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Regulation of the Innate Immune System

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Declaration

I declare that this thesis was written by me and that the work here is original research undertaken by myself. Where the work of others has been included, this has been duly acknowledged. This work has not been submitted for any other degree or professional qualification except as specified.

Sarah McGlasson

November 2014

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Abstract

The innate immune system is the first line of defence against pathogen invasion. The range of diseases that are caused by deficiencies in or deregulation of the innate immune system illustrates the importance of maintaining an effective balance between clearance of infectious agents and minimisation of inflammatory mediated tissue damage. This thesis explores the role of two proteins in the regulation of the innate immune system.

Primarily, this work investigates the effect of human β -defensin 3 (hBD3) on the response to self-DNA and pathogenic DNA. HBD3 is an antimicrobial peptide (AMP), which has been shown to have a role in regulating the immune response; increased copy number of the region containing the gene for hBD3, *DEFB103*, is linked to an increased risk of psoriasis. Additionally, a similar cationic AMP, LL37, has been shown to exacerbate the pathogenesis of psoriasis by forming an immunogenic complex with self-DNA. This lead to the hypothesis that hBD3 may also affect the innate immune response to DNA. Therefore this project investigates what effect hBD3 has on the response of the innate immune system to self and pathogenic DNA.

Flt-3 dendritic cells were used to show that whilst hBD3 increased cellular uptake of self-DNA, it did not convert self-DNA into an immune stimulus. However, hBD3 significantly exacerbated the response to bacterial DNA in a TLR9-dependent manner, also by increasing cellular uptake into FLDCs. The finding that hBD3 increased cellular uptake of both self- and pathogenic DNA suggests that at sites of infection or increased cell death, where DNA would be found in the extracellular environment, hBD3 may increase uptake into immune cells and could induce an increased immune response. Since increased hBD3 expression is induced by inflammatory stimuli, this process would cause a positive feedback loop of inflammation during bacterial infections.

In conclusion, hBD3's role in regulating the innate immune response to DNA is at the ligand-receptor level rather than affecting signalling pathways. Furthermore, hBD3 promotes the innate immune response to bacterial DNA by increasing the

efficiency of cellular uptake possibly by inducing DNA aggregation. These results implicate a possible role for hBD3 in the earliest stages of psoriatic plaque development, which is often initiated or exacerbated by an infection, and this could be investigated further.

Secondly, I investigated the innate immune function of an E3 ubiquitin ligase (E3L) not previously associated with human disease. Mutations in E3L have been identified in three microcephalic primordial dwarfism families; these patients also presented with recurrent respiratory illnesses. E3L has been implicated in the regulation of the innate immune system via interactions with signalling pathways downstream of the receptor, though its role is not clear. We hypothesised that E3L had a dual role both in regulating growth and cell division and in regulating the immune system. Primary patient fibroblasts did not demonstrate an altered cytokine response to bacterial or viral ligands, implying that E3L may have a specific function in immune cells. To investigate this further, and to provide a system to study E3L *in vivo*, two transgenic mouse lines were designed and engineered, firstly a conditional ‘knock-out’ designed to replicate some of the alternative isoforms of E3L seen in RT-PCRs, and secondly a ‘knock-in’ line to recapitulate the human mutation in exon 7 of E3L, R185X. These mouse lines should offer an insight into the developmental role for E3L, and contribute to establishing a potential role for E3L in the innate immune system.

This thesis exemplifies the complexity of the innate immune system and the regulatory pathways that interact to maintain a delicate homeostasis preventing pathogenic inflammation. Understanding these regulatory mechanisms may shed light on the pathogenicity of diseases and identification of potential targets for therapeutics.

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Abbreviations

AMP	Antimicrobial peptide
APC	Antigen presenting cell
Arg (R)	Arginine
ATP	Adenosine triphosphate
BS	Bluescript
CCR	Chemokine receptor
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CDN	Cyclic dinucleotide
cDNA	Complementary DNA
CFP	Cyan fluorescent protein
CXCL	C-X-C motif ligand
Cys (C)	Cysteine
DAMP	Damage Associated Molecular Pattern
DAPI	2-(4-amidinophenyl)-1H -indole-6-carboxamide
DDR	DNA damage response
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleotide triphosphate
ELISA	Enzyme linked immunosorbent assay
ES	Embryonic stem
Floxed	loxP-flanked
FLDC	Flt3-ligand dendritic cells
Flp	Flippase
FRT	Flippase recognition target
GFP/CFP	Green fluorescent protein/Cyan fluorescent protein
hBD(3)	Human β -defensin (3)
HD	Human defensin
HEK	Human embryonic kidney
HeLa	Henrietta Lacks
HNP	Human neutrophil peptide

i.p.	Intraperitoneal
IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factor
kbp	Kilobase pairs
LPS	Lipopolysaccharide
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility factor
mRNA	Messenger RNA
neo	Neomycin
NMP	Nucleotide monophosphate
ODN	Oligodinucleotide
PAMP	Pathogen Associated Molecular Pattern
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PI	Propidium iodide
RAW 264.7	Mouse leukemic monocyte/macrophage cell line
RNA	Ribose nucleic acid
RT-PCR	Reverse transcription PCR
siRNA	Small interfering RNA
STING	Stimulator of interferon genes
TIR	Toll-IL-1 and IL-18 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor activating factor
TRAIP	TRAF interacting protein

1. Introduction - The innate immune system

Humans, and other organisms, are subject to a constant barrage of insults by pathogens from the air and by contact; most of these are excluded from the body by physical barriers such as the skin, but when these pathogens gain access to the body they must be dealt with swiftly to prevent infection and tissue damage.

The innate immune system forms the first line of defence against pathogen invasion, after physical barriers, and recognises pathogen and damage associated molecular patterns (PAMPs/DAMPs). The innate immune system mounts a somewhat non-specific response against pathogens including the recruitment and activation of various innate immune cells, release of cytokines, chemokines and interferons, induction of an anti-pathogenic gene signature and activation of the complement system and the adaptive immune system. The adaptive immune system mounts a more specific immune response involving production of antibodies, and generates immunological memory to mount a more efficient response in the event of a second assault.

The innate immune system is the germ-line-encoded, phylogenetically ancient branch of the immune system and is conserved across evolution in varying forms from vertebrates, through invertebrates and plants down to simple multicellular organisms such as sponges (Kimbrell and Beutler, 2001). This is in contrast to the adaptive immune system which is solely found in vertebrates. There are distinct differences in the innate immune pathways that predominate in different phyla; for example the *Drosophila* innate immune system induces the release of secreted host defence peptides (Boman et al., 1972; Kounatidis and Ligoxygakis, 2012; Myllymäki et al., 2014) whereas the vertebrate innate immune involves release of a wide range of cytokines and chemokines as well as host defence peptides; however these are variations on a theme of a rapid, transient response.

Chronic inflammation underlies the pathogenesis of most diseases and dysregulation of the innate immune system has been implicated in many common and less common diseases including many autoimmune disorders. Understanding the involvement of the innate immune system in disease processes and how the system is regulated in

both healthy and pathological states may identify potential therapeutic targets. Indeed some diseases are already treated by targeting the innate immune system, however many of the treatments currently available target end points such as cytokine inhibitors, e.g. TNF inhibitors used to treat rheumatoid arthritis, and may have off-target, or non-specific effects (Taylor and Feldmann, 2009). Greater understanding of the complex pathways involved may lead to development of more specific, more effective treatments.

1.1 Cells of the innate immune system

The innate immune system consists of a large number of cell types, each adapted to a specific role within the broader system. This section discusses the development and function of the cell types of the innate immune system, chapter 3 introduces dendritic cells in more detail with particular focus on their roles in the immune response to nucleic acids.

1.1.1 Dendritic cells

The term ‘dendritic cells’ was first published in 1973 on the basis of the large dendrite (tree)-like projections characteristic of the mature cell-type (figure 1.1) (Steinman and Cohn, 1973). Over the next 25 years techniques were developed to allow sufficient DCs to be isolated for in depth investigations of their function (Banchereau and Steinman, 1998), for example by deriving DCs from bone marrow cultures (Inaba et al., 1992).

Dendritic cells (DCs) are professional antigen presenting cells (APCs) which capture antigen and present it to the adaptive immune system via the major histocompatibility complex (MHC) molecules (Mellman and Steinman, 2001; Mellman et al., 1998). Immature DCs are found in the peripheral tissues and in the blood, constantly sampling the environment. They detect PAMPs or DAMPs via pattern recognition receptors (PRRs) located on the cell surface, in the endosome or in the cytosol, and undergo a maturation process which involves up-regulation of costimulatory markers including MHCII, CD40, CD80 and CD86, secretion of cytokines, migration to lymphoid tissues and presentation of antigen to T cells (Steinman et al., 1999). DCs take up antigen in one of three ways: phagocytosis,

macropinocytosis and adsorptive endocytosis, which make them very efficient at continuously sampling the environment and presenting antigen that may be present at very low levels (Sallusto et al., 1995).

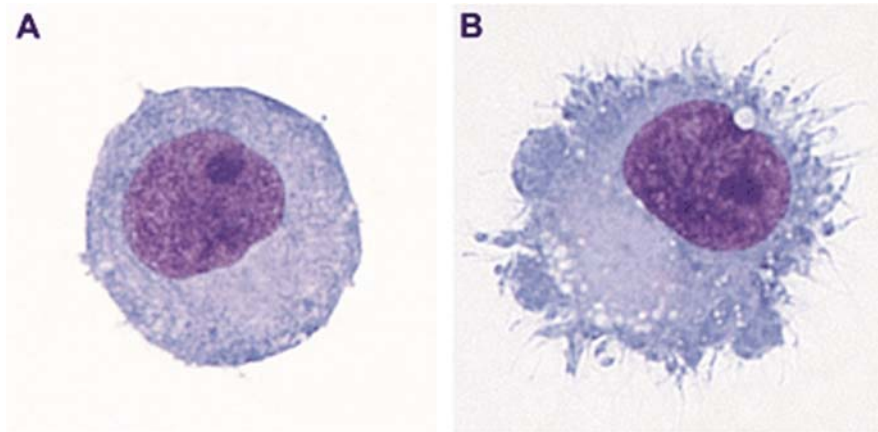


Figure 1.1 Maturation of monocyte-derived dendritic cells. Immature dendritic cell (A). Example of a dendritic cell following treatment with treated with an inflammatory stimulus (B). (O'Neill et al., 2004)

DCs are a heterogeneous group of cells with at least six subtypes in mice differing in location, migration, expression of cell surface markers and receptors, immune function and origin (Ardavin, 2003; Heath and Carbone, 2013; Shortman and Liu, 2002). Langerhans cells in the skin are derived from embryonic tissue (Hoeffel et al., 2012; Schulz et al., 2012) whereas plasmacytoid DCs and conventional (lymphoid-resident and non-lymphoid-resident) DC subsets can arise via a number of overlapping developmental pathways that are not completely understood (figure 1.2). DCs can also be derived from monocytes (monocyte-derived DCs, moDCs) generally under inflammatory conditions, but also under steady state conditions in specific sites such in mucosal tissues of the lung and intestine (Domínguez and Ardavin, 2010; Varol et al., 2007).

Dendritic cells were initially thought to be entirely myeloid-derived cells, however this was called into question when lymphoid-restricted thymic precursors were demonstrated to produce thymic CD8⁺ DCs in both mice (Ardavin et al., 1993) and humans (Galy et al., 1995). Additionally, myeloid-restricted precursors have the

potential to produce both CD8⁺ and CD8⁻ splenic DCs (Manz et al., 2001; Traver et al., 2000; Wu et al., 2001). These experiments indicate a significant amount of flexibility in the earliest developmental stages of DC development, although the predominance of myeloid precursors in the bone marrow suggests that most splenic DCs would be of myeloid origin.

1.1.1.1 Conventional DCs

Conventional DCs (cDCs) show the classical features of DCs in terms of their stellate morphology (figure 1.1), particularly following maturation, and their function as APCs. CDCs can be sub-divided into two major categories: migratory DCs and lymphoid-tissue-resident DCs.

Migratory cDCs develop in the peripheral tissues, such as the dermis, intestine, lung, liver and kidney and migrate through the lymphatic system to the draining lymph nodes (Liu and Nussenzweig, 2010). Migratory DCs are derived from common DC precursors (CDPs) in the bone marrow via pre-DCs and are thought to migrate as immature cells and mature in the peripheral tissues (figure 1.2) (Liu et al., 2009; Waskow et al., 2008).

Lymphoid resident cDC are found in the lymph nodes, spleen and thymus and are characterized by their expression of CD4 and CD8 in mice (CD4⁺, CD8⁺ and CD4⁻ CD8⁻) (Vremec et al., 2000). CD8⁺ DCs have a major role in priming CD8⁺ cytotoxic T cells (Belz et al., 2004), whilst CD4⁺ and double negative DCs preferentially cross-present antigen via MHCII to CD4⁺ T cells (Vremec et al., 2000). Lymphoid resident DCs develop from precursors in the lymphoid tissues (Naik et al., 2006) and sense antigens or pathogens that are circulating in the blood.

1.1.1.2 Plasmacytoid DCs

Plasmacytoid DCs (pDCs) are a rare cell population found in peripheral lymphoid organs and circulating in the blood and are specialized to produce large amounts of type 1 interferon rapidly in response to activation (Cella et al., 1999; Siegal et al., 1999). A number of developmental pathways are thought to lead to pDC differentiation and these may interact to contribute to a steady state population (Shortman et al., 2013). Evidence of IgH gene V(D)J rearrangement in immature

pDCs from the spleen and thymus indicates a shared early developmental stage with T cells suggesting a lymphoid-like developmental past in a proportion of pDCs (figure 1.2) (Corcoran et al., 2003). As well as producing large amounts of type I interferon, pDCs can promote tolerance (Steinman et al., 2003) and a phenotypically distinct subset of pDCs has been identified in mice which is tolerogenic. This CD9⁻ Siglec-H^{high} pDC subset is not a distinct lineage from the classical IFN- α producing CD11c⁺B220⁺CD9⁺ pDC, but rather at a different stage of maturation (Björck et al., 2011).

1.1.1.3 Langerhans cells

Langerhans DCs (LCs) were the first dendritic cell type to be identified in the human epidermal compartment, by Paul Langerhans (Langerhans, 1868), though at the time they were thought to be a component of nervous tissues. LCs were later believed to be related to melanocytes or keratinocytes before being recognized as bone marrow-derived, antigen presenting immune cells (Katz et al., 1979; Stingl et al., 1980). LCs are an essential part of the epidermal cell population for stimulating T cell responses and constitutively take up and process antigen, although they require ‘danger’ signals from surrounding keratinocytes, such as GM-CSF and IL-1, to induce maturation and migration to T cell zones (Heufler et al., 1988; Witmer-Pack et al., 1987). This system of secondary activation maintains a non-inflammatory homeostasis in the skin until a true danger signal is present. Skin exposure to UV radiation results in immune suppression which is thought to be mediated by LCs. Following UV exposure, LCs are depleted in the epidermis and the morphology of remaining LCs is altered which may affect the ability of LCs to present antigen thereby leading to immune suppression (Seite et al., 2003).

LCs have been proposed as an important barrier in the transmission of HIV infection, and the C-type lectin, langerin (CD207), which is expressed on the surface of LCs, is involved in internalisation of HIV virus particles into the Birbeck granules (Valladeau et al., 2000) of LCs where they are rapidly degraded (de Witte et al., 2007).

A distinct, unrelated subset of dermal-localised langerin positive cells (langerin⁺ dermal DCs (dDCs)) has also been identified in mice which are dependent on Flt-3 ligand, unlike epidermal LCs (Nagao et al., 2009). Langerin⁺ dDCs also migrate to lymph nodes and are thought to mediate contact hypersensitivity reactions (Bursch et al., 2007).

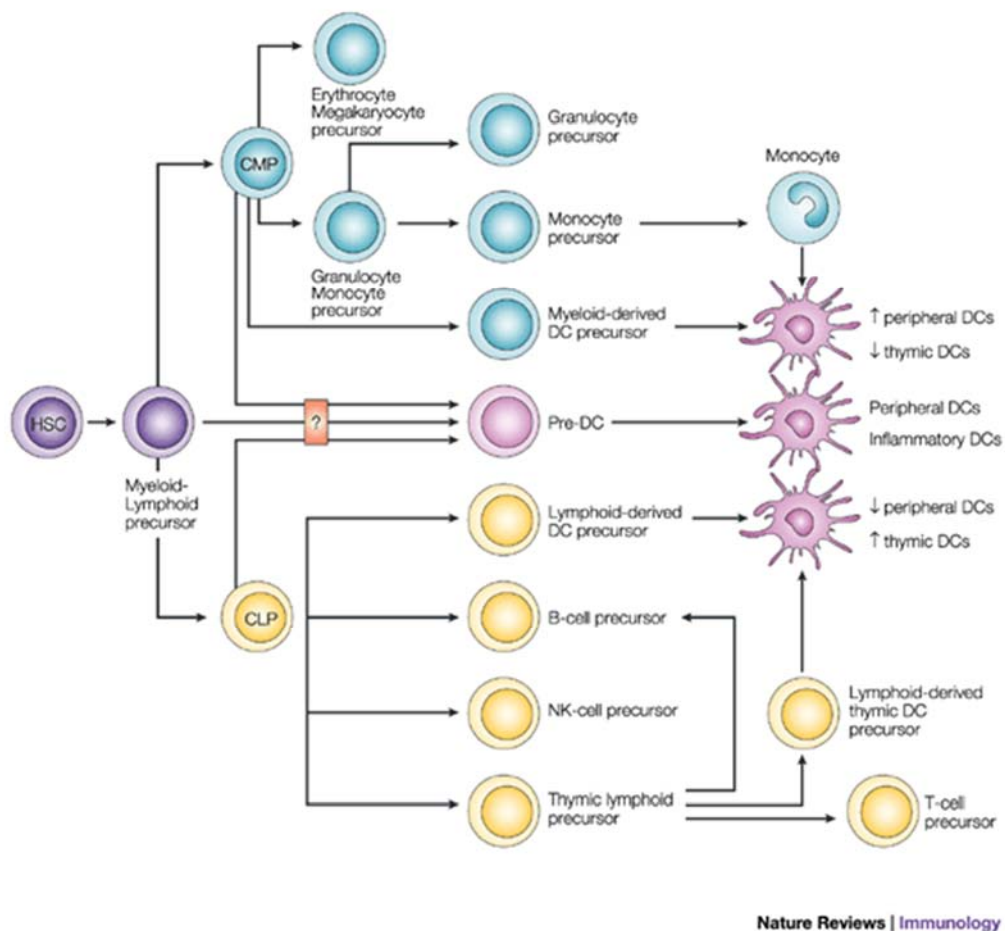


Figure 1.2 Differentiation of dendritic cells from haematopoietic stem cells occurs via a number of intermediates and overlapping pathways. Haematopoietic stem cells (HSCs) differentiate into either common lymphoid progenitors (CLPs), common myeloid progenitors (CMPs) or pre-DCs via an uncharacterised pathway. CMPs give rise to DC precursors and inflammatory monocytes can also differentiate

into DCs. There is evidence for a subset of pDCs coming from a lymphoid lineage (Ardavin, 2003).

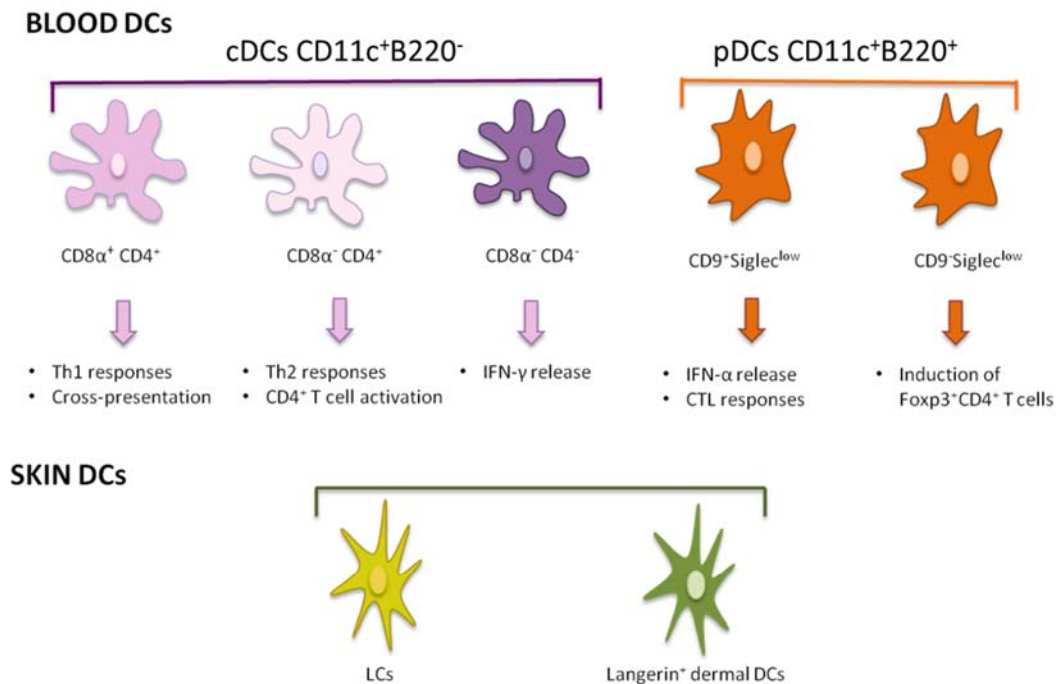


Figure 1.3 Mouse dendritic cell subsets. Circulating blood dendritic cells and divided into cDCs or pDCs which are further divided into phenotypically distinct subsets with different functions. Skin-resident DCs include epidermal Langerhans cells and dermal langerin⁺ DCs. Adapted from (Salvador, 2012)

1.1.2 Monocytes

Monocytes originate from bone marrow precursors and circulate in the blood stream. During homeostatic and inflammatory conditions monocytes migrate into peripheral tissue sites where they differentiate into macrophages or dendritic cells following stimulation with specific signals such as local growth factors, cytokines and PAMPs (Auffray et al., 2009). In the mouse, CD11b⁺Ly6C^{hi} inflammatory monocytes are rapidly recruited to sites of inflammation (Serbina et al., 2008) via a CCR2-dependent trafficking mechanism, where they differentiate into macrophages and

dendritic cells. The second major subset of monocytes expresses lower levels of CCR2 and Ly6C and high levels of CX₃CR1 (hence Ly6C^{low}CX₃CR1 designation) and are referred to as patrolling monocytes as they roll along the inner luminal wall of small blood vessels (Auffray et al., 2007). Proinflammatory cytokines and TLR ligands promote the migration of inflammatory monocytes from the bone marrow and bloodstream across the endothelium to sites of inflammation (Issekutz, 1995).

1.1.3 Macrophages

Macrophages are tissue-resident, phagocytic cells that were first identified in 1884 by Ilya Mechnikov. Macrophage translates literally to ‘big eater’, and they are named due to the first observations of these cells phagocytosing foreign objects in starfish larvae. The term macrophage includes the primitive embryonic phagocytes that Mechnikov observed, which are derived from yolk sac precursors and later spread throughout the embryo following development of the circulatory system. Definitive macrophages found later in development and in adult tissues were previously considered to be derived entirely from monocytes (van Furth and Cohn, 1968) however this view has gradually changed and whilst monocytes can give rise to macrophages, it is now thought that tissue macrophages are mostly derived from embryonic precursors and can self-renew (Ginhoux and Jung, 2014). This is supported by a human syndrome of monocytopenia, B cell, NK cell and DC deficiency with no effect on tissue resident macrophages (Bigley et al., 2011). In the embryo, phagocytic clearance of apoptotic cells by macrophages during development is essential and definitive erythropoiesis depends on DNase II expressed by the macrophages in the embryonic liver (Hopkinson-Woolley et al., 1994; Kawane et al., 2001). Macrophages have major roles in tissue development in the adult, for example in angiogenesis, bone morphogenesis and also in tumour progression and metastasis (Mosser and Edwards, 2008; Reviewed by Pollard, 2009).

Macrophages are activated following detection of PAMPs, DAMPs or cytokines by cellular receptors. In the adult, activated macrophages adopt one of two major phenotypes; they either develop into classical activated M1 macrophages following stimulation via PRRs, IFN- γ or GM-CSF; or into alternatively activated M2 macrophages following stimulation by IL-4, IL-13 or M-CSF (Martinez and Gordon,

2014). The M1 or M2 nomenclature reflects the respective involvement in activation of T_H1 or T_H2 type responses. M2 macrophages are recognised as a feature of nematode infection and allergic type response (Jenkins and Allen, 2010; Loke et al., 2002) particularly during the chronic stage of infection (Zhu et al., 2014). The development of M2 macrophages in nematode infections reflects their role in wound healing, and they express growth factors as well as anti-inflammatory cytokines including IL-10 (Ferrante and Leibovich, 2012).

1.1.4 Neutrophils

Neutrophils are the most abundant white blood cell in mammals and one of the most short-lived cell types in the body. Mature neutrophils emerge from the bone marrow and circulate in the bloodstream, they are typically the first immune cell to be recruited to an inflammatory site. Reduction in numbers of neutrophils in humans (neutropenia) leads to severe immunodeficiency underscoring their importance in the immune response (Klein, 2011; Kolaczowska and Kubes, 2013). Neutrophils bind, engulf and kill bacteria via phagosome fusion with storage granules, similar to lysosomes, containing proteolytic enzymes, antimicrobial peptides and reactive oxygen species (Borregaard and Cowland, 1997; Segal, 2005). Neutrophils also release the contents of the granules extracellularly, in a process referred to as degranulation (Lacy, 2006). Additionally neutrophils can kill via extracellular structures called neutrophil extracellular traps (NETs) which trap and kill bacteria (Brinkmann et al., 2004). NET generation (netosis) involves a specific mechanism of programmed neutrophilic cell death involving release of decondensed nuclear DNA fibres associated with antimicrobial factors normally contained in the granules (Zawrotniak and Rapala-Kozik, 2013). Neutrophils migrate rapidly to sites of infections due to their ability to migrate along shallow gradients of chemoattractants such as CXCL8 (IL-8), one of the major chemoattractants of neutrophils in humans (Hammond et al., 1995; Lin et al., 2004). Recognition of a chemoattractant molecule by cell surface receptors leads to reorganisation of the actin cytoskeleton and polarisation of the neutrophil, followed by rapid migration of the neutrophil by leading edge actin polymerisation (Cicchetti et al., 2002).

1.1.5 Mast cells and basophils

Mast cells and basophils are functionally and developmentally similar, both contain histidine and heparin rich granules and express a high-affinity IgE receptor. Mast cells are tissue resident cells, particularly found at surfaces in contact with the external environment (Urb and Sheppard, 2012). In contrast, steady-state basophils circulate in the blood and are recruited into tissues at sites of inflammation (Voehringer, 2012). Mast cells and basophils are associated with type 2 immunity to allergens and parasites and rapidly release the contents of their granules in response to activation of receptors (Voehringer, 2013). Mast cells and basophils, along with eosinophils are essential mediators of the allergic response (Stone et al., 2010).

1.1.6 Innate lymphoid cells.

Innate lymphoid cells (ILCs) are a relatively recently identified class of cells, they belong to the lymphoid lineage but do not respond in an antigen specific way as they do not express antigen specific receptors (Walker et al., 2013). ILCs are grouped into three groups which arise from a common precursor and have distinct similarities to the three major T helper cell subsets, T_H1 , T_H2 and T_H17 . Natural Killer cells are also often grouped with type I ILCs, however lineage studies suggest that they arise from a different precursor so NK cells are discussed separately below (Klose et al., 2014). ILCs are commonly found at barrier surfaces (Diefenbach, 2013), all three subgroups express IL-7R and Kit (Klose et al., 2014) as well as a specific, lineage defining transcription factor. Group 1 ILCs express T-bet transcription factor, produce type-I cytokines such as IFN- γ and TNF, associated with resolving infections. Group 2 ILCs express GATA-3 transcription factor, produce type-2 cytokines such as IL-4, IL-5, IL-9 and IL-13 and are associated with helminth infections and type-2 diseases such as asthma and allergy (Neill et al., 2010; Nussbaum et al., 2013). Group 3 ILCs express ROR γ t transcription factor, produce type-17 cytokines such as IL-17 and IL-22 as well as IFN- γ and TNF (Diefenbach, 2013). ILC3s are found particularly in the intestinal wall and are thought to be involved in epithelial barrier function. A subsets of ILC3s, LTi cells are involved in embryonic development of lymphoid organs (Killig et al., 2014).

1.1.7 Natural Killer cells

Natural killer (NK) target and kill infected cells and tumour cells and are functionally analogous to cytotoxic T cells, however they do not express antigen receptors. NK cells can distinguish infected or tumorigenic cells from healthy cells and limit the spread of the infection and tissue damage. NK cells recognise cell surface 'distress' signals, for example via the NK cell surface receptor NKG2 (Lanier, 2005).

Additionally, NK cells express a number of receptors that are inhibited by MHCI, therefore T cell evasive viruses such as herpes viruses which downregulate MHCI actually flag-up infected cells for recognition by NK cells (Parham and Moffett, 2013). MHCI recognition is also involved in detection of malignant cells which often lack expression of MHCI or express alternative forms of MHCI (Ljunggren and Kärre, 1990). NK cells express several Toll like receptors (TLRs) and recognition via TLRs induces IFN- γ production and increased cytotoxicity (Sivori et al., 2004). NK cells are widespread throughout tissues though they only represent a small fraction of the total number of lymphocytes. There are three distinct subsets of NK cells in mouse (Hayakawa and Smyth, 2006) and two subsets in human (Cooper et al., 2001), and these differ in tissue distribution and cell surface marker expression. NK cell deficiency results in severe immunodeficiency and susceptibility to a range of viruses (Orange, 2013).

1.2 Recognition of PAMPs/DAMPs

The innate immune system relies on a system of germline-encoded pattern recognition receptors (PRRs) to recognise and respond to foreign particles (table 1.1). PRRs recognise conserved molecular patterns found on microorganisms (pathogen associated molecular patterns (PAMPs)) and can also recognise endogenous molecules associated with tissue damage (damage associated molecular patterns (DAMPs)) (Takeuchi and Akira, 2010).

Table 1.1 Innate immune receptors in mice

Receptor	Ligand	Effect	References
Membrane Bound PRRs			
TLR1/2	Triacetylated lipoproteins	Proinflammatory cytokines	(Farhat et al., 2008)
	Peptidoglycans lipopolysaccharides		
TLR2	Bacterial lipopeptides	Proinflammatory cytokines	(Farhat et al., 2008)
TLR3	Viral dsDNA	Type I interferons	(Alexopoulou et al., 2001)
TLR4	LPS	Proinflammatory cytokines	(Poltorak et al., 1998)
TLR5	Flagellin	Proinflammatory cytokines	(Hayashi et al., 2001)

TLR6/2	Bacterial lipopeptides	Proinflammatory cytokines	(Farhat et al., 2008)
TLR7	ssRNA	Type I interferons	(Hemmi et al., 2002)
TLR9	CpG DNA	Proinflammatory cytokines and type I interferon	(Hemmi et al., 2000)
TLR10 (non-functional pseudogene in mice)	Viral infection	Proinflammatory/antiinflammatory	(Lee et al., 2014) (Oosting et al., 2014)
TLR11	Profilin-like protein	IL-12, proinflammatory cytokines	(Yarovinsky et al., 2005)
TLR12	Profilin	IL-12, IFN- α , IFN- γ	(Koblansky et al.)

TLR13	23S ribosomal RNA	IL-12, IL-6	(Oldenburg et al., 2012)
Cytosolic PRRs			
RIG-I	5' triphosphate RNA Long dsRNA	Type I interferon, proinflammatory cytokines	(Schlee et al., 2009)
MDA-5	Long dsRNA	Type I interferon	(Feng et al.)
LGP2	dsRNA	Type I interferon	(Li et al., 2009)
NOD1	Peptidoglycan motif – iE- DAP	Proinflammatory cytokines	(Strober et al., 2006)

NOD2	Peptidoglycan motif – MDP	Proinflammatory cytokines	(Strober et al., 2006)
Dectin-1	B(1-3)- and β (1-6)-linked glucans	Antifungal immunity	(Brown, 2006; Brown and Gordon, 2001)
Dectin-2	α -mannans	Antifungal immunity	(Saijo and Iwakura, 2011)
MINCLE	Mycobacterial glycolipid (TDM/cord factor)	Inflammatory cytokines and nitric oxide	(Ishikawa et al., 2009a)
Pyrin	Pathogen modified and inactivated Rho GTPases	Caspase 1 inflammasome activation	(Xu et al., 2014)
NLRP3	Various stimuli	Inflammasome assembly IL-1 β and IL-18	(Baroja-Mazo et al., 2014)

AIM2	dsDNA	Inflammasome assembly IL-1 β and IL-18	(Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Jones et al., 2010)
STING	CDNs	Type I IFN	(Hervas-Stubbs et al., 2014; Ishikawa and Barber, 2008; Ishikawa et al., 2009b; Zhong et al., 2008)
DAI	DNA	RIP3-mediated Necroptosis STING-mediated type I interferon	(Takaoka et al., 2007; Upton et al., 2012)
DDX41	B-DNA and CDNs	STING-mediated type I IFN	(Parvatiyar et al., 2012; Zhang et al., 2011c)
DHX9	DNA, CpG-ODNs	MyD88-mediated Pro-inflammatory response	(Kim et al., 2010b; Zhang et al., 2011b)
DHX36	DNA, CpG-ODNs	MyD88-mediated type I interferon	(Kim et al., 2010b; Zhang et al., 2011b)

Ku70	DNA	CDN synthesis Type III IFN	(Zhang et al., 2011a)
cGAS	DNA	CDN synthesis STING mediated type I interferon	(Li et al., 2013; Sun et al., 2013; Wu et al., 2013)
IFI16	dsDNA	STING-mediated type I interferon	(Kis-Toth et al., 2011; Unterholzner et al., 2010)
LRRFIP1	B-DNA, Z-DNA	B-catenin-mediated type I interferon	(Yang et al., 2010)
RNA polymerase III	Detects cytoplasmic DNA and transcribes to RNA	RIG-I mediated type I interferons	(Ablasser et al., 2009; Chiu et al., 2009)

There are two major classes of PRRs; membrane-bound receptors such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) and cytosolic receptors such as RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and pyrin and HIN domain-containing (PYHIN) receptors. An ever increasing number of cytosolic nucleic acid sensors falls into the latter class. This thesis focuses on the response to nucleic acids via TLR9 with some reference to cytosolic DNA sensing, however chapter 3 introduces the relevant PRRs in more detail so this section introduces general features of TLRs and cytosolic PRRs.

1.2.1 Membrane bound PRRs

Membrane bound PRRs are either located at the cell surface in order to detect extracellular stimuli, or on the endosomal membrane to detect stimuli in the lumen of the endosome taken up by endocytosis.

1.2.1.1 Toll-like receptors

TLRs were the first group of PRRs to be identified and their role in immune signalling was revealed by *TLR4* mutations in mice demonstrating that it is essential for the response to bacterial ligand lipopolysaccharide (LPS), and for preventing bacterial sepsis (Poltorak et al., 1998). Gene targeting of other TLR genes has shown that each TLR recognises a specific molecule, or group of molecules, and activates distinct, though overlapping, inflammatory responses. TLRs are named for their homology to the Toll pathway in *Drosophila*, although Toll is not directly activated by PAMPs, it is still activated in response to bacterial, fungal and viral infection (Ferrandon et al., 2007).

TLRs are type I transmembrane proteins which recognise ligands through the N-terminal ectodomain (ECD) which faces either the extracellular space for TLR -1, -2, -4, -5, -6, -10 and (-11, mouse only) at the cell surface, or the lumen of the endosome for TLR -3, -7/-8 and -9. Following ligand binding, TLRs recruit Toll-IL-1 and IL-18 receptor (TIR) domain-containing adapter proteins to a cytoplasmic signalling domain which initiates signalling pathways leading to transcription factor activation. The TLR ECD contains leucine-rich repeat (LRR) regions (Bell et al., 2003) and many TLR ECDs contain insertions which are thought to be important for recognition of specific patterns or for binding of accessory molecules. The crystal

structures of the TLR3 ECD (Bell et al., 2005; Choe et al., 2005), and subsequently the structures of other TLR-ligand complexes provided insights into how TLRs bind specific ligands via a large number of LRR loops and how they transduce the signal across the membrane to an intercellular adapter protein (Jin et al., 2007; Kim et al., 2007; Liu et al., 2008). The structure of the TLR1-TLR2-ligand complex showed that whilst TLR1 and TLR2 exist as monomers in the absence of the bacterial cell wall derivative lipoprotein, in the presence of lipoprotein they form a heterodimer and the ECDs form a continuous hydrophobic pocket where lipoprotein is bound (Jin et al., 2007). A single nucleotide polymorphism in TLR1 which disrupts protein interactions with TLR2 impairs the response to lipoprotein indicating that heterodimerisation of TLR1 and TLR2 is essential for receptor function (Omuetti et al., 2007). TLRs form homodimers upon activation, with the exception of TLR1 and 6 which form heterodimers with TLR2, and TLR dimerisation is thought to be required to provide a scaffold for adapter protein recruitment (Nunez Miguel et al., 2007; O'Neill et al., 2003). TLR3 has a minor protein interaction domain suggesting that ligand binding is required to stabilise the TLR3 dimer (Jin and Lee, 2008). This is supported by evidence that an oligonucleotide with a minimum of 40-50 base pairs was required for stable receptor ligand interactions as this size spans the distance between the individual TLR3 proteins and high molecular weight RNA induces IFN- β expression via TLR3 more effectively than low molecular weight RNA in various cell types (Leonard et al., 2008; Zhou et al., 2013a). However shorter dsRNA, such as siRNA, can also activate TLR3 signalling, indicating that TLR3 ligand specificity is more complex than solely ligand size (Cho et al., 2009). TLR4 is unique in requiring a co-receptor, MD-2 (lymphocyte antigen 96), which is thought to be a mechanism to deal with the extreme variability of the structure of LPS even within an individual bacterium (Jin and Lee, 2008). The crystal structure of the TLR4 and MD2 in complex with LPS-mimic eritoran showed that when eritoran binds to MD-2 there is no direct interaction between eritoran and TLR4. The structure of TLR4-MD-2 with LPS has not been solved, however binding of LPS to TLR4-MD-2 induces dimerization or aggregation and modelling suggests that this is by altering the conformation of MD-2, exposing previously hidden residues which interact with other TLR4 molecules (Kim et al., 2007; Kobayashi et al., 2006). TLR4 signalling

requires an additional co-receptor: CD14 (cluster of differentiation 14), which is also involved in signalling via TLR1/2, and can act as a co-receptor for TLR3, 6, 7, 8 and 9 (Akashi-Takamura and Miyake, 2008; Baumann et al., 2010). CD14 binds LPS even at extremely low concentrations and transfers it to the TLR4-MD2 complex and whilst it is dispensable for MyD88-mediated TLR4 signalling, it is essential for endosomal TRIF/TRAM-mediated TLR4 signalling (Jiang et al., 2005). The absolute requirement for CD14 in TRIF/TRAM-mediated TLR4 signalling is due to its role in mediating the internalisation of the LPS-receptor complex to the endosome (Zanoni et al., 2011).

There is a distinction in the structure of TLRs, determined by sequence and structure analysis, which corresponds to the type of ligand that they recognise. TLR1, 2, 4, 6 and 10 belong to the three domain subfamily, the LRR domain of these TLRs can be divided into three subdomains with distinct structures (Jin et al., 2007; Jin and Lee, 2008; Kim et al., 2007), and they bind hydrophobic ligands via hydrophobic interactions. TLR3, 5, 7, 8 and 9 all have a single domain in their LRR and bind hydrophilic proteins or nucleic acids via ionic and hydrogen bonds (Liu et al., 2008). In spite of the differences between the domain subfamilies, the structures that have been solved or modelled so far demonstrate a common 'm'-shaped dimeric structure suggesting that all TLRs dimerise to form similar structures. Homology modelling using the crystal structures of human TLR1, 2, 3, 4 and mouse TLR3, 4 as template structures to predict the structure of TLR7, 8 and 9 and ligand binding dynamics predicted that these TLRs also form dimers and the ectodomains bind to opposite sides of the ligand (Wei et al., 2009). Even without their ligands, TLRs have been shown to exist in homo- and hetero multimers, however these structures do not induce signalling (Latz et al., 2007; Triantafilou et al., 2006). Only a small number of residues act as flexible linkers between the ECD, the transmembrane domain and the cytoplasmic domain (Bell et al., 2003). This suggests that ligand binding may be required to stabilise the conformation of the ECD, and this stabilised conformation brings the cytoplasmic domains into apposition and leads to recruitment of adapter proteins.

The endosomal TLRs, with the exception of TLR3, have a longer amino acid sequence than other TLRs. The ectodomain of TLR7 and TLR9 has to be cleaved in the endolysosome to allow adapter protein activation on ligand binding. This spatial restriction mechanism has possibly evolved to limit activation of these TLRs to the endolysosome, thereby preventing activation by self-nucleic acids for example DNA contained in autoantibody complexes that are characteristically found in SLE (Ewald et al., 2008).

Ligand activated TLRs recruit TIR-domain containing adapter proteins to the cytoplasmic domain and activate distinct signalling pathways (figure 1.4). All TLRs, with the exception of TLR3, require MyD88 (Myeloid differentiation primary response gene 88) as the adapter protein, whilst TLR3 and TLR4 both use TRIF (TIR-domain-containing adapter-inducing interferon- β), also known as TICAM1 (TLR adapter molecule 1). TLR4 is the only TLR to use both MyD88 and TRIF; initially recruiting MyD88 at the cell surface and subsequently undergoing endocytosis and recruiting TRIF from the endolysosome. The recruitment of MyD88 and TRIF to TLR4 also requires the sorting adapters TIRAP (toll-interleukin 1 receptor (TIR) domain containing adaptor protein, also known as Mal) and TRAM (TRIF related adapter molecule, also known as TICAM2) respectively (Kagan and Medzhitov, 2006; Rowe et al., 2006). These adapters localise to specific cellular compartments by virtue of the phosphoinositide composition of particular membranes, when the phosphatidylinositol-4,5-bisphosphate (PIP2) binding domain of TIRAP was mutated, TIRAP was no longer recruited to the plasma membrane and LPS-induced signalling was decreased (Kagan and Medzhitov, 2006).

Recruitment of MyD88 to an activated TLR leads to the assembly of a number of effectors and formation of a complex referred to as the Myddosome (Gay et al., 2011). MyD88 recruits IL-1 receptor associated kinases (IRAKs) 1, 2 and 4 via an activated death domain (DD) associating with the death domain of IRAK4, forming stable complexes with a specific stoichiometry (Motshwene et al., 2009). The assembly of these proteins into a complex leads to IRAK4-mediated sequential phosphorylation and activation of IRAK1 and IRAK2 (Kawagoe et al., 2008). Activated IRAKs interact with TNF-receptor associated factor 6 (TRAF6), a RING-

dependent E3 ubiquitin ligase which in complex with UB13 and Uev1A polyubiquitinates target proteins through K63 linked ubiquitination (Deng et al.; Wang et al., 2001). K63-ubiquitinated TAK1 recruits TAB2 and TAB3, the regulatory components of the complex, via zinc-finger-like ubiquitin-binding domains (Adhikari et al., 2007; Sorrentino et al., 2008). NEMO, the regulatory component of the IKK complex, can recognise K63-ubiquitinated proteins and also requires K63-linked ubiquitination for activation (Ea et al., 2006; Tang et al., 2003; Wu et al., 2006). TAK1 and IKK form a complex in which TAK1 phosphorylates IKK β (Wang et al., 2001). Phosphorylation of IKK β leads to phosphorylation of the NF- κ B inhibitor I κ B α which is then polyubiquitinated through K48-linked chains and degraded. This releases NF- κ B, which translocates to the nucleus and activates transcription of genes (Karin and Ben-Neriah, 2000). Activated TAK1 simultaneously phosphorylates and activates the MAP kinases, Erk1, Erk2, p38 and Jnk, which then activate various other additional transcription factors including AP-1 (Wang et al., 2001).

Whilst the MyD88 leads to the activation of NF- κ B, with the exception of particular pDC-specific pathways (chapter 3), the TRIF pathway results in the activation of both NF- κ B and IRFs, leading to production of type I interferons. TRIF activates TAK1 via TRAF6 in a similar way to that seen for MyD88 dependent signalling. TRIF also recruits RIP1 which is ubiquitinated by pellino-1, this complex activates TAK1 leading to activation of NF- κ B and MAPK pathways (Chang et al., 2009; Ermolaeva et al., 2008; Pobeziinskaya et al., 2008). To activate IRF3 and 7, TRIF recruits a complex involving TRAF3, TANK, TANK-binding kinase 1 (TBK1) and IKKi (IKK ϵ), which phosphorylates IRF-3 and IRF-7 leading to their homo- or hetero-dimerization and translocation to the nucleus (Fitzgerald et al., 2003; Hiscott et al., 1999; McWhirter et al., 2004; Sharma et al., 2003). The activation of IRFs by TRIF is dependent on the E3 ubiquitin ligase, TRAF3 (Hacker et al., 2006; Oganessian et al., 2006; Perez de Diego et al., 2010), and although a target of TRAF3 mediated ubiquitination has not been identified, a region of TRIF has been identified containing a non-canonical TRAF binding site which may be involved in modulating TRIF ubiquitination (Nguyen et al., 2014).

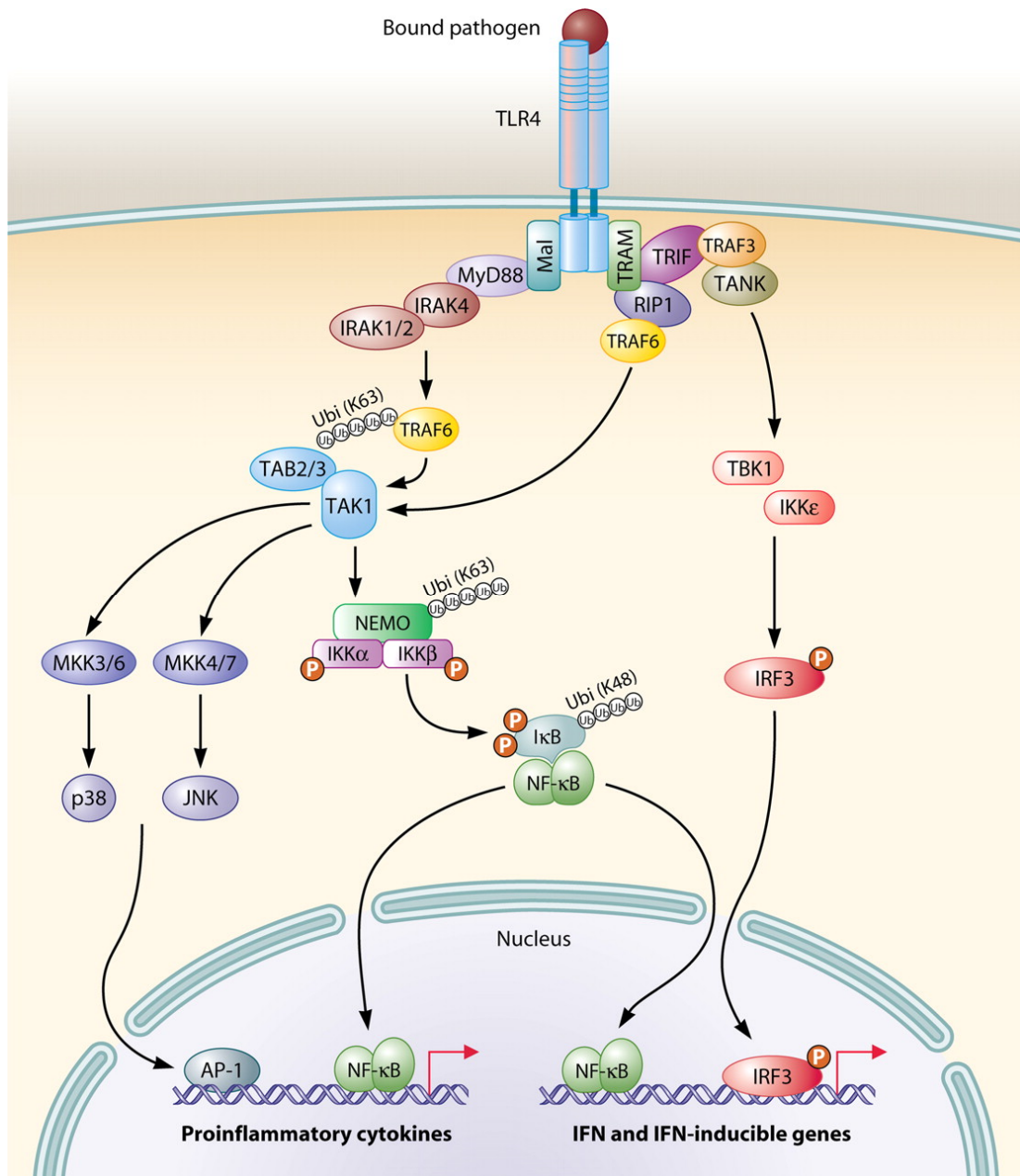


Figure 1.4 A schematic of TLR signalling pathways using TLR4 signalling to illustrate the MyD88- and TRIF-dependent pathways. (Mogensen, 2009).

1.2.1.2 C-type lectin receptors

C-type lectin receptors (CLRs) detect pathogenic carbohydrate structures such as mannose, fucose and glucan via calcium dependent carbohydrate recognition domains (CRDs) (Geijtenbeek and Gringhuis, 2009). There are seven membrane bound CLRs that are grouped into two families based on their molecular structure; type I containing multiple CRDs and type II containing a single CRD. Additionally there is a single soluble CLR, mannan binding lectin (MBL), which forms oligomers and leads to activation of the complement pathway (van Asbeck et al., 2008). CLRs are expressed by many cells types and recognition by CLRs leads to internalisation and degradation of the pathogen and presentation of antigen.

1.2.2 Cytosolic PRRs

Cytosolic PRRs exist to detect PAMPs or DAMPs that are present in the cytoplasm. The replication cycle of most RNA viruses and some DNA viruses involves cytoplasmic genome replication and transcription (den Boon et al., 2010). DNA in the cytoplasm can also be a sign of a bacterial infection or of cell damage, therefore it is detected as a damage signal by an ever increasing number of cytosolic receptors.

1.2.2.1 RIG-I like receptors (RLRs)

Retinoic-acid inducible gene-I (RIG-I) like receptors (RLRs), RIG-I (encoded by *DDX58*) and MDA-5 (Melanoma differentiation-associated gene 5, encoded by *IFIH1*), are DExD/H box RNA helicases which bind to double stranded RNA (dsRNA) via an ATP-dependent mechanism. RLRs contain a caspase activation and recruitment domain (CARD) which transduces a signal to activate NF- κ B, IRF7 and IRF3 (Yoneyama et al., 2004). LGP2 is a related RLR but lacks the CARD domain indicating that LGP-2 is a regulator rather than an effector which is supported by evidence that LGP2 interferes with activation of RIG-I and MDA-5, (Cui et al., 2001; Saito et al., 2007; Yoneyama et al., 2005). RIG-I and MDA-5 both bind the synthetic dsRNA ligand poly inosine:cytosine (poly(I:C)) but have different specificities towards different viruses (Kato et al., 2006; Loo et al., 2008; Sumpter et al., 2005). RIG-I also activates signalling in response to RNA products which have been reverse transcribed by cytoplasmic RNA polymerase III, indicating that it can act as an indirect sensor of cytoplasmic DNA (Ablasser et al., 2009; Chiu et al.). A

RIG-I-dependent response has recently been demonstrated to 5'diphosphate containing RNA (5'pp-RNA) which is found in specific viruses but not in uninfected mammalian cells (Goubau et al., 2014). RIG-I and MDA-5 both require the mitochondrially localised adapter IPS-1 (MAVS/CARDIF/VISA) to transduce the signal (Kawai et al., 2005; Kumar et al., 2006; Meylan et al., 2005; Seth et al.; Xu et al.). Association of the RLR with a ligand induces conformational changes which allow IPS-1 to bind to the CARD domain via homotypic interactions. Bound IPS-1 induces the assembly of a number of proteins including FADD and RIP1 leading to the activation of IRFs 3 and 7 by phosphorylation and the activation of NF- κ B via IKK (Kawai et al., 2005).

1.2.2.2 NOD-like receptors (NLRs)

Nucleotide oligomerisation domain (NOD) like receptors (NLRs) are a large family of receptors, 22 in humans and 33 in mice, which have a characteristic tripartite structure including a protein-protein interaction domain; a central nucleotide-binding oligomerisation domain (NOD); and a C terminal LRR domain which detects PAMPs. NLRs can be divided into four subfamilies on the basis of the protein-protein interaction domain: NLRC – containing a CARD motif, NLRP – containing a pyrin domain, NLRA – containing an acidic transactivating domain or NLRB – containing a baculovirus inhibitor domain. Alternatively they can be separated into three subfamilies on the basis of phylogenetic and structural relationships: NODs, NLRPs (NALPs) and IPAFs (Schroder and Tschopp, 2010). NLRs assemble into one class of caspase-1-activating complexes called inflammasomes and mediate maturation of IL-1 β and IL-18 in response to a wide range of PAMPs and danger signals (Lu et al., 2014). AIM2 is the only non-NLR that mediates inflammasome formation, and is explored in more detail in chapter 3 in the context of the inflammatory response to cytosolic DNA. NLRs and AIM2 recruit the adapter protein ASC (apoptosis-associated speck-like protein containing a CARD) which contains a pyrin domain and a CARD domain, via pyrin domain homotypic interactions (Masumoto et al., 1999). ASC, in turn, recruits caspase-1 through CARD homotypic interactions (Lu et al., 2014). The assembled inflammasome complex is a filamentous assembly involving stepwise nucleation-induced polymerisation with

similar features to prion polymerisation (Cai et al., 2014; Lu et al., 2014; Ruland, 2014).

1.2.2.3 Cytosolic nucleic acid sensors

TLR9 was identified as a receptor for unmethylated CpG motifs found in microbial DNA (Hemmi et al., 2000), however knock-out of TLR9 did not abolish the response to intracellularly delivered DNA leading to investigations into alternative recognition mechanisms and the identification of cytosolic nucleic acid receptors (Ishii et al., 2006). Eleven intracellular DNA receptors have been described so far the majority of which activate type I interferon signalling. Cytosolic nucleic acid receptors are examined in more detail in chapter 3.

1.3 Transcription factor activation

The major transcription factor families that are activated by the innate immune response are NF- κ B and IRF. Other transcription factors that are activated by innate immune signalling include: AP-1, a heterodimer consisting of c-jun and c-fos which is activated by the MAPK pathway; STATs 1, 2, 3, 4, 5 and 6 which can form homodimers or heterodimers in response to interferon signalling (Decker et al., 2002; Stark and Darnell Jr, 2012); XBP1 which is activated in macrophages in response to TLR signalling (Martinon et al., 2010) and CREB which is activated by a range of inflammatory stimuli (Wen et al., 2010).

1.3.1. NF- κ B

The NF- κ B is a ubiquitous transcription factor with a key role in regulation of transcription following immune activation. The NF- κ B family is composed of five proteins: p65 (RelA), RelB, c-Rel, p105/p50 (NF- κ B1) and p100/p52 (NF- κ B2) which form homo- and heterodimers and bind to DNA via a Rel-homology domain (RHD). Of the fifteen possible NF- κ B dimers, twelve have established DNA binding and transcriptional activity (Hoffmann and Baltimore, 2006) (figure 1.5). Whilst the considerable redundancy in dimer binding makes specificity difficult to study, particular dimers are known to regulate expression of specific target genes (Fusco et al., 2009; Siggers et al., 2012).

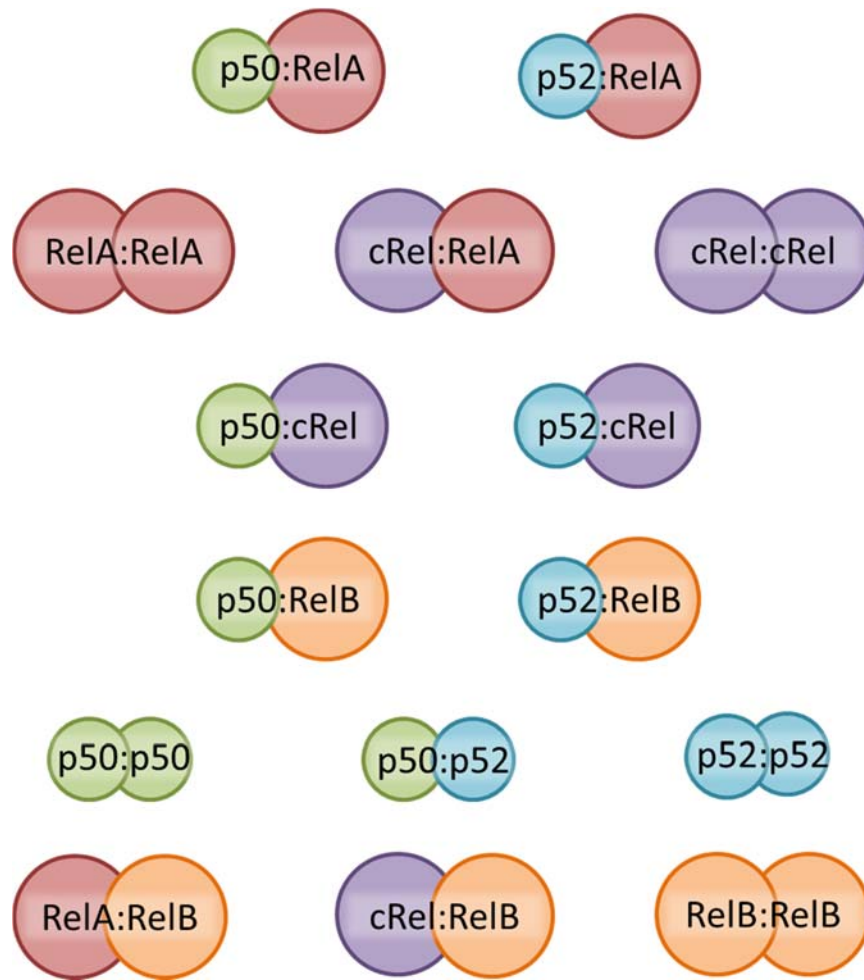


Figure 1.5 The twelve different combinations of functional NF- κ B dimers likely provide an additional level of regulation of transcription (Hoffmann and Baltimore, 2006)

The two best characterised dimers are the classical inflammatory mediator, p50:RelA, and the non-canonical p52:RelB dimer. The p50:RelA dimer is held in the cytoplasm by I κ B proteins which, upon activation of inflammatory pathways, are degraded allowing p50:Rel to translocate to the nucleus (Ghosh and Karin, 2002). In contrast p52:RelB is activated more slowly as it requires proteolytic processing of p52 from p100 (Xiao et al., 2001). Activation of canonical NF- κ B signalling by immune stimuli leads to expression of inflammatory cytokines, immune receptors, chemokines and adhesion molecules.

1.3.2. Interferon Regulatory Factors

The Interferon Regulatory Factor (IRF) family consists of nine identified proteins with a common helix-turn-helix DNA binding motif (Taniguchi et al., 2001). In addition, virally encoded IRFs (vIRF) have been identified which have a competitive negative effect on host IRF function preventing a robust antiviral response (Burýšek et al., 1999). The particular IRF family member or members that are activated depends on the type of response that has been initiated. Four of the nine IRF family members have been implicated in positive regulation of type I interferon genes; IRF1, IRF3, IRF5 and IRF7 (Honda and Taniguchi, 2006; Mamane et al., 1999; Taniguchi et al., 2001). IRF3 and IRF7 are highly similar in coding sequence and structure but differ in their expression, IRF3 is constitutively expressed whilst IRF7 is inducible via activation of the type 1 interferon receptor (IFNAR) (Marie et al., 1998; Sato et al., 1998). IRF3 only activates transcription of the *IFNB* gene whilst IRF7 activates both *IFNA* and *IFNB*, hence differential responses are induced depending on whether IRF7 is activated (Sato et al., 2000). For example, TLR4/TRIF mediated responses only activate IRF3, therefore only inducing an IFN- β response, whilst cytosolic PRRs predominantly activate IRF7 to induce both IFN- β and IFN- α (Honda and Taniguchi, 2006; Honda et al., 2005). IRFs bind to a conserved consensus sequence, the ‘interferon-stimulated response element’ (ISRE; ^A/GNGAAANNGAAACT) in the promoter region of interferon stimulated genes (ISGs) (Pine et al., 1990; Rusinova et al., 2013).

Many transcriptional events require synergy between different transcription factors, for example combinatorial binding of NF- κ B, ATF-2, c-jun, IRF3 and IRF7 is involved at the *IFN- β* promoter (Yang et al., 2004). This acts as an additional level of regulation to modulate the magnitude of an immune response, preventing an inappropriate response.

1.4 Cytokines

‘Cytokine’ is a broad ranging term for small molecules that are involved in both intracellular and intercellular cell signalling, including interleukins, tumour necrosis factor, chemokines and interferons. Cytokines are produced by immune cells as well as non-immune cells and act via receptors to affect the proliferation, movement and

response of cell populations. Cytokines can additionally have direct effects on pathogens.

1.4.1 Interleukins

The interleukin prefix (IL) is given to a large family of cytokines that was originally named as they were thought to be produced solely by leukocytes. To date more than forty interleukins have been identified and mouse models and human disease have provided insights in their function. Interleukins are reviewed in detail by (Akdis et al., 2011) and IL-1 β and IL-18 are introduced in more detail in chapter 3 in the context of inflammasome activation. The T helper 17 (T_H17) related cytokines IL-17, IL-22 and IL-23, produced by T_H17 cells, are key mediators of chronic tissue inflammation. T_H17 cells and T_H17-type cytokines drive tissue-specific autoimmune diseases such as multiple sclerosis, psoriasis, type I diabetes, rheumatoid arthritis and Crohn's disease (Qu et al., 2013). IL-22 levels have been correlated with psoriasis severity and can inhibit keratinocyte terminal differentiation, a hallmark of psoriasis (Hao, 2014). Induction of experimental autoimmune encephalomyelitis (EAE) in IL-17 deficient mice showed that whilst IL-17 was not necessary for development of EAE, the disease was less severe in the absence of IL-17 (Komiyama et al., 2006). Preliminary results from a trial using anti-IL-17 antibody in MS patients has demonstrated a reduction in formation of new lesions, suggesting that IL-17 could be a potential target for T_H17 driven disease (EudraCT number 2009-011626-34 (Baeten and Kuchroo, 2013)). IL-6 dysregulation has also been implicated in several disease processes, including autoimmune disease (Ishihara and Hirano, 2002). IL-6 is required for experimental induction of EAE (Eugster et al., 1998; Samoilova et al., 1998) and production of autoantibodies following pristane injection to induce a lupus-like disease (Richards et al., 1999).

IL-6 is commonly assayed as a measure of inflammation induced *in vitro* or *in vivo* although it can act as both a pro-inflammatory cytokine and an anti-inflammatory cytokine (Scheller et al., 2011). The induction of pro-inflammatory cytokines including IL-6 by dendritic cells is examined in more detail in chapter 3. Briefly however, production of IL-6 is mediated by NF- κ B and IRF-5 activation via a MyD88-dependent pathway from TLR9 in the late endosome (Guiducci et al., 2006).

An IL-6 deficient mouse model shows defects in the response to various pathogens and in the inflammatory response to tissue damage, demonstrating a central role for IL-6 in inflammation. Additionally, IL-6 deficient mice exhibit impaired neuronal repair, weight regulation and liver regeneration demonstrating the pleiotropic nature of this cytokine (Poli and Maritano, 2003). Dysfunction of interleukins and interleukin receptors are associated with various human autoinflammatory conditions for example; cryopyrin-associated periodic syndrome caused by dysregulation of IL-1 β production due to mutations in *NLRP3* and autoinflammatory condition of the skin and bones caused by deficiency in the interleukin-1 receptor antagonist (Aksentijevich et al., 2009; Jesus and Goldbach-Mansky, 2014).

1.4.2 Tumour necrosis factors

Tumour necrosis factors (TNFs) are a family of nineteen cytokines that can cause apoptosis in addition to affecting multiple other pathways. The best characterised member, TNF- α , is produced by a wide range of cell types and TNF- α antagonists such as rituximab, adalimumab, infliximab etc., are widely used to treat conditions with underlying inflammation such as IBD, psoriasis and rheumatoid arthritis (Ma and Xu, 2013; Pache et al., 2009; Tobin and Kirby, 2005).

1.4.3 Chemokines

Chemokines mediate chemoattraction, hence the derivation from **chemotactic cytokines**, and signal via the G-protein coupled transmembrane receptor superfamily (Harrison, 2010). Chemokines are characterised by four conserved cysteine residues and further grouped into four families, the majority fall into either the C-C or the C-X-C family which predominantly target monocytes and T cells or neutrophils, respectively (Borish and Steinke, 2003). Whilst there are a large number of chemokines (more than forty) there are fewer chemokine receptors (twenty identified), more than one of which can be expressed on a given cell type, demonstrating considerable overlap and redundancy (Pease and Williams, 2006). Additionally there is considerable promiscuity by the chemokines as to which receptor they bind to (Horuk, 2009). Activation of G-protein receptors by chemokines stimulates in a signalling cascade which culminates in integrin up-regulation and a switch from transient 'rolling adhesion' to strong adhesion of

leukocytes to blood vessel walls (Constantin et al., 2000). CXCL8 (IL-8) is the most potent chemoattractant for polymorphonuclear cells, particularly neutrophils, and is produced by a wide range of cell types in response to LPS, IL-1, TNF- α and viruses (Hoffmann et al., 2002a). Rodents lack a CXCL8 homologue, however they have other chemokines which act through the same receptors (CXCR1 and CXCR2) (Russo et al., 2014).

1.4.4 Interferons

Interferon- α (IFN- α) was the first cytokine to be identified as an antiviral molecule, and it has since been established that the IFN family of cytokines is primarily involved in the antiviral response. IFNs can directly inhibit viral replication within infected cells and activated cytotoxic T cells (Castet et al., 2002; Guidotti et al., 1996; Robek et al., 2002). There are three families of interferon: type I, type II and type III characterised by binding to the IFN- α receptor (IFNAR), the IFN- γ receptor (IFNGR) or the IFNLR1/IL10R2 complex, respectively. Type I IFNs that are expressed in humans and mice include IFN- α (which can be subdivided into thirteen subclasses), IFN- β , IFN- ϵ , IFN- κ and IFN- ω (Pestka et al., 2004). Type I interferon binding to IFNAR induces STAT1/2 activation and heterodimerisation, this complex binds to IRF-9 and induces transcription of genes with an interferon-stimulatory response element (ISRE) (Platanias, 2005). Type I interferon production leads to expression of an interferon gene signature (ISG) which is associated with infection as well as active autoimmune disease (Häupl et al., 2011; Ronnblom and Eloranta, 2013). Type I interferon production in the context of TLR9 and cytoplasmic receptor signalling in dendritic cells is explored further in chapter 3. The amplification of the IFN response by autocrine and paracrine signalling allows a rapid localised response, however aberrant signalling in autoimmune disorders can be extremely severe.

1.5 Costimulatory markers on antigen presenting cells

In addition to production of soluble cytokines, mature antigen presenting cells (APCs) express costimulatory markers at the cell surface. T cells and B cells require not only antigen presentation on MHC molecules but also a second non-specific signal, mediated by costimulatory markers which are expressed by both APCs and T- and B-cells (Harris and Ronchese, 1999). Mature DCs express low levels of CD40

(cluster of differentiation 40) in an inactivate state, however increased CD40 expression is measured as a marker activation of DCs by pathogenic molecules or on uptake of apoptotic cells (Banchereau et al., 1994). Activated DCs also up-regulate expression of CD80 and CD86 which work in tandem interacting with T-cell CD28 and CTLA-4. The interaction of costimulatory molecules and MHC:peptide complexes on the DC with CD4⁺ T cells in the lymph node is necessary for the formation of an immunological synapse, a highly stable, specialised interface which is involved in T cell priming (figure 1.6) (Rodríguez-Fernández et al., 2010; Wetzel et al., 2002)

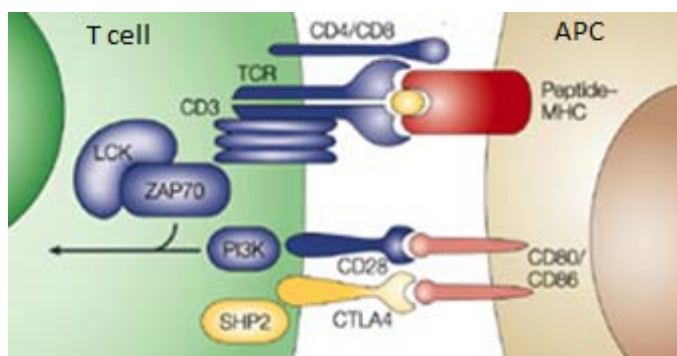


Figure 1.6 Formation of the immunological synapse (IS) between T cells and APCs. Formation of the IS requires interactions between CD80/CD86 and CD28/CTLA-4 and between the peptide:MHC complex and the T cell receptor (TCR)/CD3 complex. Adapted from (Karatas et al., 2014)

Engagement of the T cell antigen receptor (TCR) by the peptide:MHC complex and ligation of CD28 by CD80 or CD86 leads to activation of NF- κ B mediated prosurvival and proliferation pathways in the T cells (Acuto and Michel, 2003). Presentation of antigen by a mature APC leads to T cell activation stimulating the T cell to proliferate and differentiate into an effector cell via an IL-2 dependent mechanism. Activated T cells express CTLA-4 which binds to the B7 proteins (CD80/CD86) with higher affinity than CD28 and acts as an inhibitory mechanism to prevent accumulation of excessive activated T cells (Chambers et al., 2001; Tivol et al., 1995).

Activated T cells differentiate into T_H1 , T_H2 or T_H17 cells, depending on the stimulus (Mosmann and Coffman, 1989). A T_H1 response is directed against intracellular pathogens, triggering macrophages to kill phagocytosed pathogens and activating cytotoxic T cells to destroy infected cells. A T_H2 response, on the other hand, is directed against removing extracellular pathogens by stimulating B cells to produce antibodies and inducing cells to release mediators to expel microbes. The function of T_H17 cells is not entirely clear but they appear to be involved in clearance of infections when T_H1 and T_H2 responses have been ineffective. T_H17 cells produce large amounts of IL-17, IL-22 and IL-23 which induce extensive inflammation due to the wide ranging expression of IL-17 and IL-22 receptors on many cells types and the activation of immune cells by IL-23. The potent tissue inflammation induced by T_H17 cells is associated with the pathogenesis of severe autoimmune disorders (Korn et al., 2009).

1.6 Regulation of the innate immune system

This section discusses mechanism of both positive and negative regulation of the innate immune system, using some specific examples to illustrate points. The innate immune response is highly regulated at every level including transcriptional regulation via feedback, post-transcriptional regulation and post-translational regulation. The following section uses examples to illustrate the different regulatory mechanisms, and how dysregulation of pathways in human disease can aid our understanding of these processes.

1.6.1 Feedback regulation

Unchecked activation of the immune system rapidly amplifies into an excessive response leading to tissue damage and possibly chronic inflammation. To prevent excessive inflammation negative feedback mechanisms are activated alongside pro-inflammatory signalling pathways. The transcription factors that are involved in expression of pro-inflammatory cytokines following PRR activation, including NF- κ B and IRFs, also activate transcription of inhibitors. For example, micro RNA-132 (miR132), which is expressed as part of the early antiviral response to viruses, and inhibits expression of p300 which functions as a transcriptional co-activator in the antiviral response (Lagos et al., 2010). Another example is the increased expression

of the NF- κ B pathway inhibitor A20 (Tumour necrosis factor alpha-induced protein 3, TNFAIP3) following NF- κ B activation (Krikos et al., 1992). A20 is thought to inhibit NF- κ B activation by deubiquitinating NF- κ B signalling molecules. Additionally, IKK, which phosphorylates I κ B α leading to NF- κ B activation, also phosphorylates A20 increasing the ability of A20 to inhibit the NF- κ B pathway. The importance of multiple levels of control of such pathways is demonstrated by the A20 null mouse model which displays multiple organ failure and systemic inflammation and is lethal at two weeks old (Lee et al., 2000), whilst deficiency of A20 specifically in myeloid cells causes a rheumatoid arthritis-like syndrome (Matmati et al., 2011).

1.6.2 Post-transcriptional regulation

The dynamic changes in gene expression that are required following initiation of signalling pathways are often too rapid to rely entirely on changes in gene expression. A number of mechanisms exist to allow rapid changes in protein translation which don't rely on changes in gene transcription, including alternative splicing, alterations in the stability of mRNAs and differential translation of pre-existing mRNA (Carpenter et al., 2014; Ivanov and Anderson, 2013).

Alternative splicing has been shown to regulate inflammatory signalling of both pro-inflammatory and type I interferon signalling. Alternate splicing of the 5' UTR region of TLR genes in mice is common. The functional significance of this mechanism is not well understood, however it is thought to affect mRNA stability, potentially affecting translational efficiency (Wells et al., 2006). *TLR4* is the only gene that is alternately spliced to produce variable protein isoforms. Expression of an isoform lacking exon 3 is upregulated following LPS or IFN- γ stimulation, this isoform is secreted and overexpression of this isoform inhibits LPS-mediated NF- κ B activation and TNF production, leading to negative feedback regulation (Wells et al., 2006). Expression of an analogous TLR4 Δ exon3 isoform in humans is lower in patients with cystic fibrosis than in controls which is consistent with the finding that CF patients produce increased TNF in response to LPS (Jarešová et al., 2007). LPS stimulation also induces expression of a short isoform of MyD88 which can bind

TLRs and IRAK1 but cannot interact with IRAK4 thereby inhibiting downstream signalling by competitive binding (Burns et al., 2003; Janssens et al., 2002).

Cellular mRNA levels can also be altered to regulate the immune response via alterations in mRNA stability. Stimulated DCs and macrophages upregulate transcription of genes associated with a proinflammatory response, whilst simultaneously decreasing the stability of mRNA species leading to rapid degradation of specific transcripts (Elkon et al., 2010; Paulsen et al., 2013; Rabani et al., 2011).

Additionally, the translation of pre-existing mRNAs can be rapidly altered either to amplify or downregulate a response. Activation of TLR3 or TLR4 leads to TRIF mediated dephosphorylation of eukaryotic translation initiation factor 2b (eIF2b), the subunit of eIF2 which induces recycling of the complex. Increased recycling of eIF2 due to eIF2B dephosphorylation leads to increased mRNA translation (Woo et al., 2009; Woo et al., 2012). Similarly, the mRNA cap binding protein, eIF4E is dephosphorylated during viral infections which leads to down-regulation of specific mRNAs including I κ B α , leading to increased activation of NF- κ B (Herdy et al., 2012; Kleijn et al., 1996). Interestingly, recent transcriptome analysis of PBMCs has identified both deregulation of the immune system and deregulation of eIF2 as features of sporadic and genetic Parkinson's disease (Mutez et al., 2014).

1.6.3 Post-translational regulation

Post-translational modification to proteins during signalling events is required for signal transduction, with ubiquitination and phosphorylation being the major post-translational modifications which have already been introduced in terms of signal transduction from PRRs. A range of more unconventional post-translational modifications have also been described which modulate the innate immune system, including protein methylation, acetylation and nitrosylation (Mowen and David, 2014). Methylation of IFNAR-binding protein PRMT1 (protein arginine N-methyltransferase) reduces the antiviral and antiproliferative effects of IFN- α and IFN- β , suggesting that arginine methylation of IFNAR1 is required for efficient IFN signal amplification (Abramovich et al., 1997). Whilst canonical NF- κ B signalling is regulated by sequestration from the nucleus by the inhibitor I κ B α , the RelA (p65)

subunit is also regulated via reversible acetylation in the nucleus by p300 and CBP acetylases. Acetylation of specific lysines has distinct effects on the DNA binding properties of p65 and the transcriptional efficiency of the NF- κ B complex (Chen et al., 2002).

Protein function can also be regulated by the requirement for processing from an inactive precursor to an active mature peptide. For example the highly potent inflammasome cytokines IL-1 β and IL-18 both require caspase-1-mediated cleavage from precursors to mature peptides. Increased processing of pro-IL-1 β to mature IL-1 β in the absence of stimuli is linked to a number of autoinflammatory disorders including familial Mediterranean fever (Chae et al., 2006), Muckle-Wells syndrome (Hawkins et al., 2004) and neonatal-onset multisystem inflammatory disease (Aksentijevich et al., 2002), which demonstrate the necessity for peptide processing to be tightly controlled.

Overall, the innate immune system is highly regulated, and the range of human diseases arising from dysregulation illustrates the importance of multiple levels of control.

1.7 Thesis aims

This thesis investigates the role of two proteins in the innate immune system, under a broad theme of understanding more about the regulation of the innate immune system.

HBD3 has been previously shown to have a role in regulating the innate immune system in addition to chemoattractive properties and potent antimicrobial activity. This thesis specifically investigates the role of hBD3 in the response of dendritic cells to pathogenic and self-DNA to identify whether hBD3 interacts with DNA sensing pathways.

In contrast to hBD3, TRAIP has a less well defined role in the innate immune system, this study aims to identify a role for TRAIP and to understand the mechanism whereby the mutations that have been identified in PD patients lead to increased susceptibility to respiratory infections.

2. Materials and methods

2.1 Reagents

2.1.1 Sources of reagents

Table 2.1 commonly used lab solutions

Name (abbreviation)	Ingredients
1X Dulbeccos Phosphate buffered saline (PBS)	0.16 M NaCl
	3mM KCl
	8mM Na ₂ HPO ₄
	1mM KH ₂ PO ₄
	pH 7.3
20X Tris/Borate/EDTA (TBE)	1.78 M Tris base
	1.78 M Boric acid
	80 mM EDTA
50X Tris/Acetate/EDTA (TAE)	1 M Tris base
	2 M glacial acetic acid
	0.1 M EDTA
Ethylenediaminetetraacetic acid (EDTA)	0.5M EDTA
	pH 8 (using NaOH)
10X Tris buffered saline (TBS)	0.5 M Tris base
	1.5 M NaCl
	pH to 7.5 with HCl
Tris/EDTA (TE)	10 mM Tris
	1 mM EDTA
	pH to 7.5 with HCl

2.2 Cell culture

2.2.1 Cell culture reagents

Cell culture media was purchased from Gibco (Life Technologies). Sterile filtered Fetal Calf Serum (FCS) was from Sigma. Penicillin and streptomycin were provided in house and used at a final concentration of 0.35 g/ml and 0.65 g/ml respectively.

2.2.2 General cell culture techniques

All reagents were warmed to 37°C prior to cell culture. Cells were all maintained at 37°C and 5% CO₂ unless otherwise stated. All cell culture was carried out in CL1 tissue culture hood unless otherwise stated.

Adherent cells were detached by trypsinisation in a 1:1 (v:v) mixture of trypsin and versene for 5-10 minutes at 37°C following a gentle rinse with PBS. Once the cells had detached from the surface of the flask they were resuspended in media and centrifuged for 5 minutes at 301 x g. The supernatant was discarded and the cell pellet was resuspended to a single cell suspension in complete media before replating at an appropriate density.

For freezing, cells were pelleted by centrifugation for 5 minutes at 301 x g, resuspended in freezing mix (12% (v/v) DMSO in 48% F10 nutrient mix and 40% Tryptose Phosphate Broth) and transferred to a labelled cryovial. The cells were stored in liquid nitrogen. To recover cells up from liquid nitrogen pre-warmed media was added to the cryovial to defrost the cells quickly. The contents transferred to a falcon and centrifuged at 301 x g for 5 minutes. The cell pellet was resuspended in fresh complete media and plated in an appropriate size culture flask.

2.2.3 Mouse cell lines

RAW 264.7 macrophages and 3T3 fibroblasts were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin. RAW 264.7 cells were passaged by removing the culture media, vigorously hitting the flask and resuspending the detached cells in an appropriate volume of media to split them. 3T3s were passaged by the trypsin method as described above.

2.2.4 Human cell lines

HEK293 (human embryonic kidney) cells were maintained in DMEM supplemented with 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin and passaged by the trypsin method as described above.

2.2.5 Primary cells

2.2.5.1 Human dermal fibroblasts

Human dermal fibroblasts were cultured in a CL2 tissue culture hood. Fibroblasts were maintained in DMEM supplemented with 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin. Primary fibroblasts were cultured up to passage 15. Primary fibroblasts were passaged by the trypsin method as described above.

2.2.5.2 Macrophages

Femurs and tibia were taken from 8-12 week old C57Bl6/J male mice that had been culled by cervical dislocation. Under sterile conditions, the ends of the bones were removed with a scalpel and the bones were flushed with complete macrophage media (RPMI-1640 supplemented with 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin, 2 mM L-glutamine, 50 μ M β -mercaptoethanol) into a 90 mm non-tissue culture treated petri dish using a 25G needle. The cells were immediately passed through a 21G needle. The cell suspension was passed through a 70 μ m cell strainer and plated at the equivalent of one mouse per 90 mm non-tissue culture dish in 10 ml complete media supplemented with 20 ng/ml macrophage-colony stimulating factor (M-CSF, R&D). The cells were incubated at 37°C under normoxic conditions for 8-10 days after seeding. On the final day, the media was collected and spun down to obtain the suspension cells and the adherent cells were incubated with 3 mM EDTA (diluted in complete macrophage media) for 15 minutes at 37°C. The detached cells were gently rinsed off the dish and pelleted by centrifugation at 301 x g for 5 minutes. The cell pellet was resuspended in complete macrophage medium, centrifuged again to completely remove the EDTA and resuspended in macrophage medium without penicillin or streptomycin. The viable cells were counted using Trypan Blue (Gibco), plated at 2.5×10^5 – 12.5×10^5 cells/ml in a 48-well plate (Corning Costar) and incubated overnight without M-CSF before treatment.

2.2.5.3 Flt3-L Dendritic cells

Femurs and tibia were taken from 8-12 week old C57Bl6/J male mice that had been culled by cervical dislocation. Under sterile conditions, the ends of the bones were removed with a scalpel and the bones were flushed with complete FLDC media (RPMI 1640 supplemented with 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin, 2

mM L-glutamine, and 50 μ M β -mercaptoethanol) into a 90 mm non-tissue culture treated petri dish using a 25G needle. The cells were immediately passed through a 21G needle and transferred to a 50 ml falcon tube. The cells were pelleted by centrifugation at 301 x g for 5 minutes and resuspended in 1 ml per mouse of red blood cell lysis buffer (RBC, 0.168 M NH_4Cl , pH 7.2). The cells were incubated in RBC lysis buffer for 2 minutes then taken up in 20 ml complete FLDC media and pelleted as before. The cells were washed twice more in 20 ml complete FLDC media before being passed through a 70 μ m cell strainer (Corning) and pelleted for the final time before counting. 1.5×10^6 cells/ml cells were plated in T75 flasks in complete FLDC media supplemented with 20 ng/ml Flt-3L (recombinant murine Flt3-ligand, Peprotech, #250-31L). The cells were incubated at 37°C, 5% CO_2 for 8 days with a minimum of disturbance during the culture period. On day 8 the cells were gently harvested by rinsing the flask, pelleted by centrifugation at 301 x g for 5 minutes and resuspended in antibiotic-free FLDC media. The viable cells were counted using Trypan blue and plated at 2.5×10^6 cells/ml in a 48-well plate (scaled up or down accordingly) and treated immediately.

2.2.5.4 Mouse Embryonic Fibroblasts (MEFs)

The uterus was dissected from the female mouse 10.5 days after a plug was recorded and immediately placed on ice in PBS. Embryos were individually dissected from the uterus in a dish of cold PBS using a dissecting microscope. The head was removed from each embryo and discarded, and either the yolk sac or the tail was reserved for genotyping. Using curved forceps the internal organs were scooped out of the abdomen and discarded. The resulting 'shell' was washed in clean PBS and transferred to a well of a 6-well plate containing 2-3 ml MEF media (DMEM supplemented with 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin and 50 μ M β -mercaptoethanol). Once all the embryos had been processed in this way, the remaining steps were carried out in a tissue culture hood. A sterile scalpel was used to dice each embryo into small pieces and then a fine tipped pastette was used to further homogenise the tissue before being transferred to a T25 flask (Corning) containing 10 ml MEF media. MEFs were cultured at 37°C, 3% O_2 and 5% CO_2 (hypoxic conditions). The day after seeding the flask was gently rinsed with media

and the media replaced. This was repeated the following day. MEFs were passaged by trypsinisation.

2.3 Mouse colony management

2.3.1 General considerations

Mice were kept under specific pathogen free (SPF) conditions in the Transgenic Unit or the Biomedical Research Facility at the Western General Hospital. All procedures and breeding were carried out under Home Office Regulations.

TLR9^{-/-} mice were a kind gift from David Gray at the University of Edinburgh

2.3.2 DNA extraction from ear clips

Mice were ear clipped for identification purposes and the ear clips collected for genotyping. After collection the DNA was either extracted immediately or the ear clips were stored short term at -20°C. To extract DNA from ear clips, 50 µl of 25mM NaOH was added to the ear clip and incubated at 95°C for 15 minutes. The samples were then cooled on ice for 5 minutes before adding 50 µl 40 mM Tris-HCl. The samples were stored at -20°C and 5 µl was used in each PCR reaction.

2.3.3 DNA extraction from tail tips

To extract higher quality DNA from tail tips or from embryos, 500 µl tail tip buffer (1% (v/v) SDS, 0.1 M NaCl, 0.1 M EDTA, 0.05 M Tris (pH 8) made up in d.H₂O) was added followed by the addition of proteinase K (final concentration 50 µg/ml). The tubes were shaken well and incubated at 55°C overnight. Following the overnight incubation the tubes were spun to precipitate any undissolved fragments and the supernatant transferred into a clean tube. An equal volume of isopropanol was added and the sample was mixed. The DNA was spooled out into 70% ethanol, then centrifuged at full speed for 15 minutes and the supernatant was discarded. The DNA pellet was taken up in 50 µl d.H₂O. 1 µl tail tip DNA was used in PCR reactions.

2.4 Techniques used to investigate immune responses

2.4.1 Treatment of cells with ligands

Table 2.2 Ligands that were used in this study with the working concentrations, unless otherwise stated.

Ligand	Manufacturer	Working concentration
<i>E. coli</i> genomic DNA	InvivoGen Derived from <i>E. coli</i> strain K12	1 µg/ml
Mouse genomic DNA (self-DNA)	Extracted from ES cells by phenol/chloroform extraction and ethanol precipitation (2.7.1)	1 µg/ml
hBD3	Peptide Institute (#4382-s) Chemically synthesized and folded	5 µg/ml
Poly(I:C)	Invivogen	1 µg/ml

2.4.2 Transfection of DNA into FLDCs

Cells were plated at 2.5×10^6 cells/ml in a 48-well plate in 400 µl antibiotic free FLDC media. Transfection mixtures were prepared in 100 µl optimum. For example, to transfect three triplicate wells with 1 µg/ml DNA (stock at 1 mg/ml):

Prepare enough for 3.5 wells. 3.5 µl lipofectamine LTX (Invitrogen) was diluted in 171.5 µl OptiMem (Gibco)(1:50 dilution) and mixed gently. The mixture was incubated at room temperature for 5 minutes. 1.75 µl of stock DNA solution (1 mg/ml) was diluted in 173.75 µl OptiMem and added to prepared lipofectamine reaction. The reaction was mixed gently and incubated at room temperature for 20 minutes. 100 µl was added to each well with cells, mixed gently by tipping plate and incubated at 37°C overnight.

2.4.3 Induction of apoptosis in RAW 264.7 macrophages by UV irradiation

RAW 264.7 macrophages-like cells were plated into 14 cm tissue culture treated dishes. Once cells were approaching confluency (i.e. still in a log phase of growth) the media was removed and the plate was gently washed with PBS. The remaining liquid was removed and the cells (plate without lid) were exposed to 160 mJ/cm² using the energy function on a Spectrolinker XL-1500 (Spectroline). The media was replaced and the cells were cultured for 24 or 6 hours.

The media was removed from the plate and 10 ml PBS was added. The cells were removed from the plate by very gentle scraping with a cell scraper and resuspended by pipetting. The cells were counted using a haemocytometer and approximately 1x10⁵ cells were taken for annexin V/PI staining. DNA was extracted from the remaining cells as described (2.7.1), and quantified using a spectrophotometer.

2.4.4 Methylation of bacterial DNA

Methylation of *E. coli* genomic DNA was performed using CpG methyltransferase (M.Sss1, NEB) The reaction was set up as follows: 1 x NEBuffer2, 20 µg DNA, 1.28 mM S-adenosylmethionine, 160 U/ml M.Sss1, made up to 200 µl with milli-Q water (the reaction was scaled down when heat inactivating the enzyme). The reaction mixture was incubated for 2 hours at 37°C, with a gentle mix and spin after 1 hour. The methylated DNA was either phenol/chloroform extracted (2.7.1), precipitated with sodium acetate and ethanol and resuspended in 20 µl milli-Q water or the enzyme was inactivated by incubation at 75°C for 20 minutes. The final concentration of extracted DNA was measured using a spectrophotometer.

2.4.5 Labelling of DNA with CX-rhodamine

Labelling of genomic DNA was performed using the Label-IT Nucleic Acid labelling kit (CX-Rhodamine, Mirusbio). The labelling reaction was set up as follows: 1 x labelling buffer A, 5 µl label IT reagent, 5 µg DNA and made up to 50 µl with molecular grade water. The reaction was mixed well and incubated at 37°C for 1 hour with a 10 second spin at full speed after 30 minutes. The labelled DNA was purified using the G50 microspin purification columns provided with the kit and 100% recovery of DNA was assumed.

2.4.6 Sonication of genomic DNA

Sonication of genomic DNA (both ES cell DNA and *E. coli* DNA) was carried out using a Biorupter (Diagenode). 20 µg of DNA was diluted in water to a final concentration of 0.2 mg/ml in a volume of 100 µl in a 1.5ml eppendorf tube. The tubes were fitted into the lower part of the tube holder which was then screwed back onto the main section. The tube holder was completely filled with tubes containing the same volume of water as the sample. The water bath was filled with a slushy mixture of water and ice and the tube holder was fitted into the lid. The DNA was sonicated using 60 cycles of 30 seconds on/30 seconds off and the water bath was replenished with more ice every 10 minutes to keep the temperature around 4°C.

2.4.7 DNase treatment

DNase treatment of ES cell and *E. coli* genomic DNA was carried out using DNase I (recombinant, RNase-free, Roche). 5 µg DNA was mixed with 1 X incubation buffer, 1 U DNase I and the total reaction volume was taken up to 50 µl with water. The reaction was incubated at 37°C for 1 hour and the enzyme was inactivated by incubating at 65°C for 10 minutes.

2.4.8 Flow cytometry

2.4.8.1 Cells

Following overnight treatment, cells were harvested by rinsing the media around the well (FLDCs) or by EDTA treatment (macrophages) and transferred to eppendorfs. The cells were centrifuged at 1200 x g for 5 minutes. The supernatant was stored at -80°C for future ELISA analysis. The cells were resuspended in 200 µl FC buffer (1% v/v FCS in PBS) and transferred to a v-bottomed 96-well plate (Greiner CELLSTAR). A small portion of each sample was pooled and split between wells for single stained, unstained and fluorescence minus one (FMO)/isotype controls. The plate was centrifuged at 367 x g for 2 minutes, the supernatant was flicked off and the plate was blotted against tissue. To loosen the cell pellet the plate was vortexed briefly. The cell pellets was resuspended in 50 µl per well of block (Purified rat anti-mouse CD16/CD32, clone 24G2, BD Pharmingen, diluted in FC buffer at 1:250) and incubated at 4°C for 10 minutes. The antibodies were diluted in FC buffer to the appropriate concentration (see table 2.3). Following blocking the plate was

centrifuged at 367 x g for 2 minutes, the supernatant flicked off, the plate blotted and vortexed. 50 µl of the appropriate antibody staining solution was added and the plate was incubated at 4°C in the dark for 15 minutes. 100 µl of FC buffer was added to wash the cells and the plate was centrifuged, flicked, blotted and vortexed. The wash was repeated a final time and the cell pellet was resuspended in 100 µl FC buffer and transferred to a labelled FACS tube (BD Falcon). For longer term storage (1-2 days) of the samples before FC, the samples were resuspended in a final amount of 100 µl FACS fix (BD bioscience, diluted 1:10 in milliQ water), transferred to labelled FACS tubes and kept sealed at 4°C in the dark until analysis.

2.4.8.2 Spleens

Spleens were dissected from mice culled by cervical dislocation and placed into PBS on ice. Spleens were placed onto a 40 µm cell strainer (Corning) with a small amount of spleen wash buffer (SWB, 1% (v/v) FCS in PBS) and pushed through the strainer into a falcon tube using the rubber plunger from a 5 ml syringe. The strainer was washed with SWB and the tubes were centrifuged at 301 x g for 5 minutes. The supernatant was poured off and the cell pellet was resuspended in 2 ml red blood cell lysis buffer (as used for FLDC isolation) and incubated at room temperature for 2 minutes. 20 ml SWB was added and the liquid was poured into another tube leaving any clumps behind in the original tube. The cells were centrifuged at 301 x g for 5 minutes then washed in 20 ml SWB; this was repeated twice more. After the final wash the cell pellet was resuspended in 1ml SWB for counting. 1×10^6 cells were removed for staining as described above and the remainder of the cells were pelleted and frozen at -80°C.

2.4.8.3 Annexin V/PI staining

Annexin V staining was performed using the Annexin V apoptosis detection kit (Ebioscience). Approximately 1×10^5 cells were washed once in PBS, centrifuged at 1200 x g for 5 minutes in a microfuge and then washed in 1 x binding buffer diluted in distilled water and the centrifuge step was repeated. The cells were taken up in a final volume of 100 µl and 5 µl APC-conjugated annexin V was added. The cells were incubated with annexin V for 10 minutes at room temperature in the dark and then washed with 1 ml 1 X binding buffer and spun at 1200 x g for 5 minutes in a

microfuge. The cells were taken up in a final volume of 200 μ l and transferred into FACS tubes and kept on ice in the dark. DAPI was added immediately before FC analysis. The cells were analysed within 1 hour.

Table 2.3 FACS antibodies including working dilution and isotype controls (AH Armenian hamster, n.s. not specified)

Antigen	Conjugate	Clone	Manufacturer	Isotype	Dilution
CD11c	APC-eFluor 780	N418	eBioscience	AH IgG APC-eFluor 780	1:100
B220	eFluor 450	RA3-B2	eBioscience	Rat IgG2a K eFluor 450	1:40
CD80	APC	16-10A1	eBioscience	AH IgG APC	1:200
CD86	Alexa Fluor 488	GL-1	Biolegend	Rat Alexa Fluor 488 IgG2a, κ	1:200
CD40	PE	3/23	BD Bioscience	PE-CF594 Rat IgG2a, κ	1:100
MHCII	PerCP-Cy5.5	M5/114.15.2	Biolegend	Rat PerCP/Cy5.5 IgG2b, κ	1:1000
CD11b	PE	M1/70	BD Pharmingen	Rat PE IgG2b, κ	1:160
CD19	Alexa Fluor 700	eBio/D3	eBioscience	Rat Alexa Fluor 700 IgG2a, κ	1:80
Ly-6C	APC	HK1.4	eBioscience	n.s.	1:200

2.4.9 ELISA

ELISA plates (flat-bottomed 96-well plates MaxiSorp®, Nunc) were coated with 50 μ l capture antibody diluted in the appropriate buffer overnight at room temperature. The plates were washed 5 times in PBS-Tween (0.05%) and blocked for 1 hour in 1% (v/v) Reagent Diluent (TNF- α , IL-6, DuoSet), 1% (w/v) BSA in PBS (IFN- β) or

5% (w/v) BSA in PBS (IFN- α). The block was removed and 50 μ l of supernatant was added in duplicate, diluted if necessary in reagent diluent or 1% (w/v) BSA in PBS (IFN- α). Serial dilutions of standard were also added in duplicate. The plates were sealed and incubated overnight at 4°C. The plates were washed 5 times with PBS-T and 50 μ l of detection antibody diluted in the appropriate buffer was added. The plates were incubated at room temperature for 2 hours. The plates were washed 5 times with PBS-T and 50 μ l of appropriate HRP-conjugated secondary antibody was added. The plates were incubated for 20 minutes (IL-6/TNF- α) or 1 hour (IFN- α /IFN- β) and washed 5 times with PBS-T. 50 μ l of TMB (3,3',5,5'-Tetramethylbenzidine) substrate was added to each well and the plate was mixed and left to develop. The reaction was stopped by adding 25 μ l stop solution (1 N HCl) and the absorbance was measured at 450 nm using a Multiskan plate reader (Thermo Multiskan Spectrum). Details of interferon ELISAs are detailed in appendix 2, all other ELISAs were R&D duosets and carried out according to manufacturer's instructions

2.4.10 Transfection protocol for Dual luciferase assay

For dual luciferase assay the Promega dual luciferase assay kit was used to measure firefly and renilla reporter expression.

HEK293 cells were seeded at $2-3 \times 10^4$ cells in 100 μ l complete DMEM per well of a 96-well tissue culture plate 24-48 hours before transfection, so cells were 60-70% confluent at the point of transfection. Transfection mixtures were prepared in Optimem media, the media was removed from the wells and replaced with a total volume of 100 μ l. Lipofectamine 2000 was diluted 1:50 in Optimem at a volume of 50 μ l per well and incubated at room temperature for 5-10 minutes. Separately, the plasmid mixtures were prepared in a volume of 50 μ l Optimem per well and the lipofectamine mixture was added to the DNA to make a total volume of 100 μ l per well. The DNA-lipofectamine mixture was incubated at room temperature for 20 minutes and then added to the cells. The plates were incubated in the 37°C incubator for 24 hours. The media was removed from the cells and stored at -20°C for ELISA analysis. The cells were lysed using 1X passive lysis buffer provided with the kit (5X passive lysis buffer diluted in distilled water, 20 μ l per well of a 96-well plate) for 20

minutes at room temperature. 5 µl of each sample was then transferred to an opaque 96-well plate (Costar) and placed inside the machine to allow background fluorescence to fade. 1X Stop and glo reagent was prepared from 50X stop and glo reagent diluted in stop and glo buffer. Luciferase reagent was defrosted from storage at -80°C. Both solutions were equilibrated to room temperature before use. The plate was read using a FluoSTAR Omega plate reader (BMG labtech) and the data were exported from the Omega data analysis software and analysed in Excel and GraphPad Prism.

2.4.11 Immunofluorescence

For adherent cells, cells were seeded onto coverslips which had been placed into plate wells (one 16 mm coverslip per 12-well plate, two per 6-well plate). Following treatment the growth medium was removed from wells and the coverslips were gently washed in the plate with PBS 2-3 times. The cells were then fixed in the wells in 4% (v/v) paraformaldehyde (PFA) in PBS for 10 minutes at room temperature.

For suspension cells, cells were rinsed gently from the well and transferred to a 1.5 ml Eppendorf. The cells were centrifuged at 1200 x g for 5 minutes and then resuspended in 500 µl PBS. The centrifuge step was repeated and the cells resuspended in 200 µl PBS. The cell suspension was gently pipetted onto the surface of a poly-L-lysine coated slide and left to attach for 10-15 minutes (attachment could be confirmed with a tissue culture microscope). The excess PBS and unattached cells were gently tipped off and 4% PFA was gently added to the slide and incubated at room temperature for 10 minutes.

Alternatively the suspension cells were cytopspun. The cell pellet was resuspended in 100 µl 4% PFA and fixed for 10 minutes at room temperature. The cells were centrifuged at 1200 x g for 5 minutes and resuspended in 500 µl PBS, this step was repeated and the cells were resuspended in 200 µl PBS. 100 µl of this was spun onto slides at 800 rpm for 5 minutes in a Shandon Cytospin 4 (Thermo). The slides were dried at room temperature and either stored wrapped at -20°C or stained immediately.

Following fixation, excess PFA was removed and coverslips (still in the plate wells) or slides (in a slide rack) were gently washed 2-3 times in PBS.

Block and antibodies were prepared in 1% (w/v) BSA in PBS-tween (0.05% v/v). The block used depended on the animal that the secondary was raised in and was prepared as a 10% (v/v) solution of donkey or goat serum in 1% BSA in PBS-T. The samples were incubated with block for at least 1 hour at room temperature. For coverslips, the blocking step was carried out in the plate well. For slides, the block was applied to the slides and pieces of parafilm slightly smaller than the slide's attachment surface were laid gently over the block to prevent excess evaporation. Following blocking, the excess block was removed and the samples were incubated with primary antibody diluted in 1% BSA in PBS-T. For coverslips, 20 µl of primary antibody was dotted onto parafilm and the coverslips were laid cell surface down on the drops. For slides, 50-100 µl of primary antibody was applied to the slide surface and pieces of parafilm laid on top as before. Samples were incubated with primary antibody overnight at 4°C and in the dark.

Samples were washed as before (coverslips can be moved back into wells for this or dipped into 50 ml falcon tubes of PBS) and then incubated with secondary antibody diluted in 1% BSA in PBS-T for 1 hour at room temperature. Samples were washed as before and mounted in Vectashield with DAPI (coverslips mounted onto superfrost slides, slides mounted with large coverslips) (VECTOR laboratories). Excess Vectashield was blotted off if necessary using filter paper and slides were stored on a slide holder at 4°C, protected from the light with tin foil for up to 2 days before imaging.

2.4.12 *In vivo*

All *in vivo* experiments were performed according to home office regulations (Animals (Scientific Procedures) Act 1986). Wild-type C57BL/6J mice taken for bone marrow and used in *in vivo* experiments were obtained from Charles River (UK). HBD3 transgenic mice were kept in the Biomedical Research Facility. TRAIP transgenic mice were kept in individually ventilated cages in the Transgenic Facility. All *in vivo* procedures were carried out by Sheila Webb whose help was very gratefully received.

2.4.12.1 Intraperitoneal injection

WT or hBD3 transgenic (tg) mice were injected with *E. coli* DNA diluted in saline or with saline alone. The maximum volume injected intraperitoneally (i.p.) was 100 µl, injected using a 100 U insulin syringe (BD Bioscience, 100 U = 100 µl). Once injected, mice were incubated in hot boxes (28°C) and monitored for the duration of the 4 hour experiment.

2.4.12.2 Intraperitoneal lavage

The i.p. injected mice were culled by cervical dislocation and the skin was removed from the abdomen without perforating the peritoneal wall. 1 ml of PBS was injected into the left hand side of the peritoneal cavity (close to the spleen) using a 21 G needle (BD Microlance 3) with the beveled edge facing upwards. Keeping the needle in and secure, the peritoneal cavity was massaged for 30 seconds. The carcass was tilted onto the left hand side and as much as possible of the liquid was extracted. A small incision was made and the remainder of the liquid was sucked out with a fine tipped pastette. The peritoneal supernatant was centrifuged at 1200 x g for 5 minutes and the supernatant was transferred to another tube for ELISA analysis. The cells were resuspended in PBS and counted. Approximately 1×10^6 cells were stained for FACS analysis and the remainder stored at -80°C.

2.5 Biochemical methods

2.5.1 Dot blotting

250-500 ng DNA in 1-2 µl was dotted onto a nitrocellulose membrane. The DNA was cross-linked to the membrane using the 'optimal crosslink' setting on a Spectrolinker XL-1500 (Spectroline) and then the membrane was blocked in 2% (w/v) BSA in Tris-buffered saline with 0.1% (v/v) Tween (TBS-T) for at least one hour on a roller. The blot was incubated with primary antibody diluted in block overnight at 4°C on a roller. The blot was washed 3 times for 5 minutes each in TBS-T before incubation in secondary antibody diluted in block for 45-60 minutes.

2.6 RNA methods

2.6.1 RNA extraction

Cells were pelleted and used immediately or frozen at -80°C for future use. RNA was extracted from cells using an RNeasy mini kit (Qiagen). Samples were lysed in

lysis buffer (RLT buffer, supplied with kit) and passed through a Qias shredder (Qiagen) column to homogenise the samples. The sample was mixed with an equal volume of 70% ethanol (made up with RNase-free water) and applied to the spin column of the RNeasy kit. The RNA extraction was carried out following manufacturer's instruction with the inclusion of an on-column DNase1 step (RNase-free DNase set, Qiagen) for 15 minutes at room temperature. The RNA was eluted from the column with 30 µl RNase-free water. The RNA was quantified using a spectrophotometer.

2.6.2 Reverse transcription

Reverse transcription was carried out using a first strand cDNA synthesis kit (Roche). The reaction was set up according to the manufacturer's instructions using oligo (dT) and 1 µg of RNA in a total volume of 20 µl. The reaction program was as follows: 25°C for 10 minutes, 42°C for 1 hour, 99°C for 5 minutes, held at 4°C. The cDNA was either used immediately or stored at -20°C.

2.6.3 RT-PCR

The RT-PCR reaction was set up as follows: 1 x Phusion flash mix, 0.5 µM forward primer, 0.5 µM reverse primer, 1 µl cDNA and made up to 20 µl with RNase-free water. The reaction programs were as follows:

2.6.3.1 TRAIP

- i. 98°C 10s
- ii. 98°C 1s
- iii. 72°C 25s
- iv. Repeat steps 2-3 30 times
- v. 72°C 60s
- vi. 4°C hold

2.6.3.2 GAPDH

- i. 98°C 10s
- ii. 98°C 1s
- iii. 60° 5s

- iv. 72°C 15s
- v. Repeat steps 2-4 30 times
- vi. 72°C 60s
- vii. 4°C hold

2.7 DNA methods

Unless otherwise stated all PCR products were run out on a 1% (w/v) agarose gel made up in 1 X TBE buffer with ethidium bromide added to a final concentration of approximately 0.5 µg/ml. The loading dye used was TE buffer containing 15% ficoll and orange G dye. For size detection of PCR bands the 1 kb plus ladder (Invitrogen) or the gene ruler 1 kb plus ladder (Thermo Scientific) was used.

2.7.1 Extraction of DNA from mammalian cells

Cell pellets were resuspended in 500 µl lysis buffer (Tris-EDTA with 0.1% (v/v) SDS) with proteinase K (final concentration 50 µg/ml). The samples were incubated overnight at 55°C, then an equal volume of DMF/acetone (5% DMF, 70% acetone made up in MilliQ water) was added. The DNA was spooled out and washed for 1 hour in 70% Ethanol at room temperature. The DNA was either spooled out or spun out (full speed for 15 minutes) and dried until the ethanol evaporated. The DNA was resuspended in 50-100 µl water.

To further extract the DNA for particular downstream applications, phenol/chloroform extraction was used. An equal volume of phenol (Phenol:Chloroform:Isoamyl Alcohol 25:24:1, saturated with 10 mM Tris, pH8.0, 1 mM EDTA; Sigma) was added, the tube was mixed by inversion multiple times and the sample was spun in a microfuge at top speed for 1 minute. The aqueous phase was removed to a clean tube. The process was repeated. An equal volume of chloroform (Chloroform:Isoamyl alcohol 24:1; Sigma) was added, the tube was mixed by inversion multiple times and the sample was spun in a microfuge at top speed for 1 minute. The aqueous phase was removed to a clean tube. The process was repeated. Sodium acetate was added to a final concentration of 0.3 M and 2.5 times volume of cold 100% ethanol was added. The samples was mixed by inversion and spun in a microfuge at top speed for 15-20 minutes at 4°C. The supernatant was removed and the DNA pellet was washed with 70% ethanol and spun again at full

speed for 15-20 minutes. The supernatant was removed and the DNA pellet was dried for 5-10 minutes. The DNA was resuspended in an appropriate volume of water and the concentration was measured using a spectrophotometer.

2.7.2 Spectrophotometric quantification

The concentration of DNA samples was measured using a Nanodrop spectrophotometer (ND-1000, Thermoscientific). 1.2 µl of each sample was analysed and the concentration, 260/280 and 260/230 ratio were recorded. A 260/280 ratio of ~1.8 and a 260/230 ratio of 2-2.2 was used to confirm that DNA was free from contaminants.

2.7.3 Primer design

Primers were designed or checked using Primer 3 (Koressaar and Remm, 2007). Primer pairs were designed with a similar melting point. The sequences of all primers are detailed in appendix 1.

2.7.4 DNA amplification by PCR

2.7.4.1 *Taq* PCR

Standard PCR reactions were performed as follows: 1 x reaction buffer, 1.5 mM MgCl₂, 2.08 ng/µl forward and reverse primers, 0.2 mM dNTPs, 0.5 units *Taq* DNA polymerase, 1 ng plasmid DNA or up to 1 µg genomic DNA and made up to a final volume of 24 µl with d.H₂O. The standard PCR program proceeded as follows:

- i. 95°C, 2 minutes
- ii. 94°C, 30 seconds
- iii. 60°C (depending on T_m of primer), 30 seconds
- iv. 72° for 1 minute per kbp of DNA
- v. Repeat steps 2-4 for a total of 32 times
- vi. 72°C 5 minutes

2.7.4.2 *KOD taq* PCR

KOD PCR reactions were performed as follows: 1 x reaction buffer, 1 µM dNTPs, 1.5 mM MgCl₂, 3 ng/µl each primer, 1 U KOD *taq*, 1 µl plasmid DNA and made up to a final volume of 50 µl with d.H₂O. The KOD PCR program proceeded as follows for up to 1 kb target:

- i. 94°C 3 minutes
- ii. 94°C 30 seconds
- iii. 58°C 15 seconds
- iv. 68°C 2.5 minutes
- v. Repeat steps i-iv 34 times
- vi. 68°C 7 minutes
- vii. 4°C hold

2.7.4.3 Dye terminator cycle sequencing

Labelling reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and were set up as follows: 2 µl BigDye Terminator v3.1 Ready Reaction Mix (including enzyme, buffer, dNTPs and labelled terminators), 6 µl dilution buffer, 1.1 ng/µl primer, 1 µl DNA miniprep, up to 20 µl with d.H₂O. The ABI PCR program proceeded as follows:

- i. 94°C 30 seconds
- ii. 50°C 15 seconds
- iii. 60°C 4 minutes
- iv. Repeat steps i-iii 30 times
- v. 4°C 2.5 hours

Sanger sequencing was performed in-house thanks to Agnes Gallagher.

2.7.4.4 Long range PCR

Long range PCR reactions were performed using either the Expand Long Template PCR System (Roche) or PrimeSTAR max DNA polymerase (Takara).

The Expand Long Template PCR reaction was set up as follows: 1 X reaction buffer 3, 0.5 mM dNTPs, 1.5 ng/µl each primer, 0.375 µl enzyme mix, up to 500 ng template DNA and made up to 25 µl with d.H₂O. The PCR cycles were as follows:

- i. 92°C 2 minutes
- ii. 92°C 10 seconds
- iii. 60°C 30 seconds
- iv. 68°C 2 minutes for up to 3 kb, 4 minutes for up to 6 kb
- v. Repeat steps ii-iv 10 times

- vi. 92°C 15 seconds
- vii. 60°C 30 seconds
- viii. 68°C for the amount of time in iv. + 20 seconds for each successive cycle
- ix. repeat steps vi-viii 30 times
- x. 68°C 7 minutes

PrimeSTAR reactions were set up as follows: 1 X primeSTAR max mix, 2ng/μl each primer, 200 ng DNA and made up to 50 μl with d.H₂O. The PCR cycles were as follows:

- i. 94°C 2 minutes
- ii. 98°C 10 seconds
- iii. 55°C 5 seconds
- iv. 72°C 5 seconds/kb
- v. Repeat steps ii-iv 32 times

2.7.5 Crude gel extraction of DNA

Gel extraction was generally performed using a Qiagen gel extraction kit. The DNA to be extracted was run at low voltage into a 1% TBE-agarose gel for long enough to allow good separation of bands. The bands were cut out of the gel with a clean scalpel using a UV transilluminator and transferred to a clean 15 ml falcon tube. In the final step the DNA was washed from the column with milli-Q water and the concentration was quantified using a spectrophotometer.

2.7.6 Restriction digest

Restriction digest were generally performed in a volume of 20 μl but could be scaled up or down as necessary.

A typical 20 μl restriction digest with one or two enzymes was performed as follows: 1 X appropriate buffer, 1 U enzyme (or each enzyme), 1 μl plasmid miniprep DNA, made up to 20 μl with d.H₂O

For downstream applications, 1 μl SAP (shrimp alkaline phosphatase, NEB) was added to the digest and incubated at 37°C for 1 hour to dephosphorylate the ends of the DNA. The enzyme was inactivated by incubation at 65°C for 20 minutes.

2.8 Genetic engineering methods

2.8.1 Site-directed mutagenesis

Site directed mutagenesis was carried out using the QuikChange II XL site directed mutagenesis kit (Agilent). Primers (appendix 1) were designed across the site to be mutagenised with a single base pair change in each primer engineered approximately in the middle of each primer. The reaction was set up as follows: 1X reaction buffer, 10 ng plasmid DNA template, 125 ng each primer, 1 µl dNTP mix (provided with kit), 3 µl Quiksolution, milliQ water to a final volume of 50 µl. The cycling conditions were set up as follows:

- i. 95°C, 1 minute
- ii. 95°C, 50 seconds
- iii. 60°C, 50 seconds
- iv. 68°C, 1 minute per kb of plasmid length
- v. Repeat cycles 2-4 18 times
- vi. 68°C, 7 minutes

Following the cycling reaction the tubes were placed on ice for 2 minutes. 10 µl of product was run on a 1% agarose gel to check amplification of the product, if the product could not be visualised then the cycling reaction was repeated. If the product was visible then 1 µl of *Dpn* I restriction enzyme was added directly to the reaction volume and gently mixed by pipetting. The sample was spun down in a microfuge for 1 minute and incubated at 37°C for 1 hour.

XL10-gold ultra-competent cells were thawed on ice and 45 µl was aliquoted into a pre-chilled 15 ml falcon tube. 2 µl of β-mercaptoethanol was gently mixed into the tube and the cells were incubated on ice for 10 minutes, mixing gently every 2 minutes. Following digestion of the parental DNA with *Dpn* I, 2 µl of the DNA was added to the cells and gently mixed. The transformation reactions were incubated on ice for 30 minutes, heat shocked at 42°C for 30 seconds and incubated on ice for 2 minutes. 0.5 ml of prewarmed (42°C) NZY+ broth was added to the cells and incubated at 37°C for 1 hour with shaking at 225-250 rpm. The cells were plated onto agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

2.8.2 Bacterial culture

Commonly used materials for bacterial culture are detailed in table 2.5.

Table 2.4 Solutions used in bacterial culture

Solution	Details
L-Broth (Lysogeny broth)	Tryptone (10 g/L) Yeast extract (53.2 g/L) Sodium Chloride (10 g/L) Glucose (1 g/L)
SOC media (Super optimal broth)	20 g/L Tryptone 5 g/L Yeast Extract 4.8 g/L MgSO ₄ 3.603 g/L dextrose 0.5g/L NaCl 0.186 g/L KCl
L-agar (Lysogeny agar)	Tryptone (10 g/L) Yeast extract (53.2 g/L) Sodium Chloride (10 g/L) Agar (15 g/L)
Kanamycin	Final concentration 50 µg/ml
Ampicillin	Final concentration 100 µg/ml
Chloramphenicol	Final concentration 25 µg/ml

2.8.3 Growth conditions

DH5α and XL-gold bacteria were cultured at 37°C. EL-250 and EL-350 bacteria were cultured at 32°C. Liquid cultures were incubated with rotation at 225 rpm. Bacterial stocks were aliquoted from liquid culture and mixed with glycerol (1:1 mixture) in a cryovial and stored at -70°C. To recover bacteria from a glycerol stock, a sterile loop was scraped across the frozen mixture and plated out, without the glycerol stock thawing. Additionally, plasmid stocks were stored at -20°C and bacteria re-transformed as necessary.

2.8.4 Transformation

2.8.4.1 Preparation of competent bacteria

Bacteria were grown in 10 ml LB with the appropriate antibiotic overnight at the correct temperature with shaking. 5 ml of the overnight culture was transferred to 400 ml LB with antibiotic and incubated at the correct temperature with shaking for approximately 4 hours until the optical density was approximately 0.5 at 600 nm, measured using a spectrophotometer (1 ml bacterial culture in a cuvette (Kartell)). The whole culture was aliquoted into 50 ml falcon tubes (50 ml per tube) and centrifuged at 1789 x g for 5 minutes, the pellet was resuspended in 50 ml ice cold water and centrifuged again. This time the pellets were resuspended in 25 ml ice cold water and two were pooled together and centrifuged. This process was repeated until there was one tube. The cell pellet was resuspended in 20 ml ice cold 10% (v/v) glycerol and centrifuged. The cell pellet was resuspended in 150-200 µl 10% glycerol and kept on ice for immediate electroporation.

2.8.4.2 Heat-shock of competent bacteria

Competent bacteria were transformed by a heat shock process. Vials of competent bacteria were defrosted on ice and 70 µl of cells was gently transferred into a pre-chilled 15 ml falcon tube. DNA was added and gently mixed using the tip of the pipette before incubating on ice for 30 minutes. The bacteria were heat shocked at 42°C in a water bath for 45 seconds without shaking then placed on ice for 2 minutes. 1 ml pre-warmed antibiotic free SOC media was added and the bacteria were incubated for 1 hour at 37°C with shaking. 100 µl of the bacteria were spread onto LB plates with the appropriate antibiotic using a sterile spreader, the rest of the bacteria were spun down and plated onto a second plate. The plates were incubated at 37°C overnight.

2.8.4.3 Electroporation of competent bacteria

To electroporate competent bacteria 50 µl of the bacterial suspension (in glycerol) was aliquoted into an Eppendorf and gently mixed with the appropriate quantity of DNA (or nothing as a control). The whole mixture was transferred to an electroporation cuvette (Molecular BioProducts, 2 mm gap disposable pre-sterilised electroporation cuvettes with caps) and tapped to make sure the mixture was at the

bottom of the cuvette. The bacteria were electroporated at 3.5 kV. 1 ml pre-warmed LB was added to the cuvette and the bacteria were transferred to a 15 ml falcon tube and incubated with rotation for 1 hour at the appropriate temperature. Following one hour with shaking the bacteria were plated onto plates containing the appropriate antibiotic at two different dilutions and incubated overnight at the appropriate temperature.

2.8.5 Mini preparations of DNA

‘Minipreps’ were performed using the Qiaprep spin miniprep kit. Bacterial cultures were grown from a single colony in 1ml LB with selection overnight with rotation. The bacteria were centrifuged out of the supernatant at 7155 x g for 3 minutes in a microfuge. In the final step the DNA was recovered from the column with milli-Q water.

2.8.6 Maxi preparations of DNA

‘Maxipreps’ were performed with either the Qiagen plasmid maxi kit or the Qiagen endo-free plasmid maxi kit. Bacterial cultures were grown up from a single colony on a plate into a 10 ml starter culture in LB with selection overnight with rotation. The starter culture was transferred into 400 ml LB with selection and cultured overnight with rotation. The bacteria were centrifuged out of the culture medium at 6000 x g for 15 minutes at 4°C. In the final step the DNA pellet was resuspended in milli-Q water and the concentration of DNA was determined using a spectrophotometer.

2.8.7 Colony PCR

The master mix for the PCR was aliquoted into a 96-well PCR plate (Cell Projects, non-skirted) and the template for a 96-well plate was drawn onto an agar plate with the appropriate antibiotic. A single colony was picked off the master plate with a p200 pipette tip and gently touched onto the surface of the template plate, the pipette tip was then placed into the corresponding well of a 96-well plate. Once the plate was filled the 96-well plate was gently shaken and the pipette tips removed using tape. The 96-well plate was sealed and a colony PCR was performed using the standard PCR program.

2.8.8 Recombineering in EL250 and EL350

To activate the endogenous temperature sensitive recombineering machinery of EL350, before making the cells competent the flask was moved to a 42°C water bath and shaken. Gene expression is under the control of a temperature sensitive promoter which is activated by incubation at 42°C for 15 minutes. The temperature of the culture was measured using a sterilised thermometer and once it reached 42°C the flask was incubated with shaking for 15 minutes. The flask was transferred to an ice bath on a rotating plate and once the temperature was down to 4°C, the culture was incubated for 10 minutes. To induce Cre recombinase (EL-350) or FLP recombinase (EL-250) a 400 ml culture was treated with arabinose (1 in 100 volume of 10% (w/v) arabinose (Sigma)) for 1 hour at 32°C and then the bacteria were prepared for electroporation.

2.8.9 Gel extraction of DNA for ES cell targeting

Linearised DNA was run out in a 1% TAE gel, the appropriate band was cut out of the gel using a UV transilluminator, with minimal exposure, and the gel slice was put into the EluTrap Electroelution system (Whatman). The EluTrap was placed into a standard gel tank containing TAE buffer and run at 50 V overnight. The current was reversed for 15 seconds and the buffer and DNA was collected from the end chamber of the Elutrap. The DNA was precipitated using 0.3 M sodium acetate and 2.5 volumes of cold 100% ethanol and centrifuged at full speed for 30 minutes at 4°C. The supernatant was removed and discarded and the pellet was resuspended in 100 µl d.H₂O and the DNA was quantified using a spectrophotometer.

2.8.10 Sequencing

Sequencing was either performed from plasmid DNA or from PCR fragments. PCR reactions were treated with 1 µl SAP and 1 µl ExoI for 10 minutes and then the enzymes were inactivated at 65°C for 20 minutes. DNA was sequenced in house by Sanger sequencing and analysed using Sequencher (Gene Codes).

2.8.11 ES cell targeting

All of the ES cell work in this project (2.8.11-2.8.13) was very kindly carried out by Fiona Kilanowski, for which I am immensely grateful.

E14 ES cells were maintained in GMEM (Gibco) containing 10% FCS, 1mM Sodium Pyruvate (Sigma), 1 X Non-essential amino acids (Sigma), 40 μ M glutamine, 0.1 M β -mercaptoethanol, ~100 U/ml leukemia inhibitory factor (LIF, each batch individually optimised). The FCS and LIF were both batch tested in house on wild-type ES cells before use in targeting. E14 ES cells were grown in gelatin coated tissue culture flask and dishes. The culture vessels were coated for 15 minutes with 0.1% gelatin, then the excess was tipped off and the cells were added. E14 ES cells were cultured at 37°C under normoxic conditions.

To passage E14 ES cells, the media was removed, the cells rinsed with PBS and then the cells were incubated at 37°C with TrypLE Express (Gibco) for up to 5 minutes. Complete media was added up to a volume of 20 ml and the cells were centrifuged at 209 x g for 5 minutes.

2.8.12 Electroporation of ES cells

The cells from four 70-80% confluent T75 flasks were detached, pooled and counted. Approximately 1×10^7 cells were electroporated with 80-100 μ g DNA, and the same number of cells was needed as a no DNA control. The DNA was mixed with cells resuspended in 0.5 ml PBS and incubated on ice for 15 minutes. The mixture was transferred to a pre-chilled 4 mm cuvette and electroporated at 3 μ F, 0.8 kV with a time constant between 0.1-0.2 s. The cells were incubated on ice for 15 minutes following electroporation, then transferred to media and split over ten 90 mm plates.

2.8.13 Selection of transformed ES cells

Following electroporation the cells were incubated overnight without selection. The next day G418 was added to the media at a final concentration of 250 μ g/ml and the cells were cultured for 10-12 days in selection media.

2.8.14 ES cell screening

Following the selection period individual colonies were picked and transferred to individual wells of a 96-well plate. Once the well was confluent, half of the cells were frozen in freezing mix and the remaining cells were transferred to individual wells of a 24-well plate.

DNA was extracted from a confluent well of a 24-well plate. Following the wash in 70% ethanol the DNA was either spooled out or spun down at full speed in a microfuge for 10 minutes and the DNA was resuspended in 50 µl water. The ES cell DNA was stored at 4°C and 1 µl was used in genotyping PCRs.

2.8.15 Karyotyping

ES cells were karyotyped by preparing chromosome spreads and staining the G band of the chromosomes to count the number and check for abnormalities.

Colcemid (Karyomax colcemid, Gibco) was added to an exponentially growing ES cell culture to a final concentration of 10 µg/ml and the culture was incubated for 40 minutes. The cells were rinsed with PBS before adding trypsin and incubating at 37°C for 5 minutes to get a single cell suspension. The detached cells were resuspended in complete media and centrifuged at 253 x g for 5 minutes. 10 ml hypotonic solution (0.03 M potassium chloride, 0.02 M Tri sodium citrate made-up in d.H₂O) was added drop-wise whilst mixing on a vortex and the cells were incubated at room temperature for 20 minutes. The cells were centrifuged at 253 x g for 5 minutes and 10 ml fixative (3:1 mixture methanol to acetic acid) was added drop-wise whilst mixing on a vortex. The cells were incubated in fixative solution for 1 hour at room temperature and then centrifuged at 253 x g for 5 minutes. The cell pellet was resuspended in fixative solution to obtain a dense single cell suspension. The cell suspension was dropped from a height onto freshly cleaned (in fixative solution) frosted slides and allowed to air-dry. The slides were stained in KaryoMAX Giemsa stain (Gibco) diluted in Gurr buffer, pH6.8 (1 tablet in 1 L d.H₂O, Gibco).

2.8.16 Blastocyst injection

Blastocyst injections were carried out by Paul Devenney. The ES cells to be injected were grown at multiple cell densities across wells of a 24-well plate and on the day of injection, the best growing well was chosen and harvested to a single cell suspension. The cells were injected into C56BL/6J blastocysts obtained from superovulated, mated female mice. The females were sacrificed at 2.5 dpc and the 8-cell embryos and morula were harvested and cultured overnight in M16 media at 37 °C, 5% CO₂. Blastocysts were injected with 12-16 ES cells each and transferred to pseudopregnant recipient females.

2.9 Data analysis

Data was analysed using Microsoft Excel and drawn using Excel and GraphPad Prism 6. FACS data was initially analysed in FlowJo and exported to Excel/Graphpad.

Statistical analysis was performed using unpaired Students t-test to compare significant differences between treatments or χ^2 test to compare observed *versus* expected counts.

3. The response of Flt3-L dendritic cells to pathogenic and self DNA

3.1 Introduction

This chapter investigates the response of Flt3-L dendritic cells (FLDCs) to self and pathogenic DNA. Whilst the innate immune response to DNA has been intensively studied there is no comprehensive body of evidence studying the effect of DNA on a single immune cell type, the aim of this chapter was to provide a strong basis for further study on the regulation of the innate immune response to DNA by hBD3.

DNA can be sensed in the cell by a number of well characterised receptors and a number of recently described novel receptors. Since this thesis focuses on the response of dendritic cells to DNA, this introduction reviews what is known about DNA receptors that are expressed in DCs (summarised in table 3.1).

DCs express both membrane bound and cytosolic DNA receptors and cDCs and pDCs have distinct receptor expression patterns that are reflected by the respective functionality of these cell types (Naik et al., 2006). For the purpose of this thesis, only one membrane-bound DNA sensor is relevant, TLR9, as this is the only known membrane-bound DNA receptor in DCs. The TLR9-mediated response to DNA, particularly pathogenic DNA, is well characterised in DCs and the differential pathways that are triggered by TLR9 in cDCs compared to pDCs provide a mechanism for how pDCs produce large amounts of type I IFNs.

Table 3.1 Cellular DNA receptors that are expressed in DCs adapted from (Broz and Monack, 2013). CDN = cyclic dinucleotide

Receptor	Expressed in DCs?	Ligand	Response	References
TLR9	Yes: cDCs and pDCs	Unmethylated CpG motifs	Type I IFN IL-6, TNF- α Co-stimulatory markers	(Hemmi et al., 2000; Naik et al., 2005)
AIM2	Yes: BMDCs	dsDNA	Inflammasome assembly IL1 β	(Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Jones et al., 2010)
STING	Yes: FLDCs Maybe only cDCs	CDNs	Type I IFN	(Hervas-Stubbs et al., 2014; Ishikawa and Barber, 2008; Ishikawa et al., 2009b; Zhong et al., 2008)
DAI	ND	DNA	RIP3-mediated Necroptosis STING-mediated type I interferon	(Takaoka et al., 2007; Upton et al., 2012)
DDX41	Yes: cDCs	B-DNA and CDNs	STING-mediated type I IFN	(Parvatiyar et al., 2012; Zhang et al., 2011c)

DHX9	Yes: pDCs and cDCs	DNA, CpG-ODNs	MyD88-mediated Pro-inflammatory response	(Kim et al., 2010b; Zhang et al., 2011b)
DHX36	Yes: pDCs and cDCs	DNA, CpG-ODNs	MyD88-mediated type I interferon	(Kim et al., 2010b; Zhang et al., 2011b)
Ku70	Yes: ubiquitous	DNA	CDN synthesis Type III IFN	(Zhang et al., 2011a)
cGAS	Yes: FLDCs	DNA	CDN synthesis STING mediated type I interferon	(Li et al., 2013; Sun et al., 2013; Wu et al., 2013)
IFI16	Yes: GM-CSFDCs	dsDNA	STING-mediated type I interferon	(Kis-Toth et al., 2011; Unterholzner et al., 2010)
LRRFIP1	ND	B-DNA, Z-DNA	B-catenin-mediated type I interferon	(Yang et al., 2010)
RNA polymerase III	Yes: GM-CSFDCs	Detects cytoplasmic DNA and transcribes to RNA	RIG-I mediated type I interferons	(Ablasser et al., 2009; Chiu et al., 2009)

3.1.1 TLR9-dependent signalling

TLR9 is one of three endosomal TLRs in mice (TLR3, 7, 9), which sense extracellular DNA taken up via endocytosis. All murine DCs express TLR9, though pDCs express higher levels than cDCs (Naik et al., 2005). TLR9 activation in cDCs and pDCs leads to MyD88-dependent activation of IRF-5, NF- κ B and AP-1 inducing production of proinflammatory cytokines such as IL-6 and TNF- α . PDCs are capable of producing larger amounts of type I interferon following activation of TLR9 and TLR7 compared with cDCs. This is due to increased expression of the transcription factor IRF-7 in both mouse and human pDCs compared with other cell types, including cDCs (Honda et al., 2005; Izaguirre et al., 2003). TLR9-dependent IRF-7 activation is exclusively activated in pDCs leading to production of large amounts of type I interferon (figure 3.1). Whether IRF-5 and IRF-7 activation in pDCs occurs simultaneously or sequentially is not clear, whilst both transcription factors require a direct interaction with MyD88 and TRAF6 suggesting that there may be competition between the two pathways (Kawai et al., 2004; Takaoka et al., 2005), studies with synthetic ligands suggest that IRF-7 and IRF-5 are sequentially activated by TLR9 signalling from the early or late endosome respectively (Gilliet et al., 2008).

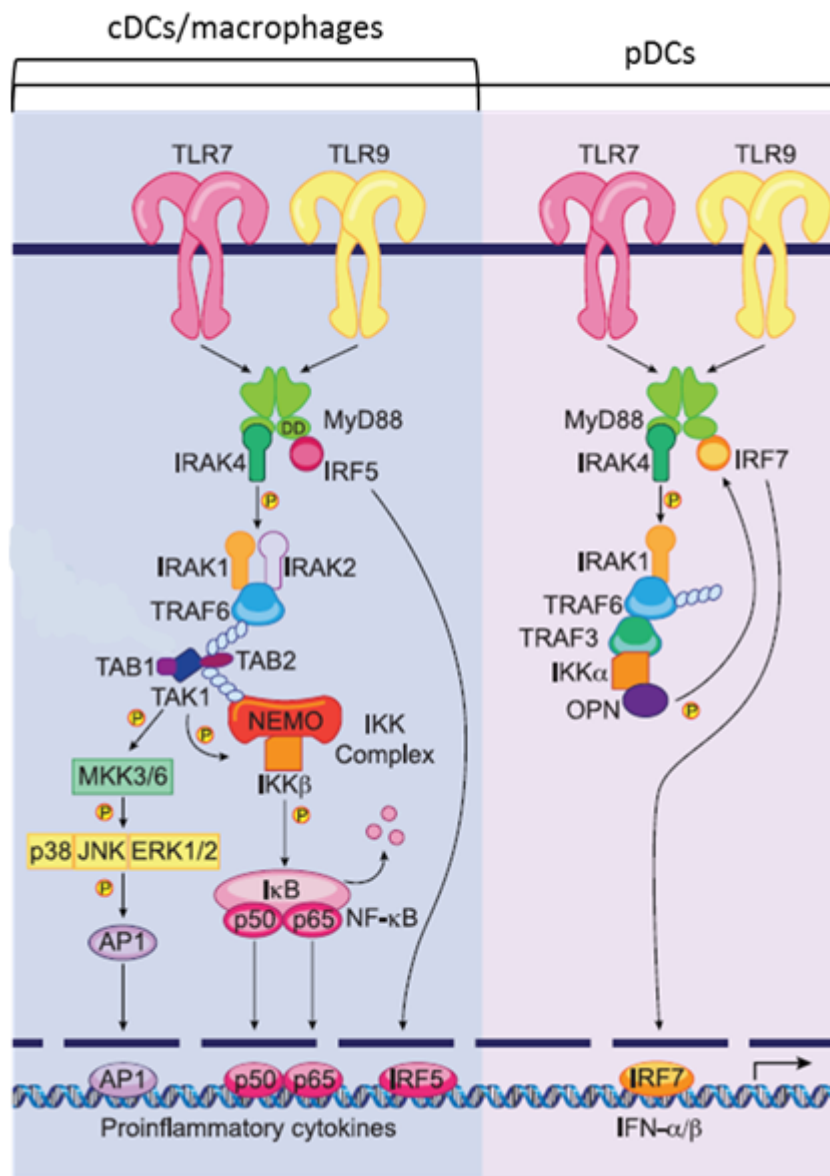


Figure 3.1 Different pathways are activated by TLR9 ligand binding in cDCs and macrophages compared with pDCs. TLR9 ligand binding activates a MyD88-dependent pathway leading to activation of AP1, NF-κB and IRF5. TLR9 activation in pDCs can also activate a unique MyD88-dependent pathway leading to activation of IRF7 and production of large amounts of IFN-α. (Adapted from Blasius and Beutler, 2010).

3.1.1.1 Intracellular trafficking and activation of TLR9

Full-length TLR9 is produced in the endoplasmic reticulum (ER) and requires the chaperone protein gp96 for correct folding (Yang et al., 2007). It was previously thought that TLR9 resided in the ER until stimulation, whereupon it would rapidly translocate to the endolysosome (Latz et al., 2004; Leifer et al., 2004). However, this model required a sensor that would sense the ligand and induce TLR9 relocalisation and activation, which could not be identified. Analysis of the carbohydrates on TLR9 revealed that it passes through the Golgi apparatus and it is now accepted that folded TLR9 is transported to the endolysosome via the Golgi apparatus (Chockalingam et al., 2009). This translocation is dependent on the multipass transmembrane protein UNC93B1, which has been confirmed by the lack of response of UNC93B1 deficient macrophages to TLR9 ligands (Tabeta et al., 2006). UNC93B1 is required for loading of full length TLR9 into COPII vesicles for ER to Golgi trafficking (Lee et al., 2013). UNC93B1 remains associated with TLR9 and regulates post-Golgi trafficking to the endolysosome which occurs via AP-2 or AP-3 mediated endocytosis from the cell surface (Engel and Barton, 2010; Lee et al., 2013) (figure 3.2). Autosomal recessive deficiency in UNC93B1 in humans has been identified in patients with herpes simplex virus-1 encephalitis (HSE), demonstrating an essential role for UNC93B1 in viral resistance (Casrouge et al., 2006).

Proteolytic cleavage of TLR9 is a key step for signalling and lysosomal cathepsin proteases and asparagine endopeptidase (AEP) are required for functional signalling via TLR9 (Bauer, 2013; Matsumoto et al., 2008; Park et al., 2008; Sepulveda et al., 2009). Proteolytic cleavage of TLR9 only occurs in the endolysosomal compartment and can be inhibited by blocking endosomal acidification (Park et al., 2008). N-terminal mutants of TLR9, which were processed normally, were non-functional demonstrating that the N-terminus is also involved in signalling. A positively charged region in the cleaved N-terminal region of TLR9 was identified which is required for CpG-DNA induced TLR9 activation (Peter et al., 2009). Therefore, a model has been proposed whereby the cleaved N-terminal fragment remains associated with the TLR9 homodimer and is involved in the conformational change that is required for TLR9 signalling (Li et al., 2012).

The process whereby TLR9 is not cleaved to a functional form until it reaches the endolysosomal compartment is a protective mechanism against the recognition of extracellular or cytoplasmic self-antigens. There has recently been evidence that neutrophils, one of the first cell types to detect infections, can express functional TLR9 at the cell surface, though it remains unclear as to whether the cell surface TLR9 is cleaved. The expression of cell surface TLR9 has implications for development of autoimmune disease, particularly since neutrophil extracellular traps (NETs) activate pDCs and amplify the immune response (Lindau et al., 2013).

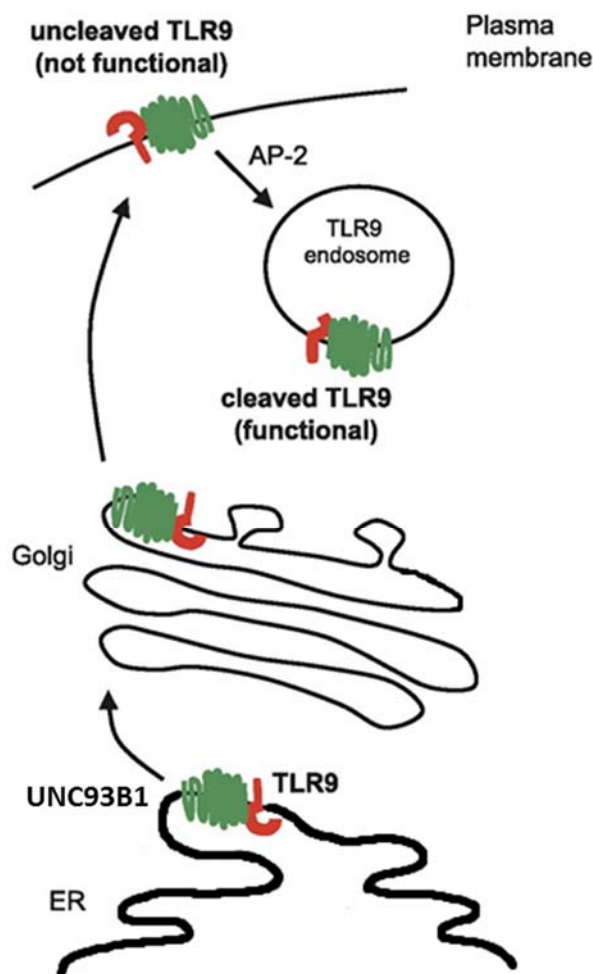


Figure 3.2 Post-translational processing of TLR9. Full length TLR9 is translated in the ER and associates with UNC93B1. This complex translocates via the Golgi

apparatus and the cell surface to the endosome where it is cleaved by cathepsins or AEP to mature TLR9. Adapted from (Lee et al., 2013).

3.1.1.2 Stimulation of TLR9 signalling

The classical ligand for TLR9 is unmethylated CpG-containing DNA (CpG-DNA) (Hemmi et al., 2000), these motifs are found at a substantially higher proportion in bacterial, viral and fungal DNA compared to mammalian DNA. The ‘compartmentalisation theory’ of TLR9 signalling suggests that it is the localisation of the DNA in the endosome, rather than the structure, which activates TLR9-dependent signalling, which is supported by evidence that chimeric TLR9 expressed at the cell surface responds to self-DNA (Barton et al., 2006).

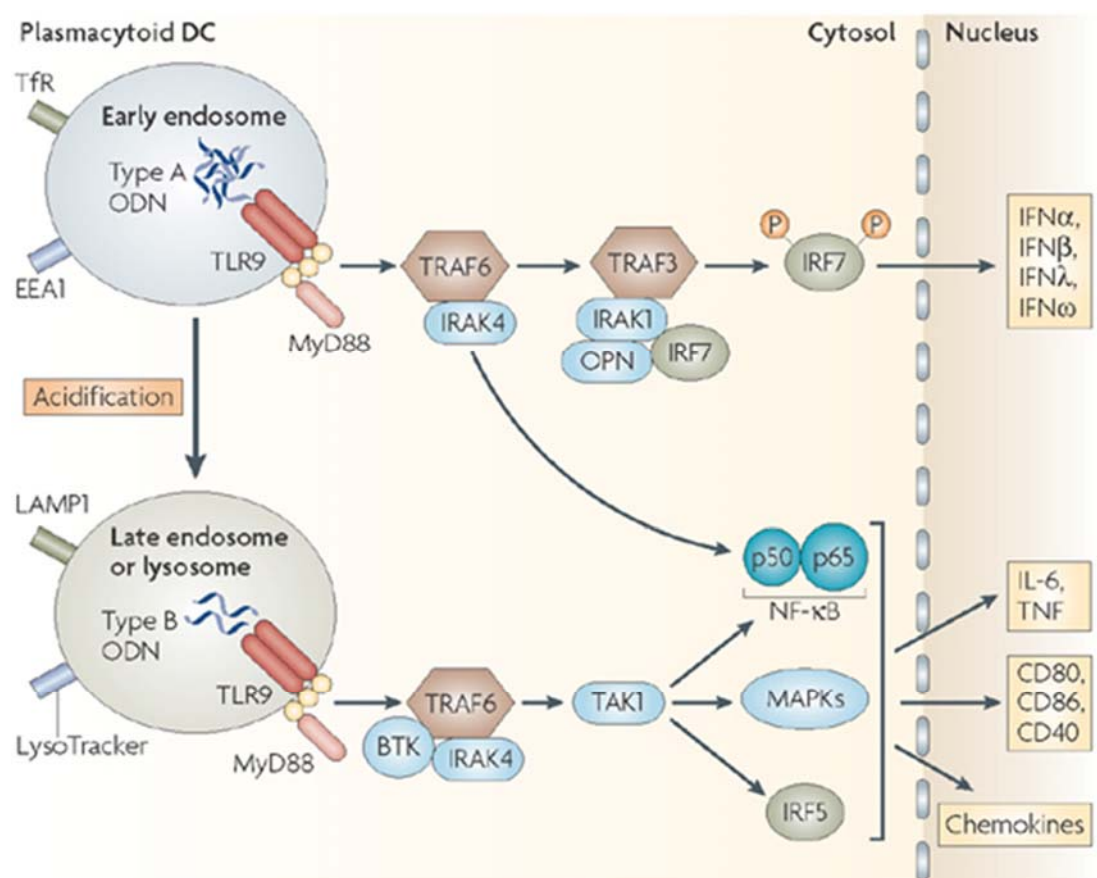
Synthetic CpG oligonucleotides (ODN) have been widely used to study TLR9-dependent immune responses and are divided into one of three classes based on the type of immune responses they induce, the cell type they stimulate and the structure of the ODN (table 3.2).

Table 3.2 The sequence of the three classes of CpG ODNs and the response that each stimulates in human pDCs (Gilliet et al., 2008; Kerkmann et al., 2003; Moseman et al., 2004) NB: Bases in capital letters are ‘natural’ phosphodiester linked whilst bases in lower case are nuclease resistant phosphorothioate linked.

Class	Sequence (5'-3')	Response
CpG-A (1585)	ggGGTCAACGTTGAgggggg (20 mer)	Type I interferon
CpG-B (1826)	tccatgacgttcctgacgtt (20 mer)	IL-6, TNF- α Co-stimulatory marker up-regulation CD40, CD80, CD86
CpG-C (2395)	tcgtcgttttc <u>gggcgc</u> :gcgccg (22 mer)	Type I interferon

The two distinct classes of CpG ODNs (A and B) stimulate a different response which is thought to be due to structure-specific cellular localisation of the ODN and

activation of different pathways. CpG-A forms aggregates due to the 3' poly-G tail, which localise to and are retained in the early endosomes stimulating robust production of IFN- α in an IRF-7 dependent manner (Kerkmann et al., 2005; Wu et al., 2004). On the other hand, CpG-B remains as linear monomers and rapidly traffics to the late endosome stimulating a proinflammatory-type response characterised by NF- κ B and IRF-5-dependent production of pro-inflammatory cytokines, up-regulation of co-stimulatory markers and production of chemokines (Guiducci et al., 2006). This effect is sequence independent and due to the oligomeric vs. monomeric state of the ODNs (Guiducci et al., 2006) (figure 3.3).



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Figure 3.3 pDCs responds differently to class A or class B CpG-ODN due to different endo-lysosomal localisation triggering distinct pathways. Multimeric

CpG-A aggregates in the early endosome, predominantly triggering an IRF7-dependent, type I IFN response. In contrast, monomeric CpG-B signals via TLR9 in the late endosome triggering a ‘proinflammatory-type’ response via IRF5, NF- κ B and MAPKs, characterised by expression of costimulatory makers, chemokines and proinflammatory cytokines (Gilliet et al., 2008).

Recent work has challenged the dogma that TLR9 is triggered by unmethylated CpG-DNA and proposed a ‘compartmentalisation theory’ whereby any DNA that is within the endolysosomal pathway will be detected by TLR9 and stimulate an immune response. This model would suggest that evolutionary pressure may have driven endosomal localisation of TLR9 to avoid sensing of host DNA and an autoimmune response (Wagner, 2008). Yasuda *et al* transfected double-stranded vertebrate DNA into the endosomes of murine FLDCs stimulating a partially TLR9-dependent pathway, and demonstrated the same effect following transfection of non-canonical phosphorothioated CpG ODNs (Yasuda et al., 2005) (Yasuda et al., 2006). FLDCs also responded to base-free phosphodiester backbone in a TLR9-dependent manner suggesting that the bases are in fact dispensable for TLR9 activation (Haas et al., 2008). TLR9-dependent stimulation of DCs by double stranded DNA-containing immune complexes was dependent on DNA containing CpG motifs, and whilst unmethylated CpG-DNA induced a larger response, methylated CpG-rich DNA also induced a response (Yasuda et al., 2009). DNA-containing immune complexes are associated with systemic lupus erythematosus suggesting that release of CpG-rich self-DNA may exacerbate the pathogenesis of SLE via TLR9 (Yasuda et al., 2009). These data together suggest that the recognition of DNA by TLR9 is simple and that TLR9 is activated by any phosphodiester DNA that is present in the endolysosome, although there are differences in the extent of activation by different structures, such as methylation, sequence and double-stranded compared with single stranded. This is still a controversial area and there is no conclusive evidence to back up a specific model of TLR9-dependent signalling.

3.1.2 TLR9-independent signalling

There are a number of cytoplasmic DNA receptors which predominantly initiate a type I interferon or inflammasome-mediated response. The cytoplasm is

predominantly a DNA-free compartment, and the presence of DNA in the cytoplasm is indicative of infection or tissue damage, therefore it is vital that cytoplasmic DNA is sensed and appropriate immune response are initiated.

DAI (DNA-dependent activator of IRFs) was the first cytoplasmic receptor described in MEFs (Takaoka et al., 2007), however it was shown to have a redundant role in certain cell-types indicating that there were other DNA sensors which activated the same response (Wang et al., 2008). Transfected poly(dA:dT) can be transcribed into 5'triphosphate RNA which is a RIG-I ligand, demonstrating another mechanism whereby cytoplasmic DNA activates type I interferon signalling indirectly (Chiu et al., 2009). Indications of TLR9-independent, MyD88-dependent pathway-mediated type I interferon production led to the identification of the DExD/H-box helicases DHX36 and DHX9 which were essential for the response to cytosolic CpG-A and CpG-B respectively (Kim et al., 2010b).

STING (Stimulator of IFN genes) was recently identified as both a signalling adaptor acting upstream of TBK1 and as a sensor of cyclic dinucleotides (CDNs) as second messengers of cytoplasmic DNA (Ishikawa et al., 2009b). *STING* deficient mice demonstrate impaired interferon induction to bacterial, viral and mammalian DNA, as well as to a number of DNA viruses (Ishikawa et al., 2009b). STING is required as an adaptor for the type I interferon response mediated by DDX41, DAI, RNA polymerase III and IFI16. More recently it was shown that STING directly recognises and is activated by the second messenger cyclic GMP-AMP (cGAMP) and activates IRF3-dependent type I interferon response. cGAMP synthase (cGAS) is a DNA sensor which catalyses production of cGAMP from cytosolic DNA (Sun et al., 2013; Wu et al., 2013).

Cytosolic DNA also stimulates assembly of the inflammasome, production of IL-1 β and IL-18 and induction pyroptotic cell death. AIM2 inflammasome activation requires DNA binding to AIM2, concurrent binding of the adapter molecule ASC, NF- κ B dependent production of pro-IL-1 β and pro-IL-18 and caspase-1 dependent cleavage to mature IL-1 β and IL-18 (Hornung et al., 2009). Pyroptosis is caspase-1 dependent apoptosis, and named for the involvement of IL-1 β and IL-18 which are pyroptotic (fever-inducing) cytokines (Cookson and Brennan, 2001).

3.1.3 Recognition of DNA and autoimmune disease

Detection of pathogenic DNA is one mechanism whereby infections are recognised and proinflammatory pathways are activated to clear the infection. However, when the immune response is aberrantly activated in response to self-DNA this can lead to the development of autoimmune disorders. Immune activation by self-DNA and self-DNA containing complexes is known to underlie the pathogenesis of diseases such as systemic lupus erythematosus (SLE) where self-DNA triggers an hyperreactive B cell response leading to production of antibodies against DNA (antinuclear antibodies) (Dorner et al., 2011). DNA-peptide complexes found in the sera of SLE patients chronically activate pDCs via TLR9 leading to IFN- α production and are deposited in blood vessels leading to extensive tissue damage (Lande et al., 2011; Means et al., 2005). Aicardi-Goutieres Syndrome (AGS), a rare recessive disorder present many clinical similarities to SLE, including production of antinuclear antibodies. Mutations have been identified in AGS in genes that are associated with nucleic acid metabolism leading to accumulation of nucleic acids that are recognised as damage signals (Crow et al., 2006a; Crow et al., 2006b; Lee-Kirsch et al., 2014; Rice et al., 2009; Rice et al., 2012). Inappropriate sensing of DNA coupled with the antimicrobial peptide LL37 by pDCs has also been described as a potential mechanism for the pathogenesis of psoriasis. Lande *et al.* demonstrated that self-DNA associated with cell death, which is normally non-stimulatory, is converted into a potent immune ligand in the presence of LL37, stimulating TLR9-dependent production of IFN- α by pDCs. Confocal imaging and flow cytometry indicated that DNA-LL37 complexes rapidly translocated into the endosomal compartment of pDCs. In the context of psoriasis, DNA released from dying keratinocytes may form a complex with LL37, which is detected by pDCs, leading to production of IFN- α (Lande et al., 2007). It is likely that, rather than a DNA-peptide complex being intrinsically more immunostimulatory, the DNA is protected from endogenous nucleases in the extracellular environment and detected in the same way as pathogenic DNA. This model is supported by findings that SLE patients with mutations in DNaseI, as well as DNaseI deficient mice, develop autoantibodies against nuclear antigens (Yasutomo et al., 2001). It is not clear how the DNA-peptide

complex enters pDCs whether by phagocytosis or receptor-mediated endocytosis (Brenicova and Diebold, 2013).

3.1.4 Chapter aims

There is clearly a great deal of complexity to the innate immune response to DNA and the differentiation between host and pathogenic DNA is essential to prevent autoimmunity. This chapter does not present novel ideas and results, but rather consolidates what is known about the immune response to DNA by studying it in a single cell type and in a consistent manner.

This chapter aims to:

- Optimise FLDC culture for the study of the immune response to DNA
- Understand the differences between the innate immune response to self vs. pathogenic genomic DNA

3.2 Results

3.2.1 Flt-3 dendritic cells for studying the response to nucleic acids

Since there are few comprehensive studies comparing the response to self and pathogenic DNA, initial experiments investigated the innate immune response to DNA. The majority of the work in this thesis was performed *in vitro* so it was crucial to identify a useful and relevant cell type.

As discussed in chapter 1, pDCs express high levels of TLR9 and produce large amounts of IFN- α in response to nucleic acid ligands. pDCs can respond to both pathogenic hypomethylated nucleic acids as well as host derived nucleic acid-containing complexes (Reviewed by Lande and Gilliet, 2010) making them the ideal model to study the responses to pathogenic and host nucleic acids in combination with hBD3.

pDCs represent an extremely rare population of cells *in vivo* (less than 1% of spleen cells), although injection of Fms-like tyrosine kinase-3 ligand (Flt3-L) greatly increases the numbers of pDCs and cDCs (Björck, 2001; Maraskovsky et al., 1996). A mixed population of cDCs and pDCs can be derived following culture of bone marrow derived cells with Flt3-L (Brawand et al., 2002; Gilliet et al., 2002). At the end of the culture period Flt3-L induced dendritic cells (FLDCs) consist of a mixed population of CD11c⁺ B220⁺ plasmacytoid DCs (pDCs) and CD11c⁺ B220⁻ conventional DCs (cDCs) (approximately 15% and 85% respectively (figure 3.4)) (Brawand et al., 2002; Gilliet et al., 2002). Using flow cytometry, FLDCs can be segregated into cDCs and pDCs on the basis of cell surface markers and activation of each population can be analysed individually by measuring the expression of cell surface costimulatory markers, CD40, CD80, CD86 and MHCII (figure 3.4).

FLDCs represent the steady state splenic DC populations, although the relative proportions of the populations are altered. The cDC population can be further divided into CD24^{hi} and CD11b^{hi}, which represent CD8⁺ and CD8⁻ splenic cDCs respectively (Naik et al., 2005) though for the purposes of this thesis cDC refers to all CD11c⁺B220⁻ cells. The different DC subsets are characterised not only on the expression of cell surface markers but also on the expression of TLRs, chemokine

receptors (CCRs) and specific responses, for example cell specific production of particular cytokines (Naik et al., 2005).

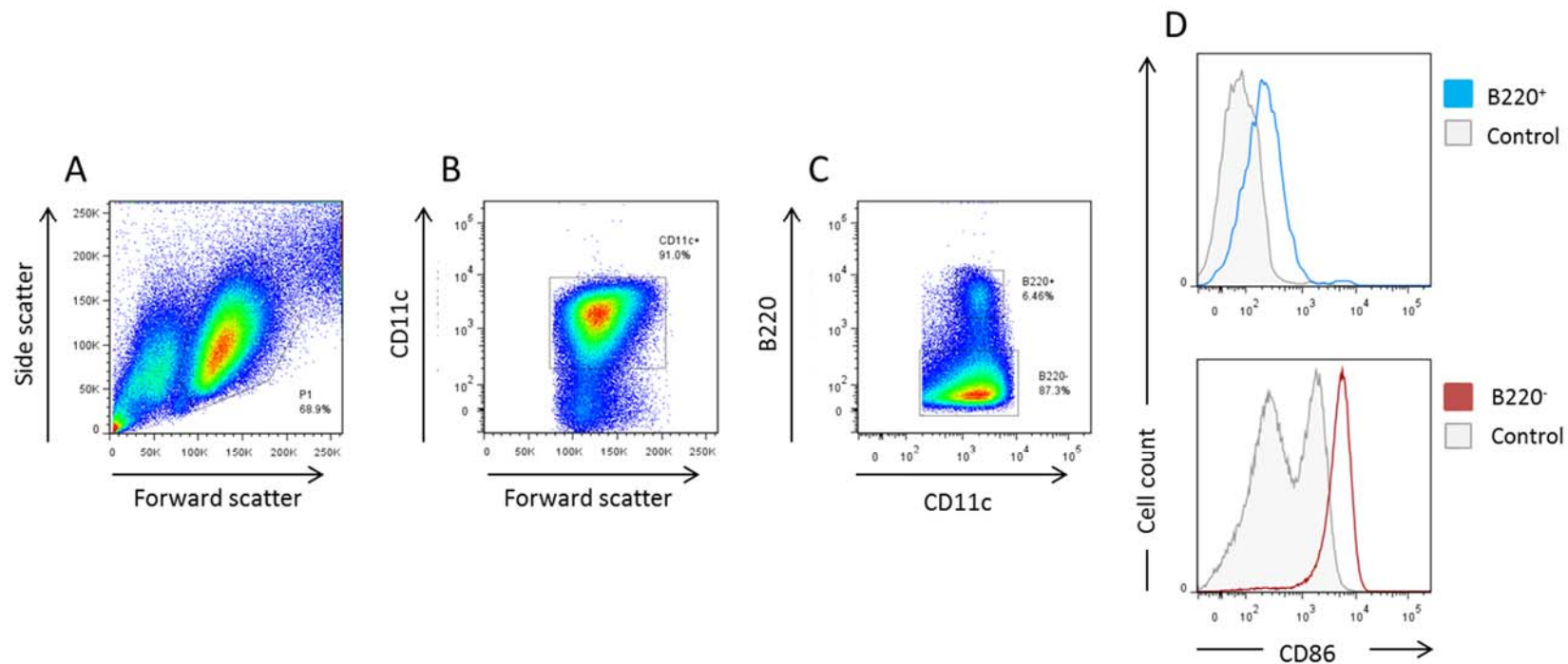


Figure 3.4 Gating strategy for flow cytometry to determine activation of cDCs and pDCs. Following overnight treatment of cultured FLDCs, they were harvested, stained with antibodies to assess costimulatory marker expression and assayed by flow cytometry. The total cell population was gated to exclude dead cells, cell debris and clumped cells on the basis of size (A, P1) and the remaining cells were gated into CD11c⁺ or CD11c⁻ (B). CD11c⁺ cells were gated into B220⁺ (pDCs) and B220⁻ (cDCs) (C) and the expression of costimulatory markers (CD86 here as a representative) was compared to untreated cells or an isotype control in both the B220⁺ population (blue) and the B220⁻ population (red) (D).

3.2.2 Bacterial DNA stimulates FLDCs

Unmethylated CpG-containing DNA induces a large response from dendritic cells via a TLR9-dependent mechanism (Hemmi et al., 2000). There are three classes of synthetic CpG oligodeoxynucleotides (ODNs) which differ in their potential to stimulate different immune cells. Class A CpG ODNs classically stimulate production of IFN- α from pDCs, however these synthetic CpG ODNs contain disproportionately high numbers of unmethylated CpG motifs in a short sequence compared to the proportion of CpG motifs found in bacteria (Dalpke et al., 2006). These synthetic ligands have been widely used to study the immune response to bacterial DNA, however there are few reports on the innate immune response to 'natural' bacterial genomic DNA.

Previous work using CpG-ODNs treating FLDCs with 1 $\mu\text{g/ml}$ initiated a robust and reproducible immune response. Therefore, FLDCs were treated with 1 $\mu\text{g/ml}$ of *E. coli* DNA and the response was analysed by ELISA and flow cytometry (figure 3.5). At this concentration of *E. coli* DNA, IFN- α and IL-6 production was significantly increased (figure 3.5 A) and expression of costimulatory markers on both pDCs and cDCs was up-regulated (figure 3.5 B). As a control, FLDCs were treated with DNase I digested *E. coli* DNA (figure 3.5 C) which failed to stimulate any response confirming that the response was specifically DNA dependent and not due to any contaminants in the DNA preparation (figure 3.5 C).

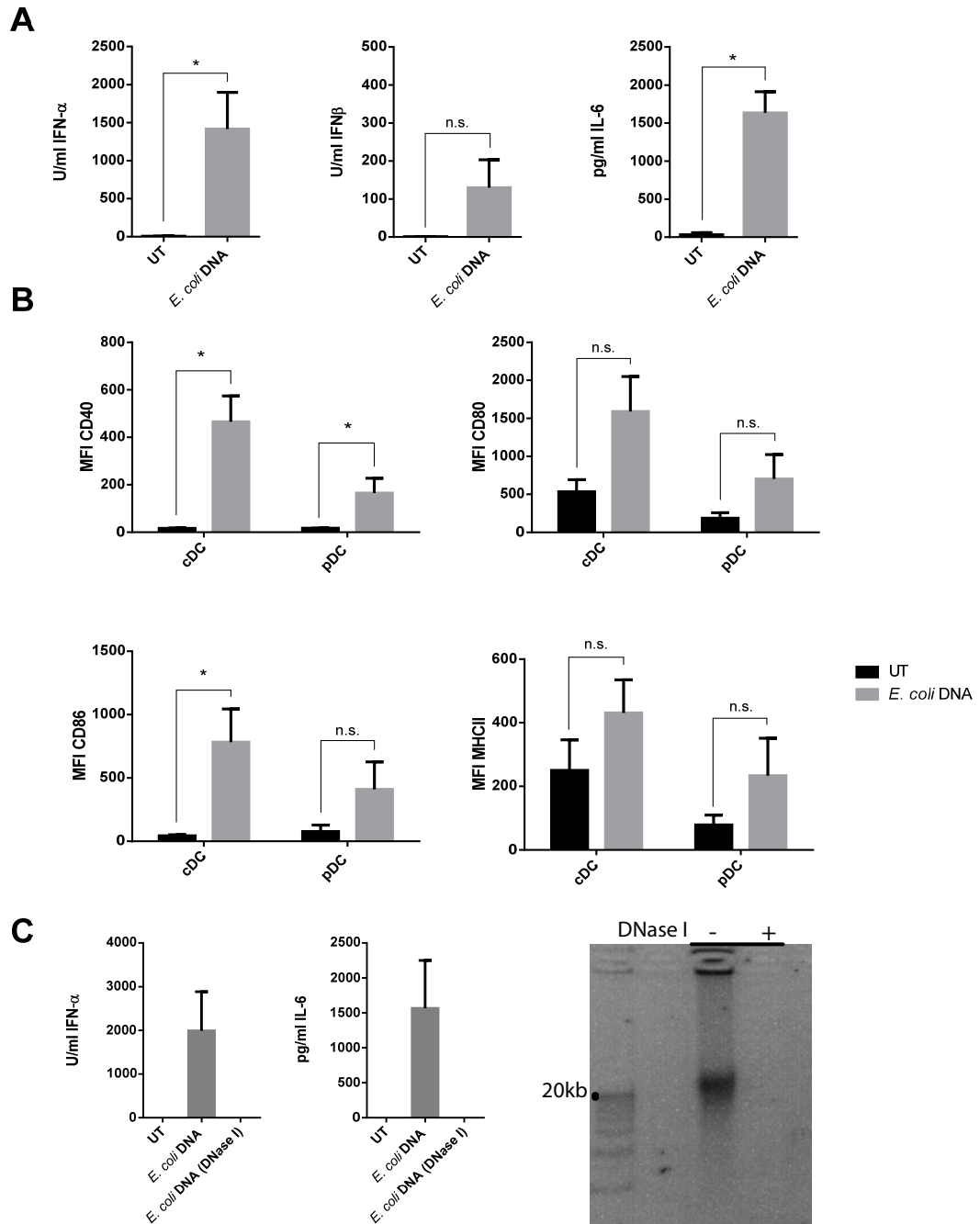


Figure 3.5 *E. coli* DNA stimulates FLDCs to produce cytokines. FLDCs were incubated with 1 $\mu\text{g/ml}$ *E. coli* DNA overnight. Cytokine production was assayed by ELISA (A) and costimulatory marker expression was analysed by flow cytometry (B). FLDCs were incubated with untreated or DNase I treated *E. coli* DNA, as demonstrated by agarose gel electrophoresis and cytokine production was measured by ELISA (C). The data shown is the mean of 4 independent experiments performed in triplicate $\pm$ standard error of the mean (A and B) or the mean of 2 independent

experiments performed in triplicate $\pm$ standard deviation of the triplicate wells (C).

* $p < 0.05$, unpaired t-test.



3.2.3 The response of FLDCs to bacterial DNA is TLR9-dependent

To confirm that bacterial DNA is stimulating FLDCs as expected via a TLR9-dependent mechanism, TLR9 deficient (TLR9^{-/-} mice were a kind gift from David Gray) FLDCs were treated with *E. coli* DNA.

TLR9^{-/-} FLDCs did not produce any IFN- α or IL-6 in response to *E. coli* DNA (figure 3.6 A) and TLR9^{-/-} cDCs did not up-regulate expression of CD86 in response to *E. coli* DNA unlike wild-type cDCs (figure 3.6 B). Although TLR9^{-/-} pDCs had a high baseline level of CD86 expression, there was no increase in CD86 expression in response to *E. coli* DNA demonstrating the increased response seen to *E. coli* DNA is TLR9-dependent in both cDCs and pDCs (figure 3.6 B).

These data together confirm that the response to *E. coli* DNA is entirely TLR9-dependent.

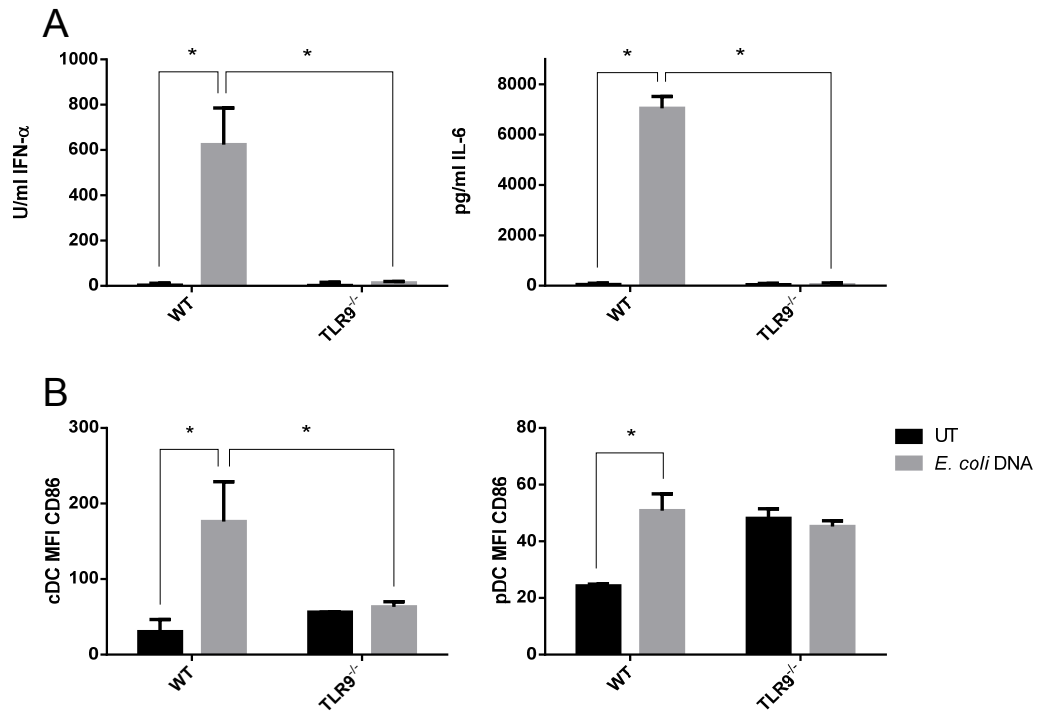


Figure 3.6 The response to *E. coli* DNA is TLR9-dependent. Wild-type or TLR9^{-/-} FLDCs were incubated with 1 μ g/ml *E. coli* DNA overnight. Cytokine production was assayed by ELISA (A) and costimulatory marker expression was analysed by flow cytometry (B). The data shown is the mean from 3 independent experiments performed in triplicate $\pm$ standard error of the independent experiments. * $p < 0.05$, unpaired t-test.

3.2.4 Transfected bacterial DNA stimulates a partially TLR9-independent response

Cytoplasmic DNA triggers a TLR9-independent innate immune response (Ishii et al., 2006; Okabe et al., 2005; Stetson and Medzhitov, 2006). Microbial DNA is found in the cytoplasm when host cells are infected, rather than it being found in the endosome when bacteria or infected cells are engulfed by phagocytosis. Stimulator of interferon genes (STING) (Ishikawa and Barber, 2008; Ishikawa et al., 2009b) and absent in melanoma 2 (AIM2) (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009) have been identified as major mediators in the response to cytoplasmic microbial DNA (Barber, 2011; Ishikawa and Barber, 2011). STING recognizes the second messenger cGAMP which is produced by cGAS synthase on detection of cytoplasmic DNA, triggering IRF-3 dependent production of type 1 interferons (Sun et al., 2013; Wu et al., 2013; Zhang et al., 2013); whilst AIM2 recognises DNA directly via a HIN200 domain leading to caspase-1 dependent cleavage of pro-IL-1 β to active IL-1 β .

To attempt to test the response of FLDCs to cytoplasmic *E. coli* DNA, DNA was delivered into FLDCs by transfection using lipofectamine LTX, a proprietary cationic-liposome formulation optimised for sensitive primary cells, and the response was measured in wild-type compared to TLR9^{-/-} FLDCs by ELISA. This experiment relied on evidence that transfection ultimately delivers ligands into the cytoplasmic via the endocytic pathway (Hoekstra et al., 2007; Tamaddon et al., 2007), however further experiments could be done to prove that transfection did indeed relocate the DNA, such as confocal microscopy using markers for cellular compartments such as endosomes and membranes.

Transfecting *E. coli* DNA precipitated a significantly larger cytokine response than untransfected *E. coli* DNA (figure 3.7). When *E. coli* DNA was transfected into TLR9^{-/-} FLDCs there was a small TLR9-independent response compared to no TLR9-independent response to untransfected *E. coli* DNA (figure 3.7). This result suggests that as well as increasing overall endocytosis, transfection may deliver some DNA to the cytoplasm, activating a TLR9-independent pathway, however further empirical evidence would be required to support this conclusion.

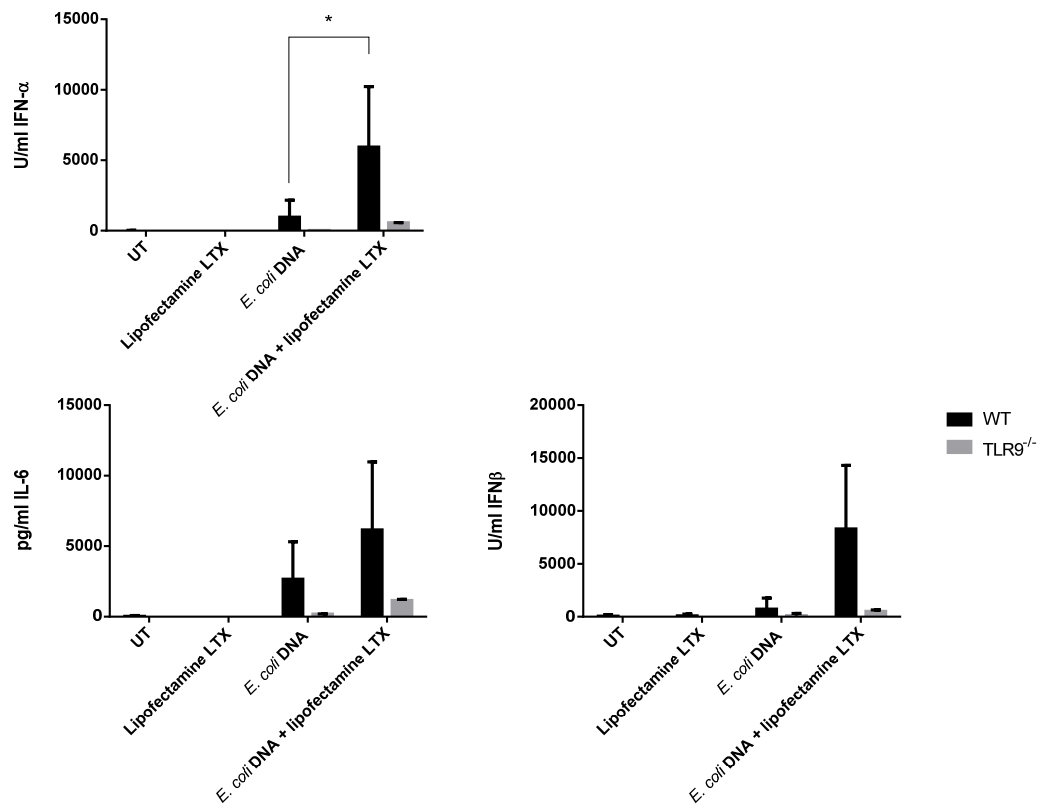


Figure 3.7 Transfection of *E. coli* DNA induces a large, partially TLR9-independent response in FLDCs.

Wild-type or TLR9^{-/-} FLDCs were incubated with, or transfected with, 1 µg/ml *E. coli* DNA overnight. Cytokine production was measured by ELISA. The data shown are the mean of 2 independent experiments performed in triplicate ± standard deviation of the replicates *p<0.05, unpaired t-test

3.2.5 Transfected self-DNA stimulates FLDCs

Mouse genomic DNA (self-DNA) was extracted from ES cell clones and purified. FLDCs were treated with self-DNA at a range of concentrations which did not induce any detectable production of IFN- α , IFN β or IL-6. However, when 1 μ g/ml of self-DNA was transfected with lipofectamine LTX into FLDCs, significant amounts of IFN- α , IFN- β and IL-6 were produced and cDCs and pDCs up-regulated expression of costimulatory markers (figure 3.8 A and B).

The response to transfected self-DNA was completely ablated following DNase I digestion (figure 3.8 C) confirming that the response to transfected self-DNA is a DNA-dependent response and not due to contaminants in the DNA preparation.

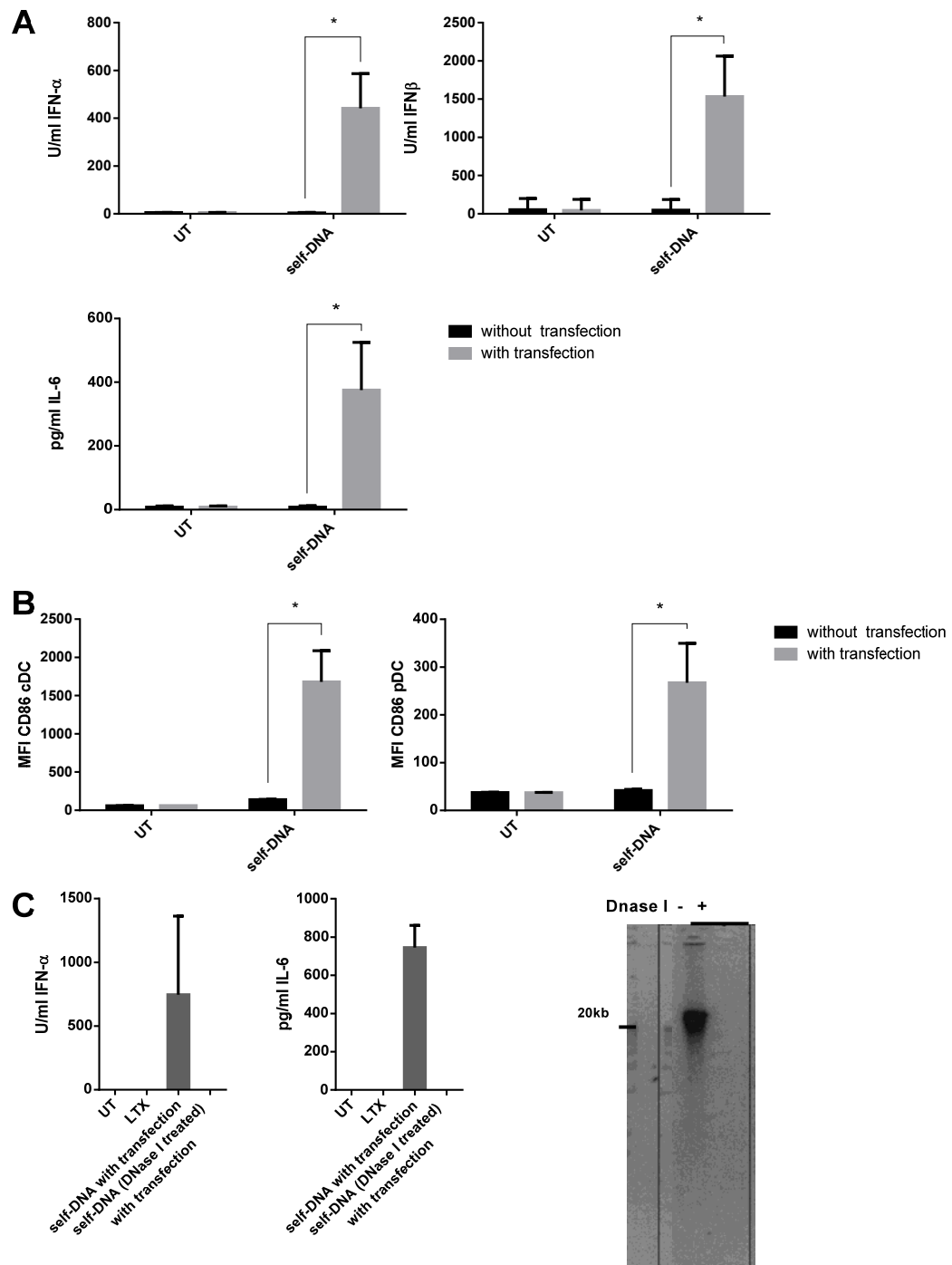


Figure 3.8 Self-DNA has to be transfected into FLDCs to induce an innate immune response. FLDCs were incubated with, or transfected with, 1 μ g/ml mouse genomic DNA overnight. Cytokine production was measured by ELISA (A) and expression of costimulatory markers on cDCs and pDCs was measured by flow cytometry (B). FLDCs were transfected with DNase I digested mouse genomic DNA

overnight and cytokine production was measured by ELISA (C). Data shown is the mean of 3 or more independent experiments performed in triplicate $\pm$ standard error (A and B) or the mean of 2 independent experiments performed in triplicate $\pm$ standard deviation of replicates (C). * $p < 0.05$, unpaired t-test

3.2.6 The response to transfected self-DNA may be TLR9-independent

To determine whether the response to self-DNA was TLR9-dependent, the response of wild-type FLDCs to self-DNA was compared with the response of TLR9^{-/-} FLDCs. There was not a difference in preliminary experiments in the production of IL-6 or IFN- α in response to transfected self-DNA in wild-type compared with TLR9^{-/-} FLDCs suggesting that this response to self-DNA is not TLR9-dependent (figure 3.9). This result is in agreement with the previous finding that self-DNA has to be transfected to induce an immune response (figure 3.8) suggesting that self-DNA induces a response via a cytoplasmic DNA receptor. In contrast however, there was a difference in the production of IFN- β in response to transfected self-DNA between wild-type and TLR9^{-/-} FLDCs. No conclusions can yet be drawn from this data since it was only performed twice using TLR9^{-/-} FLDCs but it may indicate that the production of IFN- β in response to transfected self-DNA is partially TLR9-dependent.

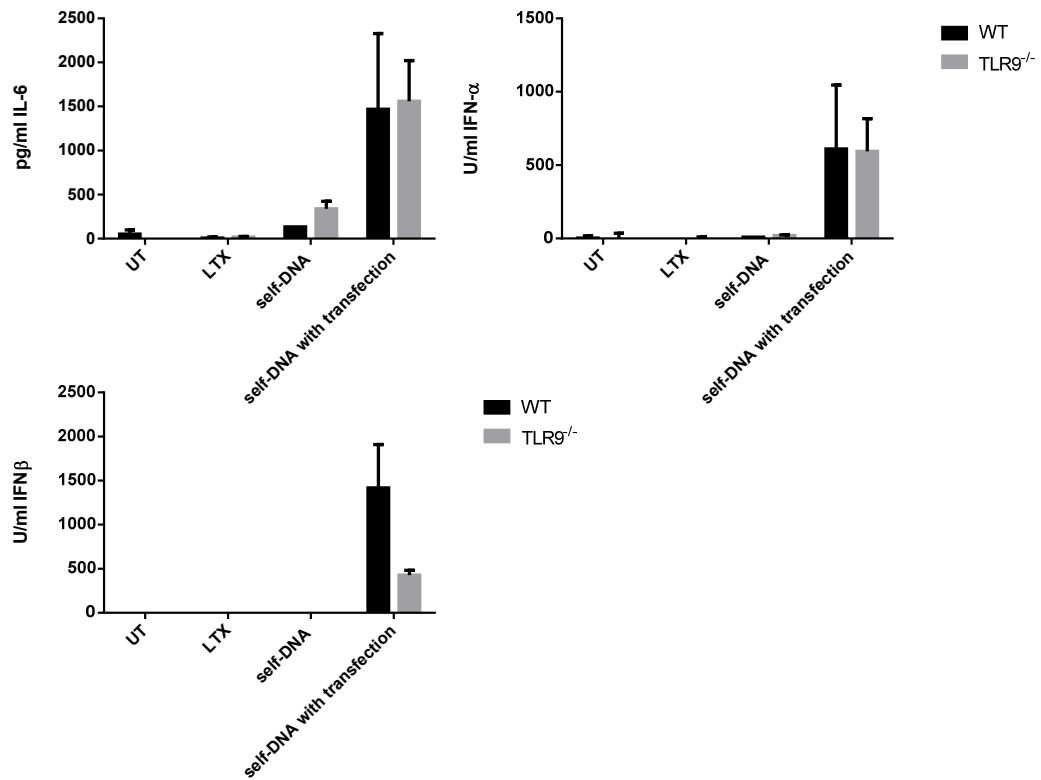


Figure 3.9 The response to transfected self-DNA is mostly TLR9-independent.

Wild-type or TLR9^{-/-} FLDCs were incubated with, or transfected with 1 µg/ml mouse genomic DNA overnight. Cytokine production was measured by ELISA. The data shown is the mean of 2 independent experiments performed in triplicate ± standard deviation of the replicates.

3.3 Discussion

This chapter has explored the immune response of Flt3-L dendritic cells to pathogenic compared with self-DNA in order to provide a strong basis for further experiments in this thesis. The following points summarise the major findings of this chapter:

- *E. coli* DNA stimulates up-regulation of costimulatory markers on cDCs and pDCs and increased production of type I interferons and IL-6
- This response is entirely TLR9-dependent
- Transfected *E. coli* DNA stimulates a large, partially TLR9-independent response
- Self-DNA has to be transfected to stimulate any response
- The response to transfected self-DNA may be partially TLR9-independent, however this experiment needs repeating.

3.3.1 Flt-3L dendritic cells respond differently to bacterial compared to self-DNA

In an effort to produce a comprehensive body of data investigating the innate immune response to DNA, the response of FLDCs exposed to either bacterial (*E. coli*) DNA or ‘self’ (mouse genomic) DNA was investigated.

FLDCs mounted a robust response to both types of DNA involving production of proinflammatory cytokines, type I interferon and up-regulation of costimulatory markers. However, in contrast to the response to *E. coli* DNA, the response to self-DNA was dependent upon transfection. This is consistent with a model whereby DNA in the cellular environment is taken up into the endocytic compartment of FLDCs by random sampling. Whilst both pathogenic and self-DNA can access TLR9 in the endocytic compartment of FLDCs, TLR9 signalling is strongly activated unmethylated CpG motifs found in microbial DNA, meaning that bacterial DNA triggers significant cytokine production and expression of costimulatory markers in FLDCs. cDCs respond via NF- κ B dependent induction of proinflammatory cytokines and costimulatory markers. pDCs activate both a unique IRF7-dependent induction of type I interferons from the early endosome, as well as an NF- κ B dependent

induction of proinflammatory cytokines and costimulatory markers from the late endosome or lysosome (Blasius and Beutler, 2010).

3.3.2 Unmethylated CpG motifs in bacterial DNA stimulate a TLR9-dependent response

Consistent with TLR9-dependent sensing of hypomethylated DNA in the endosome, TLR9 deficient FLDCs failed to respond to stimulation with *E. coli* DNA, confirming that *E. coli* DNA stimulates FLDCs via TLR9.

Additionally, methylation of *E. coli* DNA suppressed the immune response in wild-type FLDCs (chapter 4, figure 4.9), in agreement with DNA methylation being the dominant factor in sensing of DNA via TLR9 (Hemmi et al., 2000).

3.3.3 Transfection of self-DNA stimulates a TLR9-independent response, likely via a cytoplasmic receptor

Self-DNA added to the supernatant did not stimulate an immune response in FLDCs which is likely due to a low proportion of unmethylated CpG motifs meaning it is not recognised by TLR9 in the endosome following uptake by endocytosis, or may be due to inefficient uptake. However, transfection of self-DNA induced up-regulation of cytokines, type-1 interferons and increased expression of costimulatory markers on cDCs and pDCs suggesting that transfection increases uptake of self-DNA and may activate other receptors, such as cytoplasmic DNA receptors. This raises an interesting discussion about where transfection localises self-DNA so that it is recognised as an immune stimulus. The overwhelming viewpoint regarding where cationic lipid transfection targets DNA seems to be that the lipid vesicles are taken up via the endosomal pathway, and a critical part of DNA delivery is escape from the endosomal pathway into the cytoplasm (Hoekstra et al., 2007; Tamaddon et al., 2007; Xu and Szoka, 1996; Zelphati and Szoka, 1996). Whilst the data supports this model, empirical evidence obtained via methods such as confocal microscopy would establish whether this is indeed the case.

Preliminary evidence suggests that the response to transfected self-DNA is predominantly TLR9-independent, supporting a model whereby transfected self-

DNA is detected by an alternative receptor. However there were not enough repeats of this experiment to draw any firm conclusions and future priorities would include further repeats and imaging techniques to establish whether the localisation of the nucleic acids is altered following transfection.

Transfected *E. coli* DNA stimulated both TLR9-dependent and -independent responses, which supports a model whereby DNA-containing liposomes are taken up by the endosomal pathway and released into the cytosol. If liposomes were taken directly into the cytosol the response would be entirely TLR9-independent, in fact the response observed was mostly TLR9-dependent, which is likely due to transfection drastically increasing the efficiency of uptake and inducing a large TLR9-dependent response.

Although untransfected self-DNA did not induce a response in FLDCs, it was still being taken up into cells, as shown by flow cytometry analysis (chapter 4, figure 4.13). The absence of response despite uptake is consistent with a lack of an endosomal receptor that responds to methylated DNA, whilst transfected self-DNA is likely to activate a cytoplasmic receptor. To confirm that self-DNA activates a cytoplasmic receptor, endosomal signalling could be inhibited via disruption of endosomal acidification. This is discussed further in chapter 4, briefly however, preliminary experiments were unsuccessful apparently due to significant cell death.

Overall, these results are compatible with what is known about cytosolic DNA sensing. The cytoplasm should be a DNA-free compartment; exceptions to this rule are an infection or sterile tissue damage (Ahn et al., 2012; Gall et al., 2012; Hornung and Latz, 2010; Okabe et al., 2005). The host must mount an immune response to resolve either the infection or the tissue damage, which explains the presence of inflammatory pathways that respond to cytosolic DNA.

The simplest way to identify which pathway transfected self-DNA is stimulating, would be to generate receptor deficient FLDCs, in the same way that TLR9 dependency was investigated, e.g. STING^{-/-} or IPS1^{-/-} FLDCs. Receptor deficient mouse models are not always conveniently available; however there are alternative methods which may be used to identify pathways. Stimulation of some of these

pathways are characterised by production of specific cytokines which would enable identification of the pathway that is stimulated by transfection of self-DNA. For example production of mature IL-1 β and IL-18 could be measured, maturation of both of these cytokines is mediated almost exclusively by the inflammasome, therefore these are considered markers of inflammasome activation (Latz et al., 2013). Activation of STING can be measured by intracellular translocation. Activated STING forms oligomeric complexes and translocates from the ER to the perinuclear compartment, this can be used assay to measure STING activation (Ablasser et al., 2013; Ishikawa et al., 2009b).

3.3.4 Final remarks

This chapter has established a body of evidence investigating the effect of DNA on the response of cultured dendritic cells. The aim of this chapter was to establish a robust body of evidence from which to investigate the effect of hBD3 on the response to DNA, which has been successful. Whilst this is by no means an exhaustive investigation, and further experiments that could be performed have been discussed, it fulfilled its aims.

4. Investigating the role of hBD3 in regulating the innate immune response to DNA

4.1 Introduction

4.1.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) are a diverse group of short, amphipathic, antibiotic-like defence peptides. AMPs were first isolated from the giant silk moth in 1980, as heat stable peptides with potent, broad spectrum antimicrobial activity (Hultmark et al., 1980) and subsequently isolated from vertebrates, invertebrates, plants and even from microorganisms where they act as anti-fungal proteins (Hassan et al., 2012).

AMPs are typically between 12 and 50 amino acids in length with a diverse assortment of structures, however they can be grouped into five broad structure-based groups: linear, mostly helical peptides, without cysteine; linear peptides without cysteine and with a high proportion of certain residues; peptides with a single disulphide bond; peptides with two or more disulphide bonds which form a β -sheet; peptides derived from larger polypeptides with alternative functions (Boman, 1995). AMPs have many more diverse roles than purely antimicrobial functions; they have regulatory effects on both the innate and adaptive immune system, which will be discussed below in reference to specific AMPs. They also, somewhat unsurprisingly for such a highly conserved group of peptides, have functions with no obvious connections to the immune system. More than two thousand AMPs are described in the antimicrobial peptide database (Wang et al., 2009), subdivided into families according to species of origin, structure and function. This introduction focuses on defensins and more specifically hBD3 with reference to other AMPs as is relevant for the discussion of the results in this chapter.

4.1.2 Defensins

The term ‘defensin’ was first used to describe small broad spectrum antimicrobial peptides isolated from human neutrophils (Ganz et al., 1985). Similar small peptides with antimicrobial properties had been previously isolated from rabbit macrophages (Zeya and Spitznagel, 1963) and further peptides were rapidly identified as technical advances allowed better isolation and purification of proteins,

4.1.2.1 Genomic organisation of defensins

Most of the human defensin genes are distributed across five syntenic loci in two major clusters at 8p23.1, and three minor clusters: 20q11.1, 20p13 and 6p21 (figure 4.1) (Schutte et al., 2002). Individual β -defensin genes are also found on chromosomes 4 and 11 (Ensembl assembly GRCh38).

There are two regions of the cluster at 8p23.1 which are independently copy number variable with copy numbers varying from 4 to 10 copies for the 19 kb α -defensin gene locus (*DEFA1*, *DEFA3*, referred to as *DEFA1A3* since *DEFA1* and *DEFA3* differ by only one base (Aldred et al., 2005)) and from 1 to 12 copies for the 250 kb β -defensin gene cluster (*DEFB4*, *DEFB103*, (*SPAG11*), *DEFB104*, *DEFB106*, *DEFB105*, *DEFB107*) (Aldred et al., 2005; Hollox et al., 2008a; Khan et al., 2013; Linzmeier and Ganz, 2005) which also includes *SPAG11*, an alternatively spliced gene formed by head-to-tail ligation of two β -defensin genes (Yenugu et al., 2006). The similarity of the peptide structure and adjacent positioning of gene clusters suggests a common ancestry as a consequence of a duplication event. Since α -defensins are only found in mammals, it is thought that β -defensins are the ancestral defensin. Chickens only have a single gene cluster of β -defensin genes and no α -defensin genes further supporting β -defensins being the ancestral defensin (Liu et al., 1997; Patil et al., 2004; Xiao et al., 2004). Four other genes were identified in the platypus genome with a similar pattern of cysteine residues to β -defensins, three of these genes encoded the defensin-like peptides which are the major component of platypus venom, OvDLPs (*Ornithorhynchus* venom defensin-like peptides) and the remaining gene appeared to be an intermediate between β -defensins and OvDLPs. Structural resemblances between β -defensins and venom proteins called crotamines in reptiles had been noted previously (Zhao et al., 2001) and phylogenetic analysis indicates that crotamine genes evolved independently of the platypus OvDLPs (Whittington et al., 2008). The independent evolution of two families of venom proteins with similarities to defensins suggests that the structural motifs found in defensins are positively selected for evolution to toxic molecules (Fry, 2005).

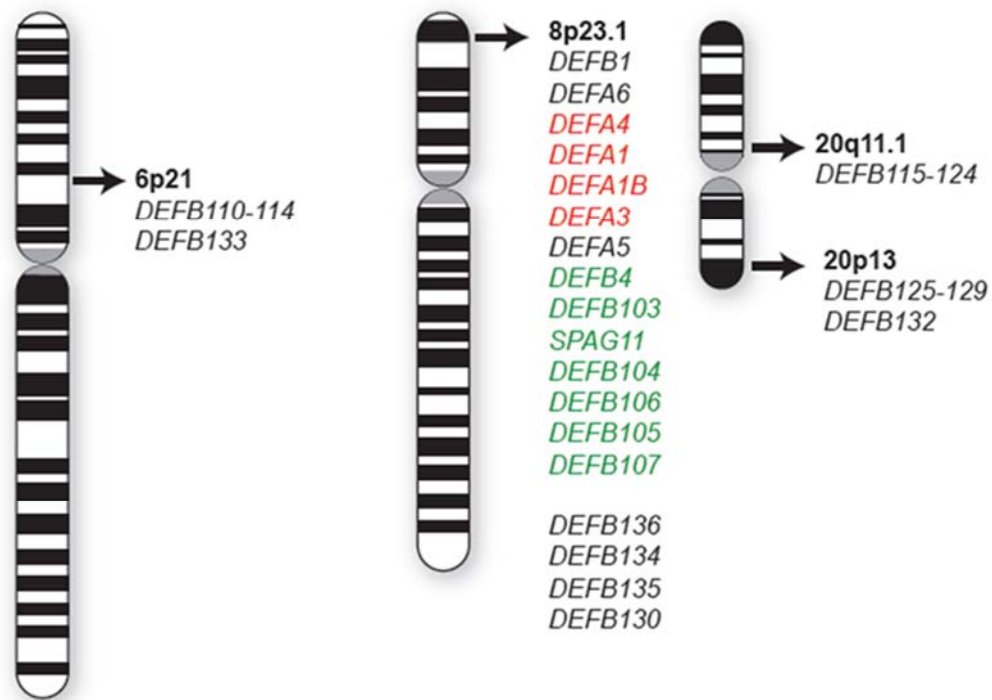


Figure 4.1 Organisation of human defensin genes across the human genome.

Human gene clusters shown in green or red text are independently copy number variable. (Based on Ensemble assembly GRCh38)

Lineage comparison of mammalian defensin genes shows evidence of a period of rapid diversification driven by positive selection following duplications from a single β -defensin-like ancestral gene. There is evidence of differences in selection pressures between mammalian lineages which has given rise to species-specific defensin genes, including a large number of rodent specific defensins (Hollox and Armour, 2008; Morrison et al., 2003; Semple et al., 2006). Mice do not have any neutrophil α -defensin genes, but have a large number of closely related cryptdins (mouse enteric alpha-defensins) which are secreted from Paneth cells into the lumen of the intestine. The mouse-lineage specificity of the cryptdins may have developed to compensate for loss of neutrophil defensins (Andersson et al., 2012), or may be a mechanism to cope with increased bacterial burden in the intestines of mice, or mouse specific pathogens.

Θ-defensin genes are primate-specific genes which arose from α-defensins following primate divergence; they are a product of two truncated α-defensin genes ligated head-to-tail (Tang et al., 1999b). Humans have a mutation in the θ-defensin gene which introduces a premature stop codon so humans do not produce θ-defensin peptides (Cole et al., 2002b).

At the gene level, most of the β-defensin genes and the enteric α-defensins (HD-5 and HD-6) are composed of two exons, the first encoding the signal sequence and pro-sequence and the second exon encoding the mature cysteine containing peptide (Mallow et al., 1996). Most α-defensins are composed of three exons with the first exon encoding a 5' UTR and exon two coding for the signal sequence and pro-sequence and exon three coding for the mature peptide (Patil et al., 2004).

4.1.2.2 Defensin peptide structure

Mammalian defensins are small (3-6 kDa, 28-42 amino acids length), cationic (net charge +1 to +11), amphipathic peptides characterised by a highly conserved triple anti-parallel β-sheet secondary structure stabilised via a framework of three disulphide bridges (Selsted and Harwig, 1989; Selsted et al., 1993; Tang et al., 1999a). The mammalian defensins are grouped into three classes: α-defensins, β-defensins and θ-defensins based on the spacing and predicted pairing of the disulphide linkages (table 4.1).

The amino acid sequence of defensins is diverse with the exception of the highly conserved cysteine residues, and each group of defensins has a characteristic amino acid consensus sequence based around the conserved cysteines (table 4.1).

Crystal structure solutions revealed that most α-defensins exist as dimers in solution and dimerise via the β-sheet (Hill et al., 1991; Pazgier et al., 2012; Zhao et al., 2012). B-defensins have been isolated as both monomers and dimers, suggesting that β-defensins may exist in multiple states which may depend on a number of physiological factors (Hoover et al., 2000; Schibli et al., 2002).

All α-defensins are produced as inactive precursors (pre-pro-HNPs), which are processed to mature peptides via two steps: signal sequence cleavage and further cleavage to remove the inhibitory pro-peptide. The pro-peptide is required for correct

folding and localisation of the peptide (Ghosh et al., 2002b; Michaelson et al., 1992; Wu et al., 2007). B-defensins are also expressed as precursor peptides with a signal and pro-sequence, however computational analysis of β -defensin precursors predicted that all β -defensins are processed in a single step by a signal peptidase to produce the mature peptide (Beckloff and Diamond, 2008; Wilson et al., 2009).

Table 4.1 The amino acid sequences of some of the best characterised mammalian defensins. Also showing the consensus sequence and the presumed cysteine pairing structure for that group of defensins.

α -defensins	
HNP-1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP-2	CYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP-3	DCYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP-4	VCSCLRVFCRRTELRVGNCLIGGVSTFTYCCTRV
HD-5	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR
HD-6	AFTCHC-RRSCYSTEYSYGTCTVMGINHRFCCL
Consensus	C-X-C-X ₄ -C-X ₉ -C-X ₉ -C-C
Cysteine pairing	C1-C6, C2-C4, C3-C5

β -defensins	
hBD1	GLGHRSDHYN C VSSGGQ C LYS A CPIFTKIQGT C YRGKAK C C K
hBD2	GIGDPVT C LKSGAI C HPVF C PRRYKQIGT C GLPGTK C C KKP
hBD3	GIINTLQKYY C RVRGGR C AVLS C LPKEEQIGK C STRGRK C C RRKK
Consensus	C -X ₆ - C -X ₄ - C -X ₉ - C -X ₆ - C - C
Cysteine pairing	C1-C5 , C2-C4 , C3-C6

θ -defensins	
RTD-1	GF C R C LG C RRGV C R C I C TR
RTD-2	GV C R C LG C RRGV C R C LG C RR
RTD-3	GF C R C I C TRGF C R C I C TR
Consensus	X ₂ - C -X- C -X- C -X ₄ - C -X- C -X- C -X ₂
Cysteine pairing	C1-C6 , C2-C5 , C3-C4

4.1.2.3 Defensin expression

Expression patterns of defensins vary both temporally and spatially. A-defensins are constitutively expressed in specific tissues, for example human neutrophil defensins 1-4 (HNP1-4) are stored in the granules of neutrophils (Ganz et al., 1985; Wilde et

al., 1989) and HD-5 and -6 are expressed in the Paneth cells of the small intestine (Jones and Bevins, 1992, 1993). B-defensins are predominantly expressed at mucosal surfaces such as the gut mucosa, the skin, the airway. HBD1 is constitutively expressed by epithelial cells (Goldman et al., 1997; Krisanaprakornkit et al., 1998; Mathews et al., 1999; McCray and Bentley, 1997; O'Neil et al., 1999; Singh et al., 1998; Valore et al., 1998; Zhao et al., 1996), whereas hBD2 and hBD3 are inducible by pathogens, including viruses, gram negative and gram positive bacteria and fungi, and by proinflammatory cytokines such as TNF- α , IL-1 α and IL-1 β ; additionally, hBD2 was shown to be increased in the BAL fluid from patients with cystic fibrosis or inflammatory lung disease (Duits et al., 2003; Garcia-Lopez et al., 2010; Harder et al., 1997; Hiratsuka et al., 1998; Kawauchi et al., 2006; Liu et al., 1998; Mathews et al., 1999; O'Neil et al., 1999; Proud et al., 2004; Singh et al., 1998; Sørensen et al., 2005; Steubesand et al., 2009).

There are distinct differences in the regulatory mechanisms for expression and synthesis of α - and β -defensins. α -defensins are constitutively expressed as inactive precursor peptides which have to undergo proteolysis steps by either MMP-7 (myeloid defensins) or trypsin (intestinal defensins) to remove the signal peptide and the inhibitory pro-region (Ghosh et al., 2002a; Harwig et al., 1992; Valore and Ganz, 1992). Mature HNPs are stored in the storage granules of neutrophils, whilst HD-5 and -6 are stored in the secretory granules of paneth cells as propeptides (Cunliffe et al., 2001; Porter et al., 1998). The anionic pro-region is essential for correct folding and cellular localisation of the α -defensin and inhibits the function by charge neutralisation. Examination of amino acid changes in α -defensin in different lineages reveals that a charge balance has to be maintained between the mature peptide and the pro-region, with amino acid substitutions in one region corresponding to amino acid changes in the other (Hughes and Yeager, 1997; Patil et al., 2004).

In contrast to the post-translational regulation of α -defensin, β -defensins are mostly transcriptionally regulated. The exception is hBD1 which is constitutively expressed, however expression of hBD1 can still be modulated by specific stimuli (Ryan et al., 2011). The hBD2 promoter region contains binding sites for NF- κ B and AP-1 (Tsutsumi-Ishii and Nagaoka, 2003; Wehkamp et al., 2004) and the similarity

between regulation of hBD2 and hBD3 suggests that *DEFB103* is regulated in a similar way.

4.1.2.4 Antibacterial function of defensins

Defensins have well characterised, broad spectrum antibacterial properties. A-defensins effectively kill a range of microbes *in vitro*, with the exception of HD-6 which has no antibacterial activity (Lehrer and Lu, 2012). However, HD-6 has been recently demonstrated to self-assemble into nanonets following binding to the bacterial cell surface, these nanonets surround and entangle bacteria. Overexpression of HD-6 in mice rescued murine lethality caused by *Salmonella* Typhimurium infection (Chu et al., 2012). Other α -defensins mediate bactericidal activity by inducing sequential permeabilisation of the outer and inner bacterial membranes, with inner membrane permeabilisation being lethal (Lehrer et al., 1989). B-defensins are too large gene family to assign a specific mode of bactericidal action. The majority of β -defensins are salt-sensitive, the exception being the most cationic member hBD3 (Taylor et al., 2008a). Both hBD3 and a fungal defensin, plectasin, have been demonstrated to bind to lipid II rich sites found at areas of active cell wall biogenesis indicating a dual mechanism for certain peptides involving both cell wall permeabilisation and inhibition of cell wall biosynthesis (Sass et al., 2010; Schneider et al., 2010). The increased antibacterial potency that correlates with increased charge and hydrophobicity of β -defensins also correlates with increased cytotoxicity towards mammalian cells. This indicates a balancing act by the host to resolve the infection without excessive causing tissue damage. Θ -defensins are also potent antimicrobials and although not expressed in mammals before the primate divergence, they are not cytotoxic to these mammals and their circular design may be an attractive option for using synthetic defensins as therapeutics (Lehrer et al., 2012; Schaal et al., 2012; Wohlford-Lenane et al., 2009).

4.1.2.5 Antiviral function of defensins

The antiviral function of defensins was observed soon after defensins themselves had been identified (Daher et al., 1986); however the mechanisms of the antiviral activity of defensins have only recently been understood. Defensins can inhibit viral infectivity by either directly affecting the virus particles by disrupting viral

envelopes, or by affecting the host cells and interfering with viral replication, for a review see (Klotman and Chang, 2006). Both HIV-1 and rhinovirus induce expression of hBD2 and hBD3 (Duits et al., 2003; Proud et al., 2004; Quinones-Mateu et al., 2003), the induction by rhinovirus relies upon active replication of the virus; a similar response was seen in the response to poly(I:C) suggesting a viral dsRNA intermediate is involved (Proud et al., 2004). HNP-1 exclusively affects enveloped viruses (Daher et al., 1986), and has a differential effect on different viral species that is thought to be dependent on the lipid composition of the viral envelope (Hristova et al., 1997).

The antiviral effect of defensins on HIV has been intensively studied; HNP1-4 all show anti-HIV activity by both direct inactivation as well as by blocking nuclear import and transcription in target cells (Wang et al., 2004) (Chang et al., 2003; Mackewicz et al., 2003; Wu et al., 2005). The effect of HNP1 on HIV-1 was dependent on serum concentration and viral MOI, with a direct viral killing mode at low concentrations of serum and low MOI, whilst at high concentrations of serum and increased MOI, HNP-1 induced the target cell to prevent viral replication (Chang et al., 2005). This mechanism suggests a mode of action whereby HNP-1 would directly kill viruses at exposed body surfaces, however during a systemic infection HNP-1 would target host cells. HBD2 and hBD3 are also anti-viral and similarly to HNP1, they have dual functionality which is dependent on concentration and serum (Quinones-Mateu et al., 2003; Sun et al., 2005). HBD2 and hBD3 interact with CXCR4, the co-receptor which HIV uses to infect CD4⁺ T cells, and induce long-term internalisation of the receptor, preventing HIV infection via this route (Feng et al., 2006; Quinones-Mateu et al., 2003; Weinberg et al., 2006). This novel mechanism for inhibition of HIV-1 infectivity has been proposed as a potential target for future anti-HIV drug design (Feng et al., 2006).

Retrocyclins (θ -defensins) also have potent antiviral activity against HIV-1, HSV-a and HSV-2 and have been intensively studied as potential therapeutics particularly in the context of preventing sexual or maternal-to-infant transmission of HIV-1 (Brandt et al., 2007; Cole et al., 2002a; Gupta et al., 2013; Owen et al., 2004; Yasin et al., 2004). Despite a premature stop codon rendering the human retrocyclin gene

inactive, expression of bioactive retrocyclin-1 could be restored in human cells by correction or read-through of the premature stop codon. Read-through of the premature stop codon was achieved by high dose treatment with aminoglycosides, and treated cells were significantly more resistant to HIV-1 suggesting that topical application of aminoglycosides may limit viral infections (Venkataraman et al., 2009).

4.1.2.6 Immunomodulatory function

4.1.2.6.1 Chemotactic effects of defensins

Both α - and β -defensins are chemoattractive for a range of immune cell types including monocytes, CD4 T cells, mast cells and immature dendritic cells (iDCs). HBD2 induces migration of immature dendritic cells, as well as memory T cells (CD4⁺ T cells), in a CCR6-dependent manner. CCL20 (MIP-3 α /LARC), a known ligand for CCR6, competes with hBD2 further supporting a requirement for the receptor CCR6 (Yang et al., 1999). HBD3 chemoattracts monocytes and CCR6 overexpressing HEK293 cells, the chemoattractive properties of hBD3 are introduced in more detail below (3.1.4.4). The secondary structure of β -defensins is remarkably similar to CCL20, the chemokine ligand for CCR6, with a similar arrangement of disulphide bonds and a high proportion of cationic residues aligned into distinct regions (Hoover et al., 2002). There is strong evidence for β -defensins competing for binding to CCR6 with CCL20, however, β -defensins chemoattract cells such as monocytes and macrophages which do not express CCR6, suggesting that this is not the only mechanism of chemoattraction (Wu et al., 2003b). HBD2 and 3, and their mouse orthologues Defb4 and Defb14, have also been demonstrated to compete for binding to CCR2 with the chemokine CCL2 (MCP-1) on macrophages and monocytes, and also to interact with CCR2 overexpressed by HEK293 cells (Röhrl et al., 2010). Human macrophages migrate into the peritoneal cavity in response to hBD2 in a SCID (severe combined immunodeficiency) mouse model. These cells are not responsive to the CCR6 ligand, CCL20, implying that human macrophages are also chemoattracted by hBD2 in a CCR6-independent manner (Soruri et al., 2007). In this system, bone marrow-derived DCs were not recruited by defensins and the authors suggest that CCR6 is not involved in defensin-mediated chemoattraction. HNP-1, HNP-3 and HD-5 chemoattract macrophages, mast cells,

naïve T cells and memory T cells but not dendritic cells (Grigat et al., 2007) although previously HNP1-4 were demonstrated to chemoattract immature dendritic cells (Yang et al., 2000). Interestingly, α -defensins can desensitise the chemotactic ability of β -defensins and vice versa suggesting that the two families of defensins regulate each other's ability to chemoattract cells (Grigat et al., 2007).

The contrasting reports on the chemotactic activity of defensins, including whether or not their activity is via CCR6 and which cells are mobilised illustrates that whilst it is clear that defensins do have chemoattractive properties, the mechanism is not yet clear. However, despite the lack of understanding of the mechanisms of chemoattraction by defensins, certain physiological processes appear to involve defensin-mediated chemoattraction including wound healing and tumour growth.

HBD2 was demonstrated to directly chemoattract endothelial cells and promote wound closure in a 3D skin wounding model and in an *in vitro* model of intestinal wound healing (Baroni et al., 2009; Otte et al., 2008). Similarly, vector-mediated hBD3 overexpression in a porcine model of diabetic wounding reduced wound healing times, apparently by increasing cell migration to the wound site (Hirsch et al., 2009).

Macrophage infiltration in oral tumours, which is associated with poor prognosis, correlates with increased hBD3 expression, and occurs in a CCR2-dependent manner, suggesting that hBD3 may be a driver of macrophage migration to tumour sites (Jin et al., 2010). Likewise, overexpression of the mouse orthologue, Defb14, increased infiltration of leukocytes into fibrosarcoma tumour tissue in a CCR6-dependent manner (Röhrl et al., 2012). Leukocyte infiltration is associated with a proangiogenic state leading to increased vascularisation and tumour growth. These results may highlight hBD3 as a therapeutic target to reduce tumour growth.

4.1.2.6.2 Pro-inflammatory effects of defensins

Defensins have proinflammatory effects in both epithelial and immune cells and as such, defensins can be grouped into a category of damage associated molecular patterns called 'alarmins', which activate the immune system. Evidence for defensins inducing chemotaxis fits with a role for defensins in promoting immune cell migration and activation. HNP-1 and HBD-1 both chemoattract and activate human

monocyte derived DCs and induced production of proinflammatory cytokines indicating that defensins may be detected by immune pathways (Presicce et al., 2009). HBD3 was shown to activate monocytes and dendritic cells via the TLR1/2 heterodimer (Funderburg et al., 2007), likewise Defb2 has been shown to activate and induce maturation of immature dendritic cells via TLR4 (Biragyn et al., 2002). HBD-2, -3 and -4 have been shown to increase production of proinflammatory cytokines by keratinocytes and increase keratinocyte migration and proliferation (Niyonsaba et al., 2007). In addition to intrinsic proinflammatory properties, hBD3 and hBD2 increased cellular uptake of both self and unmethylated CpG containing DNA, inducing a type I interferon response in human pDCs (Tewary et al., 2013). This role for defensins in converting inert molecules into immune stimulants or increasing how immunostimulatory an immune ligand is, is relatively unexplored, but not without precedent for antimicrobial peptides, as will be introduced with regards to LL37 (3.1.3).

The biological relevance of a proinflammatory role of defensins is supported by diseases such as psoriasis where increased copy number of the 8p23.1 β -defensin cluster is associated with increased risk of disease (Hollox et al., 2008b), and serum concentrations of hBD2 correlates with disease severity (Jansen et al., 2009). The published evidence strongly indicates a proinflammatory role for, particularly, β -defensins in the immune response and interestingly, the immune response to DNA.

4.1.2.6.3 Anti-inflammatory effects of defensins

In contrast to the proinflammatory function of defensins, defensins also have potent anti-inflammatory properties, attenuating the inflammatory response in a range of cell types including monocytes, macrophages and DCs. α -defensins block the release of IL-1 β from LPS treated monocytes and α -defensins released from apoptotic or necrotic neutrophils inhibit the production of cytokines from macrophages suggesting a role in downregulating the inflammatory response during cell death (Miles et al., 2009; Shi et al., 2007). A matrylisin deficient transgenic mouse (MMP-7^{-/-}), which has a reduced proteolytic capacity to convert immature intestinal α -defensins (mouse cryptdins) to their mature form, is susceptible to dextran-induced colitis and has increased levels of IL-1 β in the intestine (Shi et al., 2007). Although

proteolytic processing of enteric α -defensins is carried out by intestinal trypsin in humans, overproduction of IL-1 β in the intestine has been linked to human inflammatory bowel disease (IBD) indicating that defensins may have a role in regulating the pathogenesis of IBD (Ghosh et al., 2002b; Ligumsky et al., 1990; Ramasundara et al., 2009). The evidence that intestinal cryptdins are involved in intestinal inflammation suggests that α -defensins may down-regulate the proinflammatory response in the intestine (Shi et al., 2007). However, evidence that increased copy number and mRNA expression of *DEFA1A3* is associated with colonic Crohn's disease in humans suggests that the issue is more complex than alterations in amounts of α -defensins in the intestine (Jespersgaard et al., 2011)

B-defensins are involved in regulating the response to LPS, a TLR4 ligand found in the outer membrane of gram-negative bacteria. DEFB126, synthetic DEFB123, DEFB114 and hBD3 (and mouse orthologue Defb14) all inhibit the proinflammatory response to LPS. DEFB126, DEFB123 and DEFB114 act by binding directly to LPS, preventing binding to TLR4 (Liu et al., 2013; Motzkus et al., 2006; Yu et al., 2013). In contrast, hBD3 acts downstream of the receptor and inhibits both MyD88- and TRIF- dependent inflammatory pathways (Semple et al., 2011; Semple et al., 2010) (figure 4.3). Since the adapter proteins MyD88 and TRIF are required in all TLR pathways, these results indicate a broad role for hBD3 in regulating TLR-mediated proinflammatory pathways.

This section introduces defensins as both anti-inflammatory via suppression of inflammatory responses and proinflammatory by chemoattraction of immune cells, direct activation of immune cells or by binding to and increasing cellular uptake of ligands. The distinction between proinflammatory and anti-inflammatory appears to be dependent on the specific defensin involved as well as being ligand specific, indicating exceptionally complex mechanism of regulation that has evolved over a long period of time from an ancient ancestor.

4.1.2.6.4 Non-immune functions of defensins

As discussed previously, defensins are predominantly expressed at epithelial and mucosal surfaces, presumably to increase the effectiveness of these barriers at preventing infection. Many of human and rodent defensins are expressed in both the

male and female reproductive tracts and are detectable in cervico-vaginal and seminal fluids. However, besides being antimicrobial, defensins in the genital tracts are involved in the maturation and mobility of sperm. A mutation in *DEFB126* which causes impaired sperm function, and therefore reduced fertility, is present in a high proportion of the human population (Tollner et al., 2011). DEFB126 is adsorbed onto the sperm cell surface and aids migration through the mucosal fluids of the female reproductive tract (Tollner et al., 2008). Additionally, homozygous deletion of the 9 β -defensins gene cluster at 8p23.1, which does not include the mouse orthologue of DEFB126, lead to complete male sterility in a transgenic mouse model due to a premature acrosome reaction alongside increased fragility and decreased motility of the sperm (Zhou et al., 2013b).

4.1.3 Structure and function of cathelicidins

Cathelicidins are a varied group of AMPs characterised by an N-terminal cathelin domain that is present in the pro-form of the peptides. Primates and rodents have only one cathelicidin (hCAP18/mCRAMP) whilst other mammals have at least one, with high variability of all but the cathelin domain between species, reviewed by (Tomasinsig and Zanetti, 2005). LL37 is a cationic (net charge +6) linear helical AMP of 37 amino acids, which is thought to kill microbes by forming oligomers which disrupt and permeabilise the bacterial cell membrane (Oren et al., 1999; Zhang et al., 2010b). LL37 does not discriminate between bacterial cell membrane and eukaryotic membranes, resulting in a high level of cytotoxicity (Oren et al., 1999). Serum dampens the cytotoxic effects of LL37, but it also inhibits the antimicrobial action; analogues of LL37 which lack the N-terminal hydrophobic amino acids have a decreased cytotoxicity and also retain their antimicrobial function in serum suggesting that synthetic analogues of LL37 may be an attractive therapeutic option (Ciornei et al., 2005). Conversely, the eukaryotic cell penetrating properties of LL37 may be harnessed for delivery of nucleic acids into host cells, for example for delivery of vectors for gene targeting (Sandgren et al., 2004; Zhang et al., 2010b). LL37 also displays antiviral activity; inhibiting HIV-1 replication in peripheral blood mononuclear cells (PBMC) (Bergman et al., 2007) and vaccinia virus replication *in vitro* and mouse knock-outs of *CRAMP* (the mouse orthologue) (Howell et al., 2004). Both LL37 and mCRAMP reduced the infectivity of influenza A virus in a

mouse model of infection (Barlow et al., 2011) and LL37 additionally inhibits replication of a number of seasonal and mouse adapted influenza A strains, apparently by causing disruption of viral membranes (Tripathi et al., 2013).

LL37 has also been shown to regulate innate immune pathways in a number of ways, and expression of LL37 is in turn modulated by immune responses. Intratracheal treatment with LL37 protected mice against lethal endotoxic shock caused by LPS, and prevented activation of macrophages by bacterial components (Scott et al., 2002). Treatment with LL37 stimulated increased chemokine and chemokine receptor expression *in vitro* and *in vivo*, demonstrating a role for LL37 in the resolution of infection and tissue damage in the respiratory tract and in relevant immune cell types. Similarly, urinary tract infection induced increased expression of LL37 in human and mice and mCRAMP deficiency resulted in more severe infections, demonstrating the contribution of LL37 to urinary tract protection (Chromek et al., 2006).

LL37 expression is regulated by vitamin D-responsive elements in the promoter region and up-regulation of LL37 expression following skin wounding and inflammation is the result of activation of vitamin D in keratinocytes (Bikle et al., 1989; Dombrowski et al., 2010; Dorschner et al., 2001; Heilborn et al., 2003; Lehmann, 2009; Peric et al., 2010). Patients with the chronic skin condition rosacea express abnormally high levels of particular isoforms of LL37 in the epidermis, which promote lymphocyte chemotaxis, angiogenesis and expression of extracellular matrix proteins (Yamasaki et al., 2007; Yamasaki and Gallo, 2011). LL37 is also thought to have role in the pathogenesis of psoriasis. LL37 forms an immunogenic complex with self-DNA released from dying keratinocytes in psoriatic plaques, which activates pDCs via TLR9 to produce IFN- α (Lande et al., 2007). Similarly, LL37 forms complexes with self-RNA activating TLR7 in pDCs or TLR8 in cDCs and cytoplasmic LL37-self-DNA complexes activate a STING dependent pathway in monocytes (Chamilos et al., 2012; Ganguly et al., 2009a). Additionally, LL37 increases uptake of a synthetic 60-mer RNA-DNA hybrid leading to TLR9-dependent production of proinflammatory cytokines by FLDCs (Rigby et al., 2014).

This suggests that LL37 may have a general role in increasing uptake of nucleic acids into relevant cells, activating an innate immune response.

The parallels that can be drawn between LL37 and hBD3 in terms of structure and function explored in this section suggest that some of the properties of LL37 may be applicable to defensins and should be considered when exploring how defensins act.

4.1.4 HBD3 and Defb14

This section specifically discusses hBD3 and the mouse orthologue, Defb14. HBD3 was first isolated from keratinocytes in the psoriatic plaques of psoriasis patients (Harder et al., 2001) and has since been demonstrated to be a highly potent, broad-spectrum antimicrobial peptide with both pro- and anti-inflammatory activity and chemoattractive properties.

4.1.4.1 Expression

DEFB103 is one gene in a copy number variable β -defensin cluster on chromosome 8p21 (figure 4.1) with a worldwide diploid copy number ranging from one to nine and a modal copy number of four (Hardwick et al., 2011).

HBD3 is predominantly expressed at mucosal and epithelial surfaces and is inducible by a range of pathogens and proinflammatory cytokines and by sterile wounding (Duits et al., 2003; Garcia-Lopez et al., 2010; Harder et al., 1997; Kawauchi et al., 2006; Mathews et al., 1999; Singh et al., 1998; Sorensen et al., 2006; Sørensen et al., 2005; Steubesand et al., 2009; Zanger et al., 2011).

The 67 amino acid hBD3 prepropeptide contains a signal peptide, a conserved motif which targets proteins for secretion, however very little is known about the post-translational processing of hBD3. Interestingly, there is evidence that the preproregion has been subjected to significant selection throughout evolution suggesting that this region is important for protein function (Semple et al., 2005). HBD3 is rapidly taken up by macrophages *in vitro* into distinct vesicular structures resembling endosomes, however this has not been confirmed (Semple et al., 2011).

DefB14 is expressed in a comparable range of tissues, including the respiratory tract, spleen, liver, eye, thymus, oesophagus and tongue and is inducible by synthetic

bacterial and viral ligands (Harder et al., 2001; Röhl et al., 2008). Defb14 is also expressed in unstimulated mature dendritic cells and in both mature and immature dendritic cells following stimulation with LPS (Röhl et al., 2008) and is highly expressed throughout the male and female reproductive tracts, though Defb14 deficient mice do not show any fertility defects (unpublished data, personal communication Dr. Julia Dorin, Dr. Heather MacPherson and Tina Baker, IGMM).

4.1.4.2 Structure

HBD3 is a 45 amino acid, 5.1 kDa β -defensin with little primary sequence similarity to other β -defensins, with the exception of the conserved cysteine residues. HBD3 is the most highly charged of the defensins (net charge +11) and also has the highest charge to mass ratio (+2.1/kDa). The mouse orthologue, Defb14, shows 68% similarity to hBD3 at the protein level including conservation of the cysteine pairing that defines β -defensins (figure 4.2) (Hinrichsen et al., 2008; Röhl et al., 2008).

```
hBD3  MRIHYLLFALLFLFLVPVPGHGGIINTLQKYYCRVRGGRCAVLSSCLPKEEQIGKCSTRGRKCCRRKK
      ||:|||||..|.|||||.||.....||:|::||:|||||||:|..|||||:|..|||||:|
defb14 MRLHYLLFVFLILFLVPAPGDAFLPKTLRKFFCRIRGGRCAVLNCLGKEEQIGRCSNSGRKCCRRKKK
```

Figure 4.2 Alignment of peptide sequences of hBD3 and the mouse orthologue, Defb14. Aligned using EMBOSS needle.

The solution structure of hBD3 demonstrated formation of a dimer mediated via the β -sheet, specifically via strand β 2 (Schibli et al., 2002). This is in contrast to hBD1 and 2 which exist as monomeric (Schibli et al., 2002). Though the solution structure of Defb14 has not been solved, the high degree of conservation between Defb14 and hBD3 and the overlapping functions suggest that Defb14 has a similar structure and may also form a multimer.

4.1.4.3 Antimicrobial function

hBD3 exhibits antimicrobial action against a range of pathogens, including gram-negative and gram-positive bacteria, fungi and yeast and retains antimicrobial activity in physiological salt concentration, unlike other β -defensins (Harder et al., 2001). HBD3 forms ion channels in the membranes of *Xenopus* oocytes, suggesting

that pore formation in bacterial cell membranes could be the mechanism of bacterial killing for hBD3 (Garcia et al., 2001). Dimerisation of hBD3 introduces a large patch of hydrophobicity across the surface of the dimer which may explain the potency of hBD3 as an AMP compared to other defensins (Schibli et al., 2002). However, an artificial hBD3 peptide unable to undergo dimerisation, retained full anti-microbial activity (Boniotto et al., 2003) suggesting that the potency of hBD3 is not due to dimerisation. Additionally, the disulphide bridges in hBD3 and Defb14 are dispensable for antimicrobial activity of hBD3, though not dispensable for alternative functionality of the peptide, as will be described below (Wu et al., 2003b).

Staphylococcus aureus cells treated with hBD3 had a similar transcriptional profile to vancomycin treated *S. aureus* indicating a similar mechanism of bacterial killing by inhibition of cell wall biosynthesis, which challenges a pore-forming mechanism for the antimicrobial activity of hBD3. Additionally, hBD3 binds to lipid II rich areas of the bacterial cell membrane where active cell wall biosynthesis is occurring which may perturb lipid biosynthesis machinery (Sass et al., 2010). The published evidence together suggests a mechanism whereby the antimicrobial function of hBD3 may simply rely on hBD3 binding to anionic lipid moieties in the bacterial cell membrane, rather than on higher order structures of the peptide. Similarly, recombinant Defb14 also has potent broad spectrum microbicidal activity against Gram-negative and Gram-positive bacteria, including antibiotic resistant strains, as well as against yeast, at low concentrations (Hinrichsen et al., 2008) and at physiological salt concentrations (Taylor et al., 2008a). In contrast to hBD3, which is only effective as a full length peptide, an N-terminal Defb14 peptide fragment retains full antimicrobial activity whilst a C-terminal fragment has no antimicrobial activity (Taylor et al., 2008a).

Expression of both hBD3 and Defb14 is induced by IFN- γ indicating that hBD3 and Defb14 are an important component of the antiviral response (Garcia et al., 2001; Harder et al., 2004; Hinrichsen et al., 2008; Rohrl et al., 2008). Supporting this role in the antiviral response, hBD3 inhibits HIV-1 and HSV infection and expression of hBD3 is induced by human parvovirus infection and rhinovirus (Duits et al., 2003; Hsu et al., 2011) (Feng et al., 2006; Hazrati et al., 2006; Quinones-Mateu et al.,

2003; Weinberg et al., 2006). This indicates a broad role for hBD3 in the antiviral response.

4.1.4.4 Regulation of the innate immune system

HBD3 has both pro-inflammatory and anti-inflammatory properties. As previously mentioned, hBD3 increased intracellular uptake of self and synthetic CpG ligands inducing increased IFN- α production from pDCs (Tewary et al., 2013) and, in contrast, inhibits the immune response to TLR4 activation in a ligand and receptor independent manner (Semple et al., 2011; Semple et al., 2010).

HBD3 and Defb14 are chemotactic for cells expressing mouse CCR6, and for resident peritoneal cells (Rohrl et al., 2008). CCR6 was previously identified as the receptor responsible for the chemotactic activity of both hBD2 and hBD1 on immature DCs and memory T cells (Yang et al., 2002; Yang et al., 1999). hBD3 also chemoattracts macrophages (Garcia et al., 2001) and monocytes which do not express CCR6 via a CCR2-dependent mechanism (Röhrhl et al., 2010), as discussed above (4.1.2.6.1). The wide variation in the chemoattractive properties of different conformations of hBD3 suggests that particular disulphide bond arrangements are more effective at chemoattracting than others (Wu et al., 2003a). Substitution of the conserved cysteine residues required for disulphide bond formation rendered hBD3 chemotactically inactive (Wu et al., 2003a), however, a range of peptide derivatives of hBD3 demonstrated that full length hBD3 and Defb14 lacking all but the fifth cysteine residues retained comparable chemotactic activity to the parent peptides. Additionally, derivatives lacking disulphide bonds, but still containing cysteine residues, also retained chemotactic activity and further work demonstrated that a leucine or an isoleucine residue was essential at position 2 illustrating a requirement for specific residues rather than secondary structure (Taylor et al., 2008b; Tyrrell et al., 2010).

4.1.4.5 β -Defensin disease association

It is clear that expression of hBD3 is highly regulated and the conservation between hBD3 and Defb14 supports an essential role for this peptide. Copy number polymorphisms of the β -defensin gene cluster at 8p23.1 containing the genes encoding hBD2 (*DEFB4*), hBD3 (*DEFB103*) and hBD4 (*DEFB104*) has been

intensively studied, particularly in relation to disease susceptibility. A number of diseases have been linked to copy number polymorphisms in the β -defensin cluster, however the mechanisms leading to disease development are not well understood.

4.1.4.6 Intestinal disease

A small study correlated low copy number, and mRNA expression, of *DEFB4*, with increased risk of colonic Crohn's disease and proposed that this was due to a perturbed intestinal barrier caused by a disturbed intestinal microbiota (Fellermann et al., 2006; Wang et al., 2007). Conversely, increased colonic and ileal Crohn's disease susceptibility has also been associated with increased copy number of *DEFB4* in a much larger cohort of patients (Bentley et al., 2009). Understanding the relationship between defensin copy number and Crohn's susceptibility was further obscured by a demonstration in a small group of patients that hBD2 gene copy number had no effect on hBD2 protein levels implying that copy number does not make any difference to expression at the protein level (Aldhous et al., 2009). Copy number variation is notoriously difficult to quantify since it is measuring subtle differences in amounts of a gene region rather than identifying a changed nucleotide and the qPCR assays used in these studies may not have been sensitive enough to pick up small differences in copy number. Alternative methods could not prove any association between β -defensin copy number and Crohn's disease in a much larger cohort of patients and also highlighted significant errors in using qPCR to measure copy number variation compared to paralogue ratio based assays (Aldhous et al., 2010; Armour et al., 2007). The link between defensin gene copy number and IBD suggests an involvement of defensins in maintenance of intestinal microbiota. Whilst no direct association has been shown between β -defensin copy number and intestinal microbiota, one small study showed an association between lower β -defensin copy number and colonisation by bacterial species associated with otitis media in the nasopharynx, indicating that hBD3 does affect the microbiota in specific sites (Jones et al., 2014).

4.1.4.7 Lung disease

Polymorphisms in copy number invariant *DEFB1* are associated with COPD and cystic fibrosis (Crovello et al., 2011; Tesse et al., 2007; Vankeerberghen et al., 2005).

However, copy number variation of other β -defensin genes is not associated with COPD, asthma, lung function in cystic fibrosis patients, or general lung function (Hollox et al., 2005; Wain et al., 2014). Interestingly however, increased β -defensin copy number was associated with increased risk of smokers developing COPD (Janssens et al., 2010) implying that environmental factors play a role in risk associated with increased β -defensin copy number. *In vitro*, increased copy number of β -defensin genes correlated with increased expression of *DEFB4* in nasal epithelial cells. Whilst more than five copies had no additive effects on baseline expression indicating that this may be a threshold level, higher copy number correlated with increased expression following treatment with TNF- α and more efficient bacterial clearance (Janssens et al., 2010). This suggests that there are mechanisms of transcriptional regulation acting to prevent overexpression of defensins in the absence of an inflammatory stimulus.

4.1.4.8 Skin disease

Increased copy number of β -defensins is associated with increased risk of psoriasis, and serum levels of hBD2, in the same copy number variable gene region as hBD3 correlate with disease severity, although not with copy number (Hollox et al., 2008c; Jansen et al., 2009; Stuart et al., 2012). Psoriasis is an autoimmune disorder characterised by patches of itchy, red, scaly skin and driven, in later stages, by T cells (Prinz, 2003). A total of 36 susceptibility loci for psoriasis have been identified in European populations (Tsoi et al., 2012), several of which overlap with susceptibility loci for other autoimmune disease e.g. IL23R, IL12B (Cotsapas et al., 2011). Whilst epidemiologic studies indicate a strong genetic component is involved it is clear that environmental factors such as infection or trauma are associated with development with plaque development and exacerbation of the disease often being attributable to an environmental trigger (Chandran and Raychaudhuri, 2010).

In contrast, expression of β -defensins is down-regulated in atopic dermatitis (AD) which explains why AD patients are susceptible to infections, in contrast to psoriatic plaques which are a markedly sterile environment (Clausen et al., 2013; Nomura et al., 2003). Recently, hBD3 was demonstrated to enhance expression of genes involved in tight junction integrity via a CCR6-dependent mechanism

(Kiatsurayanon et al., 2014), which supports a previous observation of impaired tight junctions in AD skin (De Benedetto et al., 2011). Work investigating the pathogenesis of psoriasis is limited by the lack of a true mouse model of psoriasis which accurately phenocopies the human disease. Recently however, the phenotype of a humanised psoriasiform mouse was markedly improved by topical application of *DEFB4* siRNA (Bracke et al., 2014). Notably, there was no evidence that this siRNA did not target other defensins, although a BLAST search did not identify any other defensin transcript or gene targets.

4.1.5 Aims of this chapter

Apart from being a potent antimicrobial peptide, hBD3 has an established, if imperfectly understood, role in the regulation of the innate immune system. As discussed, hBD3 inhibits the response to LPS, a TLR4 ligand, and both inhibits the TLR3 dependent response to p(I:C) and exacerbates the MDA-5 dependent response (unpublished data, Dorin lab). Additionally, during the course of this study, hBD3 has been demonstrated to exacerbate the response to self-DNA by increasing cellular uptake (Tewary et al., 2013).

The effect of hBD3 on the response to both pathogenic and self-DNA is the focus of this chapter for two major reasons:

Firstly, hBD3 is an antimicrobial peptide; it induces bacterial cells death and leads to the release of potentially immunogenic bacterial components such as LPS. This bacterial cell death will also lead to the release of bacterial DNA that would be detected in the endosomal compartment, triggering TLR9 dependent signalling. Considering that hBD3 inhibits the response to LPS is it interesting to consider the effect of hBD3 on the ligand that is most likely to be found alongside LPS during a bacterial infection.

Secondly, increased copy number of the genomic region that includes the gene for hBD3 is linked to increased risk of psoriasis. The release of DNA from dying keratinocytes in the epidermis of psoriasis patients is thought to induce type 1 interferon production via a mechanism that involves LL37, a cationic AMP with a somewhat similar structure to hBD3. It is interesting to investigate whether hBD3

has a similar effect to LL37 in increasing uptake of self-DNA and inducing an aberrant immune response. Additionally, psoriasis can be triggered by a microbial skin infection. Increased expression of defensins in the skin due to increased gene copy number could amplify an immune response triggered by a DNA-defensin complex, potentially providing a second or alternative mechanism whereby increased copy number of defensin would lead to increased risk of psoriasis.

This study began with seeking to understand if hBD3 has a role in regulating the innate immune response to pathogenic DNA. Since hBD3 has been shown to have a broad role in regulating other immune pathways; the hypothesis is that hBD3 does affect the response to pathogenic DNA. This broad hypothesis was developed from the multitude of contrasting reports regarding the pro-inflammatory and anti-inflammatory properties of hBD3 and other host defence peptides. Studying the effect of hBD3 on the immune response to pathogenic DNA naturally led on to studying its effect on self DNA with a view to understanding if hBD3 may have a role in the pathogenesis of psoriasis by forming immunogenic complexes with self-DNA.

4.2 Results

4.2.1 Overexpression of hBD3 inhibits MyD88 and TRIF dependent pathways

Previous work using an NF κ B luciferase reporter assay to investigate the effect of hBD3 overexpression on NF κ B activation demonstrated that overexpression of hBD3 inhibited NF- κ B activation via both the MyD88-dependent or TRIF-dependent pathway (' β -Defensin 3 inhibits NF- κ B promoter activation following stimulation of both the MyD88 –dependent and –independent TLR4 pathways', 2011, PhD mini project) (figure 4.3). A strength of this assay is that it investigated the pathway dynamics downstream of the adapter proteins MyD88 and TRIF, therefore measuring the ligand-independent response. HEK293 cells were used as they transfect with high efficiency and whilst they express low endogenous levels of NF κ B pathway components, when a pathway components is overexpressed, expression of downstream proteins is also increased so the signalling pathway is intact. HEK293 cells have been used previously in similar assay to investigate pathway dynamics in the innate immune system (Ganguly et al., 2009b; Muroi and Tanamoto, 2008). MyD88 and TRIF are widely used as adapter proteins for innate immune receptors, and all TLRs use one or the other (Warner and Núñez, 2013), therefore our results suggest that hBD3 has a widespread role in regulating the innate immune system. It would be useful to attempt to quantify at the mRNA or protein level how much overexpression of hBD3 was achieved, however this would involve considerably scaling up the experiments from a 96-well plate format.

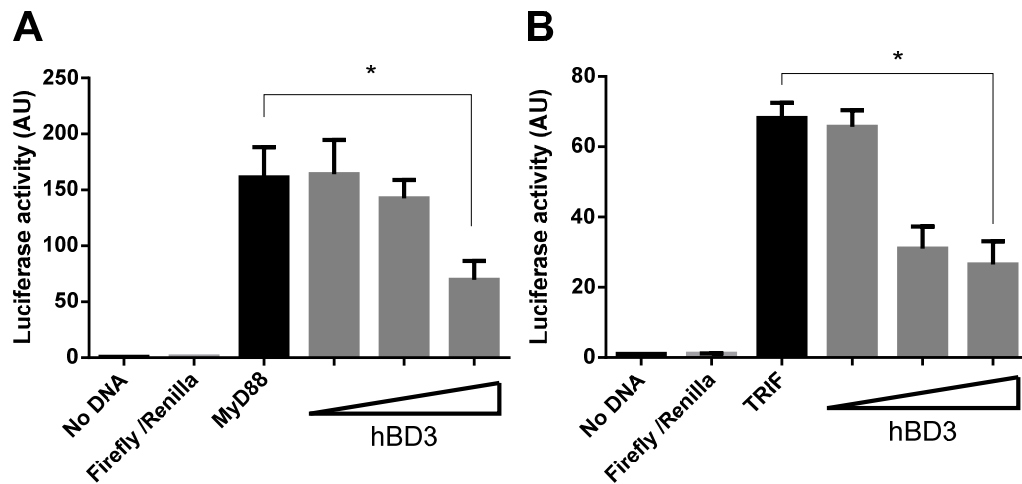


Figure 4.3 Overexpression of hBD3 inhibits ligand independent activation of NF- κ B in a concentration dependent way. HEK293 cells were transfected for 24 hours with an NF- κ B luciferase reporter construct (80 ng/well, MyD88 (A, 25 ng/well) or TRIF (B, 50 ng/well) expression construct alone (black) or with increasing amounts of hBD3 expression construct (grey, 50 ng, 100 ng or 200 ng, wedge). NF- κ B activation was measured by assaying luciferase reporter gene expression and normalising against *renilla* expression, using a renilla construct (40 ng/well). Data shown is the mean of three independent experiments performed in triplicate. Error bars are standard error of the mean of the independent experiments. * $p < 0.05$, unpaired t-test

4.2.2 HBD3 does not cause cytotoxicity in FLDCs

There are contrasting reports about defensins activating an immune response in the absence of classical immune ligands (Biragyn et al., 2002; Funderburg et al., 2007; Presicce et al., 2009); therefore it was important to establish whether hBD3 alone has an effect on FLDCs. The most striking observation when FLDCs were treated with hBD3 is that FLDCs consistently aggregated within 4 hours of treatment (figure 4.4 A). Previous publications have used this cell aggregation as a sign of cell activation (Lande et al., 2007); however flow cytometry profiles of FLDCs treated with hBD3 showed no change in the expression of costimulatory markers, as well as no difference in numbers of cDCs and pDCs compared to untreated or total numbers of FLDCs harvested in each sample (figure 4.4 B and C). Possible causes of the cellular aggregation are extensively examined in the discussion. Cell aggregation can also be sign of apoptosis, though immune cells activation and apoptosis are often intrinsically linked (Carracedo et al., 1995). A cytotoxicity assay to detect lactate dehydrogenase activity in the supernatant showed that there was no difference in the amounts of lactate dehydrogenase present in the supernatant of hBD3 treated FLDCs compared to untreated (figure 4.4 D) Lactate dehydrogenase (LDH) is a soluble enzyme normally found in the cytoplasm, upon loss of membrane integrity resulting from apoptosis or necrosis LDH is released into the extracellular supernatant. The cytotoxicity assay measures the capacity of cell culture supernatant to reduce NAD⁺ to NADH that in turn reduces iodonitrotetrazolium violet (INT) to formazan which absorbs at a wavelength 495 nm (Haslam et al., 2000; Wolterbeek and van der Meer, 2005). The LDH cytotoxicity assay confirms that 5 µg/ml hBD3 does not affect FLDC membrane integrity.

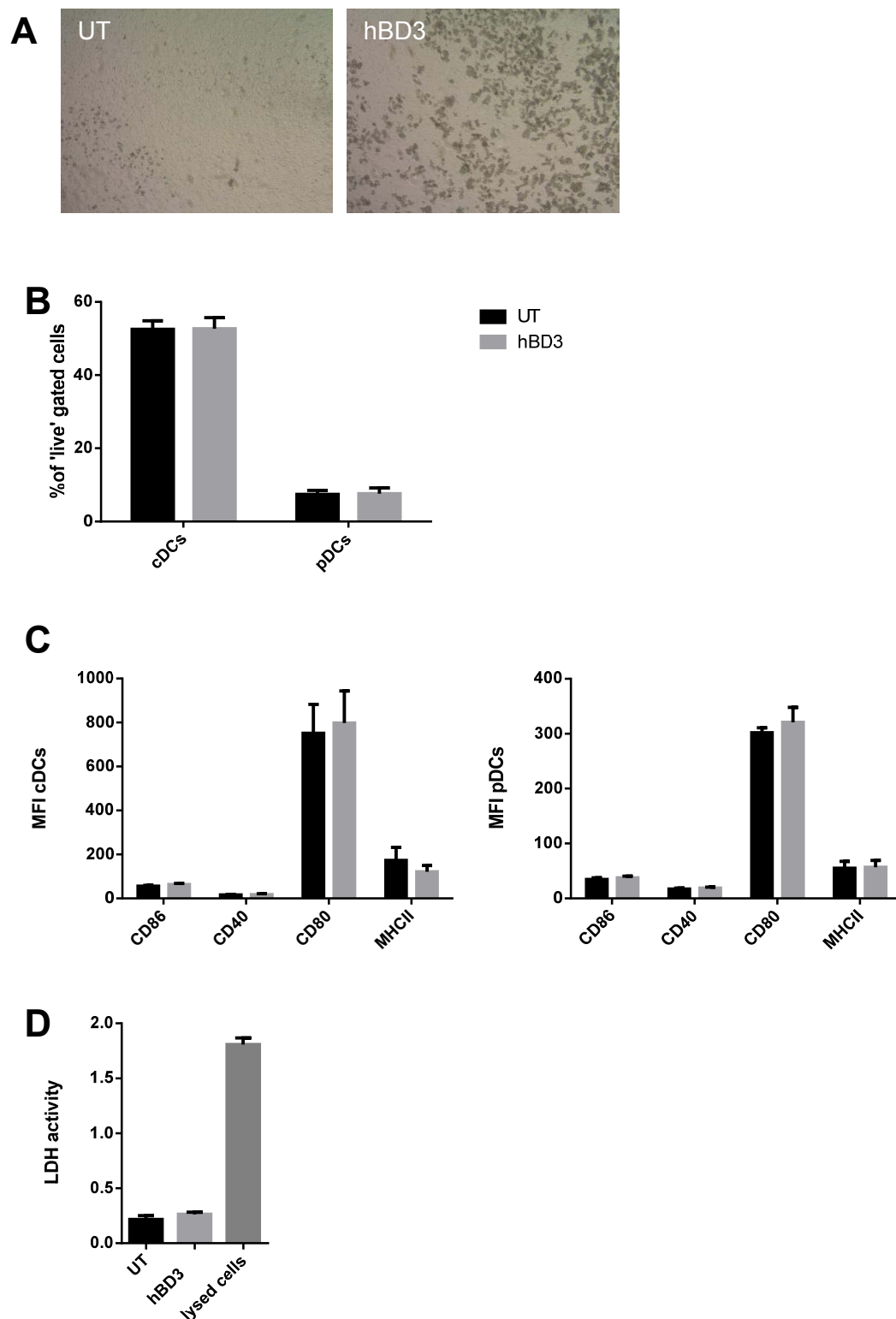


Figure 4.4 hBD3 causes FLDC aggregation but has no effect on the relative proportion of cell populations, the expression of costimulatory markers or on the viability of the cells. FLDCs were incubated with 5 μ g/ml hBD3 for 4 hours (A)

or overnight (B-D) and cells were analysed by flow cytometry. Images in A were taken using a Primovert inverted microscope with 10X objective. Image (A) is a representative image of more than 10 independent experiments. Data shown is the mean of 3 independent experiments performed in triplicate $\pm$ standard error.

4.2.3 HBD3 exacerbates the response to *E. coli* DNA

Having shown that treatment of FLDCs with *E. coli* DNA induces an innate immune response with production of cytokines and type I interferon and upregulated expression of costimulatory markers (figure 3.5); FLDCs were treated with *E. coli* DNA in combination with hBD3 (figure 4.5). Treatment of FLDCs with *E. coli* DNA in combination with hBD3 significantly increased production of IL-6 and IFN- α (figure 4.5 A), as well as expression of costimulatory markers on both cDCs and pDCs (figure 4.5 B), compared to treatment with *E. coli* DNA alone. HBD3 had no effect on the production of IFN- β in response to *E. coli* DNA (figure 4.5 A).

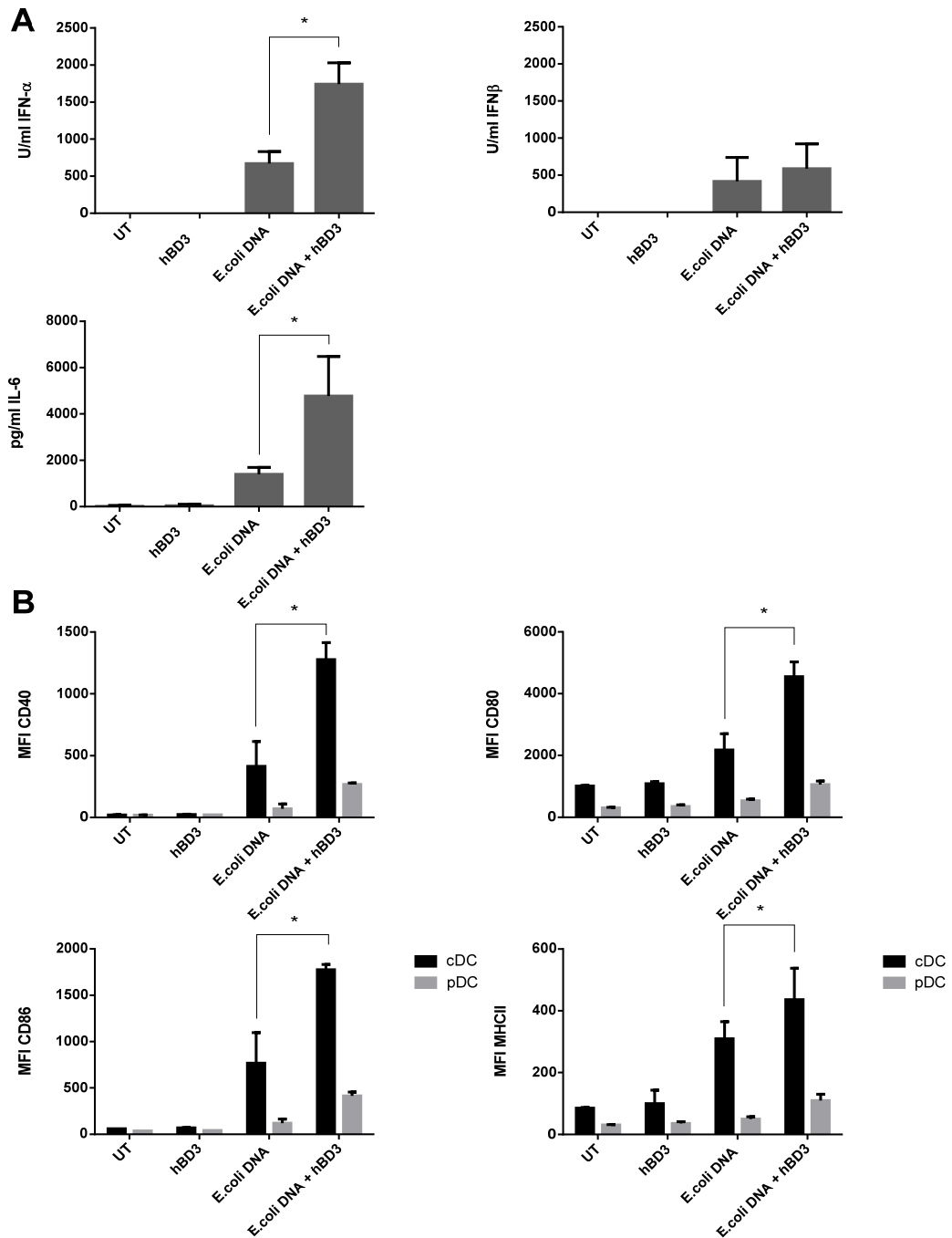


Figure 4.5 hBD3 exacerbates the response to *E. coli* DNA. FLDCs were incubated with 1 μ g/ml *E. coli* DNA with or without 5 μ g/ml hBD3 overnight. Cytokine production was measured by ELISA (A) and expression of costimulatory markers on cDCs and pDCs was measured by flow cytometry (B). Data shown is the mean of 4 independent experiments performed in triplicate $\pm$ standard error. * $p < 0.05$, unpaired t-test

4.2.4 The exacerbatory effect of hBD3 on the response to *E. coli* DNA is TLR9-dependent

The response to *E. coli* DNA without transfection has been shown to be entirely TLR9-dependent (figure 3.6) so to test whether the exacerbatory effect of hBD3 on the response to *E. coli* DNA was also TLR9-dependent, TLR9^{-/-} FLDCs were treated with *E. coli* DNA with or without hBD3 (figure 4.6). TLR9^{-/-} FLDCs treated with *E. coli* DNA and hBD3 produced no detectable IL-6 or IFN- α (figure 4.6 A) and there was no costimulatory marker expression on pDCs or cDCs above a basal level (figure 4.6 B). These data indicate that the exacerbation of the response to *E. coli* DNA by hBD3 is also entirely TLR9-dependent.

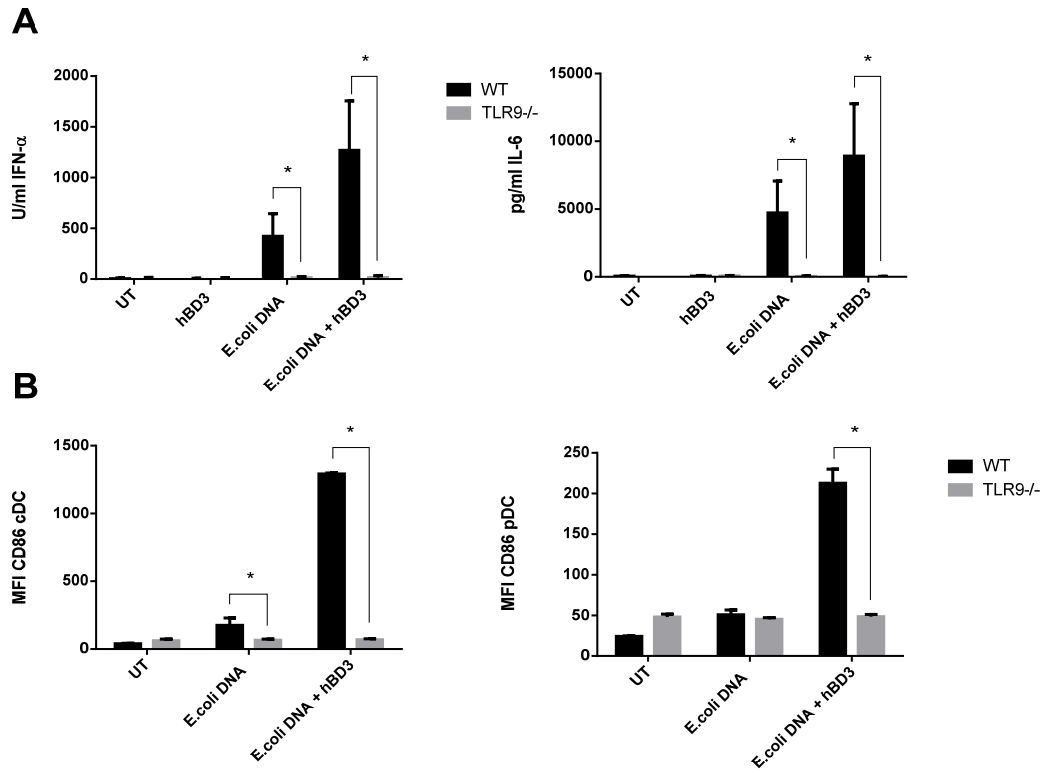


Figure 4.6 The exacerbatory effect of hBD3 on the response to *E. coli* DNA is TLR9-dependent. Wild-type or TLR9^{-/-} FLDCs were incubated with 1 μ g/ml *E. coli* DNA with or without 5 μ g/ml hBD3 overnight. Cytokine production was measured by ELISA (A) and expression of costimulatory markers on cDCs and pDCs was measured by flow cytometry (B, only CD86 shown). Data shown is the mean of 3 independent experiments performed in triplicate $\pm$ standard error. *p<0.05, unpaired t-test.

4.2.5 HBD3 has no effect on the response to self-DNA

It has been previously shown that hBD3 peptide increases the activation and production of IFN- α from pDCs by increasing cellular uptake of self DNA (Tewary et al., 2013). We found that self-DNA does not induce an immune response (figure 4.7), which Tewary et al. also demonstrated. However, in contrast to their findings, in my hands, treatment of FLDCs with self-DNA in combination with hBD3 did not affect the production of IL-6 or IFN- α (figure 4.7), implying that this combination is not immunostimulatory. Tewary *et al.* had ‘precomplexed’ the DNA with hBD3, however when self-DNA and hBD3 were ‘precomplexed’ as described in (Tewary et al., 2013) there was no increase in production of IFN- α or IL-6, demonstrating that hBD3 and self-DNA do not form a complex that is immunostimulatory to FLDCs. The differences between the experimental systems which may have caused these discrepancies are examined in the discussion.

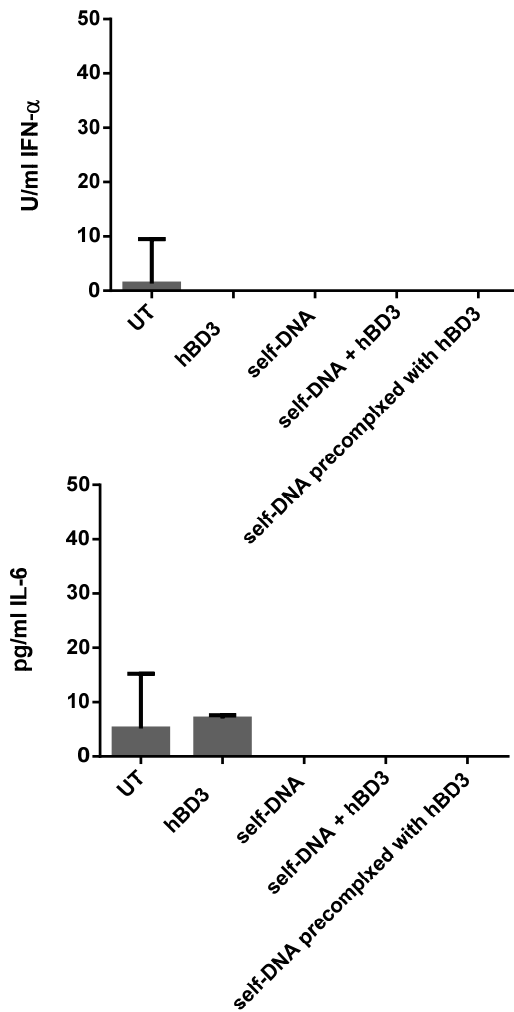


Figure 4.7 hBD3 has no effect on the response to self-DNA. FLDCs were incubated with 1 μ g/ml mouse genomic DNA alone, mixed with 5 μ g/ml hBD3 or precomplexed with 5 μ g/ml hBD3 at room temperature for 30 minutes (Tewary et al., 2013), overnight. Cytokine production was measured by ELISA. The data shown is the mean of 3 independent experiments performed in triplicate $\pm$ standard error.

4.2.6 HBD3 inhibits the response to transfected self-DNA

As previously discussed, it was found that only transfected self-DNA induced an immune response in FLDCs (figure 3.8); so FLDCs were transfected with self-DNA alone or in combination with hBD3. HBD3 significantly down-regulated the production of both IFN- α and IFN- β in response to transfected self-DNA, and decreased the production of IL-6 (figure 4.8 A) though the difference was not significant. It is notable that the combination of treatment with DNA, transfection and hBD3 did not have an effect on the activation of cDCs or pDCs in response to transfected self-DNA, suggesting that the inhibition was not caused by a decrease in cell viability, however it would be best to test cell viability directly using a cytotoxicity assay (figure 4.8 B).

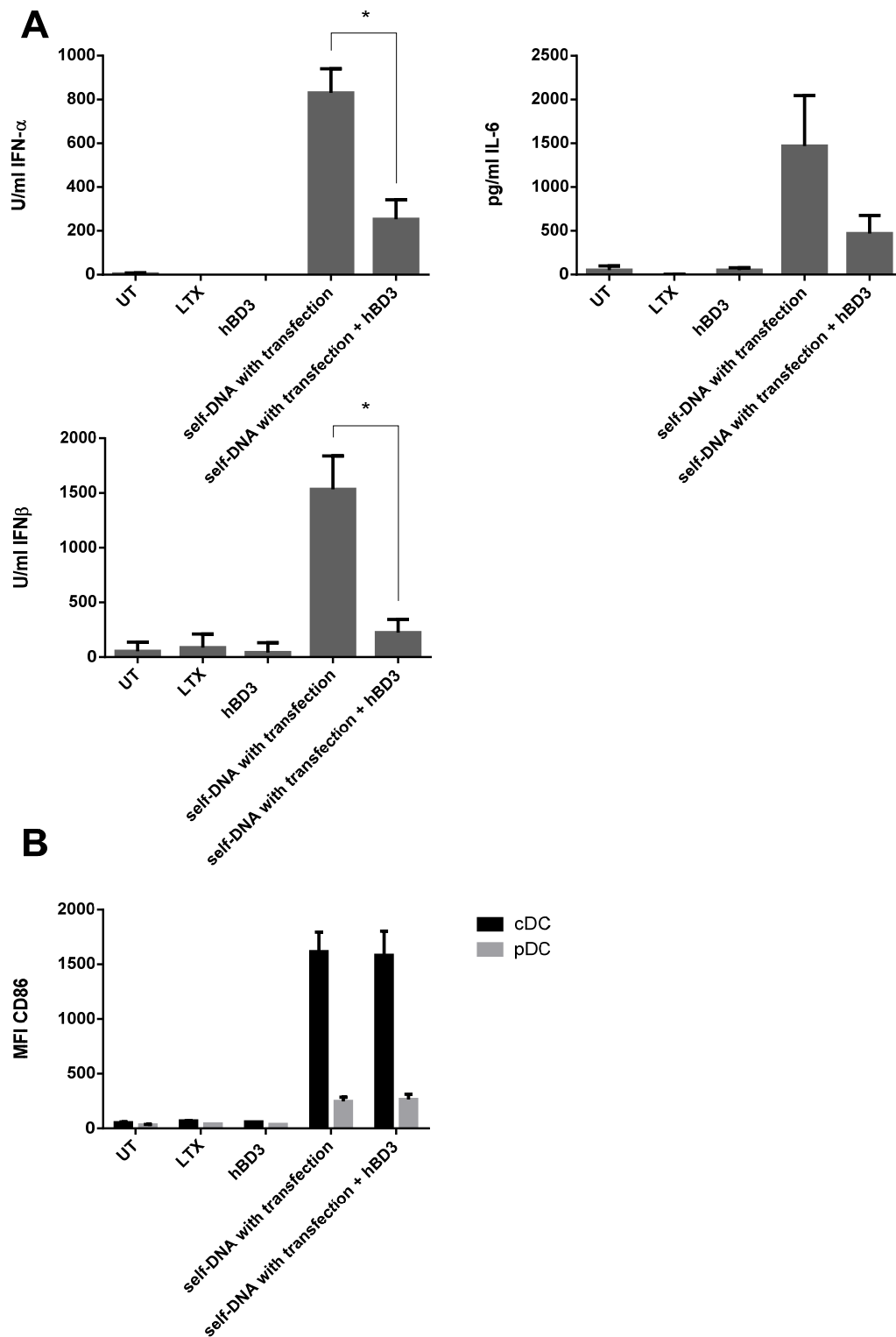


Figure 4.8 hBD3 inhibits the response to transfected self-DNA. FLDCs were transfected with 1 μ g/ml self-DNA with or without 5 μ g/ml hBD3 overnight.

Cytokine production was measured by ELISA (A) and costimulatory marker expression on both pDCs and cDCs was measured by flow cytometry (B). Data shown are the mean of 3 independent experiments performed in triplicate $\pm$ standard error. * $p < 0.05$ unpaired t-test

4.2.7 Methylated *E. coli* DNA induces a smaller immune response in FLDCs

The difference in the response to *E. coli* DNA compared to untransfected self-DNA was expected since *E. coli* DNA contains a high proportion of unmethylated CpG motifs, which are known to trigger a robust immune response via TLR9. To test whether this effect is dependent on unmethylated CpG motifs present in *E. coli* DNA, the DNA was methylated *in vitro* using CpG methyl-transferase (M.SssI), a bacterially derived enzyme which should methylate all CpG motifs in double stranded DNA. Increased methylation of the DNA was confirmed via a dot blot using an antibody against methylated CpG motifs, anti-5-methylcytosine (5-mC). Following the methylation reaction there was an increase in methylated CpG in *E. coli* DNA, as shown by the increased intensity on the dot blot, and the methylated *E. coli* DNA bound the antibody with a similar affinity to self-DNA (figure 4.9 A). When FLDCs were treated with the methylated *E. coli* DNA there was a significant reduction in the production of both IFN- α and IL-6 compared with 'native' *E. coli* DNA (figure 4.9 B). To check that it was not a contaminant in the methylation reaction that was affecting the results, a control methylation reaction was set up without the methyl-transferase enzyme, and FLDCs were treated with this DNA. There was no reduction in the response seen to the 'no methylation' control confirming that the reduction in the response to methylated *E. coli* DNA is not due to a contaminant in the methylase reaction. It would have been interesting to transfect the methylated *E. coli* DNA to see if it has the same effect as transfected self-DNA and induces cytokine production.

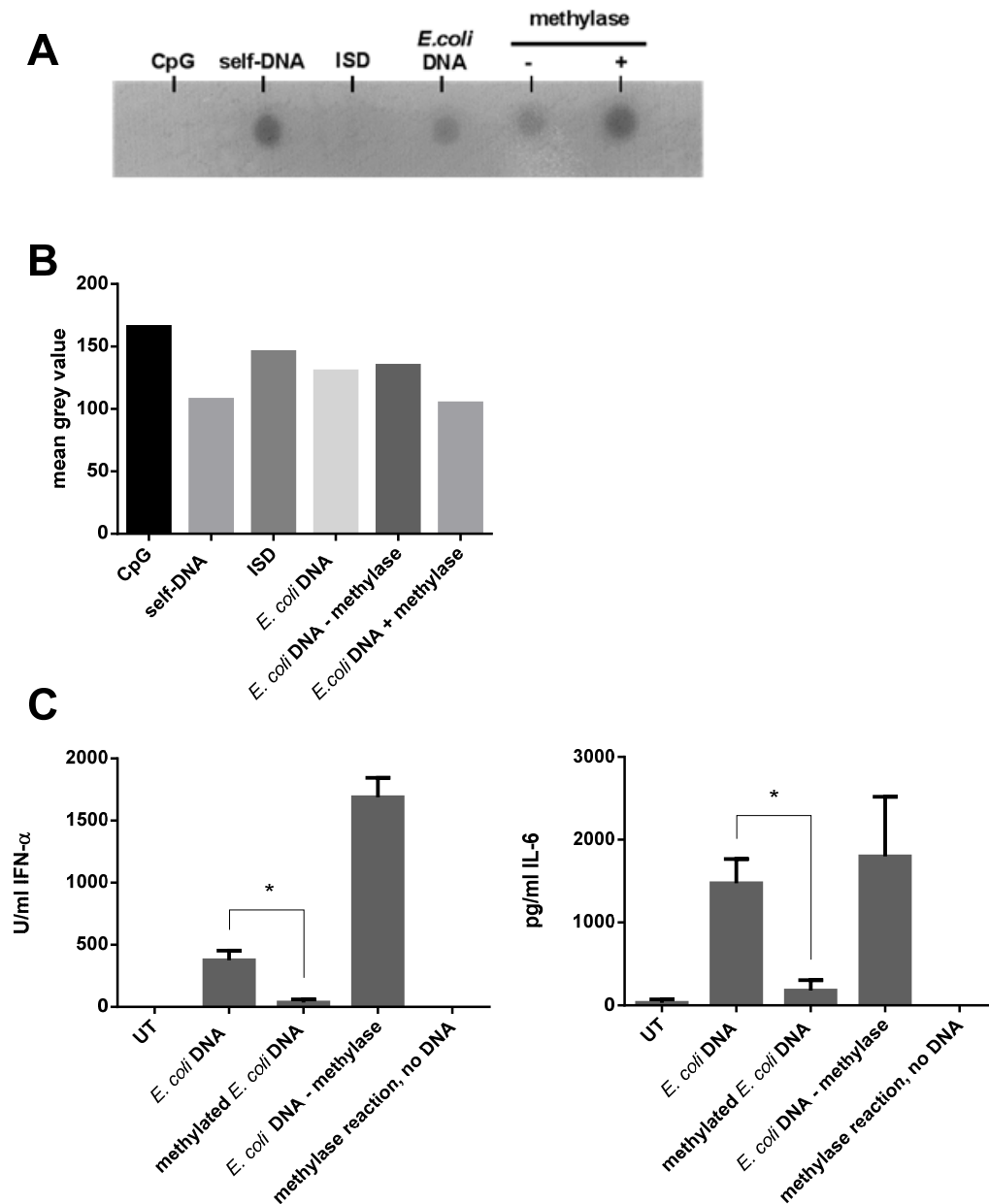
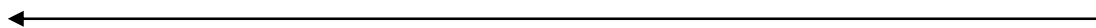


Figure 4.9 *In vitro* methylation of *E. coli* DNA makes it less immunogenic.

E. coli DNA was methylated in vitro using M.SssI. 322 ng (in 1 μ l) CpG ODN1585, self-DNA, interferon-stimulatory DNA (ISD), *E. coli* DNA or *E. coli* DNA with or without M.SssI was dotted onto a membrane and probed for methylated CpG motifs using anti-5-mC antibody, representative of 2 experiments (A). The result was quantified by measuring mean grey value of each dot, representative of 2 experiments (B). FLDCs were incubated with 1 μ g/ml *E. coli* DNA, methylated *E. coli* DNA, *E. coli* DNA without methylase or methylase reaction without DNA,

overnight. Cytokine production was measured by ELISA (C). Data shown is the mean of 3 independent experiments performed in duplicate $\pm$ standard error. * $p < 0.05$ unpaired t-test



4.2.8 HBD3 exacerbates the response to methylated *E. coli* DNA

The previous data confirm that the immune response to *E. coli* DNA is mostly due to a high proportion of unmethylated CpG motifs and by methylating these and decreasing the proportion of unmethylated CpG motifs, there was a significant decrease in the response (figure 4.10). This implies that by methylating *E. coli* DNA, it looks more like self-DNA and is therefore less immunogenic. Since hBD3 had no effect on self-DNA (figure 4.7) one might expect that hBD3 will no longer have an exacerbatory effect on methylated *E. coli* DNA. However, production of IL-6 and IFN- α was still significantly up-regulated in response to methylated *E. coli* DNA with hBD3 compared to methylated *E. coli* DNA alone (figure 4.10). This suggests that the methylated *E. coli* DNA is still recognized by TLR9 as an immune ligand and hBD3 exacerbates this response.

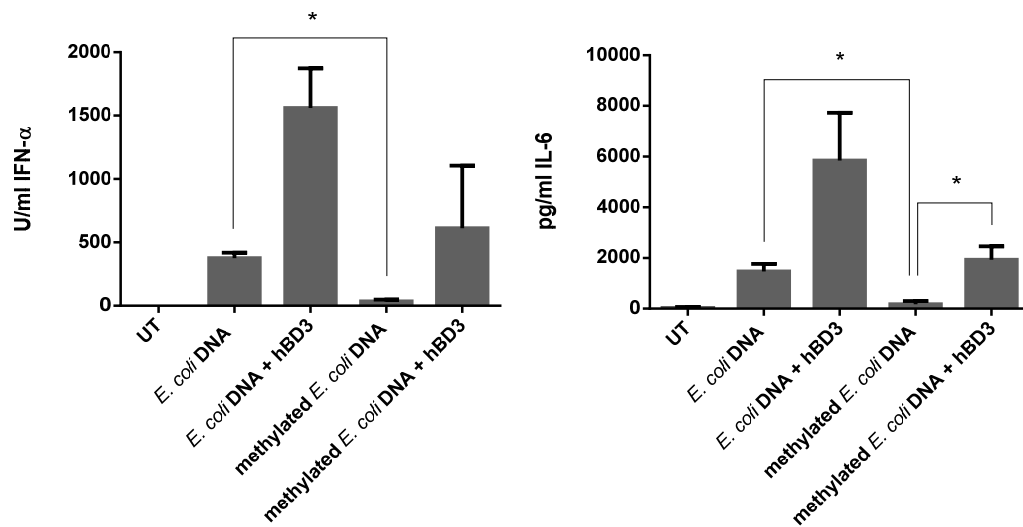


Figure 4.10 hBD3 exacerbates the response to methylated DNA. FLDCs were incubated with 1 $\mu\text{g/ml}$ *E. coli* DNA or methylated *E. coli* DNA with or without 5 $\mu\text{g/ml}$ hBD3, overnight. Cytokine production was measured by ELISA. Data shown are the mean of 3 independent experiments performed in triplicate $\pm$ standard error. * $p < 0.05$ unpaired t-test

4.2.9 HBD3 has no effect on the response to self-DNA derived from apoptotic cells

The most probable scenario when self-DNA would be found in the extracellular milieu is following cellular apoptosis, raising the possibility that self-DNA is only recognised as an immune ligand if it has particular features which marks it as 'apoptotic'. The major effect of apoptosis on DNA is to induce DNA fragmentation, which is seen as a characteristic DNA ladder on an agarose gel due to the cleavage of chromosomal DNA into internucleosomal fragments (Nagata, 2000). Firstly, apoptosis was induced in RAW 264.7 cells using UV irradiation, a portion of these cells were harvested 24 hours after irradiation and analysed by flow cytometric analysis for markers of apoptosis. UV treated and untreated cells were stained with annexin V and DAPI. Annexin V binds to phosphatidylserine (PS) on the outer leaflet of the plasma membrane where it expressed from the early stages of apoptosis, during the early stages of apoptosis there is as yet no loss of integrity of the cell membrane, therefore viability dyes such as DAPI are excluded (Vermes et al., 1995). Early apoptotic cells can be detected by annexin V⁺/DAPI⁻ whilst late apoptotic/necrotic cells can be detected as annexin V⁺/DAPI⁺ (figure 4.11 A). In this way the UV treated cells were compared to the untreated cells and clearly showed a higher proportion of both early apoptotic and late apoptotic/necrotic cells (figure 4.11 B). This confirmed that the UV treatment had worked to induce apoptosis; however when DNA was extracted from the UV treated cells at 5 or 24 hours there was no evidence of DNA laddering even though the DNA was more of a smear than DNA from untreated cells indicating some DNA fragmentation (figure 4.11 C). Transfected DNA from 24h after UV treatment did appear to stimulate increased production of IL-6 and IFN- α compared with DNA untreated from untreated cells, indicating that this DNA is more immunogenic, untransfected DNA from UV treated cells did not induce any immune response in FLDCs in the presence or absence of hBD3 (figure 4.11 D).

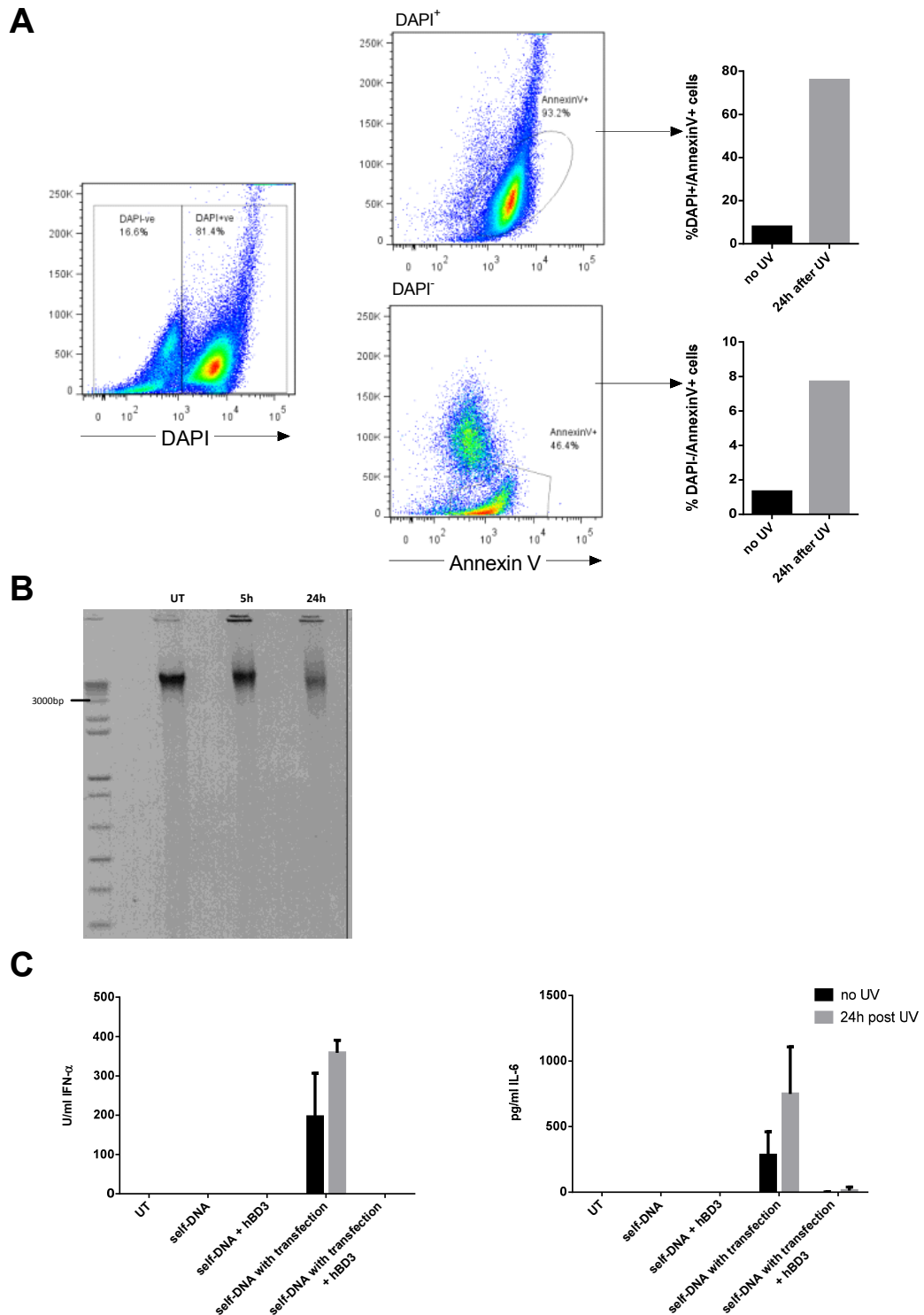


Figure 4.11 hBD3 has no effect on the response to self-DNA extracted from apoptotic cells. RAW 264.7 cells were exposed to 160 mJ/cm² UV and cultured for 24 hours. Cells were harvested and stained with annexin V and PI to measure

apoptosis using flow cytometry (A). DNA extracted from UV treated or control cells was electrophoresed in an agarose gel to look for DNA laddering (B). FLDCs were treated with 1 µg/ml DNA extracted from UV treated or untreated cells with or without 5 µg/ml hBD3 and incubated overnight. Cytokine production was measured by ELISA (C). Data shown is from one experiment performed in triplicate ± standard deviation of the replicates.

4.2.10 Using fluorescently labelled DNA to study uptake and localisation

The results previously discussed in this chapter suggest that understanding the localisation of DNA, and how hBD3 may affect this, is critical in understanding the mechanism by which hBD3 affects the response to DNA. There are no commercially available supplies of labelled *E. coli* or mouse genomic DNA so a DNA labelling kit was used to label the DNA; this kit labels double stranded DNA with a CX-rhodamine label every 20-60 base pairs.

4.2.10.1 Uptake of labelled DNA can be analysed in macrophages

The labelled DNA was tested first on a macrophage cell line, RAW 264.7 cells, to ensure that it was detectable via both fluorescent microscopy and flow cytometry. RAW 264.7 cells were used to check that the uptake of labelled DNA could be measured by flow cytometry and viewed by microscopy in a fast growing cell line before moving to primary cells. RAW 264.7 cells do not respond to *E. coli* DNA but respond to the TLR3 ligand poly(I:C) indicating that the same endosomal uptake pathways exist in RAW 264.7 as in FLDCs.

RAW 264.7 cells were plated and treated with labelled DNA for 4 hours; the cells were treated with DAPI as a viability marker immediately before flow cytometric analysis. The flow cytometry data was gated to identify live cells on the basis of exclusion of DAPI (figure 4.12 A), and then gated on what proportion of the cells was rhodamine positive (figure 4.12 B) and what the median fluorescence intensity of rhodamine was across the population of live cells (figure 4.12 C).

The proportion of live cells that were rhodamine positive increased following treatment with either labelled *E. coli* DNA or self-DNA and further increased when labelled DNA was transfected into RAW 264.7 cells as a positive control (figure 4.12). The median amount of DNA per live gated cell was also measured; there was a high level of autofluorescence in untreated cells, but even when the autofluorescence of the untreated cells is taken into account (red dotted line across figure 4.12 C) the DNA treated cells have a significantly higher MFI of rhodamine.

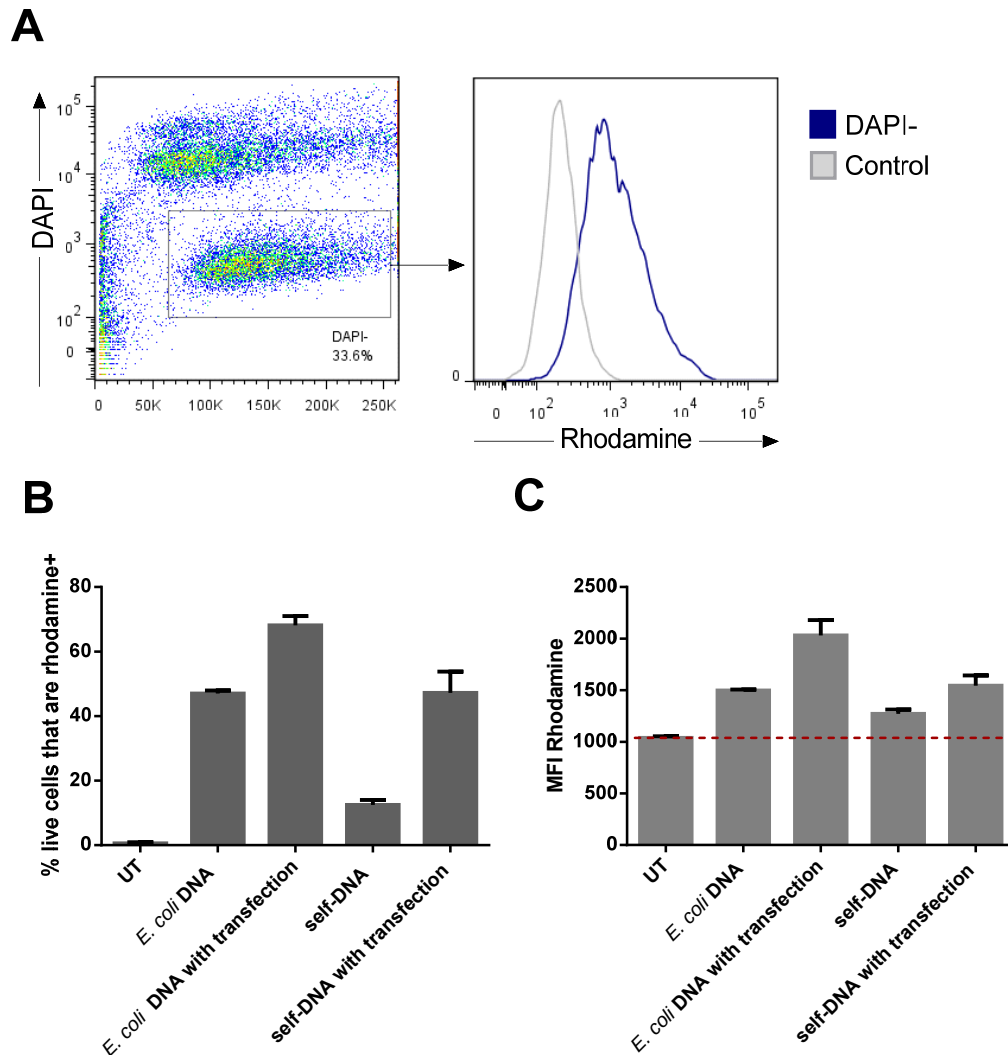


Figure 4.12 Using a cell line to optimise the use of labelled DNA. RAW 264.7 were incubated with 1 $\mu\text{g/ml}$ labelled *E. coli* or transfected with 1 $\mu\text{g/ml}$ self-DNA for 4 hours. Uptake of DNA was analysed by flow cytometry with DAPI stain as live/dead marker. (A-C) Data shown are the mean of 2 independent experiments

performed in duplicate $\pm$ standard deviation of replicates. Flow cytometry plots are representative of 2 independent experiments.



4.2.10.2 Uptake of labelled DNA can be analysed in FLDCs

The results from labelled DNA in RAW 264.7 cells confirmed that labelled DNA was a useful and effective way to look at uptake of DNA by both flow cytometry and microscopy so the same experiment was carried out in FLDCs.

FLDCs were treated with labelled DNA for 4 hours and then harvested for flow cytometry. The stains used on the FLDCs (CD11c-APC780, B220-eFluor450 and Cx-Rhodamine) were incompatible with available viability dyes so an untreated sample was stained with DAPI and the live population (DAPI⁻) correlated back to a size gated population (figure 4.13 A). There was an unexpectedly high proportion of dead (DAPI⁺) cells in both of the two independent experiments that were performed using labelled DNA; with approximately 5-20% cells in the live gated population compared to a normal live gated population of approximately 60-70%.

There was an increase in the proportion of rhodamine positive DCs following treatment of FLDCs with either *E. coli* DNA or self-DNA with or without transfection as well as an increase in the MFI of rhodamine showing that uptake of labelled DNA can also be effectively measured in FLDCs (figure 4.13 C and D).

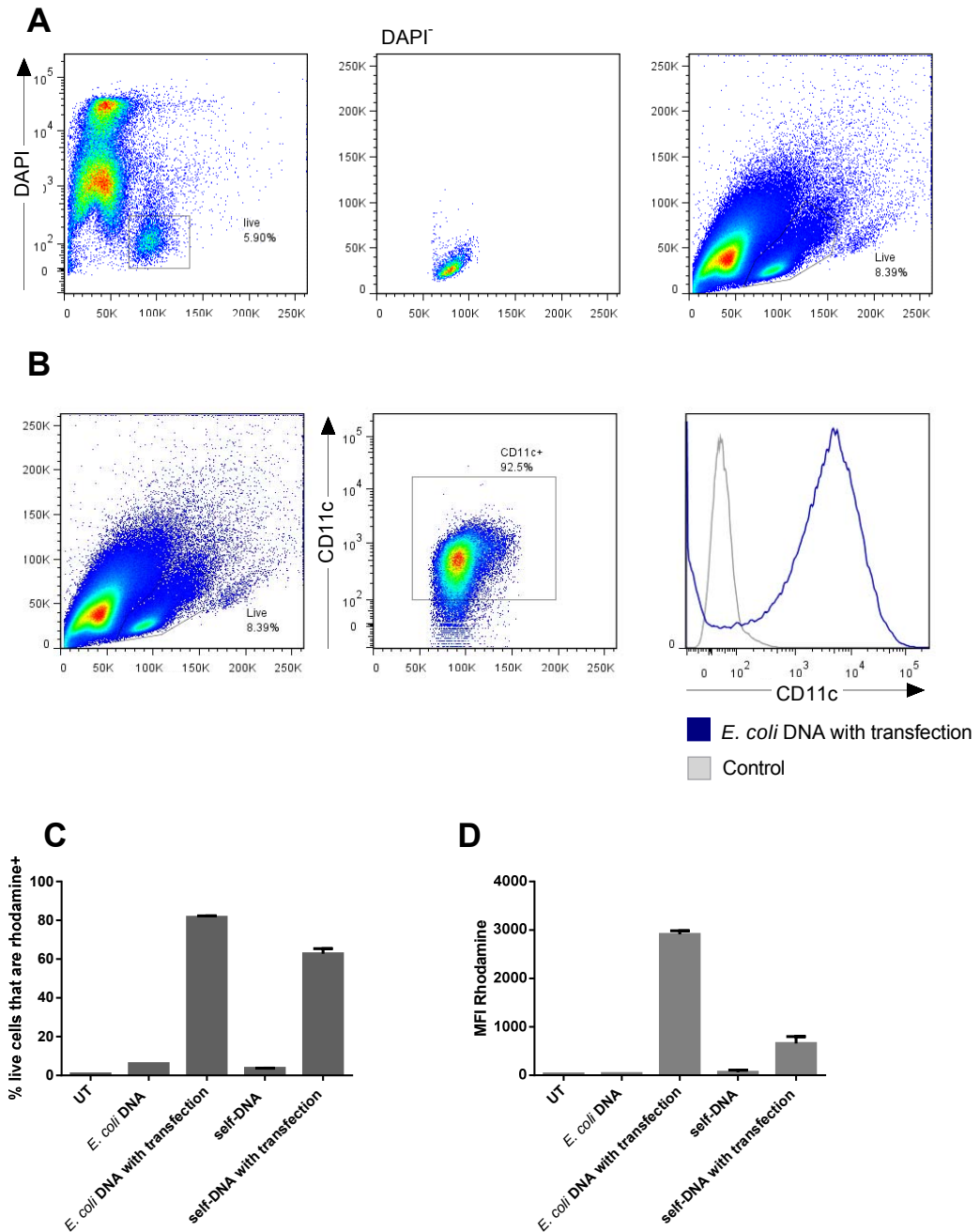


Figure 4.13 Measuring uptake of labelled DNA in FLDCs.

FLDCs were incubated with or transfected with 1 $\mu\text{g/ml}$ labelled *E. coli* or 1 $\mu\text{g/ml}$ self-DNA for 4 hours. Uptake of DNA was analysed by flow cytometry with DAPI stain as a live/dead marker (A, untreated as an example). CD11c⁺ cells were gated from the live (DAPI⁻) population and quantified for rhodamine (B, treatment with transfected *E. coli* DNA as an example). Data shown is the mean of 2 independent experiments performed in triplicate $\pm$ standard deviation of the replicates.

4.2.11 HBD3 affects uptake of both *E. coli* DNA and self DNA

Labelled DNA was used to evaluate whether hBD3 has an effect on the uptake of labelled DNA. FLDCs were treated for four hours and uptake of labelled DNA by the live, CD11c⁺ population was analysed by flow cytometry. HBD3 significantly increased the proportion of cells that were rhodamine positive i.e. hBD3 increased the proportion of FLDCs that took up labelled *E. coli* DNA and self-DNA (figure 4.14 A). HBD3 also increased the median amount of *E. coli* DNA taken up by FLDCs, as measured by MFI of rhodamine in CD11c⁺ FLDCs (figure 4.14 B). The effect of hBD3 on the amount of self-DNA taken up was not clear due to a large variability between samples treated with self-DNA alone. Since FLDCs only responded to self-DNA when it was transfected, FLDCs were transfected with labelled DNA and shortly afterwards (10 minutes) were treated with or without hBD3 and incubated for 4 hours. The results show that hBD3 significantly decreased both the proportion of cells to take up labelled DNA and the MFI of rhodamine suggesting that hBD3 inhibits uptake of transfected DNA (figure 4.14 C and D).

FLDCs treated with labelled DNA were also attached to slides by cytopinning to visualise the DNA and attempt to analyse subcellular localisation. The images show that hBD3 causes the DNA to clump together into aggregates (figure 4.14 E). Differential interference contrast (DIC) microscopy was used to enable visualisation of the cell contour, showing that these aggregates appear to enter the cell, however further investigation must be performed to confirm this, for example by taking Z-stacks using confocal microscopy.

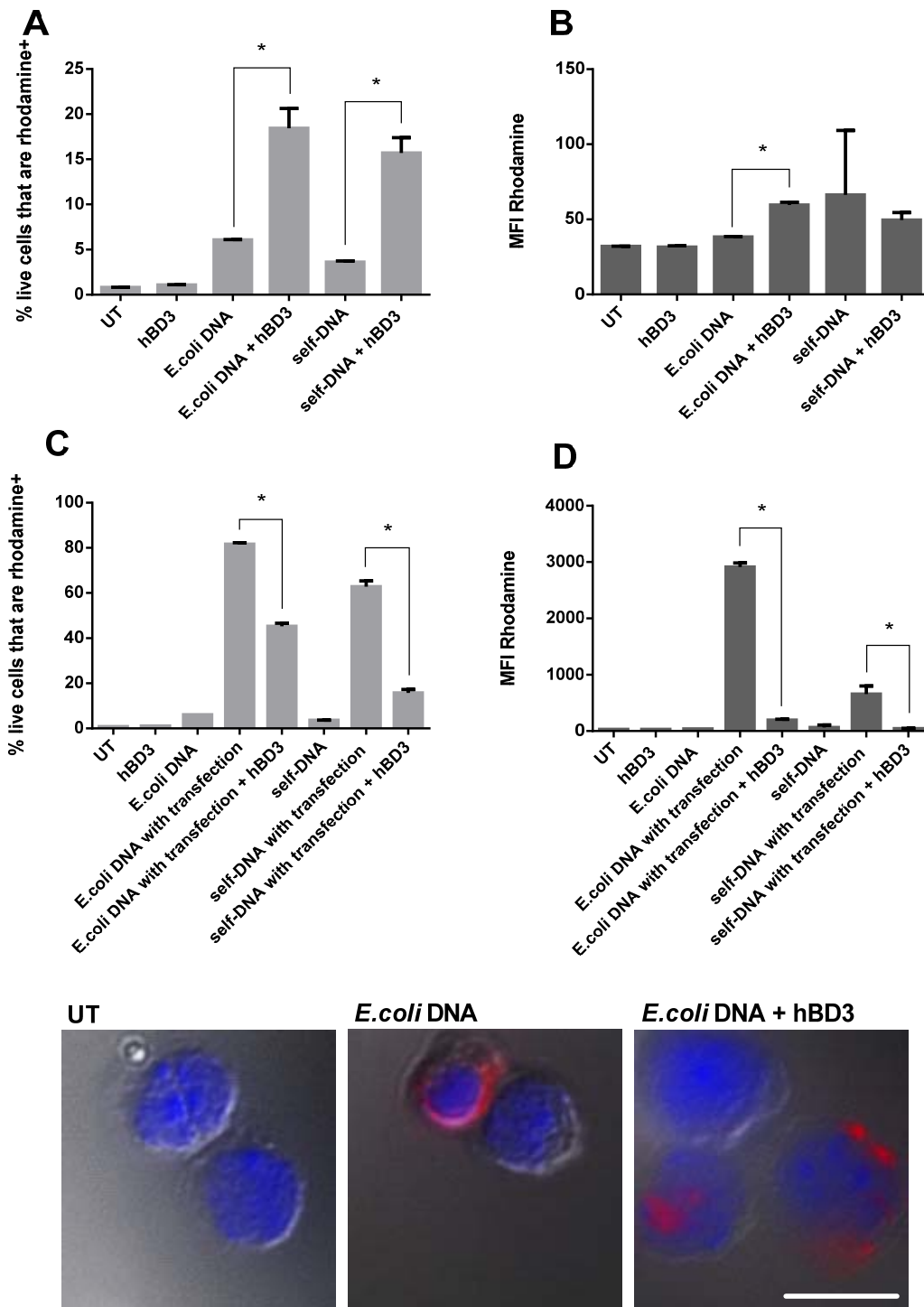


Figure 4.14 Measuring the effect of hBD3 on labelled DNA uptake.

FLDCs were incubated with, or transfected with, 1 $\mu\text{g/ml}$ labelled *E. coli* or self-DNA with or without 5 $\mu\text{g/ml}$ hBD3 for 4 hours. Uptake of DNA was analysed by flow cytometry measuring the proportion of DAPI⁺rhodamine⁺ cells and the MFI of rhodamine across the DAPI⁺ population. The data shown is the mean of 2

independent experiments performed in triplicate. Error bars are standard deviation of the replicates. * $p < 0.05$ student's t-test. Images taken using a 100x objective with fluorescent channels and DIC channels overlaid to visualise the cell membrane. Scale bar = 50 μm

4.2.12 HBD3 affects the migration of DNA in a gel shift assay

Visualising FLDCs treated with labelled DNA using fluorescence microscopy demonstrated that hBD3 had a substantial effect on the morphology of DNA. To investigate this further *E. coli* and self-DNA were mixed with a range of concentration of hBD3 and the effect of hBD3 on migration of the DNA was analysed. Both *E. coli* and self-DNA migrated into a 1% agarose gel but they were too big to accurately determine what size they were. The fact that they both appeared to run at a similar size yet mouse DNA should be much larger than *E. coli* DNA (the *E. coli* circular genome is approximately 4.6 Mbp whilst the smallest mouse chromosome is 61 Mbp) indicates that it is not possible to resolve such large sizes in an agarose gel. To be able to better resolve differences in migration of DNA with and without hBD3, the DNA was sonicated into approximately 200 bp fragments. In a single preliminary experiment, sonicated DNA induced a similar cytokine response to unsonicated DNA indicating that hBD3 has the same effect on sonicated DNA (figure 4.15 C, compared with figures 4.5, 4.7 and 4.8), however this experiment needs to be repeated to confirm that sonicated DNA has the same effect on FLDCs to non-sheared DNA. When sonicated DNA was run into an agarose gel following incubation with a range of concentrations of hBD3 there was a clear concentration-dependent effect on the migration of both *E. coli* DNA and self-DNA with hBD3 apparently trapping the majority of the DNA in the well at the highest concentration used (figure 4.15 A). When the gel was post-stained with coomassie to stain the proteins there was a correlation between where the DNA appeared to have aggregated in the gel and where the coomassie stained protein, which is assumed to be hBD3 as no other proteins were introduced (figure 4.15 B). Since all the FLDC treatments are performed in 8% serum, the experiment was repeated in 8% serum. With serum slightly more DNA migrates into the gel at the highest concentration of

hBD3 indicating that serum slightly inhibits the effect of hBD3 on preventing migration of DNA (figure 4.15 A). These results suggest that in this *in silico* system hBD3 binds to *E. coli* DNA and self-DNA, trapping the DNA in the well and preventing migration.

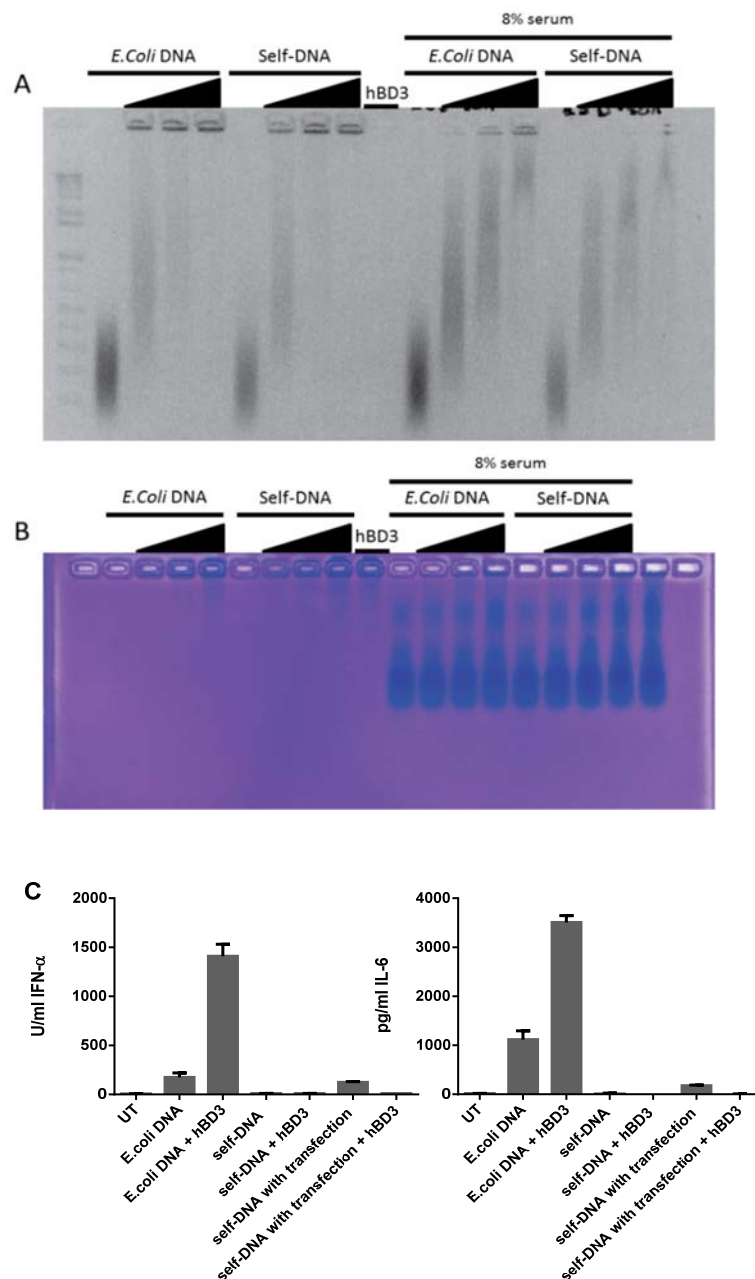


Figure 4.15 hBD3 prevents migration of sonicated DNA. DNA was sonicated to 200 bp fragments and mixed with different concentrations of hBD3 before running in

an agarose gel and visualising DNA using UV light (A) or protein using coomassie blue staining (B). The images are representative of 2 independent experiments. FLDCs were incubated with, or transfected with, 1 µg/ml sonicated *E. coli* or self-DNA with or without 5 µg/ml hBD3, overnight (C). Cytokine production was measured by ELISA. Data shown is the mean of one independent experiment performed in triplicate $\pm$ standard deviation of the replicates.



4.3 Discussion

This chapter has explored the role of hBD3 in the regulation of the innate immune response to pathogenic compared with self-DNA. The following points summarise the major findings in this chapter:

- hBD3 exacerbates the proinflammatory response of FLDCs to *E. coli* DNA in a TLR9-dependent manner, increases uptake of labelled *E. coli* DNA into FLDCs and prevents migration of *E. coli* DNA into an agarose gel.
- hBD3 has no effect on the proinflammatory response of FLDCs to self-DNA, however it increases the uptake of labelled self-DNA into FLDCs and prevents migration of self-DNA into an agarose gel.

4.3.1 A broad role for hBD3 in TLR signalling

HBD3 has been previously shown to regulate the innate immune system: down-regulating the TLR4-mediated response to LPS, chemoattracting immune cells and having differential effects on the MDA-5 or TLR3 dependent response to poly(I:C) (Semple et al., 2011; Semple et al., 2010) (Semple et al., unpublished).

Overexpression of hBD3 significantly inhibited ligand independent activation of NF- κ B via both the MyD88 and TRIF dependent pathways. By overexpressing the adapter proteins, MyD88 and TRIF, these experiments were ligand and receptor independent suggesting that hBD3 targets downstream of - or at the level of - the adapter proteins. This is supported by previous evidence that hBD3 does not inhibit the response to LPS by binding and sequestering it away from TLR4 (Semple et al., 2011). All TLRs require either functional MyD88 or functional TRIF (discussed further in the introduction) so the inhibitory effect of hBD3 on both the MyD88- and the TRIF- dependent pathway implies a broad role in regulating TLR-dependent pathways.

4.3.2 HBD3 causes aggregation of FLDCs without cytotoxicity or activation

HBD3 consistently caused aggregation of FLDCs and this effect was observed after just four hours of treatment. Aggregation of pDCs has been previously used as a marker for cell activation (Lande et al., 2007), however the expression of costimulatory markers on hBD3 cells was unchanged compared to untreated cells confirming that hBD3 does not activate FLDCs. Additionally, hBD3 treatment had no effect on the relative proportions of CD11c⁺, CD11c⁺B220⁺ or CD11c⁺B220⁻ populations. Moreover, this concentration of hBD3 did not cause cytotoxicity. However the question remained as to why treatment with hBD3 caused FLDC aggregation. *In vivo*, intraperitoneal injection of hBD3 chemoattracted Csf1r⁺ cells into the peritoneum, therefore it is possible that this cellular aggregation may be caused by a similar mechanism to chemoattraction. CD8⁻ cDCs which are functionally equivalent to CD11c⁺B220⁻CD11b^{hi} FLDCs express high levels of CCR6, whilst pDCs express intermediate levels (Brawand et al., 2002; Naik et al., 2005). This chemokine receptor is involved in chemoattraction by hBD3 *in vitro* (Röhrl et al., 2010; Taylor et al., 2008a; Tyrrell et al., 2010; Wu et al., 2003a). Alternatively, hBD3 may be stimulating expression of adhesion molecules for example DC-specific intercellular adhesion molecule-grabbing non-integrin (DC-SIGN) which is involved in mediating DC-T cell aggregation and DC migration (Geijtenbeek et al., 2000; Geijtenbeek et al., 2002). Zymosan treatment induces comparable aggregation of human monocyte derived DCs due to up-regulation of DC-SIGN expression (de la Rosa et al., 2005). The effect of hBD3 on DC-SIGN expression could be evaluated either by immunofluorescence, which would also identify whether it was involved in cell-cell contacts, or via qPCR. It would also be interesting to see whether these hBD3 treated FLDCs were more adhesive in a DC-T cell coculture.

4.3.3 HBD3 exacerbates the response to *E. coli* DNA in a TLR9-dependent manner by increasing cellular uptake

Having convincingly and reproducibly shown that hBD3 exacerbates the response to *E. coli* DNA, further investigations sought to understand the mechanism of this effect. HBD3 caused an increase in both the proportion of FLDCs that contained fluorescently labelled *E. coli* DNA after four hours and an increase in the median amount of fluorescently labelled DNA that each cell contained. The increased cellular uptake of *E. coli* DNA mediated by hBD3 potentially causes the increased immune response. HBD3 causes aggregation of the DNA as shown by both microscopy and a gel migration assay showing that hBD3 prevented migration *E. coli* DNA into an agarose gel in a concentration dependent manner. The DNA aggregates co-localised with protein staining, indicating that hBD3 co-aggregates with DNA. These results suggest that the hBD3-protein aggregates were too big to migrate into the gel. The gel migration assay is a very synthetic system for looking at the effect of hBD3 on DNA but combined with other results provides considerable evidence for hBD3 forming a complex with *E. coli* DNA. Future work should involve determining whether hBD3 is associated with these DNA aggregates by co-staining using an antibody against hBD3; or by attempting to immunoprecipitate these complexes.

The results discussed so far in this chapter provide compelling evidence for hBD3 exacerbating the response to *E. coli* DNA by increasing cellular uptake. Stimulation of TLR9 deficient FLDCs demonstrated that the exacerbatory effect of hBD3 is completely TLR9-dependent, indicating that hBD3 does not cause relocation of *E. coli* DNA to an alternative receptor. Since TLR9 is found in the endosome, presumably hBD3 increases uptake into the endocytic pathway. Pre-treatment of FLDCs with chloroquine (Rutz et al., 2004) in an attempt to verify this by preventing functionality of the endocytic pathway was unsuccessful due to significant cell death (data not shown) which probably underlies the failure of this experiment. The concentration of chloroquine used has been previously used successfully (Rigby et al., 2014), but potentially requires further optimisation. Additionally, association between TLR9 and DNA or DNA-hBD3 complexes could be shown using fluorescence resonance energy transfer (FRET). Using labelled TLR9 and labelled

DNA, interactions and interaction distances can be studied via confocal microscopy or super resolution microscopy (Horvath et al., 2009).

The DNA-hBD3 complex may not intrinsically be more immunogenic, rather it may take more time to transit through the endocytic pathway, or the complex may be more resistant to nuclease digestion than DNA alone. Either of these mechanisms would cause a larger response, simply for the reason that FLDCs would respond over a lengthened period of time. One method to test this would be to use live cell imaging to track the transit of DNA through the endocytic pathway. A major obstacle against using this technique and generally against studying subcellular localisation in FLDCs is the small size of the cells and how little cytoplasm they have. An alternative system would be to assess mRNA expression of cytokines and type I interferon over a time course to get a real-time view of whether the immune response is actually bigger or is a culmination of a longer response period due to retention in the endocytic pathway.

The finding that hBD3 exacerbates the response to *E. coli* DNA was surprising considering that the most likely context in which bacterial DNA would be found would be alongside LPS following bacterial cell death. This raised the question: why would hBD3 inhibit the response to one bacterial ligand and exacerbate the response to another. It is interesting to note that *E. coli* DNA stimulates large amounts of IFN- α as well as expression of costimulatory markers by both cDCs and pDCs; LPS does not stimulate any IFN- α production from FLDCs, since pDCs, the major IFN- α producers, do not express TLR4. The activation of pDCs and production of IFN- α is an antiviral response; IFN- α directly kills viruses and activates a subset of genes which stimulate an antiviral response, whilst expression of costimulatory markers of pDCs leads to activation of the adaptive immune response via antigen presentation. The upregulation of costimulatory markers on both cDCs and pDCs following treatment with hBD3 and *E. coli* DNA compared with *E. coli* DNA alone supports the idea of an antiviral-type response being activated. It would be intriguing to test whether these activated FLDCs were able to activate T cells in a co-culture. Since viral DNA also contains unmethylated CpG motifs this may be a conserved mechanism in responding to pathogenic DNA (Lund et al., 2003) therefore it would

also be interesting to test the effect of hBD3 on hypomethylated DNA from both viruses and fungi (Ramirez-Ortiz et al., 2008). Mitochondrial DNA (mtDNA) is also hypomethylated due to the origin of mitochondria as evolutionary symbionts. The release of mitochondrial DAMPs including mtDNA is associated with inflammation (Zhang et al., 2010a), therefore it would be valuable to investigate what effect hBD3 has on the response to mtDNA.

4.3.4 HBD3 increases uptake of self-DNA without stimulating an immune response

Contrary to recently published data in human cells, we could not find any evidence that hBD3 has an effect on the response to self-DNA; despite hBD3 enhancing the uptake of self-DNA, as shown by flow cytometry (Tewary et al., 2013). HBD3 inhibited the response to transfected self-DNA but this is likely to be an inhibitory effect on transfection rather than an inhibition of the response itself.

hBD3 was found not to have any effect on the response to untransfected self-DNA when it was added to cell cultures alongside DNA, or when it was incubated with DNA before adding to FLDCs as described in (Tewary et al., 2013). There are numerous important variations between the methods used here and the methods used by Tewary *et al.* which may explain the differences. Firstly is a potential effect of species specificity, Tewary *et al.* used a purified population of pDCs derived from human peripheral blood mononuclear cells; whereas our experiments were all done in a mixed population of mouse FLDCs. It has previously been shown that hBD3 acts in the same way as the mouse orthologue, Defb14 in inhibiting the response to TLR4 ligand LPS (Semple et al., 2010) although mouse-human differences have been previously reported in the immune response. It would be essential to ensure that this is not a species specific effect by testing the response of human peripheral blood DCs.

An alternative, or additional source of inconsistency is the source of the hBD3 peptide. Tewary *et al.* used Peprotech recombinant hBD3; a recombinant peptide has a number of issues: firstly, since it is produced in bacteria there is a risk of endotoxin contamination, though the manufacturers state that endotoxin is below threshold levels. Additionally, there is no way of proving that bacteria can fold the peptide

properly which is important as differences in hBD3 secondary structure can have different effects, particularly with regards to the regulatory function of hBD3 (Wu et al., 2003a). The peptide used in this thesis is from the Peptide Institute, it is chemically synthesised and folded so there is no risk of endotoxin contamination and it is folded via consensus disulphide bonds. Preliminary data comparing the Peprotech peptide with the peptide institute peptide proved inconclusive in demonstrating a difference between these two peptides.

Interestingly, although hBD3 did not stimulate a response to self-DNA, it increased the cellular uptake of self-DNA, which supports what Tewary *et al.* described. The gel migration assay testing the effect of hBD3 on migration of self-DNA showed a similar effect as with *E.coli* DNA. HBD3 prevented migration of self-DNA in a concentration dependent manner and protein staining showed an accumulation of peptide where there is also an accumulation of DNA. Microscope images of self-DNA in combination with hBD3 also showed hBD3 causing aggregation of self-DNA.

These data together suggest that hBD3 causes rapid, extracellular DNA aggregation and increases uptake regardless of DNA methylation. It seems likely that the hBD3-DNA complex is endocytosed with higher efficiency than DNA alone, however, whilst hypomethylated *E.coli* DNA with or without hBD3 is recognised by TLR9, there is no receptor for a self-DNA-hBD3 complex in the endosome.

A potential role for hBD3 in increasing cellular uptake of genomic DNA is extremely interesting in the context of autoimmune disease. There are a number of autoimmune diseases characterised by an aberrant response to self-DNA, for example: systemic lupus erythematosus (SLE), rheumatoid arthritis and psoriasis. In patients with SLE, DNA forms a complex with anti-DNA antibodies, these complexes induce cytokine production and deposit in tissues causing tissue inflammation (Su and Pisetsky, 2009). If these anti-DNA antibodies would still bind to DNA in complex with hBD3 which increases cellular uptake, one would predict that this would result in an increased cytokine response and more severe disease manifestation. It would be interesting to investigate this further by firstly testing whether DNA complexed with

hBD3 is still detected by anti-DNA antibodies isolated from SLE patient sera, and secondly whether this triggers a greater immune response.

Despite hBD3 increasing cellular uptake of DNA, self-DNA is not an immune stimulus so there would need to be a secondary trigger to stimulate an immune response. It has been shown that LL-37 acts by binding to self-DNA and forming a complex that induces an immune response in pDCs and monocytes (Chamilos et al., 2012; Lande et al., 2007). LL-37 levels are increased in psoriasis leading to postulation that LL37 binds to DNA released from dying keratinocytes in active psoriatic plaques leading to type I interferon production and exacerbation of the disease. Since increased copy number of the gene region containing *DEFB103* is linked to increased risk of psoriasis, hBD3 could also have a role in the development of the disease. HBD3 did not induce an immune response to self-DNA, however DNA from apoptotic cells may have specific properties that make it immunogenic. Since self-DNA in the cytoplasm is detected as a marker of cell damage inducing an inflammatory response, one would imagine that apoptotic DNA fragments would be an even more potent stimulus. Unfortunately, DNA extracted from apoptotic cells showed no indication of the apoptotic DNA fragments in multiples of 180 bp that were expected which may be due to an extraction protocol that did not favour extraction of smaller fragments (Wyllie, 1980). Moreover, preliminary data showed that whilst there was a slight increase in the response to transfected DNA from apoptotic cells, hBD3 had no effect on the response to untransfected DNA from apoptotic cells and when self-DNA was fragmented artificially using sonication, hBD3 still had no effect on the response. These results point to additional factors which may be important in making self-DNA immunogenic. It is possible that chromatin bound proteins that have been stripped away in the purification of the DNA are important in the immune response, for example, histones have been shown to regulate the innate immune response (Kobiyama et al., 2010). It would be interesting to test the effect of hBD3 on native chromatin rather than purified DNA. Alternatively, hBD3 could have been added directly to the culture of apoptotic cells and the supernatant could have been added to FLDCs directly, to avoid the process of extracting DNA.

4.3.5 HBD3 inhibited the response to transfected self-DNA, most likely by inhibiting transfection

The inhibitory effect of hBD3 on transfection efficiency of both *E. coli* and self-DNA indicates a non-specific effect of hBD3 inhibiting DNA transfection. This is not entirely surprising when considering that the likely mode of bacterial killing by hBD3 is disruption of lipid membranes, suggesting that hBD3 would disrupt liposome formation (Garcia et al., 2001; Sass et al., 2010). Additionally, liposomes are highly cationic, as is hBD3, raising a second possibility that ionic repulsion may be involved in disruption of liposomes.

4.3.6 Final remarks

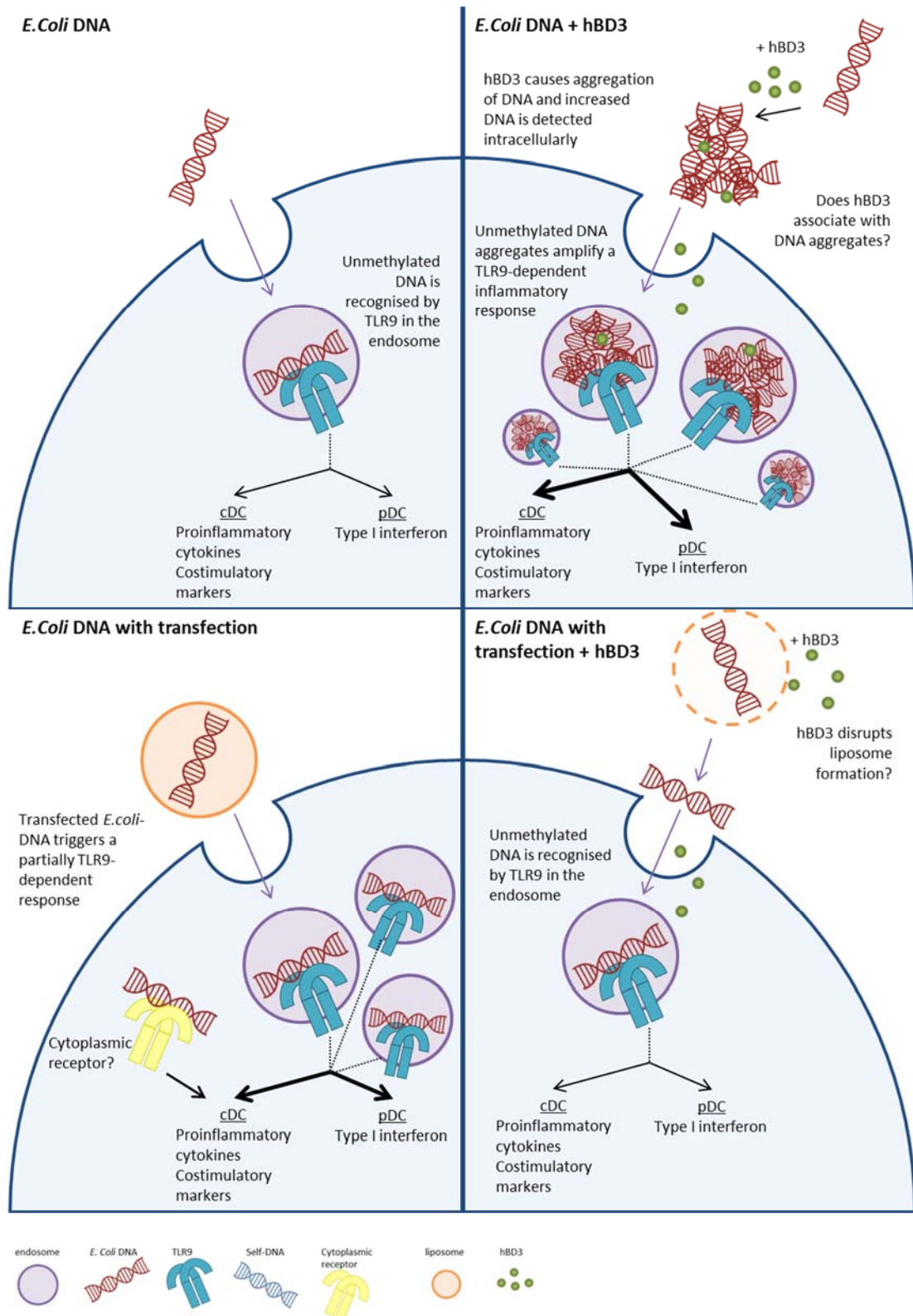
In conclusion of this chapter, hBD3 has been shown to cause aggregation of both *E. coli* DNA and self-DNA. In FLDCs, hBD3 exacerbates the response to *E. coli* DNA, by increasing cellular uptake, but also possibly by protecting the DNA from nuclease digestion, or by preventing or slowing transit through the endosomal pathway for an extended period of time. Similarly, hBD3 increases the cellular uptake of self-DNA, however this does not correspond with an increased immune response under the conditions tested, indicating that hBD3 does not convert self-DNA into an immune stimulus. This evidence suggests that hBD3 is not acting the same way as LL37, at least in this cell system, by taking self-DNA to the early endosome of pDCs and initiating a TLR9-dependent IFN- α response. Whilst hBD3 and LL37 are both highly cationic peptides, they have very different structures which may mean that they promote DNA aggregates with distinct properties and may explain the differences between how they affect the response to DNA. This could be tested using atomic force microscopy to look at the structure of the complex or size exclusion HPLC to look at the size of the complexes, as previously described (Lande et al., 2007).

It would be useful to test whether individuals with increased copy number of the β -defensin locus have increased levels of hBD3 expression and increased peptide levels in their sera; in which case it would also be relevant to determine if hBD3-DNA complexes form in early psoriatic plaques or in surrounding skin.

The potent exacerbation of the immune response to *E. coli* DNA by hBD3 raises the possibility that the role of hBD3 in the initiation of psoriasis is due to interaction with pathogenic DNA rather than self-DNA. Bacterial and viral infections can be triggers for development of psoriasis (Fry and Baker, 2007), therefore leading to the hypothesis that pathogenic DNA present in the skin due to an infection triggers an immune response involving the production of large amounts of IFN- α by pDCs and activation of immune cells. AMPs expressed in the skin including hBD3 and LL37 kill bacteria but may also cross-react with DNA from apoptotic host and bacterial cells inducing an inflammatory response which increases the severity of the psoriasis. In the latter stages of plaque development the focus shifts towards an adaptive autoimmune response instead of an innate immune response with T_H1 and T_H17 cell infiltration and activation and release of cytokines such as IL-22 and IL-17 (Baadsgaard et al., 1990; Morganroth et al., 1991; Nestle et al., 1994; Szabo et al., 1998). This hypothesis fits with a step-wise development of psoriasis from an initial trigger, via an innate immune-mediated inflammatory response to an adaptive immune-mediated autoimmune response.

HBD3 may be a target for treatment of psoriasis although removing the most potent AMP from the skin may cause further problems in terms of susceptibility to infections. By understanding how hBD3 interacts with DNA, treatments could target the complex and disrupt it, possibly improving pathogenesis.

4.4 Graphical summary



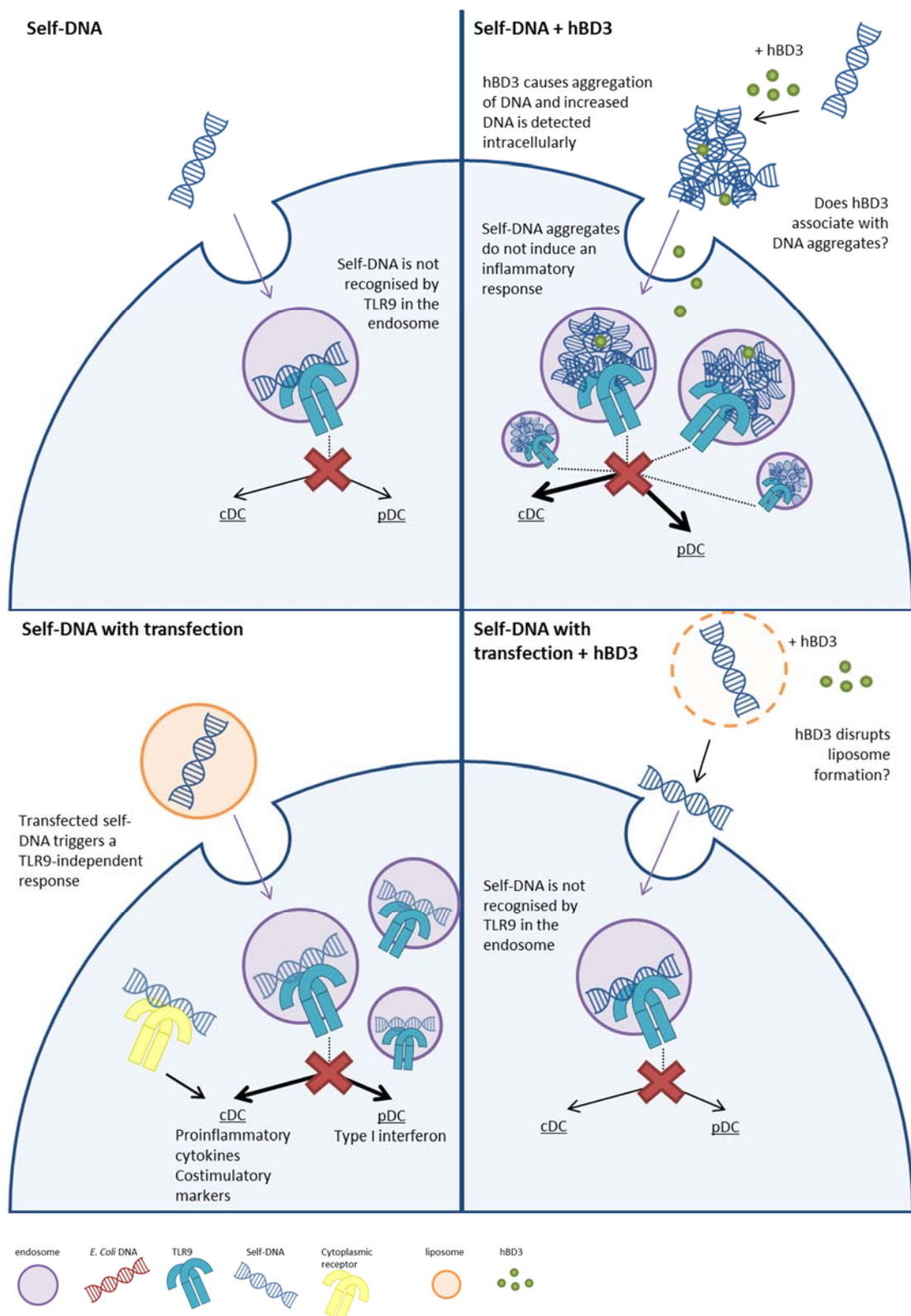


Figure 4.16 Graphical summary of the effect of hBD3 on the response of FLDCs to *E. coli* or self DNA.

- *E. Coli* DNA is taken up into FLDCs and stimulates TLR9-dependent activation of DCs and production of proinflammatory cytokines and type I interferon.
- hBD3 induces aggregation of *E. coli* DNA and increases intracellular uptake stimulating an increased TLR9-dependent response.
- Transfection of *E. coli* DNA stimulates both a TLR9-dependent response and a small TLR9-independent response via an unidentified cytoplasmic receptor.
- Additional of hBD3 decreases intracellular uptake of *E. coli* DNA compared with transfection, indicating that hBD3 may interfere with transfection.
- Self-DNA does not stimulate an immune response in FLDCs.
- Addition of hBD3 does not have any effect despite hBD3 inducing aggregation of self-DNA and increasing intracellular uptake.
- Transfected self-DNA stimulates a TLR9-independent response, likely via an unidentified cytoplasmic receptor.
- Addition of hBD3 inhibits this response and decreases intracellular uptake indicating that hBD3 interferes with transfection of self-DNA.

5. Investigating the role of hBD3 *in vivo*

5.1 Introduction

Mouse models are widely used to study inflammation, however there are a number of well-known differences between human and mouse inflammatory responses which recommend caution when using mouse models to study inflammation. When these differences are taken into account, mouse models enable the study of processes that would otherwise be impossible or highly invasive.

5.1.1 Using mouse models to study inflammation

Mouse models are widely used in the study of inflammation both to explore physiological mechanism and to assess novel therapeutics. The mouse immune system is highly similar to the human immune system in terms of cells, receptors and downstream effects, and the differences that do exist between the human and mouse systems are reasonably well understood.

There has been recent controversy over the accuracy of mouse models to represent human inflammatory processes following a gene expression study claiming that mouse responses did not accurately mimic human responses (Seok et al., 2013). However, a retaliatory study using the same data came to the opposite conclusion (Takao and Miyakawa, 2014). The difference in the conclusions reached by these two papers appears to be due to the different statistical approaches taken. Whilst Seok *et al.* focused on genes that were changed only in humans, Takao and Miyakawa focused on genes that were significantly changed in both species. Takao and Miyakawa also only included mouse studies of disease which closely replicated the human disease rather than grouping all mouse studies together and used meta-analysis of the data to look at pathways and biogroups rather than individual genes. The conclusion that can be reached by analysing both of these studies is that mice and humans may not be totally alike at the single gene level but the overall response tends to be highly comparable and mouse models of disease often closely resemble human disease. These studies suggest that careful consideration should be taken about using mouse models, however there is extensive information in publically available databases to assist with the decision. An important point to bear in mind when comparing mouse studies to human studies is the significant heterogeneity of

the human population compared to an inbred transgenic mouse strain, although even within a highly inbred strain results tend to be variable requiring large sample numbers to draw robust conclusions.

There are a vast number of mouse models available to study inflammation, the Jackson Laboratory database alone has over 1000 strains available including TLR deficient strains, type I diabetes models, cystic fibrosis models, lupus models and immune cell deficient strains (2014 Jackson Laboratory).

5.1.2 *In vivo* techniques for studying inflammation

A wide range of parameters can be measured to assess the inflammatory response of mice *in vivo*. Possibly the most informative is to look at the differences in cell populations and of expression of costimulatory markers using flow cytometry. By using multiple markers e.g. CD11c, CD11b and B220, different populations of dendritic cells and macrophages can be identified and activation of these specific subsets can be assayed by measuring cell surface expression of costimulatory markers such as CD80, CD86, CD40 and MHCII. The populations of different organs and tissues can be assayed, for example the splenic cells or the peritoneal cells (Rose et al., 2012; Wang et al., 2013a).

5.2 Results

This chapter presents a small body of preliminary work investigating the role of hBD3 *in vivo*.

5.2.1 HBD3 chemoattracts Csf1r⁺ cells into the peritoneum *in vivo*

There are numerous contradictory reports on the chemotactic effect of defensins. A number of reports have shown that hBD3 chemoattracts cells *in vitro* (Röhrl et al., 2010) and whilst hBD2 was demonstrated to chemoattract both macrophages and dendritic cells *in vivo*, and has been shown to chemoattract macrophages *in vitro*, there is no evidence hBD3 having the same effect *in vivo* (Soruri et al., 2007).

Therefore we wanted to establish whether hBD3 does affect migration of immune cells *in vivo*.

To investigate whether hBD3 chemoattracts immune cells *in vivo*, a mouse model was used which expressed ECFP (enhanced cyan fluorescent protein) in the cells that express Csf1r - monocytes, macrophages and dendritic cells (Ovchinnikov et al., 2008b). The ECFP⁺ population was gated by flow cytometry (figure 5.1 A) and a total cell count was also recorded. The total cell count from the peritoneum was increased following injection with hBD3 compared in injection with saline (figure 5.1 B). The proportion of ECFP⁺ cells was also increased by injection with hBD3 however this was not found to be statistically significant (figure 5.1 C). Whilst there was not a significant difference in the proportion of ECFP⁺ cells between treatments, there is a trend towards hBD3 increasing the proportion of ECFP⁺ cells and this combined with the statistically significant increase in total cell number implies that hBD3 chemoattracts cells *in vivo*, including Csf1r expressing cells into the peritoneum.

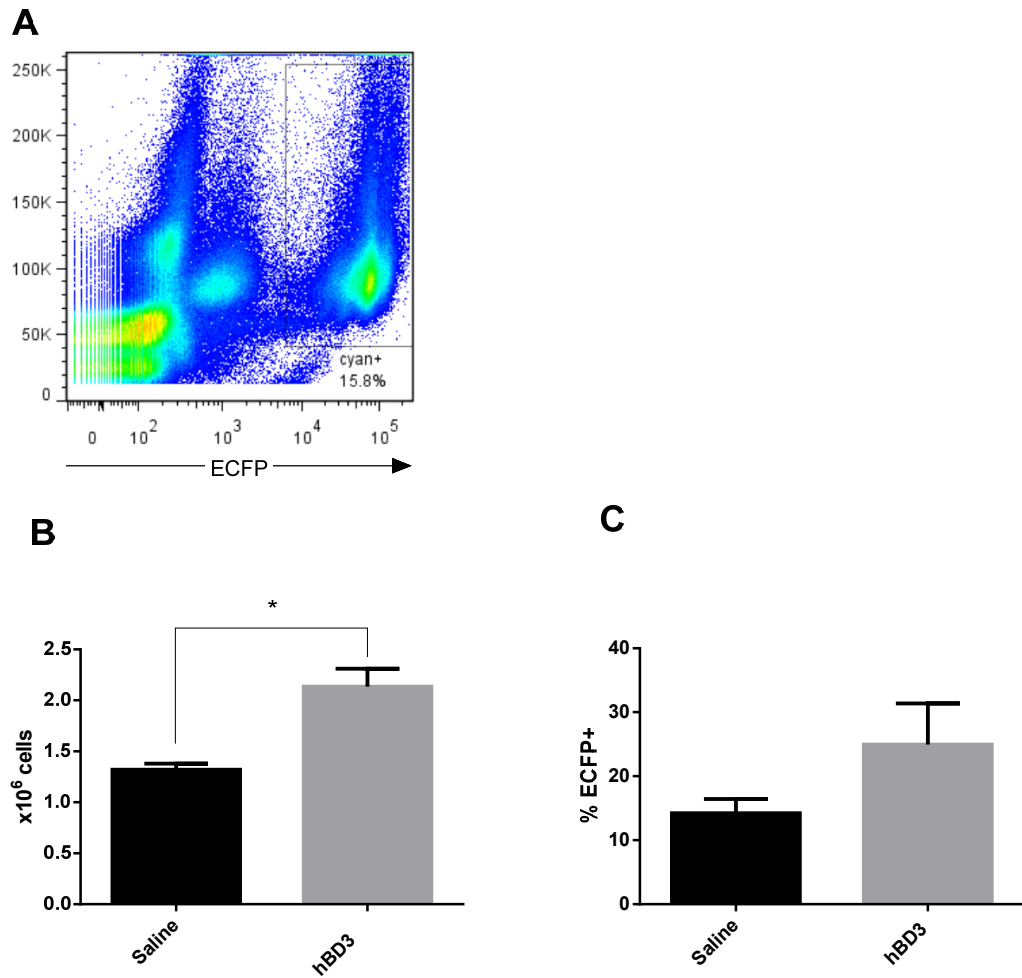


Figure 5.1 hBD3 chemoattracts Csf1r⁺ cells in the peritoneum. 10 μ g hBD3 in 100 μ l saline, or 100 μ l saline alone as a control was injected intraperitoneally into C57Bl/6J mice (8 weeks). Mice were culled after four hours and peritoneal cells were harvested via peritoneal lavage. Cells were analysed via flow cytometry gating for size against ECFP (enhanced cyan fluorescent protein) signal (A). Total cell count (B) and proportion of ECFP⁺ (C) was measured. Data represents the mean of measurements from 3 mice for each treatment $\pm$ standard deviation. * $p < 0.05$ unpaired t-test.

5.2.2 hBD3 overexpression has no effect on the response to intraperitoneal injection of *E. coli* DNA

To investigate the effect of hBD3 on the response to DNA *in vivo*, the response to intraperitoneal (i.p.) injection of *E. coli* DNA was measured in a wild-type compared an hBD3 overexpressing transgenic mouse. This mouse model was generated by random integration of a construct containing *DEFB103* and *EGFP* under the control of a ubiquitous CAG promoter (Hitoshi et al., 1991; Jun-ichi et al., 1989)(Figure 5.2). *EGFP* is preceded by an internal ribosome entry site (*IRES*) sequence so that EGFP is expressed in the same location as hBD3 but is not attached to hBD3. This is important as hBD3 a small peptide and attaching a large EGFP protein may have affected the properties of hBD3.

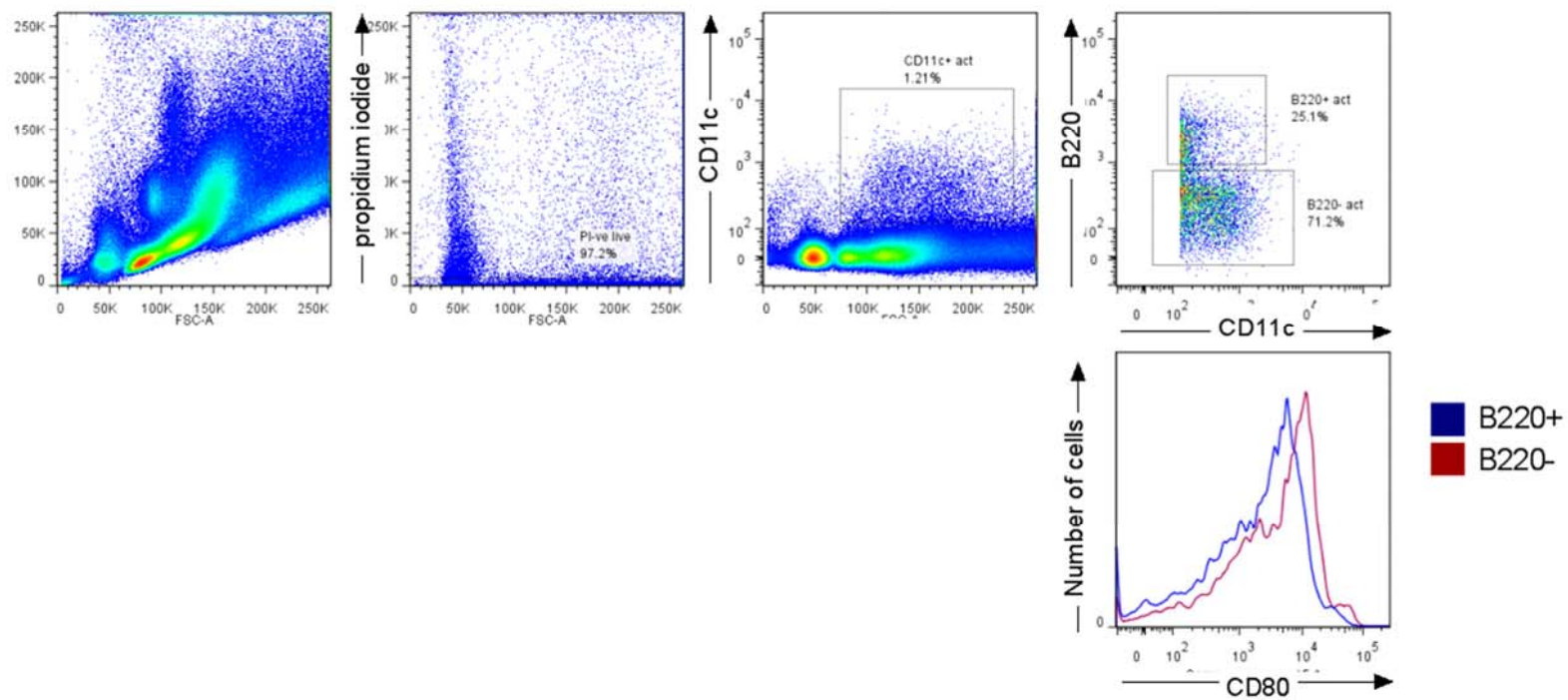


Figure 5.2 Schematic representation of the targeting construct used to generate the hBD3 overexpressing mouse model. A CAG promoter (C- CMV early enhancer element, A- promoter, first exon and first intron of chicken β -actin gene, G- the splice acceptor of the rabbit β -globin gene) (Hitoshi et al., 1991; Jun-ichi et al., 1989) drives ubiquitous expression of *DEFB103* and *EGFP*.

Following i.p. injection of the mice they were monitored for 4 hours and then culled. The peritoneal wash was collected for flow cytometry analysis (figure 5.3) and ELISA analysis of the supernatant (figure 5.4) and the spleens were collected for flow cytometry analysis (figure 5.5). The samples were stained with CD11c, B220 and CD11b to identify cDCs (CD11c⁺/B220⁻), pDCs (CD11c⁺/B220⁺) and macrophages (CD11b⁺). Expression of CD80 was also assayed to measure cell activation. There were no significant differences in the response of wild-type compared to hBD3-tg mice to *E. coli* DNA in peritoneal recruitment (figure 5.3 B) or activation (figure 5.3 C) of CD11b⁺, CD11c⁺B220⁻ or CD11c⁺B220⁺ cells. Although there were no significant differences in cytokine production in the peritoneal

supernatant, there was an increase in IL-6 and IFN- β following *E. coli* DNA injection in hBD3-tg compared to WT (figure 5.4). There were no differences in the activation of splenic CD11b⁺, CD11c⁺B220⁻ or CD11c⁺B220⁺ cells (figure 5.5). The robust *in vitro* data indicates that these preliminary *in vivo* data showing no significant effect of hBD3 *in vivo* may be due to non-optimal experimental conditions.

A



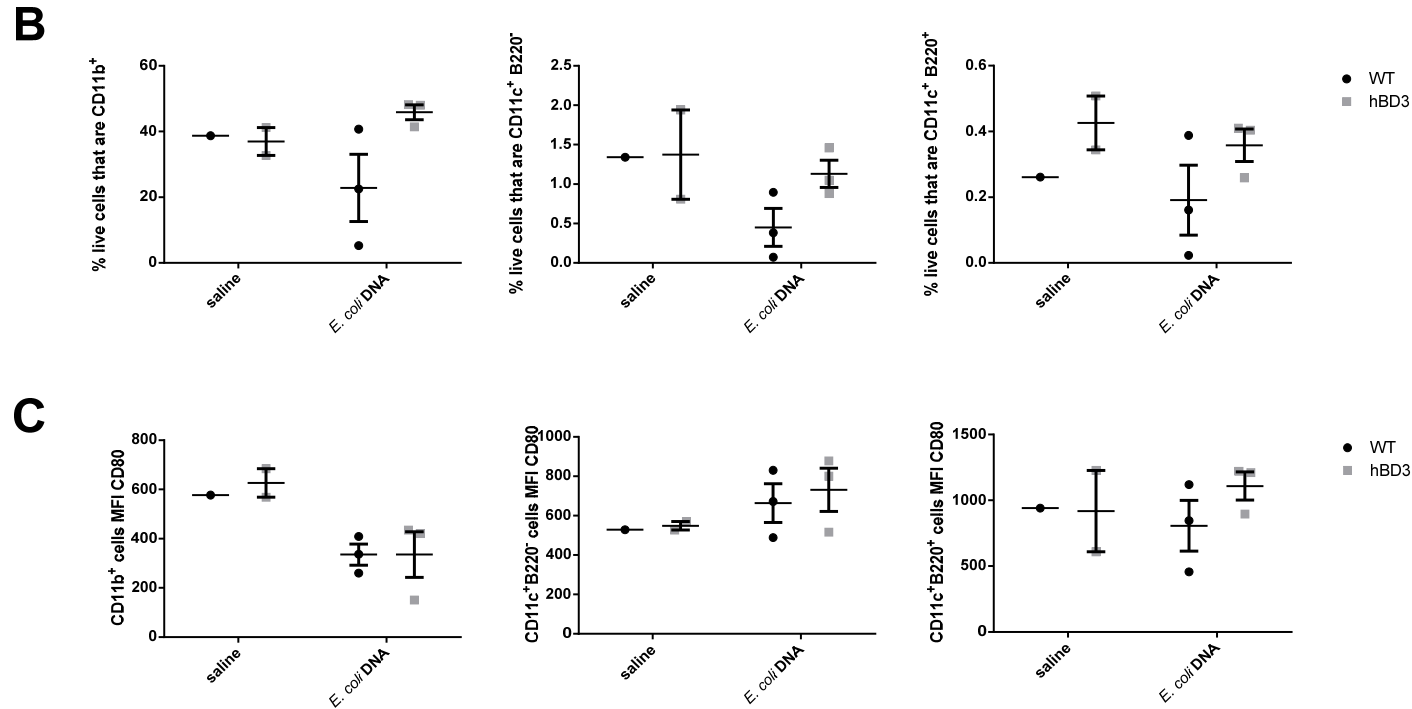


Figure 5.3 HBD3 overexpression has no effect on the recruitment of cells into the peritoneum or on the activation of immune cells following treatment with *E. coli* DNA. WT or hBD3-tg mice were injected intraperitoneally with 30 μ g *E. coli* DNA. Mice were culled after 24 hours and peritoneal lavage was collected. Peritoneal cells were analysed by flow cytometry (A) to assess cell recruitment (B) and cell activation (C). The mean of biological replicates is shown $\pm$ standard deviation.

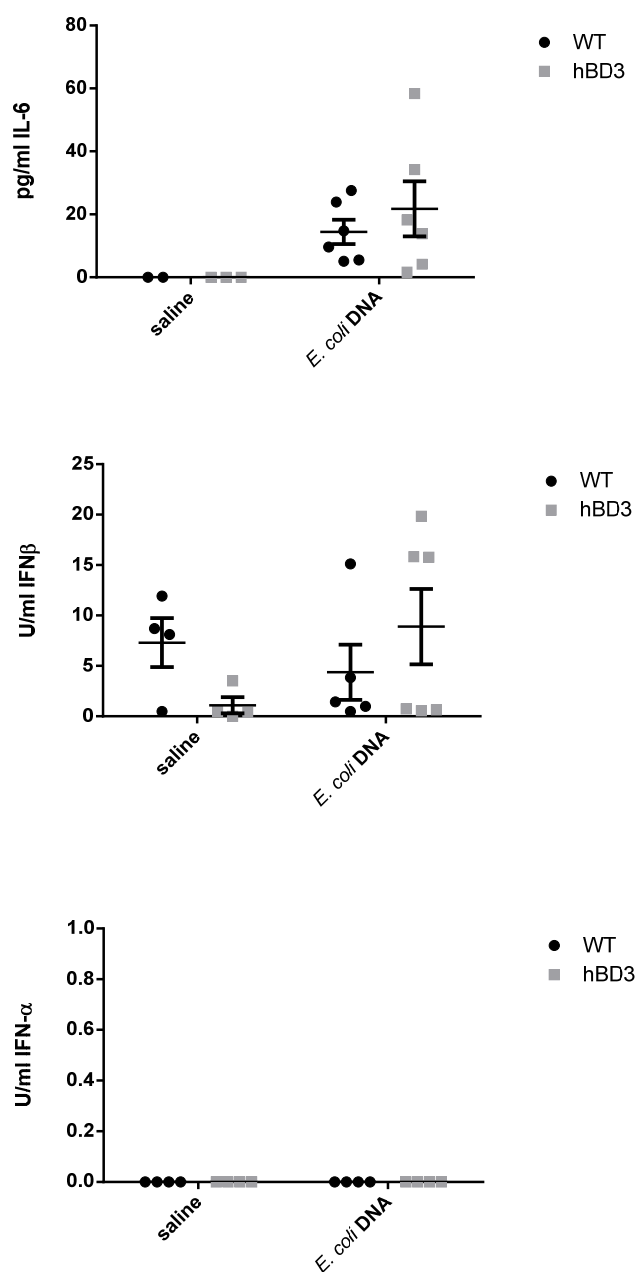
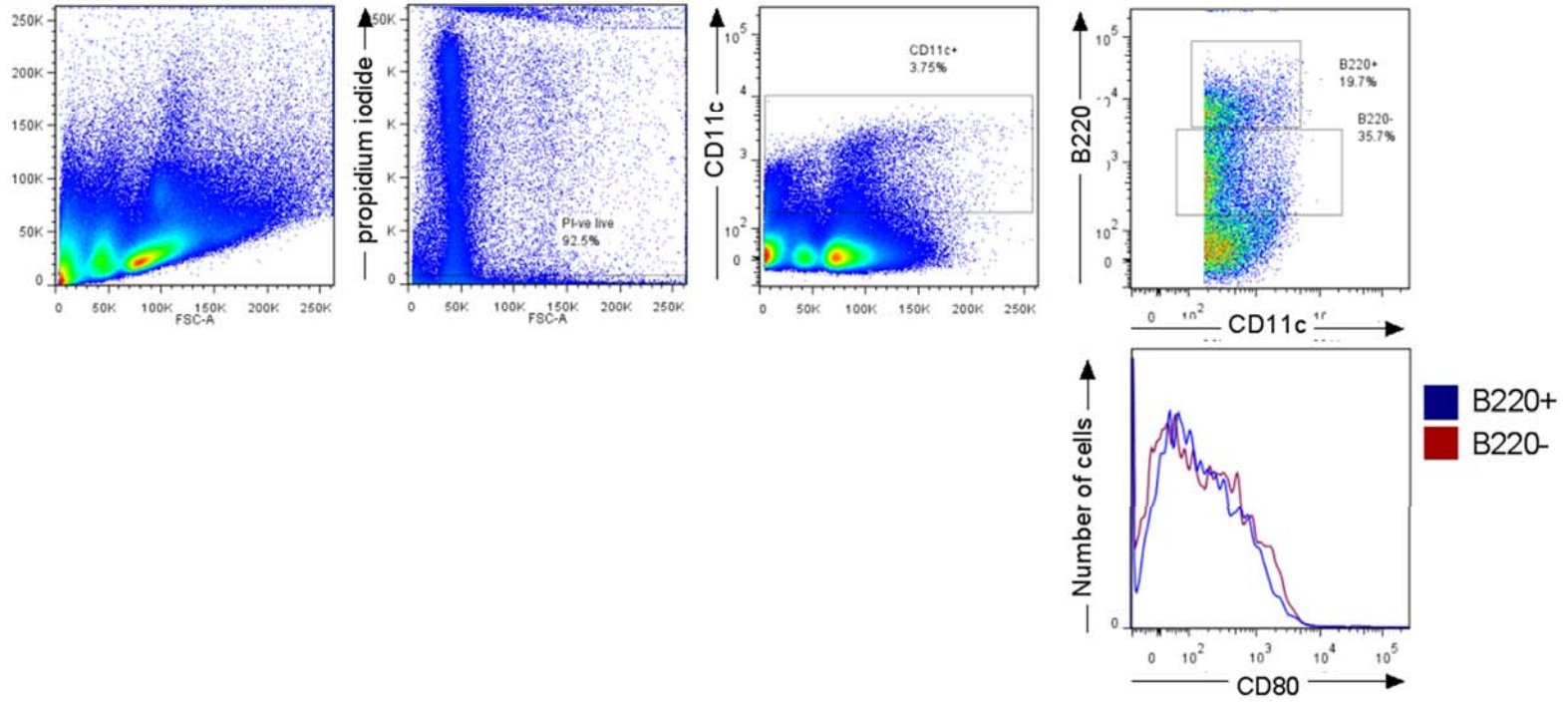


Figure 5.4 HBD3 overexpression has no effect on the production of cytokines following *E. coli* DNA injection. WT or hBD3-tg mice were injected intraperitoneally with 30 μ g *E. coli* DNA. Mice were culled after 24 hours and peritoneal lavage was collected. Peritoneal supernatant was analysed by ELISA. The mean of biological replicates is shown $\pm$ standard deviation.

A



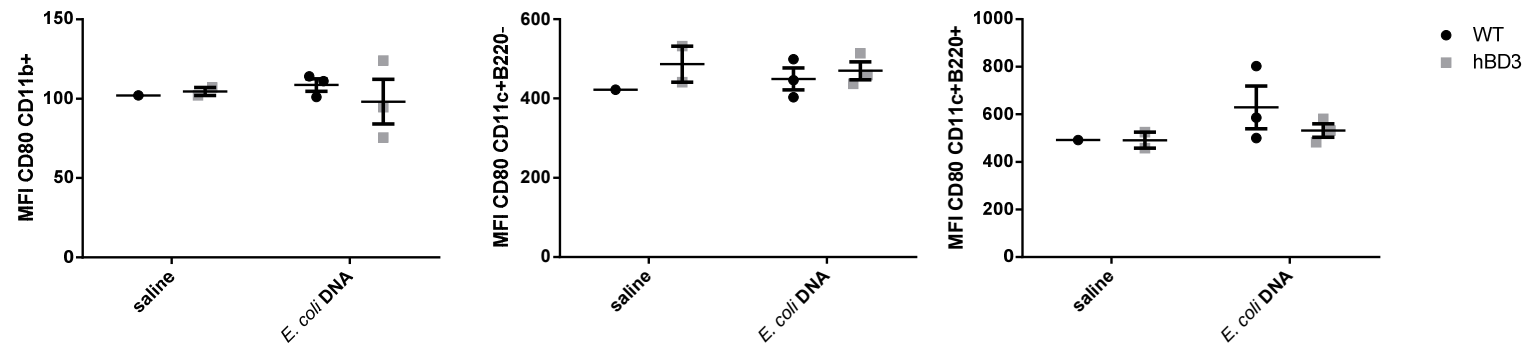
B

Figure 5.5 hBD3 overexpression has no effect on the activation of splenic cells.

WT or hBD3-tg mice were injected intraperitoneally with 30 μ g *E. coli* DNA. Mice were culled after 24 hours and spleens were collected for analysis by flow cytometry (A) to assess cell activation (B). The mean of biological replicates is shown $\pm$ standard deviation.

5.3 Discussion

The following points summarise the major findings of this chapter:

- hBD3 may chemoattract *Csf1r*⁺ cells into the peritoneum.
- hBD3 overexpression has no effect on the response to intraperitoneally injected *E. coli* DNA, though hBD3 overexpression in the peritoneum has not been quantified.
- The robust *in vitro* data suggests that the *in vivo* conditions need to be optimised to induce a basal response to *E. coli* DNA.

5.3.1 HBD3 chemoattracts *Csf1r*⁺ cells *in vivo*

In vivo, hBD3 clearly chemoattracts *Csf1r* expressing cells such as monocytes, macrophages and dendritic cells (Ovchinnikov et al., 2008a). This is consistent with hBD3 chemoattracting via CCR6 or CCR2 *in vitro* (Garcia et al., 2001; Rohrl et al., 2008; Röhrle et al., 2010). Chemoattraction of immune cells by hBD3 *in vivo* has not been shown before and supports a physiologically relevant role for hBD3 as a chemoattractant. This is particularly interesting considering the inducible nature of hBD3 expression by pathogens, proinflammatory cytokines and wounding as discussed in the introduction to this chapter, and suggests that hBD3 is involved in the recruitment of immune cells to sites of tissue damage or infection. It would be interesting to see if the *Defb14* deficient mouse model shows any defects in immune cell recruitment to any of the stimulants discussed.

5.3.2 HBD3 had no effect on intraperitoneal injection of *E. coli* DNA

An hBD3-GFP overexpressing transgenic mouse model was used as a system for avoiding having to use large amounts of hBD3 injected alongside DNA into the peritoneum. Due to the fact that self-DNA only induced an immune response when transfected; only the response to *E. coli* DNA was assayed *in vivo*. Following a literature search, no directly comparable experiment injecting bacterial DNA into the peritoneum had been performed to indicate a suitable amount of DNA to inject. Deng *et al.* injected bacterial DNA into joints and induced a measurable but non-lethal response in mice (Deng et al., 1999); however this amount of *E. coli* DNA injected

into the peritoneum of mice did not induce any significant responses in wild-type or transgenic mice. The only slight indication of a response was in the production of IL-6 which is slightly increased following *E. coli* injection and further increased in the hBD3-transgenic mouse, however these difference are not significant as there was a large amount of variation in the groups.

5.3.3 Final Remarks

It is important to establish whether the effect of hBD3 on the innate immune response to DNA can be replicated *in vivo*, to determine whether this is a viable mechanism for how hBD3 may affect the response to DNA. The hBD3 transgenic mouse model provides an extremely useful model to study the effect of hBD3 whilst avoiding the use of large amounts of synthetic peptide. Establishing what concentration of *E. coli* DNA elicits a moderate response in wild-type mice would provide a base line from which to study the effect of hBD3, and should be the next step in optimising this experiment.

6. Mutations in *TRAIP* cause primordial dwarfism with immunodeficiency

6.1. Introduction

Mutations in *TRAIP* (TRAF (TNF (tumour necrosis factor) receptor-associated factor) Interacting Protein) were identified in three patients with microcephalic primordial dwarfism (unpublished, Louise Bicknell, Matt Hurles, Bernd Wollnik). Additionally, these patients had recurrent viral and bacterial respiratory infections and one patient died from chronic lung disease. *TRAIP* is known to be an E3 ubiquitin ligase which is thought to be involved in the regulation of the innate immune system and has additionally been associated with regulation of cellular proliferation and differentiation.

This introduction reviews what is known about the structure and function of *TRAIP*. Whilst the focus of this chapter is to understand the function of *TRAIP* in the regulation of the innate immune system, this introduction reviews current evidence regarding the wider role of *TRAIP*, including the role of *TRAIP* in cellular proliferation and differentiation and how this may relate to its perturbation causing microcephalic primordial dwarfism.

6.1.1. *TRAIP* gene structure and expression

Human and mouse *TRAIP* are located on chromosomes 3 and 9, respectively. Both genes are composed of 15 exons, the human gene is 27.9 kb and the mouse gene is 21.3 kb (Ensembl assembly GRCh38, Havana Vega56). There are 4 predicted protein coding isoforms of *TRAIP*, including the full length isoform (Ensembl assembly GRCh37, Havana Vega56), however there is no evidence that the alternative transcripts code for proteins *in vivo*.

The regulation of *TRAIP* expression has not been characterised in detail, however potential transcription factor sites have been identified in the promoter region which suggest that *TRAIP* expression could be regulated in the context of the immune response and at different stages of the cell cycle. *TRAIP* has a predicted NF- κ B binding site in the 5' upstream sequence and microarray analysis demonstrated up-regulation of *TRAIP* expression following treatment of the THP-1 human monocyte

cell line with LPS (Sharif et al., 2007). Similarly, *TRAIP* expression was up-regulated in primary mouse peritoneal macrophages following treatment with various immune ligands again indicating regulation by transcriptional factors associated with immune signalling (Zhang et al., 2012). Transcription factor binding site prediction also identified an E2F binding site close to the transcriptional start site of *TRAIP* (Almeida et al., 2011) and E2F-p130 bound to the *TRAIP* promoter in serum starved human glioblastoma cells (Cam et al., 2004). The E2F-p130 complex is associated with transcriptional repression in quiescent cells and the complex is degraded as cells begin to proliferate (Smith et al., 1996).

6.1.2. TRAIP encodes an E3 ubiquitin ligase

TRAIP was first identified as a RING finger domain-containing E3 ubiquitin ligase which inhibited TRAF2-mediated NF- κ B activation (Lee et al., 1997). TRAIP has an N-terminal RING (Really Interesting New Gene) domain; a 40-60 residue structural motif identifiable by a conserved cysteine and histidine core which co-ordinates two atoms of zinc stabilising a rigid ring-like structure (Barlow et al., 1994). A number of papers unambiguously established that RING domains mediate ubiquitin ligase activity by binding to E2 ubiquitin-conjugating enzymes and promoting the direct transfer of ubiquitin to a substrate (Joazeiro et al., 1999; Kamura et al., 1999; Levkowitz et al., 1999; Lorick et al., 1999; Ohta et al., 1999; Seol et al., 1999; Tan et al., 1999; Xie and Varshavsky, 1999; Yokouchi et al., 1999). *In vitro* ubiquitination assays confirmed that TRAIP is an E3 ubiquitin ligase which autoubiquitinates and that this activity was dependent on a functional RING domain (Besse et al., 2007).

The N-terminal RING domain of TRAIP is followed by a putative coiled-coil region which is further divided into an N-terminal coiled-coil domain similar to that found in myosin heavy chains, and a C-terminal leucine zipper-like motif (Chapard et al., 2012; Lee et al., 1997) (Figure 6.1). Coiled-coil regions, including leucine-zipper motifs, are highly versatile protein domains which are associated with a wide range of biological functions, the most common being protein-protein interactions or protein-DNA interactions (Burkhard et al., 2001; Landschulz et al., 1988).

The RING domain and the coiled-coil domain of TRAIP are both highly conserved between human and mouse (100% and 89.3% similarity respectively, Figure 6.1) indicating the importance of these structures to the function of TRAIP.

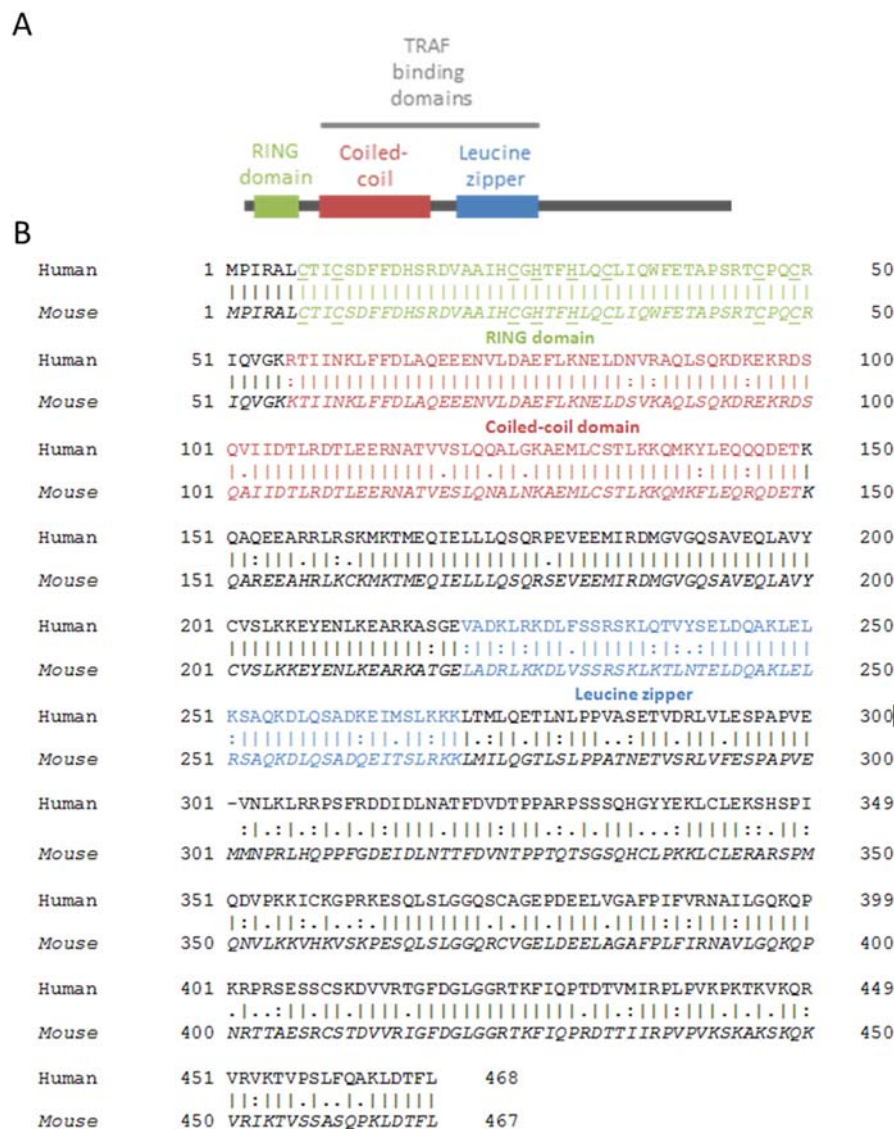


Figure 6.1 Protein domains and conservation of TRAIP between human and mouse. Schematic of the known protein domains of TRAIP (A) and the aligned sequences of human and mouse TRAIP amino acid sequences (B). Showing the RING domain (green) with conserved cysteine and histidine residues underlined, the coiled-coil domain (red) and the leucine zipper domain (blue) and the conservation of residues from human to mouse. (Assembled using EMBOSS needle and annotated using Ensembl GRCh38).

6.1.3. The mechanisms and function of ubiquitination

Ubiquitination is the post-translational attachment of ubiquitin (ub), a highly conserved 76 amino acid (8.5kDa) peptide (Goldstein et al., 1975; Schlesinger et al., 1975). The best known role for ubiquitination of proteins is targeting them for degradation by the 26S proteasome (Ciechanover et al., 1980; Hershko et al., 1980), however it is now clear that ubiquitination serves a wide range of additional purposes such as regulating protein interactions and localisation, transcription, DNA repair and signalling, and importantly, in regulation of the cell cycle (Teixeira and Reed, 2013).

Ubiquitination is a sequential process involving three classes of enzymes: ubiquitin activation enzymes (E1), ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3). These enzymes catalyse activation, conjugation and ligation of ub respectively, (Figure 6.2) and bind one or more ubiquitin peptides to lysine residues or onto the N-terminus of the substrate (Hershko and Ciechanover, 1992).

Ubiquitin activation is an energy dependent process, requiring ATP and the activating enzyme, E1 (Jentsch et al., 1990). The E1 forms a thiol ester with the carboxyl group on the C-terminal glycine residue of ubiquitin. Activated ubiquitin is then transferred to a ubiquitin conjugating enzyme (E2) which selectively interacts with E3 ligases that bind specific substrates. E2s directly influence the type of lysine that is used and are therefore involved in directing the fate of the protein (van Wijk and Timmers, 2010). There are a limited number of E1 activating enzymes which each activate a larger number of E2 conjugating enzymes which each activate a large number of substrate E3 ligases - this pyramid structure of enzymes allows vast diversity in substrate specificity.

The majority of E3 ubiquitin ligases are RING domain-containing and, in contrast to the smaller family of HECT domain E3 ligases, they mediate the transfer of ubiquitin directly from the E2 conjugating enzyme to the substrate, rather than involving an E3:ubiquitin intermediate (Joazeiro et al., 1999; Kamura et al., 1999; Levkowitz et al., 1999; Lorick et al., 1999; Ohta et al., 1999; Seol et al., 1999; Tan et al., 1999; Xie and Varshavsky, 1999; Yokouchi et al., 1999). HECT (homologous to E6-AP C-terminus) domain E3 ligases bind E2 enzymes and transiently accept ubiquitin before

transferring the peptide to the substrate (Huibregtse et al., 1995; Scheffner et al., 1995).

Proteins can be modified by single ubiquitins, multiple ubiquitins on different lysine residues or by polyubiquitin chains. The ubiquitin chains can be polymerised via either lysine 48 (K48), or lysine 63 (K63) of the ubiquitin peptide. These different modifications direct the fate of the ubiquitinated protein; broadly, K48 ubiquitination targets proteins for degradation by the 26S proteasome (Finley, 2009) whereas K63 ubiquitination is regulatory (Chen and Sun, 2009), targeting proteins to different localisations (Lauwers et al., 2009) and interestingly regulating the DNA damage response (Messick and Greenberg, 2009).

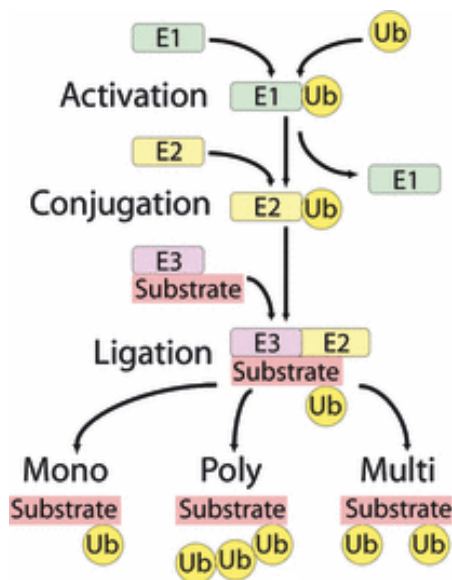


Figure 6.2 A schematic showing the process and variations of ubiquitination of a substrate. An E1 activating enzyme activates ubiquitin in an ATP-dependent step. The E1 transfers ubiquitin onto an E2 conjugating enzyme. E2s interact selectively with E3 ligases which either act as a scaffold in the transfer of ubiquitin directly from E2 to substrate (RING domain E3 ligases) or transiently form an E3:ubiquitin intermediate during the transfer step (HECT domain E3 ligases). Adapted from (Chapard et al., 2012)

6.1.4. TRAIP interactions and function

TRAIP was first identified in a yeast two-hybrid screen for TRAF1- interacting proteins; TRAIP was the only protein identified which interacted with both TRAF1 and TRAF2 and this was validated by overexpression of TRAF1 or TRAF2 in mammalian cell culture and co-precipitation of overexpressed GST-tagged TRAIP (Lee et al., 1997). A TRAIP mutant lacking the coiled-coil domain no longer bound TRAF1 or TRAF2 demonstrating that this domain is essential for TRAF interaction. Further deletion analysis established that two independent regions of the TRAIP coiled-coil region could bind TRAF proteins (Lee et al., 1997). It was concluded that TRAIP was a negative regulator of TNF- α -mediated NF- κ B activation as TRAIP over-expression inhibited TRAF2- or TNF- α -mediated NF- κ B activation, but had no effect on IL-1-mediated activation of NF- κ B. This inhibitory effect was dependent on a functional TRAF interaction domain but not on a functional RING domain (Lee et al., 1997), implying that TRAIP mediated-inhibition of NF- κ B is not dependent on ubiquitination. However, TRAIP could inhibit IL-1 β mediated NF- κ B activation in a later report (Regamey et al., 2003). These differences may be due to technical differences such as the specific IL-1 protein used, or its concentration; Regamey *et al* specifically use IL-1 β whereas Lee *et al.* do not specify which type of IL-1 was used. In contrast, Besse *et al* demonstrated that in spite of evidence of TRAIP interactions with TRAFs 1, 2, 3, 5 and 6, overexpression of TRAIP had no effect on the phosphorylation of I κ B α , which is required for NF- κ B activation (Besse et al., 2007). This suggests that negative regulation by TRAIP may be ligand-dependent. It has also been demonstrated that TRAIP negatively regulates IRF3 activation via degradative ubiquitination of TBK1 (Zhang et al., 2012). TBK1 directly phosphorylates IKK β , a catalytic subunit of the I κ B kinase complex, thereby activating NF κ B and inflammatory responses (Tojima et al., 2000). Additionally TBK1 phosphorylates AKT which regulates IRF3 activation and antiviral responses (Joung et al., 2011; Xie et al., 2011). Therefore, TRAIP-mediated ubiquitination of TBK1 negatively regulates IRF3 and NF- κ B activation.

A yeast two-hybrid screen using tumour suppressor cylindromatosis (CYLD) as bait demonstrated that CYLD interacts with the C-terminus of TRAIP and co-localises with TRAIP in the perinuclear compartment (Regamey et al., 2003). CYLD inhibits

TNF- α mediated NF- κ B activation and is dependent on the central domain of CYLD interacting with the C-terminal portion of TRAIIP. CYLD is a K63-deubiquitinase (Glittenberg and Ligoxygakis, 2007; Komander et al., 2008), the deubiquitinase domain is required for the inhibitory effect of CYLD on NF- κ B activation. Given their opposing ubiquitination roles, it raises the possibility that whether TRAIIP and CYLD regulate each other via their respective ubiquitinase and deubiquitinase functions. CYLD is known to only deubiquitinate K63-linked ubiquitin, therefore it would also be interesting to determine whether TRAIIP is a K48 or K63 ubiquitin ligase.

A different yeast two-hybrid screen focussing on spleen tyrosine kinase (Syk) interactors, also identified TRAIIP as an interacting protein (Zhou and Geahlen, 2009). Decreased expression of *SYK* is associated with poor prognosis and increased risk of metastasis in breast cancer patients (Toyama et al., 2003) and inappropriate activation of *SYK* has been implicated in the early stages of tumourigenesis (Katz et al., 2005). Syk phosphorylates TRAIIP and colocalises with TRAIIP in response to TNF- α treatment (Zhou and Geahlen, 2009) suggesting that it might be involved in regulating TRAIIP.

6.1.5. The role of TRAIIP in the immune system

The involvement of both degradative and regulatory ubiquitination in regulating the innate immune system has been extensively characterised and is nicely reviewed by (Ben-Neriah, 2002). The involvement of ubiquitination in the regulation of the innate immune system makes it likely that TRAIIP regulation acts via substrate ubiquitination. Degradative and regulatory ubiquitination of TRAF proteins is essential for the regulation of multiple innate immune pathways, including NF- κ B and IRF activation (Deng et al., 2000; Higgs and Jefferies, 2008; Huye et al., 2007; Kawai et al., 2004; Kong et al., 2007; Wang et al., 2001). TRAIIP, as an E3 ligase associated with TRAF proteins, would be likely to have a role in the regulation of the innate immune system. However, contradictory reports regarding a role for TRAIIP in NF- κ B signalling and the fact that all of these assays have involved overexpression of TRAIIP make it difficult to draw any definitive conclusions from the existing literature.

There are conflicting reports of whether TRAIIP regulates NF- κ B activation (Besse et al., 2007; Lee et al., 1997; Regamey et al., 2003). TRAIIP was demonstrated to specifically inhibit TNF- α -mediated NF- κ B in a ligand independent manner. Overexpressed TRAIIP associated with TNFR2 and CD30 signalling complexes and this was dependent on co-expression of TRAF proteins (Lee et al., 1997). However, TRAIIP had no effect on IL-1 induced NF- κ B activation which is mediated by TRAF6 rather than TRAF2 (Lee et al., 1997). Interestingly the inhibitory effect of TRAIIP was not dependent on a functional RING domain. These findings suggest that TRAIIP specifically inhibits TRAF2 mediated NF- κ B activation and that this effect is dependent on TRAIIP interacting with TRAF2 (Lee et al., 1997). In contrast to these findings, it was later demonstrated that TRAIIP could inhibit IL-1 β induced NF- κ B activation in the same cell type (Regamey et al., 2003). Further evidence from mouse fibroblasts cells stably overexpressing TRAIIP suggested that TRAIIP does not inhibit IL-1 or TNF induced phosphorylation of NF- κ B inhibitor I κ B α (Besse et al., 2007). Moreover, TRAIIP knock-down in keratinocytes did not lead to an increase in basal NF- κ B activity, although NF- κ B activity following an inflammatory stimulus was not assayed in these cells (Almeida et al., 2011). These data together suggest that the effect of TRAIIP may be cell type specific and that overexpression of TRAIIP may not represent its physiological role.

Recent evidence that TRAIIP may be involved in regulating the antiviral immune response via IRF3 extends the potential roles that TRAIIP may have in innate immune signal transduction pathways. TRAIIP-mediated degradative ubiquitination of TBK1 results in downregulation of the type 1 interferon response following viral infection (Zhang et al., 2012). RIG-I and TLR3 detect double stranded viral RNA converging on TBK1-mediated IRF3 activation and leading to production of IFN- β (Fitzgerald et al., 2003). TRAIIP induced K48-linked ubiquitination of TBK1 and subsequent degradation demonstrates a role for TRAIIP in regulating IRF3 activation and IFN- β production (Zhang et al., 2012).

6.1.6. The role of TRAIIP in proliferation and differentiation

The first evidence that TRAIIP has a role in cellular proliferation was provided by a transgenic mouse model in which exon two was replaced by a neomycin cassette,

resulting in TRAIP null mice. TRAIP null mice were lethal by E7.5, although the embryos were visibly abnormal by E6.5 with defects in cellular proliferation and an increase in apoptosis (Park et al., 2007).

Additionally, a screen for cell-cycle regulators in *Drosophila* identified a maternal lethal mutant called ‘no poles’ (*nopo*) due to the acentrosomal spindle phenotype (Merkle et al., 2009). NOPO is the *Drosophila* orthologue of TRAIP, based on conservation of function and structure, particularly of the RING domain and co-localisation of overexpressed NOPO and TRAIP in mammalian cells both spatially and temporally during G2 phase of the cell cycle (Wallace et al., 2014). NOPO interacts with bendless (BEN), a *Drosophila* E2 ubiquitin conjugating enzyme (Muralidhar and Thomas, 1993; Oh et al., 1994) indicating that NOPO, like TRAIP, functions as an E3 ubiquitin ligase and is involved in regulating the cell cycle. Recently both NOPO and TRAIP were reported to interact with polymerase η (pol η) *in vitro* via both a yeast two-hybrid assay and co-immunoprecipitation (Wallace et al., 2014). TRAIP and NOPO enhanced ubiquitination of human or *Drosophila* pol η , respectively, and TRAIP overexpression enhanced human pol η localisation to PCNA positive replication foci. Therefore, TRAIP may be involved in translesional synthesis, maintaining genomic stability by promoting ubiquitination of Y-polymerase family member pol η , positively regulating pol η activity in cell cycle progression. Pol η is an error prone DNA polymerase which bypasses DNA lesions that cause replicative polymerases to stall, thereby allowing replication to proceed. The association of pol η with the replicative complex is tightly controlled and ubiquitination has been previously shown to regulate the yeast orthologue of pol η , Rad30, by targeting it for degradation (Skoneczna et al., 2007). TRAIP-mediated ubiquitination of pol η may be an additional mechanism for regulating the association of this error prone DNA polymerase with replicating DNA.

Further evidence for a role for TRAIP in proliferation was demonstrated by high expression of TRAIP in proliferative keratinocytes compared with down-regulation as the keratinocytes began to differentiate (Almeida et al., 2011). Stable knock-down of TRAIP in keratinocytes resulted in decreased proliferation due to G1/S phase arrest indicating that TRAIP positively regulated proliferation of these cells

(Almeida et al., 2011). *TRAIP* expression in proliferating keratinocytes was also down-regulated by treatment with mTOR or PI3K inhibitors showing that expression is regulated by a target of the PI3K-Akt-mTOR pathway, one of which is the E2F family of transcription factors. As previously stated, an E2F-p130 binding site was identified in the promoter region of *TRAIP* suggesting that *TRAIP* is an E2F target gene, although a direct interaction has not been experimentally confirmed (Almeida et al., 2011; Cam et al., 2004). Interestingly, *TRAIP* expression was also increased in basal cell carcinoma which arises from the progenitor cells of the epidermis (Almeida et al., 2011; Youssef et al., 2010) and is linked to alterations in Gli1 and Gli2 activation (Gailani et al., 1996). E2F transcription factor expression is regulated by Gli1 and Gli2 (Regl et al., 2003) and since E2F-p130 is a transcriptional repressor (Smith et al., 1996), this may explain the high levels of *TRAIP* expression in these tumours. In contrast to several reports that proliferation is associated with increased expression of *TRAIP*, lymphocytes stimulated to proliferate with antigen receptors down-regulated *TRAIP* expression (Lee et al., 1997) potentially reflecting a cell-specific effect.

As well as direct effects on cell proliferation and translesional synthesis, *TRAIP* may also play a role in TNF- α -induced apoptosis (Park et al., 2007). TNF- α induces apoptosis both by inducing production of reactive oxygen species and via TNF receptor 1 (TNFR1) both of which ultimately activate caspases (Hsu et al., 1996; Hsu et al., 1995; Kim et al., 2010a; Morgan and Liu, 2010; Wang et al., 2000). Knock-down of *TRAIP* using RNA interference resulted in a dose-dependent decrease in viability and increase in activated caspase-3 following treatment with TNF- α and cyclohexamide, suggesting that *TRAIP* is required to negatively regulate TNF- α induced apoptotic pathways (Park et al., 2007).

The predicted NF- κ B binding site in the *TRAIP* promoter (Sharif et al., 2007) suggests that expression of *TRAIP* is to some extent regulated by the wide range of stimuli that lead to NF- κ B activation such growth factors, cytokines, detection of immune ligands, engagement of antigen receptors and oxidative stress; for a review see (Oeckinghaus and Ghosh, 2009). The additional evidence for an E2F binding site supports a strong link between *TRAIP* expression and proliferation, however the

questions as to whether increased *TRAIP* expression is a cause or a consequence of proliferation remains unanswered.

Whilst the studies into TRAIP-interacting proteins have been performed by over-expressing exogenous *TRAIP*; the majority of studies into the role of TRAIP in proliferation and differentiation have investigated expression of endogenous *TRAIP* or utilised knock-down of endogenous *TRAIP*. These experiments are likely more biologically relevant and the current published literature on TRAIP builds a persuasive picture of an E3 ligase which is involved in regulating cellular proliferation and differentiation. A number of interactions have been described following yeast 2-hybrid and over-expression assays and due to its ubiquitination role it is likely that TRAIP does interact with a number of proteins as part of the tightly controlled processes of cell proliferation and differentiation. However it remains to be shown whether the interactions so far described occur *in vivo* or are a by-product of overexpression of a protein containing a coiled-coil domain that has been described as ‘peptide velcro’ (O’Shea et al., 1993).

6.1.7. Primordial dwarfism

Mutations in TRAIP were identified in three microcephalic primordial dwarfism (MPD) patients also exhibiting increased respiratory infections. This section describes the clinical features of different forms of MPD, and describes in more detail the link between particular MPD genes, the DNA damage response and genome instability and how this leads to growth failure and immune deficiency.

Primordial dwarfism is a group of rare autosomal recessive single gene disorders characterized by extreme global growth failure from early development and throughout postnatal growth. In contrast to other forms of dwarfism such as achondroplasia, head size is also reduced in proportion to or smaller than their body size; hence the formal term Microcephalic Primordial Dwarfism (MPD). MPD is subdivided into distinct diseases on the basis of clinical features: microcephalic osteodysplastic primordial dwarfism (MOPD) types I/III and II, Meier-Gorlin syndrome, and Seckel syndrome. For a review of the mechanism underlying primordial dwarfism see (Klingseisen and Jackson, 2011).

6.1.7.1. MOPD type II

MOPD II is the most common type of primordial dwarfism and is caused by mutations in the pericentrin gene, *PCNT*, which encodes a centrosomal protein (Griffith et al., 2008; Piane et al., 2009; Rauch et al., 2008). MOPDII patients also exhibit skeletal abnormalities such as hip dysplasia (Karatas et al., 2014) and cerebral neurovascular abnormalities leading to complications such as moyo-moya angiopathy and intracranial aneurysms (Bober et al., 2010).

6.1.7.2. MOPD type I/III

MOPD I/III, or Taybi-Linder syndrome, represents a similar disorder to MOPD II with more severe skeletal dysplasia and brain malformations in the cerebral cortex, and is often lethal within the first year (Pierce and Morse, 2012; Sigaudy et al., 1998). MOPD I/III is caused by hypomorphic mutations in *RNU4ATAC*, coding for U4atac snRNA, a component of the U12 minor spliceosome (Abdel-Salam et al., 2011; Edery et al., 2011; He et al., 2011). U4atac snRNA is involved in splicing of less than 1% of genes, however this includes genes that are essential for DNA replication (Levine and Durbin, 2001). The severity of MOPD I/III could be due to the wide range of targets of U4atac snRNA with transcriptional consequences for many different genes arising from mutation of the non-coding RNA.

6.1.7.3. Meier-Gorlin syndrome

Meier-Gorlin Syndrome (MGS) is characterised by absent or hypoplastic patellae, distinctly small external ear structures and proportionate microcephaly (Bongers et al., 2001; Gorlin et al., 1975). Most patients have normal intellect and the growth failure can vary from mild to extremely severe (Bicknell et al., 2011a). MGS is caused by hypomorphic mutations in genes coding for components of the pre-replication complex which licenses replication origins for DNA replication during G1 of the cell cycle (Bicknell et al., 2011a; Bicknell et al., 2011b; Guernsey et al., 2011; Mendez and Stillman, 2003). It is hypothesised that if origin licensing is a rate limiting factor then the cell cycle will be lengthened causing fewer cell divisions during periods of fast cell division, such as embryogenesis and development, ultimately leading to fewer cells and therefore a smaller organism (Klingseisen and Jackson, 2011).

6.1.7.4. Seckel syndrome

Seckel syndrome is characterised by a disproportionate microcephaly at birth and often involves more severe learning disabilities, as well as distinctive craniofacial features with a receding forehead, prominent nose and small chin (Goodship et al.; Kalay et al., 2011; Majewski and Goecke, 1982). Seckel syndrome is caused by mutations in *Ataxia-Telangiectasia and RAD3 related (ATR)* and *ATR-interacting protein (ATRIP)* (O'Driscoll et al., 2003; Ogi et al., 2012) which are involved in DNA damage response signalling following replication fork stalling (Nam and Cortez, 2011).

MPD-associated genes can be grouped broadly and not mutually exclusively into replication licensing-associated genes, centriole biogenesis-associated genes and DNA damage-associated genes. Table 6.1 outlines the MPD-associated genes that are involved in the DNA damage response.

Table 6.1 MPD-associated genes that are involved in the DNA damage response.

Gene	Function	Reference
<i>ATR</i>	Master kinase of the DNA damage response Involved in sensing DNA damage and activating DNA damage checkpoints	(O'Driscoll et al., 2003)
<i>ATRIP</i>	ATR partner protein Recognises RPA-coated single stranded DNA	(Ogi et al., 2012)
<i>RBBP8/CtIP</i>	Interacts with and modulates BRCA1 during the DNA damage response	(Qvist et al., 2011)
<i>CEP152</i>	Centrosomal protein found to have a role in the DNA damage response and maintaining genome stability	(Kalay et al., 2011)
<i>DNA2</i>	Lagging strand maturation factor Replication checkpoint initiation	(Shaheen et al., 2014)
<i>XRCC4</i>	Non-homologous end joining	(Shaheen et al., 2014)
<i>Ligase IV</i>	Non-homologous end joining	(Murray et al., 2014)

6.1.8. Growth-related disorders involving defects in the DDR and genome instability

Other growth-related disorders characterised by genome instability show similarly extreme growth failure to Seckel syndrome, for example Nijmegen breakage Syndrome (NBS) (Goodship et al.; Weemaes et al., 1981) and LIG4 syndrome. NBS is caused by mutations in *NBS1* (Matsuura et al., 1997) encoding the protein nibrin which interacts with RAD50 and MRE11 in the DNA damage response (DDR) to double stranded breaks (DSBs). Defects in genes involved in non-homologous end joining (NHEJ) such as LIG4 syndrome, caused by deficiencies in DNA ligase 4, and NHEJ1 syndrome (Cernunnos/XLF syndrome) lead to growth retardation and microcephaly (Buck et al., 2006a; Murray et al., 2014).

6.1.8.1. Genome instability and immunodeficiency

Growth syndromes caused by defective DNA damage repair also commonly present with immunodeficiency of the adaptive immune system (Gennery, 2006). This phenotype is because pathways in adaptive immune development such as V(DJ) recombination, (Lieber et al., 2004), immunoglobulin class switching (Kenter, 2005; Pan-Hammarstrom et al., 2005; Petersen et al., 2001) and somatic hypermutation (Bemark et al., 2000) all require NHEJ to repair induced double-stranded breaks and generate genetic diversity of T and B cells. Hypomorphic *LIG4* mutations cause varying degrees of immunodeficiency and both V(D)J recombination and B-cell somatic hypermutation were adversely affected in patients which presented with severe combined immunodeficiency (SCID) (Buck et al., 2006b; Enders et al., 2006). Deficiency in the endonuclease Artemis (*DCLRE1C*) also presented with similar features (Bajin et al., 2013), whilst hypomorphic mutations in *DCLRE1C* are associated with development of B cell lymphoma (Moshous et al., 2003). An ever increasing body of evidence demonstrates that defects in DNA damage recognition and repair pathways lead to genome instability and adaptive immune deficiencies by preventing genetic diversity of immune cell subsets.

6.1.9. DNA damage and innate immune activation

Further to the previous section demonstrating that defective DDR likely causes defects in adaptive immune development, recent evidence suggests that DNA

damage can be detected by innate immune sensors. In this way detection of DNA damage induces an innate immune response predominantly involving production of type I interferon. Recognition of double stranded breaks in cytoplasmic DNA by MRE11, lead to STING-dependent interferon production, suggesting a similar mechanism may induce an immune response to endogenous DNA damage (Kondo et al., 2013). Activation of the innate immune system, and particularly of interferon production, following detection of DNA damage could be an evolutionary defence mechanism against viruses hijacking components of the DDR to facilitate replication (Mboko et al., 2012). The basal levels of DNA damage in a healthy individual is unlikely to cause significant changes in inflammatory response, however defective DNA repair mechanisms arising as a result of mutations in DDR genes may lead to chronic activation of the innate immune system.

Therefore, defects in the DDR may cause deficiencies in the adaptive immune response and have the potential to chronically activate the innate immune system.

6.1.10. Using mouse models to study gene function

Mouse models have been used for many years to study gene function; recently the field has opened up as new techniques have emerged allowing faster, more accurate gene targeting. This section introduces the techniques that were used in this thesis and outlines how mouse models have been previously used to study similar phenotypes. Alternative methods of genome editing and their advantages and disadvantages are also described.

6.1.10.1. Inducible gene expression

Inducible or conditional systems allow spatially or developmentally controlled expression of genes, and are particularly useful when studying a gene that is essential for development as has been shown for TRAIP (Park et al., 2007). The Cre/lox system is derived from bacteriophage P1; the Cre (cyclisation recombination) protein is a DNA recombinase which recognises a 34 bp loxP (locus of X-over of P1) site and catalyses reciprocal conservative DNA recombination between loxP sites. No host factors or genomic topological features are required for efficient Cre-mediated recombination meaning that loxP sites can be inserted into the eukaryotic genome without any co-factors (Sauer, 1998). LoxP sites are introduced into the host genome

using homologous recombineering and mice are then crossed onto a Cre line that expresses Cre under the control of specific promoters (Liu et al., 2003). Analogous to the Cre/*lox* system is the Flp/FRT system from *Saccharomyces cerevisiae*, whereby the Flippase (Flp) enzyme catalyses recombination between 65 bp FRT (Flippase recognition target) sites (Hoang et al., 1998).

6.1.10.2. Introducing specific mutations

Specific point mutations can also be introduced into the mouse genome by site-directed mutagenesis of the ES cell targeting vector and homologous recombination. By introducing mutations into conserved genes, it is possible to recapitulate and study human phenotypes.

6.1.10.3. Mouse models of growth and immunodeficiency

A number of the genes that are mutated in MPD have been targeted to generate mouse models of growth failure. Conditional ablation of *ATR* in adult mice causes age-related phenotypes and defects in stem cells renewal, whilst *ATR* null embryos die before E7.5 (Brown and Baltimore, 2003; Ruzankina et al., 2007).

Additionally, the effect of genome instability and immunodeficiency has been intensively studied in mouse models. *ATM* null mice phenocopy the growth defects and immune deficiencies of Ataxia-Telangiectasia, and most develop thymic lymphomas by the age of four months (Xu et al., 1996). A homozygous *Lig4* mutant mouse recapitulating a patient mutation (R278H (Riballo et al., 1999)) exhibits growth defects and immune defects resembling SCID as well as a high rate of thymic tumour development (Rucci et al., 2010). Artemis deficient mice produce no T cells or B-cells (Barthels et al., 2013), whilst Artemis hypomorphic mutants developed various T and B cell malignancies on a p53 null background. The malignancies are associated with misrepair of RAG-generated chromosomal breaks suggesting that the malignancies are due to defective repair rather than absence of repair (Jacobs et al., 2011). Ku80 with Ku70 make up the Ku70/80 heterodimer which binds to the ends of DSBs and is essential for NHEJ. No mutations in *Ku80* have been identified in humans, however it has been extensively studied in mice as a model for how defects in NHEJ affect cellular processes. *Ku80* null mice show severely defective V(D)J rearrangement and growth retardation (Nussenzweig et al., 1996). The similarity of

the mouse phenotypes to the patient phenotypes following disruption of these genes indicates that mice are a relevant model to use to study similar genes.

6.1.11. Objectives

Immune deficiencies in disorders involving a defective DNA damage response are not without precedence and are generally thought to be due to defective adaptive immune system development. However the patient phenotype and previously published data indicates a role for TRAIP in regulating the innate immune response. The aim of this chapter is to understand how TRAIP mutations lead to the increased susceptibility to respiratory infections and chronic lung disease seen in the patients.

6.1.12. Hypotheses

On the basis of the existing literature, the working hypotheses in the thesis were:

1. TRAIP has a role in organism growth by regulating cellular proliferation and/or the DNA damage response.
2. TRAIP has a role in the negative regulation of the innate immune response, with TRAIP deficiency resulting in over-activation of the innate immune response following exposure to immune stimuli, and possibly in a basal state.

6.1.13. Experimental outline

- To use patient dermal fibroblasts to assess the immune response.
- To generate mouse models of human MPD patients to study the function of TRAIP in a whole organism context and understand the function of TRAIP *in vivo*.
- To characterise the consequence(s) of TRAIP mutations.

6.2. Results

6.2.1. Mutations in *TRAIP* cause a common phenotype

Prior to this project starting, exome sequencing identified a recurrent homozygous mutations, c.553C>T, p.Arg185Stop, in *TRAIP* in two MPD patients (AJ093 and AJ668) (Louise Bicknell and Matt Hurles). Subsequent haplotype analysis of the two patients showed a common ancestral haplotype of 3.5 Mb covering the *TRAIP* gene indicating that these two patients have a distant common ancestor. The strong phenotypic similarity between these two patients, both in facial features and growth parameters (personal communication, Professor Andrew Jackson), and evidence of a distant common ancestor strongly suggests the mutation identified in *TRAIP* is pathogenic. Exome sequencing was utilised to identify the *TRAIP* mutation in AJ668; this data was further analysed and no other pathogenic variants were identified within the ancestral haplotype boundaries (personal communication, Dr Louise Bicknell). A third patient was identified during the course of the project by resequencing of an MPD patient (Bernd Wollnik) Parents of all three patients were healthy heterozygous carriers. The identification of a second, different mutation, c.52C>T, further strengthens the genetic evidence for *TRAIP* being the causative gene for this MPD phenotype.

A nonsense mutation c.553C>T in patients AJ093 and AJ668 resulted in a premature stop codon in exon 7 of *TRAIP* (table 6.2). At the protein level this mutation lies between the coiled-coil domains within the TRAF-binding domain (figure 6.3). A missense mutation in exon 1 in AJ1856 results in a non-conservative residue change from arginine to cysteine (table 6.2); this mutation lies in the RING domain of *TRAIP* (figure 6.3). Ancestral haplotyping showed that AJ093 and AJ668 share a common haplotype of 3.5 Mb across the *TRAIP* locus indicating a common ancestor, despite being from diverse geographical locations (UK and Italy) (haplotyping performed by Louise Bicknell).

All three cases had significantly reduced birth weight; with a mean weight of -2.6 ± 0.32 s.d. confirming intrauterine growth failure, as well as significantly reduced a mean height; -4.6 ± 0.75 s.d. and mean occipital frontal circumference (OFC); -7.6 ± 0.99 s.d., (figure 6.4). Disproportionate microcephaly was present in all three cases,

with more substantial reduction in OFC compared to the reduction in height. All patients had similar phenotypes including scaphocephaly, large low-set protruding ears and moderate developmental delay.

All three patients also had recurrent bacterial and viral respiratory infections and repeated hospital admissions. One patient died from chronic lung disease. Patient 2 also had two siblings who died as infants from unknown causes.

Table 6.2 Exome sequencing and resequencing studies identified three patients with a mutation in TRAIP. This table shows the nucleotide and amino acid change of each patient.

Patient	Mutation	Protein change	
Patient 1	c.553C>T	p.Arg185Stop	Edinburgh/WTSI
Patient 2	c.553C>T	p.Arg185Stop	Resequencing
Patient 3	c.52C>T	p.Arg18Cys	Cologne

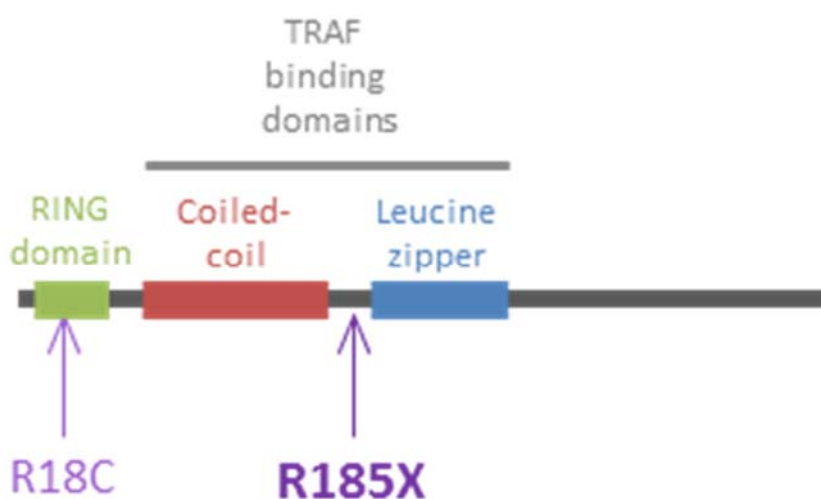


Figure 6.3 Schematic of the TRAIP protein with location of patient mutations.

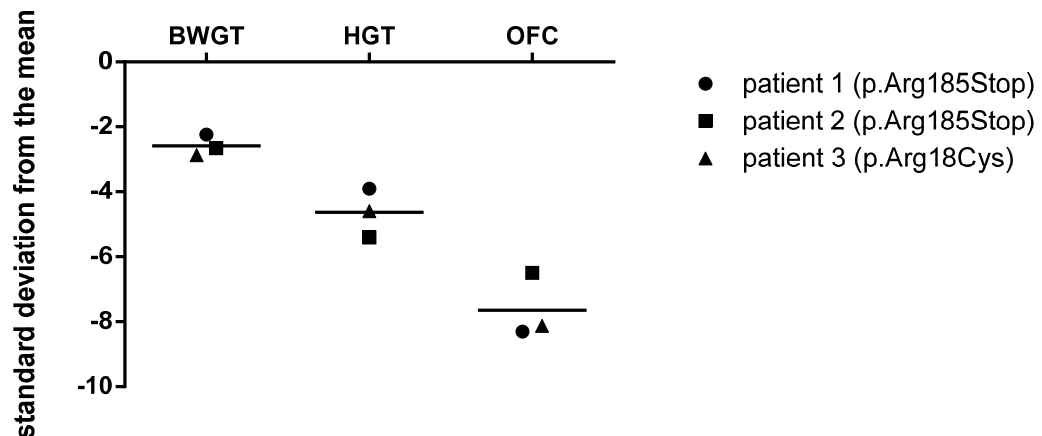


Figure 6.4 Mutations in TRAIP are associated with severe microcephaly and pre- and post-natal growth failure. Birthweight (BWGT), height (HGT) and occipital frontal circumference (OFC) of the three patients at birth (symbols) and the mean of the three patients (line). Growth parameters are plotted as Z-scores (standard deviation from the population mean for age and sex).

6.2.2. The c.553C>T mutation introduces a premature stop codon and leads to transcription of alternative splice isoforms

Reverse-transcriptase PCR (RT-PCR) performed on patient dermal fibroblasts or lymphoblastoid cells show expression of alternative isoforms of *TRAIP* in both patient cell lines, although this cannot be accurately quantified without performing qPCR. Additionally, the patient cells express unique isoforms of *TRAIP* that are not detected in wild-type controls (figure 6.5). The minus RT controls confirm that these multiple bands are not caused by contaminants and the *GAPDH* loading control shows that the same amount of cDNA was added into each reaction.

Patient 2 LCLs may express lower levels of the full length isoform (TRAIP.FL) than either wild-type or parental controls, and may express increased levels of an isoform of 1363 base pairs (bp). Sequencing of this band showed that it corresponds to an isoform with an in-frame deletion of exon 7 (TRAIP Δ 7). Patient 1 fibroblasts

expressed almost negligible levels of TRAIP.FL and lower levels of TRAIP Δ 7 than patient 2, these cells mostly expressed an isoform of approximately 1200 bp which was also expressed by patient 2 LCLs and at lower levels by heterozygous parental LCLs, however attempts to sequence this band have so far been unsuccessful. The heterozygous parents of patient 2 express low levels of the alternative isoforms detected in patient 2, including TRAIP Δ 7 and express slightly decreased amounts of TRAIP.FL compared to wild-type controls. Both patients express an isoform of *TRAIP* of approximately 1000 bp which is also expressed by the heterozygous LCLs and at very low levels by WT LCLs; again, sequencing of this band has been unsuccessful.

Immunoblotting of supernatants from patient cells could not detect any TRAIP protein in patient cells (Dr Margaret Harley, unpublished). The failure to detect any alternative protein isoforms indicates that either the antibody is specific to the full length isoform of TRAIP, or that the alternative isoforms seen by RT-PCR are not protein coding and are degraded via nonsense mediated decay.

In conclusion, mutations in *TRAIP* cause decreased levels of the full length transcript and increased expression of alternative isoforms. However, western blotting could not detect any TRAIP protein in patient cells despite low levels of transcript being amplified by RT-PCR. This suggests that expression of the full length isoform and protein is not completely depleted by the c.553C>T mutation indicating that the function of TRAIP would not be completely abrogated.

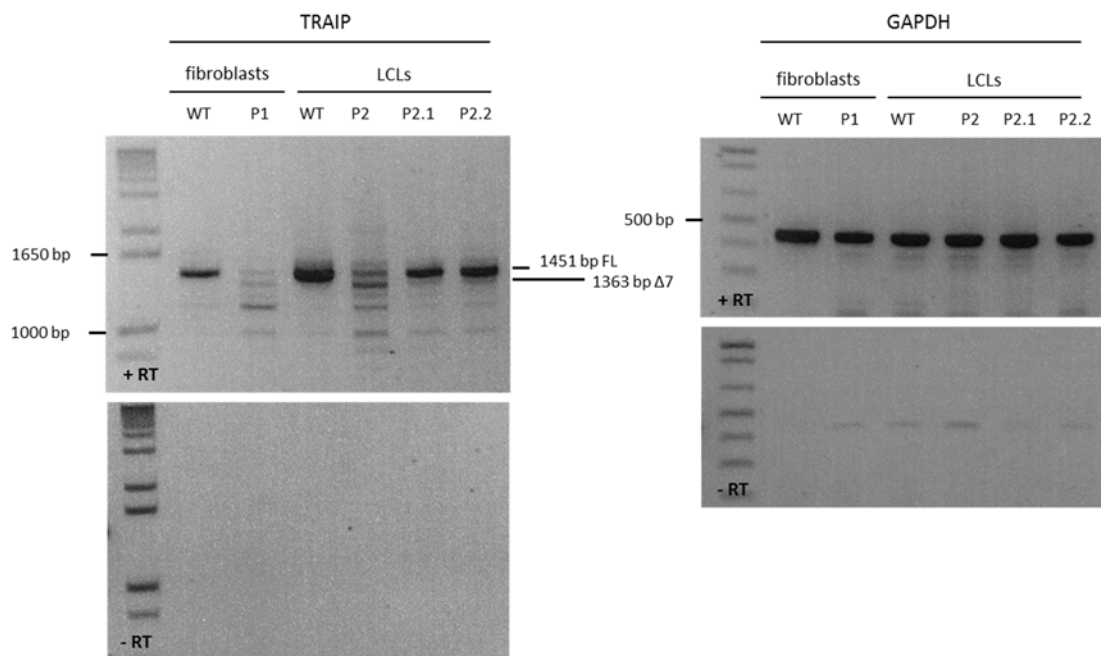


Figure 6.5 c.553C>T leads to alternative splicing. Expression of *TRAIP* in control (WT) or patient 1 dermal fibroblasts and in control (WT), patient 2 or parent 2.1/2.2 lymphoblastoid cells (LCLs) was evaluated by RT-PCR and analysed by agarose gel electrophoresis. Minus reverse transcriptase (-RT) controls were set up in parallel and expression of the housekeeping gene, *GAPDH*, was measured as a loading control.

6.2.3. The effect of p.Arg185Stop on the immune response of primary fibroblasts

Given that the patients exhibited increased susceptibility to respiratory infections, the immune response of the available primary dermal fibroblasts was assayed. Whilst fibroblasts are not the most relevant cell type to assay the immune response, they were the only patient cells available.

Primary dermal fibroblasts derived from skin punch biopsies of patients or controls were treated with synthetic ligands corresponding to PAMPs present in a bacterial infection, TLR4 ligand LPS; and a viral infection, TLR3/RIG-I ligand p(I:C). The cellular response to TNF- α was assayed due to previous reports showing that TRAIP regulates the TRAF2 dependent response to TNFR1 activation (Lee et al., 1997).

There were no consistent significant differences between patient and control fibroblasts in the production of IL-6, TNF- α or CXCL8 in response to any of the ligands (figure 6.6). The production of IL-6 was significantly increased in response to LPS in patient fibroblasts compared with control fibroblasts; conversely the production of CXCL8 was significantly decreased in response to LPS in patient fibroblasts compared with controls. Additionally, production of CXCL8 in response to TNF- α was significantly decreased in patient compared to control fibroblasts. This result is inconsistent with previous results suggesting that TRAIP is required for down regulating the immune response, however CXCL8 had not been measured in these previous reports (Lee et al., 1997; Zhang et al., 2012).

Furthermore, there was substantial variation in the response between the two wild-type control lines tested, the results from which were combined to give the values shown in figure 6.6, which suggests that the immune response of primary fibroblasts may be too variable for this to be a useful cell system to identify an immune defect.

To clarify a role for TRAIP in the immune response required a different system, leading to the design of mouse models as described in the next section. Mouse models provide the additional advantage of being isogenic systems which should remove the variation introduced by primary patient cells with an outbred variable genetic background.

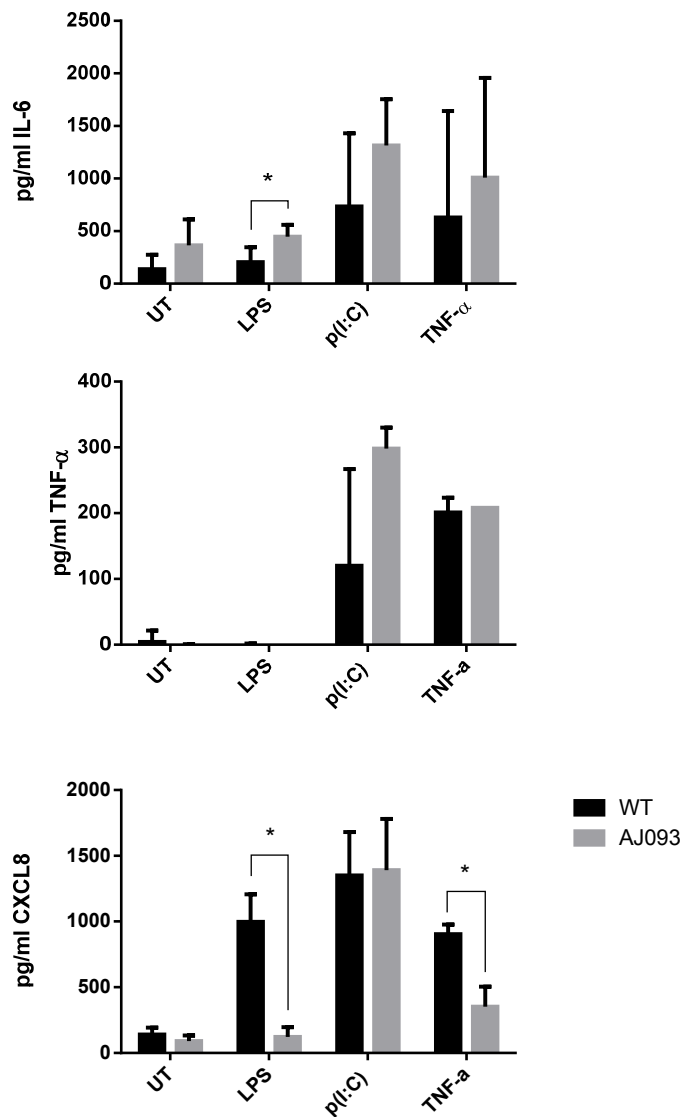


Figure 6.6 Measuring the immune response of patient dermal fibroblasts.

Primary control (WT, pool of 2 WT controls) or patient 1 fibroblasts were treated with 50 ng/ml LPS, 1 µg/ml p(I:C) or 50 ng/ml TNF-α and production of cytokines was measured by ELISA. Data shown is the mean of 1 independent experiment performed in triplicate (IFN-β) or the mean of 3 independent experiments performed in triplicate (IL-6, TNF-α, CXCL8). Error bars are standard deviation of technical replicates (IFN-β) or standard error of the independent experiments (IL-6, TNF-α, CXCL8). *p ≤ 0.05, unpaired T-test.

Two targeting constructs were designed in an attempt to recapitulate the human growth and immune phenotype. It was previously shown that a knock-out of exon 2 of *TRAIP* was lethal (Park et al., 2007) which indicates that complete loss of TRAIP is lethal.

A

human	1	GATTGAGCTTCTACTCCAGAGCCAGCGCCCTGAGGTGGAGGAGATGATC	50
			
mouse	1	AATTGAGCTCCTACTCCAGAGCCAGCGGTCTGAGGTGGAGGAGATGATT	50
human	51	GAGACATGGGTGTGGGACAGTCAGCGGTGGAACAGCTGGCTGTGTACTGT	100
		. .	
mouse	51	GAGACATGGGTGTGGGACAGTCAGCGGTGGAGCAGCTGGCTGTGTACTGC	100
human	101	GTGTCTCTCAAGAA 114	
		.	
mouse	101	GTGCCCTCAAGAA 114	

B

human	1	QIELLLQSQRSEVEEMI RDMGVGQSAVEQLAVYCVSLKK	39
mouse	1	QIELLLQSQRSEVEEMI RDMGVGQSAVEQLAVYCVSLKK	39

216

by the mutation in AJ093 and AJ668 highlighted in red (B). Assembled using EMBOSS Needle.

In outline, to achieve this, a 6.5 kb region of the *TRAIP* gene containing exons 5 to 10 was subcloned into pBS, a lone *loxP* site was inserted between exons eight and nine of the conditional construct whilst a *FRT*-flanked *neo* cassette with another *loxP* site was introduced between exons 6 and 7 in both the conditional and knock-in constructs. Sire directed mutagenesis was used to introduce the patient mutation into the knock-in targeting vector. The targeting constructs were transformed into ES cells and positive ES cells were injected into blastocysts. A schematic overview of the process is outlined below.

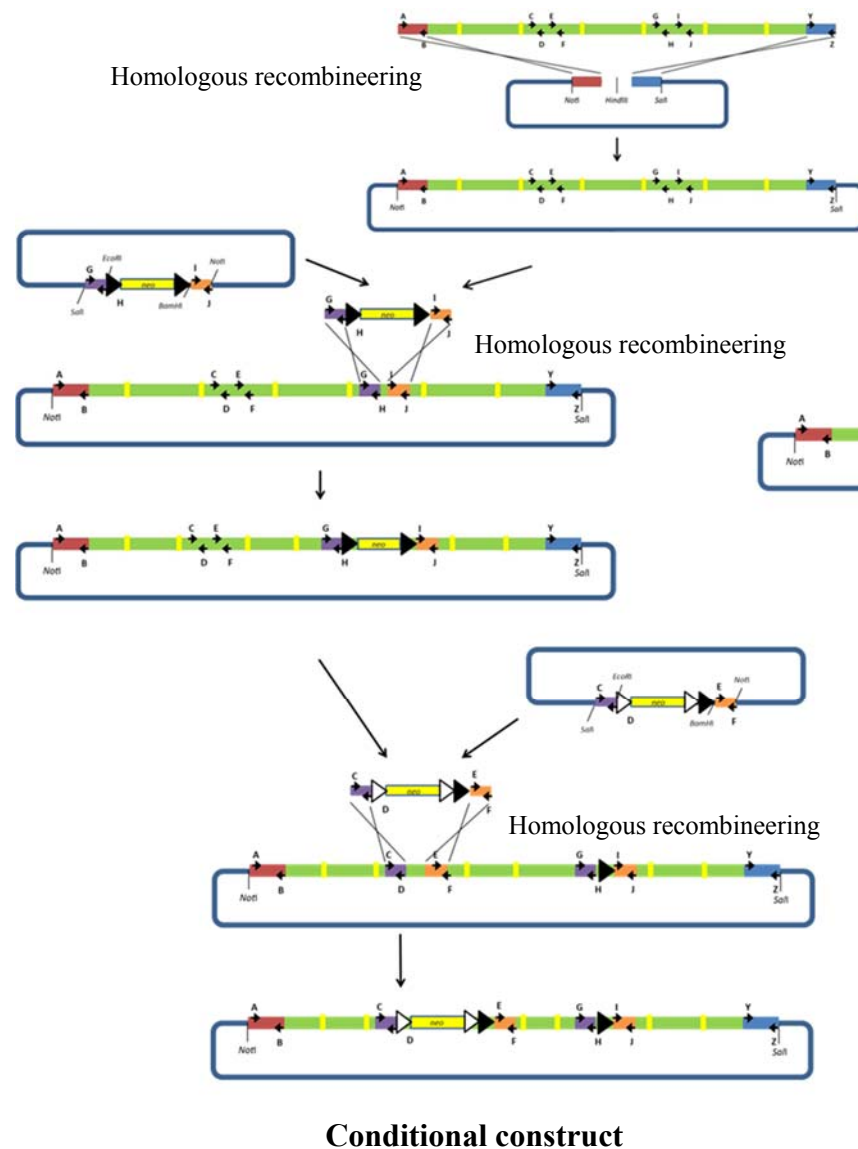
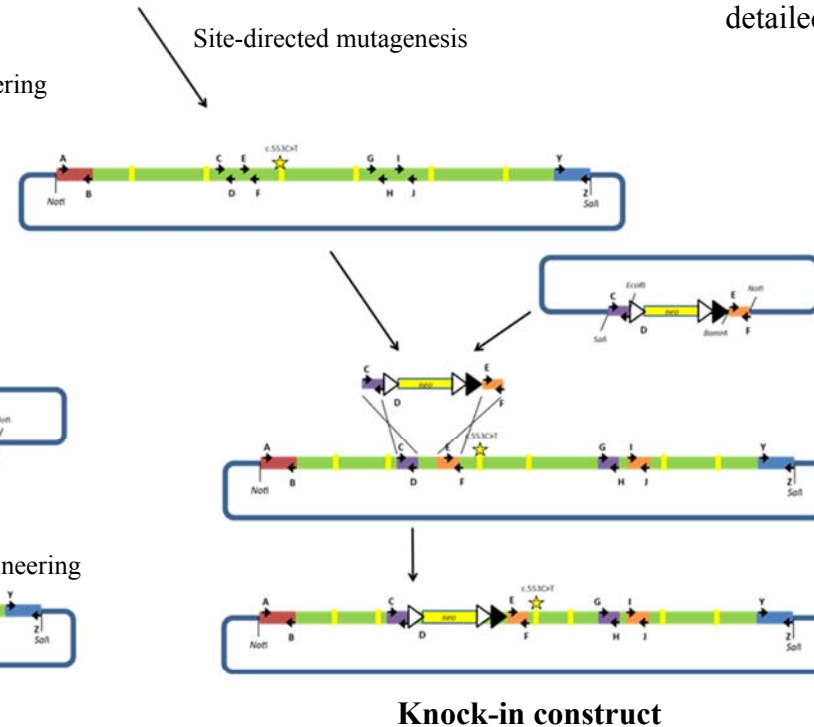


Figure 6.8 Schematic showing the complete process of engineering a conditional knock-out construct designed to remove exons 7 and 8 of *TRAIP* and a knock-in construct to recapitulate the human R185X mutation.

Arrows indicate the primers used to achieve cloning, further details are provided in the appropriate figures and primers are detailed in appendix 1



6.2.4.1. Construct design

A bacterial artificial chromosome (BAC) which contained *TRAIP* was identified using Ensembl (Adams et al., 2005). Homology arms were designed to target a 6567bp region of *TRAIP* covering exons 5 to 10, referred to henceforth as ‘X5-10’. The homology arms were designed using a repeat masked sequence of *TRAIP* to avoid repeat regions. Two sets of primers were designed to amplify the 5’ homology arm (A/B, 407 bp) and the 3’ homology arm (Y/Z, 520 bp) flanking this region. The primers used to amplify the homology arms contained restriction sites to allow cloning into digested plasmids (appendix 1). The homology arms were successfully amplified by PCR (including the smaller homology arms discussed in 6.2.4.3) (figure 6.9).

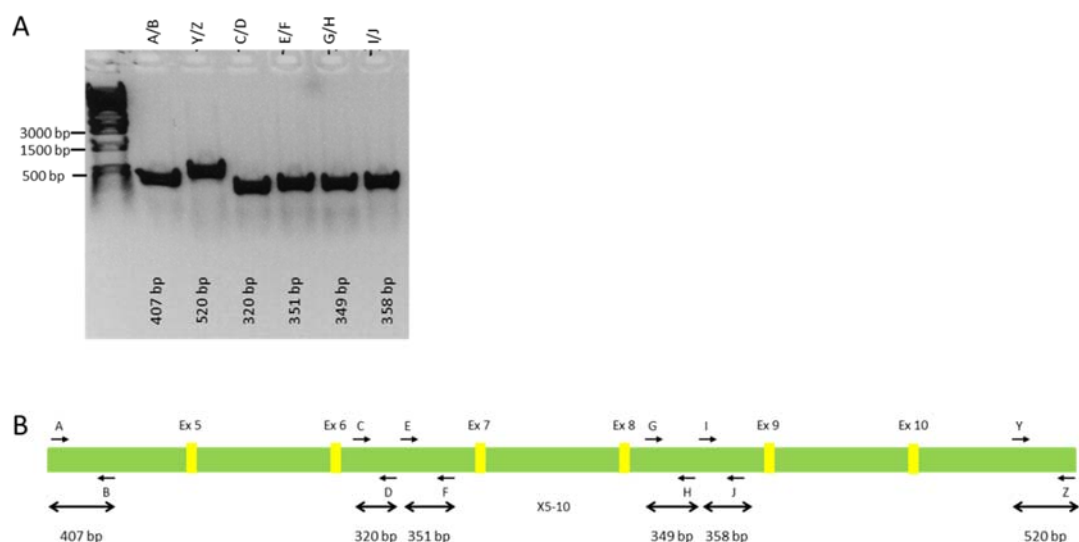


Figure 6.9 Amplification of homology arms. Homologous regions were amplified from BAC BMQ425k23 DNA using high fidelity KOD PCR and products were electrophoresed in a 1% agarose gel to confirm the correct sized fragments had been amplified (A) A schematic of *TRAIP* exons 5-10 indicating the positions of the primers used pairs used and the expected size of the product (B).

6.2.4.2. Subcloning 'TRAIP.X5-10' into pBluescript

The BAC containing X5-10 was transformed into EL350 bacteria which express the bacteriophage genes *exo*, *bet* and *gam*, part of a bacteriophage system for inserting linear double stranded DNA via homologous recombination (Lee et al., 2001).

Positively transformed EL350 were selected on the basis of chloramphenicol resistance.

Separately, the homology arms amplified from X5-10 using high fidelity PCR (figure 6.10) were ligated sequentially into pBluescript (pBS) to construct a 'retrieval plasmid' (figure 6.10 E). Colony PCRs, restriction digests and sequencing at each stage confirmed uptake of the appropriate fragment (figure 6.10)

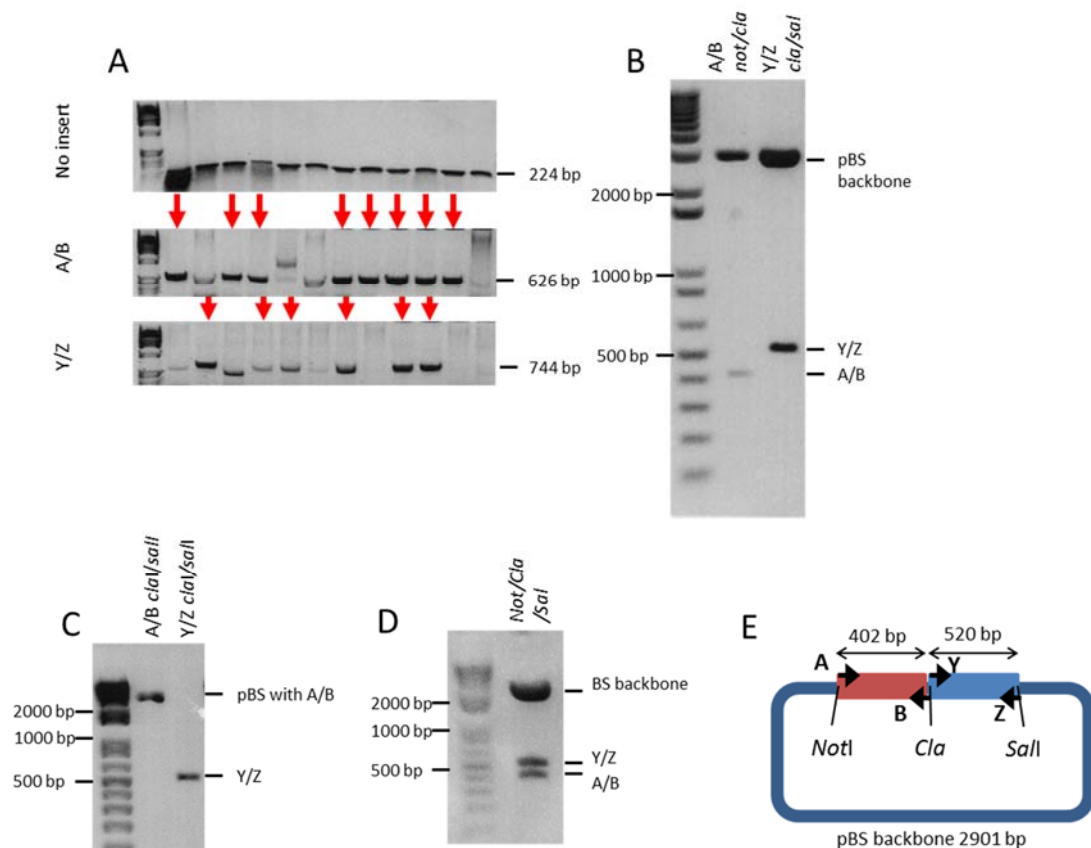


Figure 6.10 Confirmation of the retrieval plasmid. Following ligation of linearised pBS and either A/B or Y/Z, and transformation of DH5 α competent bacteria, PCR was performed across the multiple cloning site of pBluescript (pBS) using M13 primers to check correct insertion of each homology arm (224 bp - no insert, 631 bp – A/B, 744 bp – Y/Z) (A). DNA was prepared from positive clones and re-checked by restriction digest to excise the correct size fragment (B). pBS with A/B was linearised to act as the vector and Y/Z was digested out of pBS to be used as the insert (C). The linear products from C were ligated and transformed into DH5 α . The product was checked by restriction digest to demonstrate presence of both homology arms (D). A schematic showing the final retrieval plasmid containing the amplified fragments and the sizes of each restriction fragment (E).

The entire region of *TRAIP* between, and including, the homology arms (X5-10) should have been recombined from the BAC into pBS by gap repair following activation of the temperature sensitive recombineering activity in EL350. However, following five unsuccessful attempts to subclone X5-10 into pBS, the 6567 bp sequence encoding X5-10 was instead amplified from BMQ425k23 by high fidelity PCR using the original flanking primers (figure 6.11 A). X5-10 was ligated into linearised pBS (pBS.X5-10) by virtue of restriction sites introduced by primer pairs that were engineered to contain specific restriction sites, allowing directional cloning. Exon sequencing confirmed that the PCR amplification had not introduced any errors into the coding sequence of *TRAIP*.

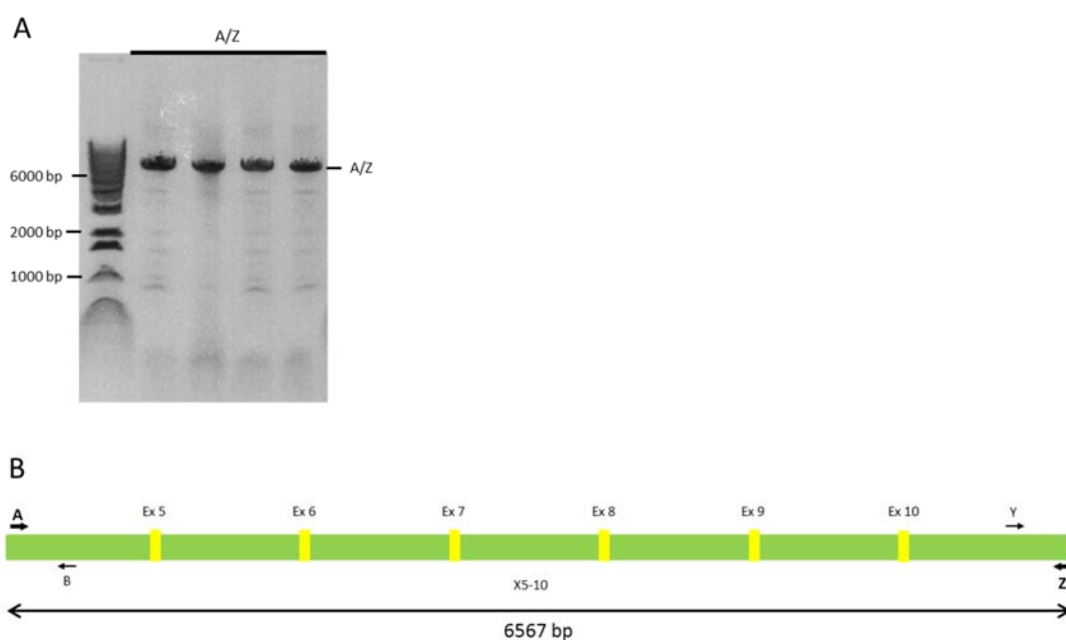


Figure 6.11 Proofreading PCR amplification of exons 5-10 of *TRAIP*. High fidelity (KOD taq) PCR was performed to amplify X5-10 from BMQ425k23 (A) and *NotI/SaII* restriction sites at the 5' and 3' ends of X5-10 were utilised to clone the region into pBS in the correct orientation as illustrated in (B)

6.2.4.3. Cloning smaller homology arms into pL451 and pL452 to construct 'mini-targeting vectors' for introduction of loxP and FRT sites.

Homology arms were amplified from bMQ425k23 using KOD proofreading PCR (figure 6.9), using primer pairs engineered to introduce specific restriction sites at the 5' and 3' ends to allow directional cloning into pL451 (C/D and E/F) (figure 6.12) or pL452 (G/H and I/J) (figure 6.13).

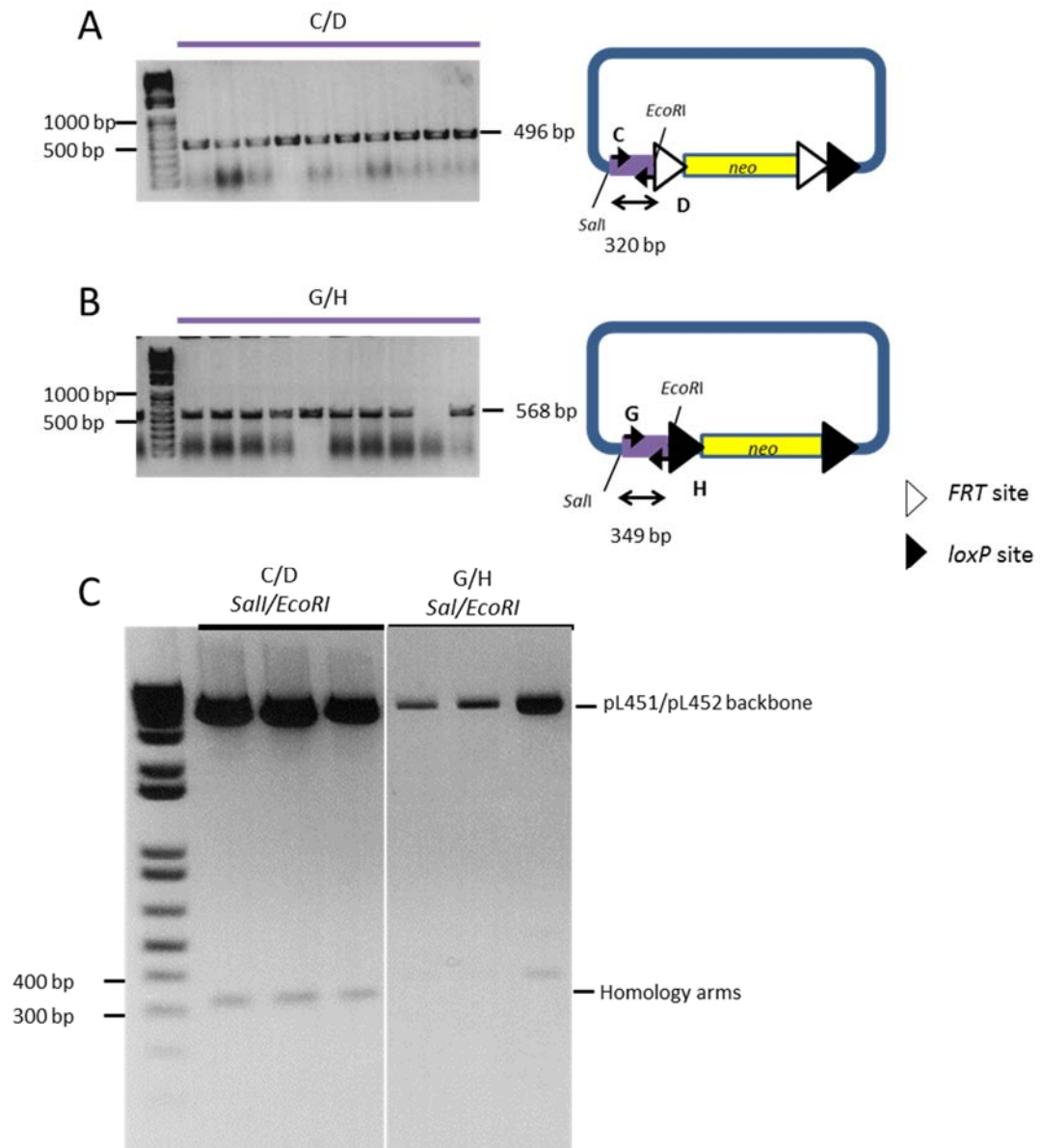


Figure 6.12 Constructing mini-targeting vectors to introduce loxP and FRT sites – subcloning the first homology arm. The homology arms C/D (A) and G/H (B) were subcloned directionally into pL451 (containing an frt flanked neo cassette and a lone loxP) or pL452 (containing a floxed neo cassette) respectively (as shown by schematics) by virtue of engineered restriction sites. M13 and internal neo primers were used to verify integration of the correct sized fragments into ampicillin resistant colonies (A, B). DNA was prepared from positive clones and validated by restriction digest to excise the correct size fragment (E, C/D 320 bp, G/H 349 bp).

The PCR products were digested, gel extracted and ligated into linearised pL451 or pL452, before transformation into DH5 α . DNA was prepared from ampicillin resistant colonies and a colony PCR was performed to check for integration of the mini-homology arms (figure 6.12 A, B, 6.13 A, B). Positive colonies were mini-prepped, validated by restriction digest (figure 6.12 C, 6.13 C) and sequenced.

Once both mini-homology arms were in the appropriate plasmid they were validated by restriction digest excision of each individual homology arm (*SalI/EcoRI* or *BamHI/NotI*) or the entire neo cassette including the homology arms (*SalI/NotI*) (figure 6.13 C), confirming that the correct size fragment was removed by the appropriate digest.

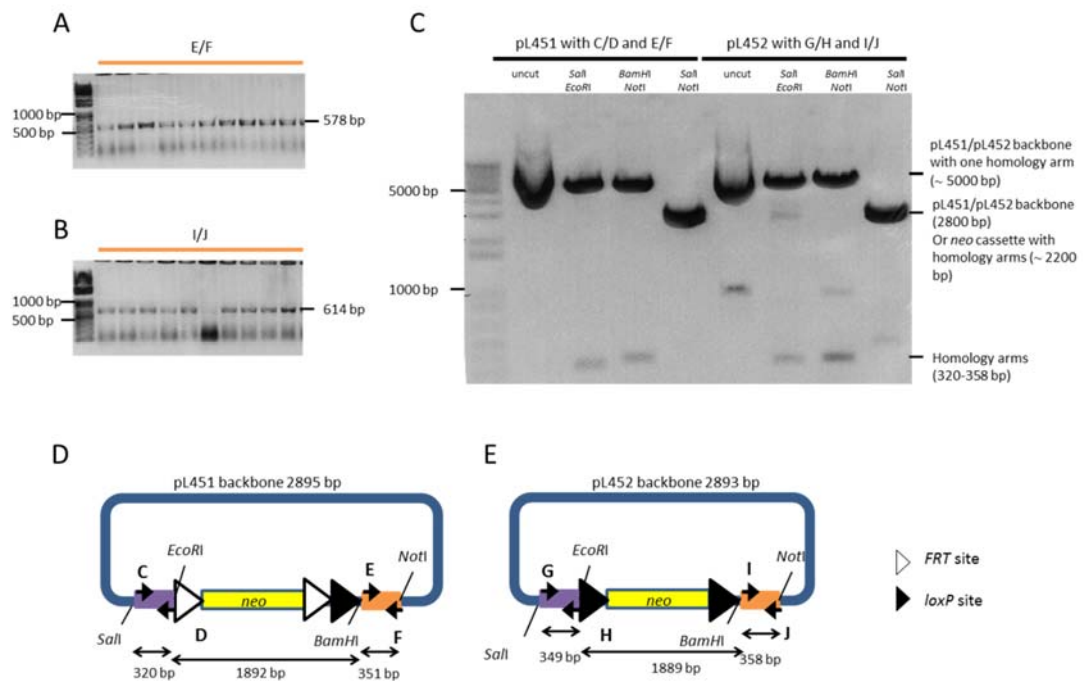


Figure 6.13 Constructing the mini-targeting vectors to introduce loxP and FRT sites – subcloning the second homology arm. The homology arms E/F (A) and I/J (B) were subcloned directionally into pL451 (containing C/D, an *frt*-flanked *neo* cassette and a lone loxP) or pL452 (containing G/H and a floxed *neo* cassette) respectively (as shown by schematics) by virtue of engineered restriction sites. M13 and internal *neo* primers were used to verify integration of the correct sized fragments into ampicillin resistant colonies (A, B). DNA was prepared from positive clones and validated by single or double restriction digest to verify excision of the correct size fragment (C). Schematics showing integration of both homology arms in either pL451 (D) or pL452 (E).

6.2.4.4. Introducing the lone *loxP* into the conditional construct

A lone *loxP* site was then introduced between exons 8 and 9 of the conditional construct by homologous recombination between pBS.X5-10 and pL452 in recombineering-active EL350, followed by arabinose induced Cre-mediated recombination

The neo cassette including homology arms (2644 bp) was excised from pL452 by digestion with *NotI* and *SalI* and co-transformed into recombineering active EL350 with pBS containing X5-10. Transformants were plated onto either ampicillin or kanamycin. Ampicillin resistance acted as a control for checking that the EL350 bacteria were competent and that transformation with pBS was successful, whilst DNA was prepared from kanamycin resistant (neo positive) clones and correct recombineering was verified by digest with *NotI* and *SalI* (figure 6.14 A). Two positive mini-preps (figure 6.14 A, red arrows) were re-transformed into DH5 α and re-plated with kanamycin to separate potential mixed clones.

The neo cassette was excised via Cre-mediated recombineering in EL350 bacteria which had been treated with arabinose to activate expression of the arabinose-inducible *cre* gene. Successful excision of the neo cassette was verified by PCR across the insertion site (using primers G and 452_flanking_R). The transformed bacteria were screened by a colony PCR across the *loxP* sites (figure 6.14 B). Without successful recombination the region across the *loxP* sites and neo cassette was too large (2800 bp) to amplify by a standard PCR with a 30 second extension time, as shown by a control PCR on the original plasmid. In the majority of colonies (18 out of 21) the PCR successfully amplified across the *loxP* generating a fragment of 1003 bp, confirming that the Cre recombinase had worked and a lone *loxP* site had been generated between exons 8 and 9 of subcloned *TRAIP* DNA. Additionally, the positive colonies were replica plated onto kanamycin plates to confirm loss of the neo cassette and concurrent loss of kanamycin resistance.

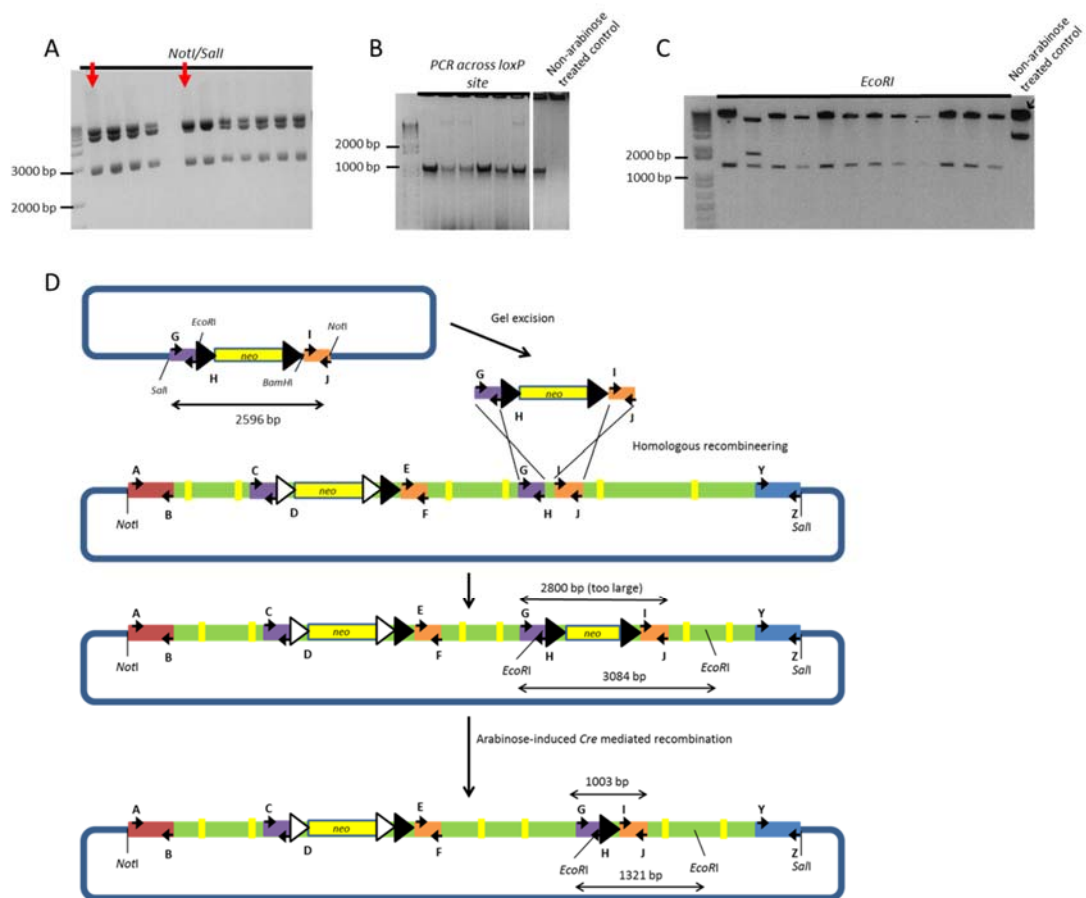


Figure 6.14 Introducing the lone loxP site between exons 8 and 9 in the targeting plasmid. The region including the homology arms and floxed neo cassette in pL452 was gel extracted, introduced into pBS via homologous recombination and validated by restriction digest with *NotI* and *SalI* (A). The floxed neo cassette was removed via Cre-mediated recombination and removal was confirmed by PCR across the loxP site (B, fragment too large to amplify by standard PCR before cre excision, 1003 bp following recombination) and by restriction digest with *EcoRI* (C, 3084 bp before recombination, 1321 bp after). A schematic demonstrates these steps leaving the final construct with pBS containing subcloned DNA with a lone loxP site (E).

DNA prepared from kanamycin resistance clones were digested with *NotI* and *SalI* however the bands were difficult to resolve as the remaining pBS backbone was a

similar size to the neo cassette with homology arms (not shown). Using NEB cutter, an *EcoRI* restriction site was identified which, in combination with an *EcoRI* immediately 5' to the loxP site allowed for better resolution of the bands. This confirmed that colonies where Cre recombinase had worked successfully had a band at 1321 bp, whereas the untreated control resulted in a fragment size of 3084 bp as it still contained two loxP sites and a neomycin selection cassette (figure 6.14 C).

6.2.4.5. Site directed mutagenesis

For the *TRAIP* knock-in vector, pBS.X5-10 and pL451 were mutated using site directed mutagenesis (Cormack, 2001). Primers were engineered containing the specific mutation (appendix 1), the constructs were mutated using circular amplification and then transformed into XL-10 gold ultracompetent bacterial cells which take up large DNA constructs with high efficiency. DNA prepared from transformed was digested with *NotI/SaII* to confirm retention of the subcloned DNA (6567 bp in pBS and 2896 bp in pL451) (figure 6.15 A) and sequenced across the mutation site (figure 6.15 B).

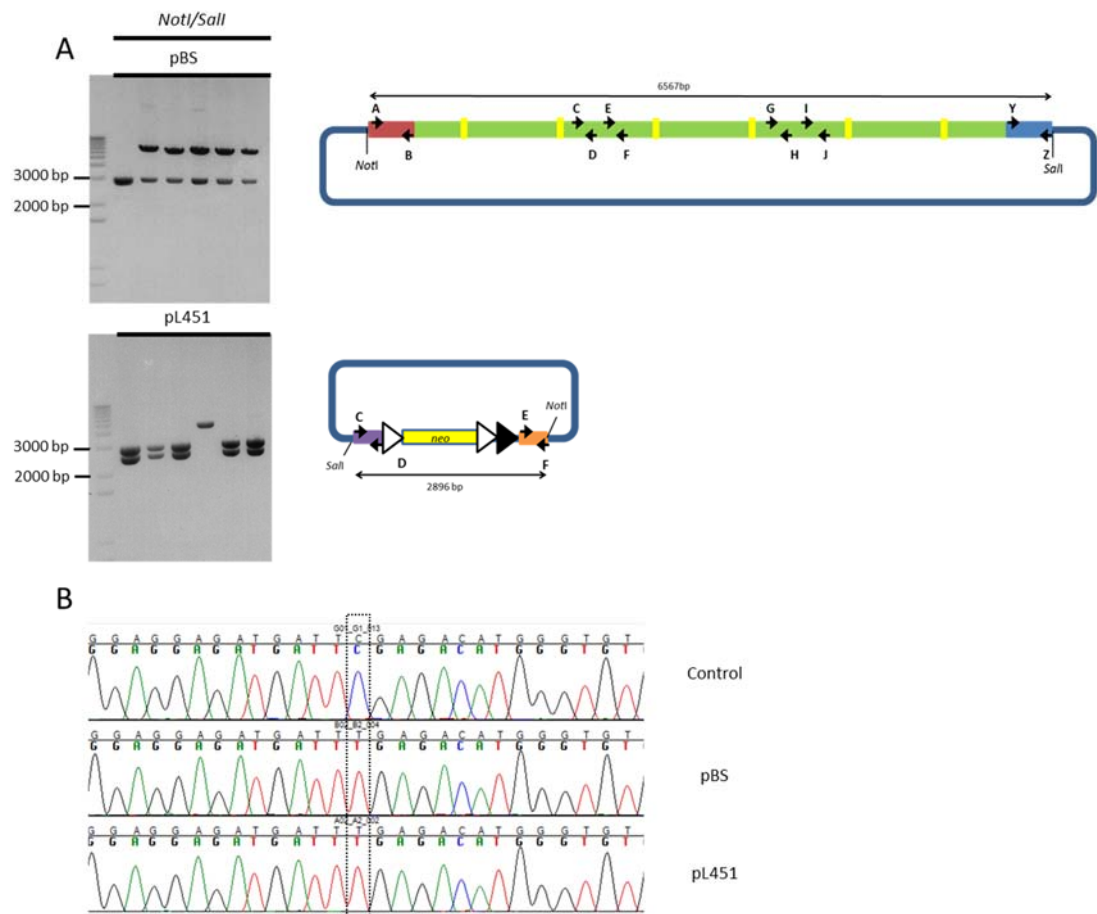


Figure 6.15 Site-directed mutagenesis. Following site directed mutagenesis, colonies were checked by restriction digest (A) and by sequencing (B) confirming introduction of C>T mutation in both pBS and pL451.

6.2.4.6. Introducing the *FRT*-flanked *neo* cassette and second *loxP* site

For both knock-in and conditional targeting vectors, the *FRT*-flanked neo cassette with a 3' loxP site was introduced between exons 6 and 7 by homologous recombineering.

Following electroporation of appropriate plasmids into heat activated EL350, DNA was prepared from kanamycin resistant colonies which must have incorporated the selection cassette. The integration of the cassette was verified by restriction digest (figure 6.16).

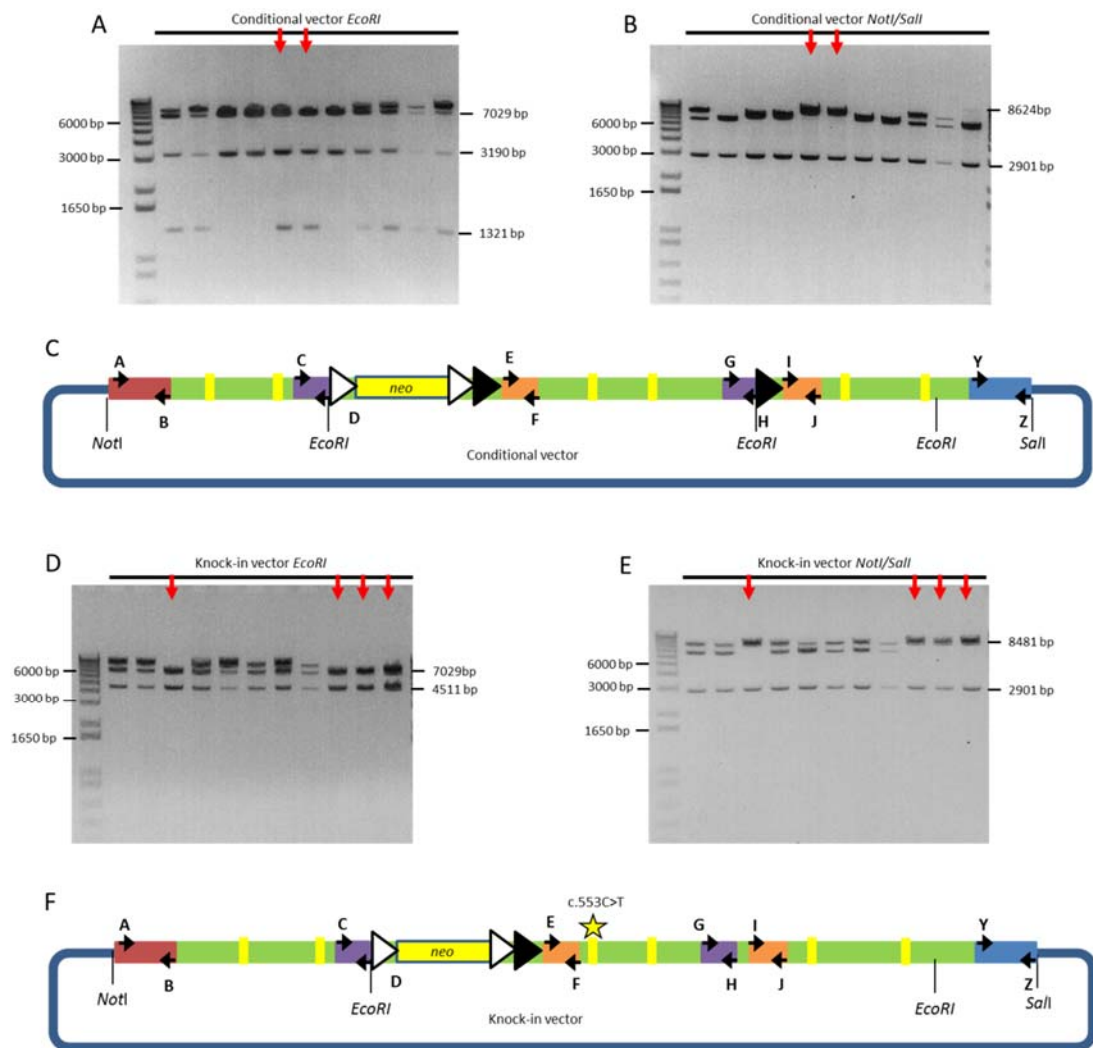


Figure 6.16 Introducing a FRT-flanked neo cassette between exons 6 and 7 of the knock-in and the knock-out vector by homologous recombineering.

Successful recombineering was verified by restriction digest with *EcoRI* (A, D) or *NotI/SalI* (B, E). The schematics show the structure of the final targeting constructs (C, F).

6.2.4.7. Verification of loxP and FRT sites in knock-out construct

The knock-out targeting construct was tested for correct Cre- or Flp-mediated DNA recombineering by either transforming the plasmid into arabinose treated EL350 bacteria, to activate Cre recombinase; or by transforming the plasmid into arabinose treated EL250 bacteria to activate Flp recombinase activity (figure 6.17). A *NotI/SalI*

restriction digest to excise the entire subcloned region of *TRAIIP* confirmed that both Cre- and Flp-mediated DNA recombineering were functional in the knock-out vector, as demonstrated by a shift in the size of 8624 bp X5-10 region to either 7257 bp after Cre-mediated DNA recombineering or 6778 bp after Flp-mediated DNA recombineering (figure 6.17 C).

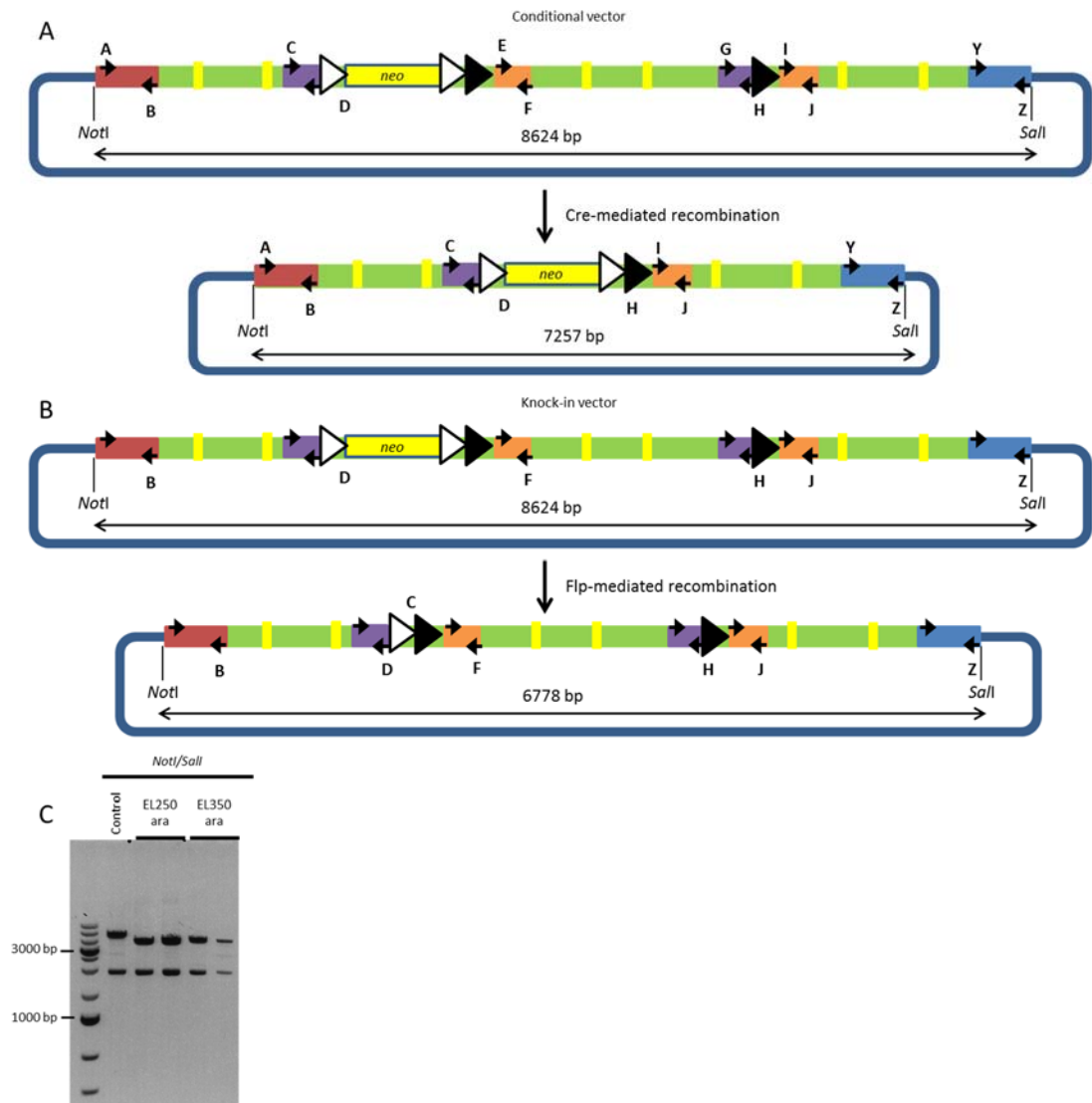


Figure 6.17 Confirmation of functional loxP and FRT sites. Arabinose activated EL250 or EL350 bacteria were transformed with the knock-out vector to check for successful Cre-mediated DNA recombination in EL350 (A) or Flp-mediated DNA recombination in EL250 (B). The correct Cre or Flp activity was verified by comparing *NotI/SalI* restriction fragments before or after treatment with arabinose (C).

6.2.4.8. ES cell targeting

Once the final targeting constructs had been checked DNA was prepared and linearised and gel extracted. DNA was quantified by spectrophotometry and then electroporated into E14 ES cells.

Following ES cell targeting, neomycin (G418) resistant clones were expanded and DNA was prepared for PCR screening. The ES cell clones were screened by long range PCR from the flanking genomic DNA into the floxed neo cassette. The left arm screen identified eight positive conditional clones and two positive knock-in clones verified by amplification of a PCR fragment of 3688 bp (figure 6.18 A). Six of the left arm positive conditional clones and both of the left arm positive knock-in clones were also positive for the right arm identified by amplification of a PCR fragment size of 5713 bp (figure 6.18 B). The positive conditional clones were additionally checked for retention of the lone loxP site and three were found to be positive with a loxP screening PCR amplifying both the WT fragment (154 bp) and a larger fragment containing the lone loxP site (252 bp) (figure 6.18 C), taking the total number of conditional ES cell clones to three.

A positive knock-in clone was transiently transfected in culture with a Flp recombinase expression construct to excise the neo cassette and further clones were picked to screen for successful recombination. A screening PCR across the *neo* cassette verified four clones where excision had been successful, demonstrated by a shift in fragment size from 2179 bp to 385 bp. One clone had no contaminating bands (red arrow) whilst the other three clones had contaminating larger bands the same size as an untreated control, which may suggest that they were mixed clones (figure 6.18 D). The exons from one positive knock-out and one positive knock-in clone were sequenced and the ES cells were karyotyped before blastocyst injection (figure 6.19)

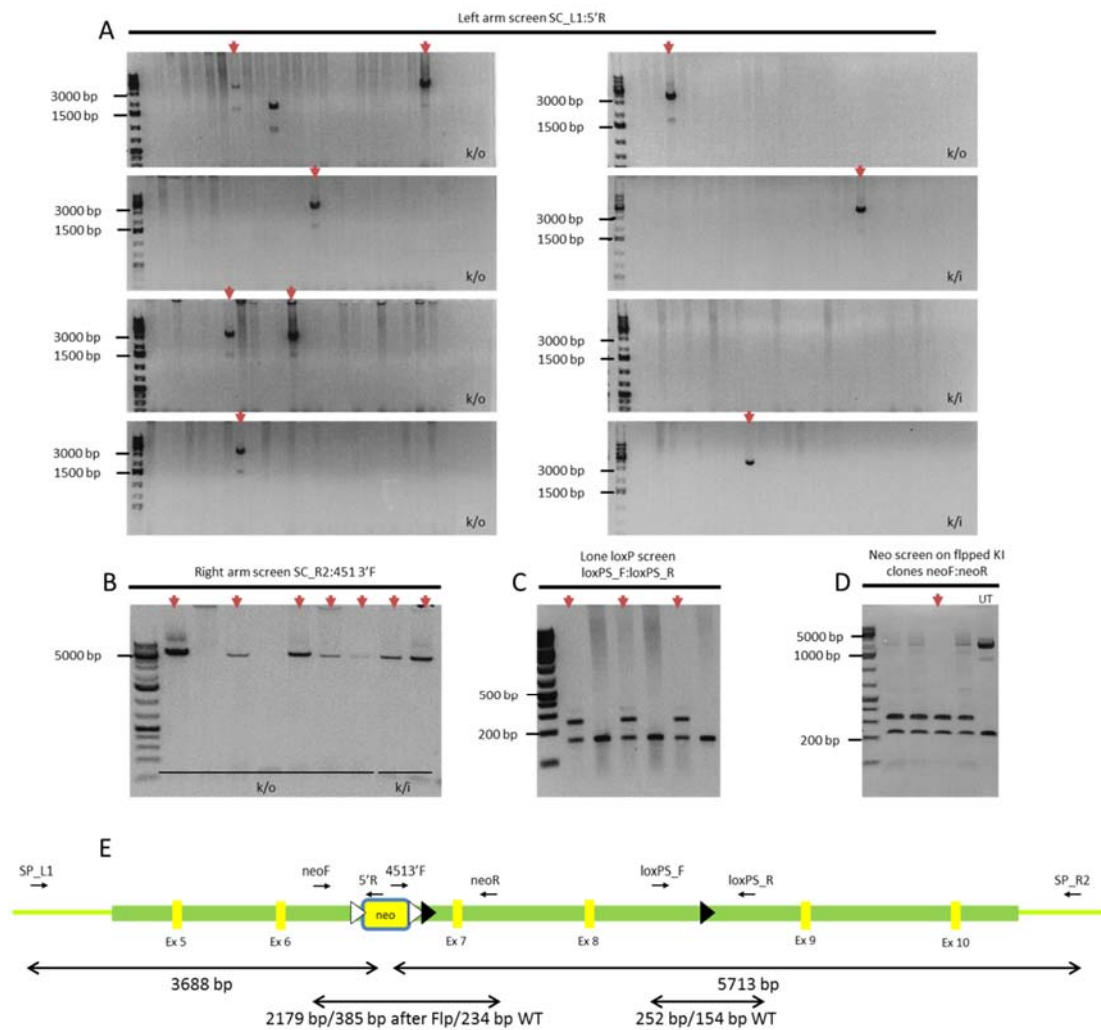


Figure 6.18 ES cell screening. G418 resistant ES cell clones were screened by long range PCR across the left arm (A) and positive clones were screened by long range PCR across the right arm (B). Positive clones were screened for retention of the lone loxP site (C). The neo cassette was excised from positive knock-in clones in culture via flp-mediated recombination and clones were screened across the neo site by PCR (D). The schematic shows the positions of the primers in relation to the exons and flanking genomic DNA, and the expected size of the PCR fragments.

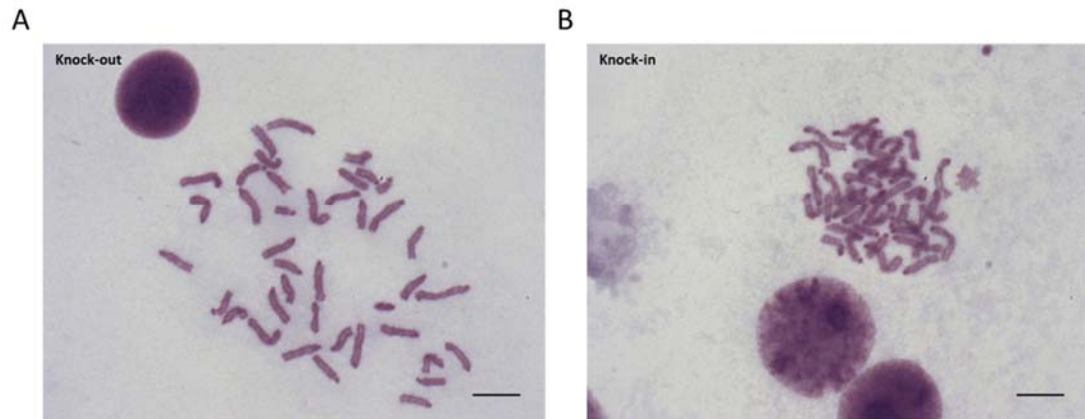


Figure 6.19 Karyotyping of positive knock-out (A) and knock-in (B) E14 clones showing correct chromosome count and no abnormalities. Images taken on a Zeiss Axioplan 2 upright microscope using a 63x lens. Scale bar = 10 μ m. Performed by Fiona Kilanowski.

The chimeric offspring from the injection of the knock-out ES cells were crossed to wild-type C57Bl6/N to test for germline transmission via transmission of agouti coat colour from the A129 ES cells. Chimera 4 successfully transmitted the targeted chromosome, and the agouti offspring were screened initially for amplification of the loxP site (figure 6.20 A). Transmission of the targeted chromosome was verified by long range PCR (figure 6.20 B and C). Eight out of twelve agouti pups were positive and were backcrossed to C57Bl6/N to create the F1 generation of conditional knock-outs.

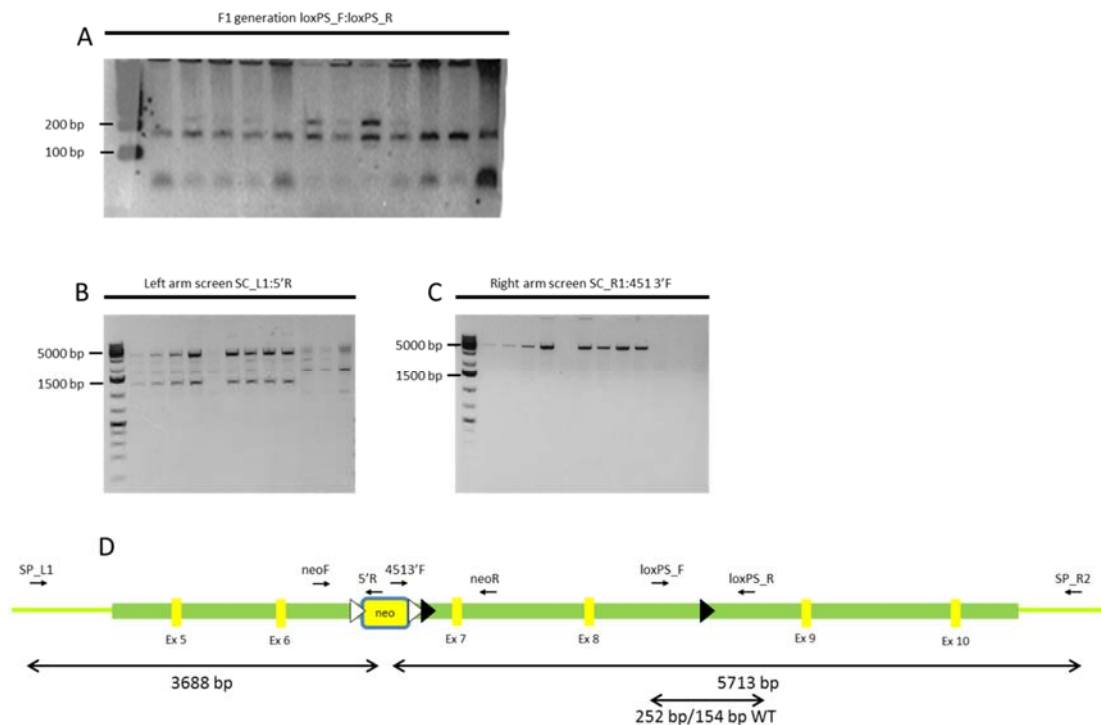


Figure 6.20 Genotyping the F1 generation. Using a loxP screen (A) and long range PCR across the left (B) and right arms (C). The schematic shows the approximate positions of the primers in relation to the exons and flanking genomic DNA, and the expected size of the PCR fragments.

6.2.5. Mouse phenotype

Ear clips were collected from each mouse to act as identification markers and for DNA preparation for genotyping.

6.2.5.1. Genotyping offspring

Offspring were genotyped initially using a screen across the lone loxP site (figure 6.21 A) amplifying either a single wild-type band of 154 bp or doublet of the wild-type band and a 252 bp band including a lone *loxP* site and a small amount of flanking DNA from pL452. Following crossing of TRAIP^{co(neo)/+} mice onto a flpe line to excise the FRT-flanked neo cassette, the offspring were also genotyped with a neo screen across the neo cassette (figure 6.21 B). A standard PCR extension time of 30

seconds did not amplify the intact neo cassette but amplified a 378 bp band following successful frt-mediated excision of the neo cassette.

6.2.5.2. Mouse breeding plan

The plan with the knock-out conditional line was to initially assess the phenotype of a ubiquitous knock-out by crossing homozygous conditional mice onto a CAG-cre line. The conditional construct could also be used to allow conditional knock-out of TRAIIP in immune cells to separate the function of TRAIIP as a potential regulator of proliferation from the role in the immune response. Figure 6.22 outlines the breeding plan.

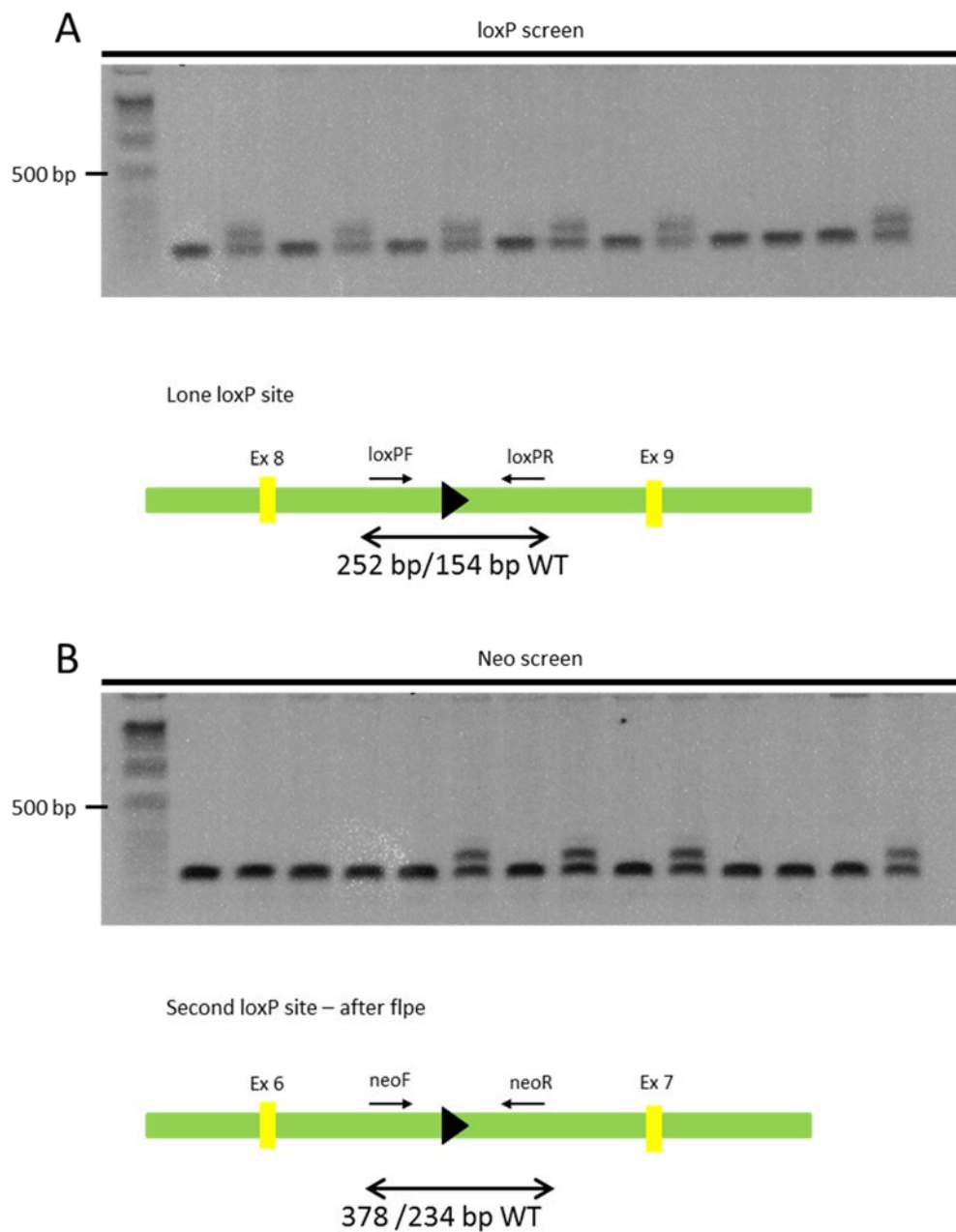


Figure 6.21 Genotyping offspring. DNA extracted from ear clips was genotyped to confirm the presence of the lone loxP site (A) or to show successful excision of the *neo* cassette following Flp-mediated DNA recombination (B).

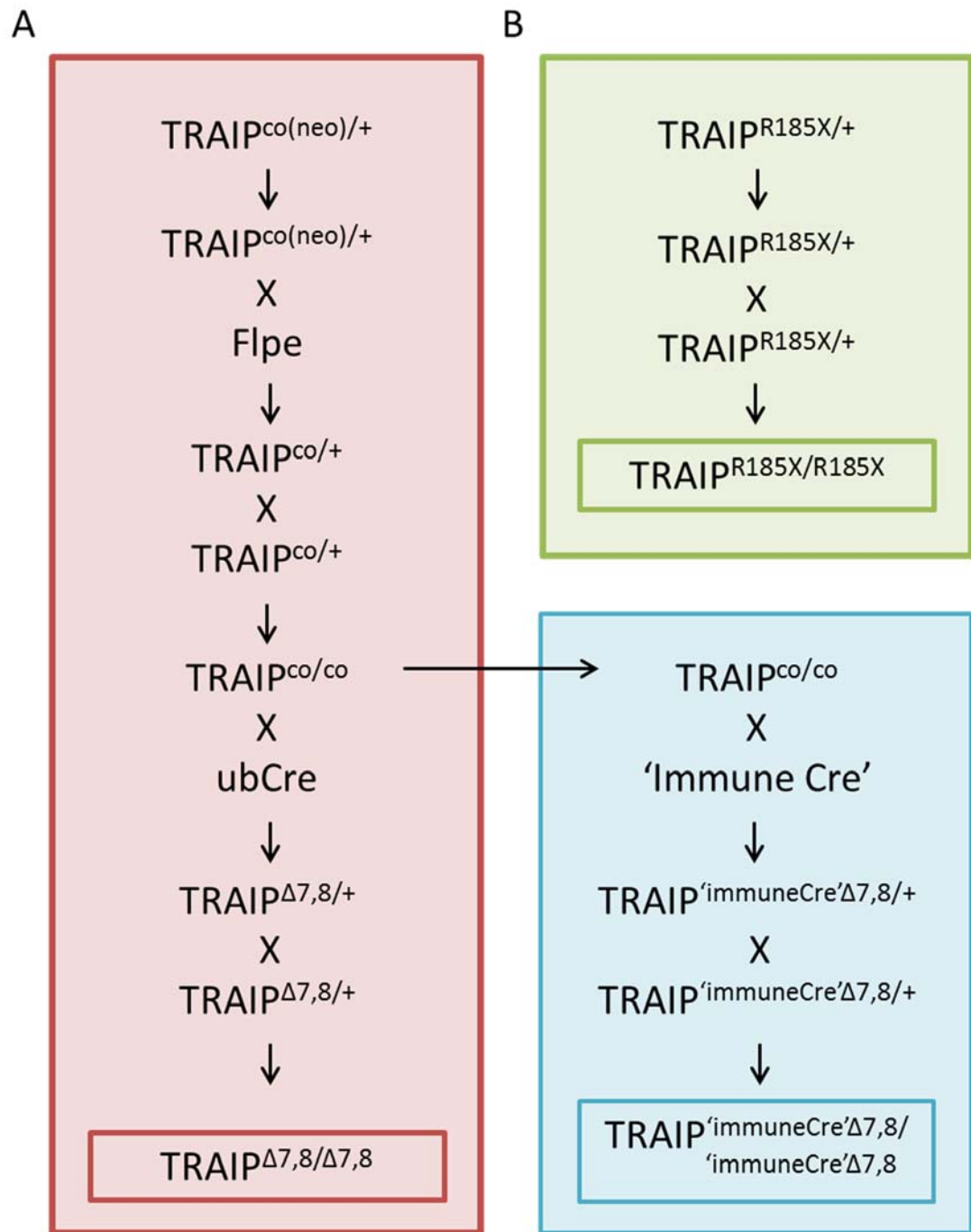


Figure 6.22 Breeding plan for TRAIP conditional knock-out and knock-in lines including nomenclature for the lines.

6.2.5.3. The *neo* cassette caused embryonic homozygous lethality in the conditional knock-out line

As mouse breeding progressed, no homozygous $\text{TRAIP}^{\text{co(neo)/co(neo)}}$ pups were genotyped from heterozygous matings, shown by using either a loxP PCR to screen for the lone loxP or a neo PCR to screen for the neo cassette. Out of a total of three litters from heterozygous parents, one would expect 25% of the pups born to be homozygous (table 6.3). Using a Chi-squared test to compare observed and expected frequencies across the litters showed that there is a significant difference between the observed and expected frequencies of homozygous pups born.

To determine the embryonic stage at which the $\text{TRAIP}^{\text{co(neo)/co(neo)}}$ offspring were lethal, plugs were set up to harvest embryos at E10.5. A total of three litters were collected at E10.5 and no homozygous embryos were identified. A Chi-squared test showed that the difference between the observed and expected frequencies was significant, demonstrating that the $\text{TRAIP}^{\text{co(neo)/co(neo)}}$ homozygous embryos are dying before E10.5 (table 6.4).

To exclude the possibility that the transcript from the neo cassette was causing lethality, the neo cassette was excised from the $\text{TRAIP}^{\text{co(neo)/+}}$ line by crossing heterozygous mice with flpe mice to activate Flp-mediated DNA recombination between the frt sites, thereby removing the neo cassette. The offspring were genotyped using both a lone loxP screen and a neo screen (figure 6.21) to identify successful Flp-mediated DNA recombination.

Further breeding of knock-in and conditional lines is on-going.

Table 6.3 Comparison of observed and expected ratios of genotypes of weaned (3 weeks or more) offspring from heterozygous (TRAIP^{co(neo)/+}) crosses.

genotype		expected	observed	P value (χ^2)
Wild-type	(TRAIP ^{+/+})	6	9	
Heterozygous	(TRAIP ^{co(neo)/+})	12	15	
Homozygous	(TRAIP ^{co(neo)/co(neo)})	6	0	* 0.0162

Table 6.4 Comparison of observed and expected ratios of genotypes of E10.5 embryos from heterozygous (TRAIP^{co(neo)/+}) crosses.

Genotype		Observed	Expected	P value (χ^2)
Wild-type	(TRAIP ^{+/+})	7	4.5	
Heterozygous	(TRAIP ^{co(neo)/+})	11	9	
Homozygous	(TRAIP ^{co(neo)/co(neo)})	0	4.5	*0.04

6.3. Discussion

6.3.1. Mutations in TRAIP cause MPD

Mutations in *TRAIP* were identified in several patients from cohort of MPD patients. These patients presented with significant growth retardation and microcephaly as well as immune deficiencies resulting in repeated hospital admissions related to respiratory bacterial and viral infections.

The identification of a shared ancestral haplotype between the two patients with c553C>T was not unexpected, even though the families are from different countries. Common ancestral haplotypes are often found associated with extremely rare, recessive autosomal mutations, and are even found in individuals from different continents (Martin et al., 2014).

6.3.2. c.553C>T causes alternative splicing

The nonsense mutation c.553C>T (p.Arg185Stop) in exon 7 of *TRAIP* results in a premature stop codon. Premature stop codons lead to expression of transcripts which are recognised and degraded via a process called nonsense mediated decay (NMD) (Chang et al., 2007). This process prevents translation of pathogenic protein isoforms, however approximately 10% of inherited diseases are caused by premature stop codons that lead to NMD (Bidou et al., 2012). c.553C>T in *TRAIP* was shown by Sanger sequencing to lack exon 7. Since exon 6 and exon 8 are in frame this isoform may be protein-coding, however western blotting of patient fibroblasts (Dr. Margaret Harley, IGMM) has not identified any alternative protein isoforms, meaning one of three things: either the protein is not translated from the Δ exon7 mRNA transcript, the antibody cannot recognise a Δ exon7 protein isoform, or protein levels are below the limit of detection. The phenotype of the patient could therefore be a severe hypomorph.

6.3.3. c52C>T may affect the function of the RING domain

The missense mutation c.52C>T (p.Arg18Cys) lies in the highly conserved RING domain of *TRAIP*. A RING domain mutant of *TRAIP* where the first cysteine was replaced with an alanine produced a non-functional protein which could no longer undergo ubiquitination (Besse et al., 2007). There are no precedents to predict how

an additional cysteine residue might affect the structure of the RING domain; however due to the level of conservation of RING domains, and the rigid secondary structure of RING domains providing a stable platform for E2 protein recognition, it is likely that the RING domain would be disrupted (Deshaies and Joazeiro, 2009). The additional cysteine residue is in one of the longer connecting loops between the two zinc co-ordinating segments (figure 6.23). An additional cysteine in this region is likely to disrupt the structure of the RING domain, and potentially the structure of the entire protein. Since this mutation is not lethal, whilst a complete lack of TRAIP is known to be lethal in mice, it is likely that the RING mutant retains some activity, which could in future be assayed via *in vitro* ubiquitination assays. The zinc binding affinity of mutated TRAIP could be investigated using electrospray ionisation-time of flight (ESI-TOF) mass spectrometry which allows observation of non-covalent and ionic interactions (Loo, 1997). This technique has been used to investigate different zinc-binding affinities of BRCA1 RING mutants (Brzovic et al., 2001), however it requires recombinant protein.

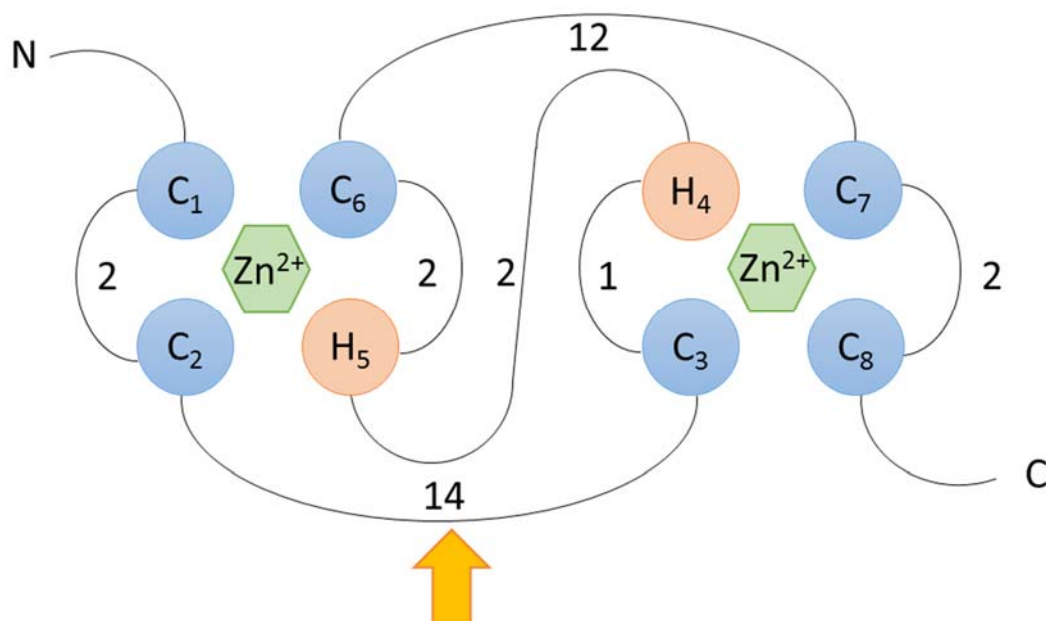


Figure 6.23 Schematic diagram of the structure of the TRAIP RING finger motif. The RING finger motif is highly conserved with cysteine and histidine residues co-ordinating two atoms of zinc. The arrow shows the position of the

p.Arg18Cys mutation in TRAIP identified in patient 3 in one of the extended loops between the two zinc co-ordinating groups.

The observation that a mutation in the RING domain of TRAIP cause a strikingly similar phenotype to introduction of a premature stop codon, suggests that decreased enzyme function is responsible for the phenotype. This supports a scenario whereby c.553C>T causes NMD of the Δ exon7 transcript resulting in a hypomorph, whilst c.52C>T results in decreased functionality of TRAIP. Essentially, both mutations are predicted to cause loss of function of TRAIP.

6.3.4. The role of TRAIP in the immune response

A consistent clinical feature of the patients with TRAIP mutations is a susceptibility to respiratory infections, however no consistent pathogen has been identified as the cause of these episodes. Patient notes contained no evidence of defects in blood cell counts as observed in MPD patients with *LIG4* mutations (Murray et al., 2014), indicating that the respiratory phenotype in TRAIP patients is not caused by leukopenia. Standard clinical tests would not identify defects in the innate immune system as they primarily assess adaptive immunity. It is unclear, in the case of TRAIP, whether patients are more susceptible to infections due to immune deficiencies or as a result of TRAIP's role in the negative regulation of innate immunity leading to an inappropriate inflammatory response to infection (Lee et al., 1997; Zhang et al., 2012).

The immune response of patient fibroblasts was assayed by stimulation with various ligands and subsequent cytokine measurement to identify signs of an altered immune response. The assays did not highlight any convincing changes in the immune response of the patient fibroblasts compared with wild-type fibroblasts, however assaying what may potentially have been the most interesting cytokine, IFN- β , due to recent evidence indicating a role for TRAIP in downregulating TBK1-dependent IFN- β production, was unsuccessful due to an inefficient ELISA protocol (Zhang et al., 2012). Although reading the end point of an immune response, the cytokine production, is often the most informative method, the immune response could be

measured using qPCR instead. Type I IFN expression is often quite transient following stimulation (personal communication Karen Makenzie) however the expression of interferon stimulated genes (ISGs) such as OAS1, IFIT1 and STAT1 can provide an accurate picture of the interferon response (Rice et al., 2013). It would be interesting to see whether the patient fibroblasts are constitutively expressing IFN- β which may indicate dysregulation of innate immune pathways and if they have an ISG signature which may indicate chronic inflammation.

Expression of the proinflammatory cytokines IL-6 and CXCL8 was also assayed, and shown to be significantly different in patient compared to control fibroblasts in response to LPS. Whilst production of IL-6 in response to LPS was significantly increased in patient fibroblasts, production of CXCL8 was significantly decreased. The production of IL-6 by dermal fibroblasts is predominantly NF- κ B dependent (Perfetto et al., 2003). Therefore, the increased response of patient cells to LPS supports evidence that TRAIP is involved in down-regulating NF- κ B activation via interactions with TRAF proteins (Lee et al., 1997).

Production of CXCL8 is also partially NF- κ B dependent, however activation of AP-1 and C/EBP are required for maximal CXCL8 production (Hoffmann et al., 2002b) raising the possibility that TRAIP may affect the activation of transcription factors other than NF- κ B. Production of CXCL8 was also significantly decreased in patient fibroblasts in response to TNF- α which is one of the most potent stimulators of CXCL8. These results together suggest that TRAIP positively regulates LPS and TNF- α induced CXCL8 production in dermal fibroblasts. Decreased production of the chemokine CXCL8, the primary role of which is chemoattraction of neutrophils, indicates that the patients may have defects in clearance of infections, similarly to individuals with neutropenia, though this would not affect cell counts (Newburger and Dale, 2013). In support of this, mice deficient in CXCR2, one of the receptors for CXCL8 and CXCR2 are susceptible to both mucosal and systemic infection by *C. albicans* and show reduced neutrophil infiltration into infected tissues (Balish et al., 1999). These mice are also more susceptible to chronic *L. monocytogenes* infection (Czuprynski et al., 1998) demonstrating the importance of this chemokine in preventing and clearing infections.

It is important to note that there was a large amount of variability between the two control fibroblast cell lines, suggesting that caution should be employed in drawing conclusions from these experiments. The variability in cell lines likely reflects natural variation due to different genetic backgrounds of the donors. Although fibroblasts are not the most relevant cell type for studying the immune response, alternative methods of studying the patient immune response would have required direct patient access to isolate more relevant immune cells from the peripheral blood such as monocytes, DCs or macrophages. Alternatively, siRNA oligos could be designed to deplete *TRAIP* mRNA in cell types such as macrophages; however it is difficult to transfect siRNA into immune cells and experiments have to be well controlled since RNA itself is an immune ligand (Sioud, 2010). By bypassing cytoplasmic immune recognition, stable knock-down of expression using short hairpin RNA (shRNA) introduced by viral vectors into the nucleus was effective in a human monocytic cell line (Lee and Reiner, 2009; Lee et al., 2004). Human monocyte-derived dendritic cells and mouse RAW 264.7 cells have been successfully transfected with short hairpin RNAs (Berger et al., 2011; Kim et al., 2011). These RNA interference techniques may be difficult to optimise to accurately recapitulate the patient genotype, but it would still be interesting to test the effect of TRAIP knock-down on the human immune response. To more accurately recapitulate the human mutations, genome editing using a technique such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) could also be utilised. There is no precedence for this technique being used in immune cell types, however it has been successfully used to introduce homozygous point mutation in mouse ES cells (Wang et al., 2013b). Mouse ES cell-derived embryoid bodies can be induced to differentiate into macrophages using conditioned media (Zhuang et al., 2012), therefore this technique would introduce mutations whilst avoiding having to perform genetic manipulations in immune cells.

These preliminary results studying the innate immune response in patient fibroblasts do not yet provide robust evidence of altered immune response in patient fibroblasts, however alternative techniques may establish whether TRAIP deficiency causes an immune phenotype.

6.3.5. Design and generation of mouse lines to recapitulate the human phenotype

The variable immune response in patient cells lead to the design and generation of two mouse lines with aim of creating isogenic systems for studying the role of TRAIP both in a whole organism context and in isolated immune cells. A mouse model also permits direct assessment of the response to infection.

TRAIP null embryos are lethal by E7.5 demonstrating that TRAIP is essential for development (Park et al., 2007), however, whilst the patient mutation introducing a premature stop codon in exon 7 substantially affects normal development, it does not cause lethality. With this in mind, two mouse lines were designed and generated to recapitulate the patient phenotype.

Mouse models of disorders of growth and immune deficiency have previously been described and accurately recapitulate the human phenotype (see 6.1.10.3). Following confirmation that the mutated codon in *TRAIP* is conserved from human to mouse, a knock-in construct was designed using site-directed mutagenesis. In parallel, a conditional knock-out construct was designed which introduced *loxP* sites flanking exons 7 and 8 allowing knock-out of these exons under the control of Cre recombinase. This knock-out construct was designed to allow conditional knock-out of TRAIP, with the aim of separating the function of TRAIP in the immune system compared to in cell replication. Since the published knock-out from exon 2 is lethal (Park et al., 2007), the conditional knock-out here was designed to knock-out exons 7 and 8 with the possibility that this may not be lethal as a constitutive knock-out, providing more scope for future studies.

No TRAIP^{co(neo)/co(neo)} offspring were born from heterozygous conditional matings. Plugs collected from heterozygous matings at E10.5 revealed that TRAIP^{co(neo)/co(neo)} embryo were dying before this age. Since there was no alteration to gene at this point other than the insertion of a FRT-flanked neo cassette in intron 6, it is likely that transcription from the neo cassette interferes with *TRAIP* expression resulting in a lethal phenotype as seen in a *TRAIP* null mouse (Park et al., 2007). Neo cassettes are driven by the phosphoglycerate kinase I (*PGKI*) promoter which is ubiquitously

expressed and has been shown to disrupt surrounding genes and exert long-range effects on genes up to 100 kb away (Pham et al., 1996). Considering that a difference in the expression of the full length *TRAIP* isoform in the heterozygous parents of patient 2 is detectable (figure 5), it would be interesting to identify whether the expression of *TRAIP* in the *TRAIP*^{co(neo)/+} is altered. Alternatively, homozygous lethality may be caused by insertion of the neo cassette into a regulatory sequence in intron six, however this region is not highly conserved, particularly not at the specific insertion site, between human and mouse making it unlikely that any regulatory domains have been disrupted.

To remove any effect that the neo cassette was having, the viable heterozygous mice were crossed onto a FLPe line. This line expresses FLPe under the control of a ubiquitous promoter thereby removing the neo cassette by FLP-mediated recombination between the FRT sites flanking the neo cassette. Whether the removal of the neo cassette restores homozygous viability is yet to be shown.

The neo cassette was removed during the culture period of the knock-in ES cells prior to ES cell injection. This does not make any difference to the overall time scale but could avoid the unnecessary investigation into a lethal phenotype that may be caused by the neo cassette rather than by targeted gene disruption.

The next steps for these mouse lines will be to establish whether there is a phenotype; the knock-in will be backcrossed and bred to homozygous, whilst the knock-out will be crossed onto a ubiquitous Cre line to remove exons seven and eight (figure 20).

6.3.5.1. Phenotyping the mouse models

Taking inspiration from other studies of growth phenotypes and/or microcephaly, a growth phenotype can be measured in a number of ways. Gross phenotype, changes in body size and skeletal abnormalities could be measured and compared to an established Seckel syndrome mouse model, a *Cenpj* hypomorph (McIntyre et al., 2012). Detailed investigation of brain size and development can be performed to investigate changes caused by *TRAIP*, in a similar way to the assays performed on

McpH1 deficient mice, for example assaying brain weight and cortical thickness (Gruber et al., 2011).

Initial investigations into the immune phenotype would involve full immune cell profiling of thymocytes and splenocytes by flow cytometry analysis as described for the *Lig4* R278H homozygous mutant mouse (Rucci et al., 2010). Since the patients do not have defects in cell counts, immune cell repertoires may also be unaffected in the mouse models. Therefore, secondary investigations should be carried out to assay whether there is any evidence of sterile inflammation indicating a basal defect in the immune response, and how the mice respond to bacterial and viral respiratory infections. Particular pathogens can be administered to induce either local respiratory infections which would mimic the most common infections seen in the patients, e.g. mouse respiratory syncytial virus (Graham et al., 1988); or systemic infections to assay the response of the entire immune system, e.g. *Pseudomonas aeruginosa* (Roy-Burman et al., 2001). For both localised and systemic infections the immune response can be measured via flow cytometry, ELISA or qPCR of a range of relevant cytokines

The conditional mouse line was generated with the aim of separating the function of TRAIP in the immune system from a growth phenotype, for example crossing to a Cre line under the control of *Csf1r* which is expressed in macrophages, or a Cre line under the control of the *CD11c* promoter which is expressed in dendritic cells (Jackson laboratory).

6.3.6. Concluding remarks

The work presented here provides a basis for future investigation of a role for TRAIP in the immune response. The effect of TRAIP on cellular proliferation and growth may well be due to a role for TRAIP in the DNA damage response and published evidence suggests that TRAIP positively regulates cellular proliferation, particularly of undifferentiated cells (Almeida et al., 2011).

Mouse models have been developed, to establish the developmental basis for how phenotypes arise from TRAIP mutations in an isogenic and relevant system. A conditional mouse model and a knock-in mouse model were designed and

successfully generated via ES cell targeting however the result of the gene targeting has yet to be determined.

These mouse models will also importantly further the understanding of the role of TRAIP in the immune response and may aid in understanding the patient phenotype. Understanding more about how TRAIP affects the immune response may also aid in understanding whether other pathogeneses may arise, leading to improved clinical management of patients.

7. Final discussion

7.1. Summary of major findings and future work

The broad objective of my thesis has been to gain increased understanding of the regulation of different aspects of the innate immune system, with the two sections of this thesis having distinct aims.

7.1.1. HBD3 and the innate immune response to DNA

The major focus of this thesis has been to investigate how hBD3 affects the innate immune response to DNA. We hypothesised that hBD3 would affect the innate immune response to DNA given that hBD3 is known to modulate other pathways in the innate immune response (Semple et al., 2011; Semple et al., 2010) and that another similar AMP, LL37, has been shown to increase uptake of self-DNA and induce a TLR9-dependent immune response in human pDCs (Lande et al., 2007). A role for hBD3 in regulating the innate immune response to DNA may have implications in disease, particularly given that *DEFB103*, encoding hBD3, is located in a copy number variable (CNV) region, increased copy number of which has been linked to increased risk of psoriasis (Hollox et al., 2008b).

Cytokine assays in mouse dendritic cells demonstrated that exposure to *E. coli* DNA and hBD3 caused an amplified inflammatory response compared with *E. coli* DNA alone. Flow cytometry demonstrated that hBD3 increases cellular uptake of DNA and fluorescence microscopy revealed that DNA aggregates formed in the presence of hBD3. Surprisingly, hBD3 also increased uptake of ‘self’ DNA but did not elicit an immune response to self-DNA. These findings together suggest that whilst hBD3 increases uptake of DNA in a non-specific manner, subsequent immune signalling is dependent on specific pathogen-associated molecular patterns. The increased response to *E. coli* DNA in the presence of hBD3, like the response to *E. coli* DNA alone, is TLR9-dependent, supporting a mechanism whereby hBD3 increases cellular uptake of *E. coli* DNA but does not affect subcellular localisation. Whether this is indeed the case, could be addressed in future experiments such as endosomal inhibition studies via various methods designed to inhibit endosomal functionality, or

by using labelled reagents to investigate binding of DNA to TLR9 with or without hBD3.

The finding that hBD3 does not induce an immune response to self-DNA, even though it increases uptake of self-DNA, suggests that hBD3 does not enhance the immunogenicity of DNA. Specific molecular patterns in the bacterial DNA, such as unmethylated CpG motifs, are still required to initiate an immune response, in contrast to immunogenic effect that LL37 has on self-DNA (Lande et al., 2007). It would be interesting to observe whether hBD3 remains associated with the DNA aggregates and whether there is any structural definition to the aggregates. DNA containing complexes have been isolated from SLE patient sera and these complexes also activate pDCs through TLR9 (Means et al., 2005) and a various putative therapies for SLE focus on reducing antibody binding to double-stranded DNA, for example topoisomerase I inhibition by campothecin reduced anti-dsDNA antibody binding (Frese-Schaper et al., 2014). If hBD3 association is necessary for DNA aggregation then hBD3 could possibly be targeted to prevent this process.

By determining whether hBD3 forms structured complexes with DNA might provide insights as to how the association might be inhibited to modulate the immune response. Super-resolution microscopy techniques such as STORM (stochastic optical reconstruction microscopy) could be employed to look at the structure and cellular localisation of the DNA aggregates (Sengupta et al., 2012). This technique allows increased resolution which is useful for looking at associated distances and also at subcellular localisation in cells such as DCs with minimal cytoplasm.

Preliminary *in vivo* experiments using an hBD3 overexpressing transgenic mouse did not produce conclusive results due to lack of a baseline response to *E. coli* DNA in wild-type mice. This was likely due to not enough DNA being injected, however time constraints did not permit further optimisation of DNA concentration. In the future experiments to establish a baseline response to *E.coli* DNA in wild-type mice should be performed, followed by an investigation into whether hBD3 overexpression affects the response.

Future experiments should include, importantly, establishing that the observed effect is not species-specific, since there are precedents for mice not recapitulating human immune responses, particularly regarding complex disease such as psoriasis and Crohn's disease and SLE (Denic et al., 2011; Gudjonsson et al., 2007; Peng, 2012; Pizarro et al., 2003), but also single gene disorders such as Familial Mediterranean Fever (FMF) (Hesker et al., 2012). Indeed, many small molecules that are effective in mice fail in human clinical trials (Perrin, 2014). Human dendritic cells can either be purified directly from peripheral blood by adhesion or sorting, or human monocytes can be stimulated with GM-CSF and IL-4 to differentiate into cDCs (Delles et al., 2002; Tedder and Jansen, 2001). Additionally, it would be interesting to test the response to DNA isolated from a range of other pathogens including other bacterial species, particular viruses and yeast, which are known to have different proportions of unmethylated CpG motifs in their genomic DNA (Dalpke et al., 2006; Krieg et al., 1998).

Finally, it is essential to establish whether this interaction between hBD3 and bacterial DNA is relevant to human disease. This work hoped to identify whether an aberrant response to DNA in the presence of increased hBD3 may be a causative factor in the development of psoriasis, providing a mechanism whereby increased *DEFB103* copy number is linked to increased risk of psoriasis. Bacterial and viral infections are a known trigger for psoriasis, therefore the work here provides a mechanism for how increased hBD3 may cause an excessive immune response to a stimulus that would normally be resolved efficiently and with minimal tissue damage. Figure 7.1 proposes a general mechanism, whereby the excessive immune response caused in the presence of increased hBD3 may lead to a self-perpetuating cycle of immune activation via activation of T_H1 and T_H17 cells and subsequent secretion of IL-17, IL-22 and IFN- γ , which are known to be involved in the pathogenesis of psoriasis (Nogales et al., 2008).

To establish whether these results are relevant to psoriasis pathogenesis, a number of experiments could be pursued using patient samples. Blood and skin samples from psoriasis patients with increased copy number of *DEFB103* could be tested to identify whether hBD3-DNA complexes are detectable, particularly at active lesions.

This would require the design of an assay to detect both DNA and hBD3 and to determine whether they are complexed. Additionally, these samples could be tested to ascertain whether increased copy number actually correlates with increased protein. This has been previously shown for hBD2 (*DEFB4*) in the same CNV region, however it should be confirmed for hBD3 (Jansen et al., 2009). Additionally, DC-T cell co-cultures could be used to establish whether DCs activated by hBD3 and bacterial DNA can activate naïve T cells and whether differentiation into a specific T cell subset is favoured (Ge et al., 2002). If increased expression of hBD3 was shown to increase the risk of psoriasis then patients could be tested for copy number, before therapeutic options were considered. Patients with increased copy number of hBD3 could potentially be treated with topical application of neutralising antibodies against hBD3, in a carrier to permeate the epidermal barrier.

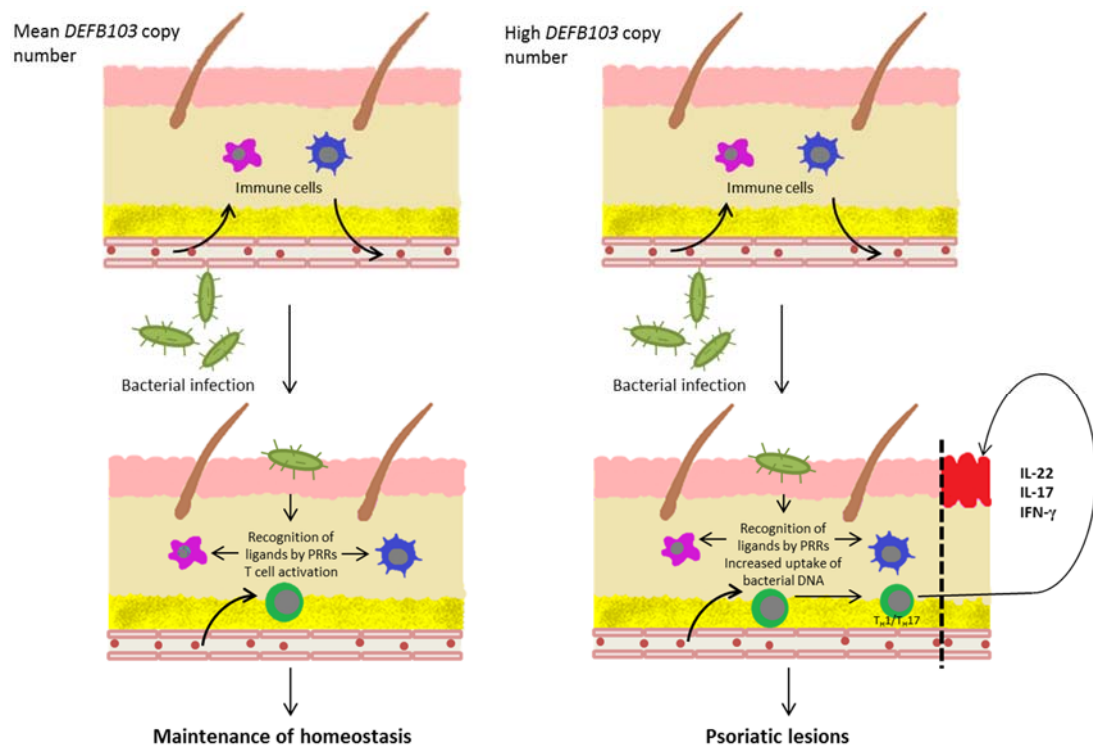


Figure 7.1 Model for how increased copy number of *DEFB103* might lead to increased risk of psoriasis via an excessive immune response to bacterial DNA released during a skin infection in the presence of increased hBD3. In individuals with a lower copy number of *DEFB103*, skin bacterial infections would be recognised by PRRs on immune cells initiating a signalling cascade leading to secretion of immune mediators, clearance of the infection and a return to tissue homeostasis. In individuals with a higher copy number of *DEFB103* we propose that bacterial DNA, a natural by-product of bacterial infections, in combination with increased hBD3 leads to an excessive immune response characterised by pDCs secreting large amounts of IFN- α and activation of DCs. This in turn may lead to activation and differentiation of naïve T cells into T_H1 and T_H17 cells which secrete IL-17, IL-22 and IFN- γ . This T_H1/T_H17 axis is known to be involved in the pathogenesis of psoriasis perpetuating an inflammatory state and affecting differentiation, function and integrity of the epidermis (Nogales et al., 2008).

7.1.2. TRAIP – a bifunctional protein?

MPD patients with mutations in *TRAIP* appear to have increased susceptibility to respiratory infections and are regularly hospitalised for treatment. Ongoing work performed in parallel with this project has identified a defect in cellular proliferation which appears to be due to defects in DNA repair pathways leading to MPD (Dr. Margaret Harley, IGMM, personal communication). Immunodeficiency characterised by decreased numbers of lymphocytes, caused by defective V(D)J recombination and NHEJ, has previously been observed in MPD patients with mutations in DNA repair pathways, for example patients with *LIG4* mutations (Murray et al., 2014; O'Driscoll et al., 2001). The lack of lymphopenia in TRAIP patients suggests that TRAIP is not involved in the DNA repair that takes place during development and maturation of the adaptive immune system. Rather, it is likely that the immune defects observed in TRAIP patients are due to TRAIP functioning in both DNA repair pathways and in the innate immune system which is consistent with E3 ligases having multiple substrates. This is further supported by published data regarding the role of TRAIP in innate immune pathways (Lee et al., 1997; Zhang et al., 2012). Patient-derived fibroblasts, which were the only patient cells available, treated with various immune stimuli did not demonstrate clear differences in cytokine production compared with wild-type fibroblasts. This may be due to significant variability in innate immune responses from different donors, with different genetic backgrounds. Alternatively, fibroblasts may well not be the ideal system to study the immune response, more suitable and widely used cells to study innate immune responses include macrophages, dendritic cells and monocytes. The lack of a suitable model to study TRAIP led to the design and generation of two mouse models to enable investigation of the developmental role of TRAIP as well as its role in innate immune regulation. Mouse models were successfully generated and should provide useful models in the future for studying the *in vivo* functions of TRAIP. The mouse models will provide an isogenic system for studying the innate immune response without the variability observed between human donors. Additionally, appropriate immune cells can be isolated e.g. DCs and macrophages, to study immune responses in a relevant system.

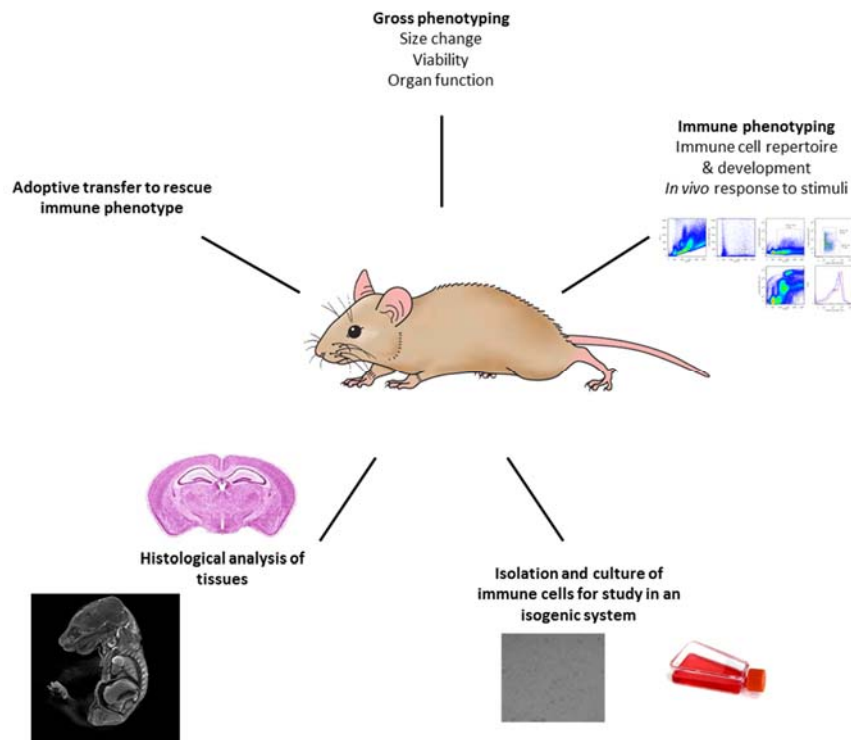


Figure 7.2 How can a transgenic mouse model of TRAIP be utilised? Mouse models are extremely useful for investigations that would otherwise be impossible with small patient samples and patient cells. Mouse models can provide a wealth of information that cannot be obtained without extremely intrusive techniques being employed, particularly with regards to studying the immune system which is notoriously variable in a genetically diverse system such as primary cell culture.

7.2. How does understanding innate immune regulation lead to therapeutic results

Studying immune pathways and regulatory mechanism using human diseases and animal models leads to novel insights into how these pathways may be targeted for therapeutic intervention. Innate immune responses can be inhibited to prevent excessive inflammation, such as that seen in diseases such as rheumatoid arthritis. TNF inhibitors are now widely used for the treatment of rheumatoid arthritis following the development of a transgenic mouse overexpressing human TNF which developed arthritis (Keffer et al., 1991). Likewise, Neonatal onset multisystem

inflammatory disease (NOMID, also known as chronic infantile neurologic cutaneous and articular (CINCA) syndrome), caused by defects in the regulation of inflammation and overproduction of IL-1 β , is treated using an IL-1 inhibitor, anakinra, or anti-IL-1 β antibody, canakinumab (Insalaco et al., 2014; Kanariou et al., 2014). In contrast the innate immune response can be boosted rather than suppressed in order to provide protection against infectious disease. A peptide has been identified which increases cytokine production by monocytes providing protection in mouse models of infection with gram-negative and gram-positive bacteria (Scott et al., 2007). Understanding how components of the innate immune system work can also lead to the development of novel therapeutics to replicate natural responses. Seven synthetic antimicrobial peptides and derivatives have so far progressed into clinical trials as antimicrobial treatments, however none have been successfully approved by the FDA, mainly because they do not prove to be more effective than classical antibiotics (Jenssen et al., 2006). However, AMPs may provide additional benefits to classical antibiotics since bacterial resistance does not develop against AMPs (Ge et al., 1999).

7.3. Concluding statement

Exploring the role of specific components in innate immune pathways, and understanding the mechanisms by which these complex pathways are regulated leads to increased understanding of disease pathogenesis and may pinpoint specific therapeutic targets.

Appendix 1. Oligonucleotides

DNA oligo	Sequence (5'→3')	Notes
TRAIP A not	GATCAT - GCGGCCGC - TGTATTTCCCAGAATCTTGC	Homology arm primers
TRAIP B' cla	GATCAT - ATCGAT - TCTGACACCCAGCAGCTCCT	
TRAIP Y cla	GATCAT - ATCGAT - TCAGGCTGGGGATAGAGAAG	
TRAIP Z' sal	GATCAT - GTCGAC - AGATACTGGTGAAGACAGGG	
TRAIP C sal	GATCAT - GTCGAC - CAGGATGAGACCAACAAGC	
TRAIP D' ecor1	GATCAT - GAATTC - CACACAGAGAGGCTCCTGGA	
TRAIP E bam	GATCAT - GGATCC - AAGTCCCTGACCTTGAAGTC	
TRAIP F' not	GATCAT - GCGGCCGC - GCACGCCCGAGCTTACGCAC	
TRAIP G sal	GATCAT - GTCGAC - TTCTCATGGCTGCTTTGCAG	
TRAIP H' ecor1	GATCAT - GAATTC - CTACCATACTGGAGGTTTGT	
TRAIP I bam	GATCAT - GGATCC - CGCCAGCAGCCCACTTTATA	
TRAIP J' not	GATCAT - GCGGCCGC - CACAGGCCAGGGTGTCTCAC	
TRAIP_451flanking_F	GGCCATGGTTCTGAGACACT	
TRAIP_451flanking_R	CCATGCAGAAAAGTGGAGGT	
TRAIP_452flanking_F	AGGGTAGGGCTGGACTGTTT	
TRAIP_452flanking_R	GAAGATTCACGGTGCCCTTA	
TRAIP_BS_F	TGCAGAATGAACTGGACAGC	
TRAIP_BS_R	TCAGACTGGCCTCATCAGTG	
TRAIP_c56t_F	GGTGGAGGAGATGATTTGAGACATGGGTGTGGG	Site directed mutagenesis
TRAIP_c56t_R	CCCACACCCATGTCTCAAATCATCTCCTCCACC	
SP_L1	CCTTTGTTTGCCTCTTGCTC	Long range PCR primers
SP_L2	GGGAATTGAATTTGGGACCT	
SP_R1	CCAGACCCTTCCACGAGATA	
SP_R2	GAGGCAAGGCACTCAAACCTC	
R185XS_F	CCGGGAATGTTTTTCAGAAT	
R185Xs_R	ACAAAAGATGATGGCAGAGG	

TRAIPex5seq	TACCCAGACAGTTCCAGCAG	
TRAIPex5R	ATGTTGGGGAGGCCTTGTTA	
TRAIPex6F	TCTCTGATGCATCCCTGGTC	
TRAIPex6	TCTCCTGCTCTATCAGCATC	
TRAIPex7	CTCATCTCTGCTGTAGAACC	
TRAIPex8F	TAGCCCTGTTTCTGACCCTG	
TRAIPex8	CACCTCATTACACATGCAGG	
TRAIPex9R	GGCATACTCGCTCTGGAGAT	
TRAIPex9	CCATATTCATGCTTAGTTGATG	
TRAIPex10F	GGTAGGGCTGGACTGTTTCT	
TRAIPex10seq	GGTCCCTAATCTCCAGAGCG	
TRAIPex10R	TTCAGAGATGGGGCAGACAG	
KI_SC_R	GCTGCATGAGCTCCTCAGAG	
4523'R	ATTGTAATACGACTCACTATA	
4523'F	ATCGCATTGTCTGAGTAGGTG	
4513'F	GCTCGACTAGAGCTTGCGG	
5'R	CGTCGAAATTCTACCGGG	
TRAIPatgF:	CCTTGAGTCCAGCCATCATGCCTATCC	RT-PCR primers
TRAIP001R:	GGCCATTGGTCAGACTCACTGTTC	
GAPDHF	GGACTCCACGACGTACTCAGCGCCAGC	
GAPDHR	GTGGATATTGTTGCCATCAATGACC	
TRAIPneoR	GTGTGCCCAAGGAAAGGTTC	Genotyping primers
TRAIPneoF	CCTTTGGCCACAGATTTCTT	
loxPS_F	GCCACCTTTTGCTCTTCTTG	
loxPS_R	TACACAGTGCGGCCTACAGA	

RNA oligos	sequence
FL.524.m	AACAGAUGAAGUCCUGGA
TRAIP.Z.1.m	GCGGCAGGAUGAGACCAA
TRAIP.246.m	CCTGCCCACAGTGTAGAAT
TRAIP.1171.m	CCTCAAGAAGGTGCACAAA

Appendix 2. ELISA antibodies

Concentrations are given as a guide; each new batch was individually optimised

ELISA	Capture antibody	Block	Standard	Detection Antibody	Conjugated secondary antibody
Mouse IFN-α	Anti-Mouse IFN- α , Clone RMMA-1 (MAb) (PBL InterferonSource) Used at 5 $\mu\text{g/ml}$ diluted in PBS	5% BSA (w/v) in PBS	Mouse IFN- α A (PBL InterferonSource) Diluted in 1% (w/v) BSA to a high standard of 1000 U/ml	Anti-Mouse IFN- α , Rabbit Serum (PAb) (PBL InterferonSource) Used at approx. 1.56 $\mu\text{g/ml}$	HRP- conjugated donkey anti-rabbit (Jackson ImmunoResearch) Used at 80 ng/ml
Mouse IFN-β	Anti-Mouse IFN- β , Clone RMMB-1 (MAb) (PBL InterferonSource) Used at 0.8 $\mu\text{g/ml}$	1% BSA (w/v) in PBS	Mouse IFN- β , mammalian (PBL InterferonSource) Diluted in 1% (w/v) BSA to a high standard of 1000 U/ml	Anti-Mouse IFN- β , Rabbit IgG, Protein A purified (PAb) (PBL InterferonSource) Used at 4.2 $\mu\text{g/ml}$	HRP- conjugated donkey anti-rabbit (Jackson ImmunoResearch) Used at 80 ng/ml
Human IFN-β	Human IFN- β , clone MMHB-1 (MAb) (PBL InterferonSource). Used at 0.5 $\mu\text{g/ml}$	1% BSA in PBS	Human IFN- β 1a, mammalian (PBL InterferonSource) Diluted in 1% BSA to a high standard of 2000 U/ml	Anti-human IFN- β , Goat IgG, Affinity purified (PAb) (PBL InterferonSource) Used at 10 $\mu\text{g/ml}$	HRP-conjugated donkey anti-goat (Santa Cruz Biotech) Used at 0.4 $\mu\text{g/ml}$

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