

Bruton's tyrosine kinase control of cytokine production in Toll-like receptor signalling

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Abstract

Inappropriate and un-regulated inflammation is the hallmark of a variety of inflammatory diseases including Rheumatoid Arthritis (RA). Central to the pathogenesis of this disease is the cytokine Tumour Necrosis Factor α (TNF), a product of activated macrophages. Macrophages are activated by numerous exogenous and endogenous ligands through Toll like receptors (TLR) leading to the activation of numerous protein tyrosine kinases (PTK). These studies have mainly described the LPS pathway and have not focused on endosomal TLRs, which are being increasingly implicated in the pathology of RA.

The work presented in this thesis describes the role of Bruton's Tyrosine Kinase (Btk) in TLR8 signalling in human primary macrophages. Using an adenovirus overexpression system and RNAi technology, Btk has been shown to control the production of TNF in R848-stimulated cells. This regulation occurred on a transcriptional level as Btk downregulation decreased the level of the primary *TNF* transcript at 2h as well as the recruitment of RNA Polymerase II to the promoter of the *TNF* gene at 1h. This rapid effect of Btk depletion on gene transcription resulted in significantly less TNF protein production at 2h following R848 stimulation.

Subsequent analysis of transcription factors potentially involved in the Btk-dependent regulation of *TNF* transcription revealed that Btk affects both NF κ B and AP-1 in TLR8 signalling. In particular, Btk downregulation decreased the binding of NF κ B to its consensus site as shown by EMSA as well as the recruitment of p65 to the *TNF* promoter or 3' enhancer region as shown by ChIP. The regulation of p65 by Btk occurred at the level of p65 phosphorylation on Serine 536 and did not affect the degradation of I κ B α . AP-1 activity in EMSA was also decreased under the Btk knockdown conditions, with cJun and ATF-2 but not cFos being involved in Btk signalling. At the same time, Btk did not alter p38, ERK and JNK MAPK activity ruling out their involvement in the Btk-dependent control of AP-1 in R848-challenged macrophages. These data extend current knowledge regarding the involvement of Tec kinases in the regulation of cytokine production under inflammatory conditions by providing a potential mechanism of transcriptional control.

Declaration

This thesis is the result of my own work with the exception of a few figures done in collaboration, as indicated. All collaborations have been acknowledged in the appropriate place within the text. The work was carried out at the Kennedy Institute of Rheumatology, Imperial College London, Faculty of Medicine and was funded by the Arthritis Research Campaign and the Kennedy Institute trustees.

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Abbreviations

Ad	Adenovirus
AP	Activator Protein
ATP	Adenosine Tri-phosphate
APC	Antigen Presenting Cell
ARE	AU-Rich Element
ATF-2	Activating transcription factor 2
BCR	B cell receptor
BMDM	Bone Marrow Derived Macrophage
Bmx	Bone marrow tyrosine kinase gene in chromosome X protein
BSA	Bovine Serum Albumin
Btk	Bruton's tyrosine kinase
CBP	CREB-binding protein
CD	Cluster of Differentiation
cDNA	Complementary DNA
ChIP	Chromatin Immunoprecipitation
CMV	Cytomegalovirus
CPE	Cytopathic Effect
CpG	Deoxycytidylate-phosphate-deoxyguanylate
CREB	cAMP response element binding protein
Ct	Threshold cycle
DAMP	Damage Associated Molecular Patterns
DC	Dendritic cell
DNA	Deoxyribonucleic Acid

DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
ds	Double stranded
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Sandwich Assay
EMSA	Electro-mobility shift assay
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorter
FCS	Foetal Calf Serum
g	gram
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte macrophage stimulating factor
h	hour/ hours
HAT	Histone acetyltransferase
HBSS	Hank's Balanced Salt Solution
HDAC3	histone deacetylase 3
HEK	Human embryonic kidney cells
HLA	Human leukocyte antigen
HRP	Horseradish Peroxidase
HSF	Human Foreskin Fibroblasts
HSP	Heat shock protein
IBD	Inflammatory Bowel Disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

IL-1R	IL-1 receptor
IκB	Inhibitor of NFκB
IKK	IκB kinase
iNOS	Inducible nitric oxide synthase
IP	Immunoprecipitation
IP-10	IFN-inducible protein-10
IRAK	IL-1R-associated kinase
IRF	Interferon Regulatory Factor
Jak1	Janus Kinase-1
JNK	c-Jun N-terminal kinase
kDa	kilo Dalton
LB	Lennox L broth base
LBP	LPS-binding protein
LPS	Lipopolysaccharide
M	Molar
m.o.i.	Multiplicity of infection
MAPK	Mitogen Activated Protein Kinase
MAPKK	MAP Kinase Kinase
MAPKKK	MAPK Kinase Kinase Kinase
Mal	Myd88-adaptor like
M-CSF	Macrophage Colony Stimulating Factor
MCP	Monocyte Chemotactic Protein
MHC	Major-Histocompatibility Complex
min(s)	minute(s)
ml	millilitre
MMP	Matrix metalloproteinase

mRNA	Messenger RNA
Myd88	Myeloid differentiating factor 88
NCoR	nuclear receptor coreceptor
NFκB	Nuclear Factor κB
ng	nanogram
NK	Natural Killer
nm	nanometre
NO	Nitric Oxide
o/n	overnight
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pen/Strep	Penicillin/Streptomycin
PH	Pleckstrin Homology
PI3K	Phosphoinositide 3-Kinases
PMA	Phorbol myristate acetate
Pol II	Polymerase II
PolyI:C	Polyinosinic-polycytidylic acid
PRG	Primary Response Genes
PRR	Pattern Recognition Receptor
PT-PCR	Primary Transcript PCR
R848	Resiquimod
RA	Rheumatoid Arthritis
RNA	Ribonucleic Acid
rpm	Revolutions per minute

RPMI 1640	Roswell Park Memorial Institute 1640
RT	Reverse Transcription
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sec(s)	second(s)
Ser	Serine
SF	Serum Free
SFK	Src Family Kinases
SH	Src Homology
si	Short interfering
SLE	Systemic Lupus Erythematosus
SMRT	the silencing mediator for retinoic acid and thyroid hormone receptor
Src	sarcoma
SRG	Secondary Response Genes
STAT3	Signal Transducer and Activator of Transcription 3
TAP	Tandem affinity purification
TCR	T cell receptor
Tec	Tyrosine kinase Expressed in hepatocellular Carcinoma
TFII-I	Transcription factor II-I
TH	Tec Homology
TIR	Toll/IL-1 Receptors
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor α
TRAM	TRIF-related adaptor molecule
TRAF6	TNF receptor associated factor 6
Treg	Regulatory T cells
TRIF	TIR-domain-containing adapter-inducing IFN- β

TTP	TrisTetraProlin
Tyr	tyrosine
UTR	Untranslated region
UV	Ultra Violet
V	Volt(s)
WB	Western Blot
WT	Wilt Type
Xid	x-linked immunodeficiency
XLA	X-linked agammaglobulinemia
µg	micro gram
µl	micro litre

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Chapter 1

Introduction

1.1 Inflammation

Inflammation, the defensive response of immune system to pathogens, irritants and damaged cells, is an extremely complex, multistep process. The biological aim of inflammation is to remove the causative agent and start the healing process of injured tissue. There are two types of inflammation; acute and chronic. The onset of acute inflammation is rapid, taking only a few days, and is predominantly mediated by cells of innate immune system and results in the resolution or chronic inflammation. Chronic inflammation, on the contrary, can last for years, engages cells from the innate and adaptive immune response and is characterised by severe tissue destruction. Therefore, chronic inflammation is a pathological condition and poses a serious threat to the organism (Janeway 2005).

Classic characteristics of inflammation are swelling, redness, pain, heat, and loss of function. Redness and increased heat are caused by widening of blood vessels (vasodilation) and elevated blood flow at the site of inflammation, swelling that results from the leakage of plasma fluid into the tissue (extravasation), increased sensitivity to pain that is believed to be caused by the release of pain mediators and is accompanied by a loss of function. Following physical injury (trauma) and infection, destruction (necrosis) of surrounding cells occurs. Necrotic cells release so called 'danger signals' such as the transcription factor high mobility group box 1 (HMGB1), heat shock proteins, hyaluronan oligosaccharides, fibronectin fragments and antibody-DNA complexes which together with pathogenic antigens stimulate neutrophils, mast cells and antigen presenting cells (APCs) (Matzinger 2002; Piccinini and Midwood 2010). Simultaneously, in response to pain, neurons secrete neuropeptides that activate mast cells, residents of the connective tissue in the mucus membranes. Mast cells in turn secrete histamine, eicosanoids and tryptases which facilitate vasodilation and movement of leukocytes out of circulatory system. The main function of APCs is the presentation of antigen to lymphocytes, recruitment of lymphocytes to the site of infection, secretion of inflammatory mediators as well as phagocytosis. Lymphocytes in turn sustain inflammation by secreting cytokines which together with the antigen activate macrophages to secrete molecules responsible for pathogen and cellular debris elimination such as proteases, reactive oxygen species (ROS), cytokines and nitrogen intermediates. Furthermore, lymphocytes are responsible for the regulation of inflammation and formation of memory cells (Nathan 2002).

1.1.1 The interplay between Innate and Adaptive Immunity

Innate immunity responds predominantly to microbes such as bacteria, fungi and viruses. It is an initial defence mechanism able to fully eradicate the pathogen preventing the activation of an adaptive response. Innate immunity is specific as it recognises pathogen-associated molecular patterns (PAMPs); highly conserved structures of microbes not produced by mammalian cells. The host cells recognise these structures through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) or mannose receptors which are encoded in the germ line and are characterised by limited diversity. Innate immunity allows a rapid and effective response against repeated or persistent infections (Janeway and Medzhitov 2002). If, however, this initial response fails to protect the host, the cells of innate immunity remain crucial for the secondary immunity as they drive the adaptive response.

The first line of defence against invading pathogens is the epidermis and mucosal membranes. Apart from forming a physical barrier to infections, epithelium also eliminates microbes by locally producing peptide antibiotics. In addition, mucosal linings of gastrointestinal and reproductive tracts contain a subset of T lymphocyte, called intraepithelial lymphocyte (IEL). IEL express antigen receptors of limited diversity recognising microbial lipids and other elements which are conserved among microbes of the same type. Upon antigen challenge, IEL release cytokines and cause killing of infected target cell (Montufar-Solis et al. 2007). Neutrophils are recruited to the site of pathogen invasion in the blood or extravascular tissue where they rapidly eliminate the microbe coated with antibodies and complement. The granule content is crucial for neutrophil function as neutrophil-specific granule deficiency causes impaired chemotaxis, receptor up-regulation and bactericidal activity, resulting in frequent and severe bacterial infections (Shiohara et al. 2004). Neutrophils do not return to circulation as they die within a few hours. Monocytes fulfil a similar to neutrophils function in rapidly fighting the infectious agent. Unlike neutrophils, circulating monocytes differentiate into macrophages which ensconce themselves in extravascular tissue where they survive for many days (Butterfield et al. 2006).

Natural killer (NK) cells are destined to recognise and kill infected and stressed cells and to secrete macrophage-activation cytokine interferon- γ (IFN- γ). NK cells are a class of lymphocytes but in contrast to T cells and B cells, they do not express T cell receptors or immunoglobulins. Instead, they produce abundant cytoplasmic granules which are full of molecules able to initiate apoptotic death of the infected cell upon discharge. The cells are activated by the NKG2D receptor and deactivated by self class I MHC proteins. They are crucial in recognition of virus infected

cells which have lost the ability to present an antigen on class I MHC molecules to T cells. Alternatively, NK cells bind to antibody-coated cells through an Fc receptor called FcγRIII (CD16) and cause their death in a process called antibody-dependent cellular cytotoxicity. Furthermore, NK cells are involved in the clearance of irreparably injured and tumor cells as they carry receptors recognising DNA damage or malignant transformation. Activated macrophages and DCs produce IL-12 which additionally enhances the effector function of NK cells (Bottino et al. 2005).

The adaptive immune system is activated when microbes or pathogenic agents break the barrier of the innate system. It is able to respond to a large variety of antigens with high specificity. The components of adaptive immunity strongly rely on and cooperate with the cells of innate immunity in antigen presentation and microbial clearance. In contrast to PRRs which recognise highly conserved microbe-derived peptides, the cells of adaptive immunity operate with an extremely diverse range of receptors designed for the immune recognition of proteins (T cells) or proteins, lipids, carbohydrates and nucleic acids (B cells). T cell and B cell receptors are produced in the somatic line during lymphocyte development and are generated randomly during the course of gene segment recombination. When the receptor encounters the target antigen, the lymphocyte undergoes clonal expansion to produce an army of cells reactive to the same antigen. These specific receptors are not inherited by the next generation of cells as opposed to PRRs which are encoded in the germline. The adaptive immune system comprises of humoral and cell-mediated immunity. The first one involves B lymphocytes and their products, antibodies, also known as immunoglobulins (Ig). Antibodies are secreted into the circulation and mucosal fluids. They specifically bind to antigens present on a microbe surface or to microbial toxins, to neutralise and eliminate them. When the antigen invades a host cell or becomes ingested by a phagocyte, then it can no longer be detected by antibodies. Cell-mediated immunity is then recruited to recognise antigens produced by intracellular microbes (Janeway 2005).

T-cell mediated immunity

Cell-mediated immunity provides a secondary defence mechanism against microbes such as intracellular bacteria, fungi and protozoa which are resistant to lysosomal degradation as well as against viruses that infect and replicate in the cytoplasm of a host cell. T lymphocytes are key cell types involved in cell-mediated immunity able to recognise host and foreign peptides presented on MHC molecules from every cell type in the body. Naive T cells constantly recirculate through lymph nodes where they scan for foreign peptides presented on dendritic cells (DCs). Upon

antigen recognition, naive T cells undergo activation accompanied by the secretion of cytokines, clonal expansion and ultimately differentiation into effector cells. Effector T cells can remain in the peripheral lymph node where they eliminate infected cells and activate B lymphocytes or they can circulate to the site of infection (Abbas 2009).

T cells themselves are not able to distinguish between extracellular and intracellular microbes. Professional APCs such as DCs, macrophages and B lymphocytes present protein antigens on class II MHC molecules as well as providing co-stimulatory signals to CD4⁺ T cells (also known as helper T cells or Th cells). Microbes internalised by professional APCs undergo digestion in intracellular vesicles facilitated by lysosomal enzymes. The CD8⁺ subset of T cells, on the other hand, responds to peptides presented on class I MHC molecules which are expressed on all nucleated cells. Class I MHC-presented antigens are produced in cytoplasm from processed viruses, phagocytosed microbes escaped from internalised vesicles, own non-functional proteins or the products of mutated genes, as in tumors. These cytoplasmic proteins are unfolded and ubiquitinated what ultimately leads to their proteolytic degradation by proteasomes (Abbas 2009).

Primed CD8⁺ T cells mature into cytotoxic T lymphocytes (CTLs) which, by secreting the content of intracellular granules, immediately induce apoptotic death of virus-infected cells. CD4⁺ T cells differentiate into subsets of effector cells such as Th1, Th2, Th17 or regulatory T cells (Treg) characterised by different sets of secreted cytokines and performed functions. Therefore, the response of CD4⁺ T lymphocytes to infection depends on the subset of an effector cell (Cooke 2006). DC-released IL-12 differentiates naive CD4⁺ T cells into the Th1 subset. Th1 cells induce a classical pro-inflammatory pathway of macrophage activation resulting in phagocyte-mediated ingestion and killing of microbes (**Figure 1.1**). The major cytokine secreted by Th1 cells is anti-viral IFN- γ , a potent activator of macrophages and B cells. IL-4-differentiated Th2 cells, on the other hand, induce an alternative pathway of macrophage activation by secreting IL-4, IL-5 and IL-13 (**Figure 1.1**). This set of cytokines inhibits microbicidal activities in macrophages and induces synthesis of extracellular matrix proteins thus promoting tissue repair by these cells. Apart from regulating macrophage function, Th2 cells stimulate phagocyte-independent eosinophil-mediated defence against helminthic parasites (Romagnani 2000). IL-5 activates eosinophils, whereas IL-4 activates B cells to produce IgE antibodies. Antigen-bound IgE antibodies in turn activate mast cells, the major effector cells of hypersensitivity (allergic) reaction. IL-17-producing T helper cells (Th17) are the newly discovered subset of CD4⁺ T cells. The significance of Th17 has been recognised in the development of multiple autoimmune diseases

such as rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease. Th17 cells, similarly to Treg cells are induced by transforming growth factor- β 1 (TGF- β). IL-6 is also required for Th17 differentiation but is inhibitory for Treg cells. Regulatory T cells maintain immune system homeostasis and tolerance to self-antigens by releasing immunosuppressive TGF- β and IL-10 (Kimura and Kishimoto 2010).

B cell-mediated immunity

The major role of B lymphocytes in immunity is the production of antibodies, which when membrane-bound serve as antigen receptors, and when secreted neutralize and eliminate microbes and their toxins in the effector phase of humoral immune responses. Antibodies recognise antigens and toxins via their variable domain (Fab region) and bind to Fc receptors on phagocytes and to proteins of complement via their constant Fc region. Naive B cells only produce membrane-bound IgM and IgD isotypes. Upon antigen recognition, a B cell proliferates into antigen-specific clones (clonal expansion) and can differentiate into an effector cell (plasma cell) which is able to secrete specific antibodies. B cell response to protein antigens is dependent on T cells that mediate isotype switching and affinity maturation whereas the response to polysaccharide, lipids and other nonprotein antigens is T cell- independent (Janeway 2005). The complement system also greatly enhances B cell activation, as the binding of C3d protein, the product of complement proteolysis, to the CD21 receptor provides second signals for B cell proliferation and differentiation (Heyman 2000). Conversely, if the antigen is a protein, effector B cells can also present the antigen to CD4⁺ T cells and provide co-stimulatory signals for T cell activation. B lymphocytes belong to professional APCs thus they are able to endocytose antigens bound to Ig receptors, process them in endosomal vesicles and ultimately present antigenic peptides on class II MHC molecules. However, unlike DCs, B cells can only activate previously differentiated effector T cells but not naive T cells (Abbas 2009).

1.1.2 Macrophages as a bridge between Innate and Adaptive Immunity

Monocytes (mononuclear phagocytes) originate from pluripotent progenitor cells in the bone marrow. The cells are subsequently released from the bone marrow into the bloodstream where they circulate for few days before migration into various tissues and organs. In the presence of growth factors such as macrophage colony stimulator factor (M-CSF) or granulocyte macrophage colony stimulator factor (GM-CSF), monocytes differentiate into macrophages (Barreda et al.

2004). Resident macrophages in the lungs are called alveolar macrophages, those in the liver are called Kupffer cells, whereas those in the central nervous system are known as microglia. Tissue macrophages share their progenitor cells with myeloid dendritic cells (DCs) and osteoclasts in the bone. Macrophages directly respond to the pathogenic agent through various PRRs and are involved in the activation of innate and adaptive immune response. Their role is to phagocytose cellular debris and microbes as well as to resolve inflammation and initiate the healing process of the inflamed tissue (Gordon 2003). The cells can be identified by flow cytometry by the expression of CD14 (lipopolisaccharide-binding protein), CD68 (sialoadhesin, a sialic acid binding lectin) or EMR1 (epidermal growth factor module-containing mucin-like receptor 1) (Khazen et al. 2005).

Upon encounter with infectious agents, resident macrophages secrete cytokines such as tumor necrosis factor α (TNF) and interleukin (IL)-1 which in turn activates endothelial cells to produce selectins - the adhesion molecules for circulating phagocytes. The expression of selectins enables binding of blood leukocytes to the endothelium and initiates a sequence of events that ultimately leads to leukocyte migration through the endothelium and to microbe digestion. The process of phagocytosis consists of the following steps: phagocytic receptor-mediated binding of a microbe to neutrophils and macrophages, internalisation of the cellular membrane containing the pathogen and forming a phagosome as well as the formation of phagolysosome where the microbe is destroyed. A lysosome contains activated enzymes whose products such as reactive oxygen species (ROS) and nitric oxide (NO) have microbicidal activities. Lysosomal proteases, on the other hand, digest microbial proteins (Underhill and Gantner 2004). There is a plethora of phagocytic receptors, including fragment crystallisable (Fc), complement (CR1, CR3 and CR4), scavenger (SRA and MARCO) and mannose (CD206) receptors, various integrins, Dectin-1 or CD14. Fc γ receptors bind to the Fc region of IgG-opsonised particles, Fc ϵ receptors bind to IgE-opsonised particles whereas Fc α receptors are specific for IgA-opsonised antigens. Other molecules that are able to act as opsonins include C3b and C3bi; enzymatic products of the complement system cascade. Complement proteins are able to opsonise pathogens through antibody-dependent and antibody-independent methods (Underhill and Ozinsky 2002).

Apart from eliminating invading agents, macrophages are also essential for clearance of apoptotic cells in the process called efferocytosis. The role of efferocytosis is the elimination of dead cells before their membrane breaks and toxic enzymes, oxidants and proteases are released into the surrounding tissue. Thus efferocytosis prevents inflammation or promotes inflammation

resolution. Ineffective efferocytosis is one of the hallmarks of the chronic vascular condition, atherosclerosis (Tabas 2010). The role of macrophages in the resolution of inflammation can be further supported by the fact that the cells trigger apoptosis of surrounding immune cells by expressing cell surface Fas ligand (FasL) and releasing active soluble FasL. The binding of FasL to a death receptor expressed on neutrophils, eosinophils, lymphocytes, monocytes and macrophages leads to target cell apoptosis. FasL expression on the cell surface is induced by phagocytosis of opsonised zymozan or apoptotic neutrophils thus promoting a positive feedback loop (Brown and Savill 1999). Macrophage digestion of apoptotic cells is accompanied by the secretion of anti-inflammatory IL-10 and the inhibition of pro-inflammatory cytokine release such as TNF, IL-1 and IL-12 (Voll et al. 1997).

Macrophages are also crucial for reparation of damaged tissue as they are a major source of growth factors including fibroblast growth factor (FGF), insulin-like growth factor (IGF-1) and transforming growth factor- β 1 (TGF- β 1). Together, these factors activate fibroblasts to secrete extracellular matrix-building proteins such as collagen. Macrophages also secrete vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) which promote angiogenesis and tissue regeneration (Butterfield et al. 2006).

Due to wide-spread tissue distribution and responsiveness to many endogenous and exogenous stimuli, macrophages vary in their phenotype (Gordon 2003). Early studies showed that infection of mice with *Mycobacterium bovis* or *Listeria monocytogenes* induced the antimicrobial activities of macrophages (Mackaness 1964). Studies since have shown that this 'classical' immune activation of macrophages is dependent on pro-inflammatory cytokines produced by Th1 and NK cells. The alternative activation of macrophages is dependent on Th2-mediated allergic, cellular and humoral responses to parasites and extracellular pathogens (Gordon 2003). Classical activation leads to the polarisation of macrophages into M1 phenotype whereas alternative activation leads to the development of M2 phenotype (**Figure 1.1**). The balance between the M1 and M2 programs is crucial for effective combating of infection. M1 cells, which are considered as microbicidal and pro-inflammatory, are activated through IFN- γ and microbial product stimulation that leads to the production of inflammatory cytokines such as TNF, IL-6, IL-1, IL-12 and synthesis of NO and ROI. M2 cells are considered as immunomodulators involved in the resolution of inflammation. They are characterised by expression of alternative activation markers and are induced by IL-4 or IL-13 (M2a phenotype), agonists of TLRs or IL-1 receptors and ligation of Fc receptors to antibody coated immune complexes (M2b phenotype) or are induced by

IL-10 and glucocorticoids (M2c phenotype) (Benoit et al. 2008). It is important to emphasise that macrophage activation is a dynamic process and that the classification of macrophages as either type M1 or M2 could be an oversimplification. It has been shown that macrophages adapt to the changing microenvironment and can initially be involved in the pro-inflammatory reactions and later participate in the resolution of inflammation (Porcheray et al. 2005). Nevertheless, the imbalance between pro-inflammatory and immunoregulatory state can pose serious health consequences. Prolonged M1 polarization caused by acute infections with *Escherichia coli* leads to tissue damage, multiple organ failure and ultimately death (Benoit et al. 2008). On the other hand, chronic infectious diseases are associated with macrophages adopting M2 phenotype. Inhibiting M2-associated cytokines such as IL-10 and IL-4 in susceptible mice infected with virulent *Brucella abortus* restores M1 phenotype and allows bacteria killing (Fernandes et al. 1996).

Finally, macrophages belong to professional APCs required for the activation of adaptive immunity CD4⁺ T cells and are the effector cells of cell-mediated immunity. Other professional APCs include DCs and B cells. DCs are derived from the same common precursor as macrophages and some argue that there are no markers, pathways of development and functions that distinguish these two cell types (Hume 2008). A network of residential DCs present in most of the tissues capturing invading microbes. Activation of resting DCs by PRR stimulation and cytokines changes their phenotype and leads to their detachment from the epithelium. Upon maturation, DCs downregulate phagocytic receptors and upregulate MHC molecules as well as co-stimulatory molecules such as CD40, CD80, CD83 and CD86 (Banchereau and Steinman 1998). Effective DCs then migrate to the lymph nodes where they present antigens to T lymphocytes through class II MHC proteins (signal 1) and provide co-stimulatory molecules, such as B7-1 (CD80) and B7-2 (CD86) (signal 2). B7 is recognised by the CD28 receptor on T cells and is essential for initiating the responses of naive T cells as interaction of TCR alone with proteins presented on MHC molecules is unable to activate T cells. Soluble antigens are also captured by DCs resident in the spleen where again they act as APCs. Conversely, upon stimulation with anti-inflammatory IL-10 or corticosteroids, resting DCs induce immune tolerance via T cell deletion or induction of regulatory T cells (Tregs). Two major types of blood circulating DCs can be distinguished by surface markers; CD11c expressing myeloid DCs and CD11c negative

plasmacytoid DCs. Although both subsets activate T cells, they express distinct PRRs and therefore differ in pathogen recognition and function (Schreibelt et al.).

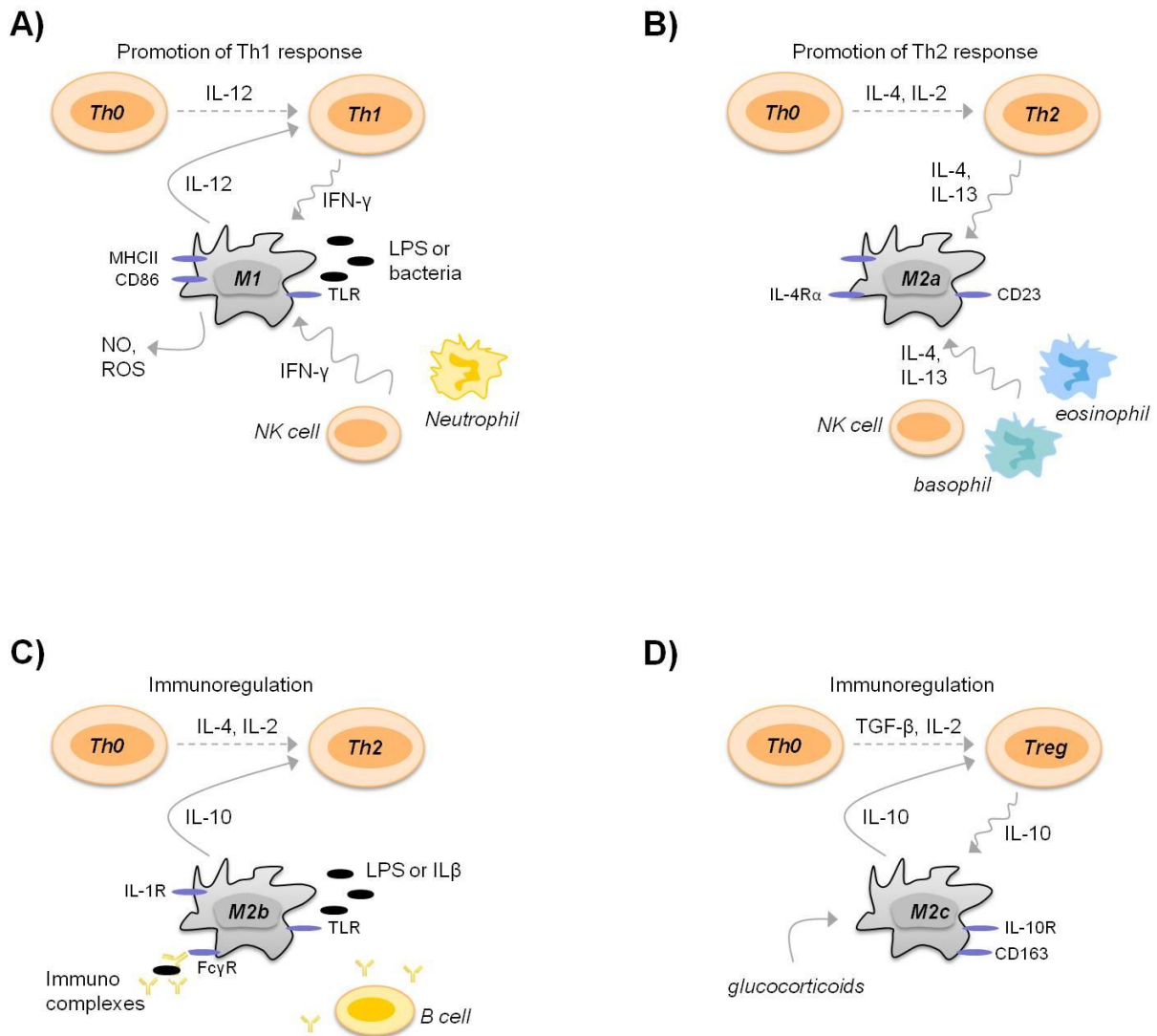


Figure 1.1. Macrophage polarization by immune cells. Macrophages polarize into M1 and M2 subsets upon encounter with pathogens and inflammatory mediators secreted by activated immune cells. IFN- γ and TLR ligands initiate classical macrophage activation. M1 in turn produce IL-12 which differentiates Th0 into Th1 cells as well as secreting microbicidal molecules such as NO and ROS (A). Alternative, M2a, polarisation of macrophages is driven by IL-4-differentiated Th2 cells, NK cells, basophils and eosinophils, all of which secrete IL-4 and IL-13 (B). Ligation of Fc receptors to antibody-covered immune complexes and LPS or IL-1 β stimulation can induce M2b polarisation (C) whereas stimulation with IL-10 mainly by TGF- β -differentiated Tregs or glucocorticoid stimulation leads to M2c polarization (D). M2 macrophages promote Th2 response thus clearance of parasites. M2c macrophages mediate the resolution of inflammation. Th, helper T cell; Treg, regulatory T cell; TLR, Toll-like receptor; TGF- β , transforming growth factor β ; NO, nitric oxide; ROS, reactive oxygen species; MHCII, class II major histocompatibility complex; IFN- γ , interferon- γ . Adapted from (Biswas and Mantovani 2010; Kimura and Kishimoto 2010).

1.2.3 Cytokines as mediators of inflammation

Cytokines are soluble cell-signalling proteins, peptides or glycoproteins that are predominantly produced by the cells of immune system. Cytokines are either secreted or expressed on the cell surface and their major role is modulation of the immune response, including activation, differentiation, deactivation or chemoattraction of target cells. They are classified as interferons, interleukins, chemokines, haematopoietic colony stimulating factors or connective tissue growth factors and tumour necrosis factors. Cytokine can act on cells both through paracrine and autocrine mechanisms by binding to the specific receptors on the cell surface. Most cytokines are short-lived and modulate only the closest microenvironment. Others, such as chemokines are relatively long acting and can attract circulating blood cells to the site of inflammation. Cytokines also modulate the action of other cytokines and disruption of cytokine network may lead to pathological consequences such as autoimmune and inflammatory diseases (Feldmann 2002). More than 50 cytokines have been identified and individual description of each is beyond the scope of this thesis. Thus, only key pro-inflammatory cytokines such as TNF, IL-6 and IFN as well as anti-inflammatory cytokine IL-10 are described in detail.

Tumor Necrosis Factor α - TNF

TNF was functionally described in mid 1970s as a macrophage derived product that caused necrosis of solid tumors (Carswell et al. 1975). The expression of TNF is induced by various factors including viruses, bacteria, parasites, tumour cells, complement or other cytokines. Monocytes and tissue macrophages are the primary source of TNF. However, this cytokine is also widely expressed in other immune cells as well as non-immune cells including fibroblasts, neurons, osteoblasts or smooth muscle cells. TNF is implicated in a variety of processes including 1) growth, differentiation and metabolism; 2) can produce cachexia characterised by loss of weight, muscle atrophy, fatigue, weakness; 3) can trigger apoptosis in malignant and transformed cells, virus-infected cells, T cells and epithelial cells; as well as 4) initiate and perpetuate inflammation. TNF regulates the production of other cytokines (IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, IL-18, IFN- γ , TGF- β), hormones (cortisol, glucagon, insulin) as well as acute phase proteins, leukotrienes, ROS, NO, platelet-activating factor and prostaglandins. TNF also modulates T cell differentiation into the Th1 subset by inducing IL-12 and IL-18 production. Th1 cells in turn produce IFN- γ that leads to the activation of macrophages and increased production of TNF (Schottelius et al. 2004) (**Table 1.1**).

Due to its central role in inflammation, it is not surprising that TNF production is regulated on every expression level including transcription, post-transcription, translation and post-translation. The transcription of *TNF* is regulated by a plethora of transcription factors including NF κ B, AP-1, Sp1 or Ets that can activate or inhibit depending on the dimer composition and which are mutually dependent (see section 1.2.4 for details). Apart from inducing transcription, pro-inflammatory stimuli also induce the stability of *TNF* mRNA, through a mechanism dependent on p38 MAPK signalling (see section 1.2.6 for details). *TNF* is approximately 3 kilobases in size, it is located on chromosome 6, is translated as a 233 amino acid, 26kDa pro-protein deficient in a classic signal peptide. Newly synthesised pro-TNF is embedded in the plasma membrane in the active form able to mediate inflammatory effects of TNF through cell-to-cell contact (Kriegler et al. 1988). Pro-TNF is cleaved to its mature 157 amino acid long form by TNF-alpha-converting enzyme (TACE). TACE is a matrix metalloproteinase whose action is not only restricted to TNF as deletion of TACE, but not TNF, is developmentally lethal in mice (Moss et al. 1997; Peschon et al. 1998). Proteolytic cleavage of membrane pro-TNF provides another checkpoint in the regulation of TNF production.

Mature 17-kDa soluble TNF forms homotrimers through the C-terminus and binds to the TNF receptors through the free N-terminus. There are at least 41 members of TNFR superfamily, including receptors for TNF (TNF-RI and TNF-RII), Fas, CD40 or RANKL. These receptors all share the extracellular ligand binding domain that contains different repeats of cysteine-rich domains (CRD), a transmembrane region and intracellular domains that largely vary between the family members. The first subclass of TNFR, to which TNF-RI belongs, possesses a death domain in their intracellular region. Following TNF binding, TNF-RI recruits the TNFR associated death domain (TRADD) adaptor molecule and the Fas-associated death domain (FADD) adaptor molecule leading to the activation of a caspase cascade and ultimately to apoptosis. Alternatively, TRADD associates with TNFR-associated factor (TRAF)2, TRAF1 and receptor interacting protein (RIP) to initiate the nuclear factor κ B (NF κ B) and mitogen-activated protein kinases (MAPK) signalling that promotes the transcription of inflammatory mediators and protects the cell against apoptosis. The second subclass of TNFR, to which TNF-RII belongs, does not contain death domains. Instead these receptors possess a sequence that facilitates association with TRAFs, called TRAF-interacting motifs (TIMs). Again, the engagement of TRAF family members leads to the activation of NF κ B and MAPK signalling (Hehlgans and Pfeffer 2005). In addition to expression on the cell surface, TNFR can be cleaved and found in a soluble form. These soluble

receptors are antagonists for TNF, inhibiting the biological activity of TNF (Moosmayer et al. 1996).

Interleukin-6 - IL-6

IL-6 (interferon- β_2), a 21-28-kDa protein, was first cloned during the course of isolation and characterisation of viral-induced protein interferon- β in human fibroblasts treated with the synthetic double-stranded RNA, PolyI:C (Weissenbach et al. 1980). IL-6, which is mainly secreted by macrophages and monocytes, is the central mediator of the production of most acute phase proteins (Gauldie et al. 1987). *Il6* knockout mice are unable to exhibit a normal immune response, the induction of acute phase proteins is largely inhibited and these mice do not lose body weight (Fattori et al. 1994). IL-6 can also induce the production of IL-8 and monocyte chemoattractant protein (MCP)-1 from endothelial cells as well as expression of adhesion molecules (Romano et al. 1997). During chronic inflammation, in addition to its role in inducing acute phase proteins, IL-6 initiates cellular immune responses and elicits a mucosal humoral response. In particular, IL-6 induces differentiation of activated B cells, which leads to the production of antibodies (Takatsuki et al. 1988). IL-6 also triggers proliferation of thymic and peripheral T cells, differentiation of Th0 into Th17 cells as well as activation of NK cells (Keller et al. 1996) (**Table 1.1**). IL-6 binds to IL-6R composed of two subunits; an alpha subunit and glycoprotein (gp) 130. Binding of IL-6 to this receptor induces cellular signalling events, such as activation of Janus protein tyrosine kinases (JAKs). Activated JAKs phosphorylate signal transducers and activators of transcription (STATs), leading to their activation, particularly STAT3. When phosphorylated, STAT3 dimerises and translocates to the nucleus leading to transcription of genes containing STAT3 response elements (Taniguchi and Takaoka 2001).

Type I and II Interferons - IFNs

Type I IFNs- IFN- α and IFN- β - are the main antiviral cytokines of the innate immune system. They are structurally unrelated to Type II IFNs such as IFN- γ . Apart from driving antiviral response, IFNs are involved in immunomodulatory activities by activating NK cells, macrophages and DCs (**Table 1.1**). IFN- α/β induce the expression, but not the activation, of p53, a crucial element required for apoptosis of cancerous cells. Therefore, IFN- α/β can contribute to tumour suppression (Takaoka et al. 2003). Binding of IFN- α/β to the receptor comprising of two subunits, IFNAR-1 and IFNAR-2, activates JAK-STAT signalling. The intracellular domains of IFNAR interacts with Tyk2 and Jak1 which subsequently tyrosine phosphorylate STAT1 and STAT2. IFN- γ signals through a receptor comprising IFNGR-1 and IFNGR-2 subunits which bind to Jak1

and Jak2 but not Tyk. The engagement of STATs results in the recruitment of IFN- regulatory factor (IRF) 9 leading to downstream gene induction in an IFN-stimulated response elements (ISRE)-dependent manner (Takaoka and Yanai 2006). One of the IFN target genes is *IP10*. The product of this gene, IFN-inducible protein 10, is a chemokine of the CXC family. IP-10 is a central chemoattractant for T cells as *Ip10*^{-/-} mice injected with a neurotropic mouse hepatitis virus displayed impaired T cell trafficking into the brain as well as impaired T cell proliferation and Th1 responses (Dufour et al. 2002). The IP-10 receptor is highly expressed on IL-2-activated T cells, but is not detectable in resting T cells, B cells, monocytes or neutrophils (Loetscher et al. 1996).

Interleukin-10 - IL-10

IL-10 was first discovered as a soluble factor, named cytokine synthesis inhibitory factor (CSIF), produced by murine Th2 lymphocytes, which inhibited the production of cytokines from pro-inflammatory Th1 cells (Fiorentino et al. 1989). Subsequent studies performed in murine and human systems revealed that IL-10 is secreted by numerous cells including Th1, Th2, Th17, cytotoxic CD8⁺ T cells, B cells and myeloid cells (O'Garra et al. 2008). IL-10 is also the key cytokine secreted by Tregs which are defined by the expression of a transcription factor Foxp3. The pivotal physiological function of IL-10 is mediating control of signals that induce inflammation in the gut as mice deficient in IL-10 developed chronic enterocolitis under pathogen free conditions (Kuhn et al. 1993). IL-10 exerts multiple anti-inflammatory actions. The major role of IL-10 is the inhibition of macrophage and monocyte effector functions including the production of NO and the production of pro-inflammatory mediators such as TNF, IL-6, IL-1, IL-12, GM-CSF or chemokines MCP1, MCP5 or CCL5 (Moore et al. 2001). In addition to downregulation of cytokine expression, IL-10 up-regulates the production of soluble TNFR and the IL-1 receptor antagonist (IL-RA) which inhibit the biological activity of TNF and IL-1, respectively (Jenkins et al. 1994; Joyce et al. 1994). Furthermore, IL-10 also inhibits the antigen presentation to T cells by down-regulating the expression of class II MHC and the co-stimulatory molecules CD80/86 in human DCs (Buelens et al. 1995) (**Table 1.1**). IL-10 signals through the IL-10 receptor in myeloid cells which is comprised of two molecules of the IL-10R1 and two molecules of the IL-10R2 chain. Jak1 and Tyk2 have been shown to be permanently associated with the IL-10R. Activation of IL-10R results in the Jak1/Tyk2-dependent recruitment of STAT1 and STAT3. STAT3 has been particularly found to mediate anti-inflammatory effects of IL-10 in human primary macrophages with examples including the inhibition of TNF and IL-6 production, the induction of soluble TNFR production and downregulation of class II MHC expression (Williams et al. 2004).

<i>Cytokine</i>	<i>Principal cell source(s)</i>	<i>Principal cellular targets and biologic effects</i>
Tumor necrosis factor (TNF)	Macrophages, DCs, T cells	Endothelial cells: activation (inflammation, coagulation) Neutrophils: activation Liver: synthesis of acute phase proteins Muscle, fat: catabolism for energy conversion, cachexia (loss of weight, weakness) Hypothalamus: fever and sleep Fibroblasts: stimulates the synthesis of collagen and collagenase for scar tissue formation
Interleukin- 1 (IL-1)	Macrophages, DCs, Endothelial cells, some epithelial cells	Endothelial cells: activation (inflammation, coagulation) Liver: synthesis of acute phase proteins Hypothalamus: fever
Chemokines (e.g IL-8)	Macrophages, Endothelial cells, T cells, Fibroblasts, Platelets	Leukocytes: chemotaxis, activation
Interleukin-12 (IL-12)	Macrophages, DCs	NK cells and T cells: IFN- γ production, increased cytotoxic activity T cells: Th1 differentiation
Interferon-γ (IFN-γ)	NK cells, T cells	Activation of macrophages Stimulation of some antibody responses
Type I IFNs (IFN-α, IFN-β)	IFN- α : macrophages, DCs IFN- β : fibroblasts	All cells: anti-viral state, increased class I MHC expression NK cells: activation
Interleukin-10 (IL-10)	Macrophages, DCs, T cells	Macrophages, DCs: inhibition of cytokine production, reduced expression of co-stimulators and class II MHC molecules
Interleukin-6 (IL-6)	Macrophages, Endothelial cells, T cells	Liver: synthesis of acute phase proteins B cells: proliferation of antibody-producing cells T cells: Th17 differentiation

Table 1.1 Key cytokines of Innate Immunity, adapted from (Abbas 2009)

1.2 Initiation of the inflammatory response by Pattern Recognition Receptors

The innate immune system is responsible for early detection and clearance of invading microbes. The PRRs used by the innate immune system to recognise microbial antigens are expressed on phagocytes, DCs, and many other cell types, including mast cells, NK cells and epithelial cells. These receptors are either secreted and play a major role in PAMP opsonisation, or are expressed on the cell membrane or intracellular compartments where microbes may be localised. TLRs, on the cell membrane and on endosomes, constitute one of the biggest families of PRRs able to recognise bacterial, fungal and viral components (PAMPs) as well as damage-associated molecular patterns (DAMPs) generated upon tissue injury such as the molecules released from necrotic cells and extracellular matrix (ECM) fragments. The activation of TLRs results in initiation of NF κ B/ activator protein-1 (AP-1) as well as the interferon regulatory factor (IRF) 3/7 pathways (**Figure 1.3**). Cytoplasmic caspase-recruiting domain (CARD) helicases, including RIG-I/ MDA5 respond to viruses which directly enter the cytoplasm, or to double stranded (ds) RNA produced during active viral replication. Nucleotide-binding oligomerization domain (NOD)-like receptors participate mainly in cytoplasmic recognition of bacteria leading to NF κ B or inflammasome activation. Antifungal immunity is mainly mediated by Dectin-1 which activates NF κ B and promotes phagocytosis. The consequence of PRR ligation is the production of pro-inflammatory cytokines and chemokines, and the induction of the complement pathways. The activation of the innate immune system also leads to the production of anti-inflammatory factors such as TGF- β , IL-10 and steroid hormones which control inflammation through a negative feedback loop mechanism (Lee and Kim 2007).

1.2.1 TLRs in inflammation

Toll proteins were first identified as crucial components in dorso-ventral development of the *Drosophila melanogaster* embryo (Anderson et al. 1985). Subsequent studies revealed that Toll proteins mediate innate immune responses against fungal infections in *Drosophila*, and that the signalling initiated by Toll activation resembles the human IL-1 receptor pathway in terms of NF κ B engagement (Gay and Keith 1991; Lemaitre et al. 1996). Human homologues of Toll receptors, termed Toll-like receptors were next cloned and tested for the induction of inflammatory mediators (Medzhitov et al. 1997; Rock et al. 1998). Today, TLRs represent the best-characterised family of PRRs, crucial for pathogen recognition and for shaping adaptive

immunity. Apart from mediating inflammatory responses, TLRs may coordinate the efficiency of phagosome formation or maturation of newly formed phagosomes as TLR signalling activates many molecules that are involved in phagocytosis such as paxilin, an actin-binding protein, or protein kinase C. It has been also shown that Rac1, a Rho family GTPase, and PI3-kinase which are required for phagocytosis are also indispensable for TLR functionality. Furthermore, TLRs regulate the expression of multiple genes that participate in microbial recognition, actin cytoskeletal reorganisation, proteolysis and antigen presentation. Stimulation of TLR4 also upregulates the expression of phagocytic receptors such as certain members of scavenger receptor family or activating Fc γ receptors. Phagocytosis is essential for microbe elimination and processing of antigens for class II MHC antigen presentation that ultimately leads to activation of CD4⁺ T cells (Underhill and Gantner 2004). To date, ten TLRs have been identified in humans, of which TLRs 1, 2, 4, 5 and 6 are expressed on the cell surface whereas TLRs 3, 7, 8 and 9 are expressed on endosomes. TLRs are type I transmembrane receptors that belong to the TLR/IL-1R superfamily which also includes receptors for IL-18 and IL-1. These receptors share cytoplasmic Toll/ IL-1 receptor (TIR) domain responsible for signal transduction but differ in the extracellular domain. The IL-1R family is characterised by the Ig-like domains whereas TLRs contain leucine-rich repeats (LRRs) required for ligand recognition (Akira and Hemmi 2003) (**Figure 1.2**).

Ligand recognition by TLRs localised on the cell membrane

TLR4 was the first human homologue described. Researchers revealed the role of this receptor for cytokine production by using a TLR4 mutated version that contained the mouse CD4 region instead of the regular ectodomain. Expression of this constitutively active TLR4 in THP-1 cell line resulted in the induction of NF κ B luciferase as well as IL-1, IL-6 and co-stimulatory molecule B7.1 (Medzhitov et al. 1997). A year later, the first TLR4 ligand, LPS, was discovered. This was achieved by genetic and functional mapping of the gene responsible for impaired response to LPS in mice (Poltorak et al. 1998). Since its discovery as the LPS receptor, TLR4 has been implicated in the recognition of mannan from *Candida albicans*,

glycoinositolphospholipids from a protozoan parasite *Trypanosoma cruzi* as well as viral envelop protein from certain viruses (Lee and Kim 2007). TLR4 has been also reported to recognise endogenous ligands such as HMGB-1 released during cell necrosis, heparin sulphate and recently Tenascin-C, all of which are components of extracellular matrix (ECM) found at high levels around injuries and in rheumatoid arthritis (Yu et al. 2006; Tang et al. 2007; Midwood et al. 2009) (**Figure 1.2**). Signalling through TLR4 depends on co-receptor MD2 to bind LPS. MD2 is also required for TLR4 dimerisation. Other components of TLR4 complex include LPS-binding protein (LBP) and CD14 where soluble plasma LBP first binds to LPS and is subsequently recruited to TLR4 by CD14 (Kawai and Akira 2010).

TLR2 was suggested to recognise LPS prior to the discovery that mutation of the TLR4 gene leads to LPS hyporesponsiveness (Kirschning et al. 1998). However, the role of TLR2 in recognition of LPS was later discarded due to the discovery of lipoprotein contamination of introduced LPS, and no responsiveness of TLR2-transfected cells to purified LPS (Hirschfeld et al. 2000; Tapping et al. 2000). TLR2 is implicated in the recognition of lipopeptides from bacteria, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, zymosan from fungi or the hemagglutinin protein from measles virus (Kawai and Akira 2010). Similarly to TLR4, TLR2 was shown to recognise endogenous HMGB-1 (Yu et al. 2006). The receptor also associates with other TLRs or non-TLR PRRs to increase its PAMPs recognition range. TLR2 generally interacts with TLR1 or TLR6. TLR1/2 heterodimers recognize a synthetic bacterial triacyl lipopeptide Pam₃CSK₄, and TLR1/6 heterodimers recognize diacyl lipopeptides such as MALP2 (*Mycoplasma*-derived macrophage-activating lipopeptide 2) (**Figure 1.2**). Additional molecules facilitate ligand recognition and discrimination. CD36 presents MALP2 and lipoteichoic acid but not zymosan to TLR2/TLR6 heterodimers (Hoebe et al. 2005) similarly to CD14 presenting LPS to the TLR4/MD2 complex. TLR2 alone poorly responds to zymosan and it requires cooperation with Dectin-1 for enhanced recognition. Dectin-1, on the other hand, requires TLR2 to induce NFκB-dependent pro-inflammatory cytokine production (Gantner et al. 2003).

TLR5 was discovered in 1998 as the receptor that recognises flagellin, the main component of flagella derived from both gram-positive and gram-negative bacteria (**Figure 1.2**). Flagellin was purified and identified by tandem mass spectrometry from the fraction of *Listeria monocytogenes* culture supernatant which induced TLR5-mediated activation of NFκB luciferase (Hayashi et al. 2001). Flagellin, unlike most other ligands, is a protein that can activate both innate and adaptive immunity. TLR5 has been found to function as endocytic receptor. Internalised flagellin can be

processed by APCs and subsequently presented on class II MHC molecules to CD4⁺ T cells. Interestingly, this function of TLR5 is independent of Myd88, an adaptor molecule crucial for TLR-induced cytokine production (Letran et al. 2011). Individuals who carry a stop codon polymorphism of TLR5 show impaired response to flagellin and are prone to developing pneumonia during infection with the flagellated bacteria *Legionella pneumophila* (Hawn et al. 2003).

Nucleic acid recognition by endosomal TLRs

TLRs 3, 7/8 and 9 are mainly localised in the endoplasmic reticulum (ER) prior to stimulation. Upon ligand internalisation, these receptors are rapidly sequestered from the ER and they traffic to endolysosomes (Kim et al. 2008). The inhibition of endolysosome acidification by chloroquine blocks TLR3, 7/8 and 9-induced responses indicating that endosomal maturation is a prerequisite for TLR activation (Sacre et al. 2008).

TLR3 has been identified as the receptor recognising dsRNA in 2001 (Alexopoulou et al. 2001). This group has shown that *Tlr3*^{-/-} mice demonstrate impaired responses to a synthetic analogue of dsRNA, polyinosinic-polycytidylic acid (PolyI:C) in terms of NFκB activation and pro-inflammatory cytokine production. Although TLR3 is predominantly expressed intracellularly, a recent report noted TLR3 expression on the cell surface in primary human endothelial cells. This endothelial cell surface TLR3 responded to small interfering (si) RNA of at least 21 nucleotides in length (**Figure 1.2**). As gene silencing through siRNA has been considered as a therapeutic tool, the potential pro-inflammatory effects of siRNA administration should be therefore carefully considered (Kleinman et al. 2008). Cellular single stranded (ss) RNA that forms hairpin loops can also act as endogenous dsRNA. Endogenous dsRNA released from necrotic cells has been shown to stimulate cultured rheumatoid arthritis synovial fibroblasts (RASFs) via TLR3 (Brentano et al. 2005a). Interestingly, CD14, which was originally identified as an LPS co-receptor, was reported to enhance TLR3's response to dsRNA (Lee et al. 2006). TLR3 is a critical component of host defence against viral infections. In response to TLR3 activation, cells produce anti-viral type I IFN and inflammatory cytokines. TLR3- and TLR9-deficient mice are predisposed to lethal infections with murine cytomegalovirus, and TLR3 deficiency in humans is linked to susceptibility to herpes simplex virus type 1 (Tabeta et al. 2004; Zhang et al. 2007).

TLR7 was initially identified to recognise the double cyclic organic molecules, imiquimod and resiquimod (R848), which have antiviral and antitumor properties, ssRNA derived from RNA viruses, synthetic poly (U) RNA and certain siRNAs (Jurk et al. 2002; Heil et al. 2004; Hornung et al. 2005) (**Figure 1.2**). TLR7 is mainly expressed on plasmacytoid pre-DCs where it serves as a sensor of infection with ssRNA (Kadowaki and Liu 2002). Recently, Beignot and colleagues have shown that endocytosed human immunodeficiency virus (HIV)-1 activates pDCs to produce type I IFN and TNF via TLR7-mediated recognition of RNA. The group therefore suggested that this adjuvant feature of HIV-1 RNA could be used for generation of vaccines for the prevention of AIDS (Beignon et al. 2005). Surprisingly, a TLR7 ligand, imiquimod appears to be an antagonist of TLR8 signalling, as it inhibits TLR8- induced TNF production from human primary macrophages (Sacre et al. 2008). TLR8 is evolutionary most similar to TLR7. In contrast to TLR7 which is mostly expressed of pDCs, TLR8 is highly expressed on monocytes (Kadowaki and Liu 2002). The receptor mediates the recognition of synthetic R848 and viral ssRNA in humans but not in mice. Its expression is upregulated after bacterial infections (Lee and Kim 2007). TLR9 recognises bacterial and viral unmethylated 2'-deoxyribo cytidine-phosphate-guanosine (CpG) DNA motifs that are rare in mammalian cells. TLR9 also responds to synthetic CpG oligonucleotides. Although native mammalian DNA has been shown to be a poor stimulator of TLR9, chromatin-containing immune complexes could stimulate TLR9 on B cells in an *in vitro* system (Viglianti et al. 2003). Positioning of the TLRs for nucleic acids in endosomal compartments protects the host from reacting to endogenous nucleic acids and consequently from developing autoimmune conditions. Another protective mechanism involves post-transcriptional modifications of eukaryotic RNA that distinguishes self and foreign nucleic acid (Tluk et al. 2009).

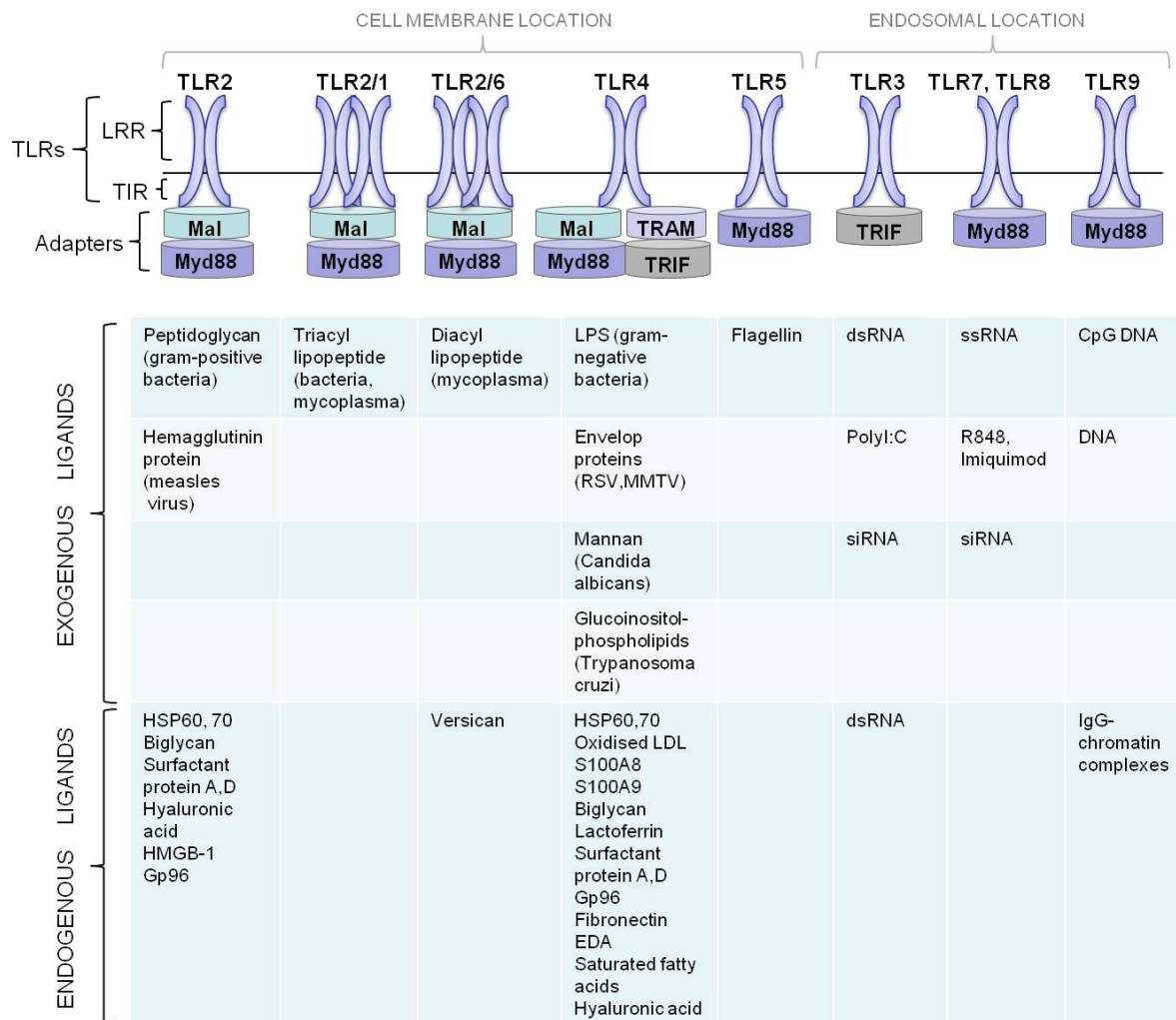


Figure 1.2 Endogenous and exogenous ligand recognition by TLRs. This figure was compiled from (Kawai and Akira 2007; Piccinini and Midwood 2010).

1.2.2 Activation of the Myd88-dependent and independent pathways by TLRs

Following stimulation of TLRs, TIR domain-containing intracellular adaptor molecules are recruited. The adaptor molecule Myd88 is used universally by all TLRs except of TLR3. MyD88 adaptor-like (Mal) molecule is additionally used as a bridging adaptor for TLR4 or TLR2/TLR1 and TLR2/TLR6 heterodimers. TLR4 engages the most complex signalling cascade as it also requires two other adaptor molecules; TIR-domain-containing adaptor inducing IFN- β (TRIF) and TRIF-related adaptor molecule (TRAM). TLR3 engages only the TRIF molecule (**Figure 1.2**). The initiation of the Myd88-dependent pathway leads to activation of NF κ B and MAPKs that ultimately results in inflammatory cytokine production. The Myd88-independent pathway, which employs the adaptor molecule TRIF, leads to activation of NF κ B and IRF3 and consequently to the production of type I IFN as well as inflammatory cytokines. In response to ligand stimulation, the cell membrane-localised TLR4 initiates the Myd88-dependent pathway. The receptor is subsequently internalised through dynamin-dependent endocytosis, traffics to endosomes and initiates TRIF-dependent pathway that leads to IRF3 and late-phase NF κ B activation (Kawai and Akira 2010) (**Figure 1.4**).

Myd88 mediates the signalling of TLRs as well as IL-1 receptors. The protein is recruited to the receptors through its carboxyl terminal TIR domain and interacts with its downstream binding partners through its amino-terminal death domain. Upon activation, Myd88 recruits IL-1 receptor-associated kinases (IRAKs) as well as TNF receptor associated factor (TRAF) 6. MyD88 first binds to IRAK4 which, in turn, phosphorylates IRAK1. IRAK1 subsequently induces TRAF6 which binds to the TAB-TAK complex and activates TAK1 protein. TRAF6 is an E3 ligase that, in conjugation with the ubiquitin-conjugating enzyme (Ubc)13/ Uev1A complex, catalyzes the synthesis of polyubiquitin linked to lysine 63 (K63) on target proteins including IRAK1, TRAF6, TAB2, TAB3 and NF κ B essential modulator (NEMO). Ubiquitination of TAB2 and 3, the adaptor proteins for TAK1, leads to TAK1 activation and subsequent initiation of the canonical NF κ B pathway and MAPKs signalling (Takeda and Akira 2004) (**Figure 1.4**). Mice deficient in Myd88 are resistant to LPS stimulation exemplified by the lack of TNF, IL-6 or IL-1 β production from macrophages and defective B cell proliferation. At the same time, activation of NF κ B and MAPK is delayed but not abolished in the *Myd88*^{-/-} macrophages (Kawai et al. 1999) indicating that the TRIF pathway can compensate for the loss of Myd88 in TLR4 signalling.

Stimulation of TLR3 or TLR4 leads to the recruitment of TRIF, and in the case of TLR4 also TRAM. The engagement of TRIF results in Myd88-independent activation of the canonical NF κ B

pathway, possibly also through a ubiquitination-dependent mechanism mediated by TRAF6. In addition, TRIF recruits receptor interacting protein (RIP)-1. RIP-1 requires adaptor TRADD and an E3 ubiquitin ligase Pellino-1 to induce NF κ B (**Figure 1.4**). Interestingly, TRAF6 deficiency has been shown to be dispensable for TRIF-dependent TLR3 and TLR4 signalling in terms of IP-10 production. In contrast, TRAF6 deficiency greatly decreased LPS-induced TNF and IL-6 production (Gohda et al. 2004). This is because the TRIF-dependent pathway also leads to the activation of the TRAF3/IKKi/TBK1 pathway and ultimately to the induction of IRF3. The consequence of the TRIF activation is the expression of IFN-inducible genes such as those encoding IFN- β , IP-10 and RANTES (regulated on activation, normal T expressed and secreted) (Kawai and Akira 2010).

Prolonged activation of TLR signalling, which engages NF κ B, MAPK and IRF pathways, leads to excessive production of pro-inflammatory cytokines that may result in multiple organ failure as seen in sepsis (Tsujimoto et al. 2008). Apart from the induction of anti-inflammatory mediators, TLR signalling is controlled by the network of regulatory molecules including phosphoinositide 3-kinases (PI3Ks) and small GTPases of the Rho family. Studies using a general PI3K inhibitor, LY294002, in the THP-1 monocytic-like cell line have demonstrated impaired activation of NF κ B upon TLR2 stimulation indicating that PI3K act as a positive regulator of TLR signalling (Arbibe et al. 2000). On the other hand, DCs derived from mice deficient in p85 α (regulatory subunit of class IA PI3K), or treated with PI3K general inhibitor, wortmannin, exhibited increased LPS-dependent IL-12 production. In this study, PI3K functioned as IL-12 suppressor via a p38-dependent signalling cascade (Fukao et al. 2002). Also the role of GTPases of the Rho family appear to be dependent on the family member as dominant-negative Rac1N17, but not dominant-negative Cdc42N17, has been shown to block NF κ B gene transactivation upon TLR2 stimulation (Arbibe et al. 2000). TLR signalling can be also regulated by its own components. For instance, newly discovered fifth TIR adaptor molecule, sterile alpha and HEAT-Armadillo motifs (SARM), has been shown to negatively regulate TRIF-dependent TLR signalling. SARM physically associated with TRIF, and SARM siRNA-mediated downregulation increased TRIF-driven cytokine induction in HEK-TLR3 and HEK-TLR4 cells (Carty et al. 2006).

1.2.3 Induction of NF κ B by TLRs

The transcription factor NF κ B has been associated with the development and perpetuation of most chronic diseases. It regulates the expression of cytokines, genes involved in apoptosis, cell

adhesion, proliferation, oxidative stress responses as well as up-regulation of co-stimulatory molecules on DCs that provides an essential signal for CD4⁺ T cell activation. Dysregulation of NF κ B has been linked to cancer, AIDS, atherosclerosis, asthma, arthritis, diabetes, Inflammatory Bowel Disease, stroke and viral infections (Gupta et al. 2010).

NF κ B forms homo- and heterodimers which are composed of molecules that contain Rel-homology domains (RHDs) required for DNA binding at specific κ B sites within the promoter and enhancer regions of a target gene. Five members of the NF κ B family have been identified in mammals. These include RelA (p65), RelB, c-Rel, p105 (NF κ B1; a precursor of p50) and p100 (NF κ B2; a precursor of p52). NF κ B is retained in the cytoplasm in unstimulated conditions in an inhibitory complex with I κ B. The I κ B family consists of I κ B α , β , γ , ϵ as well as I κ B ζ , Bcl-3 and I κ BNS. I κ B α , β , γ and ϵ inhibit NF κ B by blocking its nuclear localisation sequence and thus NF κ B translocation to the nucleus. The remaining I κ B ζ , Bcl-3 and I κ BNS are present in the nucleus where they control NF κ B-mediated gene transcription (Kawai and Akira 2007).

A heterodimer composed of RelA and p50 is the most common canonical NF κ B form activated by TLRs. Following TLR stimulation, I κ B is phosphorylated by TAK1-activated I κ B kinase (IKK) complex, then ubiquitinated and subsequently degraded by the 26S proteasome. IKK complex comprises two catalytic molecules, IKK α and IKK β , as well as a regulatory molecule NEMO. Following NF κ B release from the inhibitory I κ B complex, p65 is phosphorylated on Serines 276, 529, 536 and 311; these residues are required for p65 translocation to the nucleus and p65-mediated gene transactivation (Hayden and Ghosh 2008). p65 has been shown to be phosphorylated on Serine 276 by mitogen- and stress- activated protein kinase-1 (MSK1 and MSK2) and protein kinase A (PKA) (Zhong et al. 1998; Wang et al. 2000; Vermeulen et al. 2003). Casein kinase II was found to phosphorylate p65 on Serine 529, IKK α / β on Serine 536 whereas protein kinase C (PKC) ζ on Serine 311 (Wang et al. 2000; Duran et al. 2003; Sakurai et al. 2003; Chen et al. 2005). The initiation of the Myd88-dependent pathway, which involves TRAF6 and TAK1 molecules, leads to an early canonical NF κ B activation. The initiation of the TRIF-dependent pathway by TLR3 and TLR4 stimulation results in a late canonical NF κ B activation (Kawai and Akira 2007) (**Figure 1.4**). The inhibition of protein synthesis by cyclohexamide abrogated NF κ B activation in Myd88-deficient mice indicating that the late NF κ B induction is dependent on new protein synthesis (Covert et al. 2005). Although, similarly to Myd88, TRIF also utilises TRAF6 for TAK1 activation, this pathway also seems to be dependent on RIP1 as RIP1-deficient cells exhibit defective TLR3-mediated NF κ B activation (Cusson-Hermance et al. 2005).

The alternative, noncanonical, pathway involves the processing of p105 and p100 to p50 and p52, respectively. The long forms contain C-terminal ankyrin repeats, which similarly to I κ B proteins in the canonical pathway, bind to RHD and inhibit NF κ B nuclear translocation. Ubiquitin-dependent degradation of C-terminal region of p105 and p100 is a prerequisite for the generation of the mature NF κ B forms. Phosphorylation of p100 is also required for p52 processing. This step is accomplished by IKK α -IKK α homodimer, which does not involve IKK β or NEMO. It is unclear whether TLRs activate the noncanonical NF κ B pathway (Kawai and Akira 2007).

IKKi, another member of the IKK family, was first identified as an LPS-inducible protein. It interacts with TBK1, whose deficiency in mice leads to embryonic death (Bonnard et al. 2000). Interestingly, despite being members of the IKK family, the double *Ikki* and *Tbk1* knockout does not result in reduced NF κ B DNA binding or I κ B degradation in embryonic fibroblasts stimulated with TLR3 or TLR4 ligands (Hemmi et al. 2004). Rather IKKi and TBK1 have a vital role in type I IFN induction via the transcription factor IRF3. IKKi/TBK1-mediated phosphorylation of IRF3 allows its dimerisation and subsequent nuclear translocation. In the nucleus IRF3 binds to IFN-stimulated response element (ISRE) and together with NF κ B and ATF2/cJun regulates the expression of *IFN- β* (Fitzgerald et al. 2003). TLR3- and TLR4- induced IRF3 activation is mediated by the adaptor molecule TRIF and its binding partner TRAF3 (**Figure 1.4**). IRF3 activation is independent of Myd88 as it is intact in Myd88-deficient mice. Conversely, the production of TLR7- and TLR9- induced type I IFN in pDC depends entirely on Myd88 indicating that another pathway mediating type I IFN production is utilised by these receptors. IRF7 has been since reported to control IFN- α production via the Myd88/IRAK4/TRAF6 pathway. Simultaneously, IKKi- and TBK1-deficient pDCs do not exhibit impaired IFN- α production upon TLR7 and TLR9 stimulation (Kawai and Akira 2007).

1.2.4 NF κ B-dependent regulation of *TNF* transcription

TNF was initially recognised as a factor that could initiate apoptosis in malignant and transformed cells. Subsequent studies revealed that TNF is a potent inducer of immune responses and a central mediator of inflammation. Systemically administered TNF induces other cytokines, hormones as well as acute phase proteins, leukotrienes, ROS, NO, platelet-activating factor and prostaglandins (Schottelius et al. 2004). Because of its predominant functions, the regulation of TNF production

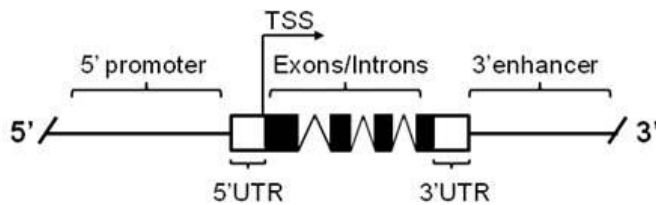
has been extensively studied and it is now clear that TNF is precisely regulated at every stage of gene and protein expression (Falvo et al. 2010).

The human *TNF* 5' promoter and downstream 3' enhancer regions contain multiple transcription factors binding sites which have been described to be involved in the TLR-driven induction of *TNF*. These include NFκB (κB) sites, Ets, cAMP-responsive element (CRE), CCAAT/enhancer binding protein β (C/EBP) and AP-1-like (Kramer et al. 1995; Liu et al. 2000) (**Figure 1.3**). NFκB is the key transcription factor involved in *TNF* transcription as the overexpression of IκBα considerably decreases TNF production from LPS-stimulated human primary macrophages (Foxwell et al. 1998). There are three confirmed NFκB binding sites in the *TNF* promoter: κB site 1 is located at position -673 base pairs (bp) upstream from the transcription start site, the cluster of κB site 2 starts at -627bp whereas κB site 3 starts at position -90bp and is localised in the core promoter of *TNF*. Additionally, κB site 4 is situated in the 3'downstream region of *TNF* at the position +2981bp. NFκB site 4 has been shown to act as enhancer of LPS-mediated TNF induction (Kuprash et al. 1999). Previous studies revealed that p65 is recruited to all four κB sites in the *TNF* promoter upon LPS stimulation (Krausgruber et al. 2010; Smallie et al. 2010). However, deletion or site-directed mutagenesis of any single κB site from the *TNF* promoter has little effect on the induction of the gene by LPS (Goldfeld et al. 1990; Udalova et al. 1998), (Tim Smallie, thesis). The canonical form of NFκB is a dimer comprising of a p65 and p50 subunits. The composition of the dimer determines the site of DNA binding as well as the function of the complex. p65/p50 heterodimer shows little variation in binding to κB sites 1, 2 and 3. p50/p50 homodimer which is considered to be inhibitory, binds with high affinity to κB site 1 and to κB site 2a. On the contrary, the p65/p65 homodimer has been shown to be strongly recruited to κB site 2 but not 2a. In murine macrophages, κB site 4 has been shown to recruit NFκB complexes of p50 and p65 subunits (Udalova et al. 1998; Kuprash et al. 1999; Liu et al. 2000; Udalova et al. 2000).

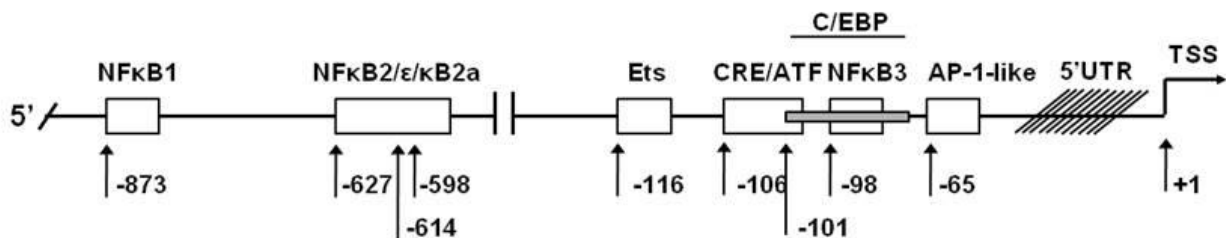
NFκB recruitment to the promoters of inflammatory genes was reported to be dependent on p38 MAPK. Particularly, the inhibition of p38 by SB203580 impaired phosphorylation and phosphoacetylation of histone H3 in LPS-treated human DCs. Histone-DNA complexes when highly packed, limit the accessibility of the genome to transcription machinery. The post-transcriptional modifications of histones such as acetylation, phosphorylation and methylation regulate chromatin folding and allow DNA-binding proteins to be recruited to gene regulatory

elements. p38 MAPK-mediated H3 phosphorylation on Serine 10 is required for NF κ B access to promoters and for the transcription of target genes (Saccani et al. 2002).

***TNF* gene**



***TNF* promoter**



***TNF* enhancer**

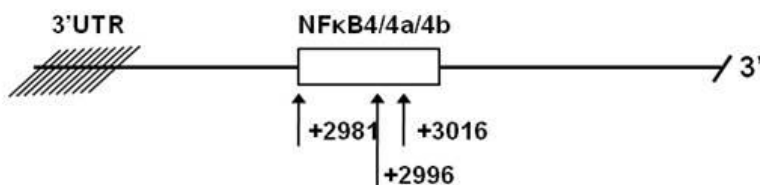


Figure 1.3 A schematic of the human *TNF* gene. *TNF* gene comprises of 5' promoter region, followed by transcription start site (TSS), a segment of 4 exons (shown as black squares), 3' UTR and 3' enhancer region. Multiple transcription sites involved in TLR signalling, including NF κ B, Ets, CRE/ATF, C/EBP, AP-1 are localised in the promoter (shown as white squares). NF κ B site 4 is localised in the 5' enhancer region. The location of the transcription factors in relation to TSS is shown numerically in base pairs.

1.2.5 Induction of MAPK by TLRs

MAPKs are involved in gene expression, mitosis, movement, metabolism, and programmed cell death. They phosphorylate specific serines and threonines of target protein substrates including other protein kinases, phospholipases, transcription factors, and cytoskeletal proteins. Apart from transcriptional regulation, MAPKs were found to regulate mRNA stability, transport and translation. Mammalian MAPK family includes members such as the extracellular-signal-regulated kinase (ERK), JUN N-terminal kinase (JNK, also known as MAPK8) and p38 (also known as MAPK14). The activation of upstream cell surface receptors leads to the initiation of a cascade of sequential phosphorylation events, starting from the phosphorylation of MAPK kinases (MAPKKs) by MAPK kinase kinases (MAPKKKs). Triggered MAPKKs then phosphorylate MAPKs on threonine and tyrosine residues which changes MAPKs conformation and leads to their enhanced substrate accessibility. Activated MAPKs can translocate to the nucleus where they regulate the activity of transcription factors such as AP-1, activating transcription factor 2 (ATF2) or CRE-binding protein (CREB) (**Figure 1.5**). MAPKs are deactivated by dephosphorylation mediated by tyrosine, serine/threonine phosphatases such as mitogen-activated protein kinase phosphatases (MKPs), also known as dual-specificity protein phosphatases (DUSPs) (Johnson and Lapadat 2002).

The ERK pathway is mainly activated by growth factors, cytokines, virus infection or carcinogens. Upon activation, the proto-oncogene GTPase Ras first recruits a MAPKKK called c-Raf, which in turn activates MAPKKs MEK1 and MEK2 to phosphorylate ERK1 and ERK2. One of the downstream targets of ERK1/2 in LPS signalling is the transcription factor Elk-1 that binds to Ets motifs in DNA (**Figure 1.5**). Phosphorylated Elk-1 also recruits serum response factor (SRF) to SRE sites in the target gene (Brook et al. 2006). TLR-activated ERK has been shown to induce the expression of the *MKP-1* gene, and the MKP-1 stabilisation through phosphorylation. The activation of MPK-1 leads in turn to JNK and p38 dephosphorylation and as a consequence, to the graduate inhibition of TNF production (Liu et al. 2007). The inhibition of ERK by a drug PD98059 reduces LPS-induced TNF and IL-1 β release but does not block IL-10 synthesis (Foey et al. 1998).

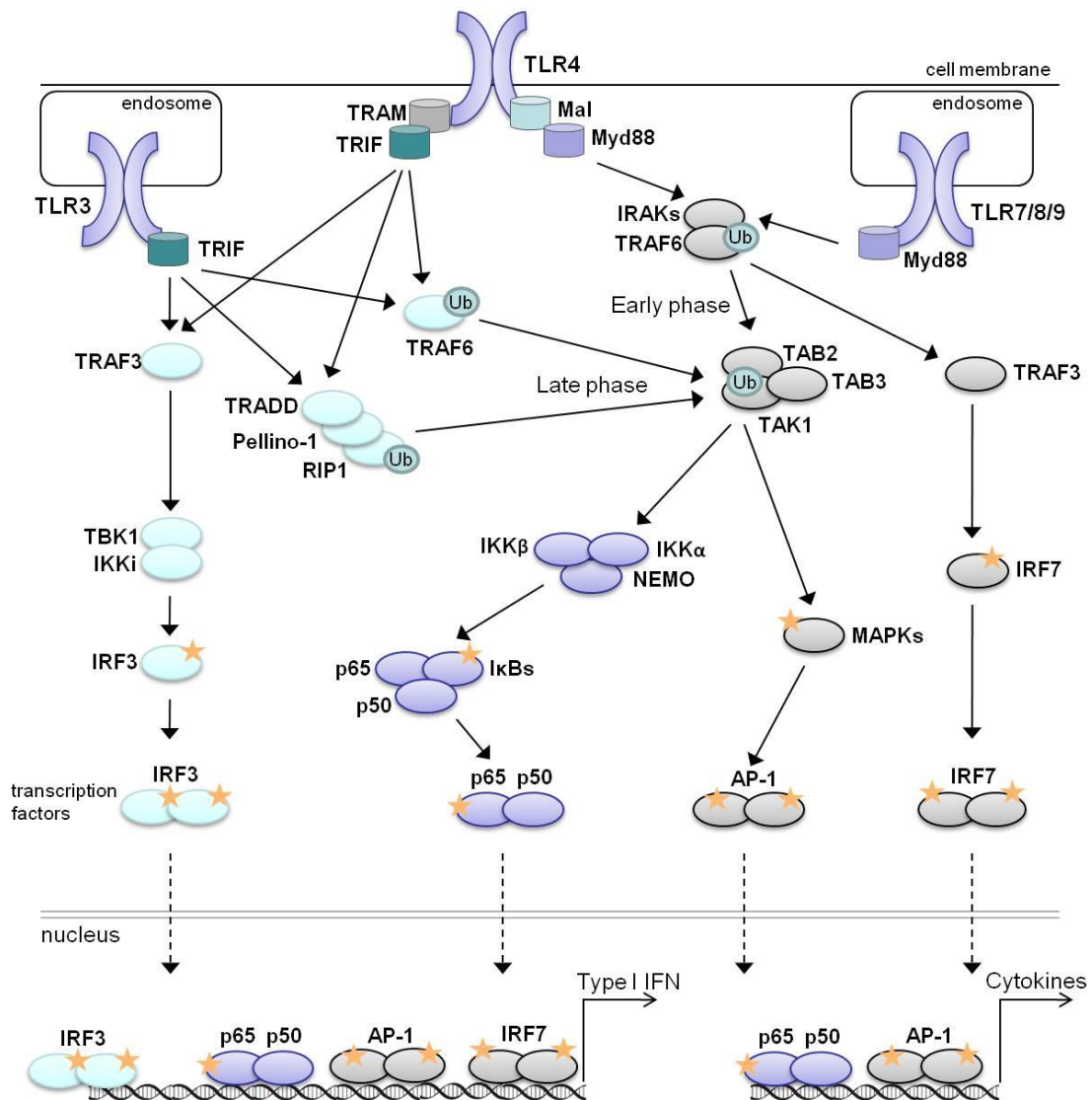


Figure 1.4 Overview of Myd88-dependent and Myd88-independent pathways. TLR4 stimulation initiates the most complex TLR signalling cascade as the receptor utilises four adaptor molecules: TRAM, TRIF, Mal and Myd88. In contrast, endosomal TLR3 uses only TRIF, whereas endosomal TLRs 7, 8 and 9 use only Myd88. The initiation of TRIF-dependent pathway by TLR4 and TLR3 induces TRAF3 which then activates the kinases TBK1 and IKKi, which phosphorylate and activate IRF3. TRIF also recruits TRAF6 and TRADD which interacts with Pellino-1 and RIP1. Ubiquitinated RIP1 and TRAF6 together activate TAB2/TAB3/TAK1 complex to initiate late NFκB signalling. TAK1 first activates IKK complex comprising of NEMO and IKKαβ, which phosphorylate IκB and thus lead to p65-p50 release from the inhibitory IκB complex. p65 is then phosphorylated and transfers to the nucleus. TAK1 is also required for MAPK and subsequent AP-1 activation. Activation of Myd88 induces IRAKs and TRAF6 which then together with Ubc13 and Uev1A ubiquitinates the TAB2/TAB3/TAK1 complex leading to early NFκB activation. The recruitment of TRAF3 to the Myd88/IRAKs/TRAF6 complex also leads to induction of IRF7. Gene transactivation by IRF3, NFκB, AP-1 and IRF7 results in type I IFN expression. NFκB and AP-1 are also required for cytokine production. Adapted from (Kawai and Akira 2010).

The JNK pathway was initially characterized as a stress-activated protein kinase (SAPK) pathway. JNK is activated by MKK7, which itself is primarily activated by cytokines and MKK4, as a result of environmental stress (Derijard et al. 1995; Tournier et al. 1997) (**Figure 1.5**). These two MAPKKs are activated by a variety of MAPKKKs including MEKK1-4 (Blank et al. 1996; Xu et al. 1996; Gerwins et al. 1997), TAK-1, apoptosis signal regulating kinases ASK1 and ASK2 (Ichijo et al. 1997; Wang et al. 1998). Of these, MEKK1-3 can also activate ERK1/2 pathway whereas TAK1 and ASK1 can also initiate the p38 pathway. JNKs are central inducers of the AP-1 transcription factor components such as cJun and ATF-2 (Gupta et al. 1996). Inhibition of JNK by a drug SP600125 prevents the induction of matrix destroying metalloproteinases (MMPs) as well as TNF and thereby blocking joint destruction in rodent models of rheumatoid arthritis. JNK inhibitors were also shown to enhance chemotherapy-induced blockage of tumor expansion (Johnson and Lapadat 2002).

p38 MAPK was originally identified as a compound involved in the biosynthesis of pro-inflammatory mediators. Apart from being activated upon TLR and cytokine receptor stimulation, the p38 MAPK pathway is also initiated by growth factors and environmental stress such as heat shock, radiation or ultraviolet light. There are four members of the p38 family: p38 α , p38 β , p38 γ and p38 δ . p38 α has been shown to be the key member implicated in the immune and inflammatory responses. p38 MAPK is activated by several MAPKKs such as MKK6 and MKK3 which were previously phosphorylated by small GTPases Rac and Cdc42 (Yong et al. 2009) or by TAK1 (Lee et al. 2000). p38 MAPK has been shown to be predominantly localised in the nucleus in a complex with MK2. The phosphorylation of MK2 by p38 exposes a nuclear export signal in the carboxyl terminal domain of MK2 thus allowing the translocation of p38 to the cytoplasm (Engel et al. 1998). p38 kinase has been reported to control the activation of numerous transcription factors including ATF-1, ATF-2, CREB, p53 and NF κ B (Yong et al. 2009) (**Figure 1.5**). In particular, the dominant negative mutant of p38 MAPK inhibited NF κ B reporter activity in LPS-stimulated human primary macrophages at the level similar to I κ B α (Campbell et al. 2004). Expression of many inflammatory mediators including TNF, IL-6, IL-1, IL-8, iNOS, COX-2, MMPs, c-Jun, c-Fos or JunB has been shown to be regulated by the p38 MAPK pathway (Yong et al. 2009).

1.2.6 p38 MAPK- dependent control of *TNF* mRNA stability

Apart from being involved in the transcriptional regulation of inflammatory genes, p38 MAPK has been implicated in the post-transcriptional regulation of gene expression by mediating the stability of mRNA (Clark et al. 2003) . During the course of transcriptional maturation, RNA Polymerase II (Pol II) adds a 7-methylguanosine cap to the 5' termini of mRNA whereas poly (A) polymerase adds a poly(A) tail to the 3' termini of mRNA. The removal of the poly (A) tail by deadenylase and subsequent decapping of the 5' termini uncovers mRNA which is then degraded by exonucleases (Wilusz et al. 2001). p38 has been shown to stabilise mRNA by inhibiting deadenylation. All p38-regulated transcripts contain adenine and uridine-rich elements (ARE) in their 3' untranslated regions (3'UTR). These elements attract ARE-binding proteins that can negatively regulate mRNA stability by promoting rapid deadenylation and subsequent decay of the transcript (Clark et al. 2003).

The importance of ARE in post-transcriptional regulation of TNF was confirmed in mice lacking TNF 3'UTR ARE. These mice exhibit chronic overproduction of TNF in serum and develop chronic inflammatory arthritis and inflammatory bowel disease (Kontoyiannis et al. 1999). The ARE-binding protein tristetraprolin (TTP) has been shown to regulate the stability of *TNF* mRNA. Several mechanisms of TTP-dependent *TNF* transcript decay were proposed and these include the proteasome pathway, the RNA processing-body (P-body) and the microRNA pathway (Deleault et al. 2008). Upon stimulation with an inflammatory agonist, p38 activates its substrate MK2 which in turn phosphorylates TTP and inactivates its destabilising function. TTP expression is enhanced upon LPS stimulation and paradoxically, activated p38 supports the stability of the *TTP* transcript (**Figure 1.5**). During the resolution of inflammation, the activity of p38 decreases and TTP is dephosphorylated. Reconstituted TTP binds to *TNF* mRNA resulting in destabilisation of the transcript. p38 MAPK can therefore play a double role by both promoting TNF production as well as preventing overproduction of TNF (Brook et al. 2006).

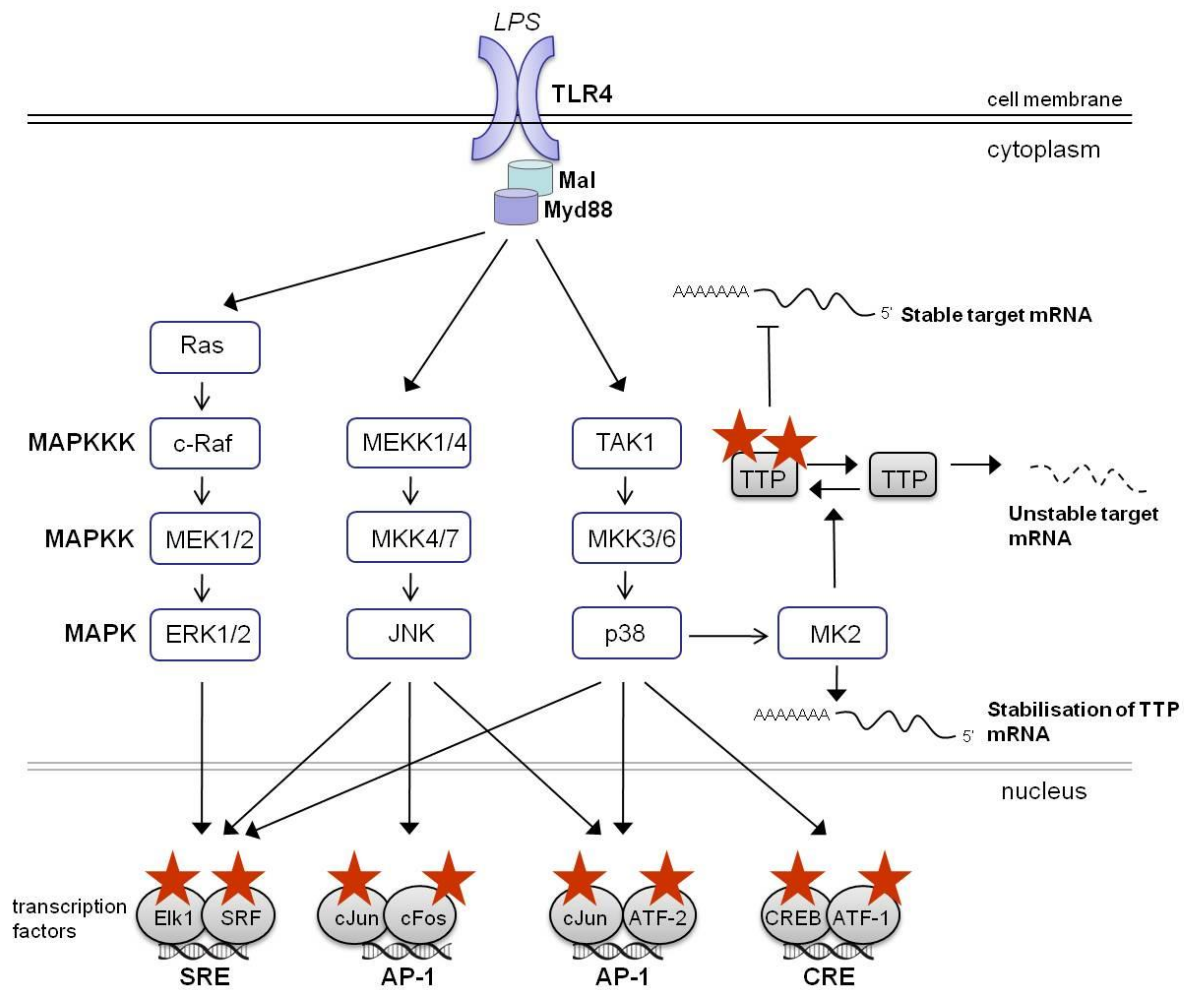


Figure 1.5 MAPK signalling pathways activated by LPS. LPS binding to TLR4 initiates Myd88-dependent pathway which leads to the activation of the MAPK signalling cascade. MAPKKK including c-Raf, MEKK1/4 and TAK1 are activated first. This activation leads to MAPKK (MEK1/2, MKK4/7 and MKK3/6) and subsequent MAPK (ERK1/2, JNK and p38) phosphorylation. MAPK mediate the activation of multiple transcription factors. In addition, cytoplasmic p38 regulates the stability of an ARE binding protein TTP via MK2. TTP is bound to ARE in the 3'UTR of many inflammatory genes (e.g. *TNF*) promoting mRNA deadenylation and subsequent degradation. MK2 deactivates TTP through phosphorylation resulting in the stability of *TNF* mRNA (adapted from (Guha and Mackman 2001; Brook et al. 2006)).

1.2.7 Induction of AP-1 by TLRs

The AP-1 family of transcription factors feature basic region-leucine zipper (bZIP) domains that facilitate dimerisation and DNA-binding domains which facilitate AP-1 binding to target 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (5'-TGAG/CTCA-3') or cAMP response elements (CRE, 5'-TGACGTCA-3'). AP-1 operates as a dimeric protein composed of members of the Jun (cJun, JunB, JunD), Fos (cFos, FosB, Fra1 and Fra2), Maf (c-Maf, MafB, MafA, MafG/F/K and Nrl) and ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2) sub-families. MAPK are implicated in AP-1 activation as well as the expression of AP-1 components (Pramanik et al. 2003). A previous report by Kristof and colleagues showed that chemical inhibition of p38 and ERK decreased LPS/IFN γ -induced AP-1 binding to the *iNOS* promoter (Kristof et al. 2001). The function of p38 in regulating AP-1 activity varies between p38 isoforms as p38 β appears to induce whereas p38 δ and p38 γ seems to inhibit or have no effect on AP-1-induced luciferase activity in human breast cancer cells (Pramanik et al. 2003). A subsequent study revealed that the activation of c-Jun and ATF-2 under genotoxic stress was inhibited by JNK inhibitors or JNK-specific siRNA in human breast cancer cells (Hayakawa et al. 2004).

cJun is regarded as one of the most important transcription activators as its deficiency leads to embryonic lethality in mice (Jochum et al. 2001). c-Jun/ATF-2 heterodimers have been reported to have a high affinity to the CRE/ATF site in the *TNF* promoter (Hayakawa et al. 2004) (**Figure 1.3**). Deletion of both Ets and CRE/ATF sites completely abolishes cJun-dependent trans-activation of the human *TNF* promoter (Kramer et al. 1995). It has been suggested recently that cJun homodimers bound to the TPA motif form a scaffold for a gene co-repressor complex which consists of the nuclear receptor corepressor (NCoR). NCoR together with other associated proteins (TBL1, transducin β -like protein 1; TBLR1, TBL1-related protein) actively represses gene transcription by cooperating with histone deacetylase (HDAC)³, an enzyme associated with inactive chromatin. TPA treatment of macrophages leads to c-Jun phosphorylation on Serine 63 and 73 resulting in the release of the co-repressor complex. De-repression of cJun homodimers facilitates the exchange of one cJun subunits into the cFos subunit. Newly formed cJun/cFos heterodimer attracts a co-activator complex comprising histone acetyl transferases (HATs) allowing gene transcription (Ogawa et al. 2004) (**Figure 1.6**). The process of NCoR clearance from cJun homodimers appears to be p65-dependent but JNK-independent in TLR signalling. Specifically, upon LPS stimulation, p65 is phosphorylated on Serine 536 to enable p65 transactivation of target genes. When bound to the *iNOS* promoter, p65 acts as a courier for IKK ϵ ,

an enzyme able to phosphorylate cJun and as a consequence to initiate NCoR clearance (Huang et al. 2009).

The role of AP-1 in inflammation is inconclusive. Cells lacking cJun or JNK demonstrate largely impaired production of monocyte chemotactic protein-1 (MCP-1) upon IL-1R stimulation probably caused by the decreased recruitment of NF κ B and Pol II to the MCP-1 gene, *Ccl2*, as well as by deficient histone modifications (Wolter et al. 2008). Downregulation of ATF-2, on the other hand, decreases LPS-induced expression of *Socs-3* in murine macrophages (Hirose et al. 2009). SOCS-3 has been described as a negative regulator of TLR4 signalling as stable transfection of *Socs-3* resulted in an inhibition of NO, TNF, IL-6, and GM-CSF release from LPS-treated murine macrophages (Berlato et al. 2002). JunB has been also reported to bind to the promoters of inflammatory genes. DCs subjected to JunB knockdown exhibit decreased

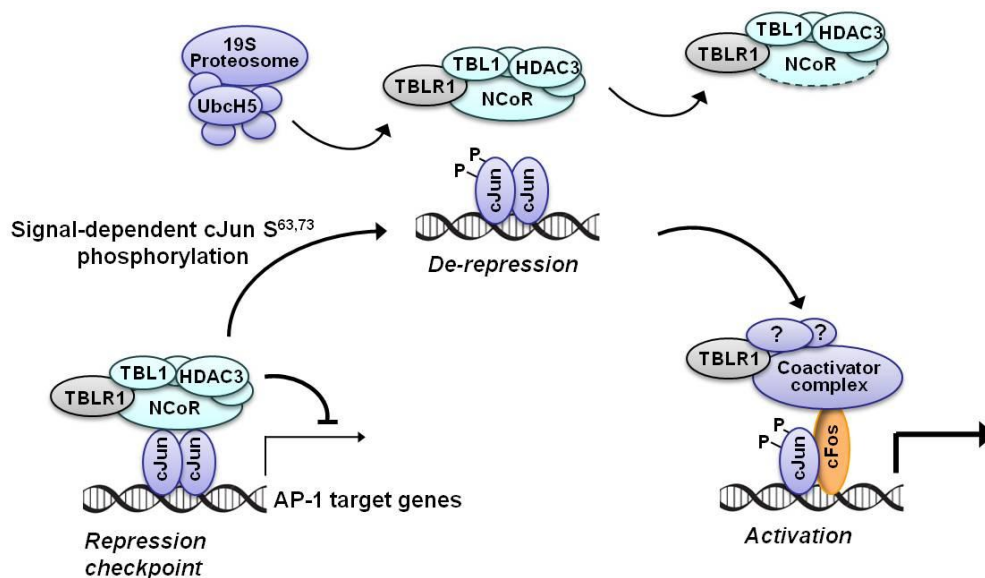


Figure 1.6 Model of de-repression of AP-1 target genes. In basal conditions, transcription of AP-1 target genes is inhibited by cJun homodimers which attract a repression complex comprising NCoR, TBL1, TBLR1 and HDAC3. Upon stimulation cJun is phosphorylated on Serine 63 and 73 resulting in TBLR1-dependent recruitment of ubiquitin noncovalent complex Ubch5, which ultimately leads to proteosomal degradation of NCoR and the release of the repression complex. cFos is then recruited to AP-1 motifs substituting one of the cJun components. cJun/cFos heterodimer binds a coactivator complex which subsequently leads to gene transcription. HDAC, histone deacetylase; TBL1, transducin β -like protein 1; TBLR1, TBL1-related protein; NCoR, nuclear receptor corepressor. Adapted from (Ogawa et al. 2004).

transcription activation of the *TNF*, *IL-6* and *IL12p40* genes upon LPS stimulation (Gomard et al. 2010) suggesting that JunB promotes inflammatory response. In contrast, *JunB/ cJun* double knockout mice develop psoriasis-like phenotype also characterised by elevated expression of *Tnf*, *Il1* and *Il6* in epidermis (Schonthaler et al. 2009) indicating that these two AP-1 components may have anti-inflammatory properties.

1.2.8 Induction of primary and secondary response genes by LPS

The recruitment of transcription factors to DNA initiates the transcription of the genes. The first step involves the binding of Pol II and chromatin modifiers. The association of chromatin-remodelling complexes reveals critical regulatory DNA regions previously restricted by tight histone-DNA assemblies. Transcription factors also recruit histone-modifying enzymes including HATs and HDACs. The histone post-transcriptional modifications such as methylation and acetylation provide a docking site for additional components of the transcriptional complex (Medzhitov and Horng 2009).

Classical model of transcription by Pol II involves initiation, elongation and termination. The initiation occurs when Pol II is recruited to the gene and when it is phosphorylated by TFIIF on Serine 5 (Ser5) of the C-terminal domain (CTD). During initiation, Pol II produces short mRNA but halts after few dozen base pairs from the transcription start site. The second phosphorylation of Serine 2 (Ser2) of the CTD mediated by the P-TEFb complex derepresses Pol II allowing the elongation event (Medzhitov and Horng 2009). Fully activated Pol II is also required for mRNA maturation as the recruitment of polyadenylation factors to the 3' termini of mRNA is impaired in the absence of Ser2 phosphorylation (Ahn et al. 2004).

LPS induces the expression of hundreds of genes which can be divided into primary response genes (PRGs) and secondary response genes (SRGs). PRGs such as *TNF*, *JUNB* and *FOS* are not dependent on novel protein synthesis as a protein synthesis inhibitor, cyclohexamide, does not affect their expression. These genes also do not require chromatin remodelling thus as a consequence they are induced within an hour of stimulation. The expression of PRGs is regulated by co-repressor complexes containing NCoR and HDAC3 which are recruited to NFκB and AP-1 sites through transcription factors p50/p50 and cJun. The induction of SRGs such as *IL6*, on the other hand, is largely delayed as they rely on *de novo* protein synthesis and chromatin modifications. The regulation of SRGs expression appears to be independent of co-repressor

complexes as NCoR is not recruited to these promoters. One possible explanation is that closed chromatin may limit the access of transcription factors to the SRGs (Ogawa et al. 2004; Ramirez-Carrozzi et al. 2006; Hargreaves et al. 2009).

The promoters of PRGs rich in guanine and cytosine have been demonstrated to possess high levels of histone H3 modifications including H3K4me3 and H3K9Ac which are associated with active chromatin. In addition, Ser5-phosphorylated Pol II is preassembled to many PRGs prior to LPS stimulation. This modified Pol II is able to produce full-length unspliced mRNA that is rapidly degraded. The engagement of TLR4 signalling leads to P-TEFb-mediated phosphorylation of Pol II on Ser2 what results in the production of mature mRNA (Hargreaves et al. 2009). This complex mechanism of gene expression allows rapid production of inflammatory mediators upon pathogen recognition. These mediators are required for pathogen clearance and activation of adaptive immunity. The induction of inflammatory genes is highly sensitive but at the same time is tightly regulated as multiple signals are required for effective gene transcription (**Figure 1.7**).

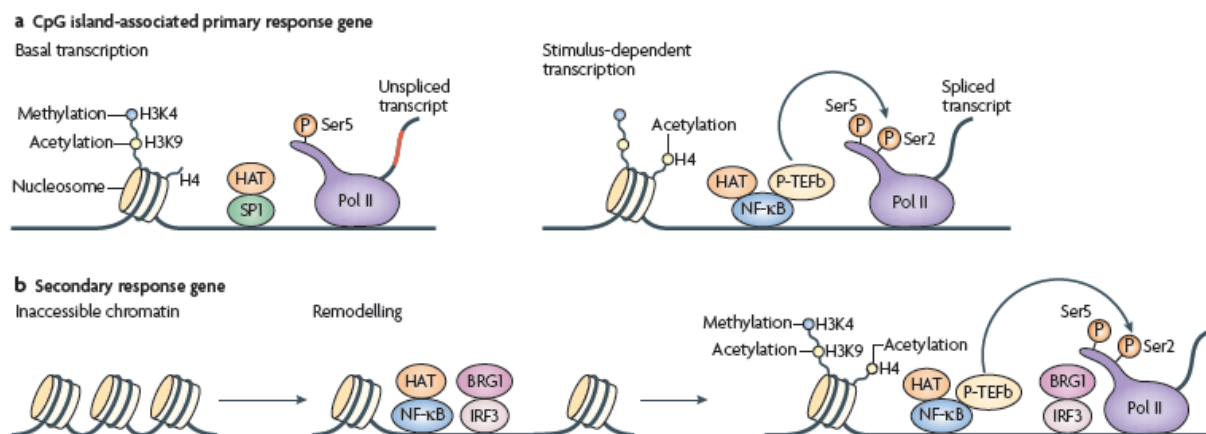


Figure 1.7 Model of inflammatory gene induction by LPS. The promoters of primary response genes (a) rich in CpG islands are characterised by the presence of H3K4me3, H3K9Ac and Serine 5- phosphorylated Pol II. Transcription factors such as Sp1 form a scaffold for HAT. In basal conditions only unspliced transcript is produced. However, upon LPS stimulation Pol II is phosphorylated on Serine 2 by P-TEFb what allows the production of mature transcript. In contrast, secondary response genes (b) require chromatin remodelling to allow transcription factor and HAT binding. Pol II, RNA Polymerase II; P-TEFb, positive transcription elongation factor b; Ser, Serine; HAT, histone acetyltransferase; Sp1, specificity protein 1, NFκB, nuclear factor κB. Adapted from (Hargreaves et al. 2009; Medzhitov and Horng 2009).

1.3 TLRs in immune tolerance and autoimmunity

Apart from playing a central role in host defence against danger, TLR activation has been implicated in the pathogenesis of many autoimmune diseases including systemic lupus erythematosus, inflammatory bowel disease, rheumatoid arthritis, type I diabetes, and multiple sclerosis. Autoimmunity is a failure of the immune system to remain unresponsive to self-antigens and non-infectious foreign antigens. Tolerance to self-antigens can be induced in lymphoid organs where immature T and B lymphocytes differentiate into mature cells, or in peripheral tissue. Immature lymphocytes which react to self-antigens undergo apoptosis, change their receptor specificity (B cells) or develop into Tregs (CD4⁺ T cells). Alternatively, some self-reactive immature lymphocytes can escape the negative selection or reprogramming and complete the maturation process in peripheral tissue. These cells are then inactivated in a process called anergy, undergo apoptosis or are inhibited by Tregs. Deficiency in FoxP3, a key regulator of Tregs, leads to the development of auto-reactive (autoimmune) diseases in humans (Gambineri et al. 2003; Stephens et al. 2005). Similarly, defects in apoptotic signalling, such as dominant mutations in the Fas death receptor pathway, leads to dysregulation of self-tolerance (Fisher et al. 1995). The principal factors in the development of autoimmune diseases are gene mutations and environmental stress. Exogenous and endogenous ligands may modulate TLR responsiveness and induce abnormal activation of TLRs under pathophysiological conditions. TLR stimulation could lead to the activation of innate immunity as well as tissue injury resulting in increased production of co-stimulators and cytokines by tissue APCs. These inflammatory conditions may break T cell anergy and promote the activation of self-reactive lymphocytes. Multiple mutations, inherited and somatic, may be needed to bypass multistep tolerance checkpoints in lymphocytes. Mutations associated with MHC genes (HLA-DR alleles) are the most common ones, however, other deficiencies have been also increasingly identified in recent years (Goodnow 2007). Examples of autoimmune diseases are presented below.

1.3.1 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease affecting 40-200 per 100,000 individuals with a 15-year survival rate of 80%. The cause of lupus is unknown but since 90% of cases occurs in women it was suggested that female hormones or genes of the X

chromosome may be implicated (Rahman and Isenberg 2008). Mutations in the MHC genes (HLA-DR2, HLA-DR3) appear to increase chance of SLE development when triggered by environmental factors. But also mutations of IRF5, programmed death 1 (PD1) receptor or complement deficiencies C1Q, C2, C4 have been shown to increase a person's chance of developing lupus (Gregersen and Behrens 2006). SLE is a Type III hypersensitivity reaction caused by antibodies reacting with self dsDNA released from the patient's apoptotic cells. Self dsDNA antibodies are present in serum of 70% patients with lupus distinguishing them from healthy individuals or RA patients and are likely to be generated as a consequence of impaired clearance of dying cells by phagocytes (Gaipal et al. 2007). It still remains unclear what activates autoimmune B cells. Endosomal TLRs, which recognise nucleic acids, have been proposed to initiate the inflammatory response resulting in the disease progression. In particular, as observed in lupus-prone MRL^{lpr/lpr} mice, the deficiency in TLR9 dramatically decreased the production of anti-dsDNA auto-antibodies (Christensen et al. 2005). In contrast to this observation, generation of *Tlr9*^{-/-} mice on different genetic background resulted in increased anti-DNA antibody production (Wu and Peng 2006). Similarly to the mice studies, the role of TLR9 in human SLE is inconclusive as the association of *TLR9* gene polymorphisms with the disease varied between studies (Hur et al. 2005; Ng et al. 2005). On the other hand, stop codon polymorphism in *TLR5* has been linked to the resistance to SLE in humans. PBMCs carrying truncated TLR5 showed impaired production of pro-inflammatory cytokines. Furthermore, this truncated TLR5 allele was preferentially transmitted to offspring who were not affected by SLE (Hawn et al. 2005). The ligand for TLR5 in SLE, whether exogenous or endogenous, has not been identified yet. T cell-mediated immunity also plays a crucial role in the pathogenesis of lupus. Enhanced antigen presenting capacity on class II MHC molecules on macrophages, DCs and B cells together with co-stimulatory signal such as CD40-CD40 ligand and CD28-B7 interactions activate auto-reactive T cells which in turn induce effector functions in phagocytes and B cells. Therefore, inhibition of CD40 ligand or a CD28-B7 contact is a potential treatment for lupus. Other drugs that are currently being evaluated in clinical trials target B cell receptors or B cell-inducing cytokines (Rahman and Isenberg 2008).

1.3.2 Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) including two major clinical forms; Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammatory condition of gastrointestinal track highly linked

to intestinal infections in a genetically susceptible host. The disease mainly affects individuals at 15 to 30 years of age. Cigarette smoking has been associated with increased risk of CD but paradoxically former smokers or non-smokers are at greater risk of UC indicating that these two forms largely differ in the disease pathogenesis and course. Genes linked to IBD include nucleotide oligomerization domain 2 (NOD2) encoding intracellular PRR, components of interleukin-23-type Th17 pathway and autophagy. No specific pathogen has been identified to drive inflammation in IBD although an antibiotics treatment appears to ameliorate the disease symptoms. Rather, antigens that are normally present in the gut trigger the inflammation. In healthy individuals, an array of pro-inflammatory cytokines limits the entry of pathogens into the mucosa whereas anti-inflammatory mediators such as TGF- β and IL-10 regulate immune responses preventing extensive tissue damage and prolonged inflammation. The healthy mucosa is also characterised by the homeostasis between Tregs and effector T cells (Th1, Th2 and Th17). In contrast, the gut in IBD shows an increased number of APCs that secrete pro-inflammatory cytokines. Enhanced levels of TNF, IL-1 β , IL-6, IL-12 has been associated with over-activation of NF κ B, a mediator in the NOD2 pathway. The inhibition of pro-inflammatory cytokines, particularly TNF, is currently the most efficacious treatment for IBD (Kaser et al. 2010). The role of TLRs in IBD has not been fully elucidated. A few researchers have demonstrated the association of TLR polymorphisms to human IBD. TLR defects may alter receptor-mediated ligand recognition and mucosal immune tolerance leading to hypo- or hyperactivity. For instance, the TLR1-R80T and TLR2-R743Q polymorphisms appear to increase the risk of developing pancolitis in UC patients (Pierik et al. 2006). Apart from affecting the disease phenotype, none of these polymorphisms affected the susceptibility to IBD. In contrast, carriers of the TLR4-D299G allele, one of the most common TLR4 mutation that leads to impaired LPS signalling and increased susceptibility to infections, also demonstrated increased risk of developing CD and UC (Franchimont et al. 2004).

1.3.3 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic disorder mainly affecting synovial joints but in 15% of patients, also causing symptoms such as lung fibrosis, renal amyloidosis, atherosclerosis and osteoporosis. Because the joint inflammation (synovitis) ultimately leads to joint destruction, deformation and loss of function, RA patients suffer from chronic pain and stiffness in joints, swelling and tenderness. The disease incidence is 1% of the global population with a higher

prevalence in older women. Although the cause of RA remains to be elucidated, it is clear that genetic factors and environmental factors such as smoking are involved. The disease has been associated with HLA-DR4 alleles and other loci including protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*), cytotoxic T-lymphocyte antigen 4 (*CTLA4*), Fc receptor for IgG (*FcγRs*) or various cytokines (TNF, IL-1, IL-10, IL-8) (McInnes and Schett 2007). Normal healthy synovial joint consists of articular capsule, which is a fibrous, avascular layer that surrounds the synovial cavity, an underlying synovial membrane inhabited mainly by fibroblasts and macrophages and cartilage that covers joint ends of bone. In pathological conditions such as RA, the synovial membrane becomes inflamed due to the massive infiltration of immune cells into joints forming a destructive tissue called the pannus. In early RA, the synovial membrane expands due to the proliferation of synovial cells and the formation of new blood vessels occurs. Angiogenesis together with enhanced expression of adhesion molecules on endothelium and the production of chemokines by resident synovial cells enables the recruitment of monocytes, T cells and B cells from the blood circulation to the synovial cavity. Monocytes mature into macrophages, become activated and produce pro-inflammatory cytokines which in turn promote a positive feedback loop and facilitates the recruitment of new immune cells. During the progression of the disease inflammatory tissue starts to invade cartilage and destroys bone (Choy and Panayi 2001).

1.3.3.1 Targeting immune cells as a treatment strategy for RA

Various cell types of the innate and adaptive immune systems are implicated in the initiation and progression of RA. Along with X-ray analysis of bone deformation, testing for the presence of rheumatoid factor (RF), an autoantibody specific for IgG Fc, in the blood is used for diagnosis. RF, however, is often absent during the first years of illness suggesting that B cells are an important component of the inflammatory response within the joints but are not necessarily the predominant cells responsible for the initiation of disease. More recently, immunoassays for the detection of autoantibodies directed against citrullinated proteins are widely used for diagnostic purposes. The presence of citrullinated proteins correlates with collagen-induced arthritis (CIA) severity and has been linked to the pathogenesis of RA (Lundberg et al. 2005). The significance of B cells in RA is further supported by the fact that the B cell specific drug Rituximab shows a benefit in 80% of subjects (Edwards et al. 2004). Rituximab is a chimeric IgG1 monoclonal antibody which binds to the CD20 molecule of B cells. The binding of the antibody initiates antibody-dependent cell-mediated cytotoxicity leading to B cell depletion (Uchida et al. 2004).

A correlation between RA and the HLA-DR4 allele (class II MHC genes), which is responsible for antigen presentation to CD4⁺ T cells, suggests that T lymphocytes also play an important role in the pathogenesis of RA. The activation of T cells influences the effector and inhibitory functions of macrophages. T cells require two signals for activation. The first one is the TCR-mediated recognition of antigens presented on class II MHC molecules on APCs. The second one is the binding of a costimulatory receptor on T cells such as CD28 or cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) to a ligand on APC such as CD80 (B7-1) or CD86 (B7-2). The inhibition of CD80 and CD86 by CTLA4Ig significantly improves the signs and symptoms of RA (Kremer et al. 2003).

Mast cells, the residents of inflamed joints, are also being recognised as an important enhancer of chronic inflammation as they induce vascular permeability, leukocyte recruitment and activation of synovial fibroblasts and macrophages (Nigrovic and Lee 2007). In response to IL-33, mast cells produce a plethora of pro-inflammatory cytokines and chemokines. The stimulation of wild type mice with IL-33 led to acceleration of CIA indicating that activated mast cells significantly contribute to the development of chronic inflammation and that targeting IL-33 may be a promising treatment strategy for RA (Xu et al. 2008).

Macrophages, the representatives of the innate immune response, are involved in both the initiation and perpetuation of RA-associated inflammation because they directly respond to injurious stimuli via PRRs. The outcome of this stimulation is the secretion of pro-inflammatory cytokines whose inhibition has proven effective in the treatment of RA (Suresh 2010). Macrophages are abundant in inflamed synovial membrane. The severity of pain and radiological joint destruction in RA correlates with the macrophage infiltration into the synovium (Mulherin et al. 1996). Furthermore, it has been shown that conventional anti-rheumatic drugs affect the effector functions of macrophages. For instance, methotrexate impairs monocyte chemotaxis and enhances the production of cytokine inhibitors whereas corticosteroids downregulate the expression of TNF, IL-1 and IL-6 (Kinne et al. 2000). Together with fibroblast-like synoviocytes (FLSs), macrophages activate adjacent cells and promote inflammation. FLSs secrete chemokines (IL-8) and cytokines (IL-6 and GM-CSF) which activate macrophages creating a positive feedback as macrophages in response to this stimulation produce FSL-activating cytokines, specifically TNF, IL-1 and IL-18 (Sweeney and Firestein 2004). Except for the interactions with FLSs, macrophages increase the proliferation of endothelial cells and therefore promote

angiogenesis, activate mast cells and neutrophils and promote the differentiation of naïve T cells into Th1/ 17 lineages. Macrophages are also closely related to bone degrading osteoclasts as both cell types have common precursor cells, monocytes (McInnes and Schett 2007). A monoclonal antibody, Denosumab, which targets receptor activator of nuclear factor- κ B ligand (RANKL), the osteoclast differentiation factor, has recently been approved for the treatment of RA. The importance of macrophages and the network with other cell types important in RA is presented in **Figure 1.8**.

1.3.3.2 Cytokines drive inflammation in RA

Cytokines, soluble mediators of inflammation, play a significant role in the chronic inflammation observed in RA. Synovial fluid and supernatants derived from whole synovial membrane cultures contain significant amounts of pro-inflammatory cytokines such as TNF, IL-1 and IL-6 as well as anti-inflammatory cytokines such as IL-10 and TGF β . However, despite the over-production of immunoregulatory mediators, the levels of IL-10 or TGF β appear to be unable to neutralise all the secreted TNF and IL-1 (Feldmann et al. 1996). TNF, which is produced mainly by monocytes, macrophages, lymphocytes and fibroblasts, is believed to be a pivotal cytokine that controls the production of both pro-inflammatory mediators (MCP1, IL-8, IL-6, GM-CSF) as well as anti-inflammatory mediators (IL-10, IL-1Ra, soluble TNFR, IL-11) (Butler et al. 1995) (Feldmann 2002). Moreover, TNF facilitates immune cell infiltration into the joint by stimulating endothelial cells to express adhesion molecules, it also decreases collagen synthesis by fibroblasts, elevates the production of MMPs, plus it activates monocytes, neutrophils and lymphocytes (McInnes and Schett 2007). Another important function of TNF is the promotion of bone erosion by acting on osteoclast differentiation and activation. Osteoclast maturation from precursor cells requires two main signals; M-CSF and RANKL. TNF induces the production of these stimulants by synovial mesenchymal cells and T cells (both M-CSF and RANKL) as well as synovial fibroblasts (RANKL). IL-1, IL-6 and IL-17 are also known to induce the expression of RANKL (Kwan Tat et al. 2004; Horwood 2008). In 1998, infliximab (Remicade; Centocor), a chimeric monoclonal antibody directed against TNF was introduced for the treatment of RA. Infliximab, despite being a humanized antibody, does not initiate the development of neutralizing antibodies and can be used for many years in therapy. Anti-TNF therapy inhibits IL-1 dependent bone and cartilage destruction, decreases the production of cytokines and chemokines in joints as well as angiogenesis. Infliximab is successful in around 60% of cases (80% if the treatment is combined with the use of methotrexate) (Feldmann 2002). The approval of this drug stimulated interest in

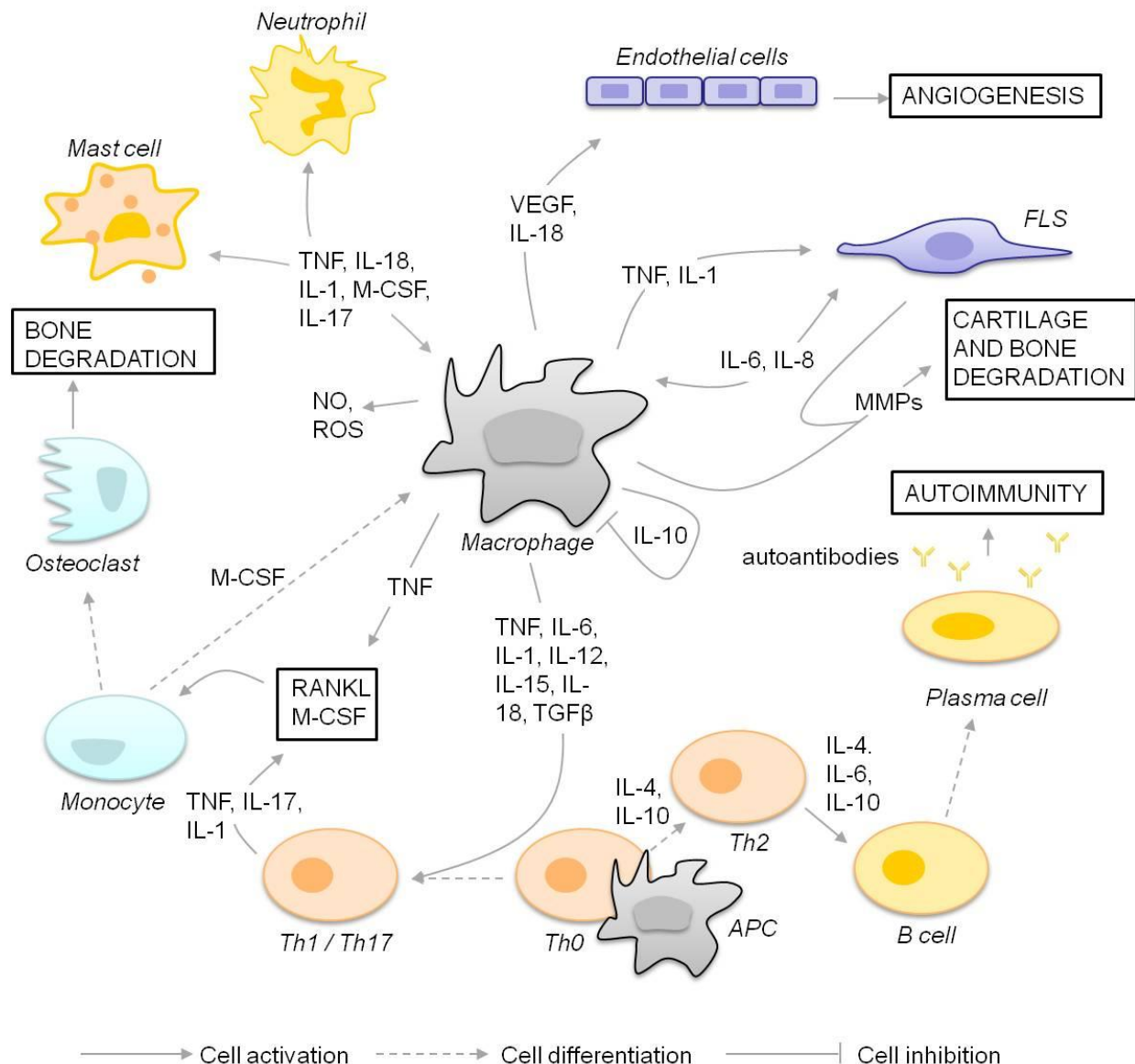


Figure 1.8 Macrophage interactions and cytokine expression in RA. Monocyte-derived macrophages are central components in RA as upon activation they secrete multiple proinflammatory mediators which increase endothelial cells proliferation and therefore promote angiogenesis, activate FLS to secrete MMPs responsible for cartilage and bone degradation, promote the polarisation of naive CD4⁺ T cells into the Th1 and Th17 subsets, or activate mast cells and neutrophils. TNF is a crucial cytokine that mediates macrophage effector functions. Among multiple roles, TNF triggers RANKL and M-CSF production, the factors which are required for monocyte differentiation into bone-degrading osteoclasts. Activated macrophages also secrete reactive oxygen species (ROS), nitric oxide (NO) which have microbicidal as well as tissue damaging activities. Macrophages can act as APCs to T cells. Cytokines secreted by macrophages also promote B cells proliferation and antibody secretion. IL, interleukin; VEGF, vascular endothelial growth factor; TNF, tumor-necrosis factor; FLS, fibroblast-like synoviocytes; MMP, matrix metalloproteinase; TGF- β , transforming growth factor- β ; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor- κ B (RANK) ligand; Th, helper T cell; NO, nitric oxide; ROS, reactive oxygen species (Sweeney and Firestein 2004; McInnes and Schett 2007; Horwood 2008).

the development of a new generation of immunomodulatory drugs, or biologics, that inhibit inflammatory cytokines. Other commonly used drugs include TNF inhibitors; Etanercept (Enbrel), Adalimumab (Humira), the IL-1 inhibitor Anakinra (Kineret) and the IL-6 inhibitor Tocilizumab (Suresh 2010).

IL-1 is secreted predominantly by activated macrophages, but also by synovial fibroblasts and activated T cells. In addition to its function in osteoclast maturation, IL-1 seems to play a major role in cartilage degradation as injection of antibodies directed against IL-1 reduced cartilage degradation in a rabbit model of RA (Joosten et al. 1996). It is now known that IL-1 β inhibits chondrocyte-dependent matrix synthesis and promotes matrix degradation by MMPs (Eberhardt et al. 2000). Depletion of IL-18, another member of IL-1 superfamily, also proved successful in ameliorating CIA in mice (Plater-Zyberk et al. 2001). IL-18 stimulates naive T cells, NK cell and neutrophil activation and is pro-angiogenic for endothelial cells (McInnes and Schett 2007).

A potential role for IL-6 in RA was suggested after the discovery that *Il6* knockout mice are resistant to antigen-induced arthritis; these mice do not develop joint swelling upon injection with methylated bovine serum albumin (Boe et al. 1999). Similarly, when arthritis was induced by type II collagen (CII), IL-6-deficient DBA/1J mice showed delayed onset and less severe disease accompanied by decreased production of anti-CII antibody and fewer bone erosions (Alonzi et al. 1998; Sasai et al. 1999). The main role of IL-6 in RA is to stimulate the production of acute phase proteins from the liver. Acute phase proteins induce pro-inflammatory cytokines, activate the complement system, mediate fever and induce neutrophil chemotaxis. IL-6 itself also stimulates lymphocyte migration by upregulating chemokine and adhesion molecule expression by endothelial cells. IL-6 is required for B cell proliferation and antibody production as well as T cell proliferation, differentiation and cytotoxicity. IL-6 can activate macrophage differentiation and induces MMP production leading to joint and cartilage degradation (McInnes and Schett 2007). Moreover, soluble IL-6R together with IL-6 activates osteoclast formation and excessive production of these factors may contribute to bone resorption (Tamura et al. 1993). Inhibition of IL-6 by Tocilizumab, a humanized monoclonal antibody specific for IL-6 receptor, suppresses disease activity and erosive progression in RA patients (Vitale et al. 2004).

The anti-inflammatory cytokine, IL-10, is a potent inhibitor of inflammation as it blocks the secretion of several cytokines, including TNF and IL-1, as well as the release of NO. IL-10 also downregulates class II MHC molecule expression on macrophages leading to T cell anergy,

decreases MMP and collagen release from synovial fibroblasts as well as induces B cell isotype switching (McInnes and Schett 2007). The importance of IL-10 has been shown *in vivo* as mice lacking IL-10 demonstrated more severe arthritis than wild type counterparts (Finnegan et al. 2003). IL-10 is present in the synovial fluid of RA patients but in concentrations that are insufficient to regulate the chronic inflammation. Because of strong immunomodulatory properties, IL-10 is considered to have a therapeutic role in RA. Unfortunately, the first clinical trials showed that RA patients did not exhibit clinical improvements when treated with exogenous IL-10 (Smeets et al. 1999).

1.3.3.4 Evidence for a role for TLRs in RA

Despite the role of cytokines in the pathology of RA, the evidence supporting the contribution of TLRs in the initiation or/and perpetuation of chronic inflammation in RA is limited. Expression of TLRs 2, 3, 4, 7 and 8 is elevated in the synovial tissue of RA patients in comparison to healthy donors (Radstake et al. 2004; Roelofs et al. 2005; Brentano et al. 2005a). Bacterial and viral infections have been proposed to contribute to the development of RA. However, no infectious pathogens have been identified and, furthermore, bacterial TLR ligands are detected both in human RA synovium and in healthy donors who exhibit no inflammatory response (Mackenzie 2005). This may be due to the fact that the pathogens could no longer be detected when the patient develops RA symptoms. Alternatively, it has been proposed that endogenous TLR ligands may instead contribute to the development of RA as a wide range of these ligands, including hyaluronan, fibronectin, DNA, HMGB-1 and Tenascin-C has been detected in RA joints (Clanchy and Sacre 2010).

Studies carried out at the Kennedy Institute revealed that TLRs 2 and 4 are present and functional in synovial membrane cells derived from RA patients and that their activation promotes inflammation in RA (Sacre et al. 2007a). Our group has subsequently demonstrated that the inhibition of MyD88 and Mal by dominant negative resulted in the blockage of TNF α , IL-6, IL-8, VEGF and MMPs spontaneous production from RA-derived cells (Sacre et al. 2007b). TLR2 has been reported to be the mediator of streptococcal cell wall (SCW)-induced joint inflammation as TLR2-deficient mice did not exhibit joint swelling or impaired cartilage matrix synthesis (Joosten et al. 2003). Similarly, an endogenous TLR4 ligand, Tenascin-C induced joint inflammation in

mice. Tenascin-C- deficient mice, on the other hand, were resistant to joint inflammation and tissue destruction during CIA (Midwood et al. 2009). The role of TLR4 in arthritis was further supported by the finding that an antagonist for TLR4 reduced disease progression in the CIA model (Abdollahi-Roodsaz et al. 2007). Interestingly, blocking TLR2 and 4 with specific antibodies did not suppress the spontaneous secretion of IL-6, one of the major mediators of inflammation, from RA synovial cultures suggesting that other TLRs may contribute to RA progression (Sacre et al. 2007b).

Observations from several groups have implicated the importance of endosomal TLRs. In particular, the inhibition of these TLRs by chloroquine, an inhibitor of endosomal acidification, or antidepressant mianserine, blocked spontaneous release of proinflammatory cytokines from RA synovial membrane cultures (Sacre et al. 2008). Moreover, patients lacking UNC93B1, a protein required for TLR3, 7, 8 and 9 delivery to endolysosomes, did not display autoreactive antibodies or develop autoimmunity (Isnardi et al. 2008). Additional analysis revealed a central role for TLR8 as the specific inhibition of TLR8 by imiquimod blocked spontaneous release of TNF from RA synovial cultures (Sacre et al. 2008). TNF is a pivotal cytokine in RA joints as its inhibition proved to be an effective treatment strategy for RA (Feldmann 2002). Furthermore, RNA released by necrotic synovial fluid cells derived from RA patients activated TLR3 on fibroblasts and resulted in the production of high levels of IFN- β , IP-10 and IL-6 (Brentano et al. 2005a). A recent study revealed that a widely used drug for the treatment of RA, Auranofin, apart from targeting TLR4 also inhibited TLR3 signalling by impairing PolyI:C-induced phosphorylation of IFR3 as well as IP-10 production (Park et al. 2010). Additionally, the administration of suppressive oligonucleotides that target TLR9 proved to be effective in ameliorating CIA symptoms in mice (Dong et al. 2004). Although these data support the possible role of endosomal TLRs in *in vitro* RA cultures, it still remains to be elucidated what could be a clinical advantage of blocking a particular TLR. Many of these studies are at the preclinical stage, however a few of them have already reached phase II clinical trials and could be soon available for the treatment of autoimmune diseases including multiple sclerosis, IBD, SLE or RA (Clanchy and Sacre 2010).

1.4 A role for Protein Tyrosine Kinases in TLR signalling

Protein tyrosine kinases (PTKs) have attracted an increasing amount of interest over past 20 years as they are clearly involved in the regulation of cytokine production in PRR signalling. PTKs form a group of enzymes which catalyse the transfer of a phosphate group from ATP to a tyrosine residue of a substrate. The group consists of 20 families of receptor and 10 families of non-receptor PTKs, representing a large proportion of the protein kinase complement of human genome, the kinome (Manning et al. 2002). First reports regarding the role of PTKs in PRR signalling showed that tyrosine phosphorylation is induced within 5 min in human monocytes and macrophages following LPS stimulation (Weinstein et al. 1991; Beaty et al. 1994). Tyrosine phosphorylation reached a maximum by 15 min and declined after 30-60 min. Treatment with Herbimycin A inhibited PTK activation as well as LPS-induced TNF production leading to the conclusion that PTKs are crucial components of TLR signalling (Weinstein et al. 1991; Mander et al. 1997). Since these initial observations four main non-receptor PTK families have been implicated in TLR signalling: the sarcoma (Src), tyrosine kinase expressed in hepatocellular carcinoma (Tec), spleen (Syk) and proline-rich (Pyk2) tyrosine kinase families.

Src family kinases

Src family tyrosine kinases (SFKs) form the largest group of non-receptor PTKs. The family consists of 9 members: Src, Fyn, Yes, Lck, Blk, Fgr, Hck, Yrk and Lyn, having distinct amino acid variations and expression patterns. For instance, Hck, Lyn and Fgr are predominantly expressed in myeloid cells, Lck in T cells, and Blk in B cells. SFKs share domain structures comprising of N-terminal unique domain which facilitates membrane localisation, then Src homology (SH) 3 domain, followed by SH2 domain and C-terminal SH1 or kinase domain (**Figure 1.9**). SFKs are retained in inactive state in the cytoplasm. This state is characterised by tyrosine phosphorylation of the kinase by COOH-terminal Src kinase (Csk) and subsequent folding of SFKs. Active SFKs are characterised by unfolded state. Activation of SFKs requires initial dephosphorylation of C-terminal Tyr499 residue followed by auto-phosphorylation of Tyr388 in the catalytic domain as well as Tyr29 in the unique domain (for the SFK Hck) (Johnson et al. 2000). Once activated, SFKs induce downstream signalling cascades by phosphorylating immunoreceptor tyrosine-based activating motifs (ITAMs), Tec kinases and Pyk2. SFKs are

involved in numerous signalling pathways including antigen receptor pathways in lymphocytes, integrin, cytokine and TLR pathways (Page et al. 2009).

SFKs are rapidly phosphorylated after TLR ligand binding (Beatty et al. 1994). LPS also induces the expression of SFKs in macrophages (Boulet et al. 1992). The importance of SFKs in regulating cytokine production was revealed through a specific inhibitor, a pyrazolo pyrimidine compound (PP2) which blocks the production of major pro- and anti-inflammatory cytokines (IL-6, TNF, IL-10, IP-10) from human primary macrophages induced with TLRs 1, 2, 4-8. Interestingly, SFKs do not seem to engage MAPKs or NFκB to control cytokine production. Rather, PP2 treatment decreases AP-1 binding to its consensus site suggesting that SFKs may induce cytokine expression by activating the transcription factor AP-1 (Smolinska et al. 2008).

Surprisingly, the deletion of Hck, Fgr and Lyn in mice did not affect LPS-induced cytokine production in macrophages (Meng and Lowell 1997). These researchers also claimed that only those three SFKs are expressed in murine macrophages. Although, this finding questioned the role of SFKs in LPS signalling it is now clear that distinct family members may have the ability to substitute for the absence of others. Three additional SFKs; Src, Yes and Fyn, have been since identified to be expressed in murine and human macrophages (Majeed et al. 2001; Smolinska et al. 2008). The potential functional redundancy among SFK family members can be explained by the fact that the kinases were depleted in the germ line providing time for the compensatory mechanisms to develop. For instance, the kinase activity of Lyn has been reported to be largely increased in Hck-deficient murine macrophages subjected to LPS stimulation (Lowell et al. 1994). Similarly, the expression of Fyn, Lyn, Yes and Syk, but not Src was found to be increased in *Hck*^{-/-} *Fgr*^{-/-} murine BMDM (Majeed et al. 2001). Temporary changes in protein levels may be more beneficial in assessing the role of a specific SFK. By using RNAi or adenovirus-mediated overexpression it has been shown that Hck is particularly important for the regulation of LPS-induced TNF production in murine or human macrophages (English et al. 1993), (Smolinska et al., manuscript submitted).

Syk

The Syk family consists of p72Syk and p70Zap. These proteins differ in structure from the Src family as they contain two tandem SH2 domains separated by interdomains (**Figure 1.9**). The tandem SH2 domains of Syk bind to ITAMs diphosphorylated by Src upon activation. In fact,

macrophages derived from *Hck/ Fgr/ Lyn* triple knockout mice demonstrated impaired ITAM phosphorylation and Syk activation (Fitzner-Attas et al. 2000). Syk has been shown to interact with ITAMs on TCR ζ , Fc ϵ R1 β and Fc ϵ R1 γ as well as to be involved in BCR signalling and FcR-mediated phagocytosis (Page et al. 2009).

Syk associates with TLRs 4 and 9 as well as Myd88 in an ITAM-independent manner. The kinase has been also shown to undergo phosphorylation upon LPS and CpG stimulation in a variety of cells including macrophages, neutrophils and fibroblasts (Page et al. 2009). In spite of that, the kinase does not seem to regulate cytokine production in these pathways as Syk-deficient macrophages produce normal amounts of TNF, IL-6, IL-1 or IL-12 in response to LPS, whereas Syk-deficient DCs show no impairment in IL-12 production in response to CpG (Crowley et al. 1997; Rogers et al. 2005). Instead, Syk appears to control chemokine production as Syk chemical inhibition or siRNA-mediated knockdown decreased the production of MCP-1 and RANTES in LPS-stimulated neutrophils and fibroblasts, respectively (Yamada et al. 2001; Arndt et al. 2004).

Pyk2

Pyk2 together with Focal adhesion kinase (FAK) form a family of proline-rich cytoplasmic tyrosine kinases. Both Pyk2 and FAK lack SH2 and SH3 domains. Rather, the kinase domain of Pyk2 is flanked by proline-rich FERM and FAT domains which mediate protein-protein interactions (**Figure 1.9**). Pyk2 is phosphorylated in monocytes and macrophages subjected to LPS stimulation (Williams and Ridley 2000). Furthermore, the inhibition of Src by PP2 abrogated TLR-induced phosphorylation of Pyk2 and its substrate, a cytoskeletal protein paxilin, in rat macrophages suggesting that Pyk2 and Src share the same signalling cascade (Hazeki et al. 2003). The role of Pyk2 in cytokine production is unclear. In human primary macrophages, the overexpression of Pyk2 or kinase dead version does not affect TNF, IL-6, IL-8 or IL-10 production following LPS stimulation (Horwood et al. 2003). In contrast, Pyk2 was required for LPS-induced IL-8 production in endothelial cells. In addition, the chemical inhibition of Pyk2 decreased p38 but not ERK MAPK phosphorylation in these cells indicating that Pyk2 is required for p38 MAPK activation in TLR signalling (Anand et al. 2008). The role of Pyk2 in regulating IL-8 production, the primary chemokine for neutrophils, suggests that Pyk2 is more likely to be involved in cellular migration than pro-inflammatory cytokine production. This hypothesis is also supported by the fact that Pyk2 is phosphorylated in response to MCP-1, one of the most potent chemokines to induce migration of monocytes. Furthermore, the inhibition of MCP-1-induced

Pyk2 phosphorylation by soluble chemokine fractalkine correlated with the inhibition of monocyte migration and impaired p38 MAPK activation (Vitale et al. 2004).

Tec family kinases – structure and function

The Tec family of tyrosine kinases is the second largest non-receptor tyrosine kinase family after SFKs. Tec family members were identified as tyrosine kinases involved in hepatocarcinogenesis (Mano et al. 1990) and 5 of them have been characterised in mammalian cells: **B**ruton's agammaglobulinemia **t**yrosine **k**inase (Btk), **b**one **m**arrow kinase gene on **X** chromosome (Bmx), **I**L-2 inducible **T**-cell **k**inase (Itk), **t**yrosine kinase **e**xpressed in hepatocellular **c**arcinoma (Tec) and **T** and **X** cell expressed **k**inase (Txk; also known as Rlk). Tec PTKs are generally expressed in hematopoietic cells but Tec, Bmx and Itk can be also found in other cell types. Btk is primarily expressed in B cells (but not in plasma cells), myeloid cells and platelets (de Weers et al. 1993; Smith et al. 1994) whereas Itk, Txk and Tec in T cells (August et al. 1994; Hu et al. 1995). The structure of Tec PTKs comprises five distinct domains (**Figure 1.9**). The N-terminal pleckstrin homology (PH) domain is responsible for membrane localisation; which is followed by the Tec homology domain which is unique for each Tec PTKs member, then by SH3 and SH2 domains which bind substrates and lastly by the kinase catalytic domain at the carboxyl tail. Exceptionally, PH domain- lacking Txk comprises a cysteine-string motif which enables anchoring the kinase to glycolipid enriched membranes (Debnath et al. 1999). Two tyrosine phosphorylation sites have been identified in the structure of Tec kinases. Phosphorylation of tyrosine 551, located in the activation loop of the catalytic domain, leads to conformational changes allowing further modifications. The second one is found in the SH3 domain of Tec kinases (tyrosine 223) and undergoes auto-phosphorylation (Park et al. 1996).

Tec PTKs are involved in multiple signalling cascades in response to the stimulation of G-protein-coupled receptors (GPCR), Fc receptors, integrins, antigen or cytokine receptors (Mohamed et al. 2009). The activation of Tec PTKs influences calcium (Ca^{2+}) response, actin remodelling, apoptosis, proliferation and gene expression. The involvement of Tec kinases in different signalling pathways is based on direct interaction with other proteins (**Figure 1.9**). For instance, it has been shown that the SH1 and PH domains of Tec PTKs interact with Fas, a death receptor involved in apoptosis. Proteins implicated in cytoskeletal reorganisation and, therefore, antigen presentation and processing such as WASP and Vav, also bind to Tec PTKs through its SH3 domain (Sharma et al. 2009). The TH domain of Tec PTKs directly interacts with the members of

the SFKs (Fyn, Hck and Lyn) whereas their SH2 domain binds to B cell receptor (BCR) signalling proteins BLNK (Mohamed et al. 2009).

The importance of Btk in the immune system was recognised after the discovery that point mutations in the *BTK* gene result in human XLA (X-linked agammaglobulinemia) (Thomas et al. 1993). Alternations affecting human *BTK* range from missense, nonsense, in-frame to frame-shift mutations that affect all Btk domains as well as the gene non-coding sequences. The number of alternations exceeds 550 unique mutations in more than 800 families. Arginine CpG codons (formed when cytosine is followed by guanine) are especially prone to mutations (Lindvall et al. 2005). Mutation of arginine (R) at position 28 to a cysteine (C) at position 28 in the PH domain leads to murine X-linked immunodeficiency (Xid) (Tsukada et al. 1993). Xid mice are characterised by a 50% reduction in their peripheral B cell population and reduced levels of IgM and IgG3. As a result of poor B cell responses, Xid mice are highly susceptible to bacterial infections (Khan et al. 1995). In humans, XLA results in more severe symptoms as patients have less than 1% of mature B cells that are present in a healthy individual (Campana et al. 1990). Moreover, double knockout of *Btk* and *Tec* in mice still gives a milder phenotype than in XLA patients suggesting Tec PTK diversity in function and organisation between different species (Ellmeier et al. 2000). Detailed analysis of the role of Btk in B cells revealed that the kinase is critical for the differentiation of pre-B cells into immature B cells as well as the differentiation of IgM^{high}IgD^{low} immature B cells into IgM^{low}IgD^{high} mature B cells. Btk is also critical for B cell proliferation and survival (Kerner et al. 1995; Khan et al. 1995; Hendriks et al. 1996).

Apart from being implicated in B cell signalling, Btk was also found to fulfil a crucial role in mast cell, erythrocyte, platelet, neutrophil, basophil and DC signalling networks (Schmidt et al. 2004). Mast cells are the key mediators of allergic reactions. Their activation, mainly through the high-affinity IgE receptor (FcεRI), leads to secretion of histamines, leukotrienes and cytokines. Btk deficiency was found to significantly reduce histamine secretion in activated murine mast cells. Both Btk and Tec were required for FcεRI-mediated TNF, IL-13 and GM-CSF production. However, in contrast to *Tec*^{-/-}-derived mast cells which exhibited decreased production of IL-4, *Btk*^{-/-}-derived cells produced increased levels of IL-4. Furthermore, Tec deficiency did not affect histamine production, but significantly impaired leukotriene C(4) levels indicating that the role of these two kinases in allergy may be dramatically different (Schmidt et al. 2009). Interestingly, activated basophils derived from *Btk* knockout mice also showed decreased production of histamine, once again confirming the role for Tec PTKs in allergic reactions (Kneidinger et al.

2008). Btk also plays a role in platelet activation by collagen. Effective platelet function is required for thrombus formation and cessation of bleeding after damage to the vasculature. Platelets derived from XLA patients demonstrated diminished aggregation, dense granule secretion and calcium mobilisation in response to collagen (Quek et al. 1998). The defects in platelet activation were further increased in double *Btk/ Tec* knockout mice as compared to *Btk*^{-/-} mice suggesting that Tec may also contribute to platelet signalling (Atkinson et al. 2003). The latest studies also describe the involvement of Btk in osteoclast differentiation and suggest a possible regulatory mechanism for osteoclastogenesis through a transcription factor NFATc1, initiation of which is mediated by RANKL (Lee et al. 2008; Shinohara et al. 2008). *Tec*^{-/-}*Btk*^{-/-} mice showed an increase in bone volume correlated with decreased osteoclast number and osteoclastic bone resorption (Shinohara et al. 2008). The impaired osteoclast bone resorption was also observed in XLA-derived cultured osteoclasts. However, XLA patients did not demonstrate increased bone density despite having defective *in vitro* osteoclast activation. Analysis of XLA patients' serum revealed that TNF, IL-6 and IL-1 levels were elevated in these patients and that stimulation of osteoclasts with these cytokines increased osteoclast activity *in vitro*, suggesting that elevated inflammatory cytokines in the XLA serum conceals defects in osteoclast function *in vivo* (Danks et al. 2011). Btk has been also involved in M-CSF receptor signalling pathway that regulate the survival of macrophages (Melcher et al. 2008) and FcγR-induced phagocytosis in macrophages (Jongstra-Bilen et al. 2008).

Tec PTKs also modulate T cell responses. T lymphocytes express Rlk, Itk and Tec, and Rlk/Itk deficiency has been found to show reduced TCR-mediated Ca²⁺ mobilisation, proliferation and cytokine production. Rlk and Itk were also required for the immune response to the infection of mice with the intracellular parasite, *Toxoplasma gondii* (Schaeffer et al. 1999). Itk is also involved in T cell development as Itk deficiency in mice decreased the number of mature T cells. Itk appears to be more critical for CD4⁺ T cell development as the lack of the kinase decreased the ratio of mature CD4⁺ versus CD8⁺ thymocytes in the homozygous mutant (Liao and Littman 1995). The study of the Itk function in T lymphocyte response to antigen recognition revealed that the kinase is rather involved in Ca²⁺ mobilisation than the formation of a stable T cell-APC synapse (Donnadieu et al. 2001). Increase in Ca²⁺ is concomitant to cytoskeleton reorganisation, the prerequisite for T cell adhesion and activation (Delon et al. 1998).

Tec PTKs are also important components of the signalling pathways used by many cytokines and growth factors. In particular, in the context of inflammation, Btk and Tec are known to be

involved in the IL-6 signalling pathway, by being constitutively associated with the gp130 molecule, a component of the IL-6 family receptor. The stimulation of gp130 with IL-6 leads to tyrosine phosphorylation of Btk and Tec kinases. The same investigators found that Tec is also activated by IL-3 and granulocyte colony-stimulating factor (G-CSF) suggesting a general role of Btk and Tec in supporting the growth and differentiation of immune cells (Matsuda et al. 1995). In addition, Bmx has been implicated in signalling via TNFR2 and VEGFR2 and to play an important role in TNF/VEGF-mediated inflammatory angiogenesis (Pan et al. 2002; He et al. 2006). *In vitro* experiments carried out on endothelial cells have shown that TNF stimulation of the cells results in the phosphorylation of Bmx whereas the introduction of dominant negative Bmx blocks TNF-induced angiogenesis, signifying a crucial role of Bmx in this developmental process (Pan et al. 2002).

Activation, regulation and cellular localisation of Btk

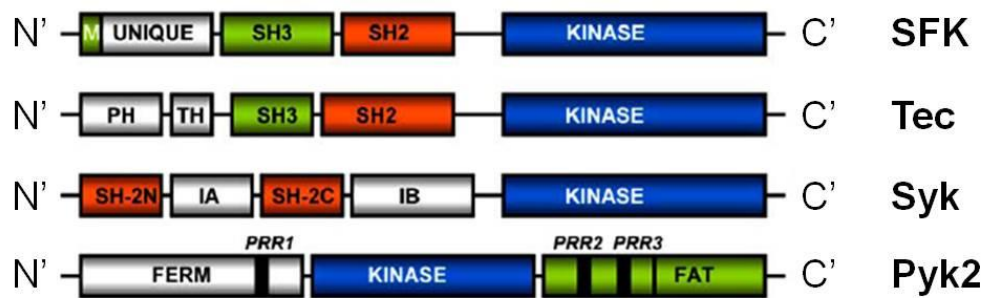
Multiple studies on B cells have shown that activation of Btk requires two steps. The first one is an association of the positively charged PH domain with negatively charged phosphate groups of phosphatidylinositol (3,4,5)-triphosphate (PIP₃); a phosphoinositide 3-kinase (PI-3K) product. This electrostatic interaction enables Tec PTKs to localise to the cell membrane and respond to the second signal, which requires the engagement with Src family kinases (Ferguson et al. 1995; Rawlings et al. 1996; Li et al. 1997). Src tyrosine kinases such as Lyn, Fyn and Hck are associated with Btk tyrosine kinase in B and T cells as assessed by means of the yeast two-hybrid system (Cheng et al. 1994). The interaction between Src kinases and Btk is mediated by the Src-homology domain (SH3) and leads to phosphorylation and activation of Btk under B cell receptor engagement (Cheng et al. 1994; Alexandropoulos et al. 1995; Wahl et al. 1997).

Although predominantly cytoplasmic, Btk has been reported to continuously shuttle between the cytosol and the nucleus. The molecular mechanisms of this translocation are, however, still unclear. Although, the PH domain of the kinase contains classical nuclear localization signals they do not seem to play a critical role in nuclear translocation as the PH deletion resulted in even distribution of Btk between cytosol and the nucleus. Nuclear shuttling also seems to be independent on the Btk kinase activity as demonstrated using kinase-defective mutant. On the other hand, an SH3-deleted mutant displayed impaired auto-kinase activity and was found

predominantly in the nucleus (Mohamed et al. 2000). The importance of the auto-phosphorylation event in nuclear shuttling can be also supported by the fact that mutation of tyrosine 223, but not tyrosine 551, retained Btk in the nucleus (Mohamed et al. 2009). The role of Btk in the nucleus remains to be elucidated. There is, however, increasing evidence that the kinase may be involved in transcriptional regulation by being implicated in transcription factor activation. For instance, Xid mice-derived B cells demonstrate impaired NF κ B signalling following BCR engagement (Petro et al. 2000). Btk has been also placed to be upstream of nuclear factor of activated T cells (NFAT) in BCR signalling (Hao et al. 2003). The kinase directly interacts with the general transcription factor TFII-I (also known as BAP-135). Upon BCR engagement, Btk phosphorylates TFII-I leading to nuclear translocation and gene transactivation (Yang and Desiderio 1997; Novina et al. 1999). Impaired production of antibodies by Xid mice-derived B cells could be explained by the fact that Btk interacts with Bright, the transcription factor that together with TFII-I initiates immunoglobulin heavy chain transcription (Webb et al. 2000; Rajaiya et al. 2006). The interaction between Btk and Bright does not seem to be dependent on Btk's catalytic function as Bright is not tyrosine phosphorylated by Btk (Webb et al. 2000). Finally, Btk has been shown to phosphorylate signal transducer and activator of transcription 5A (STAT5A) in the chicken B-cell line DT-40 and CREB during basic fibroblast growth factor (bFGF)-induced neuronal differentiation (Mahajan et al. 2001; Yang et al. 2004).

Btk expression and activity is often regulated by the factors that require Btk in order to function. For instance, the inhibition of NF κ B pathway suppresses *BTK* transcription. Further analysis revealed that both p65 and p50 NF κ B subunits bind to the *BTK* promoter where two functional NF κ B binding sites were identified (Yu et al. 2008). Moreover, Btk is negatively regulated by the transcriptional co-activator BAM11. At the same time, the kinase is required for BAM11-dependent induction of gene transcription in COS7 cells. Both PH domain and kinase activity of Btk as well as TFII-I were shown to enhance BAM11 transactivation activity (Hirano et al. 2004). Btk is also negatively regulated by protein kinase C (PKC) β which phosphorylates Btk on Serine 180 in the PH domain and disturbs Btk membrane binding in B cells (Kang et al. 2001). PKC θ , on the other hand, has been shown to activate Btk in platelets (Crosby and Poole 2002). Another protein inhibitor of Btk (IBtk) has been found to physically associate with the PH domain of Btk and to downregulate Btk kinase activity as well as Btk-mediated calcium mobilization and NF κ B transcription in B cells (Liu et al. 2001).

A)



B)

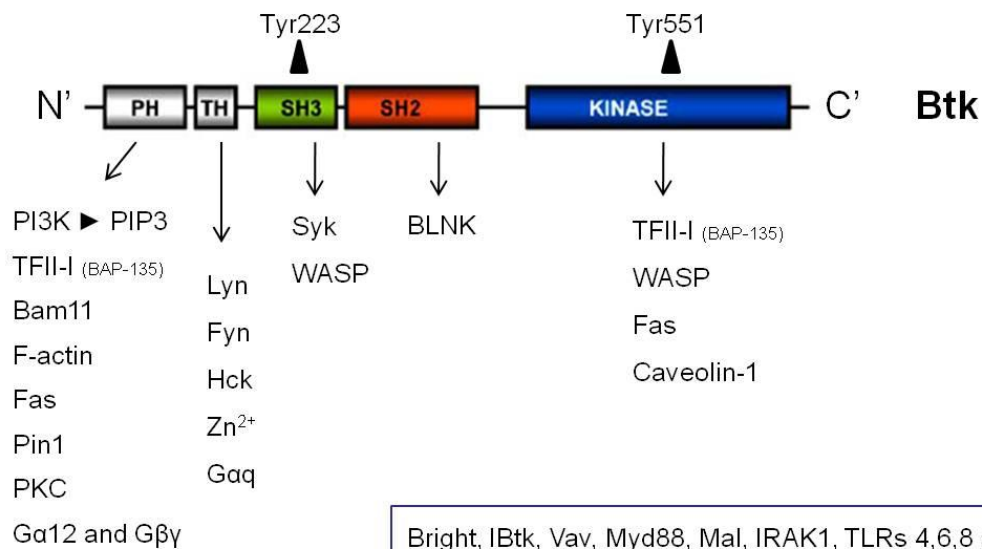


Figure 1.9 Schematic representation of domains of PTK families and Btk domains with known interacting/ regulating partners. The common feature of SFKs, Tec, Syk and Pyk2 is the kinase domain. Apart from that, SFKs and Tec share SH2 and SH3 domains. They differ, however, in the N' terminus as SFKs have a myristoylation site (M) in the unique domain, whereas Tec kinases have a membrane-associating pleckstrin homology (PH) domain and the family specific Tec homology (TH) domain. The SH2 domain of Syk is divided between interdomains A and B. The Pyk2 kinase domain is followed by two proline rich regions (PRR) and focal adhesion targeting (FAT) domains (A). Btk, a member of Tec family kinases, is activated by phosphorylation of tyrosine 551 and 223. Btk interacts with members of SFKs (Lyn, Fyn, Hck), requires Zn²⁺ binding for activity and stability, PIP3 for membrane localisation. Btk is involved in cytoskeleton reorganisation (interaction with F-actin, WASP and Vav), apoptosis (Fas), B-cell receptor signalling (BLNK), G-protein-coupled receptors or TLR. Btk activates transcription factors Bright and TFII-I as well as co-activators Bam11. At the same time, the kinase is regulated by PKC, Pin1, Caveolin-1 or IBtk (B). Not drawn to scale. Adapted from (Mohamed et al. 2009; Page et al. 2009).

1.5 A role for Tec tyrosine kinases in TLR signalling

The notion that Tec PTKs might be involved in TLR signalling was suggested by the previous finding that peritoneal macrophages derived from Xid mice exhibited reduced responses to LPS (Mukhopadhyay et al. 1999; Mukhopadhyay et al. 1999; Mukhopadhyay et al. 2002). Xid macrophages showed poor microbicidal activity, as well as produced reduced levels of NO, TNF and IL-1 β , but increased levels of IL-12 in response to LPS. Increased IL-12 production correlated with increased Th1/Th2 ratio in Xid mice, suggesting that Th1 cell responses led to delayed pathogen clearance. Further experiments revealed, however, that Xid-derived Th1 cells showed unaltered rates of pathogen clearance indicating that the delayed clearance of injected microfilaria in Xid mice was mainly attributed to defective effector functions of macrophages (Mukhopadhyay et al. 2002). The involvement of Btk in TLR4 as well as TLR2 signalling was confirmed on monocytes derived from XLA patients as these cells are characterised by reduced production of TNF and IL-1 β in response to TLR2 and TLR4 ligands, demonstrating a significant role of Btk in the expression of these pro-inflammatory cytokines (Horwood et al. 2003; Horwood et al. 2006). Simultaneously, results obtained from XLA PBMCs have shown that Btk has no impact on IL-6, IL-8 and IL-10 production when the cells were stimulated with LPS or Pam₃Cys (Horwood et al. 2006). Palmer and colleagues confirmed that Btk does not mediate the production of IL-6 and that overexpression of Bmx, another Tec kinase, leads to increased production of both TNF and IL-6 in TLR 2-, 4- and 5- stimulated human macrophages (Palmer et al. 2008). This report shows that Tec PTKs are differentially involved in the production of cytokines despite being closely related. It is presently unclear why Bmx mediates the production of both TNF and IL-6 in LPS-stimulated human macrophages, whereas Btk mediates the production of TNF, but not IL-6. It also appears that the importance of Btk in TLR signalling may be restricted to certain cell types as the kinase was found to be dispensable for TLR4-mediated activation of murine mast cells in terms of TNF and IL-6 production (Zorn et al. 2009). Similarly, Btk deficiency had no effect on LPS-induced effector functions of neutrophils such as shedding of leukocyte surface CD62L, up-regulation of surface integrins or respiratory burst. Xid neutrophils also showed unchanged progression of apoptosis upon LPS stimulation indicating that the mechanisms that promote survival of activated neutrophils are independent of Btk (Marron et al. 2010).

Further studies have provided more evidence for the involvement of Tec kinases, particularly Btk, in TLR signalling. Investigations of protein-protein interactions by means of a yeast-two hybrid system have suggested that Btk interacts with the TIR domains of TLR4, 6, 8 and 9 (Jefferies et al. 2003), however, the precise signalling mechanism of Tec activation following TLR engagement has not been elucidated. Starting from the upstream components in the TLR signalling cascade, it has been shown in the monocytic-like THP-1 cell line that Btk interacts with the adaptor proteins Myd88 and Mal under TLR4 receptor engagement (Jefferies et al. 2003). Furthermore, it has been suggested that Btk phosphorylates Mal after stimulation with TLR2 and TLR4 ligands resulting in polyubiquitination and degradation of this adaptor molecule (Gray et al. 2006; Mansell et al. 2006). Accordingly, mutations of the tyrosine residues within the TIR domain of Mal leads to impaired Mal phosphorylation and reduced interaction with Btk (Piao et al. 2008). Studies in the murine macrophage cell line RAW 264.7 further revealed that Btk associates with PI3K to mediate TLR2 signalling. Btk and PI3K constitutively interacted with each other and increasingly bound to TLR2 upon Lipoteichoic acid (LTA) stimulation. Interestingly, Btk inhibition by a drug LFM-A13 decreased MIP-2, iNOS and TNF production at an earlier time point than PI3K chemical inhibition did (Liljeroos et al. 2007). Recently, Btk was also found to interact with intracellular class II MHC molecule in LPS-challenged murine macrophages. Moreover, Btk phosphorylation was reduced in class II MHC-deficient cells and the lack of class II MHC attenuated LPS-, CpG- and PolyI:C-induced TNF, IL-6 and IFN- β production (Liu et al. 2011). Although studies regarding the position of Btk in TLR signalling have not been confirmed in human primary cells, recent investigations carried out on RA fibroblast-like synoviocytes (FLS) implicated Bmx in an interaction with integrin $\alpha 5 \beta 1$, Myd88 and focal adhesion kinase (FAK) and that the knockdown of Bmx or Mal results in decreased IL-6 production in response to LPS (Semaan et al. 2008).

Data from primary human cells concerning the downstream components implicated in Tec PTK signalling are in contradiction with data obtained from mice and cell lines. Studies performed on Xid macrophages (Doyle et al. 2005) and the THP-1 cell line (Jefferies et al. 2003) have demonstrated that LPS-activated Btk engages the NF κ B pathway by promoting p65 subunit transactivation and nuclear localization. The chemical inhibition of Btk activity also decreased p65 phosphorylation on Ser536 as well as NF κ B binding to DNA in TLR2-stimulated murine RAW264.7 (Liljeroos et al. 2007). In human XLA PBMC, however, Btk appears to function independently of NF κ B and operate through the p38 MAPK signalling pathway following

stimulation with TLR2 and TLR4 ligands (Horwood et al. 2006). Interestingly, Bmx which regulates both TNF α and IL-6 production in human macrophages does not involve either NF κ B or p38 MAPK pathways. However, similarly to Btk, Bmx maintains the stability of mRNA of the cytokines it regulates (Palmer et al. 2008). It still remains to be revealed what the exact position of Tec kinases in the TLR signalling cascade in human primary macrophages actually is. In particular, questions remain concerning the upstream localisation of Tec kinases relative to the adaptor molecules and interactions with SFK in human cells as well as the exact mechanism of Tec-dependent regulation of gene expression.

In particular, little is known about the involvement of Tec kinases in the signalling of the endosomal TLRs. The work of Jefferies and colleagues has shown that Btk had the strongest interaction with TLR8 compared to all of the other TLRs (Jefferies et al. 2003). A study from the same group further demonstrated that Btk is important for both TLR8 and 9 signalling and that it is implicated in NF κ B activity in cell lines (Doyle et al. 2007). TLR8 is an intracellular receptor that binds ssRNA obtained from viruses or released from necrotic cells. Interestingly, XLA patients demonstrate lower resistance to enteroviral infections (Winkelstein et al. 2006) whilst Xid mice suffer from higher susceptibility to acute mouse adenovirus type-1 (MAV-1) infection (Moore et al. 2004) which implies an important role for Btk in the immune response to viral infections. To further emphasise a possible role of Tec kinases in endosomal TLR signalling, the response of DCs obtained from XLA patients to ssRNA has been shown to be significantly decreased in terms of IL-6 and TNF production (Sochorova et al. 2007). This was partially confirmed by another group which observed the inhibition of TNF but not IL-6 or IL-12p70 production by DCs from XLA patients stimulated with TLR2, TLR3, TLR4 and TLR7/8 ligands (Taneichi et al. 2008). In contrast, the deficiency of Btk in B cells led to increased responsiveness to the TLR9 ligand, CpG DNA, as the cells produced elevated levels of TNF, IL-6 and IL-12p40 but less of the inhibitory cytokine IL-10 (Hasan et al. 2008). This observation was further confirmed by Lee and colleagues who also found that increased IL-12 production in *Btk*^{-/-} B cells led to greater Th1 type IgG2b and IgG3 antibody production upon TLR9 stimulation. Paradoxically, Btk deletion also decreased p65 translocation to the nucleus in these cells suggesting decreased NF κ B activation. However, decrease in NF κ B activity was independent of IL-12 and IL-10 production as *p65*^{-/-} B cells could respond similarly to wild type B cells in terms of cytokine production (Lee et al. 2008). There are no reports regarding the role of Tec kinases in endosomal TLR signalling in human macrophages.

1.6 Aims of study

1.6.1 Rationale

TLRs are crucial components of the innate and adaptive immune system. They facilitate the recognition of pathogen-derived as well as endogenous proteins and nucleic acids. There is increasing evidence that TLRs may be involved in the initiation or/and perpetuation of numerous autoimmune diseases, thus the inhibition of a particular TLR signalling pathway may prove to be a promising treatment strategy for RA, SLE or IBD (Clanchy and Sacre 2010). TLR8 seems be the central TLR in RA as its inhibition blocked spontaneous release of the predominant cytokine, TNF, from RA synovial cultures (Sacre et al. 2008).

Many studies have shown that the Tec tyrosine kinases, Btk and Bmx, control the TLR-induced production of major pro-inflammatory cytokines in monocytes and macrophages, and that the inhibition of the tyrosine kinases significantly reduces the effector functions of immune cells. The majority of these studies were performed in LPS-stimulated cells and there is limited knowledge describing the role of Tec PTKs in endosomal TLRs. The tyrosine kinases are differently involved in cytokine production which poses an interesting question regarding the regulatory mechanisms of cytokine production. For instance, Btk has been shown to regulate the production of TNF but not IL-6, IL-8 or IL-10 in human primary macrophages (Horwood et al. 2003; Horwood et al. 2006). In contrast, Bmx was reported to control both TNF and IL-6 production in LPS-stimulated cells (Palmer et al. 2008). Further studies are necessary to assess whether Btk exclusively regulates TNF production, as determined in TLR4, 2 and 5 signalling, or whether it is involved in the regulation of a broader spectrum of cytokines and chemokines in endosomal TLR signalling.

Furthermore, Btk appears to utilise the p38 MAPK pathway to control the stability of *TNF* mRNA in LPS-challenged human macrophages (Horwood et al. 2003; Horwood et al. 2006). However, other studies revealed that Btk controls NFκB activation by promoting p65 nuclear localization (Jefferies et al. 2003; Doyle et al. 2005). These studies were not, however, gene specific as they did not measure the effect of Btk on NFκB recruitment to a target gene. Furthermore, these studies did not involve cytokine measurement and most of all, they were performed in murine macrophages and cell lines. The exact control mechanism of gene expression by Tec kinases is therefore poorly understood and requires further investigation. The question remains whether Btk

controls TNF production at the transcriptional level. If so, which transcriptional factors are involved in the Btk-dependent regulation of *TNF* transcription?

Another unanswered question concerns the upstream and downstream binding partners of Tec kinases in TLR signalling. One study performed on a cell line demonstrated that Btk phosphorylates Mal (Gray et al. 2006). However, this model can not apply to all TLRs as endosomal TLRs do not utilize Mal. Due to the role of endosomal TLRs in RA, the investigation of the signalling cascades that are activated following receptor stimulation is particularly important. This investigation may reveal novel molecules that are involved in the production of pro-inflammatory cytokines and the control mechanisms that would facilitate the development of new therapeutic strategies.

1.6.2 Aims of research

1. To investigate whether Btk plays a role in the endosomal TLR3 and TLR8 signalling cascades by measuring the effect of Btk overexpression and downregulation on cytokine and chemokine production in human primary macrophages.
2. To develop a human monocytic cell line model to investigate the role of Btk in TLR3, 4 and 8 signalling. This would then be used for affinity tag purification of downstream and upstream molecules that interact with Btk following endosomal TLR stimulation.
3. To decipher the mechanisms of *TNF* gene control regulated by Btk. Which regions of *TNF* are required for Btk-dependent regulation and can Btk control the transcription of *TNF* in TLR-stimulated human primary macrophages?
4. To determine whether Btk activates transcription factors NF κ B and AP-1 to regulate *TNF* expression in TLR8-stimulated human primary macrophages.

Chapter 2

Materials and methods

2.1 Materials

All chemicals and reagents were purchased from Sigma-Aldrich (Dorset, UK) or BDH Chemicals (Dorset, UK) unless stated otherwise.

2.1.1 Tissue culture reagents and transformed cell lines

For the culture of cell lines and human primary macrophages the following tissue culture reagents were used:

- Roswell Park Memorial Institute 1640 (RPMI) medium containing 25 mM L-glutamine, Dulbecco's Modified Eagle's Medium (DMEM) with glucose, L-glutamine and Hank's Balanced Salt Solution (HBSS) were purchased from PAA, Austria. RPMI 1640 without phenol red was purchased from Gibco-Invitrogen, Paisley, UK.
- Heat inactivated foetal bovine serum (FBS) (PAA, Yeovil, UK), Penicillin/Streptomycin (Pen/Strep) (PAA), non-essential amino acids (NEAA), sodium pyruvate, bovine insulin, oxaloacetic acid (all from Sigma) were used as medium supplements.
- Cell dissociation media (Sigma) and Trypsin/EDTA (PAA) were used for sub-culturing.
- Cell culture was performed in FalconTM plastics obtained from BD Biosciences, Oxford, UK.

The list of transformed cell lines used in the project is shown in Table 2.1.

<i>Cell line</i>	<i>Description</i>	<i>Source</i>
AD-293	<ul style="list-style-type: none"> • Derived from human embryonic kidney (HEK293) cells • Allow virus production by expressing E1 gene 	Stratagene, CA, USA
HSF	<ul style="list-style-type: none"> • Human foreskin fibroblast 	Prof J. Saklatvala, KIR, London
THP-1	<ul style="list-style-type: none"> • Human monocytic leukemia cell line 	ATCC
U937	<ul style="list-style-type: none"> • Human monocytic leukemia cell line 	ATCC
Monomac-6	<ul style="list-style-type: none"> • Human monocytic leukemia cell line 	ATCC

Table 2.1 Cell lines

2.1.2 Antibodies

Antibodies for ELISA, western blotting (WB), immunoprecipitation (IP), kinase assay (KA), EMSA, TransAm, ChIP and FACS are listed in Tables 2.2 and 2.3.

<i>Specificity</i>		<i>Species</i>	<i>Application</i>	<i>Concentration</i>	<i>Source</i>
hIL-6	Coat	-	ELISA	1 µg/ml	BD Pharmingen, San Diego, USA
	Detect	-		0.5 µg/ml	
hTNF	Coat	-	ELISA	4 µg/ml	BD Pharmingen
	Detect	-		0.5 µg/ml	
hIL-10	Coat	-	ELISA	1 µg/ml	BD Pharmingen
	Detect	-		1 µg/ml	
hIP-10	Coat	-	ELISA	2 µg/ml	BD Pharmingen
	Detect	-		0.1 µg/ml	
Btk		mouse	WB	1:2000	BD Biosciences (Oxford, UK)
Hsp90		mouse	WB	1:2000	BD Biosciences
Streptavidin		rabbit	WB	1:2000	Sigma (Dorset, UK)
STAT3		mouse	WB	1:2000	New England Biolabs (UK)
Hck		mouse	WB	1:2000	BD Biosciences
GFP		rabbit	WB	1:2000	Abcam (Cambridge, UK)
IκBα		rabbit	WB	1:2000	Santa Cruz Biotechnology (CA, USA)
Phospho-p65 Ser536		rabbit	WB	1:2000	Cell Signalling Technology, Inc., MA, USA
TAK1		rabbit	WB	1:2000	Cell Signalling Technology
Phosphotyrosine clone 4G10,HRP		-	WB	1:10000	Millipore, CA, USA
Phospho-ERK Thr202/Tyr204		rabbit	WB	1:2000	Cell Signalling Technology, Inc.
Phospho-JNK Thr183/Tyr183		rabbit	WB	1:2000	Cell Signalling Technology, Inc.
Phospho-p38 Thr180/Tyr182		rabbit	WB	1:2000	Cell Signalling Technology, Inc.
Total-p38		rabbit	WB/KA	1:2000	Prof J. Saklatvala, KIR
α-Tubulin		mouse	WB	1:3000	Sigma
GAPDH			WB	1:2000	Abcam
Btk		rabbit	IP/KA	1:250	Dr M. Tomlinson, University of Birmingham, UK
IgG control		rabbit	IP/KA	1:250	Harlan Sera-Lab, UK
p50 (NFκB1) (C-19)X		rabbit	EMSA	200µg/µl	Santa Cruz Biotechnology
p65 (relA) (F-6)X		rabbit	EMSA	200µg/µl	Santa Cruz Biotechnology
p65 (ralA) (ab46540-1)		rabbit	ChIP	2µg/ml	Abcam
cJun		rabbit	EMSA/ChIP	200µg/µl/ 2µg/ml	Santa Cruz Biotechnology
cFos		rabbit	EMSA/ChIP	200µg/µl/ 2µg/ml	Santa Cruz Biotechnology
JunB		rabbit	EMSA/ChIP	200µg/µl/ 2µg/ml	Santa Cruz Biotechnology
JunD		rabbit	EMSA/ChIP	200µg/µl/ 2µg/ml	Santa Cruz Biotechnology
ATF2		rabbit	EMSA/ChIP	200µg/µl/ 2µg/ml	Santa Cruz Biotechnology

phospho-cJun	rabbit	TransAm	200µg/µl	Active Motif (CA, USA)
Pol II (sc-9001-X)	rabbit	ChIP	2µg/ml	Santa Cruz Biotechnology
IgG	rabbit	ChIP	2µg/ml	Abcam
TLR-3 FITC	mouse	FACS	1:25	Imgenex, San Diego, USA
TLR-4 FITC	mouse	FACS	1:25	Imgenex, San Diego, USA
TLR-8 FITC	mouse	FACS	1:25	Imgenex, San Diego, USA
IgG1 FITC	mouse	FACS	1:25	Imgenex, San Diego, USA
IgG2a FITC	mouse	FACS	1:25	Imgenex, San Diego, USA

Table 2.2 Primary antibodies used for WB, IP, KA, ChIP, TransAm and ELISA

<i>Specificity</i>	<i>Species</i>	<i>Application</i>	<i>Concentration</i>	<i>Source</i>
Anti-mouse IgG, HRP	sheep	WB	1:2000	Amersham, Little Chalfont, UK
Anti-rabbit IgG, HRP	donkey	WB	1:2000	Amersham, Little Chalfont, UK

Table 2.3 Secondary antibodies used for WB

2.1.3 Cytokines

Recombinant cytokines used for ELISA standards and cell culture and stimulation are listed in Table 2.4.

<i>Cytokine</i>	<i>Application</i>	<i>Concentration</i>	<i>Source</i>
IL-6	ELISA	13-10000 pg/ml	Peprtech, London, UK
TNF	ELISA	13-10000 pg/ml	Peprtech, London, UK
	Cell stimulation	40000 pg/ml	
IL-10	ELISA	13-10000 pg/ml	Peprtech, London, UK
IP-10	ELISA	13-10000 pg/ml	RnD, Minneapolis, USA
M-CSF	Cell culture	100 ng/ml	Peprtech, London, UK

Table 2.4 Human recombinant cytokines

2.1.4 TLR ligands

Toll-like receptor ligands used for cell stimulation are summarised in Table 2.5.

<i>Receptor</i>	<i>Ligand</i>	<i>Description</i>	<i>Source</i>
TLR 3	PolyI:C	synthetic dsRNA	ALEXIS, Nottingham, UK
TLR 4	LPS	lipopolysaccharide from <i>E.coli</i>	ALEXIS
TLR 7/8	R848	imidazoquinoline resiquimod	ALEXIS

Table 2.5 TLR ligands

2.1.5 Inhibitors

The list of enzyme inhibitors is presented in Table 2.6. RNA oligonucleotides for RNA interference (RNAi) are summarised in Table 2.7.

<i>Inhibitor</i>	<i>Cellular Target</i>	<i>Source</i>
100x Protease Inhibitor Cocktail (PIC)	proteases	Sigma
Protease Inhibitor Tablet (PIT)	proteases	Roche
Sodium fluoride	protein phosphatases	Sigma
Sodium Orthovanadate	protein phosphatases	Sigma
Geldanamycin	Hsp90 inhibitor	Invitrogen

Table 2.6 Enzyme inhibitors

<i>Target</i>	<i>Type</i>	<i>Catalogue Number</i>	<i>Source</i>
Btk #9	Duplex	J-003107-09	Dharmacon, IL, USA
Btk #11	Duplex	J-003107-11	Dharmacon
Non targeting	siControl TM	D-001206-13	Dharmacon

Table 2.7 RNAi oligonucleotides

2.1.6 Custom designed oligonucleotide primers

The oligonucleotide primers used in this study were all purchased from MWG (Germany) and stored at -20°C. The primers were diluted in TE buffer to a final concentration of 100pmol/μl.

2.1.6.1 PCR primers

Primers used for primary and mature transcript RT PCR, PCR and ChIP are listed in Tables 2.8, 2.9 and 2.10, respectively.

<i>Name</i>	<i>Sequence (5'-3')</i>
TNF_mature_forward (for)1	CCTGCTGCACTTTGGAGTGATCGG
TNF_mature_reverse (rev)1	GTACAGGCCCTCTGATGGCACCACC
TNF_primary_forward (PT_F2)	GCAGTCAGATCATCTTCTCG
TNF_primary_reverse(PT_R2)	AGGTACAGGCCCTCTGATGGCAC
ARP_for	CGACCTGGAAGTCCAACACTAC
ARP_rev	ATCTGCTGCATCTGCTTG

Table 2.8 Primers used for detecting primary and mature TNF transcripts in RT-PCR

<i>Name</i>	<i>Sequence (5'-3')</i>
Btk- <i>SpeI</i> _for	TCACTAGTATGGCCGCACTGATTC
Btk- <i>Sall</i> _rev	CAGTCGACGGATTCTTCATCCATG

Table 2.9 Primers used for PCR to introduce *SpeI* and *Sall* restriction sites upstream of the ATG transcription start codon and instead of transcription stop codon of human *Btk*, respectively.

<i>Name</i>	<i>Sequence (5'-3')</i>
TNFdown_for	GGCAGTCAGTAAGTGTCTCCAA
TNFdown_rev	TTACCTACAACATGGGCTACAGG
TNF TSS(+1)_for	CAGCAAGGACAGCAGAGGAC
TNF TSS(+1)_rev	TCCCGGATCATGCTTTCAGT
κBsite1for	CCACATGTAGCGGCTCTGAG
κBsite1rev	AGGCTCTTTCACCTCCCTGGG
κBsite2for	GAAGAAGGCCTGCCCCAGTGG
κBsite2rev	GGTAGCAGGGACAAGCCTGG
κBsite4for	ATATTCCTCCATCCCCCAGGAAACA
κBsite4rev	CTGCAACAGCCGAAATCTCACC
κBsite3for	GCTTGTGTGTCCCCAACTTT
κBsite3rev	GGGGAAGAATCATTCAA

Table 2.10 Primers used for ChIP

2.1.6.2 Primers used for human *Btk* gene sequencing

Table 2.11 presents a list of primers that allowed full *Btk* gene sequencing.

<i>Primer internal name</i>	<i>Sequence 5'-3'</i>
Btkseq_rev2	CAACCTGGAAGGGATAAGGG
Btkseq_rev3	GGCTCTGAGGTGTGGAACAC
Btkseq_for2	AAAAGCCACTACCGCCCTGAG
Btkseq_for3	CCCCATCTTCATCATCACTG
Btkseq_for15	TCATCTGGCTTCAGAGAAGG
Btkseq_for16	GAGCCTTTGTGCTCCCACTC

Table 2.11 Primers used for gene sequencing

2.1.7 Oligonucleotides used for EMSA

Table 2.12 presents a list of consensus oligonucleotides used for EMSA. The double-stranded oligonucleotide is designed with 5'-OH blunt ends.

<i>Name</i>	<i>Sequence (5'-3')</i>	<i>Source</i>
AP-1	CGCTTGATGAGTCAGCCGGAA	Promega
NFκB	AGTTGAGGGGACTTTCCAGGC	Promega

Table 2.12 AP-1 and NFκB consensus oligonucleotides used for EMSA

2.1.8 Restriction enzymes used for cloning

The restriction enzymes used in these studies were purchased from New England Biolabs (Hitchin, UK) and are presented in Table 2.13.

<i>Enzyme</i>	<i>Buffer</i>	<i>10x BSA</i>	<i>Temperature</i>
<i>SpeI</i>	NEB2	+	37°C, inactivation at 65°C
<i>Sall</i>	NEB3	+	37°C, inactivation at 80°C
<i>BglII</i>	NEB3	-	37°C
<i>EcoRV</i>	NEB3	+	37°C, inactivation at 80°C
<i>AleI</i>	NEB4	-	37°C, inactivation at 65°C
<i>BamHI</i>	NEB3	+	37°C

Table 2.13 Restriction enzymes and the reaction conditions

2.1.9 Adenoviral constructs

All adenoviral constructs used for these studies expressed Green Fluorescent Protein (GFP) allowing the determination of virus infection. They are listed in Table 2.14.

<i>Virus</i>	<i>Insert</i>	<i>Virus generated by</i>
AdBtk	Human Btk wild type	Mrs Brenda Mutch
AdCon	Empty control, carrying Gateway® (Invitrogen) pENTRY4.3.F vector	Mrs Brenda Mutch
AdBtk- <i>Strep</i>	Human Btk wild type with C-terminal 31amino acid SBP tag	Ms Anna Urbaniak
Ad <i>Strep</i>	Control 31 amino acid SBP tag	Dr Ale Lanfrancotti
AdBtk-NTAP	Human Btk wild type with N-terminal Protein G/TEV/SBP tag	Ms Anna Urbaniak
AdNTAP	Control Protein G/TEV/SBP tag	Ms Anna Urbaniak
AdTAK1	Human TAK1 wild type	Ms Kate Willetts
Ad5'Luc	Firefly luciferase gene driven by human TNF 5' promoter	Dr Agnes Denys
Ad5'Luc 3'UTR	Firefly luciferase gene flanked by human TNF 5' promoter and the 3'UTR	Dr Tim Smallie
Ad5'Luc 3'1037bp	Firefly luciferase gene flanked by human TNF 5' promoter and 3'1037bp sequence containing the 3'UTR plus 252bp enhancer	Dr Agnes Denys
Ad5'Luc 3'1251bp	Firefly luciferase gene flanked by human TNF 5' promoter and 3'1251bp sequence containing the 3'UTR plus 466bp enhancer	Dr Tim Smallie
Ad5'Luc 3'site 4X	Firefly luciferase gene flanked by human TNF 5' promoter and 3'1251bp sequence carrying a κ B site 4 point mutation	Dr Tim Smallie
Ad5'Luc 3' site 1/2/3/4X	Firefly luciferase gene flanked by human TNF 5' promoter and 3'1251bp sequence carrying κ B sites 1,2,3 and 4 point mutations	Dr Tim Smallie

Table 2.14 Adenoviruses

2.1.10 Solutions

2.1.10.1 General buffers

Phosphate buffered saline (PBS)	4.3 mM Na ₂ HPO ₄ 1.4 mM KH ₂ CO ₃ 1.4 mM KCl 137 mM NaCl pH 7.2
Tris buffered saline (TBS) (10x)	20 mM Tris/HCl 137 mM NaCl pH 7.6
Tris Borate EDTA buffer (TBE) (10x)	0.89 M Tris/HCl pH 8.0 0.89 M boric acid 2 mM EDTA
Sodium orthovanadate stock solution	100 mM Na ₃ VO ₄ pH 10.0 Boiled for 60 – 90 min, stored at 4°C

2.1.10.2 ELISA reagents

ELISA blocking buffer	PBS + 2% (w/v) bovine serum albumin (BSA)
ELISA stop solution	940 ml dH ₂ O + 60 ml H ₂ SO ₄ ,
ELISA wash buffer (PBS/Tween)	PBS + 0.01% (v/v) Tween-20

2.1.10.3 Protein extraction buffers for Western blotting and Immunoprecipitation

NP-40 lysis buffer	1% (v/v) NP40 20 mM Tris-Base pH 7.6 125 mM NaCl
RIPA lysis buffer	1% (v/v) NP-40 50 mM Tris-Base pH 7.6 150 mM NaCl 10 mM tetrasodium pyrophosphate 1 mM EDTA 25 mM β-glycerophosphate 0.5% (w/v) sodium deoxycholate 0.1% (w/v) SDS

10 mM DTT, 100 μ M Na₂VO₃, 5 mM NaF, 1x PIC (Table 2.6) were added freshly to the lysis buffers

2.1.10.4 SDS-PAGE and Western blotting reagents

SDS-PAGE running buffer	25 mM Tris-Base 192 mM Glycine 0.1% (w/v) SDS
SDS-PAGE resolving buffer	3 M Tris-Base pH 8.8 0.6% (w/v) SDS
SDS-PAGE stacking buffer	140 mM Tris-Base pH 6.8 0.11% (w/v) SDS
5x protein gel sample buffer (20ml)	250 mM Tris-HCl pH 6.8 10% (w/v) SDS 50% (v/v) glycerol 12.5% (v/v) β -mercaptoethanol (ME) (mix Tris, SDS, water. Heat to 50°C. Add glycerol, β -ME and bromoethanol blue)
WB transfer buffer	25 mM Tris-Base 192 mM Glycine 20% (v/v) Methanol
WB wash buffer (TBS/Tween)	TBS 0.1% (v/v) Tween-20 (Sigma-Aldrich, product no. P-1379)
Blocking buffer (BSA)	TBS/Tween 2% (w/v) bovine albumin serum
Blocking buffer (milk)	TBS/Tween 5% (w/v) skimmed milk powder

2.1.10.5 Luciferase assay reagents

Luciferase assay buffer (LAB)	1% (v/v) Triton X-100 25mM Tris-phosphate pH 7.8 8 mM MgCl ₂ 1mM EDTA 15% (v/v) Glycerol
CAT Lysis buffer	0.65% (v/v) NP40 10 mM Tris-HCl pH 8 1mM EDTA 150 mM NaCl

2.1.10.6 EMSA reagents

Buffer A	10 mM Hepes pH 7.9 10 mM KCl 0.1 mM EDTA 0.1 mM EGTA
Buffer B	20 mM Hepes pH 7.9 0.4 M NaCl 1 mM EDTA 1 mM EGTA
5% TBE gel (40ml)	5% acrylamide (6.6ml of 30% stock) 1x TBE (4ml of 5x TBE) 1.5% (v/v) glycerol 300µl 10% (w/v) APS 25µl TEMED

Buffers A and B were supplemented with 1mM DTT and 1:50 000 PIT (Table 2.6)

2.1.10.7 ChIP reagents

Cytoplasmic lysis buffer	50 mM Tris, pH 8.0 2 mM EDTA, pH 8.0 0.1% NP40 10% Glycerol
Nuclear lysis buffer	50 mM Tris pH, 8.0 5 mM EDTA 1 % SDS
Dilution buffer (DB)	50mM Tris, pH 8.0 5mM EDTA 200mM NaCl 0.5 % NP40
Washing Buffer (WB)	20mM Tris, pH 8.0 2mM EDTA 0.1 % SDS 1 % NP40 500 mM NaCl
Extraction Buffer (EB)	1X TE 2 % SDS

All the buffers were supplemented with fresh 1mM DTT and 1:50 000 PIT (Table 2.6)

2.1.10.8 One-Strep purification reagents

Cytoplasmic lysis buffer	0.5%NP-40 5mM PIPES pH 8.0 85mM KCl Alternatively: 100ug/ml of avidin, 1ug/ml of a DNase
Nuclear lysis buffer (RIPA)	1% (v/v) NP-40 50 mM Tris-Base pH 7.6 150 mM NaCl 10 mM tetrasodium pyrophosphate 1 mM EDTA 25 mM β -glycerophosphate 0.5% (w/v) sodium deoxycholate 0.1% (w/v) SDS Alternatively: 100ug/ml of avidin, 1ug/ml of a DNase
Washing buffer (WB) 1 and 2	20mM Tris 50mM NaCl (WB1) or 150mM NaCl (WB2) 0.2mM EDTA 0.2%NP-40 5% Glycerol
Elution buffer	100mM Tris pH 8 150mM NaCl 2mM Biotin

10 mM DTT, 100 μ M Na₂VO₃, 5 mM NaF, 1:50 000 PIT (Table 2.6) were added fresh to the lysis buffers

2.1.10.9 Molecular biology solutions

Lennox L broth base (LB)	20g LB (Invitrogen, Paisley, UK) 1l dH ₂ O (Autoclave before use)
Lennox L agar (LB agar)	32g LB agar (Invitrogen, Paisley, UK) 1l dH ₂ O (Autoclave, add antibiotics once cooled, and then pour into plates before it sets)
Ampicillin	100 mg/ml Ampicillin (Sigma, Poole, UK) (Diluted in autoclaved sterile dH ₂ O)

Kanamycin	30 mg/ml Kanamycin (Diluted in autoclaved sterile dH ₂ O)
SYBR Green II	Use at 10% (v/v) in DNA solutions (Molecular probes, Eugene, Oregon, USA)
DNA Hyperladders	5 µl loaded per lane (Bioline, London, UK)
Ethidium bromide	Used at a final concentration of 0.1 µg/ml (Sigma, Poole, UK)
10x DNA gel sample buffer (DNA GSB)	80% (v/v) glycerol 1% (v/v) 5xTBE 0.025% (w/v) bromophenol blue 0.025% (w/v) xylene cyanol

2.1.10.10 Adenovirus purification reagents

Phosphate buffered saline ²⁺ (PBS²⁺)	PBS 0.01% (w/v) CaCl ₂ 0.01% (w/v) MgCl ₂
Saturated Caesium Chloride (CsCl)	0.01M Tris/Base pH8 1mM EDTA CsCl added until saturated
1.34g/ml density Caesium Chloride	85% (v/v) 1.4g/ml density CsCl 15% (v/v) PBS ²⁺
1.4g/ml density Caesium Chloride	53.4% (w/v) CsCl

2.1.10.11 Kinase assay reagents

Kinase lysis buffer	20 mM HEPES pH 7.4 50 mM β glycerophosphate 2 mM EGTA 1% Triton X 100
Kinase wash buffer (3X)	60 mM HEPES pH 7.4 600 mM NaCl 60 mM β glycerophosphate 1.5 mM EDTA 1.5 mM EGTA 30 mM MgCl 0.15% Brij

10 mM DTT, 100 μ M Na₂VO₃, 5 mM NaF, 1:50 000 PIT (Table 2.6) were added fresh to the lysis buffers

2.1.10.12 Remaining reagents

MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)	5mg/ml MTT in sterile PBS
MTT lysis buffer	100g SDS, 1ml of conc. HCl per 1l of H ₂ O
Buffer '41' for Amaxa	90 mM Sodium Phosphate Buffer pH 7.2 (68.4 ml of 1 M Na ₂ HPO ₄ + 31.6 ml of 1 M NaH ₂ PO ₄) 5 mM KCl 10 mM MgCl ₂ 20 mM HEPES

2.1.11 Bacteria strains

Electrocompetent *E.coli* XL-1 Blue (Stratagene, Cambridge, UK)

Genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacIqZΔM15 Tn10* (Tetr)]

Electrocompetent *E.coli* BJ5183 EC (Q-Biogene, Cambridge, UK)

Genotype: *endA sbcBC recBC galK met thi-1 bioT hsdR* (*Str^r*)

Chemically competent *E.coli* Top10 (Invitrogen)

Genotype: *F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (Str^R) endA1 nupG*

Key to genotypes: www.invitrogen.com

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Endotoxin testing

All reagents and solutions used for siRNA transfection, cell culture and stimulation underwent endotoxin testing by *Limulus* Amebocyte assay (Biowittaker, Berkshire, UK) by Mr Sunil Modi prior to use. A solution of 0.1 EU/ml endotoxin (equivalent to 100 pg/ml) was serially diluted in LPS-free H₂O and used as a standard for calibration. Test samples, the standard and a blank (H₂O) were added to the 96-well micro plate (previously pre-equilibrated to 37°C) in 50 µl volumes in duplicate. An equal volume of *Limulus* amebocyte lysates was added to each well and incubated for 10 min at 37°C. 100 µl of substrate solution was added to each well and incubated for a further 6 min at 37°C, prior to the addition of 100 µl of stop reagent. The absorbance was read at 410 nm and the sensitivity of the assay was >10 pg/ml.

2.2.1.2 Isolation of Human Peripheral Blood Monocytes

Human Peripheral Blood Mononuclear Cells (PBMC) were isolated from buffy coat formed after centrifugation of whole blood obtained from the North London Blood Transfusion Service (Colindale, UK) through a Ficoll- Histopaque gradient. Buffy coats (approximately 50ml) were diluted 1:2 in HBSS and layered onto 20ml Lymphocyte[®]-H Solution (Cedarlane Laboratories Ltd, Ontario, Canada). The tubes were then spun at 2200rpm for 30min, PBMC layer was extracted from the interface between the bottom Ficoll-red blood cell fraction and the top HBSS fraction, washed once with HBSS at 2000rpm for 10min and resuspended in RPMI, 1% FCS prior to elutriation.

2.2.1.3 Elutriation

Monocyte fractions at a purity of greater than 80% were isolated from PBMC by centrifugal elutriation at 2500rpm in a Beckman JE6 elutriator (Beckman Coulter, High Wycombe, UK). Separation due to cell size and granularity is achieved in an elutriator by the opposing effects of centrifugal force and fluid velocity in a specialised chamber (**Figure 2.1**). A suspension of cells is

pumped into a funnel-shaped chamber at a low flow rate. The cells position where the fluid velocity force and the centrifugal force are balanced. As smaller and less dense cells are under a low centrifugal force, they are positioned close to the outlet of the chamber. Low flow rate and its gradual increase forces smaller and less dense cells to exit the chamber first, where they are collected and analysed by flow cytometry and CellQuest software. Larger cells, which are under a high centrifugal force, require a higher flow rate to overcome the centrifugal force which retains them by the inlet of the chamber. Therefore, large cells emerge later. T lymphocytes emerge from the chamber first (flow rate between 11-14ml per min) followed by monocytes (18-22ml per min) and granulocytes (>20ml per minute). Elutriation was carried out in RPMI, containing 1% FCS.

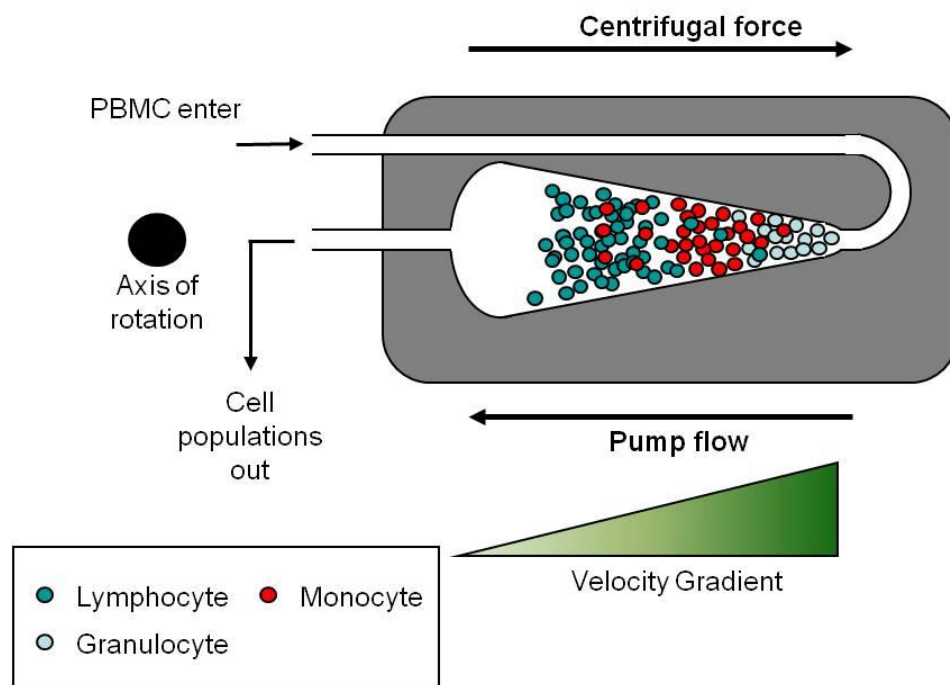


Figure 2.1 Centrifugal elutriation. Illustration shows cross section of an elutriation chamber. The chamber rotates around the axis at 2500rpm creating a centrifugal force. The peristaltic pump creates an opposing flow. The funnel shaped chamber causes the velocity of the fluid to slow, forming a defined gradient. Cells of different densities migrate to the point where the two opposing forces balance. Cell populations of increasing sizes elute with increasing flow rate overcoming limiting centrifugal force which keeps large cells by the inlet of the chamber. Monocytes are typically eluted at a flow rate of 18mls per minute. Purities of >80% are routinely obtained as determined by flow cytometry.

2.2.1.4 Production of M-CSF-derived macrophages

Primary human monocytes (>80% purity) were cultured at 1×10^6 cells / ml in RPMI 1640 supplemented with 5% heat-inactivated FCS and 1% penicillin (100U/ml) and streptomycin (100U/ml) (Pen/Strep) cocktail at 37°C in 5% CO₂. Macrophage colony stimulating factor (M-CSF) at a concentration of 100 ng/ml was added to the culture. After 96h, non-adherent cells were removed and cell dissociation media was applied onto differentiated macrophages for 45min. Cells were scraped and 5% RPMI was added to neutralise cell dissociation media. After washing, macrophages were counted and plated in experimental dishes in complete medium without M-CSF. Typically, 50% of plated peripheral blood mononuclear cells fully differentiated into macrophages.

2.2.1.5 Culture of transformed cell lines

Human monocytic leukemia cell lines THP-1, U937 and Monomac-6 were obtained from the American Type Culture Collection (ATCC). THP-1 and U937 cell lines were grown in RPMI 1640 supplemented with 5% FCS and 1% Pen/Strep at 1×10^6 cells/ ml in 75cm² flasks and sub-cultured at a ratio of 1:3 every 3 days. Cells were differentiated by the addition of phorbol-12-myristate-13-acetate (PMA) (Sigma) (10ng/ml) for 24h. Cells were then washed and plated in 96-well plates in PMA-free medium for another 24h before stimulation. Monomac-6 cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, 10 µg/ml bovine insulin, 1mM oxaloacetic acid, 1% Pen/Strep and maintained at $0.3-1.0 \times 10^6$ cells/ml in 24-well plates. Monomac-6 cells were sub-cultured at a ratio of 1:3 every 3 days. Human embryonic kidney (HEK293)-derived AD-293 cells (Stratagene, California, USA) were cultured in DMEM supplemented with 10% FCS and 1% Pen/Strep. Cells were maintained in 150cm² flasks, passaged at a confluence of 80-90% and dissociated from the bottom of the flask by applying Trypsin/EDTA solution for 5min. All the cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

2.2.1.6 MTT cell viability Assay

A colorimetric MTT assay was utilized to assess the viability of the cells. The assay is based on the ability of living cells to reduce yellow MTT (Section 2.1.10.12) to purple formazan by mitochondrial enzymes. Cells were cultured in 96-well plates, supernatants were harvested, replaced with RPMI media containing 5µg/ml MTT for 12-18h. MTT lysis buffer at equal volume was added and left for a minimum of 4 hours. Absorbance of formazan was read at 620nm using a Fluostar Omega (BMG Labtech, Aylesbury, UK) plate reader and accompanied MARS data analysis software. The absorbance values reflect the proportion of living cells.

2.2.2 Molecular biology techniques

2.2.2.1 RNA Interference

RNA interference (RNAi) is a naturally occurring process involved in gene downregulation, host defence against viruses and transposones as well as development control. There are two types of RNAi molecules; 20-25 nucleotide (nt) long small interfering (si) RNA and microRNA. SiRNA is generated from >200 nt long double-stranded (ds) RNAs by an enzyme called Dicer. Next, RNA-induced silencing complex (RISC) incorporates one strand of each siRNA and pairs it with the complementary sequence of messenger (m) RNA. This enables a component of RISC complex, argonaute, to cleave the target gene and leads to post-transcriptional gene silencing. The phenomenon of selective gene downregulation makes RNAi a widely used research technique. However, as mammalian cells develop an anti-viral phenotype upon encounter of external long (>30nt) dsRNA, only short pre-synthesised siRNAs are used for targeted gene silencing (Elbashir et al. 2001).

Human primary monocytes (5×10^6) were transfected with 100-300nM siRNA targeting Btk mRNA (Btk SMARTpool M-003107-01, Dharmacon, IL) and with non-targeting control oligonucleotide (siControl D-001206-13). The siRNA was first suspended in 100µl of buffer '41' (Section 2.1.5.11). The cells were spun and the medium was replaced with buffer '41'. Cells were immediately placed in electroporation cuvettes and subjected to electroporation using the Y-001 programme on Amaxa Nucleofector[®] Device (Lonza Cologne GmbH, Germany). Cells were then left to recover in 6-well plates for 1 day in 10% FCS RPMI media without phenol red

supplemented with 100ng/ml M-CSF. Cells were scraped, counted and plated in full 5% FCS RPMI media with phenol red and M-CSF and incubated for a further 72h prior to stimulation.

2.2.2.2 RNA extraction from primary human macrophages

The blood RNA extraction kit (Qiagen, UK) was used to extract total RNA from human primary macrophage cultures. Macrophages (0.5×10^6 per well) were initially washed with PBS. Cells were lysed in the provided RLT buffer supplemented with β -mercaptoethanol and further homogenised by use of shredder columns provided. The lysate was applied to a second RNA-binding column in order to remove impurities by extensive washing. RNA was eluted in RNase free dH₂O and stored at -80°C. Contaminating DNA was removed using the Ambion TURBO DNA-free™ kit.

2.2.2.4 Reverse Transcription of total RNA to cDNA

The AMV-reverse transcriptase kit (Promega) was used to convert total RNA to cDNA. The reaction was run at 42°C for 2h followed by 65°C for 10min to inactivate the enzyme in the following 50µl volume:

Reagent	volume
1µg RNA template	20 µl
5 x AMV buffer	10 µl
dNTP (10 mM)	2.5 µl
DTT (100 µM)	2.5 µl
Random hexamer primers	2 µl
AMV reverse transcriptase (1 U/µl)	1 µl
dH ₂ O	12 µl

2.2.2.5 Primary and Mature Transcript SYBR green PCR

Quantitative real-time PCR was used to amplify and simultaneously quantify targeted DNA in a sample by measurement of SYBR-green fluorescence. The SYBR Green I dye nonspecifically binds to nucleic acid. Upon intercalation with dsDNA the fluorescence of the dye increases 1000-

fold. The intensity of the signal increases as the target is amplified thus it is proportional to the amount of target DNA present.

RNA from human primary macrophages was extracted using the Blood RNA extraction kit (Qiagen, UK) (Section 2.2.2.2). RNA was then purified from genomic DNA with the TURBO DNAfree™ kit (Ambion) and converted into cDNA (Section 2.2.2.4). The primers for the PCR reaction were designed to distinguish between primary and mature *TNF* transcripts (Table 2.8) such that the primer set spanning exon-intron boundary was used to detect the primary transcript whereas the primer set spanning exon-exon boundary was used for the mature transcript amplification.

Samples were run in triplicate on the Rotor-Gene 6 (Corbett Research UK Ltd., Cambridge, UK). The DNA was denaturated at 95°C for 5min, and then 40 cycles of 95°C for 10sec (denaturation), 59°C for 30sec (annealing), and 78°C for 20sec (elongation). Real-time PCR reactions were set as follows:

Reagent	Volume
DNA	10µl
SYBR Premix ExTaq (2x) Lonza (Takara)	10µl
Forward Primer (Table 2.10)	0.04µl
Reverse Primer (Table 2.10)	0.04µl

Relative quantification of gene expression was determined using the comparative $\Delta\Delta C_T$ method using the RotorGene 6000 software. Briefly, threshold cycle (C_t) values of ARP were subtracted from *TNF* C_t values and additionally normalised to the untreated control. The detailed method of calculating is as follows:

$$\text{mean } C_t \text{ TNF} - \text{mean } C_t \text{ ARP} = \text{TNF } \Delta C_t$$

$$\Delta \text{TNF } C_t (\text{treated sample}) - \Delta \text{TNF } C_t (\text{untreated sample}) = \Delta\Delta C_t$$

If the reaction has a perfect amplification efficiency (E), then the template is doubled in each amplification cycle ($E=2$). The difference in C_t values will be then determined by the equation 2^n , where 'n' is the number of cycles between two C_t values of the fluorescent curves. $\Delta\Delta C_t$ values are then calculated as shown:

$$2^{-\Delta\Delta C_t} = 1 \text{ (untreated sample), as } \Delta\Delta C_t = 0$$

$$2^{-\Delta\Delta C_t} = x \text{ (treated sample)}$$

2.2.2.6 Isolation of plasmid DNA by mini- and maxi-preps

Harvesting plasmid DNA, transformed into *E.coli* was performed using mini- and maxi-prep kits purchased from Qiagen (Crawley, UK) according to the manufacturer's instructions. Briefly, bacteria were lysed in alkaline conditions allowing the separation of plasmid and genomic DNA. Pre-cleared lysates were then applied onto a silica gel membrane that selectively binds plasmid DNA. The membrane was then washed to remove any contaminants prior to elution with dH₂O. Mini-prep plasmid DNA purification yielded approximately 0.2µg/µl DNA per 1.5ml bacterial culture, whereas maxi-prep purification yielded 0.6-1.0 µg/µl DNA per 200ml culture.

2.2.2.7 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used to introduce restriction sites into the *Btk* gene through the DNA amplification process. The primers (Table 2.9) containing a restriction site and the *Btk* sequence annealed to the template in two steps thus two sets of annealing temperatures had to be optimised. The first step is when only the Btk complementary sequence of the primer annealed to the template. This results in the incorporation of the restriction sites in the Btk sequence. The second step is when the whole primer anneals to the template. The MWG-Biotech Primus 96 machine was programmed as follows:

Initial melting step:	92°C for 1min
Amplification Step 1 (5 cycles of):	92°C for 30sec
	47°C for 30sec
	68°C for 3min
Amplification Step 2 (30 cycles of):	92°C for 30sec
	64°C for 30sec
	68°C for 3min
Final elongation step:	72°C for 10min

PCR was performed using *Thermus aquaticus* (Taq) DNA polymerase called Titanium™ Taq Polymerase (Clontech, USA). Betaine (Promega) was used to increase the efficiency of the reaction as it protects the Taq polymerase from denaturation and dissolves secondary structures.

The PCR reaction was set as follows:

Reagent	Volume
DNA template (10 ng)	10 μ l
dNTPs (100 mM)	1 μ l
10X PCR buffer	9 μ l
Forward Primer	4 μ l
Reverse Primer	4 μ l
Taq DNA polymerase (5 U/ μ l)	1 μ l
dH ₂ O	61 μ l

2.2.2.8 DNA agarose gel

The efficiency of PCR, restriction digests or ligation was evaluated on 0.7% (w/v) agarose gels in 1X TBE buffer with 1/10,000 SYBR Green II (Molecular Probes, Oregon, USA). DNA Hyperladder (Bioline, London, UK) was run along with the sample DNA. 10X DNA gel sample buffer (Section 2.1.10.9) was added to the digests, and the bands were visualised using a Dark Reader Transilluminator (model DR88M, BRI Ltd., UK).

2.2.2.9 Restriction digest of DNA

DNA was digested with restriction enzymes (Table 2.13) at the temperature suggested by a supplier (New England Biolabs, MA, USA) for 2h. Reaction conditions which were used in this study are as follows:

Reagent	Volume
DNA (2 μ g)	x μ l
10x NEB buffer	1 μ l
BSA	2 mg
Enzyme (10 000U/ml)	0.5 μ l
dH ₂ O	up to 10 μ l total

The efficiency of digests was evaluated on a DNA agarose gel (Section 2.2.2.9). The bands were purified using Wizard SV Gel and PCR Clean-Up system (Promega).

2.2.2.10 Blunt- ending of 5' overhangs using *Pfu* polymerase

Pfu DNA polymerase, along with a 5' to 3' polymerase activity, also has a 3' to 5' exonuclease proofreading activity. It therefore fills in 5' overhangs with dNTPs and cleaves nucleotides from the 3' termini of a polynucleotide chain resulting in blunt-ended DNA fragments. DNA cohesive ends generated by restriction enzyme digestion were polished with the *Pfu* polymerase (Promega, Southhampton, UK) treatment at 72°C for 30min. The reaction was set as follows:

Reagent	Volume
DNA (2 µg)	40 µl
10X <i>Pfu</i> buffer	6 µl
<i>Pfu</i> polymerase (2-3 U/µl)	5 µl
dNTPs (1 nM)	6 µl
dH ₂ O	3 µl

2.2.2.11 Dephosphorylation of DNA ends

The 5' ends of digested vector DNA were dephosphorylated by shrimp alkaline phosphatase (SAP) (Promega, Southhampton, UK) treatment at 37°C for 30min followed by inactivation of the enzyme at 65°C for 15min. The reaction was set as follows:

Reagent	Volume
DNA (2 µg)	10 µl
10X SAP buffer	1.5 µl
SAP	1 µl
dH ₂ O	2.5 µl

2.2.2.12 Ligation of DNA

Prior to ligation DNA was purified using Wizard SV Gel and PCR Clean-Up system (Promega). Ligations of purified digested DNA were carried out using T4 DNA ligase (New England Biolabs, Hitchin, UK) at a molar ratio 4 (insert) to 1 (vector). The reaction was set as follows:

Reagent	Volume
Vector DNA	1 μ l
Insert DNA	4 μ l
10X T4 buffer	1 μ l
T4 ligase	1 μ l
dH ₂ O	4 μ l

Ligation mixture (4 μ l) together with the vector or insert alone controls were then transformed into the 50 μ l electrocompetent XL1-Blue *E.coli* cells as described in the next section. The efficiency of ligation was evaluated by diagnostic digestion with restriction enzymes (Section 2.2.2.9).

2.2.2.13 Transformation of electrocompetent E.coli

A maximum volume of 4 μ l DNA was transformed into 50- 80 μ l of electrocompetent *E.coli* cells (Section 2.1.11) by electroporation at 2000V. The bacteria were then allowed to recover in 0.5ml of antibiotic-free LB (Section 2.1.10.9) for an hour and plated on agar plates containing 100 μ g/ml ampicillin (Invitrogen). Bacteria were incubated at 37°C overnight prior to colony collection. The colonies were then cultured in 5ml LB broth containing 100 μ g/ml ampicillin at 37°C overnight and plasmid DNA was isolated from the whole cell lysate using miniprep kits purchased from Qiagen (GmbH, Germany) according to the manufacturer's instructions (Section 2.2.2.6). The efficiency of the ligation was evaluated by diagnostic digestion with restriction enzymes (Section 2.2.2.9).

2.2.2.14 Precipitation of DNA using Sodium Acetate and Ethanol

100 μ l of DNA was precipitated by adding 7 μ l of 3M NaAc (pH 5.2) and 321 μ l of 100% EtOH. The sample was left at -20°C for 1h then spun at 13000 rpm for 5min. The supernatant was carefully removed and the pellet was gently washed in 0.5ml 70% ice-cold EtOH to remove the leftover salt. DNA was resuspended in either dH₂O or the required buffer/media.

2.2.2.15 PCR fragment cloning using pCR-Blunt II-TOPO

The pCR-Blunt II-TOPO PCR cloning system (Invitrogen) allows rapid cloning of blunt ended PCR fragments. The TOPO vector is supplied linearized and contains single 3' thymidine overhangs together with Topoisomerase covalently bound to the vector. The vector accepts PCR fragments that terminate with a single deoxyadenosine. The linearization is facilitated by Topoisomerase I from *Vaccinia* virus. Positive selection is obtained by the inactivation of the lethal *E.coli* ccdB gene fused to the C-terminus of the LacZ fragment, permitting growth of successful recombinants upon transformation.

A 5µl reaction was set up containing 1µl of the TOPO vector, 1µl of 4x diluted salt solution (also supplied with the kit), 100ng of the PCR fragment and dH₂O. The reaction was conducted at 16°C overnight.

2.2.3 Adenoviral production

Recombinant adenoviruses are replication-defective adenoviral vectors used for gene transfection. The advantage of using this system is the high level of transgene overexpression and high efficiency of infection of a broad spectrum of cells, including non-dividing cells. In addition the adenoviral backbone can accommodate large transgenes up to 35 kb. Adenoviral infection begins with the binding of the virus to the specific receptor on the host cell membrane followed by endocytosis. Adenoviruses then escape the endosomes and translocate from the cytoplasm to the nucleus where viral transcription and replication proceeds. Recombinant adenoviruses lack early genes required for propagation therefore the infection does not result in host cell apoptosis (Luo 2007). In the current study, adenoviruses were used for transgene overexpression in human primary macrophages. Two recombination systems, AdEasyTM (Q-BIOgene, CA, USA) and Gateway® Technology (Invitrogen), were used to generate transgene expressing adenoviruses.

2.2.3.1 The AdEasyTM recombination system

The AdEasyTM recombination system involves the use of two vectors; the ‘transfer’ and ‘backbone’ plasmids. The transfer vector carries the cloned gene of interest and is used for recombination with the backbone vector. The latter contains most of the viral genome but lacks genes required for virus propagation. The homologous recombination of a *PmeI*-linearized transfer vector with a backbone vector is then conducted in modified BJ5183 bacteria. Finally, *PacI*-digested recombinant adenoviral DNA is transfected into HEK-293 cells which contain the *E1* gene required for virus propagation. The overview of the system is presented in **Figure 2.2**.

The transfer vector used in this study was pAdSK16 (9.45kb) constructed by Dr Clive Smith. The vector was derived from commercially available pAdTrack.CMV (9kb) (Q-Biogene, CA, USA) by modifying the multiple cloning site (MCS) and by removing the *EcoRI* site from the vector. The N-terminal Protein G- TEV- SBP (NTAP) tag was cloned into pAdSK16 by Ms Kate Willetts and used as a control for the tandem affinity purification protocol (Section 2.2.5). Human Btk was cloned into pAdSK16-TAP using standard molecular biology techniques (Section 2.2.2). The backbone vector used in this study was commercially available pAdEasy-1 (Q-BIOgene, CA, USA) (**Figure 2.2**).

The transfer vector was first linearized by *PmeI* as described (Section 2.2.2.9) and purified using Wizard SV Gel and PCR Clean-Up system (Promega). Electrocompetent BJ5183 *E.coli* cells (Q-Biogene, Cambridge, UK) (80µl) were co-transfected with 200ng of pAdEasy and 600ng of pAdSK16 in a total volume of 4µl of DNA (Section 2.2.2.13). After 1h incubation in antibiotic-free LB, the cells were spread onto kanamycin (0.3µg/ml)-containing agar plates and incubated at 37°C overnight. The following day representative colonies were collected and cultured in 5ml LB broth media at 37°C overnight. Plasmid DNA was extracted and digested with *PacI*. Plasmids with diagnostic digestion pattern of 3kb or 4.5kb, and 35kb were further transformed into XL-1 blue *E.coli* to increase plasmid yield. Plasmid DNA (10µg) was then extracted from bacteria, linearized with *PacI* and subsequently precipitated using the NaAc/EtOH protocol (Section 2.2.2.14) prior to transfection into AD-293 cells (Section 2.2.3.3).

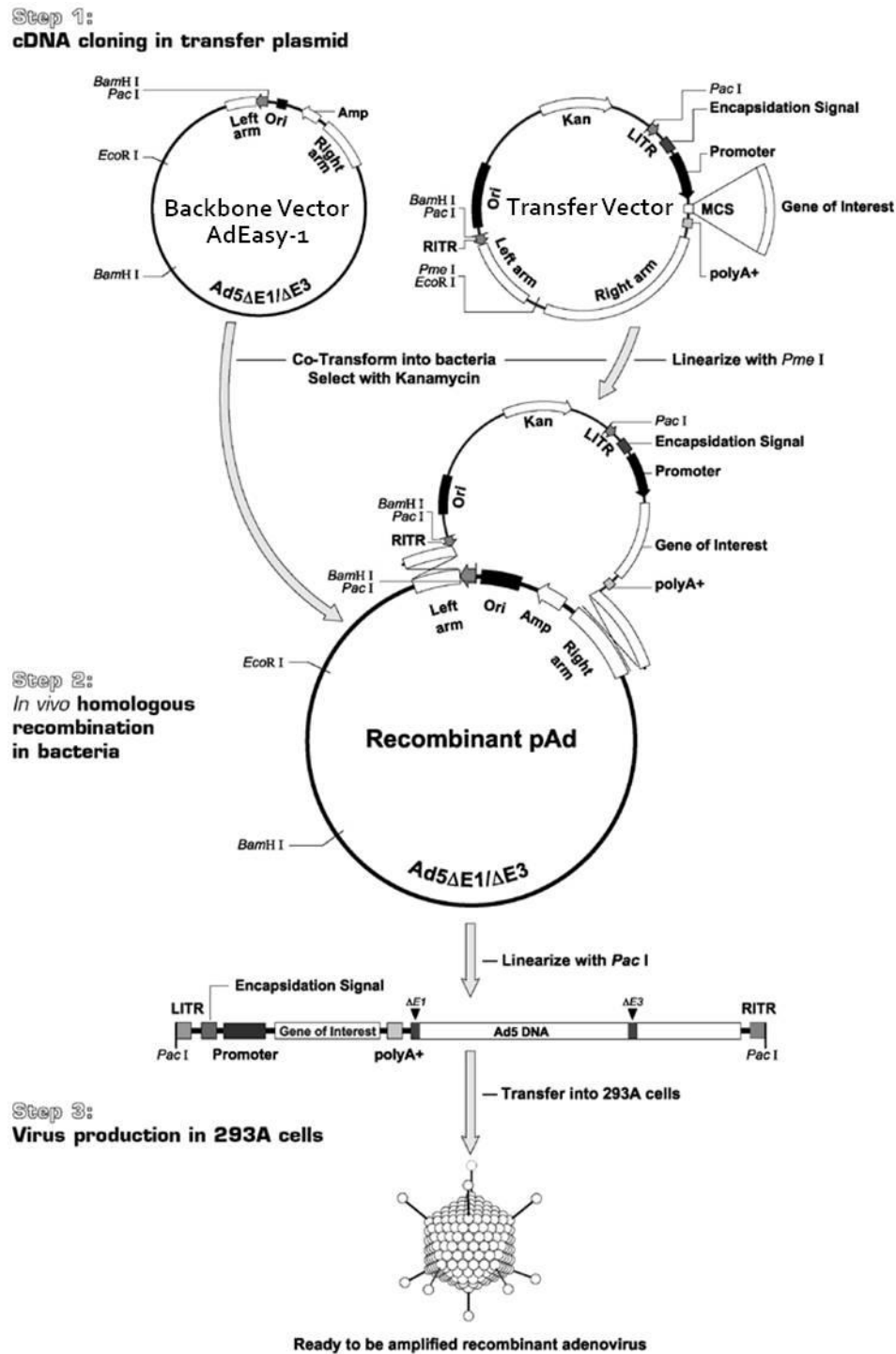


Figure 2.2 Generation of a recombinant adenovirus using AdEasy™. The AdEasy™ protocol involves three steps. In the first step, cDNA of the gene of interest is cloned into the transfer vector. The transfer vector is linearized with *PmeI* and co-transformed with the adenovirus backbone vector lacking the genes involved in virus propagation (Ad5ΔE1ΔE3) into the BJ5183 bacterial strain. Following homologous recombination plasmid DNA is isolated from bacteria, linearized with *PacI* and transfected into HEK-293 for amplification. ITR: inverted terminal repeats, LITR: left-hand ITR, RITR: right-hand ITR, MCS: multiple cloning site, Kan: kanamycin resistance, Amp: ampicillin resistance gene, Ori: bacterial origin of replication. The diagram is adapted from www.qbiogene.com.

2.2.3.2 The Gateway® recombination system

Similarly to the AdEasy™ system, the Gateway® Technology system involves the use of transfer and backbone vectors. The transfer vector does not, however, have to be linearized prior to site-specific recombination with the backbone, which increases the efficiency of virus generation. Moreover, recombined vectors do not have to be transformed into two sequential bacterial strains as only the Top10 *E.coli* strain is used for plasmid amplification. The technology is based on the lambda recombination system to facilitate transfer of heterologous DNA sequences, flanked by *att* sites, between transfer and backbone vectors. The overview of the system is presented in **Figure 2.3A**.

The transfer vector used in this study, pBENT2 containing C-terminal SBP (*Strep*) tag (4590bp), was kindly provided by Dr Irina Udalova and was utilised to generate the control adenovirus for One-*Strep* purification (Section 2.2.5.1) (**Figure 2.3B**). Human Btk was cloned into the vector using standard molecular biology techniques (Section 2.2.2). The backbone vector used in this study was pAd/PL-DEST™ (Invitrogen) (**Figure 2.3C**).

Gateway LR Clonase Enzyme mix (Invitrogen) was used for the recombination of the transfer and backbone vectors. A 10µl reaction was set up containing 2µl (300ng) of Btk-*Strep*-pBENT, 2µl (300ng) of pAdPL-DEST, 2µl of LR Clonase enzyme, 2µl of LR Clonase buffer and 2µl of TE buffer. The sample was incubated at 25°C overnight.

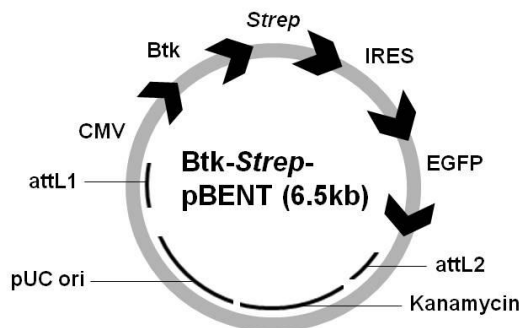
Chemically competent One Shot® Top10 *E.coli* cells (Q-Biogene, Cambridge, UK) (80µl) were transfected with 2µl of the recombination mixture. Briefly, the bacteria were incubated with DNA on ice for 30min. The cells were then subjected to 42°C heat shock for 30sec and put back on ice for 2min. The cells were left to recover in antibiotic-free LB broth (Section 2.1.10.9) for 1h at 37°C, spread onto ampicillin- containing agar plates and incubated at 37°C overnight. The following day representative colonies were collected and cultured of either 5ml ampicillin or chloroamphenicol- containing LB broth overnight at 37°C. If recombination had occurred, chloroamphenicol resistance was lost. Plasmid DNA was extracted from the bacteria cultured in ampicillin- containing LB broth media and digested with *BamHI*. Plasmids with a diagnostic

digestion pattern of 500bp, 3.5kb, and above 14kb were considered positive clones. Plasmid DNA (10µg) was then linearized with *PacI* and subsequently precipitated using the NaAc/EtOH protocol (Section 2.2.2.14) prior to transfection into AD-293 cells (see below).

A)



B)



C)

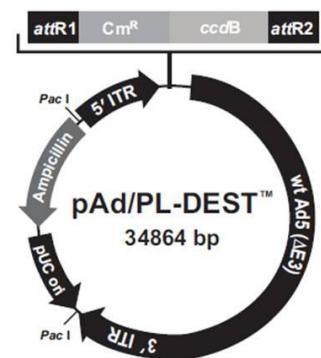


Figure 2.3 Generation of a recombinant adenovirus using the Gateway® Technology. The technology is based on the site-specific recombination between *attL* and *attR* using LR Clonase (A). The transfer vector used in this project was Btk-Strep-pBENT containing *attL* sites (B), whereas the backbone vector containing *attR* sites was pAd/PL-DEST (C). Upon recombination the chloroamphenicol resistance gene (*Cm^R*) is lost enabling the selection of positive clones. Ori: bacterial origin of replication, TK: Herpes Simplex Virus thymidine kinase polyadenylation signal, wt Ad5 (ΔE3): human adenovirus type 5 sequence with *E3* early gene deletion, ITR: inverted terminal repeats, EGFP: Enhanced Green Fluorescent Protein, CMV: Cytomegalovirus promoter. Diagrams A and C were adapted from www.invitrogen.com.

2.2.3.3 Transfection of viral genomic DNA into AD-293 cells

AD-293 cells were plated at 8×10^5 cells per well in a 6-well plate and incubated at 37°C overnight to reach 90% confluence. The cells were transfected with *PacI*-linearized and NaAc/EtOH-precipitated recombinant adenovirus vectors using Lipofectamine™ 2000 transfection reagent (Invitrogen). Briefly, 15µl of Lipofectamine 2000 was mixed with 400µl of

Opti-MEM® medium (Invitrogen) and incubated at RT for 5min. The mix was added to 100µl of DNA, agitated and left at RT for a further 30min. In the mean time the culture media was removed from AD-293 cells and replaced with 1ml of pre-warmed Opti-MEM® medium for 30min. The Lipofectamine/DNA mixture was added to the cells and replaced with DMEM/10% FCS after 2h incubation. The next day DMEM/10% FCS was removed and the cells were covered with 3ml of pre-warmed 0.75% (w/v) sea-plaque agarose; 1 x MEM, 2% (v/v) FCS and 1% (v/v) penicillin/streptomycin. The mixture was allowed to solidify at RT for 5min before continued incubation at 37°C. The cells were incubated for 10-14 days to allow virus plaques to develop in the monolayer of AD-293 cells.

2.2.3.4 Plaque purification and virus recovery

Plaque purification is essential in the production of viruses *de novo*. The plaques are formed by the infection and lysis of neighbouring cells and are easily seen by eye or with the assistance of a microscope. 8-10 plaques were picked using a 10µl Gilson pipette and filter tips and subsequently diluted in 0.5ml of serum-free DMEM. Virus-infected media was left at 37°C in an incubator overnight allowing viral dissociation from cellular debris or agar constituents as well as preliminary detection of bacterial/ yeast contamination. The 0.5ml of infected media was applied onto one well of AD-293 monolayer cultured in a 12-well plate. After 2h incubation at 37°C the virus infected media was replaced with 1ml of 2% DMEM. The cells were thereafter incubated for 3-5 days until the plate exhibited a full cytopathic effect (CPE) characterised by cell swelling and detachment due to adenoviral infection. One clone was selected for adenovirus large scale grow-up.

2.2.3.5 Adenovirus large scale grow-up

The amplification of adenovirus consisted of infection of confluent AD-293 cells plated in successively larger dishes. Initially, AD-293 cells were cultured in 6-well plates. Adenovirus from the archive stock was introduced in 100µl volume to the culture in 1ml serum- free (SF) DMEM. After 2h, 1ml of DMEM supplemented with 4% FCS and 2% Pen/Strep was added to the cells. AD-293 cells were then incubated for 2 days for a full CPE to be obtained. Viral lysate was prepared by freeze-thawing in liquid nitrogen 3 times to ensure that adenovirus was entirely

released from the cells. At the next step, AD-293 cells in T75cm² culture flask were infected with 1ml of this lysate in 5ml of SF DMEM as described above. Again cells were incubated for 2 days for a full CPE. Lysate was frozen-thawed and used for the infection of 10x T175cm² flasks containing 80-90% confluent AD-293. Cells were incubated in 20ml of 2% FCS medium for 40h until they became non-adherent but not lysed as this would prevent virus concentration. The cells were then harvested into 4 50ml (200ml of medium total). Falcon tubes and spun at 1000 rpm for 5min. The pellets were then resuspended in SF DMEM, pooled together into one tube and spun again. Supernatants were discarded and cells were re-suspended in 10ml of 0.1mM Tris (pH 8.0) and placed into 2 cryovials. AD-293 cells were then frozen-thawed and passed through a 19-gauge needle 4 times to shear chromatin. Lysates were then spun at 2000 rpm for 5min to pellet cell debris.

2.2.3.6 Purification of Adenovirus from large scale grow-up

Purification of the adenovirus was performed to remove contaminants such as viral capsid proteins and to increase the yield of infection (Cotten et al. 2003). Virus-containing supernatant was placed into a new tube, made up to 11.4ml with 0.1M Tris-HCL and to 18ml with saturated CsCl. The solution was then transferred to 9ml Sorvall ultracentrifuge tubes (RC-M150 GX, Kendro laboratory products, UK) and spun at 60 000 g overnight at 4°C. The next day, the tube was pierced with a 19-gauge needle and the virus band was drawn into the 5ml syringe. Virus material from two tubes was pooled together, injected into a new ultracentrifuge tube and filled to the top with 1.34g/ml density CsCl. The rotor was balanced and the tube was spun as before.

The band with the virus was removed in less than 2.5ml volume total and injected onto a PD10 column previously equilibrated with 25ml PBS²⁺. The virus was eluted by further addition of 3.5ml PBS²⁺ and collected into a bijou containing 10 drops of sterile glycerol. Virus was then aliquoted and frozen at -70°C.

2.2.3.7 Adeno-X™ rapid titre kit

Virus titre was determined using the AdenoX™ titre kit (BD Biosciences). AD-293 cells were seeded at 5×10^5 cells per well in a 12-well plate. The cells were cultured in 1ml of standard growth medium (DMEM supplemented with 5% FCS and 2% Pen/Strep) overnight. The next day, the viral sample dilutions (5×10^{-5} , 1×10^{-5} , and 5×10^{-6}) made up in 100µl SF DMEM were applied to each well. 28h later the medium (1ml + 100µl) was aspirated and cells were allowed to dry in a hood for 5min prior to fixation with 1ml ice-cold 100% methanol. The plate was incubated at -20°C for 10min. Methanol was aspirated and the wells were gently rinsed 3 times with 1ml PBS containing 1% (w/v) BSA. A mouse anti-hexon antibody at a 1:1000 dilution in PBS (1% BSA) was incubated with the cells for 1h at 37°C. The wells were washed 3 times with 1ml PBS (1% BSA) prior to a 1h incubation with rat anti-mouse antibody (HRP conjugate) diluted 1:500 in PBS (1% BSA). After the final wash, a colorimetric reaction was carried out using DAB working solution (10X DAB substrate 1:10 with 1X Stable Peroxidase Buffer). A minimum of 3 fields of brown/black positive cells were counted using a microscope with a 20X objective. The mean number of positive cells in each well was counted. The titre (number of viral particles per ml) was calculated from the dilution which gave around 50 positive cells per field (PC). The number of PC was multiplied by 573 (the number of fields in the well) and divided by the result of multiplication of 0.1 (virus volume diluted 1:10 in medium) by the dilution factor to determine the viral titre.

2.2.3.8 Replication competency test

To test if adenovirus regained replication competency outside AD-293 cells, a small flask of non-adherent human foreskin fibroblast (HFS) cells were infected with the virus in SF DMEM. After 2h the medium was removed and replaced with 10% FCS DMEM. Cells were incubated for 7-10 days. If a cytopathic effect was not observed, the batch of virus was used for experiments.

2.2.3.9 Adenoviral gene transduction of human macrophages

Human primary monocytes were differentiated into macrophages using M-CSF. M-CSF was essential to upregulate the low expression of the $\alpha\beta 3$ and $\alpha\beta 5$ integrins which are necessary for adenoviral entry into the cells (Huang et al. 1995). Human primary macrophages were then

detached from culture plates with cell dissociation media for 45min and subsequently scraped. They were counted and plated in experimental dishes in 5% RPMI without M-CSF and incubated overnight. Adenovirus was diluted in SF RPMI to final multiplicity of infection (moi) from 50 to 150 infectious virus particles per cell then added to the cells. After 2h medium was removed and replaced with 5% RPMI. Cells were incubated for 24-48h. The extent of viral infectivity was determined by visualisation of GFP under UV light. For double infection, cells were first infected with adenovirus at moi 100 for 2h, rested for 4h in 5% RPMI prior to secondary infection at moi 50 as described before.

2.2.4 Biochemical Techniques

2.2.4.1 Whole cell lysis

Cells designated for Western blotting analysis were first lysed in 1% NP40 buffer (Section 2.1.10.3) for 20 min on ice. Nuclei and insoluble debris were removed by centrifugation at 13000 rpm for 8 min at 4°C. Supernatants were transferred to fresh Eppendorfs. 5X denaturing protein gel sample buffer containing SDS was added and samples were boiled for 5 min prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.2.4.2 Protein concentration estimation by BCATM protein assay kit

The bicinchoninic acid (BCA) assay (ThermoScientific) was used to determine the total protein concentration in cell lysates prior to the kinase or TransAm assays. For the BCA assay, 5µl of fresh lysate was diluted in 5µl of H₂O and transferred to a 96-well U-shape Microtest plate (Becton Dickinson) in triplicate, along with a series of BSA concentrations as standard. The BCA solutions were mixed at volume ratios of 50:1 (assay reagents A:B) and 200µl of the mixture was added to each well. Samples were incubated for 30 min at 37°C and the absorbance was read at 562nm wavelength on a Fluostar Omega (BMG Labtech, Aylesbury, UK) plate reader and accompanied by MARS data analysis software.

2.2.4.3 SDS-PAGE

SDS-PAGE is a technique that allows protein separation based on molecular size. The separation occurs in an electric field. Denaturated proteins, which are negatively charged due to addition of SDS at the lysis stage, migrate toward the positively charged anode. Electrophoresis gels consist of an upper, stacking, 3% (pH 6.8) gel containing wells designated for sample loading and concentration. The bottom, resolving layer (7-10%, pH 8.8) separates the proteins. The gel was cast in the Bio-Rad Protein Cell II apparatus (Bio-Rad, Herts, UK) and submerged in a running buffer (Section 2.1.10.4). Protein samples and Full Range Rainbow molecular weight marker RPN800 (Amersham Biosciences) were loaded into individual wells and run at 55V overnight.

Gels were made as follows:

<i>Reagent</i>	<i>4% stacking gel</i>	<i>7% resolving gel</i>	<i>10% resolving gel</i>
30% Acrylamide	1.25 ml	9.33 ml	13.33 ml
Resolving buffer	n/a	15 ml	15 ml
Stacking buffer	1.5 ml	n/a	n/a
10% (w/v) APS	100 µl	250 µl	250 µl
TEMED	10 µl	25 µl	25 µl
water	7.14 ml	15.4 ml	11.4 ml
total	10 ml	40 ml	40 ml

2.2.4.4 Protein transfer

Proteins resolved by SDS-PAGE were transferred onto polyvinylidene difluoride (PVDF) (Perkin Elmer Life Sciences Inc, MA, USA) or NitroBind membrane (GE Water and Process Technologies, USA) by using a wet transfer technique. PVDF membrane was first moistened in methanol and then together with other layers placed in transfer buffer. The gel was removed from the electrophoresis apparatus and placed onto three layers of filter paper and a cloth. It was then covered by membrane and stacked into another three layers of filter paper and a cloth on the top. The structure was then compressed into a plastic cassette (Bio-Rad, UK) and placed with the membrane side facing the anode (+) into a transfer tank filled with cooled transfer buffer. The transfer was run at 100V for 90min.

2.2.4.5 Western blotting

The membrane was removed and blocked for 1h in TBS-Tween (0.1%) containing 2% BSA for the detection of phosphorylated proteins or in 5% skimmed milk for the detection of other proteins. Primary antibodies (Table 2.2) made up in the blocking buffer were then applied for a minimum of 1h at RT. The membrane was washed for 30min in frequently changed TBS-Tween (0.1%) and horseradish peroxidase (HRP) conjugated secondary antibodies (Table 2.3) at a dilution of 1:2000 were added for an hour. The membrane was washed again as before and ECLTM (Amersham Biosciences) reagent applied for 1min to visualize the reaction. The luminescence of the product was detected by placing HyperfilmTM (Amersham Biosciences) on the top of the membrane and subsequent development of exposed films in an AGFA Cruis-60 film processor (AGFA-Gaevert, Brentford, UK). If necessary, blots were re-probed with other antibodies after stripping of the membrane with Re-blot stripping solution (Chemicon International Inc, CA, USA) for 5min.

2.2.4.6 Luciferase assay

The luciferase assay involved the use of adenoviruses carrying DNA constructs consisting of the luciferase gene flanked by the 5' *TNF* promoter and different portions of the 3' *TNF* untranslated and enhancer regions (Table 2.14). The luciferase enzyme catalyses mono-oxygenation of beetle luciferin in the presence of Mg^{2+} and ATP, resulting in the release of photons (fluorescence) (Gould and Subramani 1988). This fluorescence can be measured using a luminometer, and the activity of the element regulating the luciferase gene can thereby be quantified.

Cells were cultured in 96-well plates and following infection with adenovirus containing the firefly luciferase enzyme (as detailed in Section 2.2.3.9), cells were washed in ice cold PBS then lysed on ice in 100 μ l CAT buffer (Section 2.1.10.5) for 10min. Samples were either stored at -20°C until further use, or assayed directly. 50 μ l of the lysates was transferred to a luminometer plate with 120 μ l LAB containing 1mM ATP and 0.5mM DTT per well. The cocktail was assayed in a Luminometer (Labsystems Luminoskan) by the addition of 30 μ l luciferin and the measurement of photon release over a 10sec period. Values were recorded as relative luciferase units (RLU).

2.2.5 Affinity Tag Purification and Mass Spectrometry

Mass Spectrometry (MS) is a powerful tool for the identification of novel protein-protein interactions (Vasilescu and Figgeys 2006). Although MS is a highly sensitive and effective technique, the purification of proteins still remains difficult due to the low abundance of the proteins and the instability of the protein-protein complexes *in vivo*. A commonly used technique for the purification of endogenous proteins, such as immunoprecipitation (IP), requires a high number of cells and a long purification procedure. Moreover, as the procedure is highly dependent on the antibody used for the pull-down of the bait, the yield of the purified protein varies between different proteins and antibody batches. These limitations led to the development of affinity tag purification systems based on the strong interactions of the tag with its target (Lichty et al. 2005). A novel tandem affinity purification (TAP) strategy does not rely on the use of antibodies, the proteins are purified rapidly and still remain biologically active (Puig et al. 2001). This strategy has been extensively used since 2002, when Gavin and colleagues performed a large scale purification of yeast and eukaryotic proteins. In their studies, the group tagged 1,739 genes to a TAP tag and successfully purified 589 protein assemblies providing an extensive network of biological interactions (Gavin et al. 2002).

The TAP system involves the fusion of bait to the tag and subsequent column purification of the protein complexes. The TAP tag consists of the 136 amino acid long Protein G, a cleavage site for tobacco etch virus (TEV) protease and streptavidin-binding peptide (SBP) moieties. During the first step of purification, a tagged protein together with its interacting partners binds to IgG sepharose through Protein G. The complex is then cleaved by a TEV protease. Eluted protein complexes are then sequestered by streptavidin sepharose through SBP and undergo an extensive wash in order to remove any contaminants and the remaining TEV protease. A tagged protein together with its binding partners is subsequently eluted with biotin, which competitively binds to the streptavidin column (**Figure 2.4**) (Burckstummer et al. 2006). The use of the two-step purification system decreases the background by limiting the occurrence of non-specific proteins in the elution fraction. On the other hand, the introduction of the 136 amino acid long tag increases the risk of interfering with a native conformation of the bait and may lead to impaired protein interactions.

An alternative is the introduction of a shorter version of the tag composed of only an 8 to 31 amino acid long SBP, called a *Strep* tag (www.iba-go.com). The use of the One-*Strep* system has been shown to result in excellent, cost-effective purification with good yields as compared to other affinity tags such as HIS, calmodulin-binding peptide (CBP) or FLAG (Lichty et al. 2005). The tagged bait is first purified through a *Strep*-Tactin[®] column, extensively washed and eluted with biotin in native conditions within a short period of time. The recovered material is then run on an SDS-PAGE gel and analysed by several methods including Western blotting or MS. The first approach is used to confirm the efficiency of the system as it facilitates the detection of known protein-protein interactions. The second approach is used to identify new binding partners, provided that the protein complex is adequately purified in reasonable quantity.

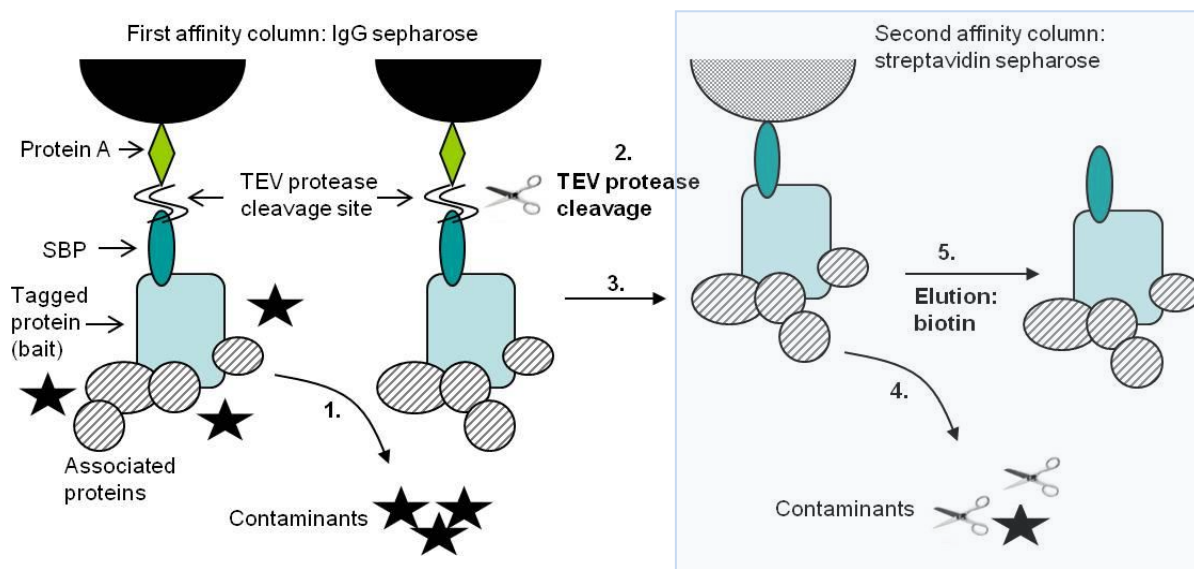


Figure 2.4 Overview of the TAP purification strategy. The full length TAP tag consists of a Protein G, which facilitates the binding to an IgG sepharose column, a TEV protease cleavage site that enables the first elution of the complex, and a streptavidin binding protein (SBP) that facilitates the binding to the second affinity column. The One-*Strep* purification process is used when the target protein is tagged to the SBP only (shaded square). The complex is eluted with biotin and submitted for mass spectrometry analysis (adapted from (Burckstummer et al. 2006)).

2.2.5.1 One-Strep purification

Human primary macrophages previously infected with *AdStrep* or *AdBtk-Strep* were plated at 7×10^6 cells per 10cm^2 Petri dish. Following stimulation the cells were washed 3 times in ice cold PBS, scraped and resuspended in cytoplasmic lysis buffer (Section 2.1.10.8). After 20min on ice the sample was spun at 12000rpm for 1min at 4°C . The supernatant was kept as the cytoplasmic fraction. The pellet was resuspended in nuclear lysis buffer and incubated with gentle agitation for 15min at 4°C . The lysate was finally spun at 13000rpm for 10min at 4°C to remove nuclear insoluble fractions. The supernatant was kept as the nuclear fraction.

Btk-Strep complexes were purified on a Bio-Spin® gravity flow chromatography column (Bio-Rad, CA, USA) containing Strep-Tactin Macroprep (IBA GmbH, Germany) resin. 200µl of the resin was applied onto a column and equilibrated by adding 400µl of washing buffer (WB). The cell lysate was then applied to the column. 10% of the Input and 10% of the Flow Through was kept for further analysis. The column was then washed 10 times with 1ml of WB. 10% of each WB fraction was kept as previously detailed. *Btk-Strep* complexes were eluted in 6 fractions of 200µl elution buffer containing 2mM Biotin. Eluted fractions were concentrated on a Vivaspinn 500 column (Sartorius Stedim Biotech, France) prior to applying onto an SDS-PAGE gel.

2.2.5.2 Silver staining of an SDS-PAGE gel

Visualisation of proteins on an SDS-PAGE gel was performed using the Pierce Silver Stain kit (Thermo Scientific, IL, USA). Proteins bind silver ions, which can be reduced under appropriate conditions and form black elementary silver (Chevallet et al. 2006). According to the manufacturer's protocol, the gel was washed in dH_2O and fixed in fixing solution (30% ethanol, 10% acetic acid) on rotation for 15min. 10% ethanol wash was added for 10min followed by 2 times 5min wash with dH_2O . The gel was then sensitised with provided Silver Stain Sensitizer and washed again with dH_2O twice for 1min. Silver Stain Enhancer was incubated with the gel for 5min followed by two brief dH_2O washes. Developer working solution was added immediately to the gel until protein bands appeared. The reaction was stopped with 5% acetic acid.

2.2.5.3 Protein identification by tandem mass spectrometry

The identification of Btk-*Strep* protein complexes by MS was performed by Dr Robin Wait. Silver stained gel bands were excised with a scalpel, and proteins were trypsin digested in gel, using an Investigator Progest robot (Genomic Solutions, Huntingdon, UK) as previously described (Wait et al. 2002).

Samples were analysed by high performance liquid chromatography coupled to electrospray ionisation tandem mass spectrometry (HPLC ESI MS/MS). HPLC was carried out on a CapLC liquid chromatography system (Waters, Manchester UK). Aliquots (6 µl) of peptide mixtures were injected onto a Pepmap C18 column (300 µm x 0.5 cm; LC Packings, Amsterdam, The Netherlands) and eluted with an acetonitrile/ 0.1% formic acid gradient to the nanoelectrospray source of a Q-ToF spectrometer (Micromass, Manchester, UK) at a flow rate of 1 µl/min. The spray voltage was set to 3500 V and data dependent MS/MS acquisitions were performed on precursor peptides with charge states 2, 3, or 4, over a survey mass range 440-1400, using argon collision gas. Product ion spectra were recorded over the range 100-1800, transformed onto a singly charged m/z axis using a maximum entropy method (MaxEnt3, Waters, UK), and centroided peaklist (pkl) files were extracted using the MassLynx routine peptide auto (Waters, Manchester UK).

Proteins were identified by correlation of uninterpreted spectra to entries in SwissProt (Release 2010_04: 516,081 entries) using Mascot (version 2.2: www.matrixscience.com). MS/MS ion searches specified up to two missed cleavages per peptide, a precursor mass tolerance of ± 100 ppm and a fragment ion mass tolerance of ± 0.5 Da. Carbamidomethylation of cysteines and methionine oxidation were specified as fixed and variable modifications respectively.

MS/MS based peptide and protein identifications were validated using Scaffold (Proteome Software Inc., Portland, Oregon: version 3.01). Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al. 2002).

2.2.6 Immunological Techniques

2.2.6.1 Flow cytometry

Flow cytometry is a technique that enables quantitative measurements on single cells at very high rates of speed. The flow cytometer consists of fluidics, optics and a detecting system. The fluidics system brings a cell into the light beam, where it is then illuminated and the scatter of light is collected by photodetectors, which are located at different positions. The one that is located in front of the illuminating beam detects the forward scatter (FSC), others that are situated at angles detect the side scatter light (SSC). FSC gives the information about the size and volume of the cell, whereas SSC photodetector is related to the surface and internal structure of the cell. Flow cytometers can also detect fluorescence signal emitted from fluorochrome- conjugated monoclonal antibodies. The illuminating beam will excite the fluorochrome which then emits light at different wavelengths. The light is then selectively collected by photodetectors, converted into electrical impulses and interpreted by the computer software.

In the present study, human monocytic-like cell lines and human primary macrophages were stained with Fluorescein isothiocyanate (FITC)-conjugated anti-TLR4, anti-TLR3, anti-TLR8, or control IgG1 or IgG2 antibodies (Imgenex). For external staining (TLR4 and IgG2 control), cells were first washed and then blocked in PBS containing 5% human serum and 0.1% sodium azide. After 30min incubation on ice, antibodies (20µg/ml) were added for another hour. Cells were washed 3 times in PBS and directly analysed on BD Biosciences LSR flow cytometer. For the staining of internal antigens (TLR3, TLR8 and IgG1 control), cells were additionally fixed in 2% paraformaldehyde for 5min on ice, washed 3 times in PBS and then permeabilized with 0.1% saponin during the blocking step.

2.2.6.2 Immunoprecipitation and phospho-tyrosine detection

Human primary macrophages (5×10^6 per time point) were plated on 10cm² Petri dishes and serum starved for 2h before TLR stimulation. Cells were lysed in 1% NP40 lysis buffer (Section 2.1.10.3) and insoluble debris and nuclei were removed by centrifugation at 13000 rpm for 8min. Supernatants were transferred to new tubes. Immunoglobulin-binding proteins A and G, which are covalently bound to SepharoseTM beads (GE Healthcare, Sweden), were mixed at equal volumes and washed 3 times in the 1% NP40 lysis buffer to remove ethanol. Supernatants were then pre-

cleared with 50µl of Protein A/G mix at 4°C for an hour to remove proteins that bind non-specifically to Protein A/G. Non-specifically bound protein complexes were further removed by centrifugation at 13000 rpm for 3min and supernatants were incubated with anti-Btk antibody (gifted by Dr M. Tomlinson) or isotype control rabbit IgG, at 4°C for an hour. Protein A/G mix was introduced to pull down antibodies conjugated with target proteins. After a 2h incubation, Sepharose- ProteinA/G- Btk complexes were isolated by centrifugation at 13000rpm for 3min, supernatants were discarded and the pellets were washed 3 times in 1% NP40 lysis buffer. All steps were performed at 4°C with constant agitation. Protein gel sample buffer was added and samples were boiled for 5min before loading on an SDS-PAGE gel. Proteins were transferred to nitrocellulose membrane as described before. The membrane was blocked in 2% BSA and incubated with HRP-conjugated 4G10 antibody directed against tyrosine phosphorylated proteins (section 2.2.4).

2.2.6.3 *In vitro* kinase assays

In vitro kinase assays utilising radiolabelled ATP were used to assess Btk auto-kinase activity or p38 activity in the presence of the specific substrate, MK2 (Millipore, Dundee, UK). Adenovirus-infected human primary macrophages (2×10^6 per time point) were subjected to TLR stimulation and lysed in 0.4ml of kinase lysis buffer (Section 2.1.10.11) for 10min on ice. Lysates were centrifuged (10 min, 13000 rpm, 4°C) and the cleared nuclear lysates was transferred to new Eppendorfs. The quantity of the protein was assessed using the BCA assay (Section 2.2.4.2). Samples were further divided into two fractions of which one was analysed for equal loading control using Western blotting analysis and the remaining 500µg of protein was used for the kinase assay.

Protein A/G-SepharoseTM beads (GE Healthcare, Sweden) were washed 3 times in 1x kinase wash buffer. The lysate and 3 µl of the IP antibody (Table 2.2) were added to the 30µl of the beads. The sample was incubated on a rotator at 4°C overnight. The supernatant was then discarded, beads were washed 4 times in 1ml of ice cold RIPA buffer followed by two washes in 1ml of ice cold 1x kinase wash buffer. The excess of the buffer was removed from the beads using an aspirator. The beads were then incubated with 30µl of kinase assay mastermix containing 10µl of 3x kinase assay buffer, 1µl (1µg) of substrate, 0.1µl of 6mM cold ATP, 0.4µl of $\gamma^{32}\text{P}$ - ATP (or an equivalent

of 4 μ Ci) and 18.5 μ l of dH₂O. The substrate was omitted in the auto-kinase assays. The reaction tubes were incubated at RT for 30min with gentle agitation. The samples were spun down to avoid lid contamination and the reaction was stopped by adding 8 μ l of 5x reducing gel sample buffer and boiling for 5min. The samples were then loaded on a 12% SDS-PAGE gel and run overnight at 55V (Section 2.2.4.3). The gel was subsequently fixed for 30min in dH₂O, methanol and acetic acid at the respective ratios 5:4:1 and dried for 2h. Enzyme activity was assessed by autoradiography.

2.2.6.4 Sandwich enzyme linked immuno-sorbent assay

Enzyme linked immune-sorbent assays (ELISA) were used for a quantitative detection of cytokines in the supernatants collected from cultured cells stimulated with TLR ligands. ELISA plates were initially coated with 50 μ l of cytokine specific antibodies (Table 2.4) for an hour at 37°C and washed 3 times in 1x PBS with 0.001% Tween20. The plates were then blocked in 2% BSA made up in 1x PBS at RT for another hour and washed as before. Samples and standard curve cytokines at concentrations ranging from 10,000-13.72pg/ml were applied overnight. After washing, wells were filled with biotin-labelled detection antibody diluted in 1x PBS and incubated for 1 hour at RT. The wells were washed and loaded with HRP/streptavidin conjugates at 1:400 dilution in 1x PBS and incubated again for 1 hour at RT. Wells were washed and 1 volume of a HRP substrate, tetramethylbenzide dichloride (TMB), was added until the visualization of the standard curve was satisfactory. To stop the reaction 1 volume of 2M H₂SO₄ was applied, absorbance was read at 450nm wavelength using a Fluostar Omega (BMG Labtech, Aylesbury, UK) plate reader and analysed using MARS data analysis software. Samples were analysed in triplicate whereas standards in duplicate.

2.2.7 Electrophoretic Mobility Shift Assay

Electrophoretic Mobility Shift Assay (EMSA) is a technique that allows the characterisation of nuclear complexes bound to a DNA motif. In the assay, nuclear extracts derived from cultured cells are incubated with double-stranded oligonucleotides that are pre-labelled at their termini with γ ³²P-ATP. The samples are then run on a non-denaturing gel. The formation of DNA-protein complexes retards the electro-mobility of the DNA probe in the gel resulting in a shift of the band.

To characterise components of the complex, specific antibodies are added to the nuclear extracts prior to the DNA binding reaction. If the antibody target is present in the DNA-protein complex this will result in an additional shift of the band, thus identifying complex components binding to the DNA probe.

2.2.7.1 Preparation of cytosolic and nuclear protein extracts

Human primary macrophages were grown at 8×10^6 cells per 10cm^2 petri dish. Following stimulation, the cells were washed twice with ice-cold PBS containing 1:50000 PIT. Cells were scraped and re-suspended in 0.5ml ice-cold hypotonic lysis buffer A (Section 2.1.10.6). Cells were kept on ice for 15min prior to addition of 32 μl NP-40 to a final concentration of 0.0064%. The samples were vortexed for 10sec and incubated on ice for an additional 5min prior to centrifugation for 1min at 12000 rpm at 4°C. Pellets were washed with 0.5ml buffer A, vortexed for 10sec and spun as previously. Hypertonic lysis buffer B (150 μl) was added to the pellets for nuclear extraction for 3h. The samples were kept on ice and occasionally vortexed to enhance lysis. Nuclear debris was removed by centrifugation for 15min at 12000 rpm at 4°C. The supernatant was collected into a new pre-cooled tube and the protein concentration was assessed by BCATM protein assay (2.2.4.2). The samples were stored at -20°C.

2.2.7.2 $\gamma^{32}\text{P}$ -ATP end-labelling of double stranded oligonucleotides

The AP-1 and NF κ B consensus double-stranded oligonucleotides (Table 2.12), each possessing 5'-OH blunt ends, were radiolabelled with $\gamma^{32}\text{P}$ -ATP by polynucleotide kinase as described in the following binding reaction:

Reagent	Volume
Consensus oligonucleotide (1.75pmol/ μl)	2 μl
T4 polynucleotide kinase buffer (Promega)	1 μl
20 μCi $\gamma^{32}\text{P}$ -ATP	1 μl
dH ₂ O	5 μl
10 x T4 polynucleotide kinase (Promega)	1 μl

The reaction mixture was incubated for 20min at 37°C followed by the addition of 1µl of 0.5M EDTA to stop the reaction and 89µl of TE (10mM Tris pH 8, 1mM EDTA) to dilute the probe. Unincorporated γ ^{32} P-ATP was removed by using a G-25 microspin column (GE Healthcare). Briefly, the column was pre-equilibrated by centrifugation for 1min at 13000rpm prior to applying the radiolabelled probe. The probe was thereafter stored at -20°C.

2.2.7.3 DNA binding reaction and EMSA gel electrophoresis

Binding reactions between radiolabelled probe and nuclear protein were set up as follows:

Reagent	Volume
Nuclear extract (5µg)	as determined by BCA assay
Probe	1µl
5x binding buffer (Promega)	2µl
Buffer B (Section 2.1.5.6)	to a total of 10µl

For the supershift experiment, 1µl of concentrated ChIP grade antibody (Table 2.2) was incubated with the nuclear extract for 30min at RT prior to the probe binding reaction.

The probe binding reaction was then left shaking at RT for 20min. DNA loading dye 5X Orange G (50µM Tris, 10µM EDTA, 65% (v/v) glycerol, 0.2% (w/v) Orange G) was added to the probe binding reaction to a final dilution of 1X. The samples were spun down and loaded on a 5% TBE gel (Section 2.1.5.6). The gel was run at 140V for 3-4h, dried for 1h on a Bio-Rad Model 583 gel dryer and exposed to HyperfilmTM overnight. The film was subsequently developed by an automatic film processor (AGFA Cruis-60, AGFA-Gaevert, Brendford, UK).

2.2.8 TransAm assay

TransAm (Active Motif) is an ELISA-based assay used for detection and quantification of transcription factor activation. The assay employs a 96-well plate pre-coated with a specific transcription site binding oligonucleotide. Nuclear extracts are applied onto the wells where

activated transcription factors (TF) bind to DNA. The presence of activated TF is then detected by a specific antibody. Addition of a secondary antibody conjugated to a horseradish peroxidase (HRP) provides sensitive colorimetric readout.

Nuclear extract prepared as described previously (Section 2.2.7.1) was applied at 5µg per well in duplicate. The activation of cJun was measured by the detection of phospho-cJun binding to a TPA-responsive element (TRE). TPA-stimulated K-562 nuclear extract was used as a positive control. The assay was performed as described by the manufacturer (Active Motif) in the kit. Tetramethylbenzide dichloride (TMB), a substrate for HRP, was added to enable the colorimetric reaction to occur. To stop the reaction, 1 volume of 2M H₂SO₄ was applied, absorbance was read at 450nm wavelength using a Fluostar Omega (BMG Labtech, Aylesbury, UK) plate reader.

2.2.9 Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) is a genomics technique that allows the detection and quantification of transcription factor (TF) recruitment to specific motifs of genes or the characterisation of unknown TF target sequences. The advantage of using ChIP is that it illustrates the real events occurring in a living cell thus taking into account chromatin complexity. Following an experimental treatment, the cells are subjected to formaldehyde crosslinking that freezes any DNA- protein interactions. The cells are subsequently lysed and the nuclear fraction containing DNA-protein complexes is isolated and subjected to DNA fragmentation by sonication. DNA-protein complexes are then immunoprecipitated by specific antibodies and intensively washed to remove any contaminants. The samples are subjected to reverse cross-linking and the released DNA is purified. Analysis of bound DNA can then be performed by PCR, microarray or sequencing (Collas 2010). For the purpose of the current project, the enrichment of the human TNF gene motifs to NFκB and AP-1 transcription factors was examined by RT-PCR. This protocol was optimised by Dr Tim Smallie. Antibodies used in these studies are listed in Table 2.2 with the primers used for RT-PCR listed in Table 2.10. A schematic representing the major steps involved in ChIP is presented in **Figure 2.5**.

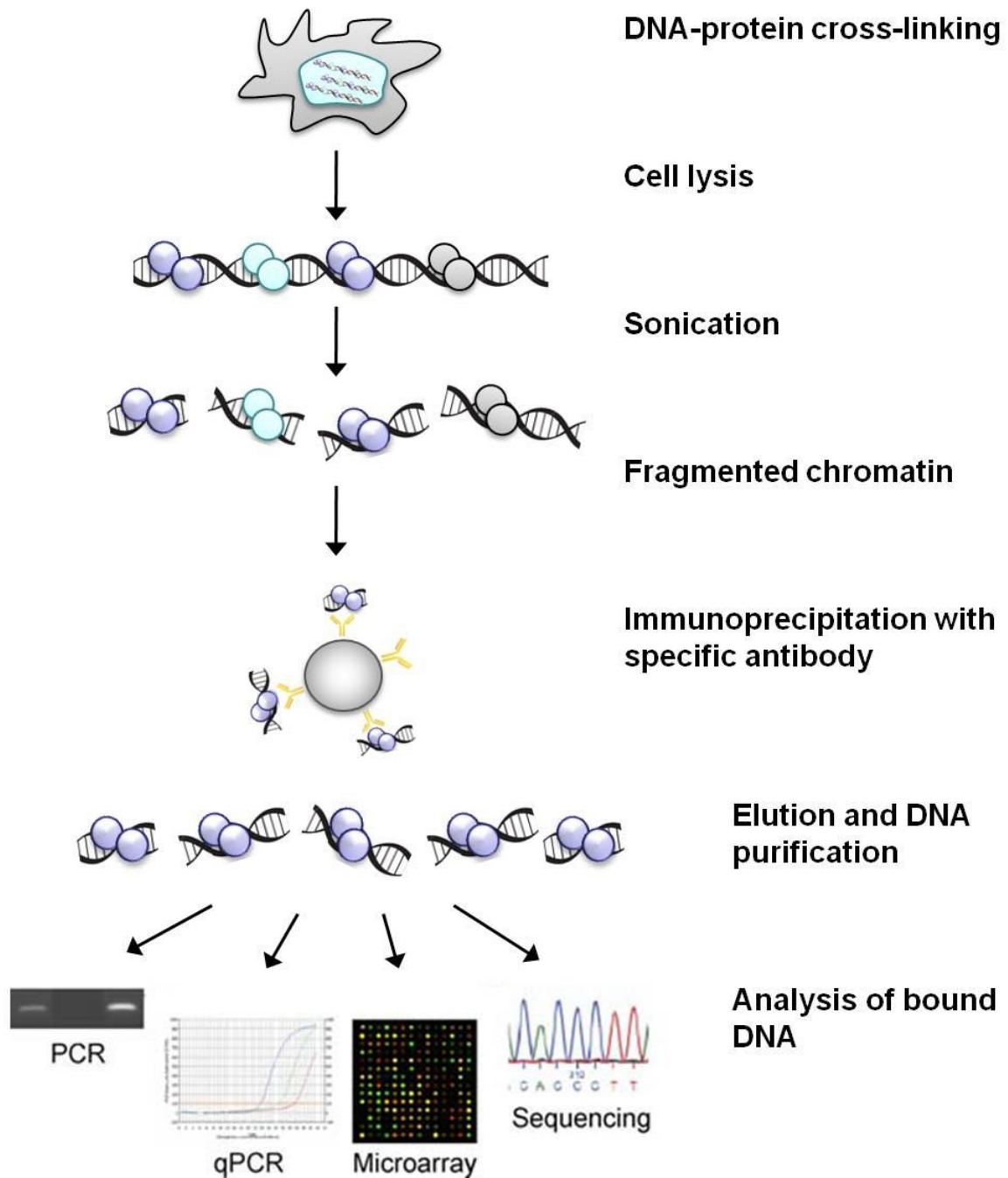


Figure 2.5 ChIP and various methods of analysis. Cells are first subjected to formaldehyde crosslinking and subsequently lysed. The nuclear fraction is extracted and the chromatin fragmented by sonication. Specific antibodies are added to immunoprecipitate DNA-protein complexes. DNA is then purified and subjected to various types of analysis. The figure is adapted from (Collas 2010).

2.2.9.1 Preparation of nuclear extracts

Human primary macrophages were cultured at 8×10^6 cells per 10cm^2 petri dish. Following experimental treatment, formaldehyde at a final concentration of 1% was added for 10min. The crosslinking reaction was stopped by addition of Tris pH 7.5 at a final concentration of 125mM. Cells were subsequently rinsed twice in ice cold PBS, scraped and then pelleted by centrifugation at 1000rpm at 4°C . 900 μl cytoplasmic lysis buffer (Section 2.1.10.7) was added and the lysate was transferred to prechilled Eppendorf Lobind tubes (Cambridge, UK). After 5min on ice the sample was spun at 3000rpm for 5min. The nuclear pellet was resuspended in 600 μl of nuclear lysis buffer and frozen at -80°C .

2.2.9.2 Sonication

Nuclear lysate was gradually thawed on ice. Chromatin was fragmented using sonication conditions optimised for human primary macrophages. A Sonics Vibra CellTM sonicator (Boston, USA) was set to 20% amplitude and a 12sec long pulse. 6 pulses were required to shear DNA into 200-800 base pair long fragments, the resolution sufficient for different primer sets to exclusively span target sequence during RT-PCR.

2.2.9.3 Immunoprecipitation

600 μl of previously sonicated sample was divided into 5 aliquots of 120 μl each. An aliquot was first diluted 10 times with DB buffer (Section 2.1.10.7). To pre-clear 80 μl of Protein G slurry was added to the diluted sample for 2h. Protein G slurry was prepared by adding Salmon Sperm DNA (Invitrogen) into Protein G Sepharose (GE Healthcare) at a ratio of 1:10, subsequently incubated at 4°C for 2h and beads washed 3 times in WB. Protein G slurry was removed from the lysate by centrifugation at 3000rpm for 1min. The supernatant was transferred to a new tube and incubated with 2 μg of IgG control antibody and then a further 60 μl of Protein G slurry for 2h. Again following incubation Protein G was spun at 3000rpm for 1min and 2 μg of a ChIP antibody directed against a transcription factor (Table 2.2) was added to the lysate. After an overnight incubation with rotation at 4°C , 60 μl of Protein G slurry was added to the sample for a maximum of 25min. The sample was spun at 13000 rpm, 300 μl of the supernatant was kept as an Input

fraction and the rest discarded. Protein G– antibody complexes were then washed 4 times in 500µl of WB and additional 3 times with TE buffer. After the final spin, TE buffer was aspirated and Protein G slurry was resuspended in 200µl of TE buffer containing 2%SDS for 15min to elute protein-DNA complexes. The sample was spun at 13000 rpm allowing the collection of the Protein G-free supernatant, which was thereafter used as a ChIP fraction. Both the Input (300µl) and ChIP fractions (200µl) were subjected to reverse crosslinking by incubation at 65°C overnight.

2.2.9.4 DNA purification

Purification of DNA from the Input and ChIP fractions was performed using the Qiaquick PCR purification kit (Qiagen). Briefly, 3 volumes of pre-warmed PB buffer were added to the fractions. The samples were then applied onto a column and spun at 13000 rpm for 1min. The columns were washed with PE buffer containing EtOH and additionally spun at 13000 rpm for 1min. DNA was eluted with 200µl (ChIP) or 300µl (Input) of pre-warmed dH₂O.

2.2.9.5 Real-time PCR analysis

DNA from both the Input and ChIP fractions was subjected to Real-time PCR. The reaction was set up as follows:

Reagent	Volume
DNA	10µl
SYBR Premix ExTaq (2x) Lonza (Takara)	10µl
Forward Primer (Table 2.10)	0.04µl
Reverse Primer (Table 2.10)	0.04µl

Samples were run in triplicate on the Rotor-Gene 6 (Corbett Research UK Ltd., Cambridge, UK). The DNA was denaturated at 95°C for 5min, and then 40 cycles of 95°C for 10sec (denaturation), 59°C for 30sec (annealing), and 78°C for 20sec (elongation) were performed. Relative quantification of gene expression was determined using the comparative ΔC_t method through the

RotorGene 6000 software. Briefly, threshold cycle (Ct) values of the Input were subtracted from ChIP Ct values. The outcome value was then raised to the power of 2.

2.2.10 Bioinformatic analysis

Genomic human Btk sequence (Ref: NG_009616.1) and human TNF sequence (Ref: NG_007462.1) were derived from www.ncbi.nlm.nih.gov. Multiple sequence alignments were performed using the ClustalW algorithm at www.ebi.ac.uk/clustalw. A search for transcription factor binding site was performed using the MatInspector software at www.genomatix.de.

2.2.11 Statistical analysis

Repeated measures ANOVA with Dunnett's post test or Student's t-test was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). A *p* value below 0.05 was considered significant.

Chapter 3 - Results I

Purification of Btk protein complexes – optimization of the *Strep*-tag system in human primary macrophages

3.1 Introduction

Increasing evidence suggests that TLRs contribute to the development of autoimmune diseases as they sense both pathogens and necrotic cell derived self-antigens (reviewed in (Marshak-Rothstein 2006)). Expression of TLRs 2, 3, 4, 7 and 8 has been shown to be elevated in the synovial tissue of rheumatoid arthritis (RA) patients in comparison to healthy donors (Radstake et al. 2004; Roelofs et al. 2005; Brentano et al. 2005a). A study carried out at the Kennedy Institute has shown that TLRs 2 and 4 are present and functional in synovial membrane cells derived from RA patients, and that their activation promotes inflammation in RA (Sacre et al. 2007a). The inhibition of endosomal TLRs by the antidepressant mianserine, on the other hand, decreased the production of TNF or IL-6 from RA synovial membrane cultures indicating that targeting endosomal TLR cascades could have therapeutic potential in RA (Sacre et al. 2008). TLR8 may be of the greatest importance as the inhibition of TLR8 by imiquimod in RA cultures resulted in the decreased spontaneous release of TNF (Sacre et al. 2008).

Protein tyrosine kinases have been recognised as mediators in TLR signalling since the discovery that LPS stimulates protein tyrosine phosphorylation in macrophages (Weinstein et al. 1991). First reports on Btk showed that Xid mice do not develop collagen induced arthritis (CIA) (Jansson and Holmdahl 1993) and that TNF and IL-1 β production from LPS-stimulated Xid macrophages is reduced (Mukhopadhyay et al. 2002). Similarly in humans, Btk is implicated in TLR4 signalling as monocytes derived from XLA patients exhibit reduced TNF production when treated with LPS (Horwood et al. 2003). In contrast, overexpression of Btk increases LPS-induced TNF production in human primary macrophages (Horwood et al. 2003). Since the initial description of the function of Btk in TLR4 signalling, the kinase has also been implicated in TLR2, 4 and 5 signalling pathways by regulating the production of TNF, but not IL-6, IL-8 or IL-10, in XLA-derived PBMCs and in AdBtk- infected human macrophages (Mukhopadhyay et al. 2002; Jefferies et al. 2003; Horwood et al. 2006) Palmer, thesis). However, despite numerous reports focusing on the Btk-dependent regulatory mechanism of cytokine production in the TLR4 pathway, relatively little is known about the involvement of Btk in the signalling of endosomal TLRs. Using the yeast two-hybrid system Jefferies and colleagues showed that TLR8 interacts more strongly with Btk than the other TLRs examined (Jefferies et al. 2003). Functional studies by Sochorova (2007) demonstrated that DCs obtained from XLA patients produced significantly less IL-6 and TNF

when treated with ssRNA (Sochorova et al. 2007). There are no reports implicating Tec kinases in endosomal TLR signalling in human macrophages.

Btk has been shown to interact with multiple proteins involved in cell proliferation, differentiation, mobility and antigen recognition. These studies mainly focused on the early signalling events of the B cell receptor (BCR) pathway, and little work has been done to identify Btk downstream components in the TLR pathway (**Figure 1.9**). The approaches used for mapping the Btk signalling network included screening of cDNA expression libraries or co-immunoprecipitation of epitope-tagged proteins. It has been shown that the SH1 and PH domains of Btk interact with the Fas protein which is a death receptor involved in apoptosis. Proteins which are implicated in cytoskeletal reorganisation and, therefore, antigen presentation and processing, such as WASP and Vav, also bind to Btk through its SH3 domain. The TH domain of Btk directly interacts with the members of the SFK (Fyn, Hck and Lyn) whereas its SH2 domain binds to BCR signalling proteins BLNK and BASH (reviewed in (Lindvall et al. 2005)). Using the yeast two-hybrid system it has also been demonstrated that Btk interacts with TIR domains of TLRs 4, 6, 8 and 9. Co-immunoprecipitation experiments also showed that Myd88 and IRAK-1 form complexes with Btk in HEK293 cells (Jefferies et al. 2003).

The objectives of this study were to determine the role of Btk in TLR3 and TLR8 signalling pathways in human primary macrophages and to identify TLR-induced Btk-protein interactions that might be relevant to TLR signalling. To achieve the latter goal an affinity tag purification approach has been used (section 2.2.5). The tandem affinity purification (TAP) system was previously successfully used for the detection of Btk interaction with Dasatinib, a drug that blocks BCR-ABL kinases, in a chronic myelogenous leukemia cell line (Hantschel et al. 2007). Due to the therapeutic potential of Btk inhibition in lymphomas, autoimmune diseases and allergic inflammation (Di Paolo 2010; Honigberg et al. 2010), continuous and thorough investigation of the Btk signalling cascade is required. Novel binding partners of the kinase could also provide attractive drug targets whose inhibition may demonstrate greater selectivity and longer duration of action.

3.2 Results

3.2.1 Overexpression of Btk results in increased TNF production in human macrophages

Prior to the investigation of Btk protein interactions in endosomal TLR cascades, it was important to ascertain the involvement of Btk in TLR3 and TLR8 signalling. It was previously demonstrated in our laboratory that adenoviral overexpression of Btk increased TNF production in TLR1/2-, TLR2/6-, TLR5- and TLR4- challenged primary human macrophages ((Horwood et al. 2003; Horwood et al. 2006) Palmer, thesis). To determine the role of Btk in TLR3 and 8 signalling in primary human macrophages, the effect of Btk overexpression on cytokine production was examined. M-CSF-differentiated macrophages were infected with adenoviruses carrying either the wild type *BTK* gene (AdBtk) or an empty vector (AdCon); both viruses also expressed green fluorescent protein (GFP) to determine infection efficiency. Cells were allowed to express the protein for a minimum of 24h prior to stimulation with either R848, LPS or PolyI:C for 18h. LPS was used as a positive control as it has previously been shown that Btk overexpression super-induces TNF with this TLR4 ligand (Horwood et al. 2006). The efficiency of infection was assessed by Western blotting. The level of GFP increased with increasing multiplicity of infection (moi) in both AdBtk and AdCon macrophages, whereas the expression of Btk was only increased in the presence of AdBtk at 10 to 150 moi (**Figure 3.1**). Subsequent analysis of cytokine production revealed that Btk overexpression significantly increased the production of TNF following either LPS or R848 stimulation (**Figure 3.2**). Btk had no effect on IL-6 secretion

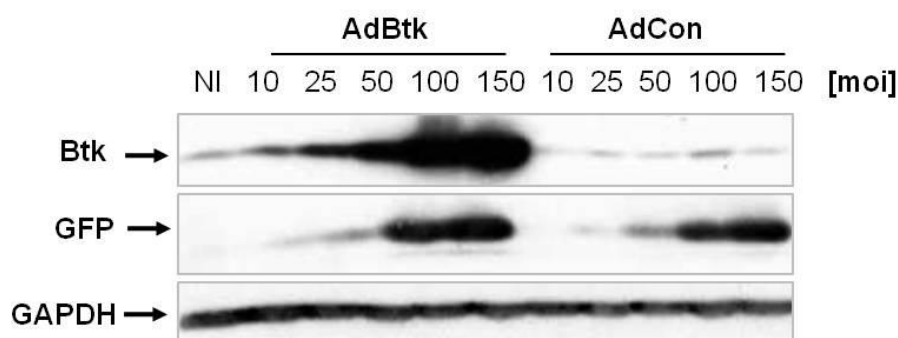


Figure 3.1 The efficiency of adenoviral gene transfer on human macrophages. Differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were infected with Btk (AdBtk) or control adenovirus (AdCon) at moi ranging from 10 -150 for 24h (section 2.2.3.9). The protein level of Btk, GFP and GAPDH was assessed by SDS-PAGE and Western blotting.

in LPS-challenged cells, whereas in R848-challenged macrophages there was a significant increase in IL-6 with AdBtk at a moi of 150; illustrating the first major difference in the role of Btk between these two TLR cascades (**Figure 3.2A/B**). No consistent changes in the LPS- or R848-induced production of IL-10 could be observed when comparing AdBtk and the control adenovirus, indicating that the Btk effect may be specific to pro-inflammatory cytokines (**Figure 3.2C**). Furthermore, Btk did not affect the secretion of IP-10, an IFN-inducible chemokine, following either TLR4 or TLR3 stimulation (**Figure 3.2D**). These data suggest that Btk signalling may be involved in the production of LPS-induced TNF and R848-induced TNF and IL-6, whilst Btk did not affect PolyI:C-induced IP-10 production. As the TLR3 pathway seems to be unaffected by Btk overexpression, this suggests that Btk is not an intrinsic part of the TLR3 signalling pathway.

3.2.2 Human monocytic-like cell lines express TLRs 3, 4 and 8 at the protein level

The identification of Btk binding proteins needs to be performed in a system that allows the purification of large protein yields. The accessibility of cell lines and their ability to grow in large volumes make them an ideal model for the optimization of the TAP method prior to employing TAP in human primary macrophages. Btk is known to be expressed in all myeloid cells as well as monocytic cell lines such as THP-1 and U937 (Smith et al. 1994; Doyle et al. 2007). There are three monocytic cell lines available in the lab (Monomac-6, THP-1 and U937) and all three were tested for TLR3, 4 and 8 expression in comparison to human primary macrophages. Unstimulated cells were stained with FITC-conjugated monoclonal antibodies directed against TLRs 3, 4 and 8 and analysed on the flow cytometer. THP-1 and U937 cell lines required treatment for 48h with phorbol-12-myristate-13-acetate (PMA) to induce differentiation into macrophage-like cells. As shown in **Figure 3.3**, human monocytic cell lines, similarly to primary cells, expressed cell surface TLR4 and intracellular TLRs 3 and 8, as the mean fluorescent intensity of the peak representing FITC-TLR staining was higher than FITC-IgG control and unstained cells. The result obtained from this experiment indicated that the human monocytic cell lines retain the phenotype of primary macrophages as they express TLR 3, 4 and 8 protein. It was therefore possible that these cell lines also maintained the native TLR signalling machinery enabling them to respond to pathogens and other danger signals. This assumption, however, had to be confirmed in a functional assay.

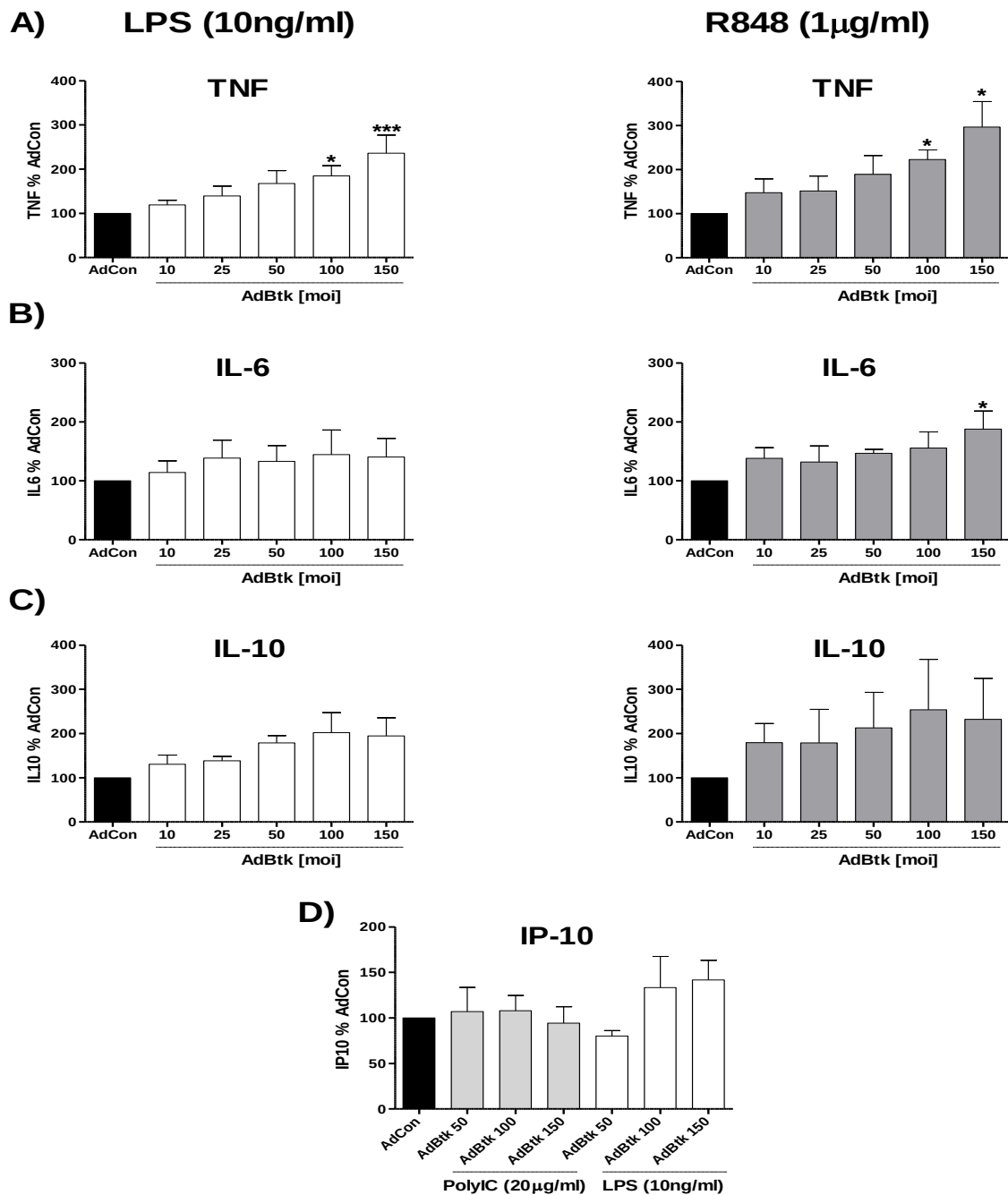


Figure 3.2 Btk overexpression results in increased TNF production in human macrophages. Differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were infected with adenovirus at moi ranging from 10 to 150 virus units per cell (section 2.2.3.9). Cells were incubated for 24hrs and stimulated with R848 (1µg/ml), LPS (10ng/ml) and PolyI:C (20µg/ml) for 18hrs. The cytokine concentration was assessed by ELISA (A-D). Control values (black bars) represent cells infected with adenovirus carrying empty vector. Btk overexpression values were normalised to the control at relevant moi. Unstimulated cells are not shown as values were undetectable. Cytokine levels are expressed as means of triplicate repeats $\pm$ SEM. Results shown are representative of at least 3 donors. Statistical analysis: repeated measures ANOVA, * $P < 0.05$, *** $P < 0.001$ as compared to stimulated AdCon.

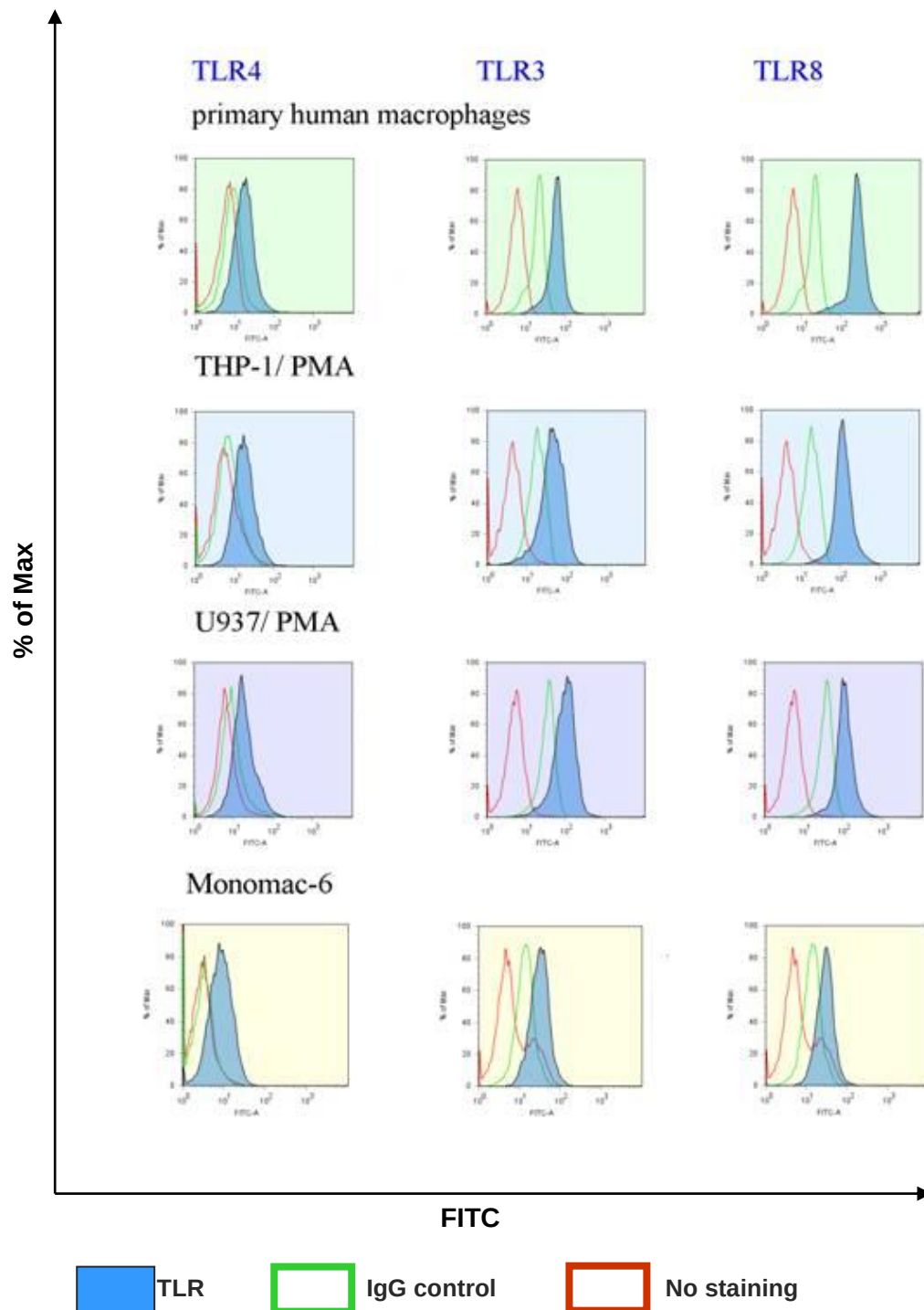


Figure 3.3 Human cell lines express TLRs 3, 4 and 8 at the protein level. Human primary macrophages, Monomac-6, PMA-differentiated THP-1 and U937 cells (0.3×10^6) were blocked in 5% human serum and stained with FITC-TLR3, 4 or 8. For internal staining (TLRs 3 and 8) cells were fixed (2% PFA) and permeabilised (0.1% saponin) before applying antibody (Section 2.2.6.1). As a control, an isotype matched relevant FITC-IgG was used. The graphs represent one of 3 independent experiments.

3.2.3 Human monocytic-like cell lines produce cytokines in response to TLR4, but not TLR3 and TLR8 stimulation

The activity of TLRs 3, 4 and 8 in Monomac-6, THP-1 and U937 cells was examined by analysing the production of cytokines from stimulated cells. Cell lines and human primary macrophages were stimulated with LPS, R848 or PolyI:C for 4, 6 and 24h. Supernatants were collected and analysed by ELISA for TNF, IL-6, IL-10 and IP-10 production. Human primary macrophages were used as a reference as they responded to all three TLR ligands (**Figure 3.4A**). Stimulation with LPS resulted in the highest TNF production at 4h followed by a decline at 24h, as opposed to the increasing secretion of IL-6, IL-10 and IP-10 over the time course followed. Activation with R848 yielded similar results to LPS stimulation with regard to IL-10 expression, however there was consistently higher production of TNF and IL-6. R848-initiated production of IP-10, a chemokine involved in the interferon pathway, differed between donors and was observed in 60% of cases. Activation of the TLR3 pathway by PolyI:C resulted in the production of IP-10 at 24h.

In contrast to primary macrophages, the human cell lines, Monomac-6, PMA-treated THP-1 and U937, all responded to LPS but failed to secrete cytokines upon R848 or PolyI:C stimulation (**Figure 3.4/3.5**). Pre-treatment of THP-1 and U937 with PMA is required for their differentiation into cells with functional characteristics of mature macrophages (Tsuchiya, 1982; Zarembek and Godowski, 2002). Stimulation with LPS triggered the production of TNF, IL-6, IL-10 and IP-10 in Monomac-6 and U937/PMA cells. The expression profile of cytokine production in these two cell lines was similar to human macrophages, as TNF production peaked at 4h, whereas IL-6, IL-10 and IP-10 production continued to increase to 24h. In contrast, the secretion of TNF, IL-6 and IP-10 peaked at 24h in LPS-stimulated THP-1/PMA cells and the production of anti-inflammatory IL-10 was below the level of detection. In the absence of PMA, U937 and THP-1 cells did not produce pro-inflammatory cytokines in response to the TLRs 3, 4 and 8 stimulations (data not shown).

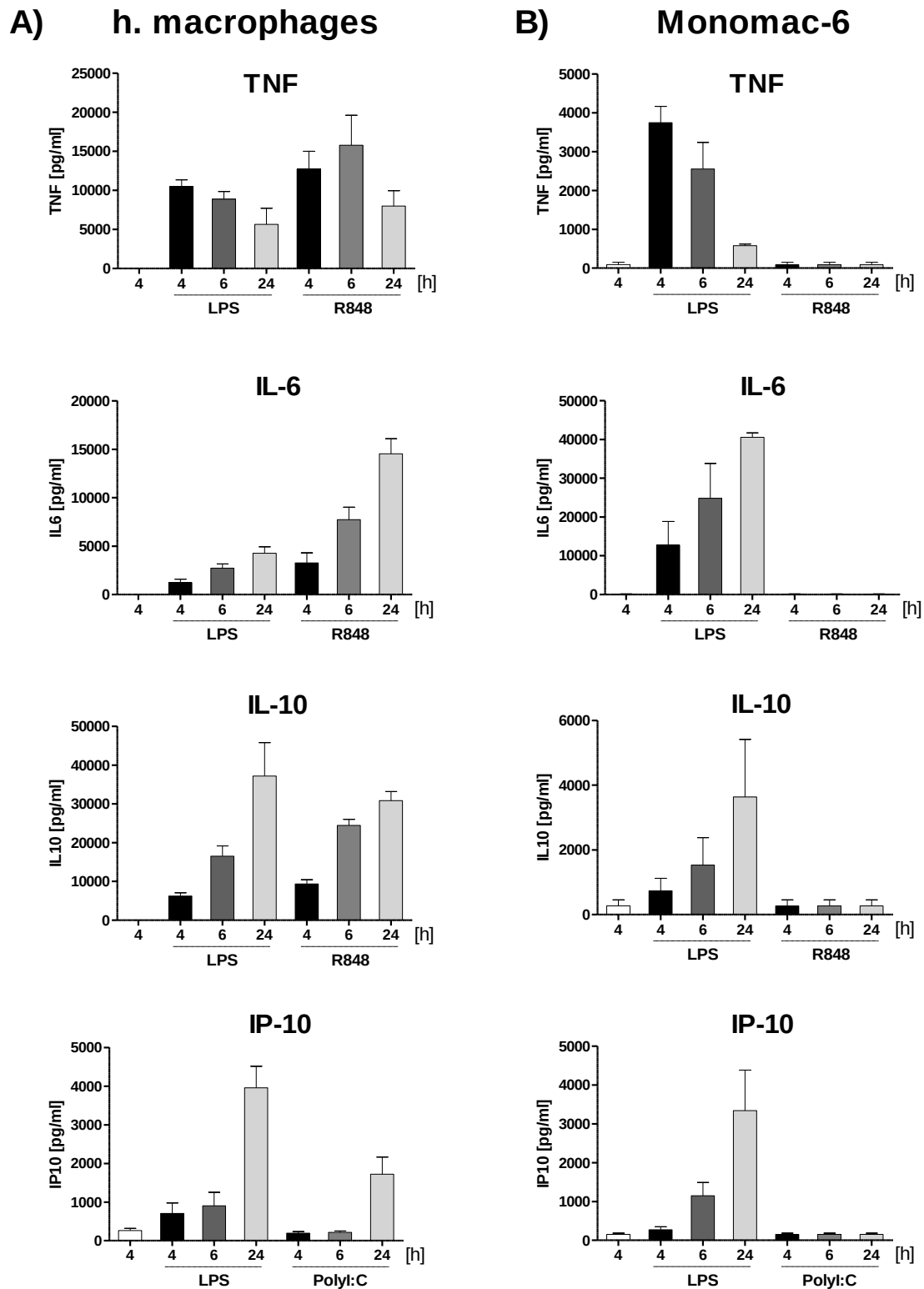


Figure 3.4 Cytokine expression pattern in human primary macrophages and Monomac-6 cells. M-CSF-differentiated human primary macrophages (h. macrophages) (A) and Monomac-6 cells (B) were plated onto 96-well plates (1×10^5 cells/well) and stimulated with 10ng/ml LPS, 1 μ g/ml R848 or 20 μ g/ml PolyI:C. Supernatants were collected after 4, 6 and 24h and analysed for TNF, IL-6, IL-10 and IP-10 by ELISA.

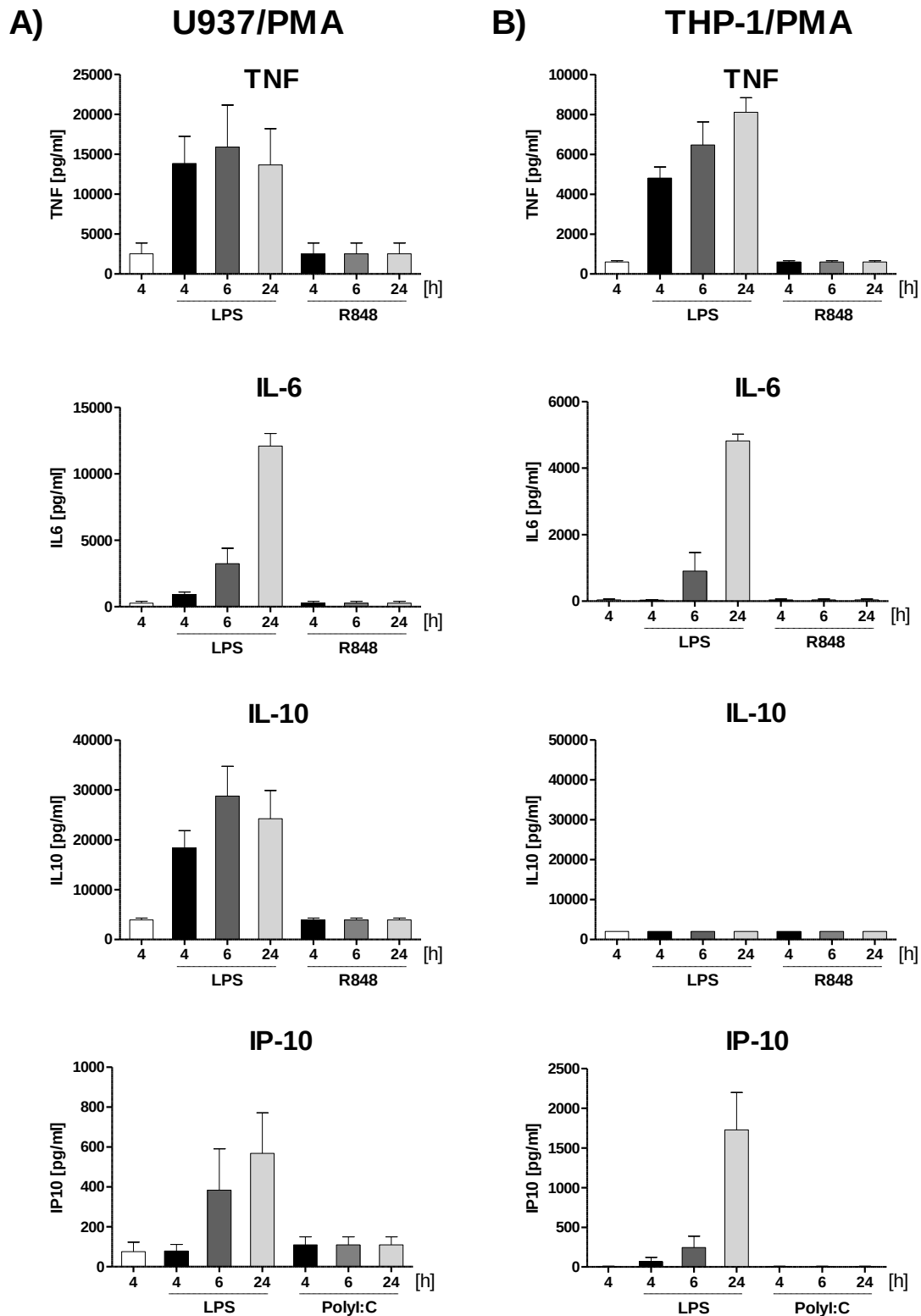


Figure 3.5 Cytokine expression pattern in PMA-treated U937 and THP-1 cells. PMA-differentiated U937 (A) and THP-1 cells (B) were plated onto 96-well plates (1×10^5 cells/well) and stimulated with 10ng/ml LPS, 1 μ g/ml R848 or 20 μ g/ml PolyI:C. Supernatants were collected after 4, 6 and 24h and analysed for TNF, IL-6, IL-10 and IP-10 by ELISA.

The data obtained in this section clearly demonstrate that there is a major discrepancy in TLR signalling between human primary macrophages and the cell lines examined. In contrast to primary macrophages, human cell lines did not respond to either R848 or PolyI:C stimulation. All the cell lines, on the other hand, secreted cytokines in response to LPS showing that only the TLR4 pathway is functional in those cells. These results were disappointing as the screened cell lines could not be used for the optimisation of the TAP system. Human primary macrophages were therefore used for the optimisation and purification of Btk complexes formed upon TLR8 stimulation.

3.2.4 Overview of the cloning strategy for the affinity tag purification system

The importance of Btk in the immune system is increasingly recognised and many Btk inhibitors are currently in clinical trials for the treatment of lymphomas (Honigberg et al. 2010). The potential benefits of Btk blockade are also seen in the treatment of rheumatoid arthritis as chemical inhibition of Btk blocked BCR-dependent B cell proliferation and autoantibody production as well as FcγRIII-dependent TNF, IL-6 and IL-1 production in macrophages (Di Paolo 2010). The recognition of the Btk signalling network is critical for the development of more specific drugs and in predicting possible side effects of Btk inhibition. The results thus far have implicated Btk in the production of TNF, and possibly IL-6, in R848-challenged human macrophages (**Figure 3.2**). Activation of TLR8 appears to be central for TNF production in RA cultures (Sacre et al. 2008), therefore it is of importance to determine whether interference with Btk or its signalling partners would have therapeutic benefit. The affinity tag purification system (see section 2.2.5 for the full description) was used to identify the Btk binding partners upon TLR8 stimulation in primary human macrophages. The protocol required cloning the gene of interest, human *BTK*, into a vector carrying either the N-terminal or C-terminal tag. Standard cloning techniques were used to incorporate *BTK* in-frame with the coding region of the tag. The ligated construct was then cloned into an adenoviral backbone and the activity of the construct was examined. The details of the cloning strategies are discussed below.

3.2.5 Cloning procedure for fusing Btk to the N- terminal full length tandem affinity purification tag.

The tandem affinity purification (TAP) system involves the fusion of the tag comprising full length N-terminal Protein G and streptavidin binding protein (NTAP) to the target gene, human *BTK*. Protein G and streptavidin binding protein (SBP) are separated with the cleavage site for tobacco etch virus (TEV) protease. The advantage of using the double tag is that the bait is purified through two subsequent columns, IgG sepharose and streptavidin sepharose, which reduces contamination from non-specific binding. The cloning process required three major subcloning steps. Firstly, *BTK* was excised from the pMB.SP vector with blunt-end cutting *AleI* within the 5'UTR and cohesive-end cutting *BamHI* within the 3'UTR of *BTK*. In order to blunt end the 3' terminus, the Pfu polymerase, which adds nucleotides to the pre-existing 3' terminus, was used (**Figure 3.6A**). The blunt-ended Btk insert was then ligated into an *EcoRV* pre-linearised and shrimp alkaline phosphatase (SAP) pre-treated NTAP-SK16 vector (**Figure 3.6B**). To ensure the efficiency of the ligation and the correct orientation of *BTK*, plasmid DNA was isolated for a diagnostic digestion with *XbaI*. Clones with the plasmid digestion patterns consisting of 2kb and 10kb size bands were sent for sequencing. The positive plasmid was then linearised with *PmeI* and subsequently co-transfected into BJ5183 *E.coli* together with the destination vector, pAdEasy-1 (**Figure 3.7**). The BJ5183 strain is designed for homologous recombination as, unlike most common laboratory strains, it is *recA* proficient, which enables recombination with high efficiency. Recombinants were identified and confirmed by restriction analysis using *PacI*. Clones with a *PacI* digestion pattern consisting of a 4.5kb and a 41kb band were considered as positives. The plasmid was then transfected into replication deficient XL-1 blue *E.coli*, to amplify the recombined adenovirus plasmid. The purified plasmid was then linearised with *PacI* and transfected into the HEK293A cell line to initiate virus production (section 2.2.3.3).

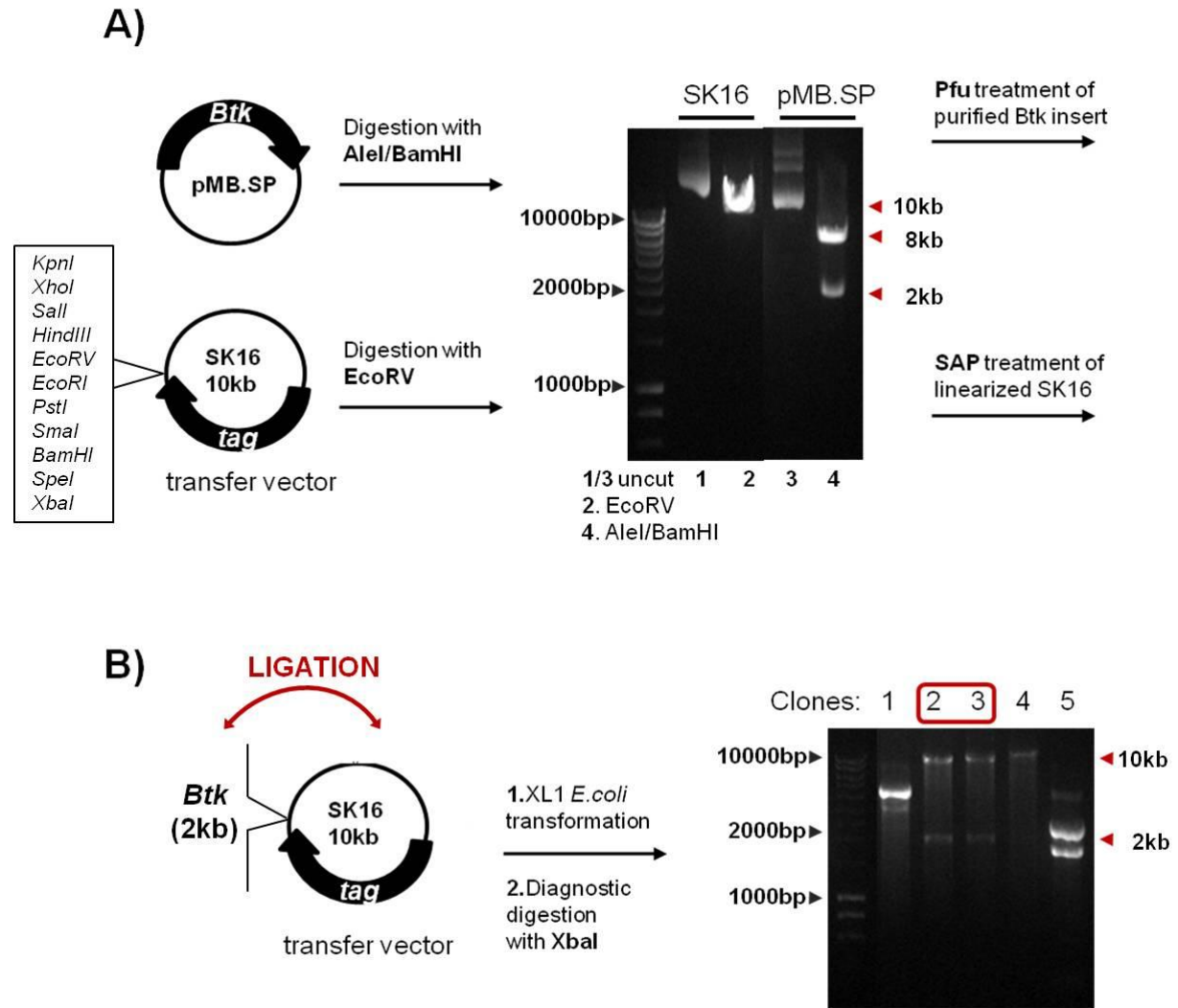


Figure 3.6 Subcloning strategy for the manufacture of Btk-NTAP-SK16 from Btk-pMB.SP. *Btk* was extracted from pMB.SP using *AclI* and *BamHI* enzymes, and then gel purified and blunt ended by Pfu polymerase (section 2.2.2). Prior to the ligation with *Btk*, the NTAP-SK16 transfer vector underwent a digestion with *EcoRV*, it was SAP treated and subsequently gel purified using Wizard SV Gel and PCR Clean-Up system (Promega) (A). The ligation reaction was performed overnight at 4°C and the construct was transfected into the XL-1 cells. The positive clones were identified by *XbaI* digestion and sequenced (B). Red arrows indicate the predicted fragment sizes of a digested Btk-pMB.SP or NTAP-SK16 plasmids (A) or the positive clones digested with *XbaI* (B).

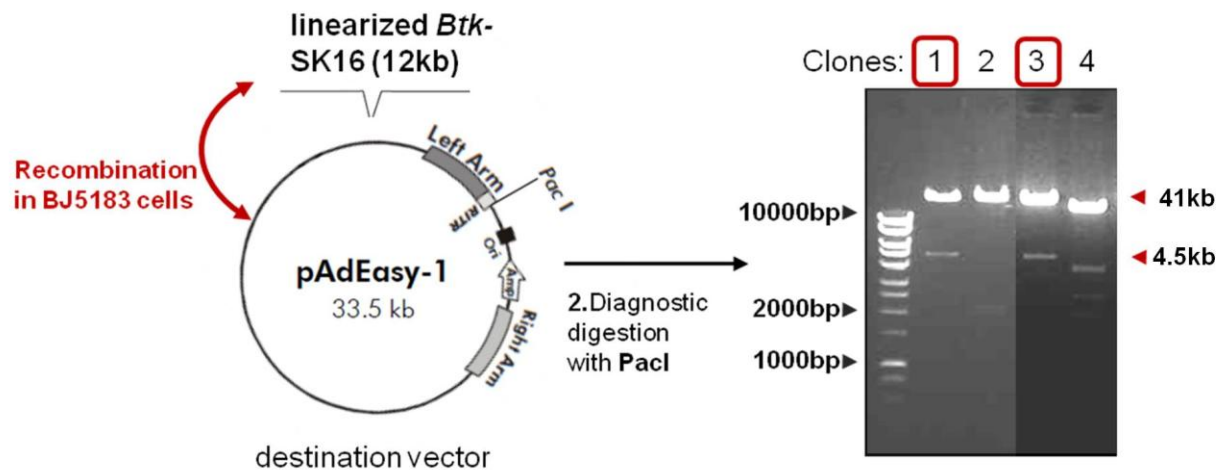


Figure 3.7 Subcloning strategy for the manufacture of Btk-NTAP-SK16-pAdEasy from Btk-NTAP-SK16. *PmeI*- linearised Btk-NTAP-SK16 was gel purified using Wizard SV Gel and PCR Clean-Up system (Promega) and co-transfected into the BJ5183 cells together with pAdEasy-1 (section 2.2.2/ 2.2.3). The recombinants were screened by using *PacI* diagnostic digestion for the predicted band sizes of 4.5kb and 41kb (red arrows). The picture of pAdEasy was adapted from www.qbiogene.com.

3.2.6 AdBtk-NTAP is unable to increase TNF protein production following LPS or R848 stimulation

The expression of Btk-NTAP was confirmed at the protein level, whereas the functionality of the construct was assessed by the ability of over-expressed Btk-NTAP to increase TNF production in human primary macrophages. Differentiated macrophages were infected overnight with either Btk-NTAP or control NTAP adenoviruses at moi ranging from 10 to 100 viral particles per cell. The cells were either lysed the next day for Western blotting analysis or stimulated with LPS or R848 for 18h for ELISA. Due to the large size of the NTAP tag (**Figure 3.8A**), Btk-NTAP (~95kDa) and endogenous Btk (78kDa) could be individually detected by the anti-Btk antibody on a Western blot indicating that the Btk-NTAP adenoviral construct was efficiently expressed (**Figure 3.8B**). However, the expression of the construct at increasing moi failed to enhance TNF production upon LPS or R848 stimulation in human primary macrophages (**Figure 3.8C**). One of the possible reasons why Btk fused to the N-terminal tag lost its activity could be the location of the tag. Since NTAP was attached to the PH domain of Btk, the tag could have disrupted the membrane localisation of the kinase and consequently affected the activity of Btk. Secondly, the size of the tag (136 amino acids in length) could have interfered with the native conformation of Btk leading to impaired protein interactions.

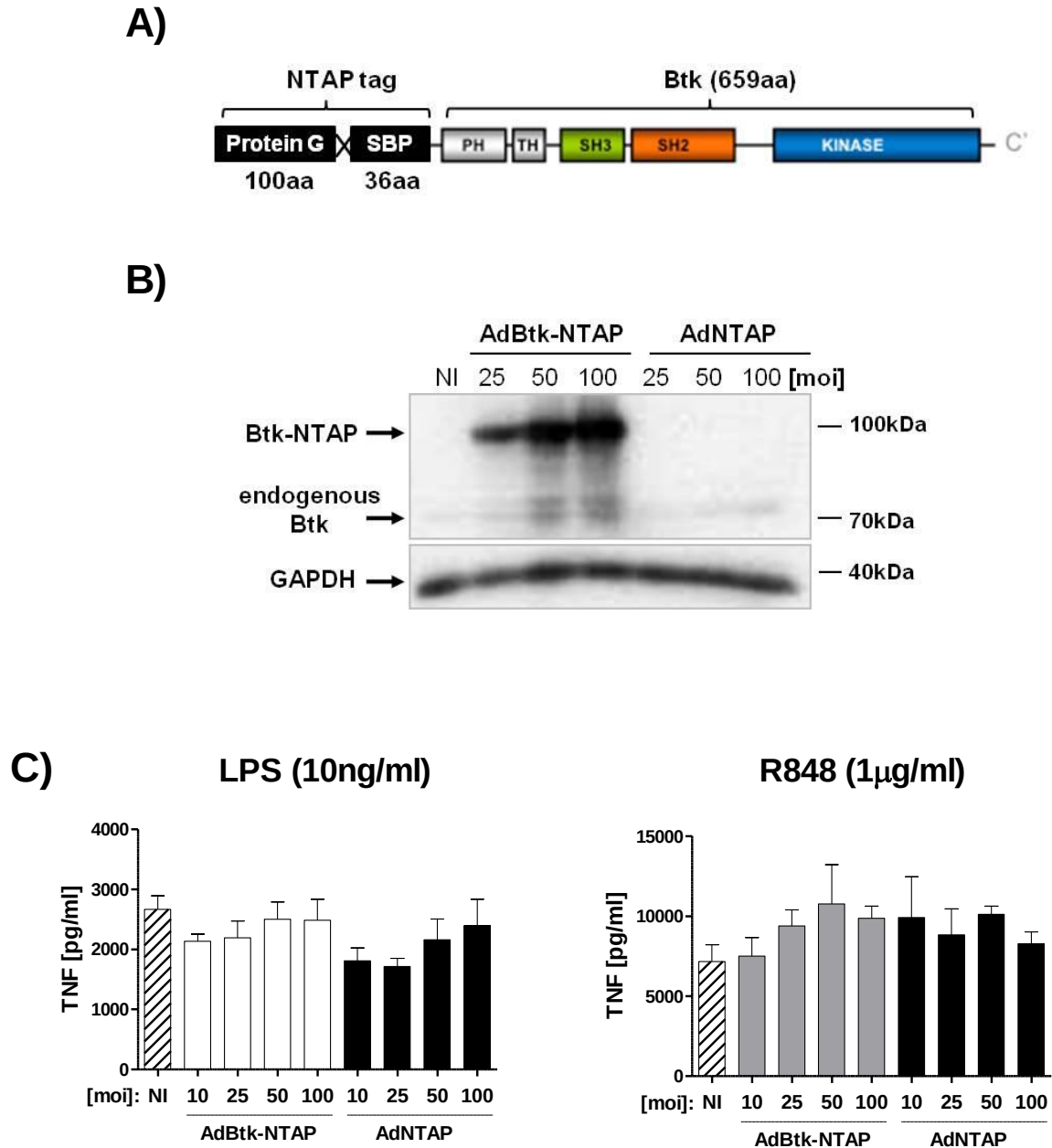


Figure 3.8 Btk-NTAP construct is expressed but does not increase LPS- and R848-induced production of TNF in human primary macrophages. Differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were infected with AdBtk-NTAP (A), control AdNTAP at moi ranging from 10-100 virus particles per cell or left uninfected (NI) (section 2.2.3.9). Cells were incubated for 24h, lysed for Western blotting (B) or stimulated with LPS (10ng/ml) or R848 (1μg/ml) for 18h. TNF production was then analysed by ELISA (C). Cytokine levels are expressed as means of triplicate repeats $\pm$ SEM. Results shown are representative of at least 3 donors. The analysis performed with Student's t-test as compared to stimulated AdNTAP did not show any statistical significance.

3.2.7 Cloning procedure for fusing Btk to the C- terminal short length *Strep*- tag

To overcome the limitations derived from the use of a large tag attached to the N-terminus of Btk, the C-terminus of Btk was next fused to the short (31 amino acids in length) tag comprising only streptavidin-binding protein (*Strep*-tag). Benefits presented by the utilisation of the One-*Strep* purification system would also include an easier and faster purification process and reduction in sample dilution.

The cloning process of fusing *Strep*-tag to the C-terminus of *Btk* required four major sub-cloning steps. The first cloning step involved the addition of an *SpeI* restriction site upstream of the *BTK* transcriptional START codon simultaneously with the addition of a *Sall* site in place of the STOP codon by PCR. This approach allowed the isolation of *BTK* from its original vector followed by its insertion into the pCRII-TOPO vector (**Figure 3.9A/B**). To monitor the efficiency of the ligation, plasmid DNA was isolated for diagnostic digestion with *SpeI* and *Sall* enzymes. Clones with plasmid digestion patterns consisting of 2kb and 3.9kb size bands were sent for sequencing. After excluding potential missense or *frame shift* mutations by sequencing, the chosen Btk-pCRII-TOPO clone was used for further sub-cloning steps (**Figure 3.10A/B**); the insertion of *BTK* into a transfer vector CTAP-pBENT by using *SpeI* and *Sall* enzymes and the subsequent recombination of a positive Btk-CTAP-pBENT with an adenoviral destination vector pAd/PL-DESTTM. Recombinants were identified and confirmed by restriction analysis using *BamHI*. The purified plasmid was then digested with *PacI* and transfected into the HEK293 cell line to initiate virus production (section 2.2.3.3).

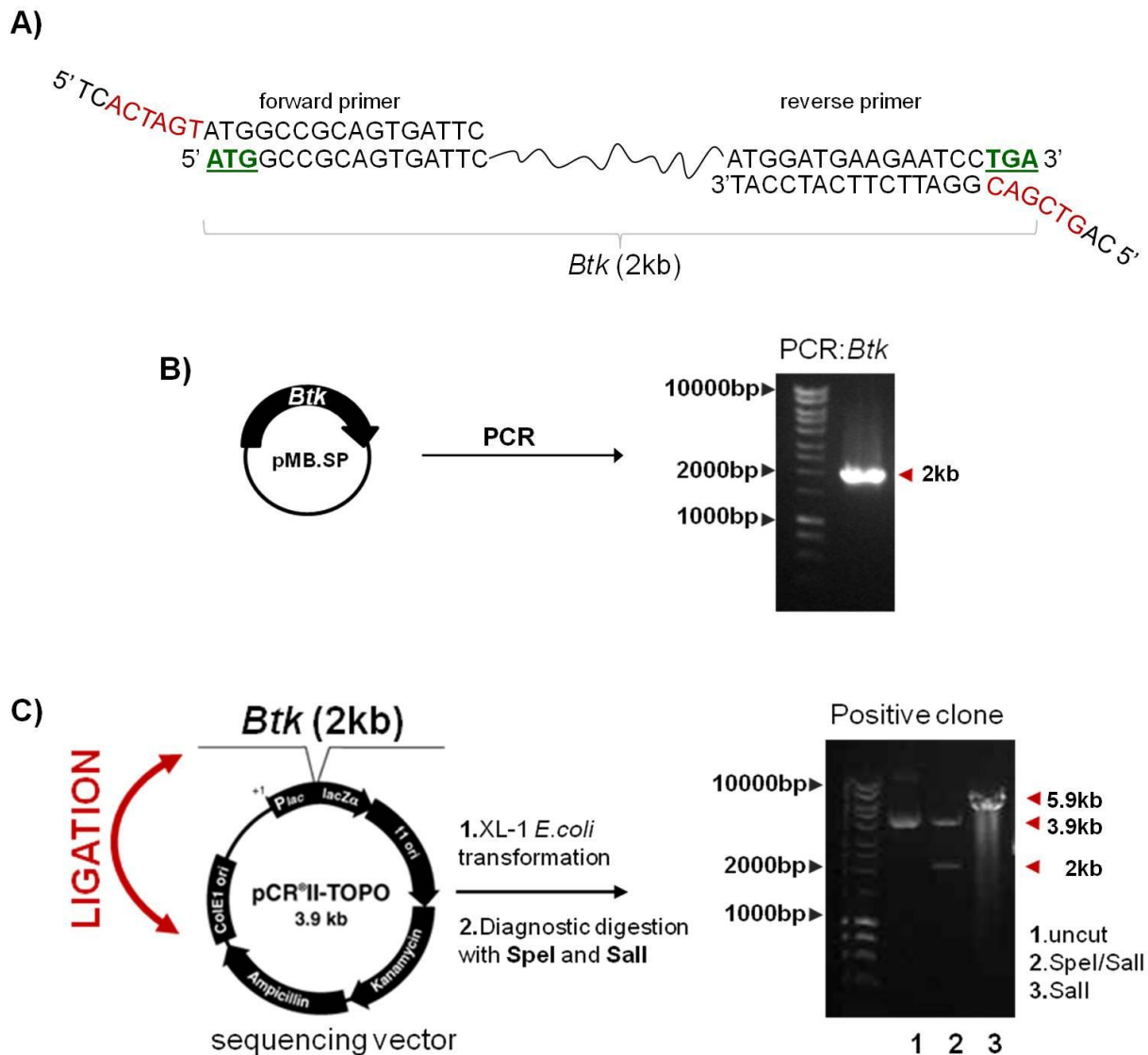


Figure 3.9 Subcloning strategy for the manufacture of Btk-pCRII-TOPO from Btk-pMB.SP. Btk was excised from pMB.SP in a PCR reaction by using a forward primer containing *SpeI* site (sequence in red) together with a reverse primer containing *Sall* site (sequence in red) (A). Transcription start and stop codons are shown in green, underlined. The purified Btk insert (B) was then ligated with pCRII®-TOPO overnight at 4°C and electroporated into the XL-1 cells. The positive clones were identified by *SpeI/Sall* or *Sall* digestion and sequenced (B). The insert was gel purified using Wizard SV Gel and PCR Clean-Up system (Promega) (section 2.2.2). Red arrows indicate the predicted size of the Btk insert (A) or fragment sizes of a digested plasmid obtained from a positive clone (B). The picture of pCRII®-TOPO was adapted from www.invitrogen.com.

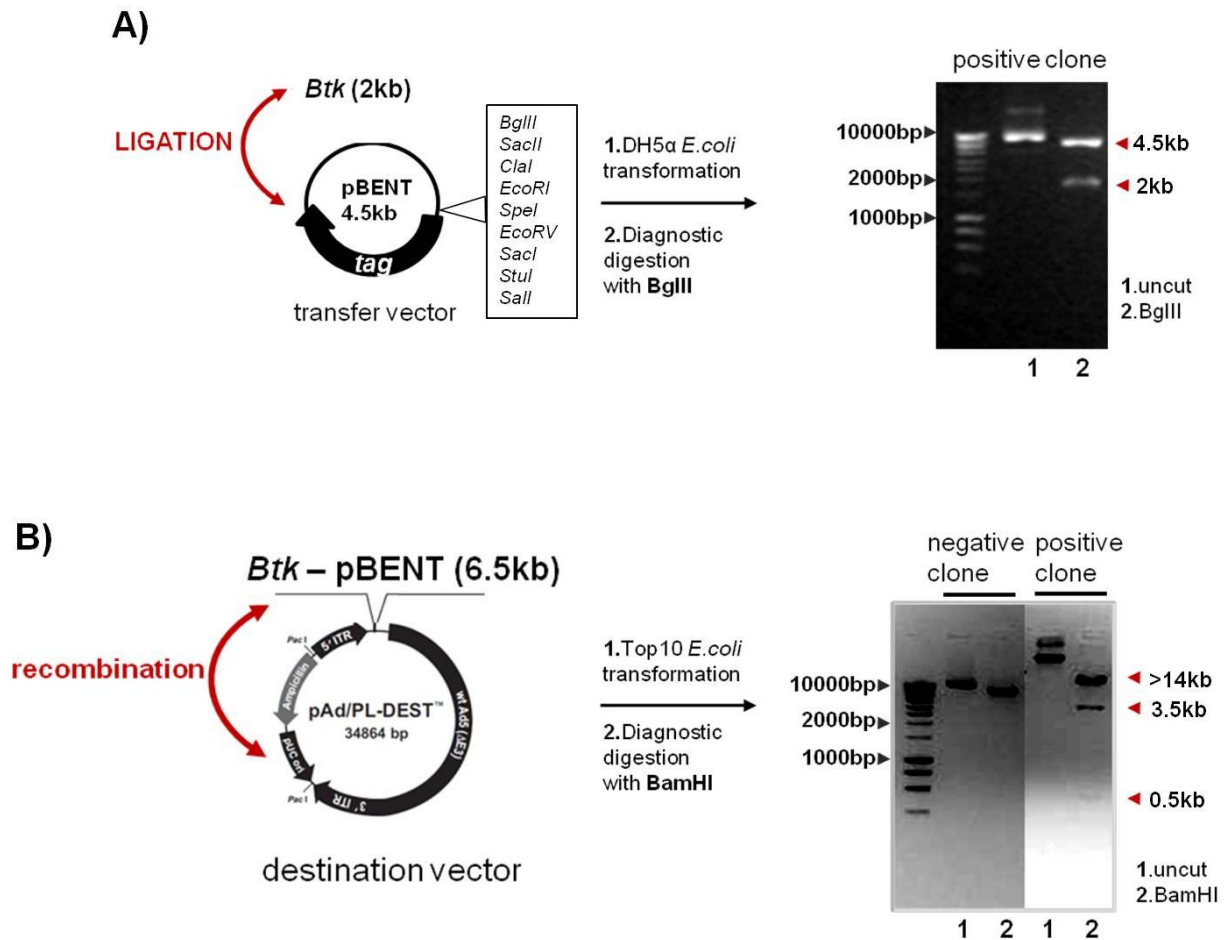


Figure 3.10 Subcloning strategy for the manufacture of Btk-Strep-pBENT-DEST from Btk-Strep-pBENT. The Btk insert was cut out from pCRII®-TOPO with *SpeI* and *SalI*, gel purified and ligated with *Strep*-pBENT by using *SpeI* and *SalI* sites present in the MCS. The construct was transfected into DH5α cells and the subclones were identified by the *BglII* digestion (A). An expression clone was generated by performing an LR recombination reaction between Btk-Strep-pBENT and a Gateway® destination vector (pAd/PL-DEST™)(B). The construct was transfected into the Top10 cells and the positive recombinants were screened by using *BamHI* digestion (B) (section 2.2.2). Red arrows indicate the predicted fragment sizes of a digested plasmid obtained from a positive clone. The picture of pAd/PL-DEST™ was adapted from www.invitrogen.com.

3.2.8 AdBtk-*Strep* increases TNF production following LPS and R848 stimulation

The expression of the Btk-*Strep* was confirmed at the protein level whereas the functionality of the construct was assessed by the ability of overexpressed Btk-*Strep* to increase TNF production in human primary macrophages. Differentiated macrophages were infected with either AdBtk-*Strep* or control Ad*Strep* at moi ranging from 10 to 100 viral particles per cell. The cells were either lysed the next day for Western blotting or stimulated with LPS or R848 for 18h and supernatants taken for ELISA. As shown in **Figure 3.11B**, the infection with AdBtk-*Strep* resulted in moi-dependent expression of Btk-*Strep*. The addition of the *Strep* tag to Btk increased the size of the kinase only by 31aa, thus the tagged and endogenous Btk were indistinguishable on a 10% SDS-PAGE gel (**Figure 3.11A**). The expression of the Btk-*Strep* construct significantly increased the production of TNF at 50 moi upon either LPS or R848 stimulation in human primary macrophages demonstrating the functionality of the construct at low moi (**Figure 3.11C**). The experiment provided data regarding the expression level of Btk-*Strep* as well as the minimum moi of AdBtk-*Strep* that could be used in the subsequent optimisation of the purification process.

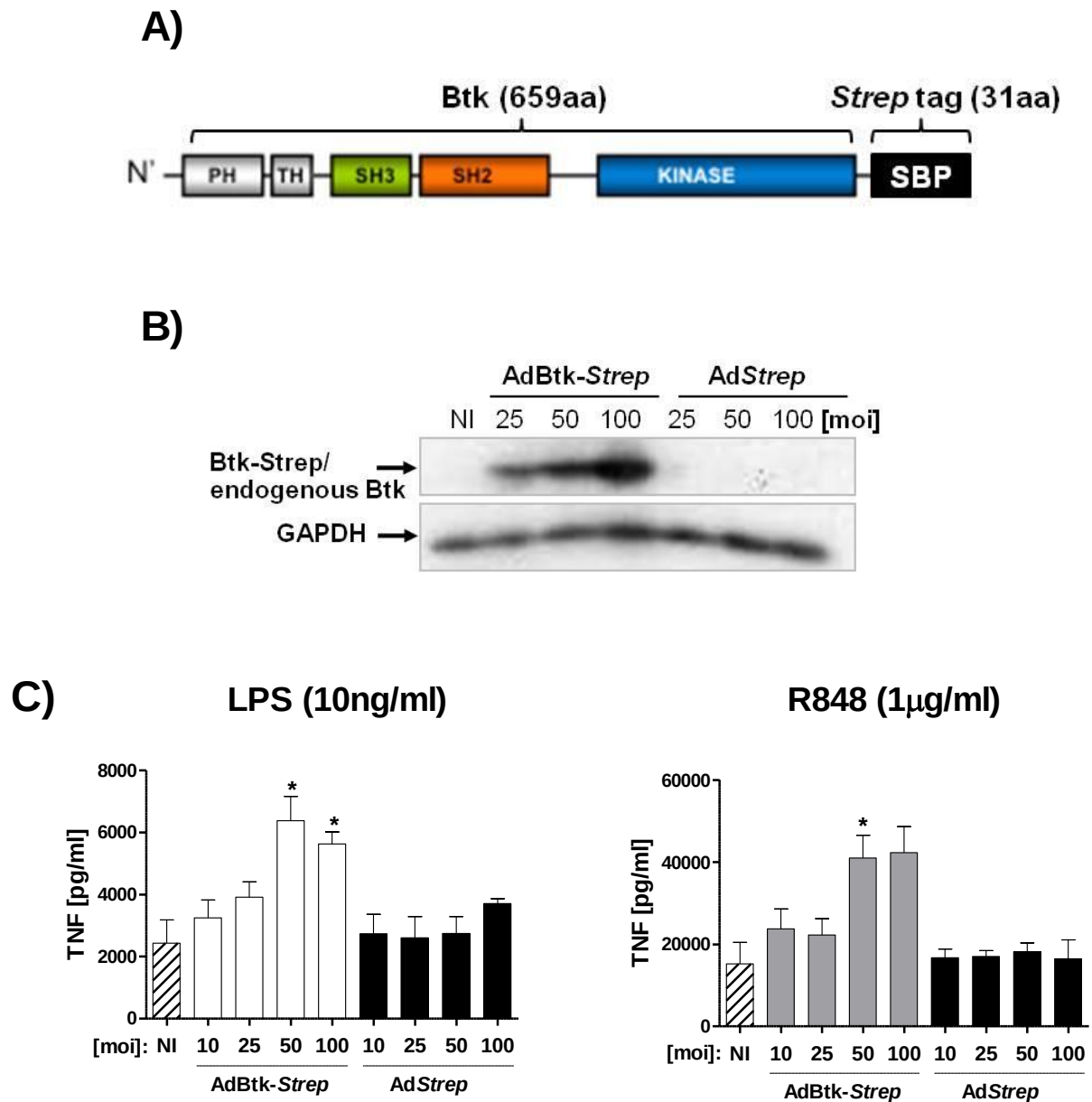
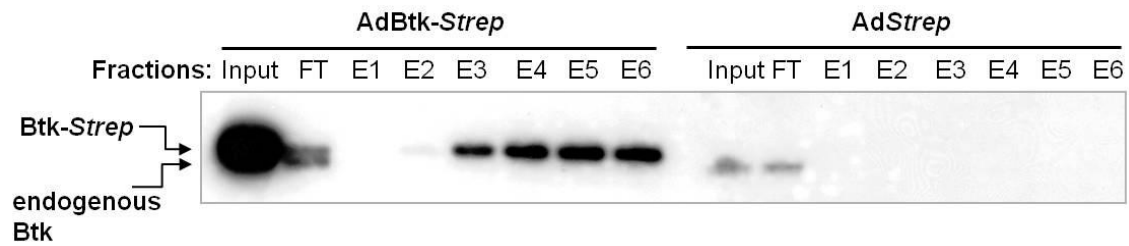


Figure 3.11 Overexpression of the Btk-Strep construct increases LPS- and R848-induced production of TNF in human primary macrophages. Differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were infected with AdBtk-Strep (A), control AdStrep at moi ranging from 10-100 virus particles per cell or left uninfected (NI) (section 2.2.3.9). Cells were incubated for 24hrs, lysed for Western blotting (B) or stimulated with LPS (10ng/ml) or R848 (1μg/ml) for 18hrs. TNF production was then analysed by ELISA (C). Cytokine levels are expressed as means of triplicate repeats $\pm$ SEM. Results shown are representative of at least 3 donors. Statistical analysis: Student's t-test, * $P < 0.05$, as compared to stimulated AdStrep.

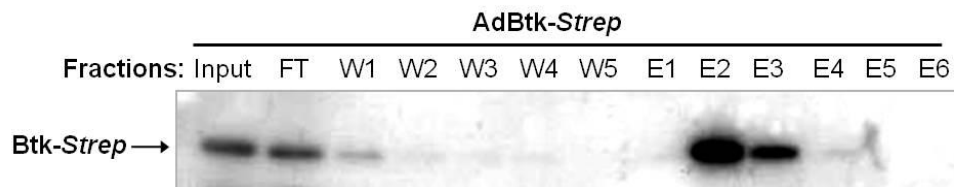
3.2.9 Btk-*Strep* is efficiently removed from the column by using competitive biotin elution

The preceding experiment proved the functionality of Btk fused to the *Strep* tag by examining the effect of Btk-*Strep* overexpression on TNF production. To determine the activity of the *Strep* tag, the ability of the Btk-*Strep* (bait) to bind to a Strep-Tactin® MacroPrep® column and the subsequent elution of the bait was assessed. Differentiated macrophages were infected with either AdBtk-*Strep* or control Ad*Strep* at 50 moi for overnight. They were allowed to express for another 24h and subsequently lysed. The total cell extract (input), was passed over 200µl of the resin (column volume, V=200µl) and washed in 0.2% NP40 (50mM NaCl) followed by a competitive elution with biotin at 0.5V (section 2.2.5.1). The eluted fractions were then run on an SDS-PAGE gel and the bands were visualised by Western blot using an anti-Btk antibody. **Figure 3.12A** illustrates that Btk-*Strep* was eluted from the column in fractions E2-6 demonstrating that the *Strep* tag could bind to tactin. The increase in biotin volume to 1V resulted in more concentrated Btk-*Strep* in the elution fractions as only fractions E2-4 had to be collected (**Figure 3.12B**). It has been previously shown in our laboratory that Btk constitutively interacts with the Src tyrosine kinase family member, Hck, and with a transcription factor involved in IL-10 signalling, Signal transducer and activator of transcription 3 (STAT3) (Maria Smolinska-Bylanska, thesis, Theresa Page, unpublished). This was shown by immunoprecipitating endogenous proteins from human primary macrophages by using either Hck or Btk antibodies. To examine if Btk-*Strep* attracted the same binding partners, and to prove the reproducibility and selectivity of the *Strep* system, the Western blot membrane from **Figure 3.12B** was subsequently probed with anti-STAT3 and anti-Hck antibodies. As shown in **Figure 3.12C**, STAT3 and Hck were present in the flow through (FT) and wash (W) fractions compared to the input, indicating that the proteins did not bind to Btk-*Strep* which remained on the column. A small portion of STAT3 and Hck were, however, eluted with Btk-*Strep* in the fraction E2 demonstrating that the proteins interacted with Btk. One possible explanation for weak detection of the interacting proteins could be that binding of Btk-*Strep* to the column represents a high affinity interaction, whereas most of the target interactions are going to be of lower affinity. Furthermore, Btk could only bind to a small fraction of STAT3 and Hck which were also involved in other signalling cascades. Independently confirming the previous results, the *Strep* system proved to be functional and efficient in identifying Btk binding partners in human primary macrophages. It was then required to optimise purification conditions to obtain the optimal number of Btk- associated proteins and to limit the occurrence of false positives.

A)



B)



C)

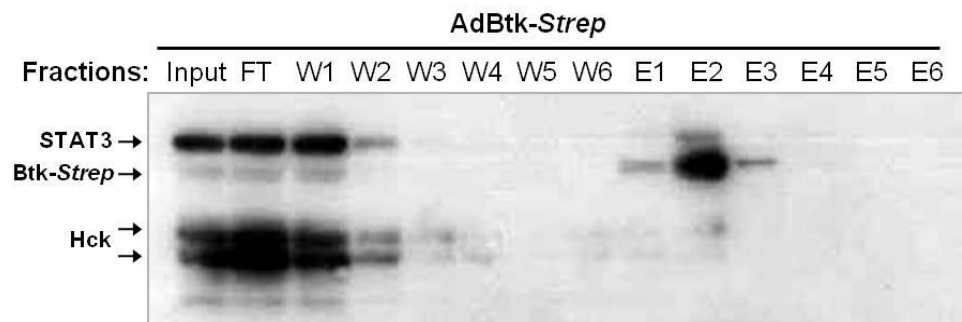


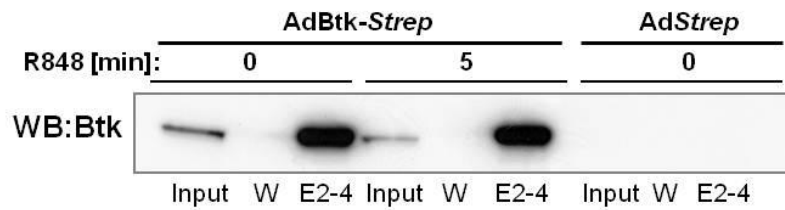
Figure 3.12 Efficient elution of Btk-Strep interacting with STAT3 and Hck from the Strep-Tactin® MacroPrep® column. 2.5×10^6 differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were infected with AdBtk-Strep or control AdStrep at moi 50 (section 2.2.3.9). After 36h the cells were washed twice with 1xPBS and lysed in 1ml of 1%NP40 lysis buffer. The lysates were applied onto 200ul of settled resin (V=1) (Input) and flow through (FT) was collected. Columns were washed with 0.2%NP40 (50mM NaCl) (W1-5), and eluted with biotin (E1-6) at V=0.5 (A) or V=1 (B) (section 2.2.5.1). Fractions were concentrated on Vivaspin 500 columns. 90% of the elution and 10% of Input, FT and each wash fraction were run on a 4-20% SDS-PAGE gel. Western blotting analysis with anti-Btk (A,B), anti-STAT3 and anti-Hck (C) antibodies was performed.

3.2.10 Optimisation of Btk-Strep purification conditions

3.2.10.1 *Mild washing conditions of Btk-Strep complexes result in high background on a silver stained gel*

A protocol previously optimised by Dr David Saliba in our laboratory for the purification of IRF5-Strep from HEK293 cells was initially used for the purification of Btk-Strep from human primary macrophages (Krausgruber et al. 2010). Differentiated macrophages were infected with either AdBtk-Strep or control AdStrep at 50 moi, overnight. The cells were allowed to express for another 24h and were subsequently stimulated for 5min with R848. This time point was chosen as a rapid Btk auto-activation under TLR4 stimulation has previously been demonstrated (Horwood et al. 2003) indicating that TLR-mediated Btk protein associations are likely to occur early. The present data also support this finding as Btk is tyrosine phosphorylated between 5-10min and auto-activated within 5min following R848 challenge (Chapter 4, section 4.2.3). Total cell lysate was then applied onto Strep-Tactin® MacroPrep® column and washed with 0.2% NP40 buffer containing a low concentration of NaCl (50mM). Btk complexes were then eluted with 1V biotin in E2-4 elution fractions. To prevent high protein background in the lanes, only 9% of the wash fraction and 90% of E2-4 was run on the SDS-PAGE gradient gel which was subsequently subjected to silver staining, whereas only 1% of the input and 10% of E2-4 were subjected to Western blotting with the anti-Btk antibody. As visualised in **Figure 3.13A**, Btk-Strep was present in the input and in the elution volume of the AdBtk-Strep infected cells but absent in the W fraction. The utilisation of the mild washing conditions resulted in the formation of multiple bands on the silver stained gel both in AdBtk-Strep and AdStrep-infected cells (**Figure 3.13B**). Despite high background, which interfered with the detection of the differences between the treatments, one distinguishable band of the size of tagged Btk was detected in AdBtk-Strep infected cells. There were, however, no differences in the band patterns between R848-stimulated and unstimulated cells. More stringent washing conditions were required to eliminate non-specific protein contamination from the Btk complexes.

A)



B)

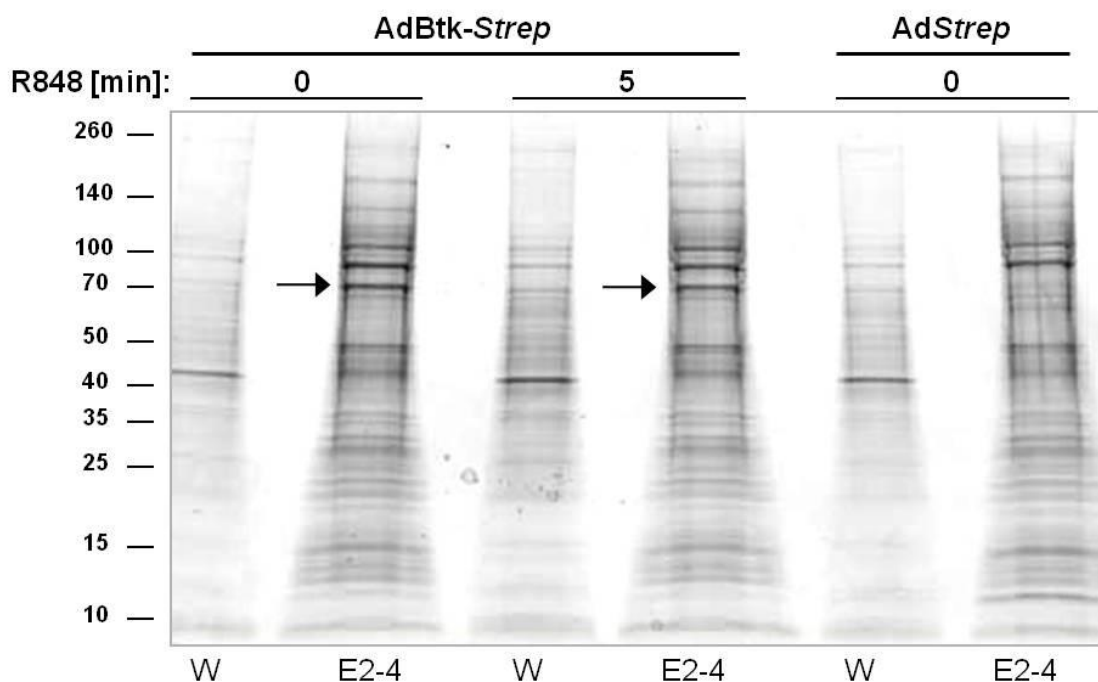


Figure 3.13 Low salt concentration in the wash buffer results in high background of *Strep* purification on a silver stained gel. 25mln differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were infected with AdBtk-*Strep* or control Ad*Strep* at moi 50 (section 2.2.3.9). After 24h cells were stimulated with 1µg/ml R848 for 5min, washed twice with 1xPBS and lysed in 1ml of 1%NP40 lysis buffer. The lysates were applied onto Strep-Tactin® MacroPrep® column and washed with 0.2%NP40 (50mM NaCl). Btk-*Strep* complexes were eluted with biotin at V=1 in elution fractions E2-4 (section 2.2.5.1). Fractions were concentrated on Vivaspin 500 columns. 1% of the input (I) and the last wash fraction (W) together with 10% of E2-4 were analysed by Western blotting with the anti-Btk antibody (A). 10% of the last wash fraction (W) and 90% of E2-4 were applied onto 4-20% SDS-PAGE gel which was subsequently subjected to silver staining (B). Two distinguishable bands of the size of tagged Btk (black arrows) were detected in AdBtk-*Strep* infected cells.

3.2.10.2 The application of Formaldehyde and more stringent washing conditions of Btk-Strep complexes results in the formation of undistinguishable bands on the silver stained gel

The use of strong washing conditions can result in the loss of actual binding partners from the bait. This is because many interactions are transient in nature and the proteins are expressed at low levels. To preserve these weak and temporary interactions, cross-linking reagents are commonly used for mapping protein-protein interactions through affinity purification (Vasilescu and Figeys 2006). Formaldehyde is a reversible cross-linker of proteins and DNA. The reagent binds closely interacting molecules by fixing amino groups of one molecule with other nearby nitrogen atoms through –CH₂– linkage and thus was chosen for the current protocol. Differentiated macrophages were infected with either AdBtk-Strep or control AdStrep and stimulated for 15min with R848. As Btk appears to be activated within 5-10min following R848 challenge (Chapter 4, section 4.2.3) it is likely that the major protein-protein interaction events occur at later time points justifying the change in R848 stimulation time from 5 to 15min as compared to the previous section. Prior to lysis, the cells were subjected to 1% formaldehyde treatment. Nuclear and cytoplasmic fractions were isolated to further clarify the lysate and reduce the appearance of multiple bands. DNase was added to the lysis buffers to eliminate the formation of protein-DNA complexes. The lysates were then applied onto Strep-Tactin ® MacroPrep ® column and washed with 0.2% NP40 buffer containing a higher concentration (150mM) of NaCl than in previous experiments. Eluted fractions were subjected to the reverse cross-linking reaction by heating overnight and resolved on a SDS-PAGE gel. Btk-Strep was present in both cytoplasmic and nuclear elution fractions of the AdBtk-Strep infected cells (**Figure 3.14A**). The use of more stringent washing conditions resulted in the reduction of band accumulation in the cytoplasmic fraction of the control samples (**Figure 3.14B**). Unfortunately, the resolution of the bands did not improve with the formaldehyde pre-treatment. On the contrary, the bands in the control and Btk-Strep lanes were smeary and consequently indecipherable, which hindered the detection of a distinguishable Btk-Strep band. These results indicate that either the utilization of the formaldehyde pre-treatment or the reverse cross-linking reaction conditions used are unable to obtain highly distinguishable bands on a silver-stained gel. This could be a result of high presence of contaminants that were also non-specifically cross-linked to Btk by the formaldehyde treatment. The use of an increased NaCl concentration in the washing buffer did, however, reduce the high background of the control samples and was therefore used for the further optimization steps.

A)



B)

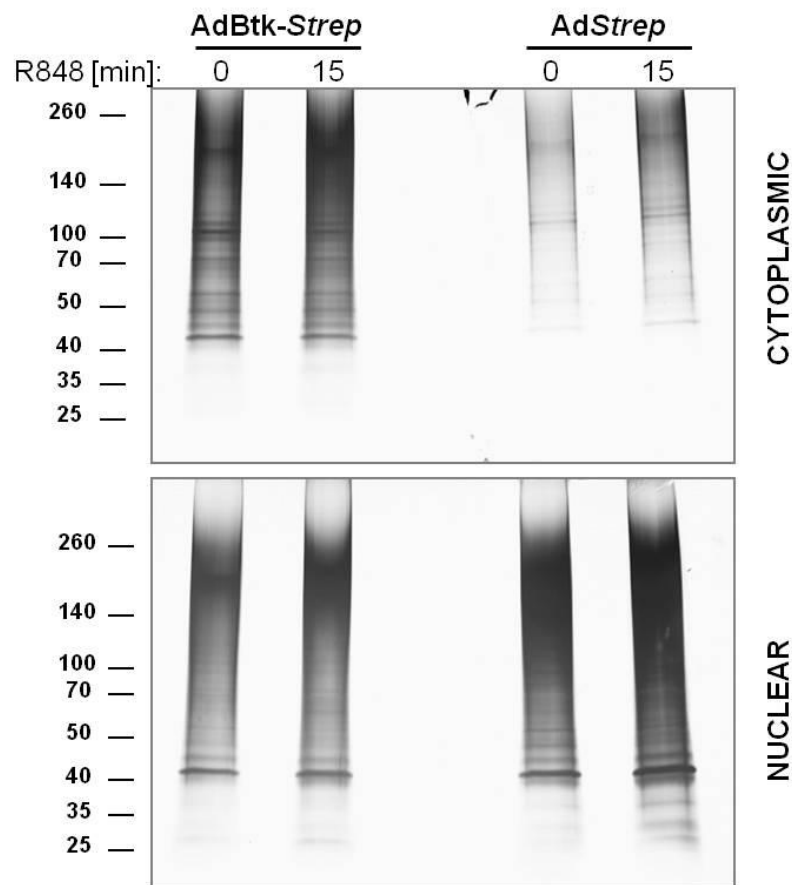


Figure 3.14 Formaldehyde treatment of macrophages together with high salt concentration in the wash buffer results in the formation of smears on a silver stained gel. 25mln differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were infected with AdBtk-Strep or control AdStrep at moi 50 (section 2.2.3.9). After 24h cells were stimulated with 1µg/ml R848 for 15min, fixed in 1% formaldehyde for 10min, washed twice with 1xPBS and lysed. Nuclear and cytoplasmic lysates were applied onto Strep-Tactin® MacroPrep® column and washed with 0.2%NP40 (150mM NaCl). Btk-Strep complexes were eluted with biotin at V=1 in elution fractions E2-4 (section 2.2.5.1). Fractions were concentrated on Vivaspin 500 columns. 90% of E2-4 were applied onto a 4-20% SDS-PAGE gel which was subsequently subjected to silver staining (A). 1% of the input (I) and the last wash fraction (W) together with 10% of E2-4 were analysed by Western blotting with the anti-Btk antibody (B).

3.2.10.3 Avidin and DNase pre-treatment of the lysates together with stringent washing conditions greatly improve the clarity of the bands on a silver stained gel

One explanation for the high background on a gel could be the contamination of the Strep-Tactin[®] MacroPrep[®] column with biotinylated proteins derived from the cells and present in the RPMI medium (Moore et al. 1967). Biotin, or Vitamin H, fulfils many crucial roles in a cell as it is necessary for cell growth, the production of fatty acids, and the metabolism of fats and amino acids. It is a cofactor for CO₂ transfer and usually attaches to its substrate via an amide bond to the ϵ -amino group of a lysine residue. These biotinylated proteins non-specifically bind to the column and are subsequently eluted with the bait (www.iba-go.com). To remove such potential contaminants avidin, which has a high affinity to biotin, was used to purify the lysates. Thus, differentiated macrophages were infected with either AdBtk-*Strep* or control Ad*Strep* and stimulated for 15min with R848. DNase and avidin were added to the nuclear and cytoplasmic lysis buffers and subsequently spun down to remove avidin-biotin complexes in the pellet. The supernatants were then applied onto a Strep-Tactin[®] MacroPrep[®] column and washed with 0.2% NP40 buffer containing 150mM NaCl. The new purification conditions resulted in the formation of distinguishable bands of the Btk-*Strep* lanes and low background in the control lanes on a silver stained gel (**Figure 3.15**). There were, however, no differences in the band patterns derived from unstimulated and R848-stimulated cells. Nevertheless, all the bands were extracted from the gel and submitted for LC-ESI-MS/MS analysis in collaboration with Dr Robin Wait (K.I.R).

The data generated from MS analysis revealed that Btk was present in 50% of the bands ranging from 40kDa to 140kDa thus masking the detection of additional proteins. The highest accumulation of Btk on the gel is shown with arrows in **Figure 3.15**. The identification of the peptide spectra with Mascot also recognised the presence of AP-3 adaptor-like complex components which are implicated in intracellular biosynthesis and transport, as well as the presence of heat shock protein (Hsp) 90. The identity of the remaining bands could not be determined due to the proportionally high presence of trypsin used for MS sample digestion and peptides of foreign origin (**Figure 3.16**).

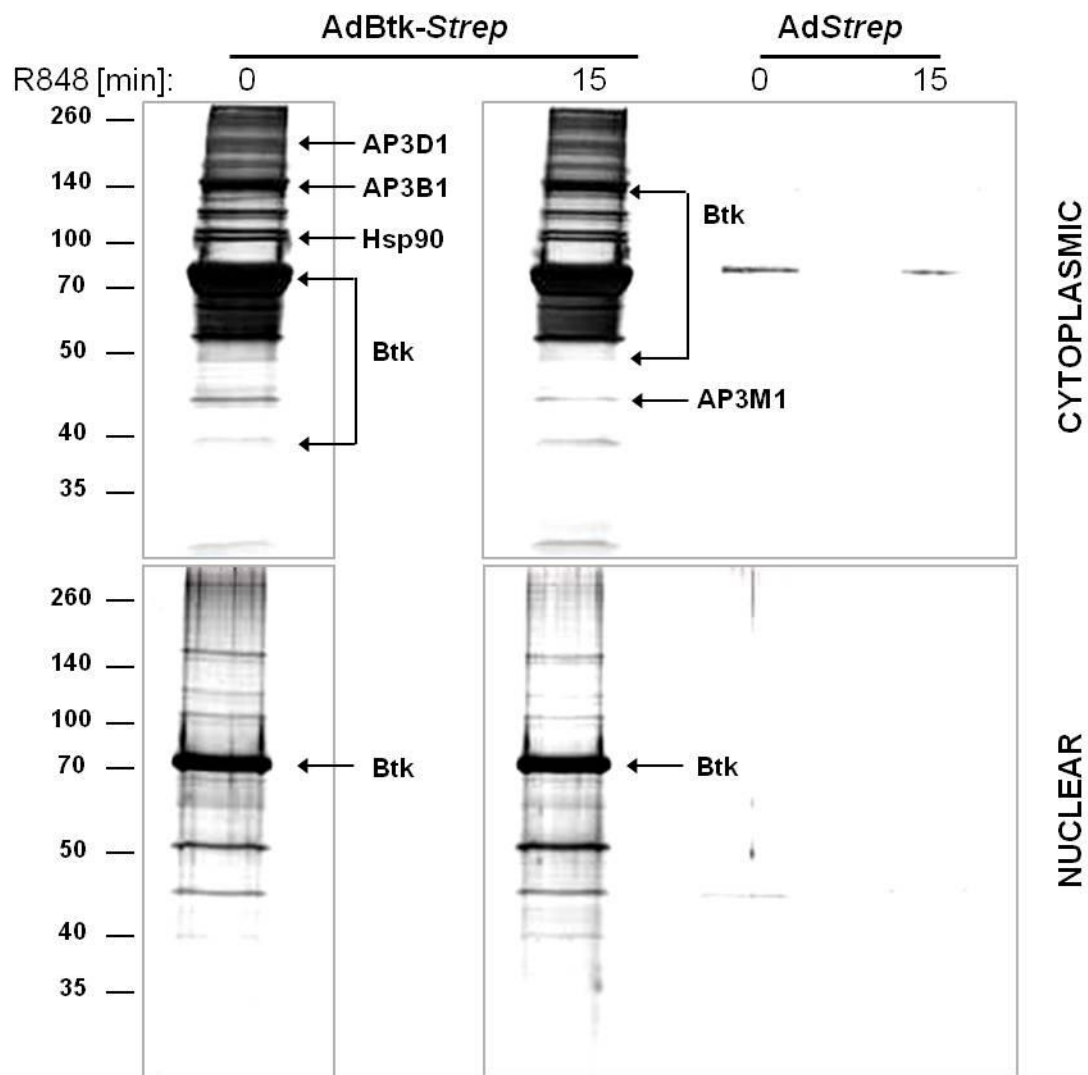


Figure 3.15 Pre-cleaning of the lysates with avidin and DNase together with high salt concentration in the wash buffer results in low background on a silver stain gel. 2.5×10^6 differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were infected with AdBtk-Strep or control AdStrep at moi 50 (section 2.2.3.9). After 24h cells were stimulated with $1\mu\text{g/ml}$ R848 for 15min, washed twice with $1\times\text{PBS}$ and lysed. Nuclear and cytoplasmic lysates were treated with $100\mu\text{g/ml}$ avidin and $1\mu\text{g/ml}$ DNase, the insoluble pellets were spun down whereas the supernatants were applied onto Strep-Tactin® MacroPrep® column and washed with $0.2\%\text{NP40}$ (150mM NaCl). Btk-Strep complexes were eluted with biotin at $V=1$ in elution fractions E2-4 (section 2.2.5.1). Fractions were concentrated on Vivaspin 500 columns. 90% of fractions E2-4 were analysed by 4-20% SDS-PAGE gel which was subsequently subjected to silver staining. All the bands were cut out of the gel and analysed by MS by Dr Robin Wait.

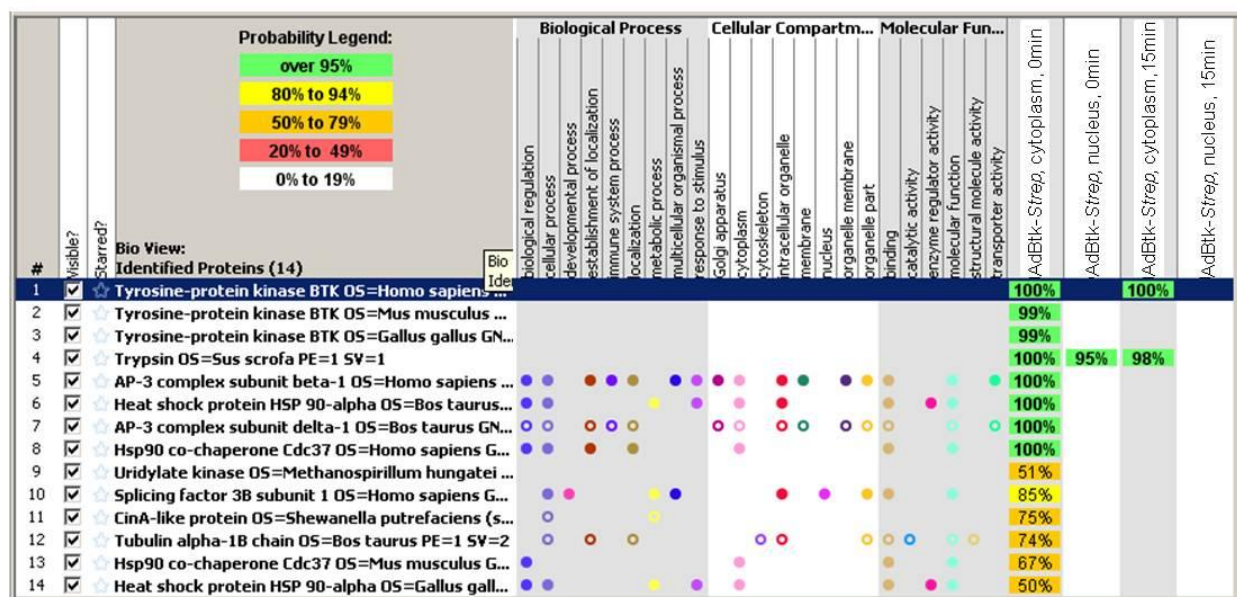


Figure 3.16 Screen print from the Scaffold analysis of the hits obtained from Btk-Strep purification. 14 proteins were identified by Mascot/Scaffold analysis of the peptide spectra. The hits included Btk, which was a predominant protein; trypsin, which was used for the protein digestion; the members of AP-3 complex and Hsp90. Hits with a probability over 95% are shown in green, whereas hits with a probability between 50-95% are shown in yellow and orange. Hits below 50% probability are not shown as they were not considered valid.

3.2.10.4 The prolongation of the R848 stimulation time to 30min does not lead to the identification of novel Btk binding partners.

Btk is known to be activated by TLRs as early as 5min post ligand binding in human primary macrophages (Horwood et al. 2003; Horwood et al. 2006). At the same time it was suggested that Btk is implicated in early stages of TLR signalling, and in overexpression systems in HEK293 and THP-1 cells it has been shown to constitutively interact with Myd88, IRAK1 and the TIR domains of TLRs 4, 6, 8 and 9. It has also been shown to phosphorylate Mal in response to LPS (Jefferies et al. 2003; Gray et al. 2006). The downstream components of Btk-mediated TLR-signalling remain uncharacterised.

The experiments above (section 3.2.10.3) did not detect differences in band patterns between unstimulated and 15min-stimulated macrophages. Using the same purification protocol as in

Section 3.2.10.3, the differences in the band patterns were further examined in unstimulated and 30min stimulated macrophages. As demonstrated in **Figure 3.17**, prolonging the R848 stimulation time of AdBtk-*Strep*-infected cells did not result in a change of band pattern as compared to unstimulated cells. Nevertheless, all the bands were extracted from the gel and analysed by LC-ESI-MS/MS and a subsequent Mascot search engine by Dr Robin Wait. The Btk-interacting proteins identified by MS included components of the transcription machinery (pre-mRNA-splicing factor 3b (SF3B4)), translation machinery (eukaryotic translation elongation factor 1 alpha 1 (EEF1A)), and also proteins involved in intracellular biosynthesis and transport (AP3), calcium-dependent exocytosis (annexin A2), and cytoskeleton reorganisation (tubulin, actin, strumpellin (KIAA0196)) (**Figure 3.18**). Many of these interactions were non-specific as EEF1A, actin, annexin2a and GAPDH were also present in the control Ad*Strep* lanes. The others, SF3B4, AP-3 or KIAA0196 have not previously been linked to TLR signalling.

The molecular chaperone, Hsp90, was once again detected in the complex and appears to be constitutively bound to Btk. In addition, the co-chaperone of Hsp90, cell division cycle protein 37 (Cdc37), was also detected. The Hsp90-Cdc37 complex has been previously shown to be crucial for TLR signalling as the pharmacological inhibition of Hsp90 led to degradation of IRAK1, and as a consequence, to the reduction of LPS-induced cytokine production. The overexpression of Cdc37, on the other hand, led to enhanced IRAK1 activation (De Nardo et al. 2005). The results suggest that Hsp90 is involved in Btk signalling but the association of Hsp90 with Btk is not TLR-dependent, since it was present both prior to and post R848 stimulation.

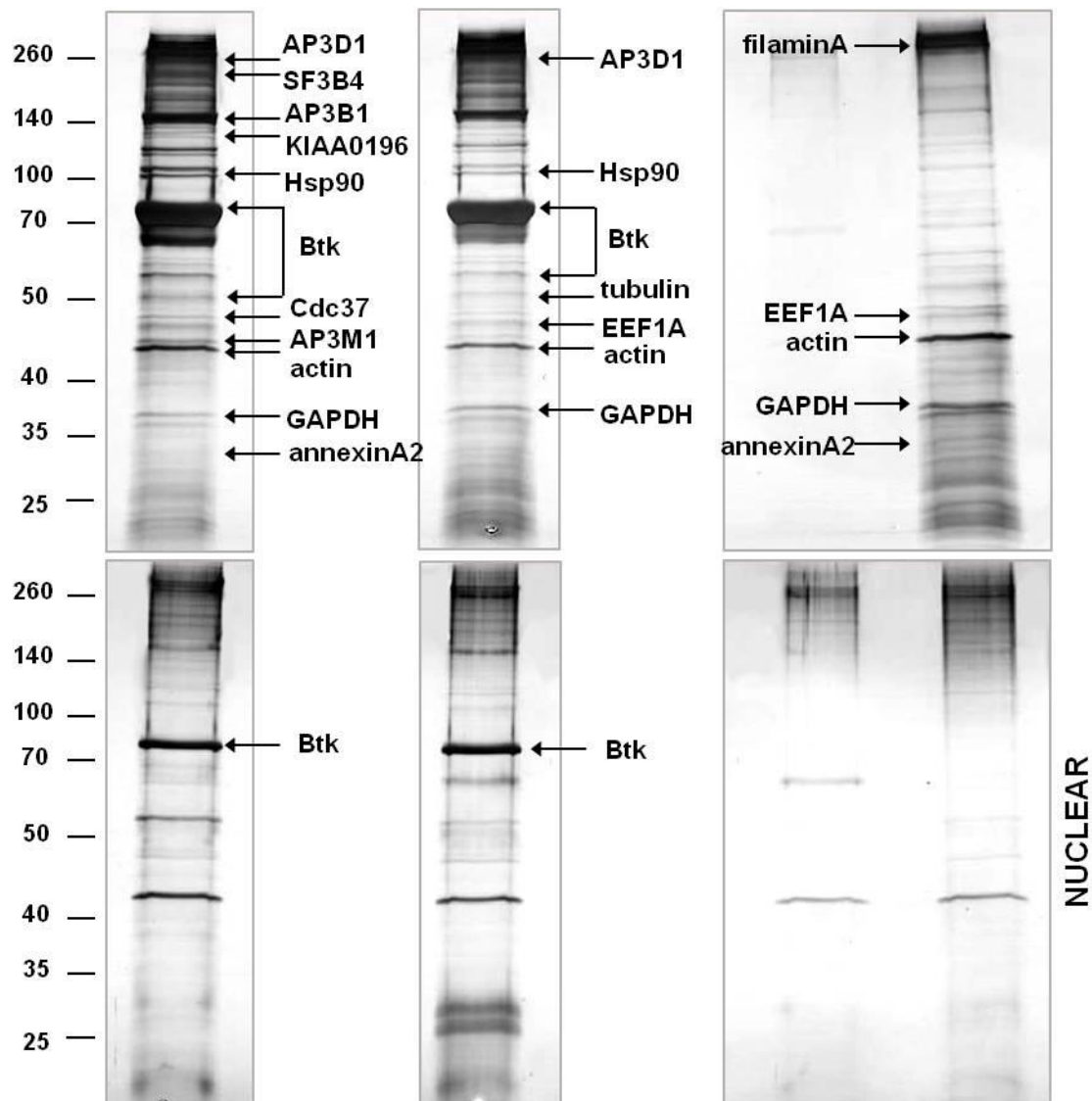


Figure 3.17 Lack of differences in the Btk-Strep band patterns between unstimulated and 30min-stimulated human primary macrophages. Differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) at 25×10^6 cells per treatment were infected with AdBtk-Strep or control AdStrep at moi 50 (section 2.2.3.9). After 24h cells were stimulated with $1\mu\text{g/ml}$ R848 for 30min, washed twice with $1\times\text{PBS}$ and lysed. Nuclear and cytoplasmic lysates were treated with $100\mu\text{g/ml}$ avidin and $1\mu\text{g/ml}$ DNase. The insoluble pellets were spun down whereas the supernatants were applied onto Strep-Tactin[®] MacroPrep[®] column and washed with $0.2\%\text{NP40}$ (150mM NaCl). Btk-Strep complexes were eluted with biotin at $V=1$ in elution fractions E2-4 (section 2.2.5.1). Fractions were concentrated on Vivaspin 500 columns. 90% of fractions E2-4 were applied onto a 4-20% SDS-PAGE gel that was subsequently subjected to silver staining. All the bands were cut out of the gel and analysed by MS by Dr Robin Wait.

#	Visible?	Starred?	Bio View: Identified Proteins (20)	Probability Legend				Biological Process				Cellular Compartment				Molecular Function				AdBtk-Strep, cytoplasm, 0min				AdBtk-Strep, nucleus, 0min				AdBtk-Strep, cytoplasm, 30min				AdBtk-Strep, nucleus, 30min			
				over 95%	80% to 94%	50% to 79%	20% to 49%	0% to 19%	cellular process	developmental process	establishment of localization	immune system process	localization	metabolic process	multicellular organismal process	response to stimulus	Golgi apparatus	cytoplasm	extracellular region	intracellular organelle	membrane	mitochondrion	nucleus	organelle membrane	organelle part	plasma membrane	binding	catalytic activity	molecular function	transporter activity	AdBtk-Strep, cytoplasm, 0min	AdBtk-Strep, nucleus, 0min	AdBtk-Strep, cytoplasm, 30min	AdBtk-Strep, nucleus, 30min	
1			Tyrosine-protein kinase																																
2			Trypsin OS=Sus crofa L																																
3			Actin, cytoplasmic OS=																																
4			Glyceraldehyde-3-phos																																
5			Elongation factor 1-alf																																
6			Plastin-2 OS=Homo sap																																
7			Alpha-enolase OS=Homo																																
8			Annexin A5 OS=Homo s																																
9			AP-3 complex subunit b																																
10			Pyruvate kinase isozym																																
11			Annexin A2 OS=Bos tau																																
12			AP-3 complex subunit d																																
13			Serum albumin OS=Bos																																
14			6-phosphogluconate de																																
15			Chloride intracellular ch																																
16			Splicing factor 3B subu																																
17			Lysine-sensitive aspart																																
18			E3 ubiquitin-protein liga																																
19			sp Q01518 CAP1_HUM																																
20			Heat shock protein HSP																																

To determine whether Hsp90 has a role in TLR signalling, the effect of Hsp90 inhibition by geldanamycin (GA) on cytokine production was assessed by ELISA. GA is an inhibitor of Hsp90 which has been widely used for determination of Hsp90 function (De Nardo et al. 2005). In the current system, GA dose-dependently decreased the production of TNF, IL-6 and IL-10 from LPS- and R848- stimulated human primary macrophages without affecting the viability of the cells (**Figure 3.19B-D**). These results suggest that even though Hsp90 interactions with Btk are not dependent on TLR stimulation, the chaperone is important for the production of cytokines induced by TLR signalling. The data also demonstrate that Hsp90 function is not specific to Btk as GA blocked the production of all three cytokines as opposed to Btk which appears to only regulate LPS-induced TNF or R848-induced TNF and IL-6 (section 3.2.1).

Hsp90, a molecular chaperone that protects the function of its client proteins, is the most abundant protein expressed in cells (Csermely et al. 1998). The molecule maintains the conformational integrity of its clients and guards them against misfolding during exposure to heat shock and other stress (Tsan and Gao 2009). Hsp90 interacts with numerous molecules involved in TLR signalling. This explains why the use of GA, which leads to destabilisation and degradation of Hsp90 client proteins, results in the inhibition of cytokine production. For instance, Hsp90 has been involved in the activation of NF κ B by directly binding to IKK and in the activation of MAPK (Broemer et al. 2004; De Nardo et al. 2005). The inhibition of Hsp90 by a small molecular inhibitor decreased the activation of JNK and ERK in RA synovial fibroblasts as well as inflammation and pannus formation in RA animal models providing foundation for the use of Hsp90 inhibitors in the future clinical trials for the treatment of RA (Rice et al. 2008). Hsp90 has been also shown to interact with another member of the PTK family, Src (Koga et al. 2006).

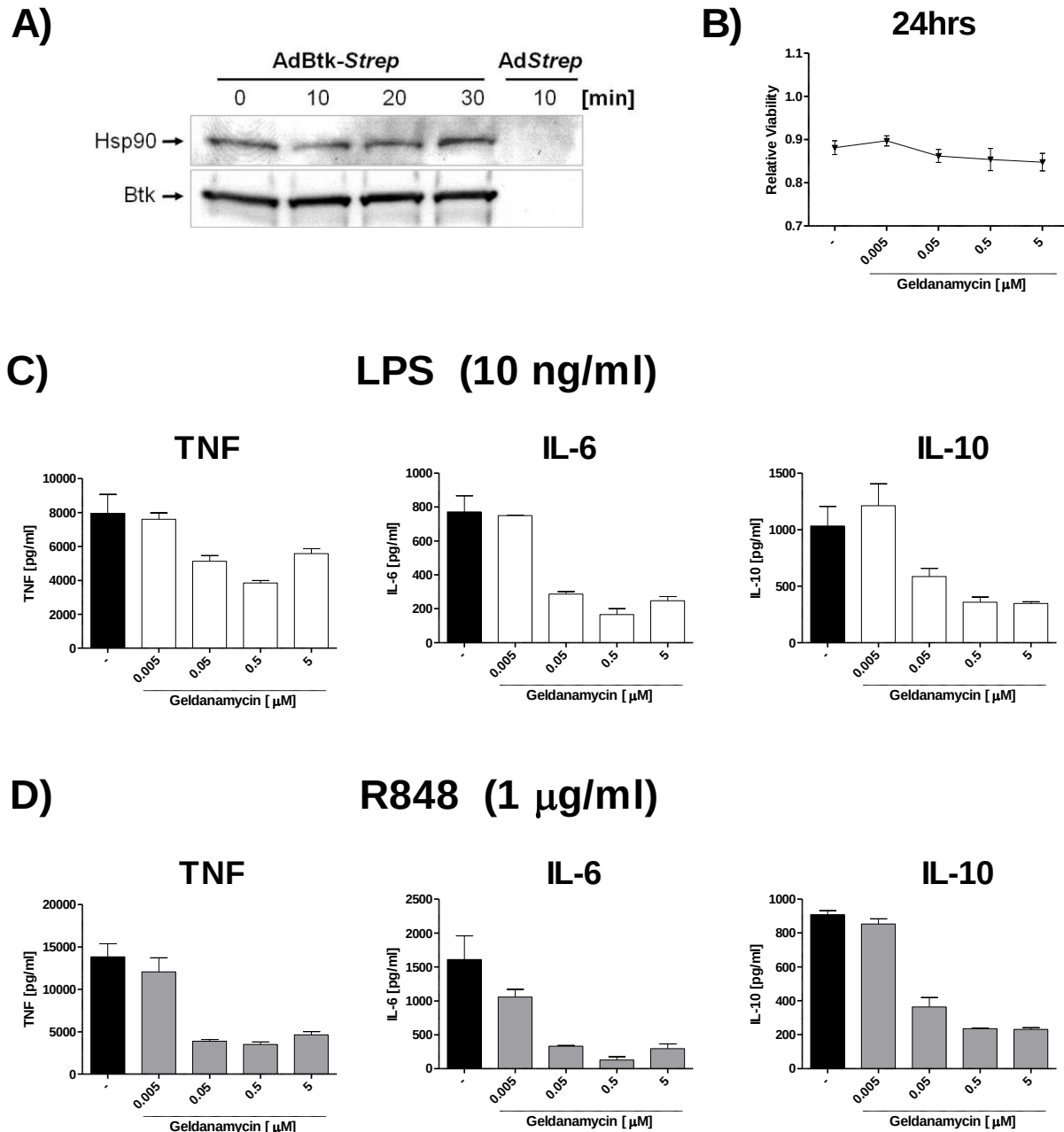


Figure 3.19 The inhibition of Hsp90 which is consistently bound to Btk decreases the cytokine production upon TLR4 and 8 stimulation. 1×10^6 differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were infected with AdBtk-Strep or control AdStrep at moi 50 (section 2.2.3.9). After 24h cells were stimulated with $1\mu\text{g/ml}$ R848 for 30min, washed twice with $1\times\text{PBS}$ and lysed. Total lysates were treated with $100\mu\text{g/ml}$ avidin and $1\mu\text{g/ml}$ DNase. The insoluble pellets were spun down whereas the supernatants were applied onto Strep-Tactin® MacroPrep® column and washed with 0.2%NP40 (150mM NaCl). Btk-Strep complexes were eluted with biotin at $V=1$ in elution fractions E2-4 (section 2.2.5.1). Fractions were concentrated on Vivaspin 500 columns and run on a 4-20% SDS-PAGE gel. Anti-Btk and anti-Hsp90 antibodies were used for Western blotting (A). Differentiated macrophages plated in 96-well plates were pre-treated for 1h with 0.005- $5\mu\text{M}$ geldanamycin and stimulated with 10ng/ml LPS or $1\mu\text{g/ml}$ R848 for 8h. The toxicity of geldanamycin was assessed by the MTT assay (B). Supernatants were collected and analysed for TNF, IL-6 and IL-10 by ELISA (C,D).

3.3 Discussion

This study has shown that Btk is required for TLR8 but not TLR3 signalling in human primary macrophages as the overexpression of the kinase increases R848-induced TNF production but not PolyI:C-induced IP-10 production. However, the approaches used to dissect Btk signalling downstream of TLR8 activation were not successful. Firstly, the monocyte-like cell lines, which are broadly used for determining the signalling mechanism of pattern recognition receptors, lack functional TLR3 and 8. Secondly, although data obtained from the use of optimised One-*Strep* system has revealed that Btk constitutively interacts with chaperone Hsp90, this interaction was not exclusive to Btk and was not dependent on TLR8 activation.

Human monocyte-like cell lines (THP-1/ PMA, U937/ PMA and Monomac-6), which were intended to be used for the optimisation of the Btk affinity purification system, failed to induce cytokine production upon TLR3 and TLR8 stimulation (**Figure 3.4/3.5**). The majority of studies regarding the role of Btk in TLR signalling have been performed on cell lines that frequently are unable to express TLRs or in which the functionality of TLRs has not been previously validated (Jefferies et al. 2003; Doyle et al. 2005; Gray et al. 2006; Doyle et al. 2007). For instance, TLR-lacking HEK293 cells transfected with tagged versions of both Btk and Myd88 or Mal were used to detect the interactions between the kinase and the adaptor molecules (Jefferies et al. 2003). The studies performed in THP-1 cells focused mainly on analysing TLR8 or TLR9-activated signalling pathways and did not refer to the ability of TLR to induce cytokine expression (Doyle et al. 2007). Interestingly, potential defects in TLR signalling in the examined cell lines did not affect the TLR4 pathway (**Figure 3.4/3.5**) which is known to be the most complex TLR pathway as it utilizes 4 adaptor molecules; TRIF, TRAM, Mal and Myd88, as compared to TLR8 which uses only Myd88 and TLR3 which uses only TRIF (Kawai and Akira 2010). A hypothetical defect in one of the adaptor molecules could be compensated by an alternative adaptor molecule allowing LPS signalling to function normally. Although THP-1 cells were unable to produce cytokines upon TLR8 activation in this study, the stimulation of undifferentiated THP-1 cells with R848 has been previously shown to effectively induce the expression of the CD1d molecule that in turn activated a subset of T cells (Salio et al. 2007). An extensive literature search did not identify any reports that would address the functionality of TLR3 and 8 in terms of cytokine production in THP-1, U937 or Monomac-6 cells.

Due to the differences in cytokine production between cell lines and primary macrophages, the investigation of Btk interacting partners was undertaken in primary cells. The initial fusion of Btk to a large N-terminal NTAP tag proved unsuccessful as AdBtk-NTAP was unable to increase LPS- or R848- induced production of TNF in human primary macrophages (**Figure 3.8**) as compared to wild type AdBtk (**Figure 3.2**). The underlying reason could be the large size of the tag (136aa) or its N-terminal location which could interfere with the PH domain binding to the membrane. The TAP tag attached to the C-terminus of Btk was previously successfully applied for the identification of Btk interaction with the inhibitor Dasatinib (Hantschel et al. 2007). Therefore it was unlikely that tag size precluded Btk from inducing TNF, rather the location of the TAP tag interfered with the activation and function of the kinase. The second attempt involved the fusion of a smaller *Strep*-tag (31aa) to the C-terminus of Btk where the kinase domain is located. Fortunately, AdBtk-*Strep* proved to be efficient in the induction of cytokine production (**Figure 3.11**) and therefore was used for the subsequent purification experiments.

The use of the One-*Strep* system has been shown to result in efficient purification with good yields as compared to other affinity tags such as HIS, calmodulin-binding peptide (CBP) or FLAG (Lichty et al. 2005). The initial purification protocol has been previously used for the purification of the transcription factor IRF5 from fixed HEK293 cells (Krausgruber et al. 2010). The purification of predominantly cytoplasmic Btk from unfixed human primary macrophages required modification of the protocol. The optimisation of the One-*Strep* system included the following steps: determination of the minimum cell number required, modification of the lysis and washing conditions as well as the adjustment of elution volumes. The final established protocol engaged the use of minimum 2.5×10^7 human primary macrophages per treatment, the use of avidin and DNase in a lysis buffer as well as the use of a wash buffer containing 150mM NaCl. Avidin, which has a high affinity to biotin was used to deplete biotinylated contaminants from the lysates, DNase to minimise the formation of protein-DNA complexes and as a consequence the purification of indirect interacting partners, and high salt to increase the stringency of the wash. Despite the visible improvements in the background level on a silver stained gel (**Figure 3.15/3.17** as compared to **Figure 3.13/3.14**), there were no differences in band patterns between R848-stimulated and unstimulated cells. This suggests that the washing conditions were not stringent enough to remove non-specific bands. Potentially, if cell number was increased together with more stringent washing conditions to remove contaminants, subtle changes in the band patterns may be identified. The average elutriation of PBMCs obtained from 10ml blood cones and subsequent differentiation of human primary monocytes allows the acquisition of on average $1-2 \times$

10^8 macrophages in total which were all used for one *One-Strep* experiment. The increase of cell number would require the pooling of the donors together, which may mask donor-dependent differences. Ideally, macrophages at the same culture time should be used for the experiment as *One-Strep* purification has to be performed on a day of lysis to retain protein interaction.

Analysis by MS demonstrated that Btk was present in the majority of the bands impeding the detection of interacting proteins (**Figure 3.15/3.17**). The problem could be removed by decreasing the expression level of Btk or by modifying the elution step. The expression of a tagged protein must, however, remain at a level high enough to compete over the endogenous Btk which also interacts with binding partners. Alternatively, to eliminate Btk in the final fraction, the biotin elution step could be avoided. Btk complex chemically fixed prior to the lysis stage could undergo reverse-cross linking on a column allowing the Btk binding partners but not Btk itself to be released from the column. The reverse cross-linking fraction could then be analysed by MS to allow the identification of more binding partners undisturbed by the presence of Btk.

Specific proteins interacting with Btk included a member of the WASH complex involved in cytoskeletal reorganisation, strumpellin, the subunits of AP3 complex known to be involved in intracellular biosynthesis and transport, transcription splicing factor SF3B4, and components of a chaperone complex, Cdc37 and Hsp90 (www.genecards.org). Btk has been long known to have a profound effect on cytoskeletal reorganisation and cell motility therefore the identification of strumpellin in the Btk complex was not surprising. The kinase interacts with F-actin through its PH domain, WASP and Vav through its SH3 domain (Lindvall et al. 2005). The function of Btk in cell motility is critical as B cells derived from Xid mice or XLA patients lose integrin-mediated cell adhesion and migration abilities (Spaargaren et al. 2003). Moreover, osteoclasts derived from XLA patients also exhibit impaired resorption activity known to be dependent on F-actin ring formation (Danks et al. 2011). On the other hand, Btk has not been previously implicated in mRNA maturation thus the detection of SF3B4 in the Btk complex was novel. The kinase has, however, been previously linked to transcription initiation as it interacts with and directly activates a transcription factor TFII-I (Novina et al. 1999), whereas another member of Tec family kinases, Bmx, has been demonstrated to tyrosine phosphorylate STATs 1, 3 and 5 (Tsai et al. 2000).

Both Hsp90 and its co-chaperone Cdc37 were found in the Btk complex. Hsp90 has a crucial role in cancer development and there are now 13 Hsp90 inhibitors in clinical trials for different types of malignancy (Kim et al. 2009). The presence of Hsp90 was not stimulation-dependent as the

chaperone was constitutively bound to Btk. In order to determine the role of Hsp90 in the TLR8 pathway, attempts to downregulate Hsp90 with siRNA directed against Hsp90 α and β were undertaken. However, as this approach did not prove successful (data not shown), potentially due to the high abundance of the protein in the cells, a drug called geldanamycin was used to inhibit Hsp90. Geldanamycin, a natural occurring antitumor antibiotic, competitively binds the substrate pocket of Hsp90 blocking the chaperone function and resulting in the degradation of its client proteins (Stebbins et al. 1997). As Hsp90 has been shown to be required for the stability or activation of the TLR pathway components including IKK, TAK1, Lck or Src (Broemer et al. 2004; Giannini and Bijlmakers 2004; Koga et al. 2006; Liu et al. 2008), it is not unexpected that the inhibition of Hsp90 led to decreased production of TNF, IL-6 or IL-10 upon TLR4 or 8 stimulation. Previous research has demonstrated that Btk did not affect IL-6 production from LPS-challenged human primary macrophages (Horwood et al. 2003), suggesting that the observed effect of Hsp90 inhibition on the cytokine secretion was not restricted to Btk alone.

In summary, the identification of Btk interacting partners downstream of TLR8 signalling did not reveal any molecules exclusively binding to Btk after R848 stimulation. Instead, the identification of Hsp90 as being constitutively bound to Btk revealed a complex protein interaction mechanism where interruption affects the production of cytokines. Furthermore, Btk overexpression was able to super-induce cytokine production upon TLR8 stimulation. Therefore the mechanism of action needs to be investigated further.

Chapter 4 - Results II

Btk differentially regulates cytokine production under TLR4 and TLR8 stimulation

4.1 Introduction

The data obtained from Btk overexpression studies suggest that the kinase may be differentially involved in TLR4 and TLR8 signalling due to the fact that Btk regulated LPS-induced TNF production as compared to R848-induced TNF and possibly IL-6 production. At the same time, Btk overexpression did not change the level of IP-10 production from PolyI:C-stimulated macrophages suggesting that the kinase is not involved in TLR3 signalling. These observations have to be, however, confirmed in another experimental setting. The TLR4 and TLR8 signalling cascades differ fundamentally in many aspects. First of all, TLR4 has been designed to detect bacterial and viral proteins whereas TLR8 responds to viral ssRNA or synthetic imidazoquinoline resiquimod (R848) stimulation. Secondly, TLR4 is primarily localised on the cell membrane as opposed to endosomal TLR8. Furthermore, the TLR4 pathway appears to be far more complex as it utilises four adaptor molecules in contrast to TLR8 which employs only Myd88. The complexity of TLR signalling network may explain why Btk does not regulate IL-6 production as other Tec family members can fulfil this role instead. For instance, Bmx and Tec kinases can be activated in response to TLR signalling (Horwood et al. 2003; Palmer et al. 2008). Recent studies in primary human macrophages and rheumatoid synovial fibroblasts (RASFs) have also demonstrated that Bmx can mediate TLR4 signalling by affecting IL-6 production (Palmer et al. 2008; Palmer et al. 2008).

TNF is believed to be a pivotal cytokine that controls the production of both pro-inflammatory and anti-inflammatory cytokines (reviewed in (Feldmann 2002)). Due to its central role in the pathology of RA and many other autoimmune and inflammatory disorders, the regulation of the *TNF* gene has been widely studied. Currently, there are two major theories describing the mechanism through which Btk could control TNF production. The first one suggests a role for Btk in transcription. Studies performed on Xid macrophages and the THP-1 cell lines have demonstrated that LPS-activated Btk engages the NF κ B pathway by promoting p65 subunit transactivation and nuclear localisation (Jefferies et al. 2003; Doyle et al. 2005). However, experiments performed in our laboratory showed that Btk does not affect an early expression of *TNF* indicating that the kinase is more likely to be involved in the post-transcriptional than transcriptional control. In particular, XLA-derived PBMCs or AdBtk-infected human macrophages did not exhibit impaired or enhanced, respectively, expression of LPS-induced *TNF* at an early 4h time-point as compared to the 18h time-point. Later reports have demonstrated no

decrease in I κ B α degradation in XLA-derived PBMCs and confirmed the positive effect of AdBtk on the stability of TNF mRNA as shown by Actinomycin D chase experiments on LPS-challenged human primary macrophages (Horwood et al. 2003; Horwood et al. 2006). The direct role of Btk in *TNF* transcription, however, has never been examined, therefore it cannot be excluded that both transcriptional and post-transcriptional mechanisms are implicated.

Work presented in this chapter focuses on the role of Btk in the production of TNF, IL-6, IL-10 and IP-10 following stimulation with the TLR4 ligand, LPS, and the TLR8 synthetic ligand, R848 in human primary macrophages. After establishing the positive role of Btk on cytokine production in R848-challenged cells, the effect of Btk downregulation on *TNF* gene expression was examined. By using primary-transcript (PT)-PCR and the Polymerase (Pol) II ChIP technique, the mechanism of Btk-dependent control of TNF expression was established.

4.2 Results

4.2.1 Major differences in general tyrosine phosphorylation patterns were detected in human primary macrophages stimulated with the TLR4 or TLR8 ligands

Protein tyrosine kinases are essential signalling mediators of macrophages cytokine responses as the inhibition of tyrosine phosphorylation by genistein and herbimycin inhibits TNF and IL-10 production from LPS- or zymozan-challenged cells ((Mander et al. 1997), John McDaid, thesis). Prior to the investigation of the role of Btk in TLR signalling, the overall tyrosine phosphorylation pattern was examined in human macrophages to establish the involvement of protein tyrosine kinases in the TLR4 and 8 signalling cascades. Cells were stimulated with LPS or R848 for 30min and lysed. The samples were then analysed by western blotting by using a 4G10 antibody directed against tyrosine phosphorylated proteins. Stimulation with the ligands triggered the phosphorylation of multiple proteins (**Figure 4.1**) providing the evidence that tyrosine kinases are active during TLR signalling. As the macrophages were obtained from the same donor, the experiment also allows a direct comparison of patterns between different stimulations. Major differences in tyrosine phosphorylation patterns were detected between LPS and R848

stimulations. This was particularly visible at the 38kDa level, where the TLR4-triggered increase in tyrosine phosphorylation was detectable at 10min, comparing to 20min with R848. Similarly, an early signal triggered by LPS was observed at around 78kDa level compared to a 10min delay for R848. Although this type of analysis does not allow identification of individual kinases or substrates, reblotting of the membrane suggested that the protein that is tyrosine phosphorylated at 38kDa molecular weight is likely to be p38 MAPK (**Figure 4.2**).

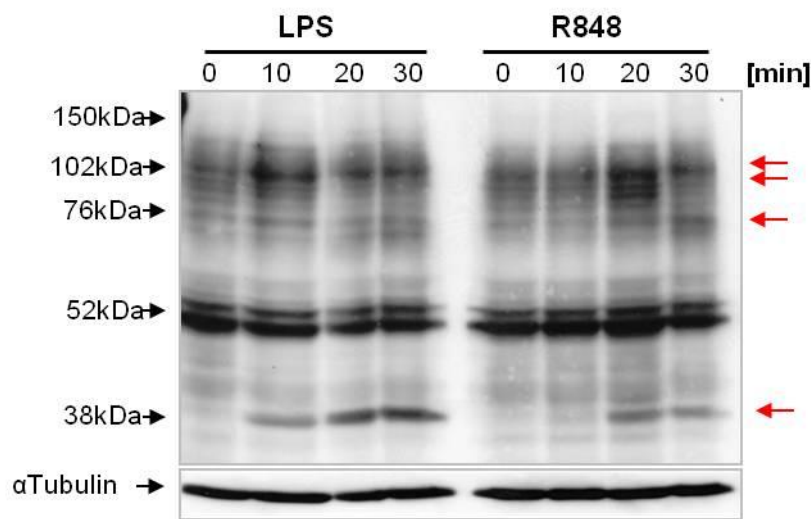


Figure 4.1 The differences in tyrosine phosphorylation patterns under TLR3, 4 and 8 stimulation. Macrophages obtained by 4 day culture of monocytes in M-CSF supplemented medium were stimulated for 30min with 10ng/ml LPS or 1 μ g/ml R848. Cells were lysed, the samples were run on 10% SDS-PAGE gel and transferred on PVDF membrane. The membrane was probed with 4G10 antibody which detects tyrosine phosphorylation. The red arrows represent different phosphorylation patterns between TLR treatments. The result is representative of 3 separate experiments.

4.2.2 Stimulation of human primary macrophages with LPS or R848 leads to the activation of MAPK and NFκB pathways.

TLR signalling triggers numerous signalling cascades that are responsible for the regulation of cytokine production. The ability of Btk to control cytokine production has previously attributed to NFκB and MAPK activation (Horwood et al. 2003; Jefferies et al. 2003). In this study, the signals initiated following stimulation of TLR4 and 8 in human primary macrophages were characterised prior to further investigation of the role of Btk in TLR signalling. M-CSF- derived human macrophages were stimulated with TLR ligands for 10, 20 and 30min and examined for the activation of MAPK as well as NFκB. As illustrated in the western blots and relative density graphs in **Figure 4.2**, the stimulation with LPS resulted in increased and sustained phosphorylation of p38, ERK and JNK MAPK from 10min mirrored by simultaneous IκBα degradation. The stimulation with R848 led to similar outcomes, although the kinetics of MAPK phosphorylation and IκBα degradation appeared to be delayed by 10min when compared to LPS. This observation could be explained by the extracellular and intracellular localisation of TLR4 and TLR8, respectively. The initiation of TLR8 pathway, in contrast to TLR4, requires the internalisation of the ligand (Kawai and Akira 2010) which could result in a delayed response. Taken together, these results highlight the substantial discrepancies in the ERK, JNK, p38 or NFκB involvement in the TLR4 and 8 pathways. Whether Btk is implicated in these different signalling cascades and whether its presence is indispensable for the cytokine production was then thoroughly investigated.

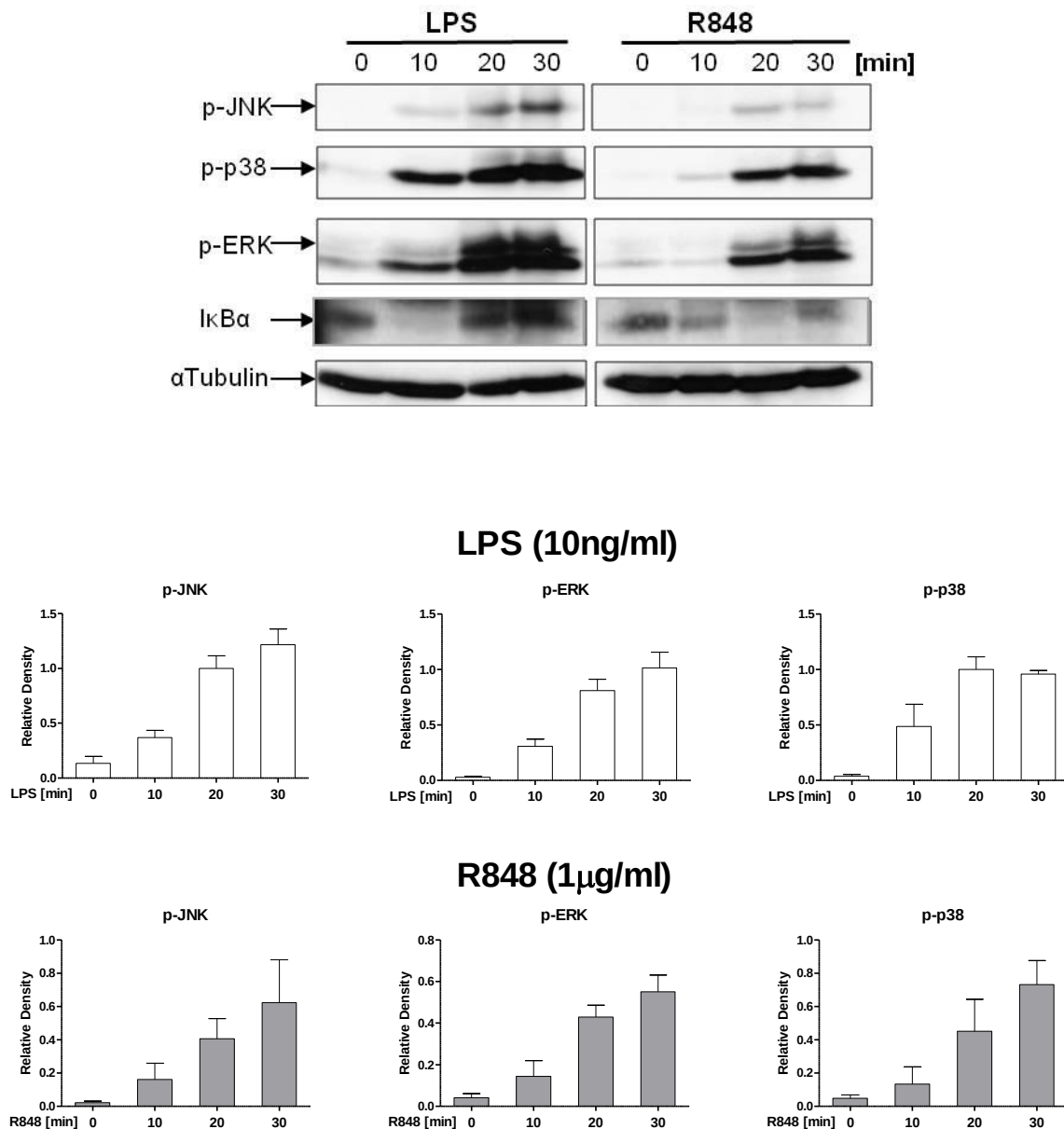


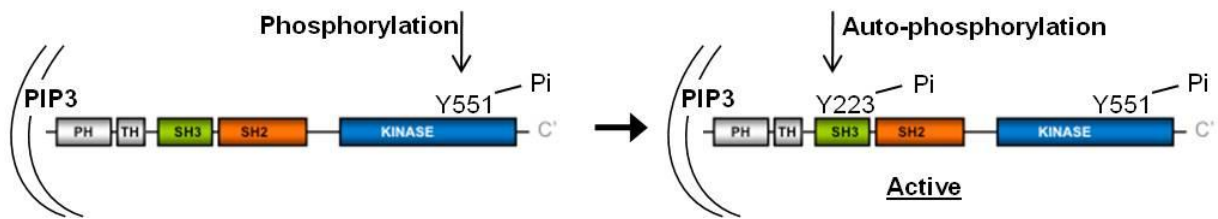
Figure 4.2 LPS and R848 activate MAPK and NFκB pathways in human primary macrophages. Macrophages obtained by 3 day culture of monocytes in M-CSF supplemented medium were plated at 1×10^6 cells and stimulated for 30min with 10ng/ml LPS or 1μg/ml R848. Cells were lysed, the samples were run on 10% SDS-PAGE gel and transferred on PVDF membrane. Subsequent probing with anti p-JNK, p-p38, p-ERK antibodies was used to detect the phosphorylation of MAPK. The membrane was then probed with anti IκBα IgG and anti α-tubulin IgG for a loading control. Between each step, the membrane was stripped and blocked in 2%BSA (phospho-westerns) or 5% milk. The blots are representative of 3 separate experiments.

4.2.3 Btk is activated in response to LPS and R848 in human primary macrophages

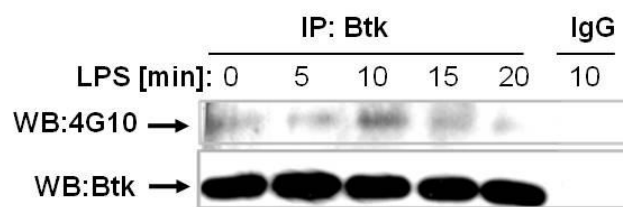
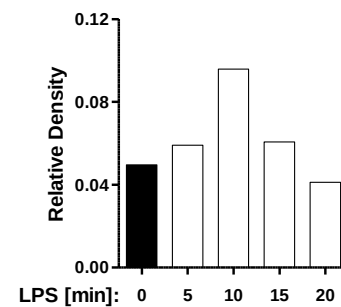
It has been described before that LPS stimulation of human macrophages leads to the rapid phosphorylation of Btk (Horwood et al. 2003) and that the kinase may play a role in endosomal TLR signalling pathways in THP-1 cell line (Doyle et al. 2007). In B cells, Btk activation requires three steps; the association of the PH domain with PIP3 to enable membrane localization, the phosphorylation on a tyrosine residue Y551 in the kinase domain by Src followed by auto-phosphorylation of a tyrosine residue Y223 in the SH3 domain (Park et al. 1996) (**Figure 4.3A**). After the comparison of the general tyrosine phosphorylation patterns and the initiation of common signalling cascades between the TLR4 and 8 pathways, the activation of Btk following TLR stimulation was examined. Due to the unavailability of anti-Btk antibodies efficient in the detection of the kinase phosphorylation state, an alternative, more technically demanding approach was chosen. Btk was immunoprecipitated from the whole cell lysate using a gifted anti-Btk antibody and its tyrosine phosphorylation state was subsequently detected by using 4G10 antibody. Stimulation with either LPS or R848 resulted in similar levels of Btk activation. The highest two-fold increase in phosphorylation was detected within 10 min of LPS stimulation and decreased after this time (**Figure 4.3B**). With R848, the phosphorylation peak remained stable between 10 and 15min (**Figure 4.3C**).

To extend previous data on Btk activation obtained by using 4G10 antibody, a Btk auto-kinase assay was performed. The assay involves immunoprecipitation of Btk from the cell lysates followed by the incubation of the isolated kinase with P^{32} radiolabelled ATP, a coenzyme which is utilised by Btk to phosphorylate itself. **Figure 4.4** demonstrates that auto-phosphorylation of the kinase occurred rapidly after LPS or R848 stimulation, reaching a peak at 5min and slowly decreasing thereafter. No signal was detected with an IgG control antibody as illustrated on the blot and confirmed in the relative density graph. In summary, these results show that Btk becomes activated in both TLR4 and 8 signalling at comparable time-points. To determine if Btk is equally involved in cytokine production in these two pathways, the differences of the Btk-dependent control of cytokine production between the TLR4 and 8 signalling cascades were next examined.

A) Model of Btk activation



B) LPS (10ng/ml)



C) R848 (1μg/ml)

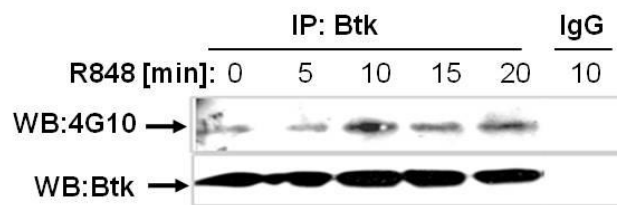
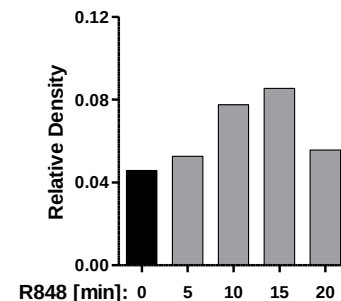


Figure 4.3 LPS and R848 stimulation phosphorylates Btk in human macrophages. Differentiated macrophages (obtained by 3 day culture of monocytes in M-CSF supplemented medium) were plated at 10×10^6 cells per time point in 5 cm^2 Petri dishes and incubated overnight. They were then serum starved for 2hrs and stimulated with 10ng/ml LPS or $1 \mu\text{g/ml}$ R848 for 20 min. The cells were lysed and Btk was immunoprecipitated (section 2.2.6.2). Samples were run on 10% SDS-PAGE gel and transferred on nitrocellulose membrane where the tyrosine phosphorylation was detected by 4G10 antibody. Anti-rabbit IgG was used as an isotype control. The blots represent 3 separate experiments.

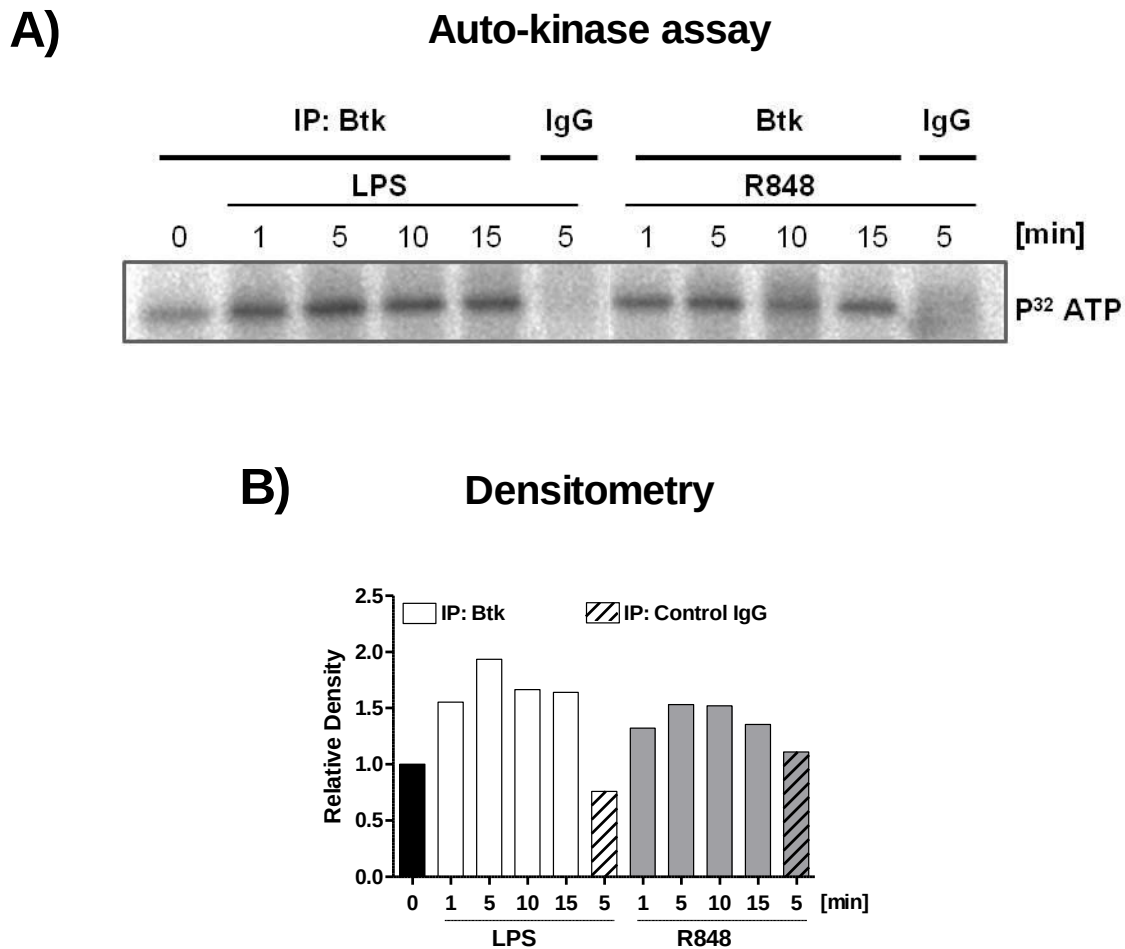


Figure 4.4 LPS and R848 stimulation leads to auto-phosphorylation of Btk in human macrophages. Differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were plated at 10×10^6 cells per time point in 10 cm^2 Petri dishes and incubated overnight in RPMI with 0.5% serum. They were then stimulated with 10 ng/ml LPS or $1 \mu\text{g/ml}$ R848 for 0, 1, 4, 10 and 15 min and lysed. Total Btk was immunoprecipitated with anti-Btk or IgG control antibodies. Following immunoprecipitation, kinase activity was assessed as described in section 2.2.6.3 (A). Relative density was calculated by normalising values from stimulated cells to unstimulated cells using AIDA software (B). The blot represents one out of 2 separate experiments.

4.2.5 Downregulation of Btk expression results in decreased TNF production in human macrophages

Previous reports have shown that the genetic dysfunction of Btk in XLA patients leads to the impaired release of TNF from LPS-stimulated PBMCs and both TNF and IL-6 from ssRNA-stimulated DC (Horwood et al. 2003; Sochorova et al. 2007). To confirm the results previously obtained with an overexpression system (Section 3.2.1), the effect of siRNA-mediated Btk knockdown on cytokine production was examined in this section. Freshly elutriated human monocytes were transfected with individual siRNA duplexes and subsequently differentiated into macrophages in the presence of M-CSF for 4 days and stimulated with TLR ligands for 18h. The efficiency of Btk knockdown was assessed by western blotting and the production of cytokine, by ELISA. To reduce off-target effects of gene silencing occurring when the siRNA sequence has homology to mRNA transcripts other than the gene of interest, two separate siRNA duplexes were first tested to downregulate *BTK* (section 2.1.6.2). Although both RNAi oligos similarly decreased Btk expression as well as TNF and IL-6 production from LPS- and R848-challenged cells (**Figure 4.5**), only the effects of the more efficient siRNA, 'Btk #9', are presented in this thesis.

The increasing concentration of siBtk#9 did not affect the efficiency of Btk knockdown as evaluated using western blotting analysis (**Figure 4.6A**). **Figure 4.6B** illustrates that after Btk targeted knockdown, the LPS and R848- mediated production of TNF was significantly decreased. The reduction was much stronger for R848 (75%) than for LPS (35%) suggesting that TLR8 signalling depends more on Btk than TLR4 signalling does. Downregulation of the kinase did not affect IL-6 production induced by LPS but surprisingly had a significant effect on R848 signalling at 200nM but not 300nM siRNA concentration (**Figure 4.6C**). The knockdown of Btk did not change IL-10 production induced by either LPS or R848 (**Figure 4.6D**) thus confirming the results obtained from the Btk overexpression study.

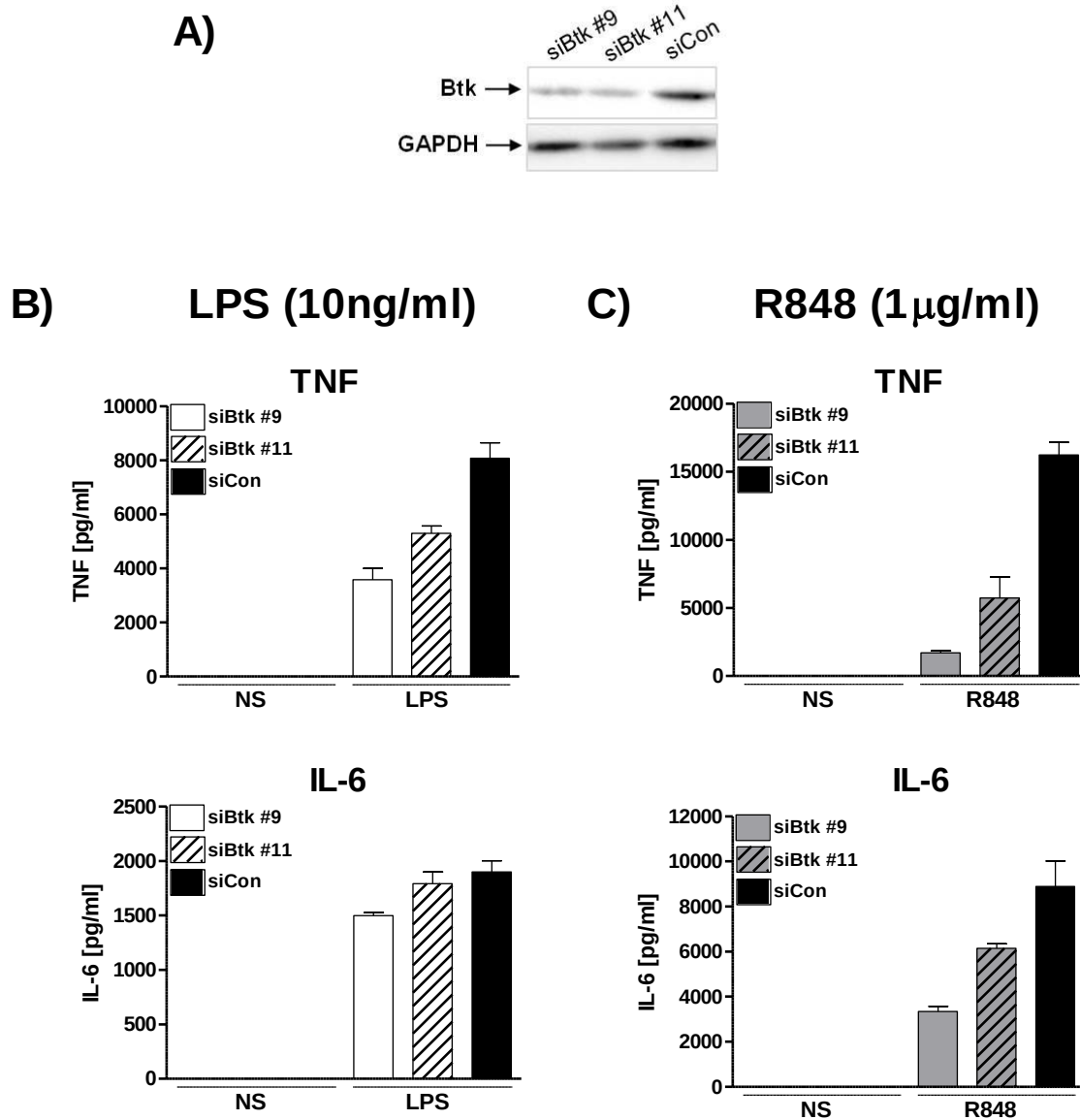


Figure 4.5 The comparison of two siRNA duplexes to downregulate Btk expression in human macrophages. Monocytes were transfected with 200nM Btk #9, Btk #11 or Control siRNA using the Amaxa Human Monocyte Nucleofector® Kit (section 2.2.2.1). After 4 days with M-CSF cells were stimulated with LPS (10ng/ml) or R848 (1μg/ml) over 18hrs. The protein level was assessed by SDS-PAGE and western blotting to ensure the efficiency of the knockdown (A) whereas TNF and IL-6 protein production was assessed by ELISA (B-C). Data are expressed as mean +/- SEM of triplicate measurements for a single donor.

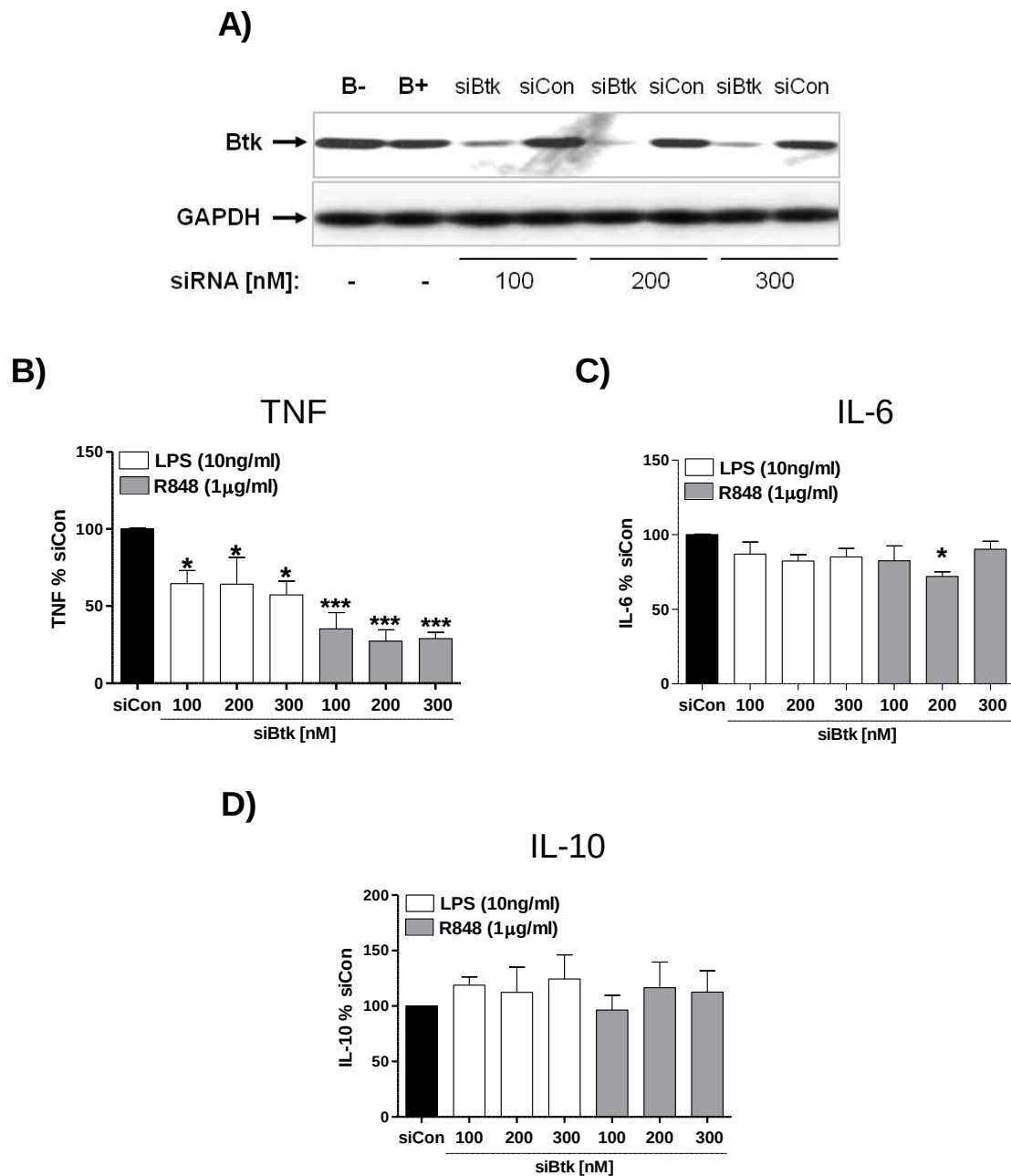


Figure 4.6 Btk knockdown results in decreased TNF production in human macrophages. Monocytes were transfected with siRNA at concentrations ranging from 100-300nM, electroporated in Amaxa buffer lacking siRNA (B+) or left untreated (B-) (section 2.2.2.1). After 3 days with M-CSF cells were either lysed or stimulated with R848 (1µg/ml) or LPS (10ng/ml) for 18h. The protein level was assessed by SDS-PAGE and western blotting (A) whereas the cytokine concentration was assessed by ELISA (B-D). Control values (black bars) represent cells transfected with non-targeting siRNA at relevant concentrations. Btk knockdown values were normalised to the control. Unstimulated cells are not shown as values were undetectable. Cytokine levels are expressed as means of triplicate repeats $\pm$ SEM. Results shown are representative of at least 3 donors. Statistical analysis: repeated measures ANOVA, * $P < 0.05$, *** $P < 0.001$ as compared to stimulated siCon.

4.2.6 Downregulation of Btk results in decreased TNF production at an early stage

The results strongly support the role of Btk in TLR8 signalling as both Btk overexpression and downregulation altered TNF and IL-6 production upon stimulation. To further establish the magnitude and the earliest time point of TNF and IL-6 production affected by Btk, a detailed time course of R848-induced cytokine production under Btk knockdown was carried out. As previously, freshly elutriated human monocytes were transfected with individual siRNA duplexes, subsequently differentiated into macrophages in the presence of M-CSF for 4 days and stimulated with TLR 4 and 8 ligands for 18hrs. Supernatants were collected every 2hrs and analysed for TNF and IL-6 production using ELISA. The results summarized in **Figure 4.7** demonstrate that TNF production was considerably decreased in the presence of Btk knockdown for both LPS- and R848-stimulated human macrophages. The decrease in TNF release was significant for as early as 4hrs for LPS and 2hrs for R848 which implies that Btk involvement in TLR8 signalling is more profound. Following Btk knockdown, the largest reduction in TNF levels in the presence of Btk knockdown was 60% at 8h under R848 stimulation as compared to 50% at 10h under LPS challenge (**Figure 4.7A**). The TLR4 pathway is the most complex one as the receptor utilises four adaptor molecules as opposed to only one, Myd88, used by TLR8. It is likely that other Tec tyrosine kinases are involved in the TLR4 pathway and that the absence of Btk is substituted by another kinase compensating for the deficit in cytokine production. IL-6 levels were also unaffected by Btk ablation with LPS, as opposed to R848 stimulation (**Figure 4.7B**). The effect of Btk on IL-6 was, however, greatly delayed as it was visible at 8h and reached the significance only at 18h post R848 stimulation. These results indicate that Btk selectively regulates cytokine expression in human primary macrophages.

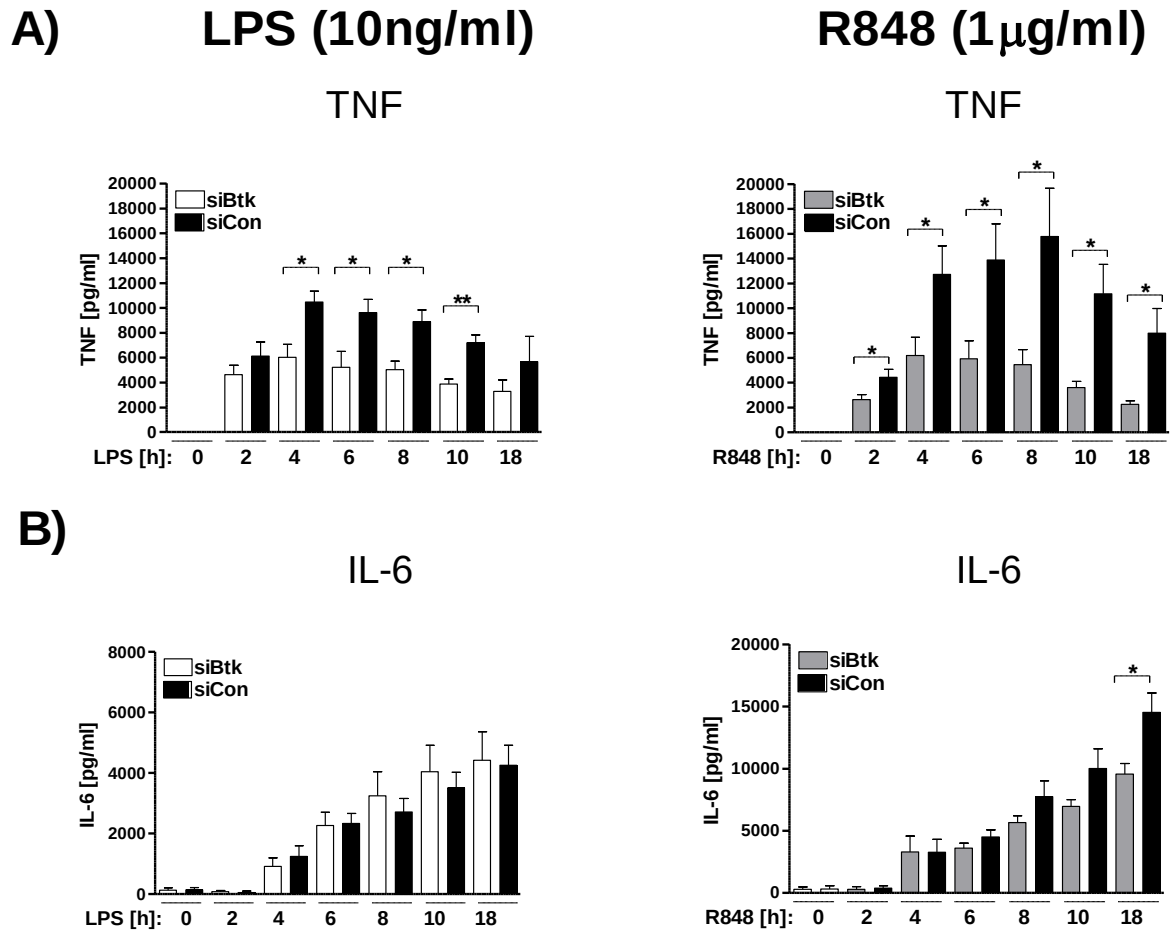


Figure 4.7 Btk knockdown affects early stage TNF production in human macrophages. Monocytes were transfected with 200nM Btk or Control siRNA using the Amaxa Human Monocyte Nucleofector® Kit (section 2.2.2.1). After 4 days with M-CSF cells were stimulated with LPS (10ng/ml) or R848 (1μg/ml) over 18hrs. The protein level was assessed by SDS-PAGE and western blotting to ensure the efficiency of the knockdown (data not shown) whereas TNF (A) and IL-6 (B) protein production was assessed by ELISA. Cytokine levels are expressed as means of triplicate repeats +/- SEM. Results shown are representative of at least 3 donors. Statistical analysis: student t-test, *P<0.05, **P<0.01, as compared to stimulated siCon.

4.2.7 Btk protein ablation affects IL-6 production upon TLR8 stimulation

It is evident from the previous results that Btk alters IL-6 production under TLR8 but not TLR4 engagement. TNF has been previously shown to regulate cytokine expression as the administration of TNF into cultured synoviocytes induces the production of TNF, IL-6 and IL-1 (Mander et al. 1997; Feldmann 2002). Therefore, it is possible that the observed decrease in IL-6 from siBtk-transfected macrophages could be a consequence of strongly reduced TNF production. To rule out this possibility, the level of TNF between siBtk- and siCon- treated macrophages was evened by adding excess TNF to the supernatants of the cultured cells. The effect of Btk downregulation on R848-induced IL-6 was then examined 8h post-stimulation by ELISA. This particular time point was chosen as the earliest reduction in IL-6 production in siBtk-transfected cells was previously observed at 8h (**Figure 4.7B**). **Figure 4.8B** confirms that the addition of TNF equilibrated the cytokine levels between the cells transfected with either siBtk or siCon. The presence of excess TNF also increased the production of IL-6 proving the bioactivity of the added cytokine (**Figure 4.8A**). Btk protein ablation decreased the production of IL-6 at 48% as compared to 31% in the TNF-treated cells following R848 stimulation. This result indicates that Btk is likely to be directly involved in IL-6 production upon TLR8 stimulation. At the same time, the effect of TNF on IL-6 production cannot be excluded as the decrease in IL-6 production upon Btk knockdown was smaller when the TNF level was evened (**Figure 4.8B**). This data supports the role of Btk in the regulation of R848-induced IL-6 secretion that again emphasises the differences of the kinase involvement in the TLR4 and 8 pathways. The Btk effect on R848-induced IL-6 production was, however, much delayed as compared to TNF suggesting that two separate mechanisms of Btk-dependent gene control may be involved. Due to the time restrictions and the availability of tools required for examining TNF regulation, further work in this chapter focused on decoding the Btk-dependent pathway involved in the regulation of TNF expression.

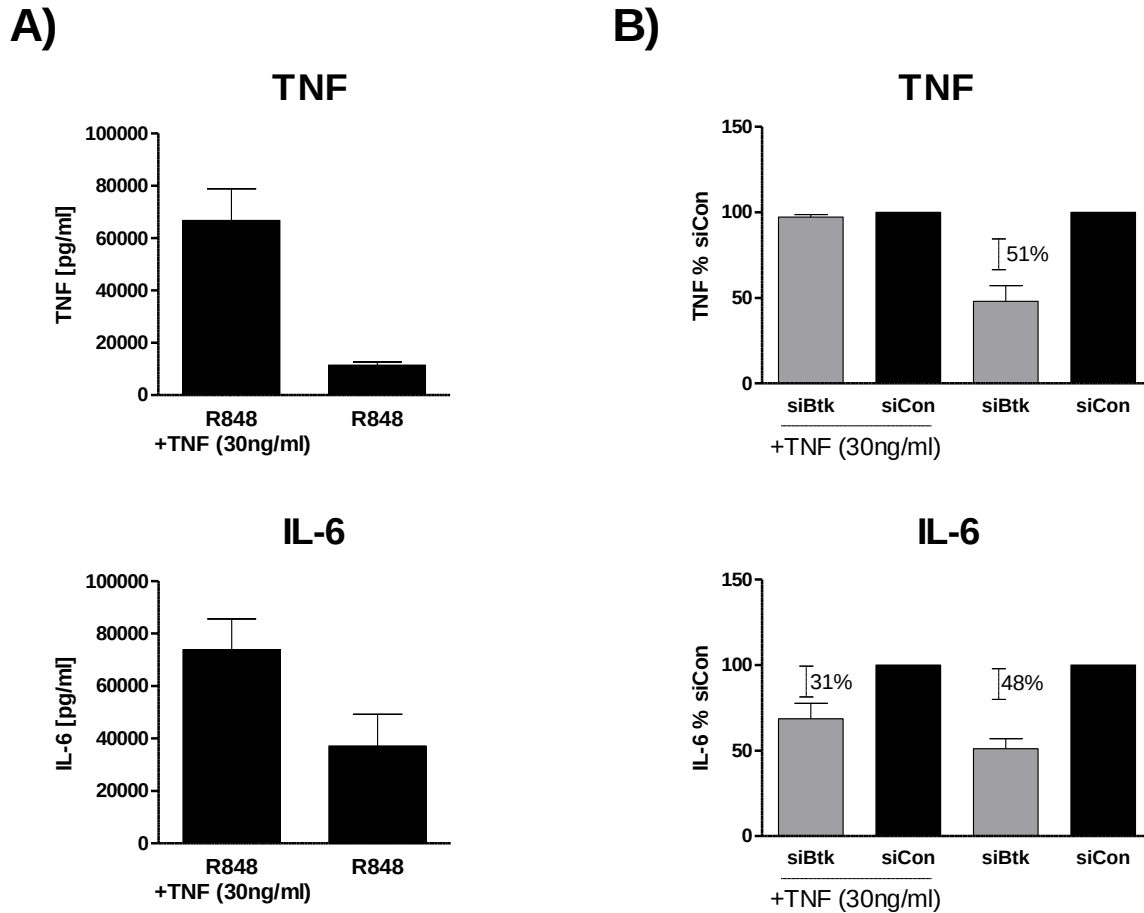


Figure 4.8 Btk knockdown directly affects IL6 production upon R848 stimulation in human macrophages. Monocytes were transfected with 200nM Btk or Control siRNA using the Amaxa Human Monocyte Nucleofector® Kit (section 2.2.2.1). After 4 days with M-CSF cells were challenged with 30ng/ml of recombinant TNF and 1µg/ml of R848 for 8 hrs. The protein level was assessed by SDS-PAGE and western blotting to ensure the efficiency of the knockdown (data not shown) whereas TNF and IL6 protein production was assessed by ELISA. Btk knockdown values were normalised to the control. Unstimulated cells are not shown as values were undetectable. Cytokine levels are expressed as means of triplicate repeats +/- SEM. Results shown are representative of at least 3 donors.

4.2.8 Btk control of TNF expression requires the 3' distant region of *TNF* to induce the Luciferase Activity

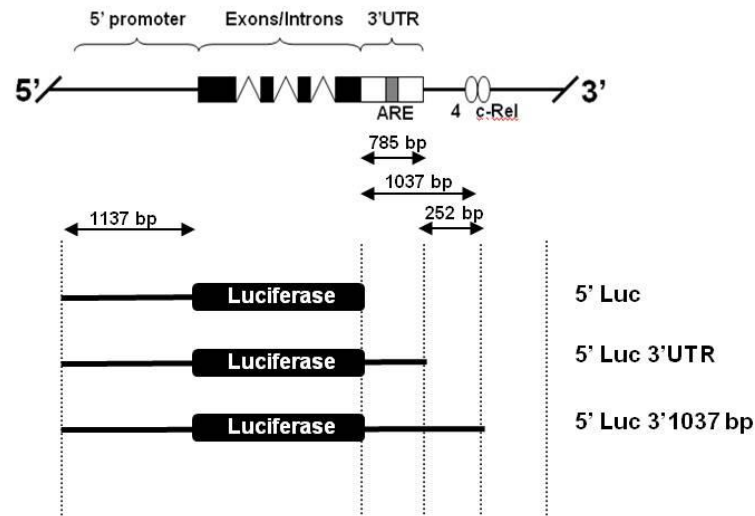
To further characterise the mechanism by which Btk determines the level of TNF, the mapping of Btk-dependent regions of *TNF* was performed using a luciferase assay. The assay involves the use of adenoviruses carrying DNA constructs consisting of the luciferase gene flanked by 5' *TNF* promoter and different lengths of the 3' *TNF* untranslated and enhancer regions. The 3' untranslated region (UTR) has been previously implicated in the destabilisation of TNF mRNA as the deletion of the adenine and uridine-rich elements (ARE) within the region increases TNF message stability in macrophages derived from *TNF*^{ΔARE} mice (Kontoyiannis et al. 1999). The 252bp enhancer region localised downstream of the 3'UTR has been implicated in the transcriptional control of TNF as p65 and IRF5 transcription factors are recruited to this region following LPS activation (Krausgruber et al. 2010; Smallie et al. 2010). The adenoviruses used in this project were 5'Luc, 5'Luc 3'UTR and 5'Luc 3'1037bp (**Figure 4.9A**). The expression of 5'Luc was the strongest as this construct lacks the 3'UTR element responsible for the negative regulation of the TNF message stability (**Figure 4.9B**). In contrast, the introduction of the 3'UTR resulted in the weakest expression of 5'Luc 3'UTR. The addition of the 252bp enhancer region to 3'UTR increased the expression of 5'Luc 3'1037bp as compared to 5'Luc 3'UTR. This was expected as 3' enhancer regions have been long known to be essential positive regulators of gene transcription (Meyer et al. 1990). As the relative levels of luciferase activities reflected the predicted TNF expression pattern, the constructs were used for further investigation of the Btk-dependent regulatory mechanism.

It has been shown before that Btk targets 3'UTR region of *TNF* following LPS stimulation, as the double infection of macrophages with AdBtk and the 5'Luc construct deprived of the 3'UTR did not result in increased luciferase activity, while infection with an adenovirus carrying the 3'UTR-containing luciferase construct did (Horwood et al. 2003). Closer analysis of the 3'region in these constructs has revealed that, in addition to the 3'UTR (785bp), the construct used by Horwood et al. (2003) also contained 252bp long enhancer region (now named 5'Luc 3'1037bp adenovirus) implicated in transcriptional regulation of *TNF*. The importance of the 252bp long enhancer region was not established at that point due to the lack of the 5'Luc 3'UTR constructs. The 5'Luc 3'UTR and 5'Luc 3'1037bp adenoviruses have been constructed in house to facilitate the investigation of specific TNF 3'elements targeted by Btk in TLR4 and 8 signalling. Human differentiated macrophages were sequentially infected with AdBtk and luciferase adenoviruses. Cells were

thereafter stimulated with either LPS or R848 for 6h and the luciferase activity was measured. As displayed in **Figure 4.10**, AdBtk did not super-induce 5'Luc indicating that the lacking 3' region is required for Btk-dependent regulation in TLR4 and 8 signalling. The addition of 3'UTR also did not result in AdBtk-regulated increase in luciferase activity. However, AdBtk elevated the expression of 5'Luc 3'1037bp as compared to AdCon signifying that the kinase is likely to target the 252bp enhancer region of the *TNF* gene.

Previous evidence from our laboratory indicated that Btk relies on 3'UTR region of *TNF* to stabilise the cytokine mRNA levels in TLR4 signalling. This was further supported by the Actinomycin D chase experiment where the rate of *TNF* mRNA decay was rescued by AdBtk (Horwood et al. 2003). Detailed inspection of the *TNF* 3' region has revealed, however, that the 3'UTR itself is not sufficient for the Btk-dependent super-induction of luciferase and that the additional enhancer region containing transcriptional elements may be required. As the 3'UTR was shown to downregulate *TNF* expression (**Figure 4.9B**), it is not surprising that Btk, a positive regulator of *TNF* production, was not dependent on this region.

A)



B) Induction of Luciferase by R848 or LPS

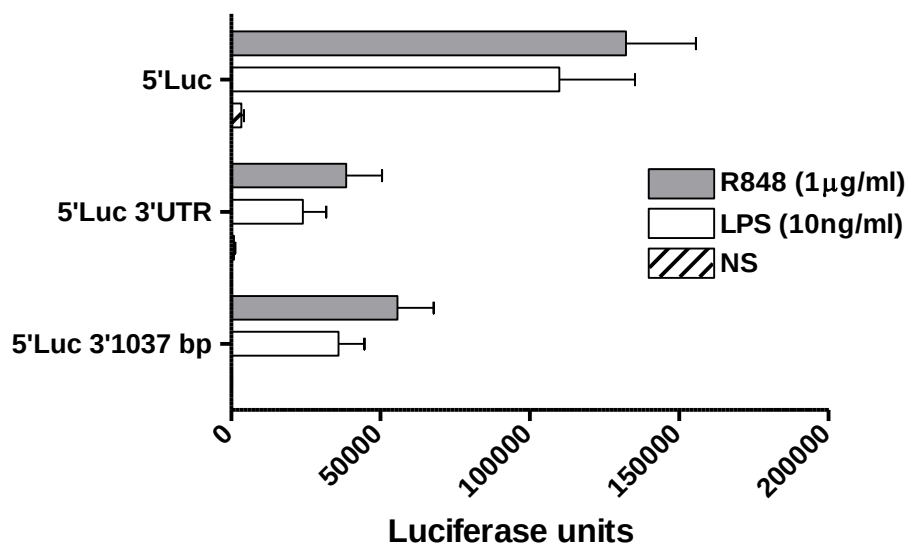


Figure 4.9 The induction of Luciferase activity by LPS or R848 in human macrophages. Differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were plated at 0.07×10^6 cells per well and incubated overnight. The cells were then infected with adenovirus carrying different luciferase constructs (A) at moi 50 and allowed to express for 24h (section 2.2.3.9). Cells were then stimulated with LPS (10ng/ml) or R848 (1μg/ml) and lysed 6h post stimulation. Luciferase activity was measured as described in section 2.2.4.6. Values are expressed as means of triplicate repeats $\pm$ SEM. Results shown are representative of at least 3 donors (B).

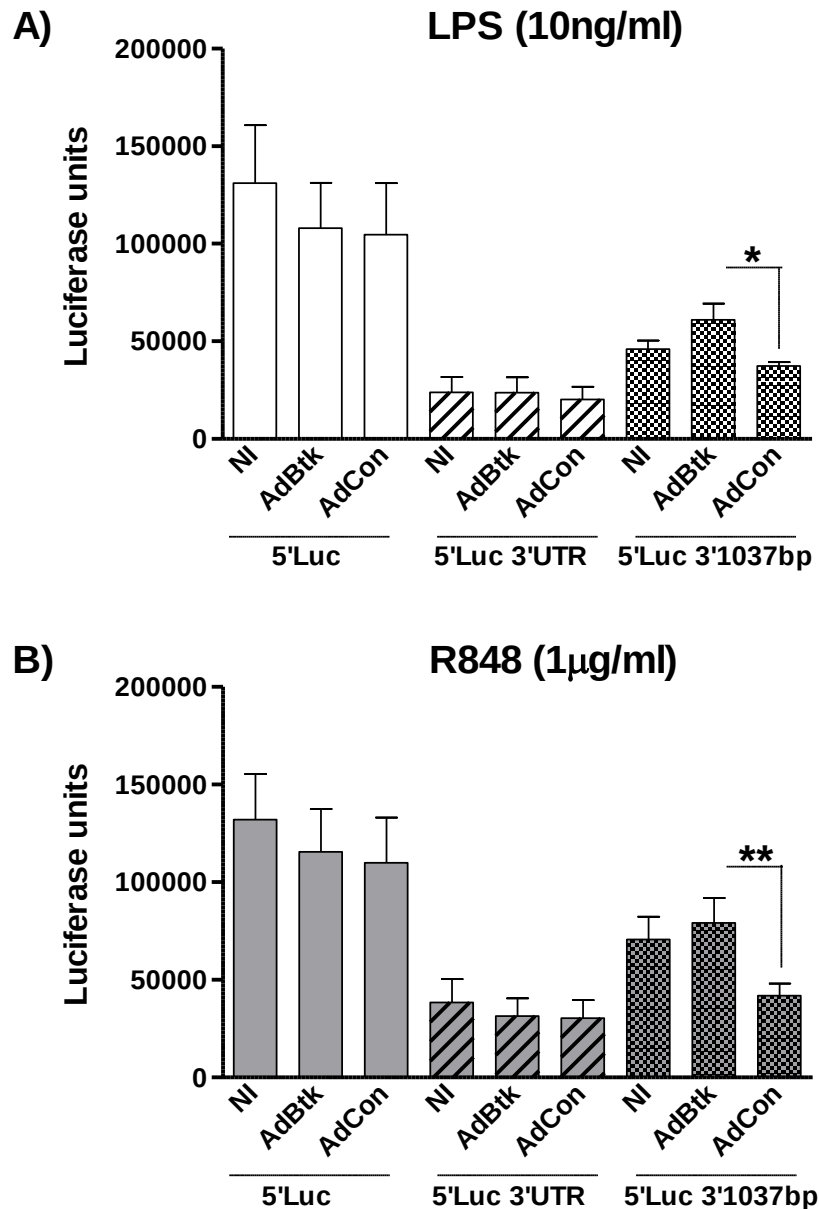


Figure 4.10 Btk requires 3' distant region of *TNF* to induce Luciferase activity in response to LPS or R848 in human macrophages. Differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were plated at 0.07×10^6 cells per well and incubated overnight. The cells were then infected with AdBtk or AdCon at moi 100 and allowed to express for 4hrs. Following primary infections cells were infected with 5'Luc, 5'Luc 3'UTR or 5'Luc 3'1037bp adenoviruses at moi 50 and allowed to express for further 24h (section 2.2.3.9). Cells were then stimulated with LPS (10ng/ml) or R848 (1μg/ml) and lysed 6h post stimulation. Luciferase activity was measured as described in section 2.2.4.6. Values are expressed as means of triplicate repeats $\pm$ SEM. Results shown are representative of at least 3 donors. Statistical analysis: repeated-measures ANOVA, * $P < 0.05$, ** $P < 0.01$ as compared to stimulated AdCon.

4.2.9 Btk regulates the transcription of *TNF* following TLR8 but not TLR4 activation

The early effect of siBtk on *TNF* protein production suggests that the kinase may regulate *TNF* expression on a level of transcription, message stability, translation or the release of soluble *TNF* from the membrane. To investigate the first hypothesis, also supported by Results 4.2.8, the levels of *TNF* primary and mature transcripts were compared by using Primary Transcript (PT) - PCR. The technique was initiated by (Lipson and Baserga 1989) and enables the detection of pre-mature mRNA by using the primer set covering intron-exon boundary as opposed to the detection of mature *TNF* transcript with primers spanning exon-exon sequence of approximately 200bp (**Figure 4.11**). Freshly elutriated human monocytes were transfected with individual siRNA duplexes, subsequently differentiated into macrophages and stimulated with either TLR4 or TLR8 ligands. Supernatants were collected every 2h up to 10h and analysed for *TNF* mRNA by using PT-PCR approach. As illustrated in **Figure 4.12**, both LPS and R848 highly induced the levels of *TNF* primary and mature transcripts within 2h. The LPS-induced *TNF* mRNA levels dropped sharply after this period as compared to R848 stimulation where the induction of the primary transcript was sustained for 4h. The introduction of siBtk decreased the synthesis of the primary *TNF* transcript by 25% and the mature *TNF* transcript by 10% at 2h following LPS stimulation (**Figure 4.12A**). The effect of Btk ablation on *TNF* mRNA was much more striking in R848-challenged macrophages as the difference amounted to 80% at 2h for both the primary and mature *TNF* transcripts (**Figure 4.12B**). The strong Btk effect on both primary and mature mRNA indicates that the kinase is involved in the regulation of *TNF* transcription upon R848 stimulation whilst Btk is less likely to participate in LPS-induced transcription. As the TLR4 cascade is considered to be much more complex, it is possible that the ablation of the kinase may be substituted by others resulting in a weaker effect on *TNF* transcription.

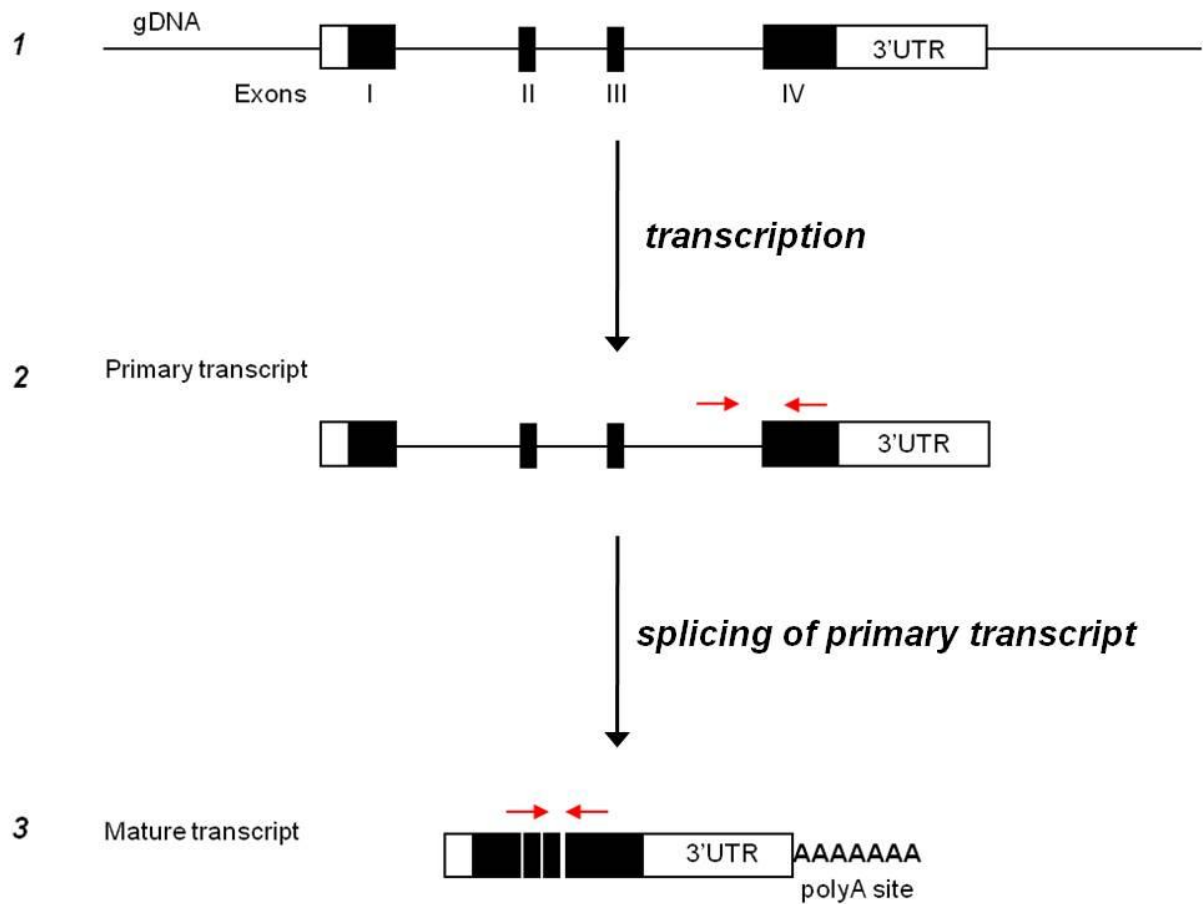


Figure 4.11 Schematic representing the synthesis of mature *TNF* transcript. Primary transcript is generated from genomic DNA during transcription. In this process the gene DNA sequence, composed of UTR regions (white boxes), coding exons (black boxes) and introns (lines between exons), is converted into mRNA sequence. The maturation of the primary transcript involves the splicing of the exons with simultaneous removal of introns and the attachment of PolyA tale. Red arrows indicate the primer binding sites for the distinction of the primary and mature transcripts in PT-PCR.

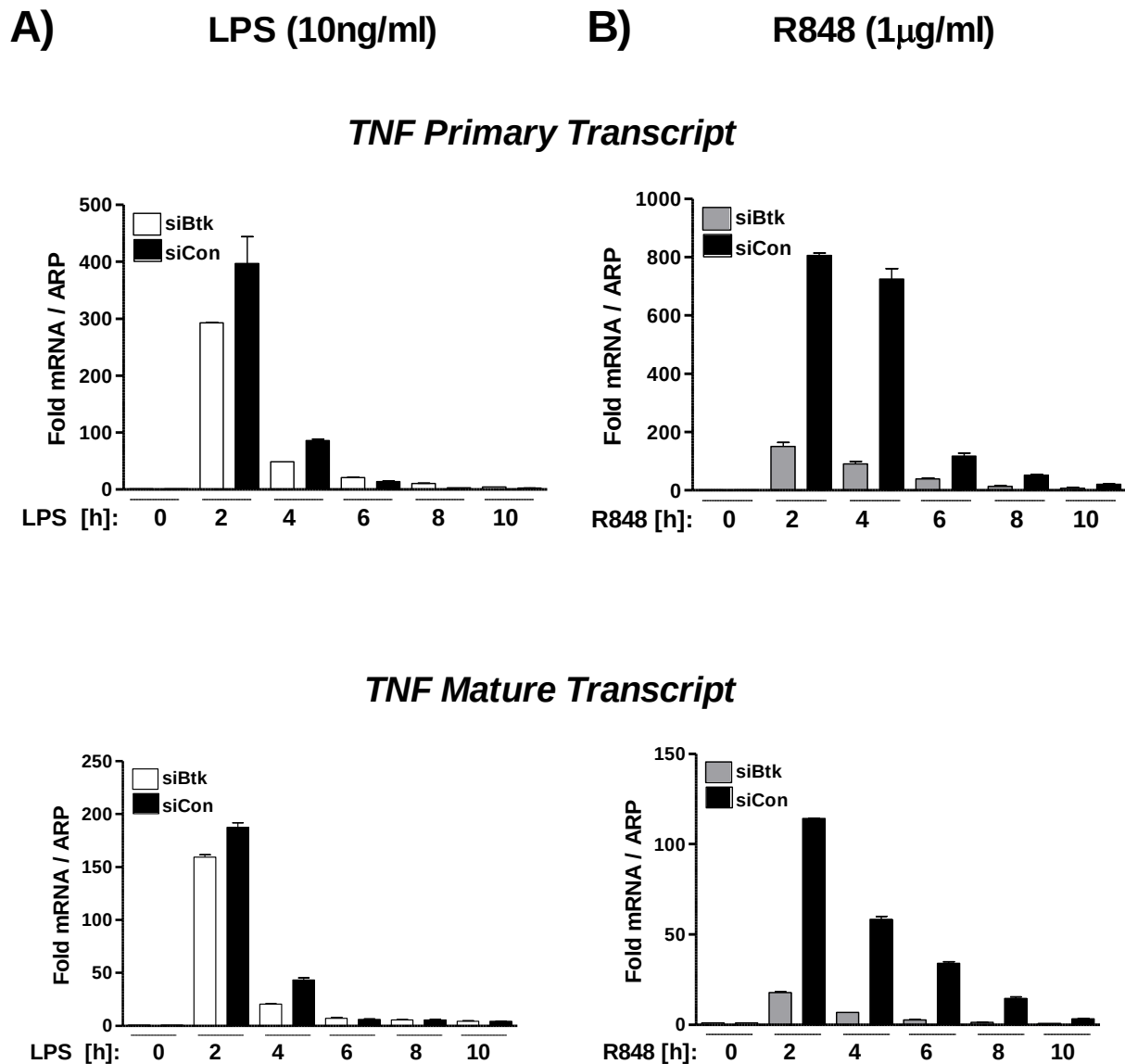


Figure 4.12 Btk knockdown affects the levels of TNF mRNA in human macrophages stimulated with R848. Monocytes were transfected with 200nM Btk or Control siRNA using the Amaxa Human Monocyte Nucleofector® Kit (section 2.2.2.1). After 4 days with M-CSF cells were stimulated with LPS (10ng/ml) or R848 (1 μ g/ml) over 10hrs. The protein level was assessed by SDS-PAGE and western blotting to ensure the efficiency of the knockdown (data not shown) whereas TNF primary (A) and mature (B) transcript levels were measured by RT-PCR using One-Step SYBR kit (section 2.2.2.5). Data are expressed as mean $\pm$ SEM of triplicate measurements for a single donor representative of 3 independent experiments using different donors.

4.2.10 Downregulation of Btk decreases the initiation of transcription as assessed by RNA Polymerase II recruitment to the upstream region of *TNF*

To further confirm the role of Btk in *TNF* transcription upon R848 stimulation, the analysis of RNA Polymerase II (Pol II) recruitment to *TNF* was performed by using chromatin immunoprecipitation (ChIP) methodology. By designing primers targeting the transcription start site (TSS) and the downstream region of *TNF*, this technique allows the identification of the effect of Btk on the transcriptional initiation and elongation (**Figure 4.13B**). The application of ChIP for human primary macrophages was optimised by Dr Tim Smallie from our laboratory and among others; it required the optimisation of sonication conditions, cell numbers and PCR primers targeting specific *TNF* sites. Sonication of the chromatin is one of the initial steps and involves the shearing of the genome in the fragments that are on average 200-1000 bp in length (**Figure 4.13A**). This guarantees that the accurate resolution can be obtained and that using primers spanning a particular DNA fragment will result in a PCR signal specific for the target sequence. The melt curve analysis was performed in every PCR run to assess the primer set specificity and the presence of non specific products such as primer dimers (**Figure 4.13E-F**). Peaks in the melt curve analysis represent the highest fluorescence signal generated by the dye binding to double stranded (ds) DNA in the PCR reaction. The presence of only one peak at around 85°C indicates specific products of *TNF* TSS (1+) or *TNF* Downstream primer sets. The analysis of PCR results also requires the normalisation of the threshold cycle (C_t) values obtained from ChIP to the C_t values obtained from the input. The input was derived from DNA remained from immunoprecipitated Pol II or IgG and therefore represents the initial size of an individual sample. Both the ChIP and input samples were subjected to the same PCR reaction (**Figure 4.13C-D**). The C_t values for the inputs were on average 8 times lower than for the ChIP samples indicating, as expected, that there were fewer *TNF* fragments in the immunoprecipitated samples than in the total nuclear extracts.

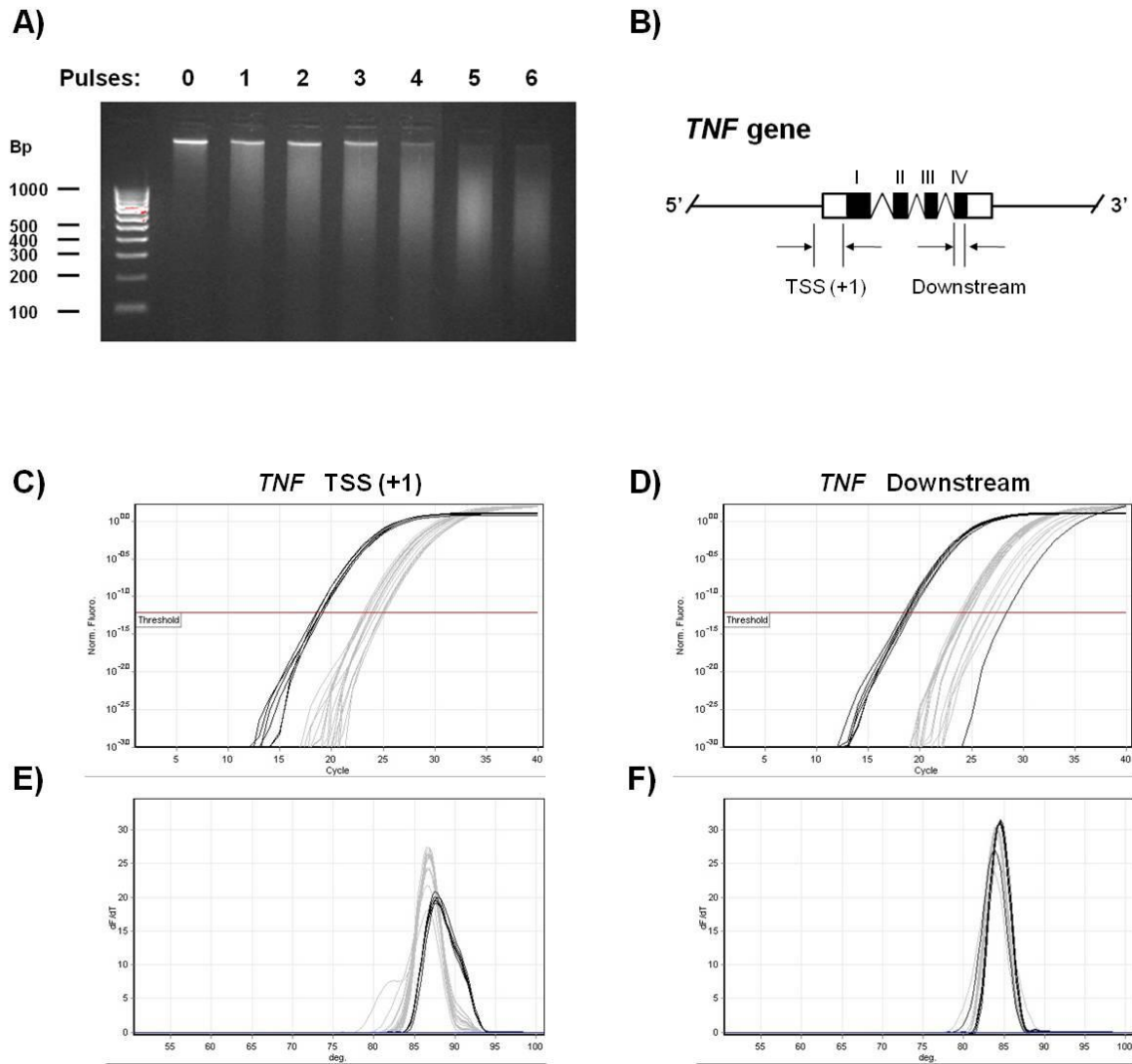


Figure 4.13 Optimisation steps required in ChIP. Fixed cells are lysed and subjected to sonication which shears the genomic DNA. To ensure that the DNA fragments are on average 200-1000bp in length, the samples are sonicated 6 times (pulses of 12 sec) as optimised by Dr Tim Smallie (A). Pol II is then immunoprecipitated from the lysates and the purified DNA is analysed by PCR with the primers covering TSS(+1) or Downstream regions (B). Only when the melt curves result in sharp peaks (E, F) and the cycle values of inputs (black lines) are lower than the cycle values of ChIP (silver lanes) on the profile runs (C,D), the results are considered as valid and further analysed. The threshold line indicates the cycle values used for the calculations (C-F) (section 2.2.9.5).

To determine whether Btk downregulation affects the initiation or the elongation of *TNF* transcription, the recruitment of Pol II to the upstream and downstream region of *TNF* was examined. Primary monocytes were subjected to transfection with individual siRNA duplexes and differentiated into macrophages in the presence of M-CSF for 4 days. Cells were stimulated with R848 for 1 and 3h and subjected to ChIP with the Pol II or the IgG control antibody. Btk was previously shown to affect *TNF* primary transcript synthesis at 2h and that the level of the mature transcript is the highest at 2h post R848 stimulation (**Figure 4.12B**). The 1h time point for the ChIP was thus chosen to detect Pol II recruitment prior the transcript elongation and termination. Interestingly, a high background level of Pol II bound to *TNF* TSS(+1) but not *TNF* Downstream was detected in resting macrophages suggesting that Pol II is constitutively bound to the upstream *TNF* region but does not elongate the transcript without stimulation (Adelman et al. 2009) (**Figure 4.14**). Activation with the TLR8 ligand increased the recruitment of Pol II to the TSS(+1) and downstream sites of *TNF*. The highest recruitment was observed at 1h and then slightly dropped at 3h. The signal for IgG antibody was negligible illustrating the specificity of the Pol II antibody. In the presence of Btk siRNA, there was substantially less recruitment of Pol II to both upstream and downstream element of *TNF* indicating that Btk affects the initiation of *TNF* transcription.

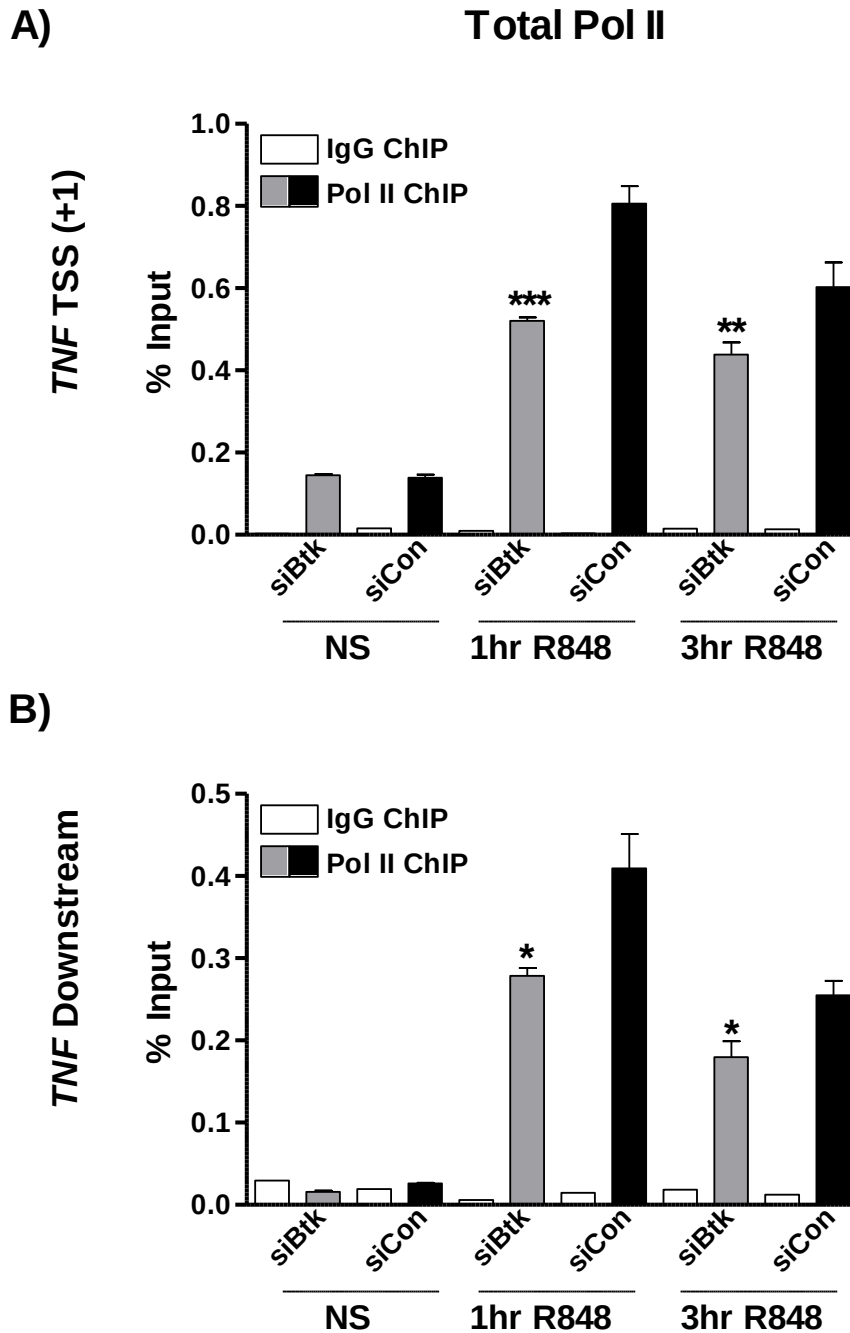


Figure 4.14 Btk knockdown inhibits the recruitment of Pol II to upstream and downstream regions of *TNF* in human macrophages stimulated with R848. Monocytes were transfected with 200nM Btk or Control siRNA using the Amaxa Human Monocyte Nucleofector® Kit (section 2.2.2.1). After 4 days with M-CSF cells were treated with R848 (1µg/ml) for 3h, fixed and lysed as described in section 2.2.9. ChIP was performed using the Pol II or IgG antibodies and primers specific for upstream (TSS+1) (A) or downstream (B) *TNF* region (section 2.2.9). Data are expressed as mean $\pm$ SEM of triplicate measurements for a single donor representative of 3 independent experiments using different donors. Statistical analysis: Student's t-test was performed on dC_t values on one donor, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to siCon.

4.3 Discussion

Since the discovery that LPS stimulates protein tyrosine phosphorylation in macrophages (Weinstein et al. 1991; Beaty et al. 1994) and that the inhibition of tyrosine phosphorylation by Herbimycin A and Genistein blocks TNF production (Mander et al. 1997), protein tyrosine kinases have been increasingly investigated in the context of TLR signalling. The endosomal TLRs which are able to detect exogenous and endogenous nucleic acids have attracted significant interest in recent years and increasing experimental data has provided evidence for their importance in cytokine expression in rheumatoid arthritis synovium (Roelofs et al. 2005; Roelofs et al. 2008; Sacre et al. 2008; Roelofs et al. 2009). Studies presented in this and the previous chapter show that Btk signalling differentially controls cytokine production in human primary macrophages in response to various TLR stimuli. In particular, Btk affects TNF and IL-6 production in R848-stimulated cells as opposed to only TNF in LPS-stimulated cells. Also, the dependence of the TLR8 pathway on Btk is more profound when compared to TLR4. At the same time, the kinase does not affect the production of IP-10 following PolyI:C stimulation indicating that Btk signalling is not implicated in the TLR3 pathway; the only TLR not to use Myd88 but to use TRIF instead. Lastly, the kinase is clearly involved in the R848-induced transcription of *TNF* as Btk protein ablation decreases the synthesis of the primary TNF transcript and Pol II recruitment to the gene.

Earlier reports concerning the role of Btk in TLR signalling have been mainly focused on the investigation of the TLR2 and 4 pathways. The current results replicate the previously published data which showed rapid phosphorylation of Btk in response to LPS stimulation in primary human and mouse macrophages (**Figure 4.3/4.4**) (Horwood et al. 2003; Schmidt et al. 2006). Extensive analysis revealed that the kinase is also phosphorylated and auto-phosphorylated in response to the TLR8 stimulus. The participation of Btk in TLR4 and 8 cascades could be explained by the fact that they both share common stages of signal transduction mechanisms, involving the adaptor protein Myd88. Btk has been previously shown to be associated with Myd88 as demonstrated in co-immunoprecipitation experiment from HEK293 cells or with Myd88-dependent TLRs 4, 6, 8 and 9 as shown in yeast two-hybrid screening (Jefferies et al. 2003). Bmx, another member of Tec family kinases, was also found to form a complex with Myd88 in LPS-challenged synovial fibroblasts isolated from rheumatoid arthritis joint tissue (Jefferies et al. 2003; Semaan et al. 2008). Myd88 is used by numerous TLRs including TLRs 2 and 4 which proved to be

dysfunctional in XLA-derived PBMC (Horwood et al. 2006) supporting the dependence of Myd88 on Btk. Furthermore, Btk has been shown to phosphorylate the adaptor molecule Mal which forms a scaffold for Myd88 in TLRs 2 and 4 signalling cascades (Gray et al. 2006). The association of Mal with Btk supports the role of the kinase in early TLR signalling events and places it in the complex with adaptor molecules (**Figure 4.15**).

The results obtained by using adenovirus overexpression and RNAi techniques demonstrated that Btk regulates cytokine production in R848-challenged macrophages (**Figure 3.2, 4.5-4.8**). The impact of the kinase on TNF release was much stronger in the TLR8 pathway than in the TLR4 one. Again, this could arise from the complexity of the TLR4 cascade which involves four different adaptor molecules, compared to only one found in TLR8 (**Figure 4.15**). It is possible, that different members of Tec family kinases are involved in one TLR cascade and substitute for the absence of another kinase rescuing the functionality of a TLR pathway. This hypothesis was supported by the observation that the expression of Tec increased in M-CSF-treated monocytes derived from XLA patients as compared to healthy donors (Horwood et al. 2003). Also in other systems, functional redundancy among Tec family members has been proposed. For instance, Btk has been shown to have an important signalling function in platelet activation. However, Btk depletion does not affect platelet-dependent bleeding tendency probably because the phosphorylation of Tec is increased as shown in collagen-stimulated platelets derived from XLA patients (Oda et al. 2000). By utilising a more complex adaptor molecule network, TLR4 could employ a wider range of Tec family kinases. For instance, Bmx has been shown to regulate both TNF and IL-6 production from human primary macrophages stimulated with either LPS or Pam3Cys indicating that one TLR pathway can involve numerous Tec family kinases which differ in function (Palmer et al. 2008).

Similarly to the LPS pathway, Btk did not influence R848-induced IL-10 production in human macrophages (**Figure 3.2, 4.5**). In contradiction to these results, Btk appears to be implicated in the production of LPS-induced IL-10 in murine macrophages emphasising the difference in TLR signalling between species (Schmidt et al. 2006). Furthermore, in contrast to the TLR4 pathway, Btk displayed a consistent effect on IL-6 production upon TLR8 stimulation suggesting that the same kinase may exhibit a different range of cytokine control in various TLR cascades. The effect of Btk on R848-induced IL-6 production was less profound than the effect on TNF. Although, the pathways distinguishably regulating TNF and IL-6 production still remain to be elucidated, it is clear that there are major differences in how these two cytokines are regulated. For instance, the

chemical inhibition of p38 MAPK resulted in decreased production of TNF but super-induction of IL-6 production from LPS-stimulated human primary macrophages (Page et al. 2010). The effect of Btk on IL-6 secretion upon R848 stimulation in human primary macrophages can be supported by studies performed on DC derived from XLA patients and confirmed by using the non-specific inhibitor of Btk, LFM-A13 (Sochorova et al. 2007).

The kinetics of TLR-induced IL-6 production was largely delayed as compared to TNF production (**Figure 4.7**). This was consistent with the classification of the *TNF* and *IL6* genes as respective primary and secondary response genes (Hargreaves et al. 2009). Primary genes do not require new protein synthesis what results in their rapid induction, whereas secondary genes depend on new protein synthesis and chromatin remodelling at their promoters what leads to their delayed kinetics (Ramirez-Carrozzi et al. 2006; Natoli 2009). Downregulation of Btk decreased early TNF production as opposed to IL-6. Although IL-6 release was detectable at 4h, Btk appeared to regulate R848-induced IL-6 production at the late 8h time point. This finding suggests that two separate mechanisms of Btk-dependent gene control may be implicated. Btk seems to be directly involved in the early stages of TNF expression as opposed to IL-6 regulation. Further work should focus on expanding on the role of Btk in the IL-6 gene expression and on establishing the involvement of transcriptional, translational and cellular release control.

In the current system Btk did not regulate IP-10 production in LPS- or PolyI:C-activated human primary macrophages (Chapter 3, **Figure 3.2**), implying that the kinase is not involved in the interferon pathway dependent on the adaptor molecule TRIF. This result disagrees with data derived from XLA DC stimulated with a TLR3 ligand where the production of TNF, but not IL-6, was significantly decreased comparing to healthy donors (Taneichi et al. 2008). Human primary macrophages do not, however, express TNF or IL-6 in response to PolyI:C. The engagement of TLR3 also does not lead to I κ B α degradation, NF κ B luciferase induction or p38 MAPK phosphorylation in macrophages (Lundberg et al. 2007). As both p38 MAPK and NF κ B pathways have been implicated in Btk-regulated cytokine expression it was not unexpected that the lack of MAPK or NF κ B activation correlated with the lack of Btk involvement in the TLR3 pathway.

Previous studies suggest that Btk-mediated TNF expression is controlled transcriptionally in an NF κ B-dependent manner. Most of the studies describing NF κ B activation were, however, done in B cells (Bajpai et al. 2000; Petro et al. 2000), on cell lines of non-myeloid origin which do not endogenously express Btk or TLR4, in mouse cells or by using LFM-A13 (Jefferies et al. 2003)

which displays high toxicity in human primary macrophages (McDaid, unpublished). The findings of this work showing the early involvement of Btk in regulating TNF production as well as the dependence of Btk on the *TNF* enhancer region in the luciferase assay suggested that Btk may be implicated in the transcription of *TNF* in R848 signalling (**Figure 4.10**). Indeed, downregulation of Btk extensively decreased the synthesis of primary *TNF* transcript under TLR8 engagement as opposed to TLR4 signalling where this effect was weaker (**Figure 4.12**). The weak effect of Btk on LPS-induced *TNF* transcription can be explained by previous studies performed in human primary macrophages where it has been suggested that Btk does not regulate the transcription, rather TNF message stability through p38 MAPK (Horwood et al. 2006).

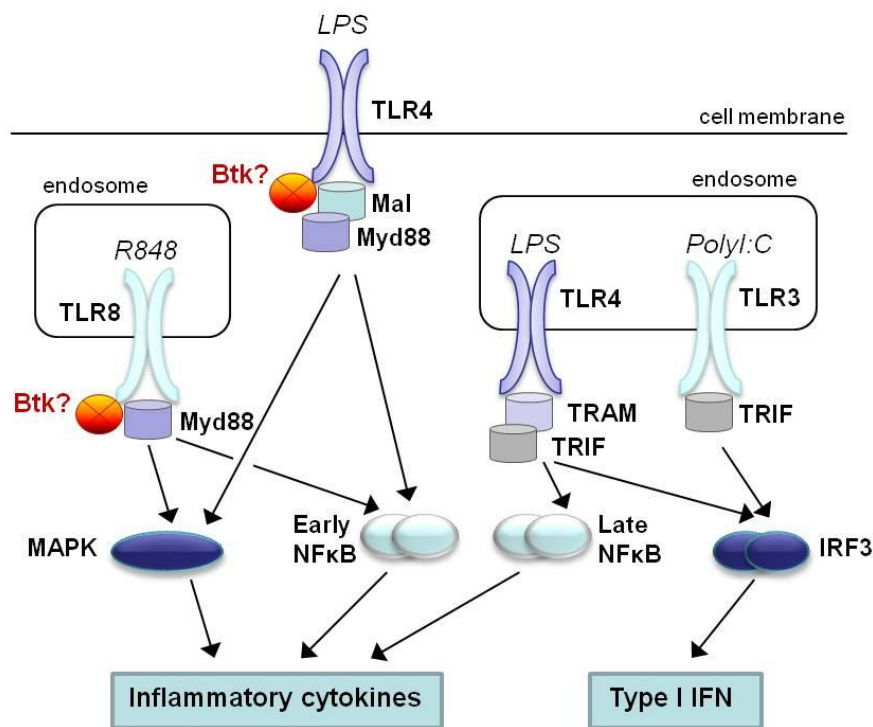


Figure 4.15 Schematic representing major differences in the signalling components of TLRs 3, 4 and 8. The initial activation of TLR4 involves the recruitment of Mal and Myd88, followed by the activation of early NFκB and MAPK. TLR4 can translocate to endosomes when it engages TRAM and TRIF to activate late NFκB and IRF3. IRF3 is also activated by PolyI:C which activates TLR3 and an adaptor molecule TRIF. TLR8 stimulation by R848 recruits Myd88 and activates MAPK and NFκB. The consequence of IRF3 activation is the production of Type I IFN, whereas the activation of MAPK and NFκB leads to the production of Inflammatory cytokines such as TNF and IL-6 (adapted from (Kawai and Akira). Btk interacts with TLRs 4 and 8 as well as with Myd88 and Mal (Jefferies et al. 2003).

The process of gene transcription includes the initiation, elongation and termination. The initiation involves the binding of multiple transcription factors to DNA which then recruits RNA Polymerase II (Pol II) and chromatin modifiers (Kadonaga 2004). In basal conditions Pol II phosphorylated on Serine 5 is bound to promoters of primary response genes and is able to produce unspliced TNF transcripts which are rapidly degraded (Hargreaves et al. 2009). This is consistent with the results obtained in the chapter as Pol II bound to the *TNF* TSS(+1) was detected in unstimulated cells (**Figure 4.14**). The stimulation with R848 increased Pol II initiation of *TNF* transcription but was strongly affected by Btk downregulation. The utilisation of the ChIP technique confirmed the role of Btk in the *TNF* transcription giving insight into how this regulation occurs. Numerous transcription binding sites are localised in the *TNF* gene including NF κ B and AP-1-like site. Whether they are implicated the Btk-dependent initiation of R848-mediated *TNF* transcription has been not described yet.

In summary, the studies presented in this chapter have revealed a critical role for Btk in the TLR-dependent regulation of cytokine and chemokine production in human primary macrophages. In particular, it has been demonstrated that Btk affects R848-triggered TNF and IL-6 production to a much higher extent than the production of TNF in LPS-stimulated cells revealing the differential role of Btk on cytokine production in various TLR pathways as well as different level of Btk dependence between TLR4 and 8. Subsequent experiments revealed that a potential mechanism that regulates Btk-dependent TNF production in R848-stimulated macrophages is related to *TNF* transcription. The clear effect of Btk on the transcription initiation also indicates that the kinase affects the components on nucleoprotein complexes, known as enhanceosomes, with transcription factors being obvious candidates undergoing this regulation. Therefore, the aim of the next chapter was to investigate the involvement of NF κ B and AP-1 transcription factors in Btk signalling.

Chapter 5 - Results III

Btk regulates TNF expression through NF κ B and AP-1 transcription factors in R848-stimulated human primary macrophages

5.1 Introduction

The results from the previous chapter clearly indicate that Btk affects the initiation of *TNF* transcription under inflammatory conditions in human primary macrophages. Transcription initiation involves the signal-dependent binding of transcription factors to target promoters, enabling the recruitment of various components of the transcription initiation machinery including Polymerase (Pol) II. The human *TNF* 5' promoter and 3' enhancer which is located downstream of the 3'UTR region contain multiple *cis*-acting elements that are binding motifs for transcription factors (TF). The motifs that have been described to be involved in the TLR-driven induction of *TNF* in macrophages include NFκB sites 1, 2, 3 and 4, Ets, cAMP-responsive element (CRE), CCAAT/enhancer binding protein β (CEB/P) and AP-1-like sites (Kramer et al. 1995; Liu et al. 2000) (**Figure 5.1**).

In the previous chapter, Btk was shown to be dependent on the 252bp long 3' enhancer region of the *TNF* gene as the removal of this region abrogated Btk-dependent super-induction of luciferase. This region contains various TF binding sites including κB site 4. NFκB is a dominant transcription factor required for the production of inflammatory cytokines (Foxwell et al. 1998; Udalova et al. 1998). Previous studies have revealed that κB sites 1, 2 and 3 in the *TNF* promoter as well as κB site 4 in the *TNF* 3'enhancer are all involved in the transcription of *TNF* upon LPS stimulation (Krausgruber et al. 2010; Smallie et al. 2010). The canonical form of NFκB is a dimer comprising p65 and p50 subunits. The composition of the dimer determines the site of DNA binding as well as the function of the complex. p65/p50 heterodimer shows little variation in binding to κB sites 1, 2 and 3. p50/p50 homodimer which is considered to be inhibitory, binds with high affinity to κB site 1 and to κB site 2a. On the contrary, the p65/p65 homodimer has been shown to be strongly recruited to κB site 2 but not 2a ((Udalova et al. 1998; Liu et al. 2000; Udalova et al. 2000). The deletion or site-directed mutation of single κB sites in the *TNF* promoter decrease, but not completely abrogate, the induction of a luciferase reporter construct (Goldfeld et al. 1990; Tsai et al. 2000; Udalova et al. 2000). Also the inhibition of NFκB by dominant negative p65 or overexpression of IκBα does not completely abolish LPS-induced TNF expression suggesting that other transcription factors may play an equally important role (Foxwell et al. 1998).

The AP-1 family of transcription factors has also been shown to regulate TNF production (Liu et al. 2000; Steer et al. 2000). The binding sites for the AP-1 family members include CRE/ATF and

AP-1-like sites localised in the core promoter of the *TNF* gene. Among the best studied AP-1 components are Jun proteins (c-Jun, JunD and JunB) which form dimers between themselves or heterodimerize with Fos proteins. Depending on the composition of the dimer, AP-1 can be inhibitory or activatory with higher or lower affinity to the AP-1-like site (Gomard et al. 2010). AP-1 members can also dimerize with components derived from other TF families such as ATF-2, a member of the ATF/cAMP response element-binding (CREB) protein family of bZIP proteins. It has been shown that c-Jun/ATF-2 heterodimer has a high affinity for the CRE/ATF site in the *TNF* promoter under genotoxic stress such as a cisplatin treatment (Hayakawa et al. 2004). Deletion of both Ets and CRE/ATF sites completely abolishes cJun-dependent trans-activation of the human *TNF* promoter (Kramer et al. 1995).

There remain unresolved questions regarding the position of Btk in the TLR signalling cascade in human primary macrophages as well as the exact mechanism of Btk-dependent regulation of gene transcription. Studies performed on Xid macrophages and the THP-1 cell line have demonstrated that LPS-activated Btk engages the NF κ B pathway by promoting p65 subunit transactivation and nuclear localization (Jefferies et al. 2003; Doyle et al. 2005). In human XLA PBMC, however, Btk appears to function independently of NF κ B, as the degradation of I κ B α remained intact, and operate through the p38 MAPK signalling pathway following stimulation with TLR2 and 4 ligands (Horwood et al. 2003; Horwood et al. 2006). Btk has been also implicated in the activation of AP-1 in LPS-stimulated murine macrophages (Schmidt et al. 2006).

The current chapter focuses on determining the role of Btk in activating NF κ B and AP-1 upon R848 stimulation in human primary macrophages. After establishing the importance of Btk in regulating the binding of NF κ B and AP-1 to their consensus sites, more detailed analysis of the involvement of p65, cJun, cFos and ATF-2 was performed. The findings in this chapter confirm the role of Btk in the initiation of *TNF* transcription and provide clues as to which signalling pathways may be involved.

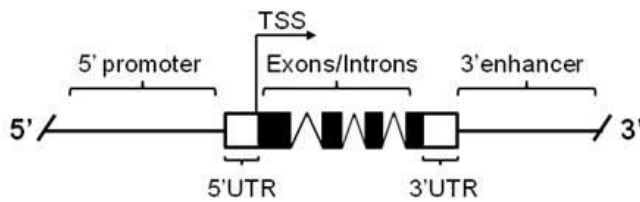
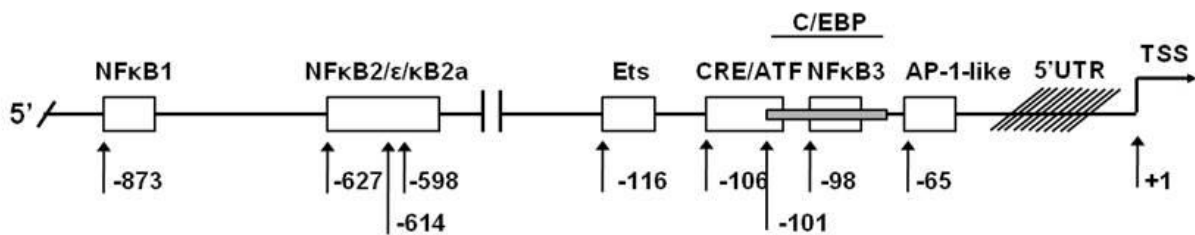
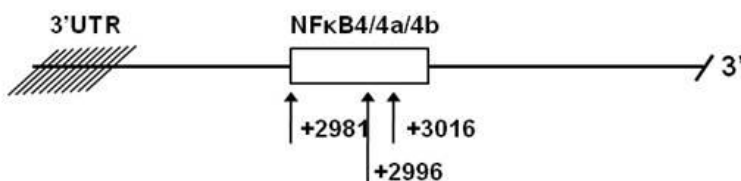
TNF* gene**TNF* promoter*****TNF* enhancer**

Figure 5.1. A schematic of the human *TNF* gene. *TNF* gene comprises of 5' promoter region, followed by transcription start site (TSS), a segment of 4 exons (shown as black squares), 3' UTR and 3' enhancer region. Multiple transcription sites involved in TLR signalling, including NFκB, Ets, CRE/ATF, C/EBP, AP-1 are localised in the promoter (shown as white squares). NFκB site 4 is localised in the 5' enhancer region. The location of the transcription factors in relation to TSS is shown numerically in base pairs.

5.2 Results

5.2.1 NF κ B mutations abrogate the Btk-dependent super-induction of TNF production under R848 stimulation.

It has recently been demonstrated in our laboratory that κ B sites located in the *TNF* 5'promoter as well as κ B site 4 located in the downstream 3'enhancer region are all implicated in the initiation of *TNF* transcription in response to LPS (Udalova et al. 1998; Krausgruber et al. ; Smallie et al. 2010). In the previous chapter, Btk was shown to be dependent on the 252bp long region of the 3'enhancer where putative κ B site 4 is located. To determine the role of NF κ B in Btk signalling, the effect of κ B mutations on the Btk-dependent induction of TNF luciferase reporter was investigated.

The luciferase viruses utilised in this experiment included a full length 5'Luc 3' 1251bp construct, a construct containing a mutant κ B site 4 (5'Luc 3' site 4X) and a construct where all κ B sites (5'Luc 3' site 1/2/3/4X) had been mutated. A construct lacking the 3'enhancer but containing the 3'UTR (5'Luc 3' UTR) was used as an additional control (**Figure 5.2**). Human M-CSF-derived macrophages were sequentially infected with AdBtk and luciferase adenoviruses. The cells were then stimulated for 6hrs with R848 and a luciferase assay was performed.

As illustrated on **Figure 5.3A**, simultaneous mutations in all the κ B sites increased the basal R848-induced luciferase expression. This effect can be explained by the fact that κ B sites are the binding motifs for both activatory and inhibitory NF κ B dimers (Udalova et al. 2000). Mutations in all 4 κ B sites must therefore affect the balance of these various dimers and perhaps promote the transcription of *TNF*. On the other hand, the site-directed mutagenesis of κ B site 4 did not alter basal levels of R848-induced TNF or abrogate the effect of Btk overexpression on luciferase induction. In fact the ability of Btk to super-induce luciferase expression was only decreased when the adenovirus carrying the construct with all the κ B sites mutated was used. This result is supported by previous research from our laboratory describing collective role of all 4 κ B sites in regulating TNF expression. In particular, in these studies, the inhibition of TNF expression by IL-10 was unaffected by one κ B mutation in the TNF luciferase reporter as compared to the mutations of all κ B sites (Tim Smallie, thesis). The inability of κ B site 4 to impair R848-mediated TNF expression and Btk signalling could derive from the fact that other unknown transcription factor sites in the 3' 1251bp long region remained intact and compensate for the NF κ B function.

To assess the efficiency of Btk overexpression in this system, an ELISA was performed on the supernatants from the R848-stimulated cells. The levels of TNF protein production and the induction by AdBtk overexpression were equal between all the luciferase viruses confirming that the results obtained from the luciferase assay were unaffected by the quality of the virus titres or the efficiency of transduction (**Figure 5.3B**). Taken together, these findings indicate that Btk requires all intact κ B sites to enhance R848-induced *TNF* transcription. The mutation of κ B site 4 alone as compared to the deletion of 252bp long region where κ B site 4 is located was insufficient to abrogate Btk signalling. The collective findings from the present and previous chapters, therefore, suggest that Btk requires NF κ B to regulate TNF production but also may involve other TF whose binding sites are located in the 1037bp long 3' enhancer.

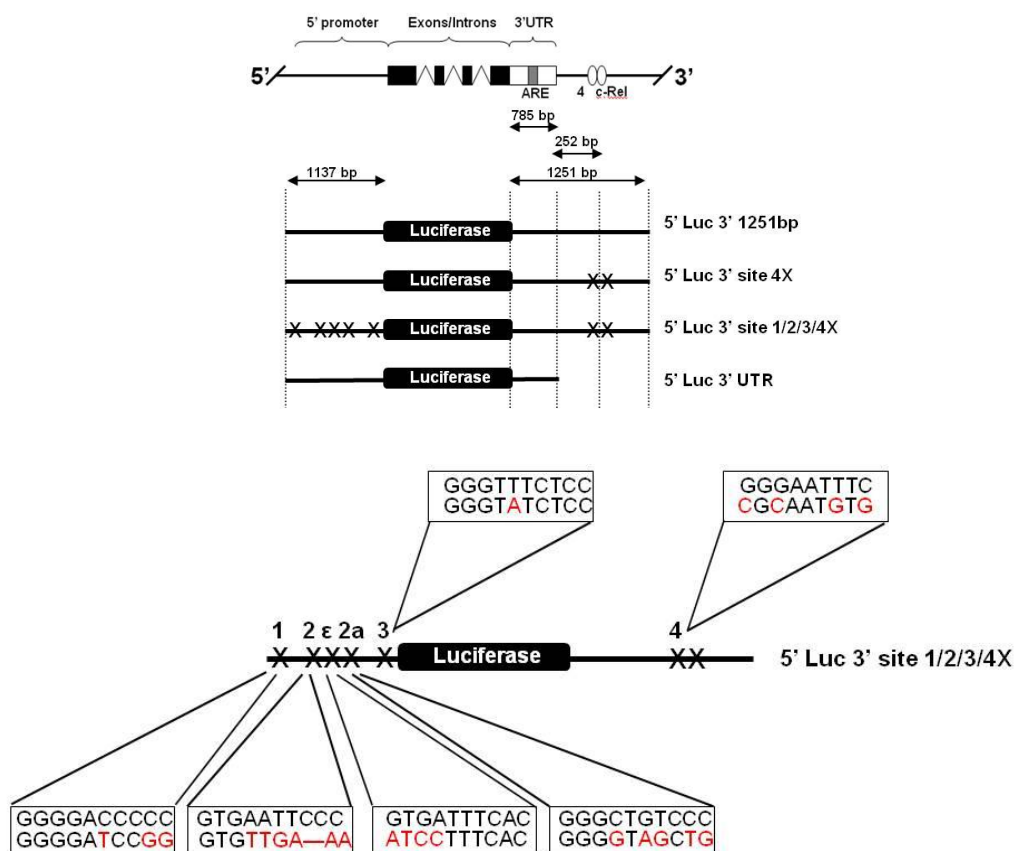


Figure 5.2 Schematic showing details of mutated κ B site adenovirus luciferase reporters. Adenoviral luciferase reporters based on the human *TNF* gene are shown in the top panel. Full length 3' 1251bp construct was used as a control for the constructs carrying either κ B site 4 or all 4 κ B sites mutated or for the construct lacking the 5' enhancer region. The NF κ B mutations are denoted as black crosses. Specific mutations of κ B site 1, cluster 2, site 3 in the 5' promoter or κ B site 4 in the 3' enhancer are shown in red and compared to wild type sequence shown in black. The mutants were generated by Dr Tim Smallie.

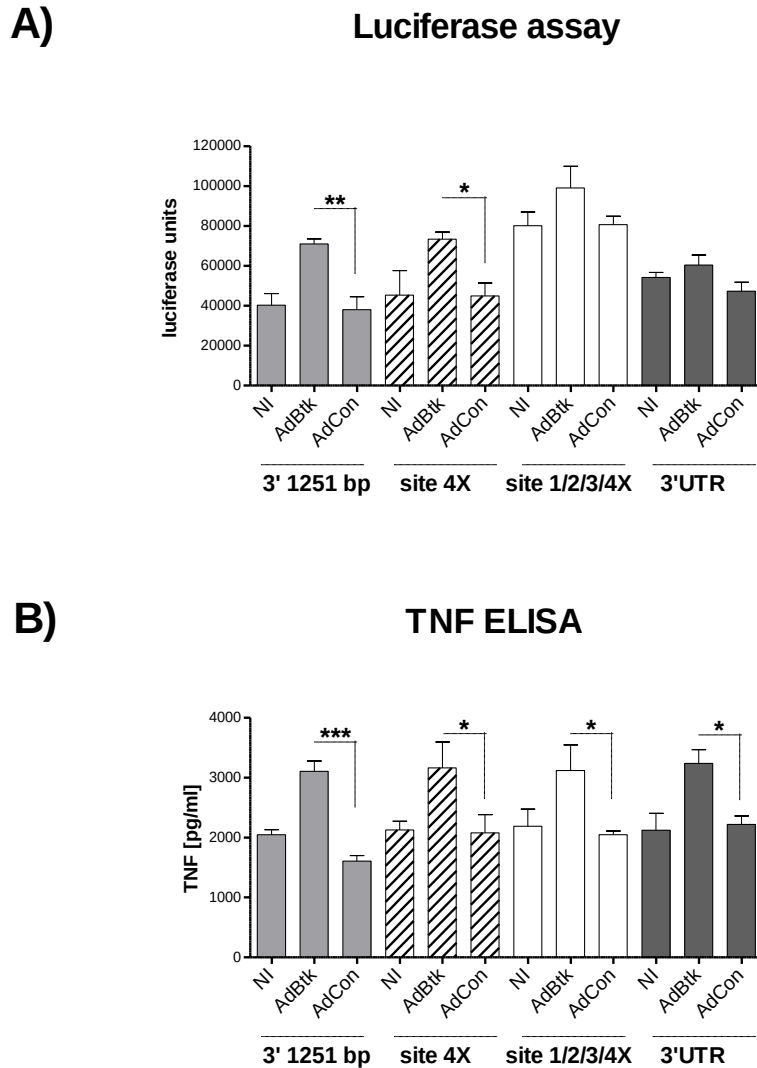


Figure 5.3 The presence of NF κ B site mutations in the TNF luciferase reporter interferes with the Btk-dependent induction of luciferase. Differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were plated at 0.07×10^6 cells per well and incubated overnight. The cells were then infected with AdBtk or AdCon at moi 100 and allowed to express for 4hrs. Following primary infections cells were infected with 5' Luc 3'1251bp, 3'site 4X, 3'site 1/2/3/4X or 3'UTR adenoviruses at moi 50 and allowed to express for further 24h (section 2.2.3.9). Cells were then stimulated with R848 (1 μ g/ml) and lysed 6h post stimulation. Luciferase activity was measured as described in section 2.2.4.6 (A). The efficiency of Btk overexpression was assessed by analysing TNF and IL-6 protein production in the same supernatants by ELISA. Values are expressed as means of triplicate repeats $\pm$ SEM. Results shown are representative of at least 3 donors. Statistical analysis: repeated measures ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to stimulated AdCon.

5.2.2 Downregulation of Btk expression reduces NFκB binding to its consensus DNA sequence following TLR8 stimulation

To further confirm the role of NFκB in Btk signalling under R848 stimulation, the effect of Btk downregulation on the binding of NFκB to its consensus site was examined. This was accomplished through using an electrophoretic mobility shift assay (EMSA). Human primary macrophages previously subjected to Btk knockdown were stimulated with R848 for 1hr and lysed. Nuclear extract was then incubated with P³² ATP- labelled NFκB oligonucleotide and run on an SDS-PAGE gel. As demonstrated in **Figure 5.4**, 1h stimulation with R848 resulted in a rapid increase of the protein:DNA complexes in cells that were not subjected to Btk knockdown. On the contrary, the binding of NFκB was abrogated when Btk was depleted from the cells indicating that the kinase is involved in the DNA binding activity of NFκB. In the presence of NFκB unlabeled probe, named as competitor, there was no accumulation of protein:DNA complexes proving the specificity of the probe. The canonical form of the NFκB complex is known to include p65-p50 heterodimers which have a high affinity for the *TNF* promoter. However, p50-p50 homodimers can also bind to the NFκB sites within the *TNF* promoter and act as transcriptional repressors (Udalova et al. 2000). Additional analysis of the NFκB supershifts revealed that the p50 and p65 members of the NFκB family were present in the complex (**Figure 5.4**). The complex is considered to be comprised of the p65/p50 heterodimer (top part) and of the p50/p50 homodimer (bottom part). Therefore, upon addition of anti-p50 antibody the whole doublet shifts up, whereas in the presence of anti-p65 antibody only the upper part of the doublet disappears. Although the results obtained from EMSA revealed the importance of Btk in the R848-driven NFκB activity, this technique utilises short consensus DNA fragments which are not gene specific and therefore one may argue that this approach does not reflect the real *in vivo* situation. Other techniques such as ChIP which considers the complexity of chromatin and is gene specific was required to determine if the defect in NFκB activation in Btk depleted cells affects *TNF* transcription.

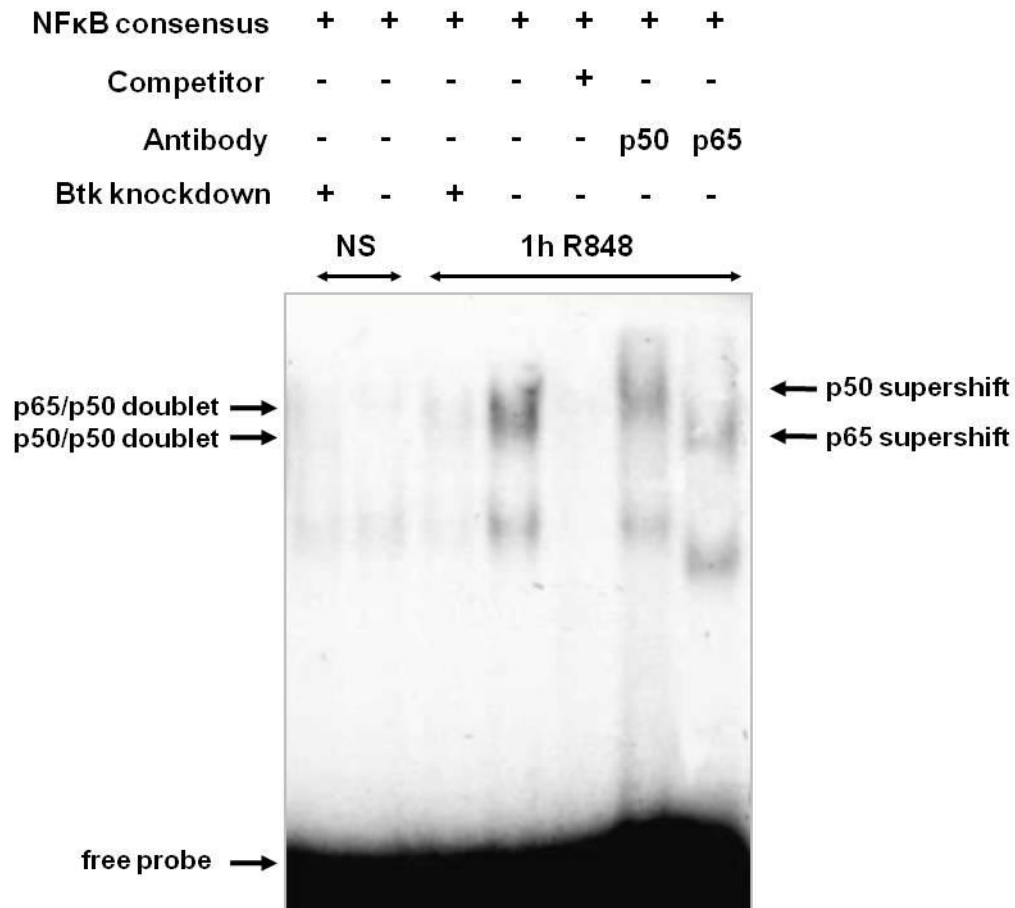
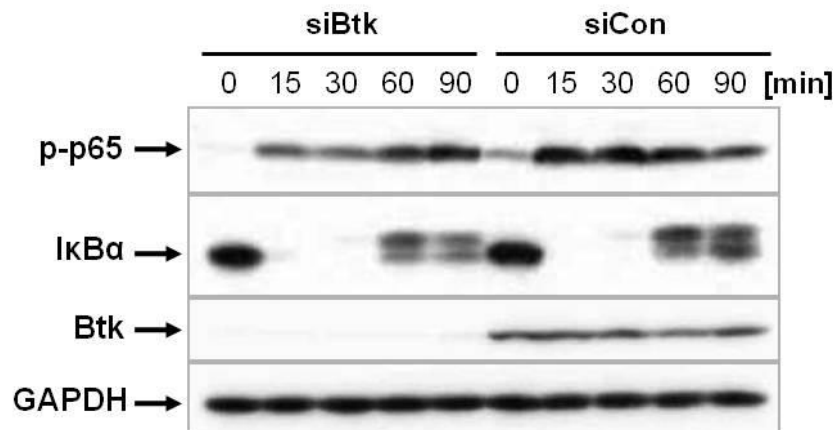


Figure 5.4 R848-mediated NFκB activity is regulated by Btk. Monocytes were transfected with 200nM Btk or Control siRNA using the Amaxa Human Monocyte Nucleofector® Kit (section 2.2.2.1). After 4 days with M-CSF cells were stimulated with R848 (1μg/ml) for 1h and 5μg of nuclear extract was subjected to EMSA with consensus probes for NFκB (section 2.2.7). A supershift assay was performed with antibodies against p50 and p65. As a control, a competitor wild type non-radiolabelled NFκB oligonucleotide was added in excess into one of the samples. The blot is a representative example of 3 independent experiments.

5.2.3 Btk downregulation increases the phosphorylation of NFκB component p65 under R848 stimulation.

Previous studies performed on XLA-derived and control PBMCs have shown that IκBα degradation is not affected in cells lacking functional Btk under LPS challenge which led to the conclusion that Btk does not affect the NFκB pathway in TLR4 signalling (Horwood et al. 2003). However, a later report has shown that p65 can be activated by IκBα-independent mechanisms marked by the phosphorylation of p65 on Serine 536 (Sasaki et al. 2005). To define whether Btk affects NFκB activation through IκBα- dependent or independent pathway in R848 signalling, the degradation of IκBα and the phosphorylation of p65 on Serine 536 was examined. Human primary monocytes were subjected to RNA interference with Amaxa Nucleofector ®Kit, differentiated into macrophages in the presence of M-CSF for 4 days and subsequently stimulated for 90min with R848. The results obtained from western blotting demonstrate that the degradation of IκBα during the course of R848 stimulation was not affected by the Btk knockdown (**Figure 5.5A**). On the other hand, the phosphorylation of p65 on Serine 536, was significantly decreased in the absence of Btk as calculated from 3 independent donors and presented on the relative density graph (**Figure 5.5B**). The effect of Btk ablation on phospho- p65 was much stronger at early time points than later, suggesting that some compensatory mechanisms were later activated. Furthermore, Btk downregulation decreased the basal level of phospho-p65. Btk and Tec have been described to be implicated in the M-CSF receptor signalling pathway required for the macrophage survival. The apoptosis rate was significantly increased in *Tec*^{-/-}*Btk*^{-/-} macrophage culture but rescued by excess M-CSF (Melcher et al. 2008). As M-CSF, which is present in the culture medium, induces NFκB binding to DNA (Oster et al. 1992) it was not unexpected that Btk ablation decreased phosphorylation of p65 even prior to R848 stimulation. These results support the role of NFκB in Btk signalling and give some more insights into how Btk may regulate the NFκB cascade. Having evidence that Btk regulates the phosphorylation of p65 which then leads to the binding of p65 to its consensus site it was reasonable to investigate further how Btk affects the binding of p65 to *TNF* upon R848 stimulation.

A)



B)

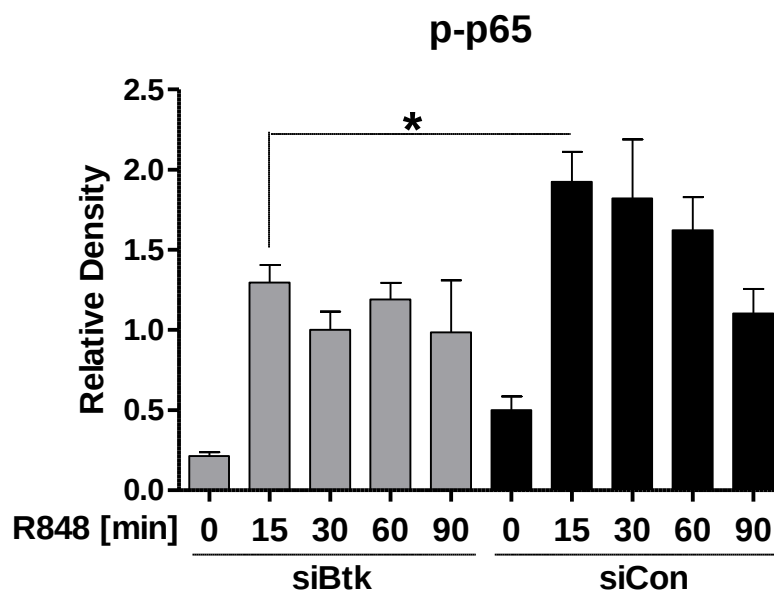


Figure 5.5 Btk knockdown impairs the phosphorylation of p65 in human macrophages stimulated with R848. Monocytes were transfected with 200nM Btk or Control siRNA using the Amaxa Human Monocyte Nucleofector® Kit (section 2.2.2.1). After 4 days with M-CSF cells were stimulated with R848 (1μg/ml) for 90min. The phosphorylation of p65 and degradation of IκBα was assessed by SDS-PAGE and western blotting with anti-p-p65 (Tyr536) and anti-IκBα antibodies (A). Relative density was calculated by normalising values from p-p65 bands to GAPDH bands at relevant time points from 3 independent experiments and using Phoretix™ 1D software (B). The blot represents one out of 3 independent experiments. Statistical analysis: Student's t-test, *P<0.05 as compared to stimulated siCon.

5.2.4 Btk abrogation affects the recruitment of p65 to the *TNF* gene following TLR8 activation.

The use of a variety of molecular biology and biochemistry techniques presented herein has provided results strongly indicating that Btk affects the transcription of *TNF* as well as NFκB activation. ChIP is a technique whose application allows a simultaneous analysis of multiple variables involved in the process of transcription as it combines data obtained from EMSA and Primary Transcript-PCR. Data presented here has shown that mutation of all 4 NFκB sites prevents the ability of Btk to up-regulate TNF. ChIP was therefore used to determine if Btk affects the recruitment of p65 to specific NFκB sites of the *TNF* 5' promoter or 3' enhancer (**Figure 5.6**).

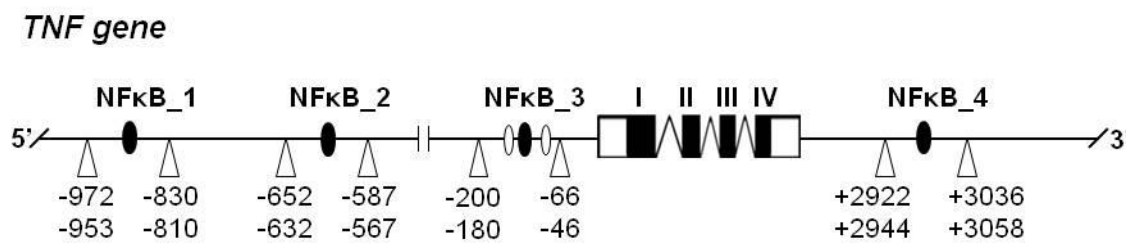


Figure 5.6 Primer sets used for ChIP. The primer sets spanning κB site 1 (NFκB_1), a cluster of κB2/ε/κB2b (NFκB_2), ATF/CRE, κB site 3 and AP-1 motifs (NFκB_3) in the 5' promoter as well as κB site 4 (NFκB_4) in the 3' enhancer of human *TNF* were used to detect the enrichment of these sites to immunoprecipitated transcription factors. Black circles illustrate the κB sites, white circles show the ATF/CRE and AP-1-like sites, whereas triangles shown the location of the primers. The primers were designed and optimised by Dr Tim Smallie.

Freshly elutriated human monocytes were transfected with individual siRNA duplexes, subsequently differentiated into macrophages in the presence of M-CSF for 3-4 days and stimulated with a TLR8 ligand for 1 and 3 hours. Cells were then fixed with formaldehyde to ensure that the transcription factors were firmly bound to DNA. Macrophages were subsequently lysed and the ChIP assay was performed with the use of IgG antibody as a control and anti-p65 antibody. The run profiles of the Real-Time PCR are illustrated in **Figure 5.7A** and show the recruitment patterns of p65 to NF κ B sites 1, 2, 3 and 4 of the *TNF* gene upon R848 stimulation. The binding of p65 to κ B sites 1, 3 and 4 at 1h increased as opposed to κ B site 2 where the recruitment of p65 was the highest prior stimulation. p65 was released from κ B site 4 at 3h but remained attached to κ B sites 1 and 3 indicating that the kinetics of p65 recruitment to various κ B motifs differs. The % Input values for the isotope control were much lower than for the p65 antibody, they did not increase and remained constant, demonstrating the specificity of the p65 antibody. In the presence of Btk knockdown the recruitment of p65 to κ B sites 2 and 4 but not to the remaining sites was significantly reduced prior to R848 stimulation. However, Btk downregulation inhibited the recruitment of p65 to all κ B sites following R848 stimulation. Although the experiment was performed on 4 independent donors resulting in similar p65 recruitment patterns, the different C_t values obtained from each blood donor prevented the data to be combined for the statistics to be applied. Therefore, only dC_t values from one representative donor were statistically analysed and the outcome presented in this report (**Figure 5.7A**). The use of melt curve analysis indicated that the PCR primers designed for different NF κ B sites resulted in a specific amplification product as only one peak at around 85-90°C was visible (**Figure 5.7B**). This data confirms that Btk acts on *TNF* transcription and that p65, an NF κ B component, is one of the transcription factors that may be involved in this regulation. Although Btk controlled the recruitment of p65 to all four κ B sites, a single mutation of κ B site 4 did not abrogate Btk- dependent regulation of *TNF* expression. These results are in line with the recent publication from our group describing the mechanism of the *TNF* transcriptional control dependent on all κ B sites. In particular, IL-10 decreased p65 recruitment to all κ B sites despite the fact that no single κ B mutation in the *TNF* luciferase construct abrogated the effect of IL-10 on *TNF* production ((Smallie et al. 2010), Tim Smallie, thesis).

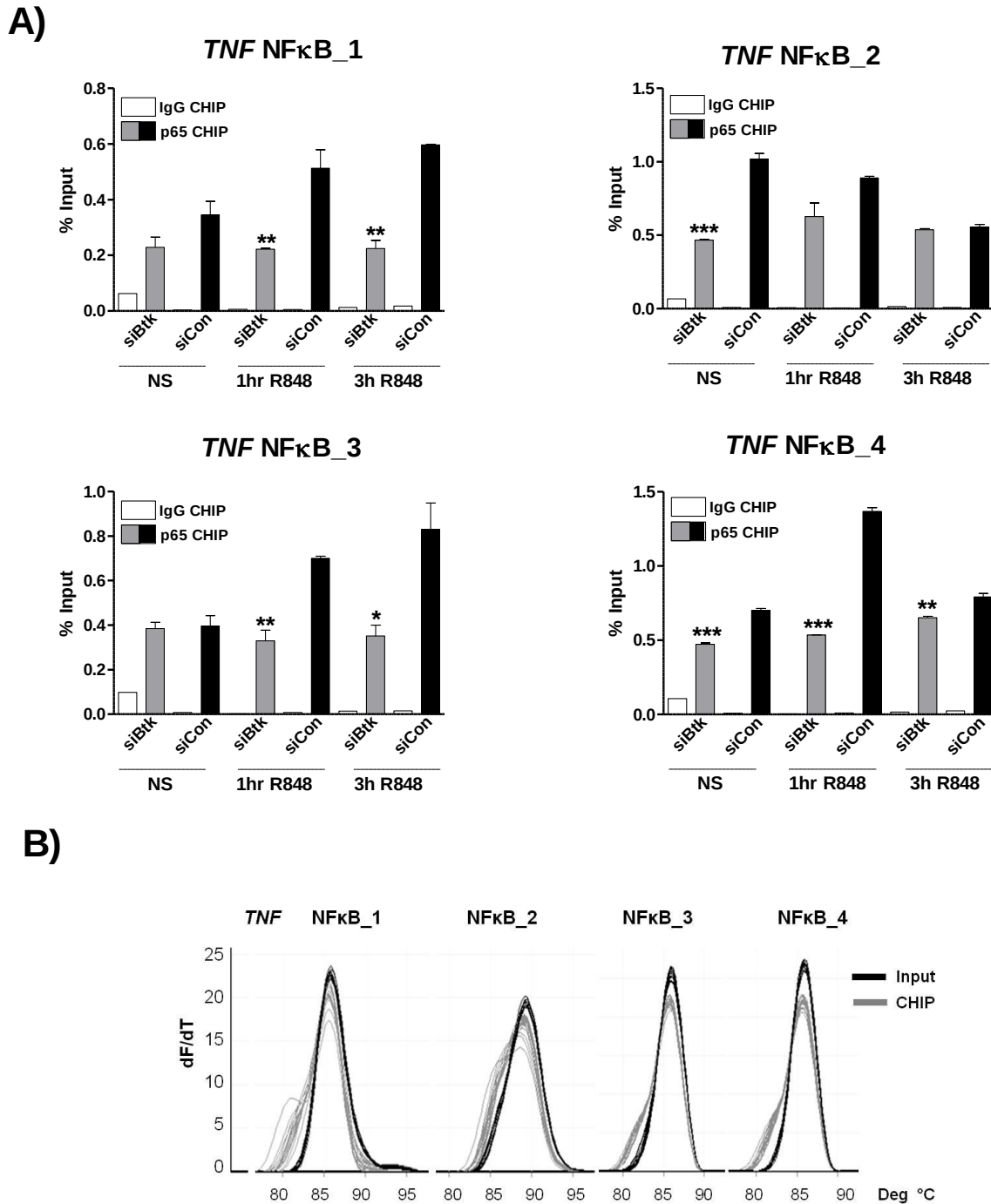


Figure 5.7 Btk knockdown inhibits the recruitment of p65 to NFκB sites of *TNF* in human macrophages. Monocytes were transfected with 200nM Btk or Control siRNA using the Amaxa Human Monocyte Nucleofector® Kit (section 2.2.2.1). After 4 days with M-CSF cells were treated with R848 (1μg/ml) for 3h, fixed and lysed as described in section X. ChIP was performed using anti-p65 or isotope antibodies with primers specific for NFκB sites 1, 2, 3 or 4 of human *TNF* gene (A) (section 2.2.9). The efficiency of the primers was assessed using melt curve analysis of immunoprecipitated DNA (ChIP) or remaining DNA (Input) (B). Data are expressed as mean $\pm$ SEM of triplicate measurements for a single donor representative of 3 independent experiments using different donors. Statistical analysis: Student's t-test was performed on dCt values on one donor, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to siCon.

5.2.5 Downregulation of Btk expression reduces AP-1 binding to its consensus DNA sequence following TLR8 activation.

Although NFκB is likely to be a major transcription factor involved in the transcription of *TNF* in macrophages, the inhibition of NFκB by dominant negative p65 or wild type IκBα adenoviruses decreases but does not completely abolish LPS-induced TNF expression (Foxwell et al. 1998; Liu et al. 2000). This observation suggests that other transcription factors may play a role in *TNF* induction by TLR ligands (**Figure 5.1**). CRE/ATF and AP-1 sites are located in close proximity to the κB site 3 in the core promoter of *TNF* (**Figure 5.8**) and studies by (Yao et al. 1997) have demonstrated that NFκB and AP-1 components interact with each other and are mutually dependent as increasing the spacing between CRE and κB site 3 abolished LPS-driven induction of *TNF*. Btk has also been shown to be important for the activation of both NFκB and AP-1 as Btk-depleted murine macrophages exhibit impaired binding of NFκB and AP-1 to the consensus sites upon LPS activation (Schmidt et al. 2006). These studies provided a basis for the hypothesis that Btk may also affect AP-1 activation upon TLR8 induction.

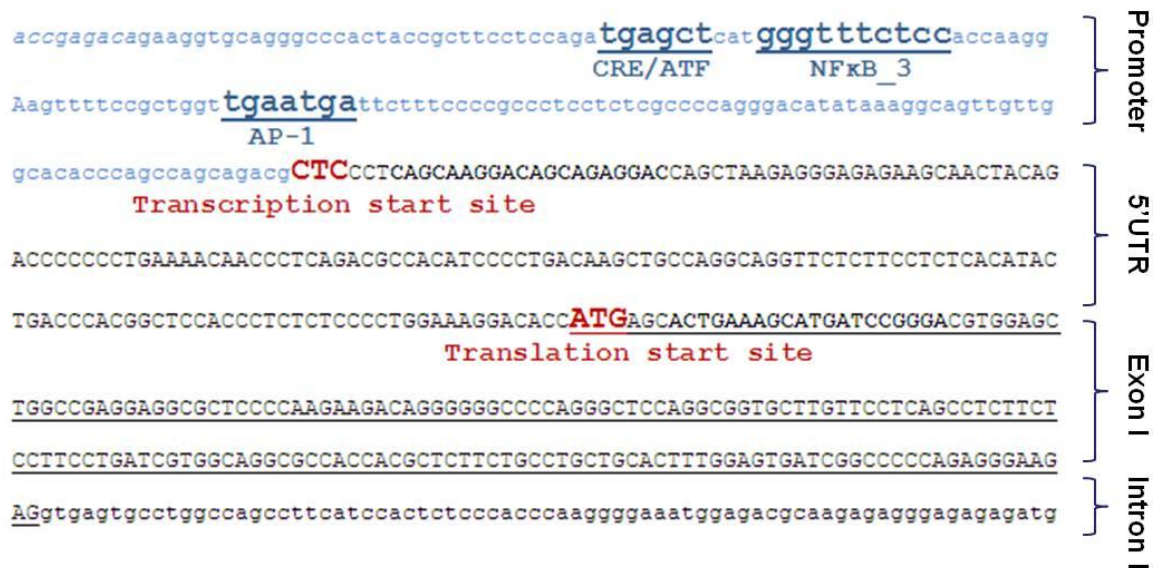


Figure 5.8 Transcription factor sites localised in the core promoter of *TNF*. NFκB site 3 (-98) is flanked by CRE/ATF (-106) and AP-1 (-65) sites (in blue, underlined) in the core promoter of *TNF*. Transcription and translation start sites are shown in red. 5'UTR is localised upstream of the first exon (underlined) followed by the first intron of *TNF*. Adapted from Mr Thomas Krausgruber.

Initially, EMSA was employed to test if Btk downregulation affects the binding of AP-1 to its consensus site. Human primary macrophages previously subjected to Btk knockdown were stimulated with R848 for 1hr and lysed. Nuclear extract was then incubated with P^{32} ATP- labelled AP-1 consensus and run on an SDS-PAGE gel. As illustrated in **Figure 5.9A**, stimulation with R848 resulted in a rapid increase of the protein:DNA complexes. The intensity of the complexes was consistently lower in the cells subjected to Btk knockdown demonstrating that Btk depletion impairs AP-1 binding. Moreover, Btk ablation changed the size of the complex as the bands were lower than in control cells. This may indicate that the absence of Btk changed the composition of the AP-1 dimer as one of the AP-1 members could no longer be efficiently activated. In the presence of an AP-1 unlabeled probe, here named as competitor, there was no accumulation of protein:DNA complexes proving the specificity of the probe.

An additional analysis of the AP-1 components has revealed that cFos, JunD and possibly cJun and JunB members of the AP-1 family were present in the complex. Antibodies against cJun and JunB blocked the formation of the DNA-protein complexes visualised as a clear complex disappearance whereas the addition of anti-cFos or anti-JunD antibodies blocked the formation of the DNA-protein complexes visualised as supershifts (**Figure 5.9A**). The absence of the complex displacement with anti-cJun and anti-JunB antibodies indicates that these antibodies were not efficient for supershift experiments. An anti-ATF-2 antibody did not cause a supershift or a band disappearance signifying that ATF-2 was not present in the AP-1 site- binding complex.

An alternative for a radiolabelled EMSA is a TransAm kit based on a sandwich enzyme- linked immunosorbent assay. Specifically, the bottom of 96-well TransAm plate is covered with AP-1 consensus sequences which are binding motifs for the AP-1 transcription factors present in the nuclear extract. By using primary antibodies against specific AP-1 components and HRP-conjugated secondary antibodies, the kit enables the measurement of the activation state of these transcription factors. For the purpose of this study, the nuclear extracts prepared previously for EMSA were analysed by TransAM for cJun activation. As illustrated in **Figure 5.9B**, the recruitment of phosphorylated cJun to the AP-1 motif increased upon R848 stimulation and was significantly decreased in the cells subjected to Btk knockdown. The control used in this experiment was TPA-stimulated K-562 extract supplied by the kit. These results demonstrate for the first time that Btk affects AP-1 binding to its consensus site in TLR8 signalling. Based on these novel findings, further characterisation of particular AP-1 components dependent on Btk was performed in more detail.

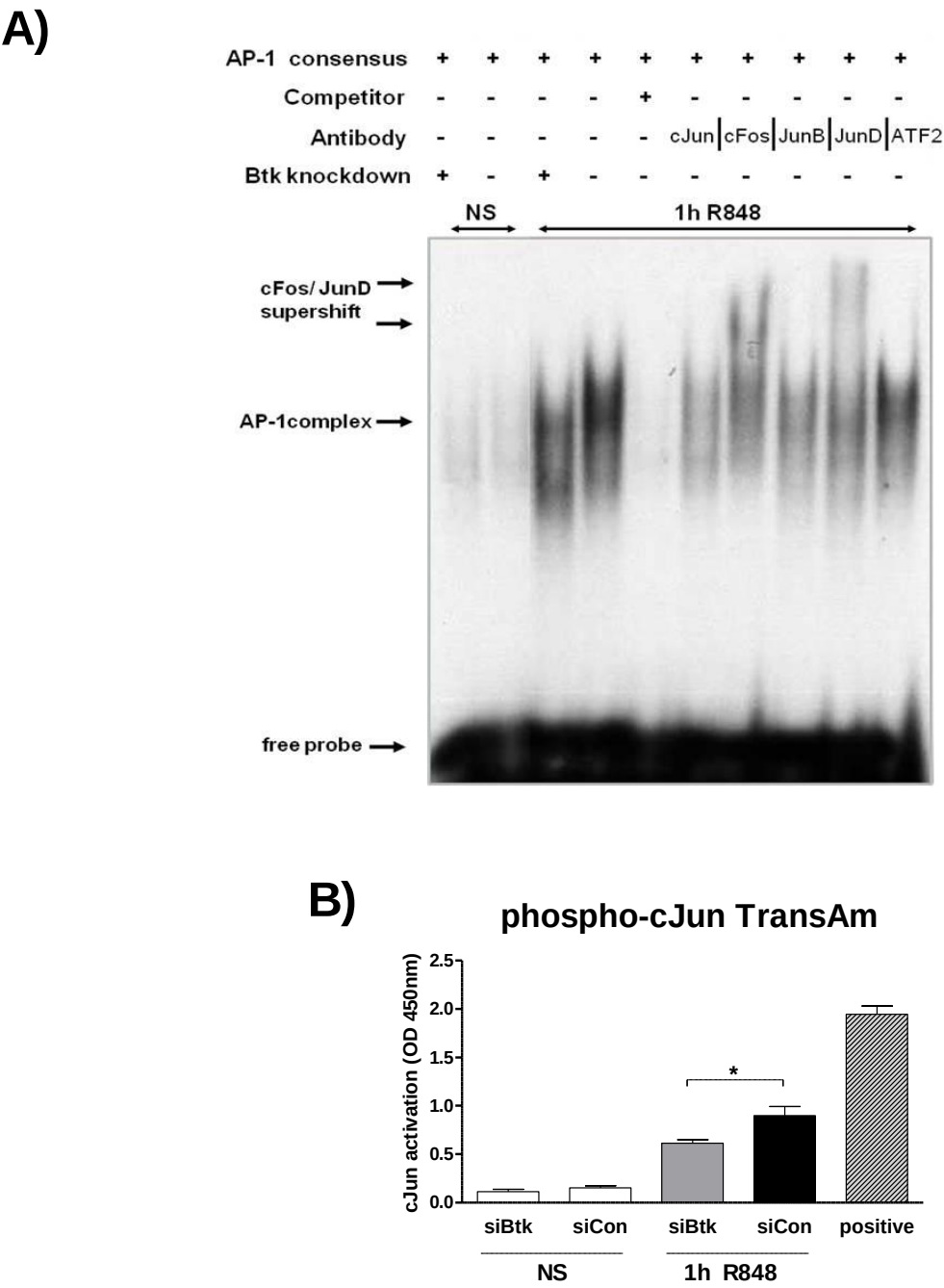


Figure 5.9 R848-mediated AP-1 activity is regulated by Btk. Monocytes were transfected with 200nM Btk or Control siRNA using the Amaxa Human Monocyte Nucleofector® Kit (section 2.2.2.1). After 4 days with M-CSF cells were stimulated with R848 (1µg/ml) for 1h and 5µg of nuclear extract was subjected to EMSA with consensus probes for AP-1 (section 2.2.7). A supershift assay was performed with antibodies against cJun, cFos, JunB, JunD and ATF-2. As a control, a competitor wild type nonradiolabelled AP-1 oligonucleotide was added in excess into one of the samples. The blot represents one out of 3 independent experiments (A). Nuclear extracts from EMSA were used for TransAm assay where anti-p-cJun antibody was applied to detect the activation of cJun (B) (section 2.2.8). Values for TransAm are expressed as means of triplicate repeats +/- SEM. Results shown are representative of 3 donors. Statistical analysis: Student's t-test, *P<0.05, as compared to stimulated siCon.

5.2.7 Btk depletion increases the basal and R848- induced recruitment of cJun and ATF-2 to the human *TNF* gene.

The ChIP technique was employed to gain an insight into the mechanisms actually occurring in the context of chromatin complexity. Specifically, the technique was used to investigate the effect of Btk on particular AP-1 components which are recruited to the *TNF* gene upon stimulation with R848. Freshly elutriated human monocytes were transfected with siRNA duplexes against Btk or control siRNA, subsequently differentiated into macrophages in the presence of M-CSF for 4 days and stimulated with R848 for 3 hours. The ChIP assay was performed with the use of IgG antibody as a control and anti-cJun, anti-ATF-2 and anti-cFos antibodies. cJun and cFos homo- and heterodimers are known to bind the AP-1-like site whereas cJun/ATF-2 heterodimers have been demonstrated to bind the ATF/CRE site of human *TNF* upon LPS stimulation (Diaz and Lopez-Berestein 2000). The primer set used was *TNF* NFκB_3 (**Figure 5.6**), as it amplifies the DNA sequence covering both CRE/ATF and AP-1 sites. The shearing of chromatin during the sonication step allows obtaining DNA fragments of 100-200bp in length. It was therefore impossible to design primers specifically for CRE/ATF or AP-1 sequence alone as the sites are clustered together and the resolution of ChIP was not sufficient at that point to distinguish between a few base pairs in the cluster (**Figure 5.8**).

The run profiles of the Real-Time PCR are demonstrated in **Figure 5.10** and show that the recruitment of total cJun and ATF-2 to the CRE/ATF-2/AP-1 site dropped upon stimulation with R848. In contrast, cFos was recruited to DNA and reached a maximum at 1h. Interestingly, the similar pattern could be observed around κB site 4 where there are no confirmed ATF/CRE or AP-1 sites. However, detailed bioinformatics analysis performed with the MatInspector programme revealed that there is a putative AP-1 site in the 3'enhancer region localised at position +3071 (**Figure 5.11**), close enough to the κB site 4 to be detected with the *TNF* NFκB_4 primer set (**Figure 5.6**). The depletion of Btk led to the enrichment of CRE/ATF/AP-1 sequence to cJun and ATF-2 at basal and 1 hour post-stimulation with no effect on cFos. A less profound but still significant effect of Btk on the AP-1 binding to the 3'enhancer region of *TNF* was observed. Unexpectedly, it was shown in **Results 4.2.8** that Btk is dependent only on the 252bp long 3'*TNF* enhancer element, but not on the downstream region where the AP-1 site is located, to induce luciferase. Together, these findings indicate that although Btk affects the recruitment of AP-1 to sites located in the 5'promotor and 3'enhancer, the deficiency of one of the AP-1 motifs does not impede Btk-induced *TNF* transcription. The values for the isotope control were considerably

lower showing the specificity of anti-cJun, anti-cFos and anti-ATF-2 antibodies. The experiment was performed on 4 independent donors resulting in similar AP-1 recruitment patterns, but the different Ct values obtained from each blood donor prevented the data to be combined for the statistics to be applied. Therefore, only dCt values from one representative donor were statistically analysed. Other tested antibodies for ChIP included anti-JunD which proved inefficient in immunoprecipitating JunD from the genome as no recruitment of this AP-1 component to *TNF* was detected by Real-Time PCR.

These data demonstrate that Btk affects cJun and ATF-2 recruitment both to the *TNF* 5' and 3' regions but has no effect on cFos. Interestingly, Btk depletion increased the recruitment of total cJun to *TNF* as shown by ChIP but decreased phospho-cJun binding to the AP-1 consensus site as shown by TransAm (**Results 5.2.6**). This discrepancy may be derived from the differences in the assays themselves as TransAm utilised the AP-1 consensus sequence whereas the primers used in ChIP were *TNF* specific. Alternatively, AP-1 activation is known to be complex as AP-1 components may be inhibitory or activatory depending on their phosphorylation status (Ogawa et al. 2004). Therefore, although Btk depletion affected the levels of total cJun and phospho-cJun recruitment to DNA differently, the final effect on *TNF* expression still resulted in decreased transcription. Interestingly, despite the negligible AP-1:DNA complex formation in unstimulated cells as visualised by EMSA (**Results 5.2.6**), there was on average 2 fold enrichment of cJun to *TNF* before stimulation as compared to R848-stimulated cells (**Figure 5.10A**). On the other hand, the recruitment of cFos to the *TNF* gene increased with R848 stimulation following the AP-1 binding pattern observed in EMSA. The decreased recruitment of c-Jun and ATF-2 to *TNF* upon R848 stimulation indicates that these AP-1 components may have an inhibitory role in TLR signalling. To test this hypothesis, numerous attempts at downregulating cJun, ATF-2 and cFos were made by using an Amaxa Human Monocyte Nucleofector® Kit and lipid based Dharmafect1 reagents. The attempts proved to be unsuccessful (data not shown) preventing further investigation of the role of particular AP-1 members in *TNF* transcription. Nevertheless, the collective results obtained from EMSA, TransAm and ChIP show that Btk regulates the activation of AP-1 upon R848 stimulation which results in the transcription of *TNF*.

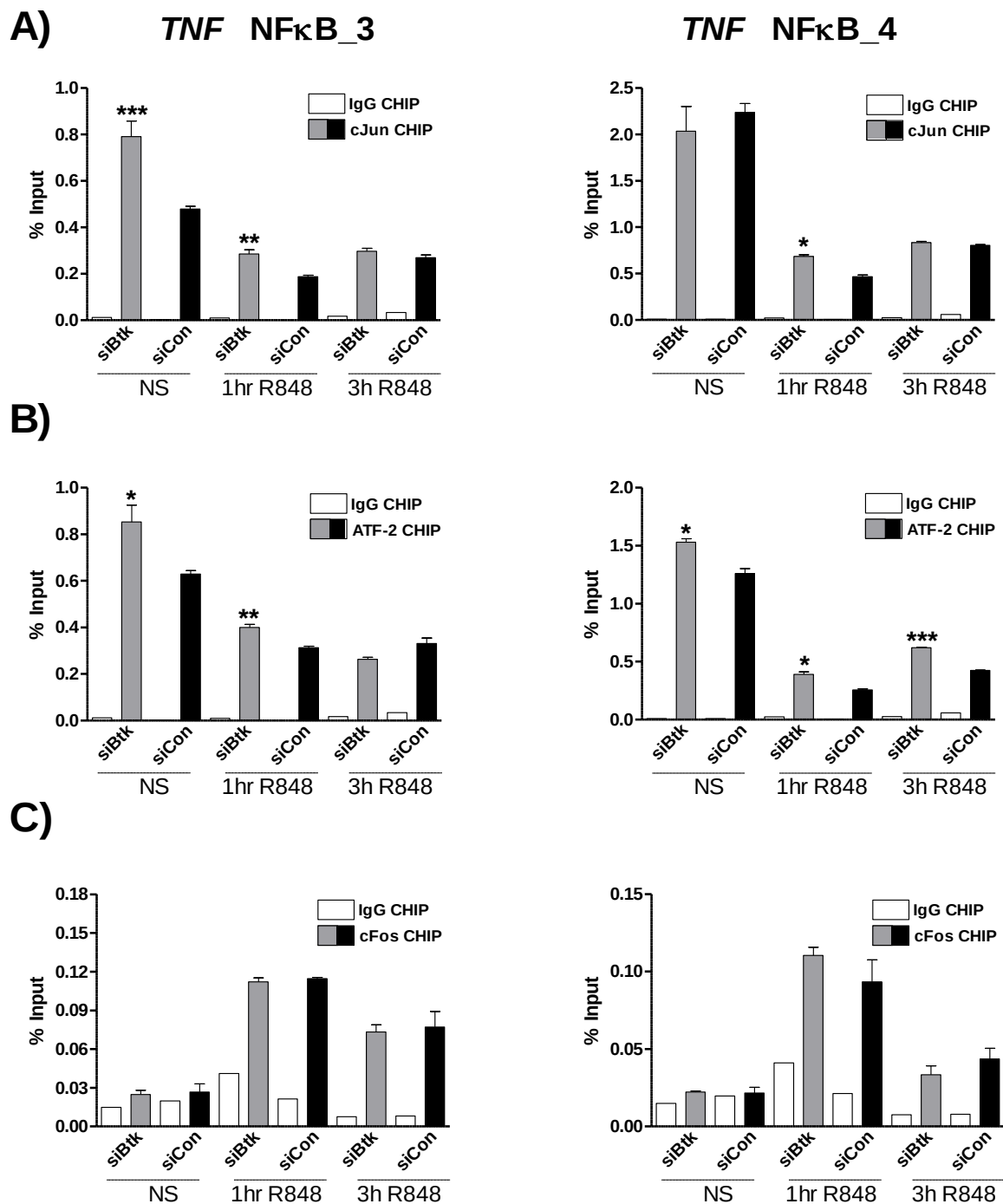


Figure 5.10 Btk knockdown increases the recruitment of cJun and ATF-2 to CRE/ATF/AP-1 site of *TNF* in human macrophages. Monocytes were transfected with 200nM Btk or Control siRNA using the Amaxa Human Monocyte Nucleofector® Kit (section 2.2.2.1). After 4 days with M-CSF cells were treated with R848 (1μg/ml) for 3h, fixed and lysed as described in section 2.2.9. ChIP was performed using anti-cJun, anti-ATF-2, anti-cFos or isotope antibodies with primers specific for CRE/ATF/AP-1 site in 5' promoter or NFκB site 4 in 3' enhancer of human *TNF* gene (A). Data are expressed as mean $\pm$ SEM of triplicate measurements for a single donor representative of 3 independent experiments using different donors. Statistical analysis: Student's t-test was performed on dC_t values on one donor, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to siCon.

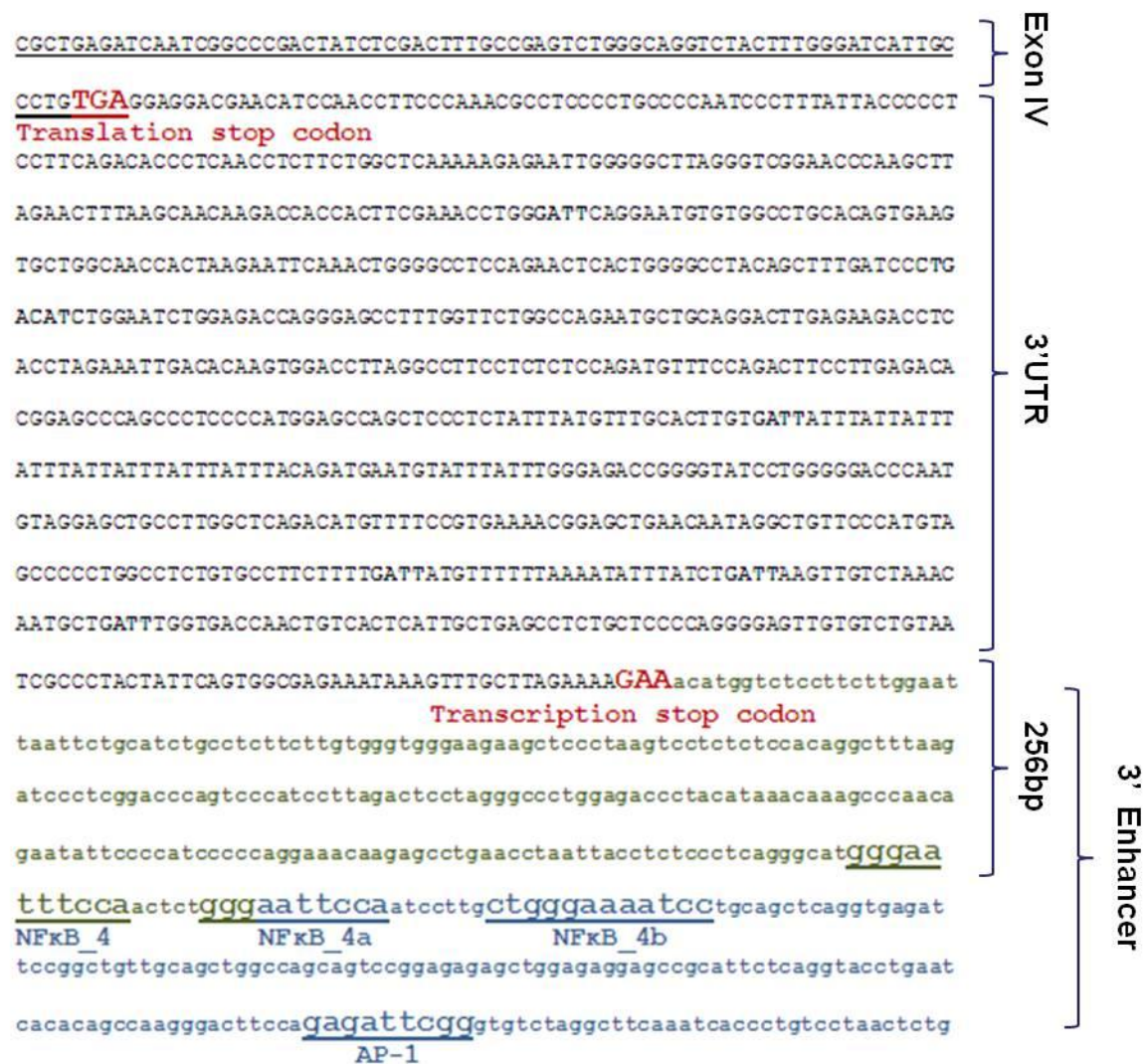


Figure 5.11 Transcription sites localised in the *TNF* enhancer. NFkB_4 cluster (+2981, +2996, +3016) is localised in high proximity to the AP-1 site (+3071) in 3' enhancer region of *TNF* (bold, underlined). The termini of 256bp region of 5'Luc 3'1037bp construct is shown in green. Transcription and translation stop sites are shown in red. 3'UTR is localised downstream of the fourth exon (black, underlined). Adapted from Dr Tim Smallie and Mr Thomas Krausgruber.

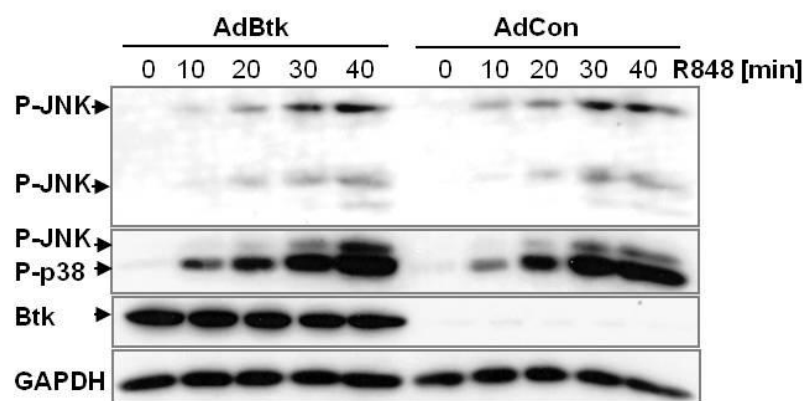
5.2.8 The activation of MAPK under R848 stimulation is not affected by Btk overexpression in human primary macrophages.

MAPK are implicated in AP-1 activation as well as expression of AP-1 components (Pramanik et al. 2003). A previous report by (Kristof et al. 2001) has shown that the chemical inhibition of p38 and ERK decreased LPS/IFN γ -induced AP-1 binding to *iNOS* promoter. The function of p38 in regulating AP-1 activity varies between p38 isoforms as p38 β appears to induce activity whereas p38 δ and p38 γ seems to inhibit or have no effect on AP-1 driven reporter genes luciferase activity (Pramanik et al. 2003). Another study revealed that the recruitment of c-Jun and ATF-2 to the *TNF* promoter under genotoxic stress was inhibited by JNK inhibitors or JNK-specific siRNA (Hayakawa et al. 2004). The involvement of MAPK in Btk signalling under TLR stimulation is still unresolved and seems to be largely dependent on the cell model and species (Horwood et al. 2006; Zemans and Arndt 2009). To examine the effect of Btk on R848-driven MAPK activation, the outcome of Btk overexpression on p38, JNK and ERK phosphorylation or p38 kinase activity was investigated. *In vitro* differentiated human macrophages were infected with adenovirus carrying the wild type *BTK* gene or an empty vector. Cells were left to express the protein for a minimum of 24h and stimulated with R848 for 40min. Western blotting analysis showed that the phosphorylation of p38, JNK and ERK increased within 10min of stimulation with R848 and that there were no significant differences in the phosphorylation patterns between AdBtk- and AdCon-infected cells (**Figure 5.12A**).

The lack of the effect of Btk on MAPK activation may have been due to the fact that the antibodies did not detect phosphorylation sites affected by Btk activity. To rule out this possibility, a kinase assay was carried out to monitor the activity of p38 as this particular kinase has previously been implicated in LPS-induced Btk signalling (Horwood et al. 2006). The assay involves the use of P³² radiolabelled ATP, a coenzyme which is utilised by p38 to phosphorylate its substrate MK2. Radiolabelled MK2 is then visualised on a SDS-PAGE gel. **Figure 5.12B** illustrates that the phosphorylation of MK2 was apparent at 10min, peaked between 20 and 30min followed by a gentle drop at 40min. The activation pattern of p38 visualised in the kinase assay differed from the results obtained in western blotting, where the phosphorylation of p38 stably increased and was the highest at 40min. Nevertheless, there was again no change in MK2 phosphorylation under the excess of Btk providing further evidence that activation of p38 is not affected by Btk under R848 stimulation (**Figure 5.12A/B**). This result indicates that it is unlikely that Btk regulates AP-1 through MAPK activation. Other molecules regulating AP-1 activation

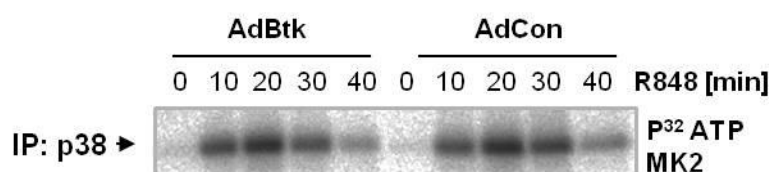
such as TAK1 (Shim et al. 2005), or AP-1 stability and degradation such as members of the Src family kinases; respective c-Abl and CSK (Xie and Sabapathy 2010) may be therefore implicated in Btk signalling instead.

A)



B)

Kinase assay



C)

MK2 Phosphorylation

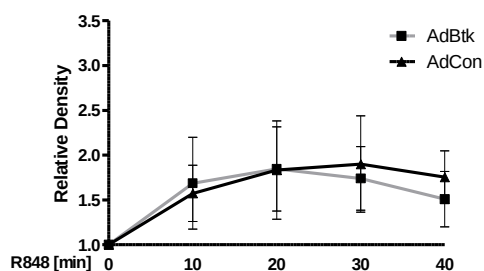


Figure 5.12 Btk overexpression does not affect MAPK activation under R848 stimulation. Differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were infected with adenovirus at 100 moi (section 2.2.3.9). Cells were incubated for 24h and stimulated with R848 (1 μ g/ml) for 40min. The phosphorylation of p38, ERK and JNK was assessed by SDS-PAGE and western blotting (A) whereas the activation of p38 was assessed by a radioisotope kinase assay using MK2 as a substrate (B) (section 2.2.6.3). Relative density of the bands derived from the p38 kinase assay was calculated by normalising values from stimulated cells to unstimulated cells using PhoretixTM 1D software (C). The blots represent one out of 3 independent experiments.

5.2.9 R848-induced TNF and IL-6 production is dependent on TAK1 in human primary macrophages.

Transforming growth factor (TGF) beta-activated kinase 1 (TAK1) has been described to be implicated in IL-1, TNF and TLR4 signalling (Landstrom 2010). The first evidence demonstrating the importance of this kinase in LPS signalling, provided by Irie and colleagues, showed that co-transfection of the TAK1 dominant negative vector together with an NF κ B reporter into murine macrophages decreased LPS-induced luciferase activity (Irie et al. 2000). It was thereafter demonstrated that the activation of AP-1 is significantly impaired in *Tak1^{m/m}* MEF cells stimulated with LPS (Shim et al. 2005) implicating that TAK1 controls both NF κ B and AP-1 activities. Although there is no literature addressing the role of TAK1 in tyrosine kinase signalling, the findings describing the dependence of NF κ B or AP-1 on TAK1 led to a question regarding the potential role of TAK1 in Btk signalling. In order to investigate this possibility, the effect of dominant negative TAK1 K63W (TAK1 DN) and wild type TAK1 (TAK1 WT) adenoviruses on TNF and IL-6 production was examined. The TAK1 DN construct was previously successfully used for determining the role of TAK1 in TGF β signal transduction (Yamaguchi et al. 1995). Human differentiated macrophages were infected with the adenovirus at moi ranging from 50 to 150 virus particles per cell. The cells were subsequently stimulated with R848 for 18h to measure TNF, IL-6 or IL-10 production. The level of TAK1 protein was monitored by western blotting and showed that both TAK1 DN and WT adenoviruses efficiently overexpressed TAK1 as compared to AdCon (**Figure 5.13A/B**). The overexpression of TAK1 WT moi- dependently increased the production of both TNF and IL-6 from R848-stimulated macrophages. This was mirrored by an moi-dependent drop in cytokine secretion in cells infected with AdTAK1 DN (**Figure 5.13C/D**). Surprisingly, only AdTAK DN significantly decreased the production of IL-10 which was not accompanied by the super-induction of IL-10 by AdTAK WT. This observation is consistent with data obtained from LPS-stimulated macrophages (Dr Lynn Williams, personal communication). These data demonstrate that the kinase is involved in the production of both TNF and IL-6 thus revealing a novel role for TAK1 in TLR8 signalling. Furthermore, the effect of TAK1 on cytokine production resembles the effect of Btk on TNF and IL-6 expression upon R848 stimulation.

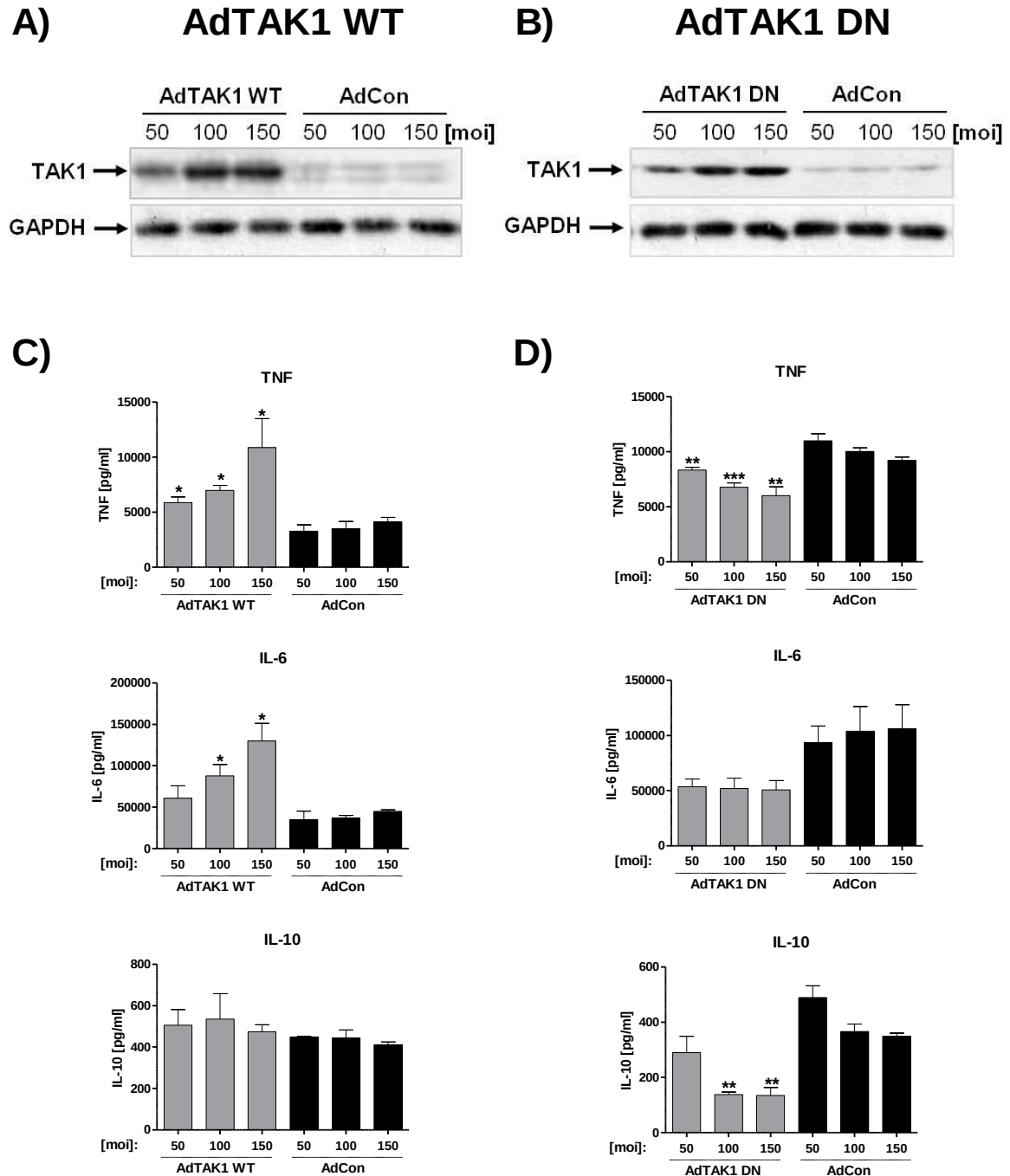


Figure 5.13 TAK1 regulates the production of TNF and IL-6 under R848 stimulation. Differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were infected with TAK1 wild type (AdTAK1 WT), TAK1 dominant negative (AdTAK1 DN) or control adenovirus (AdCon) at moi ranging from 50 -150 virus particles per cell (section 2.2.3.9). Cells were incubated for 24hrs and stimulated with R848 (1 μ g/ml) for 18hrs. The protein level was assessed by SDS-PAGE and western blotting (A-B) whereas the cytokine concentration was assessed by ELISA (C-D). Unstimulated cells are not shown as values were undetectable. Cytokine levels are expressed as means of triplicate repeats $\pm$ SEM. Results shown are representative of at least 3 donors. Statistical analysis: Student's t-test, * P <0.05, ** P <0.01, *** P <0.001 as compared to stimulated AdCon at relevant moi.

5.2.10 Overexpression of TAK1 WT increases NFκB and AP-1 binding to the consensus DNA sequences following TLR8 activation.

To further characterise the role of TAK1 in TLR8 signalling and to gather evidence about the potential interactions between TAK1 and Btk, the effect of TAK1 WT overexpression on NFκB and AP-1 binding to the consensus DNA fragments was assessed by EMSA. Human differentiated macrophages were infected with either AdTAK1 WT or AdCon, subsequently stimulated with R848 for 1h and lysed. Nuclear extract was then incubated with P³² ATP- labelled NFκB or AP-1 oligonucleotides and run on an SDS-PAGE gel. **Figure 5.14** shows that the formation of NFκB or AP-1 complexes increased upon stimulation in AdCon- infected cells. In the presence of overexpressed TAK1 the binding of NFκB to DNA also considerably increased and was higher than control both before and after stimulation. Comparable results were found in the AP-1 EMSA, as TAK1 overexpression significantly increased the recruitment of AP-1 to its consensus site at both time points. The high presence of NFκB and AP-1 complexes in unstimulated cells previously infected with TAK1 indicates that TAK1 was slightly pre-activated and could be explained by the fact that the kinase is known to be induced by many stimuli such as growth factors, environmental stress and inflammatory cytokines (Landstrom 2010). The pre-activation of TAK1 is therefore unavoidable as M-CSF is added to the culture medium for monocyte differentiation and low levels of inflammatory cytokines are secreted by cells upon adenovirus infection. In the presence of NFκB or AP-1 unlabeled probes, named as competitor, there was no accumulation of protein:DNA complexes proving the specificity of the probes. TAK1 signalling appears to follow the same pattern as Btk which also affected the binding of NFκB and AP-1 to DNA.

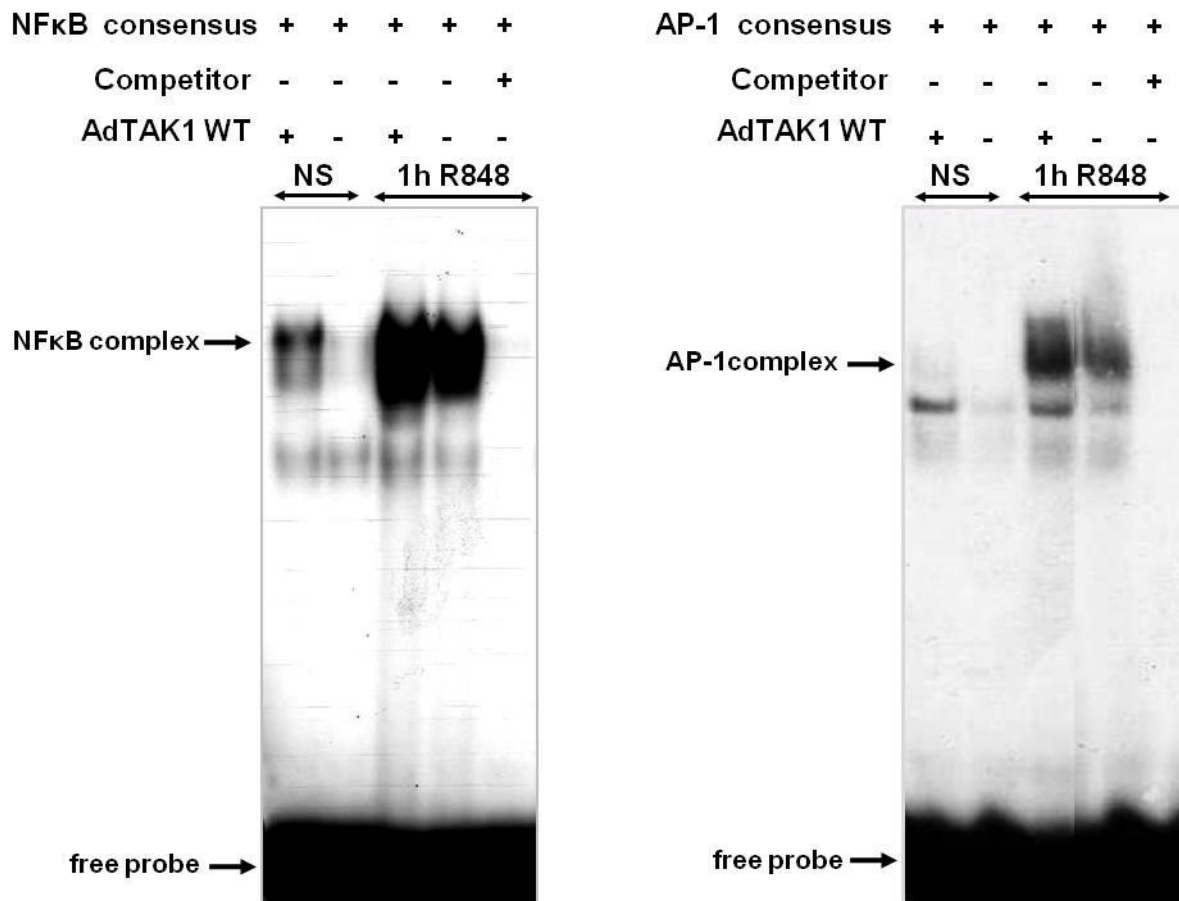


Figure 5.14 R848-mediated NFκB and AP-1 activity is regulated by TAK1. Differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were infected with TAK1 wild type (AdTAK1 WT) or control adenovirus (AdCon) at moi 100 (section 2.2.3.9). Cells were incubated for 24hrs and stimulated with R848 (1μg/ml) for 1hrs. Cells were lysed and 5μg of nuclear extract was subjected to EMSA with consensus probes for NFκB or AP-1 (section 2.2.7). As a control, a competitor wild type nonradiolabelled NFκB or AP-1 consensus was added in excess into one of the samples. The blots represent one out of 2 independent experiments.

5.2.11 Btk-induced TNF and IL-6 production is dependent on TAK1 in human primary macrophages.

Both TAK1 and Btk affected the binding of AP-1 and NF κ B to DNA as well as the secretion of TNF and IL-6 under TLR8 stimulation suggesting that these two kinases might share the same signalling pathway in human primary macrophages. To validate this hypothesis, the effect of TAK1 DN adenovirus on Btk-dependent super-induction of TNF and IL-6 was examined in R848-stimulated cells. Human differentiated macrophages were sequentially infected with AdBtk or AdCon followed by the infection with AdTAK1 DN or AdCon. The cells were then stimulated with R848 for 18h and the efficiency of the adenoviral transfection or the level of cytokine production was measured by western blotting or ELISA, respectively. **Figure 5.15A** shows that both AdTAK1 DN and AdBtk expressed efficiently and that the double infection did not affect the expression of either of the proteins. Infection with AdTAK DN significantly decreased the production of R848-induced TNF and IL-6, whereas AdBtk significantly increased the production of TNF and to a lesser extent the production of IL-6. Double infection with AdTAK1 DN and AdBtk significantly abrogated Btk-induced TNF and IL-6 production (**Figure 5.15B**). These results provide further evidence that both TAK1 and Btk are required for R848-induced TNF and IL-6 production and that they co-operate with each other as dysfunctional TAK1 impaired the Btk effect on cytokine production.

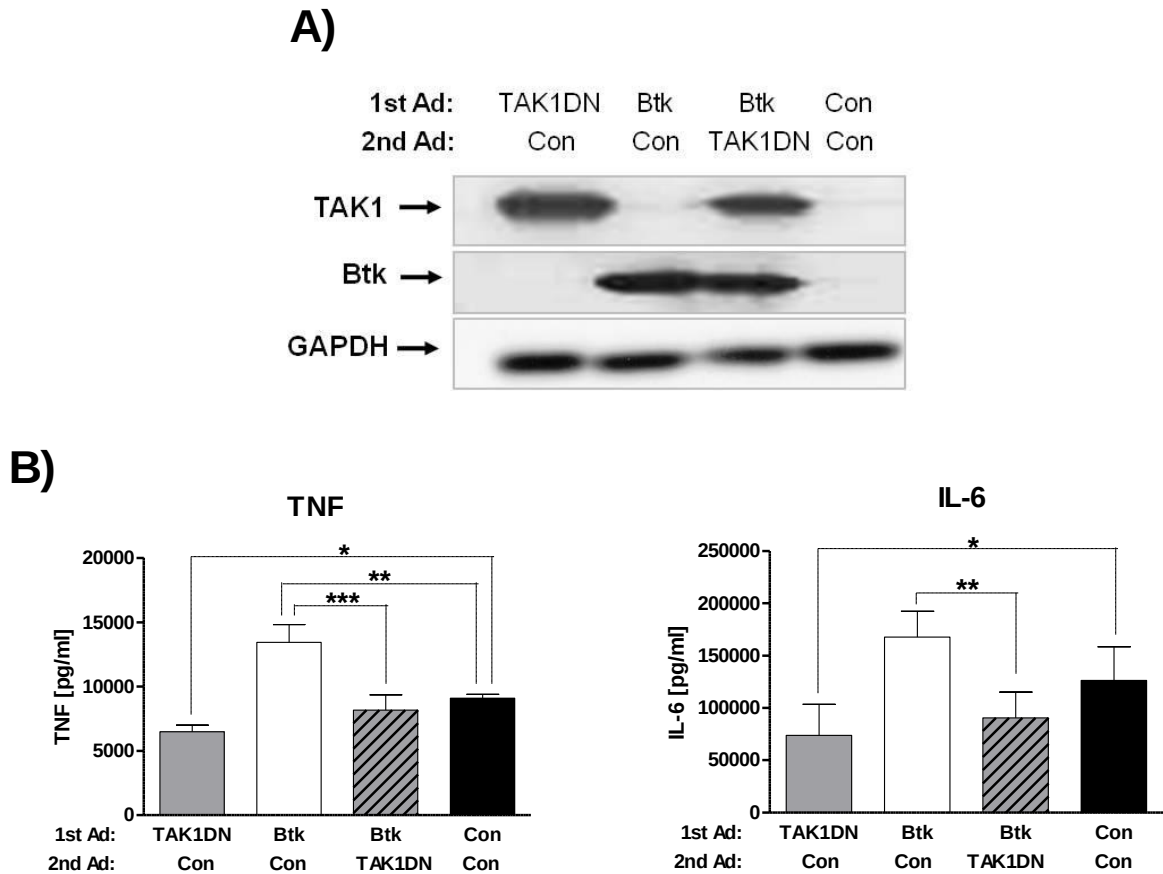


Figure 5.15 Btk-induced TNF and IL-6 production is dependent on TAK1. Differentiated macrophages (obtained by 4 day culture of monocytes in M-CSF supplemented medium) were plated at 0.07×10^6 cells per well and incubated overnight. The cells were then infected with AdBtk or AdCon at moi 100 and allowed to express for 4hrs. Following primary infections cells were infected with AdTAK1 DN or AdCon at moi 50 and allowed to express for further 24h (section 2.2.3.9). Cells were then lysed for western blotting or stimulated with R848 ($1 \mu\text{g/ml}$) for 18h. The efficiency of Btk and TAK1 overexpression was determined on a protein level (A) and the level of TNF and IL-6 secreted by the infected cells was measured by ELISA (B). Values are expressed as means of triplicate repeats $\pm$ SEM. Results shown are representative of at least 3 donors. Statistical analysis: repeated measures ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

5.3 Discussion

In these studies, the involvement of Btk in the R848-mediated control of *TNF* transcription via two families of transcription factors, namely NFκB and AP-1 has been proposed. Furthermore, it is evident from the results that Btk does not engage p38, ERK or JNK to regulate AP-1 activity, rather signalling through other kinases, such as TAK1. These data extend current knowledge regarding the involvement of Tec kinases in the regulation of cytokine production in response to R848 by providing a potential mechanism of transcriptional control.

Much of the research in previous years has been focused on indentifying downstream components of the Btk signalling pathway initiated by TLRs localised on plasma membranes. The conflicting results, describing the effect of Btk on transcription factors such as NFκB, appear to be largely dependent on the cell system used and still remain unclear (Doyle and O'Neill 2006; Horwood et al. 2006). Few studies have focused on the Btk-dependent induction of NFκB under endosomal TLR stimulation (Doyle et al. 2007). The data reported here has shown that the kinase is clearly involved in the activation of NFκB under R848 stimulation since it affected the formation of protein:DNA complexes as visualised in EMSA experiments (**Figure 5.4**). Furthermore, the data strongly demonstrate for the first time that Btk affects the recruitment of NFκB to the *TNF* gene in a natural genomic state (**Figure 5.7**) and that mutations of NFκB sites 1, 2, 3 and 4 in the TNF reporter construct significantly abrogate Btk-induced luciferase activity (**Figure 5.3**).

Downregulation of Btk also decreased the phosphorylation of p65 on Serine 536, an event which is considered to be independent of the IκBα pathway (Sasaki et al. 2005) and did not affect the degradation of IκBα under R848 stimulation (**Figure 5.5**). This is consistent with results obtained by Lee and colleagues showing that B cells derived from *Btk*^{-/-} mice did not display impaired IκBα degradation following TLR9 engagement (Lee et al. 2008). In common with the results reported here, Btk also affected the p65 phosphorylation on Ser 536 but not IκBα degradation in LPS-stimulated Xid macrophages or HEK293-TLR4 cells (Doyle et al. 2005). The activation of p65 requires two distinctive steps; one is the release of p65 from the inhibitory IκBα complex which allows nuclear translocation whilst the other is post-translational modification including the phosphorylation of p65 on Serine 536. It is this phosphorylation event that is required for transactivation of gene expression (Doyle et al. 2005). p65 can be phosphorylated on Serine 536 by IKKα and IKKβ (Sakurai et al. 2003). Mutation of Ser536 to alanine has been reported to abolish the interaction of p65 with coactivators p300 and CREB-binding protein (CBP) and as a

consequence to decrease transcriptional activation (Chen et al. 2005). Neither of these molecules has been described to interact with Btk. Alternatively, Btk has been described to directly phosphorylate CREB at Serine 133 during neuronal differentiation indicating that the kinase activity of Btk is not only restricted to tyrosine phosphorylation and that the kinase can directly interact with transcription factors (Yang et al. 2004). The present results show that Btk is more likely to be involved in p65 transactivation than its release from the inhibitory I κ B complex.

Although NF κ B is a major regulator of *TNF* transcription, it has been shown before that the inhibition of the NF κ B pathway does not fully abrogate the production of TNF (Foxwell et al. 1998; Liu et al. 2000). Similarly in the current system, the mutations of all four NF κ B sites in the TNF reporter construct did not decrease the production of luciferase under R848 stimulation (**Figure 5.3**). Taken together, these data imply that there are other families of transcription factors involved in the regulation of *TNF* transcription in TLR signalling. The AP-1 family has been described to be controlled by NF κ B, as the transient transfection of human pancreatic tumor cell line with mutant I κ B α impeded both the AP-1 luciferase activity as well as the expression of cFos (Fujioka et al. 2004). Studies by Schmidt and colleagues have also revealed that Btk deficiency decreases the activity of AP-1 in TLR4- and TLR9- stimulated murine macrophages (Schmidt et al. 2006) thus pointing to AP-1 as a likely candidate factor in Btk-mediated TLR8 signalling. Current findings also show that the downregulation of Btk affected the activity of AP-1, specifically interfering with phospho-cJun binding to the AP-1 motif (**Figure 5.9**). These results justified further investigation of the specific involvement of cJun, ATF-2 and cFos in the Btk-dependent regulation of TNF production. Using ChIP, it was demonstrated that the downregulation of Btk increased both the basal and R848-induced cJun and ATF-2, but not cFos, recruitment to the *TNF* 5' promoter (**Figure 5.10**) where well documented CRE/ATF/AP-1 sites are located (Steer et al. 2000). Surprisingly, cJun, ATF-2 and cFos also bound to the 3' enhancer near κ B site 4 although a putative AP-1 binding site has not been reported previously. However, detailed bioinformatics analysis with MatInspector software revealed a possible AP-1 binding motif in close proximity to NF κ B site 4. The new AP-1 site is located in the 3' enhancer region which was shown in the luciferase experiment to be independent on Btk indicating that although Btk regulates the recruitment of cJun and ATF-2 to the AP-1 motif in the 3' enhancer, the site is not important for Btk-mediated *TNF* transcription.

The unexpected enrichment of the cJun and ATF2 at the *TNF* gene in unstimulated macrophages and their release upon R848 stimulation suggests that these AP-1 components may be negative regulators of TNF production. AP-1 has been previously shown to have anti-inflammatory

properties as *JunB*^{-/-} *cJun*^{-/-} mice develop psoriasis-like phenotype also characterised by elevated expression of TNF, IL-1 and IL-6 in epidermis (Schonthaler et al. 2009). Unfortunately, as the attempt to downregulate cJun, ATF-2 or cFos in human primary macrophages proved to be unsuccessful, the possible function and the mechanism of action of these AP-1 components in inflammatory conditions can be only speculated. One possible explanation for this is the *co-repression* hypothesis. Data from a number of laboratories have established that under basal conditions, the promoters of many TLR-dependent genes in macrophages are occupied by co-repressor complexes comprising histone deacetylase 3 (HDAC3), which is associated with inactive chromatin and consequently with gene repression. It has been shown recently that a cJun homodimer can also be a part of such co-repressor complexes. Upon stimulation, cJun is phosphorylated, which ultimately leads to the release of HDAC3 complex followed by the substitution of one of the cJun homodimer components with cFos. The rearrangement of the dimer components results in the recruitment of a coactivator complex and subsequent transcription of the gene (Newell et al. 1994; Ogawa et al. 2004; Huang et al. 2009). Current data strongly agrees with this hypothesis as the recruitment of cJun to the *TNF* gene fell, whereas the recruitment of cFos rose upon R848 stimulation, supporting the rearrangement of a cJun homodimer into cJun/cFos heterodimer theory (**Figure 5.10**). On the other hand, the recruitment of phosphorylated cJun to the AP-1 consensus site increased upon R848 stimulation, once again being in agreement with the *co-repression* hypothesis (**Figure 5.9**). Additional downregulation of Btk increased the recruitment of cJun and ATF-2 to the *TNF* promoter, potentially augmenting the repressor function of cJun homodimers resulting in decreased TNF production. Conversely, downregulation of Btk resulted in the decreased recruitment of phosphorylated cJun to the AP-1 motif upon R848 stimulation, potentially impairing the activator function of cJun, once again resulting in decreased TNF production (**Figure 5.16**).

Independent studies have shown that MAPK are crucial for TNF expression as they regulate both transcription and mRNA stability of *TNF*. Btk has been previously shown to employ p38 MAPK in the regulation of TNF production following TLR4 receptor engagement in human macrophages (Horwood et al. 2003). At the same time, studies performed on murine macrophages have shown that Btk does not affect the phosphorylation of MAPK upon LPS stimulation (Schmidt et al. 2006). As evident from the current results, Btk overexpression did not alter R848-induced phosphorylation of p38, JNK or ERK indicating that the Btk-regulated AP-1 and NFκB activity is not mediated through MAPK (**Figure 5.12**). In common with the results reported here, selective activation of p38, JNK or ERK by transiently expressing upstream MAPK kinases induced *TNF*

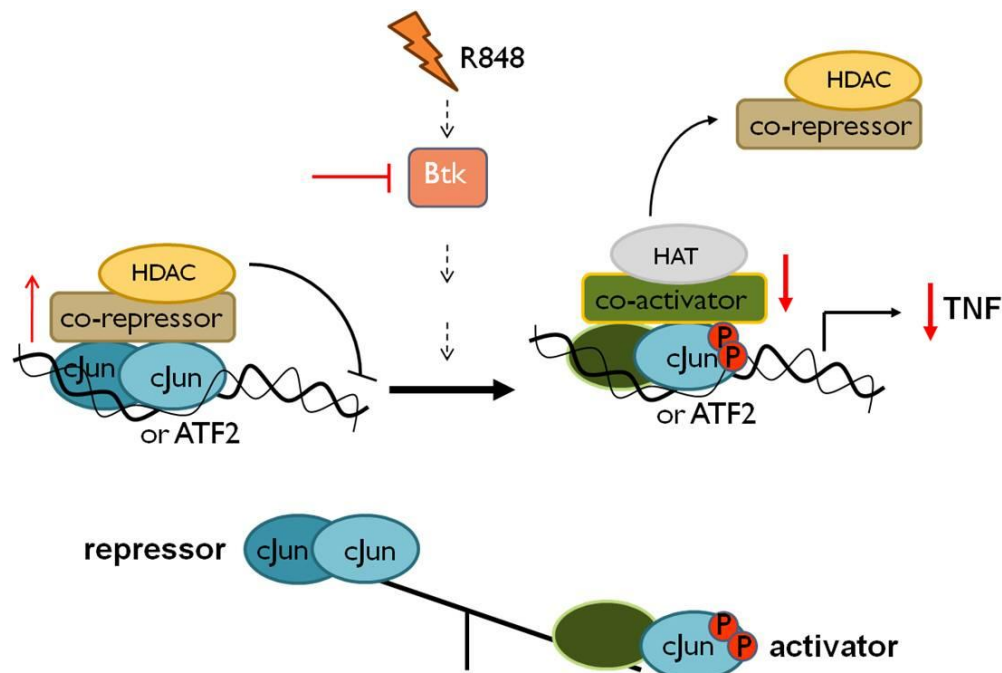


Figure 5.16 Model of Btk-dependent de-repression of AP-1 target genes in R848-stimulated macrophages. In basal conditions, cJun homodimers are bound to the promoter of *TNF*. These form a scaffold for the co-repressor complex involving HDAC which is associated with inactive chromatin and gene repression. The engagement of TLR8 leads to Btk-mediated phosphorylation of cJun and subsequent exchange of one cJun subunits into another AP-1 component. Newly formed heterodimer attracts a co-activator complex and HAT what leads to *TNF* transcription. Btk downregulation increases the repressor function of cJun homodimers and decreases the activator function of phosphorylated cJun ultimately leading to impaired TNF production. HDAC, histone deacetylase; HAT, histone acetyl transferase.

transcription independently on *cis* acting elements such as NFκB or AP-1 motifs in the *TNF* promoter (Zhu et al. 2000). The question remains how Btk regulates AP-1 components if this is not through MAPK. Firstly, Btk could act on AP-1 indirectly through the NFκB pathway, which has been shown to be crucial for AP-1 activity as IKKε phosphorylates cJun following TLR4 engagement leading to NCoR clearance and gene de-repression (Huang et al. 2009). Secondly, Btk could affect AP-1 directly as the kinase is able to shuttle to the nucleus and interact with transcription factors such as TFII-I or CREB (Novina et al. 1999; Mohamed et al. 2000; Yang et al. 2004). Lastly, Btk could employ other pathways to indirectly regulate AP-1 such as TAK1 which has been shown to activate AP-1 (Shim et al. 2005), c-Abl or CSK involved in AP-1 stability or degradation, respectively (Xie and Sabapathy 2010).

One of the molecules implicated in Btk signalling could be TAK1 whose phosphorylation has been shown to be triggered by many stimuli including growth factors, environmental stress or

inflammatory cytokines (Landstrom 2010). It has been demonstrated that TAK1 activates AP-1 under IL-1R or under TLR4 engagement (Shim et al. 2005; Yu et al. 2008) becoming an obvious candidate to be involved in the Btk pathway. Initial experiments demonstrated that TAK1 behaves similarly to Btk as AdTAK1 WT increased TNF and IL-6 production whereas AdTAK1 DN decreased the cytokine production under R848 stimulation (**Figure 5.13**). Furthermore, AdTAK1 WT considerably increased the binding of NFκB and AP-1 to their consensus DNA motifs once again mimicking the Btk effect (**Figure 5.14**). The ultimate experiment has revealed that Btk-dependent super-induction of TNF and IL-6 is dependent on TAK1 (**Figure 5.15**). TAK1 was originally identified as a MAPKKK (Yamaguchi et al. 1995) lying upstream of p38 MAPK in LPS signalling in the THP-1 cell line. This may be in disagreement with the current hypothesis that Btk employs TAK1 to regulate AP-1 and NFκB independently of MAPK. Experiments performed in human primary macrophages revealed, however, that TAK1 does not activate p38, JNK or ERK MAPK upon TLR4 engagement (Lynn Williams, manuscript in preparation). The activation of MAPK by TAK1 in TLR8 signalling has not been investigated but similarly to TLR4 pathway may be absent. TAK1 has been previously shown to be differentially phosphorylated by IL-1 and TGF-κ stimulation indicating that the TAK1 mode of action is stimuli-dependent (Sorrentino et al. 2008; Yu et al. 2008; Yamazaki et al. 2009).

Data presented in this chapter further strengthens the hypothesis established in Chapter 4 that Btk regulates the initiation of *TNF* transcription. Following the engagement of TLR8, Btk activates NFκB and AP-1 leading to the recruitment of these transcription factors to the *TNF* promoter and 3' enhancer region. p38, JNK or ERK MAPK are not involved in Btk signalling under TLR8 engagement in human primary macrophages. TAK1 also activates NFκB and AP-1 resulting in TNF and IL-6 production thus resembling the Btk effect on cytokine production. However, the nature of the Btk interaction with TAK1 remains to be further investigated.

Chapter 6

Concluding discussion

6.1 Concluding discussion

Inflammation, the defensive response of the immune system against pathogens, irritants and damaged cells, is a tightly regulated process. If the immune response fails to eradicate a causative agent or if the immune regulatory mechanisms fail to resolve the immune response, the inflammation persists and becomes a pathological chronic condition which poses a serious threat to the host. The initiation of the immune response involves the detection of antigen by pattern recognition receptors such as TLRs. The immune system is designed to recognise both endogenous and exogenous molecules but not to be activated by self antigens. Failure to do so results in the development of autoimmune diseases such as RA, SLE or IBD in which perpetuating chronic inflammation leads to severe tissue damage. Macrophages, abundant in the inflamed synovial membrane, appear to be involved in both the initiation and development of RA. In particular, they directly respond to injurious stimuli via TLRs and are the main source of the pivotal cytokine TNF. Expression of TLRs 2, 3, 4, 7 and 8 is elevated in the synovial tissue of RA patients (Radstake et al. 2004; Roelofs et al. 2005; Brentano et al. 2005a) and the blockade of TLRs has been considered as an attractive treatment strategy for the treatment of various autoimmune conditions (Clanchy and Sacre 2010). Endosomal TLRs may be of particular interest as their inhibition with the antidepressant mianserine or with chloroquine, an inhibitor of endosomal acidification, blocked spontaneous release of TNF from RA synovial membrane cultures (Sacre et al. 2008). TLRs on the cell membrane have been extensively investigated and it is now clear that non-receptor tyrosine kinases including Src, Syk, Pyk2 and Tec family kinases are implicated in the TLR4 pathway as they regulate the production of various cytokines and chemokines following LPS stimulation. Whether tyrosine kinases fulfil the same role in endosomal TLRs has been poorly investigated and still remains unclear.

These studies focused on the role of Btk, a member of the Tec family of tyrosine kinases, in endosomal TLR signalling in human primary macrophages. The major finding of this work is that Btk is an essential component of TLR8 signalling as Btk abrogation abolished TLR8-induced TNF production. Btk downregulation or overexpression also modulated IL-6 production from R848-challenged human primary macrophages. The initial results demonstrated a rapid tyrosine phosphorylation and auto-phosphorylation of Btk in either LPS- or R848-stimulated macrophages (**Figure 6.1**). This was in line with previous research showing activation of Btk within 5-10 min following LPS challenge in human and mouse macrophages (Horwood et al. 2003; Schmidt et al.

2006). The effect of Btk on IL-6 and TNF production was previously observed by Sochorova et al (2007) who found that DCs obtained from XLA patients exhibited decreased response to ssRNA stimulation in terms of cytokine production. The later finding was not, however, consistent with the work performed by Taneichi et al (2008) who did not record any changes in IL-6 production from XLA-derived DCs stimulated with R848. Taneichi et al also found an impaired XLA DC response to LPS. In contrast, Sochorova et al did not observe any decrease in TNF production in LPS-stimulated XLA DCs. This discrepancy could be the result of different experimental settings (control, time course or type of TLR8 ligand). It is also possible that both workers obtained blood samples from different XLA patients and that the occurring mutations could cause different phenotypic changes. The number of unique mutations detected in the *BTK* gene of XLA patients currently exceeds 550 in more than 800 unrelated families. Although most of the investigated patients have the classical severe form of XLA, there are few exceptions (Lindvall et al. 2005). The effect of Btk on cytokine production would, therefore, have to be established in wild type DCs subjected to Btk inhibition or overexpression. Surprisingly, the deficiency of Btk in murine B cells led to increased responsiveness to the TLR9 ligand as the cells secreted elevated levels of TNF, IL-6 and IL-12p40 but less inhibitory cytokine IL-10 (Hasan et al. 2008; Lee et al. 2008). In human primary macrophages, Btk overexpression or downregulation did not alter IL-10 production from R848- or LPS-challenged cells. This would suggest that Btk depletion does not exhibit a consistent anti-inflammatory effect among the cells of the immune system. Nevertheless, the cumulative effect of Btk depletion is an impaired immune response to enteroviral infections as observed in XLA patients, or higher susceptibility to acute mouse adenovirus type-1 as observed in Xid mice (Moore et al. 2004; Winkelstein et al. 2006).

Another key observation of this work is that the dependence of TLR8 on Btk is greater than for TLR4 signalling. First of all, Btk was involved in R848-induced TNF and IL-6 production as opposed to only TNF in LPS-stimulated human primary macrophages. Furthermore, siRNA-mediated Btk downregulation almost completely abrogated TNF production in TLR8 but not in TLR4 signalling. Previously, Jefferies et al (2003) reported using yeast two-hybrid system that Btk interacts with TIR domains of TLR4, 6, 8 and 9 with the strongest association with TLR8 suggesting that Btk may be an indispensable member of Tec PTKs involved in TLR8 signalling. Indeed, to date no other Tec family member has been associated with the TLR8 pathway. In contrast, TLR4 has been found to depend on Bmx to induce TNF and IL-6 production in human primary macrophages and rheumatoid synovial fibroblasts (Palmer et al. 2008; Palmer et al. 2008). Unlike the role of Btk in TLR4 and TLR8 signalling, the kinase did not affect PolyI:C –induced

IP-10 production suggesting that Btk is not involved in the TLR3 pathway in human primary macrophages. The major difference between TLRs 4, 8 and TLR3 is that the latter does not utilise the adaptor molecule Myd88. Btk has been previously associated with Myd88-dependent signalling as it controls TNF and IL-1 β production upon TLR1/2, TLR2/6 and TLR4 engagement (Horwood et al. 2006)(Christine Palmer, thesis). The lack of Btk effect on TLR3 signalling is not only restricted to human macrophages, as myeloid DCs obtained from XLA patients or treated with a Btk inhibitor LFM-A13 exhibited normal production of TNF and IL-6 following TLR3 stimulation (Sochorova et al. 2007). Btk has been also found to physically interact with Myd88 as well as to phosphorylate the Myd88-bridging molecule, Mal, in response to LPS stimulation (Jefferies et al. 2003; Gray et al. 2006) further supporting the hypothesis that Btk is limited to the Myd88-dependent signalling.

Detailed investigation of Btk-dependent signalling cascades revealed that the kinase does not regulate MAPK activation in R848-stimulated human primary macrophages. In particular, Btk overexpression did not alter p38, JNK or ERK phosphorylation levels or p38 kinase activity up to 40 min post stimulation. These results were consistent with studies published by Olsson and Sundler describing no effect of LFM-A13 on p38 MAPK phosphorylation in LPS-stimulated murine macrophages (Olsson and Sundler 2006). Similarly, studies in *Btk*^{-/-} BMDM showed that Btk deficiency did not reduce p38 MAPK phosphorylation (Schmidt et al. 2006). In addition, another Tec family member, Bmx, also appears to signal independently of p38 in terms of regulating LPS-induced *TNF* and *IL-6* message stability in human primary macrophages (Palmer et al. 2008). These observations contradict a previously published study from our laboratory where the phosphorylation of p38 MAPK was found to be defective in LPS-stimulated PBMCs derived from XLA patients (Horwood et al. 2003). One of the possible explanations for the discrepancy between the current and XLA-derived studies could be the use of different model systems and different TLR stimuli. It is likely that p38 activation is already saturated following TLR8 engagement. Therefore, the overexpression of Btk could not increase the levels of p38 phosphorylation any further and the effect of Btk on p38 activation could have been missed. Further, more detailed studies performed in cells obtained from XLA patients are necessary to explain some of these data.

Instead of MAPK activation, Btk regulated the induction of the NF κ B pathway in TLR8 signalling as Btk downregulation decreased the phosphorylation of p65 on Serine 536 (**Figure 6.1**). This regulation was independent of the I κ B α pathway as the level of I κ B α degradation was unchanged.

The current data is consistent with an earlier observation made in Xid-derived BMDM which showed reduced levels of phosphorylated p65 at Ser536 and normal patterns of I κ B α degradation post LPS stimulation (Doyle et al. 2005). The current findings are also comparable with the results obtained in B cells derived from *Btk*^{-/-} mice which did not display impaired I κ B α degradation following TLR9 engagement (Lee et al. 2008). IKK α and IKK β have been found to phosphorylate p65 on Ser536. This post-transcription modification of Ser536 as well as Ser311 or Ser276 has been shown to be required for the association of p65 with the co-activator CBP/p300 and histone acetyltransferases (HATs) and thus for the p65 target gene expression (Hayden and Ghosh 2008). Whether Btk is involved in these phosphorylation events directly or indirectly has to be examined further. It is, however, possible that the kinase activates p65 itself as Btk has been described to directly phosphorylate CREB at Serine 133 during neuronal differentiation (Yang et al. 2004).

Btk ablation also impaired R848-induced NF κ B binding to DNA. This observation is in line with studies performed in endothelial cells overexpressing Btk as well as in murine and human cell lines treated with LFM-A13 (Jefferies et al. 2003). In particular, Jefferies and colleagues showed that TLR4-HEK293 cells transfected with the K430R Btk mutant exhibited reduced NF κ B luciferase activity following LPS stimulation. Furthermore, the treatment of human myeloid THP-1 cells with LFM-A13 decreased the binding of NF κ B to DNA post LPS treatment. Similarly, NF κ B luciferase activity was decreased in HEK293 transfected with either CD4-TLR8 or CD4-TLR9 and dominant negative mutants of Btk (Doyle et al. 2007). The interpretation of these data, has to be, however, viewed with caution as non-myeloid HEK293 cells do not express endogenous Btk or TLRs, whereas LFM-A13 has been found to also inhibit Jak2 and Polo-like kinase (PLK) activity as well to reduce viability of primary human macrophages (van den Akker et al. 2004; Uckun et al. 2007) (John McDaid, thesis). The link between Btk and NF κ B-regulated *TNF* transcription has not been previously investigated in the literature.

TNF is a pivotal cytokine that controls the production of both pro-inflammatory and anti-inflammatory mediators (Butler et al. 1995; Feldmann 2002). The crucial role of TNF in the development of autoimmune diseases has been highlighted by the fact that anti-TNF therapies proved to be successful in the treatment of RA, IBD and the skin disease, psoriasis. Because of its predominant functions, the regulation of TNF production has been extensively studied and it is now clear that TNF is precisely regulated at every stage of gene expression (Falvo et al. 2010). This study for the first time demonstrated that Btk is a central regulatory component of *TNF*

transcription in TLR8 signalling, as Btk downregulation almost completely abolished the synthesis of the primary *TNF* transcript. Btk knockdown also significantly decreased Pol II recruitment to the 5' promoter of *TNF* indicating that Btk affects the initiation of *TNF* transcription in TLR8 signalling. Transcription initiation involves the signal-dependent binding of transcription factors to target promoters, enabling the recruitment of various components of the transcription initiation machinery including Pol II. The human *TNF* 5' promoter and downstream 3' enhancer regions contain multiple transcription factor binding sites which have been described to be involved in TLR-driven induction of TNF including κ B sites, CRE and AP-1-like sites (Kramer et al. 1995; Liu et al. 2000).

Studies here have shown that Btk-mediated induction of TNF was dependent on the 3' enhancer region of *TNF* where the putative κ B site 4 is located. Additional analysis revealed that mutation of all four κ B sites in the 3' and 5' *TNF* regions abolished Btk-mediated super-induction of luciferase indicating that the kinase requires NF κ B to regulate *TNF* transcription. This hypothesis was confirmed using the ChIP technique which showed for the first time that Btk mediates p65 recruitment to *TNF* as Btk downregulation inhibited R848-induced p65 binding to the gene (**Figure 6.1**). Although inhibitory p50-p50 dimers have been found to be constitutively bound to DNA, the signal-dependent activation of NF κ B increases the binding of activatory p50-p65 heterodimers to the target genes (Hoberg et al. 2006). In basal conditions p50 homodimers provide a scaffold for a co-repressor complex comprising the silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) and histone deacetylase 3 (HDAC3) which is associated with gene repression. Upon stimulation, IKK α phosphorylates SMRT to initiate p50-p50-HDAC3 displacement and p50-phospho-p65 recruitment to the κ B site. Phosphorylation of p65 allows p300 to load and subsequently acetylate p65 at Lys310, which is required for full NF κ B transcription activity (Chen et al. 2002b; Hoberg et al. 2006).

The components of the transcription factor AP-1 such as cJun, ATF-2 and cFos were also found to bind to *TNF*, however the recruitment of cJun and ATF-2 dropped upon R848 stimulation in comparison to cFos and p65. One possible explanation is that cJun-cJun dimers are constitutively bound to AP-1 target genes just like p50-p50 dimers are bound to the κ B sites. cJun homodimers attract a co-repressor complex comprising nuclear receptor coreceptor (NCoR) and HDAC3, an enzyme associated with inactive chromatin. Stimulation of macrophages leads to c-Jun phosphorylation on Serines 63 and 73 resulting in the release of the co-repressor complex and

substitution of one cJun subunit with a cFos subunit. The newly formed cJun/cFos heterodimer attracts a co-activator complex comprising HATs allowing gene transcription (Ogawa et al. 2004) (**Figures 5.16 and 6.1**). Btk downregulation increased cJun and ATF-2 binding to *TNF* at both basal and post-stimulation conditions but did not affect cFos activity. Therefore, Btk ablation increased the levels of cJun-cJun-dependent co-repressor complexes that ultimately led to decreased *TNF* transcription. Btk has been previously linked to AP-1 activation as Btk-depleted BMDM exhibited impaired AP-1 binding to its consensus site upon LPS and CpG DNA stimulation (Schmidt et al. 2006). The current data expand on this finding by revealing the role of Btk in the recruitment of a particular AP-1 component to human *TNF* in whole genome setting taking into consideration the complexity of chromatin, thus reflecting the real *in vivo* situation.

Apart from affecting the basal recruitment of cJun to *TNF*, Btk also regulated the recruitment of phospho-cJun to an AP-1 consensus site upon R848 stimulation. However, Btk overexpression did not affect the activation of MAPKs which are considered to be the major upstream regulators of AP-1. This observation is in line with studies performed on the Src family kinase, Hck, which also regulated AP-1 activity through a MAPK-independent pathway in LPS-stimulated human primary macrophages (Maria Smolinska, manuscript submitted). The mechanism by which Btk controls AP-1 activation in TLR8 signalling therefore remains unclear. It could be that Btk acts on AP-1 activation indirectly by regulating the NF κ B pathway. CRE/ATF and AP-1 sites are located in close proximity to the κ B site 3 in the core promoter of *TNF* and studies by (Yao et al. 1997) have demonstrated that NF κ B and AP-1 components interact with each other and are mutually dependent as increasing the spacing between CRE and κ B site 3 abolished LPS-driven induction of *TNF*. It has been shown that p65 recruitment to κ B sites located at close proximity to the cJun-binding sites allows cJun phosphorylation and subsequent gene de-repression (Huang et al. 2009). In particular, in response to TLR4 stimulation, p65-p50 heterodimers bind to the *inos* promoter and recruit IKK ϵ which in turn phosphorylates cJun and initiates NCoR clearance. IKK ϵ has been shown to be the central regulator of cJun activation in this instance as IKK ϵ downregulation completely abolished cJun phosphorylation on Serines 63 and 73 and NCoR clearance. Interestingly, phosphorylation of Serine 536 on p65, the one dependent on Btk, creates a docking site for recruitment of IKK ϵ . Furthermore, NCoR clearance has been shown to be JNK-independent (Huang et al. 2009), once again supporting the hypothesis that Btk regulates AP-1 activation through the NF κ B but not the MAPK pathway (**Figure 6.1**).

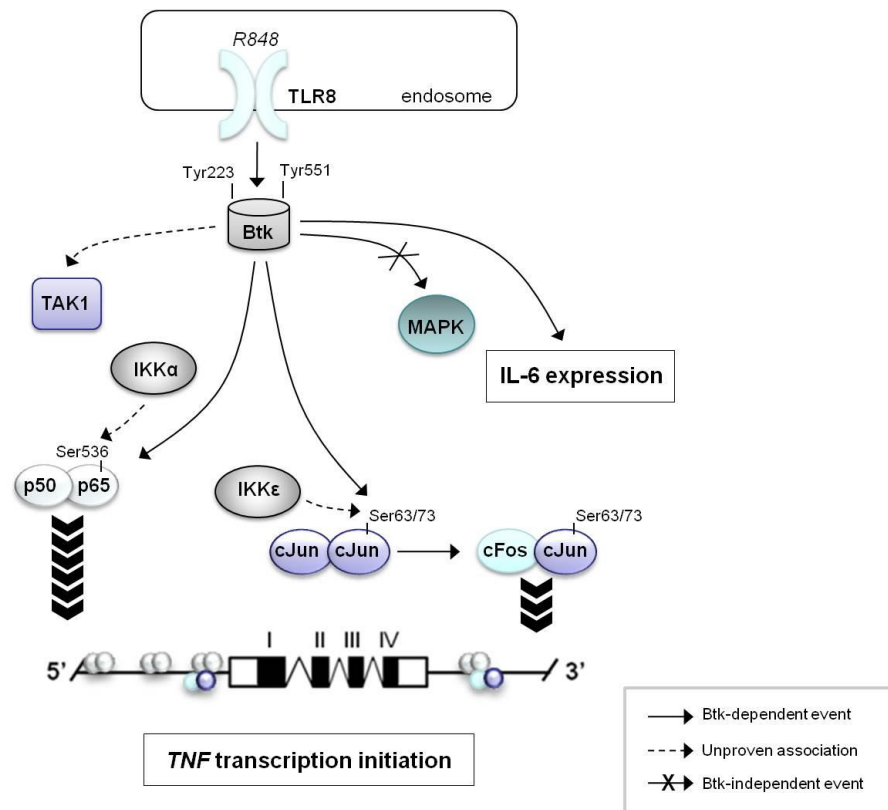


Figure 6.1 Proposed mechanism of Btk signalling in TLR8-induced cytokine production.

Another interesting finding of this work was that the monocytic-like cell lines do not carry functional TLRs 3 and 8. The present data has revealed that although THP-1, U937 and Monomac-6 cells express TLRs3 and 8 on the protein level they do not secrete TNF, IL-6, IL-10 or IP-10 upon PolyI:C or R848 stimulation. Nevertheless, these cells are routinely used for deciphering the endosomal TLR pathways. For instance, THP-1 cells were previously used for determining the role of Btk in TLR8 or TLR9- activated signalling pathways but did not refer to the ability of TLR to induce cytokine production (Doyle et al. 2007). Similarly, U937 were recently used for TLR3 and TLR9 studies which did not measure cytokine levels upon stimulation. U937 cells did, however, respond to CpG DNA and PolyI:C in terms of NO production and *IFN-β* mRNA expression (Motoyama et al. 2009). The current results imply that, although the examined cell lines are widely used as a model for the investigation of macrophage biology, they do not always reflect the results obtained from primary cells. Therefore, the results obtained from cell line models have to be viewed with caution and the use of primary cells should be considered instead to investigate the endosomal TLR pathways.

In summary, the data obtained from TLR-challenged human primary macrophages provide further insight into the molecular mechanisms used by Btk to control cytokine production. In particular, Btk proved to be the central regulator of TNF in TLR8 signalling as Btk downregulation dramatically reduced *TNF* transcription and as a consequence TNF protein production from R848-stimulated cells. Cumulative data show that in response to R848 stimulation Btk becomes rapidly phosphorylated on Tyr551 and auto-phosphorylated on Tyr223. Btk signals upstream of NF κ B, AP-1, possibly TAK1 but not MAPK to regulate the initiation of *TNF* transcription as well as IL-6 expression. Activated Btk mediates p65 phosphorylation on Ser536, possibly through IKK α , leading to NF κ B translocation to the nucleus and gene transactivation. Btk also mediates cJun phosphorylation that facilitates rearrangement of cJun homodimers into cJun/cFos heterodimers and consequently *TNF* transcription. IKK ϵ may be required for cJun de-repression as the kinase is able to phosphorylate cJun on Ser63/73 (**Figure 6.1**).

These findings are of particular importance as TNF is the major pro-inflammatory cytokine able to regulate the production of other inflammatory mediators (IL-1, IL-6, IL-12), acute phase proteins, ROS, NO and prostaglandins (Schottelius et al. 2004). Anti-cytokine therapies have proven highly efficacious in the treatment of RA, SLE and IBD (Suresh 2010). However, not all RA patients respond equally well to the anti-TNF therapy indicating that therapies that simultaneously target several cytokines may be more beneficial in these patients. For instance, chemical inhibition of particular non-receptor tyrosine kinases may prove to be a more systemic and a cheaper alternative than existing antibody-based biological treatments. The current findings propose a mechanism of *TNF* gene control and provide molecular targets that could be targeted in the course of development of more efficient anti-inflammatory therapeutics.

6.2 Future work

6.2.1 Positioning Btk in the TLR8 signalling network

Previous published results have shown that Btk interacts with Myd88 and Mal under TLR4 engagement and that Btk phosphorylates Mal in the THP-1 cell line (Jefferies et al. 2003; Gray et al. 2006). As the TLR8 pathway does not utilize Mal it becomes clear that Btk-Mal interactions are not important in TLR8 signalling. Therefore, Btk-Myd88 interactions may be of more

importance. However, the requirement for and hierarchy of Btk-MyD88 interaction has not been proved in TLR8 signalling. Thus, the effect of Myd88 deletion and mutation on Btk phosphorylation can be pursued by means of *Myd88* knockout mice and adenoviruses carrying murine and human mutated versions of Myd88 (both available in house). The results will clarify if Myd88 is functionally associated with Btk, and if so, whether it is located upstream or downstream of Btk in TLR8 signalling. The outcome would then provide further evidence for the role of Btk in Myd88-dependent signalling, such as TLR and IL-1R, and possibly help to explain the effect of Btk on downstream signalling components.

Current results suggest that Btk shares a signalling cascade with TAK1 as both kinases mediate AP-1 and NF κ B activation as well as TNF production in R848-challenged human primary macrophages. Furthermore, Btk-dependent TNF super-induction was abrogated in the presence of dominant-negative TAK1. TAK1 is known to be activated by TRAF6 which in turn is activated by the Myd88/IRAK pathway in LPS-challenged cells (Landstrom 2010). Btk, on the other hand, has been shown to interact with Myd88, TLR4 and IRAK1 but not TRAF6 (Jefferies et al. 2003) suggesting that it could be located upstream of TAK1. To confirm this hypothesis, the effect of Btk downregulation on TAK1 or TRAF6 activation could be investigated thus adding more detail to a potential Btk-TAK1-NF κ B pathway.

6.2.2 The role of Btk in transcription factor activation

From this study it is clear that Btk regulates p65-mediated *TNF* transactivation in R848-stimulated human primary macrophages. One of the Btk-dependent mechanisms which facilitates p65 nuclear translocation and recruitment to *TNF* is the phosphorylation of p65 on Ser536. Further studies should be conducted to determine whether Btk affects other p65 activation events. The transactivation activity of NF κ B downstream of I κ B degradation has been found to be regulated on several levels. The phosphorylation of p65 at Ser276 by protein kinase A (PKA) or by mitogen- and stress-activated protein kinases MSK1 and MSK2 is one example (Zhong et al. 1998; Vermeulen et al. 2003). This particular phosphorylation event has been found to promote the interaction of p65 with the transcription co-activators p300 and CREB-binding protein (CBP) (Zhong et al. 1998). Similarly, the phosphorylation of p65 at Ser536 by IKK α and IKK β has been found to be required for p300/CBP association (Sakurai et al. 2003; Chen et al. 2005). PKC ζ can phosphorylate p65 at Ser311 and PKC ζ deficiency blocks p65 interaction with the co-activator

CBP as well as Pol II recruitment to the *IL-6* promoter in response to TNF (Duran et al. 2003). The association with the co-activator CBP/p300 and histone acetyltransferases (HATs) results in acetylation of p65 at Lysine 310 and promotion of target NFκB gene transcription (Chen et al. 2002b; Hayden and Ghosh 2008). Future experiments should focus on deciphering whether Btk interacts with any of the kinases that have been implicated in the transcriptional activity of p65 or whether Btk phosphorylates p65 itself. The outcome would not only explain the role of Btk in NFκB signalling but also shed more light onto general control mechanisms of *TNF* transcription in TLR signalling.

Studies presented in this thesis have shown a role for Btk in the regulation of AP-1 activation and also provided insight into the mechanism of this regulation. Although Btk has been found to regulate total cJun recruitment to *TNF*, further ChIP analysis should be performed to assess whether the recruitment of phospho-cJun to *TNF* is also affected. If so, this would provide a basis for further validation of the co-repression hypothesis. Firstly, the presence of NCoR at the human *TNF* gene has to be confirmed. It would be then interesting to examine if Btk regulates NCoR clearance from the cJun-cJun co-repressor complex in R848 signalling. This could be achieved by performing NCoR ChIP in the presence of Btk knockdown.

p65 bound to the *inos* promoter has been found to recruit IKKε which phosphorylates cJun on Ser63 and Ser73 upon LPS stimulation (Huang et al. 2009). The interaction between p65 and IKKε is dependent on phosphorylation of p65 on Ser536. Studies should be conducted to establish whether Btk affects IKKε-mediated cJun activation as Btk regulates p65 recruitment to *TNF* as well as p65 phosphorylation. Furthermore, cJun-binding sites are located in close proximity to κB site 3 in the *TNF* promoter; this is a prerequisite for the AP-1-NFκB collaboration. This question could be answered by examining the effect of Btk knockdown on phospho-cJun recruitment to *TNF* in the presence of inhibited or down-regulated p65.

Finally, there is a possibility that Btk itself could be found in the DNA-binding complexes. The kinase has been found to constantly shuttle between the nucleus and the cytoplasm (Mohamed et al. 2000). Moreover, the kinase interacts with the transcription factors TFII-I, STAT5A and Bright (Webb et al. 2000; Mahajan et al. 2001; Rajaiya et al. 2006). TFII-I is a general transcription factor whose binding sites in *TNF* can be identified using the MatInspector programme. Confocal microscopy could be used to establish whether Btk shuttles to the nucleus upon R848 stimulation. An adenovirus expressing Btk conjugated with GFP is available in house and can be used for

imaging of Btk cellular localisation in human macrophages. Subsequent immunoprecipitation of Btk-TFII-I complexes would then provide further evidence for Btk direct involvement in gene transcription.

6.2.3 Mechanism of Btk-dependent regulation of IL-6 expression

Btk has been found to regulate IL-6 production in R848-stimulated human primary macrophages. However, the effect of Btk downregulation on IL-6 was largely delayed as opposed to TNF indicating that there are differential mechanisms of Btk mediated induction of IL-6 expression as compared to TNF expression. The *IL6* gene, in contrast to *TNF*, is considered as a secondary response gene as it is transcribed later than primary response genes due to the limited chromatin accessibility and the requirement for new protein synthesis (Saccani et al. 2001). Although the mechanisms that distinguishably regulate IL-6 expression are poorly understood, it is clear that factors involved in the induction of the *IL6* promoter activity include NFκB, AP-1, NF-IL6, C/EBPβ and CREB (Keller et al. 1996; Hershko et al. 2002). Since Btk has been implicated in NFκB and AP-1 activation, it is possible that the kinase regulates *IL6* transcription in R848 signalling. However, as Btk depletion decreased IL-6 levels after as late as 8h post R848 stimulation (2h for TNF) it is more likely that Btk affects other stages of IL-6 gene expression. It would be also interesting to see the effect of Btk downregulation on Brahma-related gene1 (Brg1) recruitment to the *IL6* promoter. Brg1 is a subunit of switch/sucrose nonfermenting (SWI/SNF) complexes which are required for nucleosome remodelling and chromatin accessibility to transcription factors (Ramirez-Carrozzi et al. 2006). Brg1 has been shown to be recruited to secondary response genes such as *IL6* upon LPS stimulation in human primary monocytes (Chen and Ivashkiv 2010). Alternatively, Btk may affect *IL-6* mRNA stability following R848 stimulation similarly to the way it is able to regulate *TNF* message stability in LPS-stimulated macrophages (Horwood et al. 2003).

6.2.4 Btk as a drug target in RA

Btk inhibition has been considered as an attractive treatment approach against B cell diseases and a small molecular inhibitor PCI-32765 is now undergoing clinical evaluation for the treatment of B-cell lymphoma (Honigberg et al. 2010). In the recent publication by Di Paolo and colleagues, Btk specific inhibition by CGI1746 blocked BCR-dependent B cell proliferation and autoantibody

production as well as FcγRIII-dependent TNF, IL-6 and IL-1 production in macrophages derived from rodent arthritis models (Di Paolo 2010). Further experiments are required to validate if Btk inhibition ameliorates RA in humans. For instance, the effect of Btk downregulation with siRNA on spontaneous release of pro-inflammatory cytokines from RA synovial cultures could be first investigated. The design of selective Btk inhibitors would then allow the investigation of the effect of anti-Btk therapy in RA and other autoimmune diseases in larger preclinical and clinical studies.

6.2.5 Implication of future work

In conclusion, the future objectives outlined above will add more detail into not only the role of tyrosine kinases in controlling cytokine production but also into understanding the molecular mechanisms that control TNF production under inflammatory conditions. The development of advanced molecular techniques such as ChIP has already accelerated the process of understanding gene regulatory networks including the interplay between co-repressor and co-activator complexes as well the factors involved in chromatin remodelling. The current project has proven that by investigating the role of one tyrosine kinase in cytokine production, it is possible to reveal unknown mechanisms that control cytokine gene expression. For instance, the current results have exposed a differential kinetics of AP-1 component recruitment to human *TNF* upon TLR8 engagement. Future studies would therefore shed more light into the role of Btk in *TNF* transcription as well as provide novel drug targets whose inhibition could be more specific and cost-effective.

Chapter 7

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