IL-6 and IL-8 promoters which were transcriptionally activated, TNFα + IL-17 stimulation had no significant effect on histone modifications or pol II (S2P) recruitment at a region of DNA in NHEKs which has previously been defined as an IL-6 enhancer in primary human macrophages; a region 25kb upstream of the IL-6 transcriptional starts site (TSS) (Qiao *et al.*, 2013). In primary human macrophages Qiao *et al*. identified several regions upstream of the IL-6 TSS, which showed high levels of H3K27 acetylation in response to IFNγ + LPS stimulation. These regions also demonstrated high DNAseI sensitivity, in keeping with them being potential gene enhancers. These regions included a region 25kb upstream of the IL-6 TSS which demonstrated increased recruitment of CREB binding protein (CBP, a known histone acetyl transferase co-activator) as well as the transcription factor, p65. Together these findings suggest the region 25kb upstream of the IL-6 TSS is an enhancer in primary human macrophages. In comparison, the findings in NHEKs suggest that this region does not perform the same function in keratinocytes following TNFα + IL-17 stimulation. For example TNFα + IL-17 stimulation did not increase recruitment of pol II (S2P) to the IL-6 enhancer region in NHEKs, nor was there any increased acetylation of H3K27; stimulation which would be expected to increase both pol II recruitment and H3K27ac to a true enhancer region (Calo and Wysocka, 2013). Furthermore, in NHEKs the ratio of H3K4me1 to H3K4me3 was lower at the "IL-6 enhancer" region compared to the IL-6 promoter region, approximately 1:25 and 1:5 respectively at 4h after TNFα + IL-17 stimulation; true enhancer regions generally show greater levels of enrichment of H3K4me1 compared to H3K4me3 (Heintzman *et al.*, 2009). In addition to these findings, it was demonstrated that this region also had relatively low enrichment of Brd4 (section 4.3.6). In contrast enhancers, especially "super enhancers", often demonstrate high levels of Brd4 binding (Brown *et al.*, 2014). Although enhancer regions from macrophages and keratinocytes have not been directly compared previously, enhancers regions have previously been shown to be cell-type specific (Heintzman *et al.*, 2009), therefore it is not unexpected that the same DNA sequence may have different regulatory roles in macrophages and keratinocytes.

5.5. Future work

In a model of cutaneous inflammation relevant to psoriasis it was demonstrated that TNFα + IL-17 stimulation resulted in chromatin remodelling facilitating histone hyperacetylation and pol II (S2P) binding at the promoter regions of IL-6 and IL-8. These findings provide mechanistic insight into how stimuli relevant to psoriasis regulate inflammatory responses through changes in the epigenome and could lead to the identification of novel targets for psoriasis therapy. A number of key questions, however, remain:

- Stimulation with TNFα + IL-17 increased acetylation of specific lysine residues on histones at IL-6 and IL-8 promoters and pol II (S2P) recruitment traced with these changes, but which other epigenetic modifications (permissive or repressive) are key regulators of cutaneous inflammation?
- In addition to changes in the epigenome driving IL-6 and IL-8 expression, what changes in the epigenome are responsible for their repression; a decrease in permissive marks (as observed with IL-8) or an increased in repressive marks or a shift in balance between the two?
- Which additional factors and co-activators are involved in the recruitment of pol II (S2P) to target promoters? Could inhibition of binding of these factors and co-activators to the epigenome represent novel therapeutic targets?

5.6. Conclusions

- Consistent with the positive effects of TNFα + IL-17 stimulation on IL-6 and IL-8 expression in NHEKs, stimulation increased permissive (but not repressive) histone modifications, including lysine acetylation, at promoter regions of effector genes.
- Epigenetic changes induced by TNFα + IL-17 stimulation preceded maximal IL-6 and IL-8 expression.
- Recruitment of pol II (S2P) tracked with changes in histone acetylation at IL-6 and IL-8 promoters.
- The decline in histone acetylation at the IL-8 promoter at 4h post-stimulation could account for the subsequent decline in IL-8 expression.
- There was greater enrichment of pol II (S2P) at the IL-8 promoter than at the IL-6 promoter in both unstimulated and TNFα + IL-17 stimulated NHEKs, this

may explain the higher expression of IL-8 compared to IL-6 in both basal and inflammatory states.

- Enrichment of acetyl-H3 and H3K9ac was approximately 5-10 fold higher at the IL-6 promoter than for the IL-8 promoter, corresponding with increased binding of Brd4 at the IL-6 promoter (sections 4.3.3 and 4.3.4). This suggests these genes are differentially regulated by epigenetic writers and erasers.
- There were difference in kinetics of histone modifications at IL-6 and IL-8 promoters, with maximal changes around 4h and 1h respectively. These differences were not obviously reflected in differences in expression of these 2 genes over time, suggesting additional factors may also be involved in regulating their expression.

Chapter 6.

Gene arrays: investigating the effects of BET inhibition in the *in vitro* model of cutaneous inflammation

6. Gene array: investigating the effects of BET inhibition in the *in vitro* model of cutaneous inflammation

6.1. Introduction

Psoriasis represents a complex interplay between genetic predisposition, the environment and inflammatory responses (section 1.2). While the most widely accepted model of psoriasis suggests hyperproliferation of keratinocytes (which themselves may be dysfunctional or hypersensitive to stimuli) is driven by an over-active immune system, the underlying changes which regulate intracellular signalling and inflammatory feedback loops are not fully understood. Knowledge of differentially expressed genes (DEGs) in psoriasis may aid our understanding of the changes in signalling pathways between disease and non-disease states and identify candidates for further discovery of pathogenesis or treatment targets. Advances in array technology have transformed this field, for example, the Illumina human HT-12 v4.0 BeadChip array allows high throughput screening of over 47,000 transcripts simultaneously and comparisons between samples (*e.g.* involved vs. uninvolved psoriatic skin or unstimulated vs. stimulated cells) enables DEGs to be identified and potentially candidates for further investigation to aid understanding of the pathogenesis of psoriasis or as novel targets for therapy. Although a number of individual psoriasis array studies have previously been published (Kulski *et al.*, 2005; Yao *et al.*, 2008; Swindell *et al.*, 2012), more recently a robust meta-analysis of 5 studies has been undertaken (Tian *et al.*, 2012). Tian *et al.* uncovered 677 upregulated and 443 downregulated genes across the 5 transcriptomic studies analysed, which the authors termed the meta-analysis derived ("MAD-5") transcriptome.

Although it is known that BET inhibitors have anti-proliferative and anti-inflammatory effects in *in vitro* and *in vivo* models of inflammation and cancer, thus providing rationale for their use in psoriasis, to date there have been no array studies in keratinocytes, skin or models of psoriasis using BET inhibitors. In section 4.3.2, it was demonstrated that I-BET151 blocked induction of IL-6 and IL-8, key cytokines in the pathogenesis of psoriasis, in TNFα + IL-17A (IL-17) stimulated normal human epidermal keratinocytes (NHEKs), which could be accounted for by the inhibition of BET protein recruitment to IL-6 and IL-8 promoter regions in the presence of I-BET151. TNFα + IL-17 stimulation of NHEKs was previously shown to induce expression of key genes thought to be central to the pathogenesis of psoriasis (Chiricozzi *et al.*, 2011); we hypothesised that BET proteins would be involved in the

regulation of some of these genes and that I-BET151 treatment would modulate their expression. We therefore undertook microarray studies to examine the effects of TNFα + IL-17 stimulation in NHEKs and the effects of I-BET151 in this model of cutaneous inflammation. Finally, we analysed genes which were differentially regulated with TNFα + IL-17 stimulation and I-BET151 treatment and compared them to the MAD-5 transcriptome to determine the overlap and understand their relevance to psoriasis.

6.2. Aims

- To investigate gene expression responses in keratinocytes following stimulation with TNFα + IL-17 at early (6h) and late (24h) time points post-stimulation.
- To determine the effect of I-BET151 on gene expression in keratinocytes stimulated with TNFα + IL-17 at early (6h) and late (24h) time points post stimulation.
- To understand the overlap of genes which are both sensitive to TNFα + IL-17 stimulation, and also I-BET151 treatment, and how these relate to known DEGs in psoriasis and to genes known to be involved in the pathogenesis of psoriasis.

6.3. Results

6.3.1. Data quality assessment and generation of differentially expressed gene lists

In section 3.3.2, it was demonstrated that stimulation of NHEKs with TNFα + IL-17 induced IL-6 and IL-8 expression, with maximal mRNA levels detected at 6h post-stimulation. Furthermore, IL-6 and IL-8 responses could be inhibited by I-BET151 and the inhibition could be accounted for by the reduction in BET protein and pol II (S2P) binding to the promoter regions of IL-6 and IL-8 (sections 4.3.4 and 4.3.5). In order to understand the effects of TNFα + IL-17 stimulation on other early and late gene responses and to investigate the effects of I-BET151 treatment in this cell model, NHEKs were treated with the following 8 conditions:

1. Mock/unstimulated (*i.e.* media change), 6h stimulation
2. TNFα (10ng/ml) + IL-17 (100ng/ml), 6h stimulation
3. TNFα (10ng/ml) + IL-17 (100ng/ml) + I-BET151 vehicle (DMSO (1:1200)), 6h stimulation
4. TNFα (10ng/ml) + IL-17 (100ng/ml) + I-BET151 (1μM), 6h stimulation
5. Mock/unstimulated (*i.e.* media change), 24h stimulation
6. TNFα (10ng/ml) + IL-17 (100ng/ml), 24h stimulation
7. TNFα (10ng/ml) + IL-17 (100ng/ml) + I-BET151 vehicle (DMSO (1:1200)), 24h stimulation
8. TNFα (10ng/ml) + IL-17 (100ng/ml) + I-BET151 (1μM), 24h stimulation

Based on power calculations, using pilot qRT-PCR data, and cost effectiveness analysis, it was determined that array data from 12 donors would yield sufficient power to detect meaningful changes in gene expression after stimulation with TNFα + IL-17 in the presence or absence of I-BET151 (section 2.8). In total, NHEKs derived from 14 consecutive donors were treated; excess donors were treated to allow for any problems with growth, infections or outlier responses *etc*. No donors demonstrated macroscopic problems with growth or infection. RNA was therefore extracted for each condition from all 14 donors and qRT-PCR, using primers to IL-6 and IL-8, was undertaken (Table 6-1 and Table 6-2). All 14 donors, demonstrated upregulation of both IL-6 and IL-8 at 6h after stimulation with TNFα + IL-17, as previously observed (section 3.3.2), with many also demonstrating sustained induction of IL-6 and IL-8 at 24h after TNFα + IL-17 stimulation. I-BET151 had an inhibitory effect on IL-6 and IL-8 responses to stimulation at 6h in all donors, apart from donors XII and XIV in which I-BET151

appeared to potentiate the IL-6 response to stimulation at 6h (table 6.1). It is unknown why donors XII and XIV had outlying responses to I-BET151, but for this reason the other 12 donors were selected as the 12 for further microarray analysis.

All 96 samples had RNA integrity number (RIN) values above 8, therefore all samples were suitable for microarray analysis which was performed using Illumina human HT-12 v4.0 microarray platform which contains over 47,000 probes (section 2.8). Three different methods were used for the quality assessment of arrays. Firstly, the mean absolute difference of each array from all other arrays was considered - a threshold for outlier detection was determined from the distribution of mean absolute differences. Secondly, the principal component analysis (PCA) was considered for outlier detection - outliers being determined by visual inspection and expert judgement. Finally, the array intensity distribution was assessed - an array will be marked as an outlier by this method if the distribution tests are significantly different from the pooled intensity distribution of the other arrays. Only one array failed any of these quality control metrics, and this single case was only considered an outlier by the mean absolute difference method, and passed PCA and array intensity distribution quality assessments; all 96 arrays were therefore retained for analysis. There were on average approximately 33% of probes detectable per array and in total, 23794 probes (50.28% of the total number of probes) were retained (detection p value < 0.01 on at least 1 array).

PCA demonstrated that variance was mainly accounted for by treatment with I-BET151, with length of treatment with I-BET151 also contributing to the variance (Figure 6-1A). As I-BET151 had such a marked effect, the effect of other components, *e.g.* TNFα + IL-17 stimulation, was difficult to observe. For this reason, we undertook a further PCA excluding conditions which contained I-BET151 treatment (*i.e.* conditions 4 and 8). This allowed greater separation of other conditions and showed separation between TNFα + IL-17 stimulated and unstimulated conditions (Figure 6-1B). Furthermore, TNFα + IL-17 stimulation with or without DMSO (*i.e.* conditions 2, 3, 6 and 7, above) grouped closely together, suggesting DMSO had very limited effect.

In keeping with these findings, when formal contrasts were performed, DMSO was found not to regulate expression of any transcripts analysed regardless of the cut-offs specified (fold change, FCH, and p-value) (Table 6-3). TNFα + IL-17 stimulation regulated 46 - 1277 transcripts at 6h and 80 - 2338 transcripts at 24h, depending on the cut-offs specified, Table 6-3. In comparison, and in keeping with results from PCA

plots (Figure 6-1), I-BET151 regulated a greater number of transcripts compared to TNFα + IL-17 stimulation for any given FCH and p-value (Table 6-3).

There were good reasons to believe that inclusion of genes with equal or greater fold-changes than IL-6 and IL-8 would be of biological relevance as these cytokines are known to play key roles in the pathogenesis of psoriasis (section 1.2.5) and have been shown to be regulated by TNFα + IL-17 in an *in vitro* model of cutaneous inflammation (Chiricozzi *et al.*, 2011). Furthermore, I-BET151 was shown to inhibit induction of IL-6 and IL-8 in response to TNFα + IL-17 stimulation in the model (section 4.3.2). It was therefore decided to select a cut off threshold so that both IL-6 and IL-8 were included in subsequent analyses. Table 6-4 shows the absolute FCH in IL-6 and IL-8 responses, after stimulation with TNFα + IL-17 and the effect of I-BET151 treatment in TNFα + IL-17 stimulated NHEKs, each at 6 and 24h. A FCH cut-off of 1.3 would capture changes in IL-6 and IL-8 for all conditions, except the effect of I-BET151 treatment in TNFα + IL-17 stimulated NHEKs at 24h (absolute FCH = 1.2995). However, the I-BET151 effect on induction of IL-8 after TNFα + IL-17 stimulation at 24h was unclear, as it appeared to have an overall inhibitory effect as determined by microarray but an overall potentiation effect on IL-8 when measured by qRT-PCR, although other results generally showed good concordance, (Table 6-4). For this reason, we chose not to lower the cut off threshold further to include this value. As the threshold for FCH was set relatively low (1.3) to include biologically relevant changes in IL-6 and IL-8, we chose a stringent threshold for p-value of 0.01 (adjusted for multiple testing) to ensure changes were robust. Table 6-5 shows the number of transcripts with an absolute FCH ≥ 1.3 and p-value ≤ 0.01; there were approximately 10 times the number of transcripts regulated by I-BET151 compared to TNFα + IL-17 stimulation at either timepoint (3614 and 260, respectively at 6h; and 4289 and 436, respectively at 24h). I-BET151 modulated ~ 7.7% and 9.1% of the total number of probes in the array (~47000) at 6 and 24 h respectively, indicating that effects of the compound were discrete, not global. Although more transcripts were upregulated by TNFα + IL-17 stimulation than downregulated, a similar number of transcripts were either up or downregulated by I-BET151.

	IL-6, Fold change (Log_2)			
Donor	**TNFα + IL-17 6h**	**TNFα + IL-17 24h**	**I-BET151 6h**	**I-BET151 24h**
I	3.36	4.01	-1.82	-0.63
II	2.00	2.58	-2.05	-0.24
III	1.88	0.89	-0.93	-1.99
IV	1.42	2.24	-0.89	-0.16
V	2.60	0.28	-1.09	-1.56
VI	3.95	4.41	-1.57	-0.68
VII	1.87	3.14	-0.04	-2.16
VIII	3.43	3.64	-0.15	-1.65
IX	4.53	-0.16	-1.03	-0.62
X	1.83	3.07	-1.11	-1.53
XI	2.29	2.95	-1.66	-0.71
XII	2.29	3.86	0.25	-0.26
XIII	1.63	3.03	-0.82	-1.08
XIV	2.98	3.70	0.13	-1.28

Table 6-1: qRT-PCR analysis of IL-6 expression in TNFα + IL-17 stimulated human keratinocytes and the effect of I-BET151

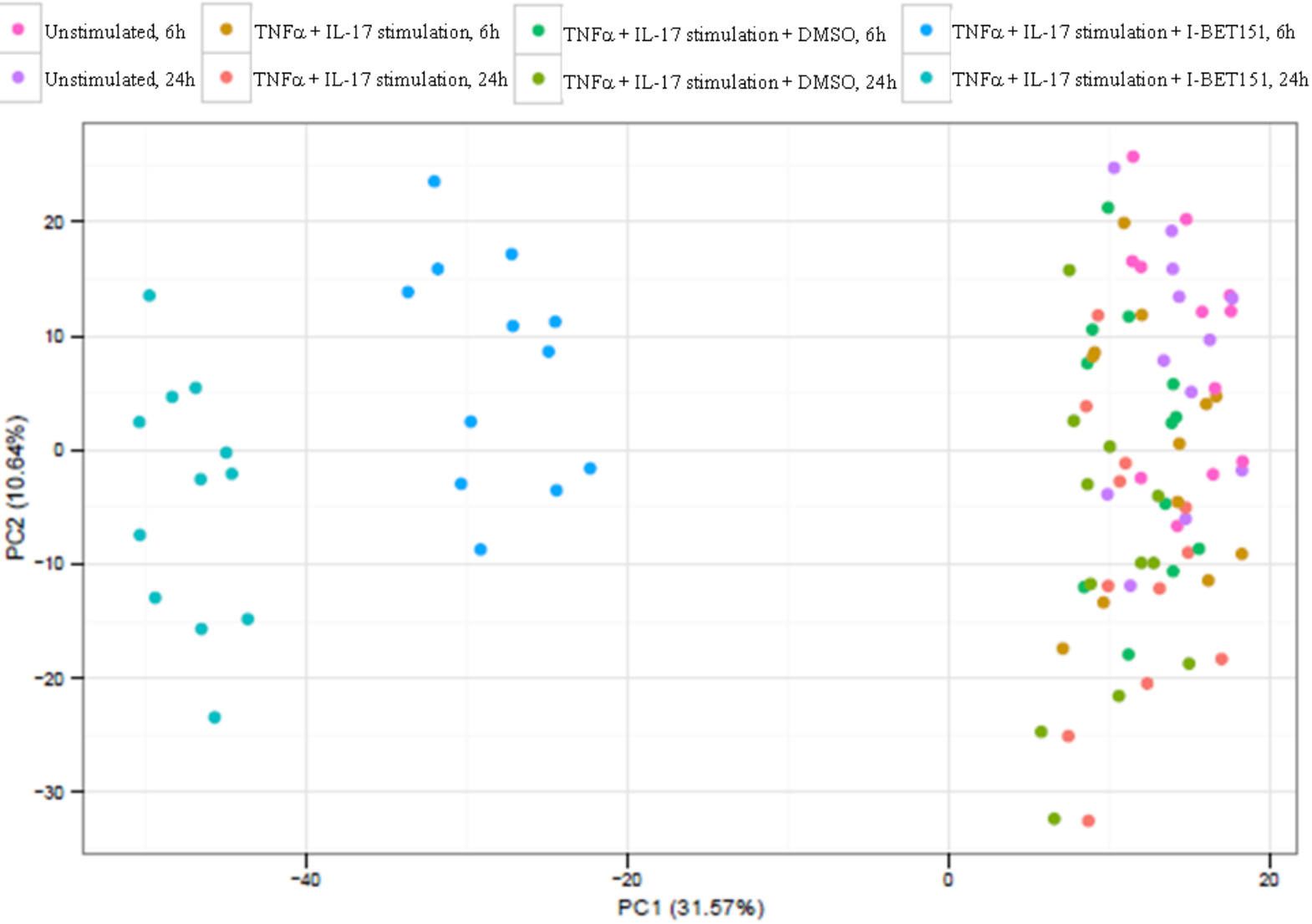
NHEKs derived from 14 donors were treated with TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. NHEKs were also stimulated with TNFα (10ng/ml) + IL-17 (100ng/ml) in the presence of I-BET151 (1uM) or vehicle (DMSO, 1:1200). Cells were harvested at 6h or 24h post-stimulation and expression of IL-6 mRNA determined by qRT-PCR. Columns 2 and 3 show IL-6 fold change (Log_2) in stimulated NHEKs compared to mock stimulated NHEKs at 6 and 24h after stimulation, respectively. Columns 4 and 5 show IL-6 fold change (Log_2) in TNFα + IL-17 + I-BET151 treated NHEKs compared TNFα + IL-17 + vehicle treated NHEKs at 6 and 24h after stimulation, respectively.

	IL-8, Fold change (Log_2)			
Donor	**TNFα + IL-17 6h**	**TNFα + IL-17 24h**	**I-BET151 6h**	**I-BET151 24h**
I	6.24	7.35	-1.46	0.33
II	5.38	6.07	-2.60	-0.01
III	7.18	6.89	-0.96	0.72
IV	5.70	4.88	-1.77	0.67
V	5.33	5.55	-1.21	0.27
VI	7.64	8.19	-2.29	-0.44
VII	5.57	5.18	-1.27	0.83
VIII	5.14	6.10	-0.27	0.64
IX	5.73	6.26	-0.95	0.98
X	4.74	5.31	-2.29	0.11
XI	7.76	6.61	-2.22	0.38
XII	6.64	7.75	-0.54	-0.16
XIII	6.58	6.73	-1.42	0.38
XIV	6.35	7.26	-0.84	0.24

Table 6-2: qRT-PCR analysis of IL-8 expression in TNFα + IL-17 stimulated human keratinocytes and the effect of I-BET151

NHEKs derived from 14 donors were treated as per Table 6-1 and expression of IL-8 mRNA determined by qRT-PCR. Columns 2 and 3 show IL-8 fold change (Log_2) in stimulated NHEKs compared to mock stimulated NHEKs at 6 and 24h after stimulation, respectively. Columns 4 and 5 show IL-8 fold change (Log_2) in TNFα + IL-17 + I-BET151 treated NHEKs compared TNFα + IL-17 + vehicle treated NHEKs at 6 and 24h after stimulation, respectively.

A)


Unstimulated, 6h
TNFα + IL-17 stimulation, 6h
TNFα + IL-17 stimulation + DMSO, 6h
TNFα + IL-17 stimulation + I-BET151, 6h
Unstimulated, 24h
TNFα + IL-17 stimulation, 24h
TNFα + IL-17 stimulation + DMSO, 24h
TNFα + IL-17 stimulation + I-BET151, 24h
PC2 (10.64%)
PC1 (31.57%)
20
10
0
−10
−20
−30
−40
−20
0
20

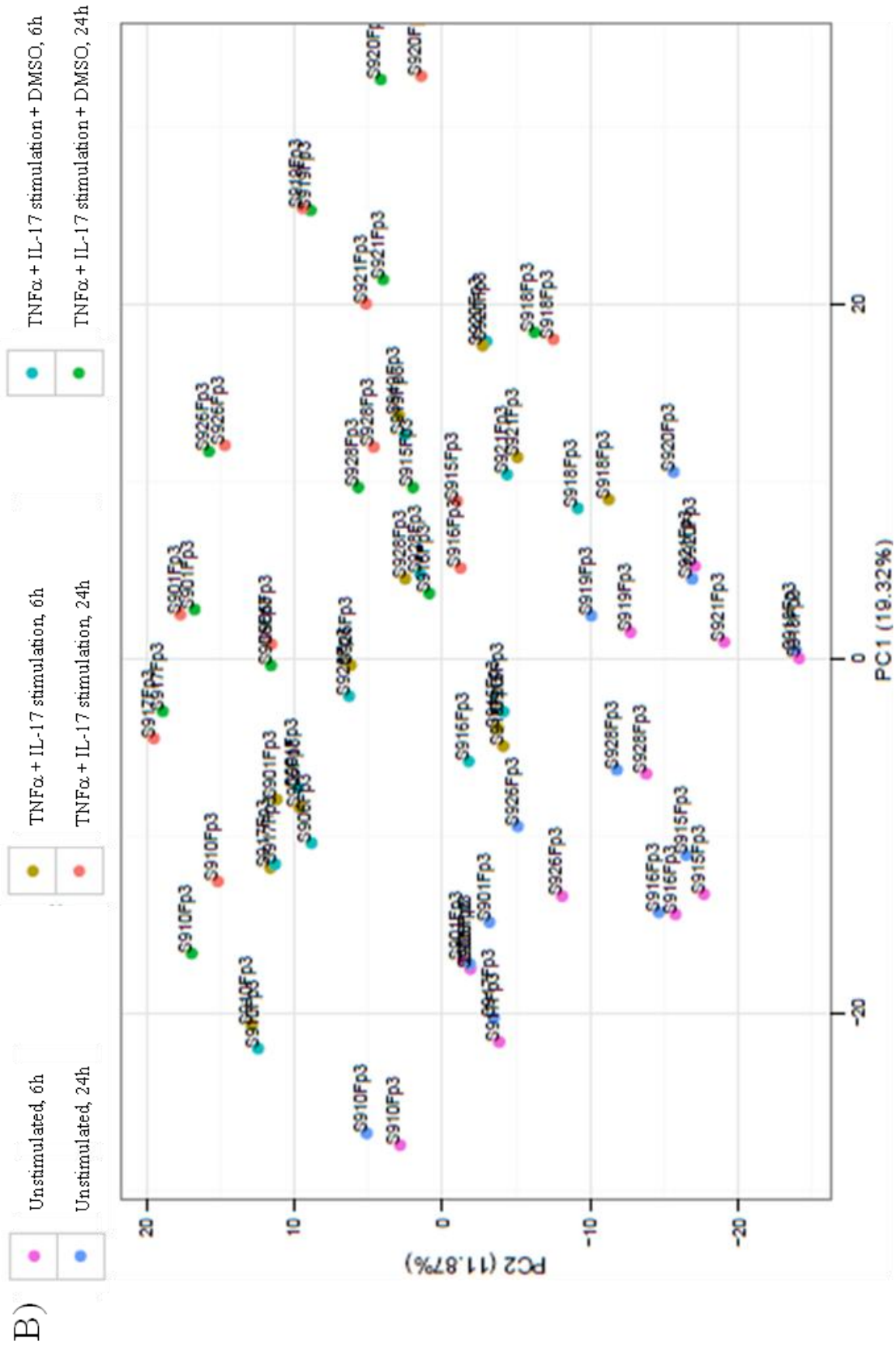


Figure 6-1: Principal component analysis (PCA) plots of data

PCA plot A) containing data from all 96 samples (*i.e.* 12 donors, each stimulated under 8 conditions (section 6.3.1). B) Data from all samples, except conditions containing I-BET151 treatment. Numbers in PCA plot B, represent different donors.

Fold change		2.0	2.0	2.0	1.5	1.5	1.5
p-value		**0.05**	**0.01**	**0.001**	**0.05**	**0.01**	**0.001**
1) TNFα + IL-17 stimulation	6h	49	47	46	114	107	101
2) DMSO	6h	0	0	0	0	0	0
3) TNFα + IL-17 stimulation + I-BET151 minus DMSO	6h	409	409	409	1601	1600	1594
4) TNFα + IL-17 stimulation	24h	84	82	80	219	211	200
5) DMSO	24h	0	0	0	0	0	0
6) TNFα + IL-17 stimulation + I-BET151 minus DMSO	24h	734	734	732	2262	2258	2245

Table 6-3: Number of differentially regulated probes, for a range of cut-offs, for each contrast

Table shows the number of differentially regulated probes for a given fold change and p-value (adjusted for multiple testing) for each contrast, shown in the left column. Contrast "TNFα + IL-17 stimulation" is the comparison between TNFα + IL-17 stimulated NHEKs compared to mock stimulated NHEKs (*i.e.* conditions 1 vs. 2 and 5 vs. 6 (section 6.3.1). Contrast "DMSO" is the comparison of TNFα + IL-17 stimulated NHEKs in the presence or absence of DMSO (*i.e.* conditions 2 vs. 3 and 6 vs. 7 (section 6.3.1). Contrast "TNFα + IL-17 stimulation + I-BET151 minus DMSO" is the comparison of TNFα + IL-17 stimulated NHEKs in the presence or absence of I-BET151 or DMSO (*i.e.* conditions 3 vs. 4 and 7 vs. 8 (section 6.3.1).

A) IL-6

Condition	Time	Microarray IL-6, fold change (Log_2)	Microarray IL-6, fold change (absolute)	qRT-PCR IL-6 fold change (Log_2)
TNFα + IL-17 stimulation	6h	0.5632	1.4775	2.5655
TNFα + IL-17 stimulation	24h	0.6364	1.5545	2.5069
TNFα + IL-17 stimulation + I-BET151 treatment	6h	-0.4496	1.3657	-1.0964
TNFα + IL-17 stimulation + I-BET151 treatment	24h	-0.4836	1.3982	-1.0843

B) IL-8

Condition	Time	Microarray IL-8, fold change (Log_2)	Microarray IL-8, fold change (absolute)	qRT-PCR IL-8 fold change (Log_2)
TNFα + IL-17 stimulation	6h	4.7150	26.2637	6.0827
TNFα + IL-17 stimulation	24h	4.6950	25.9022	6.2594
TNFα + IL-17 stimulation + I-BET151 treatment	6h	-1.5030	2.8343	-1.5586
TNFα + IL-17 stimulation + I-BET151 treatment	24h	-0.3780	1.2995	0.4056

Table 6-4: IL-6 and IL-8 responses to TNFα + IL-17 stimulation and I-BET151, as measured by microarray and qRT-PCR

Table shows the effect of TNFα + IL-17 stimulation compared to mock stimulated NHEKs at 6 and 24h (*i.e.* conditions 1 vs. 2 and 5 vs. 6 (section 6.3.1) as well as the effect of I-BET151, compared to vehicle, in TNFα + IL-17 stimulated NHEKs at 6 and 24h (*i.e.* conditions 3 vs. 4 and 7 vs. 8 (section 6.3.1) on **A**) IL-6 responses and **B**) IL-8 responses. Microarray and qRT-PCR values are mean data (N=12).

Condition	Time	Up	Down	Total
TNFα + IL-17 stimulation	6h	222	38	260
TNFα + IL-17 stimulation	24h	334	102	436
TNFα + IL-17 stimulation + I-BET151 treatment	6h	1621	1993	3614
TNFα + IL-17 stimulation + I-BET151 treatment	24h	2159	2130	4289

Table 6-5: Number of probes regulated by TNFα + IL-17 stimulation and I-BET151, with fold change ≥ 1.3 and p-value ≤ 0.01

Table shows the number of probes regulated by TNFα + IL-17 stimulation compared to mock stimulated NHEKs at 6 and 24h (*i.e.* conditions 1 vs. 2 and 5 vs. 6 (section 6.3.1) as well as the number of probes regulated by I-BET151 in TNFα + IL-17 stimulated NHEKs compared to NHEKs stimulated with TNFα + IL-17 + DMSO at 6 and 24h (*i.e.* conditions 3 vs. 4 and 7 vs. 8 (section 6.3.1), with fold change ≥ 1.3 and p-value ≤ 0.01.

6.3.2. TNFα + IL-17 stimulation of keratinocytes, upregulated expression of genes relevant to psoriasis

In NHEKs, TNFα + IL-17 stimulation differentially regulated expression of 260 probes at 6h, compared to mock stimulated NHEKs (222 upregulated and 38 downregulated) and 436 probes at 24h (334 upregulated and 102 downregulated) (Table 6-5). Figure 6-2, shows that 174 probes were upregulated at both 6 and 24h and 23 probes were downregulated at both 6 and 24h, demonstrating that TNFα + IL-17 stimulation had some sustained effects. The effect of TNFα + IL-17 stimulation on keratinocytes has previously been investigated and was found to upregulate expression of many genes involved in the pathogenesis of psoriasis (Chiricozzi *et al.*, 2011). To understand how reproducible the effects of TNFα + IL-17 stimulation were, the two data sets were compared using the same cut-offs and timepoint as those specified by Chiricozzi *et al*. (*i.e.* FCH ≥ 1.3, false discovery rate (FDR) = 0.3, 24h stimulation). This comparison demonstrated that there was overlap of 157 probes between the 2 datasets, but over 200 probes featured in only one or the other of the arrays (Figure 6-3). Furthermore, although Chiricozzi *et al*. did not report on genes which were downregulated by TNFα + IL-17 stimulation, we found 116 probes were negatively regulated by TNFα + IL-17 stimulation (Figure 6-3).

DEGs at 6h (217 genes) and 24h (355 genes) after TNFα + IL-17 stimulation in NHEKs were compared to the MAD-5 transcriptome; there was an overlap of 59 genes at 6h (Figure 6-4) and 97 genes at 24h (Figure 6-5). Figure 6-4 shows that of the 59 genes regulated by TNFα + IL-17 stimulation in NHEKs at 6h, the majority (54 genes) were upregulated by TNFα + IL-17 stimulation and also upregulated in the MAD-5 transcriptome. Figure 6-5 shows that of the 97 genes regulated by TNFα + IL-17 stimulation in NHEKs at 24h, the majority (80 genes) were upregulated by TNFα + IL-17 stimulation and also upregulated in the MAD-5 transcriptome; interestingly there were 5 genes downregulated by TNFα + IL-17 stimulation which were also downregulated in the MAD-5 transcriptome. These findings demonstrated that at each timepoint, approximately 25% of genes regulated by TNFα + IL-17 stimulation, featured in the MAD-5 transcriptome with the same direction of expression and that approximately 5 and 8% of DEGs in the MAD-5 transcriptome were regulated by TNFα + IL-17 stimulation in NHEKs at 6h and 24h respectively.

Table 6-6 and Table 6-8 show the top 10 upregulated genes in response to TNFα + IL-17 stimulated NHEKs, at 6h and 24h respectively, which included a number of genes known to be upregulated in psoriasis, *e.g.* IL-8, IL-36G, DEF4A/DEF4B, CXCL1, CCL20 and LCN2; and a psoriasis susceptibility loci has recently been identified near NFKBIZ (Tsoi *et al.*, 2015). In addition to overlap of DEGs at 6 and 24h post-TNFα + IL-17 stimulation, there was considerable overlap in upstream regulators of DEGs at these two timepoints (Table 6-7 and Table 6-9) with 4 of the 5 top upstream regulators featuring in both datasets (TNF, NFKB, IL1A and IFNG).

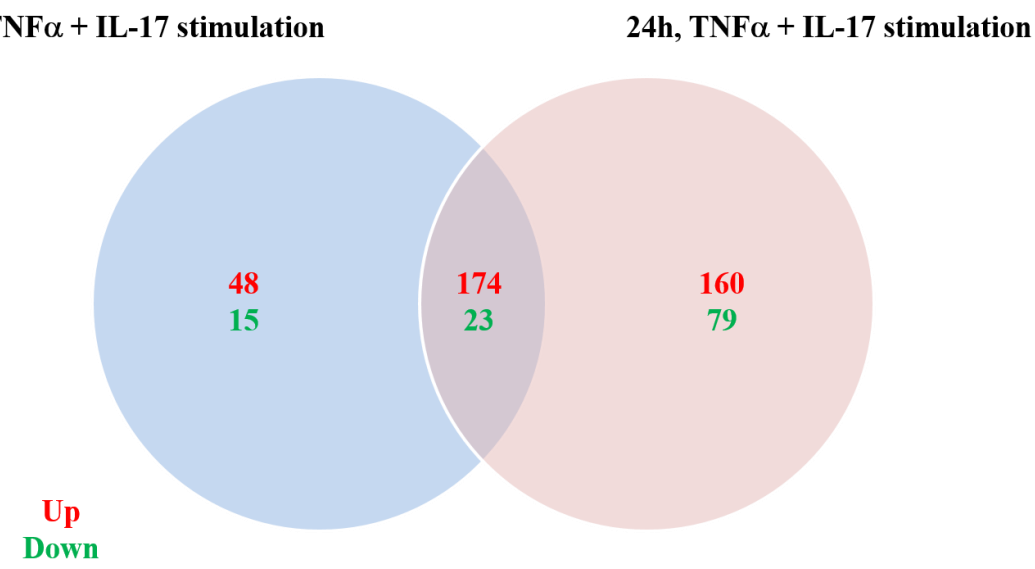


Figure 6-2: Overview of effects of TNFα + IL-17 stimulation at 6 and 24h

Venn diagram shows the number of probes regulated by TNFα + IL-17 stimulation in NHEKs at 6h and 24h after stimulation, compared to mock stimulated NHKEs, with fold change ≥ 1.3 and p-value ≤ 0.01.

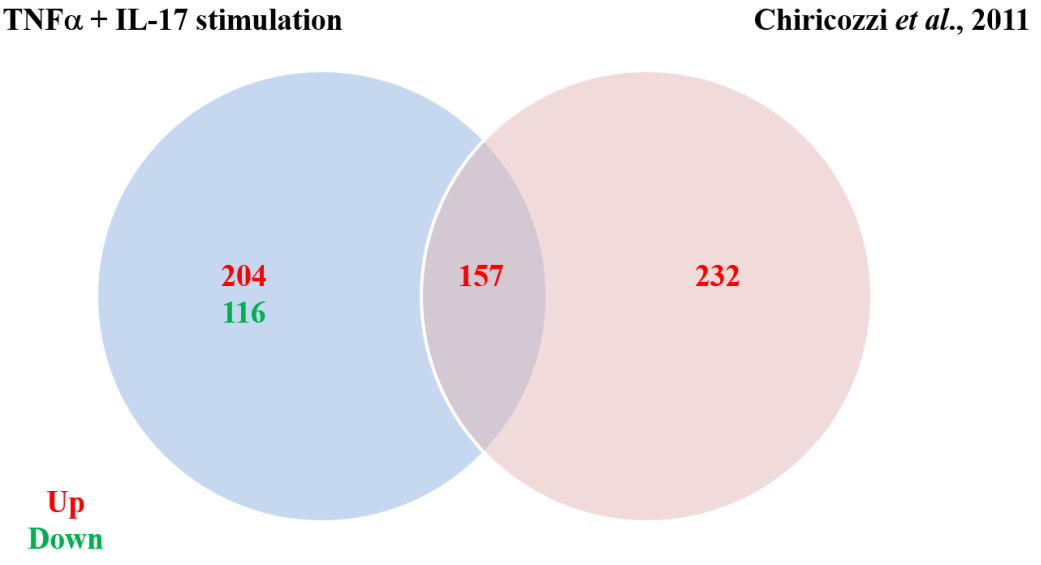


Figure 6-3: Comparison of effects of 24h stimulation with TNFα + IL-17 to a published data set using the same stimuli and timepoint

Venn diagram shows the number of probes regulated by TNFα (10ng/ml) + IL-17 (100ng/ml) in NHEKs compared to a published data set of NHEKs stimulated with TNFα (10ng/ml) + IL-17 (200ng/ml) (Chiricozzi *et al.*, 2011). For both datasets, NHEKs were stimulated for 24h and cut-offs were as follows: fold change ≥ 1.3 false discovery rate = 0.3. N.B. Chiricozzi *et al*. only reported on upregulated genes.

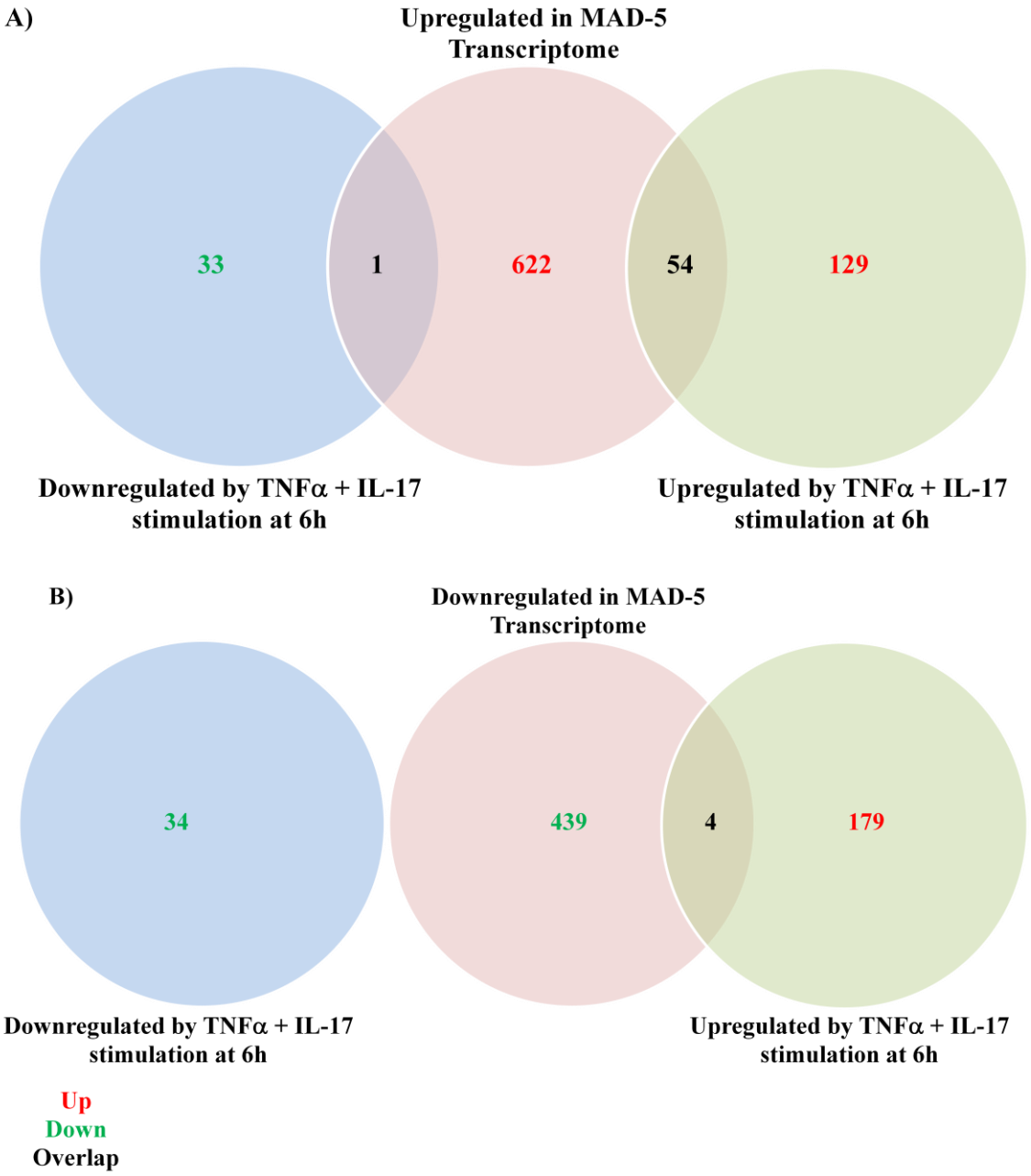


Figure 6-4: Overview of genes regulated by TNFα + IL-17 stimulation in keratinocytes at 6h and included in the MAD-5 psoriasis transcriptome

Venn diagram shows overlap of genes regulated by TNFα + IL-17 stimulation in NHEKs at 6h and either **A**) upregulated in the MAD-5 psoriasis transcriptome (Tian *et al.*, 2012) or **B**) downregulated in the MAD-5 psoriasis transcriptome.

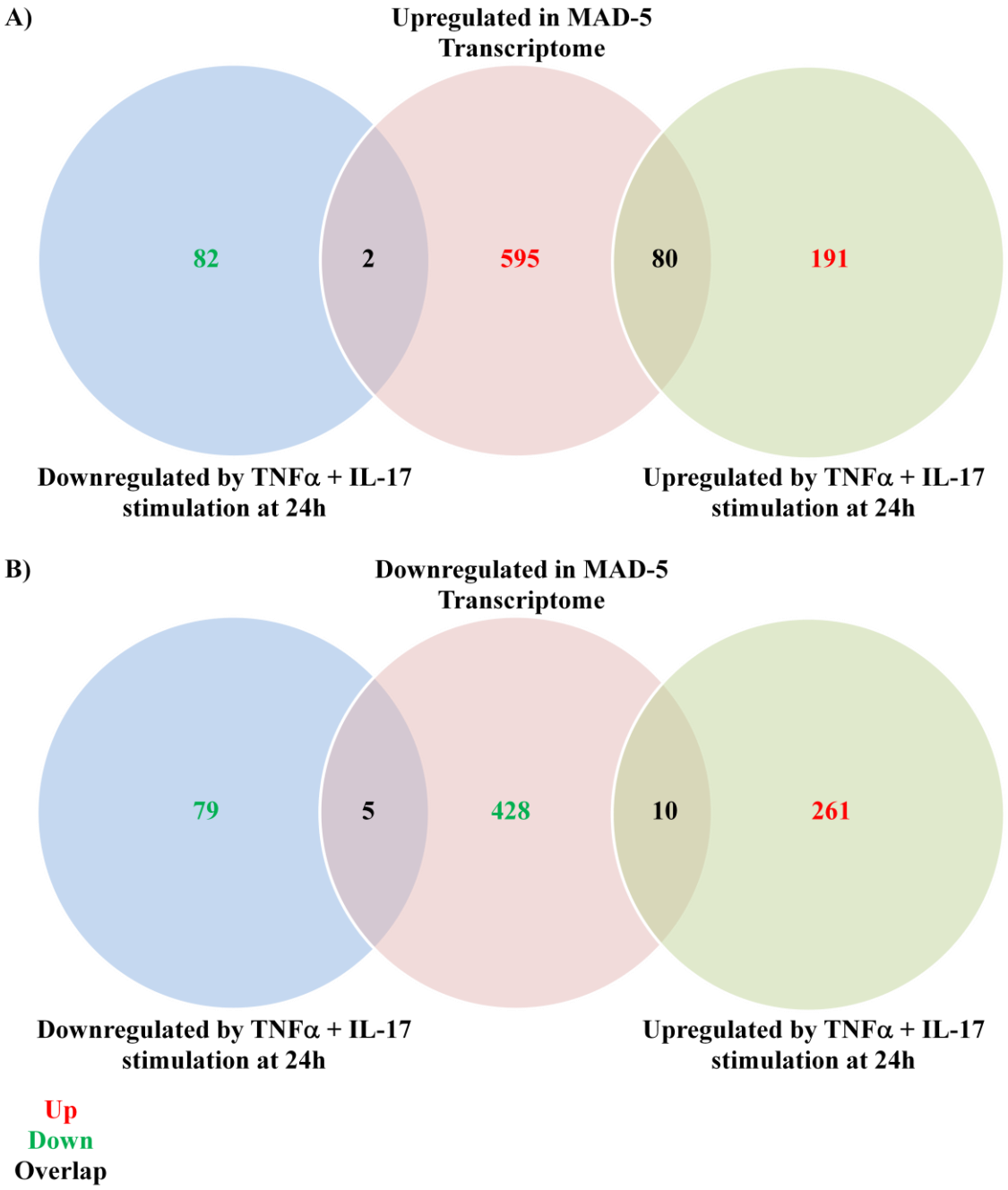


Figure 6-5: Overview of genes regulated by TNFα + IL-17 stimulation in keratinocytes at 24h and included in the MAD-5 psoriasis transcriptome

Venn diagram shows overlap of genes regulated by TNFα + IL-17 stimulation in NHEKs at 24h and either **A**) upregulated in the MAD-5 psoriasis transcriptome (Tian *et al.*, 2012) or **B**) downregulated in the MAD-5 psoriasis transcriptome.

Symbol	Description	Fold change (Log_2)
IL-8	Interleukin-8	4.72
IL-36G	Interleukin-36, gamma	4.17
CXCL1	Chemokine (C-X-C motif) ligand 1	3.89
CCL20	Chemokine (C-C motif) ligand 20	2.92
CSF2	Colony Stimulating Factor 2	2.91
DEFB4A/DEFB4B	Defensin, beta 4A/4B	2.91
SPRR2A	Small Proline-Rich Protein 2A	2.82
SPRR2F	Small Proline-Rich Protein 2F	2.55
NFKBIZ	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, zeta	2.45
ZC3H12A	Zinc Finger CCCH-Type Containing 12A	2.32

Table 6-6: Top 10 upregulated genes at 6h after TNFα + IL-17 stimulation, compared to mock stimulated NHEK

Upstream regulators, 6h	Predicted activation or inhibition
TNF	Activated
NFKB	Activated
IL1A	Activated
IFNG	Activated
IL1B	Activated

Table 6-7: Top 5 upstream regulators of differentially expressed genes at 6h after TNFα + IL-17 stimulation

Upstream regulators were predicted using IPA.

Symbol	Description	Fold change (Log_2)
DEFB4A/DEFB4B	Defensin, beta 4A/4B	5.32
SPRR2A	Small Proline-Rich Protein 2A	5.02
IL-36G	Interleukin-36, gamma	4.78
IL-8	Interleukin-8	4.70
SPRR2F	Small Proline-Rich Protein 2F	4.48
CXCL1	Chemokine (C-X-C motif) ligand 1	3.90
SPRR2E	Small Proline-Rich Protein 2E	3.83
SAA1	Serum Amyloid A1	3.55
CCL20	Chemokine (C-C motif) ligand 20	3.01
LCN2	Lipocalin 2	2.97

Table 6-8: Top 10 upregulated genes at 24h after TNFα + IL-17 stimulation, compared to mock stimulated NHEKs

Upstream regulators, 24h	Predicted activation or inhibition
TNF	Activated
IL1A	Activated
NFKB	Activated
EHF	Activated
IFNG	Activated

Table 6-9: Top 5 upstream regulators of differentially expressed genes at 24h after TNFα + IL-17 stimulation

Upstream regulators were predicted using IPA.

6.3.3. I-BET151 differentially regulated expression of genes principally involved in p53 signalling and cell cycle, these had most relevance to cancer but also to dermatological diseases

In TNFα + IL-17 stimulated NHEKs, I-BET151 differentially regulated expression of 3614 probes at 6h (1621 upregulated and 1993 downregulated) and 4289 probes at 24h (2159 upregulated and 2130 downregulated) (Table 6-5). Figure 6-6, shows that 884 probes were upregulated at both 6 and 24h and 1063 probes were downregulated at both 6 and 24h, demonstrating that I-BET15 had some sustained effects. In some instances I-BET151 had differential effects at 6 vs. 24h; 21 probes were upregulated at 6h but downregulated at 24h, and in 40 probes the opposite was observed. Table 6-10 and Table 6-11 show the top 10 upregulated and downregulated genes in response to I-BET151 treatment in TNFα + IL-17 stimulated NHEKs, at 6h and 24h respectively. In order to understand the gross functions of the ~4000 differentially regulated probes at each timepoint, data was mined using Ingenuity® Pathway Analysis (IPA).

As shown in Figure 6-7, at 6h post treatment with I-BET151, compared to vehicle, in TNFα + IL-17 stimulated NHEKs, the highest canonical pathway was p53 signalling ($p = 1.91 \times 10^{-8}$), but canonical pathways involving cell cycle also featured very prominently (*e.g.* cell cycle: G1/S checkpoint regulation, $p = 2.78 \times 10^{-7}$; cell cycle: G2/M DNA damage checkpoint regulation, $p = 3.95 \times 10^{-7}$ and also cell cycle control of chromosomal replication, $p = 2.13 \times 10^{-6}$ amongst other pathways in cell cycle). Interestingly, the role of IL-17A in psoriasis also featured in the top 20 canonical pathways ($p = 7.13 \times 10^{-5}$) and 7 of the 8 genes regulated by I-BET151 within this pathway were inhibited (Figure 6-8). Given the canonical pathways affected by I-BET15 in TNFα + IL-17 stimulated NHEKs, it was logical the top diseases and biological functions were related to these pathways: 839 genes were included in the cell death and survival dataset, and 981 genes in the cancer dataset. Interestingly, 291 genes were included in the dermatological diseases and conditions dataset and 305 genes in the protein synthesis dataset (Figure 6-9). IPA was also used to analyse interactions within the differentially regulated gene dataset at 6h, by linking genes through their relationships. The most number of differentially regulated genes were included in the "Cell cycle, cellular assembly and organisation, DNA replication, recombination and repair" network, (Figure 6-10, and the majority of genes in this network were downregulated by I-BET151 treatment at 6h.

Similar to 6h, the I-BET151 DEG list at 24h included canonical pathways involving the cell cycle (*e.g.* cell cycle control of chromosomal replication, $p = 2.81 \times 10^{-8}$; role of CHK proteins in cell cycle checkpoint control, $p = 2.02 \times 10^{-6}$; and cell cycle: G2/M DNA damage checkpoint regulation, $p = 2.6 \times 10^{-6}$) (Figure 6-11). Again cancer and cell death and survival were at or near the top of diseases and biological functions affected by I-BET151, with 1219 and 948 genes in these datasets respectively (Figure 6-12). Of note, the dermatological diseases and conditions dataset also featured at 24h (403 genes) and also a further dermatological dataset appeared: hair and skin development and function (124 genes). Again, IPA was also used to analyse interactions within the differentially regulated gene dataset at 24h. The most number of differentially regulated genes were included in the "Amino acid metabolism, small molecule biochemistry, post-translational modification" network (Figure 6-13) and the majority of genes in this network were downregulated by I-BET151 treatment at 24h. Interestingly downregulation of MYC, an oncogenic transcription factor involved in cellular proliferation and apoptosis, was at the core of this network.

In order to understand the relationships between DEGs at 6 and 24h after I-BET151 treatment in TNFα + IL-17 stimulated NHEKs, IPA was used to predict upstream regulators of DEGs at 24h and these were compared to regulators of DEGs at 6h. Table 6-14 shows the top 10 inhibited regulators at 6h and the overlap with predicted regulators of DEGs at 24h after I-BET151 treatment in TNFα + IL-17 stimulated NHEKs. Seven of the key regulators at 6h, were also predicted to be inhibited at 24h, based on the DEGs dataset at 24h. It was logical that key regulators were involved in similar processes as DEGs *e.g.* inflammation (IL1A & B and NFKB1), cell cycle (*e.g.* cyclin D1, E2F2) and cancer (*e.g.* MYC) (Table 6-14). The observation that IL1A, IL1B and NFKB1 were among the top most inhibited regulators at 6 and 24h provided further rationale for investigating the effects of BET inhibitors in cutaneous inflammation.

Therefore, DEGs at 6h (2926 genes) and 24h (3372 genes) after I-BET151 treatment in TNFα + IL-17 stimulated NHEKs were compared to the MAD-5 transcriptome; there was an overlap of 286 genes at 6h (Figure 6-14) and 365 genes at 24h (Figure 6-15). Figure 6-14 shows that of the 286 genes modulated by I-BET151 treatment in TNFα + IL-17 stimulated NHEKs at 6h, the majority (190 genes) were upregulated in the MAD-5 transcriptome but downregulated by I-BET151 and 24 genes which were

downregulated in the MAD-5 transcriptome were upregulated by I-BET151. Figure 6-15 shows that of the 365 genes modulated by I-BET151 treatment in TNFα + IL-17 stimulated NHEKs at 24h, 228 genes were upregulated in the MAD-5 transcriptome but downregulated by I-BET151 and 59 genes which were downregulated in the MAD-5 transcriptome were upregulated by I-BET151. These findings demonstrated that of the 1120 up or downregulated DEGs in the MAD-5 transcriptome, approximately 19% and 26% of genes were differentially modulated by I-BET151 treatment in TNFα + IL-17 stimulated NHEKs, at 6h and 24h respectively.

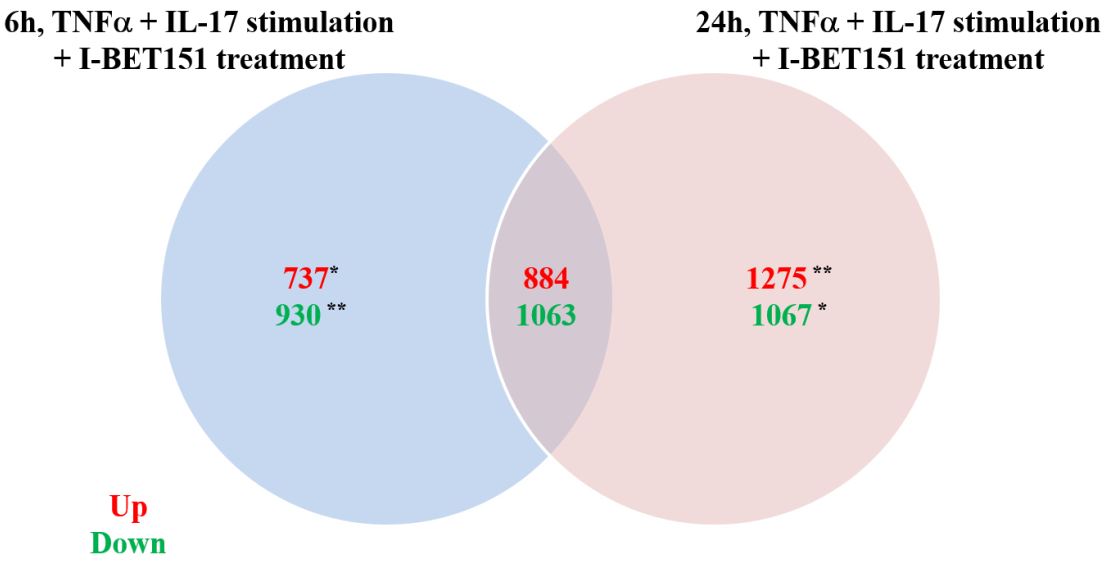


Figure 6-6: Overview of effects of I-BET151 in TNFα + IL-17 stimulated keratinocytes at 6 and 24h

Venn diagram shows the number of probes regulated by I-BET151, compared to vehicle, in TNFα + IL-17 stimulation of NHEKs at 6h and 24h, with fold change ≥ 1.3 and p-value ≤ 0.01. *Includes 21 probes upregulated at 6h but downregulated at 24h. **Includes 40 probes downregulated at 6h but upregulated at 24h.

Symbol	Description	Fold change (Log_2)	Type
CSF2	Colony Stimulating Factor 2	-3.19	Cytokine
FST	Follistatin	-3.05	Other
DKK1	Dickkopf-related protein 1	-3.05	Growth factor
C15orf52	Chromosome 15 Open Reading Frame 52 (Uncharacterised)	-2.89	Other
IL-1A	Interleukin-1, alpha	-2.76	Cytokine
EPN3	Epsin 3	-2.52	Other
IL-1B	Interleukin-1, beta	-2.44	Cytokine
ADAMTS1	A disintegrin and metalloproteinase with thrombospondin motif, 1	-2.31	Peptidase
IL-36G	Interleukin-36, gamma	-2.30	Cytokine
IFIT3	Interferon-Induced Protein With Tetratricopeptide Repeats 3	-2.14	Other
HIST2H2AA3/HIST2H2AA4	Histone cluster 2, H2aa3	3.23	Other
HIST1H2BD	Histone cluster 1, H2bd	3.05	Other
HCFC1R1	Host cell factor C1 regulator 1 (XPO1 dependent)	2.93	Other
HIST1H4H	Histone cluster 1, H4h	2.93	Other
HIST2H2AC	Histone cluster 2, H2ac	2.78	Other
HIST2H2BE	Histone cluster 2, H2be	2.60	Other
HIST1H1C	Histone cluster 1, H1c	2.44	Other
TRNP1	TMF1-regulated nuclear protein 1	2.31	Other
H2AFJ	H2A histone family, member J	2.31	Other
MT1X	Metallothionein 1X	2.26	Other

Table 6-10: Top 10 down and upregulated genes at 6h after I-BET151 treatment, compared to vehicle, in TNFα + IL-17 stimulated keratinocytes

Molecule type was defined by IPA.

Symbol	Description	Fold change (Log_2)	Type
FST	Follistatin	-5.00	Other
UBE2C	Ubiquitin-conjugating enzyme E2 C	-3.80	Enzyme
CDC20	cell-division cycle protein 20	-3.48	Other
DKK1	Dickkopf-related protein 1	-3.16	Growth factor
PRC1	Protein Regulator of cytokinesis 1	-3.14	Other
OAS2	2'-5'-oligoadenylate synthetase 2	-3.14	Enzyme
CCNB2	cyclin B2	-3.09	Other
SERPINB2	Serpin peptidase inhibitor, clade B, member 2	-3.08	Other
TOP2A	DNA topoisomerase 2-alpha	-3.05	Cytokine
KRT34	Keratin 34	-2.97	Other
HIST2H2BE	Histone cluster 2, H2be	3.28	Other
HIST2H2AA3/HIST2H2AA4	Histone cluster 2, H2aa3	2.937	Other
CTGF	Connective tissue growth factor	2.94	Growth factor
HIST1H2BG	Histone cluster 1, H2bg	2.74	Other
HIST1H2AC	Histone cluster 1, H2ac	2.64	Other
HIST1H2BD	Histone cluster 1, H2bd	2.62	Other
TXNIP	Thioredoxin interacting protein	2.60	Other
HIST2H2AC	Histone cluster 2, H2ac	2.40	Other
CCL20	Chemokine (C-C motif) ligand 20	2.38	Cytokine
TUBB3	Tubulin, beta 3 class III	2.35	Other

Table 6-11: Top 10 down and upregulated genes at 24h after I-BET151 treatment, compared to vehicle, in TNFα + IL-17 stimulated keratinocytes

Molecule type was defined by IPA.

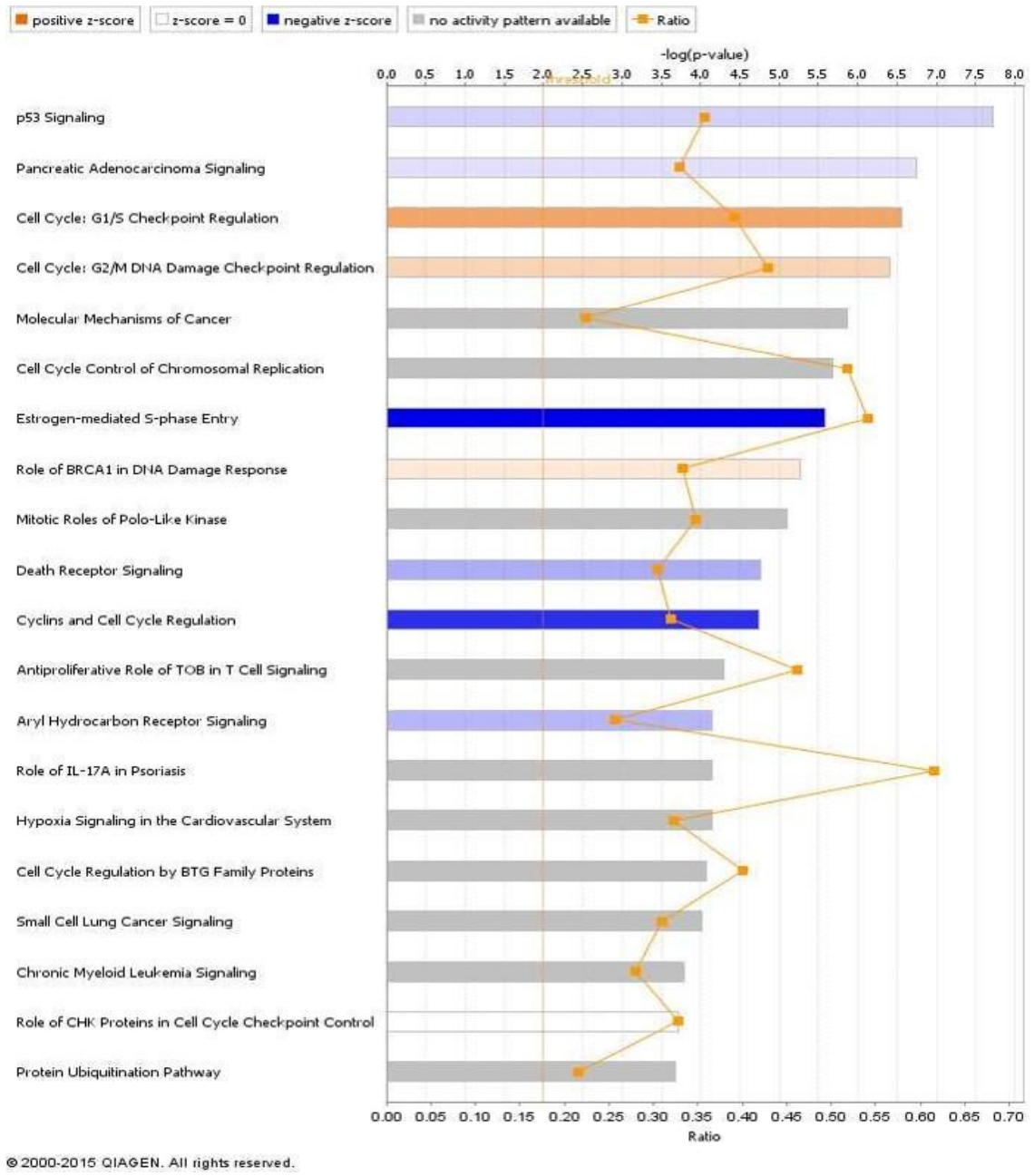


Figure 6-7: Top 20 Ingenuity® Pathway Analysis "canonical pathways" differentially modulated by I-BET151 in TNFα + IL-17 stimulated keratinocytes at 6h

Expression profiles are shown as log mean values, compared to DMSO in TNFα + IL-17 stimulated keratinocytes at 6h, and were considered significant when the adjusted p-value (threshold) was ≤ 0.01 and used as input into IPA. The orange line depicts the ratio (number of pathway relevant proteins in the data set divided by the total number of proteins in that pathway); 35 out of 98 genes were included in the p53 signalling pathway and 8 out of 13 genes were included in the role of IL-17A in psoriasis pathway (Figure 6-8). Bars in orange, blue or grey predict an increase, decrease or undetermined change in a pathway's activity, respectively. This is based on the activation Z-score, which infers the activation state of predicted transcriptional regulators of a particular pathway using expression patterns of differentially expressed genes within that dataset.

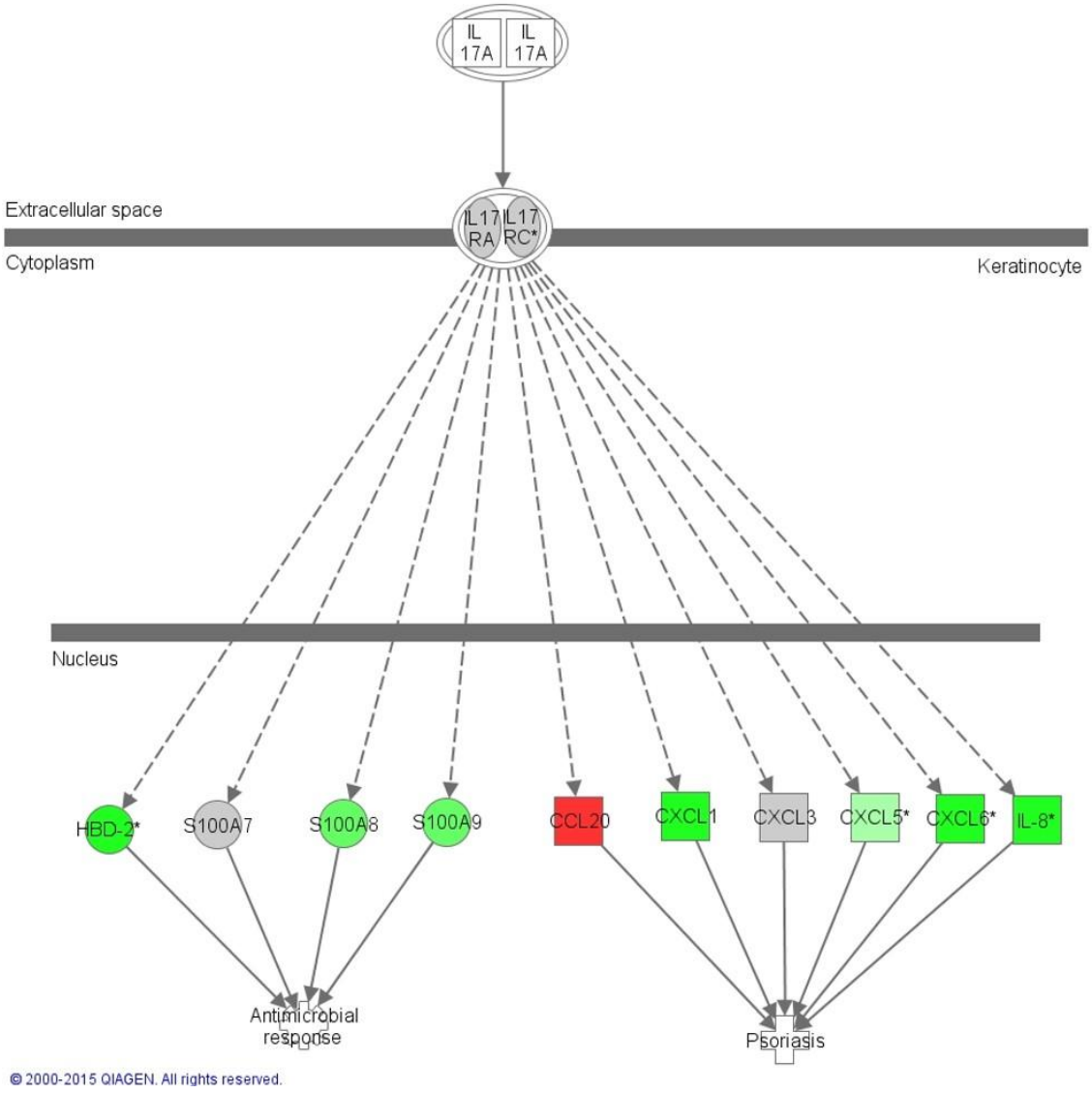


Figure 6-8: Effects of I-BET151 treatment, compared to vehicle, in TNFα + IL-17 stimulated keratinocytes in Ingenuity® Pathway Analysis' "role of IL-17A in psoriasis pathway"

Squares represent cytokines, horizontal ovals are transmembrane receptors and circles are other molecules. The intensity of red and green indicates the degree of up or downregulation, respectively, at 6h after treatment with I-BET151, compared to vehicle, in TNFα + IL17 stimulated NHEKs. *indicates gene was represented by more than one probe.

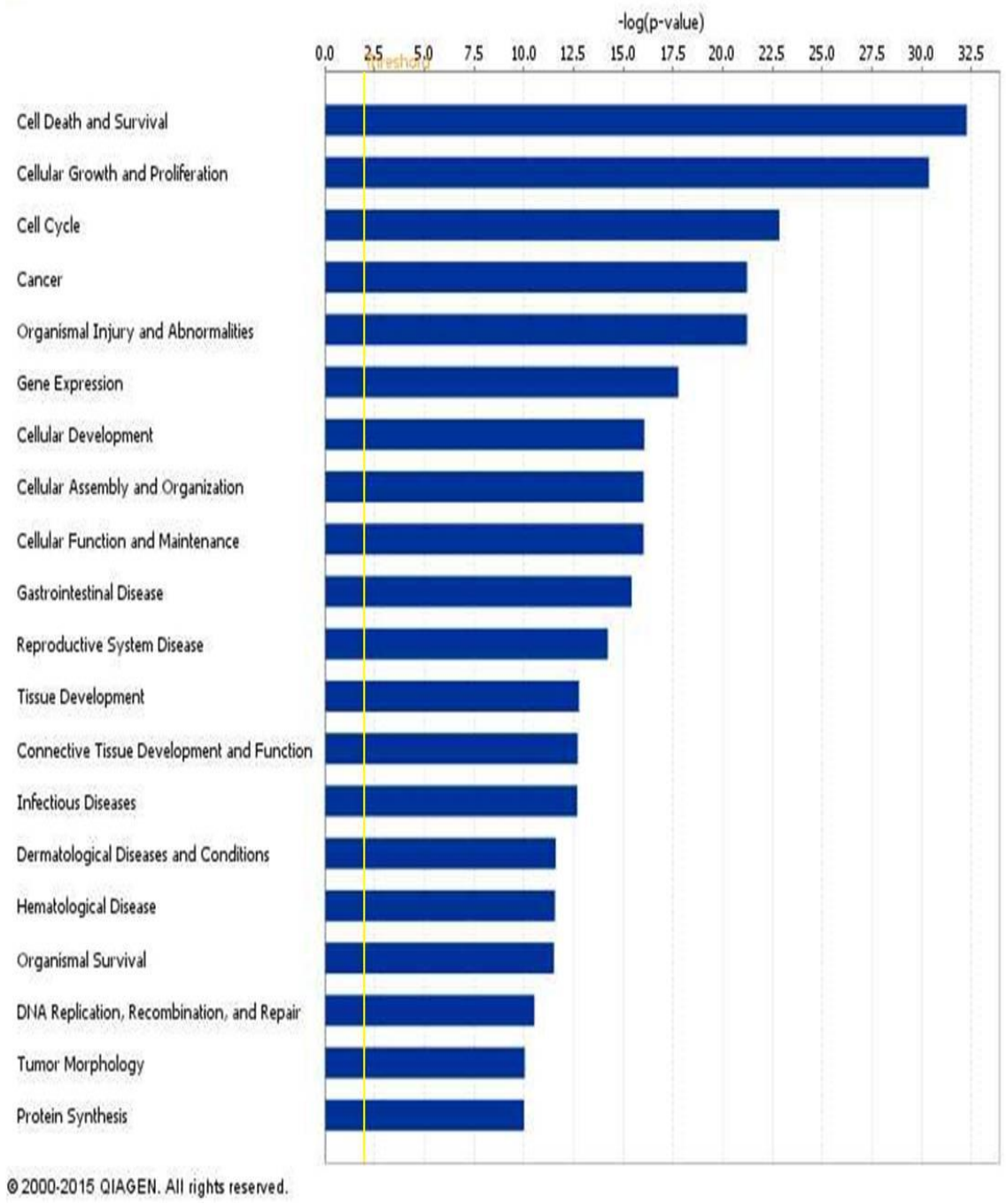


Figure 6-9: Top 20 Ingenuity® Pathway Analysis "diseases and biological functions" differentially modulated by I-BET151 in TNFα + IL-17 stimulated keratinocytes at 6h

Expression profiles are shown as log mean values, compared to DMSO in TNFα + IL-17 stimulated keratinocytes at 6h, and were considered significant when the adjusted p-value (threshold) was ≤ 0.01 and used as input into IPA. The yellow line indicates the significance, $p < 0.01$ (*i.e.* all datasets shown were significant). 839 genes were included in the cell death and survival dataset, 981 genes in the cancer dataset, 291 genes in the dermatological diseases and conditions dataset and 305 genes in the protein synthesis dataset.

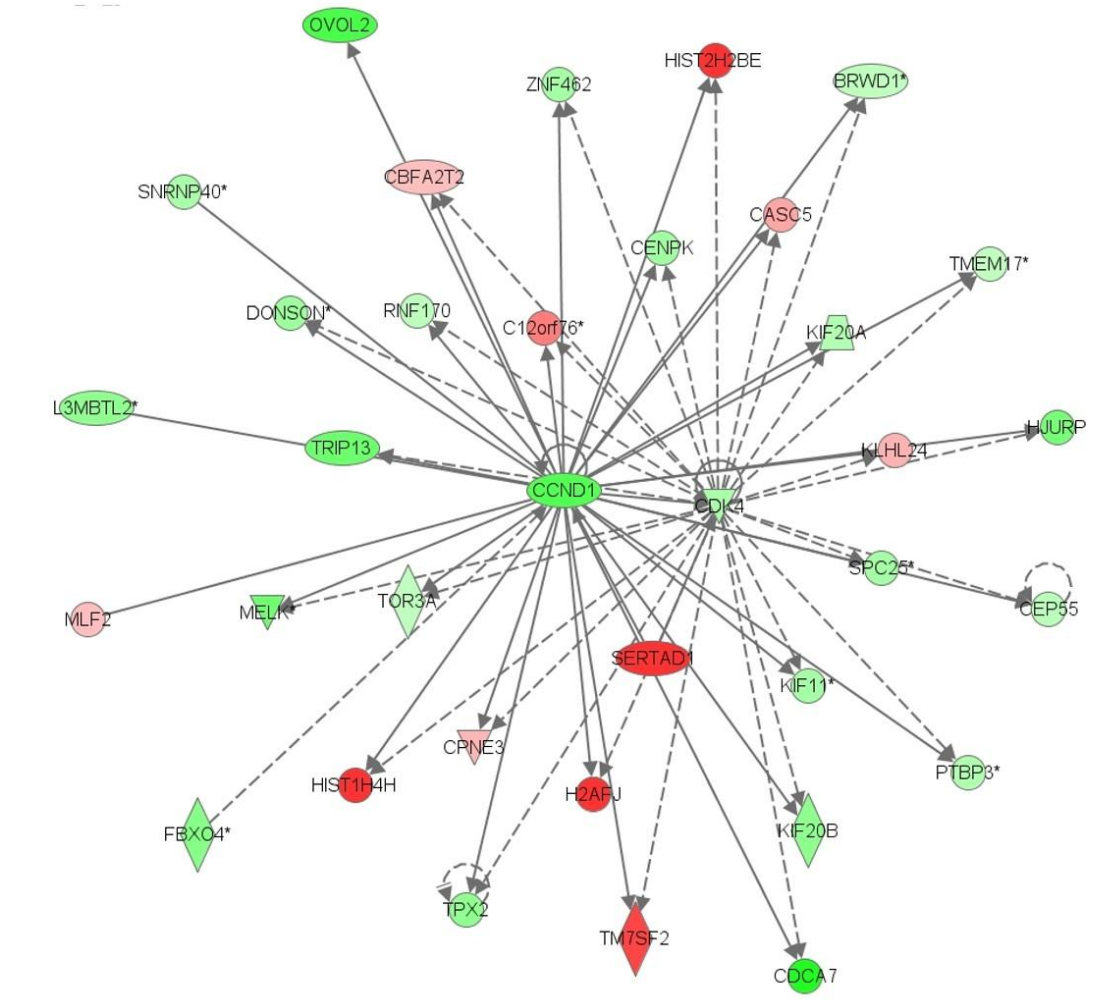


Figure 6-10: Interaction of genes modulated by I-BET151 in TNFα + IL-17 stimulated keratinocytes at 6h

IPA revealed that the greatest number of differentially regulated genes grouped into the "Cell cycle, cellular assembly and organisation, DNA replication, recombination and repair" network, shown above with biological relationships between indicated. Solid lines indicate direct interactions whereas dashed lines indicate indirect relationships. Inverted triangles represent kinases, vertical diamonds are peptidases, ovals are transcription regulators, trapeziums are transporters and circles are other molecules. The intensity of red and green indicates the degree of up or downregulation, respectively, at 6h after treatment with I-BET151, compared to vehicle, in TNFα + IL17 stimulated NHEKs.

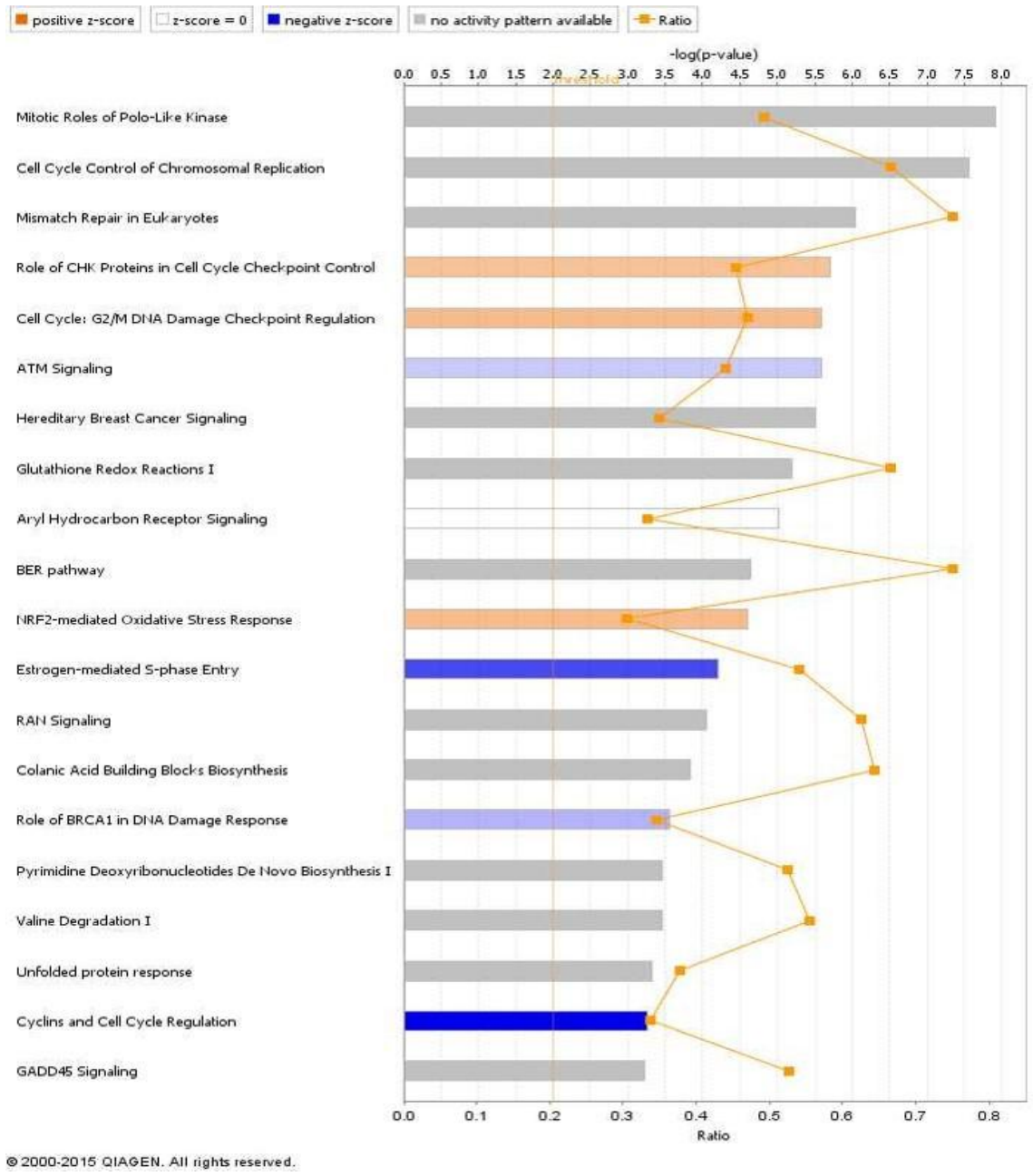


Figure 6-11: Top 20 Ingenuity® Pathway Analysis "canonical pathways" differentially modulated by I-BET151 in TNFα + IL-17 stimulated keratinocytes at 24h

Expression profiles are shown as log mean values, compared to DMSO in TNF□ + IL-17 stimulated keratinocytes at 24h, and were considered significant when the adjusted p-value (threshold) was ≤ 0.01 and used as input into IPA. The orange line depicts the ratio (number of pathway relevant proteins in the data set divided by the total number of proteins in that pathway). Bars in orange, blue or grey predict an increase, decrease or undetermined change in a pathway's activity, respectively. This is based on the activation Z-score, which infers the activation state of predicted transcriptional regulators of a particular pathway using expression patterns of differentially expressed genes within that dataset.

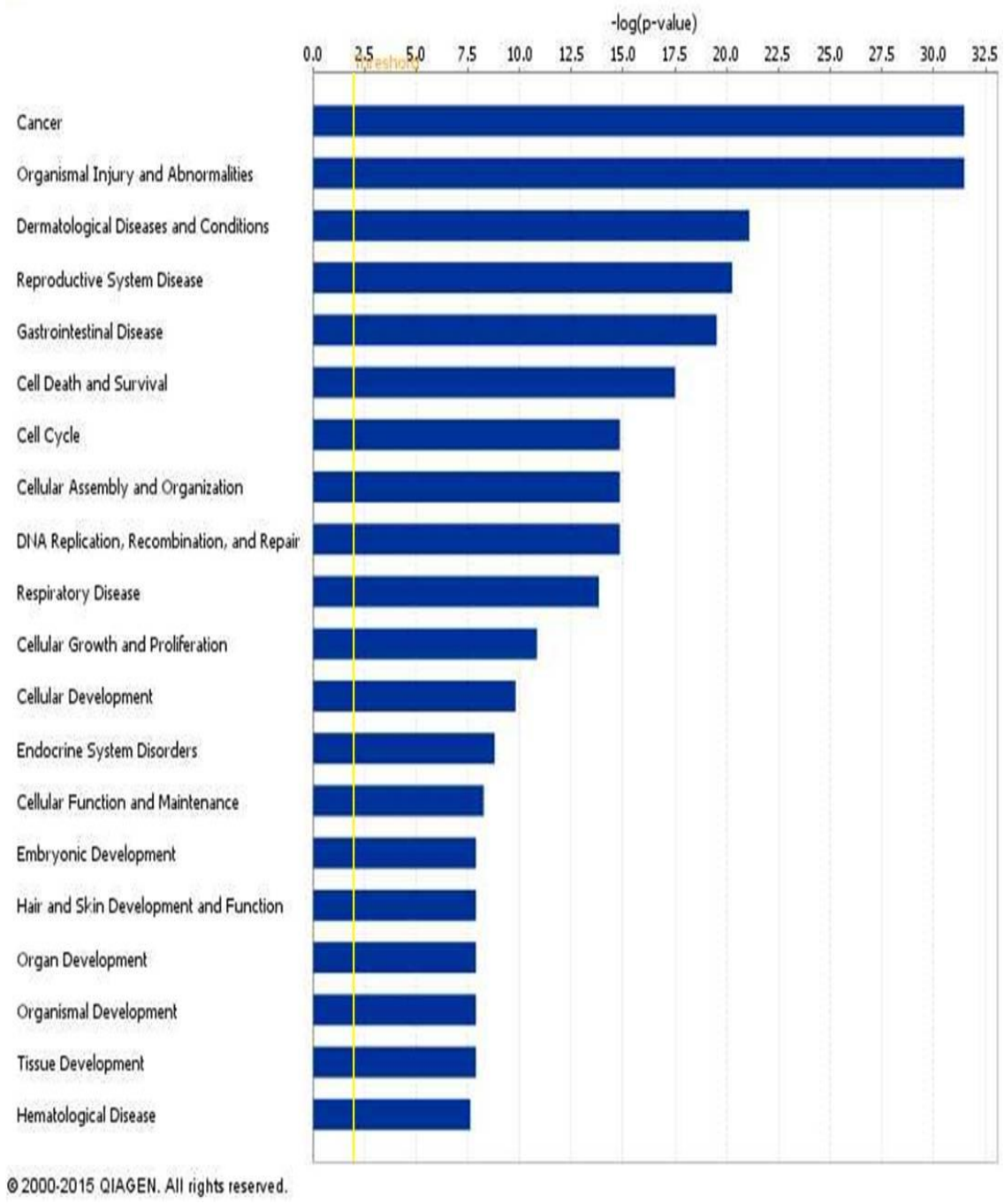


Figure 6-12: Top 20 Ingenuity® Pathway Analysis "diseases and biological functions" differentially modulated by I-BET151 in TNFα + IL-17 stimulated keratinocytes at 24h

Expression profiles are shown as log mean values, compared to DMSO in TNFα + IL-17 stimulated keratinocytes at 6h, and were considered significant when the adjusted p-value (threshold) was ≤ 0.01 and used as input into IPA. The yellow line indicates the significance, $p < 0.01$ (*i.e.* all datasets shown were significant). 1219 genes were included in the cancer dataset, 403 genes in the dermatological diseases and conditions dataset, 948 genes in the cell death and survival dataset and 124 genes in the hair and skin development and function dataset.

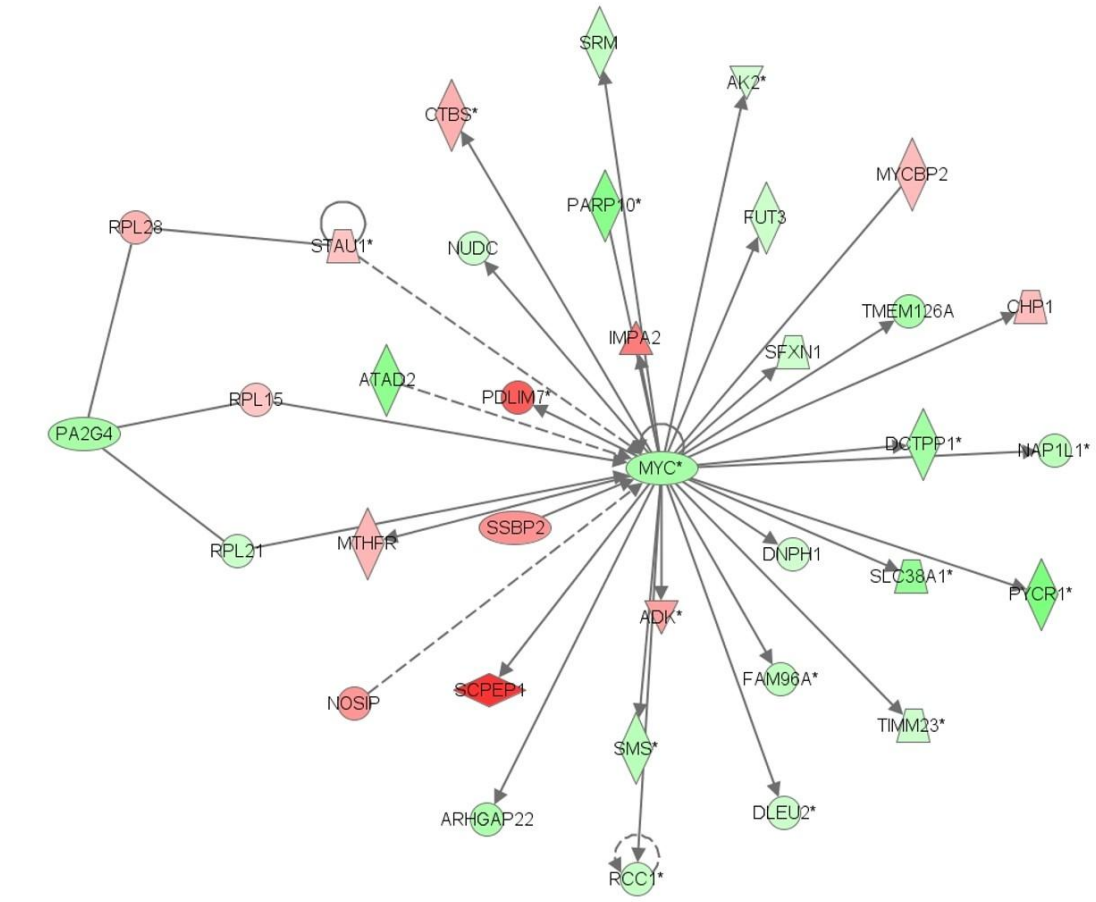


Figure 6-13: Interaction of genes modulated by I-BET151 in TNFα + IL-17 stimulated keratinocytes at 24h

IPA revealed that the greatest number of differentially regulated genes grouped into the "Amino acid metabolism, small molecule biochemistry, post-translational modification" network, shown above with biological relationships between indicated. Solid lines indicate direct interactions whereas dashed lines indicate indirect relationships. Inverted triangles represent kinases, vertical diamonds are peptidases, horizontal diamonds are enzymes, ovals are transcription regulators, trapeziums are transporters and circles are other molecules. The intensity of red and green indicates the degree of up or downregulation, respectively, at 24h after treatment with I-BET151, compared to vehicle, in TNFα + IL17 stimulated NHEKs.

Symbol	Gene name	Fold change (Log_2), 6h	Activation Z-Score, 24h	Inferred activation, 24h
CSF2	Colony stimulating factor 2	-3.19	-7.14	Inhibited
IL1 (A, B)	Interleukin 1 (alpha, beta)	-2.76, -2.44	-2.95, -2.11	Inhibited
TRIB3	Tribbles pseudokinase 3	-1.99	2.53	Activated
TP63	Tumor protein p63	-1.85	-2.46	Inhibited
IL1RN	Interleukin 1 receptor antagonist	-1.65	4.61	Activated
CCND1	Cyclin D1	-1.04	-2.97	Inhibited
E2F2	E2F transcription factor 2	-0.96	-2.18	Inhibited
MYC	V-myc avian myelocytomatosis viral oncogene homolog	-0.80	-5.38	Inhibited
IKBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	-0.80	2.22	Activated
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	-0.75	-2.47	Inhibited

Table 6-12: Top 10 inhibited regulators at 6h, in response to I-BET151 in TNFα + IL-17 stimulated keratinocytes, and inferred activation/activation Z-score at 24h predicted using Ingenuity® Pathway Analysis

Table shows top 10 regulators inhibited by I-BET151 treatment in TNFα + IL-17 stimulated NHEKs at 6h. Inferred activation state at 24h of these regulators was based on the IPA-predicted activation Z-score at 24h. The activation Z-score infers the activation state of predicted transcriptional regulators of a particular pathway using expression patterns of differentially expressed genes within the (24h) dataset.

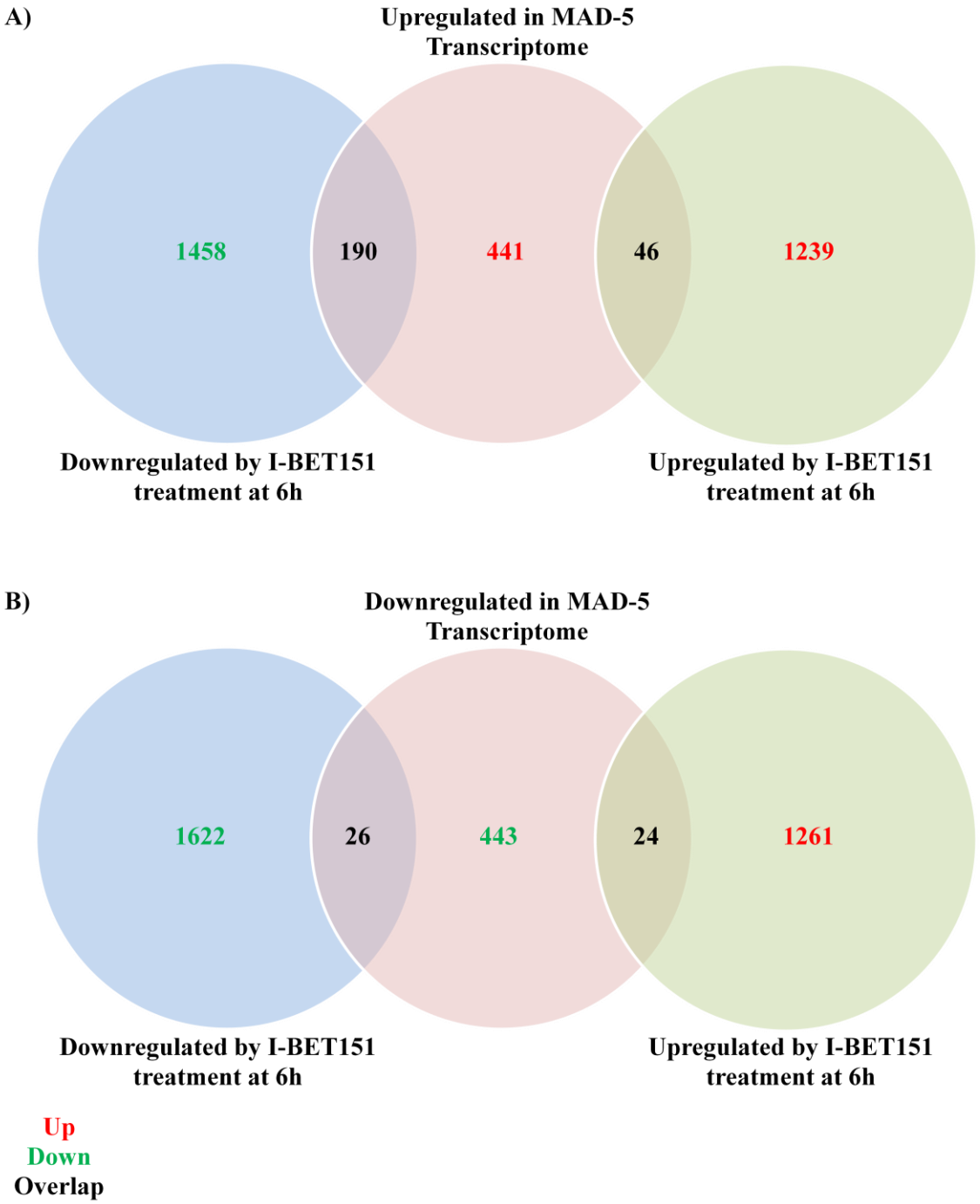


Figure 6-14: Overview of genes modulated by I-BET151 in TNFα + IL-17 stimulation in keratinocytes at 6h and included in the MAD-5 psoriasis transcriptome

Venn diagram shows overlap of genes modulated by I-BET151 in TNFα + IL-17 stimulated NHEKs at 6h and either **A**) upregulated in the MAD-5 psoriasis transcriptome (Tian *et al.*, 2012) or **B**) downregulated in the MAD-5 psoriasis transcriptome.

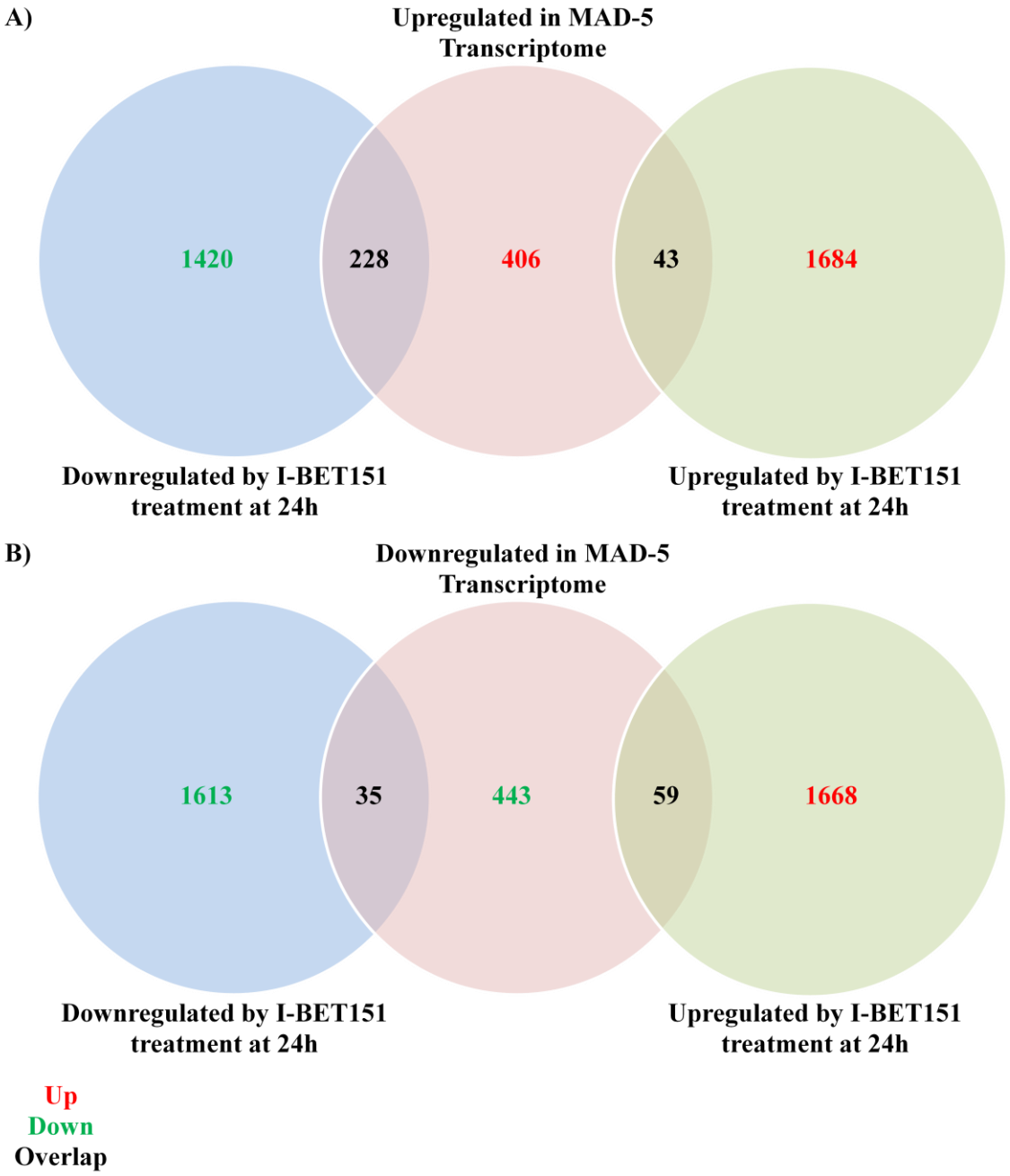


Figure 6-15: Overview of genes modulated by I-BET151 in TNFα + IL-17 stimulation in keratinocytes at 24h and included in the MAD-5 psoriasis transcriptome

Venn diagram shows overlap of genes modulated by I-BET151 in TNFα + IL-17 stimulated NHEKs at 24h and either **A**) upregulated in the MAD-5 psoriasis transcriptome (Tian *et al.*, 2012) or **B**) downregulated in the MAD-5 psoriasis transcriptome.

6.3.4. I-BET151 modulated a sub-set of genes, induced by TNFα + IL-17 stimulation in keratinocytes, which are known to be differentially expressed in psoriasis

Having observed that I-BET151 in TNFα + IL-17 stimulated NHEKs, regulated expression of genes involved in p53 signalling, cell cycle and cellular growth and proliferation, which are known to be involved in the pathogenesis of psoriasis and that many of the differentially regulated genes are involved in "dermatological diseases and conditions", we hypothesised that there would be an overlap of genes which were regulated by TNFα + IL-17 stimulation and sensitive to I-BET151 treatment and also relevant to the pathogenesis of psoriasis.

Figure 6-16 shows that of the 260 probes regulated by TNFα + IL-17 stimulation in NHEKs at 6h, 168 were modulated by I-BET151. Of these 168, 121 probes were upregulated by TNFα + IL-17 stimulation but downregulated by I-BET151 and 6 were downregulated by TNFα + IL-17 stimulation but upregulated by I-BET151. Figure 6-17 shows that of the 436 probes regulated by TNFα + IL-17 stimulation in NHEKs at 24h, 280 were modulated by I-BET151. Of these 280, 169 probes were upregulated by TNFα + IL-17 stimulation but downregulated by I-BET151 and 25 were downregulated by TNFα + IL-17 stimulation but upregulated by I-BET151. These findings demonstrated that 48.4% and 44.4% of genes regulated by TNFα + IL-17 stimulation in NHEKs were differentially modulated by I-BET151 treatment, at 6h and 24h respectively.

Figure 6-18, shows that 174 probes were upregulated by TNFα + IL-17 stimulation a both 6 and 24h, while 1063 probes were downregulated by I-BET151 treatment at both 6 and 24h; with an overlap of 70 probes, which represented 59 unique genes. To understand the gross functions of the 59 DEGs, data was mined using Ingenuity Pathway Analysis (IPA). Perhaps unsurprisingly, the top diseases and biological functions, that genes grouped into was dermatological diseases and conditions dataset (32 genes were included in this data set) (Figure 6-19). Interestingly, DEGs also grouped immune cell function trafficking and inflammatory responses, both relevant to the pathogenesis of psoriasis (Figure 6-19). Of these 32 genes, 26 had roles in psoriasis according to analysis in IPA (Figure 6-20) which derives this information from primary literature, in *e.g.* journal articles, review articles and textbooks, as well as publicly available databases.

In comparison, 22 probes were downregulated by TNFα + IL-17 stimulation a both 6 and 24h, while 884 probes were upregulated by I-BET151 treatment at both 6 and 24h; with an overlap of only 1 probe, which represented the connective tissue growth factor gene (Figure 6-21).

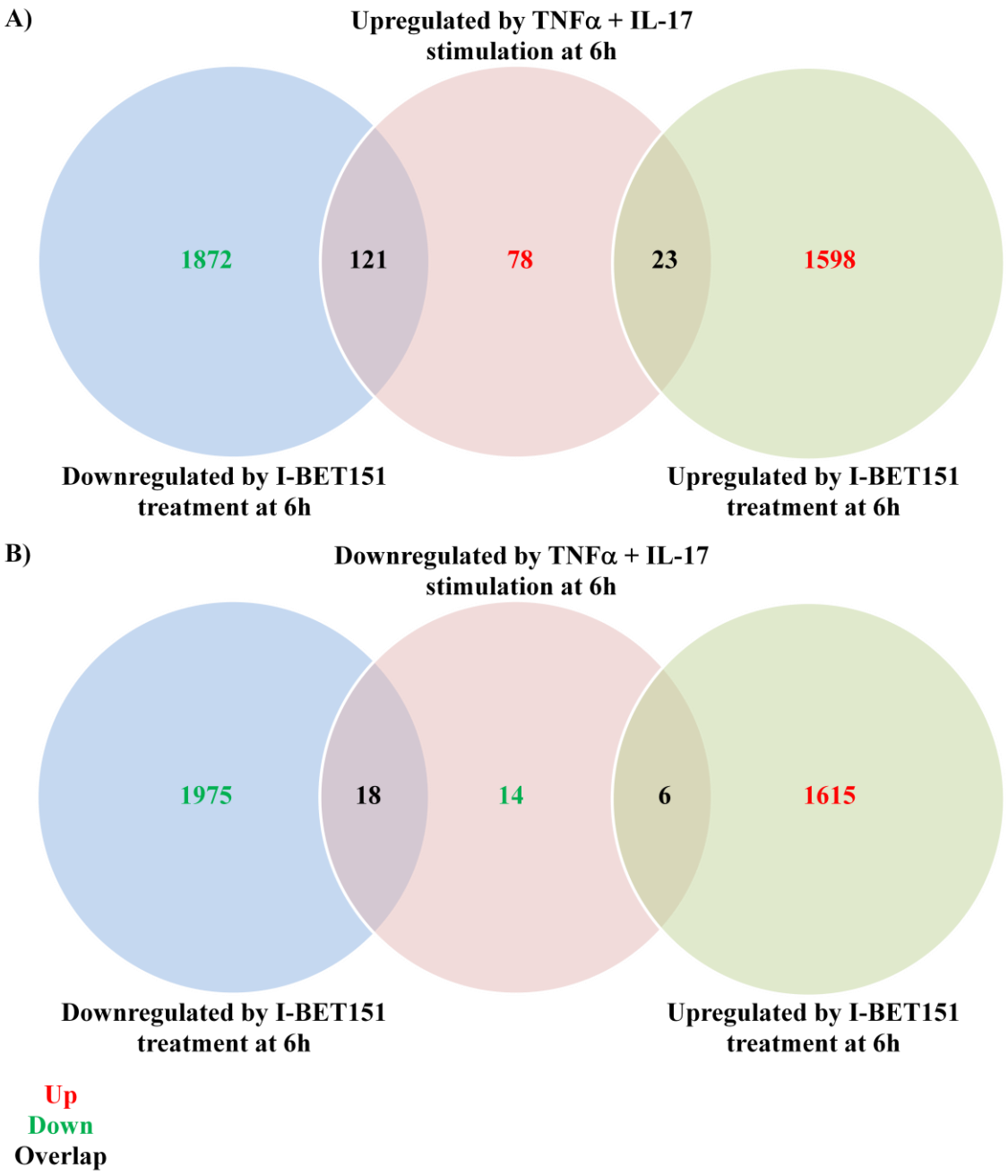


Figure 6-16: Overview of probes modulated by I-BET151 and TNFα + IL-17 stimulation in keratinocytes at 6h

Venn diagram shows overlap of probes modulated by I-BET151 in TNFα + IL-17 stimulated NHEKs at 6h and either **A**) upregulated in TNFα + IL-17 stimulated NHEKs or **B**) downregulated in TNFα + IL-17 stimulated NHEKs.

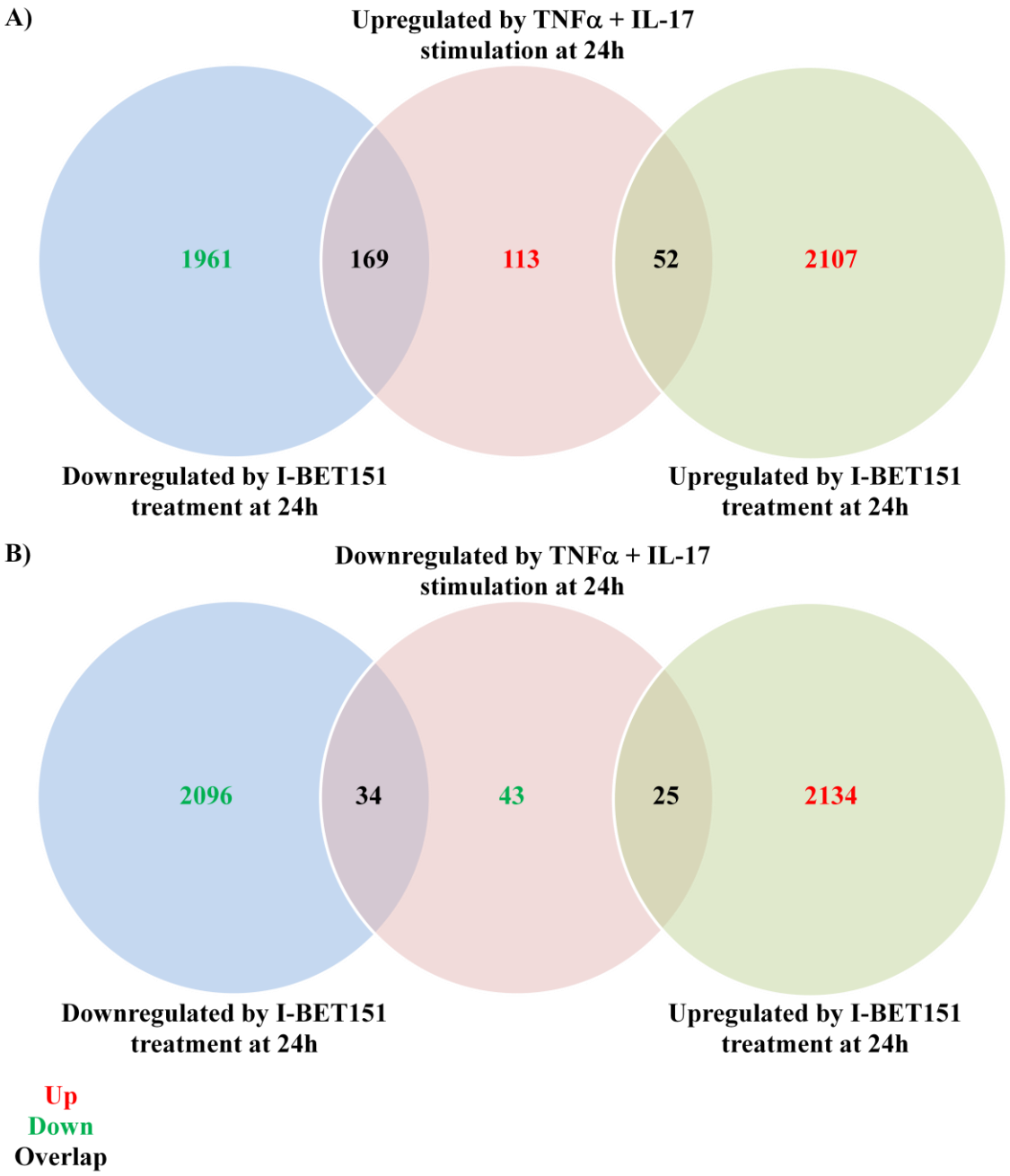


Figure 6-17: Overview of probes modulated by I-BET151 and TNFα + IL-17 stimulation in keratinocytes at 24h

Venn diagram shows overlap of probes modulated by I-BET151 in TNFα + IL-17 stimulated NHEKs at 24h and either **A**) upregulated in TNFα + IL-17 stimulated NHEKs or **B**) downregulated in TNFα + IL-17 stimulated NHEKs.

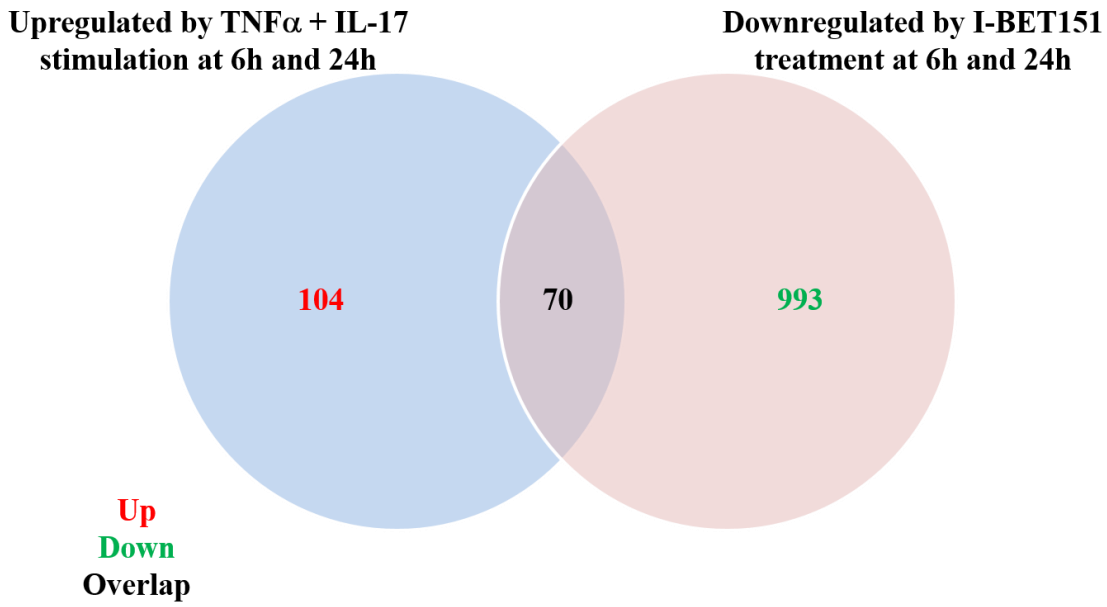


Figure 6-18: Overview of probes positively regulated by TNFα + IL-17 stimulation and sensitive to I-BET151 treatment

Venn diagram shows the number of probes upregulated by TNFα + IL-17 stimulation at both 6 and 24h and the number of probes downregulated by I-BET151 treatment at both 6 and 24h. The 70 overlapping probes represented 59 unique genes. For both datasets, cut-offs were as follows: fold change ≥ 1.3 and p-value ≤ 0.01.

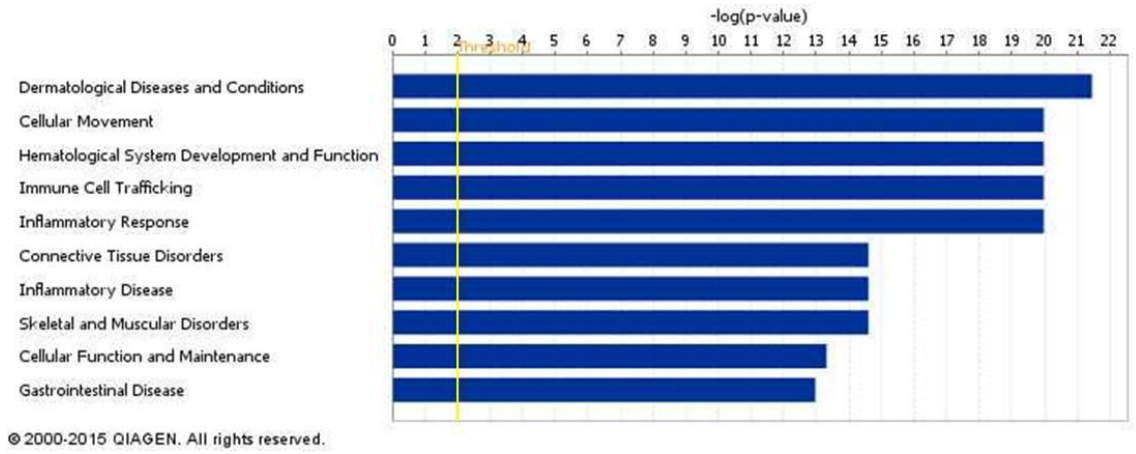


Figure 6-19: Top 10 Ingenuity® Pathway Analysis "diseases and biological functions" of genes positively regulated by TNFα + IL-17 stimulation and downregulated by I-BET151 treatment in keratinocytes (*i.e.* overlap of genes from Figure 6-18).

The yellow line indicates the significance $p < 0.01$ (*i.e.* all datasets shown are significant), 32 genes were included in the dermatological diseases and conditions dataset.

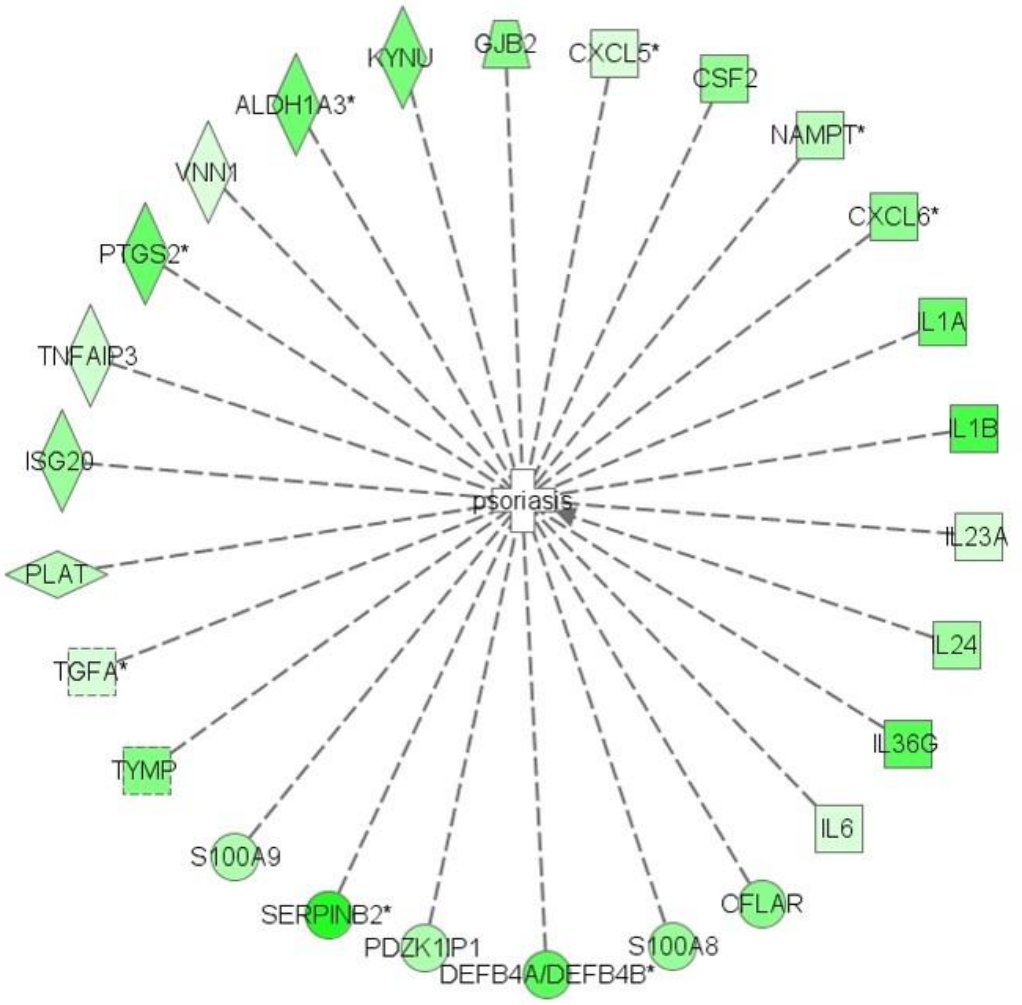


Figure 6-20: Overview of the 26 genes positively regulated by TNFα + IL-17 stimulation, downregulated by I-BET151 treatment and included in a psoriasis network defined by Ingenuity® Pathway Analysis

Diagram shows genes, and their functions, upregulated by TNFα + IL-17 stimulation at both 6 and 24h and downregulated by I-BET151 treatment at both 6 and 24h (*i.e.* overlap of genes from Figure 6-18 and which were included in an-IPA defined psoriasis network. Squares represent cytokines, trapeziums are transporters, vertical diamonds are peptidases, horizontal diamonds are enzymes, interrupted squares are growth factors, and circles are other molecules. The intensity of green indicates the degree of downregulation at 24h after treatment with I-BET151, compared to vehicle, in TNFα + IL17 stimulated NHEKs. *indicates gene was represented by more than one probe.

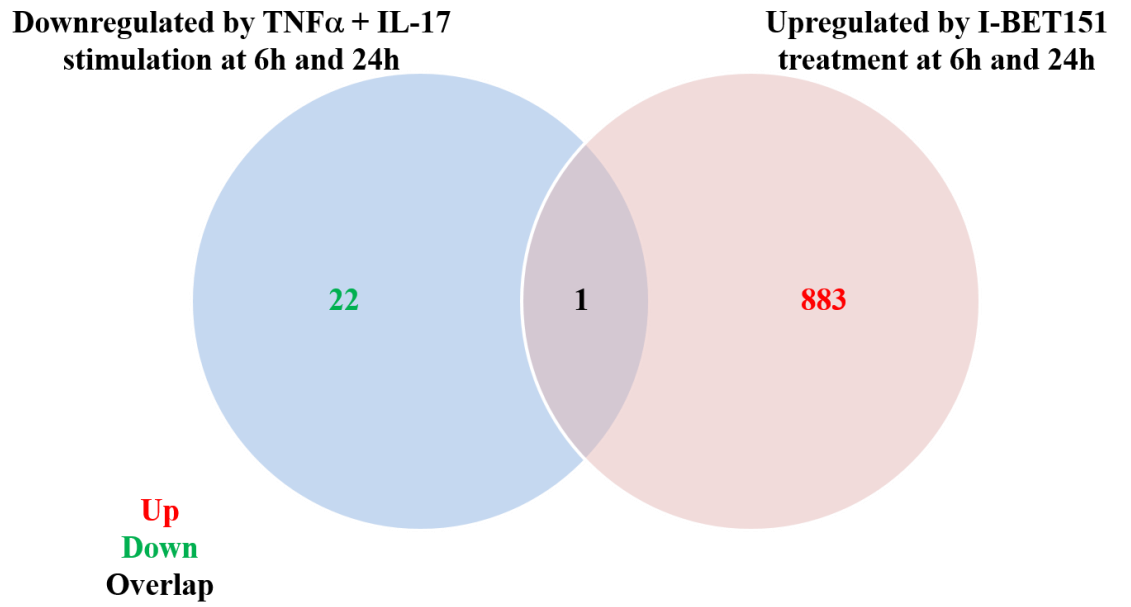


Figure 6-21: Overview of probes differentially regulated by TNFα + IL-17 stimulation and I-BET151 treatment

Venn diagram shows the number of probes downregulated by TNFα + IL-17 stimulation at both 6 and 24h and the number of probes upregulated by I-BET151 treatment at both 6 and 24h. For both datasets, cut-offs were as follows: fold change ≥ 1.3 and p-value ≤ 0.01.

6.3.5. Overlap of genes differentially regulated by TNFα + IL-17 and I-BET151 compared to published MAD-5 psoriasis transcriptome

In addition to investigating the overlap of genes which were upregulated by TNFα + IL-17 stimulation at both 6 and 24h and downregulated by I-BET151 treatment at both 6 and 24h and which were included in an IPA-defined psoriasis network, the overlap with a published psoriasis transcriptome was also investigated. The meta-analysis derived ("MAD-5") transcriptome is a previously published meta-analysis of 5 transcriptomic studies, which defined a total of 677 upregulated and 443 downregulated genes across the 5 studies (Tian *et al.*, 2012). Of the 677 upregulated genes in the MAD-5 transcriptome, 27 overlapped with the 59 genes upregulated by TNFα + IL-17 stimulation at both 6 and 24h and downregulated by I-BET151 treatment at both 6 and 24h (Figure 6-22 and Table 6-13).

The genes upregulated by TNFα + IL-17 stimulation at both 6 and 24h and downregulated by I-BET151 treatment at both 6 and 24h and included in the IPA psoriasis network (26 genes, Figure 6-20) was compared to those included in the MAD-5 transcriptome (27 genes, Table 6-13). This analysis identified 13 overlapping genes, but 13 and 14 genes featured only in the IPA or MAD-5 datasets respectively (Table 6-14).

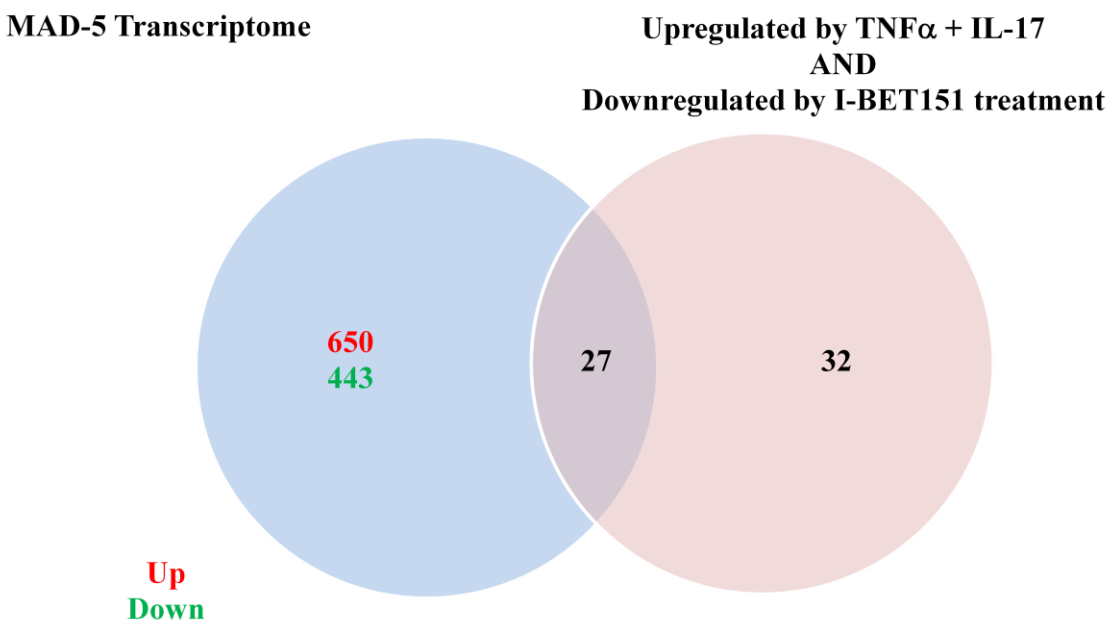


Figure 6-22: Overview of genes positively regulated by TNFα + IL-17 stimulation, downregulated by I-BET151 treatment and included in the MAD-5 psoriasis transcriptome

Venn diagram shows overlap of genes included in the MAD-5 psoriasis transcriptome (Tian *et al.*, 2012) which were also upregulated by TNFα + IL-17 stimulation at both 6 and 24h and downregulated by I-BET151 treatment at both 6 and 24h (*i.e.* overlap of genes from Figure 6-18).

		Fold change (Log_2)				
Gene	**Description**	**MAD-5**	**TNFα + IL-17 6h**	**TNFα + IL-17 24h**	**I-BET151 6h**	**I-BET151 24h**
Cytokines						
IL1F9	Interleukin 1 family, member 9	5.45	4.17	4.78	-2.30	-2.28
CXCL2	Chemokine (C-X-C motif) ligand 2	3.28	1.72	1.39	-0.91	-0.45
IL1B	Interleukin 1, beta	2.43	1.75	2.22	-2.44	-2.55
NAMPT	Nicotinamide phosphoribosyltransferase	1.84	0.44	0.65	-0.42	-0.90
IL1RN	Interleukin 1 receptor antagonist	1.43	1.16	1.20	-1.65	-1.36
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	1.17	0.85	0.94	-1.20	-0.93
Enzymes						
PLAT	Plasminogen activator, tissue	2.85	0.64	0.99	-0.73	-0.91
FUT2	Fucosyltransferase 2	2.02	0.39	0.64	-0.45	-0.62
BIRC3	Baculoviral IAP repeat-containing 3	1.11	0.97	0.84	-0.95	0.60
CYB5R2	Cytochrome b5 reductase 2	1.01	0.49	0.97	-0.77	-1.08
Peptidases						
KYNU	Kynureninase	6.17	1.05	1.26	-1.20	-1.57
VNN1	Vanin 1	3.4	0.41	0.53	-0.46	-0.47
ISG20	Interferon stimulated exonuclease gene 20kDa	2.39	0.40	0.79	-0.56	-1.36
ALDH1A3	Aldehyde dehydrogenase 1 family, member A3	2.37	1.21	1.80	-1.70	-1.92
Growth factors						
TYMP	Thymidine phosphorylase	3.09	0.47	1.16	-0.89	-0.84

TGFA	Transforming growth factor, alpha	1.48	0.65	0.75	-0.48	-0.52
Transporters						
RHCG	Rh family, C glycoprotein	6.3	0.73	1.70	-0.69	-1.76
Transcription regulators						
PRDM1	PR domain containing 1, with ZNF domain	1.64	0.75	0.78	-1.28	-1.25
Other						
DEFB4A	Defensin, beta 4A	7.59	2.91	5.32	-1.89	-2.27
S100A9	S100 calcium binding protein A9	5.18	1.61	2.87	-0.93	-1.11
MPZL2	Myelin protein zero-like 2	3.64	0.56	0.60	-1.12	-0.71
S100A8	S100 calcium binding protein A8	2.3	1.47	2.56	-0.94	-1.40
SERPINA3	Serpin peptidase inhibitor, clade A, member 3	2.29	0.96	0.88	-0.75	-0.71
PDZK1IP1	PDZK1 interacting protein 1	2.29	0.52	1.41	-0.55	-1.09
NOD2	Nucleotide-binding oligomerization domain containing 2	1.82	0.70	0.88	-0.80	-0.92
MYO1B	Myosin IB	1.66	0.48	0.63	-0.69	-0.68
NDRG4	NDRG family member 4	1.26	0.63	1.17	-0.50	-0.53

Table 6-13: Overview of the 27 genes positively regulated by TNFα + IL-17 stimulation, downregulated by I-BET151 treatment and included in the MAD-5 psoriasis transcriptome

Identity of the 27 genes (Figure 6-22) included in the MAD-5 psoriasis transcriptome (Tian *et al.*, 2012) which were also upregulated by TNFα + IL-17 stimulation at both 6 and 24h and downregulated by I-BET151 treatment at both 6 and 24h (*i.e.* overlap of genes from Figure 6-18). Fold changes (Log_2) are shown for each condition: "TNFα + IL-17" represents that effect of TNFα + IL-17 stimulation compared to mock stimulated and "I-BET151" represents the effect of I-BET151 treatment, compared to vehicle, in TNFα + IL-17 stimulated NHEKs, fold changes for MAD-5 were as published (Tian *et al.*, 2012). Molecule type was defined by IPA.

Included in IPA and MAD-5 datasets	Included in IPA dataset	Included in MAD-5 dataset
KYNU	CXCL5	IL1F9
VNN1	IL6	CXCL2
ISG20	IL23A	IL1RN
ALDH1A3	IL1A	TNFSF10
IL1B	IL36G	FUT2
NAMPT	IL24	BIRC3
TYMP	CSF2	CYB5R2
TGFA	CXCL6	RHCG
PLAT	TNFAIP3	PRDM1
DEFB4A	PTGS2	SERPINA3
S100A9	GJB2	NOD2
S100A8	SERPINB2	MYO1B
PDZK1IP1	CFLAR	NDRG4
		MPZL2

Table 6-14: Overview of genes differentially regulated by TNFα + IL-17 stimulation and I-BET151 treatment (*i.e.* overlapping gene from Figure 6-18) and overlap with genes included in an Ingenuity® Pathway Analysis-defined psoriasis network and/or MAD-5 psoriasis transcriptome

Peptidases are shown in green, cytokines in red, growth factors in blue, enzymes in purple, transporters in orange, transcription regulators in maroon and others in black. Molecule type was defined by IPA.

6.4. Discussion

For the first time, the effects of a BET inhibitor on global gene expression in a cell model of cutaneous inflammation were investigated. In TNFα + IL-17 stimulated NHEKs, I-BET151 modulated expression of genes involved in the cell cycle, p53 signalling and inflammation; pathways which are involved in the pathogenesis of psoriasis. Both I-BET151 and TNFα + IL-17 affected expression of genes which have previously been shown to be differentially expressed in psoriasis. Furthermore we demonstrated that many genes which were upregulated by TNFα + IL-17 stimulation and down-regulated with I-BET151 treatment, overlapped with genes which have been shown to be both upregulated in psoriasis and involved in its pathogenesis. These findings suggest that i) BET proteins regulate the expression of genes relevant to psoriasis and ii) BET proteins may represent novel targets for the treatment of psoriasis.

The effects of TNFα + IL-17 stimulation of NHEKs have previously been investigated in a microarray study (Chiricozzi *et al.*, 2011) which showed some discordance with our dataset. There are number of factors which may contribute to the discrepancies. Firstly, primary keratinocytes were used, therefore donor variability may account for some of these differences. As our dataset had 3 times the number of donors it is likely to be more robust (N=4 in Chiricozzi *et al*.'s dataset and N=12 in our dataset) and although there were some heterogenic responses, for logistical reasons our array focused on uniform responses at this stage (section 6.3.1). It would however be important to validate a sub-set of our DEGs through qRT-PCR. Secondly, a specified FDR of 0.3 could suggest that some of the observations reported by Chiricozzi *et al*. are due to chance and may include false positives (Chiricozzi *et al.*, 2011). Thirdly, Chiricozzi *et el*. used a IL-17 concentration of 200ng/ml compared to 100ng/ml in our experiments. Although it was previously observed that there were no signification differences in IL-6 or IL-8 induction in response to simulation with IL-17 at concentrations between 100 – 400 ng/ml (data from preceding MRes, as discussed in section 3.3.1), it is possible that other analytes showed greater sensitivity to IL-17 stimulation at increasing concentrations. Finally, although Chiricozzi *et al*. also used Illumina human HT-12 microarray platform for analysis, the version was not specified therefore probes may have varied (Chiricozzi *et al.*, 2011). However despite these potential differences, the overlap between the 2 datasets was good (157 probes) suggesting that TNFα + IL-17 stimulation had robust and reproducible effects in different hands. Furthermore, many

of the genes regulated by these stimuli, which are key cytokines in the pathogenesis of psoriasis, were also known to be differentially expressed in psoriasis.

In NHEKs, TNFα + IL-17 stimulation regulated expression of 260 probes (217 genes) and 436 probes (358 genes), with a fold change ≥ 1.3 and p-value ≤ 0.01, at 6h and 24h post-stimulation respectively. At each timepoint, approximately 25% of the DEGs were also included in the MAD-5 psoriasis transcriptome (Tian *et al.*, 2012). There was also clear overlap of probes (174 upregulated and 23 downregulated) regulated by TNFα + IL-17 stimulation at both 6h and 24h, this demonstrated stimulation had sustained effects. In keeping with this finding, 7 of the top 10 genes upregulated by TNFα + IL-17 stimulation at 6h were also in the top 10 at 24h and included both cytokines (*e.g.* IL-8) and anti-microbial peptides (*e.g.* DEFB4A) known to be increased and to play key roles in psoriasis. Consistent with the overlap of DEGs at 6 and 24h after stimulation with TNFα and IL-17, 4 out of the top 5 predicted upstream regulators of DEGs at 6h (TNF, NFKB, IL1A and IFNG) were also in the top 5 predicted upstream regulators of DEGs at 24h, and all these factors are key drivers of the pathogenesis of psoriasis (Perera *et al.*, 2012; Lowes *et al.*, 2014). Furthermore approximately 5 and 8% of DEGs in the MAD-5 transcriptome were regulated by TNFα + IL-17 stimulation in NHEKs at 6h and 24h respectively. These are reasonable figures given that our array included only one cell type whereas the MAD-5 transcriptome was derived from array data of whole skin. These findings suggest the TNFα + IL-17 *in vitro* model of cutaneous inflammation was a valid model to study the expression of genes known to be dysregulated in psoriasis.

As BET inhibitors have previously been shown to have anti-inflammatory and anti-proliferative effects (Filippakopoulos *et al.*, 2010; Nicodeme *et al.*, 2010; Dawson *et al.*, 2011), we hypothesised that I-BET151 would regulate the expression of some key genes involved in the pathogenesis of psoriasis and these would compromise genes regulated in other cell types (Filippakopoulos *et al.*, 2010; Nicodeme *et al.*, 2010; Dawson *et al.*, 2011) but also some novel cell-type specific genes. This hypothesis was supported by the finding that, of the 1120 up or downregulated DEGs in the MAD-5 transcriptome, approximately 19% and 26% of genes were differentially modulated by I-BET151 treatment in TNFα + IL-17 stimulated NHEKs, at 6h and 24h respectively. Furthermore, approximately 10% of genes regulated by I-BET151 in the model of cutaneous inflammation were also included in the MAD-5 transcriptome. DEGs were

compared in TNFα + IL-17 stimulated NHEKs with or without I-BET151 and it was found that 48.4% and 44.4% of genes regulated by TNFα + IL-17 stimulation in NHEKs were differentially modulated by I-BET151 treatment, at 6h and 24h respectively. Furthermore, the top I-BET15 inhibited regulators at 6h overlapped considerably with predicted regulators of DEGs at 24h, demonstrating that I-BET151 had sustained effects, and interestingly inhibited regulators (IL-1A & B, and NFKB1) that are known to be key regulators of inflammation in psoriasis (Perera *et al.*, 2012; Lowes *et al.*, 2014). In addition Cyclin D1 was also one of the most inhibited regulators of 6 and 24h DEGs (Table 6-12). Cyclin D1 is known to have a role in regulating the cell cycle, has been shown to be over expressed in lesional psoriatic skin and interestingly is downregulated in response to currently prescribed anti-psoriatic treatments, including phototherapy (Abou *et al.*, 2010). Furthermore TP63 was also a key regulator of genes inhibited by I-BET151 (Table 6-12); TP63 has been demonstrated to regulate keratinocyte differentiation, a process known to be dysregulated in psoriasis (Kouwenhoven *et al.*, 2015). Of particular note, two of top upstream regulators (IL1A and NFKB) of DEGs in TNFα + IL-17 stimulated NHEKs (Table 6-7 and Table 6-9) were also the most sensitive to I-BET151 treatment in TNFα + IL-17 stimulated NHEKs (Table 6-12). Collectively these findings support the hypothesis that BET proteins are involved in regulating expression of keratinocyte genes, which have roles in inflammation and proliferation, and suggests BET inhibitors may have a role in treating cutaneous inflammation.

In addition to identifying key signalling pathways, DEGs were interrogated using R Software and IPA to define genes of particular interest; the subset of genes upregulated by TNFα + IL-17 stimulation at both 6 and 24h but downregulated by I-BET151 treatment at both 6 and 24h was compared to an IPA-defined psoriasis network as well as the MAD-5 psoriasis transcriptome. Using this approach, 59 genes were found to be consistently upregulated by stimulation but downregulated by compound at both timepoints. 32 of the 59 genes grouped into IPA's dermatological diseases and conditions dataset and this was the top diseases and biological functions group, encompassing the most number of DEGs. The 59 DEGs were also included in other IPA-defined diseases and biological functions groups including immune cell function trafficking and inflammatory responses, both relevant to the pathogenesis of psoriasis. Of the 59 genes, 26 also featured in an IPA-defined psoriasis network and 27 were in the MAD-5 transcriptome and, perhaps of most interest, there were 13 genes common to

both of these two datasets. Many of the proteins encoded by these 13 genes are known to have key roles in psoriasis, for example:

- S100A8 and S100A9 encodes S100 calcium binding protein A8 and A9, respectively, which as the name suggests binds calcium but also has anti-microbial and chemotactic effects as well as roles in regulating epidermal differentiation and activation of immune cells (Wilsmann-Theis *et al.*, 2015). Interestingly S100A8/A9 have both been proposed to be biomarkers for psoriatic arthritis activity (Hansson *et al.*, 2014). S100 proteins are some of the most upregulated genes in psoriasis (Yao *et al.*, 2008); stimulation with TNFα + IL-17 increased expression of S100A8 by 2.8 and 5.9 fold at 6h and 24h respectively and I-BET151 inhibited expression by 1.9 and 2.6 fold at 6h and 24h respectively. Stimulation with TNFα + IL-17 had even greater effects on S100A9, increasing expression by 3.1 and 7.3 fold at 6h and 24h respectively and I-BET151 inhibited expression by 1.9 and 2.2 fold at 6h and 24h respectively.
- DEFB4A encodes defensin-β4, an anti-microbial peptide with broad-spectrum activity against bacterial, fungal, and viral pathogens. β-defensins also have chemotactic effects on dendritic and T-cells (Ganz, 2003). β-defensins are some of the most upregulated genes in psoriasis (Suarez-Farinas *et al.*, 2010); stimulation with TNFα + IL-17 increased expression of DEFB4A by 7.5 and 39.9 fold at 6h and 24h respectively and I-BET151 inhibited expression by 3.7 and 4.8 fold at 6h and 24h respectively.
- TGFA encodes transforming growth factor alpha, a growth factor that has been shown to enhance keratinocyte migration and upregulate expression of toll-like receptors 5 and 9 in keratinocytes, leading to enhanced secretion of IL-8 and β-defensins (Cha *et al.*, 1996; Miller *et al.*, 2005); stimulation with TNFα + IL-17 increased expression of TGFA by 1.6 and 1.7 fold at 6h and 24h respectively and I-BET151 inhibited expression by 1.4 and 1.4 fold at 6h and 24h respectively.
- KYNU encodes kynureninase, a peptidase involved in tryptophan degradation; upregulation of tryptophan metabolism confers anti-microbial effects in cells including macrophages and disruption of this pathway can affect T-cell proliferation (Ito *et al.*, 2004). KYNU is one of the most upregulated genes in psoriasis (Suarez-Farinas *et al.*, 2010); stimulation with TNFα + IL-17 increased

expression of KYNU by 2.1 and 2.4 fold at 6h and 24h respectively and I-BET151 inhibited expression by 2.3 and 3.0 fold at 6h and 24h respectively.

- VNNI encodes Vanin 1, a pantetheinase involved in pantetheine (the cysteamine amide analogue of vitamin B_5) metabolism. This enzyme is induced by T_H1 and T_H17 psoriasis-associated cytokines, may have roles in inflammation and epidermal differentiation and has previously been described as a potential target in psoriasis treatment (Jansen *et al.*, 2009); stimulation with TNFα + IL-17 increased expression of VNNI by 1.3 and 1.4 fold at 6h and 24h respectively and I-BET151 inhibited expression by 1.4 and 1.4 fold at 6h and 24h respectively.
- NAMPT (PBEF1) encodes nicotinamide phosphoribosyltransferase, which is involved in nicotinamide adenine dinucleotide (NAD) biosynthesis thus may regulate inflammation and cellular proliferation, NAMPT is also known to have cytokine functions and induces expression of IL-6, TNFα and VEGF (Xie *et al.*, 2014); stimulation with TNFα + IL-17 increased expression of NAMPT by 1.4 and 1.6 fold at 6h and 24h respectively and I-BET151 inhibited expression by 1.3 and 1.9 fold at 6h and 24h respectively.
- TYMP (PD-ECGF) encodes thymidine phosphorylase, involved in pyrimidime metabolism and is known to act as an angiogenic growth factor and promotes angiogenesis in vivo and stimulates the growth and chemotaxis of cells in vitro (Akiyama *et al.*, 2004); stimulation with TNFα + IL-17 increased expression of TYMP by 1.4 and 2.2 fold at 6h and 24h respectively and I-BET151 inhibited expression by 1.9 and 1.8 fold at 6h and 24h respectively.

In addition to the overlapping 13 genes included in both the IPA-defined psoriasis network and the MAD-5 psoriasis transcriptome, 13 genes only featured in the IPA network and 14 genes only in the MAD-5 transcriptome (Table 6-14). There are a number of reasons why there was not complete concordance between the two datasets. As previously discussed, the information in IPA is manually curated from a wide variety of published data sources. Although IPA is not without some faults (Henderson-Maclennan *et al.*, 2010) and it relies on published data, and publication bias is possible, the careful curation is likely to mean the IPA-defined psoriasis network may define a broader set of genes relevant to psoriasis than a purely array-based meta-analysis dataset. For example, it is known that microarrays can have low sensitivity compared to PCR-based methods (Chiricozzi *et al.*, 2011). Indeed, when we compared IL-6 and IL-

8 expression profiles, based on microarray and qRT-PCR results, qRT-PCR results consistently demonstrated greater sensitivity compared to array data (Table 6-4. Chiricozzi *et al*. previously reported TNFα +IL-17 stimulation of NHEKs had an additive effect on upregulating expression of some genes (*e.g.* LCN2) when measured by microarray, but that the effect was synergistic when measured by RT-PCR, demonstrating the latter method had greater sensitivity. Interestingly 8 of the 13 genes (*i.e.* CXCL5, IL6, IL13A, IL1A, IL36G, IL24, CSF2 and CXCL6 (Table 6-14) included in the IPA dataset but not the MAD-5 dataset are cytokines and many of these cytokines including IL-6, in particular, are known to have key roles in psoriasis (Perera *et al.*, 2012; Saggini *et al.*, 2014). I-BET151 inhibition of IL36G may also be of particular interest as deficiency in IL-36 receptor antagonist is known to underlie pustular psoriasis (Marrakchi *et al.*, 2011; Sugiura *et al.*, 2013), BET inhibitors may therefore represent novel therapy for this potentially life threatening endotype of psoriasis. Stimulation of NHEKs with TNFα + IL-17 increased expression of IL36G by 17.3 and 27.5 fold at 6h and 24h respectively and I-BET151 inhibited expression by 4.9 and 4.8 fold at 6h and 24h respectively. The discrepancy in cytokines being excluded in DEGs sets in array data has previously been addressed with the explanation that "expression of these (inflammatory) genes is usually low on the Affymetrix gene array platform and hence fold change is not accurately measured. Most analysis pipelines filter out low abundance genes so they may be excluded from the statistical analysis" (Suarez-Farinas *et al.*, 2010); a similar explanation for the lack of many cytokines in the MAD-5 transcriptome was made by its authors (Tian *et al.*, 2012). In addition variance in DEGs amongst different transcriptomic studies may be a factor in excluding potentially relevant DEGs, as illustrated by connective tissue growth factor (CTFG), below. Furthermore, DEGs in psoriasis are compounded by variations in numbers of relevant cells, which may also be in low abundance, that express the gene in the sample (section 1.2.7).

The 14 genes included in the MAD-5 dataset but not included in the IPA-defined psoriasis network, may represent interesting candidates for further study given that they are also upregulated by TNFα + IL-17, key psoriasis cytokines.

Interestingly there was only one gene, CTFG, which was downregulated by TNFα + IL-17 stimulation at both 6 and 24h and upregulated by I-BET151 treatment at both 6 and 24h; stimulation with TNFα + IL-17 decreased expression of CTFG by 1.7 and 1.9 fold at 6h and 24h respectively and I-BET151 increased expression by 7.7 and 3.3 fold

at 6h and 24h respectively. Although CTFG was not included in either the IPA-defined psoriasis network or in the MAD-5 transcriptome, it has previously been found to be downregulated in psoriatic lesional skin, compared to non-lesional skin (Manczinger and Kemeny, 2013), and although its role in psoriasis has not yet identified it was identified as a central node in a network analysis of DEGs in psoriasis (Manczinger and Kemeny, 2013). Connective tissue growth factor is involved in angiogenesis and acts downstream of TGF-β and in addition to being downregulated in psoriasis, stimulation with key psoriasis-related cytokines (IFNγ + TNFα) has previously been demonstrated to downregulate CTFG in lung endothelial cells (Laug *et al.*, 2012). Thus CTGF may be a further gene of interest to investigate in the pathogenesis of psoriasis.

I-BET151 modulated ~ 7.7% and 9.1% of the total number of probes in the array (~47000) at 6 and 24 h respectively, indicating effects of the compound were discrete, not global. These discrete effects were in keeping with the effects of BET inhibitors in other models of inflammation and cancer (Nicodeme *et al.*, 2010; Dawson *et al.*, 2011). Although I-BET151 diminished expression of many genes implicated in psoriasis, the majority of the compound sensitive genes (~90%) did not overlap with the MAD-5 transcriptome. Analysis of these genes (non-overlapping with MAD-5 transcriptome) in IPA revealed that many were involved in the cell cycle (*e.g.* cell cycle: G1/S checkpoint regulation and cell cycle: G2/M DNA damage checkpoint regulation; both sets had predicted activation scores at 6h after I-BET151 treatment in TNFα + IL-17 stimulated NHEKs, with the latter also being predicted to the activated at 24h) and p53 signaling, with cancer, cell cycle and cell death and survival being some of the top disease and biological functions. These findings are consistent with previously published data, which demonstrated that BET inhibitors have anti-cancer effects (Filippakopoulos *et al.*, 2010; Dawson *et al.*, 2011) and indeed BET inhibitors are currently in phase I/II clinical trials for the treatment for a variety of cancers (Basheer and Huntly, 2015). Interestingly, IPA revealed that downregulation of MYC was the central node in the network featuring the most number of DEGs at 24h after I-BET151 treatment in TNFα + IL-17 stimulated NHEKs (Figure 6-12. Again these findings are consistent with previously published data which showed that BET inhibition led to downregulation of MYC expression in *in vitro* and *in vivo* models of mixed-lineage leukaemia (MLL)-fusion leukaemia, multiple myeloma and neuroblastoma (Dawson *et al.*, 2011; Delmore *et al.*, 2011; Wyce *et al.*, 2013). Although BET inhibitors have previously been demonstrated to inhibit MYC expression in other cell types, it was not necessarily

predictable that the same would occur in TNFα + IL-17 stimulated NHEKs, as BET inhibitors are known to have cell-type specific effect, in particular on MYC expression. Thus, for example, although BET inhibitors blocked expression of MYC expression in leukaemic cells, as described above, there were minimal effects on MYC expression in fibroblasts (Zuber *et al.*, 2011). Inhibition of Brd4 binding, by BET inhibitors, to cell-type specific enhancers has previously been hypothesised to account for these cell-type specific effects (Shi and Vakoc, 2014). Given that I-BET151 did inhibit MYC mRNA expression and modulate expression of genes involved in the cell cycle and other cancer related pathways in keratinocytes, this could provide rationale for investigating the role of BET proteins and the effects of BET inhibitors in suitable models of keratinocyte-based cancers. In this respect, keratinocyte-derived basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the most common types of non-melanoma skin cancer, with incidences of ~76/100, 000 person-years and ~23/100, 000 person-years, respectively, in England (Lomas *et al.*, 2012). Whereas BCCs are generally indolent, slow growing and rarely metastasizes, untreated SCCs can penetrate underlying tissue, invade lymph nodes, and metastasise to distant sites. Over expression of MYC has previously been described in both BCCs and SCCs, this provides further rationale for investigating the effects of BET inhibitors in models of these keratinocyte-based cancers (Freier *et al.*, 2006; Toll *et al.*, 2009).

In section 4.3.2, it was demonstrated that I-BET151 had a greater maximal effect and was more potent in inhibiting IL-6 protein release in response to TNFα + IL-17 stimulation in NHEKs, compared to effects on IL-6 mRNA expression. As previously discussed, HDAC inhibitors have previously been shown to have differential effects on inhibiting IL-6 mRNA vs. protein responses which could be accounted for by the observation that histone deacetylase (HDAC) inhibitors decreased IL-6 mRNA stability (section 4.4) (Grabiec *et al.*, 2012). We hypothesise that BET inhibitors may have similar posttranscriptional effects to HDAC inhibitors, to account for the differences that were observed. As discussed, these effects could have been through I-BET151 i) directly binding and inhibiting the function of proteins/molecules with post-transcriptional roles *e.g.* mRNA translation or protein trafficking, or ii) inhibiting expression of these proteins/molecules. Interestingly, I-BET151 modulated the expression of molecules involved the IPA-defined "protein synthesis" diseases and functions pathway at 6h post-treatment, Figure 6-8, and the network involving the greatest number of DEGs at 24h was "amino acid metabolism, small molecule

biochemistry, post-translational modifications", Figure 6-12. Further interrogation of the "protein synthesis" pathway in IPA at 6h post I-BET151 treatment, revealed that all sub-categories within this group (*e.g.* synthesis of protein, expression of protein and translation of protein) had negative activation Z-scores (-1.7, -1.7 and -1.2, respectively) suggesting that these pathways are all downregulated by I-BET151. These findings support the hypothesis that BET inhibitors can inhibit expression of proteins involved in mRNA translation and may explain the differential effects of I-BET151 on IL-6 mRNA vs. protein. Additionally it is possible that I-BET151 could directly bind and inhibit the function of these protein(s). However, direct inhibition by I-BET151 compared to inhibition of expression is less likely, given the high specificity of BET inhibitors and the observation that no non-BET binding partners have previously been described in the literature, as previously discussed in section 4.4.

I-BET151 impacted expression of ~3000 genes with ~10% included in the MAD-5 psoriasis transcriptome. As BET inhibitors have previously been shown to bind to BET proteins with high affinity without any known non-BET binding partners (Nicodeme *et al.*, 2010; Klein *et al.*, 2014), it is likely that BET proteins are involved in the regulation of many genes in keratinocytes, included ones relevant to psoriasis. In sections 4.3.4 and 4.3.5, it was demonstrated that I-BET151 inhibition of IL-6 and IL-8 expression could be explained by the reduction in BET proteins binding to IL-6 and IL-8 promoter regions in the presence of I-BET151. Although a similar mechanism may account for how I-BET151 inhibits the expression of a number of these other genes in keratinocytes, other underlying mechanisms are possible. For example, some will be secondary effects, *e.g.* some genes will be downstream of genes directly regulated by BET proteins; expression of other genes may be inhibited by I-BET-151 through disruption of Brd4/*pTEFb* and Brd4/p65 interactions (Jang *et al.*, 2005; Huang *et al.*, 2009; Schroder *et al.*, 2012). Finally, in addition to blocking BET proteins binding to gene promoter regions, I-BET151 may also block BET proteins binding to keratinocyte enhancer regions which may result in the downregulation of a variety of cell-specific genes as previously demonstrated in other cell types (Delmore *et al.*, 2011). It would be of particular interest to understand the underlying mechanism(s) by which BET inhibitors block expression of key regulators of inflammation (*e.g.* IL1A & B and NFKB1) (Table 6-12). Of note, as BET inhibitors modulate expression of a wide variety of genes, many not involved in the pathogenesis of psoriasis, topical application of BET inhibitors (small molecules) may be a method to avoid widespread systemic

effects. In this respect, topical application of JQ1, a BET inhibitor, was recently shown to inhibit psoriasis-like cutaneous inflammation in an imiquimod-induced mouse model of psoriasis (Nadeem *et al.*, 2015).

6.5. Future work

In summary I-BET151 treatment in TNFα + IL-17 stimulated NHEKs altered expression of a variety of genes, including ones known to be upregulated in psoriasis and with known roles in its pathogenesis. In addition a number of DEGs, whose functions have not been previously characterised in psoriasis, were also identified and may be interesting candidates for further investigation. The sensitivity of psoriasis related genes to the BET inhibitor supports a biological model for psoriasis pathogenesis, in which epigenetic processes, including histone acetylation, are involved in the psoriasis phenotype and that these processes can be dynamic and reversible. The effect of the BET inhibitor highlights the potential for targeting epigenetic processes, and in this case specifically BET proteins are implicated as targets. Collectively the data highlight the area of epigenetics as a potential route to identify novel disease modifying therapies. Furthermore, many DEGs sensitive to I-BET151 treatment were involved in the cell cycle, p53 signalling and MYC regulated pathways, in keeping with previously published data of BET inhibitors in other model systems, thus providing rationale for studying the potential effects of BET inhibitors in keratinocyte-based skin cancers.

Using these datasets, a number of further analyses would be of interest:

- Using IPA to interrogate datasets, what are the predicted downstream effects of DEGs regulated by TNFα + IL-17 and I-BET151, and how do these relate to the pathogenesis of psoriasis and disease resolution?
- How do genes regulated by I-BET151 treatment relate to changes in gene expression after psoriasis treatments which are in current use? Such an analysis may provide further justification for investigating the effects of BET inhibitors in psoriasis, and may identify potential novel therapeutic opportunities and combination treatments?
- In addition to taking a Venn diagram approach to compare DEGs to published datasets, *e.g.* to psoriasis or cancer transcriptome, using a gene set enrichment analysis (GSEA) approach allows whole datasets or pathways to be compared, not just individual genes (Suarez-Farinas *et al.*, 2010). Although the list of genes identified through the Venn diagram approach are of interest, taking a

GSEA approach may enrich for a wider set of genes and identify particular pathways or functions for further investigation into the role in psoriasis or its treatment.

- qRT-PCR validation of array for IL-6 and IL-8 already performed, it would be important to validate array of other target genes *e.g.* from Table 6-14, in the form of qRT-PCR from the same 12 donors used in the array. It would also be interesting to understand protein expression profile of validated genes in response to TNFα + IL-17 stimulation and I-BET151 treatment.

6.6. Conclusions

- There was considerable overlap of DEGs regulated by TNFα + IL-17 stimulation of NHEKs at 6h and 24h post-stimulation and of the top 5 upstream regulators of DEGs at each timepoint, 4 were common to both datasets (TNF, NFKB, IL1A and IFNG) and known to regulated inflammatory responses in psoriasis.
- I-BET151 treatment of TNFα + IL-17 stimulated NHEKs resulted in ~3000 DEGs at both 6 and 24h post-treatment. DEGs were involved in cell cycle, p53 signalling and inflammation, in keeping with previously published anti-proliferative and anti-inflammatory effects of BET inhibitors in other *in vitro* and *in vivo* models of disease.
- Regulators of I-BET151-sensitive DEGs included IL1A & B and NFBK1, which are known key regulators of inflammatory circuits in psoriasis. Furthermore, of the 1120 up or downregulated DEGs in the MAD-5 transcriptome, approximately 20% of genes were differentially modulated by I-BET151 treatment in TNFα + IL-17 stimulated NHEKs, at 6h and 24h respectively. These findings supported the rationale for evaluating I-BET151 in a model of cutaneous inflammation.
- 59 genes were upregulated by TNFα + IL-17 stimulation but downregulated by I-BET151 treatment, at both 6 and 24h. Of these 59 genes, 26 were included in an IPA-defined psoriasis network and 27 were included in the MAD-5 psoriasis transcriptome. 13 genes were included in both datasets, with known roles in psoriasis, and other genes may warrant further investigation for their potential roles in the pathogenesis of psoriasis or as potential novel target for treatment.
- The observation I-BET151 modulated expression of genes involved in cell cycle, inflammation and cancer pathways in keratinocytes could provide

rationale for investigating the effects of BET inhibitors in models skin cancer and other non-psoriatic inflammatory skin disease.

- I-BET151 modulated expression of genes involved in protein synthesis and all sub-categories within this group (*e.g.* synthesis of protein, expression of protein and translation of protein) had negative activation Z-scores suggesting that these pathways were all downregulated by I-BET151. This may explain the differential effects of I-BET151 on IL-6 mRNA vs. protein responses to TNFα + IL-17 stimulation in NHEKs.
- The sensitivity of psoriasis related genes to I-BET151 supports a role for epigenetic mechanisms in regulating the psoriasis phenotype and highlights the potential for targeting epigenetic processes; specifically BET proteins were implicated as targets in treating cutaneous inflammation. This highlights the area of epigenetics as potential targets for novel disease modifying treatment.

Chapter 7.

The effects of exogenous factors in the *in vitro* model of cutaneous inflammation

7. The effects of exogenous factors in the *in vitro* model of cutaneous inflammation

7.1. Introduction

Genetic susceptibility is known to contribute to inflammatory skin diseases; monozygotic twins show a concordance of 65–72% for psoriasis, *versus* 15–30% in dizygotic twins (Bowcock and Cookson, 2004) and genome wide association studies have identified 41 psoriasis susceptibility loci in individuals of European ancestry (Tsoi *et al.*, 2012; Tsoi *et al.*, 2015). However, 36 of the 41 previously discovered psoriasis susceptibility loci were predicted to account for only approximately 14% of the total variance of psoriasis risk or approximately 22% of its estimated heritability (Tsoi *et al.*, 2012), which together with the observation that monozygotic twins do not show complete concordance for psoriasis, implies non-genetic factors must also contribute to disease.

As previously discussed in section 1.4, epidemiological studies implicate environmental factors including alcohol and drugs, *e.g.* beta-blockers (β-blockers) and lithium, in the development and exacerbation of psoriasis. For example, the relative risk of developing psoriasis was 1.73 (95% confidence interval, 1.16–2.58) in those who consumed ≥ 2.3 alcoholic drinks/week compared with those who did not drink any alcohol (Qureshi *et al.*, 2010). Many investigators also report that alcohol consumption can exacerbate pre-existing psoriasis (Higgins, 2000) and that abstinence can induce remission (Vincenti and Blunden, 1987). Incidence of lithium exacerbation or induction of psoriasis ranges between 3.4 – 45%, is not necessarily dose related and latency periods of 20 weeks (mean) for exacerbation and 48 weeks (mean) for induction of psoriasis are reported (Rongioletti *et al.*, 2009). An analysis of the UK-based General Practice Research Database, showed that patients who received 5 or more prescriptions of lithium had an odds ratio of 1.68 (95 confidence interval: 1.18-2.39) for developing psoriasis compared to those who did not receive lithium (Brauchli *et al.*, 2009). β-blockers are also widely accepted to both provoke and exacerbate existing psoriasis, although latency can be over 1 year (Rongioletti *et al.*, 2009), not all patients exposed to β-blockers will develop psoriasis and discontinued use of the offending drug will induce remission of β-blocker-induced psoriasis in the majority of patients (Arntzen *et al.*, 1984). In addition to drugs, infections *e.g.* Streptococcal Group A (*S pyogenes*) throat infections and stress are also implicated as risk factors in the development and exacerbation of psoriasis (Peters *et al.*, 2000).

Specific environmental factors have also been associated with particular psoriasis phenotypes. For example, patients with high alcohol intake have a tendency towards a more inflamed psoriasis phenotype (Higgins and du Vivier, 1992), whereas patients taking lithium are often reported to develop generalised plaque or pustular psoriasis (Lowe and Ridgway, 1978; White, 1982; Jafferany, 2008).

Increased knowledge of genetic components may lead to greater understanding of psoriasis pathogenesis. However genetic susceptibility, unlike environmental insults, may be irreversible and unavoidable. Although it is unknown how environmental factors interact with genetic components and the adaptive and innate immune systems to produce the different disease phenotypes in psoriasis, greater understanding of the underlying mechanisms could lead to the development of novel therapies to target these mechanisms. Although a variety of mechanisms may exist; it has previously been shown that environmental factors can alter the epigenome which may then impact on phenotype. For example, Park *et al*. were the earliest investigators to demonstrate that ethanol treatment, in rat derived hepatocytes, resulted in histone hyperacetylation (Park *et al.*, 2003), and ingestion of alcohol in rats *in vivo* also leads to enhanced histone acetylation in the liver and spleen but not in brain or kidney (effects in skin were not investigated) (Kim and Shukla, 2006). In a macrophage model of acute alcoholic hepatitis, ethanol and its principal metabolite, acetate, enhanced cytokine responses to pro-inflammatory stimuli through hyperacetylation of histones at the promoter region of inflammatory genes (Kendrick *et al.*, 2010).

Given that environmental factors can influence the psoriasis phenotype, and therefore potentially gene expression, this raised the hypothesis that in the normal human epidermal keratinocytes (NHEKs) model of cutaneous inflammation, exogenous factors would potentiate expression and secretion of pro-inflammatory cytokines. Furthermore, potentiation of inflammatory responses by exogenous factors might be through chromatin remodelling *e.g.* via hyperacetylation thus promoting altering gene expression.

To test this hypothesis, the effects of clinically relevant concentrations of a variety of exogenous factors (*e.g.* ethanol, lithium and beta-blockers), implicated in the risk of developing or exacerbation of psoriasis, were examined in the NHEKs model of cutaneous inflammation. Expression of pro-inflammatory cytokines in response to a variety of stimuli and underlying changes in chromatin were investigated.

7.2. Aims

- To investigate whether exogenous factors, relevant to the psoriasis phenotype, can alter inflammatory responses in the NHEKs model of cutaneous inflammation and understand if these effects are stimulus specific (*e.g.* cytokine *vs.* non-cytokine stimuli).
- To investigate epigenetic mechanisms underlying modulation of inflammatory responses by exogenous factors and test whether these might represent potential therapeutic targets.

7.3. Results

7.3.1. Acetate, but not ethanol, potentiated the IL-6 response to TNFα + IL-17 stimulation

Psoriasis patients who consume excess alcohol have a tendency towards a more severe, widespread and inflamed phenotype (Monk and Neill, 1986; Higgins and Du Vivier, 1994b). This raised the hypothesis that ethanol would potentiate pro-inflammatory cytokine responses in NHEKs, as previously shown in ethanol treated macrophages (Kendrick *et al.*, 2010). To understand whether ethanol or acetate (one of ethanol's principal metabolites, section 1.4.2), could potentiate responses to inflammatory stimuli, primary human NHEKs were incubated with ethanol or acetate for 7 days prior to stimulation with TNFα + IL-17. The incubation time with acetate was previously shown to enhance subsequent responses to stimuli in macrophages (Kendrick *et al.*, 2010). Serum levels of TNFα and IL-17 are both increased in patients with psoriasis and correlate with disease activity (Takahashi *et al.*, 2010) and was earlier demonstrated to regulate expression of genes relevant to psoriasis (sections 3.3.1and 6.4). Media supernatants were collected after 48h and IL-6 and IL-8 levels in the conditioned media were measured by ELISA. The ethanol concentration (86mM, 0.5%, 400mg/dL) used was equivalent to five times the legal blood ethanol concentration for driving in the UK and the acetate concentration (1mM) was equivalent to the corresponding serum concentration observed in individuals metabolising ethanol at the concentration used (Mascord *et al.*, 1992). In a study of over 700 drunken drivers in Finland, offenders had blood ethanol and acetate levels similar or higher than the concentrations assayed, suggesting the concentrations used were clinically relevant (Roine *et al.*, 1988).

As shown in Figure 7-1, pre-incubation with acetate (1mM) for 7 days potentiated the release of IL-6 from primary human NHEKs in response to stimulation with TNFα + IL-17 for 48h (~50% enhancement compared to control, $p<0.01$). On the other hand, acetate did not potentiate IL-8 release in response to TNFα + IL-17 stimulation. Although acetate appeared to have an inhibitory affect on IL-8 release, this was not significant. In comparison to acetate, pre-incubation with ethanol did not significantly potentiate IL-6 or IL-8 responses to TNFα + IL-17 stimulation. Neither ethanol nor acetate affected basal IL-6 or IL-8 secretion, in unstimulated NHEKs.

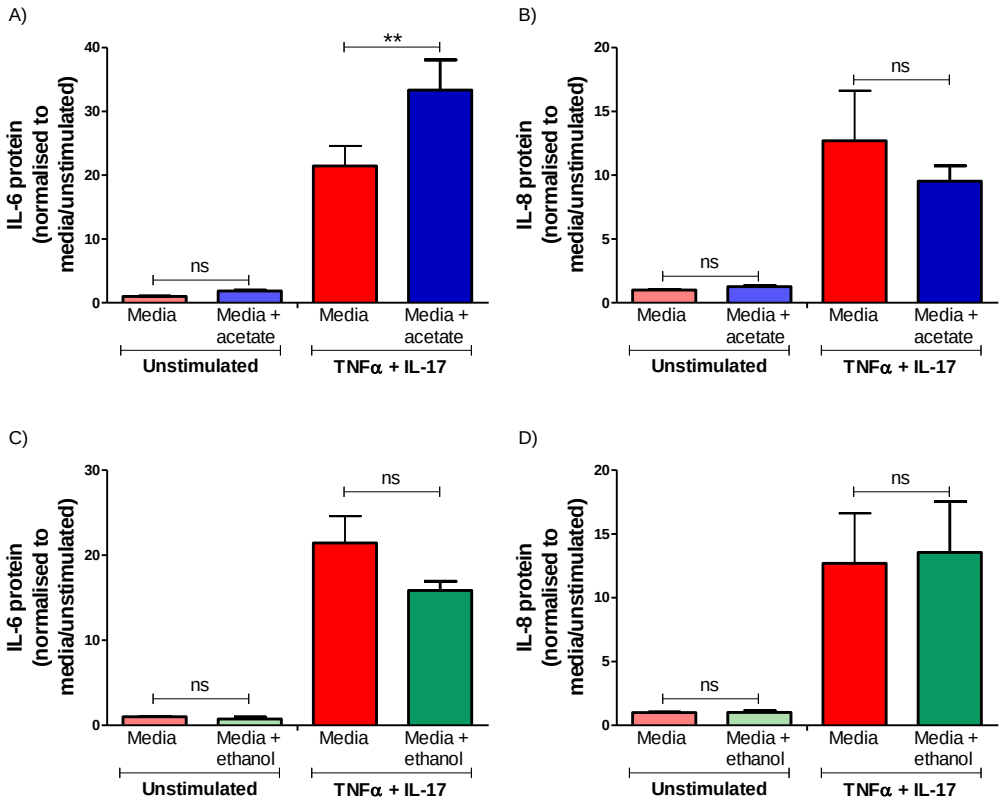


Figure 7-1: Acetate but not ethanol potentiated IL-6 secretion in response to TNFα + IL-17 stimulation

NHEKs were incubated with media, 1mM acetate (**A** and **B**) or 86mM (0.5%, 400mg/dL) ethanol (**C** and **D**) or for 7 days prior to stimulation with TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. Supernatants were collected 48h post-stimulation and secreted IL-6 (**A** and **C**) and IL-8 (**B** and **D**) levels were measured by ELISA. (N=3, with each donor performed in triplicate repeat Analysis by one-way ANOVA with Dunnett's post-test, comparing against the relevant media control; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points (normalised to media/unstimulated control) ± SEM . The absolute values for secreted IL-6 and IL-8 (Mean ± SEM) were 613 pg/ml ± 127 and 1003 pg/ml ± 177, respectively in TNFα + IL-17 stimulated, media treated NHEKs.

7.3.2. Keratinocytes demonstrated low expression of enzymes involved in ethanol metabolism

Having observed that ethanol, unlike its principal metabolite acetate, did not potentiate the IL-6 response to TNFα + IL-17 stimulation we hypothesised that NHEKs may not metabolise ethanol. *In vivo*, ethanol is metabolised to acetaldehyde by alcohol dehydrogenase (ADH), mainly ADH1A, ADH1B and ADH1C. Acetaldehyde is subsequently oxidised to acetate by aldehyde dehydrogenase (ALDH), mainly ALDH1 and ALDH2 (section 1.4.2).

To investigate this hypothesis, expression of ADH and ALDH in NHEKs was determined by qRT-PCR using Taqman® gene expression assays, with a hepatocellular carcinoma cell line (HepG2) as a comparative positive control.

As shown in Figure 7-2, NHEKs demonstrated significantly lower expression of all isoenzymes of both ADH and ALDH involved in ethanol metabolism, compared to HepG2 cells suggesting that there is little metabolism of ethanol in keratinocytes.

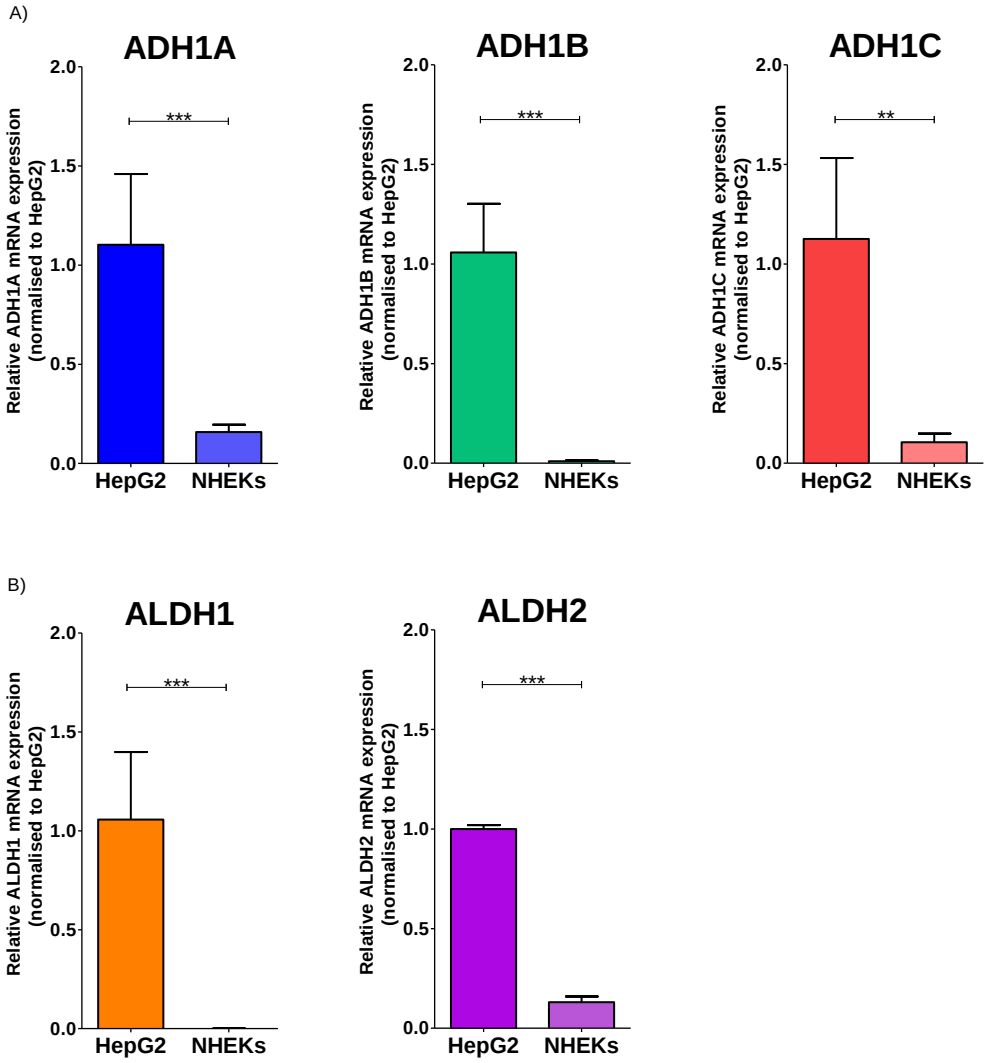


Figure 7-2: Keratinocytes demonstrated low expression of enzymes involved in ethanol metabolism

Taqman® gene expression assays were used to compare NHEKs expression of isoenzymes of ADH (**A**) and ALDH (**B**) to HepG2 cells. (N=3, with each donor performed in triplicate repeat) Analysis by t-test; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points (relative to HepG2 positive control) ± SEM.

7.3.3. Acetate potentiated the IL-6, but not IL-8, mRNA responses to TNFα + IL-17 stimulation

Incubation of NHEKs with acetate for 7 days prior to stimulation enhanced the level of secreted IL-6 detected in conditioned medium in response to TNFα + IL-17 stimulation. We hypothesised that acetate-enhanced levels of IL-6 detected in conditioned medium following TNFα + IL-17 stimulation may reflect at least in part an increase in IL-6 mRNA production. To test this hypothesis, primary human NHEKs were grown in the presence of 1mM acetate for 7 days prior to stimulation with TNFα + IL-17. As expression of IL-6 and IL-8 mRNA was previously demonstrated to be maximal at 6h after stimulation (section 3.3.2), NHEKs were harvested at various timepoints around 6h post-stimulation (3-12h) and IL-6 and IL-8 mRNA levels measured by qRT-PCR.

As shown in Figure 7-3, pre-treatment with acetate (1 mM) for 7 days significantly enhanced IL-6 mRNA production at 6-10h post TNFα + IL-17 stimulation, but interestingly this effect was reduced at 12h post-stimulation. In contrast, pre-treatment with acetate appeared to inhibit IL-8 mRNA production at all timepoints, although this was only significant at 6h, 10h and 12h post- TNFα + IL-17 stimulation. Pre-treatment with acetate did not affect basal production of IL-6 or IL-8 mRNA at any of the timepoints investigated, in unstimulated NHEKs.

These findings are in keeping with the differential effect of acetate on IL-6 and IL-8 cytokine release post-TNFα + IL-17 stimulation and suggest that acetate could mediate this effect at least in part through transcriptional responses.

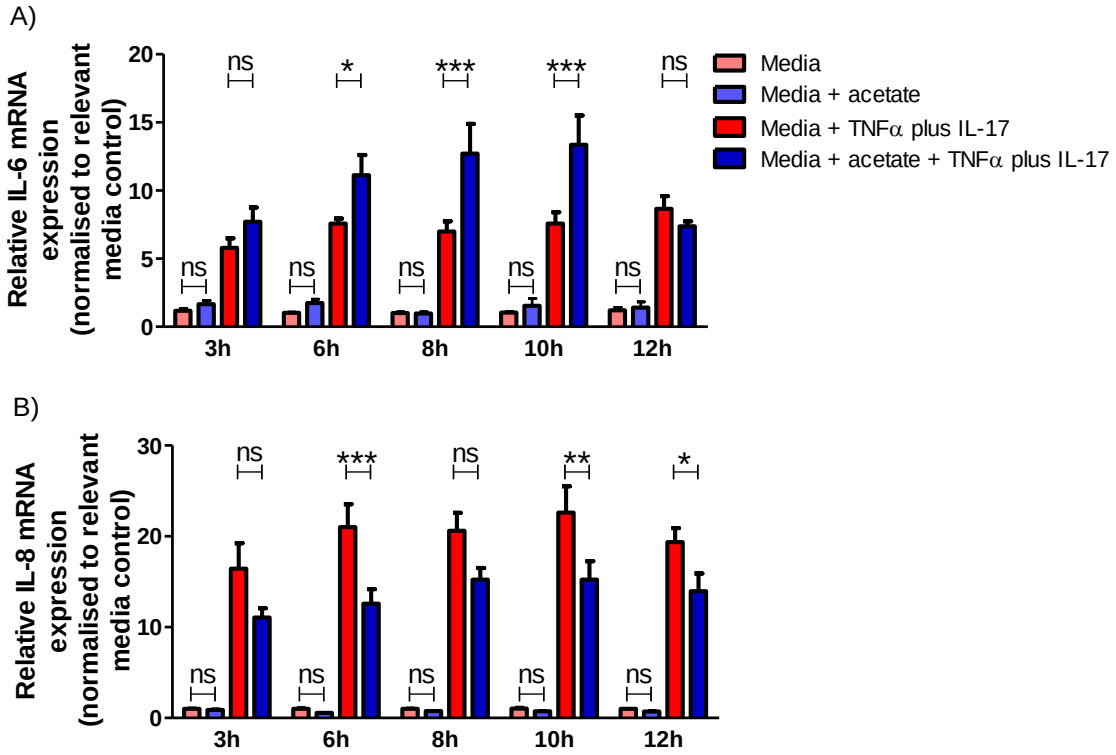


Figure 7-3: Pre-treatment with acetate potentiates the IL-6 mRNA response to TNFα + IL-17 stimulation and attenuates the IL-8 mRNA response to TNFα + IL-17 stimulation in a time dependent manner

Primary human NHEKs were incubated with or without 1mM acetate for 7 days prior to stimulation with TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. Cells were harvested for mRNA at indicated timepoints post-stimulation and IL-6 (**A**) and IL-8 (**B**) mRNA levels were measured by qRT-PCR. (N=3, with each donor performed in duplicate repeat) Analysis by two-way ANOVA with Bonferroni post-test; *$p<0.05$, **$p<0.01$, ***$p<0.001$. Graphs show mean of data points (normalised to unstimulated media control within each timepoint) ± SEM.

7.3.4. The potentiation by acetate of the IL-6 response to TNFα + IL-17 stimulation was concentration dependent

Having observed that 7 days' pre-treatment of primary human NHEKs with 1mM acetate potentiated the subsequent IL-6 response to TNFα + IL-17 stimulation, we sought to understand the concentration range of acetate for which this potentiation could be observed. NHEKs were therefore incubated with increasing concentrations of acetate (0.2 – 1mM) for 7 days prior to stimulation with TNFα + IL-17, reflecting physiological concentrations observed *in vivo*. Cells were harvested 6h post-TNFα + IL-17 stimulation for mRNA extraction to determine IL-6 and IL-8 mRNA levels (measured by qRT-PCR). Media supernatants were collected from parallel cultures of NHEKs 48h post- TNFα + IL-17 stimulation and secreted IL-6 and IL-8 levels were measured by ELISA.

As shown in Figure 7-4, the potentiation by acetate of IL-6 expression in response to TNFα + IL-17 stimulation appeared to be concentration dependent. For effects on IL-6 mRNA, the potentiation was significant for both 0.6mM acetate ($p<0.05$) and 1mM acetate ($p<0.05$), but at the secreted protein level this was only significant for 1mM acetate ($p<0.05$). Acetate did not potentiate or attenuate IL-8 mRNA production or secreted protein in response to TNFα + IL-17 stimulation, at any of the concentrations of acetate assayed. Furthermore, acetate did not potentiate or attenuate basal IL-6 or IL-8 mRNA or secreted protein production, in unstimulated NHEKs, at any concentration.

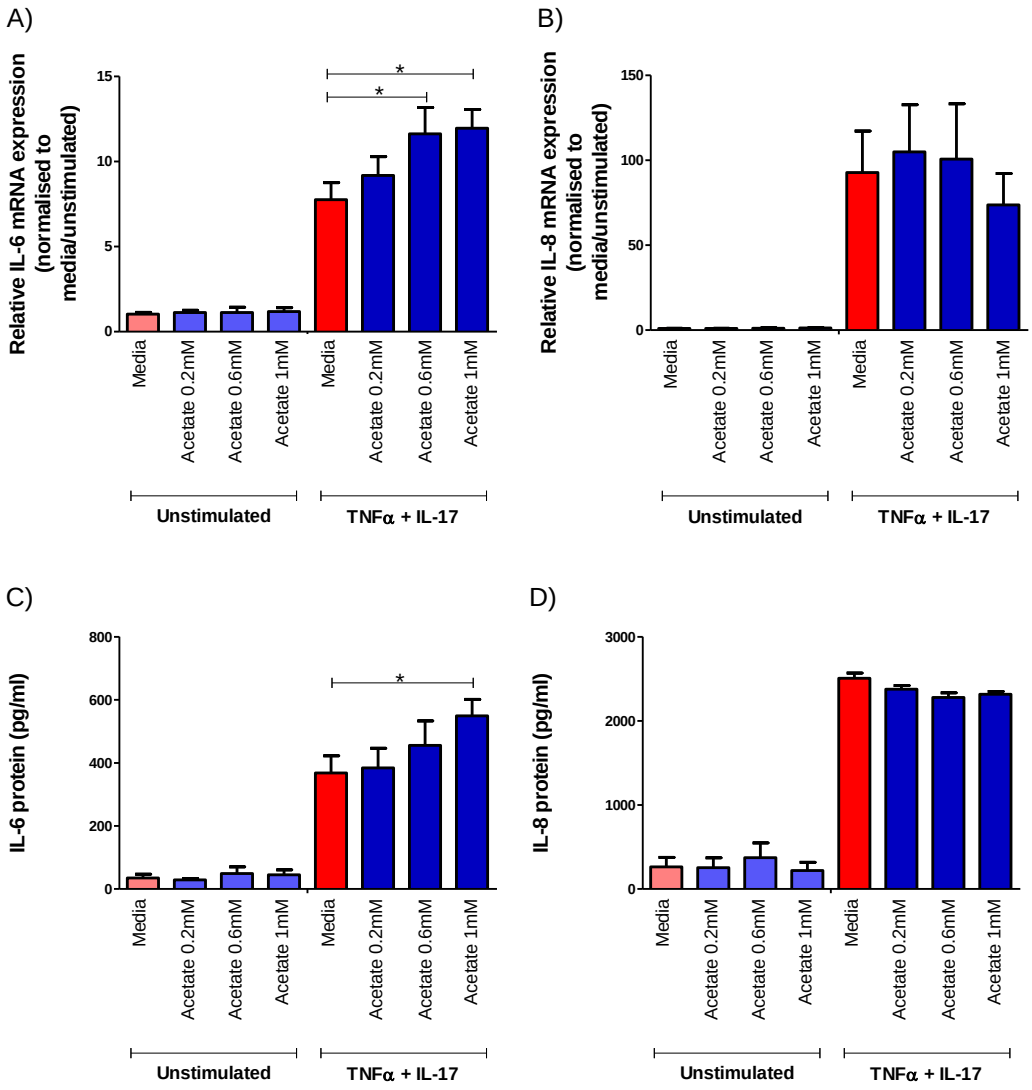


Figure 7-4: Acetate demonstrated concentration-dependent potentiation of IL-6 expression in response to TNFα + IL-17 stimulation

NHEKs were incubated with acetate (0.2 - 1mM) or media for 7 days prior to stimulation with TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. Cells were harvested 6h post-stimulation and IL-6 (**A**) and IL-8 (**B**) mRNA measured by qRT-PCR. Media supernatants were collected 48h post-stimulation and secreted IL-6 (**C**) and IL-8 (**D**) levels were measured by ELISA. (for mRNA: N=3, with each donor performed in duplicate repeat; for protein: N=3, with each donor performed in triplicate repeat) Analysis by one-way ANOVA with Dunnett's post-test, comparing against the relevant media control; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points (for mRNA, levels were normalised to unstimulated media control) ± SEM.

7.3.5. In comparison to acetate, lithium and propranolol did not potentiate the IL-6 response to TNFα + IL-17 stimulation

In addition to ethanol, lithium and propranolol have also been implicated in the development and exacerbation psoriasis (Brauchli *et al.*, 2009; Rongioletti *et al.*, 2009) and we hypothesised that these may enhance pro-inflammatory responses to TNFα + IL-17 stimulation. To address this, NHEKs were pre-incubated with 1mM lithium chloride, 1mM lithium carbonate or 150ng/ml propranolol hydrochloride (propranolol) for 7 days prior to stimulation with TNFα + IL-17. The concentration of lithium assayed was equivalent to the therapeutic concentration used clinically (BNF, 2015) and the concentration of propranolol was equivalent to the concentration required to achieve physiological beta-receptor blockade (Duff *et al.*, 1986). As there are often long latency periods between lithium or β-blocker exposure and development or exacerbation of psoriasis (Rongioletti *et al.*, 2009), and 7 days exposure to acetate was necessary to demonstrate potentiation of inflammatory responses, the effects 7 days incubation with lithium or β-blocker prior to stimulation was assayed. Although lithium is prescribed as either lithium carbonate or lithium citrate, for the treatment of bipolar disorder and depression (BNF, 2015), lithium chloride has previously been shown to induce keratinocyte proliferation, a key feature of psoriasis, through inhibition of glycogen synthase kinase 3 (GSK-3) (Hampton *et al.*, 2012). For this reason, the potential effect of lithium chloride on inflammatory responses in NHEKs was also investigated. NHEKs were harvested 6h post-TNFα + IL-17 stimulation for mRNA (measured by qRT-PCR). Supernatants were collected 48h post-stimulation and secreted IL-6 and IL-8 levels were measured by ELISA.

As shown in Figure 7-5, unlike acetate, neither lithium chloride, lithium carbonate nor propranolol potentiated IL-6 or IL-8 responses (mRNA or secreted protein) to TNFα + IL-17 stimulation. Although propranolol appeared to potentiate expression of IL-6 mRNA in response to TNFα + IL-17 stimulation, this effect was not significant and this did not translate into potentiation of secreted IL-6 protein. In unstimulated NHEKs, lithium chloride, lithium carbonate and propranolol had no significant effect on basal production of IL-6 or IL-8 mRNA or secreted protein. These findings suggest that, while both lithium and β-blockers are implicated as risk factors in the development and exacerbation of psoriasis, they may act through alternative mechanisms to alcohol.

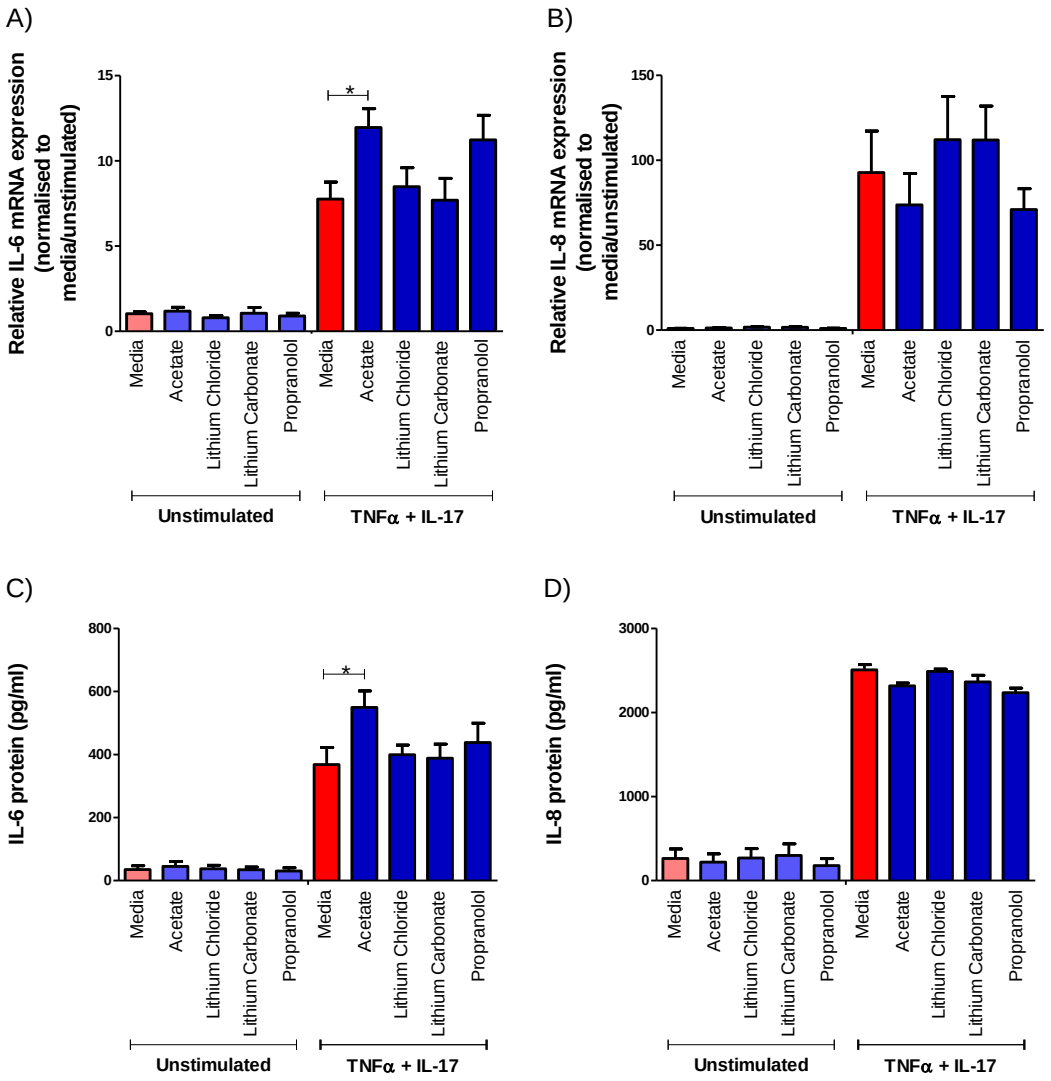


Figure 7-5: Pre-incubation with lithium or propranolol had no significant effect on IL-6 or IL-8 cytokine secretion in response to TNFα + IL-17 stimulation

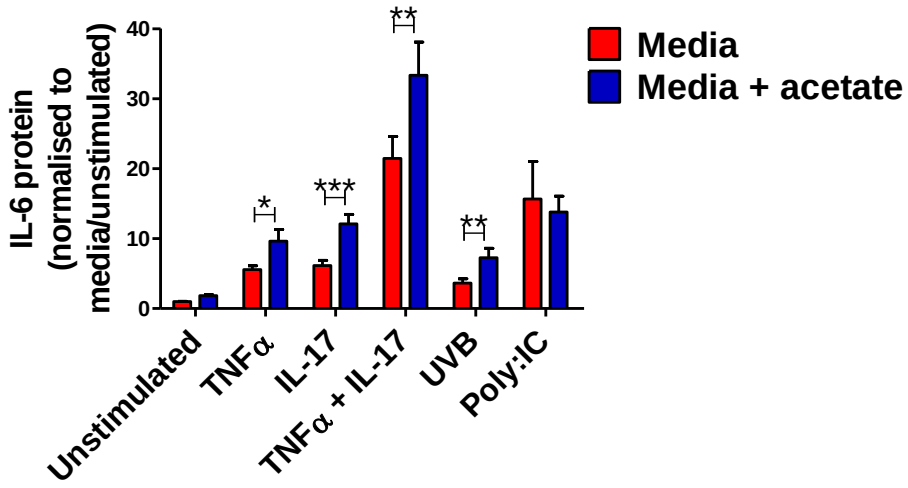
NHEKs were incubated with acetate (1mM), lithium chloride (1mM), lithium carbonate (1mM) or propranolol (150ng/ml) or media for 7 days prior to stimulation with TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. Cells were harvested 6h post-stimulation and IL-6 (**A**) and IL-8 (**B**) mRNA measured by qRT-PCR. Supernatants were collected 48h post-stimulation and secreted IL-6 (**C**) and IL-8 (**D**) levels were measured by ELISA. (for mRNA: N=3, with each donor performed in duplicate repeat; for protein: N=3, with each donor performed in triplicate repeat) Analysis by one-way ANOVA with Dunnett's post-test, comparing against the relevant media control; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points (for mRNA, levels were normalised to unstimulated media control) ± SEM.

7.3.6. IL-6 responses were more sensitive to potentiation by acetate compared to IL-8 responses

To determine whether potentiation by acetate of inflammation was specific to TNFα + IL-17 stimulation, NHEKs were grown in the presence of 1mM acetate for 7 days prior to stimulation with cytokine or non-cytokine stimuli including TNFα, IL-17, TNFα + IL-17, UVB or poly:IC or mock. Supernatants were collected 48h post-stimulation and secreted IL-6 and IL-8 levels were measured by MSD®.

As shown in Figure 7-6, IL-6 and IL-8 were both induced by all stimuli. In addition to acetate potentiating the secretion of IL-6 in response to TNFα + IL-17 stimulation ($p<0.01$), as previously observed, acetate also significantly potentiated IL-6 secretion in responses to TNFα alone ($p<0.05$) and IL-17 alone ($p<0.001$). Interestingly acetate also potentiated IL-6 secretion in response to non-cytokine stimuli (UVB; $p<0.01$, but not poly:IC). In comparison, acetate potentiated the secretion of IL-8 in response to TNFα alone ($p<0.05$) and UVB ($p<0.01$), but not IL-17 alone or TNFα + IL-17, as previous demonstrated.

A)



B)

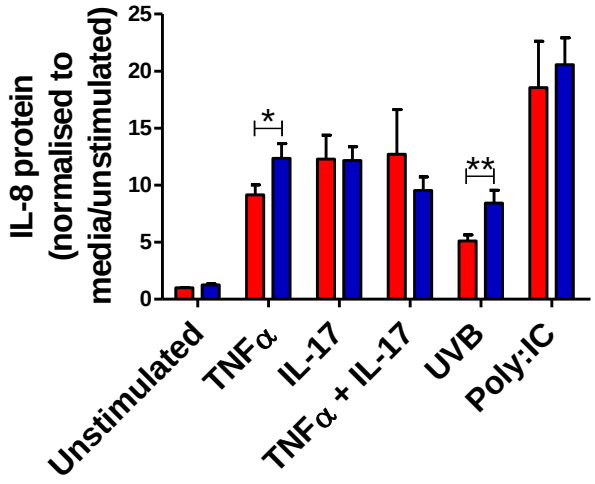


Figure 7-6: Acetate potentiated secretion of IL-6 and IL-8 in response to cytokine and non-cytokine stimuli; IL-6 was more sensitive to potentiation

NHEKs were incubated with or without 1mM acetate for 7 days prior to stimulation with TNFα (10ng/ml), IL-17 (100ng/ml), TNFα (10ng/ml) + IL-17 (100ng/ml), UVB (3SED) or poly:IC (0.5μg/ml). Supernatants were collected 48h post-stimulation and secreted IL-6 (**A**) and IL-8 (**B**) levels were measured by MSD®. (N=6, with each donor performed in triplicate repeat) Analysis by two-way ANOVA with Bonferroni post-test; *p<0.05, **p<0.01, ***p<0.001; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points (normalised to media/unstimulated control) ± SEM.

7.3.7. Prolonged exposure of NHEKs to acetate prior to stimulation with TNFα + IL-17 is necessary for potentiation of IL-6 secretion

Previously, incubation of NHEKs with acetate for 7 days was shown to enhance IL-6 inflammatory response to TNFα + IL-17 stimulation (Figure 7-1), in keeping with acetate's effect in a macrophages (Kendrick *et al.*, 2010). In order to understand time-dependent effects of acetate exposure, NHEKs were grown in the presence of 1mM acetate for 2 days prior to stimulation with TNFα, IL-17 or TNFα + IL-17. Supernatants were collected after a further 48h and secreted IL-6 and IL-8 levels were measured by ELISA.

As shown in Figure 7-7 and in contrast to acetate exposure for 7 days (Figure 7-1) exposure of NHEKs to acetate for only 2 days, was insufficient to demonstrate potentiation of the IL-6 secreted protein response to TNFα, IL-17, or TNFα + IL-17 stimulation, although there were trends for enhancement these were not significant. Equally, although there were general trends for inhibition, there were no significant effects on IL-8 responses after 2 days of exposure to acetate, in the presence or absence of subsequent stimulation.

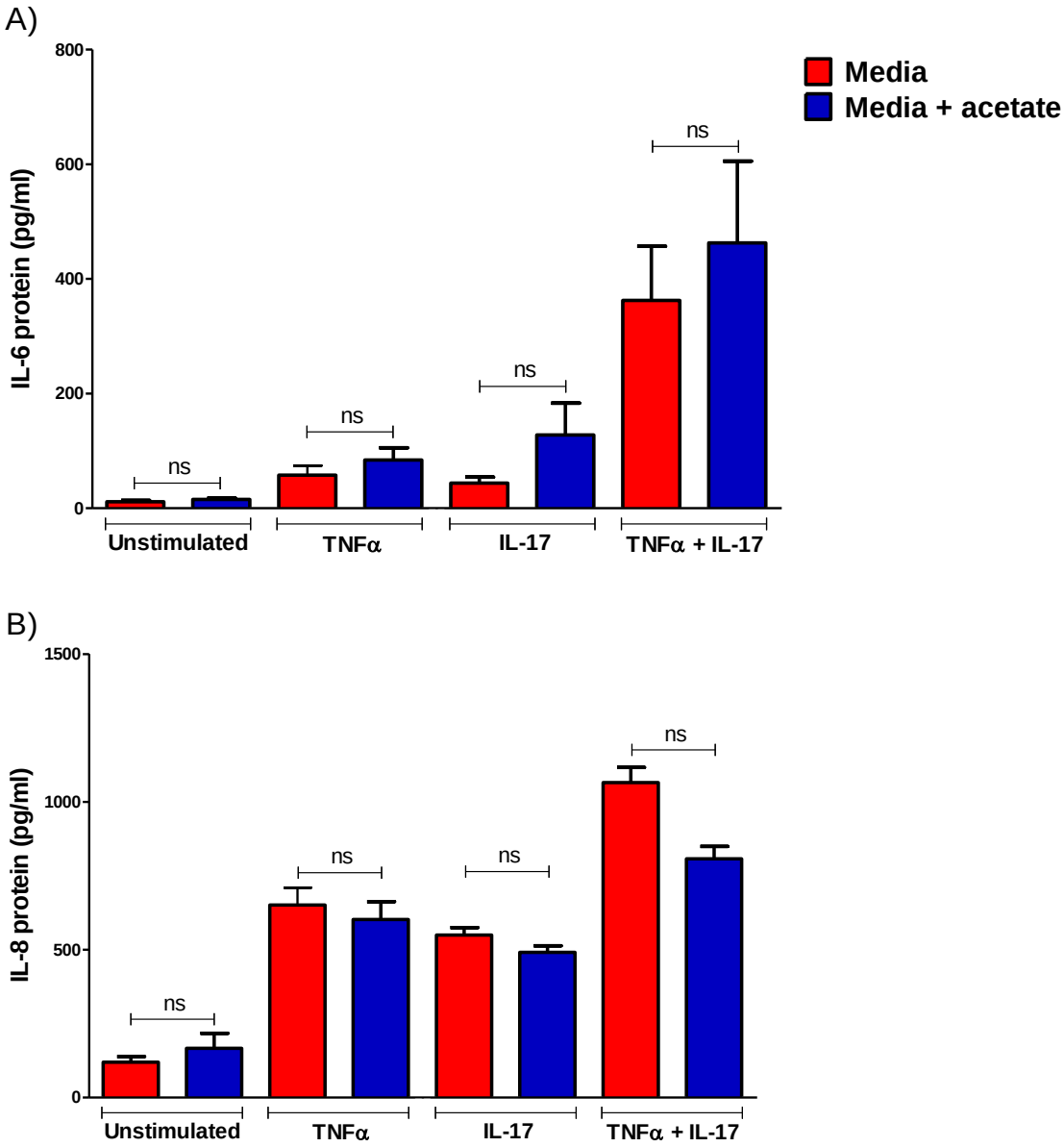


Figure 7-7: Exposure to acetate for 2 days was insufficient to significantly affect pro-inflammatory responses

NHEKs were incubated with or without 1mM acetate for 2 days prior to stimulation with TNFα (10ng/ml), IL-17 (100ng/ml) or TNFα (10ng/ml) + IL-17 (100ng/ml). Supernatants were collected 48h post-stimulation and secreted IL-6 (**A**) and IL-8 (**B**) levels were measured by ELISA. (N=3, with each donor performed in triplicate repeat) Analysis by one-way ANOVA with Dunnett's post-test, comparing against the relevant media control; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points ± SEM.

7.3.8. Acetate reduced keratinocyte metabolism and total protein in the presence of specific pro-inflammatory stimuli

In addition to excessive inflammation, psoriasis is also characterised by excessive epidermal keratinocyte proliferation. We therefore hypothesised that acetate may enhance NHEKs proliferation and cellular metabolism. To test this hypothesis, NHEKs were grown in the presence of 1mM acetate for 7 days prior to stimulation with both cytokine and non-cytokine stimuli including TNFα, IL-17, TNFα + IL-17, UVB or poly:IC. 48h later effects on cellular metabolism (measured by MTT) and total protein (measured by SRB, which is highly correlated with cell density) (Skehan *et al.*, 1990), were determined.

As shown in Figure 7-8: The effects of acetate on cellular metabolism and total protein were stimulus specific, pre-incubation with acetate had no effect on total protein or cellular metabolism in unstimulated NHEKs compared to control. On the other hand, pre-incubation with acetate had a significant inhibitory effect on total protein ($p<0.001$), but not cellular metabolism in TNFα + IL-17 stimulated cells. In the presence of poly:IC stimulation, acetate inhibited both cellular metabolism ($p<0.01$) and total protein ($p<0.01$).

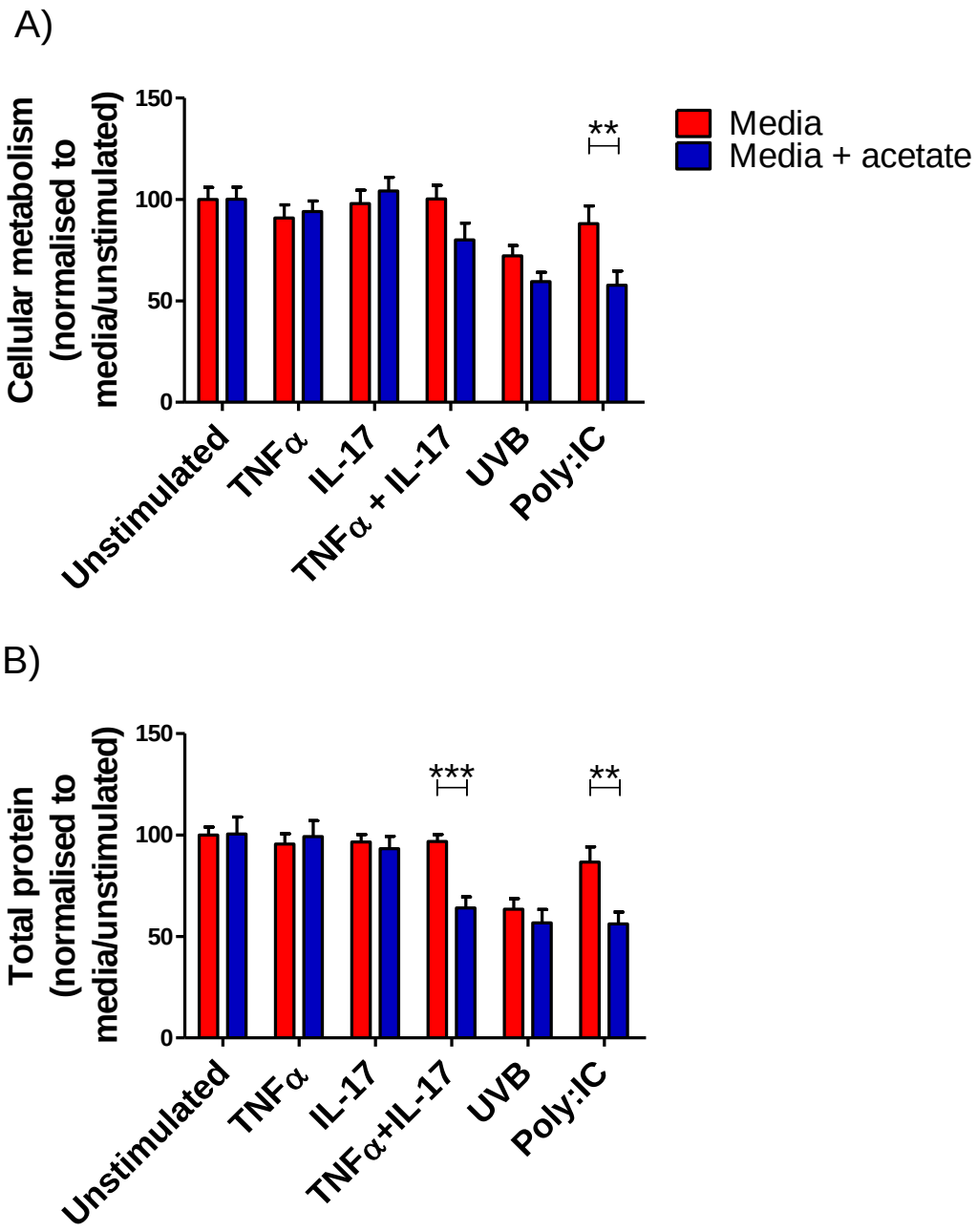


Figure 7-8: The effects of acetate on cellular metabolism and total protein were stimulus specific

NHEKs were pre-incubated with or without 1mM acetate for 7 days prior to stimulation with TNFα (10ng/ml), IL-17 (100ng/ml), TNFα (10ng/ml) + IL-17 (100ng/ml), UVB (3SED) or poly:IC (0.5μg/ml) or mock. 48h post-stimulation effects on NHEKs metabolism (**A**) (measured by MTT) and total protein (**B**) (measured by SRB) were determined. (N=6, with each donor performed in triplicate repeat) Analysis by two-way ANOVA with Bonferroni post-test; *p<0.05, **p<0.01, ***p<0.001; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points (normalised to media/unstimulated control) ± SEM.

7.3.9. At the IL-6 promoter, acetate exposure prior to stimulation resulted in enhancement of active epigenetic epitopes and RNA polymerase II recruitment in TNFα + IL-17 stimulated NHEKs

The observation that exposure of NHEKs to acetate for 7 days, prior to stimulation with TNFα + IL-17, enhanced IL-6 expression (mRNA and protein) raised the hypothesis that an epigenetic mechanism was involved, as previously demonstrated in a macrophage model of acute alcoholic hepatitis (Kendrick *et al.*, 2010). Since TNFα + IL-17 stimulation of NHEKs resulted in an increase in permissive epigenetic epitopes (acetyl-H3, H3K9ac, H3K27ac and H3K4me3) but not a repressive epitope (H3K4me1) at the IL-6 promoter (section 5.3.1), and acetate enhanced IL-6 responses to TNFα + IL-17 stimulation, it was hypothesised that acetate treatment would lead to an increase in permissive marks at the IL-6 promoter thus facilitating gene expression.

To explore this hypothesis, chromatin immunoprecipitation (ChIP) studies were undertaken to understand acetate's effects on the epigenome. NHEKs were grown in the presence of 1mM acetate for 7 days prior to stimulation with TNFα + IL-17. As IL-6 (and IL-8) mRNA expression was maximal at 6h post-TNFα + IL-17 exposure (Figure 7-3), and it is likely that any chromatin remodelling and epigenetic changes would precede maximal transcription, NHEKs were harvested at 1h and 4h post-TNFα + IL-17 stimulation for ChIP studies. We were particularly interested to investigate whether acetate modulated the following permissive epigenetic marks that were identified to be increased by TNFα + IL-17 stimulation (sections 5.3.1 and 5.3.2): global acetylation of histone H3, Acetyl-H3; acetylation of the lysine 9 residue on histone H3, H3K9ac; acetylation of the lysine 27 residue on histone H3, H3K27ac; and tri-methylation of the lysine 4 residue on histone H3, H3K4me3. Chromatin was selectively immunoprecipitated with antibodies to:

- RNA polymerase II, phosphorylated (and therefore activated) at its serine 2 residue (pol II (S2P))
- epigenetic epitopes known to positively regulate gene expression: Acetyl-H3, H3K9ac, H3K27ac and H3K4me3
- epigenetic epitopes known to have repressive effects on gene expression: mono-methylation of the lysine 4 residue on histone H3, H3K4me1
- IgG was used as a negative control and α-pan H3 a positive control

Co-immunoprecipitated DNA was assayed by real-time PCR (qPCR) for enrichment of the IL-6 promoter region, using specifically designed primers directed at the IL-6 promoter region (Qiao *et al.*, 2013), in media + acetate treated NHEKs compared to media alone. Enrichment of a particular region of DNA (*e.g.* IL-6 promoter) reflects the extent to which this region was associated with the histone modification or transcription factor/protein of interest.

As previously discussed in (section 5.3.1), we observed enrichment of pol II (S2P) at the IL-6 promoter after TNFα + IL-17 stimulation (4h, $p<0.05$), Figure 7-9. Perhaps more notably, pre-incubation with acetate enhanced the recruitment of pol II (S2P) to the IL-6 promoter (4h, $p<0.05$) in TNFα + IL-17 stimulated NHEKs. In addition pre-incubation with acetate also significantly enhanced the TNFα + IL-17 stimulated active epigenetic epitopes at 4h (Acetyl-H3, $p<0.001$; H3K9ac, $p<0.01$; H3K27ac, $P<0.001$; H3K4me3, $P<0.001$) in TNFα + IL-17 stimulated NHEKs. By comparison, pre-incubation with acetate had no significant effect on the repressive H3K4me1 mark.

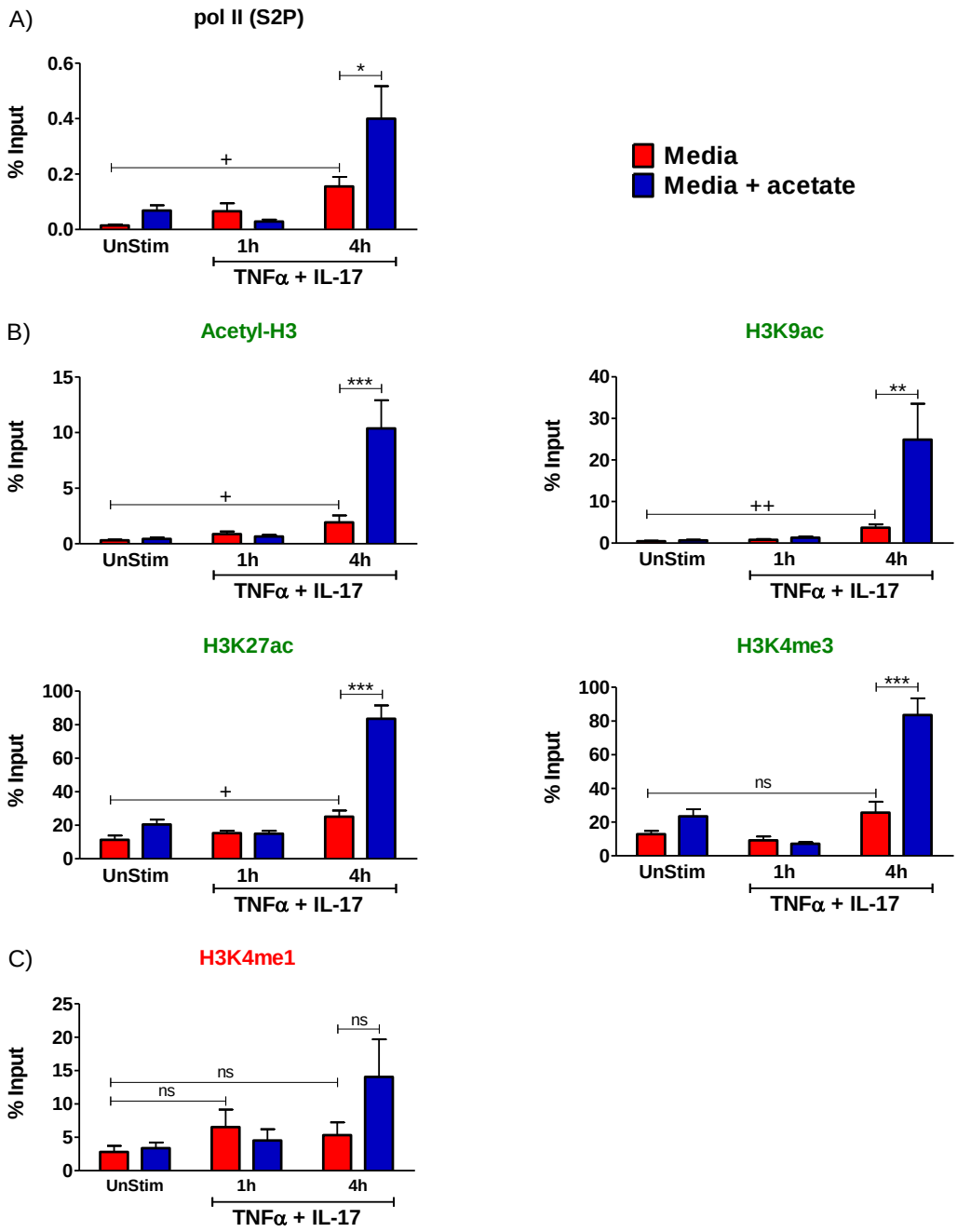


Figure 7-9: At the IL-6 promoter, pre-incubation with acetate enhanced active epigenetic epitopes and pol II (S2P) recruitment

NHEKs were incubated with or without 1mM acetate for 7 days prior to stimulation with TNFα (10ng/ml) + IL-17 (100ng/ml) or mock stimulated. NHEKs were harvested at 1h and 4h post-stimulation and ChIP undertaken using antibodies to pol II (S2P) (**A**), epigenetic epitopes that positively regulate gene expression, highlighted in green (**B**) and epigenetic epitopes with repressive effects on gene expression, highlighted in red (**C**). Quantification of co-immunoprecipitated DNA was performed by qPCR, using primers to the IL-6 promoter region. (N=3) Analysis by one-way ANOVA with Dunnett's post-test, comparing against the unstimulated media control; $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$. Analysis by two-way ANOVA with Bonferroni post-test, comparing against the relevant media control; *p<0.05, **p<0.01, ***p<0.001. Data points show mean (after subtracting background/non-specific binding and normalised to an input control) ± SEM.

7.3.10. In comparison to effects at the IL-6 promoter, at the IL-8 promoter acetate exposure decreased active epigenetic epitopes and RNA polymerase II recruitment in TNFα + IL-17 stimulated keratinocytes

Having observed that NHEKs exposed to acetate for 7 days prior to stimulation with TNFα + IL-17 reduced IL-8 mRNA expression (at 6h post stimulation, $p<0.001$, Figure 7-3) it was hypothesised that, in contrast to the positive effects at the IL-6 promoter, acetate would have an inhibitory effect on pol II (S2P) recruitment and overall repressive effects on the epigenome at the IL-8 promoter. In particular it was hypothesised that acetate would augment enrichment of the epigenetic marks that were identified to be altered by TNFα + IL-17 stimulation at the IL-8 promoter (section 5.3.2).

Co-immunoprecipitated, purified DNA samples (prepared as per section 7.3.9) were assayed by qPCR for enrichment of the IL-8 promoter region (using specifically designed primers, (Costa *et al.*, 2013)) in media + acetate treated NHEKs compared to media alone.

As shown in Figure 7-10, acetate significantly reduced recruitment of pol II (S2P) to the IL-8 promoter post-TNFα + IL-17 stimulation (1h, $p<0.05$). In addition, acetate significantly reduced the TNFα + IL-17 stimulated increase in active epigenetic epitopes at 1h (Acetyl-H3, $P<0.05$; and H3K4me3, $P<0.05$). Acetate also reduced the TNFα + IL-17 stimulated increase in H3K9ac and H3K17ac; although not significant, this trend was observed at both 1h and 4h post-TNFα + IL-17 stimulation. Interestingly acetate significantly reduced the repressive H3K4me1 mark (1h, $p<0.05$, although this was not significant at 4h).

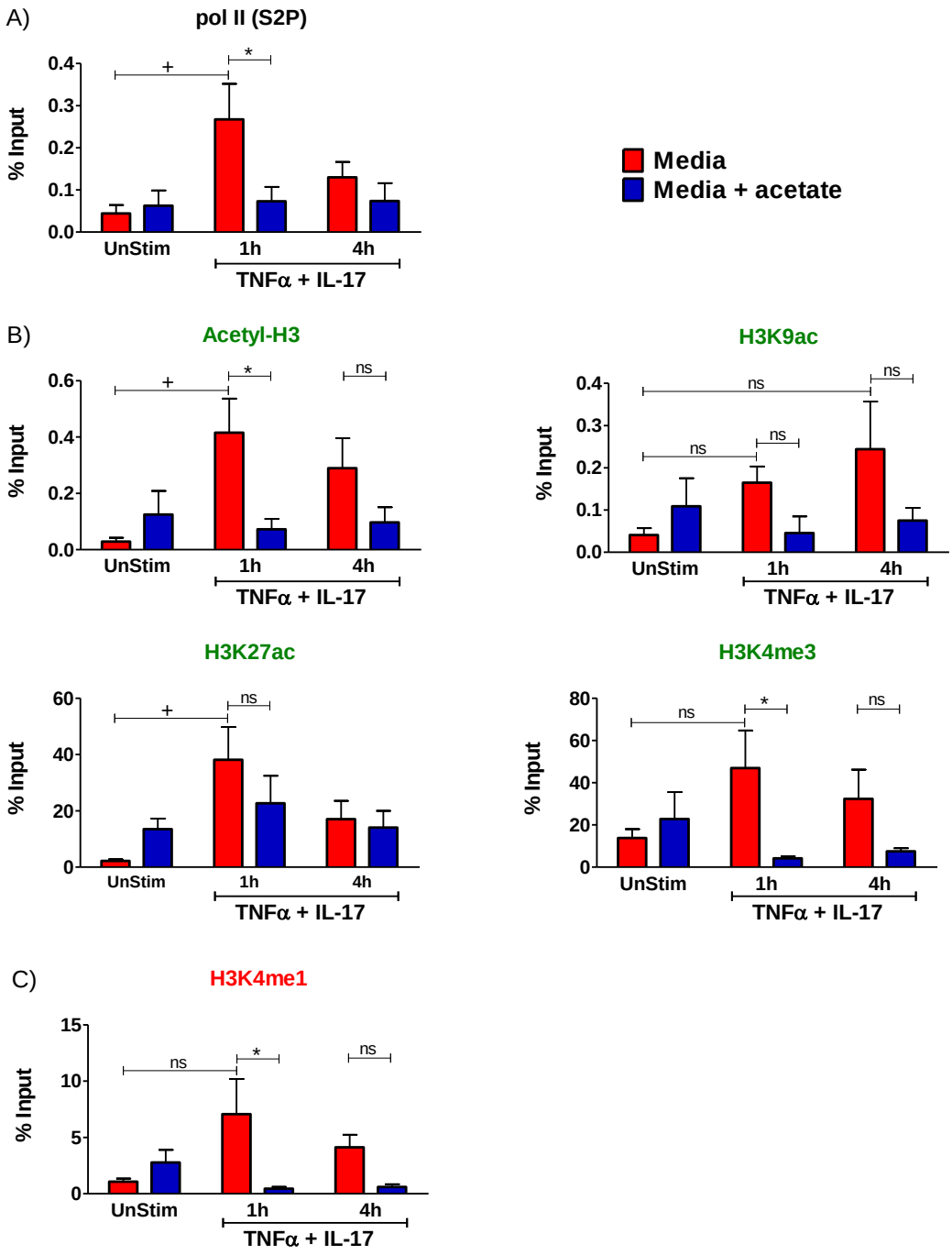


Figure 7-10: At the IL-8 promoter, pre-incubation with acetate decreased active epigenetic epitopes and pol II (S2P) recruitment

See Figure 7-9 for experimental details. NHEKs were harvested at 1h and 4h post-stimulation and ChIP undertaken using antibodies to pol II (S2P) (**A**), epigenetic epitopes that positively regulate gene expression, highlighted in green (**B**) and epigenetic epitopes with repressive effects on gene expression, highlighted in red (**C**). Quantification of co-immunoprecipitated DNA was performed by qPCR, using primers to the IL-8 promoter region. (N=3) Analysis by one-way ANOVA with Dunnett's post-test, comparing against the unstimulated media control; $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$. Analysis by two-way ANOVA with Bonferroni post-test, comparing against the relevant media control; *p<0.05, **p<0.01, ***p<0.001. Data points show mean (after subtracting background/non-specific binding and normalised to an input control) ± SEM.

7.3.11. At the IL-6 enhancer, acetate had little effect on epigenetic epitopes and no effect on pol II (S2P) recruitment

Having observed that in ChIP studies at the IL-6 promoter acetate enhanced recruitment of pol II (S2P) as well as an increase in active epigenetic epitopes, which could explain the potentiation of IL-6 expression in response to TNFα + IL17 stimulation, we hypothesised that similar mechanisms might occur at the IL-6 enhancer region. As an IL-6 enhancer has not been previously described in keratinocytes, we assayed the effects of acetate at a region previously described as an IL-6 enhancer in macrophages (Qiao *et al.*, 2013)

Co-immunoprecipitated, purified DNA samples (prepared as per section 7.3.9) were assayed by qPCR for enrichment of a region previously described as an IL-6 enhancer in macrophages, using specifically designed primers directed at this region (Qiao *et al.*, 2013) in media + acetate treated NHEKs compared to media alone.

As shown in

Figure 7-11, and in contrast to effects at the IL-6 promoter, acetate had little significant effect at the IL-6 enhancer, only the active epitope H3K27ac demonstrated a significant enhancement at 1h and 4h post-TNFα + IL-17 stimulation ($p<0.05$ at both timepoints) and recruitment of pol II (S2P) to this region was not affected by acetate.

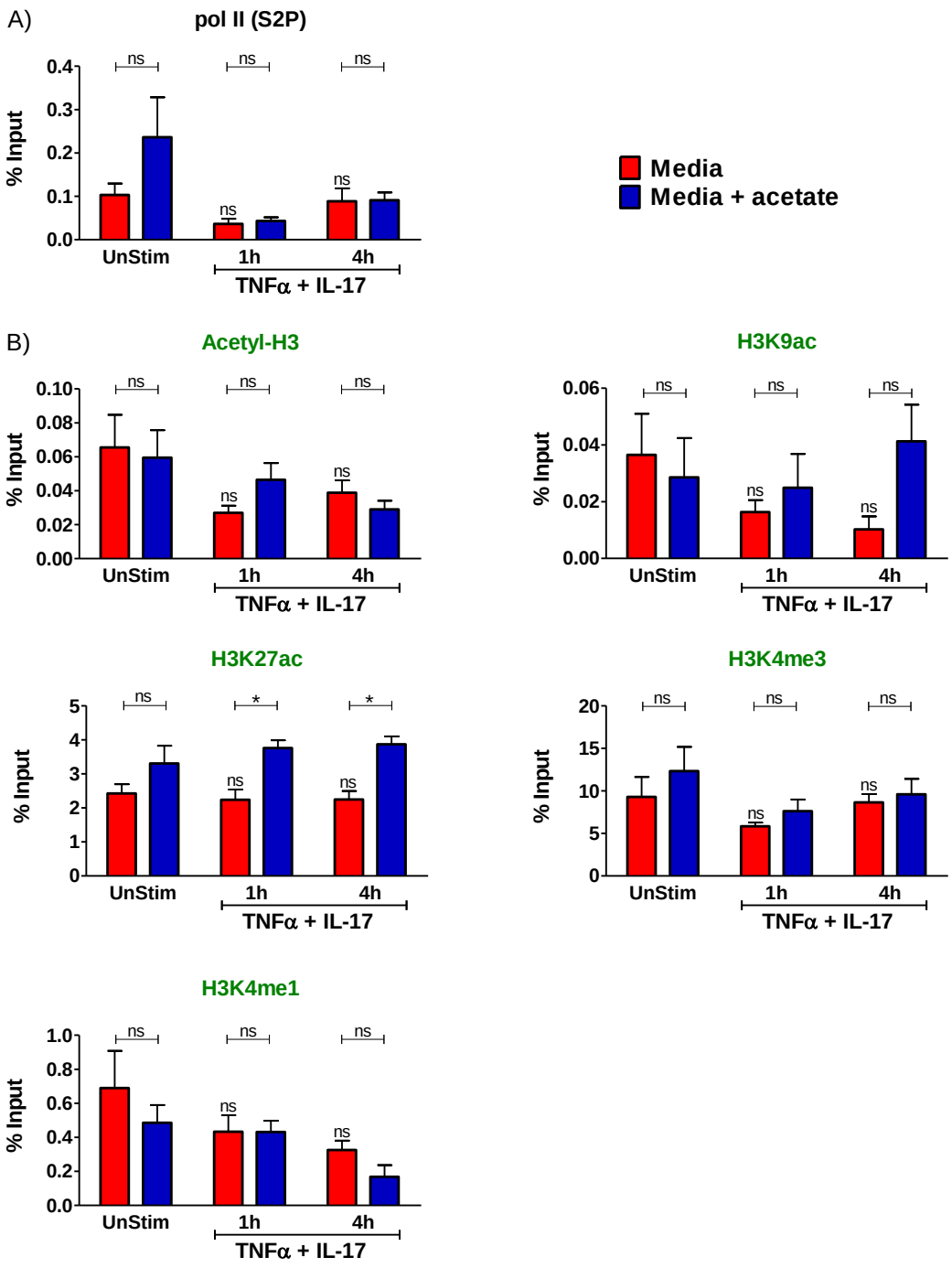


Figure 7-11: At a region previously defined as an IL-6 enhancer in macrophages, pre-incubation with acetate had relatively little effect

See Figure 7-9 for experimental details. NHEKs were harvested at 1h and 4h post-stimulation and ChIP undertaken using antibodies to pol II (S2P) (**A**) and epigenetic epitopes that positively regulate gene expression, highlighted in green (**B**). Quantification of co-immunoprecipitated DNA was performed by qPCR, using primers to the potential IL-6 enhancer region. (N=3) Analysis by one-way ANOVA with Dunnett's post-test, comparing against the unstimulated media control; $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$. Analysis by two-way ANOVA with Bonferroni post-test, comparing against the relevant media control; *p<0.05, **p<0.01, ***p<0.001. Data points show mean (after subtracting background/non-specific binding and normalised to an input control) ± SEM.

7.3.12. Exposure of NHEKs to acetate did not alter histone acetyl transferase (HAT) or histone deacetylase (HDAC) enzyme activity or expression of acetyl-coA synthetase (ACSS) enzymes

Since acetate treatment resulted in hyperacetylation of histones and specific lysine residues at the IL-6 promoter, we hypothesised that acetate might upregulate expression of ACSS enzymes or alter the balance of HAT *versus* HDAC activity to favour hyperacetylation, similar to the mechanism observed in a model of acute alcoholic hepatitis (Kendrick *et al.*, 2010). To test this hypothesis, NHEKs were incubated with 1mM acetate for 7 days, nuclear extracts were prepared and HAT and HDAC enzyme activity determined. HAT activity was measured using a kit that detects acetylation of a substrate by HAT enzymes, using acetyl-coA as a substrate; the release of co-A in the reaction can be detected as a measurable colour change (section 2.10). HDAC activity was measured using a kit that detects deacetylation of an acetylated lysine residue; in subsequent reactions the deacetylated lysine residue results in release of a chromophore or fluorophore which can be detected by a quantifiable colour change (HDAC 1,2,6,10,11) or fluorescence (HDAC 1,2,6,8,10,11) (section 2.10). Acetate effects on ACSS1 and ACSS2 expression were quantified by qRT-PCR/Taqman® gene expression assays.

As shown in Figure 7-12, acetate exposure had no significant effect on total HAT or HDAC enzyme activity, compared to control NHEKs grown in media alone. Furthermore, acetate exposure did not have any significant effect on ACSS1 or ACSS2 expression, compared to control NHEKs grown in media alone (Figure 7-13).

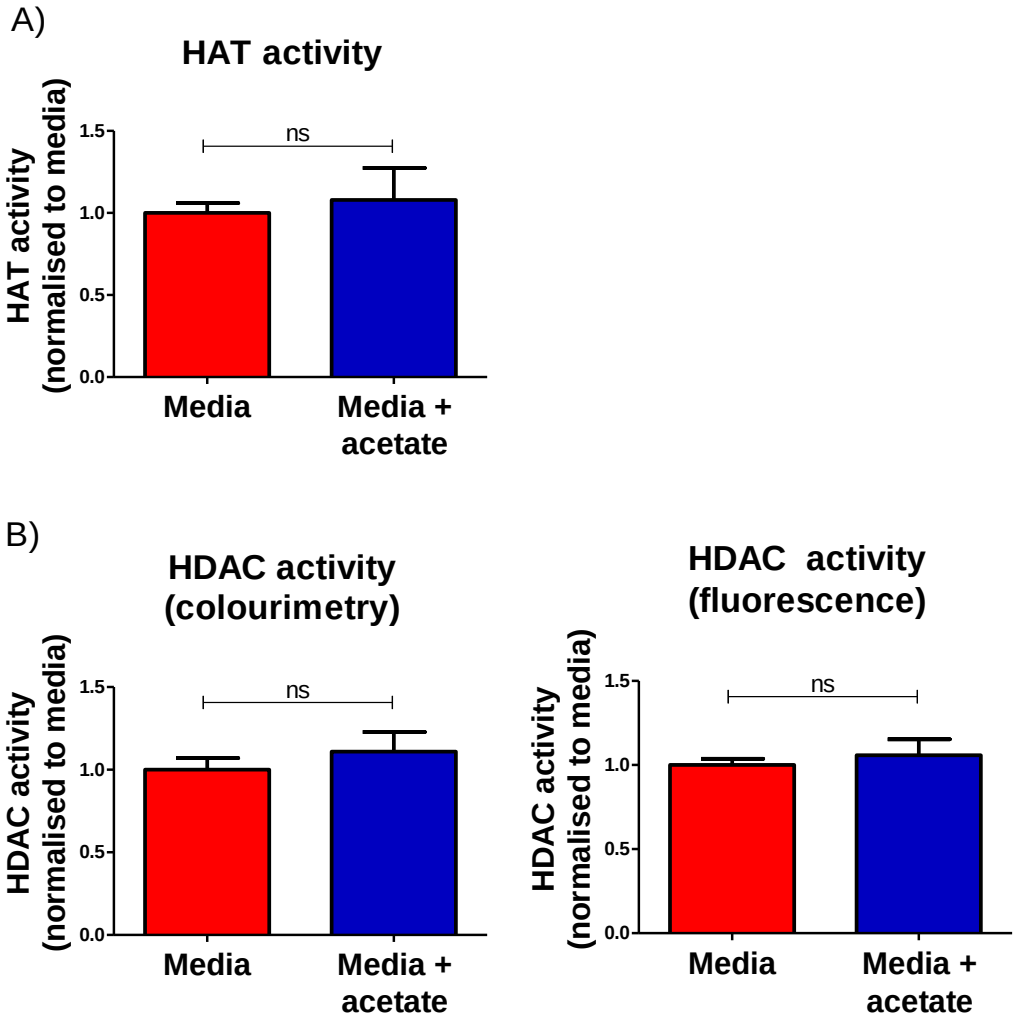


Figure 7-12: pre-incubation with acetate had no significant effect on HAT or HDAC enzyme activity

NHEKs were incubated with or without 1mM acetate for 7 days and effects on HAT (**A**) enzyme activity was determined using a kit based on colour change after release of co-A from acetyl-coA by HAT enzymes. HDAC (**B**) enzyme activity was measured using a kit producing a change in colour (HDAC 1,2,6,10,11) or fluorescence (HDAC 1,2,6,8,10,11) after deacetylation of an acetylated substrate by HDAC enzymes. (N=3, with each donor performed in triplicate repeat) Analysis by t-test; *$p<0.05$, **$p<0.01$, ***$p<0.001$. Graphs show mean of data points (normalised to media control) ± SEM.

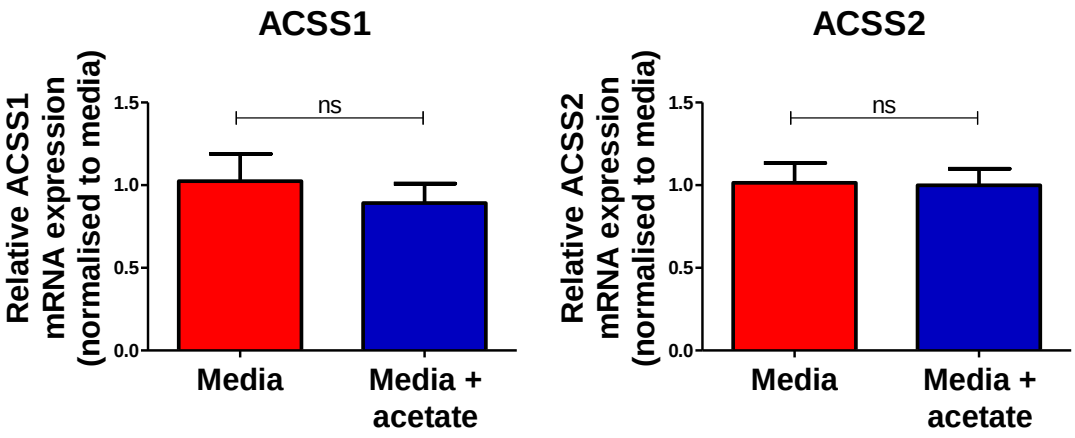


Figure 7-13: Pre-incubation with acetate did not affect ACSS expression

NHEKs were incubated with or without 1mM acetate for 7 days and ACSS mRNA expression was measured by qRT-PCR/Taqman® gene expression assays. (N=3, with each donor performed in triplicate repeat) Analysis by t-test; *p<0.05, **p<0.01, ***p<0.001. Graphs show mean of data points (normalised to media control) ± SEM.

7.3.13. I-BET151 attenuated acetate's potentiation of the IL-6 response to TNFα + IL-17 stimulation

Potentiation of the IL-6 response to TNFα + IL-17 stimulation together with hyperacetylation of histone residues at the IL-6 promoter, following pre-incubation with acetate, suggests histone hyperacetylation is a key component of the underlying mechanism that modulates gene expression. In sections 4.3.4 and 4.3.5, it was demonstrated that BET proteins were involved in the TNFα + IL-17 induced IL-6 and IL-8 expression. These finding raised the hypothesis that BET proteins might also be involved in the acetate potentiated phenotype. To test this hypothesis we utilised I-BET151, which specifically inhibits binding of BET proteins to acetyl lysine residues (section 1.3.7), to investigate whether I-BET151 would inhibit acetate's potentiation of the IL-6 response to TNFα + IL-17 stimulation.

NHEKs were incubated with 1mM acetate for 7 days prior to stimulation with TNFα + IL-17, either in the presence or absence of I-BET151 or vehicle (DMSO). Paired cultures of NHEKs were harvested 6h post-TNFα + IL-17 stimulation for mRNA (measured by qRT-PCR). Supernatants were collected 48h post-stimulation and secreted IL-6 and IL-8 levels were measured by ELISA.

A shown in Figure 7-14, acetate treatment enhanced the expression of IL-6 in response to TNFα + IL-17 stimulation ($p<0.01$) although interestingly the effect was less significant in vehicle (DMSO) treated NHEKs ($p<0.05$), for both IL-6 mRNA and protein. I-BET151 reduced IL-6 expression with a greater effect on protein than mRNA, as previously shown (section 4.3.2), but also in NHEKs pre-treated with 1mM acetate for 7 days prior to stimulation ($P<0.001$). Furthermore, I-BET151 treatment blocked potentiation of IL-6 by acetate in the TNFα + IL-17 stimulated model. Interestingly the inhibition of protein secretion appeared maximal with 0.3μM I-BET151 as inhibition was comparable for both concentrations of I-BET151.

I-BET151 also had an inhibitory effect on expression of IL-8 in response to TNFα + IL-17 stimulation, although the inhibition was not as great as for IL-6 at the protein level, as previously demonstrated. IL-8 protein was inhibited in acetate treated cells ($p<0.01$ for 0.3μM I-BET151) and the inhibition of IL-8 was more significant at a higher concentration of I-BET151 ($p<0.001$ for 1μM I-BET151). There was also inhibition of IL-8 mRNA in response to TNFα + IL-17 stimulation in both acetate exposed and non-

exposed NHEKs ($p<0.05$). Consistent with our previous results, acetate did not significantly affect basal IL-6 or IL-8 mRNA or secreted protein in NHEKs not stimulated with TNFα + IL-17. Furthermore, I-BET151 had no significant effect on basal levels of IL-6 or IL-8, in unstimulated NHEKs (either in the presence or absence of acetate).

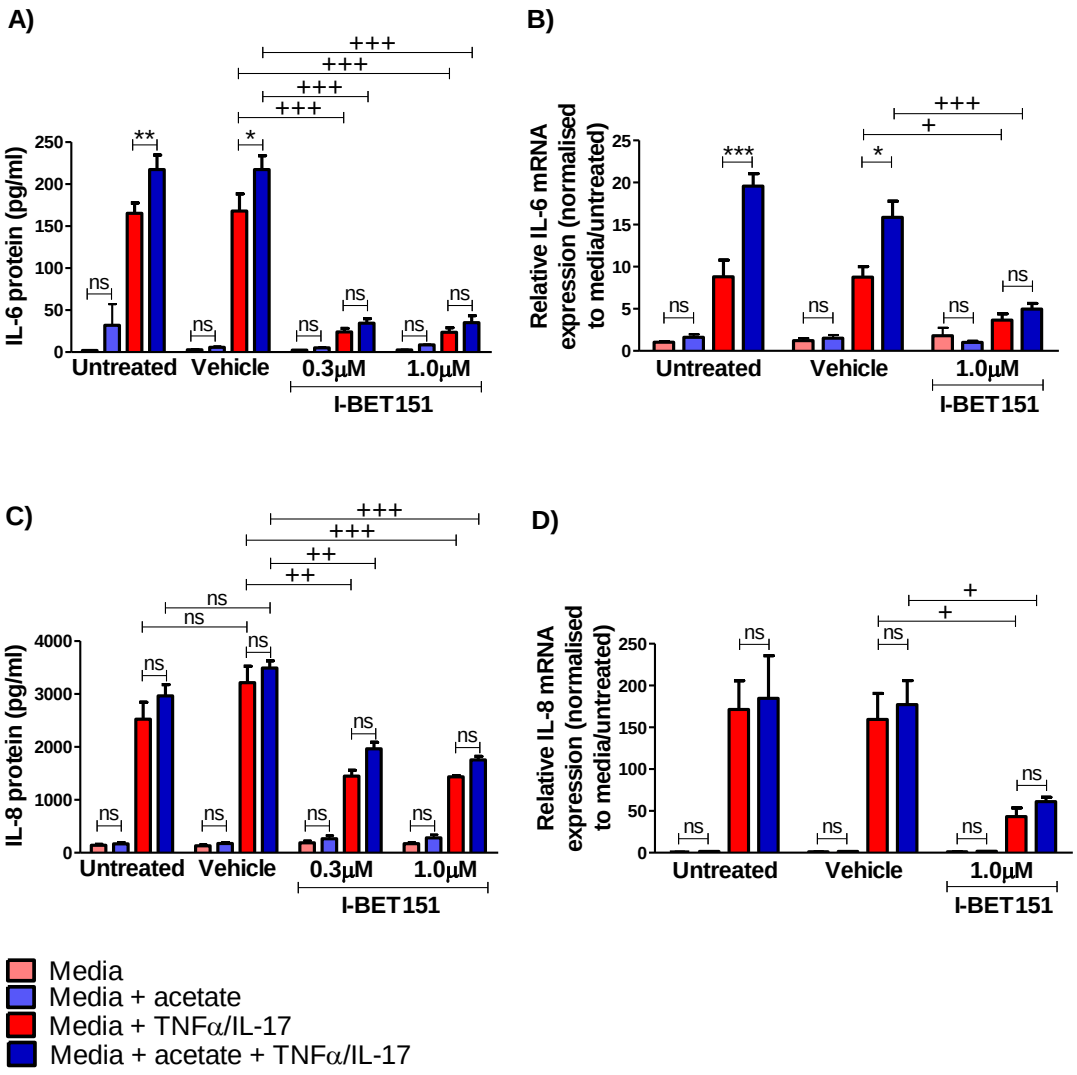


Figure 7-14: I-BET151 an inhibitor of BET protein interaction with acetylated lysine residues blocks acetate's potentiation of the IL-6 and IL-8 response to TNFα + Il-17 stimulation

NHEKs were incubated with or without 1mM acetate for 7 days prior to stimulation with TNFα (10ng/ml) + IL-17 (100ng/ml) or mock, in the presence or absence of I-BET151 or vehicle (DMSO, 1:1200). Secreted IL-6 (**A**) and IL-8 (**C**) protein was measured by ELISA, 48h post-stimulation (for protein: N=3, with each donor performed in triplicate repeat). IL-6 (**B**) and IL-8 (**D**) mRNA was measured by qRT-PCR, 6h post-stimulation, (for mRNA: N=3, with each donor performed in duplicate repeat). Analysis by two-way ANOVA with Bonferroni post-test, comparing against media control; *p<0.05, **p<0.01, ***p<0.001. Analysis by one-way ANOVA with Dunnett's post-test, comparing against vehicle control; $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001. Graphs show mean of data points (normalized to media/untreated for mRNA) ± SEM.

7.4. Discussion

For the first time in a model of cutaneous inflammation relevant to psoriasis we demonstrate that pre-incubation with acetate, a principal metabolite of ethanol, used at clinically relevant concentrations significantly enhanced the keratinocyte IL-6, but not IL-8, response to TNFα + IL-17 stimulation, at both the mRNA and secreted protein level. These findings could be accounted for by the chromatin changes, induced by acetate, at the IL-6 promoter, which facilitated recruitment of pol II (S2P) thus enhancing IL-6 expression. In contrast acetate did not enhance permissive epigenetic epitopes or pol II (S2P) recruitment to the IL-8 promoter. These findings provide mechanistic insight into how environmental factors, implicated in disease, interact with the genome to influence the disease phenotype. Specifically this may explain how alcohol predisposes patients to an exaggerated inflammatory responses resulting in development or exacerbation of psoriasis, could lead to identification of novel targets for therapy and could be used to educate patients about the hazards associated with excess alcohol consumption.

Our finding that acetate could modify epigenetic epitopes is in keeping with previously published data, where it has been shown that ethanol can mediate its effects on the epigenome through acetate (Park *et al.*, 2003; Park *et al.*, 2005). Our data showed that in NHEKs, ethanol had no significant effect on IL-6 or IL-8 responses to TNFα + IL-17 stimulation and it was demonstrated that NHEKs showed low expression of enzymes, ADH and ALDH, involved in ethanol metabolism. This finding is consistent with previously published data which showed expression of class 1 ADH proteins, measured by western blot analysis, was approximately 30-fold higher in the liver compared to the skin (Cheung *et al.*, 1999). Furthermore, class 1 and 2 ALDH protein expression was also higher in the liver compared to the skin; approximately 22-fold and 12-fold, respectively (Cheung *et al.*, 1999; Cheung *et al.*, 2003). In addition it has previously been calculated that ~96 to 98.8% of total ADH activity in the human body is confined to the liver and only 0.05% occurs in the whole of the skin and that the specific enzyme activity of skin derived ADH is ~190 fold lower than in liver for ethanol metabolism (Saleem *et al.*, 1984). Together these findings suggests there is very little metabolism ethanol in skin as a whole, compared to the liver, and therefore very little/negligible in individual keratinocytes. This is likely to explain why ethanol had no effect on pro-inflammatory responses, unlike its principle metabolite acetate.

We found that the magnitude of enhancement of IL-6 expression in response to TNFα + IL-17 stimulation was dependent on the concentration of acetate, during the pre-incubation period prior to stimulation. This suggests that potentiation of the cutaneous inflammatory response may correlate with the degree of alcohol consumption and interestingly, in support of this suggestion, the severity of psoriasis has previously been shown to correlate with alcohol intake (Zou *et al.*, 2015). In addition, psoriatic skin and serum is rich in both TNFα and IL-17 and has also been shown to correlate with disease severity (Takahashi *et al.*, 2010), thus the cytokine milieu in psoriasis provides the necessary environment for acetate enhancement of cutaneous inflammation.

The enhancement of IL-6 expression by acetate is in keeping with a model of acute alcoholic hepatitis in which it was shown that acetate enhanced the IL-6 response to lipopolysaccharide (LPS) in a human monoblastic cell line (Kendrick *et al.*, 2010). However, Kendrick *et al*. also observed potentiation of IL-8 (and TNFα) in responses to LPS, which we did not observe in our system. Furthermore, Kendrick *et al*. only described potentiation effects of ethanol and acetate; no inhibitory effects or null effects on gene expression were described, the specificity of ethanol/acetate effects on different genes was therefore unclear. These differences could be due to the differences in cell type or the stimulus. For example, in our system we demonstrated that in NHEKs stimulated with TNFα alone or UVB, pre-incubation with acetate could potentiate both IL-6 and IL-8 expression; LPS may have had a similar effect in our model system. However, as LPS is derived from gram negative bacteria, it would be difficult to justify the clinical relevance of investigating LPS in a cutaneous inflammatory model relevant to psoriasis. It is interesting that in the KC model acetate potentiated IL-6 expression in response to TNFα + IL-17 stimulation, but not IL-8 as this could be consistent with what is observed clinically. As previously discussed, psoriasis patients with high alcohol consumption tend to have a more inflammatory phenotype, which would fit with enhanced IL-6 release. In psoriasis, IL-6 is involved in differentiation and activation of naïve T-cells into T_H17 cells, resulting in IL-17 release; IL-17 can induce further IL-6 production from non-immune cells, including keratinocytes and fibroblasts (Saggini *et al.*, 2014). Thus, ethanol/acetate could enhance the positive feedback loop between the adaptive and innate immune system. On the other hand IL-8 is important in neutrophil recruitment in psoriasis (Gillitzer *et al.*, 1996). Therefore if alcohol or its metabolites potentiated IL-8 production *in vivo*, one might expect to more commonly

observe a pustular psoriasis phenotype in patients with high alcohol consumption, which is not the case (Higgins and du Vivier, 1992).

Our results showed that lithium, although implicated as a risk factor in the development and exacerbation of psoriasis, did not potentiate the IL-6 or IL-8 response to TNFα + Il-17 stimulated NHEKs, nor basal IL-6 and IL-8 levels in unstimulated NHEKs. These findings are in keeping with previously published data, where it was shown that lithium chloride did not potentiate release of IL-6 or IL-8 protein in unstimulated keratinocytes derived from patients with psoriasis or healthy controls (Ockenfels *et al.*, 1996). However the authors did demonstrate that lithium potentiated basal levels of transforming growth factor alpha (TGFα) in keratinocytes derived from healthy controls. We did not assay the effects of acetate on TGFα; it is therefore difficult to make any direct comparisons for this particular response. However these results may need to be interpreted with caution, as the concentration of lithium used by Ockenfels *et al*. was 1.5mM; *in vivo* a serum-lithium concentration over 1.5mM is associated with toxicity (BNF, 2015). Additionally, recently it was reported lithium chloride increased keratinocyte proliferation, through inhibition of glycogen synthase kinase 3 (GSK-3) (Ohteki *et al.*, 2000; Hampton *et al.*, 2012). Hampton *et al*. demonstrated that clinically relevant concentrations of lithium lead to increased keratinocyte proliferation and that this could be reproduced by either pharmacological inhibition of GSK-3 or retroviral transduction of GSK-binding protein, a known endogenous inhibitory binding partner of GSK-3, which resulted in both increased nuclear translocation of Nuclear factor of activated T cells 2 (NFAT2) and transcriptional activation of NFAT (Hampton *et al.*, 2012). These findings suggest that while both lithium and ethanol can exacerbate or provoke psoriasis, this may be through different underlying mechanisms. However, these findings may need to be interpreted with caution as Ockenfels *et al*. and Hampton *et al*. both investigated the effects of lithium chloride and not the compounds which are used clinically *i.e.* lithium carbonate and lithium citrate.

We also observed that propranolol did not potentiate pro-inflammatory responses to TNFα and IL-17 stimulation. Although beta-blockers are implicated as a risk factor for the development of psoriasis, the underlying mechanism is unknown. It is suggested that it may be through decreased levels of cyclic adenosine monophosphate, as a result of beta-blockade. This is thought to lead to decreased levels of intracellular calcium and therefore reduced differentiation and increased proliferation, as seen in psoriasis

(Wu *et al.*, 2014). Therefore beta-blockers may influence the psoriasis phenotype through positive effects on proliferation rather than inflammation as we had initially hypothesised.

Interestingly, acetate enhanced both IL-6 and IL-8 secreted protein secretion in response to UVB stimulation. Epidemiological studies suggest that alcohol consumption is a risk factor for sunburn, as well as non-melanoma and melanoma skin (Saladi *et al.*, 2010). Furthermore it has been shown that alcohol consumption causes a significant decrease in minimal erythemal dose (MED) (Darvin *et al.*, 2013). An MED is defined as the smallest amount of UV irradiation required to achieve just perceptible erythema (redness) of the skin, 24h after exposure, and is widely used in clinical practice as a visible indicator of a biological response. A reduction in MED therefore indicates that less irradiation is required to cause erythema of the skin. The precise mechanism by which alcohol sensitises the skin to UV irradiation and contributes to skin cancer is unknown but it has been hypothesised that metabolites of ethanol, especially acetaldehyde, form highly reactive molecules which generate reactive oxygen species (ROS) upon UVB exposure which can lead to DNA damage (Saladi *et al.*, 2010). Our *in vitro* data showing that pre-incubation of keratinocytes with acetate sensitises them to UVB supports the clinical finding that ethanol, or its metabolites, sensitises the skin to UV irradiation and a reduction in MED *in vivo*. Although we have shown that acetate induced enhancement of the IL-6 response to TNFα + IL-17 stimulation can be explained by changes to the epigenome, we have not shown that this is necessarily the case with the potentiation by acetate of the inflammatory response to UVB stimulation.

We showed that NHEKs exposure to acetate for only 2 days was insufficient to potentiate a subsequent IL-6 response to TNFα + IL-17 stimulation. In a model of acute alcoholic hepatitis, it was shown that prolonged exposure to ethanol (6 days) or acetate (2 days) was necessary for the induction of ACSS enzymes, responsible for the conversion of acetate to acetyl-coA, which the authors found was essential for the facilitation of histone hyperacetylation (Kendrick *et al.*, 2010). However in our system prolonged acetate exposure for 7 days had no effect on ACSS expression, HAT or HDAC activity. In contrast to Kendrick *et al*.'s observations in a monoblastic cell line, ethanol nor acetate treatment did not significantly affect total HDAC activity in rat-derived hepatocytes (Park *et al.*, 2005). This suggests that either i) changes in HDAC activity are not responsible for ethanol/acetate induced changes in acetylation or ii) because individual HDACs (and HATs) target particular histone residues, it is plausible

that specific HDAC(s) may be inhibited by ethanol/acetate thereby contributing to the net increase in acetylation of specific lysine residues.

Our mechanistic ChIP studies showed that the differential effects of acetate on IL-6 and IL-8 responses to TNFα + IL-17 stimulation could be explained by the changes seen at the promoter regions of these two genes. In NHEKs, incubation with acetate for 7 days had no effect on basal expression of IL-6 or IL-8, in unstimulated cells. Entirely in keeping with this finding, acetate also had no significant effects on epigenetic marks or pol II (S2P) recruitment to IL-6 or IL-8 promoters in unstimulated cells. Pre-incubation with acetate only had an effect on the epigenome in conjunction with stimulation. At the IL-6 promoter, acetate treatment prior to TNFα + IL-17 stimulation resulted in significant enhancement of acetylation of histone H3 (as well as H3K9ac and H3K27ac), H3K4me3 and recruitment of pol II (S2P). It has previously been shown that there is a degree of specificity to ethanol-induced histone acetylation; ethanol treatment of hepatocytes increased acetylation of histone H3 at lysine residue 9, but had little effect on acetylation of lysine residues 14, 18 and 23 (Park *et al.*, 2005). This specificity was also demonstrated *in vivo* in rats exposed to ethanol; and acetate was shown to have similar effects (Park *et al.*, 2005). Park *et al*. also demonstrated that H3K9ac was increased at the ADH I promoter, this is consistent with their finding that ethanol exposure upregulates expression of this gene and consistent with our finding that acetate mediates its effects, in part, through increased H3K9ac at target gene promoters.

Interestingly in a model of acute alcoholic hepatitis, the authors demonstrated that ethanol exposure alone (without LPS stimulation) lead to increased histone acetylation at the IL-6 promoter (Kendrick *et al.*, 2010). Kendrick *et al*. did not investigate the effects of acetate on histone acetylation at gene promoter regions; it may therefore be difficult to make direct comparisons with our results. However, in our system we showed that acetate enhanced pol II (S2P) recruitment and histone and lysine (residues 9 and 27) acetylation at the IL-6 promoter only in the presence of stimulation (TNFα + IL-17), but not in baseline unstimulated cells as demonstrated in the acute alcoholic hepatitis model. Our findings are internally consistent; acetate did not potentiate basal IL-6 production in unstimulated cells, which is in keeping with the observation that acetate had no effect on pol II (S2P) recruitment or histone acetylation at the IL-6 promoter in unstimulated cells. In contrast to effects at the IL-6 promoter, at the IL-8 promoter pre-incubation with acetate had the opposite effect in that there was a decrease

in histone and lysine acetylation and inhibition of pol II (S2P) recruitment following stimulation with IL-17 + TNFα. As previously discussed (section 1.3.6) BET proteins are known to bind acetylated lysine residues found on histones, orchestrate recruitment of transcription factors and proteins involved in RNA polymerase II activation, thus BET proteins can regulate gene expression. Since the modulation of inflammatory responses by acetate could be explained by changes in histone acetylation and pol II (S2P) recruitment, BET proteins may also be involved in underlying mechanisms.

Of interest, in addition to modulating histone acetylation, pre-incubation with acetate also affected histone methylation, in TNFα + IL-17 NHEKs. For example, at the IL-6 promoter acetate significantly enhanced H3K4me3, a permissive epigenetic mark, but significantly decreased H3K4me3 at the IL-8 promoter. Our observation that acetate can affect histone methylation is supported by the finding that ethanol treatment of rat-derived hepatocytes has been shown to alter the methylation status of lysine residues on histones (Pal-Bhadra *et al.*, 2007). Interestingly, Pal-Bhadra *et al*. demonstrated that ethanol affected methylation patterns in a lysine-residue specific manner; ethanol induced an increase in global dimethylation on lysine 4 on histone-H3 (H3K4me2) but a decrease in global dimethylation on lysine 9 on histone-H3 (H3K9me2). The local effects of acetate on histone acetylation and methylation at IL-6 and IL-8 promoters are entirely consistent with the differential effects of acetate on IL-6 and IL-8 responses to TNFα + IL-17 stimulation and provide mechanistic insight into how environmental stimuli regulate cutaneous inflammation.

As an IL-6 enhancer has not been defined in keratinocytes, we investigated the effects of acetate at a region previously defined as an IL-6 enhancer in macrophages (Qiao *et al.*, 2013); pre-incubation with acetate had little significant effect on the epigenome at this region and had no effect on pol II (S2P) recruitment. However, as this region demonstrated little enrichment of H3K27ac, H3K4me1 and Brd4, it is unlikely to function as IL-6 enhancer region in keratinocytes, as previously discussed in sections 4.4 and 5.4. It is therefore difficult to conclude if the effects of acetate were solely through chromatin remodelling at the IL-6 promoter region and not at other regulatory regions.

As a global change in ACSS expression, or indeed HAT or HDAC activity, could not necessarily explain the potentiation of IL-6 *versus* the repression of IL-8 responses by acetate nor the changes in histone methylation in our data, we hypothesised that local

changes at the level of individual gene promoters or enhancers driven by acetate induced activation or inhibition of specific epigenetic writers (*e.g.* acetylases or methylases) and/or erasers (*e.g.* deacetylases or demethylases) would be necessary to account for these differences. Our finding that acetate had a differential effect on the epigenome at the IL-6 *versus* IL-8 promoters is consistent with a model of alcohol-induced cardiac hypertrophy, in which ethanol-exposed mice demonstrated increased expression of the transcription factor NKX2.5 involved in cardiac development and hypertrophy with a corresponding increase in H3K9ac at the NKX2.5 promoter(Peng *et al.*, 2015). In contrast, there was no increase in H3K9ac at the RPL13A promoter, a gene whose expression was not enhanced with ethanol treatment (Peng *et al.*, 2015). This supports our hypothesis that the phenotypic effects of ethanol, or its metabolites, can be explained by localised chromatin remodelling at individual gene promoters. To account for the differential epigenetic modifications at the IL-6 *versus* IL-8 promoters, we hypothesise that acetate modulated the recruitment of epigenetic writers (*e.g.* HATs) and erasers (*e.g.* HDACs) to individual gene promoters. In support of this hypothesis, it has previously been shown that in nuclear extracts prepared from rat derived hepatocytes, both ethanol and acetate exposure lead to the selective enhancement of acetylation of H3 at lysine 9 (H3K9ac), suggesting that ethanol or acetate could selectively modulate activity of epigenetic writers and erasers with specificity for particular histone residues (Park *et al.*, 2005). More recently, in a mouse model of alcohol-induced cardiac hypertrophy, ethanol-induced localisation of specific members of the HAT family (p300, CBP, PCAF, SRC1 and GCB5) to the NKX2.5 promoter and that ethanol enhanced the binding of a specific subset of these (p300, CBP, PCAF and SRC1 but not GCB5), corresponding to enhance expression of NKX2.5. In addition anacardic acid, a pan-HAT inhibitor, reduced binding of p300 and PCAF with corresponding attenuation of H3K9ac. (Peng *et al.*, 2015). To explain the finding that acetate also had a differential effect on histone methylation at IL-6 and IL-8 promoters, we hypothesised that acetate would also affect recruitment of methylases and demethylases to specific genes. Consistent with this hypothesis, in ChIP studies of ethanol treated rat-derived hepatocytes showed that H3K4me2 was enriched at the promoter region of genes which were upregulated with ethanol treatment, whereas H3K9me2 was enriched at the promoter region of genes which were downregulated with ethanol treatment, although the underlying mechanism could not be explained (Pal-Bhadra *et al.*, 2007). Together these findings suggest ethanol, or acetate, can induce a variety of epigenetic modifications at the promoter region of specific genes by directing

recruitment of particular epigenetic writers (and potentially erasers). This mechanism could explain the differential effects of acetate (on both histone acetylation and methylation) at IL-6 and IL-8 promoters that we observed.

Interestingly, similar to the effects of acetate, IFNγ enhanced IL-6, but not IL-8, protein response to TNFα + IL-17 stimulation in NHEKs (section 3.3.1). As discussed in section 3.4, in macrophages, pre-treatment with IFNγ synergistically upregulated subsequent LPS-induced response of IL-6 (Qiao *et al.*, 2013). Qiao *et al*. demonstrated this was mediated through IFNγ induced recruitment of HAT enzymes (p300 and CBP) to the IL-6 promoter, resulting in increased acetylation of H3K27, thus facilitating IL-6 expression; the effects of IFNγ on IL-8 responses to LPS stimulation were not investigated. Together these findings demonstrate that IL-6 response is more sensitive to potentiation by added stimuli, *e.g.* acetate and IFNγ, and that these different stimuli mediate their potentiation through a common pathway *i.e.* histone hyperacetylation (*e.g.* H3K27ac) at the IL-6 promoter, this likely to be through the recruitment of particular HAT enzymes (*e.g.* p300 and CBP) specifically to the IL-6 promoter, ultimately resulting in increased IL-6 expression.

I-BET151 treatment inhibited acetate enhancement of IL-6 response (mRNA and protein) to TNFα + IL-17 stimulation. Whilst this finding does not necessarily provide additional understanding of the underlying mechanisms involved, it supports the hypothesis that these involve BET proteins which are a key, rate-limiting step and a potential therapeutic target in alcohol-provoked psoriasis In this respect I-BET151, may block BET proteins binding either to the acetylated histones at gene regulatory regions or to other proteins involved in transcription *e.g.* acetyl-p65 or *pTEFb*, as previously discussed (section 4.4). The hypothesis that histone hyperacetylation (which may involve BET proteins) plays a key role in enhancing gene expression in response to ethanol/acetate treatment is supported by the observation that anacardic acid (a pan-HAT inhibitor) attenuated alcohol-induced enhancement of NKX2.5 expression in a model of alcohol-induced cardiac hypertrophy (Peng *et al.*, 2015). Peng *et al*. demonstrated anacardic acid decreased hyperacetylation of H3K9 as well as p300 and PCAF binding at the NKX2.5 promoter. Interestingly our results showed that the vehicle (DMSO) also reduced the potentiation of the IL-6 response in TNFα + IL-17 stimulated NHEKs. Two studies implicate DMSO in affecting histone acetylation, or binding to the acetyl-lysine residue, and potentially may explain effects of DMSO in the

acetate/TNFα + IL-17 model. Firstly DMSO may have HDAC inhibitor activity; it has previously been shown that DMSO can induce cellular differentiation and it was through this observation that HDAC inhibitors were developed, based initially on the structure of DMSO (Marks and Breslow, 2007). Secondly, DMSO has been shown to bind within the acetyl-lysine binding cavity of BET proteins. However as the concentration of DMSO used in our studies (11.8mM) was low compared to the DMSO IC_{50} for Brd2 and Brd4, 255.9mM and 218mM respectively, based on peptide displacement assays (Philpott *et al.*, 2011), this second possible explanation is perhaps less likely to account for the effects of DMSO in the acetate/TNFα + IL-17 model.

7.5. Future work

It would be interesting to understand in our system the localised, underlying mechanisms at the IL-6 *versus* IL-8 promoter regions which give rise to the observed differences on the epigenome and pol II (S2P) recruitment to these two regions. We propose a number of possible underlying hypotheses/future work:

1) We demonstrated that exposure to acetate for only 2 days was insufficient to potentiate the IL-6 response. Could acetate affect the expression or activation of transcription factors or co-factors involved in IL-6 or IL-8 expression? In addition to histone acetylation, could acetate act as a substrate (through the conversion to acetyl-coA by ACSS) for the acetylation of other transcription factors (*e.g.* p65) involved in gene expression? Does any delay in expression or activation explain why 2 days' exposure of NHEKs to acetate is insufficient to observe acetate's effects on responses to stimulation?
2) We showed that acetate's potentiation of inflammatory responses was both gene and stimulus specific, and demonstrated differential effects at target gene promoters. What are the underlying mechanisms; do particular stimuli have localised effects at the level of gene promoters (or enhancers) *e.g.* through differential recruitment of epigenetic writers (*e.g.* HATs) and erasers (*e.g.* HDACs)?
3) BET proteins are known to bind acetylated lysine residues on histones, which are augmented by acetate. Therefore, are BET proteins involved in underlying mechanisms which might explain how acetate affects gene expression?

4) Which other genes, other than IL-6 and IL-8, are enhanced or repressed by acetate treatment prior to TNFα + IL-17 stimulation in our model and how do these genes relate to psoriasis pathogenesis?

7.6. Conclusions

- Acetate, a principal metabolite of ethanol, potentiated IL-6, but not IL-8 expression in response to TNFα + IL-17 stimulation.
- Ethanol did not potentiate IL-6 or IL-8 expression in response to TNFα + IL-17 stimulation; NHEKs demonstrated low expression of enzymes involved in ethanol metabolism.
- Lithium and propranolol did not potentiate IL-6 or IL-8 expression in response to TNFα + IL-17 stimulation.
- IL-6 expression induced by both cytokine and non-cytokine stimuli, were more sensitive to potentiation by supra-basal exposure to acetate, compared to IL-8.
- In the TNFα + IL-17 stimulation model, at the IL-6 promoter, acetate exposure resulted in chromatin remodelling with histone hyperacetylation and enhancement of pol II (S2P) recruitment, thus promoting IL-6 expression. This was consistent with the effect of acetate to potentiate IL-6 mRNA expression. Acetate had little effect at the IL-6 enhancer.
- In comparison in the TNFα + IL-17 stimulation model, at the IL-8 promoter acetate exposure resulted in chromatin remodelling with histone deacetylation and inhibition of pol II (S2P) recruitment, thus inhibiting IL-8 expression. This was consistent with acetate's inhibitory effects on IL-8 mRNA expression.
- The effect of acetate on pro-inflammatory responses, through epigenetic modifications, may explain how alcohol predisposes patients to an exaggerated inflammatory response resulting in exacerbation of their psoriasis and provide mechanistic insight into the regulation of cutaneous inflammation by defined environmental signals.

Chapter 8.

Concluding remarks

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Psoriasis represents a complex interplay between genetic predisposition, the environment and inflammatory responses. Although how these factors interact to influence the psoriasis phenotype is incompletely understood, there is increasing evidence that alterations in the epigenome, including aberrant histone acetylation, plays an important role in regulating inflammatory skin conditions, including psoriasis. In addition, there is little documentation about the function of BET proteins or the effects of BET inhibition in keratinocytes. However, given that psoriasis is characterised by chronic inflammation and excessive keratinocyte proliferation, and that BET inhibitors are known to have anti-proliferative and anti-inflammatory effects in other cell types, it was hypothesised that epigenetic modifications and BET proteins would be involved in regulating inflammatory gene responses to cytokines relevant to psoriasis and that BET inhibitors would block the pathogenic inflammatory response to stimuli in keratinocytes.

This project set out to evaluate the role that epigenetics, including histone acetylation and BET epi-reader proteins, plays in regulating cutaneous inflammation and inflammatory skin disease and how exogenous factors relevant to disease might alter inflammatory responses through underlying changes in the epigenome of effector genes.

The main tool used to investigate epigenetic modifications was an *in-vitro* keratinocyte model of cutaneous inflammation (Chiricozzi *et al.*, 2011) which was further developed by investigating the effects of TNFα + IL-17 stimulation on secreted cytokine responses and evaluating the model for pre-clinical testing of potential therapeutic compounds. This work demonstrated that stimulation of normal human epidermal keratinocytes (NHEKs) with TNFα + IL-17, key cytokines in the pathogenesis of psoriasis, induced expression of IL-6 and IL-8 (mRNA and protein). Consistent with these findings we demonstrated through chromatin immunoprecipitation (ChIP) studies, for the first time, that TNFα + IL-17 stimulation induced histone modifications including global hyperacetylation of histone-H3, as well as acetylation of specific lysine residues associated with active promoters (*e.g.* H3K9ac and H3K27ac), at the promoter regions of IL-6 and IL-8. In contrast, TNFα + IL-17 stimulation had no significant effect on a repressive epigenetic epitope, H3K4me1. Collectively these data demonstrate that there is dynamic, gene-specific modulation of the epigenome, inducible by disease relevant stimuli, regulating gene expression in human keratinocytes.

The model of cutaneous inflammation was then used to study potential new therapies. We initially demonstrated ciclosporin, a compound used clinically to treat psoriasis, inhibited IL-6 and IL-8 induction in response to TNFα + IL-17 stimulation, illustrating that the *in vitro* model of cutaneous inflammation could be used for pre-clinical evaluation of compound effects. Following on from this, we chose to explore the role of the BET epi-reader proteins in the model, which are known to bind acetylated lysine residues, found on histones via bromodomains and recruit transcription factors, thereby regulating gene expression. We showed that TNFα + IL-17 stimulation resulted in transient and co-ordinated recruitment of BET proteins (Brd2, Brd3 and Brd4) and RNA polymerase II to the promoter regions of IL-6 and IL-8, consistent with the positive effects of stimulation on histone acetylation and IL-6 and IL-8 mRNA expression. Small molecule BET bromodomain inhibitors have been discovered which exhibit anti-proliferative and anti-inflammatory effects in *in vitro* and *in vivo* models of cancer and inflammation, therefore we hypothesised that BET inhibitors would inhibit inflammatory responses in keratinocytes. I-BET151, a specific small molecule BET inhibitor, potently inhibited IL-6 induction by TNFα + IL-17 but had a weaker and more variable effect on IL-8. Moreover, chromatin immunoprecipitation (ChIP) studies showed that I-BET151 reduced recruitment of Brd4 to the IL-6 promoter and Brd3/4 to the IL-8 promoter, with corresponding decreases in RNA polymerase II binding at both promoters, in TNFα + IL-17 stimulated NHEKs; these findings were consistent with the inhibitory effects of I-BET151 on IL-6 and IL-8 mRNA expression. Additionally, we hypothesised that I-BET151 would be most effective during the phase of chromatin remodelling induced by the TNFα + IL-17 stimulation, and thus be active even when added after stimulation. This mechanism is in contrast to drugs which block the stimulation event or proximal cytokine receptor-signalling. In support of this hypothesis, I-BET151 continued to have an inhibitory effect on IL-6 and IL-8 expression when added after stimulation (up to 2h for IL-6/-8 mRNA and up to 24h for IL-6 protein), which also more realistically reflects how psoriasis treatments are used clinically. In addition, given that levels of IL-6 and IL-8 are high in lesional psoriatic skin, reflecting active transcription of these genes, then chromatin around the promoter region of IL-6 and IL-8 is likely to be in an "active/open" (euchromatin) state and accessible to compound. These novel findings demonstrate that stimuli relevant to disease induce chromatin remodelling, provide mechanistic insight into the role of BET proteins in regulating cutaneous inflammatory responses and highlight that BET

proteins could represent novel targets for psoriasis treatment. This concept is supported by recently published findings that JQ-1, another BET inhibitor, inhibited psoriasis-like cutaneous inflammation in an imiquimod-induced mouse model of psoriasis (Nadeem *et al.*, 2015).

Global gene expression array experiments were also undertaken to investigate the wider effects of TNFα + IL-17 stimulation in NHEKs and I-BET151 treatment in the model, and to place the results for IL-6 and IL-8 into a broader context. Comparisons of genes regulated by TNFα + IL-17 in NHEKs to a previously published psoriasis datasets (the MAD-5 psoriasis transcriptome), demonstrated that > 25% of genes regulated by these stimuli were also differentially expressed in psoriasis (Tian *et al.*, 2012). In TNFα + IL-17 stimulated NHEKs, I-BET151 modulated expression of genes mainly involved in cell cycle, p53 signalling and inflammation, and of the 1120 up or downregulated genes in the MAD-5 transcriptome ~20% of genes were differentially modulated by I-BET151 treatment in TNFα + IL-17 stimulated NHEKs. Specifically, I-BET151 inhibited expression of key regulators of inflammation in psoriasis, including IL1A, IL1B and NFKB. Comparisons of genes regulated by TNFα + IL-17 stimulation and sensitive to I-BET151 treatment, with an Ingenuity® Pathway Analysis (IPA)-defined psoriasis network and the MAD-5 psoriasis transcriptome, revealed a number of overlapping genes as well as ones which were regulated by stimulation and differentially expressed in psoriasis but have not previously been characterised in the pathogenesis of psoriasis. These genes may represent interesting candidates for further investigation. Together these findings suggest the *in vitro* model of cutaneous inflammation is a valid cell model for studying pathogenic pathways, regulation of expression of genes involved in cutaneous inflammatory responses relevant to psoriasis and supports the hypothesis that BET proteins are involved in regulating cutaneous inflammation and that BET inhibitors may represent novel therapy for the treatment of psoriasis.

The global gene expression array studies with I-BET151 in TNFα + IL-17 stimulated NHEKs, also showed that I-BET151 inhibited expression of genes encoding proteins involved in protein synthesis and post-translational modification. These findings may explain the differential effects of I-BET151 on IL-6 mRNA *versus* protein responses to TNFα + IL-17 stimulation, and support the hypothesis that BET inhibitors impair expression of molecules with post-transcriptional roles, as previously suggested with HDAC inhibitors (Grabiec *et al.*, 2012). In addition to I-BET151 regulating expression

of genes involved in the cell cycle and p53, downregulation of MYC was at the centre of an IPA network encompassing the most number of I-BET151-sensitive genes, in keeping with previously published effects on BET inhibitors on MYC expression (Dawson *et al.*, 2011; Delmore *et al.*, 2011; Wyce *et al.*, 2013). The role of BET proteins and BET inhibitors in keratinocyte-derived cancers, including basal cell carcinoma and squamous cell carcinoma, the most common non-melanoma skin cancers in which over-expression of MYC is implicated, therefore warrants further investigation.

Exogenous factors, including alcohol, are implicated in the development and exacerbation of psoriasis. Alcohol and its principal metabolite, acetate, was previously shown to enhance pro-inflammatory responses to stimuli in macrophages, through epigenetic modifications and we hypothesised similar processes existed in keratinocytes. Pre-treatment with acetate resulted in concentration-dependent potentiation of IL-6 expression, but not IL-8, in response to TNFα + IL-17 stimulation compared to control. Consistent with this, chromatin immunoprecipitation studies showed pre-treatment of keratinocytes with acetate significantly enhanced the TNFα + IL-17 stimulated increase in permissive epigenetic epitopes and RNA polymerase II recruitment to the IL-6 promoter, but not to the IL-8 promoter. On the other hand, ethanol did not significantly potentiate IL-6 or IL-8 responses to TNFα + IL-17 stimulation in keratinocytes. Keratinocytes exhibited low expression of enzymes involved in ethanol metabolism, which probably accounted for the lack of effect of ethanol in the model. Although lithium and propranolol have also been implicated in the development and exacerbation of psoriasis, pre-treatment of NHEKs with these compounds did not result in any potentiation of subsequent inflammatory responses, suggesting these factors may act through alternative mechanisms to ethanol/acetate. The enhancement of pro-inflammatory responses by acetate, through epigenetic modifications, may explain how alcohol predisposes patients to an exaggerated inflammatory response resulting in development or exacerbation of psoriasis and could be used to educate patients about the hazards associated with excess alcohol consumption. These data provide mechanistic insight into the regulation of cutaneous inflammation by defined environmental signals and could lead to identification of novel targets for therapy. In addition, these findings could explain how environmental factors interact with genetic predisposition in other common complex diseases *e.g.* cancer, diabetes and cardiovascular disease.

TNFα + IL-17 stimulation had a consistently synergistic effect on IL-8 mRNA induction in all donors, but donor-dependent effects on IL-6 induction. In addition, we showed that both acetate and IFNγ enhanced the IL-6, but not IL-8, response to TNFα + IL-17 stimulation. These findings suggest that i) IL-6 responses are more sensitive to enhancement by additional disease relevant stimuli and ii) IL-6 and IL-8 are differentially regulated at the transcriptional level. Interestingly, TNFα + IL-17 simulated NHEKs demonstrated greater enrichment of Brd4 and p65 at the IL-6 promoter than at the IL-8 promoter, and changes in p65 binding tracked with the kinetics of Brd4 binding at the IL-6 promoter. These findings could suggest NF-κB plays a greater role in regulating IL-6 expression, compared to IL-8 expression, consistent with previous findings in Jurkat T cells (Khalaf *et al.*, 2013). They also raise the hypothesis that NF-κB (p65)-regulated genes are more sensitive to enhancement by additional stimuli and that Brd4/p65 may mediate enhancement of gene expression through binding to the promoter, of effectors genes, which are hyperacetylated in response to pathogenic stimuli *e.g.* acetate or IFNγ (Qiao *et al.*, 2013).

In summary we have further developed an *in vitro* keratinocyte model of cutaneous inflammation to study the regulation of genes involved in pathogenic pathways and to evaluate novel targets/drugs. Disease relevant stimuli induced dynamic, gene-specific epigenetic changes, with co-ordinated recruitment of BET proteins (Brd2, Brd3 and Brd4) and RNA polymerase II, at the promoter region of effector genes. In addition acetate a principal metabolite of ethanol, which is implicated in the pathogenesis of psoriasis, modulated pro-inflammatory responses to stimulation through gene-specific changes in the epigenome. IL-6 and IL-8 responses to stimuli were found to be differentially sensitive to potentiation by additional disease relevant factors, *e.g.* acetate and IFNγ, and it was observed that there was increased enrichment of Brd4/p65 at the IL-6 promoter than at the IL-8 promoter, raising the hypothesis that NF-κB (p65)-regulated genes are more sensitive to additional stimuli and that Brd4/p65 are involved in underlying mechanisms in alcohol provoked-inflammatory skin disease, consistent with previous findings in IFNγ + LPS stimulated macrophages (Qiao *et al.*, 2013). Pharmacological profiling of a specific BET inhibitor was supported by gene array data and putative mechanisms validated through ChIP studies. The sensitivity of psoriasis related genes to BET inhibition, supports a biological model in which epigenetic process are involved in the pathogenesis of psoriasis and that these processes can be dynamic, subject to reversal and potentially targetable by compounds thereby altering

the phenotype. In addition drugs currently used in the treatment of psoriasis to induce remission are associated with changes in the epigenome (Roberson *et al.*, 2012; Gu *et al.*, 2015), relapse in disease may therefore also be associated with potentially reversible epigenetic processes. The effect of BET inhibition highlights the potential for targeting epigenetic processes, and in this case specifically BET proteins are implicated as targets. Collectively the data highlight the area of epigenetics as a potential route to discover novel disease modifying therapies.

8.1. Future directions

It would be interesting to investigate whether there are specific expression quantitative trait loci (eQTLs) associated with donor-dependent variability in keratinocyte inflammatory responses, whether these are associated with particular disease endotypes or whether these represent potential biomarkers for sensitivity to therapy. In the UK, the British Association of Dermatologists' Biologic Interventions Register (BADBIR) and Psoriasis Stratification to Optimise Relevant Therapy (PSORT) (http://www.psort.org.uk/; Burden *et al.*, 2012) studies could provide suitably large cohorts to link genome wide association studies (GWAS) and epigenome wide association studies (EWAS) to gene expression data for specific genes.

ChIP-sequencing (ChIP-Seq) could be an informative screening method to investigate where, for example, Brd4 (other BET proteins), p65 or acetyl marks map within the genome in order to further understand their regulatory roles. Advances in ChIP-Seq technology using low cell numbers could allow this to be performed in activated primary keratinocytes in future (Mundade *et al.*, 2014; Jakobsen *et al.*, 2015).

Future investigations into how disease relevant cytokine stimuli *e.g.* TNFα, IL-17 and IFNγ, influence the balance between epigenetic writers and erasers to regulate the epigenome, how environment factors affects this balance and which genes are most sensitive to exogenous stimuli, this may reveal novel targets for therapy.

Further data-mining of the gene array analysis generated here may identify candidates for further investigation into the pathogenesis of psoriasis or as novel targets for its treatment. In addition this may provide further rationale for investigating the role of BET proteins and BET inhibitors in suitable models of keratinocyte-derived skin cancers.

Chapter 9.

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9. References

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