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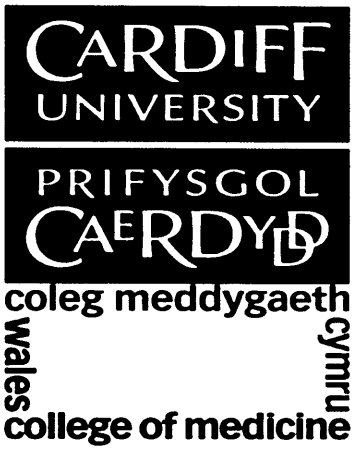

REGULATION OF INTERLEUKIN-18 SIGNALLING IN DENDRITIC CELLS

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Thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy

Ph.D. 2008

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Dedicated to:

Yannis Vallianos

For always being there for me
And for his love, motivation and true belief in me

And to my family:

Eleni, Dimitrios and Haralambos Koutoulakis

Thank you for your unconditional love, support and encouragement in everything I do

I love you all, and could not have achieved this without you.
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Abstract

This study demonstrates the mechanism of regulation of IL-18 function by the pro- and anti-inflammatory cytokines, TNFα and TGFβ1 respectively. The importance of the antagonistic interaction between these two cytokines, to control IL-18 signalling, has been demonstrated using an *in vitro* model of dendritic precursor cells, which have the ability to produce IFNγ and potentially mature upon stimulation with IL-18. The ability of TNFα to sensitise the cells and promote IL-18-induced IFNγ production was suppressed in the presence of TGFβ1, in part via a mechanism of IL-18 receptor regulation. TNFα stimulation increased the levels of both mRNA and surface protein of IL-18 receptor, whereas the addition of TGFβ1 resulted in 50% reduction of the surface expression of the receptor. Further work confirmed the counter effects of these cytokines on IL-18 signalling, through p38 MAPK activation and T-bet expression. The importance of TNFα and TGFβ1 in controlling the maturation process of dendritic cells, by regulating their early IL-18-induced IFNγ production, led to the hypothesis that blocking IL-18 could dampen Th1 immune response in chronic inflammatory conditions, through the regulation of dendritic cell maturation. Therefore, a soluble recombinant human heterodimeric receptor was generated and was confirmed to bind strongly to IL-18. Preliminary *in vitro* work showed that this soluble decoy receptor was active and able to suppress IL-18 function. Further studies to investigate the effects of this receptor *in vivo* may lead to the development of a potential anti-cytokine therapy for chronic inflammation.

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List of Abbreviations

γc	cytokine receptor common γ subunit
A_{260}	Absorbance at 260nm
A_{280}	Absorbance at 280nm
aa	Amino acids
ANOVA	Analysis of variance
AP-1	Activator protein-1
APC	Antigen presenting cells
ATP	Adenosine triphosphate
BMP	Bone morphogenic protein
bp	Base pair
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary DNA
CHO	Chinese hamster ovarian cell
CIA	Collagen-induced arthritis
CLP	Common lymphoid progenitors
CMP	Common myeloid progenitor
cpm	Counts per minute
DC	Dendritic cell

DMEM	Dulbecco's modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribonucleotide triphosphates
DTT	Dithiothreitol
ECL™	Enhanced chemiluminescence
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-related kinase ½
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
Fc	Fragment crystallizable
FITC	Fluoroisothiocynate
g	Gravitational force
GM-CSF	Granulocyte macrophage colony-stimulating factor receptor
GTP	Guanidine triphosphate
HLA-DR	Human leukocyte antigen-DR
HPC	Haematopoietic progenitor cells
HRP	Horseradish peroxidase
HSC	Haematopoietic stem cells
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecules

ICE	IL-1-B converting enzyme
ICSBP	IFN-consensus sequence-binding protein
iDC	Immature dendritic cell
IFNγ	Interferon-γ
IFNγR	Interferon-γ receptor
IGFBP	Insulin-like growth factor binding protein
IKK	IκB kinase
IL	Interleukin
IL-18	Interleukin-18
IL-18BP	IL-18 binding protein
IL-18R	Interleukin-18 receptor
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRAK	IL-1 receptor associated kinase
JNK	c-jun N-terminal kinase
kb	Kilobase
Kd	Dissociation constant
kDa	Kilodaltons
KO	Knock out
LAP	Latency associated peptide
LB	Luria broth
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoarrtactant protein-1
MCS	Multiple cloning site
mDC	Mature dendritic cell

MEK	MAPK/ERK kinase
MEK	Mitogen-activated kinase
MHC I/II	Major histocompatibility complex I/II
min	minutes
MIP	Macrophage-inflammatory protein
Mo-DC	Monocyte derived dendritic cell
mRNA	Messenger ribonucleic acid
MS	Multiple Sclerosis
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide or Methylthiazolyldiphenyl-tetrazolium bromide
MyD88	Myeloid differentiation primary response gene 88
NFκB	Nuclear factor-kappa B
NIK	NFκB-inducing kinase
NK	Natural killer
NKT	Natural killer cell
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PKC	Protein kinase C
PMN	Polymorphonuclear neutrophils
PPR	Pattern recognition receptors
Pre-DC	Precursor dendritic cell
Pro-IL18	Precursor of IL18
RA	Rheumatoid Arthritis

RAG	Recombination activating gene
rh	Recombinant human
rm	Recombinant mouse
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
R-SMAD	Receptor regulated SMAD
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second
SGBS	Simpson-Golabi-Behmel syndrome
shIL-18R-Fc	Soluble human Interleukin-18 receptor Fc
siRNA	Small interfering RNA
STAT	signal transducer and activator of transcription
TBE	Tris-Borate ethylenediaminetetraacetic acid
T-bet	T-box expressed in T cells
TBS	Tris buffered saline
TCR	T cell receptor
TEMED	Tetra methylethylenediamine
TGFβ1	Transforming growth factor-β1
TGFβR	Transforming growth factor beta receptor
Th	T helper
Thr	Threonine
TIR	Toll-IL-1 receptor

TM	Transmembrane
TNFR	Tumor necrosis factor receptor
TNFα	Tumour necrosis factor α
TRAF-6	TNFR-associated factor-6
TReg	Regulatory T cells
Tyr	Tyrosine
v/v	Volume/volume
w/v	Weight/volume
WT	Wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

Chapter 1

1 General Introduction

1.1 Principles of Innate and Adaptive Immunity

The principles of immunity were acknowledged back in ancient civilisations, where Thucydides wrote about the plague of Athens during the Peloponnesian War in 430BC, and noted that people who had recovered from a previous attack of the disease could foster the sick, without being infected for a second time. However, the origin of immunology as a scientific theory is relatively new, and arose quite recently with the discovery of vaccination by Edward Jenner (1796). Nowadays, the rate of development in the field has revealed the complexity of the immune system and the major goal of research in immunology is the regulation of immune responses, i.e. whether to suppress them, when unwanted or to stimulate them, when necessary to fight infectious diseases.

1.1.1 Bacterial infection induces inflammation by activating innate immunity

As soon as pathogens enter the epithelial surfaces, which are considered to be the first line of defence against infection, they are recognised, ingested and killed by macrophages and neutrophils, through the production of phagolysosomes and respiratory burst (Hampton et al., 1998). Tissue damage and cytokine release in response to lipopolysaccharide (LPS), induce an inflammatory response, where inflammatory cells such as neutrophils, monocytes, eosinophils and lymphocytes enter the site of infection to initiate killing of pathogens. The activation of the kinin system increases vascular permeability for the complement system of plasma proteins (complement components and anaphylatoxins) to enter from circulation and start bacterial killing, through osponisation of pathogens and recruitment of inflammatory cells (Lambris et al., 2008). At the same time the coagulation system is triggered in response to damage in blood vessels and leads to the formation of a clot that prevents any microorganisms to enter the blood stream (Sun, 2006). Cytokines of the early immune response induce the expression of co-stimulatory signals on

macrophages and dendritic cells, enabling them to prime lymphocytes and initiate the slower and more specific adaptive immune response.

1.1.2 Activation of specialised antigen presenting cells bridges innate and adaptive immunity

Dendritic cells (DCs) are the most potent specialised antigen presenting cells (APCs). These cells function as effector cells in innate immunity, play a role in the induction of peripheral immunological tolerance and activate adaptive immunity by taking up bacterial antigens at the site of infection and presenting them to T cells; thereby inducing their clonal expansion and their differentiation into effector cells regulating the types of T cell responses (Banchereau and Steinman, 1998).

The functional diversity of DCs depends on the different DC subsets and lineages and on the functional plasticity of DCs at the immature stage. The $CD34^+$ haematopoietic stem cells (HSC) in the bone marrow differentiate into common myeloid progenitors (CMP) and common lymphoid progenitors (CLP). The CMPs further differentiate into $CD34^+CLA^+$ and $CD34^+CLA^-$ that become $CD11c^+CD1a^+$ Langerhans immature DCs (iDCs) upon their migration to epidermis, and $CD11c^+CD1a^-$ interstitial iDCs upon their migration to dermis and other tissues, respectively (Strunk et al., 1997, Ito et al., 1999). There are also another two types of DC precursor cells (pre-DCs) that come from the HSCs during haematopoiesis; the CMP-derived monocytes (myeloid pre-DC1) and the CLP-derived plasmacytoid cells (lymphoid pre-DC2) (Liu et al., 2001b).

DCs are the most potent activators of naïve T cells. Upon encountering an antigen (Ag), immature DCs residing in the peripheral tissues migrate to lymphoid organs, where they mature into professional antigen-presenting cells capable of the efficient activation of T cells. Pattern recognition receptors (PPRs), such as Toll-like receptors (TLRs), expressed by immature DCs (iDCs) facilitates microbial antigen (Ag) recognition and induction of their maturation process (Kaisho and Akira, 2001). Maturing DCs rapidly lose their endocytic ability, undergo an increase in the

expression and stability of major histocompatibility complex class (MHC) I- and II-peptide complexes, up-regulate expression of adhesion and co-stimulatory molecules (CD40, CD54, CD80, CD83 and CD86) and secrete pro-inflammatory cytokines such as interleukin (IL)-18, IL-1, IL-12, IL-6 and IL-23 that facilitate conversion of naïve T cells into effector cells. Maturing DCs express chemokine receptors, such as CCR7, that drive their migration into T cell areas of the draining lymph nodes, where they screen for antigen-specific naïve T cells and stimulate them to initiate primary immune responses and the differentiation into armed effector T cells (Sallusto and Lanzavecchia, 2000).

There are two groups of signals that activate iDCs to promote T helper (Th) 1, Th2 Th17 or Treg cell differentiation. Pathogenic molecules such as LPS, bacterial CpG DNA and double stranded viral RNA, along with T cell signals such as CD40 ligand and interferon (IFN)-γ, are signals that activate iDCs to produce IL-18 and IL-12 in order to drive Th1 cell responses or IL-1, IL-21, IL-23, TGFβ and IL-6 to induce Th17 cell responses (Mills, 2008). Conversely, inhibition of iDC maturation by the anti-inflammatory molecules TGFβ1, IL-10, prostaglandin E-2 (PGE-2) and corticosteroids, suppress IL-12 production driving Th2 or regulatory T (TReg) cell responses (Kaliński et al., 1999).

1.2 Cytokine Networks Drive T Cell-Mediated Immunity

1.2.1 Cytokines determine lineage commitment of T helper cells and their effector functions

The Th1-Th2 theory, which has hitherto supported the adaptive $CD4^+$ T effector cell responses (Mosmann and Coffman, 1989, Murphy and Reiner, 2002), has been recently revisited. Depending on the cytokine milieu, $CD4^+$ T helper (Th) precursor cells (Th0 or naïve $CD4^+$ T cells) differentiate into the three lineages of Th1, Th2 and the recently identified Th17 cells, all three of which produce different cytokines to facilitate the adaptive immune response. Th17 is the third lineage of $CD4^+$ T cells that has been recently identified (Bettelli et al., 2006) and is the only additional subset of effector $CD4^+$ T cells to be described since the original discovery of Th1 and Th2 cells. Th17 cells produce distinct effector cytokines such as IL-17 (or IL-17A), IL-17F, and IL-6, and promote clearance of pathogens not targeted by Th1 and Th2. Th17 cells are distinct from Th1 and Th2, but similar to TRegs they require TGFβ for their development and action (Weaver et al., 2006).

Differentiation of Th1 cells, which are important for the clearance of intracellular pathogens, release IFNγ and IL-12 effector cytokines (Hsieh et al., 1993, Scharton and Scott, 1993), whereas Th2 cells that are responsible for clearing parasites, produce IL-4, IL-5, IL-10 and IL-13 (Mosmann and Coffman, 1989, Min et al., 2004, Shinkai et al., 2002). IFN-γ produced by Th1 cells promotes the differentiation of Th0 to Th1 cells, and inhibits the proliferation of Th2 cells. On the contrary, IL-4 produced by Th2 cells can drive Th2 cell proliferation, and IL-10 can indirectly inhibit the secretion of IFNγ by Th1 cells (Fitch et al., 1993).

1.2.2 Interferon-γ production influences the functional differentiation of $CD4^+$ T cells and the commitment to Th1 lineage

As described above, interferon (IFN)-γ is a critical Th1 cytokine that induces the differentiation of naïve $CD4^+$ T cells into Th1 effector cells that mediate cellular

immunity against infection. Mice deficient in IFNγ are highly susceptible to both intracellular bacterial infections, such as Listeria (Harty and Bevan, 1995), Salmonella (John et al., 2002), and viral or protozoan infections (i.e. Toxoplasma and Leishmania) (Dalton et al., 1993, Huang et al., 1993, Jouanguy et al., 1999). Decreased levels of IFNγ production resulting from gene deficiencies in IL-12, IL-23 and IFNγ pathways are associated with increased susceptibility to mycobacterial and Salmonella infections in humans (Filipe-Santos et al., 2006, Casanova and Abel, 2002, de Jong et al., 1998). IFNγ is also involved in protection of the host against tumour growth (Ikeda et al., 2002, Rosenzweig and Holland, 2005). Apart from the protective role of IFNγ in host defence, the aberrant up-regulation of IFNγ has been associated with the pathogenesis of chronic inflammatory and autoimmune diseases, such as inflammatory bowel disease (IBD), multiple sclerosis (MS), and diabetes Mellitus (Bouma and Strober, 2003, Neurath et al., 2002, Skurkovich and Skurkovich, 2003).

During immunological synapse, i.e. when an APC comes in contact with a lymphocyte, IFNγ binds to its receptor on the surface of the cells and induces STAT signalling. In $CD4^+$ T cells, phosphorylation and nuclear translocation of STAT-1, together with TCR and CD28 co-stimulatory signals, induces T-bet transcription factor expression (Lighvani et al., 2001), which is important in driving Th1 differentiation (Szabo et al., 2000). T-bet-deficient mice lack Th1 cells and exhibit a large Th2 compartment (Szabo et al., 2002). T-bet induces two transcription factors, Hlx (Mullen et al., 2002) and Runx3 (Djuretic et al., 2007) to regulate IFNγ transcription. T-bet and Runx3 bind to IFNγ promoter to induce IFNγ transcription, but they also bind to the IL-4 silencer to facilitate commitment to Th1 lineage (Djuretic et al., 2007). Hlx promotes chromatin accessibility at the IFNγ promoter (Mullen et al., 2002) and facilitates the expression of the IL-12Rβ2 chain (Afkarian et al., 2002). Binding of IL-12 (p35 and p40) to IL-12Rβ1 and IL-12Rβ2, subsequently induces phosphorylation of Jak2/Tyk2 signalling and nuclear translocation of STAT4 (Trinchieri et al., 2003), which is able to induce IL-18R (Ahn et al., 1997, Nakahira et al., 2001), thereby conferring responsiveness to IL-18

by mature Th1 cells. IL-12 also signals through p38 MAPK, and loss of its activity in T cells by using a specific pharmacological inhibitor or p38 MAPK deficiency inhibits IFNγ production in response to IL-12, but not in response to T cell receptor (TCR) stimulation (Berenson et al., 2006). The IL-12-driven component of Th1 immune response results in effector cells that produce IFNγ through either TCR-dependent or TCR-independent (IL-12 and IL-18) pathways (Robinson et al., 1997, Yang et al., 1999). The combination of IFNγ, IL-12 and IL-18 signals can optimise activation and expansion of Th1 cells (Grogan and Locksley, 2002, Ho and Glimcher, 2002, Murphy and Reiner, 2002).

1.2.3 IFNγ-inducing cytokine Interleukin-18

As described above, the production of IFNγ is induced by IL-18 in synergy with other Th-1 pro-inflammatory cytokines such as IL-12, IL-2, IL-15 and IL-23 (Robinson et al., 1997, Okamoto et al., 2002, Nakahira et al., 2002, Okazawa et al., 2004). IL-18 is a member of the IL-1 superfamily (Dinarello, 1999), together with IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra) and the recently described IL1F5 to IL-1F10 cytokines (Dinarello, 2004, Gracie et al., 2003). IL-18, previously known as IFNγ-inducing factor (IGIF), was initially described as an unidentified soluble factor present in the serum of *Mycobacterium bovis* BCG-infected mice challenged with lipopolysaccharide (LPS). This cytokine was able to induce high levels of IFNγ in cooperation with IL-2 in macrophage-depleted murine splenocytes (Nakamura et al., 1989). The cloning of IL-18 from a murine liver cell library generated from heat-killed *Propionibacterium acnes* and LPS challenged animals, has resulted in the sequencing of a 192 amino acid precursor polypeptide lacking a conventional signal peptide and a 157 amino acid mature protein that had the ability to induce IFNγ production in T cells (Okamura et al., 1995b). Cloning of the human IL-18 from normal human liver cDNA libraries has revealed 65% homology with the murine IL-18 and subsequent expression of the cloned cDNA in *E. coli* has resulted in the purification of the rhIL-18 that also induced IFNγ production in mitogen-activated PBMCs (Ushio et al., 1996).

1.2.3.1 Role of IL-18 in host defence and disease

Studies utilising IL-18 deficient mice (Takeda et al., 1998, Wei et al., 1999) have demonstrated the important role of IL-18 in host defence and chronic inflammation. IL-18 knock-out (ko) mice have demonstrated increased susceptibility to *Leishmania major* (Wei et al., 1999, Ohkusu et al., 2000), *Streptococcus pneumoniae* (Lauw et al., 2002) *Mycobacterial* (Sugawara et al., 1999), *Plasmodium berghei* (Singh et al., 2002) and *Cryptococcus neoforms* (Kawakami et al., 2000) infection. In the animal model of Collagen induced arthritis (CIA), the incidence and severity of disease was significantly decreased in IL-18 ko mice compared to the heterozygote or wild type (wt) counterparts (Wei et al., 2001). Similar result was obtained with the experimental model of MS of autoimmune encephalomyelitis (Shi et al., 2000). In these two studies protection has been attributed to the impaired capability of leukocytes to produce tumour necrosis factor (TNF)-α and IFNγ due to the IL-18 deficiency. Mice deficient in IL-18 have reduced NK cell response and lower levels of IFNγ production (Takeda et al., 1998). Mice deficient in IL-18R also demonstrated defective NK cell activity, showed decreased levels of IFNγ production and impaired Th1 development (Hoshino et al., 1999). Loss of IL-18 was also beneficial for animals with experimental autoimmune diabetes (Lukic et al., 2003) and with 2,4,6-trinitrobenzene sulfonic acid-induced colitis (Kanai et al., 2001).

1.2.3.2 Regulation of IL-18 expression and production

The transcriptional regulation of IL-18 gene expression has been reviewed by Gracie *et al.*, 2003. The first 2 of the 7 exons that comprise the IL-18 gene are non-coding. The promoter activity upstream of exon 2 acts constitutively, whereas that of exon 1 is up-regulated by LPS in activated macrophages and T cell lines (Tone et al., 1997). As opposed to all the other cytokines, the two promoters of IL-18 are TATA-less and G+C poor type, which could explain why IL-18 is expressed in various cell types, even non-immune cells. The 3'-untranslated region (UTR) of human IL-18 mRNA lacks the AUUUA destabilisation sequence, which could be responsible for the constitutive mRNA expression of IL-18 in PBMCs and mouse splenic macrophages

(Puren et al., 1999). The IFN-consensus sequence-binding protein (ICSBP) and PU.1 transcription factors have been identified upstream of exon 1 and 2, respectively and are important for the activation of IL-18 promoter, as they are found to be up-regulated by IFNγ (Kim et al., 1999, Weisz et al., 1994, Sharf et al., 1997, Shackelford et al., 1995). Activator protein 1 (AP-1) and ICSBP elements are also induced by IFNγ stimulation of macrophages (Kim et al., 2000b).

IL-18 requires post-translational enzymatic processing in order to be biologically active. It is produced as a 24kDa inactive precursor (pro-IL-18) that lacks a signal peptide. Pro-IL-18 is cleaved by IL-1β-converting enzyme (ICE; caspase-1) after Asp35 to generate the 18kDa biologically active mature form of IL-18 (Ghayur et al., 1997, Gu et al., 1997). The importance of IL-18 activation by ICE is demonstrated in splenocytes isolated from ICE knock-out mice which do not produce IFNγ production upon LPS stimulation (Fantuzzi et al., 1998). Additionally, mice deficient in ICE are protected from ischemic acute renal failure (Melnikov et al., 2001). Similar to IL-1β, another mechanism of IL-18 secretion is through the ATP regulated P2X-7 receptor, as P2X-7R-deficient macrophages do not produce IL-18 upon ATP stimulation (Perregaux et al., 2000). DCs constitutively produce IL-18 upon T cell interaction via a CD40 signal, since an agonistic antibody against CD40 also stimulates IL-18 secretion (Gardella et al., 1999). Proteinase-3 release of active IL-18 has also been implicated in regulating IL-18 secretion in the presence of LPS in IFNγ primed oral epithelial cells (Sugawara et al., 2001).

1.2.3.3 IL-18 receptor and signalling

The IL-18 receptor consists of an IL-18Rα chain responsible for extracellular binding and an IL-18Rβ chain responsible for signal transduction (Torigoe et al., 1997, Hoshino et al., 1999, Parnet et al., 1996). The IL-18Rα and IL-18Rβ chains both belong to the IL-1R super-family and have an extracellular domain consisting of Ig-folds (~340aa), a signal peptide (~15aa), an intracellular domain (~200aa) that is homologous to the cytosolic motif of the Drosophila Toll protein and a transmembrane domain (~220aa) (Parnet et al., 1996, Born et al., 1998, Debets et al., 2000). The presence of IL-18 activates its receptor through the heterodimerisation of the two structurally related but distinct Ig-like chains, to form a complex required for IL-18 signal transduction (Born et al., 1998, Debets et al., 2000). A study by (Kato et al., 2003) using NMR spectroscopy and mutants for the 50 surface-exposed residues of IL-18, has revealed two residues on IL-18 (sites I and II) that are important in binding of IL-18 to IL-18Rα (Fig.1.1) and a third one that is involved in the cellular response and potentially binds to IL-18Rβ but not to IL-18/IL-18Rα. Another study looking at the carbohydrate recognition activities of IL-18 in KG-1 cells revealed the presence of a single GPI-anchored protein, CD48, that forms a complex with IL-18/IL-18Rα and immediately binds to IL-18Rβ to induce IFNγ production (Fukushima et al., 2005).

IL-18Rα is expressed on Th1, NK cells, as well as macrophages, dendritic cells, neutrophils, basophils, endothelial cells, smooth muscle cells, synovial fibroblasts, chondrocytes and epithelial cells (Yoshimoto et al., 1998, Nakamura et al., 2000, Gerdes et al., 2002, Leung et al., 2001, Möller et al., 2002, Sims, 2002, Gutzmer et al., 2003). Upon binding of IL-18 to IL-18Rα, IL-18Rβ is recruited to form a higher affinity signalling complex (Fig. 1.2) (Kim et al., 2001b, Debets et al., 2000). The approximation of the cytoplasmic Toll-IL-1 receptor (TIR) domains of the receptors triggers signal transduction (O'Neill, 2000), via the recruitment of the myeloid differentiation (MyD)-88 adaptor molecule and auto-phosphorylation of the IL-1R-associated kinase (IRAK) (Wesche et al., 1997, Kanakaraj et al., 1999, Adachi et al.,

1998). IL-18, thus, shares common downstream signalling pathways with important regulatory molecules, such as Toll-like receptor (TLR) that are implicated in regulating IL-18 expression (Seki et al., 2001, Akira et al., 2001), providing feedback loops to control IL-18 function. IRAK then dissociates from the receptor complex to interact with the adaptor protein tumour necrosis factor receptor (TNFR)-associated factor 6 (TRAF) (Cao et al., 1996, Kojima et al., 1998). Phosphorylation of NFκB-inducing kinase (NIK) and rapid degradation of IκB, through activation of IκB kinases 1 and 2 (IKK1/2), result in the release of the p50 and p65 components of NFκB and its subsequent nuclear translocation (Robinson et al., 1997, Matsumoto et al., 1997) to regulate IFNγ expression, as shown in KG-1 cells (Kojima et al., 1999).

Additional signalling pathways have been described in IL-18 stimulated human NK92 cells through the activation of p38 MAPK and extracellular signal-related kinases p44 and p42 (ERK1/2) (Kalina et al., 2000, Yang et al., 2001, Wyman et al., 2002, Lee et al., 2004, Shapiro et al., 1998). IL-18 has also been shown to signal through MAPK-ERK kinase 4 (MEKK4) in Th1 cells (Yang et al., 2001). Additionally, $Tyk2^{-/-}$ mice demonstrated decreased NK cell activity and IFNγ production in response to IL-18 implicating an alternative signalling pathway similar to IL-12 (Shimoda et al., 2002).

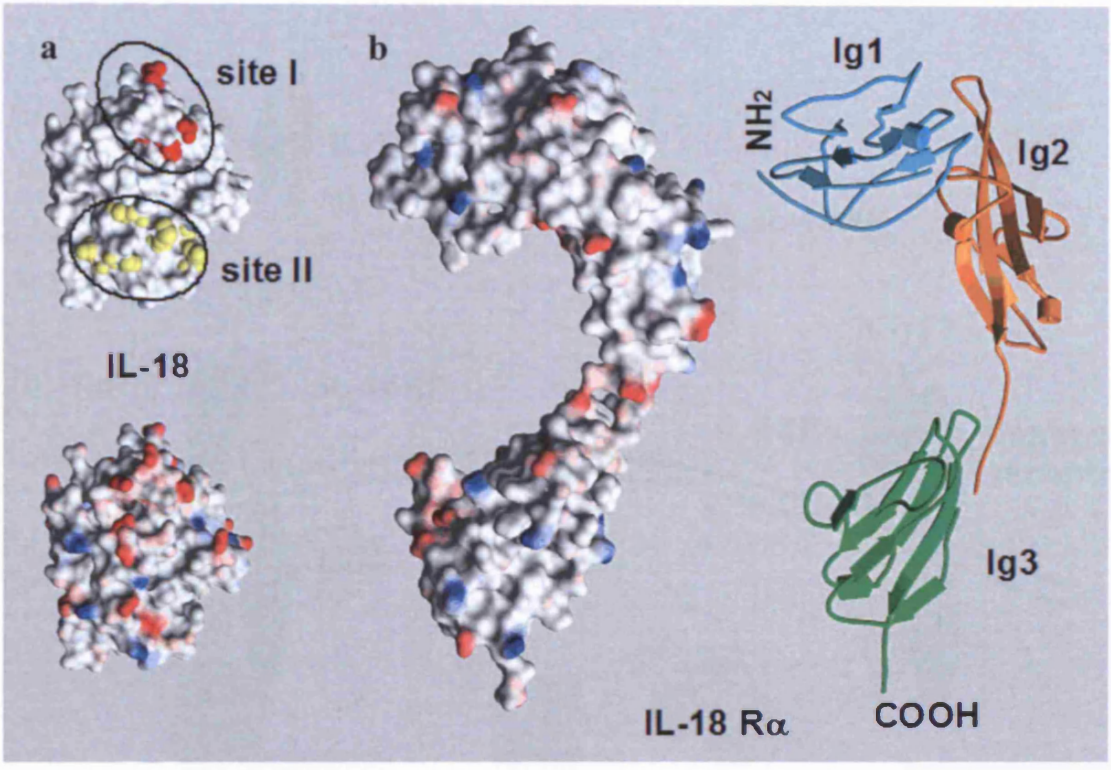


Figure 1.1: Modelled crystal structures of IL-18 and IL-18Rα.

Crystal structure of IL-18 (NMR derived from structure PDBj 1j0s) (**a**). Surface representation of IL-18 site I and II residues responsible for binding IL-18Rα. IL-18Rα model based on IL-1β-IL-RI structure (EMBL-EBI entry 1itb) (**b**). Ribbon drawing of the NMR structure of IL-18Rα showing the corresponding Ig-like domains that constitute its extracellular domain Distribution of the electrostatic potential of IL-18 (**a**, bottom) and IL-18Rα (**b**, left), where blue corresponds to residues with positive potential and red to negative potential residues (figure provided by Dr. K. Beck).

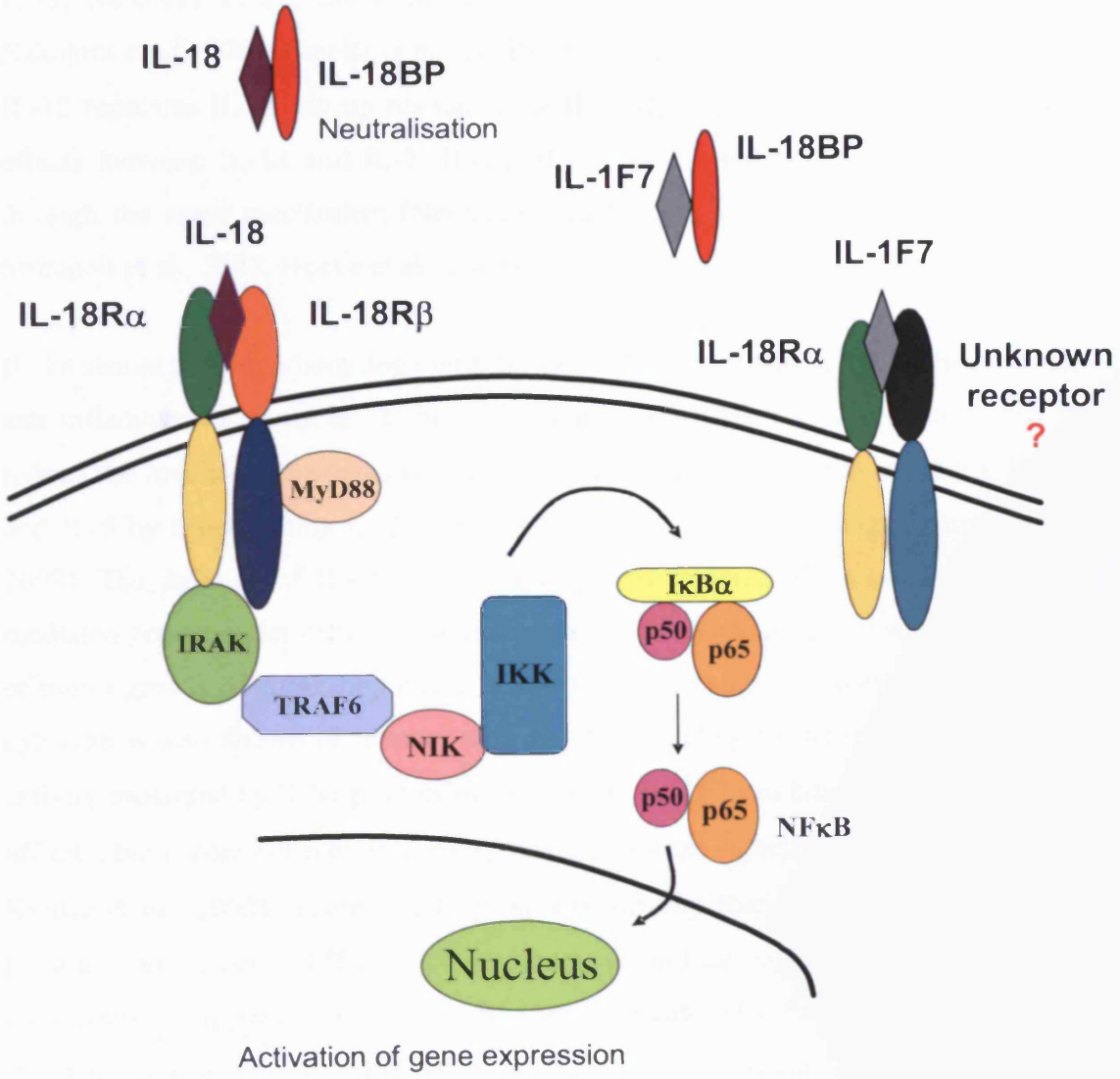


Figure 1.2: Regulation of IL-18 signalling pathway

The expression of IL-18Rα and IL-18Rβ chains is up-regulated by cytokine signals such as IL-2 and IL-12, but inhibited by IL-4 (Yoshimoto et al., 1998, Hoshino et al., 1999, Sareneva et al., 2000, Smeltz et al., 2001, Neumann and Martin, 2001, Nakahira et al., 2001, Smeltz et al., 2002). It has been extensively demonstrated that IL-12 regulates IL-18 via up-regulation of IL-18Rβ expression, and the synergistic effects between IL-18 and IL-2, IL-12, IL-15, IL-21 and IL-23 can be explained through the same mechanism (Neumann and Martin, 2001, Strengell et al., 2002, Strengell et al., 2003, Hoeve et al., 2003).

IL-18 shares sequence homology with IL-1F7, which is considered to be member the anti-inflammatory member of the IL-1 family, as it has recently been shown to reduce the levels of LPS-induced pro-inflammatory cytokines such as TNFα, IL-1α and IL-6 by translocating to the nucleus after caspase-1 processing (Sharma et al., 2008). The function of IL-1F7 is currently poorly understood. *In vivo*, adenoviral-mediated gene transfer of IL-1F7 in murine tumours results in significant suppression of tumor growth, demosrating a role in anti-tumor immunity (Gao et al., 2003). This cytokine is also shown to interact with IL-18BP leading to the reduction of IL-18 activity measured by IFNγ production (Fig. 1.2). IL-1F7 can bind IL-18Rα with low affinity, but it does not recruit IL-18Rβ chain to initiate signalling (Bufler et al., 2002, Kumar et al., 2002). Therefore, there is a possibility that IL-18Rα binds to other ligands and recruits different co-receptors to induce signal transduction. This hypothesis is supported by evidence in a mouse model of inflammatory encephalitis that gene deletion of IL-18Rα results in resistance to disease, whereas IL-18 gene deletion does not change susceptibility to disease in these mice (Gutcher et al., 2006).

1.2.3.4 Natural inhibitors of IL-18

There are several natural inhibitors for IL-18, such as soluble IL-18Rα and IL-18 binding protein (BP). The levels of the sIL-18Rα in the human serum are not known but *in vitro* assays have demonstrated that this soluble receptor binds IL-18 with low affinity and is only able to neutralise its function at an 80-fold molar excess of IL-18 and in the presence of IL-18Rβ (Reznikov et al., 2002). Conversely, IL-18BP can neutralise IL-18 activity down to 50% at equimolar concentrations, reaching complete inhibition at a 2-fold molar ratio *in vitro* (Kim et al., 2000a, Kim et al., 2002). In addition to IL-18BP, there are several viral proteins identified that are able to suppress IL-18 activity *in vitro*, such as p13 a homologous protein to IL-18BP that is encoded by *ectromelia poxvirus* (Born et al., 2000), and two *Molluscum contagiosum* viral proteins, MC53/54 (Xiang and Moss, 1999). However, both *in vitro* and *in vivo* studies have demonstrated that IL-18BP inhibits IL-18- and LPS-induced IFNγ production respectively (Novick et al., 1999, Aizawa et al., 1999), indicating that it is a more potent antagonist for IL-18 and it will be discussed in detail below.

IL-18BP is a novel naturally occurring inhibitor that is constitutively expressed and secreted to bind IL-18 with high binding affinity (Kd~400pM) to suppress IL-18-induced IFNγ synthesis and regulate Th1 immune response (Kim et al., 2000a, Novick et al., 1999, Aizawa et al., 1999). IL-18BP binds only mature IL-18 and not pro-IL-18 according to ELISA and plasmon resonance binding studies (Novick et al., 2001, Kim et al., 2000a). Apart from IL-18 it can also bind IL-1F7 (Bufler et al., 2002). IL-18BP is not a soluble form of IL-18Rα protein but it is distinct from IL-1 super-family member proteins, as it comprises of one rather than three Ig-like domains with only one amino acid identity found in the third Ig domain of IL-18Rα (Colotta et al., 1994, Kim et al., 2002). Located on the human chromosome 11q13, the IL-18BP gene is highly expressed in immunologically active tissues such as spleen and intestine, and it encodes for at least four isoforms (IL-18BPa, b, c and d) derived from mRNA splice variants. There are two isotypes of the murine IL-18BP (Kim et al., 2000a). The isoforms that retain their Ig domain intact are the ones that

are able to bind and neutralise IL-18 function, such as human IL-18BPa and c, but not b and d, whereas both murine IL-18BP isoforms c and d can bind and neutralise IL-18 (Kim et al., 2000a). Using mutational studies, two (glutamic acid 35 and lysine 89) residues on IL-18 are identified that are important for binding both IL-18Rα and IL-18BP and result in neutralisation of IL-18 function (Kim et al., 2002, Kim et al., 2001a).

IFNγ is implicated in the transcriptional regulation of IL-18BP transcript and protein release that is confirmed using histone deacetylase inhibitor sodium butyrate in a human colon carcinoma epithelial and a keratinocyte cell line, and in several intestinal cell lines (Paulukat et al., 2001). The promoter of IL-18BP has two IFNγ regulatory elements, indicating a negative feedback loop for the regulation of IL-18-induced IFNγ through the activation of IL-18BP (Hurgin et al., 2002).

IL-18BP is found elevated in several chronic inflammatory and autoimmune diseases such as rheumatoid arthritis (RA), Crohn's disease, hepatitis C and in chronic liver diseases (Corbaz et al., 2002, Möller et al., 2002, Kawashima et al., 2001, Möller et al., 2001, Bresnihan et al., 2002, Kaser et al., 2002, Ludwiczek et al., 2002). The levels of IL-18BPa in healthy human serum are 2.15 ± 0.15 ng/ml (range 0.5-7 ng/ml), whereas in patients with sepsis and acute liver failure they increase to 21.9 ± 1.44 ng/ml (range 4-132 ng/ml) (Novick et al., 1999). The levels of IL-18 in healthy subjects is 64 ± 17 pg/ml and ~85% was in free form, whereas in sepsis the levels of both IL-18 and IL-18BPa are elevated (1.5 ± 0.4 ng/ml and 28.6 ± 4.5 ng/ml respectively), but there is still some free IL-18 in the serum of the patients that is higher compared to the healthy individuals (Novick et al., 1999). This indicates that administration of an exogenous antagonist to inhibit IL-18 could be beneficial in reducing the circulating IL-18 activity.

1.2.4 Pro-inflammatory cytokine Tumour Necrosis Factor-α

Tumor necrosis factor (TNF)-α was initially identified in 1975 as an endotoxin-induced glycoprotein in the serum that caused haemorrhagic necrosis of sarcomas transplanted into mice (Carswell et al., 1975) and the TNFα cDNA was cloned later in 1985 (Pennica et al., 1985). Physiological levels of TNFα play a fundamental role in the host defence to bacterial, viral and parasitical infections, but inappropriate or excessive production contributes to the pathogenesis of autoimmune disorders, such as RA. TNFα together with IL-6 and IL-8 are produced by human synovial cells stimulated with IL-17, implicating the importance of Th17 cells in regulating TNFα to promote inflammation in RA (Fossiez et al., 1996, Kotake et al., 1999).

IL-18 is also found in significant levels in RA synovium (Gracie et al., 1999), where it has the ability to sustain Th1 immune response and promote TNFα production leading to tissue injury. In particular, IL-18 induces CIA by activating and attracting neutrophils through the production of TNFα, which in turn induces the synthesis of leukotriene B4 (LTB4) (Canetti et al., 2003), a well known chemoattractant of neutrophils (Dahlén et al., 1981, Ford-Hutchinson, 1990). Thus, TNFα has been an attractive target for immunotherapy for RA. Monoclonal antibodies (i.e. infliximab and adalibumab) and soluble TNF receptor:Fc fusion protein (i.e. etanercept) have been used in clinical trials for effectively reducing the symptoms and signs of RA. Blockade of TNFα results in amelioration of cartilage or bone damage (Bathon et al., 2000, Lipsky et al., 2000). TNFα therapy is found to be safe for most patients although a higher risk of mycobacterial infection has been implicated (Keane et al., 2001).

TNFα is produced predominantly by activated macrophages and T lymphocytes as a 26kDa type II transmembrane precursor arranged in stable homotrimers. It is displayed on the plasma membrane and is proteolytically cleaved by the metalloprotease TNFα converting enzyme (TACE) between alanine -1 and valine +1, to produce a biologically active 17kDa mature form of TNF (Kriegler et al., 1988)

that exists as a 51kDa trimer in solution (Smith and Baglioni, 1987). Both membrane-bound and soluble form of TNFα can bind to TNF-R1 (TNF receptor type 1; CD120a; p55/60) and TNF-R2 (TNF receptor type 2; CD120b; p75/80) resulting in the release of SODD (Silencer of death domain protein) and the formation of a receptor proximal complex consisting of adaptor proteins TRADD (TNF receptor-associated death domain protein), TRAF2 (TNF receptor-associated factor 2), RIP (receptor-interacting protein) and FADD (Fas-associated death domain protein). These molecules then in turn recruit enzymes such as caspase-8 and IKKβ to the complex where they are activated leading to the activation of NFκB, p38 MAPK and JNK (Wajant et al., 2003).

A small amount of research has also been documented looking at the effect TNFα on IL-18 signalling and function. In T lymphocytes and NK cells, IL-18 directly stimulates the gene expression and synthesis of TNFα (Puren et al., 1998). IL-18 neutralisation during carrageenan-induced acute inflammation *in vivo* completely suppresses TNFα expression (Leung et al., 2001). Recent reports have indicated the role of these two cytokines in the pathogenesis of several inflammatory diseases. IL-18 is present in significant levels in RA synovium, where it promotes Th1 immune responses and TNFα production (Gracie et al., 1999). IL-18 knockout mice show reduced incidence and severity of CIA compared to wild type and are associated with suppressed TNFα production and Th1 immune responses *ex vivo*, which is completely reversed by the administration of rmIL-18 (Wei et al., 2001). In addition, this cytokine promotes the inflammatory response in CIA by activating neutrophils through the production of TNFα, which in turn induces the synthesis of leukotriene B4 (LTB4), resulting in the recruitment of neutrophils at the site of inflammation (Canetti et al., 2003). It has also been shown that IL-18 induces human $CD4^+$ T cell chemotaxis *in vitro* and mononuclear cell recruitment *in vivo* (Komai-Koma et al., 2003). The expression of CD54 on dendritic cells causes naïve T cells expressing LFA-1 (CD11a:CD18 heterodimeric $\alpha_L\beta_2$ integrin) to adhere strongly and cease migration at the site of inflammation. The up-regulation of CD54 induced by IL-18 has been detected on PBMCs (Yoshida et al., 2001). Specifically, monocyte-derived

DCs (Mo-DCs) isolated from human PBMCs express IL-18R and are chemoattracted to IL-18. In these cells, IL-18R is up-regulated by IFNγ but not TNFα, and IL-18 induces CD54 (ICAM-1) expression pronounced by IFNγ stimulation (Gutzmer et al., 2003). In addition, KG-1 cells (Kohka et al., 1998) and RA synovial fibroblasts (Morel et al., 2001) also show increased CD54 expression in response to IL-18.

The biological importance of TNFα as a regulator of immunity has been validated by TNF or TNF receptor deficient studies. It can either contribute to or protect the host from tissue damage in chronic inflammatory conditions. This differential role of TNFα depends on the type of the tissue, the cellular context, the TNF-R expression and the timing and duration of TNFα action *in vivo*.

TNF, IL-1 and IL-10 are shown to increase expression of TNFR2, but down-regulate the expression of TNFR1 (Kalthoff et al., 1993, Winzen et al., 1992, Winzen et al., 1993). TNFR1 deficiency in mice leads to resistance to lethal doses of LPS or *Staphylococcus aureus* enterotoxin B. However, infection with *Listeria monocytogenes* in these mice results in lethality, as they are impaired to clear the pathogen (Pfeffer et al., 1993, Rothe et al., 1993). TNF-deficient mice are protected against cerebral malaria and display increased Th1 immune response, demonstrating the contribution of TNFα in the pathogenesis of parasitic infections through the ICAM-1 (CD54)-dependent recruitment of mononuclear cells (Rudin et al., 1997). On the contrary, local production of TNFα and IL-1β is enhanced by IL-17 in the lungs and resulted in neutrophils recruitment and clearance of *Klebsiella pneumoniae* infection (Ye et al., 2001).

A two-edge role of TNFα *in vivo* is also demonstrated in mouse models of liver regeneration after partial hepatectomy (surgical removal of the 70% of the liver). TNF-R1 knock-out mice show decreased hepatocyte DNA synthesis, indicating that TNFα is involved in liver regeneration (Yamada et al., 1997). Conversely, TNFα through TNF-R1 contributes to liver destruction in models of acute hepatotoxicity (Bradham et al., 1998). Additionally, the differential role of TNFα in neuronal

diseases is prominent as demonstrated in a murine model of retinal ischemia, where TNF-R1 exacerbated tissue damage, whereas TNF-R2 signalling protected the host through activation of PKB/Akt (Fontaine et al., 2002). The complex mechanism of action and pathophysiological responses of TNFα, demonstrated by *in vivo* and clinical studies, reveal the importance of this cytokine in regulating immunity.

1.2.5 Anti-inflammatory cytokine Transforming Growth Factor-β1

TGFβ1 exists as an inactive precursor form of 100kDa produced from the dimerisation of 50kDa monomers. This dimeric precursor is cleaved by furin proteases to yield the 25kDa active TGFβ1 dimer, which remains coupled to the remaining portion of its own pro-form, the so-called latency-associated peptide (LAP, 75kDa) forming the secreted latent form of TGFβ1 (Annes et al., 2003, Nunes et al., 1995). A large latent complex is formed by other proteins binding the latent form of TGFβ, such as latent TGFβ-binding protein (LTBP) or α2 macroglobulin important in targeting TGFβ to the ECM or associated with circulating TGFβ (Annes et al., 2003). Intergrin-family members (Munger et al., 1999, Annes et al., 2004), oxygen or nitrogen free radicals (Vodovotz et al., 1999), and heat (Barcellos-Hoff and Dix, 1996) are involved in the process of dissociation and degradation of LAP proteins to activate latern TGFβ1 by mechanisms that are not fully understood (Massagué, 1990, Annes et al., 2003). Hydrophobic interactions and an inter-subunit disulphide bond stabilises the active form of TGFβ1 in a homodimer (Hinck et al., 1996).

Active TGFβ1 then binds TGFβ-type II receptor (TGF-βRII), which then transphosphorylates the type I receptor (TGF-βRI) to phosphorylate receptor-regulated SMADs (R-SMADs), that is SMAD2 and SMAD3, at two serine residues within their C-terminus. The latter form heteromeric complexes with a common-partner SMAD (Co-SMAD), known as SMAD4 (Shi and Massagué, 2003, Wrana et al., 1994). Accumulation of the SMAD2/SMAD4 and SMAD3/SMAD4 complexes to the nucleus to control gene expression by binding to the promoter regions of

TGFβ1-responsive genes (Hata et al., 1997). The N-terminal regions of SMAD3 and SMAD4 are able to bind directly to the core sequences of GTCT or AGAC of DNA, but SMAD2 does not bind. SMADs bound to the promoter are responsible for maintaining a balance in gene expression by recruiting both transcriptional co-activators and co-repressors (Derynck et al., 1998, Liu et al., 2001a, Prunier et al., 2003, Takeda et al., 2004, Wotton and Massagué, 2001). The TGFβR remains activated for ~3-4 hours following initial phosphorylation and continuous activation of the receptor results in the accumulation of complexes within the nucleus that regulate gene expression (Inman et al., 2002). TGFβ1 signal transduction is also mediated through p38 MAPK activation in a SMAD-independent mechanism (Bhowmick et al., 2001, Yu et al., 2002). TGFβ1 suppresses IFNγ production (Bellone et al., 1995, Bright and Sriram, 1998, Espevik et al., 1987, Pardoux et al., 1999, Sudarshan et al., 1999) partly through the inhibition of T-bet transcription factor expression (Gorelik et al., 2002, Lin et al., 2005, Neurath et al., 2002).

In contrast to pro-inflammatory cytokines, TGFβ1 is the most potent immunosuppressive cytokine that is produced by almost all leukocytes, especially NK cells (Horwitz et al., 1997, Letterio and Roberts, 1998) and it has been shown to play an important role in the regulation of T cell and DC functions (Rubtsov and Rudensky, 2007), as it is a negative regulator of IFNγ production by T and NK cells (Dennler et al., 2002, Letterio and Roberts, 1998). It has recently been documented that IL-18 in combination with IL-12 or IL-15 are able to antagonise the TGFβ1 signalling pathway in NK cells via the down-regulation of TGFβRII, SMAD2, and SMAD3 (Yu et al., 2006). The balance between pro- and anti-inflammatory cytokines correlates with the level of inflammation in chronic inflammatory conditions such as RA, as demonstrated by the levels of TNFα, IL-1β, IL-6 and TGFβ1 in the joint of DBA/1 mice with CIA (Marinova-Mutafchieva et al., 2006). TGFβ1 is thus one of the dominant ant-inflammatory cytokines that ameliorates the inflammatory response induced by the pro-inflammatory cytokines by inhibiting the maturation of DCs and promoting the differentiation of $CD4^+CD25^+Foxp3^+$ TRegs

(Prud'homme and Piccirillo, 2000, Peng et al., 2004, Fantini et al., 2004, Li et al., 2006).

The anti-inflammatory effect of TGFβ1 has also been demonstrated by the group of A. Flavell, who has used dnTGFβRII-transgenic mice using a transgene expressing only the extracellular and transmembrane domains of TGFβRII to block TGFβ1 signalling in NK cells, resulting in the production of large amounts of IFNγ responsible for Th1 development and protection from *Leishmania major* infection (Laouar et al., 2005). TGFβ1 was also shown to inhibit the IL-18-induced IFNγ expression in a murine NK cell line (Hayashi et al., 2003). Additionally, TGFβ1 suppresses IFNγ responses in primary murine $CD4^+$ T cells by inhibiting the phosphorylation of IFNγ-induced Jak-Stat signalling proteins and by inhibiting the induction of the T-bet and IRF-1 transcription factors (Park et al., 2005).

However, IFNγ has also been shown to inhibit TGFβ1 signalling via the up-regulation of SMAD7, which competes for SMAD3-mediated downstream signalling that is induced by TGFβ1 (Ulloa et al., 1999). Another mechanism of inhibition of TGFβ1 signalling by IFNγ proposed is blocking SMAD transcriptional activity via the sequestration of the nuclear coactivator p300/CREB by Stat-1, which prevents it from associating with SMADs (Ghosh et al., 2001). Another study has provided evidence that endogenous IFNγ can negatively regulate TGFβ1 signalling during the wound healing process. IFNγ-deficient BALB/c mice exhibit increased levels of TGFβ1 expression and signalling at wound sites (Ishida et al., 2004).

1.3 Overall Aims of the Thesis

Findings from both *in vivo* and *in vitro* studies have supported the idea that the IL-18-induced IFNγ derived from dendritic cells contributes to infection control through the activation of Th1 immunity. However, aberrant expression of IL-18 has been implicated in the pathology of many inflammatory and autoimmune diseases (Dinarello, 2007). Whereas the central role of IL-18 in immune regulation is clear, the detailed mechanism giving rise to the response is in many cases not understood. The complexity of cytokine and cellular interactions in the human body makes it difficult to decipher the role of cytokine action on individual cell populations. Therefore, simple single-cell model systems that allow the uncomplicated investigation of the synergistic or antagonistic interactions between cytokines in isolation must be employed that provide important mechanistic clues. In order to enhance the patient's immune response, the provision of IFNγ and TNFα was highlighted as a potentially useful combination for cancer and HIV therapy in Phase I/II clinical trials (Demetri et al., 1989, Agosti et al., 1992). This combination implicates IL-18 signalling as a potential target for therapeutic intervention. This led to the hypothesis that the understanding of the regulation of IL-18 signalling by other cytokines could provide checkpoints that can be targeted to improve anti-cytokine therapy with the aim to control chronic inflammation and autoimmunity. These may be elucidated in *in vitro* model systems.

The aim of this project was primarily to understand the mechanisms of regulation of IL-18 function in order to devise novel strategies for the blockade of IL-18-driven inflammation. Work in the study has focused on investigating the IL-18 signalling and ways in which this can be regulated by stimulatory or inhibitory cytokines, such as TNFα and TGFβ1. Based on the concerns of lymphoid cell impurities in primary DCs, which could account for the IL-18-induced IFNγ production, the *in vitro* model of myeloid pre-dendritic KG-1 cells were utilised in studies within this project to establish the role of cytokine combinations on the myeloid-derived IFNγ production in response to IL-18.

IL-18, TNFα and TGFβ1 are important cytokines driving autoimmunity and inflammation that play important roles in regulating dendritic cell differentiation and action. The fundamental question that needs to be answered in order to study the role of IL-18 in the pathogenesis of autoimmune and inflammatory diseases is what makes this cytokine protective or harmful for the host. To investigate this, I studied the role of IL-18, TNFα and TGFβ in pre-dendritic cells. I hypothesised that IL-18 drives dendritic cell responses by inducing IFNγ production, that is is promoted by TNFα and inhibited by TGFβ1. Blockade of IL-18 using a soluble decoy receptor could be a potential strategy of reducing IL-18-induced inflammation.

To conclude, the overall aims of my thesis are:

- To investigate the mechanism of regulation of IL-18-induced IFNγ production in dendritic precursor cells by studying the role of the pro- and anti-inflammatory cytokines TNFα or TGFβ1.

- To attempt the blockade of IL-18-induced inflammatory response in pre-dendritic cells by generating a high affinity soluble decoy receptor.

Chapter 2

2 Materials and Methods

All materials were purchased from Sigma-Aldrich Foundation UK, unless otherwise stated within the description of the methods.

2.1 Cell Culture and Maintenance

2.1.1 KG-1 cells

The human bone marrow derived acute myelogenous leukemia (AML) pre-dendritic cell line KG-1 was obtained from the American Type Culture Collection (ATCC-LGC Promochem, UK) and maintained in complete RPMI 1640 (stands for Rosewell Park Memorial Institute) growth medium containing L-Glutamine and 25mM HEPES (Gibco, Invitrogen Ltd., UK) and supplemented with 10% heat-inactivated FBS (fetal bovine serum, Gibco, Invitrogen Ltd., UK), 50UI/mL penicillin (Gibco, Invitrogen Ltd., UK) and 50µg/mL streptomycin (Gibco, Invitrogen Ltd., UK) at 37°C with humidified air with 5% CO_2. Cells were grown in suspension and maintained in T-75 flasks (Greiner Bio-One Ltd., UK) at a density between 2 x 10^5 and 1 x 10^6 viable cells/mL. Cells were passaged by centrifugation at 300 x g for 5 minutes at room temperature for media renewal every 2 to 3 days and were split 1:2 to 1:4 for re-seeding.

2.1.2 Chinese Hamster Ovarian (CHO) Cells

The epithelial-like CHO (Chinese Hamster ovary) and the fibroblast-like African green monkey kidney COS-7 cell lines were maintained in D-MEM (Dulbecco's modified Eagle's medium) containing 4500mg/L Glucose and L-Glutamine (Gibco, Invitrogen Ltd., UK) supplemented with 10% FBS, 50UI/mL penicillin and 50µg/mL streptomycin (all Gibco, Invitrogen Ltd., UK) at 37°C with humidified air with 5% CO_2. Cells were passaged on attaining 70% to 90% confluency and split 1:3 to 1:5 for reseeding. Cells were detached with 1.5mL of 0.5% Tryspin-EDTA (Gibco, Invitrogen Ltd., UK) per T-75 flask (Greiner Bio-One Ltd., UK). Trypsin activity

was stopped by addition of 15mL full growth media and cells were collected by centrifugation at 300 x g for 5 min at room temperature. Cells were re-suspended in growth medium and re-seeded at a density of 1 - 3 x 10^4cells/cm^2.

2.1.3 COS-7 Cells

The fibroblast-like African green monkey kidney Cos-7 cell line was derived from CV-1, a simian cell line (*Cercopithecus aethiops*), by transformation with an origin-defective mutant of SV-40 (Gluzman, 1981). Cos-7 cells were cultured in the same conditions as the CHO cells (section 2.1.2).

2.1.4 Freezing, Storing and Thawing Cells

Freezing and storing cell line stocks

KG-1 suspension cells were harvested and Cos-7 or CHO adherent cells were trypsinised from a T75 flask (Greiner Bio-One Ltd., UK) and centrifuged at 300 x g for 5 minutes. Cells were then resuspend in 3 ml of full growth medium containing 10% DMSO and 1ml aliquots of the cell suspension (about 2.5 x 10^6 cells/ml) were placed into freezing vials on ice. The samples were transferred to a pre-cooled freezing polycarbonate container Nalgene® Mr. Frosty (Sigma-Aldrich, UK) containing isopropyl alcohol and foam insert that provided repeatable 1 °C/min cooling rate required for successful cryopreservation of cells. After storing at -80°C overnight, the frozen cells were transferred to liquid nitrogen.

Thawing frozen cell line stocks

The vials containing the frozen cells were taken out of the liquid nitrogen and immediately kept at 37°C. The thawed cell suspension was then transferred into 10 ml of pre-warmed full growth medium and the cells were pelleted by centrifugation. The cell pellet was resuspended in 10ml fresh full growth medium and cultured in a T25 flask (Greiner Bio-One Ltd., UK).

2.2 Cell Stimulation with Cytokines and Growth Factors

2.2.1 KG-1 Cell Bioassay

To examine the amount of IFNγ produced from IL-18 stimulated KG-1 cells, the cells were cultured at a density of 2 x 10^5 cells/well in a U-bottom 96 well plate, together with increased concentrations of recombinant human IL-18 (MBL International Corporation Ltd., Japan) for 24 or 48 hours. The supernatant was collected for IFNγ ELISA (section 2.6). In some tests, KG-1 cells were pre-treated with different concentrations of TNF-α and/or TGFβ1 for 24 hours. The pre-treated cells were washed with full medium to get rid of the residual TNFα and TGFβ1 before their stimulation with 50 or 100ng/ml of IL-18.

2.2.2 ERK1/2 and p38 MAPK Phosphorylation Assays

KG-1 cells were cultured at a concentration of 2 x 10^6cells/ml and stimulated with 50 or 100 ng/ml of IL-18. 2 x 10^5cells were harvested after stimulation with IL-18 for 5, 10, 15, 30 and 60 minutes; the cells without stimulation were used as time 0 control. In the experiment to study the role of ERK1/2 and p38 MARK in IFNγ production induced by IL-18, the cells were treated with increased concentrations of ERK1/2 specific inhibition (PD98059, Invitrogen Ltd., UK) and p38 MAPK specific inhibitor (SB203580, Invitrogen Ltd., UK) for 30 minutes and then stimulated with 50ng/ml IL-18 for 72 hours. The cell culture supernatants were collected and stored at -20°C to be used for IFNγ ELISA (see section 2.6). The cells were lysed with Cell Signalling Buffer I (refer to Table 2.4 for buffer constitution) to be used for Western Blotting as described in section 2.5.

2.2.3 T-bet Expression in KG-1 Cells

To investigate the regulation of T-bet expression in KG-1 cells by pro- and anti-inflammatory cytokines, 1.5 - 3 x 10^7cells/ml were added to each well of a 12 well plate containing stimulation with the appropriate concentrations of TNF-α or IL-18

and/or TGFβ1 for 24h. The cells were then harvested by centrifugation at 300 x g for 5min, washed once in PBS and resuspended in 50-100μl of Cell Signalling Extraction Buffer I (refer to Table 2.4 for buffer constitution).

2.2.4 IL-18Rα and IL-18Rβ Expression in KG-1

To examine the expression levels of human IL-18Rα and IL-18Rβ mRNA and cell surface protein, the cells treated with TNF-α and/or TGFβ1 at a density of 5 x 10^6cells/ml for RNA analysis and 1 x 10^7cells/ml for FACS analysis in 6-well plates for 16 or 24 hours. The cells were collected by centrifugation at 300 x g for 5min and washed with PBS to be used for total RNA extraction (section 2.4) or FACS staining (section 2.7).

Cytokine	Cat. No.	Source	Stock Concentration
rhIL-18	PHC0186	BioSource, Germany, S.A.	100μg/ml
rhTNF-α	11343013	ImmunoTools, Germany	10μg/ml
rhTGFβ1	T1654	Sigma-Aldrich, Inc. UK	1μg/ml

Table 2.1: List of cytokines and growth factors used for KG-1 stimulation.

2.3 MTT KG-1 Cell Proliferation Assay

MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide or Methylthiazolyldiphenyl-tetrazolium bromide] (Sigma-Aldrich, UK) is a water soluble tetrazolium salt yielding a yellowish solution. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes (SLATER et al., 1963). This water insoluble formazan can be solubilized using isopropanol or other solvents and the dissolved material is measured spectrophotometrically yielding absorbance as a function of concentration of converted dye. The cleavage and conversion of the soluble yellow dye to the insoluble purple formazan has been used to develop an assay system for measurement of cell proliferation. Active mitochondrial dehydrogenases of living

cells will cause this conversion. Dead cells do not cause this change. Modification has improved the sensitivity (Denizot and Lang, 1986).

In this experiment, MTT working solution was prepared in sterile PBS (5mg/ml), filtered through a 0.2 μm filter and stored at 4°C for frequent use or frozen for extended periods. Proliferation assays were carried out in triplicates and 100μl of cells were plated at 1 x 10^5cells/well in flat bottom 96 well plates and 50μl of appropriate cytokine stimulation in complete medium was added to each of them. 150μl of complete growth medium alone (without cells) was used as a negative control. After stimulation, 38μl of MTT was added into 150μl cell suspension or complete growth medium as negative control and incubated for 4 hours. At the end of the incubation period the cells were checked microscopically; live cells metabolised MTT and converted it into a black substance intracellularly. 75μl of cell medium was carefully removed from each well without disturbing the cells. The converted dye was solubilised with 100μl of acidified cell lysis buffer (20% w/v SDS, 50% v/v NN Dimethylformamide, 2.5% acidic mix, pH 4.7 and water up to 50ml,) i.e. containing 2.5% of acidic mix (80% v/v acetic acid glacial, 0.025N HCl, distilled water to 100ml) and incubated overnight in the dark at 37°C with humidified air and 5% CO_2. Absorbance at the wavelength of 540nm was measured using a Microplate reader (EL311, BioTek Instruments, Winoosky, VT, USA).

2.4 RT-PCR and Real-time PCR for mRNA Detection

2.4.1 Isolation of Total RNA

Human KG-1 cells were cultured at a concentration of 5 x 10^6cells/ml and stimulated as described in section 2.2.1 for 16 or 24 hours. After stimulation cells were pelleted by centrifugation for 5 min at 300 x g. The total RNA was isolated and purified from any contaminating reagents such as DNA, DNases and other cytoplasmic contaminants, using the RNeasy® Mini protocol for isolation of tRNA from animal cells (Qiagen, UK). The supernatant was carefully removed and cells were disrupted by the addition of 350 μl RLT buffer, a highly denaturating guanidine isothiocynate

were added to 10µl of each 1:20 diluted (in water) cDNA sample. Target sequence-specific oligonucleotide primer pairs were then added to a final concentration of 20ng/ml (usually 2.5µl of a 40µg/ml solution) and distilled water added to 49µl final volume. 1µl (5U/µl) of recombinant GoTaq® Flexi DNA polymerase from *Thermus aquaticus* was finally added (Promega, UK). The 50µl reaction mixture was subjected to thermal cycling under the following standard conditions: Denaturation at 95°C for 1 min, primer binding at 56°C for 2 min and sequence extension at 72°C for 3 min for 35 cycles; 1 cycle of 72°C for 10 min.

PCR was also performed on cDNA and genomic DNA samples without the requirement for reverse transcription. In these experiments, 0.1ng - 50ng of target cDNA or genomic DNA were mixed with 2µl 50mM $MgCl_2$, 10µl 10x concentrated PCR buffer (200mM Tris-HCl, pH 8.3; 500mM KCl), 2µl of dNTP (10mM each dATP, dCTP, dGTP, dTTP), 20ng/ml of each specific primer, 1µl (5U/µl) of GoTaq® Flexi DNA polymerase and distilled water to 50µl. The same cycling conditions described above were used.

2.4.4 TaqMan Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, UK) (see section 2.4.1), genomic DNA was digested with RNase free/DNase I and reverse transcribed to cDNA using Superscript™ II RNase H⁻ reverse transcriptase (Invitrogen, Paisley, UK) as described in section 2.4.3. Negative control samples (no first strand synthesis) were prepared by performing reverse transcription reactions in the absence of reverse transcriptase. Real-time PCR amplification of IL-18Rα, IL-18Rβ and human acidic ribosomal phosphoprotein P0 (h36B4) was performed using AmpliTaq Gold® PCR Master Mix (Applied Biosystems, UK).

The mastermix was prepared on ice containing 1.25U AmpliTaq Gold® DNA Polymerase, 0.2mM dNTP mix, 2mM $MgCl_2$, 1 x TaqMan buffer, 1µl of diluted cDNA (0.5 - 10ng per reaction) and the appropriate concentrations of gene-specific

primers and probes listed in Table 2.2 in a total volume of 25µl made up with nuclease-free double distilled water. The reactions were carried out using an ABI Prism® 7700 sequence detection system (Perkin Elmer Applied Biosystems, Fostor City, CA). The amplification conditions were the same for all the different genes (94°C for 7 min; 40 cycles of 94°C for 30sec, 55°C for 45 sec and 72°C for 45 sec; 72°C for 7 min). cDNA levels during the linear phase of amplification were normalised against h36B4 endogenous controls.

Absolute quantitation using a standard curve with known concentration for each set of reactions was used. Plasmids containing the full length hIL-18Rα and hIL-18Rβ were used for the generation of the standard curve starting from 6.43 and 23.6ng per reaction respectively. Determination was carried out in triplicates and expressed as a mean copy number per total RNA ± SD. The primers (f, forward; r, reverse; Sigma Genosys Co., UK) and dual-labelled TaqMan® probes (Applied Biosystems, UK) that were used to detect expression of the corresponding human genes are listed in Table 2.2. Different combinations of primer-pair and probe concentration matrices (Table 2.1) were used to optimise the annealing temperatures, extension times and cycle numbers.

Oligonucleotide Name	Oligonucleotide Sequence
IL-18Rα forward	$^{5'}$CAA CAG CAC ATC ATT GTA TAA GAA CTG$^{3'}$
IL-18Rα reverse	$^{5'}$CAA GAA GAA CCG GAA CTA TAT TAC TGC$^{3'}$
IL-18Rβ forward	$^{5'}$CCG CAT CAC ATA AGC AAG ACC $^{3'}$
IL-18Rβ reverse	$^{5'}$GAT TCG GTT GCT CCT TTC CAC$^{3'}$
IL-18Rα Probe	$^{5'}$6 - FAM - CCG AGT TTG AAG ATC AGG GGT ATT ACT CCT GCG TG - TAMRA$^{3'}$
IL-18Rβ Probe	$^{5'}$6 - FAM - CTC AGC TGC CAA AGT GAT GTA CAA AGT CCA G - TAMRA$^{3'}$

Table 2.2: Primer and probe sequences used for real time PCR of hIL-18Rα and Rβ.

Gene	Primer f/r [nM]	Probe [nM]
Human 36B4	300/300 nM	100 nM
Human IL-18Rα	600/600 nM	150 nM
Human IL-18Rβ	300/150 nM	150 nM

Table 2.3: Optimised concentrations of primers and probes used for real-time PCR.

2.5 Pull down Assay and Western Blotting for Detection of Protein Expression in Cell and Cell Culture Media

Protein G Pull Down Assay

Protein G Sepharose™ 4 Fast Flow (GE Healthcare; Amersham Biosciences, UK) is recombinant Streptococcal protein G lacking albumin-binding region, produced in *E. coli* and coupled using the Cyanogen Bromide Activation (CNBr) method to Sepharose 4 fast Flow, which is a highly cross-linked 4% agarose derivative with unique chemical and physical stability. The molecular weight of the ligand is about 17,000 Da and has two binding sites for IgG. The binding capacity of the Protein G Sepharose™ 4 Fast Flow for IgG depends on the source species of the particular Immunoglobulin (refer to Appendix 8.3 for a Table with the relative binding strengths of antibodies from various species to Protein G as measured in a competitive ELISA test). Protein G binds specifically to the Fc portion of IgG from most mammalian species. In this experiment, Protein G Sepharose 4 Fast Flow was used for the isolation and precipitation of shIL-18Rα, shIL-18Rβ and shIL-18Rαβ-Fc for their subsequent detection with Immunoblotting.

1 ml of stably transfected CHO cell culture media containing shIL-18Rα-Fc, shIL-18Rβ-Fc or shIL-18Rαβ-Fc was centrifuged at 300 x g for 5 min to get rid of cell debri and mixed with 30μl of already balanced Protein G Sepharose™ 4 Fast Flow beads incubated rotating overnight at 4°C. In order to balance the Protein G Sepharose™ 4 Fast Flow, 500μl of beads were washed 3 times with 1ml sterile PBS, pH 7.4 at 3,300 x g for 1min and resuspended in the appropriate volume of PBS. The following day, the samples containing the beads were washed 3 times with 500μl PBS by centrifugation at 3,300 x g for 1 min in order to discard the non-bound material. After the final wash, the pellet comprising of the beads bound to the protein of interest was resuspended in 25μl PBS plus 25μl of 2x Laemmli buffer (0.2M Tris-HCl, pH 6.8; 4% w/v SDS; 1mM EDTA; 30% glycerol; 0.3% w/v bromoethanol blue; 5% β-mercaptoethanol). The beads with the reducing sample buffer were incubated

at 90°C for 5 min so that the protein is released from the beads. After spinning at 16,100 x g for 10 min, the 50µl supernatant containing the protein precipitated with the beads was carefully removed to be used for SDS-PAGE (section 2.11) and subsequently Immunoblotting using antibodies against IL-18Receptor α or β chain or IgG-Fc (see Table 2.5 for a list of antibodies). The pelleted beads were discarded.

To test the efficiency of the purified shIL-18Rα-Fc, shIL-18Rβ-Fc or shIL-18Rαβ-Fc to bind rhIL-18, the same procedure was followed after different concentrations of the receptors were incubated with 50ng/ml rhIL-18 for 1 h at 37°C, prior to the addition of Protein G Sepharose™ 4 Fast Flow beads. Then a monoclonal specific antibody against IL-18 (refer to Table 2.5) was used to detect the amount of IL-18 precipitated with the shIL-18R-Fc using the pull down assay.

Western blotting

The cells were lysed with the appropriate lysis buffer (Table 2.4) and the protein concentration was determined by BCA assay (section 2.4.1). Following SDS-PAGE (section 2.11), gels were placed onto Protran® nitrocellulose membrane (Schleicher and Schuell, Germany) and proteins were transferred to the membrane in 1x transfer buffer (192mM Glycine, 20% methanol, 25mM Tris-HCl, pH 8.3) for 2h at 25mV, 160mA using X-Cell II blot module in Novex XCell SureLock™ Mini-Cell system, power supply Novex PowerEase® 500 (Invitrogen, UK). The membrane was removed and blocked overnight at 4°C in 5% non-fat dried milk (NFDM, Marvel dried milk powder) in TBS (137mM NaCl, 20mM Tris-HCl, pH 7.4) containing 0.05% Tween® 20, unless otherwise indicated. The membrane was washed three times between incubations with TBS/0.05% Tween® 20 for 5 min. Primary antibody incubations were performed overnight at 4°C (Table 2.5) and all the secondary horseradish perosidase (HRP) - conjugated antibody incubations were performed for 90 minutes at room temperature. When the primary antibody was Biotin-conjugated, 2µg/ml Extravidin-Peroxidase was for detection. Proteins were visualised by chemiluminescence using ECL Plus™ Western blotting reagents (GE Healthcare, Amersham Biosciences). It consists of a lumigen PS-3 acridan substrate, which is

converted to an acridinium ester intermediate when catalyzed by HRP. The ester intermediate reacts with peroxide in alkaline conditions and emits light, which was detected by autoradiography (Hyperfilm ECL, Amersham Biosciences). Solution A (substrate solution containing Tris buffer) and solution B (acridan substrate solution in dioxane and ethanol) of the kit were mixed in 40:1 ratio.

Antibody stripping and re-probing of membranes

In order to re-probe an already probed membrane with a different set of antibodies, the stripping procedure was followed. The membrane was washed in TBS / 0.05% Tween® 20 to get rid of the ECL detection reagents and was rolled into a 50 ml falcon tube with 20ml Stripping Buffer [4ml 10% SDS; 1.25 ml 1M Tris-HCl, pH 6.8; 140µl β-ME; 14.6ml distilled water] rotating for 30 min at 50°C. After the 30 min incubation, the membrane was washed in TBS / 0.05% Tween® 20 a few times and was blocked to be used for Immunoblotting as described above.

Cell Lysis Buffer	Buffer Constitution
Cell Signalling Buffer I	20mM HEPES, 150mM NaCl, 1% NP-40, 1mM Na_3VO_4, 20mM β-glycerophosphate, 20mM P-nitrophenol phosphate, 2mM NaF, 0.25% Na-deoxycholate, 1mM EGTA, 1mM PMSF, 10μg/ml leucipeptin, 10μg/ml aprotinin, and 10ml glycerol and 1 tablet of Complete Protease Inhibitor Cocktail (Roche Applied Science, UK)
1 x RIPA Buffer	5ml of the 2 x RIPA stock, 0.5M NaF, 0.5M EDTA, 0.2M $NaH_2PO_4 \cdot 2H_2O$, 5% v/v ethylene glycol and 1 tablet of Complete Protease Inhibitor Cocktail (Roche Applied Science, UK) 1 x RIPA buffer was made fresh for each experiment from the 2 x RIPA stock (100mM HEPES; pH 7.4, 300mM NaCl, 2% TritonX-100, 1% Na-deoxycholate and 2% SDS)
Cell Lysis Buffer II	50mM Tris-HCl; pH 8.0, 150mM NaCl, 1% NP-40 (Roche Applied Science, UK), 0.5% Deoxycholate, 0.1% SDS and 1 tablet of Complete Protease Inhibitor Cocktail (Roche Applied Science, UK) (Fukushima et al., 2005)

Table 2.4: List of different cell lysis buffers used in experiments.

1° Antibody [final conc.]	**Ig Type**	**Cat. No.**	**Source**
PhosphoDetect™ Anti-human p38 MAP Kinase ($pThr^{180}$, $pTyr^{182}$) [1/1000]	Rabbit IgG	506119	Calbiochem®, UK
Anti-human p38 MAP Kinase (341-360 amino acids) [1/1000]	Rabbit IgG	506123	Calbiochem®, UK
Anti-mouse, rat and human Tbet (Th1-specific T box transcription factor) [2μg/ml]	Mouse IgG1	14-5824	eBioscience, Inc.
Anti-human β-tubulin [1/500]	Mouse IgG1	T4026	Sigma-Aldrich, UK
Anti-human IL-18Rα [0.2μg/ml]	Goat IgG	AF840	R&D Systems, UK
Anti-human IL-18Rβ [0.2μg/ml]	Goat IgG	AF118	R&D Systems, UK
Anti-human IgG-Fc (Fc specific)-Biotin [1/1000]	Mouse IgG2a	B3773	Sigma-Aldrich, UK
Anti-human IL-18 [1/500]	Mouse IgG	D3D4	In-home (Dr. Wei)
2° Antibody [final conc.]	**Ig Type**	**Cat. No.**	**Source**
Anti-Rabbit IgG-HRP [0.2μg/ml]	Swine IgG	P0399	DakoCytomation, UK
Anti-goat IgG-HRP [0.25μg/ml]	Rabbit IgG	P0449	DakoCytomation, UK
Anti-mouse IgG-HRP [1/1000]	Rabbit IgG	P0260	DakoCytomation, UK

Table 2.5: List of antibodies used for the Immunoblotting experiments.

2.5.1 Protein Concentration Determination

Cell lysates were sonicated if required and centrifuged at 15,000g for 10min. The protein concentration in the supernatants was determined using the bicinchoninic acid (BCA) protein based assay (Smith et al., 1985), where BCA is mixed with cupric sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) solution (Pierce International). In the presence of protein, Cu^{2+} is reduced to Cu^{+} in an alkaline environment. One monovalent cuprous cation is chelated by two molecules of BCA, forming a purple-coloured reaction product (Fig. 2.1). This reaction is mediated by cysteine, tryptophan and tyrosine residues, by peptide bonds and additionally by overall protein structure (Wiechelman et al., 1988). 10µl of protein of unknown concentration were mixed with 190µl of protein assay reagent containing 4% $CuSO_4$ in a 96-well microtitre plate (Greiner Bio-One Ltd., UK). Samples were incubated at 37°C for 30 minutes and the plate was read at 540nm on a 96-well microplate reader (EL311, BioTek Instruments, Winoosky, VT, USA). Protein concentration was calculated using known concentrations of BSA at 31.25, 62.5, 125, 250, 500, 1000, 2000 µg/ml on the same plate to generate a standard curve.

$$\text{Protein} + Cu^{2+} \xrightarrow{OH?} Cu^{1+}$$

$$Cu^{1+} + 2\text{BCA} \longrightarrow \text{purple-coloured BCA - } Cu^{+}\text{ chelate}$$

Figure 2.1: Reactions taking place in BCA assays.

2.5.2 Densitometric Analysis

Where required, quantitative analysis of Western blots was performed using Image Quant software (Amersham Biosciences). Densitometric values represent the ratio of the value of each sample normalised against appropriate housekeeping controls for each sample, in order to measure the relative increases or decreases obtained for samples in the presence and absence of stimulation.

2.6 Sandwich ELISA for the Detection of Soluble Protein in Cell Culture Media

The cell supernatants were placed into in U-bottom 96 well plates and stored at -20°C to be used for ELISA. In this study, the levels of cytokines in culture supernatants were measured by ELISA, using paired antibodies listed in Table 2.6. The cytokine levels [pg/ml] were determined according to the standard curve. Briefly, anti-cytokine monoclonal antibodies were diluted from 2μg/ml to 10μg/ml in coating buffer (0.1M $NaHCO_3$, pH 8.2) and 50μl/well applied to high protein binding (600ng of IgG per cm^2) flat bottom 96 well ELISA microplate (Greiner Bio-one Ltd., UK) at 4°C overnight.

The plates were washed three times with PBS / 0.05% Tween® 20 as washing buffer, and blocked with 100μl/well of 10% FCS in PBS for 2 hours at 37°C, then washed three times with washing buffer. 50μl of the supernatant from the cell culture and diluted standard cytokine were added to the plate at 37°C for 2 hours or at 4°C overnight. After washing five times with washing buffer, 50μl of biotinylated anti-cytokine detecting antibody (1μg/ml) was added and incubated for 1-2 hours at 37°C. The plates were washed six times with washing buffer before adding 50μl of the extravidin-peroxidase (2μg/ml) and incubated for 1 hour at 37°C.

The plates were washed six times and the blue colour was developed by adding 50μl of SureBlue™ TMB microwell peroxidase substrate (KPL, USA) for 10-30 minutes and changed to yellow with 50μl of TMB stop solution (KPL, USA). The absorbance of the stopped reaction was read at a wavelength of 450nm using a FLUOstar OPTIMA microplate based multi-detection reader (BMG Labtech, Germany) and protein concentration was calculated by the OPTIMA BMG Labtech software based on the standard curve.

Antibody [final conc.]	**IgG Type**	**Cat. No.**	**Source**
Anti-human IFNγ [2μg/ml]	Mouse IgG1κ	MD-1	eBioscience, Inc.
Anti-human IFNγ-Biotinylated [0.5μg/ml]	Mouse IgG1κ	13-7319	eBioscience, Inc.
Anti-human IL-18Rα [1μg/ml]	Goat IgG	AF840	R&D Systems, UK
Anti-human IL-18Rβ [1μg/ml]	Goat IgG	AF118	R&D Systems, UK
Anti-human IgG-Fc (Fc specific) - Biotinylated [1/1000]	Mouse IgG2a	B3773	Sigma-Aldrich, UK
Anti-human IgG-Fc [0.8μg/ml]	Goat IgG	G-102-C	R&D Systems, UK
Anti-human IL-18Rα [1μg/ml]	Mouse IgG1	MAB840	R&D Systems, UK
Anti-human IL-18Rα [1μg/ml]	Goat IgG	AF840	R&D Systems, UK
Anti-human IL-18Rβ [1μg/ml]	Goat IgG	AF118	R&D Systems, UK
Anti-goat IgG-HRP [0.5μg/ml]	Rabbit IgG	P0449	DakoCytomation, UK
Anti-human IL-18 mAb [0.5μg/ml]	Mouse IgG1	125-2H	MBL International Corporation
Anti-human IL-18 mAb - Biotinylated [0.5μg/ml]	Rat IgG2a	159-12B	MBL International Corporation
Protein Standards	**Sensitivity**	**Cat. No.**	**Source**
rhIFNγ	10-0.16ng/ml	11343536	ImmunoTools, Germany
rhIgG1-Fc	20-0.6ng/ml	110-HG	R&D Systems, UK
rhIL-18	2000-31pg/ml	PHC0186	BioSource, Germany

Table 2.6: List of antibodies and standards used for the ELISA experiments

2.7 Flow Cytometry for Detection of Cell Surface Protein Expression

KG-1 cells with or without stimulation were counted using a haemocytometer to adjust the cell density to 1 x 10^7cells/ml in FACS buffer (1 x PBS, pH 7.4, 2.5% FCS, 5mM EDTA); 50 µl of the cell suspension was mixed with 1 µl of the fluorochrome-conjugated antibody for 30min at 4°C in the dark. Subclass controls were used at the same concentration to adjust for non-specific antibody binding and in the case of multiple colour staining, compensation controls (combination of a single positive fluorochrome and subclass control reagents) were used to adjust for spectral overlap (refer to Table 2.7 for a list of antibodies used for staining). The cells were topped up with 1ml of FACS buffer and centrifuged at 300 x g at 4°C. The supernatant was discarded and the cell pellet was resuspended by gentle vortexing. The cells were washed again for two more times and resuspended in 300µl of FACSFix buffer (FACS buffer containing 2% paraformaldehyde). The samples were analyzed the within 24 hours by collecting 10,000-25,000 events using a BD FACSCalibur™ flow cytometer (BD biosciences).

Antibody [final conc.]	**IgG Type**	**Cat./clone No**	**Source**
Anti-human IL-18Rα-PE [0.5μg/ml]	Mouse IgG1	FAB840P	R&D Systems, UK
Anti-mouse IgG1-PE (isotype-matched control for IL-18Rα) [0.5μg/ml]	Mouse IgG1	PPV-06	Euro BioSciences, Germany
Anti-human IL-18Rβ [0.5μg/ml]	Mouse IgG1	MAB1181	R&D Systems, UK
Anti-mouse IgG1-FITC (as secondary for IL-18Rβ) [14μg/ml]	Rabbit IgG1	672321	ICN
Anti-human CD80-FITC	Mouse IgG1	MEM-233	ImmunoTools, Germany
Anti-human CD14-FITC	Mouse IgG1	MEM-18	ImmunoTools, Germany
Anti-human CD58-FITC	Mouse IgG1	MEM-63	ImmunoTools, Germany
Anti-human CD50-FITC	Mouse IgG1	MEM-171	ImmunoTools, Germany
Anti-human CD54-FITC	Mouse IgG2b	1H4	ImmunoTools, Germany
Mouse IgG1-FITC isotype-matched control	Mouse IgG1	PPV-06	ImmunoTools, Germany
Mouse IgG2b-FITC isotype-matched control	Mouse IgG2b	PFR-02	ImmunoTools, Germany

Table 2.7: List of antibodies used for FACS analysis.

2.8 Construction of Expression Plasmids for shIL18Receptor

2.8.1 Amplification of Target DNA and TA Cloning into pCR®II

The soluble forms of human IL18Rα and IL18Rβ were cloned into expression vectors obtained from Invitrogen. The cDNA sequence of shIL18Rα-Fc and shIL18Rβ-Fc was amplified by using standard PCR with the proofreading *Pfu* DNA polymerase (Promega, UK) for higher fidelity of DNA amplification and lower error rate. The pcDNA3.1A and pBlaCrepA backbone vectors containing the full length human IL18Rα and IL18Rβ sequences respectively (constructed by Dr. Wei XQ) were used as templates for amplification.

The amplification primers (Sigma-Genosys Ltd, UK see Table 2.8) were designed to incorporate restriction enzyme sites to both the 5' and 3'-ends of the cDNA sequences in order to assist in the sub-cloning of the insert. Kozak consensus sequence was also added to the 5'-end of the primers after the restriction sites and prior to the start codon (ATG) in order to improve the mammalian protein expression of the cloned cDNA fragments. Four additional nucleotides were incorporated at the beginning of the 5'-end of both primers for efficient cleavage, as restriction enzymes may not cleave DNA if the recognition site is less than 3 nucleotides from the start of the DNA fragment. The amplified products were run on a 1% agarose gel to confirm the correct product size as described in section 2.8.4.

The *Pfu* DNA polymerase-generated blunt-end shIL18Rα and shIL18Rβ fragments were purified as described in section 2.8.5 using the QIAquick® PCR purification kit and amplified using a standard PCR reaction with *Taq* DNA Polymerase, which added a single 3'-A overhang residue to the each end of the PCR products. The A-tailed PCR products were then TA cloned into the pCR®II vector (Invitrogen Corp., UK; Appendix 8.2.1) to confirm sequence identity and in frame expression of the insert. After ligation (section 2.8.6) the products were transformed into DH5α *E. coli* competent cells and the recombinant plasmids with the inserts were selected with blue-white screening as described in section 2.8.8. Glycerol stocks were prepared

from the colonies grown and the DNA purified using the QIAprep® Miniprep DNA purification kit according to the manufacturer's protocol (see section 2.8.9). Purified DNA was tested with *EcoR* I, *Bgl* II and *Hind* III for shIL18Rα and *EcoR* I, *Bgl* II and *BamH* I for shIL-18Rβ restriction enzyme digests as described in section 2.8.2. The concentration of the resulting plasmid was measured as described in section 2.4.2, before sequencing to confirm identity of the sequence using the T7 and SP6 promoter sequencing primers (see section 2.8.10).

Primer Name	**Primer Sequence**
shIL18Rα-*BglII-EcoRI*-Kozak forward	5'ATC TAG ATC TGA ATT CCA CAA CCA **TG**A ATT GTA GAG AAT TAC3'
shIL18Rα-*BglII* reverse	5'TGA TAG ATC TTC TTG TGA AGA CGT GGC CTG GGA TA3'
shIL18Rβ-*BglII-EcoRI*-Kozak forward	5'GAT GAG ATC TGA ATT CCA CAA CCA **TG**C TCT GTT TGG GCT GGA T3'
shIL18Rβ-*BglII* reverse	5'GCA CAG ATC TTC TCT TTT CTT TCA GTT GGA CG3'

Table 2.8: List of primer sequences encoding the soluble form of IL18Rα and Rβ with the appropriate restriction endonuclease sequences at the 5'-end (the start codons are indicated in bold red).

2.8.2 Restriction Endonuclease Digestion of DNA

Restriction enzymes, also referred to as restriction endonucleases, are enzymes that recognize short, specific (often palindromic) DNA sequences. They cleave double-stranded DNA (dsDNA) at specific sites within or adjacent to their recognition sequences. Several restriction endonucleases with their supplied buffers (Promega, UK) were used to digest DNA into required fragments. For a list of the enzymes used and their buffers refer to Table 2.9. All the restriction endonuclease reactions of dsDNA were carried out for 2 hours at 37°C. In general, up to 1μg of the cloned and

purified DNA was digested in the presence of 2µl of 10 x supplied Buffer, 1-2µl of the enzymes (10U/µl) and water to a volume of 20µl.

Restriction Endonuclease	10x Buffer	Recognition Sequence
BamH I	E	5'- G / GATCC -3'
Bgl II	D	5'- A / GATCT -3'
EcoR I	H	5'- G / AATTC -3'
EcoR V	D	5'- G / ATATC -3'
Hind III	E	5'- A / AGCTT -3'
Kpn I	J	5'- GGTAC / C -3'
Xba I	D	5'- T / CTAGA -3'
Xho I	D	5'- C / TCGAG -3'

Table 2.9: List of restriction endonucleases used and their buffers and restriction sequences. 10 x Buffer E was used for all the digestions with restriction enzyme combinations used in this project [i.e. *BamH* I and *EcoR* I; *BamH* I and *Hind* III; *EcoR* I and *Hind* III].

2.8.3 Dephosphorylation of DNA

The dephosphorylation reaction was performed directly after the restriction endonuclease digestion and purification of the vector and before ligation with the insert. During ligation, DNA ligase will catalyze the formation of a phosphodiester bond between adjacent nucleotides only if one nucleotide contains a 5'-phosphate group and the other a 3'-hydroxyl group. Recircularization of plasmid DNA can therefore be minimized by removing the 5' phosphates from both ends of the linear DNA with calf intestinal phosphatase (Seeburg et al., 1977, Ullrich et al., 1977, Ullrich et al., 1992). As a result, neither strand of the duplex can form a phosphodiester bond. However, a foreign DNA segment with 5'-terminal phosphates can be ligated efficiently to the dephosphorylated plasmid DNA to give an open

circular molecule containing two nicks. Because circular DNA (even nicked circular DNA) transform much more efficiently than linear plasmid DNA, most of the transformants will contain recombinant plasmids. So, 40µl of purified eluted vector DNA (1 - 3µg) (see section 2.8.5 for DNA purification) were mixed with 2µl (1U/µl) of CIAP, 5µl of 10 x reaction buffer [50mM Tris-HCl (pH 9.3 at 25°C), 1mM $MgCl_2$, 0.1mM $ZnCl_2$ and 1mM spermidine] (Promega, UK) and water to 50µl. The reaction was incubated for 2 hours at 37°C and purification of the dephosphorylated plasmid DNA followed prior to ligation.

2.8.4 Agarose Gel Electrophoresis

Nucleic acids were routinely analysed by agarose gel electrophoresis. Agarose gels were prepared at a concentration range from 1% to 1.5% depending on the size of the fragment and amount of DNA. For the digestion of plasmid DNA during sub-cloning and analysis of PCR products 1% agarose gels were used. For the process of optimisation of qPCR primers 1.5% agarose gels were used. Gels were made up by dissolving the required amount of agarose dry powder (Invitrogen, UK) in 0.5 x TBE buffer from the 5 x TBE stock [450mM Tris-HCl, pH 8.4; 450mM Boric acid; 10mM Ethylenediamine tetra-acetic acid (EDTA)] by heating in a microwave oven. The melted gels were allowed to cool to 45°C and 500ng/ml final concentration of ethidium bromide was added. The gels were cast in tanks with combs where they were allowed to set for 30 min. The gels were then submerged in TBE buffer and the well-forming combs were removed. The DNA samples were mixed with 6 x loading buffer (0.75% bromophenol blue; 150mM Tris-HCl, pH 8.0; 6mM EDTA; 30% glycerol) and loaded into the relevant wells on the gel. 250µl of 1kb DNA ladder (1µg/µl) (Invitrogen, UK, Appendix 8.1.1) was mixed with 250µl of 6 x loading buffer and 1000µl of TE (25mM Tris-HCl, pH 8.0; 1mM EDTA) and 6µl of that was loaded on the gel. The gels were run at a constant current of 10mA/cm gel-length or a constant voltage of 1-5V/cm of gel-length until the bromophenol blue had migrated the required distance. The gels were then analysed and photographed under ultra-violet light (254nm wavelength).

2.8.5 Agarose Gel DNA Extraction and Simple DNA Purification

The QIAquick® Gel Extraction Kit protocol (Qiagen, UK) using a microcentrifuge was followed to extract and purify DNA from standard agarose gels in TBE [450mM Tris-HCl pH 8.4, 450mM Boric acid, 10mM Ethylenediamine tetra-acetic acid (EDTA)] buffer (section 2.8.4). All centrifugation steps were carried out at 15,700 x g in a conventional table-top microcentrifuge. DNA bands were visualised with Ethidium Bromide by short-wave UV illumination. The relevant DNA band was excised with a clean, sharp scalpel and placed in an already weighed eppendorf tube. The eppendorf tube containing the gel slice was weighed again and the weight of the gel slice was estimated. Three volumes of QG buffer were added to one volume of gel (100µl ~ 100mg) and incubated at 50°C for 10 min. After the gel slice was dissolved completely, the colour of the mixture was turned into yellow, as QG buffer contains a pH indicator which yellow at pH < or = 7.5 and orange or violet at higher pH, allowing optimal pH for DNA binding. The same method was used for purifying cDNA after digestion, vector dephosphorylation or Klenow blunt-ending of DNA without having to incubate the samples 50°C for 10 min, as there is no gel to dissolve.

One volume of isopropanol was then added to increase the yield of DNA fragments <500bp and >4kb. The sample was applied to the QIAquick® column already placed into a 2ml collection tube and centrifuged for 1 min. The flow-through was discarded and 500µl of QG buffer were added to remove all the traces of agarose. If the DNA was to be used for salt sensitive applications, such as blunt-end ligation, 750µl of PE buffer was added for 2 - 5 min to the column before centrifuging for 1 min to wash. The flow-through was discarded and an additional centrifugation step was performed to remove the residual ethanol containing PE buffer. The QIAquick® column was placed into a clean microcentrifuge tube and 40µl of water was added to the centre of the membrane for 1 min, which was then centrifuged for 1 min for the elution of bound DNA.

2.8.6 DNA Ligation

Following restriction digestion and purification, T4 DNA Ligase (Invitrogen, UK) was used to join DNA fragments with staggered or blunt ends and to repair nicks in double-stranded DNA having 3'-hydroxyl and 5'-phosphate ends. The enzyme is isolated from *E. coli* lambda lysogen NM989. A molar ratio of 3:1 insert to vector was used for the rapid ligation of DNA inserts to the appropriately digested and purified plasmid DNA to produce circular recombinant molecules. Approximately 75ng insert and 25ng vector were mixed and incubated with 1µl of T4 ligase (5 U/µl) and 4µl of 5X DNA Ligase Reaction Buffer [250 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT and 25% (w/v) polyethylene glycol-8000] to a total volume of 20µl with water. Control ligations, replacing the cDNA fragments with water but retaining the plasmid DNA were also set up to calculate the degree of self-ligation. Reactions without T4 DNA ligase and insert were used as negative controls to check for partial digestion of the plasmid. Ligations were incubated either overnight at 14°C or at room temperature for 1 hour. The resulted circular DNA plasmids were immediately used for bacterial transformation.

2.8.7 Cloning of shIL18Rα and shIL18Rβ into the pcDNA4/TO-IgG1Fc Vector

For the purpose of Protein A purification of the shIL-18Rα, Rβ and Rαβ, they had to be Fc-tagged and expressed in CHO cells. To do that the shIL18Rα and shIL18Rβ were inserted into a pcDNA4/TO/myc-HisA vector (Invitrogen Ltd., UK; Appendix 8.2.2) manipulated to contain an IgG1-Fc sequence (provided by Wei XQ). Construction of this vector for transfection required multiple steps which have been illustrated in Fig 2.2.

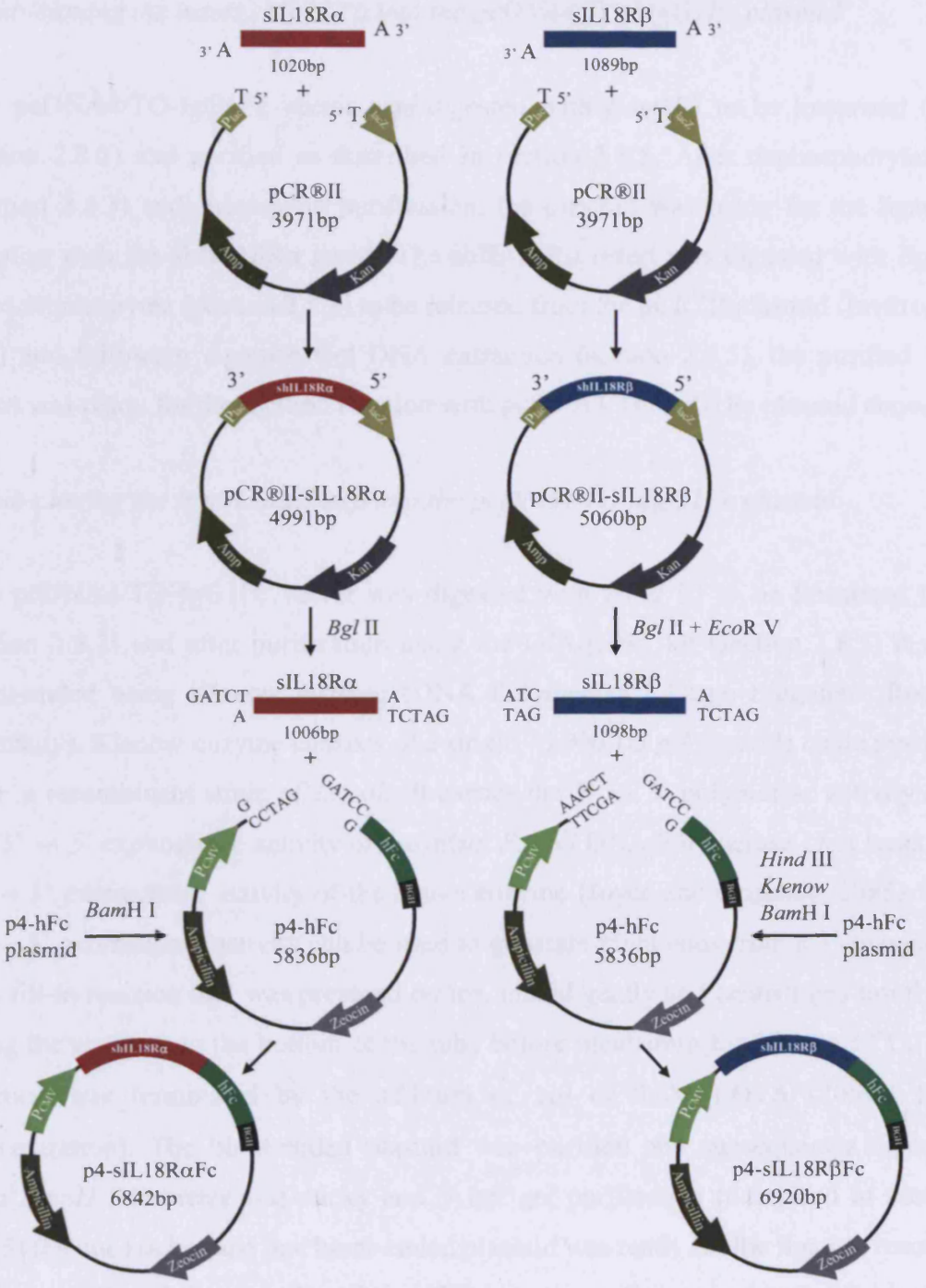


Figure 2.2: Cloning summary of shIL-18Rα-Fc and shIL-18Rβ-Fc.

Summary of the procedure carried out for the generation of constructs encoding inserts for shIL-18Rα-Fc **(a)** and shIL-18Rβ-Fc **(b)** to be expressed in CHO cells for the expression and purification of shIL-18Rα-Fc and shIL-18Rβ-Fc.

Sub-cloning the insert shIL18Rα into the pcDNA4/TO-hIgG1Fc plasmid

The pcDNA4/TO-IgG1Fc vector was digested with *BamH* I to be linearised (see section 2.8.2) and purified as described in section 2.8.5. After dephosphorylation (section 2.8.3) and subsequent purification, the plasmid was ready for the ligation reaction with the shIL-18Rα insert. The shIL-18Rα insert was digested with *Bgl* II restriction enzyme (section 2.8.2) to be released from the pCR®II plasmid (Invitrogen, UK) and following Agarose Gel DNA extraction (section 2.8.5), the purified 1kb insert was ready for the ligation reaction with pcDNA4/TO-IgG1Fc plasmid above.

Sub-cloning the insert shIL18Rβ into the pcDNA4/TO-hIgG1Fc plasmid

The pcDNA4/TO-IgG1Fc vector was digested with *Hind* III to be linearised (see section 2.8.2) and after purification using the QIAquick® kit (section 2.8.5) it was blunt-ended using Klenow enzyme (DNA Polymerase I Large Fragment; Roche, Germany). Klenow enzyme consists of a single 75,000 Da polypeptide chain purified from a recombinant strain of *E. coli*. It carries the 5' → 3' polymerase activity and the 3' → 5' exonuclease activity of the intact *E. coli* DNA Polymerase I but lacks the 5' → 3' exonuclease activity of the native enzyme (Joyce and Grindley, 1983). The 5' → 3' exonuclease activity can be used to generate blunt ends from a 3'-overhang. The fill-in reaction mix was prepared on ice, mixed gently and centrifuged briefly to bring the contents to the bottom of the tube before incubation for 30 min 37°C. The reaction was terminated by the addition of 1μl of 0.6M EDTA (20mM final concentration). The blunt-ended plasmid was purified and subsequently digested with *BamH* I to create one sticky end. After gel purification (described in section 2.8.5) the one sticky- and one blunt-ended plasmid was ready for the ligation reaction with the shIL-18Rβ insert. The shIL-18Rβ insert was digested with *Bgl* II together with *EcoR* V (as described in section 2.8.2) (Invitrogen, UK). Agarose Gel DNA extraction followed (section 2.8.5) for the purification of the 1kb insert, which was ready for the ligation reaction as described in section 2.8.6.

2.8.8 Transformation of DH5α E. Coli Competent Cells

Subcloning Efficiency™ DH5α™ Competent *E. coli* Cells (Invitrogen, UK) were used for routine subcloning into plasmid vectors. Initially, a heat shock method was used whereby 5µl (approximately 500ng of DNA) of the ligation mixture was mixed with 50µl competent bacterial cells and incubated on ice for 30 min in an eppendorf tube. The DNA-cell mixture was then incubated at 37°C for 40 seconds before a second incubation for 2 min on ice. 1ml of 2 x YT broth (Invitrogen, UK) was added to the transformation mix and this was then incubated at 37°C with vigorous shaking for 1-2 hour. The bacteria were pelleted by centrifugation at 13,000 x g for 10 seconds at room temperature and resuspended in 100µl of 2 x YT broth. The bacterial cells were then plated onto 85mm petri dishes (Sterilin) containing Luria-Bertani (LB) agar (10g/L bacterial peptone 140, 5g/L yeast extract autolysed low sodium, 5g/L NaCl and 12g/L agar, pH 7.5) and 100µg/ml Ampicillin. Plates were incubated overnight at 37°C and the following day was examined for the growth of colonies of transformed bacteria. The bacterial cells that contained the plasmid with the ampicillin resistant gene will survive in the LB-agar containing ampicillin.

For the TA-cloning experiments using the pCR®II plasmids that carry the X-gal gene, the LB-agar contained 50µg/ml isopropyl thio-β-D-galactoside (IPTG) (Invitrogen, UK) and 40µg/ml 5-bromo-4-chloro-3-indolyl-β-D galactoside (X-gal) (Invitrogen, UK). This allowed the selection of bacteria carrying the recombinant plasmids by the appearance of white colonies. Those not carrying an inserted DNA fragment retained the ability to metabolise galactose analogues and therefore produced blue colonies due to the presence of IPTG and X-gal.

2.8.9 Plasmid DNA purification

Plasmid DNA was isolated using the QIAprep® Spin Miniprep kit protocol (Qiagen) using a microcentrifuge. The procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto the QIAprep® silica membrane in the presence of high salt (Vogelstein and Gillespie, 1979) and the efficient washing and elution of purified plasmid DNA. Colonies carrying a putative plasmid of interest were inoculated into 3ml of 2 x YT broth containing 100μg/ml ampicillin and grown with vigorous shaking for 16 hours at 37°C.

a). Harvesting and resuspension: At the end of the growth period, 2ml of the bacterial culture were harvested into an eppendorf tube and centrifuged at 16,100 x g for 2 minutes at room temperature. The bacterial cell pellet was resuspended in 250μl of P1 buffer with RNase A added to it.

b). Bacterial cell lysis: The resuspended bacterial pellets were then lysed in 250μl of NaOH/SDS containing P2 buffer in the presence of RNase A (Birnboim and Doly, 1979, Birnboim, 1983). SDS caused solubilisation of the phospholipids and protein components of the membrane leading to cell lysis and release of cell contents. The alkaline environment was suitable for the denaturation of the chromosomal and plasmid DNA and proteins. Optimised lysis time was important for the maximum release of plasmid and not chromosomal DNA.

c). Lysate neutralisation and adjustment to high-salt binding conditions: 350μl of the high-salt N3 buffer was added to the lysate and mixed thoroughly and gently to ensure complete precipitation and avoid contamination of the plasmid DNA with the chromosomal DNA. This step resulted in the neutralisation of the lysate and the adjustment in high-salt binding conditions, which caused precipitation of denatured proteins, Sodium Dodecyl Sulphate (SDS), cell-wall bound chromosomal DNA and cellular debris. The smaller plasmid DNA renatured correctly and stayed in solution. The lysates were then cleared by centrifugation for 10 min at 15,700 x g.

d). DNA adsorption to the QIAprep® membrane: The supernatants were then applied to QIAprep® columns with silica-gel membrane for selective-adsorption of plasmid DNA in high-salt buffer and elution in low-salt buffer. Binding of DNA was thus ensured, while centrifugation for 1 min at 15,700 x g resulted in the release of RNA, cellular proteins and metabolites in the flow-through, which was discarded.

e). Washing of plasmid DNA: To efficiently remove all endonucleases the column was washed with 500µl PB buffer for 1 min at 15,700 x g and another wash with 750µl of ethanol-containing PE buffer ensured efficient salt removal. To remove the residual ethanol from the wash buffer that may inhibit subsequent enzymatic reactions, the flow-through was discarded with an additional 1 min centrifugation step.

f). Elution of plasmid DNA: The purified plasmid DNA was then eluted in 50µl TE buffer (25mM Tris-HCl, pH 8.0; 1mM EDTA) to the centre of the membrane, let stand for 1 min and centrifuged for 1 min to be eluted into a clean 1.5 ml microcentrifuge tube. These so-called 'mini-prep' generated DNA samples were stored at -20°C and were suitable for subsequent restriction enzyme digestion. 5 to 20µg of plasmid DNA was obtained from the 2 ml overnight culture, depending on plasmid copy number per cell, the individual insert in a plasmid, growth medium used for the bacterial culture, the elution volume and the elution incubation time.

2.8.10 DNA Sequencing

All sequencing was performed by the Wellcome Trust Central Biotechnology Services support facility (Henry Wellcome Building, Cardiff University, UK) using the ABI Prism® 3100 Genetic Analyser (Applied Biosystems, UK). Sequencing reactions were prepared according to the ABI Prism BigDye® Terminator v3.1 Cycle sequencing kit protocol (Applied Biosystems, UK). This involves the use of a ready reaction mix containing dNTPs, AmpliTaq DNA Polymerase, $MgCl_2$ and dye terminators. The concentration of the purified plasmid DNA was measured as described in section 2.4.2. The reaction mix consisted of 4µl of 5 x BigDye®

Terminator ready reaction mix, 2µl of 5 x BD3.1 sequencing buffer, 0.5 - 1µg of plasmid DNA, 20pmol of forward or reverse primer (for sequencing primers see Table 2.10) and DNase-free water to make the reaction up to 20µl. Samples were briefly vortexed and spun before being subjected to 94°C for 1 min and 30 cycles of 94°C for 15 sec, 50°C for 30 sec, 60°C for 5 min.

DNA extension products from the PCR were precipitated to remove any unincorporated BigDye® Terminators by adding 1µl of 3M sodium acetate and 1µl 0.5M EDTA. The samples were briefly vortexed and washed with 80µl of 95-100% chilled ethanol to remove any remaining residual salts prior to centrifugation at 16,000 x g for 15 min. The supernatant was decanted and 200µl of 70% chilled ethanol was added. Another centrifugation step followed and the supernatant was decanted to add 100µl of 70% chilled ethanol to the samples.

Samples were sent to the Wellcome Trust Central Biotechnology Services support facility, where they were spun in a Fisher AccuSpin Micro bench top centrifuge at 15,700 x g for 2 min. The samples were then placed in a Thermo Savant DNA110 SpeedVac on a medium setting and run until dry. For denaturation, the samples were resuspended in 10µl of ABI HiDi® Formamide (Applied Biosystems, UK), briefly vortex-mixed and then spun in bench top centrifuge as before. The samples were subsequently pipetted into ABI MicroAmp 96-well reaction plate (Applied Biosystems, UK) and Sequencing Analysis was carried out on an ABI 3130xl Genetic Analyzer using ABI Foundation Data Collection Version 3.0 (Applied Biosystems, UK). Resulting sequencing chromatograms were analysed using the computer software program, ABI Sequence Analysis Version 5.2 (Applied Biosystems, UK). The data were examined for sequence identity for the detection of mutations on the National Centre for Biotechnology Information (NCBI) database using the search engine BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Primer Name	Primer Sequence
T7	$^{5'}$TAA TAC GAC TCA CTA TAG GG$^{3'}$
SP6	$^{5'}$ATT TAG GTG ACA CTA TAG$^{3'}$
CMV forward	$^{5'}$CGC AAA TGG GCG GTA GGC$^{3'}$
BGH reverse	$^{5'}$TAG AAG GCA CAG TCG AGG$^{3'}$

Table 2.10: List of primer sequences used for sequencing.

2.9 Cell Transfection and Cloning

2.9.1 Generation of transiently transfected Cos-7 cells and stably transfected CHO cells expressing shIL-18Rα-F, shIL-18Rβ-Fc and shIL-18Rαβ-Fc

The GeneJuice® transfection reagent (MERCK Biosciences, Novagen, UK), composed of a non-toxic cellular protein and a small amount of a novel polyamine, was used for the efficient DNA transfer in both stable and transient transfection of Cos-7 and CHO cells respectively with plasmids containing shIL-18Rα-Fc and shIL-18Rβ-Fc.

The day before transfection, 2 x 10^5 cells in mid-log phase were plated per well in a 6-well plate and in 5 ml of complete growth D-MEM media. Cells were incubated overnight at 37°C (5% CO_2) to reach 50 - 80% confluence before transfection. GeneJuice® alone was used as a negative control. For each well to be transfected, 3µl of GeneJuice® was added into 100µl of serum-free media in a sterile tube, mixed thoroughly by vortexing and incubated at room temperature for 5 min. The DNA to be transfected was at a concentration of 0.5 - 1 µg/µl, so that 1µg DNA was added to GeneJuice®/serum-free medium mixture, mixed by gentle pipetting and incubated at room temperature for 15 min. The entire volume of GeneJuice®/DNA mixture was added drop-wise to the cells in 3ml of complete growth medium for Cos-7 cells and serum-free medium for CHO cells. The plate was gently rocked to ensure even

distribution of the drops. The transfection mixture was removed after 6 h incubation to be replaced with 5 ml of full growth medium.

Transient transfection of Cos-7 cells

After 48 h incubation of Cos-7 cells at 37°C (5% CO_2) following transfection, the supernatant was harvested and centrifuged at 300 x g for 5 min to get rid of any cell debri. The supernatant was used for characterisation assays to confirm expression and secretion of shIL-18Rα-Fc, shIL-18Rβ-Fc and shIL-18Rαβ-Fc.

Stable transfection of CHO cells

For stable CHO cell line selection, 24 h after transfection the cells were transferred in 75mm Petri-dishes and sub-cultured in complete growth medium plus 700μg/ml Zeocin for about 3 weeks, allowing for growth and selection of the desired cells. 48 single Zeocin resistant colonies were picked up using a tip with 40μl PBS and transferred into 24-microwell plates containing full growth medium. Cells were expanded for 10 more days and half of these were used for functional analysis in order to determine the highest expression colonies. The highest expression colonies were then expanded in a T75 flask (Greiner Bio-One Ltd., UK) and half of the cells were frozen *in situ* (section 2.1.4) and the rest were expanded for protein purification using HiTrap™ Protein A Affinity Chromatography (section 2.10).

2.10 Single Step Affinity Purification of the Soluble Human IL-18 Receptor

Chinese hamster ovarian (CHO) cells transfected with either shIL-18Rα-Fc, shIL-18Rβ-Fc or shIL-18Rαβ-Fc (desribed in section 2.9.1) were expanded in five T75 flasks (Greiner Bio-One Ltd., UK) in complete growth media, before they were cultured in 1L of D-MEM media containing 4500mg/L Glucose and L-Glutamine and supplemented with 10% Ultra low IgG fetal bovine serum, 50UI/mL penicillin and 50μg/mL streptomycin (all Gibco, Invitrogen Ltd., UK). Conditioned media

were collected after 7 to 10 days of culture and centrifuged at 4,000 x g for 20 minutes at room temperature to pellet cell debris.

The supernatant was filtered using 0.2μm pore size polyethersulfone (PES) membrane filter units (Fisher Scientific, VivaScience AG, Germany) and 1 litre of media was immediately used for purification by single step affinity chromatography using 1mL HiTrap™ Protein A HP column (Amersham Biosciences, GE Healthcare, UK) coupled to an ÄKTAprime™ plus chromatography system. Purification was carried out at 4°C and the column was equilibrated in buffer A (20mM Sodium Phosphate Buffer, pH 7.4) at flow rate 1mL/min. The shIL-18R-Fc culture media was applied overnight at a flow rate 0.7mL/min. After loading, the column was washed with buffer A (20mM Sodium Phosphate Buffer, pH 7.4) at a flow rate of 1mL/min until stable conductivity was reached. Buffer B (0.1M Glycine-HCl, pH 3.0) was applied at a flow rate of 1mL/min to elute the bound material in 1mL fractions that were neutralised in 50 - 200μl buffer C (2M Tris-HCl, pH 9.0). After elution was complete, the HiTrap™ Protein A matrix was regenarated with 20% ethanol in buffer A.

Forty fractions were collected and analysed for the amount of protein present using BCA assay as described in section 2.5.1. The fractions containing the purified protein were pooled and buffer exchanged by dialysis (changed 4-5 times every 4h) into sterile PBS, pH 7.4 (phosphate buffered saline, Dulbecco A tablets, Oxoid, Basingstoke, UK) using Spectra/Por® Biotech regenerated cellulose dialysis membrane with MWCO of 15,000Da (Spectrum Laboratories Inc. SDS-PAGE analysis and Coomassie staining (Bio-Safe™ Coomassie stain, Bio-Rad Laboratories, UK) were used to confirm the presence of shIL-18R and estimate the purity of the protein purified. The purified protein (0.5 - 2.0mg/mL) was aliquoted and stored at -80°C to avoid loss of activity due to repeated freeze-thaw.

2.11 Sodium Dodecyl Sulphate-Polyacrilamide Gel Electrophoresis (SDS-PAGE) Analysis and Coomassie Blue Staining

Protein concentration was determined as described in section 2.5.1 by BCA assay. Samples of 15µl or 20 - 50µg cell lysates were added with the appropriate amount of 2x Laemmli sample buffer (0.2M Tris-HCl, pH 6.8; 4% w/v SDS; 1mM EDTA; 30% glycerol; 0.3% w/v bromoethanol blue; 5% β-mercaptoethanol) and denatured at 95°C for 5 minutes. Proteins were separated on a pre-cast 4-20% Tris-Glycine polyacrilamide gel (1.5mm x 15well Novex®, Invitrogen, UK) in 1 x running buffer (25mM Tris-HCl, pH 8.3; 190mM glycine; 0.1% SDS) and at 125V (limit 40mA) for 90 minutes or until proteins had sufficiently separated, using a Novex X-Cell SureLock Mini-Cell system, power supply NovexEase 500 (both Invitrogen, UK). 6µl of a pre-stained molecular weight marker (SeeBlue Plus2 prestained standard, Invitrogen, UK; Appendix 8.1.2) was also loaded to visualise protein separation. Proteins were visualised by Coomassie staining using Bio-Safe™ Coomassie stain (Bio-rad Laboratories, UK) or were transferred to nitrocellulose for Western blotting (section 2.5 *Western Blotting*).

2.12 Statistical Analysis

The standard deviation of the mean was calculated for each group of samples done in triplicates. Statistical significance was calculated using one-way ANOVA with Tukey-Kramer or Bonferroni multiple comparisons post-tests. One-way ANOVA compares the means of three or more groups, assuming that the data are sampled from Gaussian population. If the overall one-way ANOVA P value is small then it is likely that the differences observed are not due to random sampling. This means that at least one but not necessarily all the means differ from the rest. Post tests were performed to identify where these differences lied. Probability values of $P > 0.05$ were not considered significant (ns), whereas P values of * $P < 0.05$ were considered statistically significant, ** $P < 0.01$ very significant and *** $P < 0.001$ highly significant compared to the unstimulated control unless otherwise stated.

Chapter 3

3 Regulation of IL-18 signalling by TNFα

3.1 Background

In chronic inflammatory conditions, host immune responses are orchestrated by networks of cytokines that determine the outcome of the disease. The important role of IL-18 in regulating chronic inflammation has been demonstrated in several disease models (Gracie et al., 2003, Dinarello and Fantuzzi, 2003, Dinarello, 2007). Investigating the mechanisms of interplay between IL-18 and other cytokines is important in identifying critical interactions that could provide novel therapeutic approaches for the treatment of chronic inflammatory diseases.

Studies carried out to date have paid special attention to the combinational effect of IL-18 and IL-12, due to their remarkable synergy. This synergy results in the induction of IFNγ by B and T lymphocytes, NK cells, macrophages and DCs (Takeda et al., 1998, Yoshimoto et al., 1997, Munder et al., 1998, Okamura et al., 1995b, Fukao et al., 2000, Stober et al., 2001). The synergy of IL-18 and IL-12 occurs mainly through the up-regulation of IL-18R expression on T cells, Th1 cells and B cells after IL-12 stimulation (Yoshimoto et al., 1998). Mice treated with IL-18 together with IL-12 show significantly higher incidence and severity of CIA compared to each cytokine alone. However, the mechanism of action of IL-18 to promote CIA differs from that of IL-12 (Leung et al., 2000). Thus, in order to examine IL-18 signalling, without synergistic effects from IL-12, we used human monocyte-like dendritic precursor KG-1 cells that do not respond to IL-12 (Konishi et al., 1997).

As described in the general introduction, IL-18 was initially characterised by its ability to induce IFNγ production in mice with endotoxin shock (Nakamura et al., 1989, Okamura et al., 1995a, Okamura et al., 1995b). Based on this characteristic of IL-18, the human myelomonocytic cell line, KG-1 has been used to establish a simple, accurate and sensitive bioassay to study the role of IL-18 in regulating the immune response in both humans and mice (Konishi et al., 1997, Taniguchi et al.,

1998). This simplified model system of pre-dendritic KG-1 cells that respond to IL-18 for the production of IFNγ is an important tool for studying the role of other cytokines on the function of IL-18 to induce the maturation of DCs.

KG-1 was isolated from the bone marrow of a 59-year old Caucasian male patient with erythroleukemia (FAB M7) undergoing myeloblastic relapse, which eventually developed into myelogenous leukaemia (Koeffler and Golde, 1978). A subline, KG-1a, was established by serial passage of KG-1 (Koeffler et al., 1980), which is arrested at a less differentiated stage than KG-1 based on criteria such as surface phenotype, gene expression and growth factor unresponsiveness (Fukuda et al., 1981, Furley et al., 1986). Like the committed $CD34^+$ myeloid DC/macrophage progenitors of DC (Ryncarz and Anasetti, 1998), KG-1 cells are MHC $II^+CD34^+CD86^+$, whereas KG-1a are MHC $II^-CD34^+CD86^-$ (St Louis et al., 1999).

The level of bioactive IL-18 in culture supernatants, cellular extracts and body fluids can be determined by measuring the amount of IFNγ produced in the culture supernatant of KG-1 cells using ELISA. In this model system, IL-18 has been shown to dose-dependently induce IFNγ production. Another sensitive and useful IL-18 assay using human mitogen-stimulated PBMCs has also been established (Ushio et al., 1996), but apart from the difficulty of isolating PBMCs, the variability between PBMC donors may present difficulties in the establishment of a standard range for the bioassay. Moreover, PBMCs have also been shown to produce IFNγ in response to human IL-12 stimulation (Kobayashi et al., 1989), as opposed to KG-1 cells (Konishi et al., 1997), making the KG-1 assay a simpler model to study IL-18 function.

The important role of IFNγ in the control of intracellular pathogens has been demonstrated in $IFN\gamma^{-/-}$ and IFNγ $receptor^{-/-}$ mice, and in studies using anti-IFNγ antibodies (Schroder et al., 2004). Initially, it was believed that only lymphoid derived cells such as $CD4^+$ T helper type I (Th1) lymphocytes, $CD8^+$ cytotoxic T cells and NK cells produced IFNγ. However, it has recently been demonstrated that

myeloid derived cells such as mature DCs also produce IFNγ (Fukao et al., 2000, Pashenkov et al., 2000, Rescigno et al., 2000).

Mature dendritic cells (DCs) are professional antigen-presenting cells and the key players that drive the immune response in chronic inflammatory conditions. These cells activate adaptive immunity by taking up bacterial antigens and presenting them to naïve T cells; thereby inducing their clonal expansion and differentiation into antigen-specific effector T cells. The process of DC maturation is regulated by various co-stimulatory factors and cytokines including IL-18 and TNFα. KG-1 cells are known to express CD54, which is up-regulated by IL-18 (Kohka et al., 1998). As previously mentioned, the up-regulation of CD54 by IL-18 has been described on other human cell lines, such as PBMCs (Yoshida et al., 2001), PBMC-derived dendritic cells (Gutzmer et al., 2003) and RA synovial fibroblasts and endothelial cells (Morel et al., 2001). Both IL-18 and TNFα induce the maturation of dendritic precursor KG-1 cells by up-regulating the expression of the co-stimulatory molecules CD83, B7.1 (CD80), B7.2 (CD86), CD40, CD54 and the major histocompatibity complex class II (MHC II) (Li et al., 2004b). Due to the difficulty of isolating homogenous monocyte or DC populations from PBMCs, we chose KG-1 cells that have similar multipotent properties to PBMCs and the capacity to mature into both macrophages (Koeffler, 1983) and dendritic-like cells (St Louis et al., 1999, Ackerman and Cresswell, 2003). KG-1 cells are therefore considered to be a good model for studying human DC-specific processes (Berges et al., 2005).

The interplay between IL-18 and TNFα has been reported in inflammatory synovitis, where IL-18 promotes the release of TNFα, which in turn up-regulates the expression of IL-18 in fibroblast-like synoviocytes (Gracie et al., 2003). It has also been reported that IL-18 induces the synthesis of TNFα from non-$CD14^+$ isolated PBMCs (Puren et al., 1998). Moreover, TNFα has been shown to up-regulate the expression of IL-18R on KG-1 cells (Nakamura et al., 2000, Wu et al., 2003).

Groups studying the role of TNFα in regulating IL-18 signalling have focussed on the regulation of IL-18 receptor expression. TNFα has been shown to up-regulate the

expression of IL-18R on KG-1 cells (Nakamura et al., 2000, Wu et al., 2003). However, it is not clear whether the induction of the IL-18 receptor complex is a direct or indirect consequence of TNFα. Recent studies have shown that TNFα regulates both IL-18 and IL-18R mRNA expression in a time dependent manner in fully differentiated human SGBS (Simpson-Golabi-Behmel syndrome) adiposities (Wood et al., 2005) and in lung and renal epithelial cells (Krásná et al., 2005). Work by (Nakamura et al., 2000) has investigated the expression of IL-18R using a large panel of hematopoietic cell lines and has shown that 24 out of 39 cell lines tested express IL-18R but only KG-1 showed the ability to produce IFNγ, GM-CSF and IL-6 upon IL-18 stimulation. This cell line responds to IL-18 and produces IFNγ in the absence of IL-12 (Konishi et al., 1997), which makes it a less complicating bioassay to characterise IL-18R and study the signal transduction mechanisms of IL-18. Nakamura *et al.*, 2000, were also the first group that presented evidence that TNFα has modulating effects on IL-18R expression. Thus, in this study KG-1 cells were used as a model system to study the role of TNFα in regulating IL-18 signalling.

Recent studies on the molecular mechanisms regulating INFγ gene expression in T cells have shown the importance of the p38 mitogen-activated protein kinase (MAPK) pathway. Several phase II-III clinical trials are underway to evaluate selective inhibitors of p38 MAPK, particularly for the treatment of RA (Kumar et al., 2003, Palladino et al., 2003, Miwatashi et al., 2005). IFNγ expression in Th1 cells depends upon the p38 MAPK signalling pathway (Rincón et al., 1998) and is induced by IL-18 and IL-12 (Berenson et al., 2006). Another group showed that IL-12 activates p38 MAPK but not ERK1/2 that is required for normal IFNγ expression in activated T cells (Zhang and Kaplan, 2000).

In mature DCs, IL-12 induces a basal level of p38 MAPK activity that is enhanced by IL-18 and is required for IFNγ production (Fukao et al., 2000). More importantly, a recent study done in KG-1 cells has demonstrated that IL-18 activates p38 MAPK and NFκB to induce T-bet (T-box expressed in T cells) expression and function (Bachmann et al., 2007). T-bet is a Th1-specific transcription factor required for the

generation of Th1 cells through IFNγ expression, as it was established by Glimcher *et al.*, 2007, through the generation of T-bet deficient and overexpressing mice (Szabo et al., 2000, Szabo et al., 2002, Glimcher, 2007). T-bet is potently induced by IFNγ in human monocytes and myeloid dendritic cells (Lighvani et al., 2001). DCs from mice deficient in T-bet show significantly impaired capabilities to produce IFNγ (Lugo-Villarino et al., 2003b).

The objective of this study was to examine the role of TNFα in the expression of IL-18R in the KG-1 cell-based model system in correlation with IL-18-induced signalling and DC maturation. Understanding the mechanism by which IL-18 mediates its effects and is regulated by other cytokines will be of importance in considering therapeutic approaches to prevent the maturation of dendritic cells by targeting IL-18 signalling.

3.1.1 Aims and Objectives

1. In order to study IL-18 signalling, the initial objective of this project was to confirm that our KG-1 cell bioassay model system is functional and the cells respond to IL-18 stimulation to secrete IFNγ.

2. To investigate the role of the pro-inflammatory mediator, TNFα, in regulating IL-18 signalling by studying its effects on IL-18R expression in KG-1 cells.

3. To investigate the consequences of TNFα regulation of IL-18R on IL-18 downstream signalling in KG-1 cells. Specifically, to determine whether IL-18 induced p38 MAPK or ERK1/2 phosphorylation and whether the activation of these kinases would be required for the subsequent IFNγ production in the KG-1 cell bioassay.

4. Also, to define the role of TNFα in regulating T-bet transcription factor expression in KG-1 cells.

5. To better understand the mechanism of DC maturation induced by TNFα and IL-18 stimulation.

3.2 Results and Discussion

3.2.1 IL-18-induced secretion of IFNγ in the KG-1 cell model system

The simple and sensitive KG-1 bioassay was used for the detection of IFNγ secreted in the culture media of the cells after stimulation with IL-18 (Konishi et al., 1997). In order to confirm the functionality of our assay, KG-1 cells were stimulated with increasing doses of IL-18 for 48 hours and the amount of IFNγ secreted in the cell supernatant was quantified using ELISA. The result in figure 3.1 confirmed that IL-18 induced IFNγ production in KG-1 cells in a dose dependent manner, reaching a significant increase of 2ng/ml IFNγ in response to 100ng/ml IL-18 stimulation compared to unstimulated.

3.2.2 Role of IL-18 in pre-dendritic KG-1 cell maturation

Following the confirmation that the cells respond to IL-18, we examined the effect of this cytokine on the maturation of the pre-dendritic KG-1 cells by investigating the expression of co-stimulatory molecules. Human dendritic cells are a heterogeneous population derived from the differentiation of two distinct subpopulations of haematopoietic progenitor cells (HPC); the $CD14^+$ monocytes (Zhou and Tedder, 1996, Pickl et al., 1996, Kiertscher and Roth, 1996) and the $CD34^+$ bone marrow cells. Lymphoid DCs derive from $CD34^+CD10^+$ HPC (Galy et al., 1995), whereas myeloid DCs from $CD34^+CD86^+$ HPC (Ryncarz and Anasetti, 1998). Mature DCs are typically characterised by the expression of MHC class I and II, the B7 family co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2), and the DC lineage marker CD83 (Hart, 1997).

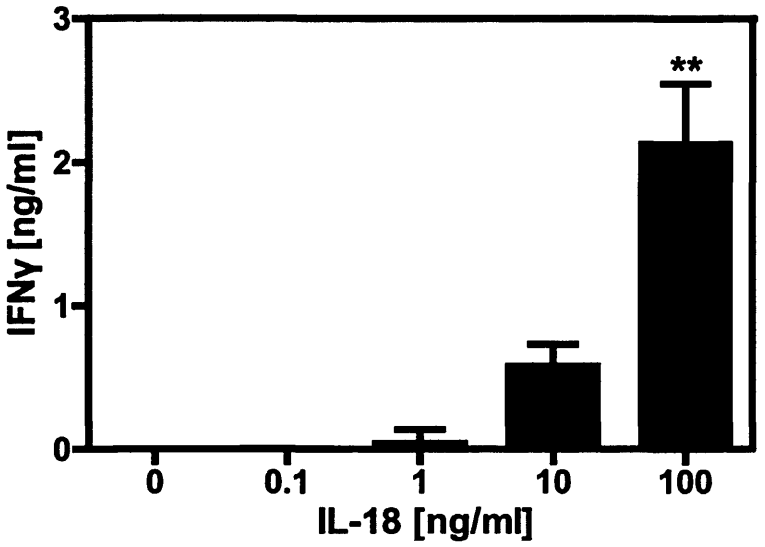


Figure 3.1: IL-18 induced the production of IFNγ in KG-1 cells in a dose-dependent manner.

KG-1 cells were stimulated with different concentrations of IL-18 for 48h. IFNγ produced in the culture media was determined by ELISA. Data shown are expressed as mean ± SD of triplicates of a representative experiment. ** $P < 0.01$ by Tukey-Kramer post-test compared to unstimulated cells.

It is known that IL-18 stimulation facilitates the maturation of the $CD34^+$ pre-dendritic KG-1 cells by up-regulating CD83, HLA-DR (MHC II) and CD54 by day 3. Marked increase of the co-stimulatory molecules CD83, CD80, CD86, CD40, CD54 and HLA-DR upon IL-18 or TNFα stimulation for 9 days has been reported. This effect was likely not due to endogenous TNFα production as it was not prevented in the presence of an antibody against TNFα (Infliximab) (Li et al., 2004b).

Flow cytometry was used to examine the effect of IL-18 on the expression of co-stimulatory molecules on KG-1 cells one day after their stimulation. CD80 is a co-stimulatory molecule expressed on professional APCs that belongs to the B7-CD28 family and is crucial for the regulation of activation and tolerance of T cell immunity. IL-18 only slightly up-regulated CD80 expression on KG-1 cells after one day of stimulation (Fig. 3.2a). It was also confirmed that KG-1 cells do not express the monocyte marker CD14 and thus they belong to the $CD34^+$ bone marrow lineage pre-dendritic cells (Fig. 3.2b).

To investigate the ability of KG-1 cells to express adhesion molecules, we used FITC-conjugated antibodies against human CD54, CD50 and CD58 and performed FACS. CD58 was expressed on KG-1 cells but IL-18 did not have any effect on the up-regulation of this molecule (Fig. 3.2c). Interestingly, the staining with CD50 revealed two sub-populations of KG-1 cells with opposite expression levels before and after stimulation with IL-18 (Fig. 3.2d). This could be due to the differential characteristics of the cells as a result of the differentiation and maturation of precursor DCs induced by IL-18. As expected, IL-18 up-regulated the expression of CD54 on KG-1 cells (Fig. 3.2e). As shown in figure 3.2e, the priming of the cells with TNFα prior to IL-18 stimulation further induced the up-regulation of CD54 expression on these cells indicating that the interaction between IL-18 and TNFα may contribute to the inflammatory response.

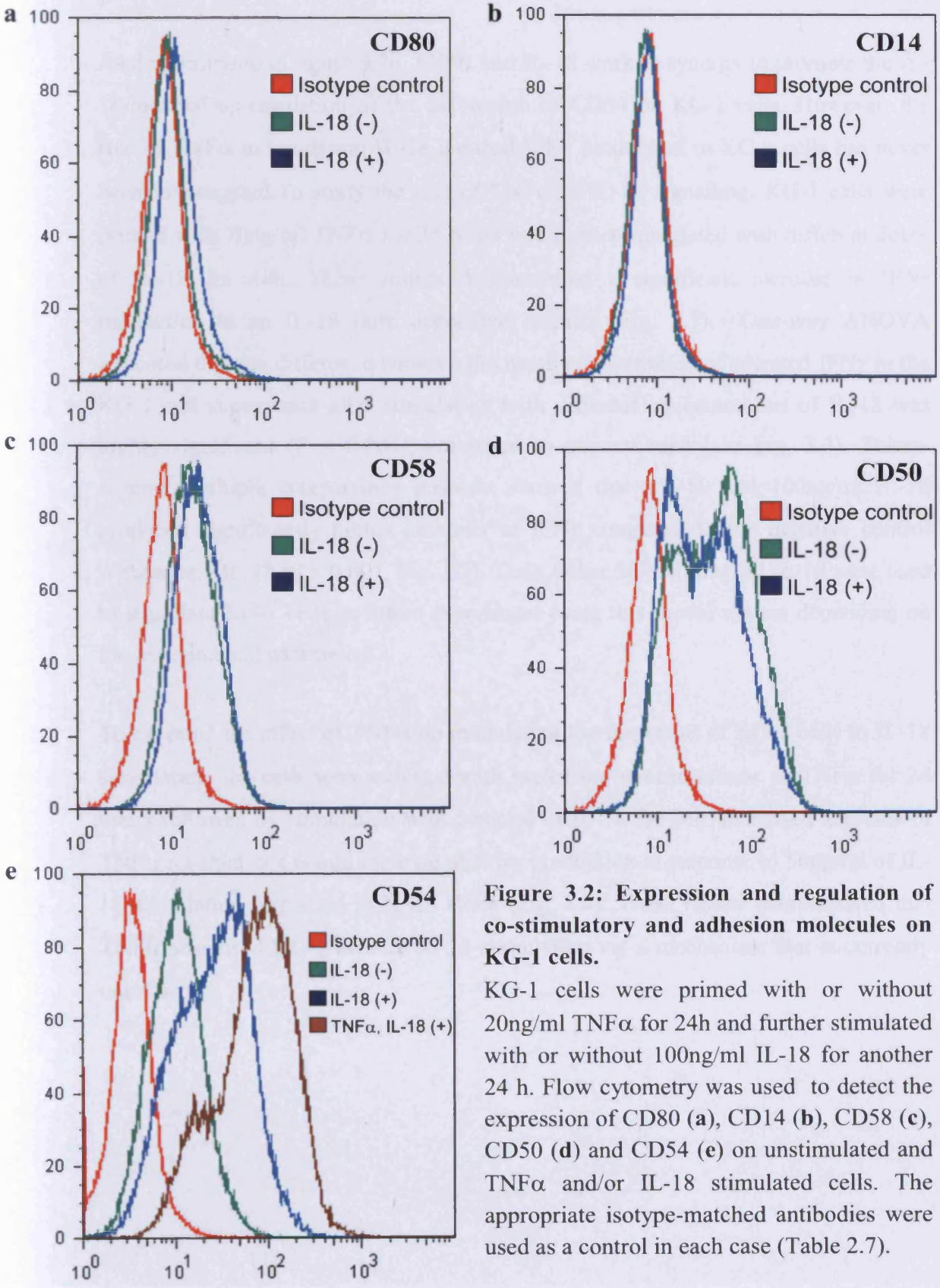


Figure 3.2: Expression and regulation of co-stimulatory and adhesion molecules on KG-1 cells.

KG-1 cells were primed with or without 20ng/ml TNFα for 24h and further stimulated with or without 100ng/ml IL-18 for another 24 h. Flow cytometry was used to detect the expression of CD80 (**a**), CD14 (**b**), CD58 (**c**), CD50 (**d**) and CD54 (**e**) on unstimulated and TNFα and/or IL-18 stimulated cells. The appropriate isotype-matched antibodies were used as a control in each case (Table 2.7).

3.2.3 TNFα increased the sensitivity of KG-1 cells to IL-18 stimulation

As demonstrated in figure 3.2e, TNFα and IL-18 work in synergy to promote the IL-18-induced up-regulation of the expression of CD54 on KG-1 cells. However, the role of TNFα in regulating IL-18-induced IFNγ production in KG-1 cells has never been investigated.To study the role of TNFα in IL-18 signalling, KG-1 cells were primed with 20ng/ml TNFα for 24 hours and further stimulated with different doses of IL-18 for 48h. These results demonstrated a significant increase in IFNγ production in an IL-18 dose dependent manner (Fig. 3.3). One-way ANOVA indicated that the difference between the mean concentration of secreted IFNγ in the KG-1 cell supernatant after stimulation with different concentrations of IL-18 was highly significant ($P < 0.001$), compared to unstimulated (see Fig. 3.3). Tukey-Kramer multiple comparisons post-test showed that 25, 50 and 100ng/ml IL-18 produced significantly higher amounts of IFNγ compared to the negative control without any IL-18 ($P < 0.001$, Fig. 3.3). Thus, either 50 or 100ng/ml IL-18 were used to stimulate KG-1 cells in future experiment using this model system depending on the experimental parameters.

To examine the effect of TNFα on modulating the responses of KG-1 cells to IL-18 stimulation, the cells were cultured with increasing concentrations of TNFα for 24 hours followed by stimulation with 50ng/ml of IL-18 for 24h. Increased amounts of TNFα resulted in a 6-fold increase of IFNγ production in response to 50ng/ml of IL-18 stimulation compared to IL-18 alone (Fig. 3.4). These results demonstrated that TNFα sensitised KG-1 cells to IL-18 stimulation via a mechanism that is currently unknown.

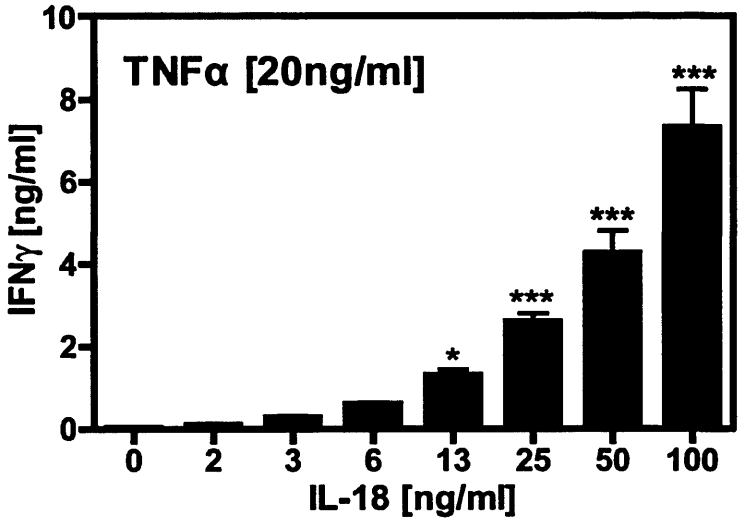


Figure 3.3: IL-18 stimulated IFNγ production in TNFα primed cells in a dose-dependent manner.

KG-1 cells were stimulated with 20ng/ml TNFα for 24h followed by stimulation with different concentrations of IL-18 for 48h. IFNγ produced in the culture media was determined by ELISA. Data shown are expressed as mean ±SD of triplicates of a representative experiment. * $P < 0.05$; *** $P < 0.001$ by Tukey-Kramer post-test compared to unstimulated cells.

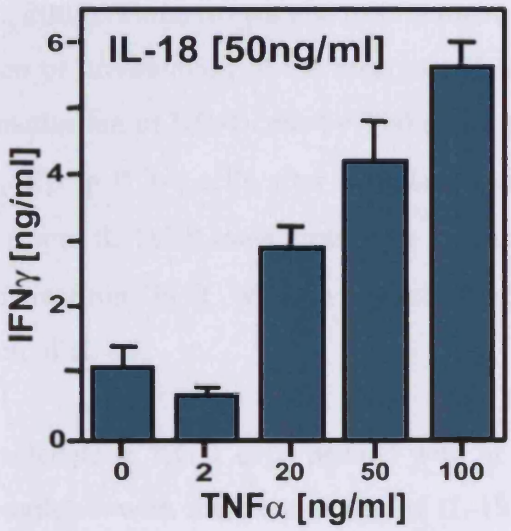


Figure 3.4: TNFα dose-dependently increased the sensitivity of KG-1 cells to IL-18 stimulation.

KG-1 cells were stimulated with increasing amounts of TNFα for 24h followed by stimulation with 50ng/ml of IL-18 for 24h. IFNγ produced in the culture media was determined by ELISA. Data shown are expressed as mean ±SD of triplicates of a representative experiment.

3.2.4 Mechanism of regulation of IL-18 signalling by TNFα

3.2.4.1 TNFα significantly up-regulated IL-18Rα and IL-18Rβ mRNA expression

It has been reported that TNFα up-regulates IL-18R expression on the surface of KG-1 cells (Wu et al., 2003). Thus, we wanted to investigate whether this effect was due to gene expression or presentation of the receptor. Therefore, in order to study the mechanism of sensitisation of KG-1 cells by TNFα, we examined the expression of IL-18Rα and IL-18Rβ in KG-1 cells after stimulation with TNFα. The level of expression of IL-18Rα and IL-18Rβ transcripts were measured by semi-quantitative PCR and quantified by real time PCR. We also assessed the effect of IL-18/TNFα on endogenous expression of IL-18.

Total RNA was extracted from KG-1 cells primed with or without 20ng/ml TNFα for 24h and then stimulated with different doses of IL-18 for 24h or 48h. Using primers specific for human IL-18, we showed that IL-18 transcript was not up-regulated in TNFα-primed cells (Fig. 3.5). We showed that the stimulation of TNFα-primed KG-1 cells with IL-18 up-regulated the gene expression of IL-18Rα, indicating that IL-18 has an effect on the transcriptional regulation of IL-18Rα. Conversely, IL-18 did not have any major effect on the gene expression of IL-18Rβ (Fig. 3.5), which was already expressed at very high levels.

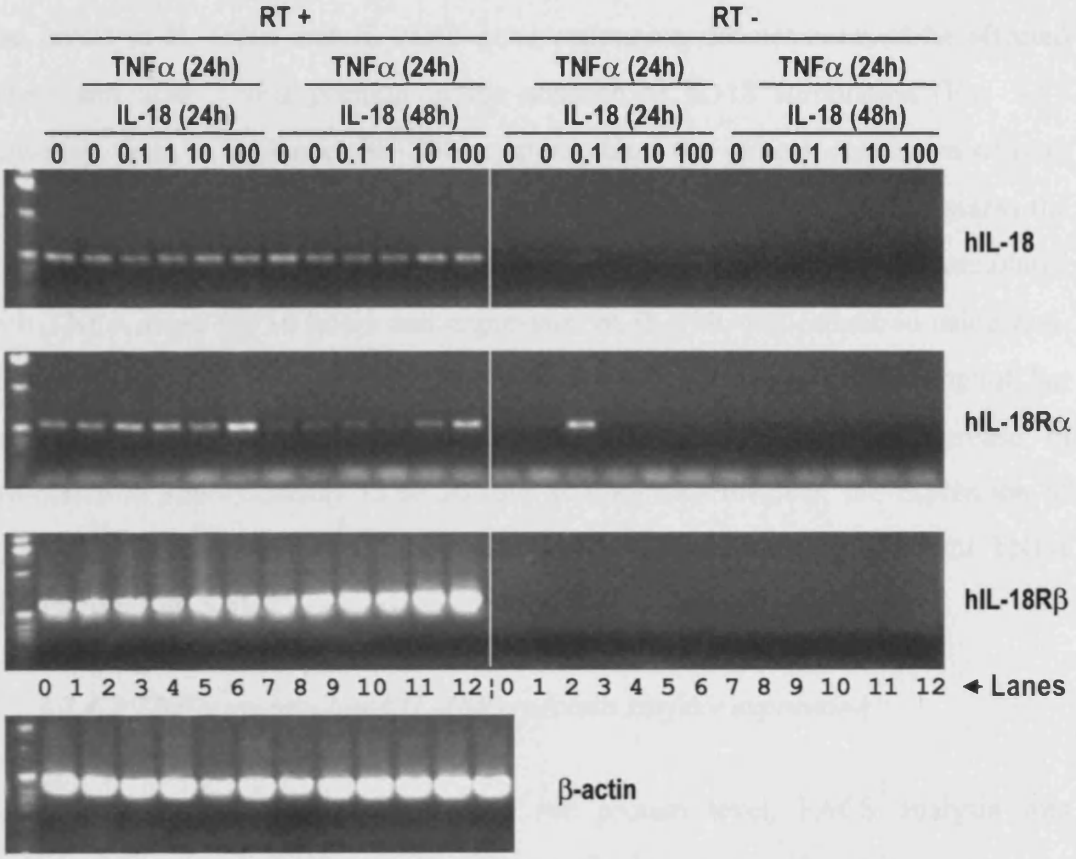


Figure 3.5: Regulation of hIL-18, hIL-18Rα, hIL-18Rβ and shIL-18Rα gene expression in TNFα primed KG-1 cells stimulated with IL-18.

Cells were stimulated with or without TNFα (20ng/ml) for 24h and then with IL-18 (0, 0.1, 1, 10, 100 ng/ml) for 24h or 48h. Gene expression was detected by PCR. Samples derived from RT-PCR of total RNA without reverse transcriptase were used as a cDNA negative control. Lanes: 0 [1kb DNA Ladder]; 1 [cells only (24h)]; 2-6 [20ng/ml TNFα (24h) + 0, 0.1, 1, 10, 100 ng/ml IL-18 (24h)]; 7 [cells only (48h)]; 8-12 [20ng/ml TNFα (24h) + 0, 0.1, 1, 10, 100 ng/ml IL-18 (48h)].

The levels of IL-18Rα and IL-18Rβ gene expression did not seem to be affected before and after TNFα priming in the absence of IL-18 stimulation (Fig. 3.5). However, there is evidence that TNFα up-regulates the protein expression of both IL-18Rα and IL-18Rβ on KG-1 cells (Wu et al., 2003). In order to better assess the effect of TNFα on the gene expression of the receptor, KG-1 cells were stimulated with TNFα alone for 16 hours and expression of IL-18R was measured using real-time PCR (Fig. 3.6). IL-18Rα was increased significantly in response to 10ng/ml, but not 1ng/ml TNFα stimulation, apparently reaching the maximal increase in expression at approximately 15 to 20-fold at 10ng/ml. However, the expression of IL-18Rβ transcript showed a modest increase of 2-fold only at 100ng/ml TNFα stimulation (Fig. 3.6).

3.2.4.2 TNFα up-regulated IL-18Rα protein surface expression

To confirm expression of IL-18Rα at the protein level, FACS analysis was performed by using specific anti-IL-18Rα antibodies. A significant increase in the membrane form of IL-18Rα (Fig. 3.7a), but not IL-18Rβ (Fig. 3.7), was detected on the cells after stimulation with TNFα. As mentioned above, a study by Wu *et al.*, 2003 has shown that TNFα induces the expression of both IL-18Rα and IL-18Rβ on KG-1 cells. These results together with our gene expression studies indicated that TNFα sensitised KG-1 cells to IL-18 stimulation via the up-regulation of IL-18R.

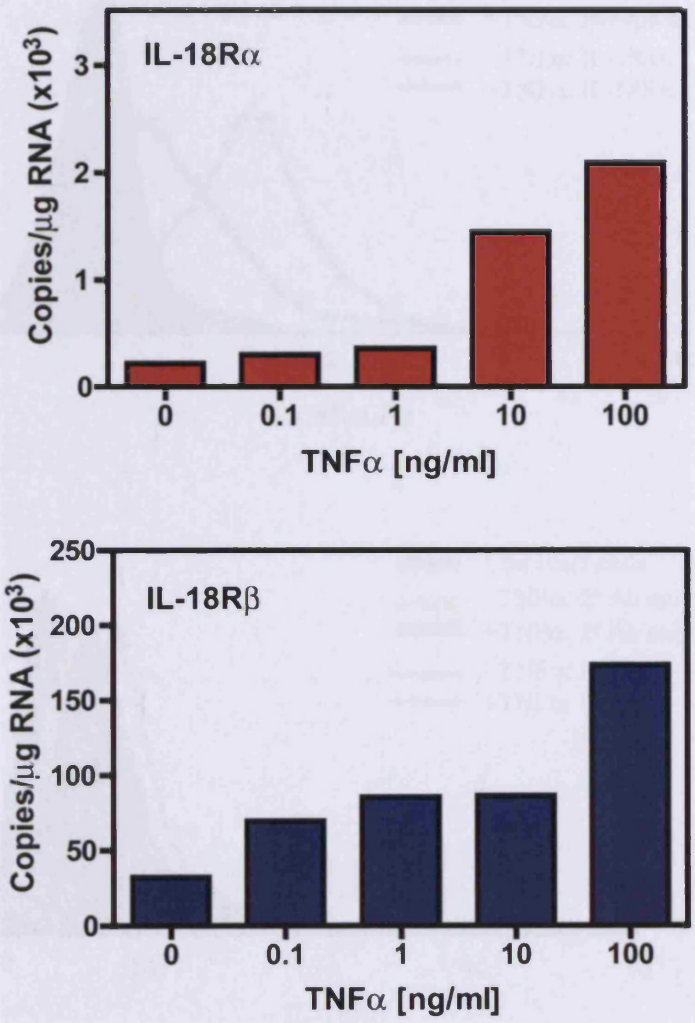


Figure 3.6: IL-18Rα mRNA transcript was upregulated by 10ng/ml TNFα treatment, while IL-18Rβ was not.

KG-1 cells were stimulated with increasing doses of TNFα for 16h. hIL-18Rα (**a**) and hIL-18Rβ (**b**) mRNA expression was quantified by qPCR using TaqMan assay. Data shown are expressed as means of duplicates of a representative experiment.

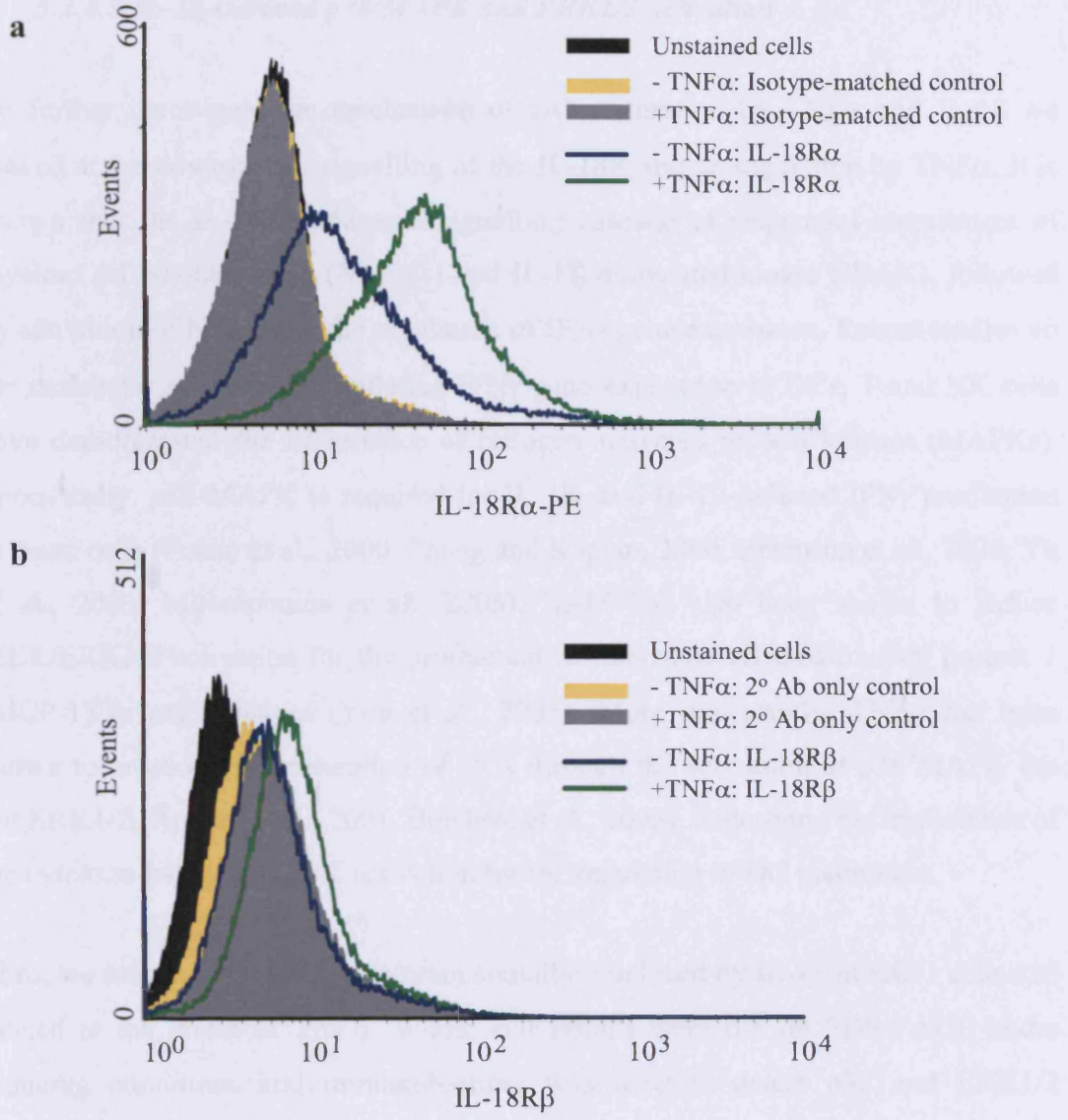


Figure 3.7: IL-18Rα, but not IL-18Rβ, protein surface expression was induced by TNFα in KG-1 cells.

KG-1 cells were stimulated with 10ng/ml TNFα for 24h. hIL-18Rα (**a**) and hIL-18Rβ (**b**) protein expression was quantified by FACS analysis. For the detection of IL-18Rα chain, a directly conjugated anti-IL-18Rα-PE mAb was used and compared to an isotype-matched Ab control. For IL-18Rβ chain, a mAb against IL-18Rβ was used and detected with a secondary (2°) anti-mouse IgG-FITC Ab. In this case, the 2° Ab alone was used as a control.

3.2.4.3 IL-18-induced p38 MAPK and ERK1/2 activation

To further investigate the mechanism of DC maturation by TNFα and IL-18 we looked at the downstream signalling of the IL-18R and its regulation by TNFα. It is known that the IL-18R induces a signalling cascade of sequential recruitment of myeloid differentiation 88 (MyD88) and IL-1R-associated kinase (IRAK), followed by activation of NFκB and the regulation of IFNγ gene expression. Recent studies on the molecular mechanism regulating IFNγ gene expression in DCs, T and NK cells have demonstrated the importance of mitogen-activated protein kinases (MAPKs). Specifically, p38 MAPK is required for IL-18- and IL-12-induced IFNγ production in these cells (Fukao et al., 2000, Zhang and Kaplan, 2000, Berenson et al., 2006, Yu et al., 2003, Mavropoulos et al., 2005). IL-18 has also been shown to induce MEK/ERK1/2 activation for the production of monocyte chemoattractant protein 1 (MCP-1) in macrophages (Yoo et al., 2005). More importantly, TNFα has been shown to promote the maturation of DCs through the activation of p38 MAPK but not ERK1/2 (Arrighi et al., 2001, Boislève et al., 2005), underlying the importance of the cytokine-induced MAPK activation for the regulation of DC maturation.

Thus, we investigated the downstream signalling induced by IL-18 in KG-1 cells and looked at the effect of TNFα. Whole cell lysates were run on SDS-PAGE under reducing conditions and immunoblotting was used to detect p38 and ERK1/2 MAPKs. A rabbit polyclonal antibody against a conserved sequence of human p38α, β, γ MAPK phosphorylated at Thr^{180}/Tyr^{182} was used to detect the activated form of p38 MAPK. A rabbit polyclonal antibody against the total human p38 MAPK (341-360) was used as a loading control. For the detection of the activated form of ERK1/2 (p44/p42) phosphorylated at Thr^{202}/Tyr^{204} rabbit anti-human antibody was used and the rabbit anti-human antibody for the total ERK1/2 was used as a loading control.

We found that IL-18 induced ERK1/2 and p38 MAPK phosphorylation within 10 minutes. The peak phosphorylation of ERK1/2 was seen at 15 minutes and p38

MAPK at 30 minutes after IL-18 stimulation (Fig. 3.8). As expected, the priming of the cells with TNFα resulted in the earlier 15 minute peak activation and more pronounced phosphorylation of p38 MAPK, possibly not only through the direct induction of p38 MAPK activation by TNFα, but also through the mechanism of up-regulation of IL-18R expression described above. TNFα priming did not seem to have an effect on ERK1/2 phosphorylation.

3.2.4.4 IL-18-induced activation of p38 MAPK but not ERK1/2 was necessary for IFNγ expression

The use of a specific p38 MAPK inhibitor *in vitro* profoundly inhibited the phenotypic and functional maturation of immature DCs obtained from peripheral blood, whereas an ERK1/2 inhibitor had no effect (Nakahara et al., 2004). Using these specific inhibitors for p38 MAPK (SB203580) (Cuenda et al., 1995) or for ERK1/2 (PD98059) (Alessi et al., 1995), we determined whether these signalling molecules are required for IL-18-induced IFNγ production in KG-1 cells and examined the effect of TNFα priming in this process. IL-18-induced IFNγ production was only blocked by a specific p38 MAPK inhibitor (SB203580) (Fig. 3.9), but not ERK1/2 inhibitor (PD98059) (Fig. 3.10), both in the TNFα-primed (Fig. 3.9b; Fig. 3.10 b) and non-primed (Fig. 3.9a; Fig. 3.10a) KG-1 cells. The results demonstrated that p38 MAPK is a key cell signalling molecule for IL-18-induced IFNγ production in human pre-dendritic KG-1 cells.

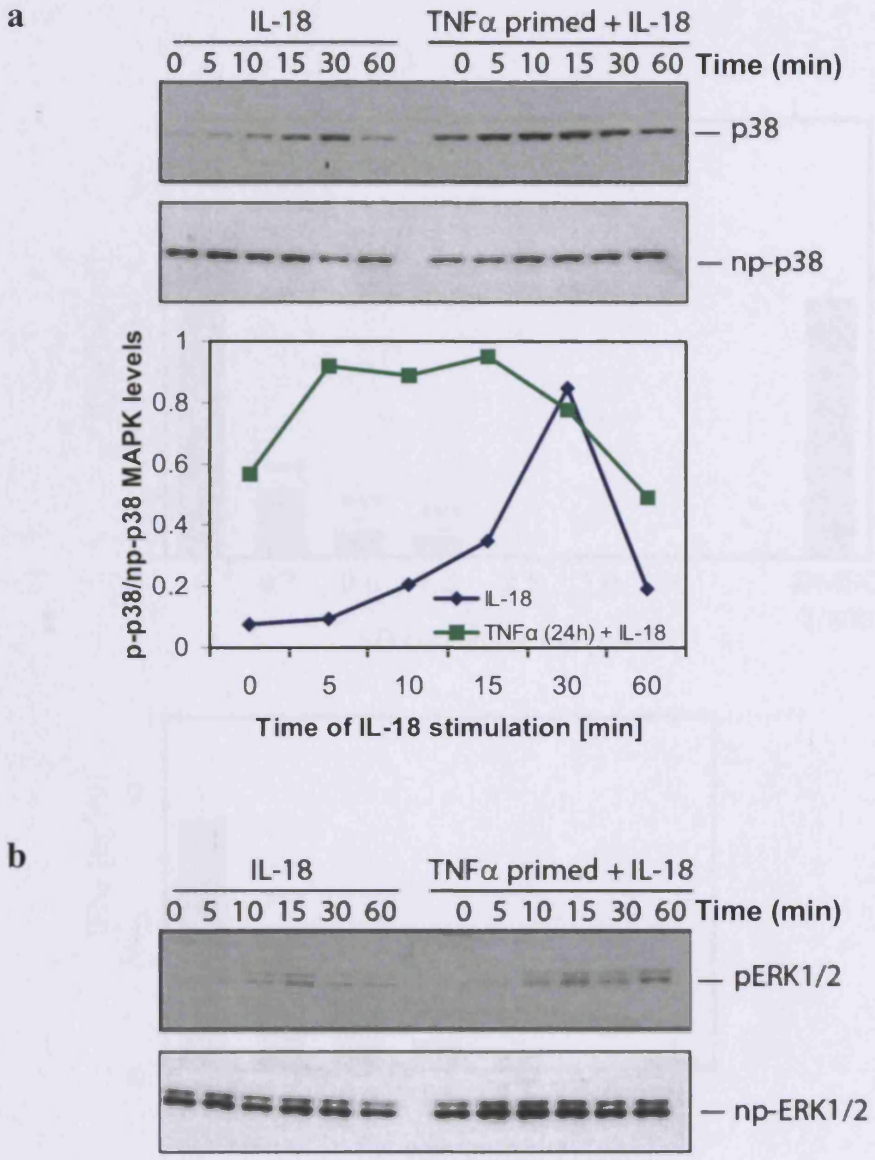


Figure 3.8: IL-18 induced rapid activation of p38 MAPK and ERK1/2.

KG-1 cells were stimulated without or with 20ng/ml TNFα for 24h followed by stimulation with 100ng/ml of IL-18 for 5, 10, 15, 30 and 60 minutes or without IL-18 (0 min). In the case of TNFα priming, 0 min represented the level of p38 or ERK1/2 after TNFα stimulation and before the addition of IL-18. Whole cell lysates were prepared and Western blotting was performed using phospho- and non-phospho-specific anti-human p38 MAPK (**a**) and anti-human ERK1/2 (**b**) rabbit polyclonal antibodies (designated as p and np respectively). The non-phosphorylated MAPK antibodies were used as loading controls. Densitometric analysis is shown in (a).

3.2.4.5 IL-18 induced T-bet expression that was up-regulated by TNFα

To better understand the downstream signalling events involved in the regulation of IFNγ expression by IL-18 and TNFα for the induction of DC maturation we examined the expression of the T-bet transcription factor in KG-1 cells. T-bet is a recently discovered member of the evolutionary conserved T-box transcription factor family that controls Th1 lineage commitment (Szabo et al., 2000).

T-bet also prominently determines the function of dendritic cells and is a crucial gene transcription factor for IFNγ production. IFNγ is potently induced by T-bet in human monocytes and myeloid DCs (Lighvani et al., 2001), and DCs isolated from T-bet gene knockout mice failed to produce IFNγ (Lugo-Villarino et al., 2003b). A recent study by Bachmann *et al.* using KG-1 cells, demonstrated that IL-18 directly activates the expression and function of T-bet via the activation of p38 MAPK (Bachmann et al., 2007). However, ERK1/2 did not phosphorylate T-bet protein *in vitro* (Hwang et al., 2005).

In order to confirm that the expression of T-bet in KG-1 cells is induced by IL-18 and to look at the effect of TNFα priming, cells were stimulated with different doses of TNFα for 24 hours and/or IL-18 for another 24 hours. T-bet expression was detected by Western blotting using an affinity purified anti-human T-bet antibody on whole KG-1 cell extracts after stimulation with increasing doses of IL-18. A basal level of T-bet expression was detected. IL-18 alone, at a concentration of 100ng/ml, induced higher expression of T-bet (Fig. 3.11). TNFα alone did not have any major effect on T-bet expression, whereas, as expected, TNFα priming of IL-18 stimulated KG-1 cells further induced the expression of T-bet (Fig. 3.11). From these results we could conclude that IL-18 could induce IFNγ production in KG-1 cells via activation of p38 MAPK and T-bet expression, but not ERK1/2, and this signalling cascade could be further promoted by TNFα priming.

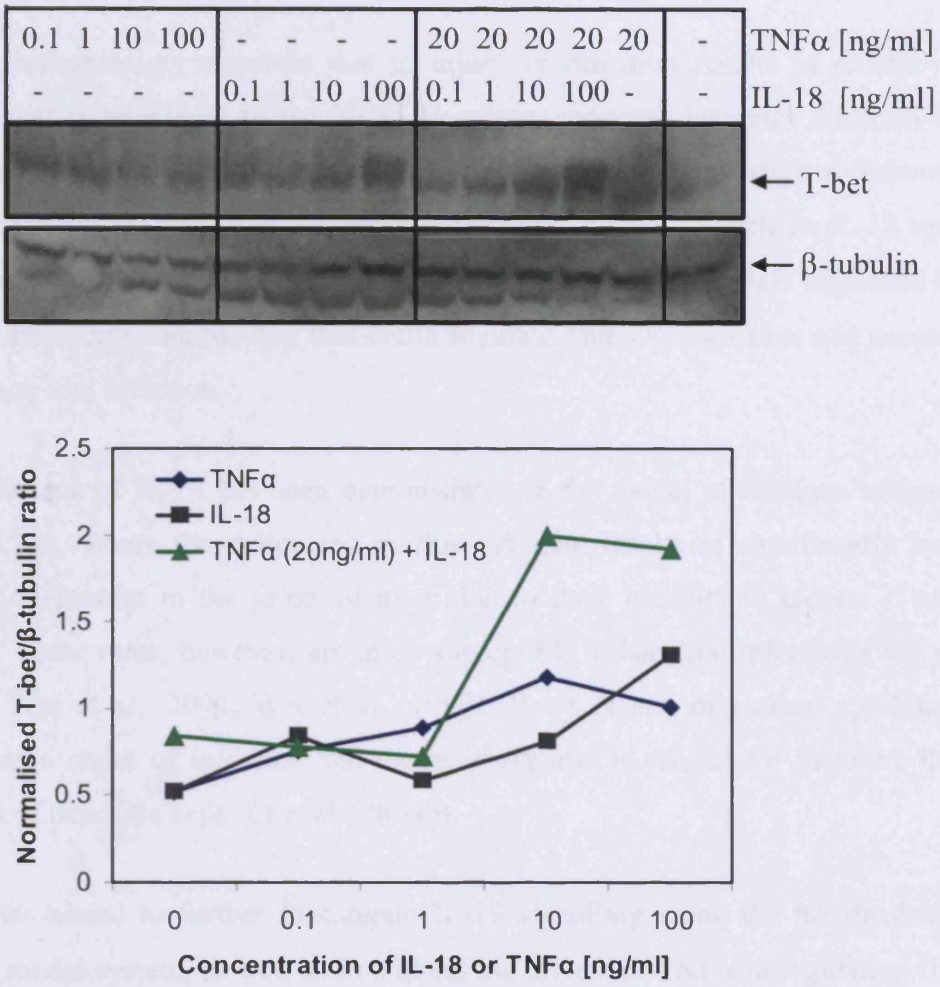


Figure 3.11: IL-18 induced T-bet expression in KG-1 cells that was up-regulated by TNFα priming.

KG-1 cells were stimulated with or without TNFα for 24h followed by stimulation with different concentrations of IL-18 for another 24h. Whole cell lysates were run under reducing conditions and a specific anti-human T-bet antibody was used for Western blotting. Antibody for β-tubulin was used as a loading control. Densitometric analysis is shown below the Western blot.

3.3 Conclusions

A normal inflammatory response due to injury or infection results in protective immunity that is beneficial to the body. However, recurrent bacterial infection in chronic inflammatory conditions can lead to uncontrolled inflammation and severe tissue damage that is promoted by pro-inflammatory cytokines such as IL-18 and TNFα. Understanding the mechanisms of interplay between cytokines is important in providing therapeutic approaches that could regulate immune responses and control inflammation and infection.

The significance of IL-18 has been demonstrated in the model of collagen induced arthritis (CIA), where the deficiency of this cytokine results in significantly less inflammation present in the joints of mice due to their inability to induce T cell activation. These mice, however, are more susceptible to bacterial infection (Wei et al., 2001, Wei et al., 2004, Wei et al., 1999). IL-18 is one of earliest cytokines produced as a result of infection and tissue injury and is critical for inducing the maturation of dendritic cells (Li et al., 2004b).

This chapter aimed to further investigate IL-18 signalling using the pre-dendritic KG-1 cell model system, as well as to address the effects of TNFα in regulating IL-18 signalling and thus promoting the maturation process of DCs. This work has demonstrated the ability of KG-1 cells to respond to IL-18 for the production of IFNγ and the ability of TNFα to sensitise these cells to produce more IFNγ in response to IL-18.

Data obtained from the flow cytometry experiments demonstrated some up-regulation of CD80 expression on KG-1 cells after stimulation with IL-18 for only 24 hours, suggesting that IL-18 has the ability to potentially initiate the maturation process of the pre-dendritic KG-1 cells. Similarly, it has been demonstrated by Gutzmer *et al.* that Mo-DCs derived from PBMCs, after stimulation with GM-CSF and IL-4 do not show any up-regulation in the expression of CD80 or CD40 after 2

days of stimulation with IL-18. These cells also show little up-regulation of CD83, CD86, HLA-DR expression (Gutzmer et al., 2003). Thus, it could be speculated that IL-18 does not have an effect on the maturation of DCs during acute inflammatory responses, whereas in conditions of chronic inflammation, where it is constantly present, it promotes immunity by inducing the maturation of DCs.

IL-18 might play a potential role in immunoregulation by inducing maturation of dendritic precursor cells infiltrated into the tissues, and it may also mediate the infiltration of these inflammatory cells. This cytokine has been shown to act as a chemoattractant of $CD4^+$ T cells in polarized morphology *in vitro* and of synovial $CD4^+$ T cells from patients with RA *ex vivo*. Injection of rhIL-18 in the foot pad of DBA/1 mice resulted in an increase of mononuclear cell infiltrate *in vivo* (Komai-Koma et al., 2003). IL-18 also has a direct migratory effect on Mo-DCs derived from PBMCs in Boyden chamber experiments and induces the up-regulation of the intercellular adhesion molecule CD54 (Gutzmer et al., 2003). In this study, levels of CD54 were found to be significantly up-regulated in the presence of IL-18 in KG-1 cells, with an even greater increase after priming with TNFα. Since the FACS results above suggested the capacity of IL-18 to induce DC maturation and immune cell infiltration at sites of inflammation, we further investigated IL-18 signalling and regulation by TNFα.

PCR experiments using primers against both IL-18Rα and Rβ demonstrated that TNFα regulated the mRNA expression of IL-18Rα, but not IL-18Rβ. However, this technique is non-quantitative and therefore experimentation using quantitative PCR was required to confirm such an effect. IL-18Rα mRNA expression appeared to be up-regulated in the presence of 10ng/ml TNFα, whereas IL-18Rβ mRNA expression was only significantly up-regulated at a much higher concentration of 100ng/ml TNFα compared to the physiologically relevant one, suggesting a mechanism of post-transcriptional or post-translational modification of IL-18Rβ by TNFα in the body. This could be due to the fact that the IL-18Rβ transcript is so much more abundant than IL-18Rα that high doses of TNFα are required to induce its

expression. Flow cytometry experiments confirmed the up-regulation of protein expression of IL-18Rα on KG-1 cells, demonstrating a mechanism of IL-18 regulation by TNFα through the direct up-regulation of the receptor gene and protein expression.

Experiments using phosho-specific antibodies against p38 MAPK and ERK1/2 demonstrated that IL-18 signalled through the activation of both of these two distinct kinases. Priming of the cells with TNFα prior to IL-18 stimulation induced only p38 MAPK and not ERK1/2, possibly through up-regulation of the IL-18 receptor, suggesting the importance of p38 MAPK signalling in the IL-18 induced inflammatory response. Additionally, the use of specific inhibitors in the KG-1 bioassay that block the phosphorylation of these molecules demonstrated that only p38 MAPK activation and not ERK1/2 was important for mediating IL-18-induced IFNγ production. Moreover, antibodies against T-bet, a transcription factor important for IFNγ gene expression, revealed that this IL-18-induced expression of IFNγ was promoted by TNFα priming of KG-1 cells, possibly through the mechanism demonstrated in figure 3.12.

IL-18 is a critical player in chronic inflammation, since it can promote both Th1 and Th2 cell responses depending on the presence or absence of IL-12 in the micro-environment and on the genetic background (Wei et al., 2004). As shown in this study and illustrated in figure 3.12, IL-18 plays a central role in the control of inflammation through p38 MAPK- and T-bet-activated IFNγ production, a signalling cascade enhanced by TNFα via a mechanism of up-regulation of IL-18R expression. Blocking TNFα by using a soluble TNFα receptor (Etanercept) or neutralising antibodies results in reduction of inflammation, especially in RA. TNFα blockers have been widely used in clinical trials to suppress the activity of TNFα, which could then inhibit the IL-18-induced maturation of DCs via the mechanism demonstrated in this study (Fig. 3.13).

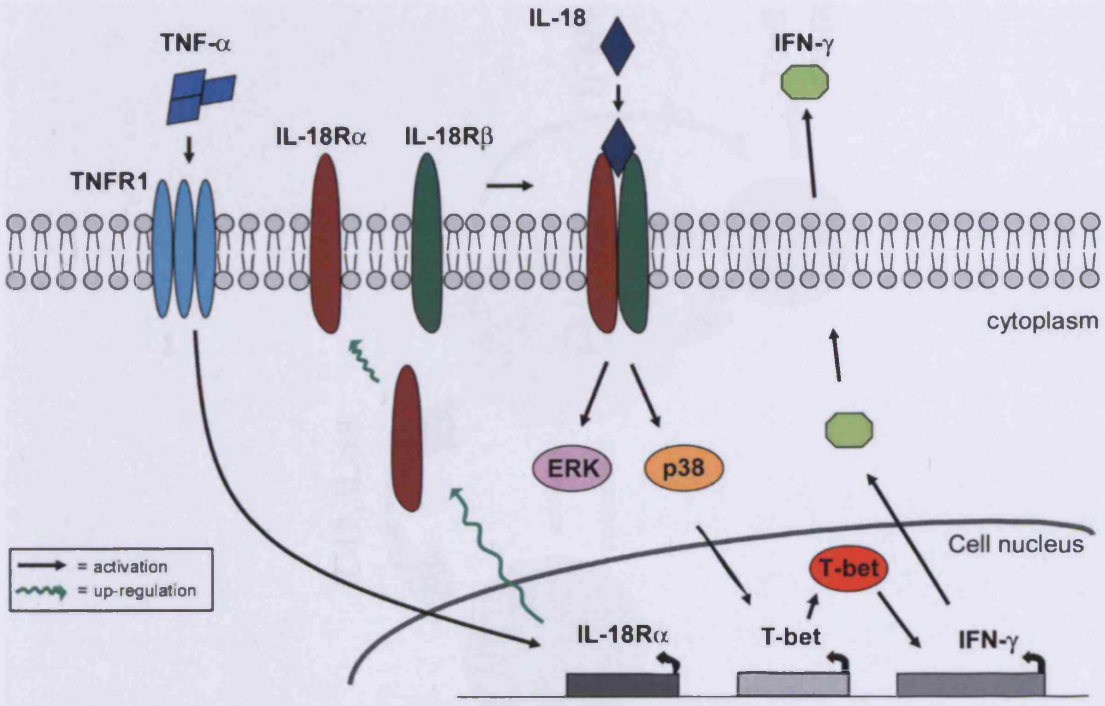


Figure 3.12: Mechanism by which TNFα enhances the IL-18-induced inflammatory response.

IL-18 signals through p38 MAPK and T-bet to activate IFNγ production. This signalling is enhanced by TNFα, via a mechanism of up-regulation of IL-18Rα expression.

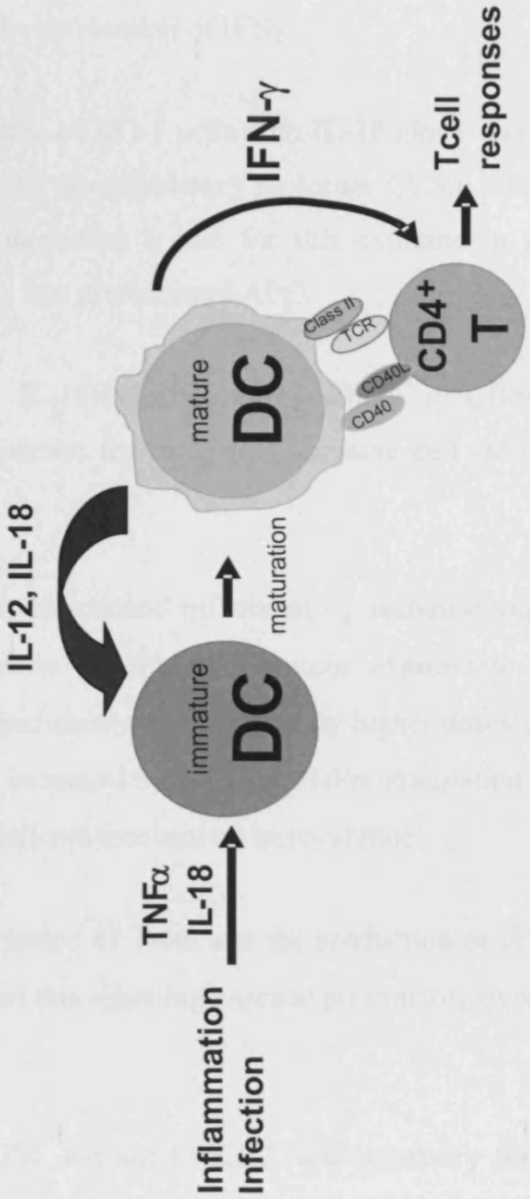


Figure 3.13: TNFα promotes the IL-18-induced maturation of dendritic precursor cells into professional antigen-presenting cells that promote both Th1 and Th2 cell responses.

3.3.1 In summary

- The KG-1 bioassay was used to demonstrate that the cells respond to the stimulation with IL-18 by production of IFNγ.

- Even the short stimulation of KG-1 cells with IL-18 alone was enough to induce some up-regulation of the co-stimulatory molecule CD80, which is found to be expressed on APCs, suggesting a role for this cytokine in the maturation of dendritic precursor cells into professional APC.

- TNFα promoted the IL-18-induced up-regulation of CD54 expression; an adhesion molecule important for mediating immune cell infiltration at sites of inflammation.

- TNFα promoted the IL-18-induced inflammatory response via a mechanism of up-regulation of IL-18Rα mRNA and protein expression. IL-18Rβ mRNA expression was only significantly up-regulated by higher doses of TNFα, whereas protein expression was increased by 20ng/ml TNFα stimulation (Wu et al., 2003), suggesting a post-translational mechanism of regulation.

- IL-18 induced the expression of T-bet and the production of IFNγ in KG-1 cells. TNFα priming promoted this signaling cascade presumably by sensitizing cells to IL-18.

- Activation of p38 MAPK, but not ERK1/2, was necessary for IFNγ production induced by IL-18. The phosphorylation of p38 MAPK by IL-18 was promoted by TNFα.

- In conclusion, this study demonstrates that IL-18 plays a central role in the control of inflammation through p38 MAPK- and T-bet-activated IFNγ production. This signalling is enhanced by TNFα via a mechanism of up-regulation of IL-18R expression.

Chapter 4

4 Regulation of IL-18 Signalling by TGFβ1

4.1 Background

The antagonistic interaction between pro- and anti-inflammatory cytokine signalling pathways operating in human DCs and the outcome of which one will prevail determines the prognosis of chronic inflammatory diseases and cancer. The prevalence of signalling of an active anti-inflammatory cytokine such as TGFβ1 that results in the suppression of pro-inflammatory immune activation by IL-18 and TNFα would be important in maintaining a balance to prevent the aberrant activation of DCs that leads to chronic inflammation. In the previous chapter, we investigated the role of the pro-inflammatory cytokine TNFα in regulating IL-18 signalling and function. In this chapter, we aim to study the effect of the anti-inflammatory cytokine TGFβ1 in controlling IL-18 signalling in the presence of TNFα.

Several studies have shown that TGFβ1 inhibits the activation and maturation of DCs by suppressing the up-regulation of T cell co-stimulatory molecules on the surface of DCs and reducing their capacity for antigen presentation. Other contrasting *in vitro* and *in vivo* TGFβ1 knock-out studies have shown that TGFβ1 is required for the function and differentiation of the immature epithelial-associated DCs (Langerhans cells) from their progenitor cells (Strobl and Knapp, 1999, Borkowski et al., 1996). Epithelial Langerhans cells (LCs) were the first population of immature DCs to be characterised for their maturation process occurring during migration from peripheral tissues to lymphoid organs (Steinman, 2007, Randolph et al., 2008). TNFα has been demonstrated to induce maturation of immature DCs generated *in vitro* from $CD34^+$ progenitors (Szabolcs et al., 1996, Caux et al., 1996, Ryncarz and Anasetti, 1998, Canque et al., 1998, Luft et al., 1998), but this maturation of DCs is suppressed by TGFβ1 even in the presence of TNFα (Yamaguchi et al., 1997, Strobl et al., 1997, Zhang et al., 1999, Geissmann et al., 1999). An analysis of the global gene expression profile of LPS-treated bone marrow-derived DCs, when their maturation process is inhibited by TGFβ1, has

demonstrated that IL-18 mRNA expression is up-regulated by TGFβ1 (Fainaru et al., 2007). This suggests a link between TGFβ1 and IL-18 signalling in DCs.

The importance of TGFβ1 in regulating IFNγ production was established by TGFβ-deficiency studies. BALB/c TGFβ1$^{-/-}$ mice develop spontaneous CD4^{+} T cell-mediated necroinflammatory liver disease and show increased levels of IFNγ in the liver (Rudner et al., 2003). IFNγ plays a critical role for the development of this disease as TGFβ1-IFNγ-double deficiency results in the protection of BALB/c mice (Gorham et al., 2001). Constitutive IFNγ signalling in tissues isolated from TGFβ$^{-/-}$ mice indicates the importance of TGFβ1 in inhibiting IFNγ production (McCartney-Francis and Wahl, 2002).

In a recent study by Yu *et al.*, the pro-inflammatory cytokines IL-12 and IL-18 or IL-15 were able to antagonise the immunosuppressive response of TGFβ1 to inhibit IFNγ production through the down-regulation of TGFβRII, SMAD2 and SMAD3 in both NK-92 and primary human NK cells. IL-18 alone did not affect SMAD2 and SMAD3 expression in these cells. They also showed that TGFβ1 suppresses IL-18/IL-12-induced IFNγ production by inhibiting pro-inflammatory cytokine-induced T-bet expression through SMAD signalling (Yu et al., 2006). It appears therefore that the respective signalling pathways are coupled through negative feedback.

Besides canonical SMAD-mediated transcription, TGFβ1 activates other SMAD-independent signalling pathways such as p38 MAPK pathways. This MAPK activation can be independent of SMAD signalling as indicated by SMAD4$^{-/-}$ studies or expression of dominant negative SMADs. A study performed by the group of YE Zhang in 2002 demonstrated that cells carrying mutated TGFβRI that are defective in SMAD activation are able to induce p38 MAPK phosphorylation in response to TGFβ1 (Yu et al., 2002). However, TGFβ-induced activation of MAPK can also result in SMAD activation allowing convergence of TGFβ-induced SMAD and MAPK pathways (Derynck and Zhang, 2003).

TGFβ1 is known to suppress the expression of IFNγ (Espevik et al., 1987, Bellone et al., 1995, Bright and Sriram, 1998, Pardoux et al., 1999, Sudarshan et al., 1999, Yu et al., 2006) in part through the inhibition of the T-bet transcription factor (Gorelik et al., 2002, Neurath et al., 2002, Lin et al., 2005, Park et al., 2007). T-bet deficient DCs are impaired in their capacity to produce IFNγ in response to stimulation with IL-18 and IL-12. These mice are also incapable of activating Th1 immune response of adoptively transferred T cells *in vivo* (Lugo-Villarino et al., 2003a). However, the expression of T-bet is in turn controlled by IFNγ in both DCs (Lugo-Villarino et al., 2003a) and $CD4^+$ T cells (Park et al., 2005), providing a positive feedback loop to maximise Th1 immunity. The study by Park *et al.*, also showed that TGFβ1 inhibits the induction of T-bet expression via IFNγ in these cells.

It has only recently been established that IFNγ is produced by antigen presenting cells (Frucht et al., 2001). The role of TGFβ1 in the regulation of IL-18-induced signalling pathway resulting in IFNγ production by pre-dendritic cells has never been investigated. Studying the molecular mechanisms that form the basis of the antagonistic relationship between pro- and anti-inflammatory cytokines is important for understanding the delicate balance between tolerance and autoimmunity.

4.1.1 Aims and Objectives

1. To understand the mechanism by which combinations of pro- and anti-inflammatory cytokines can act synergistically or competitively to modulate pre-dendritic KG-1 cell function.

2. To examine the role of the anti-inflammatory cytokine TGFβ1 in regulating IL-18-induced IFNγ production in the KG-1 cell bioassay model system.

3. To investigate the effect of TGFβ1 stimulation in regulating the protein and gene expression of IL-18 receptor induced by TNFα.

4. To study the antagonistic relationship between the pro- and anti-inflammatory cytokines, TNFα and TGFβ1, in regulating the IL-18-induced p38 MAPK activation and T-bet expression.

4.2 Results and Discussion

4.2.1 TGFβ1 suppresses IL-18-induced IFNγ production in KG-1 cells

4.2.1.1 TGFβ1 inhibited the stimulatory effect of TNFα in IL-18 signalling

From the results described in chapter 3, it is apparent that the pro-inflammatory cytokine IL-18, especially when combined with TNFα, is a strong inducer of IFNγ production in pre-dendritic KG-1 cells. To examine the effect of TGFβ1 in the regulation of IL-18 signalling in the presence of TNFα, two experiments were performed using the human pre-dendritic KG-1 cell line (Fig. 4.1). Initially, TGFβ1 was added together with TNFα in the priming phase of the experiment for 24 hours prior to the stimulation with IL-18 for another 24 hours and IFNγ production was measured by ELISA. In the second experiment, TGFβ1 was added together with IL-18 to stimulate the TNFα-primed KG-1 cells to produce IFNγ. By adding TGFβ1 either with TNFα in the priming-phase or with IL-18 in the subsequent stimulation phase, we aimed to dissect the effect of this anti-inflammatory cytokine in different aspects of the regulation of IL-18 signalling.

TGFβ1 significantly suppressed the IL-18-induced IFNγ production in a dose dependent manner both when given prior to IL-18R induction and also when given simultaneously with IL-18 (Fig. 4.1). A concentration of 300pg/ml of this cytokine was sufficient to induce a significant reduction of IFNγ in both cases. Interestingly, TGFβ1 showed a stronger suppressive effect on the response to IL-18 when it was added at same time with TNFα. These results demonstrated that TGFβ1 inhibited the IL-18-induced upregulation of IFNγ to nearly 100% at 10ng/ml, despite the presence of the pro-inflammatory cytokine TNFα. However, as shown in figures 3.1 and 3.3, KG-1 cells did not produce any IFNγ in the absence of IL-18, indicating that although TGFβ1 can very potently suppress IFNγ production, it did not reach full inhibition.

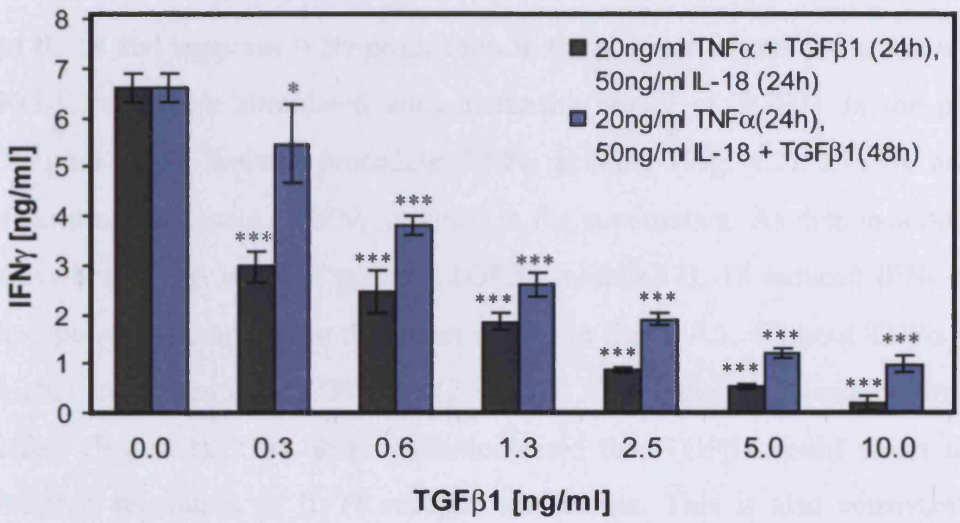


Figure 4.1: TGFβ1 inhibited the IL-18-induced IFNγ production in TNFα-primed KG-1 cells in a dose dependent manner.

KG-1 cells were primed with 20ng/ml TNFα in the presence or absence of different concentrations of TGFβ1 for 24 hours and further stimulated with IL-18 in the presence or absence of different doses of TGFβ1 for 24 or 48 hours as indicated. TGFβ1 inhibited IFNγ production both when added with TNFα in the priming phase (■) and with IL-18 in the subsequent stimulation phase (■). The amount of IFNγ produced in the culture media was analyzed by ELISA. Data shown are expressed as mean ±SD of triplicates of a representative experiment ($P<0.0001$, one-way ANOVA). * $P < 0.05$; *** $P < 0.001$; by Bonferroni post-test compared to cells without TGFβ1.

4.2.1.2 TGFβ1 suppressed the IL-18 response less potently in the absence of TNFα

To examine whether TGFβ1 was able to inhibit the strong pro-inflammatory capacity of IL-18 and suppress IFNγ production in the absence of cell activation with TNFα, KG-1 cells were stimulated with increasing doses of TGFβ1 in the presence of 50ng/ml IL-18 without preceding TNFα priming (Fig. 4.2). ELISA was used to determine the levels of IFNγ secreted in the supernatant. As demonstrated in figure 4.2 when TNFα was not present, TGFβ1 inhibited IL-18-induced IFNγ production less potently compared to the effect shown in figure 4.1. Without TNFα priming, a higher concentration of TGFβ1 (2.5ng/ml) was required to induce an inhibitory effect (Fig. 4.2). This observation indicated that TGFβ1 could affect the TNFα-induced regulation of IL-18 receptor expression. This is also consistent with the strong suppressive effect when added during cell priming as compared to addition of TGFβ1 during induction of IL-18 signalling (see Fig. 4.1).

However, before undertaking the investigation into IL-18R, we firstly examined whether the addition of IL-18 subsequent to TGFβ1 treatment had the ability to abrogate the inhibitory effect of TGFβ1. To investigate this, KG-1 cells were primed with 20ng/ml TNFα for 24 hours. The following day the cells were washed and stimulated with 10ng/ml TGFβ1 for 0 (0 min represents the addition of TGFβ1 and IL-18 at the same time), 15, 60, 120, 240 minutes prior to the subsequent addition of 20 or 50ng/ml IL-18 for another 24 hours. TNFα (20ng/ml) cells primed for 24 hours were stimulated with 20 or 50ng/ml IL-18 for another 24 hours and were used as controls. The amount of IFNγ produced in the culture media was analyzed by ELISA (Fig. 4.3).

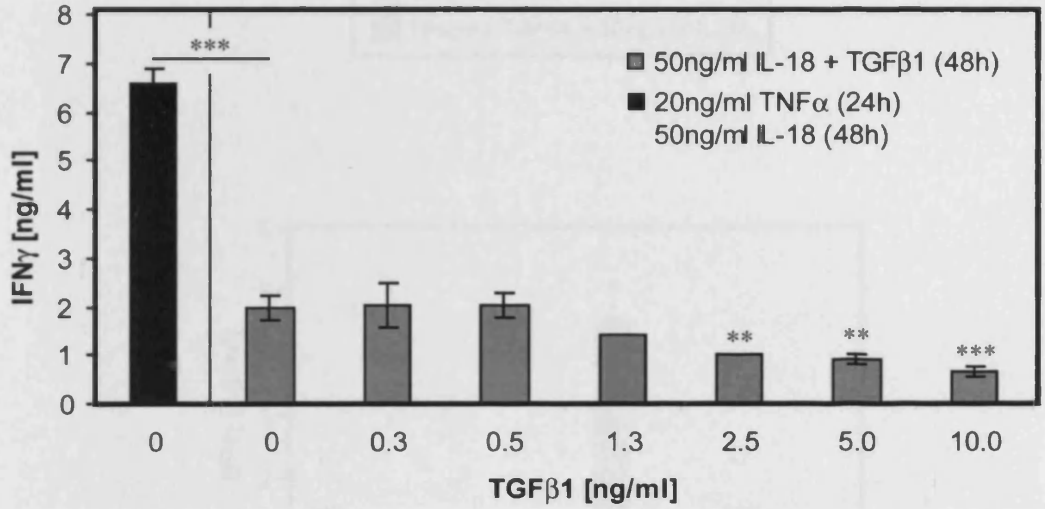


Figure 4.2: TGFβ1 less potently inhibited the IL-18-induced IFNγ production in non-TNFα-primed KG-1 cells.

KG-1 cells were stimulated with 50ng/ml IL-18 and different concentrations of TGFβ1 for 48 hours (■). Cells primed with 20ng/ml TNFα (24h) and stimulated with 50ng/ml IL-18 (48h) was used as a control (■). The amount of IFNγ produced in the culture media was analyzed by ELISA. Data shown are expressed as mean ±SD of triplicates of a representative experiment. ** $P < 0.01$; *** $P < 0.001$; by Bonferroni post-test compared to control.

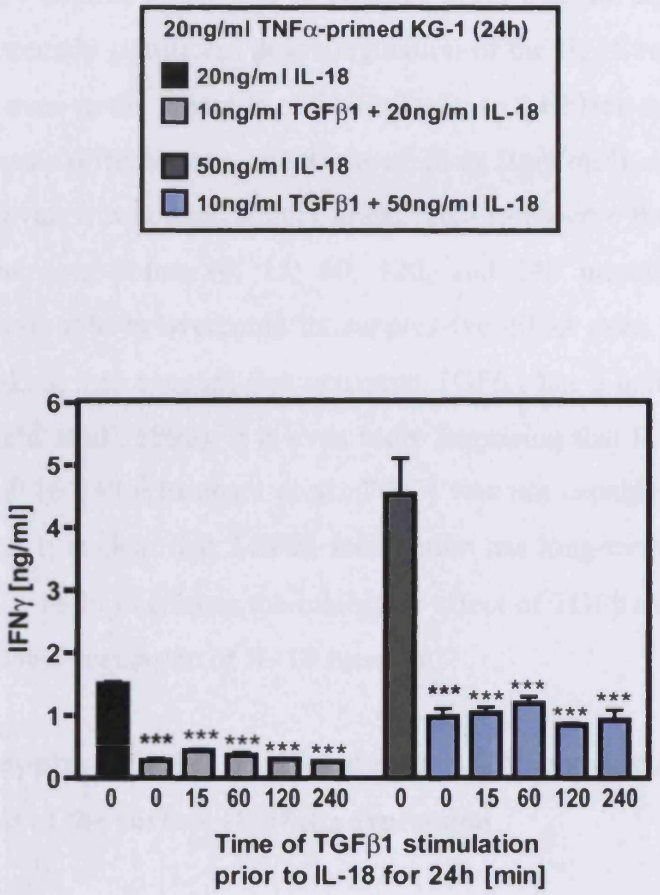


Figure 4.3: The addition of IL-18 was not sufficient to abrogate the suppressive effect of TGFβ1 in TNFα-primed KG-1 cells.

KG-1 cells were primed with 20ng/ml TNFα for 24 hours. Next day, the cells were washed and stimulated with 10ng/ml TGFβ1 for 0 [0 min represents the addition of TGFβ1 and IL-18 at the same time], 15, 60, 120, 240 minutes and then the subsequent addition of 20 (■) or 50 (■) ng/ml IL-18 for another 24 hours. TNFα (20ng/ml) primed cells for 24 hours stimulated with 20 (■) or 50 (■) ng/ml IL-18 for another 24 hours were used as controls. The amount of IFNγ produced in the culture media was analyzed by ELISA. Data shown are expressed as mean ±SD of triplicates of a representative experiment ($P<0.0001$, one-way ANOVA vs. control). * $P < 0.05$; *** $P < 0.001$ by Bonferroni post-test compared to control.

It was a necessary control to use and to confirm again that the addition of TGFβ1 resulted in an extremely significant down-regulation of the IL-18 response to induce IFNγ production even in the presence of TNFα (refer to the black and dark grey bars corresponding to two different concentrations of 20 or 50ng/ml IL-18 respectively in figure 4.3). However, it was interestingly unexpected to observe that the addition of IL-18 at different time-points (0, 15, 60, 120, and 240 minutes) after TGFβ1 stimulation was not able to overcome its suppressive effect even when added 240 minutes later. Taking into account that activated TGFβ1 has a half-life of only 2-3 minutes (Wakefield et al., 1990), it is even more surprising that IL-18 with a much longer half-life of 16-24h (Hosohara et al., 2002) was not capable of reversing the phenotype. Hence, it is clear that TGFβ1 stimulation has long-term effects on cells. The inability of IL-18 to overcome the inhibitory effect of TGFβ1 in this assay may be linked to the down-regulation of IL-18 receptor.

4.2.2 TGFβ1 suppressed the IL-18-induced IFNγ production via down-regulation of the surface IL-18Rα expression

4.2.2.1 TGFβ1 did not have any effect on the regulation of the IL-18R transcript induced by TNFα

To further investigate the mechanism of TGFβ1-induced down-regulation of IL-18 response in KG-1 cells, the mRNA levels of both IL-18Rα and β chains were examined by PCR. Standard PCR showed that 10ng/ml TGFβ1 has decreased IL-18Rα mRNA expression but did not seem to have any effect on the gene expression of IL-18Rβ in the presence of 20ng/ml TNFα (Fig. 4.4a).

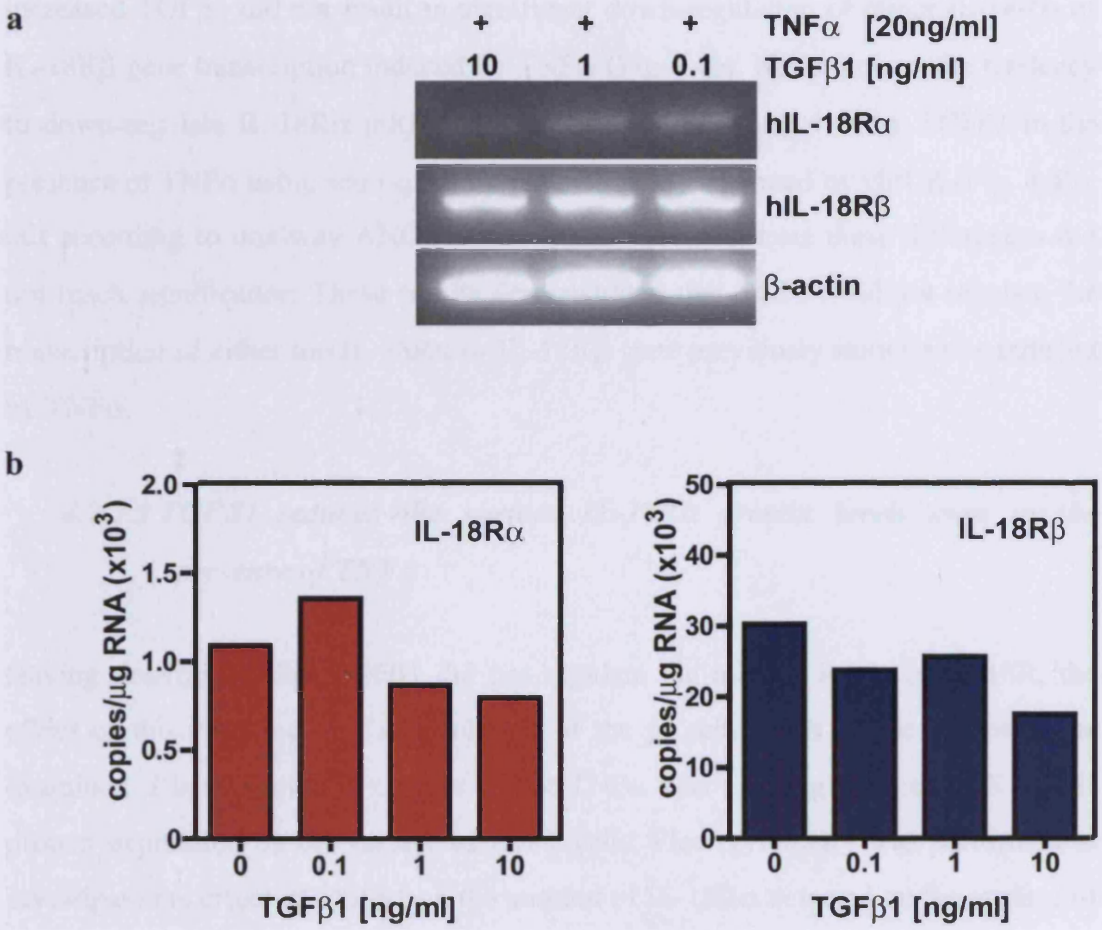


Figure 4.4: TGFβ1 did not regulate either IL-18Rα or Rβ mRNA.

KG-1 cells were stimulated with 20ng/ml TNFα and increasing doses of TGFβ1 for 24h. hIL-18Rα and hIL-18Rβ mRNA expression was examined initially by PCR (**a**) and quantified by real time PCR using TaqMan assay (**b**). Data shown are expressed as means of duplicates of a representative experiment.

To substantiate the down-regulation of IL-18Rα we used quantitative PCR. However, increased TGFβ1 did not result in significant down-regulation of either IL-18Rα or IL-18Rβ gene transcription induced by TNFα (Fig 4.4b). Nevertheless, the tendency to down-regulate IL-18Rα mRNA levels observed in figure 4.4a by TGFβ1 in the presence of TNFα using semi-quantitative PCR was confirmed by QPCR (Fig. 4.4b), but according to one-way ANOVA and Bonferroni post-tests these differences did not reach significance. These results demonstrated that TGFβ1 did not regulate the transcription of either the IL-18Rα or IL-18Rβ gene previously shown to be induced by TNFα.

4.2.2.2 TGFβ1 reduced the surface IL-18Rα protein levels even in the presence of TNFα

Having determined that TGFβ1 did not regulate the mRNA levels of IL-18R, the effect of this cytokine on the regulation of the protein levels of the receptor was examined. I have shown in chapter 3 that TNFα was a strong inducer of IL-18Rα protein expression on the surface of KG-1 cells. Flow cytometry was performed to investigate the effect of TGFβ1 on the amount of IL-18Rα detected on the surface of KG-1 cells stimulated with TNFα. KG-1 cells were stimulated with 20ng/ml TNFα and increasing doses of TGFβ1 for 24h (Fig. 4.5).

The amount of IL-18Rα induced by TNFα on the cell surface was reduced in response to 10ng/ml TGFβ1 stimulation even in the presence of TNFα (Fig. 4.5a). FACS data were represented as a percentage of the relative cell number in a gated area (R1) in relation to different doses of TGFβ1 in figure 4.5b. TGFβ1 dose dependently reduced the TNFα-induced expression of IL-18Rα on the surface of the cells. The levels of the TNFα-induced surface expression of IL-18Rα were reduced to approximately 50% by TGFβ1 (Fig. 4.5b).

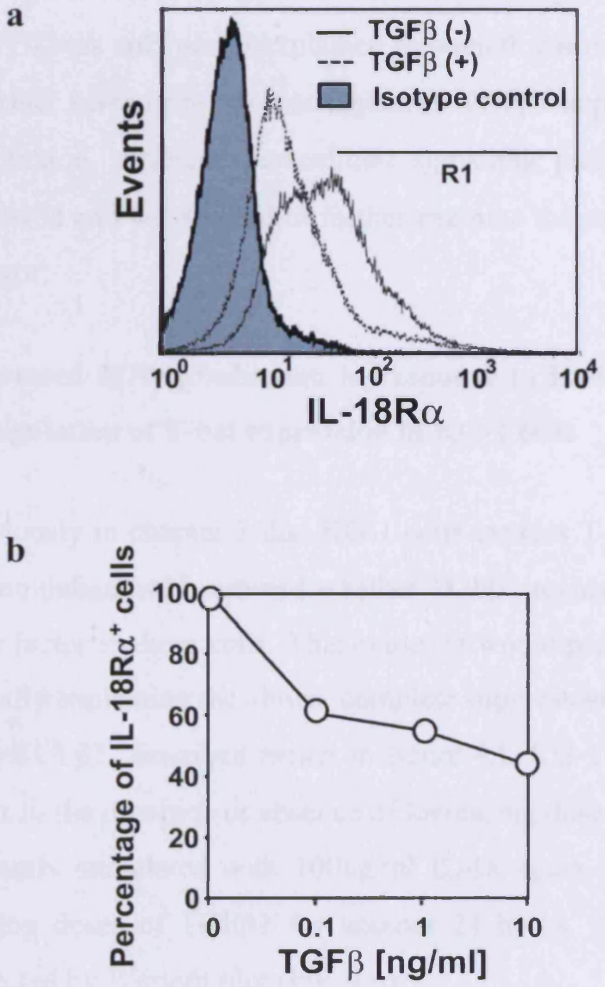


Figure 4.5: TGFβ1 induced the down-regulation of IL-18Rα surface expression in KG1 cells.

Cells were stimulated with 20ng/ml TNFα and different doses of TGFβ1 for 24h. The detection of IL-18Rα protein levels was determined by flow cytometry using an anti-human IL-18Rα-PE antibody. TGFβ1 (10ng/ml) reduced expression of IL-18Rα compared to the isotype-matched control (**a**). FACS results were represented as a percentage of the relative cell number in a gated area (R1) in relation to different doses of TGFβ1 (**b**).

These results did not completely explain the almost 100% inhibition of the IFNγ production induced by TGFβ1 in response to IL-18 stimulation in TNFα-primed KG-1 cells (Fig. 4.1). The mechanism of the anti-inflammatory regulation of the IL-18 response by TGFβ1 was only partly explained through down-regulation of the IL-18Rα levels. To further investigate the mechanism of TGFβ1 suppression of IL-18-induced IFNγ production, different intracellular signalling pathways induced by TGFβ1 were considered and we decided to further examine the expression of the T-bet transcription factor.

4.2.3 TGFβ1 suppressed IFNγ production in response to IL-18 stimulation via the down-regulation of T-bet expression in KG-1 cells

Having shown previously in chapter 3 that KG-1 cells express T-bet in response to TNFα and IL-18 stimulation, we examined whether TGFβ1 regulated the expression of this transcription factor in these cells. This evidence would provide an additional mechanism, potentially explaining the almost complete suppression of IL-18-induced IFNγ production by TGFβ1, described earlier in figure 4.1. KG-1 cells were primed with 20ng/ml TNFα in the presence or absence of increasing doses of TGFβ1 for 24 hours and subsequently stimulated with 100ng/ml IL-18 again in the presence or absence of increasing doses of TGFβ1 for another 24 hours. The level of T-bet expression was detected by Western blot (Fig. 4.6).

T-bet protein expression was down-regulated by TGFβ1 stimulation, and more effectively so when TGFβ1 was added during the priming phase together with TNFα (Fig. 4.6). These results suggested that TGFβ1 inhibited IL-18-induced IFNγ production via a dual mechanism of down-regulation of both IL-18R and T-bet expression.

4.2.4 The inhibitory effect of TGFβ1 on IL-18 signalling was not due to decreased viability of KG-1 cells

At this point, it was necessary to confirm that all the suppressive effects observed in the presence of TGFβ1 were not due to any toxicity of the employed cytokine dosages on KG-1 cells. MTT *in vitro* toxicity assay was performed using increasing concentrations of TGFβ1 for 24 hours (Fig. 4.7c). TNFα did not affect the viability of the cells (Fig. 4.7b) and neither did IL-18, which was used as a negative control (Fig. 4.7a). Increasing densities of cells without any stimulation were also used to verify the functionality of the assay (Fig. 4.7d). This experiment confirmed that the anti-inflammatory effect of TGFβ1 to suppress IL-18 function via the down-regulation of IL-18R and T-bet expression was not a result of toxicity.

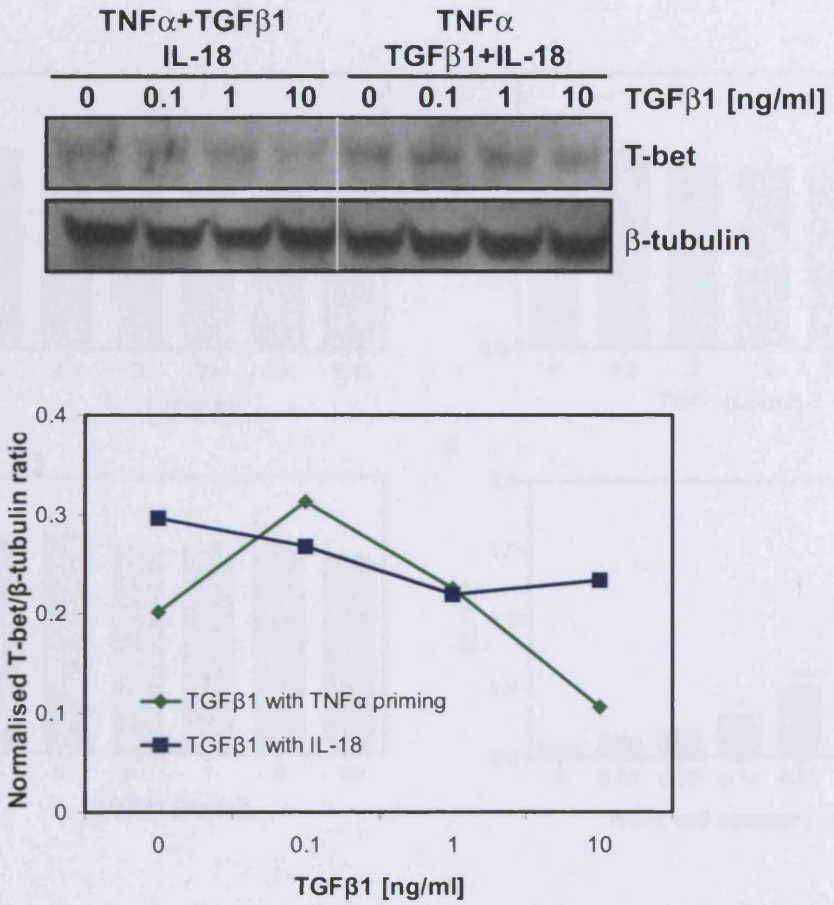


Figure 4.6: TGFβ1 induced down-regulation of T-bet expression in IL-18 stimulated KG-1 cells despite the presence of TNFα.

Cells were primed with 20ng/ml TNFα in the presence or absence of increasing doses of TGFβ1 for 24 hours. The following day, the cells were washed and further stimulated with 100ng/ml IL-18 in the presence or absence of different concentrations of TGFβ1 for another 24 hours. Whole cell extracts were subjected to SDS-PAGE under reducing conditions and a specific anti-human T-bet antibody was used for Western blotting. Antibody for β-tubulin was used as a loading control. Densitometric analysis was performed and shown below the Western blot.

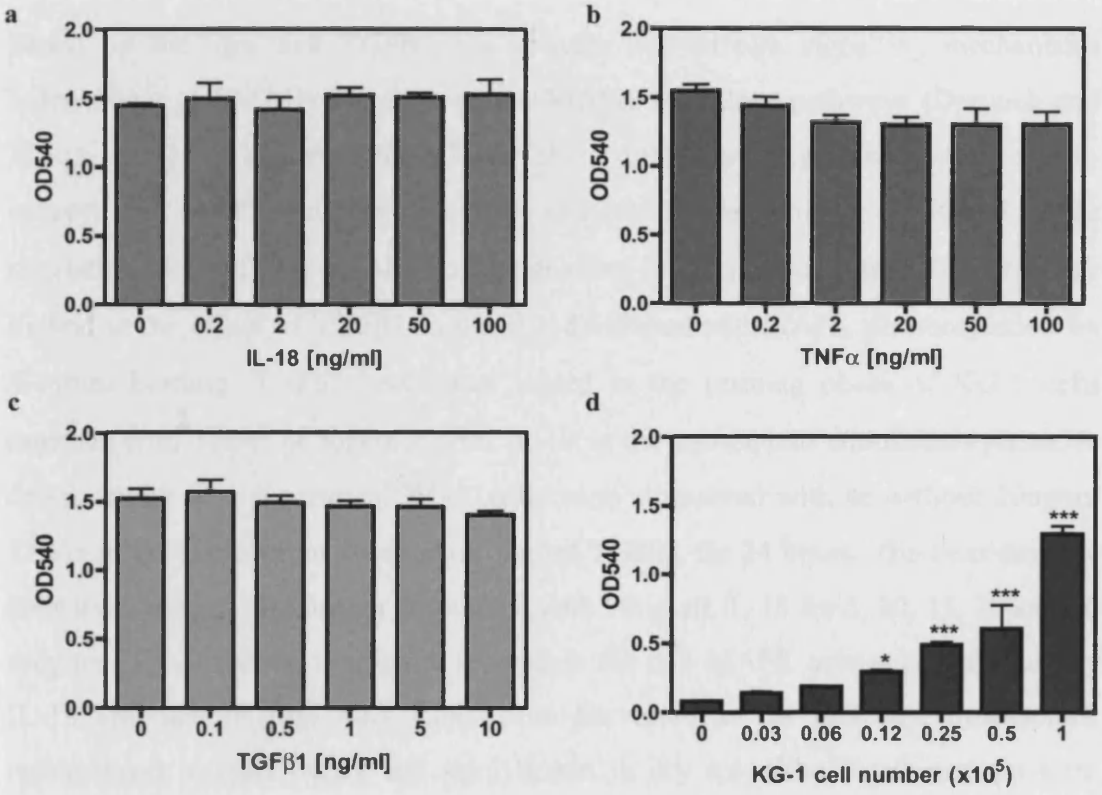


Figure 4.7: TGFβ1 stimulation did not affect KG-1 cell viability.

MTT proliferation assay was performed using $1x10^5$ KG1 cells for stimulation with increasing doses of IL-18 (**a**), TNFα (**b**) or TGFβ1 (**c**) for 24 hours. Increasing number of KG-1 cells without any stimulation was used as a control ($P<0.0001$, one-way ANOVA) (**d**). Data shown are expressed as mean ±SD of triplicates of a representative experiment. * $P < 0.05$; *** $P < 0.001$ by Bonferroni post-test. P values refer to comparisons with unstimulated cells (**a-c**), or when no cells were present (**d**).

4.2.5 TGFβ1 antagonised the IL-18-induced p38 activation even in the presence of the pro-inflammatoty cytokine TNFα

Based on the idea that TGFβ1 can activate downstream signalling mechanisms independent of SMADs but through the MAPK signalling pathways (Derynck and Zhang, 2003), we addressed the effect of this cytokine on the activation of the IL-18-induced p38 MAPK pathway. To better characterise the function of TGFβ1 in the regulation of the TNFα-primed IL-18 signalling in the pre-dendritic KG-1 cells, we looked at the effect of TGFβ1 in the IL-18-induced p38 MAPK phoshorylation by Western blotting. TGFβ1 was either added at the priming phase of KG-1 cells together with TNFα or together with IL-18 at the subsequent stimulation phase. In detail, in the first experiment KG-1 cells were stimulated with or without 20ng/ml TNFα in the presence or absence of 5ng/ml TGFβ1 for 24 hours. The next day, the cells were washed and further stimulated with 50ng/ml IL-18 for 5, 10, 15, 30 and 60 minutes. The 0 minute time-point represents the p38 MAPK activation without any IL-18 stimulation (Fig. 4.8). Cells were harvested at the indicated time-points, resuspended in lysis buffer and snap frozen in dry ice. Whole cell extracts were separated by SDS-PAGE and Western blotting was performed using phospho- and non-phosho- specific p38 MAPK antibodies as described in section 3.2.4.3 of the previous chapter.

The Western blot in figure 4.8a and the densitometry analysis of this result representing the ratio of phosphorylated over non-phosphorylated p38 MAPK (Fig 4.8b) has demonstrated that the addition of TGFβ1 in the priming phase of stimulation of KG-1 cells reduced the activation of p38 MAPK for the first 10 minutes of stimulation with 50ng/ml IL-18. The levels of p38 MAPK activation reached similar levels after 15 minutes and up to 60 minutes of IL-18 stimulation (Fig. 4.8). This result could indicate that the delayed activation of p38MAPK by TGFβ1 stimulation could be the outcome of the down-regulation of the IL-18Rα expression by TGFβ1 described earlier (Fig. 4.5).

To further address the suppressive function of TGFβ1 in the regulation of IL-18 signalling, a similar experiment was performed where TGFβ1 was added together with IL-18 to stimulate p38 MAPK activation in TNFα-primed KG-1 cells. Specifically, KG-1 cells were stimulated with 20ng/ml TNFα for 24 hours. The following day, the cells were washed and stimulated with or without 5ng/ml TGFβ1 followed by immediate simulation of 50ng/ml IL-18 (Fig. 4.9). The levels of p38 MAPK activation were determined using Western blotting as described above. As a control, TGFβ1 or IL-18 alone was used to stimulate the cells for 30 minutes in the presence of absence of TNFα priming (last three lanes in Fig. 4.9).

This result showed that stimulation of pre-dendritic KG-1 cells with TGFβ1 alone for 30 minutes did not activate p38 MAPK (Fig. 4.9). Moreover, the same figure demonstrated that the presence of TGFβ1 delayed the phosphorylation of p38 MAPK induced by IL-18 in the first 15 minutes in TNFα-primed KG-1 cells. The results in figures 4.8 and 4.9 collectively indicate that TGFβ1 suppressed the activation of the p38 MAPK signalling pathway either by interfering directly or indirectly as a result of the down-regulation of IL-18R expression.

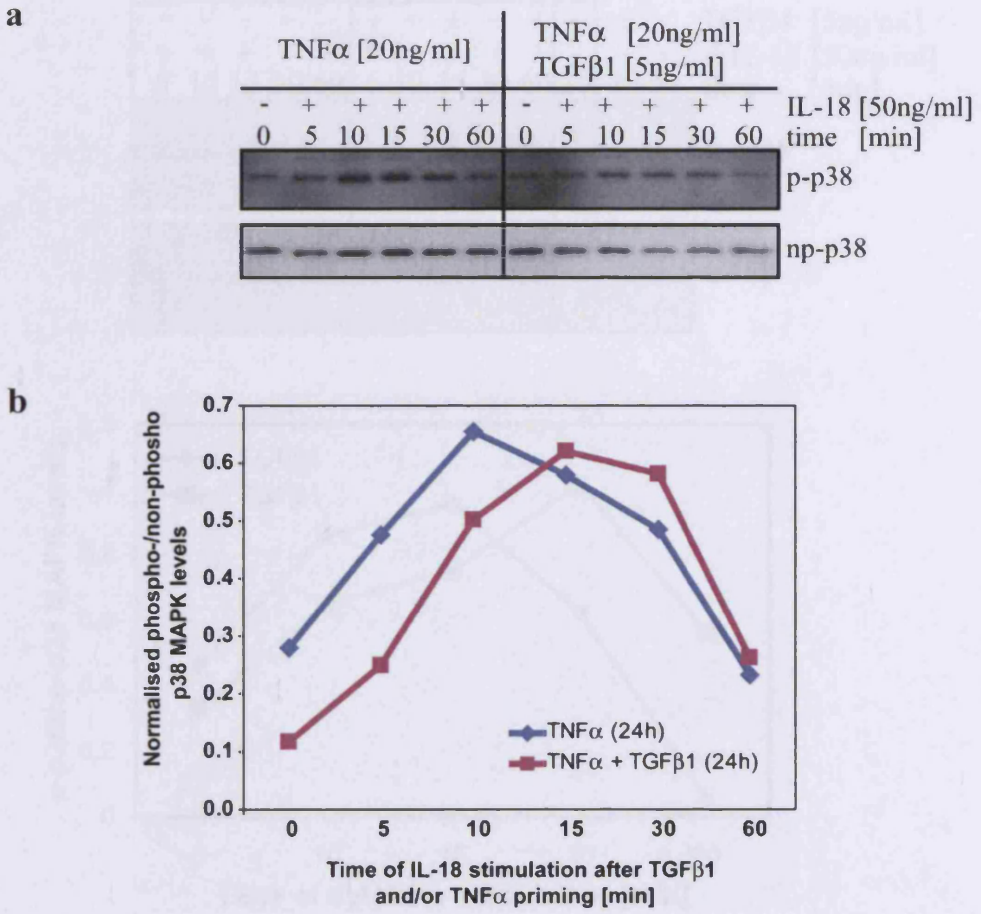


Figure 4.8: TGFβ1 delayed the IL-18-induced phosphorylation previously promoted by TNFα.

KG-1 cells were stimulated with 20ng/ml TNFα in the presence or absence of 5ng/ml TGFβ1 for 24h. The next day stimulation with or without 50ng/ml of IL-18 followed for 0, (before the addition of IL-18) 5, 10, 15, 30 and 60 minutes. Whole cell lysates were prepared and Western blotting was performed using phospho- and non-phospho-specific anti-human p38 MAPK rabbit polyclonal antibody (designated as p and np, respectively). The non-phosphorylated p38 MAPK antibody was used as a loading control (**a**). The degree of p38 phosphorylation (normalised to np-p38) was quantified by densitometry (**b**).

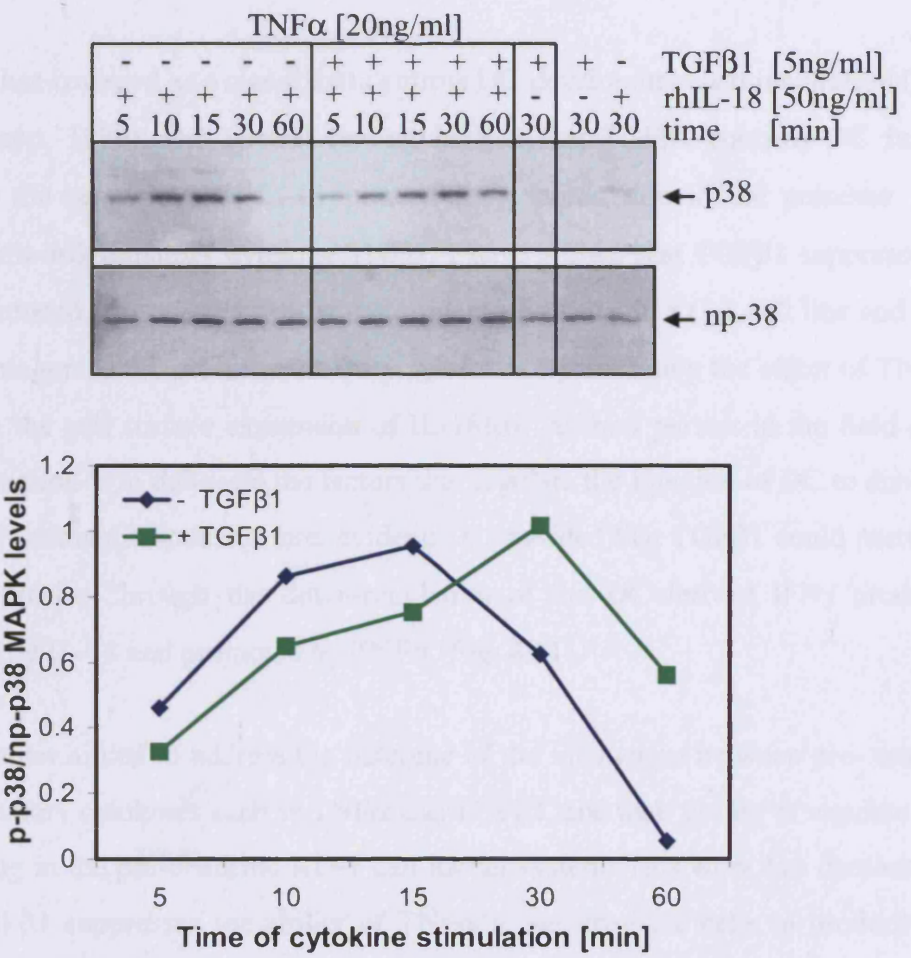


Figure 4.9: TGFβ1 delayed the IL-18-induced activation of p38 MAPK in TNFα-primed KG-1 cells.

Cells were stimulated with or without 20ng/ml TNFα for 24h, followed by stimulation with or without 5ng/ml TGFβ1 in the presence of 50ng/ml IL-18 for 5, 10, 15, 30 and 60 minutes. TGFβ1 or IL-18 stimulation alone for 30 minutes, in TNFα-primed or non-primed cells were used as controls. Whole cell lysates were prepared and Western blotting was performed using phospho- and non-phospho-specific anti-human p38 MAPK rabbit polyclonal antibody (designated as p and np respectively). The non-phosphorylated p38 MAPK antibody was used as a loading control. Densitometric analysis was performed and shown below the Western blotting.

4.3 Conclusions

TGFβ1 has emerged as a signal that controls DC development and maturation (Strobl and Knapp, 1999). Our central finding here is that TGFβ1 controls DC function through the regulation of IL-18-induced IFNγ production in the presence of the strong pro-inflammatory cytokine TNFα. I have shown that TGFβ1 suppressed the IL-18-induced IFNγ production in the human pre-dendritic KG-1 cell line and that it could antagonise the pro-inflammatory cytokines by inhibiting the effect of TNFα to increase the cell surface expression of IL-18Rα. A chief pursuit in the field of DC differentiation is to delineate the factors that regulate the function of DC to drive Th1 and Th2 immune response. Here, evidence is provided that TGFβ1 could participate in this process through the down-regulation of the DC-derived IFNγ production induced by IL-18 and promoted by TNFα (Fig. 4.11).

This chapter aimed to address the outcome of the interaction between pro- and anti-inflammatory cytokines such as TNFα and TGFβ1, and their ability to regulate IL-18 signalling in the pre-dendritic KG-1 cell model system. This work has demonstrated that TGFβ1 suppresses the ability of TNFα to sensitise the cells to produce more IFNγ in response to IL-18 and inhibits IFNγ production to almost 100%. To investigate the mechanism of the antagonistic action of TGFβ1 against TNFα, we studied the effect of these two cytokines in the regulation of the IL-18R expression. The expression of the IL-18R transcript induced by TNFα was not altered by TGFβ1, suggesting a mechanism of post-transcriptional or post-translational modification of IL-18R by TGFβ1. Flow cytometry experiments demonstrated that TGFβ1 induced a dose-dependent down-regulation of the protein expression of IL-18Rα on KG-1 cells, suggesting a mechanism of antagonistic interaction between TNFα and TGFβ1 to control IL-18 signalling through the regulation of IL-18Rα expression (Fig. 4.10). Western blotting analysis has also been attempted during the course of this study to investigate the total protein expression levels of IL-18R in KG-1 cells, but technical problems related to antibody specificity or sensitivity of the assay were encountered. Moreover, antibodies against T-bet, a transcription factor important for IFNγ gene

expression, revealed that TGFβ1 suppressed the IL-18-induced expression even in the presence of TNFα possibly through the mechanism described above and schematically demonstrated in figure 4.10.

Using Western blotting and a phosho-specific antibody to detect p38 MAPK we showed that TGFβ1 delayed the activation of p38 MAPK induced by IL-18. Priming of the cells with TNFα in the presence of TGFβ1 prior to IL-18 stimulation suppressed the phosphorylation of p38 MAPK possibly through the down-regulation of the receptor. However, IL-18-induced p38 activation was not completely inhibited upon TGFβ1 treatment. The use of specific inhibitors and Western blotting would be an appropriate experiment to test whether TGFβ1 also utilised the p38 MAPK signalling pathway to inhibit the IL-18-induced IFNγ production in KG-1 cells. The inhibitory effect of TGFβ1 was not due to decreased proliferation of KG-1 cells.

To conclude, TGFβ1 was able to almost completely inhibit IL-18-induced IFNγ production in KG-1 cells. However, the mechanism of almost full inhibition of IL-18 signalling was only partially explained from the results obtained in this chapter. In view of the results obtained in this chapter, neither the down-regulation of IL-18Rα expression, nor the reduction in T-bet expression or p38 phosphorylation would be enough to explain the almost complete inhibition of IFNγ production in KG-1 cells. Further experiments are required to elucidate the mechanism behind the strong immunosuppressive effect of TGFβ1 on IL-18 signalling in KG-1 cells.

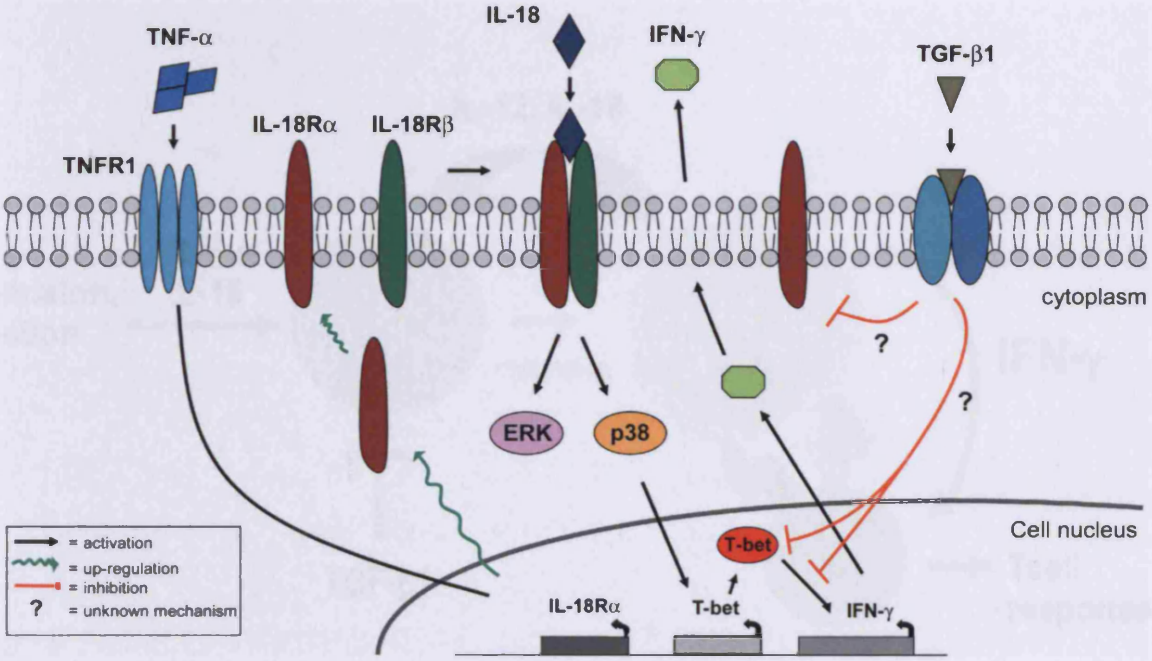


Figure 4.10: Mechanism by which TGFβ1 suppresses the IL-18-induced inflammatory response.

IL-18 signals through p38 MAPK and T-bet to activate IFNγ production. This signalling is enhanced by TNFα and suppressed by TGFβ1 via a mechanism of counter-regulation of IL-18R and T-bet expression.

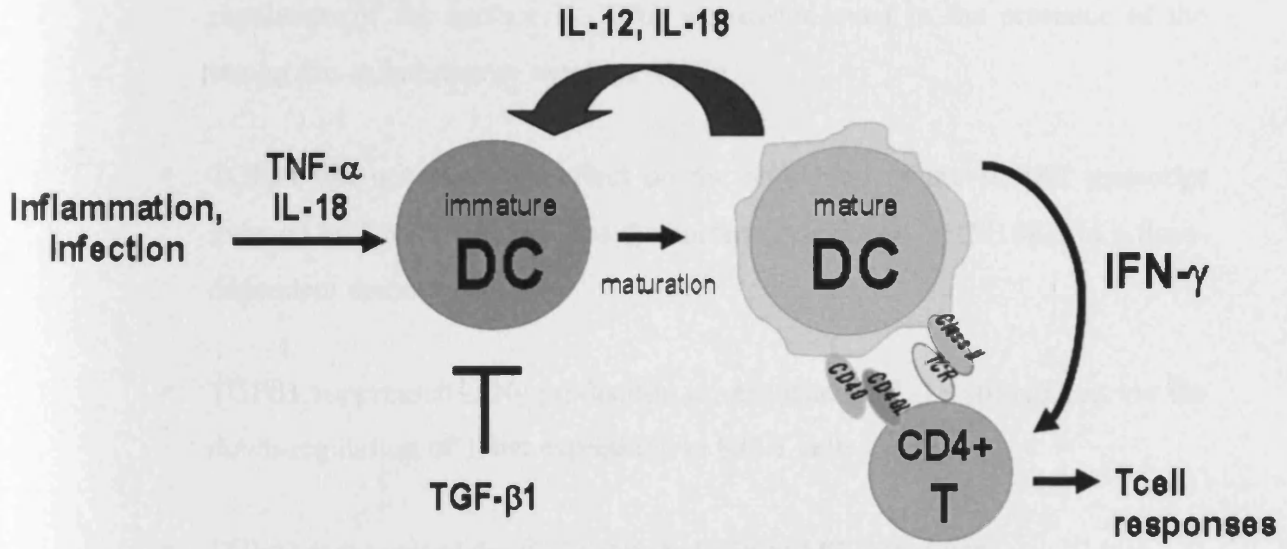


Figure 4.11: TGFβ1 inhibits the IL-18-induced maturation of dendritic precursor cells into professional antigen-presenting cells that are able to promote both Th1 and Th2 cell responses.

4.3.1 In summary

- TGFβ1 suppressed the IL-18-induced IFNγ production in KG-1 cells by inhibiting the stimulatory effect of TNFα in IL-18 signalling. However, TGFβ1 suppressed the IL-18 response less potently in the absence of TNFα.

- TGFβ1 suppressed the IL-18-induced IFNγ production via the down-regulation of the surface IL-18Rα expression even in the presence of the strong pro-inflammatory cytokine TNFα.

- TGFβ1 did not have any effect on the regulation of the IL-18R transcript induced by TNFα, but reduced the surface expression of IL-18Rα in a dose-dependent manner.

- TGFβ1 suppressed IFNγ production in response to IL-18 stimulation via the down-regulation of T-bet expression in KG-1 cells.

- TGFβ1 antagonised the IL-18-induced p38 MAPK activation.

- The inhibitory effect of TGFβ1 on IL-18 signalling was not due to decreased viability of KG-1 cells.

Chapter 5

5 Generation of a Human Soluble Heterodimeric Decoy Receptor for IL-18

5.1 Background

5.1.1 IL-18 is a potential therapeutic target in many inflammatory diseases

This chapter addresses the possibility of inhibiting the IL-18 pro-inflammatory response by directly neutralising IL-18 function in the KG-1 *in vitro* cell model system (Konishi et al., 1997) through the generation of a soluble decoy heterodimeric receptor with high affinity to IL-18. Several *in vitro* and *in vivo* studies of different disease models established the contribution of IL-18 to the pathogenesis of many clinical conditions such as rheumatoid arthritis, type I diabetes, multiple sclerosis, systemic lupus erythematosus, Crohn's disease, psoriasis, graft-versus-host disease, ischemia, atherosclerosis, and chronic hepatitis (Dinarello and Fantuzzi, 2003, Dinarello, 2007, Boraschi and Dinarello, 2006, Gracie et al., 2003).

There are several anti-cytokine therapies available in clinical trials targeting different cytokines in different inflammatory and autoimmune conditions, such as anti-TNFα monoclonal antibodies (infliximab, adalimumab, nerelimomab, golimumab), chimeric soluble type I or II TNFα receptors (etanercept, PEG-rhsTNF-RI, lenercept), soluble TNFα binding protein, anti-IFNγ antibodies, IFNγ/IgG-Fc fusion protein; anti-IL-12p40 monoclonal antibody, IL-1 receptor antagonist (IL-1ra; anakinra), anti-IL-1β monoclonal antibody (ACZ885), IL-1Trap; IL-1RI/IgG-Fc fusion protein; anti-IL-6R antibody (MRA, tocilizumab), anti-IL-15 monoclonal IgG1 antibody (AMG 714, Amgen), TGF-βRII/IgG-Fc fusion protein, and many others. However, patients with no or only partial responses are not uncommon, and they can experience a recurrence of the inflammatory disease upon discontinuation of treatment. This exemplifies the clinical necessity for the further generation of novel and pathogenesis-led therapeutic approaches.

IL-18 has been demonstrated to be a potential therapeutic target in several studies of IL-18 gene-targeting using animal disease model, as described in chapter 1. Different approaches have been developed to inhibit IL-18 in experimental models and in clinical trials for inflammatory and autoimmune diseases that include the use of neutralising antibodies against IL-18, IL-18 receptor blocking antibodies, IL-18 binding protein (IL-18BP), and caspase-1 inhibitors.

5.1.2 Neutralising antibodies to IL-18

With the aim to target IL-18 inflammatory response, a rabbit polyclonal anti-mouse IL-18 IgG has been generated and used in the model of Collagen Induced Arthritis (CIA) to demonstrate a significant decrease in the disease progression of established synovitis in wild type DBA/1 mice (Plater-Zyberk et al., 2001). A study using the model of streptococcal cell wall (SCW)-induced arthritis in C57BL/6 or BALB/c mice shows that the i.p. injection of a rabbit anti-mouse IL-18 antibody results in significant suppression of joint inflammation and decreased concentrations of endogenous IL-18 and synovial TNFα and IL-1β (Joosten et al., 2000). IL-18 is highly associated with intestinal inflammation in patients with Crohn's disease and an anti-mouse IL-18 antibody i.p. treatment in the model of dextran sulphate sodium (DSS)-induced colitis of C57BL/6 or BALB/c mice has resulted in a dose-dependent reduction in the severity of the disease (Siegmund et al., 2001). The generation of the first anti-IL-18 complete human antibody that blocks IL-18 signalling by binding to the potential association site with IL-18Rβ has recently been reported. This antibody inhibits the production of IFNγ in KG-1 cells, providing a potential therapeutic approach for IL-18-induced chronic inflammatory diseases (Hamasaki et al., 2005). Treatment of BALB/c mice inoculated with *Propionibacterium acnes* and challenged with LPS with anti-IL-18 mouse antibodies results in prevention of hepatic toxicity and endotoxemia (Okamura et al., 1995b). Neutralisation of IL-18 using mouse anti-IL-18 antibody treatment protects against LPS-induced myocardial dysfunction in C57BL/6 mice (Raeburn et al., 2002).

5.1.3 IL-18 binding protein

IL-18BP is in clinical trials for rheumatoid arthritis and other autoimmune diseases. A recent study by Faggioni et al. has demonstrated that a fusion protein of human IL-18BP linked to human IgG1 Fc (IL-18BP:Fc) has the ability to bind human, mouse and rat IL-18 with a Kd of 0.3-5nM and neutralise its function. Mortality is significantly reduced in mice treated with IL-18BP:Fc ten minutes before the administration of an *E. coli*-derived LPS with a lethal dose of 90% and decreased levels of IFNγ were detected in these mice. Treatment with IL-18BP:Fc even six days before LPS challenge is able to abrogate the LPS-induced IFNγ production, due to the long half life of the Fc fusion proteins in plasma (Faggioni et al., 2001). The same study has also demonstrated that IL-18BP:Fc reduces expression of Fas -ligand and hepatic injury in these mice. IL-18BP:Fc also prevents *P-acnes*-induced granuloma formation and reduces the production of the chemokines macrophage-inflammatory protein (MIP)-1α and MIP-2 in the liver. IL-18 mediates the hepatic damage caused by intravenous injection. IL-18BP:Fc protects against the Concanavalin A, *Pseudomonas aeruginosa* exotoxin A or anti-Fas agonistic antibody-induced liver damage and results in the reduction of Fas-ligand mRNA expression (Faggioni et al., 2001). IL-18BP:Fc has also been demonstrated to reduce the severity of CIA, (Banda et al., 2003), which is consistent with studies using neutralising anti-IL-18 antibodies. Other experimental autoimmune disease models have demonstrated the ability of sIL-18BP:Fc to ameliorate disease progression, by inhibiting IL-18 (Sivakumar et al., 2002, Zaccone et al., 2005).

5.1.4 Caspase-1 inhibitors to block IL-18 maturation

Recent therapeutic developments that may indirectly interfere with IL-18 activity include the inhibition of caspase-1 or caspase-1-activating multiprotein complexes known as inflammasomes (Randle et al., 2001, Siegmund, 2002, Faubel and Edelstein, 2005, Linton, 2005, Cornelis et al., 2007). Orally active caspase-1 inhibitors such as VX-740, or pralnacasan, and VX765 have gone into clinical trials for their efficacy as anti-inflammatory drugs (Clinical trial databases:

www.centerwatch.com and www.biospace.com) (Stack et al., 2005, Wannamaker et al., 2007). Pralnacasan, a masked aldehyde, was the first ICE (IL-1β converting enzyme) inhibitor to enter phase II clinical trial as an anti-inflammatory agent for the treatment of rheumatoid arthritis, but it was suspended after the detection of long-term liver abnormalities in treated animals. VX-765 is a reversible covalent caspase-1 inhibitor that has been in trials for the treatment of rheumatoid arthritis, osteoarthritis and psoriasis (World Intellectual Property Organisation website: http://www.wipo.int/about-wipo/en/what_is_wipo.html). This drug has been reported to be effective in blocking the hyper-reactivity of monocytes to inflammatory stimulation in patients with familial cold auto-inflammatory syndrome (FCAS), in which there is excessive caspase-1 activation due to a mutation in the cryopyrin (NALP3), gene a member of the nucleotide-binding oligomerisation domain (NOD)-like receptor family (NLR) (Stack et al., 2005).

5.1.5 Generation of a soluble human IL-18/IgGFc decoy receptor to block IL-18

The generation of a soluble fragment of murine IL-15Rα has been demonstrated to successfully inhibit the onset of CIA in susceptible DBA/1 mice (Ruchatz et al., 1998). Moreover, the chimeric soluble TNFR fusion protein, etanercept, is currently in Phase III clinical trials for the treatment of RA. However, the generation of a decoy soluble heterodimeric receptor to bind IL-18 with high affinity and neutralise its function has never been attempted. According to plasmon resonance studies, IL-18Rβ cannot bind on its own to either IL-18 or IL-18Rα, whereas, when IL-18 is present, IL-18Rβ is capable of binding to IL-18Rα, forming a ternary complex (Kato et al., 2003). This ternary complex shows higher affinity binding of IL-18 compared to binding of IL-18 to IL-18Rα alone (Kato et al., 2003). The main aim of this study was to develop a soluble heterodimeric IL-18Rαβ-Fc (shIL-18Rαβ-Fc) decoy protein that can bind IL-18 with higher affinity compared to either shIL-18Rα-Fc or shIL-18Rβ-Fc (Fig. 5.1). Thus, this new decoy protein would block IL-18 function, providing a potential therapeutic approach for targeting IL-18 mediated inflammatory responses that normally result in disease pathogenesis.

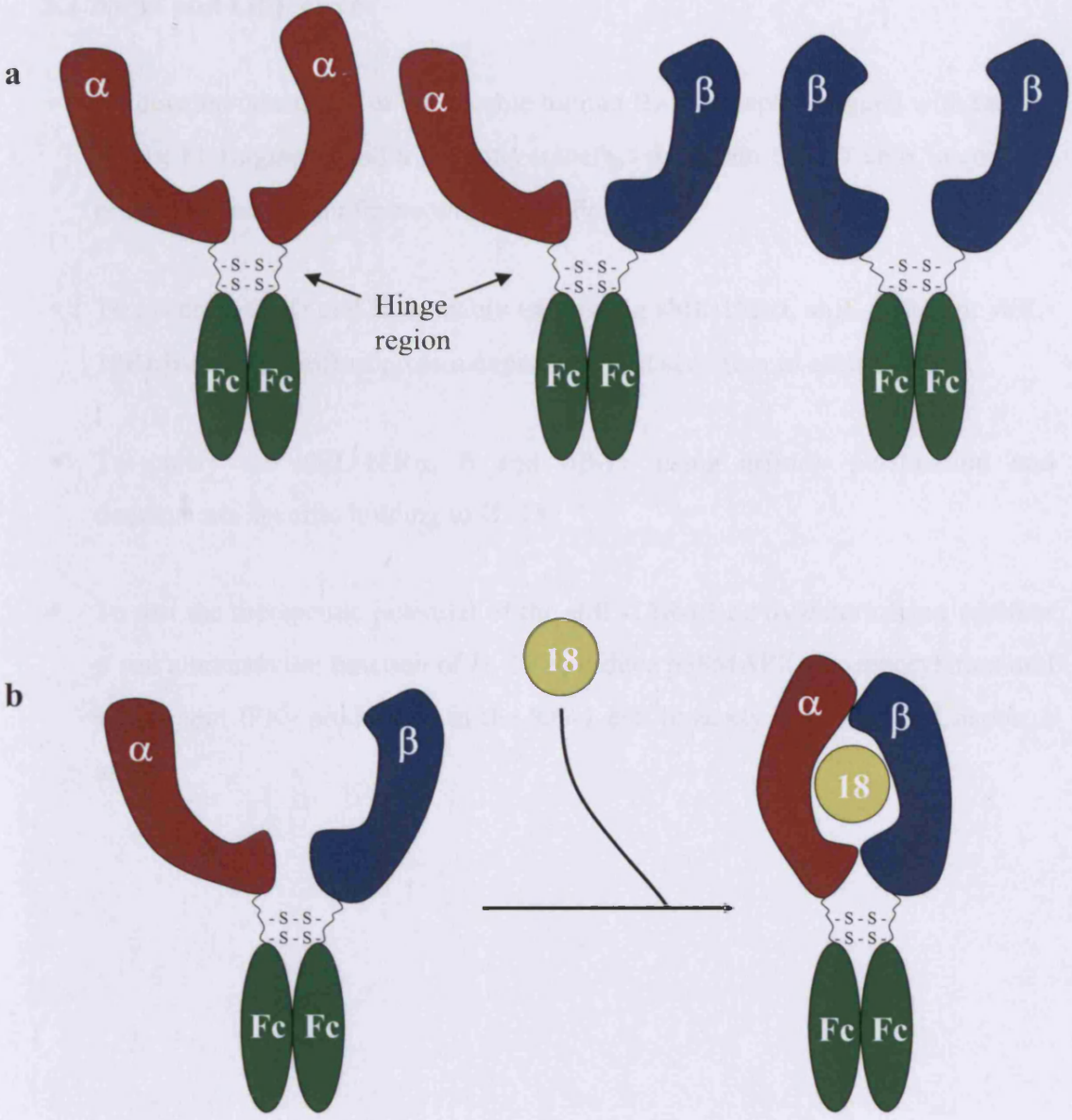


Figure 5.1: Schematic diagram of the soluble human IL-18 receptor α and β heterodimeric decoy protein tagged with hIgG1Fc.

A mixture of shIL-18Rαα-Fc, shIL-18Rββ-Fc homodimers and shIL-18Rαβ-Fc heterodimers will be formed through binding of the disulphide bonds present in the hinge region of the Fc fragment (**a**). The heterodimeric shIL-18Rαβ-Fc is expected to bind IL-18 with higher capacity compared to the homodimers (**b**).

5.2 Aims and Objectives

- To develop constructs of the soluble human IL-18 receptor, tagged with human IgG1κ Fc fragment, and transiently transfect them into COS-7 cells to confirm protein expression in frame with IgG1–Fc.

- To generate CHO cell lines stably expressing shIL-18Rα, shIL-18Rβ, or shIL-18Rαβ-Fc and confirm protein expression and secretion in each cell line.

- To purify the shIL-18Rα, β and αβ-Fc using affinity purification and demonstrate specific binding to IL-18.

- To test the therapeutic potential of the shIL-18Rαβ-Fc by determining whether it can attenuate the function of IL-18 to induce p38MAPK phosphorylation and subsequent IFNγ production in the KG-1 cell bioassay described in Chapter 3 and 4.

5.3 Results and Discussion

5.3.1 Design and Preparation of pCR®II-shIL-18Rα and pCR®II-shIL-18Rβ Expression Constructs

5.3.1.1 Isolation of Soluble Human IL-18Rα and IL-18Rβ cDNA

In order to amplify the shIL-18Rα and shIL-18Rβ cDNA for cloning, primers flanking the extracellular immunoglobulin domains of the human full length IL-18Rα and IL-18Rβ (Fig. 5.2a and b) were designed to encode the soluble IL-18Rα and Rβ, respectively. In order to assist sub-cloning and control the orientations of the inserts, the forward primers for both receptors were designed to encode *Bgl* II and *Eco*R I sites and the reverse primers a *Bgl* II site. To facilitate the initial binding of the mRNA to the small subunit of the ribosome during the initiation of translation and improve expression levels of the inserts, the Kozak consensus sequence (CACAACC) was also added directly upstream of the start codon of each of the forward primers (refer to Table 2.8 for primer sequences).

Plasmid DNAs previously confirmed to contain the correct inserts encoding the full length human IL-18Rα and β (pcDNA3.1/hIL-18Rα and pBla/hIL-18Rβ plasmids provided by Wei XQ) were used as a template in the ensuing PCR reaction in order to generate PCR products with the restriction sites and Kozak sequences added to them. The cDNA used for the initial amplification of the full length hIL-18Rα and Rβ in these plasmids was generated from KG-1 cells, as these cells are shown to express both receptors (Nakamura et al., 2000, Wu et al., 2003, Zhang et al., 2003). A proofreading DNA polymerase was used to ensure that no mutations were introduced within the sequence. Clear bands observed at 1020bp and 1089bp on a 1% agarose gel (Fig. 5.3a and b) correspond to the mRNA sequence encoding the soluble part of the full length IL-18Rα and IL-18Rβ, respectively (see Fig. 5.2a and b).

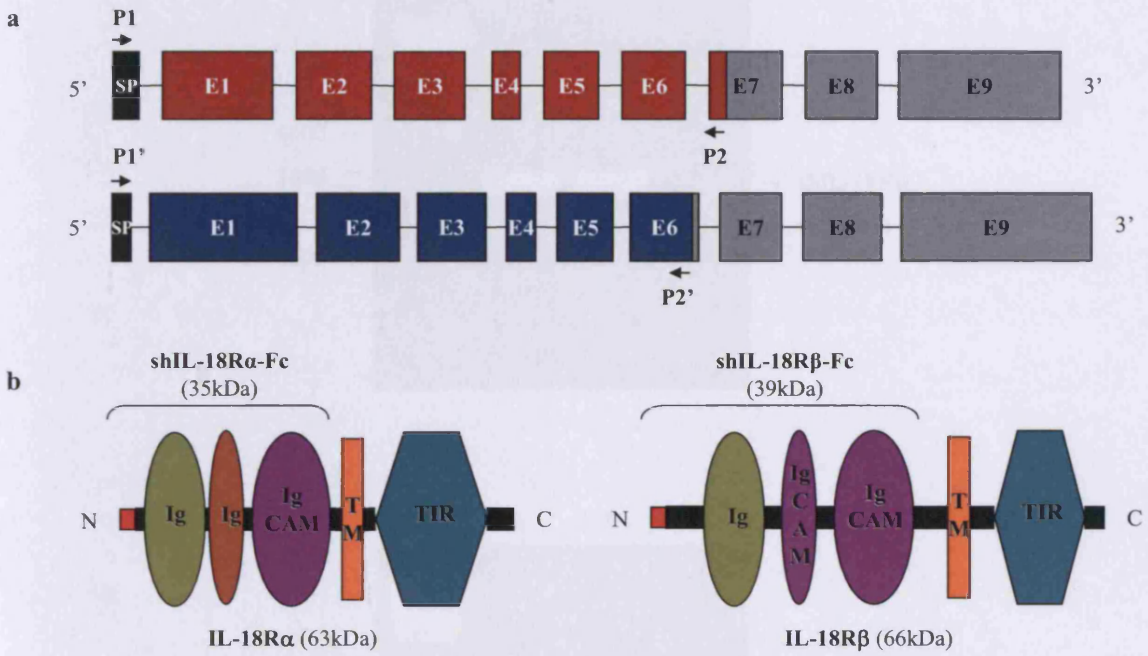


Figure 5.2: Diagram showing the genomic and protein representation of the full length human IL-18Rα and IL-18Rβ.

The hIL-18Rα (top) and hIL-18Rβ (bottom) gene is represented with the exons numbered E1-9 and indicated by solid boxes (**a**). The primers flanking the soluble region of each receptor are also indicated. The full length preceptors are also demonstrated in (**b**), indicating the conserved fragments and the part that constitutes the soluble part of the receptor encoded from the corresponding genes in (**a**). Derived from Genbank core nucleotide entry NM003855 and NM003853, for IL-18Rα and β respectively (SP, signal peptide; E1-9, exon; P1, forward primer; P2, reverse primer; Ig CAM, immunoglobulin cell adhesion molecules; TM, transmembrane domain; TIR, Toll-Interleukin 1 receptor).

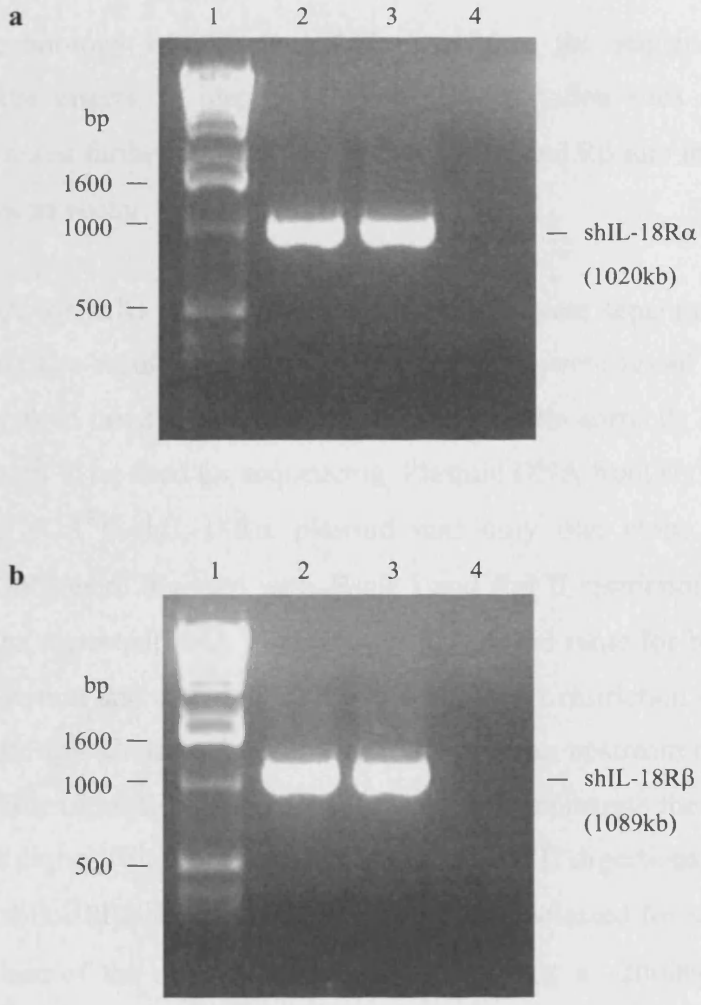


Figure 5.3: Amplification of human soluble IL-18R mRNA with *Pfu* DNA polymerase from plasmid DNA containing the full length receptor.

The PCR products for shIL-18Rα (**a**) and shIL-18Rβ (**b**) were run on 1% agarose gel. Lanes indicate 1kb DNA ladder (1), PCR fragment (2, 3) and water as negative control (4).

5.3.1.2 Restriction Digestion Analysis of the TA-cloned DNA Encoding the shIL-18Rα and Rβ Fragments

TA cloning technology was implemented to confirm the sequence identity and homology of the inserts, to identify the inserted restriction sites designed in the primers and to assist further sub-cloning of shIL-18Rα and Rβ into the pcDNA4/TO-IgG1Fc expression vector.

Amplified DNA for shIL-18Rα-Fc and shIL-18Rβ-Fc were separately inserted into pCR®II vectors. The resulting bacterial DNA plasmids were tested with restriction digestion analysis in order to confirm the insertion of the correctly sized DNA and identify the clones to be used for sequencing. Plasmid DNA from six different clones containing the pCR®II-shIL-18Rα plasmid and only one clone containing the pCR®II-shIL-18Rβ were digested with *Eco*R I and *Bgl* II restriction enzymes (Fig. 5.4a and b). The expected DNA fragment sizes were the same for both orientations of fragment insertion and are listed in Table 5.1. *Eco*R I restriction sites are located in the MCS (Multiple Cloning Site) of the pCR®II vector, upstream and downstream of the PCR product insertion site, and were used to demonstrate the presence of the insert in all the clones (Fig. 5.4a). According to the *Bgl* II digestions, clones 3 and 6 contained the shIL-18Rα insert and thus clone 3 was selected for sequencing (Fig. 5.4b). In the case of the only pCR®II-shIL-18Rβ clone, a ~2000bp fragment was released after *Bgl* II digestion that led to the hypothesis that one of the two *Bgl* II restriction sites designed in the primers was missing and thus the plasmid DNA of clone 8 was used for sequencing (Fig. 5.4b).

Restriction Enzyme	**Expected Band Sizes for pCR®II-shIL-18Rα (4991bp)**	**Expected Band Sizes for pCR®II-shIL-18Rβ (5060bp)**
*Eco*R I	21, 1015 and 3955 bp	21, 1084 and 3955 bp
Bgl II	997, 1006 and 2988 bp	983, 1089 and 2988 bp

Table 5.1: Restriction digestion products of pCR®II-shIL-18Rα and pCR®II-shIL-18Rβ plasmids.

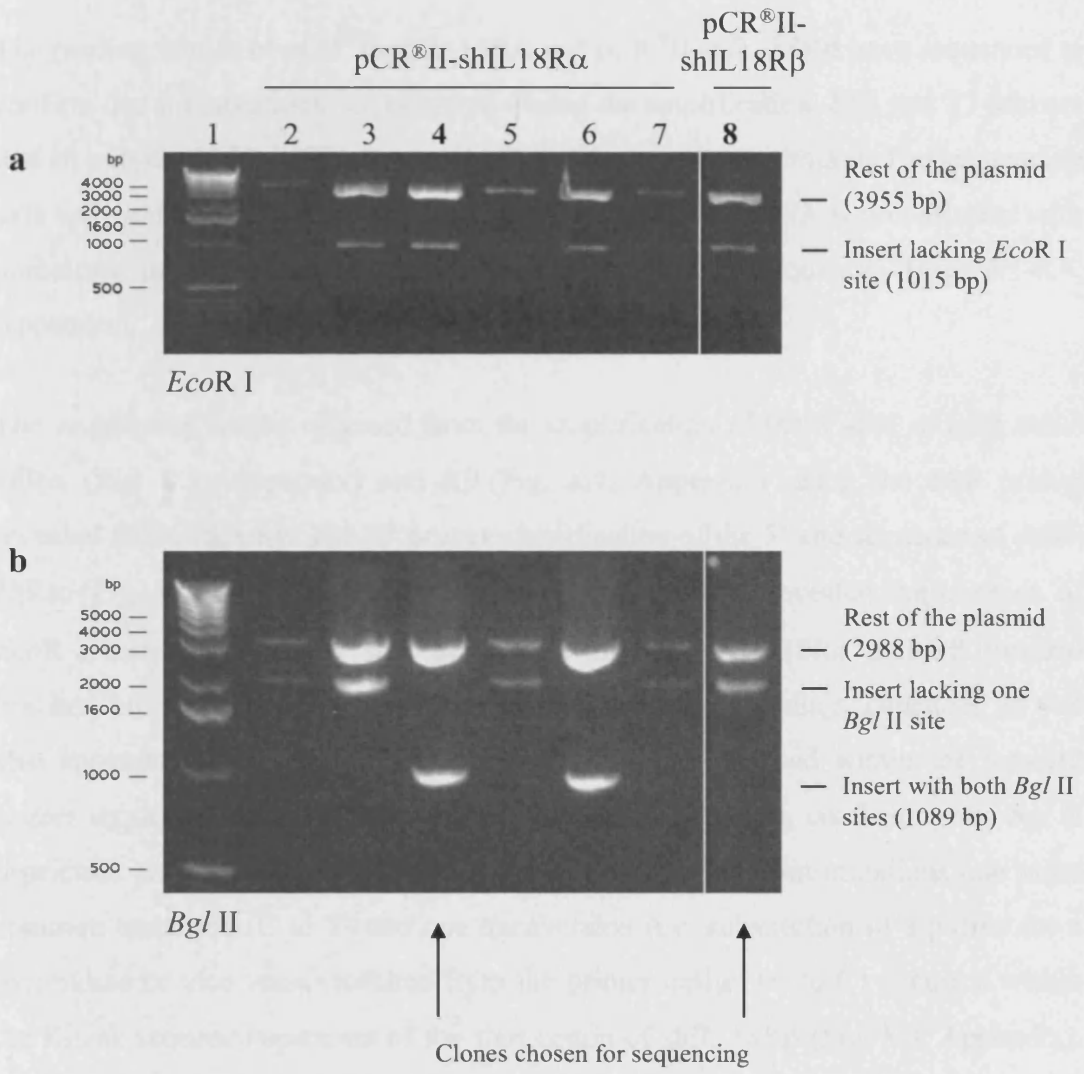


Figure 5.4: Restriction digests of pCR®II-shIL18Rα and pCR®II-shIL18Rβ plasmid DNA.

The restriction enzymes *Eco*R I (**a**) and *Bgl* II (**b**) were used to demonstrate the expected fragments, as listed in Table 4.1. Lanes indicate 1kb DNA ladder (1), pCR®II-shIL18Rα (2-7) and pCR®II-shIL18Rβ (8).

5.3.1.3 Confirmation of Sequence Identity

The reading frames of pCR®II-shIL-18Rα and pCR®II-shIL-18Rβ were sequenced to confirm that no mutations had occurred during the amplification. SP6 and T7 primers that sit outside of the MCS were used to amplify sequences within it. The sequencing data were analysed and the identity of shIL-18Rα and Rβ cDNA was confirmed with homology to the known corresponding *Homo sapiens* sequences (Fig. 8.1-8.4, Appendix).

The sequencing results obtained from the amplification of the 3'-end of both shIL-18Rα (Fig. 8.2, Appendix) and Rβ (Fig. 8.4, Appendix) using the SP6 primer revealed 100% identity. The T7 primer amplification of the 5'-end sequence of shIL-18Rα (Fig. 8.1, Appendix) and Rβ (Fig. 8.3, Appendix) revealed the absence of *Eco*R I restriction enzyme sites designed within the shIL-18Rα and Rβ forward primers, but showed 100% homology to the downstream coding sequences. It was also apparent that the *Bgl* II restriction enzyme site designed within the forward primer sequence of shIL-18Rβ was lost (Fig. 8.3, Appendix), confirming the *Bgl* II restriction analysis shown in Figure 5.4b. Moreover, two point mutations one more common transition (C to T) and one transversion (i.e. substitution of a purine for a pyrimidine or vice versa) resulted from the primer design (A to C) occurred within the Kozak sequence upstream of the start codon of shIL-18Rβ (Fig. 8.3, Appendix). These single base substitutions lie within the Kozak short recognition sequence in the non-coding region of the gene and are not expected to have any major consequences on gene expression. One possibility is that they can result in a slight reduction in the expression levels of the construct and could be a reason of the lower expression levels of shIL-18Rβ compared to shIL-18Rα that was later shown in figure 5.7a.

5.3.2 Design and Preparation of pcDNA4/TO-shIL-18Rα-IgG$_1$Fc and pcDNA4/TO-shIL-18Rβ-IgG1Fc Expression Constructs

5.3.2.1 Sub-cloning of Sequences Encoding the Fragments of hIL-18Rα and hIL-18Rβ into pcDNA4/TO-IgG$_1$Fc Expression Vectors

The sub-cloning of shIL-18Rα and shIL-18Rβ into the pcDNA4/TO-IgG$_1$Fc expression plasmid (provided by Wei XQ) was implemented for eukaryotic expression i.e. to generate stably transfected CHO cell lines expressing high levels of soluble human IL-18Rα, Rβ and Rαβ, under the CMV promoter. Expressed proteins would be in frame with the Fc fragment of IgG$_1$ and could be purified using Protein A affinity purification (Andersson et al., 1989, Nelson et al., 1987, Boshart et al., 1985).

As demonstrated in figure 2.2, the pCR®II-shIL-18Rα DNA plasmid was digested with *Bgl* II (Fig. 5.5a) and the pCR®II-shIL-18Rβ plasmid was digested with *Bgl* II and *Eco*R V (Fig. 5.5c) to release the inserts, which were gel extracted (Fig. 5.5b and d) for ligation into the expression vector pcDNA4/TO-IgG$_1$Fc. The pcDNA4/TO-IgG$_1$Fc plasmid was linearised with *Bam*H I digestion (Fig. 5.5a) and purified (Fig. 5.5b) to be used for ligation with shIL-18Rα. For ligation with shIL-18Rβ, the pcDNA4/TO-IgG$_1$Fc plasmid was digested with *Hind* III, followed by *Klenow* DNA polymerase treatment to fill in the recessed 3' ends of the linearised vector and finally *Bam*H I digestion generating one blunt and one sticky end (see Fig. 2.2), with subsequent gel purification (Fig. 5.5d). The fragments were inserted downstream of the CMV promoter and upstream of the IgG1-Fc fragment. Once within the expression vectors, the resulting constructs pcDNA4/TO-shIL-18Rα-IgG$_1$Fc and pcDNA4/TO-shIL-18Rβ-IgG$_1$Fc were transformed into DH5α bacterial cells to produce the required plasmid DNAs, which were purified and test digested before sequencing.

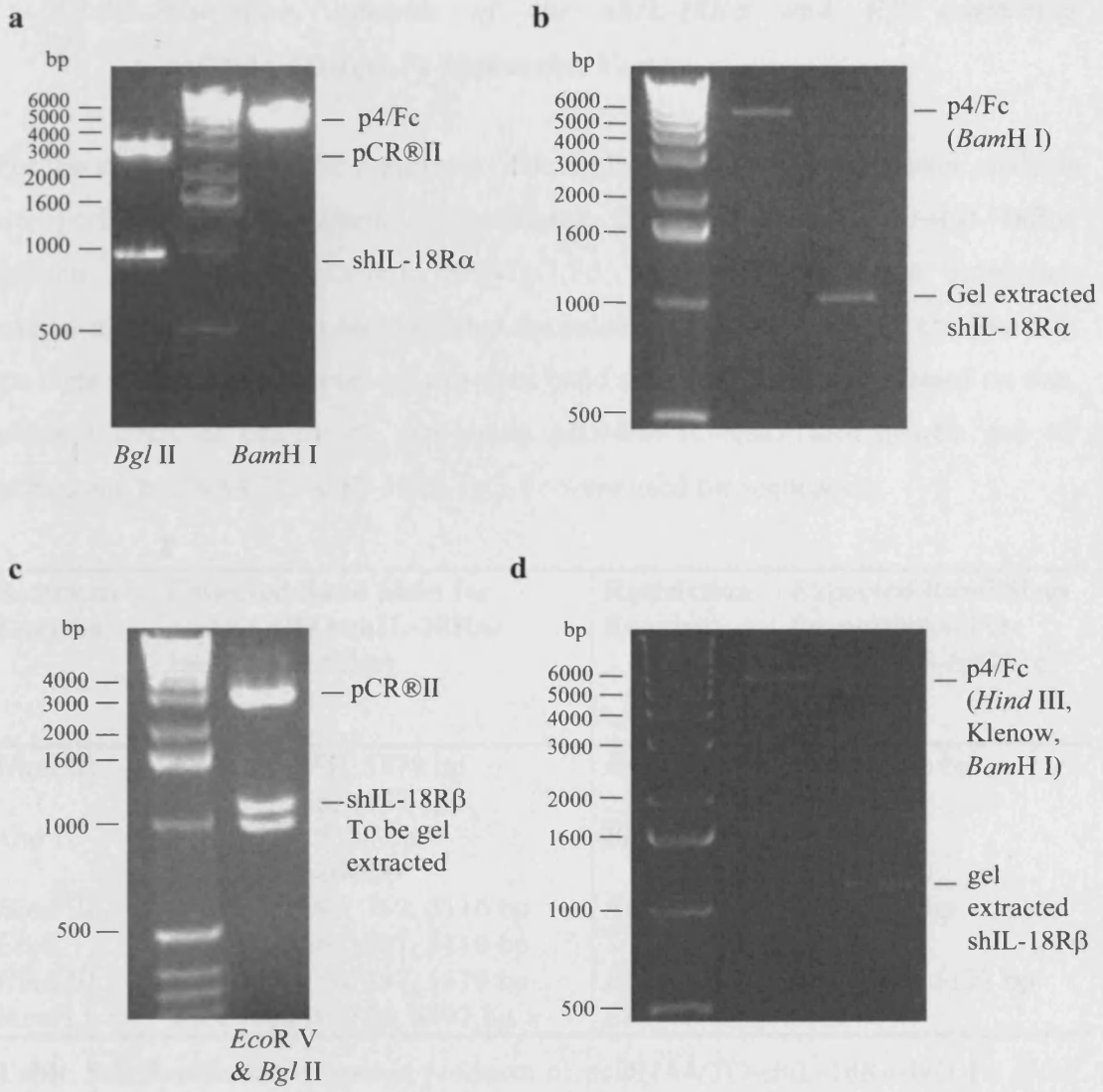


Figure 5.5: Transferring shIL-18Rα and shIL-18Rβ inserts from pCR®II plasmids into pcDNA4/TO-IgG1Fc.

shIL-18Rα was released from pCR®II-shIL-18Rα with *Bgl* II (**a**) and gel extracted (**b**) to be inserted into the purified pcDNA4/TO-IgG1Fc. In the same way, shIL-18Rβ was released from pCR®II-shIL-18Rβ with *Eco*R V and *Bgl* II (**c**) and gel extracted (**d**) to be inserted into the purified pcDNA4/TO-IgG1Fc. The top of the two bands around 1100bp in part (c) corresponds to the shIL-18Rβ fragment.

5.3.2.2 *Restriction Analysis of the shIL-18Rα and Rβ containing pcDNA4/TO-IgG$_1$Fc Expression Vector*

For the identification of the plasmid containing the correct insert, restriction analysis was performed before sequencing. Restriction digests of pcDNA4/TO-shIL-18Rα-IgG$_1$Fc and pcDNA4/TO-shIL-18Rβ-IgG$_1$Fc with the appropriate restriction enzymes (Fig. 5.6a and b) demonstrated the colonies containing the correct inserts in the right orientation based on the expected band sizes (see Table 5.2). Based on this, plasmid DNA of colony #3 expressing pcDNA4/TO-shIL-18Rα-IgG$_1$Fc and #5 expressing pcDNA4/TO-shIL-18Rβ-IgG$_1$Fc were used for sequencing.

Restriction Enzyme	**Expected Band Sizes for pcDNA4/TO-shIL-18Rα-IgG1Fc (6842bp)**	**Restriction Enzyme**	**Expected Band Sizes for pcDNA4/TO-shIL-18Rβ-IgG1Fc (6920bp)**
Hind III	*5-3':* 266, 697, 5879 bp *3-5':* 79, 266, 6497 bp	*Eco*R I	1798, 5122 bp
Xho I	*5-3':* 1510, 5332 bp *3-5':* 978, 5864 bp	*Bam*H I	6920 bp (linearised)
Hind III / *Eco*R I	*5-3':* 266, 697, 769, 5110 bp *3-5':* 79, 266, 1387, 5110 bp	*Bgl* II	824, 6096 bp
Hind III / *Bam*H I	*5-3':* 110, 156, 697, 5879 bp *3-5':* 79, 110, 156, 6497 bp	*Bam*H I / *Eco*R I	783, 1015, 5122 bp

Table 5.2: Restriction digestion products of pcDNA4/TO-shIL-18Rα-IgG$_1$Fc (both potential orientations are shown) and pcDNA4/TO-shIL-18Rβ-IgG$_1$Fc plasmids.

shIL-18Rβ-Fc were detected in the culture media compared to the negative control of non-transfected cells, with shIL-18Rβ-Fc being expressed at about 8 times lower levels (0.248ng/ml) compared to shIL-18Rα-Fc (2.071ng/ml). The difference in the expression levels between the two receptors could be due to the difference in the sensitivity of the assay or due to mutations detected within the Kozak sequence of the shIL-18Rβ plasmid described above in section 5.6.3. Significantly increased levels of shIL-18Rαβ-Fc were also detected compared to shIL-18Rα-Fc and shIL-18Rβ-Fc alone. The expression of shIL-18Rα and shIL-18Rβ in frame with hFc in the protein G concentrated culture media of Cos-7 transfected cells was also evaluated using an ELISA assay employing capturing with polyclonal antibodies against hIL-18Rα and hIL-18Rβ, respectively, and detecting with an antibody against Fc. The results demonstrated 40-50 fold increased levels of shIL-18Rα-Fc (Fig. 5.7b) and 5-8 fold increase in the level of shIL-18Rβ-Fc (Fig. 5.7c) in single or double transfected cells compared to the IgG-Fc vector and non-transfected control groups, which were similar to the numbers obtained from the IgG-Fc ELISA in figure 5.7a, where shIL-18Rα-Fc indicated 30-fold and shIL-18Rβ-Fc 3.6-fold increase compared to Cos-7 alone.

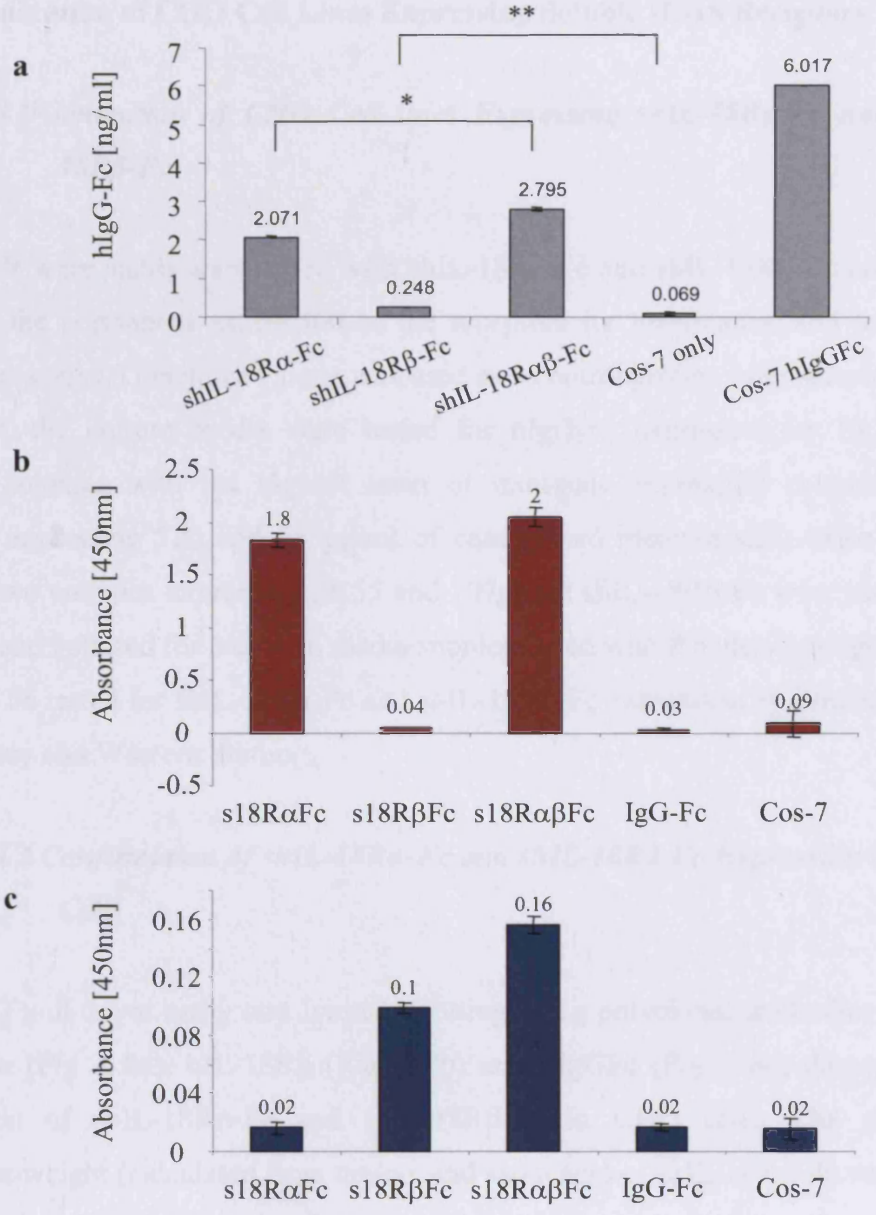


Figure 5.7: Confirmation of in-frame expression of shIL-18R with hIgG1-Fc in Cos-7 cells.

Cos-7 cells were transiently transfected with either shIL-18Rα-Fc alone, shIL-18Rβ-Fc alone, shIL-18Rα-Fc together with shIL-18Rβ-Fc or a control vector (pcDNA4/TO-IgG1Fc). Cos-7 cells with Genejuice® alone were used as a negative control group. Cell culture media were harvested after transfection and concentrated using Protein G pull down assay. ELISA detecting human IgG-Fc was used to confirm the in-frame expression of the plasmids with the Fc fragment (**a**). ELISA capturing with polyclonal anti-hIL-18Rα (**b**) and hIL-18Rβ (**c**) antibodies and detecting with an antibody against Fc were also used to confirm shIL-18Rα and shIL-18Rβ expression and in frame with Fc. Data shown are expressed as mean ±SD of triplicates of a representative experiment. * $P < 0.05$; ** $P < 0.01$ by student's t-test.

5.3.4 Generation of CHO Cell Lines Expressing Soluble IL-18 Receptors

5.3.4.1 Generation of CHO Cell lines Expressing shIL-18Rα-Fc and shIL-18Rβ-Fc

CHO cells were stably transfected with shIL-18Rα-Fc and shIL-18Rβ-Fc constructs to allow the permanent expression of the receptors for purification and testing in functional assays. Genejuice® alone was used as a control group. After selection with Zeocin™, the culture media were tested for $hIgG_1Fc$ expression by ELISA, to identify colonies with the highest level of transgene expressing colonies. Two colonies expressing 720 and 955pg/ml of conditioned medium shIL-18Rα-Fc and another two colonies expressing 98.55 and 107pg/ml shIL-18Rβ-Fc were identified, selected and cultured for 3 days in media supplemented with the ultra-low IgG serum media to be tested for shIL-18Rα-Fc and shIL-18Rβ-Fc expression by Protein G pull down assay and Western blotting.

5.3.4.2 Confirmation of shIL-18Rα-Fc and shIL-18Rβ-Fc Expression in CHO Cells

Protein-G pull down assay and Immunoblotting using polyclonal antibodies against hIL-18Rα (Fig. 5.8a), hIL-18Rβ (Fig. 5.8b) and hIgGFc (Fig. 5.8c) demonstrated expression of shIL-18Rα-Fc and shIL-18Rβ-Fc in CHO cells. The expected molecular weight (calculated from amino acid sequence) of shIL-18Rα-Fc was about 81kDa and of shIL-18Rβ-Fc about 85kDa. However, due to post-translational modification by glycosylation, the chains migrated in SDS-PAGE with an apparent molecular mass of about 95kDa (Fig. 5.8).

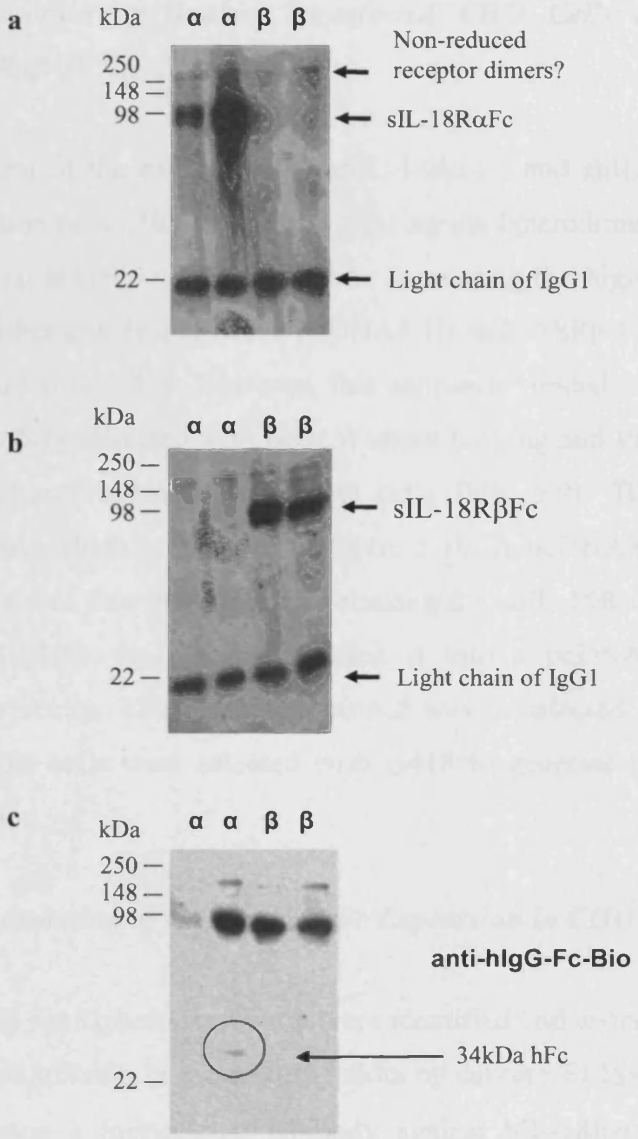


Figure 5.8: Generation of cell lines expressing shIL-18Rα-Fc and shIL-18Rβ-Fc.

CHO cells were stably transfected with shIL-18Rα-Fc and shIL-18Rβ-Fc to allow permanent expression of the receptors. Proteins were isolated by Protein G pull down assay, separated by SDS-PAGE and subjected to immunoblotting using goat polyclonal antibodies against hIL-18Rα (**a**), hIL-18Rβ (**b**) and mouse monoclonal anti-hIgGFc (Fc-specific) (**c**). This demonstrated the expression of shIL-18Rα-Fc and shIL-18Rβ-Fc in CHO cells.

5.3.4.3 Generation of Double Transfected CHO Cells Expressing ShIL-18Raβ-Fc

After confirmation of the expression of shIL-18Rα-Fc and shIL-18Rβ-Fc in CHO cells, the generation of a CHO cell line expressing the heterodimeric shIL-18Raβ-Fc was attempted. To achieve this, the cell line expressing the highest levels of shIL-18Rα-Fc was further transfected with a pcDNA3.1B-shIL-18Rβ-Fc (provided by Wei X-Q) and selected with G418. However, this approach yielded very low expression of the shIL-18Raβ-Fc detected with both Western blotting and Protein G pull down assay of the culture supernatant of CHO cells (Fig. 5.9). Thus, an alternative approach was used which is outlined in figure 5.10. A pcDNA3.1A-shIL-18Rα-Fc expression vector was thus generated by releasing the shIL-18Rα-Fc insert from the pcDNA4/TO-shIL-18Rα-IgG$_1$Fc and ligating it into a pcDNA3.1MycHisA (see Appendix 8.2.3) vector. The resulting plasmid was transfected into pcDNA4/TO-shIL-18Rβ-IgG1Fc cells were selected with G418 to generate the shIL-18Raβ-Fc expressing cell line.

5.3.4.4 Confirmation of shIL-18Raβ-Fc Expression in CHO Cells

The colonies with the highest expression were identified and tested for heterodimeric shIL-18Raβ-Fc expression in the culture media by capture ELISA, where the target was captured using a monoclonal antibody against hIL-18Rα and subsequently detected with a monoclonal antibody against hIL-18Rβ, as shown in figure 5.11a. Decreasing dilutions of culture media demonstrated an increase in shIL-18Raβ-Fc compared to the negative control expressing shIL-18Rα-Fc only (Fig. 5.11b).

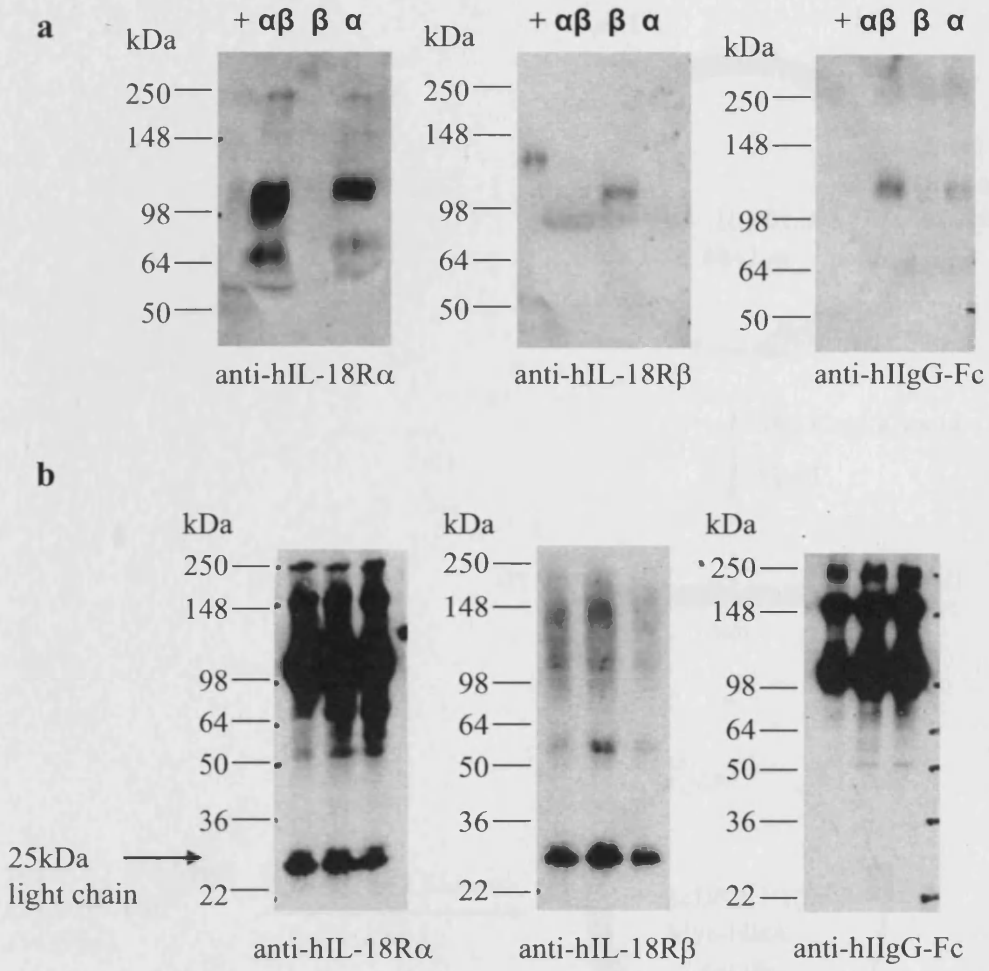


Figure 5.9: Low expression of shIL-18Rβ-Fc transfected in shIL-18Rα-Fc CHO cells.

CHO cells expressing shIL-18Rα-Fc were stably transfected with shIL-18Rβ-Fc. Western blotting of culture supernatant of a colony expressing shIL-18Rα-Fc (α), β-Fc (β), and αβ-Fc (αβ) (**a**). Protein-G pull down assay of the culture media of three of the colonies (each corresponding to each of the three lanes on the Western blot) expressing the highest shIL-18Rαβ-Fc levels (**b**). Proteins were separated by SDS-PAGE and analysed by immunoblotting using goat polyclonal antibodies against hIL-18Rα, hIL-18Rβ and mouse monoclonal anti-hIgGFc (Fc-specific) to demonstrate expression shIL-18Rαβ-Fc in CHO cells. 84ng of another purified shIL-18[Rαβ]2-Fc decoy receptor consisting of two shIL-18Rαβ-Fc chains, where IL-18Rα and Rβ have been cloned in frame (provided by Wei XQ; designated as +) was used as positive control in (**a**).

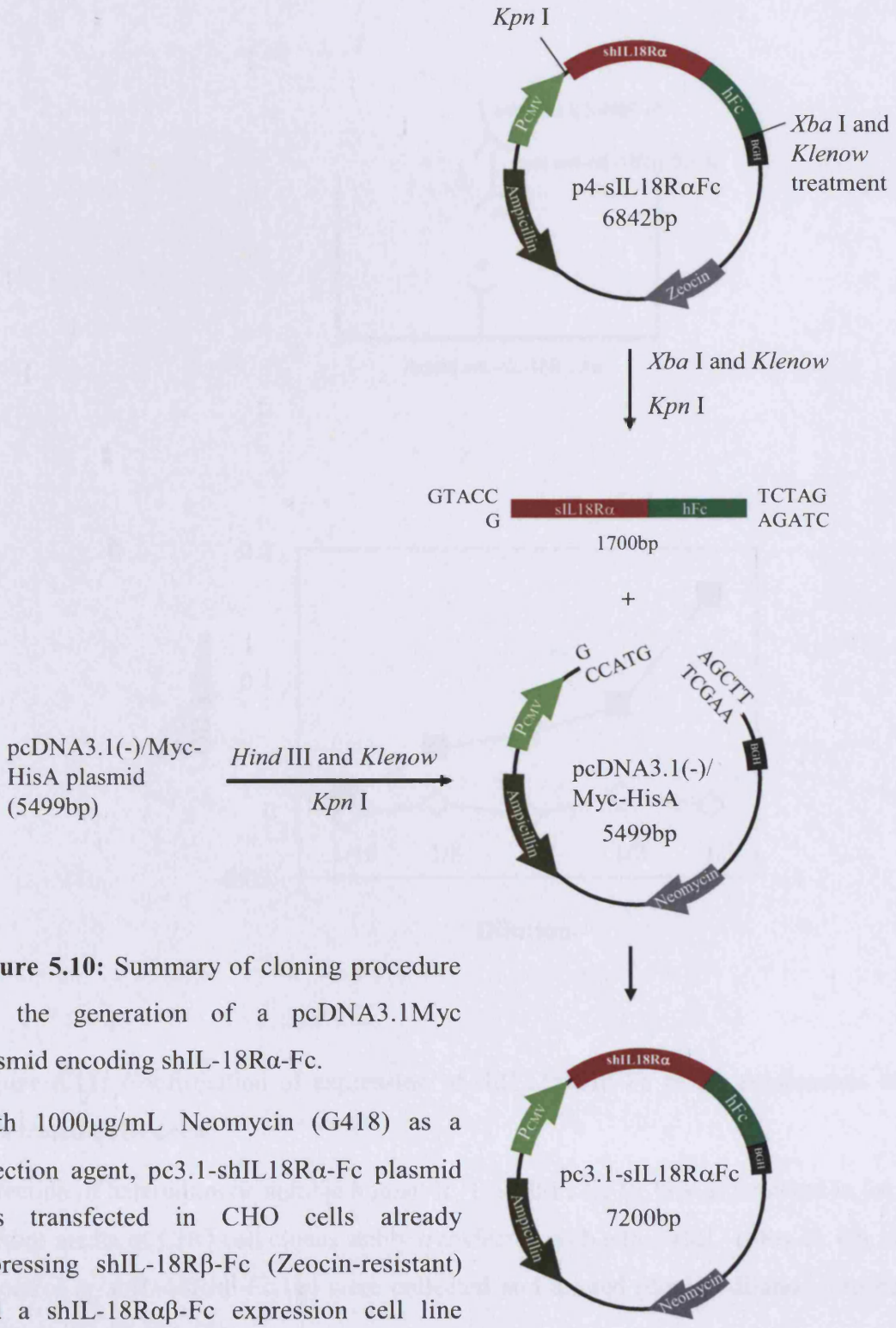


Figure 5.10: Summary of cloning procedure for the generation of a pcDNA3.1Myc plasmid encoding shIL-18Rα-Fc.

With 1000μg/mL Neomycin (G418) as a selection agent, pc3.1-shIL18Rα-Fc plasmid was transfected in CHO cells already expressing shIL-18Rβ-Fc (Zeocin-resistant) and a shIL-18Rαβ-Fc expression cell line was generated for the purification of the heterodimeric receptor.

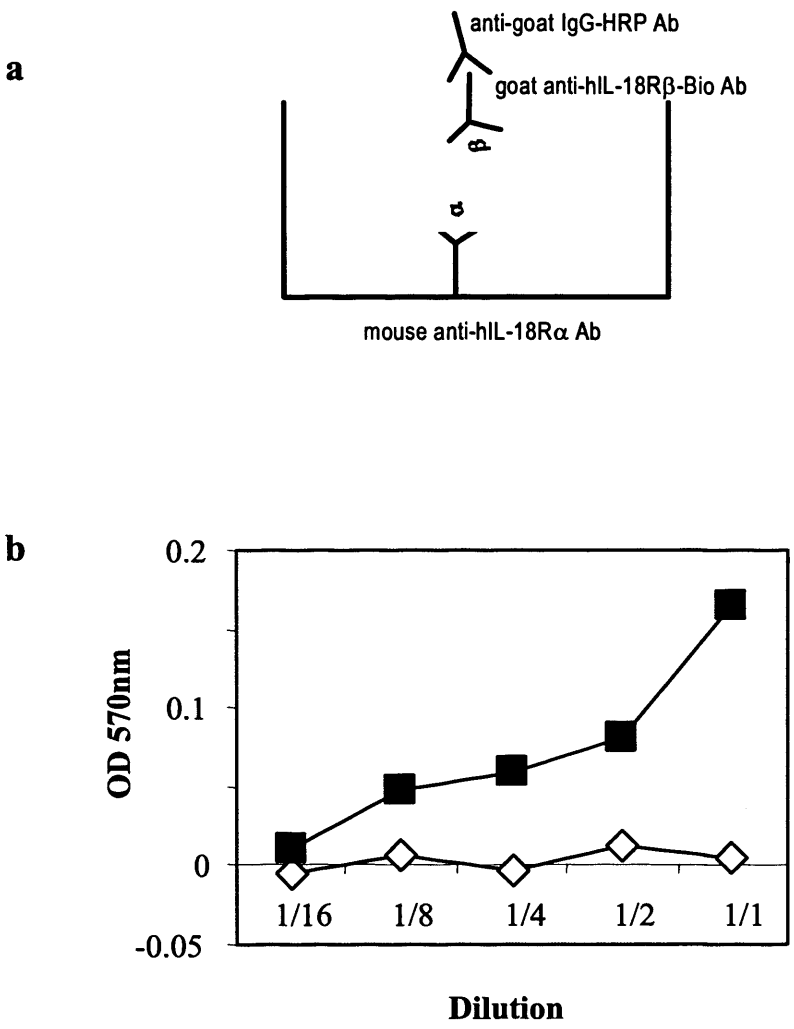


Figure 5.11: Confirmation of expression of shIL118Rαβ-Fc in the supernatant of transfected CHO cells.

Detection of heterodimeric soluble human IL-18Rαβ-Fc by ELISA as depicted in (**a**). Culture media of CHO cell clones stably transfected with either shIL-18Rα-Fc (◊) as a control or shIL-18Rαβ-Fc (■) were collected and diluted (double dilutions) to be measured by ELISA (**b**).

5.3.5 Purification of shIL-18Rα-Fc, shIL-18Rβ-Fc and shIL-18Rαβ-Fc for Use in Functional Assays

To further study the effect of shIL-18Rαβ-Fc in neutralising an IL-18-induced inflammatory response, the cell lines expressing shIL-18Rα-Fc, shIL-18Rβ-Fc and shIL-18Rαβ-Fc were cultured in ultra low IgG serum media and the respective recombinant proteins were purified using Protein A affinity purification. Protein A is derived from a strain of *Staphylococcus aureus*, and contains five regions that bind to the Fc region of IgG. Protein A is coupled to Sepharose so that one molecule of Protein A binds to at least two molecules of IgG_1, in this case with high affinity.

Fractions with the highest amount of protein as determined using the BCA assay were run on an SDS-PAGE gel for confirmation of the correct protein size and then pooled together. Estimation of the purity of the pooled protein by serial dilutions using SDS-PAGE revealed >97% purity (Fig. 5.12a and b). SDS-PAGE after Coomassie Blue staining revealed two distinct bands of ~95 and ~200kDa for the purified shIL-18Rα-Fc and shIL-18Rβ-Fc, corresponding to the monomer and dimer, respectively (Fig. 5.12a). In the case of shIL-18Rαβ-Fc, two bands also appeared at ~95 and ~200kDa that corresponded to shIL-18Rα-Fc and shIL-18Rβ-Fc monomers under reducing conditions and dimers under non-reducing conditions (Fig. 5.12b). These dimers were comprised of heterodimers as well as homodimers. The purified shIL-18Rα-Fc under reducing conditions was used for Western blotting to confirm the band identity (Fig. 5.12c). Both biotinylated antibody for human IgG-Fc and goat anti-hIL-18Rα polyclonal antibody were used to confirm the size of the monomeric shIL-18Rα-Fc at ~95kDa and the dimer at ~200kDa. The dimer was seen although it was run under reducing conditions, indicating a possible incomplete reduction of the proteins run on the gel. As the gel was run under reducing conditions, the band at 34kDa corresponds to the heavy Fc chain of IgG, which was only detected in the case of the anti-IgGFc blot. This band disappears as expected in the case of anti-IL-18Rα detection, where another band of 64kDa appears to be visible that is likely to correspond to shIL-18Rα without the Fc component.

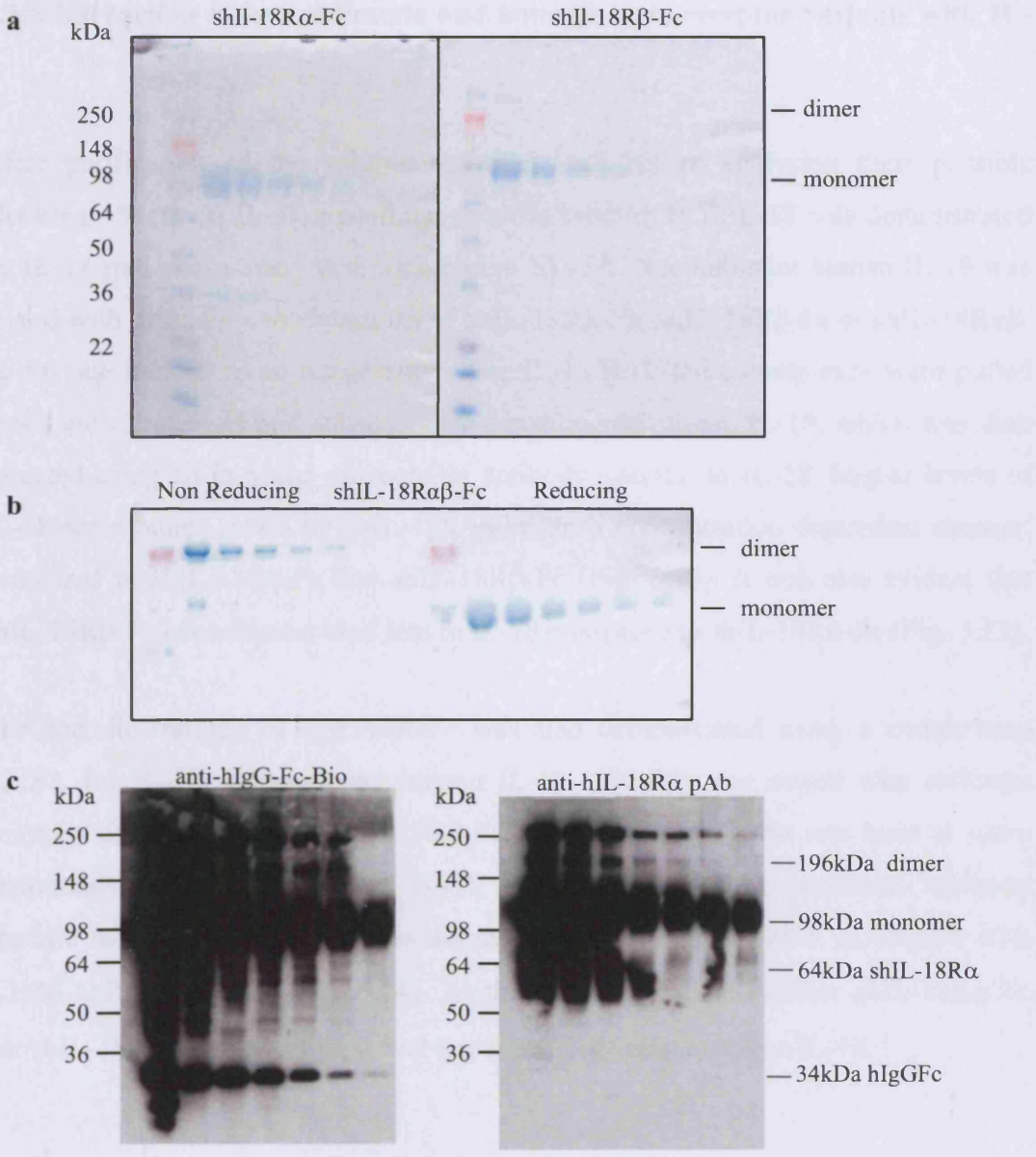


Figure 5.12: Analysis of the purified soluble human IL-18 receptor.

shIL-18Rα-Fc and shIL-18Rβ-Fc were purified using Protein A affinity chromatography (Serial dilution by factor 2 starting from 5µg) and visualised under reducing conditions with SDS-PAGE by Coomassie blue staining (**a**). The detection of shIL-18Rαβ-Fc under non-reducing and reducing conditions revealed the dimerisation of the heterodimeric receptor (**b**). Western blot under reducing conditions of the purified shIL-18Rα-Fc serially diluted and detected with antibodies for IgG-Fc and IL-18Rα (**c**).

5.3.6 Interaction of heterodimeric and homodimeric receptor variants with IL-18

After purification of the soluble receptors and before analysing their possible blocking effects on IL-18 signalling, specific binding to rhIL-18 was demonstrated by IL-18 pull down assay and competition ELISA. Recombinant human IL-18 was mixed with different concentrations of shIL-18Rα-Fc, shIL-18Rβ-Fc or shIL-18Rαβ-Fc for one hour at room temperature. The IL-18/IL-18R-Fc complexes were pulled down with Protein G and subsequently denatured to release IL-18, which was then detected using an in-house monoclonal antibody specific to IL-18. Higher levels of IL-18 were pulled down by shIL-18Rαβ-Fc, in a concentration dependent manner, compared to shIL-18Rα-Fc and shIL-18Rβ-Fc (Fig. 5.13). It was also evident that shIL-18Rβ-Fc homodimers bind less to IL-18 compared to shIL-18Rα-Fc (Fig. 5.13).

The specific binding of shIL-18R-Fc was also demonstrated using a competition ELISA for IL-18. Recombinant human IL-18 (100pM) was mixed with different amounts of shIL-18Rα-Fc, shIL-18Rβ-Fc or shIL-18Rαβ-Fc for one hour at room temperature. The unbound free IL-18 was detected with monoclonal antibody specific to IL-18. Free IL-18 was almost completely absent after incubation with 6.3nM shIL-18Rαβ-Fc (Fig. 5.14). At these concentrations neither shIL-18Rα-Fc nor shIL-18Rβ-Fc, respectively, had any significant effect on free IL-18.

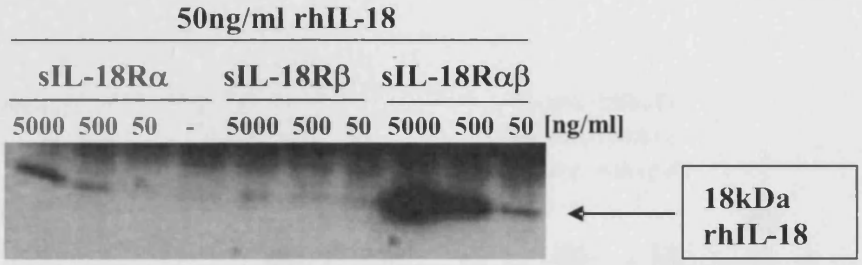


Figure 5.13: shIL-18Rαβ-Fc binds to IL-18 with higher capacity compared to the homodimers.

50ng/ml of recombinant human IL-18 was mixed with different concentrations of either shIL-18Rα-Fc or shIL-18Rβ-Fc or shIL-18Rαβ-Fc for one hour at room temperature. The IL-18/IL-18R complexes were then pulled down with Protein G and subsequently denatured to release IL-18, which was detected using in home mouse monoclonal anti-human IL-18 antibody.

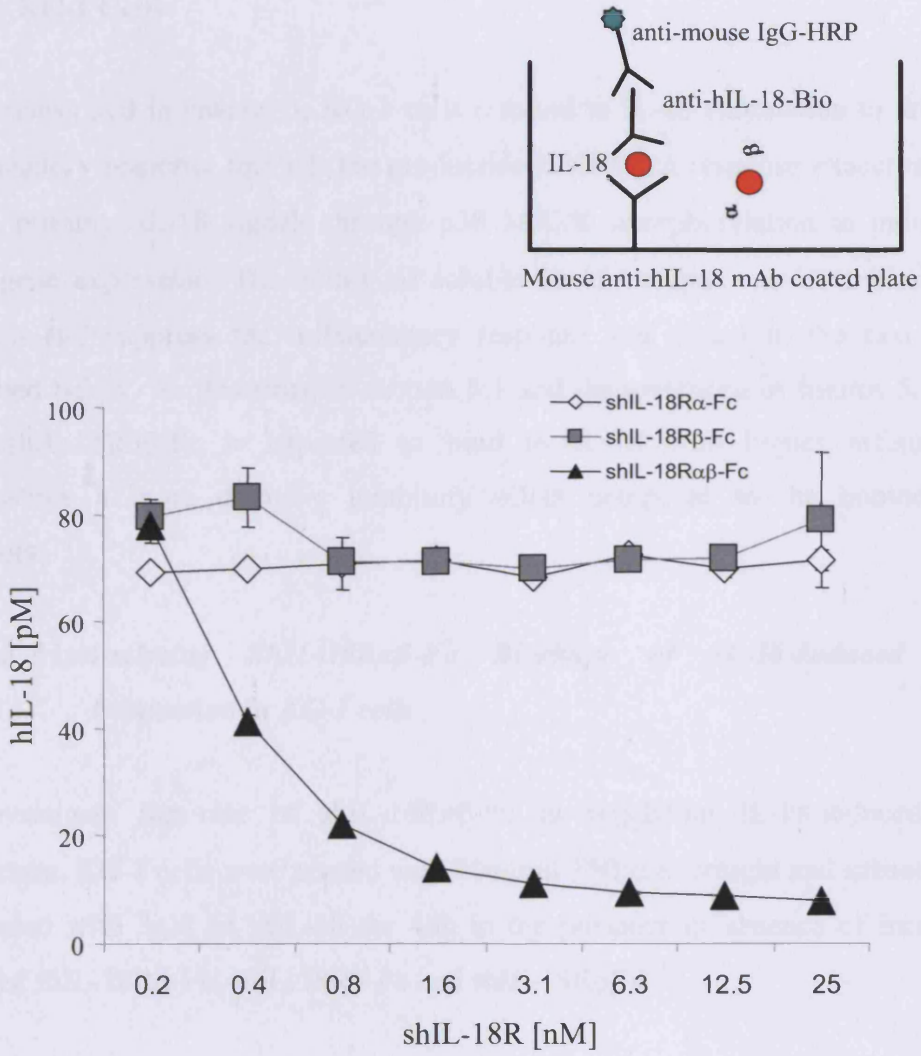


Figure 5.14: shIL-18Rαβ-Fc can bind and neutralise IL-18.

IL-18 can bind shIL-18Rαβ-Fc heterodimer, but not the shIL-18Rα-Fc or shIL-18Rβ-Fc homodimer, suggesting that both Rα and β chain are required for IL-18 to bind. 100 pM of recombinant human IL-18 was mixed with different concentrations of shIL-18Rα-Fc, shIL-18Rβ-Fc or shIL-18Rαβ-Fc for one hour at room temperature. The mixture was then transferred to ELISA plate coated with anti-hIL-18 for the unbound IL-18 to be detected using a biotynilated anti-hIL-18 Ab as illustrated. The concentration of unbound IL-18 was calculated based on rhIL-18 used as a standard.

5.3.7 Testing the Ability of shIL-18Rαβ-Fc to Neutralise the IL-18 Response in KG-1 Cells

As demonstrated in chapter 3, KG-1 cells respond to IL-18 stimulation to drive the inflammatory response through the production of IFNγ; a response exacerbated by TNFα priming. IL-18 signals through p38 MAPK phosphorylation to induce the IFNγ gene expression. The ability of soluble IL-18 receptors to neutralise IL-18 function and suppress the inflammatory response was tested in the two assays described below. As described in section 5.1 and demonstrated in figures 5.13 and 5.14, shIL-18Rαβ-Fc is expected to bind to IL-18 with higher affinity and demonstrate a more dramatic inhibitory effect compared to the homodimeric receptors.

5.3.7.1 Analyzing ShIL-18Rαβ-Fc Blockage of IL-18-Induced IFNγ Production in KG-1 cells

To investigate the role of shIL-18Rαβ-Fc in regulating IL-18-induced IFNγ production, KG-1 cells were primed with 20ng/ml TNFα overnight and subsequently stimulated with 3nM of rhIL-18 for 48h in the presence or absence of increasing doses of shIL-18Rα-Fc, shIL-18Rβ-Fc and shIL-18Rαβ-Fc.

The level of IFNγ secreted into the culture media in response to IL-18 was determined by ELISA. Increasing concentrations of shIL-18Rα-Fc or shIL-18Rβ-Fc alone did not have any effect on IFNγ produced from KG-1 cells (Fig. 5.15a). However, IFNγ production was partially suppressed by increasing concentrations of shIL-18Rα-Fc or shIL-18Rβ-Fc in the presence of 25nM shIL-18Rβ-Fc or shIL-18Rα-Fc respectively (Fig. 5.15b). More interestingly, IFNγ production was almost completely blocked by12.5nM shIL-18Rαβ-Fc in KG-1 cells stimulated with 3nM rhIL-18 (Fig. 5.15c). Thus, shIL-18Rαβ-Fc was shown to be a potent inhibitor of human IL-18.

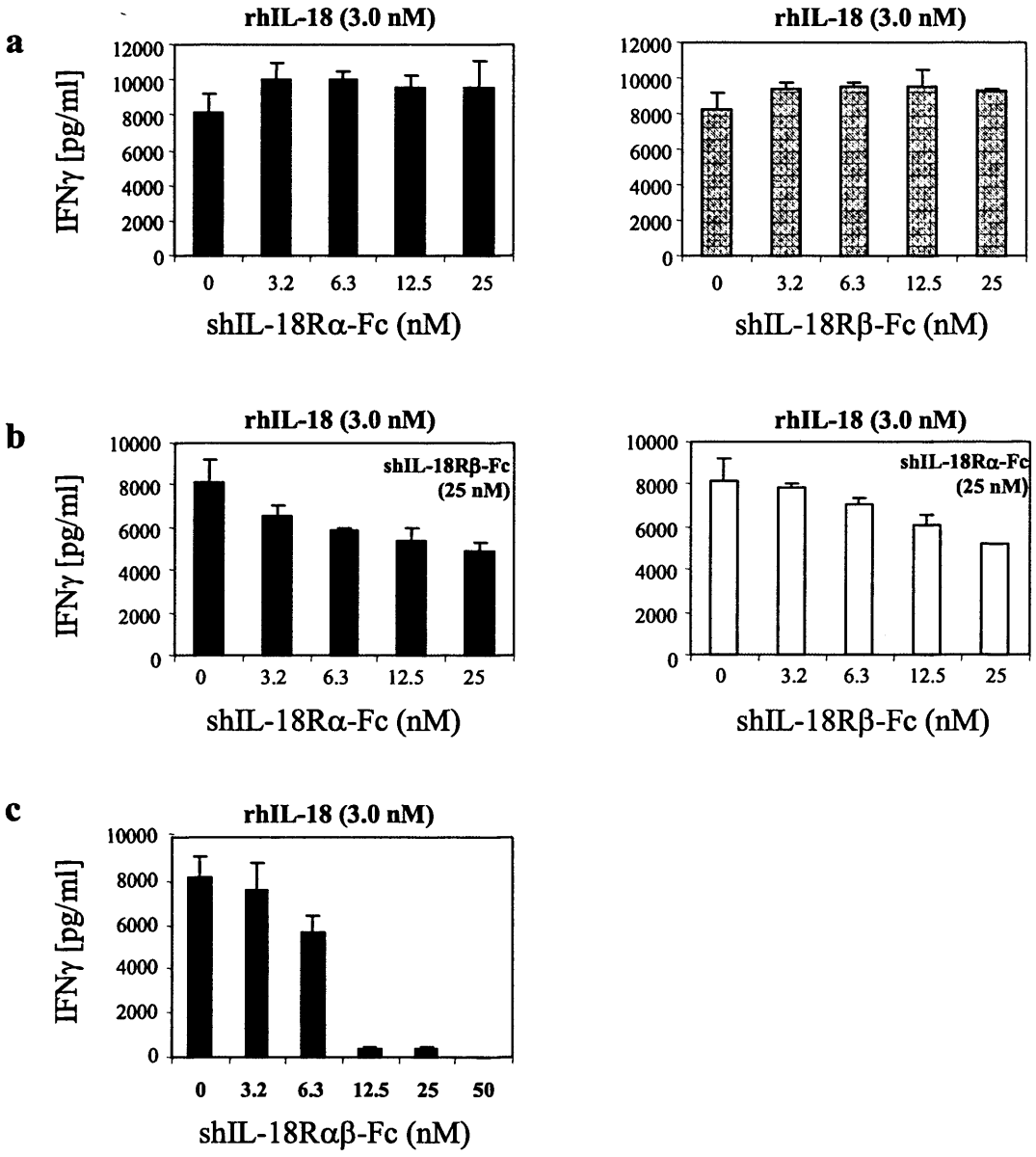


Figure 5.15: shIL-18Rαβ-Fc blocked human IL-18 activity *in vitro*.

KG-1 cells were primed with TNFα (20ng/ml) overnight before stimulation with recombinant human IL-18 (3nM), together with increasing doses of either shIL-18Rα-Fc in the absence (**a**, left) and in the presence (**b**, left) of 25nM shIL-18Rβ-Fc or shIL-18Rβ-Fc in the absence (**a**, right) or in the presence (**b**, right) of shIL-18Rα-Fc, or shIL-18Rαβ-Fc (**c**) for 48h. IFNγ secreted in the culture media of KG-1 cells in response to IL-18 was determined by ELISA.

5.3.7.2 Demonstration of Reduced IL-18-Induced p38 MAPK Phosphorylation in the presence of shIL-18Rαβ-Fc

To further evaluate the blocking effects of shIL-18Rαβ-Fc on IL-18 signalling, p38 MAPK phosphorylation was detected by Western Blotting. Recombinant human IL-18 (3nM) was mixed with different concentrations of shIL-18Rα-Fc, shIL-18Rβ-Fc or shIL-18Rαβ-Fc (starting from equimolar ratios of IL-18 and receptor proteins) for 30min at 37°C to allow complex formation. The mixture of IL-18 and soluble IL-18R-Fc was then added to stimulate the cells for 30min to allow initiation of IL-18 signalling and p38 phosphorylation. Cells were also treated with IL-18 alone as a positive control. Polyclonal antibodies against Thr^{180} and Tyr^{182} of phosphorylated p38 and against total p38 phosphorylation, as a loading control, were used for immunoblotting. A clear reduction in p38 phosphorylation was evident in the presence of shIL-18Rαβ-Fc in a dose dependent manner (Fig. 5.16a). Doses of 12.5nM or higher showed substantial inhibition, that was consistent with the results of IFNγ production (Fig. 5.15). ShIL-18Rα-Fc also resulted in a reduction of p38 activation, though less dramatic than that seen with shIL-18Rαβ-Fc, whereas shIL-18Rβ-Fc did not cause any major changes in IL-18-induced p38 phosphorylation compared to the positive control of IL-18 only (Fig. 5.16a). Densitometric band analysis was performed using ImageQuant and the 'Rolling Ball' background subtraction method, in order to normalise the band density for phosphorylated p38 to that of total p38. The results confirmed a 1.3-fold reduction of p38 phosphorylation in the presence of 25nM shIL-18Rα-Fc and a 2.7-fold reduction in the presence of 25nM shIL-18Rαβ-Fc when compared to 25nM shIL-18Rβ-Fc (Fig. 5.16b).

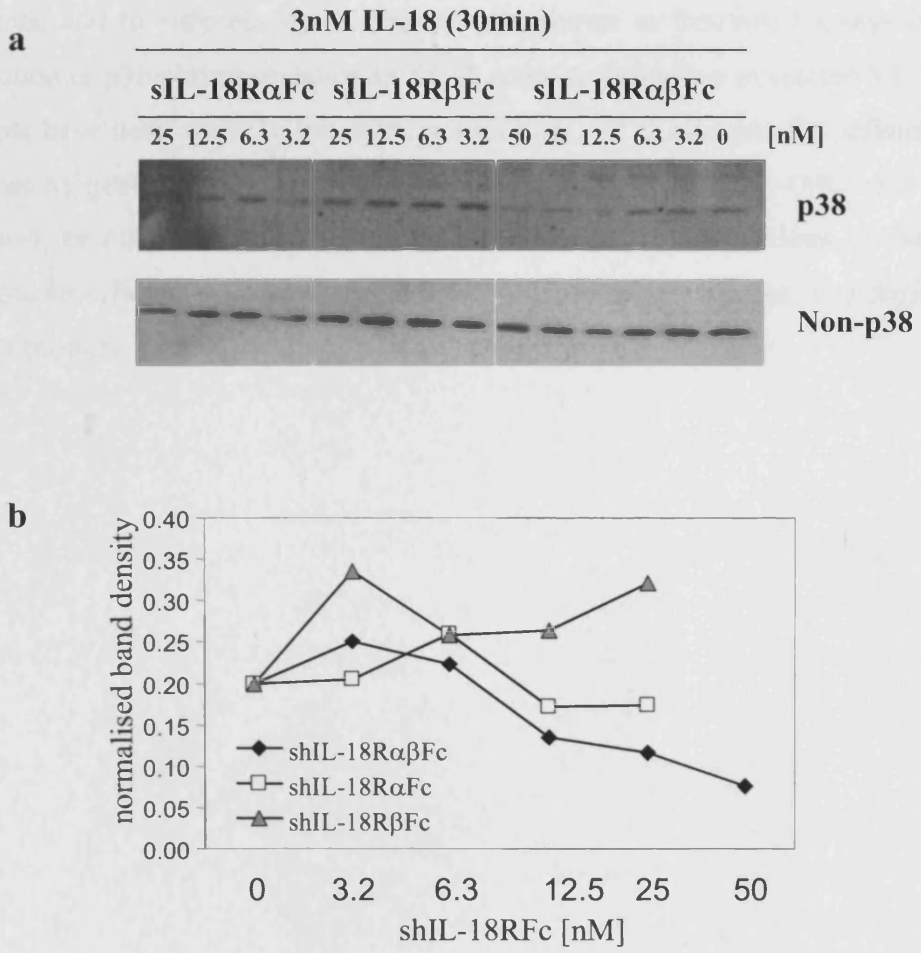


Figure 5.16: shIL-18Rαβ-Fc reduced IL-18-induced p38 activation.

IL-18 (50ng/ml) was mixed with different concentrations of shIL-18Rα-Fc, shIL-18Rβ-Fc and shIL-18Rαβ-Fc for 30 min to allow complex formation, prior to stimulation of KG-1 cells for 30 min. Lysate proteins were resolved on SDS-PAGE gel and Western blotting was performed using the anti-p38 phospho-specific antibody (**a**). An antibody against the non-phosphorylated p38 was used as a loading control. The intensity of p38 phosphorylation was quantified by densitometry (**b**). The graph shows the relative densitometric values of the p38 phosphorylated in the presence of different concentrations of soluble IL-18 receptor.

Data from both assays described above clearly demonstrated the ability of shIL-18Rαβ-Fc to bind to IL-18 with higher affinity compared to the homodimeric receptors, and to suppress IL-18 response, as shown in functional assays of IFNγ production or p38 phosphorylation in KG-1 cells. As described in section 5.1, several attempts have been made to block the action of IL-18 to alleviate the inflammatory response by generating monoclonal antibodies against IL-18 or IL-18R, inhibitors of caspase-I, or other soluble IL-18 receptors. However, no approaches to date have managed to efficiently block the IL-18 pro-inflammatory response to a degree that holds a promise for a successful therapeutic application.

5.4 Conclusions

The aim of the work described within this chapter was to develop a soluble human IL-18 IgG-Fc fusion decoy heterodimeric receptor that directly targets IL-18 function, using homodimeric receptors as a control group. Several therapeutic approaches have been developed to date that target pro-inflammatory cytokines, with anti-TNFα therapy being the most successful, especially for rheumatoid arthritis. However, non- or partially- responding patients provide the need for further anti-cytokine interventions that can ameliorate disease progression, either by themselves or in combination with other therapies.

Constructs encoding shIL-18Rα-Fc, shIL-18Rβ-Fc and shIL-18Rαβ-Fc were successfully developed and stably expressed in cell lines. Protein expression and secretion was confirmed prior to shIL-18R-Fc purification using Protein A affinity chromatography. Purity of the protein was demonstrated by SDS-PAGE and Coomassie blue staining. Different binding affinities and/or stability of the complex were confirmed by ELISA, and functional assays were used to demonstrate the interaction of shIL-18Rαβ-Fc with IL-18 as compared to homodimers of the IL-18 subunits. *In vitro* studies successfully demonstrated the inhibition of rhIL-18-induced IFNγ production using low levels of decoy heterodimeric receptor. It would be beneficial to test the efficacy of shIL-18Rαβ-Fc in naturally produced IL-18 from U937 or THP-1 cells. This analysis was initially attempted, but it could not be completed due to time limitations. Additional *in vitro* binding studies (e.g. surface plasmon resonance using the BIAcore system) are required in order to characterise binding kinetics of the pure heterodimeric shIL-18Rαβ-Fc. However, pure heterodimeric receptor is essential for this assay, rather than a mixture of homo- and heterodimers we have obtained by using Protein A affinity purification. Thus, more specific purification methods need to be employed to accomplish this. One approach will be the conjugation of large amounts of functional IL-18 on a sepharose column followed by affinity purification of the heterodimeric receptor, which will be expected to bind to the IL-18 of the column with higher affinity compared to shIL-

18Rαα-Fc and shIL-18Rββ-Fc homodimers. Another, more complex, approach is the conjugation of monoclonal antibodies specific for IL-18Rα and IL-18Rβ to sepharose beads. The anti-IL-18Rα column will purify the heterodimeric mixture from shIL-18Rββ-Fc, and the eluate containing shIL-18Rαα-Fc and shIL-18Rαβ-Fc will be applied to the anti-IL-18Rβ column. ShIL-18Rαα-Fc will be discarded as it cannot bind to anti-IL-18Rβ antibody and purified fractions containing pure sIL-18Rαβ-Fc could theoretically be obtained. A homogeneous population of shIL-18Rαβ-Fc could increase the efficacy of the decoy receptor and potentially induce complete inhibition at a two-fold molar excess, similar to IL-18BP (Kim et al., 2000a), compared to a four-fold that is currently achieved. The next step would then be to test the efficacy of the shIL-18Rαβ-Fc *in vivo*. A good animal model system would be the mouse CIA model, since IL-18 deficiency has been shown to result in decreased incidence and severity of CIA (Wei et al., 2001).

There are several benefits of using this therapeutic approach compared to other strategies used so far. In this case, as little as 12.5 nM shIL-18Rαβ-Fc was enough to almost completely inhibit IL-18 function *in vitro*, whereas several *in vitro* and *in vivo* studies have shown that milligram levels of anti-IL-18 monoclonal antibodies are required to block IL-18 function (Hamasaki et al., 2005, Fantuzzi et al., 1998, Okamura et al., 1995b, Plater-Zyberk et al., 2001). Although monoclonal antibody therapeutic approaches were considered to be highly safe in terms of toxicity, recent Phase-I clinical trials using anti-CD28 antibody in the United Kingdom have resulted in exacerbated inflammatory responses and high mortality rates. Additionally, a decoy heterodimeric receptor could potentially be more beneficial compared to IL-18BP, which is already present in high levels in the blood, making it less efficacious as a therapeutic target. Thus, based on primary *in vitro* results obtained in this chapter, shIL-18Rαβ-Fc demonstrates a potential therapeutic molecule for reducing inflammatory responses by specifically targeting the active form of IL-18.

5.4.1 In summary

- Constructs of the shIL-18Rα-Fc, shIL-18Rβ-Fc and shIL-18Rαβ-Fc were successfully developed and CHO cell lines stably expressing the receptors.

- After confirmation of protein expression and secretion by Western blotting and ELISA, shIL-18Rα-Fc homodimers, shIL-18Rβ-Fc homodimers and shIL-18Rαβ-Fc heterodimers were purified from these cell lines, and the purity of the proteins assessed by SDS-PAGE and Coomassie blue staining.

- Binding studies showed that IL-18 binds to shIL-18Rαβ-Fc and with higher binding affinity compared to the homodimers.

- *In vitro* functional assays using the KG-1 cell model system demonstrated the ability of the shIL-18Rαβ-Fc decoy protein to strongly bind to IL-18 and neutralise its function and signalling.

- These functional assays also confirmed the need for both the IL-18Rα and IL-18Rβ chain of IL18R to form a stable complex with IL-18 and that mixing of free subunits is sufficient to achieve complex formation to a degree.

Chapter 6

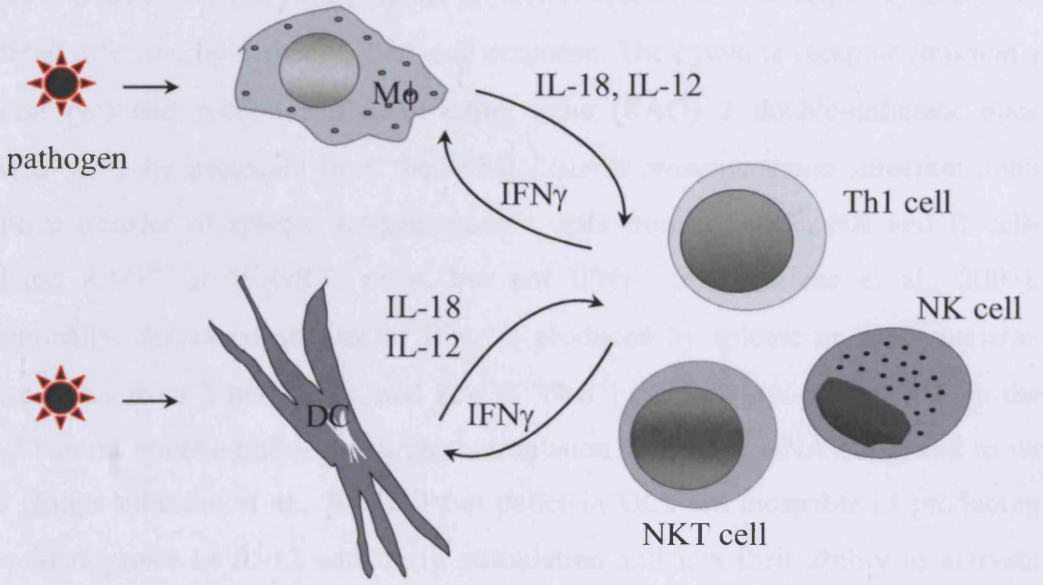
6 Final Discussion

6.1 Background

The central dogma in cell-mediated immunity is the activation of dendritic cells and macrophages by lymphoid cells through cytokines and co-stimulatory signals that result in an increase of antigen presentation and the elimination of intracellular pathogens (Mackaness, 1971). Thus, αβT cells (Scharton-Kersten et al., 1998, Andersson et al., 1998), γδT cells (Kasper et al., 1996), NKT cells (Emoto et al., 1999) and NK cells (Scharton and Scott, 1993) account for the early release of IFNγ following their activation by IL-18 and/or IL-12 that are produced by myeloid cells (Fig. 6.1a). However, the origin of early (innate) IFNγ when antigen-specific T cells are barely present and the source of signals by which lymphoid cells are primed have remained ill-defined. The IL-18 and IL-12-induced production of IFNγ by myeloid cells during the early phase of the immune response following bacterial infection offered a potential explanation for the initiation of the antimicrobial activity (Fig. 6.1b), during the time period when antigen-specific T cells are scarcely present (Frucht et al., 2001).

The idea pushed forward in the last 15 years of the autocrine activation of macrophages (Fultz et al., 1993, Puddu et al., 1997, Fenton et al., 1997, Munder et al., 1998, Fukao et al., 2001, Ohteki et al., 2001, Schindler et al., 2001), neutrophils (Yeaman et al., 1998) and dendritic cells (Fukao and Koyasu, 2000, Fukao et al., 2000, Fukao et al., 2001, Ohteki et al., 2001) by IFNγ has been controversial. In 2005, two studies have demonstrated that the increased amount of IFNγ shown to be produced by myeloid cells is a result of small numbers of contaminating lymphoid cells (Schleicher et al., 2005, Laouar et al., 2005). This disagreement leads to the necessity of sensitive single-cell cytokine detection methods of pure myeloid or lymphoid populations to solely investigate the regulation of IFNγ production by other cytokines and its function to control Th1 immune responses.

(a) Classical model



(b) 'Jump start' model

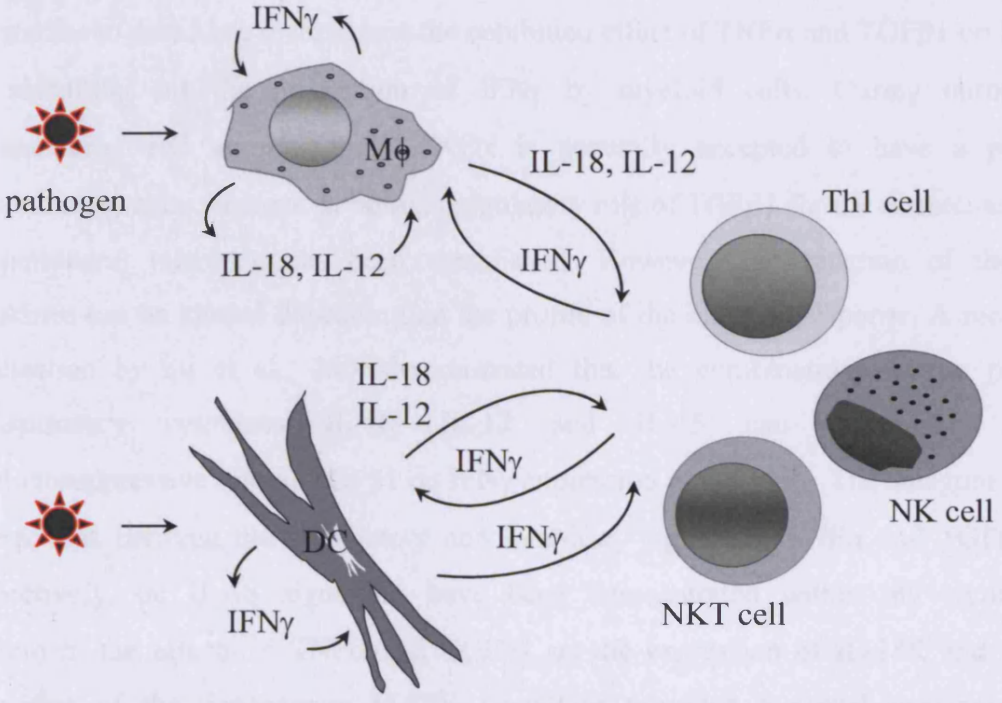


Figure 6.1: Models of interaction between APCs and lymphoid cells.

The classical model (**a**), where IFNγ is of mere lymphoid origin and 'jump start' model (**b**), where both lymphoid and myeloid cells produce IFNγ to drive Th1 immune response (Adapted from Frucht *et al.*, 2001).

In spite of the concerns about cell purity, promising results have been provided using adoptive transfer studies, regarding the protective role of DC-derived IFNγ to control bacterial infection by activating Th1-cell response. The cytokine receptor common γ subunit (γc) and recombination-activating gene (RAG) 2 double-deficient mice ($RAG2^{-/-}\gamma c^{-/-}$) are protected from the lethal *Listeria monocytogenes* infection upon adoptive transfer of splenic antigen-specific cells from T, NKT, NK and B cell-depleted $RAG^{-/-}$ or $IFN\gamma R^{-/-}$ mice, but not $IFN\gamma^{-/-}$ mice (Suzue et al., 2003). Additionally, decreased amount of IFNγ is produced by splenic or bone marrow-derived DCs from $T\text{-}bet^{-/-}$ mice, and $RAG2^{-/-}Tbet^{-/-}$ mice are not protected from the lethal listeria infection after the *in vivo* stimulation with CpG DNA compared to wt DCs (Lugo-Villarino et al., 2005). T-bet deficient DCs are incapable of producing IFNγ in response to IL-12 and IL-18 stimulation and lost their ability to activate adoptively transferred Th1 cells (Lugo-Villarino et al., 2003a).

No studies to date have investigated the combined effect of TNFα and TGFβ1 on IL-18 signalling for the production of IFNγ by myeloid cells. During chronic inflammation and autoimmunity, TNFα is generally accepted to have a pro-inflammatory role, whereas an immunoregulatory role of TGFβ1 for the maintenance of peripheral tolerance has been established. However, the function of these cytokines can be altered depending on the profile of the immune response. A recent publication by Yu et al., 2006 demonstrated that the combinations of the pro-inflammatory cytokines IL-18, IL-12 and IL-15 can antagonise the immunosuppressive role of TGFβ1 on IFNγ expression in NK cells. The antagonistic interactions between the stimulatory and inhibitory signals of TNFα and TGFβ1, respectively, on IL-18 signalling have been demonstrated within my studies. Moreover, the effects of TNFα and TGFβ1 on the expression of IL-18R and the activation of the downstream MAPK signalling provided a partial mechanistic understanding of how IL-18 signalling is regulated. The regulation of the expression levels of T-bet by combinations of IL-18, TNFα and TGFβ1 was also studied here, as T-bet has been previously demonstrated to be an important upstream transcription

factor involved in regulation of IFNγ production by DCs required for the optimal activation of antigen-specific Th1 cells (Lugo-Villarino et al., 2003a).

Redundancy has made it difficult to define the optimal cytokine-targets for blockade, and single anti-cytokine therapies, such as anti-TNFα, have been proven partially effective. In order to neutralise IL-18 function, a soluble decoy receptor was generated that could potentially be used in combination with the existing anti-TNFα therapy for the treatment of autoimmune diseases. The second rationale for this was to generate a reagent that could be used for interfering with IL-18 signalling in cell assays to work out the contribution of IL-18 to complex processes. The overall objective of this project was, therefore, to understand the molecular mechanisms of regulation of IL-18 signalling by other cytokines and identify potential therapeutic targets for blocking IL-18 function.

6.2 The stimulatory effects of TNFα on the IL-18-induced IFNγ production in dendritic precursor cells

Dendritic cells are central cells of the immune response as they are at the frontline of defence against pathogens. DC maturation is considered to be an innate response that leads to adaptive immunity, as DCs migrate from peripheral tissues to lymphoid organs. It is now clear that immature DCs (iDCs) are important for maintaining peripheral tolerance (Steinman et al., 2003), whereas mature DCs (mDCs) are the most potent antigen-presenting cells for priming naïve T cells (Banchereau and Steinman, 1998). It has recently been demonstrated that iDCs isolated from mouse bone marrow express low levels of IFNγ, which is up-regulated in mDCs that are generated with LPS stimulation in the presence of IL-4 and GM-CSF. These cells are effective in priming Th1 immune response (Xia et al., 2007).

IL-18 is a crucial cytokine that bridges innate and adaptive immunity (Maxwell et al., 2006), drives maturation of DCs (Li et al., 2004a) and is a more potent IFNγ-inducing factor than IL-12 (Okamura et al., 1995b). Thus, we could speculate that the correlation between the 'early' (innate) IFNγ produced from iDCs in response to IL-

18 and the IFNγ derived from the mDCs as part of the acquired immune response could be an indication of the maturation stage of DCs. The ability of a pro-inflammatory cytokine, other than IL-12, to regulate IL-18 function was investigated, by examining the role of TNFα on IL-18 signalling.

Primarily, using an *in vitro* model of myeloid pre-dendritic KG-1 cells it was shown that stimulation with IL-18 induced IFNγ production. Stimulation with IL-18 alone for 1 day did not induce any major up-regulation of DC maturation markers on these cells, indicating that the conditions in this assay yielded immature DCs with the potential to mature after longer stimulation as discussed in chapter 3. IL-18 significantly augmented the expression of CD54 (ICAM-1) that was amplified by TNFα, providing an indication of potential increased migratory capabilities required for iDC migration and induction of maturation, and likely mediating the mDC-T cell interactions. This study demonstrated that TNFα stimulation 24 hours prior to IL-18 resulted in a dose-dependent, up to 6-fold, increase in the levels of IFNγ.

The mechanism of the strong stimulatory effect of TNFα was investigated by looking at IL-18 signalling. TNFα stimulation resulted in significant up-regulation of both the IL-18Rα and IL-18Rβ transcripts quantified by QPCR. IL-18Rα copy numbers were significantly increased in response to 10ng/ml TNFα, whereas IL-18Rβ required 10 times more TNFα to show a significant change. Furthermore, IL-18Rα expression changed by about 20-fold upon stimulation, whereas IL-18Rβ increased only 2-fold. The abundance of the transcript for IL-18Rβ as compared to IL-18Rα (several orders of magnitude difference) suggests that expression of IL-18Rβ in physiological conditions can be considered constitutive. My results also demonstrated increased levels of IL-18Rα, but not IL-18Rβ on the surface of the cells upon treatment with TNFα by FACS analysis, and therefore established a direct connection between regulation of IL-18Rα-chain expression and functional receptor on the cell surface. As IL-18Rα-chain is essential for ligand binding this shows that regulation of the IL-18 signalling pathway could occur through limiting IL-18Rα-chain expression. Previous studies have shown that after binding of IL-18 to the IL-

18Rα, the accessory chain, IL-18Rβ is recruited into a signalling complex that induces signal transduction (Debets et al., 2000, Kim et al., 2001b). Whether IL-18Rβ is present on the surface of KG-1 cells in the absence of the complex formation or not, has not been clarified so far. We could speculate that IL-18Rβ could be constitutively expressed in the cell cytoplasm and upon complex formation it would translocate to the membrane to bind to IL-18/IL-18Rα and initiate signalling. This hypothesis supports our result of the low levels of detection or absence of IL-18Rβ on the surface of the cells.

The work within chapter 3 also addressed the effects of TNFα on the downstream signalling of IL-18 using Western blotting for p38 and ERK1/2 MAPK, and T-bet transcription factor. IL-18 induced T-bet expression in KG-1 cells that was up-regulated by TNFα priming. IL-18 was also shown to induce rapid ERK1/2 and p38 MAPK phosphorylation within 10 minutes. The priming of the cells with TNFα resulted in the earlier activation and more pronounced phosphorylation of p38 MAPK, possibly not through the direct induction of p38 MAPK activation by TNFα but through the mechanism of up-regulation of IL-18R expression described above. TNFα priming did not result in any major alteration of ERK1/2 phosphorylation. Using specific inhibitors for each MAPK it was demonstrated that p38 MAPK but not ERK1/2 was required for the IL-18-induced IFNγ production in human pre-dendritic KG-1 cells.

In summary, these experiments demonstrated the ability of TNFα to promote the pro-inflammatory role of IL-18 by sensitising DCs to IL-18. These data provided a mechanism for the synergistic action of these two cytokines on immature DCs to ultimately drive DC maturation and induce Th1 immune response, likely through a positive feedback loop that allows a potent response to infection or results in autoimmunity (Fig. 6.2).

6.3 The inhibitory role of TGFβ1 on IL-18 signalling in TNFα-primed dendritic precursor cells

As it was discussed earlier, the bridge between innate and adaptive immunity are DCs. These cells control the balance between tolerance and autoimmunity by regulating Th1 immune responses. Maturation of DCs is the critical process by which iDCs take up the microbial antigen at the site of infection and deliver it to lymphoid tissues. In lymphoid tissues mDCs (professional APCs) present the antigen to T cells inducing their clonal expansion. As described in Figure 6.2, IL-18 and TNFα are important pro-inflammatory cytokines that drive the process of DC maturation, whereas several studies have demonstrated that TGFβ1 is a potent immunosuppressive cytokine that inhibits the maturation of DCs (refer to section 4.1). However, the mechanism of TGFβ1 to block DC maturation has not been completely delineated.

Targeting cytokines to maintain the balance that is required for the sufficient generation of APCs is important in controlling inflammation. There are several disadvantages of targeting single cytokines for the treatment of inflammatory and autoimmune diseases. In RA, for example, therapies using either antibody for IL-18 or IL-18BP are currently in Phase I clinical trials. These, however, do not exclusively bind IL-18 but also other proteins, making it impossible to predict their *in vivo* targeting. TNFα therapies such as infliximab, adalimumab (anti-TNF antibody) and etanercept (TNFR-Fc fusion protein) have been in widespread clinical use, but they increase the risk of infection (tuberculosis) and malignancy (McInnes and Schett, 2007). TGFβ antagonists such as monoclonal antibodies and antisense oligonucleotides have been in clinical trials for the treatment of fibrosis and cancer (Yingling et al., 2004). Several studies have indicated that blocking or overruling TGFβ by targeting its signalling using kinase inhibitors might significantly improve the efficacy of immunotherapy (Yingling et al., 2004). Understanding the mechanism of cytokine interactions for the pathogenesis of the disease is important to develop appropriate combination therapies with higher efficacy.

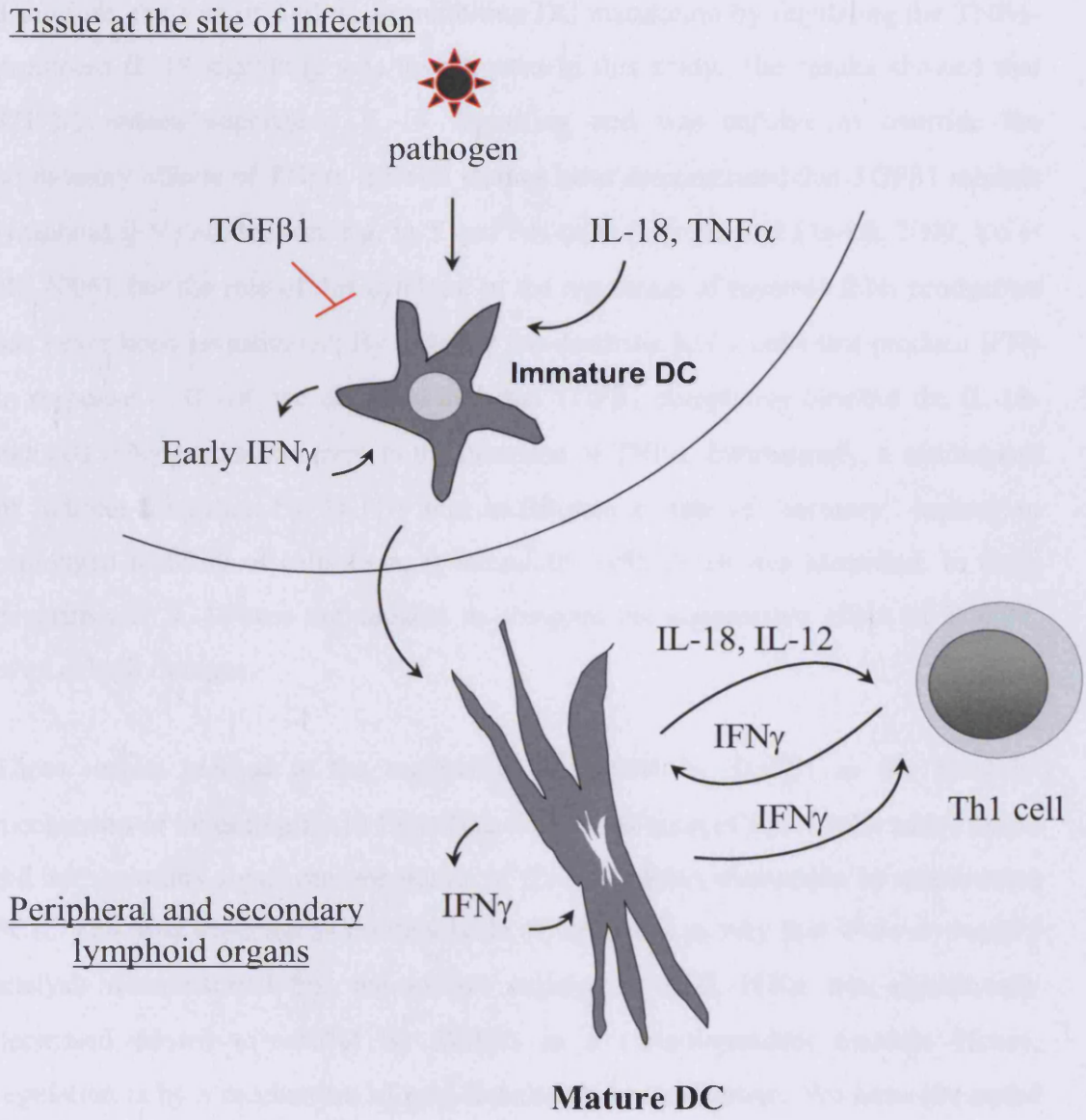


Figure 6.2: Model of dendritic cell maturation by their IL-18-induced IFNγ production: Role of TNFα and TGFβ1.

At the site of infection and upon the presence of microbial antigens, IL-18 induces early IFNγ production from iDCs. The levels of early IFNγ are up-regulated by TNFα, promoting maturation of DCs and their translocation to lymphoid organs as professional APCs, in order to induce Th1 immune response and provide the bridge between innate and adaptive immunity. This process is inhibited by TGFβ1.

Therefore, the role of TGFβ1 in inhibiting DC maturation by regulating the TNFα-promoted IL-18 signalling was investigated in this study. The results showed that TGFβ1 indeed suppressed IL-18 signalling and was capable to override the stimulatory effects of TNFα. Several studies have demonstrated that TGFβ1 inhibits lymphoid IFNγ production, e.g. in T and NK cells (Gorelik and Flavell, 2000, Yu et al., 2006), but the role of this cytokine in the regulation of myeloid IFNγ production has never been investigated. By utilising pre-dendritic KG-1 cells that produce IFNγ in response to IL-18, we demonstrated that TGFβ1 completely blocked the IL-18-induced IFNγ production even in the presence of TNFα. Interestingly, a mechanism of indirect inhibition by TGFβ1 that establishes a state of "memory" leading to prolonged inability of cells to be re-stimulated with IL-18 was identified. In these experiments, IL-18 was not capable to abrogate the suppressive effect of TGFβ1, even at high dosages.

These results pointed to the regulation of IL-18R by TGFβ1 as the potential mechanism of inhibiting IL-18 signalling. The stimulation of KG-1 cells with TGFβ1 did not show any significant regulation of IL-18R mRNA expression by quantitative PCR. This was expected as the timescale of inhibition is very fast. Flow cytometry analysis demonstrated that the surface expression of IL-18Rα was significantly decreased (down to ~50%) by TGFβ1 in a dose-dependent manner. Hence, regulation is by a mechanism of post-translational modification. We have attempted to investigate receptor shedding by proteolysis but were unsuccessful. Also regulation was only partial in all experiments carried out. This partial inhibition of the receptor has also identified a secondary mechanism of inhibition of IL-18 signalling by TGFβ1. It is well known that TGFβ1 suppresses the induction of IFNγ expression in lymphoid cells partly via the inhibition of T-bet expression (Yu et al., 2006). Western blotting data in this study has shown that TGFβ1 suppressed the IL-18-induced T-bet expression despite the presence of TNFα. This result provided a possible mechanism that accounted for the other 50% inhibition of IFNγ production by TGFβ1. Since studies have demonstrated that this cytokine can signal through SMAD-independent pathways such as p38 MAPK (Bhowmick et al., 2001, Yu et al.,

2002), Western blotting was used to examine the effect of TGFβ1 in the IL-18-induced p38 MAPK expression in KG-1 cells. TGFβ1 was shown to delay the activation of p38 MAPK induced by IL-18 together with TNFα. Nevertheless, p38 phosphorylation occurred within 10 min of IL-18 treatment and therefore suggests that some functional IL-18 receptor is indeed present on cells. Hence, neither the modulation of IL-18R nor the effect on p38 MAPK activation fully explained complete and sustained inhibition by TGFβ1.

The mechanism for the inhibition of IFNγ production by TGFβ1 in KG-1 cells appears to be a combination of events taking place simultaneously. As TGFβ1 has a short half life (2-3min), the fact that the reduced levels of IFNγ could not be reconstituted with the addition of IL-18 (Fig. 4.3), indicated that TGFβ1 acts rapidly by possibly regulating the IFNγ promoter, rather than by directly inactivating IL-18 protein. TGFβ is known to signal through SMAD2 and/or SMAD3, which then bind and activate a common SMAD4 protein to translocate to the nucleus and regulate the expression of target genes, by binding to their promoters recruiting activating or repressive complexes (Massagué, 1998, Massagué and Wotton, 2000). Yu *et al.*, 2006, have used chromatin immunoprecipitation (ChIP) to demonstrate that TGFβ1 directly inhibits IFNγ transcription by activating SMAD3/4 which then binds to IFNγ promoter and represses transcription. TGFβ1 has also been shown to inhibit the expression of T-bet, STAT4, and IL-12Rβ2 (Gorelik and Flavell, 2000, Lin et al., 2005) that are essential for IFNγ expression. Conversely, IL-18, IL-12 and IL-15 have been shown to suppress the ability of NK cells to respond to TGFβ1, via down-regulation of the surface and gene expression of TGFβ1 receptor and SMAD2/3, and by partially rescuing T-bet expression (Gorelik and Flavell, 2000, Yu et al., 2006). This could explain why T-bet expression is not completely blocked by TGFβ1 in our study.

Another potential explanation of the almost complete inhibition of IFNγ by TGFβ1 could be through an indirect mechanism of post-translational modification of IL-18. Mature or pro-IL-18 is known to be cleaved by caspase-3 at Asp71-Ser72 and

Asp76-Asn77, resulting in biologically inactive peptide (Akita et al., 1997). It would be interesting to examine the possibility that TGFβ1 induces the activation of caspases, and particularly caspase-3, to inactivate IL-18 protein. A hypothesis like this could be tested by using antibodies against caspases-3 to look at the expression levels before and after TGFβ1 stimulation by Western blotting.

In summary, the results in this chapter documented the inhibitory effects of TGFβ1 on IL-18 signalling and provided a possible mechanism that leads to the inhibition of myeloid IFNγ production via the down-regulation of IL-18R. The presence of the strong inflammatory cytokine, TNFα, was unable to abrogate the suppressive effect of TGFβ1. However, neither the down-regulation of IL-18Rα expression on the surface of KG-1 cells, nor the decrease of T-bet expression or delay in p38 phosphorylation observed in the presence of TGFβ1 resulted in the same extend of inhibition as the one observed in IFNγ production. Further experiments are required to elucidate the mechanism behind the strong immunosuppressive effect of TGFβ1 on IL-18 signalling in KG-1 cells. Blocking p38 activation or ERK1/2 MAPK activation using specific inhibitors could show whether inhibition of any of these signalling cascades would inhibit IFNγ production to the same extend as TGFβ1. Incomplete inhibition could indicate the involvement of SMAD signalling in regulating IFNγ production. Under these circumstances, either siRNA studies blocking SMADs or competition binding studies of SMADs on IFNγ promoter could provide further understanding of the mechanisms behind the immunosuppressive role of TGFβ1 on IL-18 signalling and on the regulation of IFNγ production in myeloid-derived pre-DCs.

6.4 Generation of a soluble decoy receptor to neutralise IL-18 function

Early research with synovial cultures from RA patients has provided evidence that TNFα is an important cytokine that drives the inflammatory response (Feldmann and Maini, 2003). Therapeutic approaches based on anti-TNF antibodies or soluble recombinant TNFR-IgGFc protein have resulted in significant improvement in both

RA (Elliott et al., 1994, Moreland et al., 1997) and IBD (van Dullemen et al., 1995). However, 50% of patients with chronic RA do not respond to TNFα inhibitors leading to the necessity for blockade of other pro-inflammatory cytokines such as IL-1, IL-6 and IL-15 that potentially drive the disease process. Other therapies have targeted the co-stimulatory molecules that are critical for the interaction between T and DC cells with the aim to prevent T cell activation and have been shown to improve symptoms in clinical trials for RA (Kremer et al., 2003).

Targeting IL-18 appears to be an attractive approach for therapy, as IL-18 is not only responsible for DC maturation, but is also a pro-inflammatory cytokine found in high levels in joints of patient with RA. A high affinity soluble decoy receptor would be a potentially effective way for neutralising IL-18 activity. In order to attempt the generation of such a recombinant protein, constructs were developed for expression of the extracellular ligand binding portions of IL-18R as a fusion protein with Fc derived from human IgG1. Cos-7 cells were transiently transfected with the plasmids and expression was confirmed using IgG1-Fc and hIL-18Rα or hIL-18Rβ ELISA in the culture supernatant of cells. CHO cells were stably transfected with shIL-18Rα-Fc, shIL-18Rβ-Fc plasmids and selected using the antibiotic Zeocin®. Expression was confirmed by Protein G pull down assay and Western blotting using antibodies against hIL-18Rα or hIL-18Rβ and hIgG1-Fc. The shIL-18Rαβ-Fc expressing cell line was generated by transfecting the CHO cells already stably expressing shIL-18Rβ-Fc with shIL-18Rα-Fc and selected with the antibiotic Neomycin®. The two homodimeric shIL-18Rα-Fc and shIL-18Rβ-Fc and the heterodimeric shIL-18Rαβ-Fc proteins were purified using Protein A affinity purification. The purity (>97%) and identity of proteins was demonstrated by SDS-PAGE with Coomassie blue staining and Western blotting.

Purified proteins were used in binding studies (competition ELISA and pull down assay) to show that the shIL-18Rαβ-Fc heterodimer binds IL-18 with higher binding capacity compared to the respective homodimers. *In vitro* functional assays using the KG-1 cell model system demonstrated the ability of the shIL-18Rαβ-Fc decoy protein to neutralise its function to produce IFNγ, and activate p38MAPK signalling.

These results suggest the feasibility of a therapeutic approach based on a decoy soluble receptor to IL-18. Apart from the therapeutic potential of shIL-18Rαβ-Fc, it could be utilised as a diagnostic tool to test the role of IL-18 in several disease models or it could facilitate studies examining IL-18-induced DC maturation.

Several *in vitro* and *in vivo* studies have shown that milligram levels of anti-IL-18 monoclonal antibody are required for blocking IL-18 function. Preliminary data in chapter 5 have shown that 12.5nM shIL-18Rαβ-Fc was sufficient to almost completely inhibit rhIL-18 function *in vitro*. However, *in vivo* function depends significantly on the systemic stability of the recombinant protein in addition to its effectiveness to interact with the target molecule. Neutralising antibody therapies represent an attractive approach as they offer the potential to select binding site and affinity in order to optimise efficacy. Therapeutic approaches targeting the receptor with the use of antibodies or a specific antagonist have also been utilised, although shared binding of IL-18Rα with other ligands such as IL-1F7 could reduce the specificity of this approach. Small molecule approaches such as caspase-1 inhibitors or inhibitors of IL-18 signalling can be beneficial, as they provide advantages in oral delivery, patience tolerance and cost. However, they provide limited specificity for IL-18 as other members of the IL-1 super-family may also be inhibited. Opposing to all the approaches listed above, targeting IL-18 using soluble decoy receptors offers a direct high affinity binding approach with specificity for IL-18.

6.5 Implications for Cytokine Therapy

It is important to point out that the data presented in this work were obtained from studies utilizing a single experimental *in vitro* model of a myeloid dendritic precursor cell line. Considering the enormous diversity of cytokine and inflammatory cell profile in different inflammatory diseases and the environment-dependent properties of these cytokines in different types of inflammatory response, application of these results to more complex *in vivo* experimental or clinical models should be made cautiously. Cytokines do not act in isolation within the inflammatory response to limit the cause of inflammation and lead to the repair of damaged tissue. To

overcome the complexity of the action of cytokines mediated on target cells, a simplified assay was utilised to study the interactions of these cytokines in isolation, in order to target key signalling molecules that could be critical in regulating DC maturation and function.

The findings reported here not only introduce a potential mechanism of receptor regulation by pro- and anti-inflammatory mediators to drive chronic inflammation or immunosuppression, but also provide preliminary evidence for the usefulness of a potential therapeutic option, IL-18 inhibition using a decoy receptor, in anti-cytokine therapy. This approach is particularly attractive because of the various degrees of specificity and efficacy reported for current anti-cytokine therapies or disease relapse i.e. anti-TNFα therapy in RA. Moreover, given that IL-18 inhibition overrides chronic inflammation in response to infection or autoimmunity in *in vivo* models (Boraschi and Dinarello, 2006), this may also be a promising therapeutic approach for combination therapy.

6.6 Future Avenues for Investigation

Cytokines work in synergy to regulate immune responses during infection and inflammation. A global cytokine analysis and the examination of the molecular mechanism involved in the interplay between the cytokines that are important for the regulation of dendritic cell function will be beneficial for the clinical diagnostic or prognostic assessment of chronic inflammatory conditions and for providing a direction for potential anti-inflammatory therapies.

Experiments from this project have provided a mechanism for the immunostimulatory effects of TNFα and the immunosuppressive role of TGFβ1 on IL-18 signalling. The methods used in this study are simple and effective in quantifying mRNA and protein expression to study cell signalling. However, the nature of the experiments limited their scope to specific questions and a limited number of constituent proteins only. The complexity of the field has grown exponentially with the latest advances in cell biology. A combination of standard

techniques, used in this study, with novel higher capacity methods now available, could provide a global perspective of cell signalling interactions responsible for triggering DC maturation.

In this study flow cytometry was used to look at alterations in the expression levels of IL-18 signalling receptor proteins in response to IL-18/TNFα and/or IL-18/TGFβ1 stimulation. Microscopic techniques such as immunocytochemistry could be used to detect the location or movement of IL-18R protein within the cell upon stimulation. An alternative approach to follow the movement of IL-18R expression within cells would be to transfect CHO cells, which do not express the receptor, with the IL-18R gene inserted into a GFP/YFP expression vector.

Additionally, phosphospecific antibodies and inhibitors were used to indicate that p38 MAPK is phosphorylated downstream of IL-18 signalling and is required for IFNγ production. This work did not investigate a number of key signalling pathways linked to the cytokines employed for stimulation of cells including JAK/STAT, SMAD, NFκB. Furthering this work, the KG-1 cell system could be utilised as a model of DC maturation to identify the differences in phosphorylation patterns and expression levels of several proteins in response to stimuli within a cell extract using a pool of validated antibodies and Western blotting.

Additional work within chapters 3 and 4, investigated the effects of TNFα and or TGFβ1 on the IL-18 induced T-bet transcription factor in KG-1 cells. Using T-bet siRNA techniques, IL-18 has been recently shown to induce T-bet expression in KG-1 cells (Bachmann et al., 2007), but the effect of TNFα and TGFβ1 has never been investigated. The same study by Bachmann et al. has utilised p38 MAPK-specific inhibitor (SB203580) to demonstrate that p38 MAPK activation is required for T-bet mRNA and protein expression to regulate IFNγ production in these cells. My data have confirmed that stimulation of KG-1 cells with IL-18 activates T-bet expression, which was up-regulated in the presence of TNFα and suppressed in the presence of TGFβ1 stimulation. However, it is unclear whether T-bet is the only p38 MAPK target important for IFNγ regulation. The requirement of nuclear translocation of T-

bet has been revealed in $CD4^+$ T cells of T-$bet^{-/-}$ mice for this action (Matsuda et al., 2007). Using Mo-DCs from T-$bet^{-/-}$ mice it would be interesting to investigate the nuclear translocation of T-bet upon stimulation with IL-18/TNFα and/or TGFβ1 in the presence or absence of p38 MAPK inhibitors. It would be interesting to utilise a technique that uses β-galactosidase, which has been cut into two inactive fragments that get activated when they come together and produce a chemiluminescent signal (Villalobos et al., 2007). Tagging one fragment with a nuclear localisation signal (NLS) and the other with T-bet we could theoretically detect the translocation of T-bet in transfected cells upon stimulation.

Work in chapter 5 resulted in the generation of a soluble human decoy receptor for IL-18. Pull down assay and competition ELISA showed that indeed shIL-18Rαβ-Fc can bind IL-18 with higher affinity compared to the respective homodimers. Further experiments using plasmon surface resonance spectroscopy would have provided more information about the affinity and kinetics of the IL-18/shIL-18Rαβ-Fc interactions. *In vitro* functional assays, using the KG-1 model system demonstrated the ability of this heterodimeric receptor to block IL-18 signalling and inhibit IFNγ production. Time limitations only allowed preliminary experiments, which however, provided promising results for the functional activity of shIL-18Rαβ-Fc leading to the necessity of testing this protein *in vivo*. Initially, the toxicity of this compound should be tested both *in vitro* and *in vivo*. After determining that it is not toxic for the animals, it would be essential to determine the half life of the heterodimeric receptor in mice. Mice could be injected intravenously and the levels of the decoy receptor could be measured in the blood at different timepoints. Clinical studies investigating the ability of this protein to dampen the inflammatory response using the model of CIA and ameliorate the disease would be promising for therapy.

7 References

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YOSHIMOTO, T., TAKEDA, K., TANAKA, T., OHKUSU, K., KASHIWAMURA, S., OKAMURA, H., AKIRA, S. & NAKANISHI, K. (1998) IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN-gamma production. *J Immunol,* 161, 3400-7.

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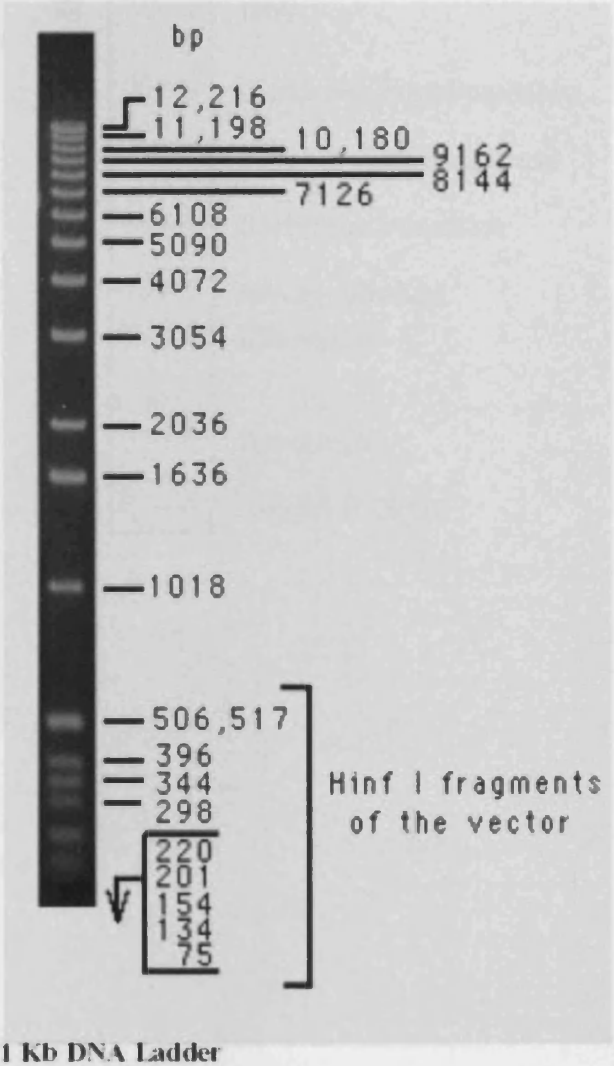
8 Appendices

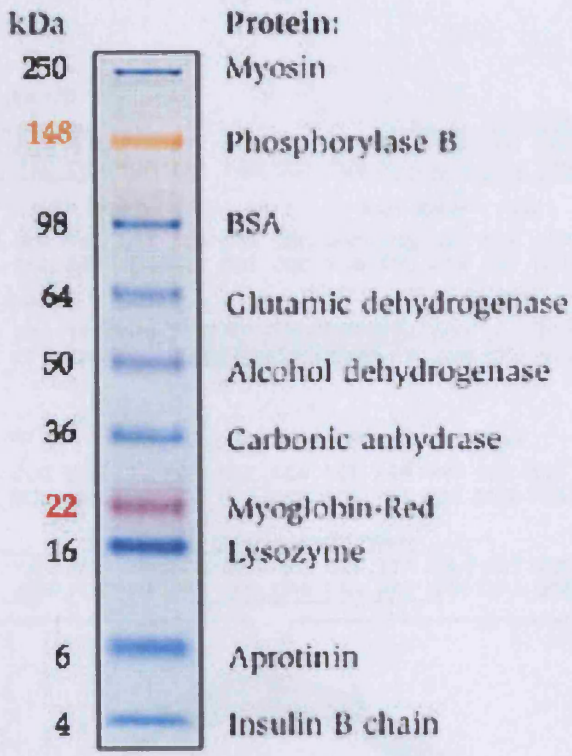
8.1 Molecular Weight Standards

8.1.1 1kb DNA ladder (Invitrogen™,UK)



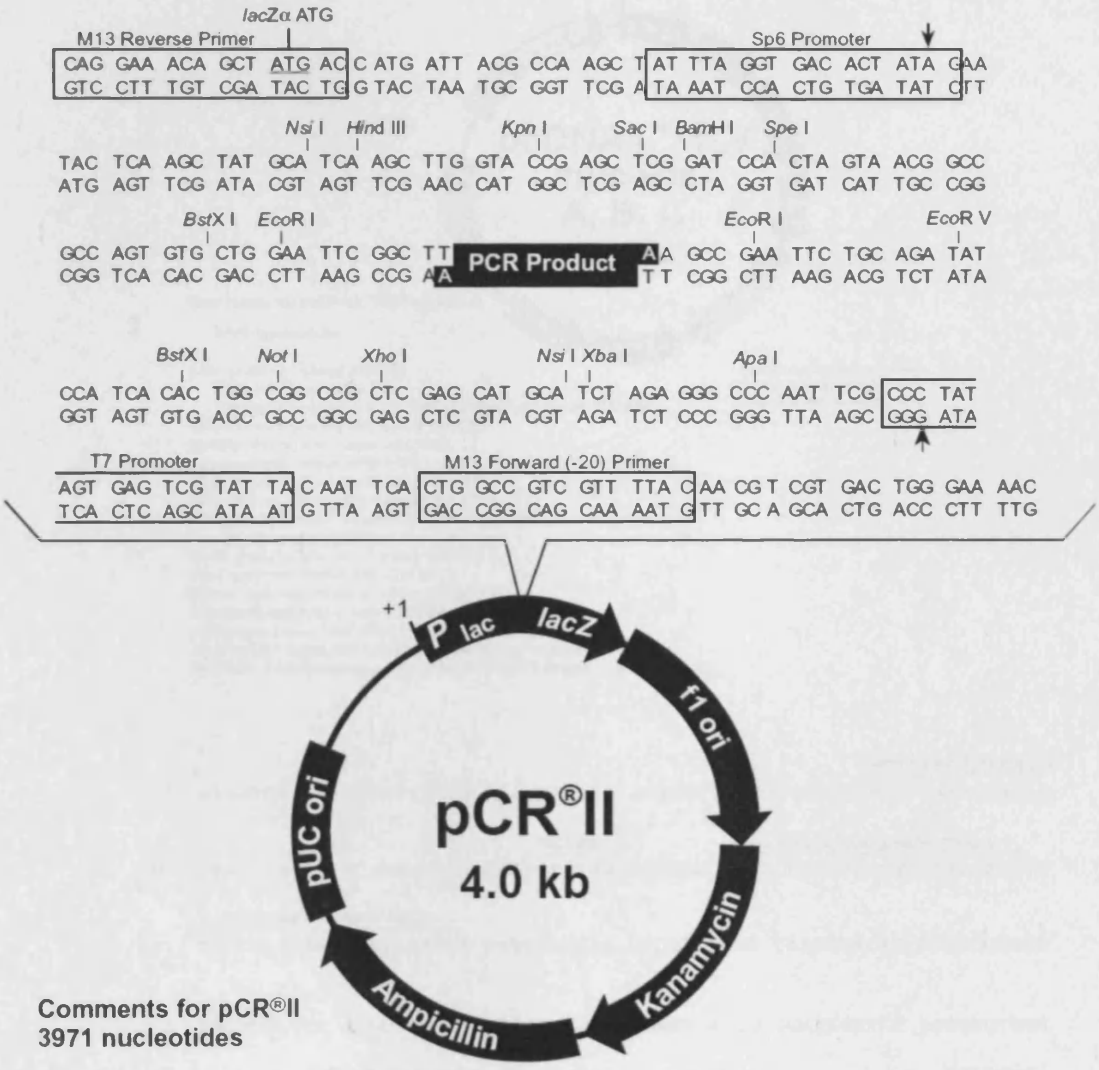
1 Kb DNA Ladder
0.5 µg/lane
0.9% agarose gel
stained with ethidium bromide

8.1.2 SeeBlue Plus2 pre-stained standard, (Invitrogen™, UK)



8.2 Vector Maps and Multiple Cloning Sites

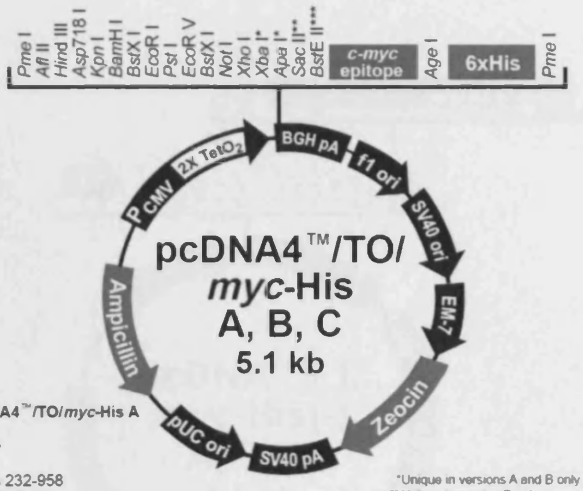
8.2.1 pCR®II Vector (Invitrogen™, UK)



Comments for pCR®II
3971 nucleotides

LacZα gene: bases 1-587
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
T7 promoter: bases 404-423
M13 (-20) Forward priming site: bases 431-446
f1 origin: bases 588-1025
Kanamycin resistance ORF: bases 1359-2153
Ampicillin resistance ORF: bases 2171-3031
pUC origin: bases 3176-3849

8.2.2 pcDNA4/TO/myc-HisA Vector (Invitrogen™, UK)



Comments for pcDNA4™/TO/*myc*-His A

5151 nucleotides

CMV promoter: bases 232-958
TATA box: bases 804-810
Tetracycline operator 2 (2X $TetO_2$) sequences: bases 820-859
CMV Forward priming site: bases 769-789
Multiple cloning site: bases 968-1069
c-myc epitope: bases 1073-1102
Polyhistidine (6xHis) tag: bases 1118-1135
BGH reverse priming site: bases 1158-1175
BGH polyadenylation sequence: bases 1164-1388
f1 origin: bases 1434-1862
SV40 promoter and origin: bases 1867-2211
EM-7 promoter: bases 2253-2319
Zeocin™ resistance gene: bases 2320-2694
SV40 early polyadenylation sequence: bases 2824-2954
pUC origin: bases 3337-4010 (complementary strand)
bla promoter: bases 5016-5114 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4155-5015 (complementary strand)

*Unique in versions A and B only
** Unique in version B only
*** Unique in version C only

CMV Forward priming site

```
721  AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG
```

TATA box; Tetracycline operator ($TetO_2$)

```
781  GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CCCTATCAGT GATAGAGATC
```

Tetracycline operator ($TetO_2$)

```
841  TCCCTATCAG TGATAGAGAT CGTCGACGAG CTCGTTTAGT GAACCGTCAG ATCGCCTGGA

901  GACGCCATCC ACGCTGTTTT GACCTCCATA GAAGACACCG GGACCGATCC AGCCTCCGGA
```

Pme I* *Afl* II *Hind* III *Asp*718 I *Kpn* I *Bam*H I *Bst*X I* *Eco*R I

```
961  CTCTAGCGTT TAAACTTAAG CTTGGTACCG AGCTCGGATC CACTAGTCCA GTGTGGTGGA
```

Pst I *Eco*R V *Bst*X I* *Not* I *Xho* I *Xba* I *Apa* I

```
1021 ATTCTGCAGA TATCCAGCAC AGTGGCGGCC GCTCGAGTCT AGAGGGCCCT TC GAA CAA
                                                              Glu Gln
```

c-myc epitope; *Age* I; Polyhistidine (6xHis) region

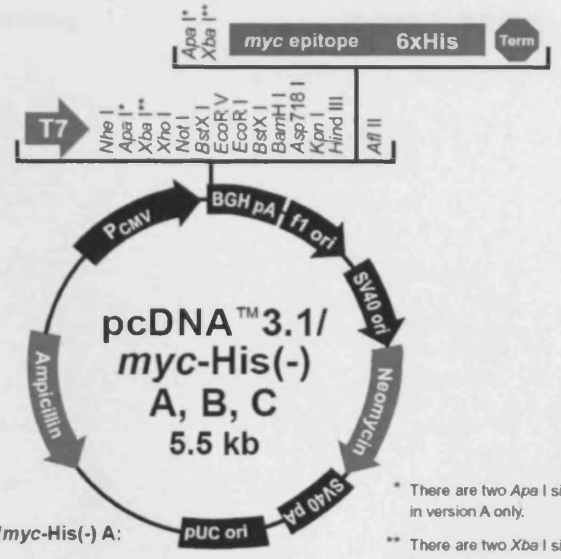
```
1079 AAA CTC ATC TCA GAA GAG GAT CTG AAT ATG CAT ACC GGT CAT CAT CAC
     Lys Leu Ile Ser Glu Glu Asp Leu Asn Met His Thr Glu His His His
```

Pme I*; BGH Reverse priming site

```
1127 CAT CAC CAT TGA GT TTAAACCCGC TGATCAGCCT CGACTGTGCC TTCTAGTTGC
     His His His ***
```

*Note that there are two *Pme* I sites and two *Bst*X I sites in the polylinker.

8.2.3 pcDNA™3.1Myc-His(-)A Vector (Invitrogen™, UK)



Comments for pcDNA™3.1/*myc*-His(-) A:
5522 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1006
myc epitope: bases 1007-1036
Polyhistidine tag: bases 1052-1069
BGH reverse priming site: bases 1113-1130
BGH polyadenylation signal: bases 1116-1343
f1 origin: bases 1389-1817
SV40 promoter and origin: bases 1844-2152
Neomycin resistance gene: bases 2227-3021
SV40 polyadenylation signal: bases 3195-3325
pUC origin: bases 3708-4381
Ampicillin resistance gene: bases 4526-5386 (complementary strand)

pcDNA™3.1/*myc*-His A MCS

```
                T7 promoter/priming site                                  Hind III          Kpn I          BamH I
861  ATTAATACGA CTCACTATAG GGAGACCCAA GCTGGCTAGT TAA GCT TGG TAC CGA GCT CGG
                                                    Ala Trp Tyr Arg Ala Arg
                                  BstX I  EcoR I            EcoR V          BstX I   Not I
922  ATC CAC TAG TCC AGT GTG GTG GAA TTC TGC AGA TAT CCA GCA CAG TGG CGG CCG
     Ile His *** Ser Ser Val Val Glu Phe Cys Arg Tyr Pro Ala Gln Trp Arg Pro
     Xho I     Xba I          Apa I   Sfu I          myc epitope
976  CTC GAG TCT AGA GGG CCC TTC GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT
     Leu Glu Ser Arg Gly Pro Phe Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn
               Age I          Polyhistidine tag                Pme I
1030 ATG CAT ACC GGT CAT CAT CAC CAT CAC CAT TGA GTTTAAACCC GCTGATCAGC
     Met His Thr Glu His His His His His His ***
     BGH Reverse priming site
1083 CTCGACTGTG CCTTCTAG
```

8.3 Specificity Guide of polyclonal IgG to protein A and protein G

Specificity Guide

Species	**Protein A binding**	**Protein G binding**
Human		
IgA	variable	-
IgD	-	-
IgE	-	-
IgG_1	++++	++++
IgG_2	++++	++++
IgG_3	-	++++
IgG_4	++++	++++
IgM*	variable	-
Mouse		
IgG_1	+	++++
IgG_{2a}	++++	++++
IgG_{2b}	+++	+++
IgG_3	++	+++
IgM*	variable	-
Rabbit	++++	+++
Rat		
IgG_1	-	+
IgG_{2a}	-	++++
IgG_{2b}	-	++
IgG_3	+	++

** Purify using HiTrap IgM Purification HP columns.*
† Purify using HiTrap IgY Purification HP columns.
+ Relative binding strength
- weak or no binding

The relative binding strengths of polyclonal IgG to protein A and protein G are listed in the specificity guide as measured in a competitive ELISA test. The amount of IgG required to give a 50% inhibition of binding of rabbit IgG conjugated with alkaline phosphatase was determined (Taken and adapted from the GE Healthcare, UK website http://www6.gelifesciences.com).

8.4 Sequencing Results

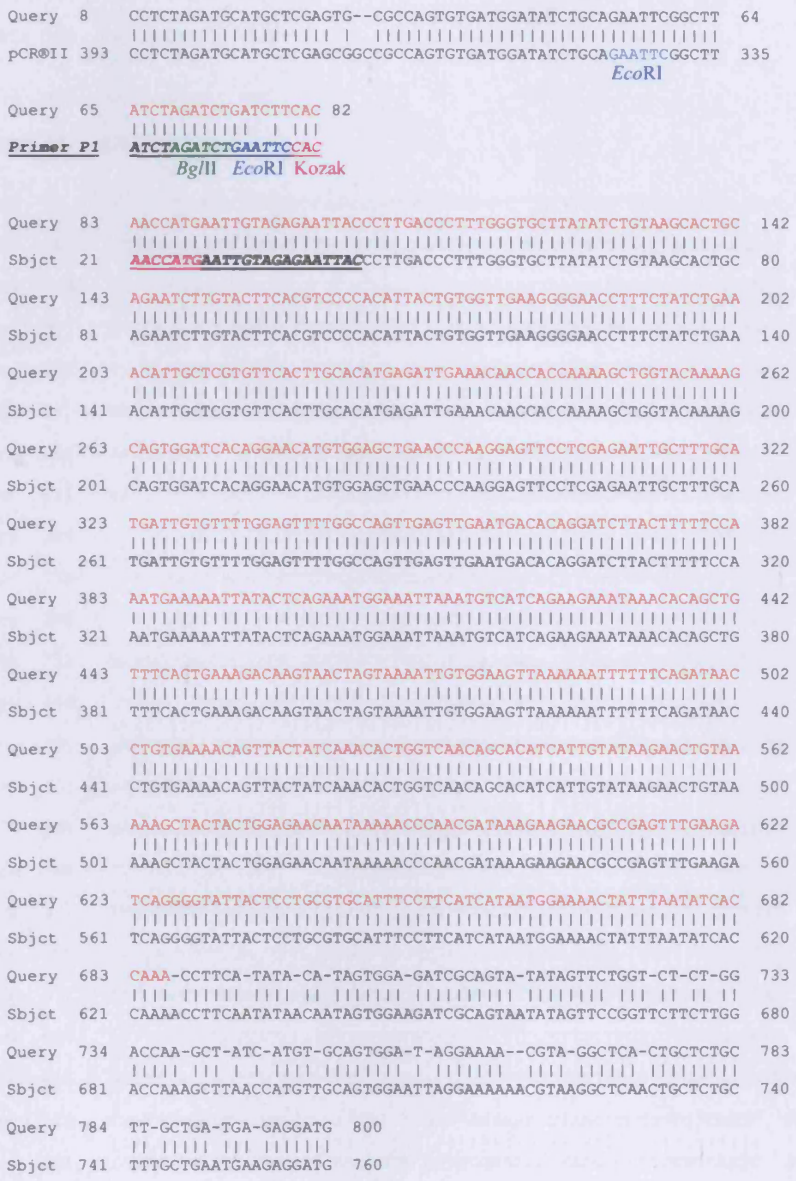


Figure 8.1: Sequencing data of shIL-18Rα TA cloned into pCR®II vector.

Using the T7 sequencing primer, homology was confirmed with the soluble part of the human hIL-18Rα mRNA sequence obtained from GenBank (NM003855). Figure also confirms the correct *Bgl* II, but not *EcoR* I, restriction enzyme sites within the forward primer for shIL-18Rα-Fc. The gaps in the coding sequence are an artefact due to the fact that it is towards the end of the readable sequence.

```
Query  6     AGCTATGCATCA-GCTTGGTACCGAGCTCGGATCCAAAAGTAACGGCCGCCAGTGTGCTG  64
             ||||||||||||  ||||||||||||||||||||||  ||||||||||||||||||||||
pCR®II 265   AGCTATGCATCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTG  324
                       HindIII
Query  65    GAATTCGGCTT  75
             ||||||||||||
pCR®II 325   GAATTCGGCTT  335
             EcoRI

Query  76    TGATAGATCT   85
             ||||||||||
Primer P2    TGATAGATCT
                 BglII

Query  86    TCTTGTGAAGACGTGGCCTGGGATATCAGCCATGTCTGCTTTTCTCACCAAGATGAAGCT  145
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1011  TCTTGTGAAGACGTGGCCTGGGATATCAGCCATGTCTGCTTTTCTCACCAAGATGAAGCT  952

Query  146   TTTGGTGTCTGTGCCTCCCGTGCTGGCCACAGTGCAATTATATAAAACATTTAGATTGCT  205
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  951   TTTGGTGTCTGTGCCTCCCGTGCTGGCCACAGTGCAATTATATAAAACATTTAGATTGCT  892

Query  206   TTCACCAATATTTTCAATTCTCAATACTTTTGAAGCATGCCATTTGCCTTCTGGAGTCAT  265
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  891   TTCACCAATATTTTCAATTCTCAATACTTTTGAAGCATGCCATTTGCCTTCTGGAGTCAT  832

Query  266   AATTCTCATTTCTTTCTCTTCATGTATATTAGGATCCGATCCATTTTCTTCCCCGAACAT  325
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  831   AATTCTCATTTCTTTCTCTTCATGTATATTAGGATCCGATCCATTTTCTTCCCCGAACAT  772

Query  326   CCAATAAATTACATCCTCTTCATTCAGCAAAGCAGAGCAGTTGAGCCTTACGTTTTTTCC  385
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Sbjct  771   CCAATAAATTACATCCTCTTCATTCAGCAAAGCAGAGCAGTTGAGCCTTACGTTTTTTCC  712

Query  386   TAATTCCACTGCAACATGGTTAAGCTTTGGTCCAAGAAGAACCGGAACTATATTACTGCG  445
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Sbjct  711   TAATTCCACTGCAACATGGTTAAGCTTTGGTCCAAGAAGAACCGGAACTATATTACTGCG  652

Query  446   ATCTTCCACTATTGTTATATTGAAGGTTTTGGTGATATTAAATAGTTTTCCATTATGATG  505
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  651   ATCTTCCACTATTGTTATATTGAAGGTTTTGGTGATATTAAATAGTTTTCCATTATGATG  592

Query  506   AAGGAAATGCACGCAGGAGTAATACCCCTGATCTTCAAACTCGGCGTTCTTCTTTATCGT  565
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Sbjct  591   AAGGAAATGCACGCAGGAGTAATACCCCTGATCTTCAAACTCGGCGTTCTTCTTTATCGT  532

Query  566   TGGGTTTTTATTGTTCTCCAGTAGTAGCTTTTTACAGTTCTTATACAATGATGTGCTGTT  625
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  531   TGGGTTTTTATTGTTCTCCAGTAGTAGCTTTTTACAGTTCTTATACAATGATGTGCTGTT  472

Query  626   GACCAGTGTTTGATAGTAACTGTTTTCACAGGTTATCTGAAAAAATTTTTTAACTTCCAC  685
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  471   GACCAGTGTTTGATAGTAACTGTTTTCACAGGTTATCTGAAAAAATTTTTTAACTTCCAC  412

Query  686   AATTTTACTAGTTACTTGTCTTTCAGTGAAACAGCTGTGTTTATTTCTTCTGATGACATT  745
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Sbjct  411   AATTTTACTAGTTACTTGTCTTTCAGTGAAACAGCTGTGTTTATTTCTTCTGATGACATT  352

Query  746   TAATTTCCATTTCTGAGTATAATTTTTCATTTGGAAAA-GTAAGATCCTGTGTCATTC  802
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Sbjct  351   TAATTTCCATTTCTGAGTATAATTTTTCATTTGGAAAAAGTAAGATCCTGTGTCATTC  294
```

Figure 8.2: Sequencing data of shIL-18Rα TA cloned into pCR®II vector.

Using the SP6 sequencing primer, homology was confirmed with the soluble part of the human hIL-18Rα mRNA sequence obtained from GenBank (NM003855). Figure also confirms the correct *Bgl* II restriction enzyme site within the reverse primer for shIL-18Rα-Fc. The gaps in the coding sequence are an artefact due to the fact that it is towards the end of the readable sequence.

Figure 8.3: Sequencing data of shIL-18Rβ TA cloned into pCR®II vector.

Using the T7 sequencing primer, homology was confirmed with the soluble part of the human hIL-18Rβ mRNA sequence obtained from GenBank (NM003853). Figure also confirms the loss of both *Bgl* II and *EcoR* I restriction enzyme sites within the forward primer for shIL-18Rβ-Fc. The gaps in the coding sequence are an artefact due to the fact that it is towards the end of the readable sequence.

```
Query  7     AGCTATGCATCA-GCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTG  65
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pCR®II 265   AGCTATGCATCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTG  324
                         HindIII
Query  66    GAATTCGGCTT  76
             ||||||||||||
pCR®II 325   GAATTCGGCTT  335
              EcoRI

Query  77    GCACAGATCT   86
             ||||||||||
Primer P2    GCACAGATCT
                BglII

Query  87    TCTCTTTTCTTTCAGTTGGACGGACTGGGTTGTGTTTCCAATGGAGTTCTGGACAAAGCA  146
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Sbjct  1552  TCTCTTTTCTTTCAGTTGGACGGACTGGGTTGTGTTTCCAATGGAGTTCTGGACAAAGCA  1493

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Sbjct  1492  AACAAACTTCCTGCGAAGATCACGCTGAGTGACTTTTTCCAAGATGATATTACGCTCAAT  1433

Query  207   GATTTCATCCTTTAAAGTGGATTTAATACTTTTCGCCTCAGGTACTGAGACTTCCCACTC  266
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Query  267   TAGGTCAGAATCTTTGATGTACCATTTTATGACAGGGTTAAAGACCCTTTCAAAGCCAAA  326
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1372  TAGGTCAGAATCTTTGATGTACCATTTTATGACAGGGTTAAAGACCCTTTCAAAGCCAAA  1313

Query  327   TCGTGCTTTGCAGCTAATAGTTAAAGGCTTTCCAAGTTCTACTTCCAGTGTGTCCTCGAC  386
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Sbjct  1312  TCGTGCTTTGCAGCTAATAGTTAAAGGCTTTCCAAGTTCTACTTCCAGTGTGTCCTCGAC  1253

Query  387   AGGATCCAGAATATCTGGTTTGAGTTTAGTGTCTCCCACAATGGTTCTCACTTGAACAAC  446
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1252  AGGATCCAGAATATCTGGTTTGAGTTTAGTGTCTCCCACAATGGTTCTCACTTGAACAAC  1193

Query  447   AGCTCTGACTGTCCACGAACTCACAGTATCCGACTGAGTGTAATCACATACATATGTGCC  506
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1192  AGCTCTGACTGTCCACGAACTCACAGTATCCGACTGAGTGTAATCACATACATATGTGCC  1133

Query  507   CTGGTGATAGTCATAAACTTCATCCACTACGATTCGGTTGCTCCTTTCCACAGAGAGGAG  566
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1132  CTGGTGATAGTCATAAACTTCATCCACTACGATTCGGTTGCTCCTTTCCACAGAGAGGAG  1073

Query  567   TTT-CCATTCTTGTACCAGGT-ACCGCTGGACTTTGTGCATCACTTTGGCAGCTGAGACT  624
             ||| ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  1072  TTTTCCATTCTTGTACCAGGTTACCGCTGGACTTTGTGCATCACTTTGGCAGCTGAGACT  1013
```

Figure 8.4: Sequencing data of shIL-18Rβ TA cloned into pCR®II vector.

Using the SP6 sequencing primer, homology was confirmed with the soluble part of the human hIL-18Rβ mRNA sequence obtained from GenBank (NM003853). Figure also confirms the correct *Bgl* II restriction enzyme site within the reverse primer for shIL-18Rβ-Fc. The gaps in the coding sequence are an artefact due to the fact that it is towards the end of the readable sequence.

```
Query  229  AACCATGAATTGTAGAGAATTACCCTTGACCCTTTGGGTGCTTATATCTGTAAGCACTGC  288
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Sbjct  21   AACCATGAATTGTAGAGAATTACCCTTGACCCTTTGGGTGCTTATATCTGTAAGCACTGC  80

Query  289  AGAATCTTGTACTTCACGTCCCCACATTACTGTGGTTGAAGGGGAACCTTTCTATCTGAA  348
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  81   AGAATCTTGTACTTCACGTCCCCACATTACTGTGGTTGAAGGGGAACCTTTCTATCTGAA  140

Query  349  ACATTGCTCGTGTTCACTTGCACATGAGATTGAAACAACCACCAAAAGCTGGTACAAAAG  408
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Sbjct  141  ACATTGCTCGTGTTCACTTGCACATGAGATTGAAACAACCACCAAAAGCTGGTACAAAAG  200

Query  409  CAGTGGATCACAGGAACATGTGGAGCTGAACCCAAGGAGTTCCTCGAGAATTGCTTTGCA  468
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201  CAGTGGATCACAGGAACATGTGGAGCTGAACCCAAGGAGTTCCTCGAGAATTGCTTTGCA  260

Query  469  TGATTGTGTTTTGGAGTTTTGGCCAGTTGAGTTGAATGACACAGGATCTTACTTTTTCCA  528
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261  TGATTGTGTTTTGGAGTTTTGGCCAGTTGAGTTGAATGACACAGGATCTTACTTTTTCCA  320

Query  529  AATGAAAAATTATACTCAGAAATGGAAATTAAATGTCATCAGAAGAAATAAACACAGCTG  588
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  321  AATGAAAAATTATACTCAGAAATGGAAATTAAATGTCATCAGAAGAAATAAACACAGCTG  380

Query  589  TTTCACTGAAAGACAAGTAACTAGTAAAATTGTGGAAGTTAAAAAATTTTTTCAGATA-C  647
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  381  TTTCACTGAAAGACAAGTAACTAGTAAAATTGTGGAAGTTAAAAAATTTTTTCAGATAAC  440

Query  648  CTGTGAAAACAGTTACTATCAAACACTGGTCA-CAGCACATCATTGTATA-GAACTGTAA  705
            |||||||||||||||||||||||||||||||| ||||||||||||||| |||||||||
Sbjct  441  CTGTGAAAACAGTTACTATCAAACACTGGTCAACAGCACATCATTGTATAAGAACTGTAA  500

Query  706  AA-GCTACTACTGGAGAACAATAAAAACCCA-CGATA--GA-GA-CGCCGAGTTTGA-GA  758
            || ||||||||||||||||||||||||||| |||||  || || ||||||||||| ||
Sbjct  501  AAAGCTACTACTGGAGAACAATAAAAACCCAACGATAAAGAAGAACGCCGAGTTTGAAGA  560

Query  759  TCAGGG-TAT-ACTC-TGC-TGCATT-C-TTCATCATA-TG-AA--CTATT-A-TATCAC  806
            |||||| ||| |||| ||| |||||| | ||||||||| || ||  ||||| | ||||||
Sbjct  561  TCAGGGGTATTACTCCTGCGTGCATTTCCTTCATCATAATGGAAAACTATTTAATATCAC  620

Query  807  CAA  809
            |||
Sbjct  621  CAA  623
```

Figure 8.5: Sequencing data from pcDNA4/TO-IgG1Fc plasmid confirming shIL-18Rα insertion.

Sequencing data from the multiple cloning site (MCS) of the pcDNA4/TO-IgG1Fc vector using the CMV forward sequencing primer confirming homology with the soluble part of the human hIL-18Rα mRNA sequence obtained from GenBank (NM003855). The gaps in the coding sequence are an artefact due to the fact that it is towards the end of the readable sequence.

```
Query  228  AACCATGCTCTGTTTGGGCTGGATATTTCTTTGGCTTGTTGCAGGAGAGCGAATTAAAGG  287
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Sbjct  601  AGAGGAATTTGTCTTATTTTGTGATTTACCAGAGCCACAGAAATCACATTTCTGCCACAG  660

Query  408  AAATCGACTCTCACCAAAACAAGTCCCTGAGCACCTGCCCTTCATGGGTAGTAACGACCT  467
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Sbjct  661  AAATCGACTCTCACCAAAACAAGTCCCTGAGCACCTGCCCTTCATGGGTAGTAACGACCT  720

Query  468  ATCTGATGTCCAATGGTACCAACAACCTTCGAATGGAGATCCATTAGAGGACATTAGGAA  527
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721  ATCTGATGTCCAATGGTACCAACAACCTTCGAATGGAGATCCATTAGAGGACATTAGGAA  780

Query  528  AAGCTATCCTCACATCATTCACGACAAATGTACCCTTCACTTTTTGACCCCAGGG-TGAA  586
            |||||||||||||||||||| ||||||||||||||||||||||||||||||||| ||||
Sbjct  781  AAGCTATCCTCACATCATTCAGGACAAATGTACCCTTCACTTTTTGACCCCAGGGGTGAA  840

Query  587  TAATTCTGGGTCATATATTTGTAGACCCAAGATGAT-A-GAGCCC-TATGATGTAGCCTG  643
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Sbjct  841  TAATTCTGGGTCATATATTTGTAGACCCAAGATGATTAAGAGCCCCTATGATGTAGCCTG  900

Query  644  T-GTGTCA-GATGATTT-AGAAGT-AAGCCC-AGACAA-TGCATCCTGTGAGTATTCCGC  697
            | |||||| |||||||| |||||| |||||| |||||| ||||||||||||||||||||||
Sbjct  901  TTGTGTCAAGATGATTTTAGAAGTTAAGCCCCAGACAAATGCATCCTGTGAGTATTCCGC  960

Query  698  ATCACATAAGCAAGACCTACTTCTTGGGAGCACTGG  733
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Sbjct  961  ATCACATAAGCAAGACCTACTTCTTGGGAGCACTGG  996
```

Figure 8.6: Sequencing data from pcDNA4/TO-IgG1Fc plasmid confirming shIL-18Rβ insertion.

Sequencing data from the multiple cloning site (MCS) of the pcDNA4/TO-IgG1Fc vector using the CMV forward sequencing primer identity with the soluble part of the human hIL-18Rβ mRNA sequence obtained from GenBank (NM003853). The gaps in the coding sequence are an artefact due to the fact that it is towards the end of the readable sequence.

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Query  250  ACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCC  309
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Query  310  CCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTG  369
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Sbjct  146  CCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTG  205

Query  370  GACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTG  429
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Sbjct  206  GACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTG  265

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Sbjct  266  CATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGGGTGGTCAGC  325

Query  490  GTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCC  549
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Sbjct  326  GTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCC  385

Query  550  AACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGA  609
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Sbjct  386  AACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGA  445

Query  610  GAACCACAGGTGTACACCCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTCAGC  669
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Sbjct  446  GAACCACAGGTGTACACCCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTCAGC  505

Query  670  CTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGGAGAGCAA  729
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Sbjct  506  CTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGT-GGGAGAGCAA  564

Query  730  TGGGC  734
            |||||
Sbjct  565  TGGGC  569
```

Figure 8.7: Sequencing data from the multiple cloning site (MCS) of the pcDNA4/TO-IgG1Fc vector.

The CMV forward primer was used for sequencing of the pcDNA4/TO-IgG1Fc vector to confirm the homology of the sequence with the human IgG1-Fc constant region sequence obtained from GenBank (E01700; subject). The gaps in the coding sequence are an artefact due to the fact that it is towards the end of the readable sequence.

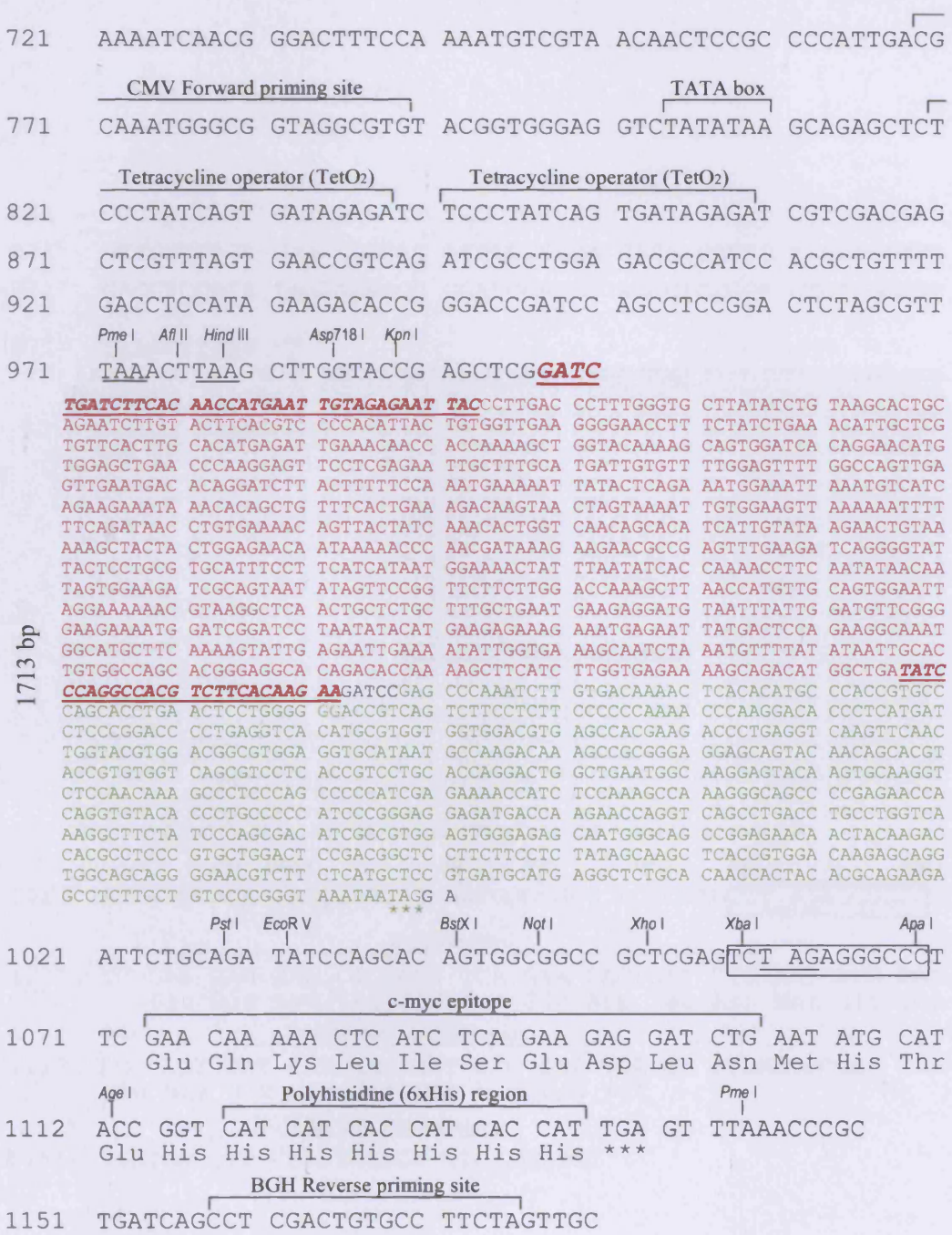

Figure 8.8: Sequencing data confirming in frame insertion of shIL-18Rα-Fc into pcDNA4/TO-IgG1Fc.

Sequence data from the multiple cloning site (MCS) of the pcDNA4/TO-IgG1Fc vector confirming in frame expression of the shIL-18Rα-Fc insert (**red**) with the Fc part of the IgG1 (**green**) already in the vector.

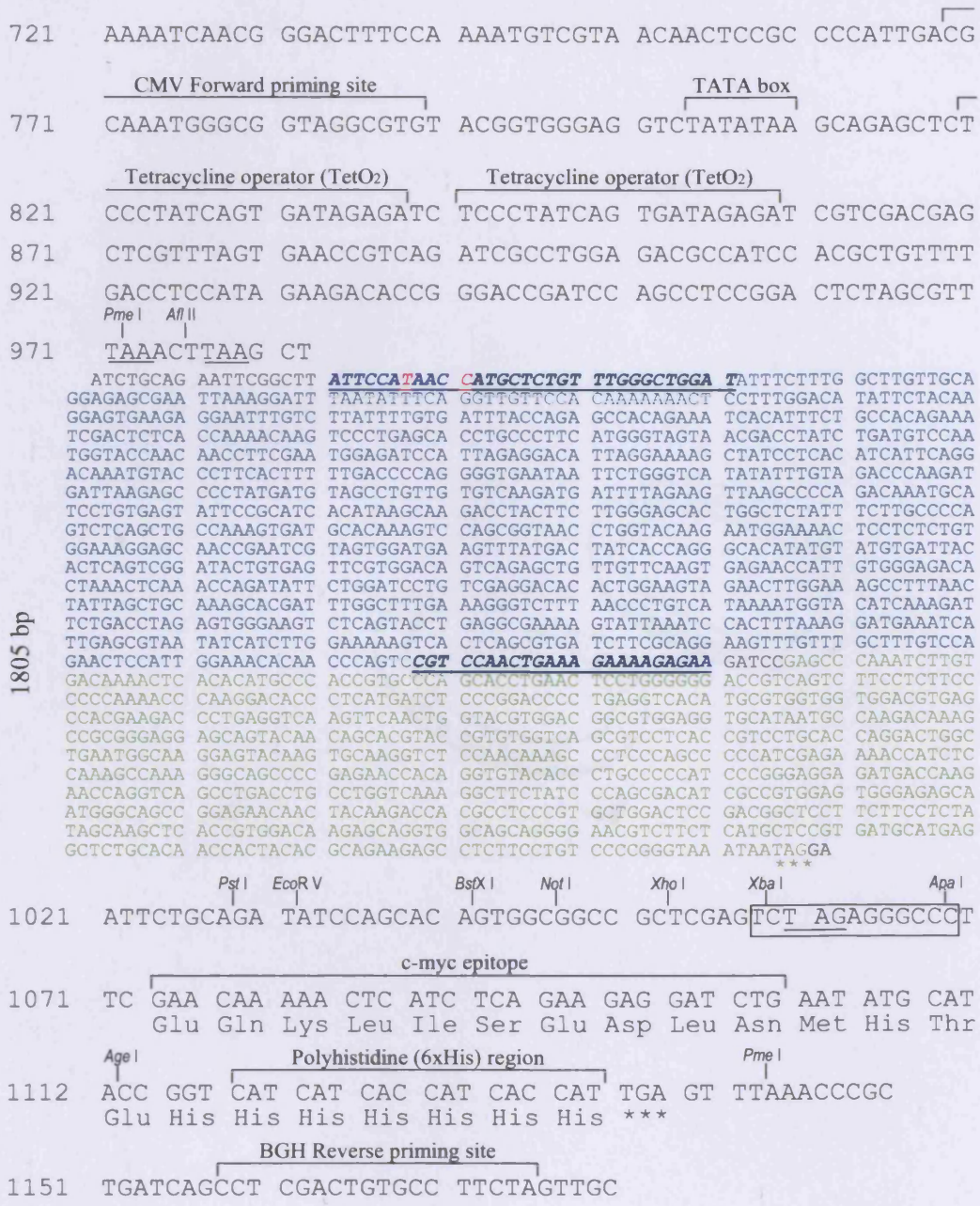

Figure 8.9: Sequencing data confirming in frame insertion of shIL-18Rβ-Fc into pcDNA4/TO-IgG1Fc.

Sequence data from the multiple cloning site (MCS) of the pcDNA4/TO-IgG1Fc vector confirming in frame expression of the shIL-18Rβ-Fc insert (blue) with the Fc part of the IgG1 (green) already in the vector. Base-mutations are shown in pink.

(GITC)-containing buffer which immediately inactivates RNases to ensure isolation of intact RNA), with 14.3M β-Mercaptoethanol (β-ME) added to it ensure efficient lysis. Cell lysates were homogenised through a QIAshredder spin column by cenrifugation for 2 min at maximum speed. Homogenisation shears the high molecular weight genomic DNA and other cellular components to reduce the viscosity of the cell lysates produced by disruption. One volume of ethanol was then added to the homogenised lysates to create conditions that provide selective binding of RNA to RNeasy mini columns. The samples were then applied to the RNeasy mini columns for total RNA to bind to the RNeasy silica-gel-based membranes by centrifugation.

After washing with 320µl of RW1 buffer, to eliminate DNA contamination, on-column DNase digestion with an RNase-free Deoxyribonuclease (DNase) I Amplification Grade (Invitrogen, UK) was performed. DNaseI is purified from bovine pancreas and digests single- and double-stranded DNA to oligodeoxy-ribonucleotides containing a 5'-phosphate. 10µl of DNase I stock solution was mixed with 70µl of buffer RDD and applied directly onto the RNeasy silica-gel membrane for 15min at room temperature. After a series of washes with buffers RW1 and RPE, the contaminants were efficiently washed away and the purified RNA was eluted in 40µl of RNase-free water by spinning at 8000 x g for 1 min. With this procedure, up to 100µg of all RNA molecules longer than 200 nucleotides were isolated. The purity of RNA was estimated and the concentration of RNA was measured using a scpectrophotometer and the A_{260}/A_{280} ratio as described in section 2.4.2.

2.4.2 RNA and DNA Concentration Measurement

To measure the RNA and DNA concentration and estimate the purity of the samples the A_{260}/A_{280} nm wavelength ratio was measured using a DU 800 Spectrophotometer 2.0. The samples were diluted at 1:50 and 1:100 in water for RNA and DNA respectively. Nucleic acids absorb at A_{260} nm, whereas both nucleic acids and aromatic amino acids (tyrosine, tryptophan and phenylalanine) absorb at A_{280} nm, which indicated the presence of protein contamination. Thus the A_{260}/A_{280} ratio was

used to estimate the purity of the RNA and DNA samples; a ratio A_{260}/A_{280} of 1.7 to 1.9 was desired when purifying nucleic acids. A ratio less than 1.7 indicated contamination by protein or organic chemicals. The concentrations of RNA and DNA were then calculated based on the following formulas:

- RNA concentration in μg/ml = OD_{260} x 40μg/ml x Dil. Factor

- DNA concentration in μg/ml = OD_{260} x 50μg/ml x Dil. Factor

2.4.3 Reverse Transcribed Polymerase Chain Reaction (RT-PCR)

Reverse transcribed polymerase chain reaction (RT-PCR) was performed using a commercially available Superscript™ II Reverse Transcriptase (RT) kit (Invitrogen, UK). Superscript™ II RT is purified from *E. coli* engineered to contain the modified *pol* gene of Moloney Murine Leukemia Virus (M-MLV) RT with reduced RNase H activity, higher yields of cDNA and increased thermal stability and specificity (Kotewicz et al., 1985, Gerard et al., 1986). Reverse transcription of RNA into cDNA was performed in two steps using 1ng - 5μg of total RNA, isolated as described in section 2.4.1. In the first denaturation step, 1μl of 100ng/μl random primers (Invitrogen, UK) were mixed with 11μl (5μg) of total RNA equalised in water, denatured at 70°C for 10min and then quickly chilled on ice. For the second amplification step, the denatured 12μl solution was mixed with the following reagents supplied with the kit: 4μl of First-Strand buffer (250mM Tris-HCl, pH 8.3; 375mM KCl; 15mM $MgCl_2$), 2μl of 0.1 M DTT, 1μl of Superscript™ II RT (200 U/μl) and 1μl of dNTP mix (10mM of each dATP, dCTP, dGTP, dTTP) (Promega, UK). This mix was incubated at 25°C for 10 min, 42°C for 50 min, 70°C for 15 min (to inactivate the reaction) and finally 4°C for ever. This protocol allowed annealing of the random primers and conversion of the total RNA (via reverse transcriptase) into cDNA.

Following reverse transcription, 2μl of 50mM $MgCl_2$, 2μl of dNTP (10mM each) and 10μl of 10x concentrated PCR buffer (200mM Tris-HCl, pH 8.3; 500mM KCl)

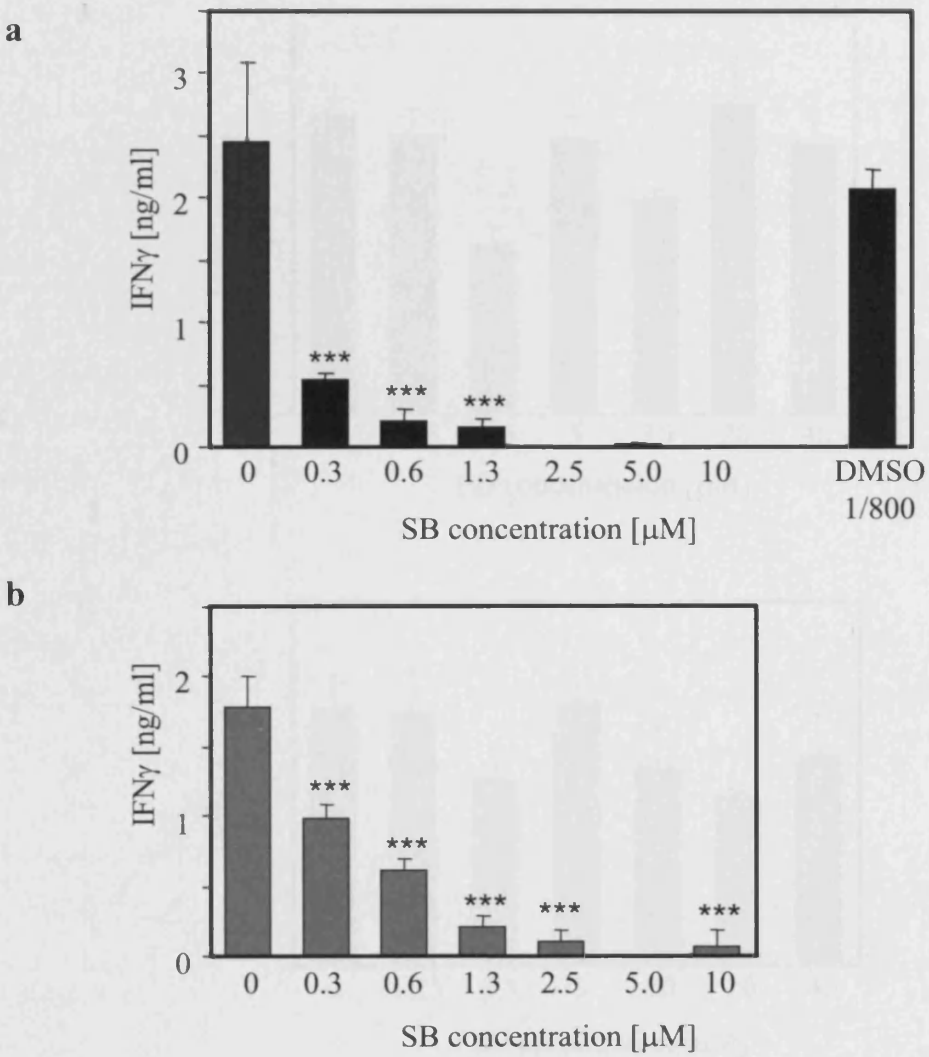

Figure 3.9: Blocking p38MAPK phosphorylation using a specific inhibitor (SB203580) completely abolished IFNγ production.

KG-1 cells were incubated with increasing concentrations of the inhibitor for 30 minutes. After that the cells were treated with 20ng/ml TNFα (**a**) or without TNFα (**b**) for 24h. The next day, the cells were further stimulated with 100ng/ml IL-18 for 72h. IFNγ secreted in culture media was quantified by ELISA. DMSO was used as a control in a dilution equivalent to the higher amount of DMSO that the inhibitors were diluted in. Data shown are expressed as mean ±SD of triplicates of a representative experiment. *** $P < 0.001$ by Tukey-Kramer post-test compared to cells without inhibitor treatment.

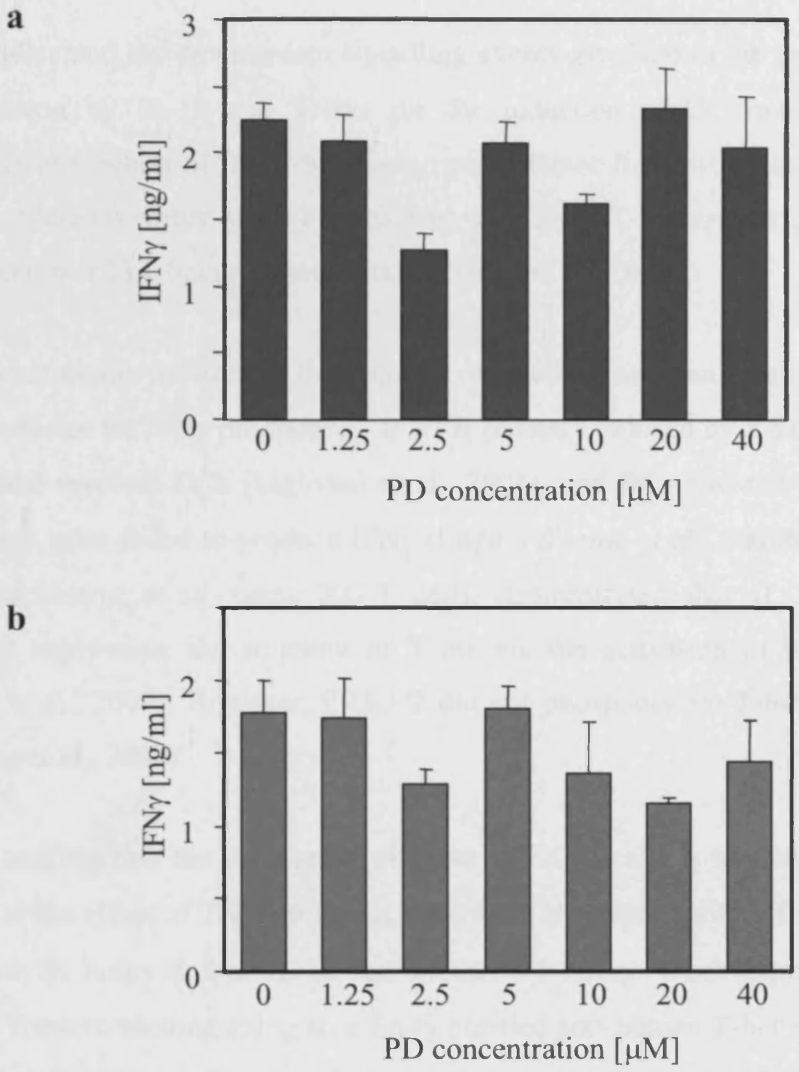

Figure 3.10: Blocking ERK1/2 phosphorylation using a specific inhibitor (PD98059) did not have any significant effect on IFNγ production.

KG-1 cells were incubated with increasing concentrations of the inhibitor for 30 minutes. After that the cells were treated with 20ng/ml TNFα (**a**) or without TNFα (**b**) for 24h. The next day, the cells were further stimulated with 100ng/ml IL-18 for 72h. IFNγ secreted in culture media was quantified by ELISA. Data shown are expressed as mean ±SD of triplicates of a representative experiment. Non-significance obtained by Tukey-Kramer post-test compared to cells without inhibitor treatment.

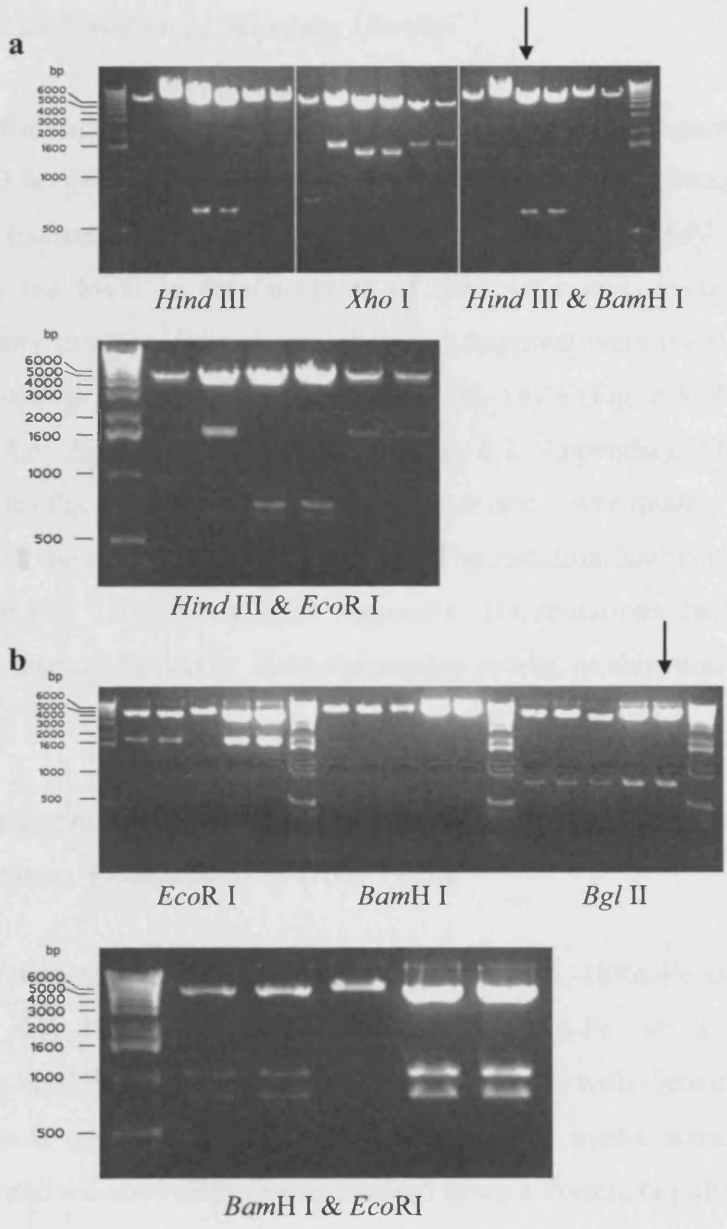

Figure 5.6: Identification of the pcDNA4/TO-shIL-18Rα-IgG1Fc and pcDNA4/TO-shIL-18Rβ-IgG1Fc DNA plasmids containing the right insert.

Restriction digest of pcDNA4/TO-shIL-18Rα-IgG1Fc plasmid DNA using the restriction enzymes *Hind* III, *Xho* I, *Hind* III & *Bam*H I and *Hind* III and *Eco*R I (**a**), and pcDNA4/TO-shIL-18Rβ-IgG1Fc using *Eco*R I, *Bam*H I, *Bgl* II and *Bam*H I and *Eco*RI (**b**). Refer to Table 5.2 for the expected sizes of the fragments. Arrow indicates the clones that contained the correct insert and were used for sequencing.

5.3.2.3 Confirmation of Sequence Identity

To confirm the correct identity of shIL-18Rα and shIL-18Rβ fragments inserted into pcDNA4/TO-IgG$_1$Fc and their homology to the known corresponding human sequences, sequencing analysis was performed. Forward CMV primers sitting upstream of the MCS in the backbone of the vector and reverse BGH primers complementary to a site downstream of the Fc fragment were used. Analysis of the sequencing results confirmed the identity of shIL-18Rα (Fig. 8.5, Appendix), shIL-18Rβ (Fig. 8.6, Appendix) and hIgG$_1$-Fc (Fig. 8.7, Appendix). The dashes in the sequencing results indicate missing bases due to the lower quality sequence at the beginning and the end of the sequencing run. The resulting final construct sequences are shown in Fig. 8.8 and 8.9 in the Appendix. The mutations discussed in section 5.2.1.3 were also confirmed by these sequencing results, as shown in Fig. 8.8 and 8.9 (Appendix).

5.3.3 Confirmation of In-frame Expression of shIL-18R with hIgG1-Fc by Transient Transfection of COS-7 cells

COS-7 cells were transiently transfected with either shIL-18Rα-Fc alone, shIL-18Rβ-Fc alone, shIL-18Rα-Fc together with shIL-18Rβ-Fc or a control vector (pSecTaq2B-IgG1Fc, provided by Wei XQ). Cos-7 cells with Genejuice® alone were also used as a negative control group. Cell culture media were collected after transfection and recombinant proteins isolated using a Protein G pull down assay.

Protein G is a cell wall component of Group G *Streptococci* and has the ability to bind most mammalian immunoglobulins (Ig). The amount of Ig captured is dependent on the concentration of Ig in the starting sample and the type and source of the Ig. Protein G binds to human IgG$_1$ with high affinity and is suitable to use for a small scale IgG purification for antibody labelling and IgG-tagged protein isolation. After purification using Protein G, an ELISA detecting human IgG-Fc or IL-18R was used to evaluate the level of expression and to confirm the in-frame expression of the plasmids with the Fc fragment (Fig. 5.7a). Significant levels of shIL-18Rα-Fc and

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LI, J., MBOW, M. L., SUN, L., LI, L., YANG, G., GRISWOLD, D. E., SCHANTZ, A., SHEALY, D. J., GOLETZ, T. J., WAN, J. & PERITT, D. (2004b) Induction of dendritic cell maturation by IL-18. *Cell Immunol,* 227, 103-8.

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