Interleukin-12 signalling pathways in human T lymphocytes

Verónica Athié-Morales

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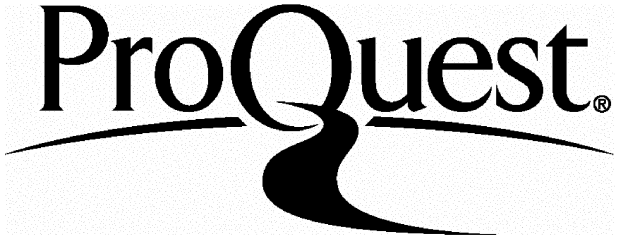
Lymphocyte Activation Laboratory
Imperial Cancer Research Fund
44 Lincoln's Inn Fields
London WC2A 3PX



ABSTRACT

The aim of the work that constitutes the present PhD thesis was to elucidate the intracellular signalling pathways downstream of the cytokine Interleukin-12 (IL-12) in human T lymphocytes. IL-12 plays a key role in the onset of the cellular immune response by driving the differentiation of naive Th cells into Th1 cells. The important role of IL-12 in human immune responses has been emphasised by recent reports that mutations of the IL-12 receptor (IL-12R) are found in immunodeficient patients suffering from recurrent *Mycoplasma sp.* and *Salmonella sp.* infections. IL-12 exerts its function through interaction with the IL-12R that leads to the activation of the Janus kinases Tyk2 and Jak2. IL-12 also induces tyrosine phosphorylation and DNA binding of STAT4 (Signal Transducer and Activator of Transcription 4), an essential response for Th1 cell differentiation. We have extensively studied the regulation of the transcription factor STAT4 in response to IL-12, using PHA-activated human T cells isolated from healthy donors as a model. These studies include: a) characterisation of other possible signals that induced in response to IL-12, could regulate the activation of STAT4; b) a comparison between the characteristics of STAT4 activation in response to IL-12 and to Interferon-α (IFN-α); and c) study of the mechanism by which IL-12 induced STAT4 activation is potentiated by IL-2.

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Publication

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Abbreviations

AICD	activation induced cell death
AP-1	activation protein-1
APC	antigen presenting cell
BAD	Bcl-2/ Bcl-X_L-antagonist, causing cell death
BCG	Bacille Calmette-Guérin
BSA	bovine serum albumin
CAT	chloramphenicol acetyl transferase
CBP	CREB binding protein
CCR-	chemokine receptor (e.g. CCR-1)
CD	cluster of differentiation
CD40L	CD40 ligand
Ci	curie
CIS	cytokine inducible SH2 containing protein
CLL	chronic lymphocytic leukaemia
CNS	central nervous system
CNTF	cyliary neutrophilic factor
COOH	carboxy
CpG-DNA	DNA containing CpG
CSF-1	colony-stimulating factor-1
CT-1	cardiotrophin-1
CTL	cytolytic T lymphocyte
DAG	dyacyl glycerol
DC	dendritic cell
DNA	deoxyribonucleic acid
DNFB	2,4-dinitro-1-fluorobenzene
ds	double stranded
DTH	delayed type hypersensitivity
ECL	enhanced chemiluminescence
EDTA	ethylendiaminetetraacetic acid
EGF	epidermal growth factor
eIF-2α	eukaryotic initiation factor 2 α-subunit
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
EPO	erythropoietin
Erk	extracellular signal regulated kinase
FACS	flow activated cell sorting
FcγR-GAS	Interferon γ receptor response element
FH	Forkhead
FITC	fluorescent isothyocyanate
FSC	forward scatter
g	unit for gravity
GAS	IFN-γ activated sequence

GAS-STAT4	oligonucleotide with the optimal DNA binding sequence for STAT4
GAP	GTPase-activating protein
γc	common gamma chain
G-CSF	granulocyte colony-stimulating factor
GDP	guanine diphosphate
GEF	guanine nucleotide exchange factor
GH	growth hormone
GM-CSF	granulocyte-macrophage colony-stimulating factor
gr	gram
Grb2	growth factor receptor bound protein 2
GRR	IFN-γR response
GSK3	glycogen synthase kinase-3
GST-	glutathione S transferase
GTP	guanine triphosphate
H7	1-(5-Isoquinolinylsolfonyl)-2-methyl-piperazine
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HIV	human immunodeficiency virus
HSs	hypersensitivity site
IFN-	interferon (e.g. IFN-α)
IFNAR1	IFN-α receptor 1
IFNAR2	IFN-α receptor 2
^{125}I	iodo-125 isotope
Ig	Immunoglubulin (e.g. IgG)
IKK	IκB kinases
IL-	Interleukin (e.g. IL-12)
IL-12p35	35 kDa subunit of IL-12
IL-12p40	40 kDa subunit of IL-12
IL-12p70	bioactive IL-12
IPTG	isopropyl β-D thio-galactopyranoside
IRF-	IFN regulatory factor (e.g. IRF-1)
IRS	insulin receptor substrate
ISGF-3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated regulatory elements
JAB	Jak-binding protein
Jak	Janus protein tyrosine kinase
JH	Jak homology
JNK	Jun N-terminal kinases
kb	kilobase
kd	dissociation constant
kDa	kilodalton
l	litre
LAK	lymphokine activated killer
LCMV	lymphocytic choriomeningitis virus
LIF	leukaemia inhibitory factor
LPS	lipopolysacharide

LT	lymphotoxin
LY294002	2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
M	molar
m	milli (10^{-3})
μ	micro (10^{-6})
mAb	monoclonal antibody
MAPK	Mitogen Activated Protein Kinase
MAPKKK	MAPK kinase kinase
MBP	myelin basic protein
MCM	mini chromosome maintenance
MCMV	murine cytomegalovirus
MDBK	Madin-Darby bovine kidney
Mek	Mitogen activated Erk kinase
MHC	major histocompatibility complex
MIP-	mitogen inflammatory protein (e.g. MIP-1α)
MLK	mix-lineage protein kinase
ml	millilitre
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
n	nano (10^{-9})
NF-AT	nuclear factor of activated T cells
NF-κB	nuclear factor of κB
NH2	amino
NK	natural killer
NLS	nuclear localisation signal
NP-40	Nonidet-P40
NPC	nuclear pore complex
OSM	oncostatin M
p	pico (10^{-12})
p38 MAPK	MAPK of 38 KDa
p70S6K	40S ribosomal protein S6 kinase
^{32}P	phosphorus-32 isotope
PAGE	polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocyte
PBL-T cells	Peripheral blood T lymphoblasts
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PD098059	2'-Amino-3'-methoxyflavone
PdBu	Phorbol 12,13-dibutyrate
PDE-3B	phosphodiesterase-3B
PE	phycoerythrin
PH	plekstrin homology
PHA	phytohemagglutinin
PI	Phosphatidyl inositol
PI3-K	Phosphatidyl inositol 3-kinase

PI(3,4)-P_2	Phosphatidyl inositol 3,4-diphosphate
PI(4,5)-P_2	Phosphatidyl inositol 4,5-diphosphate
PI(3,4,5)-P_3	Phosphatidyl inositol 3,4,5-triphosphate
PIAS	protein interacting with activated STAT
PKB	Protein Kinase B
PKC	Protein Kinase C
PKD	Protein Kinase D
PKR	dsRNA-dependent protein kinase
PMA	phorbol-12-myristate-13-acetate
PRL	prolactin
PTB	protein tyrosine binding
-R	-receptor (e.g. IL-12R)
r	recombinant (e.g. rIL-12)
RBD	Ras binding domain
RNA	ribonucleic acid
RO-31-8220	3-(1-(3-(Amidinothio)propyl-1H-indol-3-yl)-3-(1-methyl-1H-indol-3-yl)maleimide
SAPK	stress activated protein kinases
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulphate
SFV	Semliki Forest virus
SH	Src homology
SHP	SH2 containing protein tyrosine phosphatase
SIE	Sis-inducible element
SIEM67	the M67 variant of SIE
SIF	sis-inducible factor
SOCS	suppressor of cytokine signalling
Sos	Son of sevenless
SRF	serum response factor
SSC	side scatter
SSI	STAT-induced STAT inhibitor
STAT	Signal Transducer and Activator of Transcription
TAD	transactivation domain
Tc	T cytotoxic
TcF	ternary complex factor
TcR	T cell receptor
TGF-	transforming growth factor (e.g. TGF-β)
Th	T helper
Thp	T helper precursor
tk	thymidine kinase
TLR	Toll-like receptor
TNF-	tumour necrosis factor (e.g. TNF-β)
TPO	thrombopoietin
U	unit
v.	volume
VSV	vesicular stomatitis virus

X-SCID	X-linked SCID

Synonymous

CD16	FcγRIIIA
CD25	IL-2Rα chain
CD56	N-CAM
IL-2Rγ	common γ chain (γc)
IL-18Rα	IL-18R/ IL-1R related protein
IL-18Rβ	IL-1R accessory protein-like
IRF-9	p48
JNK	SAPK
MAPK	MEK
MAPKKK	MEKK
MEK	MKK
MKK4	SEK1
PKB	Akt
PKD	PKCμ
SOS	JAB/ SSI/ CIS

CHAPTER 1

Introduction

Cytokines are small (~15-30 kDa) glycoproteins that control a variety of important biological responses related to hematopoiesis and immune functions, including cell growth, differentiation and survival (for review see [1, 2]). Interleukin 12 (IL-12) is a cytokine that was originally isolated as a factor able to induce Interferon-gamma (IFN-γ) production by human peripheral blood lymphocytes, augment the cytotoxicity of natural killer (NK) cells as well as enhance T cell proliferation and lymphocyte activated killer (LAK) functions in response to Interleukin-2 (IL-2) [3-6]. Later studies have shown that IL-12 plays a key role in the differentiation of peripheral T cells, notably a population known as T helper 1 (Th1) cells. This T cell subpopulation is important for protective cell-mediated immune responses against a variety of intracellular pathogens. The aim of the experiments presented in this thesis was to characterise the signalling pathways induced by IL-12 in human T lymphocytes. The general characteristics of IL-12 as well as cytokines related to this work such as IL-2, IFN-α and IFN-γ are described. Activation of the Signal Transducer and Activator of Transcription 4 (STAT4) in response to IL-12 is essential for Th1 cell differentiation [7-9]. The STAT signalling pathway is therefore reviewed.

1.1 Immune responses to pathogen infections

Early studies of *in vivo* immune responses to pathogens described infectious agents with a predisposition to induce either antibody-mediated or cell-mediated (inflammatory) forms of immunity, with little or no overlap between the two. Defence against infectious intracellular microorganisms, such as viruses and some types of bacteria and protozoan, tends to be dominated by cell-mediated forms of immunity. This response is characterised by cellular cytolytic activity and the production of cytokines such as IFN-γ and tumour necrosis factor β (TNF-β). In contrast, resistance to extracellular forms of pathogens, for example helminths, is often associated with humoral responses, in which high levels of pathogen-specific immunoglobulins (Ig) are generated in an attempt to neutralise the foreign organism. Most pathogens are preferentially susceptible to one type of host immunity. The immune response elicited following an encounter with the infectious agent will dictate whether the host will resist or succumb to that infection as well as to subsequent infections with the same pathogen. Initiation of the wrong response can lead to the unimpeded spread of the infectious agent, resulting in severe hosts pathology and, in some cases, death [10].

1.2 Some cells of the immune response: T lymphocytes and NK cells

1.2.1 T lymphocytes

T lymphocytes are bone-marrow-derived cells that mature in the thymus and constitute 65-80% of human peripheral blood lymphocytes. Each T lymphocyte recognises a unique antigen by means of a cell-surface T cell receptor (TcR) of single

specificity, generated by random combination of receptor gene segments and selection of non-self specificity during thymic development. The TcR is a disulphide-linked heterodimer of either α and β chains (85-99% of T cells) or γ and δ chains. Each TcR recognises a specific antigen from a protein, which is presented by the major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APC). α/β T cells fall into two classes according to their expression of the cell surface proteins CD4 or CD8, which act as co-receptors for MHCII and MHCI molecules, respectively. $CD4^+$ T cells are named T helper cells (Th), recognise foreign antigens displayed in the context of MHCII molecules and have a primarily regulatory role through cytokine secretion. $CD8^+$ T cells are called T cytotoxic cells (Tc), recognise MHCI bound foreign antigen on the surface of cells infected with viruses or intracellular pathogens and destroy such cells preventing the spread of infection.

1.2.2 NK cells

NK cells are a population of bone marrow-derived large granular lymphocytes, representing approximately 5-15% of all peripheral blood lymphocytes. Most NK cells can be characterised by expression of low levels of CD56 (N-CAM) and high levels of CD16 (FcγRIIIA). However, a small population (10%) express high levels of CD56 and are $CD16^-$ or $CD16^{dim}$, and may represent a distinct functional subset. NK cells play important roles as both effectors and regulators in innate defence against intracellular pathogens and tumours, and in the regulation of the adaptive immune response to pathogens. NK cells display cytotoxicity without obvious antigen specificity and do not require activation or sensitisation to be cytolytic. They respond to various stimuli, either alone or in combination, with enhanced proliferation, gene expression, cytokine production and cytotoxicity. Such stimuli include surface structures on target cells, ligation of CD16, certain microbial

products and the cytokines IL-12, IL-2 and tumour necrosis factor-α (TNF-α).

1.3 T lymphocyte differentiation

1.3.1 T helper cell differentiation

After emigration from the thymus to the peripheral immune organs, $CD4^+$ Th cells are termed naive Th precursor (Thp) cells. Thp cells are considered functionally immature, although they can proliferate in response to appropriate antigens and are capable of secreting IL-2. Thp activation requires at least two separate signals. The first signal is derived by the TcR/ CD3 complex, after its interaction with antigen/ MHC molecules on the APC. The second signal is produced by a number of co-stimulatory or accessory molecules typified by the CD28/ B7, OX40, and LFA-1/ ICAM receptor-ligand pairs. IL-2 secretion, T cell clonal expansion and T cell differentiation follow T cell activation, which are crucial to the induction of an immune response.

A little over a decade ago, it was discovered that naive mouse $CD4^+$ Th lymphocytes, upon receiving an antigenic stimulus, differentiated into two distinct subsets defined both by their function and their unique cytokine profiles [11]. These subsets, Th1 and Th2 are responsible for cell-mediated inflammatory immunity and humoral responses, respectively [11, 12]. This division of labour fits nicely with previous demonstrations that an organism tends to mount either a cell-mediated or humoral response, but not both, in response to pathogens. The functions of Th cells can largely be explained by the cytokines they secrete. Resting T cells do not transcribe cytokine genes, but cytokines are rapidly induced following activation through the TcR and co-stimulatory receptors (reviewed by [13]). The

hallmark cytokine of Th1 cells if IFN–γ and Th1 cells also produce IL-2, TNF-β and lymphotoxin (LT), cytokines that mediate delayed type hypersensitivity (DTH) responses and macrophage activation. The signature cytokine of Th2 cells is interleukin 4 (IL-4), and Th2 cells also secrete IL-5, IL-6, IL-10 and IL-13, cytokines that help B cells to proliferate and differentiate an are critical for humoral-type immune responses [11, 14-16]. A schematic representation of Th cell differentiation is shown in Figure 1.1.

The expression of additional proteins following T cell activation contribute to the achievement of specific Th1 and Th2 cell functions. Comparison of gene expression profiles of Th1 and Th2 cells using high-density oligonucleotide arrays resulted in the identification of 215 differentially expressed genes, encoding proteins involved in transcriptional regulation, apoptosis, proteolysis as well as cell adhesion and migration (Table I) [17]. However, the preferential or unique expression of surface antigens such as cytokine receptors, homing receptors and chemokine receptors occurs late in the course of Th cell differentiation, reason why the best way to phenotypically distinguish the two Th subsets is by their unique cytokine profiles.

The Th1/ Th2 paradigm extends to other species including human where clear-cut human Th1 and Th2 clones have been generated (originally reviewed by [18-20]). However, simultaneous production of IL-2, IL-4 and IFN–γ can be observed in populations of human Th cells [15]. Many other differences between the mouse and human Th1/ Th2 system also exist. These include distinct signalling pathways required for Th cell differentiation as well as differences in the way humans and mice respond to the same pathogen (see below and [21]). The present work was developed using polyclonally activated human T cells isolated from healthy donors as a model. Therefore the majority of the information presented in this section will refer to the human system. Some relevant differences between the human and mouse systems, primarily in the signal transduction pathways regulated by IL-12 and IFN–α will be discussed.

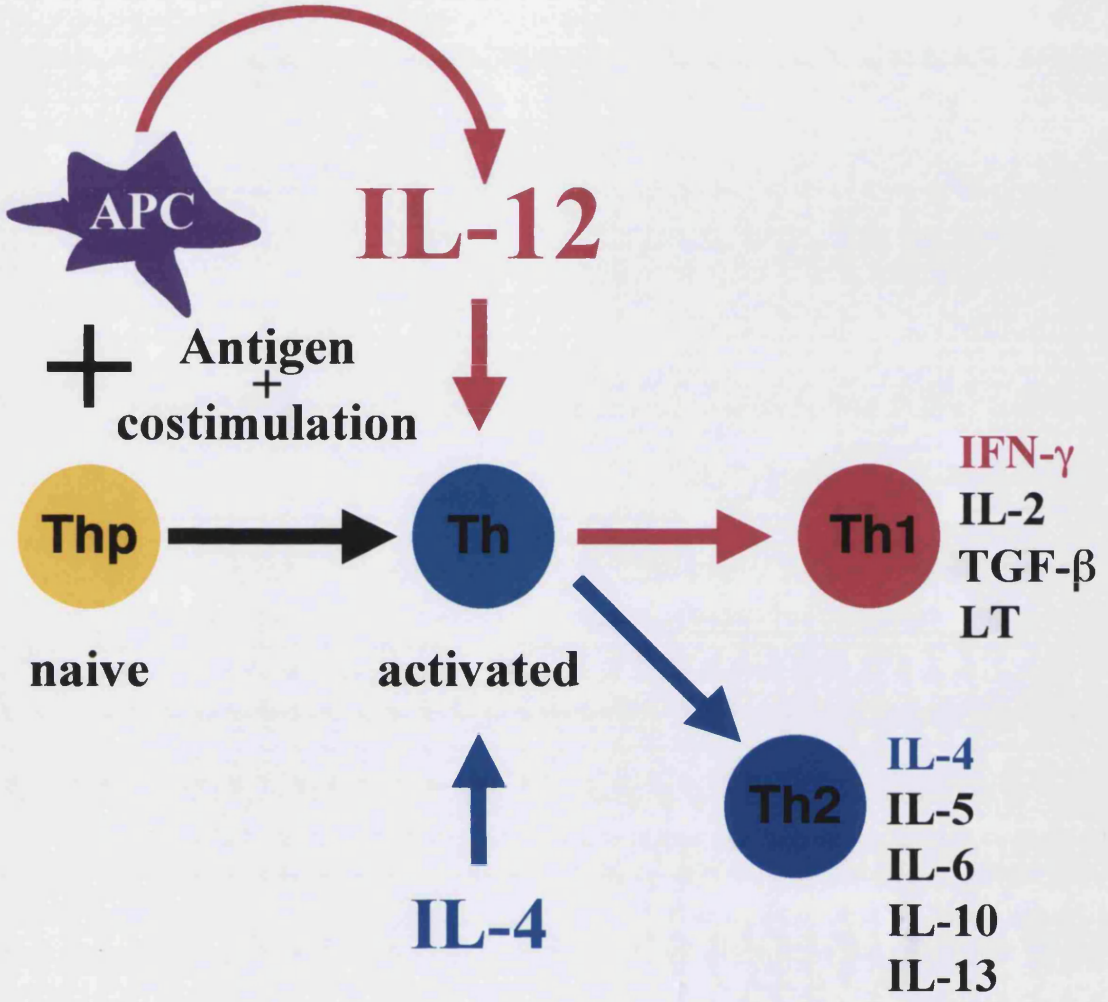


Figure 1.1) T helper cell differentiation.- T helper (Th) cells are activated by antigen and co-stimulatory signals, provided by the antigen presenting cells (APC). The polarisation towards the Th1 or Th2 phenotype is influenced by several factors, including non-MHC genetic polimorfisms, ligand-TcR interaction, antigen dose and mode of antigen administration. However, the dominant regulators of Th cell differentiation are undoubtedly cytokines. IL-12 and IL-4 drive the differentiation of naive T cells into the Th1 and Th2 cell phenotypes, respectively. Th subsets are characterised by the cytokines they secrete. Th1 cells secrete IFN-γ, IL-2, TGF-β and LT, while Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13. See text for further details.

Cytokines, growth factors and receptors

Th1	**IFN-γ**	**oncostatin M**	**leptin**	**LT-β**	**EGF-like GF**
	IL-12Rβ2	**IL-18R**	**TNF-R1,2**	**LIF-R**	
Th2	**IL-10R**				

Transcriptional regulation

Th1	**ets-1**	**NF-IL6β**	**STAT1**	**IRF-1**	**GATA-1**
	c-myb	**ICSBP**	**E2F-4**	**IRF-7A**	
Th2	**HOX-1A**	**BF-2**	**EGR2,α**	**GATA-3**	**GCF/ TCF-9**

Adhesion and migration

Th1	**MIP-1α,β**	**p-cadherin**	**CXCR3**	**L-selectin**	**MIG**
	CD97	**CD2**			
Th2	**TARC**	**CD6**	**ICAM-2**	**integrin-β_7**	

Apoptosis and proteolytic systems

Th1	**CD26/ DPPIV**	**perforin**	**granzyme B**	**TRAIL**	**granzyme H**
	pre-granzyme 3	**UPAR**	**cystatin C**	**elastase inhibitor**	**caspase 8**
Th2	**protease M**				

Enzymes and other signalling molecules

Th1	**CD69**	**PKC-L**	**Itk**	**pim-1**	**CD38**
	IFN-induced GBP-1,2	**CD40-L**	**MAPKKK5**	**PGE2-R EBP**	**MNK-1**
Th2	**PTP-α,ζ**	**EBV-induced GPCR EB1 2**	**GPCR EDG1**	**GPCR GPR6**	**thromboxane A2 receptor**

Metabolic pathways

Th1	**anexin III**	**metallothionin**	**cyclophilin**	**GTP cyclohydrolase**
	serine-pyruvate aminotransferase	**phosphodi-esterase 4B**	**acetyl-CoA synthetase**	**apolipoprotein E-R2**
Th2	**terminal transferase**	**adenylate cyclase**	**spliceosomal protein SAP61**	**aldehyde dehydrogenase 7**

Table I) Differentially expressed genes in Th1 and Th2 cells.- Comparison of the gene expression profiles of human Th1 and Th2 cells using high-density oligonucleotide arrays. Some examples of the 215 differentially expressed genes are listed. See Rogge L. et al, Nature genetics, 2000, 25 (1), 96-101 and text for further details.

1.3.2 T cytotoxic cell differentiation

Polarisation of T cells originally concentrated on $CD4^+$ Th cells. $CD8^+$ Tc cells also differentiate in the periphery. $CD8^+$ Tc cells are not spontaneously cytotoxic but have to be activated to develop their cytotoxic potential. It is recognised that $CD8^+$ Tc cells may not only function as "killer" cells but are also able to produce cytokines that modify the immune response. $CD8^+$ Tc cells differentiate in a similar manner to $CD4^+$ Th cells, resulting in Tc1 and Tc2 subsets in both human and mice [22-24]. Alloreactive Tc1 and Tc2 cells can be generated from naive $CD8^+$ T lymphocytes in the presence of IL-12 or IL-4, respectively [23]. As in the case of Th1 and Th2 cells, Tc1 cells secrete IFN–γ while Tc2 cells produce IL-4 and IL-5. However, the fundamental functional dichotomy in the Tc subsets has yet to be demonstrated, because Tc1 and Tc2 cells are more closely related as compared to Th1 and Th2 cells. Tc2 cells are readily generated *in vivo* and can be found in samples from HIV (human immunodeficiency virus) patients and lymphocytic choriomeningitis virus (LCMV) infected mice [22, 25]. However, while naive $CD4^+$ Th cells can equally differentiate into Th1 or Th2 cells, naive $CD8^+$ Tc cells show a strong preference for differentiating into Tc1 cells. *In vitro*, generation of Tc2 cells requires substantial amounts of IL-4 and anti-IFN–γ (reviewed by [26]). Tc2 cells generally produce significantly less IL-4 than Th2 cells and can continue to produce IFN–γ, whereas Th2 cells lose this capacity [23]. The cytokine pattern expressed by Tc1 is consistent with the cytotoxic function of $CD8^+$ Tc cells, but the cytokines produced by Tc2 do not fit well with a cytotoxic function. Tc2 cells share many functions with Tc1 cells. Both subsets are cytotoxic, mainly through the perforin- and Fas-mediated pathways and induce inflammation accompanied by similar cellular infiltrates (reviewed by [26]). Tc1 and Tc2 cells effectively kill resting or activated B cells. This may explain why Tc2 do not provide cognate B cell help but induce antibody production probably through a bystander mechanism based on IL-4 and IL-5 secretion. Although the exact function of the Tc2 subset

requires further examination, it has been proposed that it may contribute to the diversity of effector functions by allowing strong cytotoxicity to coexist with strong Th2-mediated antibody responses (reviewed by [26]).

1.4 Interleukin-12

1.4.1 Structure of IL-12

IL-12 (IL-12p70) is a heterodimeric cytokine composed by two disulfide-linked subunits designated IL-12p35 and IL-12p40, which are encoded by unrelated genes. IL-12p35 shares homology with IL-6 and granulocyte colony stimulating factor (G-CSF). IL-12p40 is not homologous to other cytokines but belongs to the hematopoietin receptor family and most resembles the extracellular domain of the IL-6 receptor α-subunit and the ciliary neutrophilic factor (CNTF) receptor (reviewed by [27, 28]). However, no evidence has appeared of the existence of membrane-associated forms of IL-12 or either of its subunits. IL-12p35 is expressed constitutively in most cells, but high expression in murine dendritic cells (DC) and human blood monocytes, requires cell activation [29, 30]. IL-12p40 expression is inducible and restricted to a subset of hematopoietic cells including B cells, macrophages, granulocytes and DC (reviewed by [27, 28]). Neither IL-12 subunit alone displays any biological activity, although a IL-12p40 homodimer may function as an IL-12 antagonist that binds to the IL-12R but does not mediate a biological response. Bioactive IL-12 requires expression of both IL-12p35 and IL-12p40 in the same cell (reviewed by [27, 28]). The crystal structure of monomeric human IL-12p40 and the IL-12p70 complex have been recently determined (Figures 1.2 A and B) [31]. These studies confirm that IL-12 is structurally similar to class I cytokine receptor complexes.

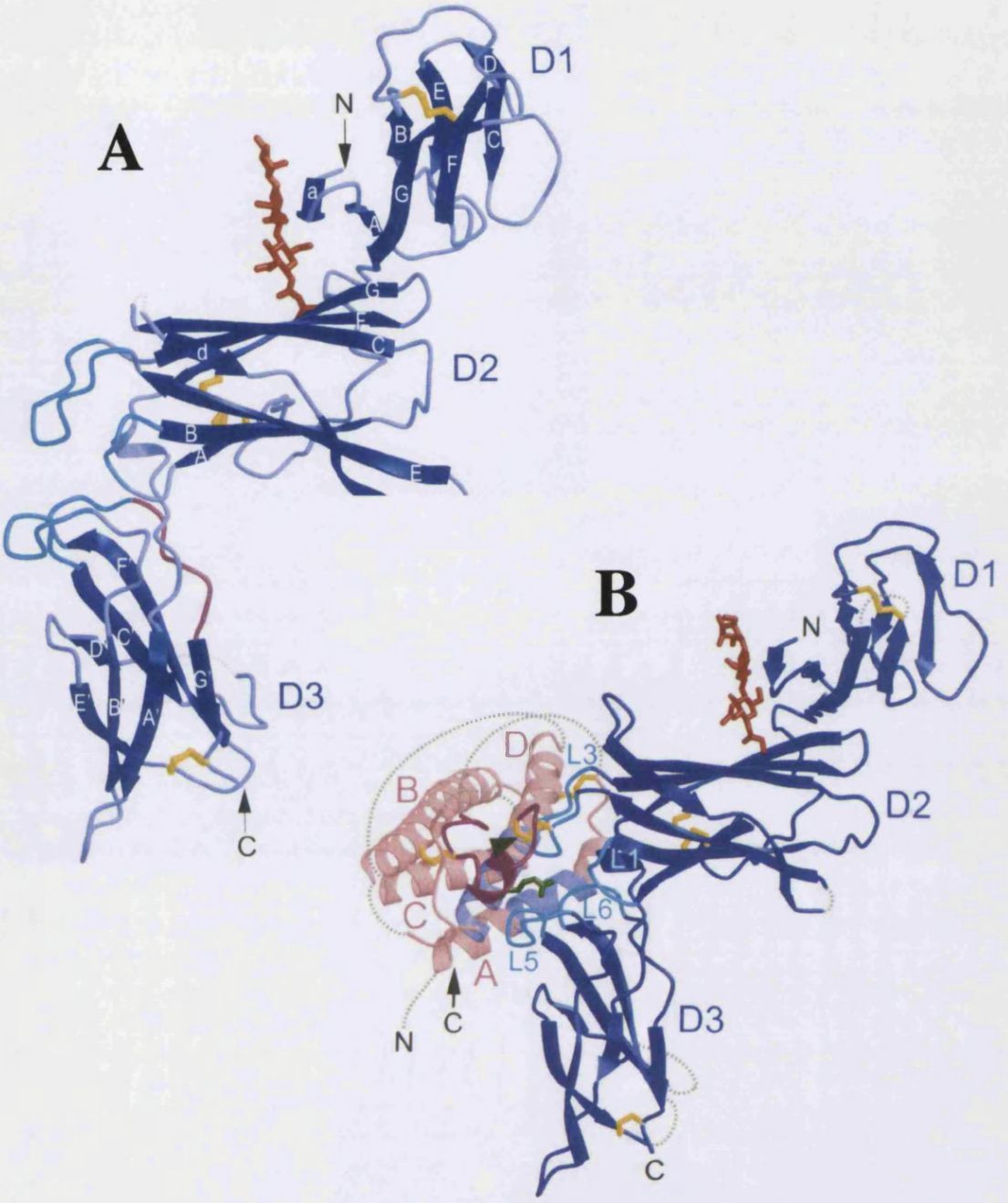


Figure 1.2) Crystal structure of IL-12p40 and IL-12p70.- A) IL-12p40 resembles a soluble class I cytokine α-receptor. N- and C- termini are labelled N and C, respectively, and the three domains are labelled D1, D2 and D3. The interchain disulfide bonds (yellow), an N-linked sugar modification (red) and the WSXWS motif (magenta) are shown. The p40 loops that contact p35 (turquoise) are located at the apex between D2 and D3. Flexible loop regions are indicated by dotted lines. **B)** IL-12p70 resembles a soluble class I cytokine-receptor complex. Ribbon diagram of the IL-12p70 complex: p35 (pink) with helices labelled A-D, p40 (blue) with domains labelled D1-D3, the interchain disulfide bond (yellow, arrowhead), intramolecular disulfide bonds (yellow), an N-linked glycosylation (red) and N- and C-termini labelled N and C, respectively. The structural epitope is also highlighted: residues from p40 loops 1, 3, 5 and 6 (turquoise) contact p35 residues from the AD helical face (lavender) and the disulfide bond loop (magenta). The p35 R189 side chain (green) extends from helix D and is buried within the p40 loops. These figures were made using MOLSCRIPT and Raster3D. Reproduced from Yoon C. et al, EMBO J., 2000, 19(14), 3530-3541.

1.4.2 Regulation of IL-12 production

Bioactive IL-12 is produced by APCs, primarily macrophages and DC by T cell-dependent and independent mechanisms. Bacteria, viruses, yeast and intracellular parasites act in a T cell-independent manner, apparently through recognition by APCs of molecular microbe hallmarks such as lipopolysacharydes (LPS), double stranded RNA (dsRNA) or unmethylated DNA containing CpG (CpG-DNA) motifs (reviewed by [32]). TLR-2 and TLR-4, members of the Toll-like receptor (TLR) family, function as LPS receptors and it has been proposed that other TLR family members may recognise distinct microbe markers (reviewed by [33]).

Activated T cells induce the production of IL-12 by APCs through CD40/ CD40L (CD40 ligand) and MHCII-TcR interactions during antigen presentation [34-39]. T cells defective in CD40L up-regulation only stimulate APCs for reduced production of IL-12, highlighting the importance of CD40/ CD40L interactions [40]. Recent studies have shown that optimal IL-12 production requires the co-ordinated action of both microbial products and CD40/ CD40L-derived signals [30, 41]

The induction of IL-12 is also modulated by numerous cytokines. Especially important is IFN–γ that in addition to directly enhancing transcription of IL-12p35, primes both IL-12p35 and IL-12p40 chains for enhanced transcription induced by LPS and other stimuli [42, 43]. IFN–γ also synergises with CD40/ CD40L for optimal IL-12 production [41].

The gene encoding the IL-12p40 subunit was initially recognised to be controlled through proximal cis-acting elements interacting with NF-κB (nuclear factor κB) family members [44]. Analyses of the IL-12p40 proximal promoter have demonstrated the presence of C/ EBP protein interactions immediately down-stream of the NF-κB target site [45]. Additionally, the transcription factor Ets may synergistically interact with NF-κB to control IL-12p40 expression [46, 47]. IL-12

production in response to bacterial LPS, CD40 ligation and IL-12 is mediated by activation of NF-κB in DCs [48].

Certain microbe products, cytokines and pharmacological agents can inhibit IL-12 production. Prostanoids, cholera toxin and heat liable toxin respectively secreted by some helminths, *V. cholera* and enterotoxigenic *E. coli,* as well as HIV and measles infections negatively regulate IL-12 production [49-53]. Monocytes and DC previously activated with endotoxin become unable to produce IL-12 following further stimulation [54]. Pathogen mediated ligation of the macrophage Fcγ or complement receptors also appear to decrease IL-12 production [55, 56]. IL-10 is an important physiological inhibitor of IL-12 induction [42, 57, 58]. Although the mechanism has not been elucidated, IL-10 may act by destabilising IL-12p40 mRNA via clustered AU-rich elements present within the 3'UTR, as has been shown for TNF-α [59]. Retinoids, acetyl salicylic acid and 1,25 dihydroxyvitamin D3 block induction of IL-12 by inhibiting NF-κB activation [60-62].

1.4.3 IL-12 receptor

The IL-12 receptor (IL-12R) consists of two chains IL-12Rβ1 and IL-12Rβ2 both members of the gp130 subgroup of the cytokine receptor superfamily [63-65]. Genetic disruption studies in mouse and analysis of patients with mutations in either IL-12Rβ1 or IL-12Rβ2 have demonstrated that both chains are required for functional IL-12 signalling [66-71]. IL-12Rβ1 and IL-12Rβ2 are independently expressed since both knock out mice show normal expression levels of the other chain and subjects with IL-12Rβ1 deficiency have normal up-regulation of IL-12Rβ2 in response to cytokines [66, 71, 72]. In humans IL-12Rβ1 is expressed in Th1 and Th2 cells as well as monocytes, NK cells, B cells and DCs, while IL-12Rβ2 expression is restricted to Th1 cells, NK cells and DCs ([48, 73] and

reviewed by [27]).

Using radiolabelled IL-12 ($^{(125\text{-}I)}$IL-12) and human PHA-activated T cells, high affinity (Kd = 5-20 pM, 100-1,000 sites per cell), medium affinity (Kd = 50-200 pM, 200-1,000 sites per cell) and low-affinity (Kd = 2-6 nM, 1,000-5,000 sites per cell) IL-12 binding sites have been detected (reviewed by [27]). The cDNAs of both IL-12Rβ1 and IL-12Rβ2 have been clonned from human and mouse. On the cell surface, each of the two recombinant IL-12R subunits occurs as dimers/ oligomers. The formation of these structures is ligand-independent. When expressed in recombinant form on COS cells, each human subunit binds radiolabelled IL-12p70 with only low (nM) affinity. Coexpression of IL-12Rβ1 and IL-12Rβ2 is required for the generation of human high-affinity (pM) IL-12p70 binding sites [65]. Using various IL-12p70 antagonists that differentially inhibit the interaction of IL-12p70 with the IL-12Rβ1 and IL-12Rβ2 subunits, it has been proposed that binding of IL-12p70 to the high affinity IL-12R complex involves multiple interaction sites [74]. IL-12p70 seems to interact with IL-12Rβ1 primarily via domains on the IL-12p40 subunit and with IL-12Rβ2 via a heterodimer specific region of the IL-12p70 to which the IL-12p40 and IL-12p35 subunits may both contribute [74].

A model for the interaction of IL-12 with the IL-12R has been proposed based on the crystal structure of IL-12p70 and the structural and sequence similarities of IL-12/ IL-12R with the IL-6/ gp130 system (Figure 1.3) [31]. This model proposes that IL-12Rβ1 or IL-12β2 would bind to IL-12p70 along the IL-12p35 helical face (AC in Figure 1.3). Two of these complexes would then interact to form a pseudo-symmetric heteroligomer containing two IL-12p70s, and one of each of the IL-12Rβ1 and IL-12Rβ2 subunits. Binding of the initial IL-12p70 to IL-12Rβ1 may cause conformational changes that might enhance the affinity of the resulting complex for an incoming IL-12p70/ IL-12Rβ2 complex. Alternatively, the first IL-12p70/ IL-12Rβ1 complex may bind the second IL-12p70, and the resulting

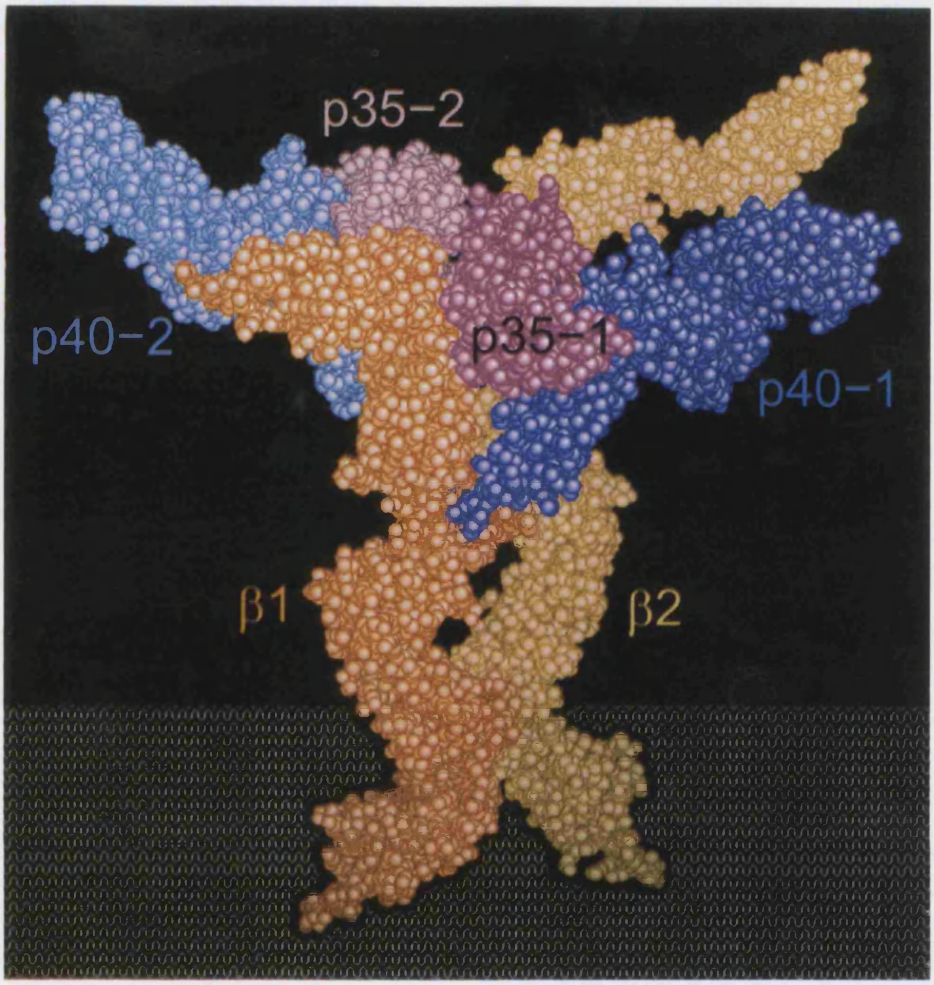


Figure 1.3) Hexameric model of the IL-12 signalling complex.- The model is composed of two IL-12p70 heterodimers juxtaposed in approximately 2-fold symmetry bound to one each of IL-12Rβ1 and IL-12Rβ2 extracellular domains in a position analogous to the low-affinity binding sites on growth hormone receptor (GHR) and erythropoietin binding protein (EBP). The IL-12Rβ1 and IL-12Rβ2/ IL-12p70-binding regions were modelled using gp130 co-ordinates (PDB code 1BQU). The other domains in IL-12Rβ1 and IL-12Rβ2 were made by duplication and manual placement of fibronectin domains from gp130, with the exception of an N-terminal Ig-like domain in IL-12Rβ2, which was constructed using IL-12p40 D1. This figure was made using QUANTA and GRASP. Reproduced from Yoon C. et al, EMBO J., 2000, 19(14), 3530-3541.

complex would then present a pre-formed binding site with high affinity for IL-12Rβ2 [31]. It is thought that this complex would be further stabilised by interactions between IL-12Rβ1 and IL-12p40 (p40-2 in Figure 1.3), since IL-12p40 seems to have a measurable affinity for IL-12Rβ1 [74]. In either scenario IL-12Rβ1 would act as an affinity converter to facilitate formation of a higher affinity IL-12 hexameric signalling complex [31].

The IL-12Rβ1 chain lacks tyrosine residues in its cytoplasmic domain and is then thought incapable of transducing IL-12-mediated signals [65, 75]. Rather the IL-12Rβ2 chain is proposed to mediate IL-12 signalling apparently through the phosphorylation of a tyrosine residue at position Y800 (IL-12Rβ2Y800) [75]. Although the phosphorylation of IL-12Rβ2Y800 in response to IL-12 has not been demonstrated, transient expression of an IL-12Rβ2Y800 mutated construct (IL-12Rβ2Y800F) blocks IL-12-induced transcriptional activity (see below for more details and [75]).

1.4.4 Regulation of IL-12R expression

Naive T cells do not express IL-12Rβ1 and IL-12Rβ2. T cell activation by antigen presentation *in vivo* as well as TcR triggering by anti-CD3 antibodies or activation with PHA (phytohemagglutinin) *in vitro* induce the expression of both IL-12Rβ1 and IL-12Rβ2 [76, 77]. Although the signals that control TcR mediated up-regulation of IL-12Rβ1 and IL-12Rβ2 have not been elucidated it is clear that they only induce low levels of both chains [78-80]. Different pieces of data indicate an important role for CD28 engagement in maximal induction of both IL-12Rβ1 and IL-12Rβ2 expression. Co-stimulation provided by B7-2 transfected CHO cells or APC enhances anti-CD3 induced IL-12Rβ1 and IL-12Rβ2 expression [80].

Likewise, inhibition of CD28/ B7 interactions with blocking antibodies or through CTLA-4 engagement decreases anti-CD3 induced IL-12Rβ1 and IL-12Rβ2 expression [79, 80]. Moreover, CD28 null T cells induce lower levels of IL-12Rβ1 and IL-12Rβ2 following TcR cross-linking with anti-CD3 antibodies or activation with APC bearing specific peptides [79, 81]. The requirement of CD28 co-stimulation varies with different populations. Co-stimulatory signals are more important for naive T cells than memory T cells or Th1 cell clones [78]. Similarly, $CD8^+$/ $CD28^-$ T cells express higher IL-12Rβ1 and IL-12Rβ2 mRNA and produce more IFN–γ than $CD4^+$/ $CD28^-$ T cells [81].

CD28 engagement may have a direct effect on IL-12Rβ1 and IL-12Rβ2 induction by increasing mRNA stability, as it does for IFN–γ, IL-2, IL-2Rα chain and IL-2Rβ chain [82-84]. However, an indirect role through the regulation of cytokine production has also been proposed. Induction of IL-12Rβ1 and IL-12Rβ2 by priming with anti-CD3 and anti-CD28 in mouse cells can be reduced by addition of anti-IL-2/ IL-2R or anti-IFN–γ antibodies, respectively [80]. Similarly, the low levels of IL-12Rβ1 and IL-12Rβ2 induced in CD28 null and IFN–γ null T cells can be respectively recovered by exogenous IL-2 and IFN–γ [79-81].

Once TcR and co-stimulatory signals have induced IL-12Rβ1 and IL-12Rβ2, the levels of expression of both chains are further modulated by cytokines. The participation of IL-2 in the regulation of IL-12R in T cells suggested by studies of CD28 co-stimulatory signals is supported by IL-2 up-regulation of IL-12Rβ1 and IL-12Rβ2 in NK cells [79-81, 85].

The regulation of IL-12Rβ2 is the focal point of early maintenance or loss of IL-12 responsiveness in differentiating Th cells [77, 86]. After T cell activation and initial induction, the expression of IL-12Rβ1 is maintained in both Th1 and Th2 cells.

However, IL-12Rβ2 expression can be either enhanced by the presence of IL-12 or rapidly lost when cells are primed in the presence of Th2 driving cytokines (i.e. IL-4) in both human and mouse systems [77, 86]. IL-12 plays an important role in the up-regulation of its own receptors. Administration of IL-12 in mice leads to increased IL-12Rβ2 expression and endogenous levels of IL-12 have proven to be sufficient for IL-12Rβ2-induced expression [40, 87, 88]. Moreover, mice with reduced production of IL-12 show decreased levels of IL-12Rβ2 and re-exposure of Th1 cells to IL-12 results in up-regulation of IL-12Rβ2 even in the absence of antigen stimulation [77, 79, 86].

IFN–α and IFN–γ up-regulate IL-12Rβ2 expression [77, 86, 87, 89]. The effect of IFN–α is exclusive to human T cells, while IFN–γ primarily induces IL-12Rβ2 in mouse T cells, but may have a minor role in human T cells [77, 86, 89, 90]. Studies in IL-12 null mice have shown that endogenous levels of IFN–γ are sufficient to induce IL-12Rβ2 expression [40, 91]. These studies also show that the effect of IL-12 and IFN–γ on IL-12Rβ2 induction are independent and complementary [40]. Since IFN–γ responsiveness is lost in IFN–γ stimulated T cells, the effect of IFN–γ on the induction of IL-12Rβ2 might be relevant during T cell priming but not at later stages of Th cell development [92-94].

IL-4 and other Th2-derived cytokines negatively regulate IL-12Rβ2 expression. The relevance of IL-4 has been highlighted by *in vivo* studies showing than BALB/c mice which overexpress IL-4 are not able to express IL-12Rβ2 and their Th cell responses are therefore bias to the Th2 phenotype [87]. Transforming growth factor–β (TGF–β) blocks IL-12Rβ1 and IL-12Rβ2 expression when is present at the time of priming but not in IL-12R expressing T cells [95-99]. IL-10 also has a negative effect on IL-12Rβ2 expression [95].

1.4.5 Biological functions of IL-12

Studies in IL-12 and IL-12R deficient mice have demonstrated an essential role for endogenous IL-12 in promoting the generation of Th1 cell responses leading to optimal IFN-γ production [66, 71, 100-102]. These responses are important in promoting immunity to a number of intracellular pathogens including bacteria, fungi and protozoan. Studies using IL-12 deficient mice have shown the direct participation of IL-12 in host defence against microbes such as *L. major*, *T. gondii*, *M. tuberculosis*, *L. monocytogenes* and *C. neoformans* (reviewed by [27]). Similarly, patients with deficiencies in IL-12 signalling by mutations in IL-12 and IL-12Rβ1 suffer from Bacillus Calmette-Guérin (BCG) or chronical *Mycobaterium sp.* and *Salmonella sp.* infections, demonstrating the requirement of IL-12 for adequate immune responses against these microorganisms [67-69, 103]. Mutations in IL-12Rβ2 are found in atopic patients, which show exaggerated Th2 cell responses due to disrupted Th1/ Th2 balance [70].

One of the most important properties of IL-12 is the ability to prime T and NK cells for the production of large amounts of IFN–γ [3, 104-108]. This activity of IL-12 is central to many effects seen when IL-12 is administered *in vivo* and provides a mechanism whereby IL-12 plays an important role in innate, as well as adaptive, immunity [105, 109-117]. In response to IL-12, NK cells can produce high amounts of IFN–γ, which activates macrophages and enhances their bactericidal activity, representing a mechanism of T cell-independent macrophage activation during the early phases of innate resistance.

IL-12 has weak proliferative effects on T cells when used alone, but it potentiates the proliferation of T and NK cells induced by various mitogens, for example IL-2 [3, 118-121].

IL-12 enhances the generation of cytolytic T lymphocyte (CTL) responses and LAK cells, and potentiates the cytotoxic activity of CTLs and NK cells [3, 104, 122, 123]. Some of the effects of IL-12 on cell-mediated cytotoxicity are due to its ability to induce increased formation of cytoplasmic granules and induction of transcription of genes encoding cytotoxic granule-associated molecules such as perforin and granzymes [108, 124].

The attraction of leukocytes to tissues is essential for the host response to infections. Extravasation of effector T cells into tissues is mediated by a series of adhesive interactions between specific ligands expressed on the T cell surface with their respective adhesion molecules expressed on the vascular endothelium [125]. Recent studies have shown that IL-12 up-regulates the expression of adhesion molecules and chemokine receptors such as E- and P-selectin ligands, integrin-α6, CCR-5, CXCR-3 and CCR-1 [126-133]. This suggests an important regulatory role of IL-12 in leukocyte trafficking.

IL-12 enhances its own responses through direct up-regulation of IL-12Rβ2 expression as well as by suppressing the production of TGF-β, which inhibits IL-12 responsiveness [40, 77, 86-88, 134, 135]. IL-12 limits its own effects through a negative feedback mechanism by inducing the production of IL-10, which inhibits IL-12 production [114, 136-138]. Finally, IL-12 can either enhance or inhibit humoral immunity dependent on the Ig isotype and the stimulus to antibody formation. IL-12 enhances the Th1-associated IgG2a, IgG2b and IgG3 responses and suppresses the Th2-associated IgG1 and IgE responses [114, 139-146]. Some of these responses are partially mediated by IL-12-induced IFN-γ.

1.5 Interleukin-2

IL-2 is a 15 kDa glycoprotein mainly produced by activated $CD4^+$ Th1 cells but small amounts may also be liberated by $CD8^+$ T cells, NK cells and activated B cells. In T cells, IL-2 production depends on TcR activated signalling pathways, together with CD28 co-stimulatory signals.

1.5.1 The IL-2 receptor

IL-2 exerts its functions through interaction with the high affinity IL-2 receptor (IL-2R) composed of α (CD25), β and γ subunits. The β chain is also used by IL-15 and the γ chain (also know as the common γ chain (γ c)) is shared by IL-2, IL-4, IL-7, IL-9 and IL-15 [147-151]. The IL-2Rα chain constitutes the low affinity IL-2R (Kd~10-8M). Both the IL-2Rβ chain and the γc bind weakly to IL-2 (Kd~10-9M) and together form the intermediate affinity IL-2R. Interaction of the β/ γc complex with the IL-2Rα chain is required for the formation of the high affinity IL-2R complex (Kd~10-11M) (reviewed by [152-154]). While IL-2Rα chain is required for high affinity binding of IL-2, the β and γc chains are involved in signal transduction. High affinity IL-2Rs are predominantly expressed on activated T cells and NK cells but have also been described on monocytes and B cells.

IL-2Rα chain plays an essential role in IL-2-mediated responses and the development of normal immune responses. This has been demonstrated by the finding that a truncation mutant of IL-2Rα results in a human immunodeficiency characterised by increased susceptibility to viral, bacterial and fungal infections [155]. In addition, gene targeting analysis reveals that IL-2Rα chain deficient mice lack all IL-2 responses and show identical immunodeficiencies to those of IL-2-deficient mice (see below) [156-158]. Disruption of the IL-2Rβ chain has a more

severe impact than that of IL-2 or IL-2Rα [159]. This is because the IL-2Rβ is also a component of the IL-15 receptor. Thus in IL-2Rβ null mice both autoimmunity associated with T cells hyperactivation and NK cell deficiencies are detected.

In humans, mutation in the γc subunit are the cause of the X-linked (X-) severe combined immunodeficiency (SCID) (X-SCID), characterised by profoundly decreased numbers of T and NK cells and by normal numbers of non-functional B cells ([154, 160, 161] and reviewed by [162, 163]). X-SCID is a fatal syndrome unless treated by bone marrow transplantation. In γc disrupted mice a profound block in the development of both early progenitor B and T cells is detected and the mice do not have a peripheral immune system [164-166]. The pathogenesis associated with the γc mutant mice are complex as these mice reflect disrupted IL-7 signalling in early lymphoid progenitors together with the associated loss of IL-2, IL-4 and IL-5 signalling in the periphery [157, 167-170].

1.5.2 IL-2 –induced signalling pathways

In T cells IL-2-mediated heterodimerisation of the IL-2Rβ and γc subunits induces protein tyrosine kinase-dependent pathways involving the Src family tyrosine kinases LcK and Fyn and the Janus kinases Jak1 and Jak3 [171-177]. Signalling cascades initiated by the action of IL-2-induced tyrosine kinases include activation of Ras [178-180], the transcription factors STAT3 and STAT5 [181-185], and the regulation of Phosphatidyl inositol 3-kinase (PI3-K) (reviewed by [186]). The activation of Jak1 and Jak3 is now generally accepted to be the first step in IL-2R signalling. This leads to phosphorylation of three tyrosine residues on the IL-2Rβ chain, which are vital for signal transduction: Tyr338, Tyr392 and Tyr510 [183, 187]. The activation of STAT3 and STAT5 in response to IL-2 will be discussed in more detail in section 1.10. IL-2 regulation of the Ras and the PI3-K signalling

pathways is briefly described in the following sections. A schematic representation of IL-2-induced signalling pathways is shown in Figure 1.4.

1.5.2.1 The Ras/ Mitogen Activated Protein Kinase pathway

Ras is a guanine nucleotide binding GTPase whose activity is controlled by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs promote the transition of Ras from the GDP-bound inactive state to the GTP-bound active conformation. GAPs stimulate the low intrinsic GTPase activity of Ras causing the hydrolysis of bound-GTP to GDP thereby returning Ras to the inactive GDP-bound estate. Ras is a key component of the complex regulatory networks stimulated by many cytokines as well as TcR triggering in T cells (for review see [188]). The regulation of Ras and its downstream effectors in response to IL-2 in T cells is described.

In T lymphocytes the first step in the Ras pathway in response to IL-2 is the tyrosine phosphorylation of the adapter molecule Shc. Shc associates with IL-2Rβ–pTyr338 via its protein tyrosine binding (PTB) domain and is tyrosine phosphorylated by active Jak1 [189, 190]. Tyrosine phosphorylation of Shc enables the recruitment of a second adapter protein Grb2 (growth factor receptor bound protein 2) into the plasma membrane through its SH2 domain. Sos is a GEF for Ras that constitutively associates with the SH3 domains of Grb2. Therefore, formation of the Shc-Grb2 complex recruits Sos to the plasma membrane, enabling the catalytic activity of Sos to activate the Ras-GTPase. [191, 192]. Active GTP-bound Ras interacts with the serine/ threonine kinase Raf-1 through the Ras binding domain (RBD) of Raf-1. This interaction recruits Raf-1 to the plasma membrane where it is activated. Active Raf-1 then phosphorylates and stimulates the kinases Mek1,2 (Mitogen activated Erk kinase1,2). Mek1,2 are dual-specificity kinases, which in turn activate the mitogen-activated protein kinases (MAPKs) Erk1,2

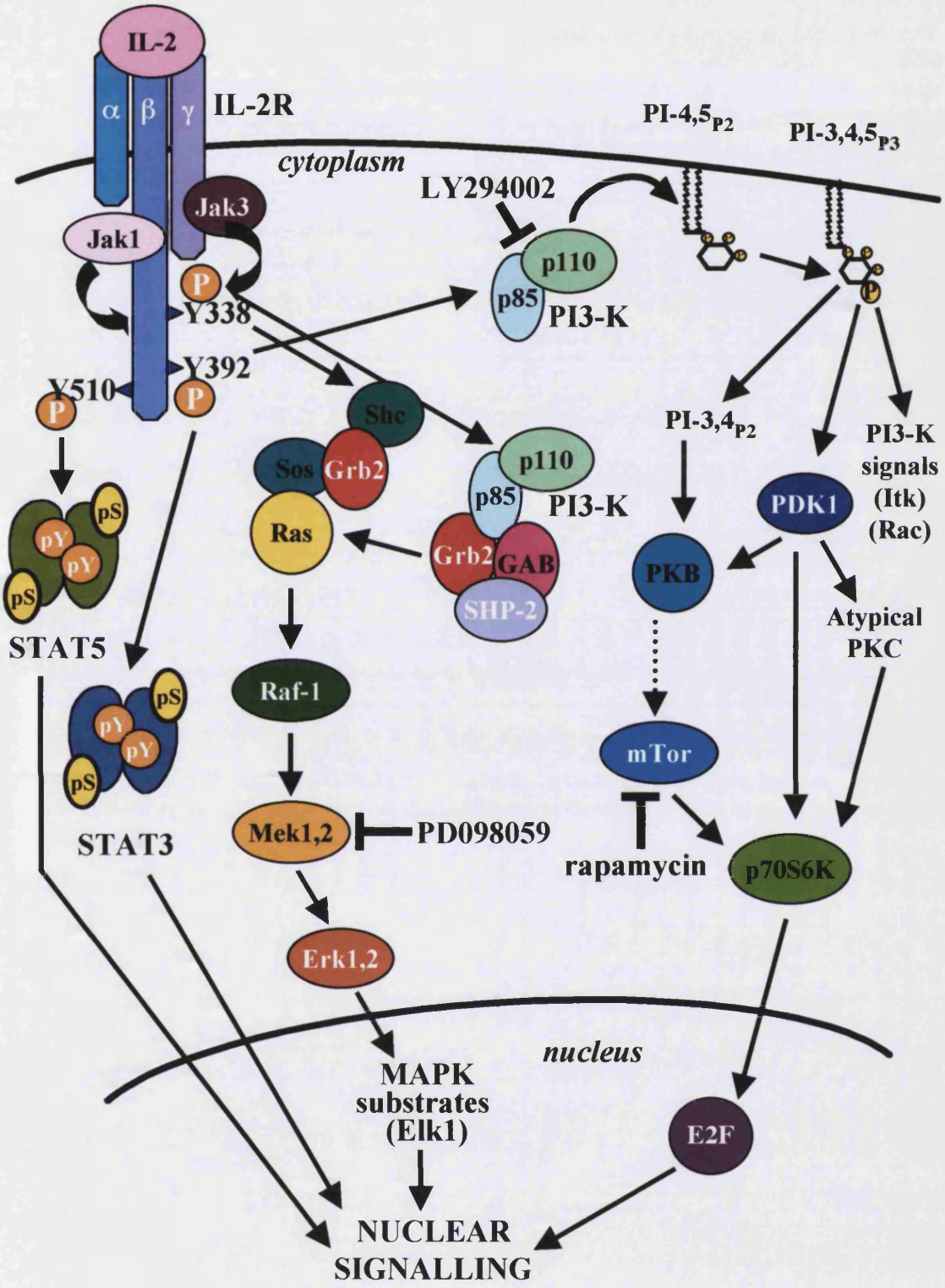


Figure 1.4) IL-2-induced signalling pathways.- Integration of the Jak/ STAT, Ras/ MAPK and PI3-K signalling pathways in T cells. The first step in the activation of IL-2-induced signalling pathways is the activation of the Jak kinases Jak1 and Jak3. This leads to the phosphorylation of three specific tyrosine residues (Tyr338, Tyr392 and Tyr510) on the cytoplasmic domain of the IL-2Rb chain. The phosphorylation of these residues is respectively associated with the activation of the Ras/ MAPK, PI3-K and STAT signalling pathways, and the formation of the Grb2/ PI3-K/ SHP-2/ GAB complex. **The levels at which the LY294002 compound, PD098059 compound and rapamycin inhibit the respective pathways are indicated.** See text for further details.

(extracellular signal regulated kinase1,2), through phosphorylation on both Thr183 and Tyr185. The downstream targets of Erk1,2 include a number of transcription factors such as Elk-1 and STAT3, the activity of which is enhanced by phosphorylation. Elk-1 is a ternary complex factor (TcF) that regulates the serum response factor (SRF). The expression of genes such as c-fos and the Egr family are controlled by TcF/ SRF (reviewed by [193-195]).

The activation of the Raf-1/ Mek/ MAPK pathway in response to Ras is important because it regulates thymocyte differentiation [196]. Mice deficient in Erk1 have a defect in thymocyte positive selection despite the expression and function of Erk2, indicating that these two MAPKs have unique non-redundant roles in Ras signalling pathways [197].

Together with Erk1,2, the MAPKs family also includes the p38 MAPK and the c-Jun N-terminal kinases (JNK) (also known as stress activated protein kinases = SAPKs). MAPKs are dual specificity kinases that can phosphorylate both serine/ threonine and tyrosine residues. The MAPK subfamilies are regulated by different extracellular stimuli. While Erk1,2 are rapidly activated by growth factors and cytokines, JNK and p38 MAPK are typically activated by environmental stress, LPS and proinflammatory cytokines, such as IL-1β and TNF-α. However, it has been recently demonstrated that JNKs and p38 MAPK can also be activated by TcR-derived signals and by several cytokines including IL-2, IL-3, IL-7, erythropoietin (EPO), thrombopoietin (TPO), colony stimulating factor-1 (CSF-1) and granulocyte-macrophage colony stimulating factor (GM-CSF) [198-208].

MAPK pathways form a cascade of kinases, where each downstream kinase serves as a substrate for the upstream activator (Figure 1.5). Substrate specificity is achieved through direct enzyme-substrate interactions (reviewed by [209]). JNK is activated by MKK4 (also known as SEK1) and MKK7 by phosphorylation on Thr183 and Tyr185. These enzymes respectively have three and six isoforms each

differentially regulated by upstream MAPKKK (reviewed by [210]). MKK4 is primarily activated by environmental stress and MKK7 is predominantly activated by cytokines (TNF and IL-1) (reviewed by [211]). JNK phosphorylates the c-Jun transcription factor and increases AP-1 transcription activity. Other JNK substrates include the transcription factors JunD, JunB, ATF-2, ATFa, Sap-1, NF-AT4 and Elk-1 ([212-214] and reviewed by [211, 215-217]).

The p38 MAPK is primarily activated in response to MKK3 and MKK6 by phosphorylation on Thr180 and Tyr182, but MKK4 can also phosphorylate and activate p38 MAPK [218-222]. p38 MAPK substrates include the kinases MAPKAP-2, MAPKAP-3 and MNK1/2, as well as the transcription factors ATF-2, CREB, CHOP, MEF2C, SAP-1 and Elk-1 [212, 213Freshney, 1994 #1308, 214, 218, 219, 223-229].

Different MAPKKK have been reported to activate JNK and/ or p38 MAPK, including members of the MEKK group (MEKK1-4), the mix-lineage protein kinase group (MLK1-3, DLK and LZK), the ASK group (ASK1-2), TAK1 and TPL2. However, the effects of all these kinases on MKK3, MKK4, MKK6 and MKK7 activation derives from *in vitro* overexpression systems and no physiological activators have been defined. Loss of JNK activation in response to a variety of stimuli in cells with disrupted MEKK1 points to this kinase as the most suitable upstream effector of JNK (reviewed by [211]).

Gene targeted disruption of JNK1, JNK2 and p38 MAPK in mice shows that these kinases have an important role in the balance between Th1 and Th2 cell differentiation [230, 231]. They also participate in T cell development in the thymus and efficient activation of peripheral T cells [230-232]. JNK1 deficient mice have enhanced Th2 cell development due to increased IL-4 production, suggesting that JNK1 acts as a negative regulator of IL-4 production [230]. JNK2 null mice show reduced Th1 cell responses and decreased expression of IL-12Rβ2,

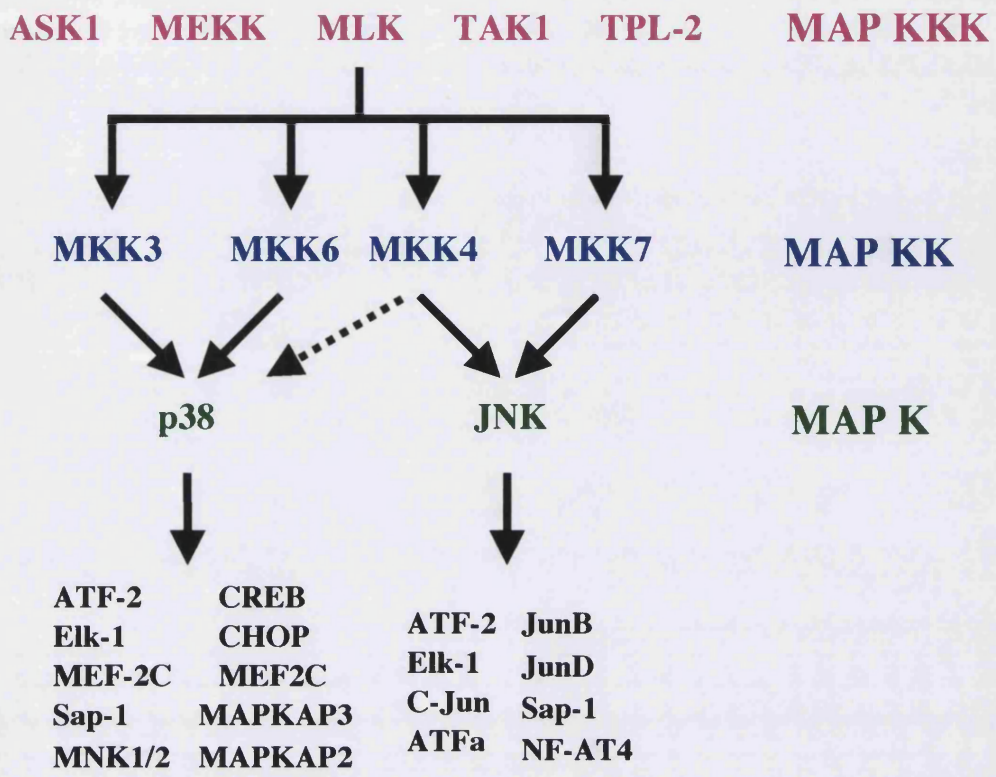


Figure 1.5) Stress-activated MAPK signalling modules.- The JNK and p38 MAPK are activated by dual phosphorylation on Tyr and Thr residues caused by members of the MAPKK group of protein kinases. The MAPKK are activated, in turn, by phosphorylation mediated by a group of MAPKKK. Stress-activated MAPK signalling modules can be created through the sequential actions of MAPKKK a MAPKK, and a MAPK.

indicating that JNK2 activation is involved in IL-12Rβ2 gene expression and IL-12 responsiveness [231]. Activation of p38 MAPK is necessary for adequate IL-12 production. This is demonstrated by impaired Th1 cell responses in MKK3 deficient mice and p38 MAPK dominant negative expressing transgenic mice, as well as enhanced Th1 cell responses in transgenic mice expressing constitutively active MKK6 [233, 234]. Reduced Th1 cell responses in the MKK3 deficient mice are due to defective IL-12 production by macrophages and DCs [234]. This is in agreement with *in vitro* studies showing the partial inhibition of IL-12p40 promoter activity by specific p38 MAPK inhibitors in macrophage cell lines [234]. The activation of p38 MAPK in response to LPS and CpG-DNA motifs, suggests a possible mechanism for the induction of IL-12 expression in response to these microbial products [235, 236]. JNK and p38 MAPK are also implicated in cytokine biosynthesis, cell transformation, stress responses and apoptosis.

1.5.2.2 The PI3-K pathway

Phosphorylation of IL-2RβY392 in response to IL-2-activated Jak1, results in association of p85, the regulatory subunit of PI3-K [237, 238]. This interaction is thought to mediate the plasma recruitment and consequent activation of the p110 catalytic subunit of PI3-K. Activation of PI3-K results in the phosphorylation of the phospholipid Phosphatidyl inositol(4,5)diphosphate (PI(4,5)-P_2) to generate Phosphatidyl inositol(3,4,5)triphosphate (PI(3,4,5)-P_3). Increases in PI(3,4,5)-P_3 activate the serine/ threonine protein kinase B (PKB) (also known as Akt). Furthermore, PI3-K signals are required and sufficient for PKB activation in T cells [239]. IL-2 regulates the transit of peripheral T cells through the G_1/ S phase cell cycle checkpoint. PI3-K signals alone are not sufficient to induce cell cycle entry in T cells [240]. However, one important G_1 checkpoint is the activation of the E2F transcription factors. Both PI3-K and PKB signals are necessary and sufficient to

transcriptionally activate E2Fs in T cells [240]. The ribosomal S6 kinase p70S6K is important in E2F regulation in lymphocytes. Accordingly, the PI3-K/ PKB/ p70S6K pathway acts as an essential link between the IL-2R and the cell cycle machinery [241].

Activation of PKB seems to mediate anti-apoptotic signals in many cell types. Although the specific mechanism has not been described, some components of the apoptotic machinery have been reported to be targets of PKB. These include the Bcl-2 family protein BAD (Bcl-2/ Bcl-X_L-antagonist, causing cell death), human caspase-9, the Forkhead (FH) transcription factors and IκB kinases (IKKs) (reviewed by [242]). Regulation of FH and IKKs is involved in the transcriptional regulation of apoptotic (such as Fas ligand) and anti-apoptotic genes. Other downstream targets of PKB include the glycogen synthase kinase-3 (GSK3), phosphodiesterase-3B (PDE-3B), mammalian target of rapamycin (mTOR) and insulin receptor sybstrate-1 (IRS-1) (reviewed by [242]). Curiously, despite the well-established role of PI3-K/ PKB in survival signalling in many cell types, inhibition of PI3-K in T cells results in cell cycle arrest and not cell death [243, 244].

Recently, it has been shown that IL-2 regulates the activity of the tyrosine kinases Itk and Tec via activation of PI3-K [245]. Itk and Tec belong to the Btk family of non-receptor tyrosine kinases which defining feature is the presence of a plextrin homology (PH) domain at their N-terminus. Although the physiological role and cellular context in which the Btk kinases act have not been defined, these enzymes seem to play central but diverse modulatory roles in various cellular processes. They are activated in response to cytokine receptors, growth factor receptors, G-protein coupled receptors, integrins and antigen receptors. The versatile roles of Btk kinases are explained by their protein-protein and lipid-protein interaction modular structures. In addition to the PH domain, these enzymes have a C-terminal kinase domain (SH1), one SH2 and one SH3 domains (reviewed by [246-248]).

Some studies indicate that beside its main role in T cell proliferation, IL-2 also regulates T cell shape and motility. IL-2 has been shown to provoke membrane ruffling of T cells through PI3-K derived signals [249]. The GTPase Rac-1 seems to be involved in IL-2/ PI3-K regulated membrane ruffling, because dominant negative Rac-1 inhibits IL-2-induced lamellipodia in T cells [249].

1.5.3 Biological functions of IL-2

IL-2 induces proliferation and controls survival of activated T cells and NK cells. IL-2 facilitates induction of cytolytic effector T cells including CTL and LAK cells and induces NK cell cytotoxicity. It also promotes T cells immunoregulatory and proinflammatory functions by directly inducing the production of cytokines such as IFN-γ, TNF-α and IL-6 as well as certain adhesion molecules.

IL-2 deficient mice show normal numbers of T cells and NK cells and only partial and weak immune defects [156, 250]. One surprise from these studies was that loss of IL-2 did not have severe defects on T cell proliferation *in vivo*. It is now known that this is because another cytokine, IL-15 can compensate for IL-2 loss in the context of T cell proliferation and NK cell differentiation ([157, 251] and reviewed by [252]). However, IL-2 deficient mice die at very young age (3-4 weeks) due to anaemia and inflammatory bowel disorder. Different hypotheses have been proposed to explain the phenotype of IL-2-deficient mice. One possible explanation is that IL-2-deficient T cells fail to undergo a normal process of apoptosis (reviewed by [253-255]). It has also been proposed that IL-2 is involved in peripheral tolerance through the elimination of self-reactive T cells by regulation of activation induced cell death (AICD) (reviewed by [255]). A third possibility and the currently favoured view, is that IL-2 acts by modulating the expression of a subset of immunomodulatory T cells (reviewed by [256-258]).

Synergistic interactions between IL-12 and IL-2 seem to be important in Th1 cell differentiation. IL-2 and IL-12 have been shown to synergise for the induction of IFN–γ, granzyme B, perforin and cytotoxicity of NK cells as well as proliferation and cytotoxicity of $CD8^+$ T cells [104, 108, 122, 259, 260].

1.6 Interferons

Interferons (IFNs) were originally discovered and characterised as soluble proteins that induce anti-viral activity in target cells [261]. They are classified in type I and type II IFNs and are collectively know as type II cytokines. Both types of IFNs exhibit potential anti-viral activity. Type I IFNs are more widely expressed and hence play a much more important role in the innate immune response to viral infections, while type II IFNs have a preferential role in the subsequent acquire immune response. IFNs act on the target cell, not on the virus, to confer a state of resistance to viral infectivity at one or more phases of the viral replication cycle.

Beside their anti-viral activity, these cytokines exert a number of other diverse biological effects. They inhibit the proliferation of normal and transformed cells, regulate differentiation, mediate host responses to various pathogens, and modulate the immune system through actions such as activating NK cells and macrophages (reviewed by [262, 263]).

1.6.1 Type I Interferons

Type I IFNs are a family of monomeric secreted proteins that have been categorised into IFN-α, IFN-β and IFN-ω based on their immunogenic properties and amino

acid sequences. IFN-α and IFN-ω species are mainly secreted by leukocytes, whereas IFN-β is primarily produced by fibroblasts. The family of IFN-α genes is now known to consist of at least 14 different members, which encode 12 different proteins. The IFN-α species are partially homologous in amino acid sequence (75-80%) and generally display a high level of species specificity in their biological properties (reviewed by [263]).

In general, exposure of cells to viruses and dsRNA induce the production of IFN–α and IFN–β. The ratio of production of these IFNs by stimulated cells varies with the tissue of origin and the species of the organism [264]. Studies using IFN–β null mice have shown that the production of IFN–α by certain cells is dependent on IFN–β [265].

1.6.2 The IFN-α/β receptor

The IFN-α/β receptor (IFN-α/β–R) is composed by two subunits: IFN-α receptor 1 (IFNAR1) and IFN-α receptor 2 (IFNAR2), both members of the cytokine receptor class II family [266]. IFNAR2 has three splice variants, two membrane bound: IFNAR2c and IFNAR2b with longer and shorter intracellular domains, respectively and IFNAR2a, which is a soluble secreted form [266, 267]. Only IFNAR2c is thought to be competent for signalling through the Jak/ STAT pathway (see below and [268]). IFNAR2c seems to be the major ligand-binding component of the receptor complex, because it has an affinity for several IFN–α subtypes, as compared to IFNAR1, which plays a more important role in signal transduction (for review see [262, 263]).

The presence of both IFNAR1 and IFNAR2 is required for high affinity IFN-α/β binding, the IFN-α/β-dependent activation of Jak kinases and IFN-α/β-induced anti-viral activities [266, 267, 269-275]. High affinity IFN-α/β–R are constitutively expressed on naive T cells. Following T cell activation the number of receptors augments paralleling the increase in cell size, to give nearly constant density of IFN-α/β–R [276, 277].

1.6.3 Biological functions of IFN-α/β

The biological relevance of IFN–α/β *in vivo* has been studied through targeted disruption of the IFNAR1 gene [271]. Mice lacking IFNAR1 do not show overt abnormalities but are unable to cope with viral infections, despite otherwise normal immune responses [271]. These mice mount normal Th and Tc mediated immune responses and produce normal amounts of neutralising antibodies (IgM and IgG) following viral infections suggesting a deficiency in innate rather than acquire immunity [271].

In vitro studies have implicated IFN–α/β as important mediators in the regulation of lymphocyte development, immune responses and the maintenance of immunological memory of Tc cells [278-282]. IFN–α/β enhance the expression of MHCI molecules therefore promoting the development of $CD8^+$ T cell responses and are involved in IgG class switch [283-285]. Most important for the present work, IFN–α has been proposed as an alternative cytokine to IL-12 for driving Th1 cell differentiation in humans (see below and [21, 286]).

1.6.4 Interferon type II

IFN–γ also known as Type II IFN, is a noncovalent homodimer that consists of two identical 17 kDa polypeptide chains. IFN–γ is induced by immune and inflammatory stimuli and is produced by both innate and adaptive immune cells, including NK cells, $CD8^+$ T cells and $CD4^+$ T cells. NK cells are an important source of IFN–γ during early stages of infection and can secrete IFN–γ upon initial activation. In contrast $CD4^+$ T cells produce little IFN–γ on primary activation and require cytokine-dependent differentiation to acquire this capacity (reviewed by [287]). The control of IFN–γ production will be discussed in more detail within the context of Th1 cell differentiation further below (section 1.11.10).

1.6.5 The IFN-γ receptor

The IFN–γ receptor (IFN–γR) is composed of two type I integral membrane proteins named IFN–γR1 and IFN–γR2. IFN–γR are expressed on nearly all cell types and display strict species specificity in their ability to bind IFN–γ (reviewed by [287]). IFN–γR1 plays important roles in mediating ligand binding, ligand trafficking through the cell and signal transduction, while IFN–γR2 plays only a minor role in ligand binding but is required for signalling (reviewed by [262]). High affinity IFN-γR formed by two molecules of each IFN–γR1 and IFN–γR2, are constitutively expressed on naive T cells [277]. Following IFN–γ binding the receptor-ligand complex is internalised, the complex dissociates and IFN–γR1 is recycled back to the cell surface (reviewed by [287]). However, the number of high affinity receptors is approximately twofold lower following T cell stimulation [277]. This is due to IFN–γ-induced desensitisation by down-regulation of IFN–γR2 mRNA and protein expression [92-94]. This mechanism occurs in T cells

but not in all cell types and is thought to prevent the anti-proliferative effects of IFN–γ, especially on Th1 cells.

1.6.6 Biological functions of IFN-γ

Inactivation of the IFN–γR by gene targeting in mice, together with studies of naturally occurring human genetic defects have defined the roles of IFN–γ *in vivo*. In humans complete deficiencies of both IFN-γR1 and IFN-γR2 as well as partial deficiencies of IFN-γR1 have been described (reviewed by [103]). These deficiencies are characterised by selective susceptibility to infection with mycobateria of low-grade virulence and sometimes also *Salmonella sp.*. Patients with partial deficiencies develop curable disseminated infections, while individuals with complete deficiencies suffer from severe and often fatal infections [103]. IFN-γR1 null mice have no overt abnormalities, and their immune system appears to develop normally. These animals display a greatly impaired ability to resist infection by a variety of microbial pathogens including *L. monocytogenes*, *L. major* and several mycobacterial species, such as *M. bovis* and *M. avium* [288, 289]. However, these mice develop normal Th and Tc responses to these pathogens [288, 289]. IFN-γR1 null mice are also susceptible to vaccinia virus infection [271].

IFN-γ promotes both specific and non-specific mechanisms of host defence against infectious agents and tumours. IFN-γ activates microbicidal mechanism of macrophages that are responsible for the control and elimination of intracellular infectious pathogens. It can also exert profound anti-proliferative effects on a variety of normal and neoplastic cells. IFN–γ is one of the major cytokines that respectively up-regulates and induces the expression of MHCI and MHCII proteins on a variety of leukocytes and epithelial cells (reviewed by [285, 290]). In addition

IFN–γ regulates humoral immune responses by effecting IgG heavy chain switching and regulates the production of other immunomodulatory cytokines such as IL-12 and TNF–α [43, 44, 291].

Comparative studies between mice deficient in receptors for type I and type II IFNs have shown that IFN–α/β and IFN-γ play separate and complementary roles in response to specific viruses. While the response against vesicular stomatitis virus (VSV) or Semliki Forest virus (SFV) primarily require IFN–α/β effects, clearance of other viruses such as vaccinia and LCMV requires the co-operation of both IFN–α/β and IFN-γ [271].

1.7 The Jak/ STAT signalling pathway

The molecular characterisation of IFNs-regulated gene expression lead to the discovery of the Janus kinases/ Signal Transducers and Activators of Transcription (Jak/ STAT) signalling pathway (reviewed by [262, 292-295]). This pathway has proved to be integral to both types of IFNs and to all cytokines whose receptors are members of the cytokine receptor superfamily.

1.7.1 The Jak family of tyrosine kinases

Unlike growth factor receptors and the TGF-β/ activin family of receptors, which respectively have intrinsec tyrosine and serine/ threonine kinase activity, cytokine receptors lack intrinsec catalytic activity. Rather, they associate with a structurally unique class of tyrosine kinases, the Janus kinases (Jak). Four mammalian Jaks have been identified: Jak1, Jak2, Jak3 and Tyk2 (reviewed by [262, 292, 294]).

Genetic analyses in other organisms have also revealed the presence of Jaks in *Drosophila*, zebra fish and chicken [296-299]. Jak1, Jak2 and Tyk2 are ubiquitously expressed. Jak3 expression is predominantly limited to myeloid and lymphoid cell types. Jak3 is inducibly expressed upon T cell activation and in activated monocytes and B cells [300-303].

Structural analyses of all Jak kinases revealed seven homologous motifs termed Jak homology domains (JH1-7) (Figure 1.6 A). The most significant is the tandem kinase motif, whereby a pseudokinase domain (JH2 domain) is flanked COOH to a functional kinase domain (JH1 domain). Only JH1 appears to be catalytically active, but both JH1 and JH2 are required for full activity (reviewed by [292, 294, 304]). Jaks are constitutively associated with many cytokine receptors, however this association is augmented following ligand stimulation in several systems. The mechanism underlying this augmentation has not been identified. The N-terminal region of the Jaks and the box1/ box2 motifs within the receptor cytoplasmic tails are crucial in mediating Jak/ receptor association [305-309].

Initially the functional importance of Jak kinases in cytokine signalling was shown using mutagenised cell lines that lacked biological responsiveness to IFNs (reviewed by [262, 292, 294]). Jak1- or Tyk2-deficient cells were unresponsive to IFN-α/β, whereas Jak1- or Jak2-deficient cells did not respond to IFN-γ [310-312]. Complementation assays then demonstrated the requirement of Jak1/ Tyk2 and Jak1/ Jak2 for full IFN-α/β and IFN-γ signalling, respectively [310-312].

All cytokine type I receptors studied to date are associated with Jaks and require the Jak kinases activity for induction of down-stream signals (Table II). For example, IL-12 requires the activation of Tyk2 and Jak2, which are respectively associated with IL-12Rβ1 and IL-12Rβ2 [313, 314]. IFN-γ activation requires both IFN-γR1 and IFN-γR2 subunits to be associated with Jak kinases (reviewed by

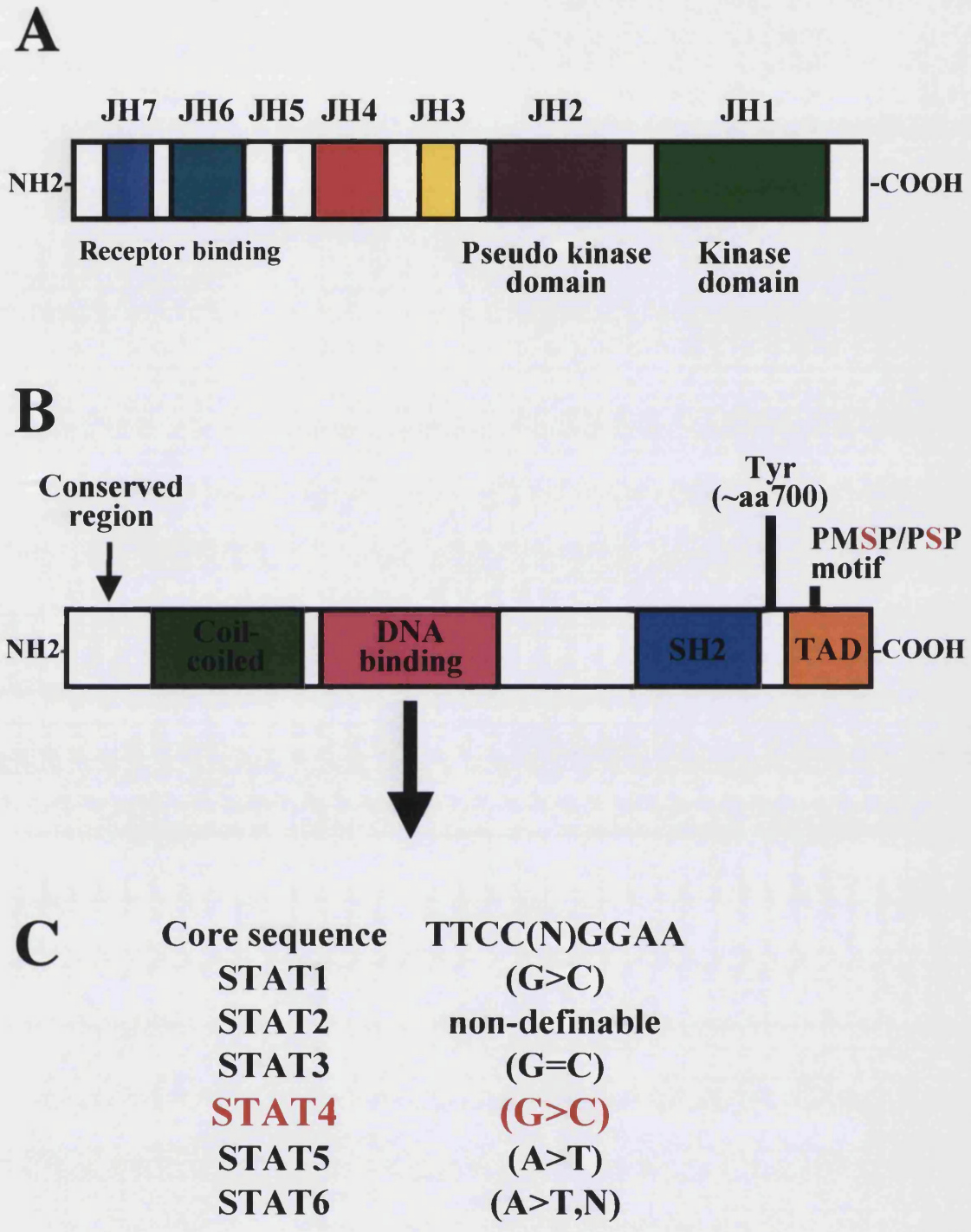


Figure 1.6) Structure of Jaks and STATs.- **A)** Mammalian Jaks structure. The Jak homology (JH) domains 1-7 are indicated. JH1, the kinase domain and JH2 the pseudokinase domain are required for full Jak activity. The N-terminal region is involved in association with cytokine receptors. **B)** Mammalian STATs. The conserved N-terminal region, coil-coiled domain involved in dimer-dimer interactions (green), DNA-binding domain (fuchsia), Src homology domain 2 (SH2, blue) required for STAT dimerisation and the transactivation domain (TAD) are indicated. Following cytokine stimulation, STATs are phosphorylated on a critical tyrosine residue located at approximately residue 700. STAT1, STAT3, STAT4 and STAT5 are also phosphorylated on a serine residue localised within the PMSP/ PSP motif in the TAD. **C)** The core DNA sequence for STATs binding as well as the preferred binding sequence for each STAT are listed. See text for further details.

Receptor Subtypes	Activated Jaks	Activated STATs
Type I cytokines		
Cytokines that share γc		
IL-2, IL-7, IL-9, IL-15	Jak1, Jak3	STAT5A, STAT5B, STAT3
IL-4	Jak1, Jak3	STAT6
IL-13*	Jak1, Jak2, Tyk2	STAT6
Cytokines that share βc		
IL-3, IL-5, GM-CSF	Jak2	STAT5A, STAT5B
Cytokines that share gp130		
IL-6, IL-11, OSM, CNTF, LIF, CT-1	Jak1, Jak2, Tyk2	STAT3
Cytokines with gp130-related receptors		
IL-12	Jak2, Tyk2	STAT4
leptin	Jak2, Tyk2	STAT3
Cytokines with homodimeric receptors		
Growth hormone	Jak2	STAT5A, STAT5B, STAT3
PRL, EPO, TPO	Jak2	STAT5A, STAT5B
Type II cytokines		
IFN-α, IFN-β	Jak1, Tyk2	STAT1, STAT2, STAT3, STAT4, STAT5
IFN-γ	Jak1, Jak2	STAT1
IL-10	Jak1, Tyk2	STAT3

Table II) A summary of the Jaks and STATs that are activated by different hematopoietin cytokines.- *IL-13 does not share the γc chain but uses the IL-4Ra chain. Abbreviations: IL-: interleukin; GM-CSF: granulocyte-macrophage colony stimulating factor; OSM: oncostatin M; CNTF: cyliary neutrophilic factor, LIF: leukaemia inhibitory factor; CT-1: cardiotrophin-1; PRL: prolactin; EPO: erythropoietin; TPO: thrombopoietin; IFN-: interferon.

[262, 263]). Jak1 specifically associates with the IFN-γR1 chain, while Jak2 associates with the IFN-Rγ2 chain [315-317]. Similarly, Jak1 and Tyk2 that respectively associate with IFN–αR2 and IFN–αR1, are phosphorylated in response to IFN–α [272, 274]. All IL-2 related cytokines function by activating Jak1 and Jak3. While the common γc associates with Jak3, the IL-2Rβ-chain and all unique transducer subunits of IL-2 related cytokines associate with Jak1 [172, 173, 318, 319].

Ligand binding induces homo- or heterodimerisation of cytokine receptor chains. This dimerisation juxtaposes Jaks associated with each receptor chain, allowing transphosphorylation and Jak activation. This conclusion is supported by biochemical evidence that Tyk2 is not phosphorylated in mutant cell lines that lack Jak1 after IFN–α/β stimulation [311]. Similarly, IL-2 and γc-dependent cytokines cannot activate Jak1 in the absence of Jak3, and Jak1 phosphorylation in response to IFN-γ requires Jak2 [320]. However, no all Jaks are interdependent for tyrosine phosphorylation and consequent activation. In Jak1-deficient cells reconstituted with kinase dead Jak1, IFN-γ can still induce sub-optimal Jak2 phosphorylation and low levels of downstream signals [321]. The model proposed for IFN-γ signalling is that Jak2 phosphorylates itself and Jak1 [322]. Auto-phosphorylation of Jak2 also occurs in the case of cytokine receptors that homodimerise, such as growth hormone (GH) and EPO and those in which only Jak2 is required for receptor association as is the case of IL-3, IL-5 and GM-CSF [322].

1.7.2 Biological functions of Jaks

The essential role of Jaks *in vivo* has been demonstrated by targeted gene disruption in mice. Jak-1 deficient mice are small, fail to nurse and die within 24 hours of birth

[323]. Neurones from these mice fail to respond to gp130-dependent cytokines (IL-6, IL-11, CNTF, cardiotrophin-1 (CT-1), oncostatin M (OSM) and leukaemia inhibitory factor (LIF)) and die by apoptosis. This failure is accompanied by a strikingly reduced number in sensory neurones and therefore seems to be the cause of perinatal lethality. Lymphopoiesis but not myelopoiesis is severely impaired due to loss of IL-7 receptor signalling. Together with impaired responses to gp130-dependent cytokines, Jak1-deficient cells fail to respond to IFNs and γc-dependent cytokines (IL-2, IL-4, IL-7, IL-9 and IL-15) [323].

Jak2 null mice exhibit embryonic lethality, due to the absence of definite erythropoiesis [324, 325]. As expected, Jak2-deficient cells fail to respond to EPO, TPO, IL-3, GM-CSF and IFN-γ [324, 325]. Jak2-deficient foetal liver cells have the ability to reconstitute T cells in sublethally irradiated Jak3-deficient mice (deficient in T and B cells, see below), indicating that Jak2 is not required for T or B cell development [324].

As mentioned in section 1.5.1, X-SCID in humans is caused by mutations in the γc shared by IL-2, IL-4, IL-7, IL-9 and IL-15 ([154, 160, 162] and reviewed by [163]). Since Jak3 associates with the γc, it was hypothesised that disruption of Jak3 would cause a similar phenotype [173]. Indeed, some patients with SCID due to Jak3 mutations have been identified [326, 327]. Accordingly, Jak3- and γc-deficient mice exhibit identical immunological phenotypes, characterised by deficient NK, B and T cell lymphopoiesis and loss of all cellular responses mediated by γc-dependent cytokines (IL-2, IL-4, IL-7, IL-9 and IL-15) [164-166, 328-330].

Tyk2 null mice do not show developmental abnormalities but have reduced responses to IFN–α/β and IL-12 for STATs tyrosine phosphorylation and IFN–γ production, respectively [331, 332]. These mice have reduced anti-viral activities, inability to clear vaccinia infections and reduced T cell responses to LCMV challenge [331, 332]. High concentrations of IFN–α/β or IL-12 moderately recover

induction of MHCI expression and Th1 cell differentiation, respectively, indicating that Tyk2 is only partially required for responses to these cytokines [332].

1.7.3 STATs

Seven mammalian STAT genes have been cloned: STAT1-6, with two different genes encoding STAT5A and STAT5B [293]. STAT1 and STAT3 have two naturally occurring splice variants (α and β). The β splice variants are C- terminal truncated forms. Invertebrate STATs have also been described in *Drosophila, C. elegans* and *Dyctostelium* [333-335]. Somatic cell genetics indicate Jaks and STATs belong to a common conserved pathway required for IFN signalling (reviewed by [262, 292-295]). Biochemical and molecular analysis revealed STATs are direct substrates of Jak kinases and are tyrosine phosphorylated in cytokine activated cells. All STAT proteins contain the following domains: highly conserved NH2 terminal domain, conserved COOH SH2 domain, COOH tyrosine phosphorylation site, a DNA binding domain and a conserved COOH terminus (Figure 1.6 B).

1.7.4 Prototypical model for Jak/ STAT activation

The binding of a ligand to its receptor induces homo- or heterodimerisation of cytokine receptor chains [1]. This dimerisation juxtaposes Jaks associated with each receptor chain, allowing trans-phosphorylation and Jak activation. Jak activation leads to the tyrosine phosphorylation of receptors that generates docking site(s) for STATs via the STAT SH2 domain. Subsequently, recruited STATs are phosphorylated by activated Jaks on the COOH tyrosine phosphorylation site. This allows homo- and hetero-dimerisation of STATs through reciprocal SH2-phosphotyrosine interactions. Active dimerised STATs translocate into the nucleus where they bind to high affinity DNA motifs containing the consensus palindromic

inverted repeat sequence TTC(N_{2-4})GAA [336-338]. Each individual STAT has particular DNA binding affinity for specific sequences (Figure 1.6 C). This depends not only on the STAT DNA binding domain itself but also on the different homo and heterodimer combinations that it forms [338-341]. STAT dimers can also polymerise through mutual N-terminal interactions to bind DNA co-operatively, enabling the recognition of variations of the consensus site [342-344]. The conserved COOH terminus domain acts as a transactivation domain (TAD) (reviewed by [293, 345]). A schematic representation of the prototypical model of Jak/ STAT activation is shown in Figure 1.7.

1.7.5 STATs Serine phosphorylation

Beside the essential role of tyrosine kinases, the requirement for serine/ threonine kinases in signal transduction pathways is well recognised (for review see [346]). Comparing the C- termini of STAT1, STAT3 and STAT4, it was noted that a PMSP motif between aminoacids at positions 720 and 730 of these molecules is perfectly conserved [347]. In addition, STAT5A and STAT5B contain a conserved PSP motif in an equivalent position [348]. PMSP and PSP sequences are potential serine phosphorylation sites for proline-directed kinases, particularly the MAPK family [349, 350]. The recognition of these motifs suggested the possibility of a second post-translational modification by serine phosphorylation on the PMSP/ PSP motifs of STAT proteins. The use of phospho-peptide mapping, phospho-amino acid and mass spectrometry analyses, as well as the development of specific anti-phosphoserine antibodies has lead to the description of serine phosphorylation in STAT1, STAT3, STAT4 and STAT5 [184, 185, 348, 351-354]. Most studies have concentrated on the biochemical and functional analysis of serine phosphorylation of STAT1 and STAT3, but little is known about STAT4 and STAT5 serine phosphorylation.

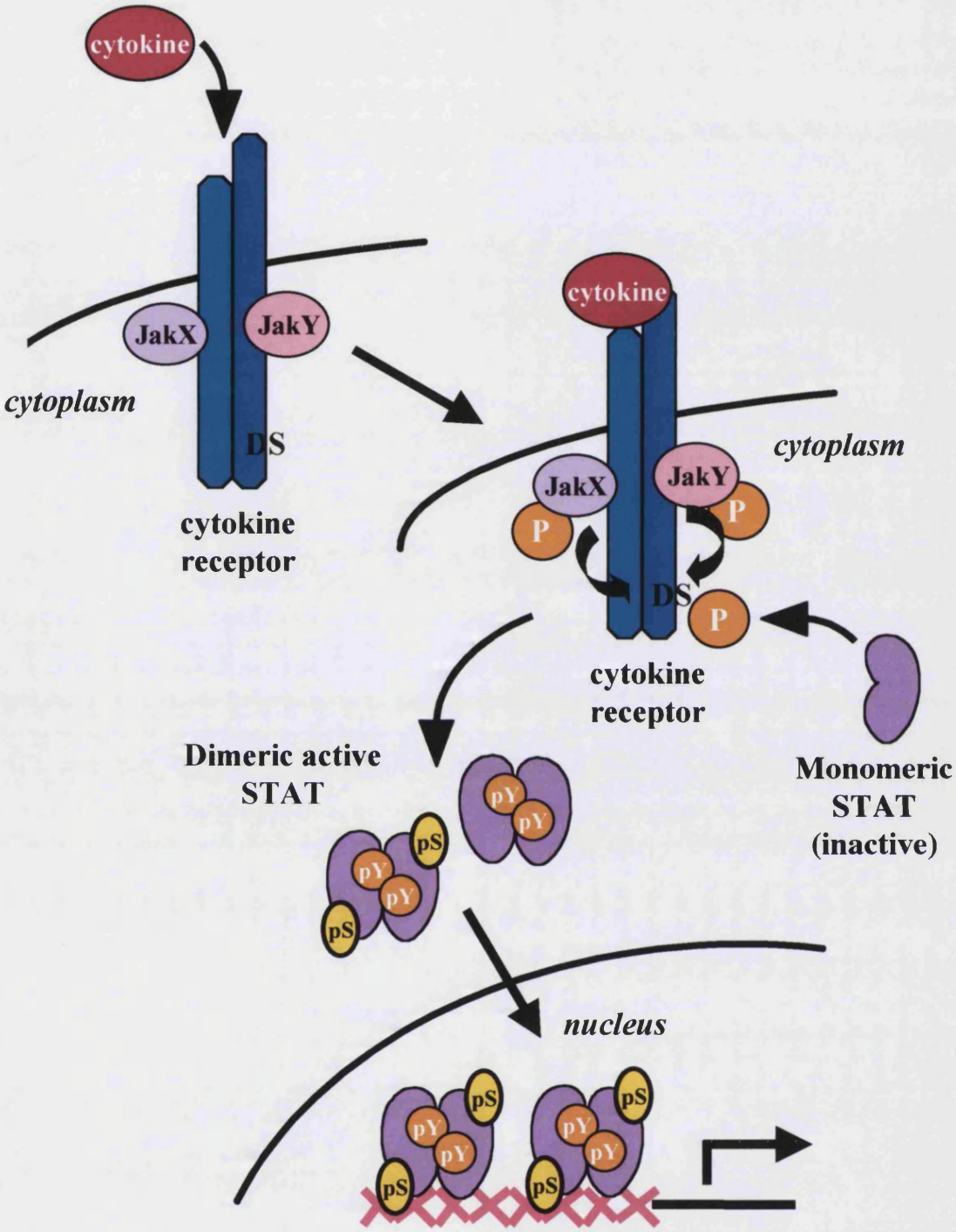


Figure 1.7) Prototypical model for Jak/ STAT activation.- The cytokine interacts with the extracellular domains of its corresponding receptor chains. The cytokine/ cytokine receptor interaction leads to activation of the receptor associated Jaks (referred as JakX and JakY). Active JakX and JakY phosphorylate tyrosine residues contained in the cytoplasmic tail of the cytokine receptor which serve as docking sites (DS) for monomeric inactive STATs. Phosphorylation of the putative STATs DS allows recruitment of the STAT into the cytokine/ cytokine receptor complex. Active JakX and JakY then phosphorylate STATs on tyrosine residues (pY), leading to formation of homodimers though mutual pY-SH2 domain interactions. Some STATs are also phosphorylated on serine residues (pS). Active dimeric STATs translocate into the nucleus where they interact with specific DNA binding sequences. See text for further details.

STAT1 is phosphorylated on Ser727 within the PMSP motif in response to IFN–α, TcR/ BcR engagement, IFN–γ, LPS and environmental stress in fibroblasts, lymphocytes and macrophages, respectively [347, 354-356]. The serine phosphorylation site of STAT3 also occurs on Ser727 within the PMSP motif. STAT3-α Ser727 phosphorylation is induced in response to epidermal growth factor (EGF), insulin, OSM, LIF, IL-6, IFN–α, IL-2 and TcR engagement in fibroblasts and lymphocytes, respectively [185, 347, 357, 358]. STAT5A is constitutively phosphorylated on Ser725 within the PSP motif and also on Ser779 [348, 359]. The constitutive levels of STAT5A-pS725 and STAT5A-pS779 are enhanced following prolactin (PRL) stimulation [348, 359]. One serine that is phosphorylated in STAT5B is Ser730 within the PSP motif, and is induced by PRL stimulation [348].

Serine phosphorylation of STAT1-αSer727 (STAT1-αpS727) is essential for maximal transcriptional activity of STAT1. This has been demonstrated by assaying IFN–γ induction of the endogenous IFN regulatory factor-1 (IRF-1) gene in cell complementation studies using the STAT1-αS727A mutant in STAT1 deficient cells [347]. The link between serine phosphorylation of STAT1-αS727 and increased transcription was strengthened by studies showing that stimuli causing serine phosphorylation increased the expression of a STAT1 target gene following further stimulation with IFN–γ [354, 355, 360, 361]. STAT1-α serine phosphorylation is also essential for IFN–γ and IFN–α-mediated anti-viral responses [347, 354, 355, 362, 363]. Transient transfection studies using the STAT3-αS727A mutant indicate that serine phosphorylation of STAT3-αS727 is required for maximal transcriptional activity [347].

The increased transcriptional activity of STAT1-αpS727 is possibly due to protein interactions occurring preferentially after serine phosphorylation. Transcriptional

co-factors and co-activators are attractive candidates for such STAT-interacting proteins (reviewed by [364]). For example, serine phosphorylation of STAT1-αS727 regulates the interaction of tyrosine phosphorylated STAT1 dimers with MCM5, a member of the mini chromosome maintenance (MCM) family involved in DNA replication [365].

Serine phosphorylation of STATs seems to be differentially required for the transcription of specific genes. Phosphorylation of STAT1-αS727 is required for maximal transcriptional activity and anti-viral effects of the STAT1 homodimer in response to IFN–γ and IFN–α [347, 354, 355, 362, 363]. However, it is dispensable for the assembly and transcriptional activity of the STAT1/ STAT2/ IRF-9 (ISGF-3) complex in response to IFN–α. ISGF-3 functions in STAT1 deficient cells after complementation with either STAT1–β that lacks the entire C-terminus or the STAT1-αS727A mutant [311, 362]. Similarly, cell complementation studies using the STAT1-αS727A mutant in STAT1 deficient fibroblasts have recently shown that serine phosphorylation of STAT1-α is essential for the transcription of endogenous GBP-1, but only partially required for the transcription of IRF-1, SOCS-1 and SOCS-3 [366].

A role for serine phosphorylation in STAT inactivation has also been proposed [357]. Naturally occurring C- truncated variants of STAT1 and STAT3, as well as short variants of STAT5 exhibit constitutive tyrosine phosphorylation or delayed tyrosine dephosphorylation and prolonged DNA binding [343, 367-369]. Evidence derived from *in vitro* studies using STAT3-αS727A mutants or serine kinase inhibitors are in agreement with this proposal. STAT3-αS727A mutants show basal tyrosine phosphorylation as well as enhanced EGF-induced tyrosine phosphorylation [357]. Similarly, STAT5A-S725A mutants show prolonged DNA binding activity in response to PRL [359]. Inhibition of the MEK kinases involved

in STAT3-αS727 phosphorylation or general block of serine kinases also resulted in enhanced and prolonged STAT3 and STAT5 tyrosine phosphorylation [357, 370]. Cell complementation analysis with STAT3-αS727A mutants in STAT3-deficient cells have not been reported. These studies are required to conclusively define which of the proposed roles for STAT3-α serine phosphorylation is involved in STAT3-dependent responses.

The serine and tyrosine phosphorylation of STAT molecules can be linked or independently regulated, depending on the stimulus and cell type. Serine phosphorylation of non-tyrosine phosphorylated STAT1-αS727 and STAT3-αS727 are induced by TcR and BcR triggering in human and mouse lymphocytes [185, 356]. Similarly, regulation of STAT1-αS727 phosphorylation by LPS and IFN–γ in monocytes and fibroblasts is independent of STAT1-α tyrosine phosphorylation [354, 371]. Important cellular functions have been described for non-tyrosine phosphorylated STATs. For example, serine but not tyrosine phosphorylation of STAT1-α (STAT1-αpS727) is required for control of basal levels of caspases and consequent regulation of programmed cell death in fibroblasts [372]. Phosphotyrosine-dependent serine phosphorylation events have also been reported. IFN–γ-induced serine phosphorylation of STAT1-αS727 in mouse fibroblasts requires tyrosine phosphorylation and intact SH2 domain interactions [366]. Serine phosphorylation of STAT3-αS727 in human fibroblasts requires Jak2 activity and is preceded by tyrosine phosphorylation [371, 373].

Although serine phosphorylation of STAT proteins can be induced independently of tyrosine phosphorylation, to date there are no reports of cytokine receptor-derived signals than can induce tyrosine phosphorylation without serine phosphorylation of a particular STAT. Therefore, a unidirectional exploitation of the possibility to uncouple tyrosine and serine phosphorylation seems to predominate in the STAT system. This suggests that serine phosphorylation alone

is a means of priming STATs for an unaltered transcriptional response once a second stimulus causing tyrosine phosphorylation is received. From the current literature we would infer primed STATs to cause increased transcription in most cases, but the negative effect proposed for STAT3 and STAT5 serine phosphorylation complicates the issue and demands further clarification (reviewed by [374]).

1.7.6 Biological functions of STATs

Two different kinds of studies have highlighted the biological relevance of STATs *in vivo*. To date each STAT gene has been eliminated by gene targeting disruption in mice and at least one human abnormality is caused by reduced levels of STAT protein [9, 375, 376].

STAT1-deficient mice exhibit a selective and severe defect in IFNs-dependent immune responses against virus and microbial pathogens [377, 378]. The defect in IFN–γ signalling shows striking resemblance to the IFN–γR1 knock out mice (section 1.6.6), suggesting that there are a few STAT1-independent signals stimulated by IFN–γ [288, 289, 377, 378]. STAT2 null mice have increased susceptibility to viral infection and loss of the IFN–α/β autocrine/ paracrine loop. Lack of STAT2 is more relevant for fibroblast than for macrophage function [379]. However, STAT1- and STAT2-deficient mice retain the ability to respond to other cytokines and have no apparent abnormalities in development. The phenotype of these mice demonstrates the importance of STAT1 and STAT2 in IFN–γ and IFN–α/β signalling, respectively and their role in antiviral activities.

STAT3-deficient mice die early in embryogenesis [380]. The timing of degeneration of STAT3 null embryos coincides with the onset of STAT3 expression in visceral

endoderm in wild type mice [381]. This suggests that lethality in STAT3-deficient mice may be due to a defect in visceral endoderm function, such as nutritional insufficiency [382]. The role of STAT3 in adult tissues has been assessed by conditional knock out of the STAT3 locus in a cell type specific manner using the Cre-loxP system [382-384]. Mice lacking STAT3 in T cells show a defect in proliferation in response to IL-2 and IL-6 [382, 383]. STAT3-deficient macrophages are unresponsive to IL-10, show polarised immune responses towards the Th1 phenotype and develop chronic enterocolitis with age [384].

STAT5A deficient mice are notably impaired in their response to PRL, resulting in defective mammary gland development and lactogenesis [385]. STAT5B null mice show that this molecule mediates the sexually dimorphic effects of GH in the liver [386]. STAT5A/ STAT5B double knock out mice show multiple hematopoietic defects explained by loss of cytokine induced proliferation in response to PRL, GH, EPO, IL-2 and members of the IL-3 family (IL-3, IL-5 and GM-CSF) [387-389].

STAT6 null mice are viable and develop normally but have specific defects in IL-4 and IL-13 responses [390-393]. The essential role of STAT6 in Th2 cell differentiation is illustrated by the lack of Th2 cell development in response to IL-4 in these mice. Lack of Th2 cell responses explains why these mice are protected from antigen-induced airway hyper-responsiveness and cannot mount an immune response to helminthic parasites [394-396]. For example, STAT6 mice are unable to expulse the gastrointestinal nematode parasite *N. brasiliensis* [396]. These mice also have enhanced tumour rejection since lack of Th2 cell responses and derived cytokines allows enhanced Th1 cell responses [397, 398]. STAT6 null T lymphocytes do not up-regulate MHCII, CD23 and IL-4Rα expression in response to IL-4 and B lymphocytes are defective in Ig class switch to IgG1 and IgE [390-393].

The biological function of STAT4 is of particular interest for the studies conducted in the present work. To date, STAT4 is the only STAT that has been associated with human deficiencies. Severely reduced levels of STAT4 protein have been found in patients with Sezary syndrome, a leukaemic phase of cutaneous T cell lymphoma that is characterised by the proliferation of clonally derived Th2 cells [9]. The presence of exaggerated Th2 cell responses may be the result of reduced Th1-derived inhibitory signals.

STAT4 deficient mice are viable and fertile, with no detectable defects in hematopoiesis. Most importantly, the phenotype of these mice demonstrates the essential role for STAT4 in IL-12 mediated Th1 cell differentiation [7, 8]. STAT4 null T cells are dramatically impaired in their ability to develop into Th1 cells in response to IL-12. STAT4 deficient NK cells fail to produce IFN–γ or kill target cells [7, 8]. Studies with these mice confirm that the capacity of IL-12 to promote the production of IFN–γ by T and NK cells, is required for resistance to intracellular pathogen infections. For example, STAT4-deficient mice are unable to control challenge with *T. gondii* and die during the acute phase of infection [399]. IL-12/ STAT4 responses are also critical for protective Th1 cell responses following *L. major* infection. This is demonstrated by development of large non-healing lesions in *L. major* infected STAT4 null mice [400]. Although Th1/ Th2 mixed responses are found following infection with *T. cruzi*, IL-12/ STAT4-induced Th1 cell responses are critical for the clearance of this pathogen. STAT4 deficient mice are highly susceptible to *T. cruzi* infection, showing increased parasitemia levels relative to wild type mice and 100% mortality [401].

Interestingly, the generation of STAT4/ STAT6 double knock out mice has shown a STAT-independent pathway for Th1 cell development [402]. These mice are unable to generate Th2 cells supporting the critical role of STAT6 in Th2 cell development. Distinctively from the STAT4 knock out mice, STAT4/ STAT6 double deficient lymphocytes are capable of generating IFN–γ producing Th1 cells,

though the amount of IFN–γ produced is still approximately four fold less than in IL-12 stimulated wild type cells [402]. This reduction is not due to a decrease in the number of IFN–γ producing cells but rather in the amount of IFN–γ produced per cell. These results suggest that STAT4 is not absolutely required for the generation of IFN–γ producing Th1 cells but is instead necessary for promoting optimal IFN–γ production from Th1 cells (see below and [403]).

1.7.7 STATs and oncogenesis

Studies of clinical tumour samples and *in vitro* transformation analysis have found a relationship between constitutively activated STATs and human oncogenesis (reviewed by [404-410]). Studies in cell lines using anti-sense molecules or dominant negative STAT protein encoding constructs, and STAT knock out mice, have shown that STATs play an important role in controlling cell cycle progression and apoptosis. STAT1 is involved in growth arrest, in promoting apoptosis and is implicated as a tumour suppressor. STAT3 and STAT5 promote cell cycle progression and cellular transformation and prevent apoptosis. Several lines of evidence suggesting a relationship between constitutive activation of STATs and oncogenesis have been recently reviewed (see [404-410]).

Deregulated serine/ threonine phosphorylation of STAT molecules is also seen in diverse oncological conditions. For example, constitutive serine phosphorylation of STAT1-α and STAT3-α is observed in chronic lymphocytic leukaemia (CLL) and lymphomas in humans [409, 411-413]. These observations have clinical significance since treatment of CLL patients with fludarabine, the most effective chemotherapeutic agent against CLL, leads to a specific loss of STAT1 protein expression. This drug also inhibits IFN–α-induced STAT1 activation and decreases STAT1 mRNA levels *in vitro* [414]. Serine phosphorylation of STAT3-α is

necessary for cell transformation by v-src, because the oncogenic potential of constitutively active STAT3 is reduced by overexpression of serine mutants of STAT3-α or inhibition of STAT3-α serine phosphorylation [415-417].

1.7.8 Association of STATs with other proteins

STATs associate with a number of proteins such as non-STAT transcription factors, co-activators, negative regulatory proteins and proteins involved in nuclear translocation (Table III). These STAT interacting proteins function to modulate STAT signalling at various stages and mediate the cross-talk of STATs with other signalling pathways (reviewed by [364, 418, 419]).

Different mechanisms for transcriptional activation by STATs have been proposed [418]. Active STATs may bind to their own DNA target site to directly drive transcription. Alternatively, they may form a transcriptional complex with a non-STAT transcription factor. These STAT/ non-STAT complexes would trigger transcription by binding to a STAT or a non-STAT DNA binding element, or through interactions with clustered independent binding sites [418]. Therefore, these complexes provide a means to modulate the DNA binding specificity of active STATs and enable the regulation of gene expression to be controlled simultaneously by multiple input. Association of IRF-9 (formerly p48), to the STAT1/ STAT2 complex is the best-documented example for modulation of DNA binding specificity (reviewed by [293, 418, 419]). IFN–α stimulation induces the activation of STAT1 and STAT2 leading to the formation of STAT1 homodimers and STAT1/ STAT2 heterodimers. However, STAT1 homodimers and STAT1/ STAT2 heterodimers translocate to the nucleus where they bind to IRF-9. The resulting trimers, named IFN-stimulated gene factor 3 (ISGF-3) in the case of the STAT1/ STAT2 heterodimer, bind to IFN-stimulated regulatory elements (ISREs) [420, 421]. STAT1/ STAT2 heterodimers and STAT1 homodimers without association with

IRF-9 drive gene transcription through gamma activated sequence (GAS) elements (reviewed by [262, 304, 422]).

Active STATs bind to DNA sites distant from the RNA polymerase II initiation sites. Co-activators are histone acetyltransferases involved in chromatin remodelling (reviewed by [423]). The co-activators CREB binding protein (CBP)/ p300 serve as bridges to facilitate contact with the RNA polymerase II and the basic transcription machinery. STAT1, STAT2, STAT5A, STAT5B and STAT6 have been shown to interact with CBP/ p300 by their COOH terminal domain, resulting in a moderate increase of transcription factor activity in transient transfection assays [424-428]. This suggests that the association of STATs with CBP/ p300 is required for STATs to interact with the basic transcriptional machinery to initiate gene transcription. As mentioned above phosphorylation of STAT1-αS727 controls its interaction with MCM5 [365]. However, studies of the association of STAT1 with CBP/ p300 found no evidence for an influence of STAT1-αpS727 [425].

Although the nuclear translocation of STATs following cytokine stimulation has been recently confirmed using confocal microscopy and immuno-fluorescent staining or GFP-tagged STAT molecules, the mechanism involved is far from being defined [429, 430]. High molecular weight transcription factors are imported into the nucleus by active transport through the nuclear pore complex (NPC) (reviewed by [431, 432]). Molecules utilising this nuclear import require specific nuclear localisation signals (NLS) that are absent in STATs (reviewed by [431, 432]). The physical association between STAT1 and NPI-1, which is part of the nuclear transport machinery, suggests a possible mechanism for the nuclear translocation of STATs [433].

STAT	Interacting Protein	Induced gene/ Function
Modification of DNA-binding specificity or co-operative binding to adjacent sites		
STAT1/ STAT1	IRF-9	association with IRF-9 modifies STATs DNA binding specificity from GAS to ISRE sites
STAT1/ STAT2	IRF-9	
STAT1	Spi	ICAM-1 by IFN-γ
STAT1	USF-1	*CIITA* by IFN-γ
STAT3	Spi	C/ EBPδ by IL-6
STAT3	c-Jun	α_2-microglubulin by IL-6 *c-fos* by different ligands matrix metalloproteinase-1 by OSM vasoactive intestinal peptide by CNTF
Enhanced transcriptional activity		
STAT5	glucocorticoid receptor	β-casein by glucocorticoid + PRL?
STAT1	p300/ CBP	
STAT2	p300/ CBP	
STAT5	p300/ CBP	
STAT5	Nmi	enhanced association to CBP?
Inhibition of DNA binding		
STAT1	PIAS1	
STAT3	PIAS3	
Nuclear translocation?		
STAT1	NPI-1	

Table II) Association of STATs with other proteins .- Association with non-STAT transcription factors modulates the DNA binding specificity of active STATs. Interactions with co-activators facilitates the interaction of active STATs with the transcriptional machinery. Association with molecules such as PIAS and NPI-1 have been respectively proposed to inhibit DNA binding of active STATs and to participate in the nuclear translocation of STATs. Examples of some of these interactions are presented. See text for further details.

1.7.9 Negative regulation of the Jak/ STAT signalling cascade

Possible mechanisms by which cytokine/ STAT responses are terminated have been emerging recently. Some evidence suggests the participation of nuclear phosphatases and/ or proteolytic degradation. The cellular distribution of STAT1 following IFN-γ stimulation has been studied in fibroblasts using methionine labelling [434]. A constant cycling of active tyrosine phosphorylated STAT1 molecules into the nucleus, followed by the quantitative return to the cytoplasm of non-phosphorylated STAT1 molecules has been seen, suggesting the existence of a nuclear tyrosine phosphatase that inactivates STAT1 [434]. However, proteolytic degradation of IFN-γ–induced STAT1 and IL-3-induced STAT5 dimers following ubiquitinylation has also been described in fibroblasts [435, 436]. Recently, the effect of the proteosome inhibitors MG132 and lactacystin has been addressed in myeloid (32Dcl) and T (Kit225 and CTLL) cells [437]. Tyrosine-phosphorylated forms of STAT4, STAT5 and STAT6 respectively induced by IL-12, IL-3 and IL-4, were significantly stabilised upon addition of these inhibitors. However, IFN-α regulated STAT1 and STAT2 were not [437]. Therefore, different mechanisms seem to be involved in the inactivation of each particular STAT. This idea is supported by the presence of a small amphipathic α-helical region in STAT5 but not in other STATs, which is required for proteosomal degradation [437].

The SH2 containing protein tyrosine phosphatases (SHP) SHP-1 and SHP-2 provide another level of negative regulation. SHP-1 and SHP-2 negatively regulate the tyrosine phosphorylation of cytokine receptors and Jak kinases, ensuring signal termination after cytokine removal [438-441]. It has been reported that SHP-1 and SHP-2 are associated with the IFN–αR in fibroblasts and become activated following IFN–α stimulation [438, 442, 443]. SHP-1 is also involved in negative regulation of EPO, GM-CSF and G-CSF [444, 445]).

Recently, a novel family of Suppressors of Cytokine Signalling (SOCS) has been implicated in the negative regulation of the Jak/ STAT signalling pathway (reviewed by [418, 446-448]). SOCS are also known as CIS, JAB or SSI. To date seven family members (SOCS1-7) have been identified. These proteins have a centrally located SH2 domain and are defined by the presence of a homologous COOH terminal motif named the SOCS box. SOCS expression is induced upon cytokine stimulation and these proteins then function in a classical negative feedback loop. SOCS inhibit Jak-mediated phosphorylation of STATs by direct interaction with either the Jak kinases themselves or the STAT docking sites on the corresponding receptors [449-454]. The SOCS box mediates an interaction with the elongin BC complex. This complex has been implicated in the regulation of protein stability. Although the physiological consequence of this interaction is not defined, it has been proposed that the SOCS box may help to target associated proteins such as Jaks for degradation [445]. SOCS are involved not only in negative feedback loops but also in negative cross-talk regulation. For example, IFN-γ and IFN-β mediated inhibition of IL-4/ STAT6 activation in monocytes and fibroblasts occurs through a STAT1 dependent mechanism involving the induction of SOCS-1 [455, 456]. Similarly, it has been proposed that IL-10 inhibits IFN-α and IFN-γ-induced STAT responses in monocytes through the induction of SOCS-3 [457].

The Protein Inhibitors of Activated STATs (PIAS) are another family of proteins involved in negative regulation of STATs (reviewed by [418]). To date four PIAS proteins have been identified through database search and cDNA library screening. PIAS1 and PIAS3 have been found to associate with STAT1 and STAT3, respectively. PIAS proteins are constitutively expressed and associate with tyrosine phosphorylated STATs preventing their DNA binding and gene regulation [458-460].

1.8 IFNα/β signalling through the Jak/ STAT pathway

Binding of IFN–α/β to the high affinity IFN–αR induces receptor oligomerisation and consequent activation of Jak1 and Tyk2, which are respectively associated to IFNAR2c and IFNAR1. Jaks activation leads to STATs recruitment and consequent tyrosine phosphorylation of STAT1, STAT2, STAT3, STAT4 and STAT5 ([353] and reviewed by [262, 304, 422]). The subsequent formation of STAT1/ STAT1 homodimers as well as STAT1/ STAT2 and STAT1/ STAT3 heterodimers has been shown (reviewed by [262, 422]). Many studies have concentrated on the gene transcription regulation by STAT1, STAT2 and STAT3, but less is known about genes activated by STAT4 and STAT5 in response to IFN–α/β in human cells.

As mentioned above, STAT1 homodimers and STAT1/ STAT2 heterodimers can drive gene transcription by binding to GAS sequences or can associate with IRF-9 to form ISGF-3 that initiates transcription through ISREs ([420, 421] and reviewed by [262, 304, 422]). STAT3 homodimers and STAT1/ STAT3 heterodimers form sis-inducible factors (SIFs) that bind to the c-sis-inducible elements (SIE) originally described in the c-fos promoter [304].

Based on studies of IRF-9-null human cells, ISGF-3 is by far the most important factor activating the majority of IFN–α/β-inducible genes [461, 462]. ISGF-3 triggers anti-viral responses in cells by inducing several endogenous enzymes that antagonise viral replication, including PKR, the 2-5A system and the Mx proteins (reviewed by [262]). PKR is a dsRNA-dependent protein kinase that plays a pivotal role in establishing the anti-viral state by inhibiting protein synthesis and is required for IFN-dependent activation of NF-κB and IRF-1 (reviewed by [463, 464]). The 2-5A system activation leads to extensive cleavage of single stranded RNA ([465-468] and reviewed by [469-471]). The Mx proteins are GTPases of the

dynamin family that interfere with viral replication, impairing the growth of influenza and other negative-strand RNA viruses at the level of viral transcription and at other steps (reviewed by [472, 473]).

1.9 IFN-γ signalling through the Jak/ STAT pathway

The activation of the Jak/ STAT signalling pathway in response to IFN–γ is the most complete model of cytokine signalling to date (reviewed by [262, 287]). Briefly, functionally active IFN–γ is a homodimer that binds to two IFN–γR1 subunits, thereby generating binding sites for two IFN–γR2 subunits. Within the symmetrical signalling complex, the intracellular domains of the receptor subunits are brought into close proximity, together with the inactive Jaks they carry. Auto- and trans-phosphorylation then sequentially activate Jak2 and Jak1. Activation of Jak2 occurs first and is needed for the subsequent activation of Jak1, which has a structural as well as enzymatic role. Once activated, the receptor-associated Jaks phosphorylate Y440 near the C- terminus of IFN–γR1, which serves as a docking site for STAT1. Two latent STAT1 molecules then bind and are phosphorylated on Tyr701 near the C- terminus. The phosphorylated STAT1 proteins dissociate from the receptor and form a reciprocal homodimer, which translocates to the nucleus apparently through a mechanism dependent on the GTPase activity of Ran/ TC4 [433]. STAT1 homodimers directly bind to GAS elements of IFN–γ-inducible genes.

The ability of STAT1 to activate gene expression may also be modulated by its interaction with other transcription factors. For example, IFN–γ induces STAT1 homodimer-IRF-9 complexes that interact with ISRE and induce the 9-27 gene expression [462, 474]. Induction of the ICAM gene by IFN–γ depends on

interaction of STAT1 and the transcription factor Spi [475]. IFN–γ also induces the 2-5A system, RNAse L, PKR and the Mx proteins (reviewed by [262]). Thus cell type specific gene induction by IFN–γ may be explained, at least in part by the ability of additional cell-specific positive and negative factors that modulate the actions of STAT1.

1.10 IL-2 signalling through the Jak/ STAT pathway

Interaction of IL-2 with the high affinity IL-2R induces receptor heterotrimerisation and triggers a rapid increase in the recruitment of Jak3 and activation of both Jak1 and Jak3. Jak1 and Jak3 associate with IL-2Rβ and the γc, respectively [173-175]. However, it has been recently shown that Jak3 can also associate with the IL-2Rβ chain [309]. This Jak3/ IL-2Rβ interaction is thought to bridge the intracellular domains of the IL-2Rβ and γc chains resulting in maximal receptor signalling. The γc chain-associated Jak3 is required for IL-2 proliferative responses [476-478]. Phosphorylation of IL-2RβY510 leads to the recruitment and consequent activation by tyrosine phosphorylation of STAT5A and STAT5B [181-184]. IL-2 also activates STAT3 in T lymphocytes [182, 183, 185].

Active STAT5A and STAT5B bind to similar core GAS sequences and specific binding sequences for each STAT5 have not been demonstrated. Interestingly, purified STAT5A appears to have a higher DNA binding affinity than STAT5B [479]. In addition to binding as a dimer, STAT5A binds to DNA as a tetrameric form while STAT5B preferentially binds DNA as dimers [479].

IL-2 achieves its biological functions through the induction of a variety of cellular genes including those encoding for IL-2Rα, IL-2Rβ, proteins involved in cell cycle

progression and anti-apoptotic proteins. The expression of some of these genes is regulated at the transcriptional level, in part through STAT5. The best-studied example is the induction of IL-2Rα chain, which requires the co-operation of active STAT5 with the transcription factor Elf-1 (reviewed by [480]). STAT5A and STAT5B function interchangeably in the induction of some of these genes. This has been demonstrated by the lack of expression of cyclin D2, cyclin D3 and cdk6 in STAT5A/ STAT5B double knock out mice, which is not revealed in the single STAT5A and STAT5B knock out mice [388, 481, 482].

1.11 The IL-12/ STAT4 response and its role in Th1 cell differentiation

1.11.1 The link between IL-12, STAT4 and Th1 cell differentiation

Initial *in vitro* studies showed that IL-12 was able to induce the differentiation of Th cells into the Th1 cell phenotype [483-486]. These findings were later confirmed in an *in vivo* mouse model in which injection of IL-12 and antigen resulted in the development of Th1 cell specific responses [487]. In contemporary studies STAT4 was isolated by two different groups using degenerative-PCR or low-stringency hybridisation, both of which were based on homology with the SH2 domain of other STAT proteins [488, 489]. The relationship between IL-12 and the Jak/ STAT signalling pathway was discovered by activation of Jak2 and Tyk2 in human activated T lymphocytes and NK cells in response to IL-12 [313]. IL-12 was then shown to induce the tyrosine phosphorylation and DNA binding of STAT4 in both mouse and human T cells [76, 490]. Finally, gene targeting disruption of IL-12, IL-12Rβ1 and STAT4 in mice, defined the essential role for the IL-12/ STAT4 response in Th1 cell differentiation [7, 8, 66, 100-102]. These findings have been

extended to the human system through studies of patients with mutations in either IL-12, IL-12Rβ1 or IL-12Rβ2, as well as those with low protein expression levels of STAT4 (section 1.4.5 and [9, 67-70])

1.11.2 STAT4 protein expression

Unlike other STATs that are ubiquitously expressed, STAT4 is restricted to lymphoid cells, myeloid cells and spermatogonia [73, 488, 489]. STAT4 expression is inducible in T cells, macrophages and DCs but is constitutive in NK cells [73, 76]. T cells express STAT4 following TcR triggering with anti-CD3 antibodies or by T cell stimulation with PHA [76]. It has been recently shown that STAT4 is expressed in murine DCs in a maturation dependent manner in both $CD8^+$ and $CD8^-$ populations [491]. In monocytes and mature DCs STAT4 expression is induced in response to LPS and IFN–γ [73]. IL-4 and IL-10 block expression of STAT4 in monocytes and during DC maturation but not in mature DCs [491]. The constitutive expression of STAT4 in NK cells seems to be regulated by IL-2 [85]. The signalling pathways involved in the regulation of STAT4 protein expression have not been explored.

1.11.3 Factors that influence Th cell differentiation

The polarisation of antigen-specific $CD4^+$ Th1 and Th2 cells by antigen priming is influenced by several factors, including non-MHC genetic polimorfisms, ligand-TcR interaction, antigen dose and mode of antigen administration [492-499]. However, the dominant regulators of Th cell differentiation are undoubtedly cytokines. IL-12 and IL-4 drive the differentiation of naive T cells into the Th1 and Th2 cell phenotypes, respectively. The requirement of IL-12 and IL-4 has been

demonstrated unequivocally by the phenotype of mice that lack these cytokines, their receptors or the effector molecules down-stream of their receptors, as well as by similar deficiencies in human patients (sections 1.4.5 and 1.7.6).

Recent studies have shown that the type of invading microorganism as well as microenvironmental tissue factors strongly influence the cytokines produced by APCs, specially DCs (reviewed by [500-502]). Specific DC subsets located in different microenvironments in the spleen appear to bias Th subset development by eliciting IL-12 and IL-4. In humans, monocyte-derived $CD11c^+$ DCs polarise naive T cells predominantly towards the Th1 phenotype, while the $CD11c^-$ DC subset induces T cells to predominantly produce Th2 cytokines. In mice splenic lymphoid $CD8a^+$ DCs produce IL-12 in response to microbes and IFN–γ, whereas splenic myeloid $CD8a^-$ DCs induce IL-4 production (reviewed by [503]).

Each particular Th driving cytokine controls a set of specific intracellular processes that lead to the commitment of either Th1 or Th2 cells. Different mechanisms may explain the molecular basis for phenotype commitment. These include differential cytokine signalling, activation of specific transcription factors, chromatin remodelling of Th1- and Th2-specific genes, exclusive cytokine receptor expression and expression of a distinct set of cytokine genes (reviewed by [504-507]). Significant progress has been made in identifying the transcription factors that control the development of Th2 cells, but very little is known about the molecular bases of Th1 cell differentiation. The present work has concentrated on the signalling pathways activated in response to IL-12 and their implications in Th1 cell differentiation. The molecular basis of Th2 cell differentiation have been recently reviewed [506, 507].

1.11.4 The IL-12/ STAT4 response and Th1 cell differentiation

Diverse studies in mouse and human systems both *in vitro* and *in vivo* have indicated the three main ways in which IL-12 drives Th1 cell differentiation. 1) IL-12 promotes the differentiation of naive T cells, during initial encounter with an antigen, into a population of Th1 cells capable of producing large amounts of IFN–γ following activation; 2) it serves as a co-stimulus required for maximal secretion of IFN–γ by differentiated Th1 cells responding to specific antigen; and 3) IL-12 stimulates the development of IFN–γ producing Th1 cells from populations of resting memory T cells interacting with an antigen to which they have been previously exposed (reviewed by [506-508]). Although STAT4 is essentially required for IL-12-induced Th1 cell differentiation, little is known about the exact mechanism by which activation of STAT4 leads to Th1 cell differentiation [7-9]. The mechanism leading to IFN–γ production following Th1 cell differentiation is not well defined either. Moreover, at the beginning of the present work no other pathways induced in response to IL-12 had been described.

Although a model for IL-12-induced Th1 cell differentiation is still far from complete, current data can be summarised as follows. Th1 cell differentiation occurs when naive T cells are primed with antigen presented by an APC in the presence of IL-12. Apparently, high doses of antigen are required ([498] and reviewed by [10]). Signals derived from the antigen-TcR interaction induce the expression of IL-12Rβ1 and IL-12Rβ2 [76, 77]. T cell activation also induces the expression of CD40L on the T cell (reviewed by [509]). The interaction of CD40L with CD40 on the surface of the APC induces expression of IL-12p40 and consequent IL-12 production by the APC [34-37]. CD40/ CD40L interactions up-regulate the expression of B7 on the APC allowing its interaction with CD28 on the surface of the T cell (reviewed by [509]). CD28/ B-7 co-stimulatory signals up-regulate the expression of IL-12Rβ1 and IL-12Rβ2 on the T cells [78-81]. The levels of IL-12Rβ2 are further

up-regulated by IL-12 [40, 77, 86-88]. In the absence of IL-12, IL-12Rβ2 is expressed at very low levels and becomes undetectable few days after T cell stimulation [77, 86].

Interaction of IL-12 with IL-12Rβ1/ IL-12Rβ2 induces the tyrosine phosphorylation and activation of Jak2 and Tyk2. The IL-12Rβ1 chain contains three cytoplasmic tyrosine residues at positions Tyr678, Tyr767 and Tyr800, which are believed to be phosphorylated by active Jak2 and Tyk2 [75]. Tyr800 has been proposed as the STAT4 docking site. Although the phosphorylation of IL-12Rβ2Y800 in response to IL-12 has not been demonstrated, transient expression of an IL-12Rβ2Y800 mutated construct (IL-12Rβ2Y800F) blocks IL-12-induced transcriptional activity [75]. Moreover, a phosphopeptide containing the IL-12Rβ2pY800 can precipitate STAT4 and block STAT4 activation in response to IL-12 [75, 510]. According to the general model for STATs activation, recruitment of STAT4 to IL-12Rβ2pY800 would allow its tyrosine phosphorylation by Jak2 and Tyk2, followed by STAT4 homodimerisation and consequent DNA binding [76]. STAT4 is further modified by serine phosphorylation by an unknown mechanism [353].

Some STAT4 target genes were uncovered during the development of the present work. These include the Ets family transcription factor Erm, which is induced in wild type Th1 cells but not in STAT4-deficient T cells [511]. The role of this factor in Th1 cell differentiation is still unknown. IL-12 stimulation induces the mRNA expression of IRF-1 [512, 513]. This is thought to be mediated by STAT4, which can bind and transactivate reporter genes bearing the IRF-1 promoter sequence [512, 513]. Interestingly, IRF-1-deficient mice are defective in their Th1 cell responses [514, 515]. However, it is still unclear if the defect is intrinsic to the T cells, because IRF-1 null APCs do not function normally. The interleukin 18 (IL-18) receptor (IL-18R) (also known as IL-18Rα) has been proven to be a STAT4

regulated gene since the IL-12 induced mRNA expression of IL-18R is impaired in STAT4 null splenocytes [516]. As will be discussed further below, the induction of IL-12Rβ2 in response to IL-12 is thought to be mediated by STAT4.

1.11.5 The IFN–α/ STAT4 response

It was originally believed that IL-12 was the only cytokine capable of activating STAT4 and consequently of driving Th1 cell differentiation. IFN-α was later shown to fully activate STAT4 in human T lymphocytes [89, 353]. Controversy originated when IFN-α was reported to be unable to activate STAT4 in mouse systems [517, 518]. It is now recognised that while IL-12 activates STAT4 in both mouse and human systems, the activation of STAT4 in response to IFN-α is limited to humans [89, 519]. Recent studies have shown the molecular basis for the species specificity of the IFN-α/ STAT4 response [519, 520]. STAT4 is not directly recruited to the IFN-αR in response to IFN-α but requires interactions involving STAT2 [519]. As yet, it has not been shown whether recruitment of STAT4 to STAT2 is directly mediated by STAT2 or indirectly by an intermediate adaptor. The inability of IFN-α to activate STAT4 in mouse systems is explained by a C-terminal sequence divergence between human and mouse STAT2, which is sufficient to disrupt the recruitment of STAT4 [520].

1.11.6 Genes that are commonly activated by IL-12 and IFN–α

Molecules such IRF-1 and granzyme B, as well as Th1 specific cytokine receptors (IL-12Rβ2 and IL-18R) and homing receptors (integrin-α6/β1, CCR-1, MIP-1α and MIP-1β) are commonly induced by IL-12 and IFN-α [77, 89, 132, 512, 513, 516,

521-524]. The participation of STAT4 is suggested in the case of IRF-1 and has been proved for the IL-18R [512, 513, 516]. However, the mechanisms involved in the IL-12 and IFN–α regulation of the other genes have not been described.

Several pieces of evidence indicate that the up-regulation of IL-12Rβ2 in response to IL-12 and IFN–α is mediated by STAT4. These include: 1) low expression levels of IL-12Rβ2 in STAT4 null mice [516]; 2) failure of IFN-α to up-regulate IL-12Rβ2 in T lymphocytes from Sezary syndrome patients, which express extremely low to undetectable levels of STAT4 protein and IL-12Rβ2 mRNA [9]; and 3) coincident activation of STAT4 and induction IL-12Rβ2 expression by IFN-α in human but not in mouse systems [86, 89].

1.11.7 IFN–α and Th1 cell differentiation

IFN-α has been shown to drive Th1 cell differentiation in human *in vitro* systems [77, 89]. These *in vitro* studies showed that IFN–α can induce Th1 cell differentiation in the presence of the Th2 driving cytokine IL-4 [77, 89]. Whereas IL-12Rβ2 expression is lost in Th2 cells, IFN–α receptors are constitutively expressed on human T cells [77, 86, 276, 277]. Taken together, these data have prompted some investigators to propose that IFN–α may have a constant capacity for promoting Th1 cell responses in humans [21, 286, 508]. The putative capacity of IFN–α to induce Th1 cell responses in humans was also suggested to explain the observation that human Th cells show less exclusive polarisation of IFN–γ and IL-4 than mouse Th cells [506]. The studies presented in Chapter 5 were developed to deepen our understanding of the activation of STAT4 by IFN–α as well as to compare the IL-12/ STAT4 and the IFN–α/ STAT4 responses.

1.11.8 Other activators of STAT4

Recently, STAT4 was shown to be activated by the urokinase-type plasminogen activator in smooth muscle and endothelial cells, and by IL-2 in NK cells [525, 526]. The mechanism involved in the expression of STAT4 by smooth muscle and endothelial cells, as well as the biological relevance of STAT4 activation in these cells are unknown. In the case of NK cells, a role for STAT4 in controlling c-myc expression in response to IL-2 has been proposed by the interaction of STAT4 with a GAS site within the c-myc promoter [527].

1.11.9 Cytokines that synergise with IL-12 for Th1 cell differentiation

Recently IL-18, an IL-1 related factor was shown to be a selective activator of IFN–γ in Th1 but not Th2 cells [528-530]. The differential expression of the IL-18R in Th1 but not Th2 cells explains the capacity of this cytokine to induce downstream signals only in Th1 cells (reviewed by [531-533]). The signalling cascades activated by IL-18 stimulation include activation of IRAK, NF-κB and TRAF6 but not of STAT4 [534-536]. The activation of some of these molecules seems to be important for Th1 cell responses. For example, IRAK-deficient mice have defective IL-18-mediated NK and Th1 cell responses to pathogens *in vivo* [537]. IL-18 null mice show reduced LPS-induced IFN–γ production after priming with *P. acnes* and mice deficient in both IL-18 and IL-12 have a more severe defect in IFN–γ production than either strain alone [538]. These phenotypes are explained by the finding that IL-18 synergises with IL-12 for IFN–γ production apparently through induction of the IFN–γ promoter by a co-operative NF-κB, STAT4, AP-1 as well as by mutual up-regulation of receptors. IL-12 induces the up-regulation of IL-18R expression while IL-18 induces the up-regulation of IL-12Rβ2 expression [516, 539].

1.11.10 Regulation of IFN–γ production during Th1 cell differentiation

The most important hallmark of Th1 cell responses is the induction and production of IFN-γ. Once secreted, IFN–γ potentiates Th1 cell responses by inducing IL-12 production by APCs. A general consensus is that IL-12 cannot directly induce IFN-γ production. IL-12, as well as IFN-α induce low amounts of IFN-γ in human T cells, while IL-12 induces very low to undetectable levels in mouse T cells [89, 511, 521, 522, 540-542]. Rather these cytokines synergise with either TcR triggering or IL-18 for optimal IFN-γ induction [522, 540, 541]. The essential role of STAT4 in Th1 cell differentiation originally suggested that this transcription factor would directly participate in the induction of IFN–γ gene expression. Several pieces of evidence indicate that activation of STAT4 is not sufficient or absolutely required for IFN-γ gene induction: 1) IFN-γ production can be induced by TcR triggering in STAT4 null T cells [7, 8]; 2) TcR triggering and IFN-γ itself (in mouse systems) that do not activate STAT4, induce IFN-γ expression [89, 231, 233, 540, 541]; 3) IFN-γ production is present in the STAT4/ STAT6 double knock out mice [402]; and 4) activation of STAT4 by IFN-α in Th2 cells is not accompanied by IFN-γ production [77].

Beside the inability of STAT4 to directly induce IFN–γ gene expression, the levels of IFN–γ in STAT4 null mice are reduced [7, 8, 402]. A recent study has shown that the presence of STAT4 is equally required for induction of IFN–γ in $CD4^+$ and $CD8^+$ T cells in response to IL-12, while STAT4 is only required by $CD4^+$ T cells in response to TcR engagement [542]. Similarly, STAT4 is expressed in mature DCs and its presence is crucial for IL-12 induced IFN–γ production and protection from *T. gondii* infection [491]. Taken together these data suggest that STAT4 may be playing an indirect role in the up-regulation of IFN–γ production, possibly through the induction of other transcription factors and/ or by increasing IFN–γ

promoter accessibility through chromatin remodelling. DNAse I hypersensitivity sites (HSs), which reflect local changes in gene accessibility, have been observed in the first and third introns of the IFN–γ gene in Th1 but not, in Th2 cells [543, 544].

Recently, T-bet a novel Th-1 specific transcription factor that controls the expression of IFN–γ has been described [545]. Ectopic expression of T-bet could not only induce endogenous IFN–γ production when introduced at an early stage into developing Th2 cells, but could also induce IFN–γ production and repress IL-4 and IL-5 production in otherwise committed Th2 cells. T-bet is expressed in response to TcR engagement but has been proposed to be regulated by STAT4 (reviewed by [506, 507]).

Optimal induction of IFN-γ seems to require activation of both the proximal and the distal elements of the IFN-γ promoter [546, 547]. Footprinting studies have detected ATF/ CREB, STAT, NF-κB; NF-AT and AP-1 binding sites within the IFN-γ promoter [344, 540, 546-550]. The activation of STAT4 by IL-12, together with the activation of NF-κB and AP-1 in response to IL-18 may explain their synergy for induction of IFN-γ [540]. Similarly, the synergy with TcR triggering may be based on the activation of ATF-2 and NF-AT [233, 541].

1.12 Aims

The aims of the present work were to deepen our current understanding of the characteristics of the IL-12/ STAT4 response in human T lymphocytes. At the beginning of this project little was know about the regulation of STAT4 and indeed of any other signalling pathways activated in response to IL-12. We initially characterised the IL-12/ STAT4 response in polyclonally activated human T cells

obtained from healthy donors and in the lymphoblastoid cell line Kit225 (Chapter 3). At that time, studies on other STAT molecules had proposed serine phosphorylation as an important post-translational modification to regulate the function of STATs. The serine phosphorylation of STAT4 had been described [353]. However, the STAT4 serine phosphorylation site as well as the responsible serine kinase had not been explored. We then set to characterise the participation of some well-known signalling pathways involving the activation of serine/ threonine kinases, in the regulation of the IL-12-induced STAT4 serine phosphorylation. Signalling pathways such as the Ras/ MAPK, PI3-K and protein kinase C (PKC) were studied (Chapter 4). These studies allowed us to discard the participation of these known signalling pathways and to propose the existence of a novel serine kinase that is involved in the regulation of STAT4.

Although the activation of STAT4 by IFN–α had been reported, the characteristics of this response were not studied [353]. Since activation of STAT4 is essential for Th1 cell differentiation, it has been proposed that IFN–α could regulate Th1 cell differentiation in humans [21, 286, 508]. We describe here the characteristics of the activation of STAT4 in response to IFN–α and also performed a direct comparison between the IFN–α/ STAT4 and the IL-12/ STAT4 responses (Chapter 5). We found that the IL-12/ STAT4 response is significantly more sustained than the IFN–α/ STAT4 response. Patients with normal IFN–α responses but impaired IL-12 responses due to mutations in the IL-12Rβ1 chain, cannot mount adequate Th1 cell responses against intracellular pathogens [67-69]. We propose that the differential kinetics of STAT4 activation in response to these two cytokines may explain the inability of IFN–α to drive Th1 cell differentiation in the absence of IL-12 in human *in vivo* systems. Our data indicate that if IFN–α was indeed able to drive Th1 cell differentiation in humans, this cannot be explained only in terms of activation of STAT4 (Chapter 5).

IL-2 plays an essential role in the generation of the immune response by ensuring T cell clonal expansion. A specific role for IL-2 in Th cell differentiation has not been defined. We studied the effect of the cytokine IL-2 on the IL-12/ STAT4 response (Chapter 6). Here we found that the IL-12/ STAT4 response can only be sustained for several days in the presence of IL-2. This is due to a direct effect of IL-2 on the expression of the IL-12R. These data suggest an important role for the cytokine IL-2 in Th1 cell differentiation.

CHAPTER 2

Materials and Methods

2.1 Chemicals, Cells, Cytokines and Inhibitors

Unless specified, all chemicals were from Sigma. Human peripheral blood derived lymphocyte T cells obtained from healthy donors were isolated by Lymphoprep (Nycomed) density-gradient centrifugation. At this stage cells were either used for stimulation experiments (referred as primary T cells) or activated *in vitro* with PHA (Murex Biotech) for four days and maintained in human rIL-2 (Chiron) for 1 week using standard protocols (referred as PBL-T cells). The human IL-2-dependent T cell line Kit225 [551], which expresses both chains of the IL-12R [63, 552], was maintained in RPMI-1640 medium with 10% heat-inactivated foetal calf serum and 20 ng/ ml human rIL-2. PBL-T cells and Kit225 cells were arrested in a quiescent state by washing twice in RPMI-1640 medium and replacing in RPMI-1640 medium with 10% heat-inactivated foetal calf serum in the absence of human rIL-2 for 24-48 hours. When indicated, cells were incubated with one of the following inhibitors for 30 minutes at 37 °C prior to stimulation: H7 (1-(5-Isoquinolinylsolfonyl)-2-methyl-piperazine); PD098059 (2'-Amino-3'-methoxyflavone) (New England Biolabs); LY294002 (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) (Biomol Research Laboratories); or RO-31-8220 (3-[1-[3-(Amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3yl)maleimide) (Roche Discovery Welwyn, Ltd., UK). Unless specified, stimulation was with either 20 ng/ ml human rIL-2, 20 ng/ ml human rIL-12 (Hoffman-La Roche, Nutley, N.J.), 10^3 U/ ml IFN-α (Welferon: a cocktail of IFN-α subtypes) (a gift from Dr. Ian Kerr,

Biochemical Regulatory Mechanisms Laboratory, ICRF, London) or 50 ng/ ml Phorbol 12,13-dibutyrate (PdBu) (Calbiochem). The anti-CD3 monoclonal antibody (mAb) OKT-3 was used for stimulation of primary T cells at 2.5 ng/ ml (Hybridoma Development Unit ICRF). For pulse stimulation assays, cytokine was removed after initial pulse by spinning (30 seconds 10,000 X g) and re-suspending the cells in pre-warmed RPMI-1640 twice, before further incubation in the presence or absence of cytokine.

2.2 Total cell lysates

$0.5\text{-}1x10^7$ cells/ ml were lysed in 1% Nonidet-P40 (NP-40) (BDH), 50 mM Hepes (pH 7.4), 10 mM NaF, 10 mM Iodoacetamide, 75 mM NaCl (BDH), 1 mM phenylmethylsulphonyl fluoride, 1 mM Na_2VO_3, and 1 μg/ ml each pepstatin, chymostatin, leupeptin and antipain. Cell nuclei and debris were eliminated by spinning 5 minutes at 10,000 X g. Proteins were acetone precipitated and re-suspended in reducing sample buffer.

2.3 Cell fractionation

Cytoplasmic lysates were obtained by gentle re-suspension of the cell pellet in low salt buffer containing 0.15% NP40, 10 mM Hepes (pH 7.9), 15 mM KCl (Fisons), 2 mM $MgCl_2$ (BDH), 0.1 mM EDTA (BDH), 10 mM NaF, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na_2VO_3 and 1 mM dithiothreitol. The cytoplasmic lysate was separated by spinning 1 minute at 10,000 X g. NaCl was added to a final 120 mM. Nuclei were washed twice in low salt buffer (NP-40-free) and lysed in high salt buffer (20 mM Hepes (pH 7.9), 20% (v/ v) glycerol (BDH), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 10 mM NaF, 1 mM

phenylmethylsulphonyl fluoride, 1 mM Na_2VO_3, 1 mM dithiothreitol) in a wheel mixer for 30 minutes at 4 °C. Proteins were acetone precipitated and re-suspended in reducing sample buffer.

2.4 Affinity precipitation of DNA binding proteins

Affinity precipitation of DNA binding proteins was performed with the following oligonucleotide sequences: the Interferon γ receptor response element (FcγR-GAS), GTATTTCCCAGAAAAGGAAC (GRR) [553]; the Sis-inducible element (SIE) of the c-fos promoter GTCGACATTTCCCGTCAATC [554]; the M67 variant of SIE (SIEM67) GTCGACATTTCCCGTAAATC [555]; and the optimal binding sequence for STAT4 (GAS-STAT4) GTGGCTTTCCGGGAATCCTTG [344, 556]. Oligonucleotides of the 3' and 5' complementary strands of each sequence where synthesised at the Oligonucleotide Synthesis Service at ICRF. 5' strands contain a biotin moiety at their 5' end. To obtain double stranded oligonucleotides, the 3' and 5'-biotin strands were re-suspended to 1μg/ μl with Tris-EDTA and equal volumes were mixed. The mixture was incubated at 95 °C in a water bath for 10 minutes and left to slowly cool to room temperature. Double stranded oligonucleotides were diluted to 0.1 μg/ μl with Tris-EDTA, 75 mM NaCl and kept at 4 °C.

$2x10^7$ cells/ ml were stimulated and lysed in 1% NP-40, 50 mM Tris (pH 7.9), 10 mM NaF, 100 mM EDTA, 10% glycerol, 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na_2VO_3, 1 mM dithiothreitol and 1 μg/ ml each pepstatin, chymostatin, leupeptin and antipain. Cell nuclei and debris were eliminated by spinning 5 minutes at 10,000 X g. Lysates were diluted with free-NaCl lysis buffer to 15 mM NaCl and pre-cleared with 20 μl streptavidin agarose beads in a wheel mixer for 15 minutes at 4 °C. Pre-cleared lysates were incubated

with 1 μg of corresponding double stranded oligonucleotide and 30 μl streptavidin agarose beads for 2 hours at 4 °C in a wheel mixer. Beads containing the oligonucleotide bound proteins were washed three times with cold lysis buffer and re-suspended in reducing sample buffer. All spins were at 5,000 X g for 5 minutes.

2.5 Affinity precipitation of peptide binding proteins

A synthetic phosphopeptide corresponding to amino acids 336-444 (T-S-F-G-pY-D-K-P-H) of the IFN-γR (IFN-γR-pY440) [557] was synthesised by Nicola O'Reilly and Elisabeth Li at the ICRF Oligopeptide Synthesis Service. The IFN-γR-pY440 peptide was diluted to 1 mg/ 750 μl in 50 mM KH2PO4/ K2HPO4 (pH 7.5) and coupled to Affigel-10 activated ester agarose (BIORAD) at 25 μM (1 mg of peptide per 200 μl beads). Affigel 10 was washed once as a 1:1 (v/ v) solution in cold distilled water and re-suspended with cold 50 mM KH2PO4/ K2HPO4 (pH 7.5) (1:1 v/ v), prior to binding. Peptide and beads were mixed and incubated over night in a wheel mixer at 4 °C. Supernatant was eliminated and uncoupled sites were blocked by incubating with 0.1 M ethanolamine (pH 8.0) and 50 mM KH2PO4/ K2HPO4 (pH 7.5) (1:5 v/ v) in a wheel mixer for 1 hour at 4 °C. Beads were washed three times and re-suspended to 1:1 (v/ v) in cold PBS/ 0.05% NaN3 and stored at 4 °C. Control beads were produced by blocking the free ester sites of Affigel 10 with 0.1 M ethanolamine (pH 8.0) and 50 mM KH2PO4/ K2HPO4 (pH 7.5) (1:5 v/ v) as described above. All spins were for 5 minutes at 5,000 X g at 4 °C.

The IFN-γR-pY440 peptide matrix was used to affinity precipitate STAT1 and STAT3. $2x10^7$ cells/ ml were stimulated and lysed in 1% NP-40, 50 mM Tris (pH 7.9), 10 mM NaF, 100 mM EDTA, 10% glycerol, 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na2VO3 and 1 μg/ ml each pepstatin, chymostatin, leupeptin and antipain. Cell nuclei and debris were eliminated by

spinning 5 minutes at 10,000 X g. Lysates were pre-cleared with 30 µl control beads in a wheel mixer for 15 minutes at 4 °C. Pre-cleared lysates were incubated with 50 µl IFN-γR-pY440-coupled Affigel 10 beads in a wheel mixer for 2 hours at 4 °C. Beads were washed three times with cold lysis buffer and re-suspended in reducing sample buffer. All spins were at 5,000 X g for 5 minutes.

2.6 Affinity precipitation using GST-fusion proteins

The Ras binding domain (RBD) of c-Raf (aa 1-149) fused to glutathione-S-transferase (GST) (RBD-GST) (a gift from Barbara Marte) [558] which specifically recognises active Ras or the full length Grb2 protein fused to GST (Grb2-GST) [559] were coupled to glutathione coated agarose beads. These matrices were used to affinity precipitate active Ras-GTP complexes and proteins interacting with the SH2 or SH3 domains of Grb2, respectively.

To prepare the GST-fusion protein matrices, *E. coli* bearing RBD-GST or Grb2-GST encoded on the pGEX2 plasmid were grown to OD_{550} = 0.6-0.8 in 400 ml L-Broth medium containing 50 mg/ ml ampicillin. Fusion protein expression was induced using isopropyl β-D thio-galactopyranoside (IPTG) for 4 hours. Cultures where centrifuged and re-suspended at 4 °C in 10 ml PBS containing 1% Triton X-100, 2 mM EDTA and 1 mM phenylmethylsulphonyl fluoride. Bacterial cells were lysed by sonication and cell debris pelleted by spinning at 20,000 X g for 10 minutes at 4 °C. Glutathione-agarose beads were washed three times and re-suspended to 50% (v/ v) solution in cold 2 mM EDTA/ PBS. Washed beads were used for initial purification of the fusion proteins by incubating with the lysate in a wheel mixer for 2 hours at 4 °C. The suspension was then centrifuged at 3,000 X g for 30 seconds and washed twice with lysis buffer and twice with 2 mM EDTA/ PBS. The protein was eluted from the beads using 25 mM glutathione in Tris buffer

(pH 8.0) and its OD_{280} was measured. Fractions containing the fusion protein were collected and dialysed in Visking tubing (Mr cut-off 5000-8000 Daltons) against 2 mM EDTA/ PBS for 12 hours at 4 °C, with three changes of buffer. Finally, fusion proteins were re-coupled to glutathione-agarose beads at 10 mg/ ml and stored in 50% glycerol, 50 mM Hepes (pH 7.4) and 50 mM NaCl at –20 °C.

For affinity precipitation, the total lysate of $2x10^7$ cells obtained as described (section 2.2), was pre-cleared with 10 µl glutathione agarose beads and incubated with 10 µl of RBD-GST or Grb2-GST beads respectively, in a wheel mixer for 2 hours at 4 °C. Beads were washed three times in cold lysis buffer and proteins were eluted in reducing sample buffer. The lysis buffer was supplemented with 10 mM $MgCl_2$ for RBD-GST affinity precipitation.

2.7 Protein Standardisation

Where indicated, the protein content in the cell lysates was standardised using the BioRad protein assay (BioRad) according to manufacturer's instructions. Briefly, 5-10 µl of cell lysate were mixed with 1 ml of BioRad staining solution (diluted 1:5) and incubated for 5 minutes at room temperature before its OD_{595} was measured. Protein content was calculated against a bovine serum albumin (BSA) standard curve. Volumes were adjusted with lysis buffer and equal protein content per sample was then used for affinity precipitation with double stranded biotinylated oligonucleotides or acetone precipitation.

2.8 Western blotting

Proteins were separated on sodium dodecyl sulphate (SDS)- polyacrylamide gel electrophoresis (PAGE) (Protogel from National Diagnostics) using the following conditions: for PKB, PKD and STATs 7.5% acrylamide/ 0.03% bis; for Shc 11% acrylamide/ 0.03% bis; for Ras 15% acrylamide/ 0.03% bis; and for MAPKs Erk1,2 15% acrylamide/ 0.075% bis. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore) and blocked in either 3% BSA or 5% dried milk, both in 0.05% Tween 20 (BDH)/ PBS. The following antibodies were used: anti-STAT4 C- (C20), anti-PKD pan (D-20) (Santa Cruz Biotech.); anti-STAT1 pan mAb, anti-STAT3 pan mAb, anti-STAT5 pan mAb, anti-Erk1/2 pan mAb (Transduction Labs); anti-Shc pan, anti-Sos1, anti-PKB pan, anti-STAT3-αpSer727 (Upstate Biotech); anti-active Erk1/Erk2 (anti-Erk1/Erk2pThrp183Tyr185) (Promega); anti-STAT3pTyr705, anti-STAT5pTyr694, anti-PKBpSer473 (New England Biolabs); anti-Ras pan mAb (Oncogene); anti-p85 (U5) mAb (Hybridoma Development Unit ICRF). The anti-PKDpSer916 was raised by Sharon Matthews [560]. The anti-STAT1-αpSer727 [354] was a generous gift from Dr. Thomas Decker (Institute of Microbiology and Genetics, Vienna Biocenter, Vienna, Austria). Horse radish peroxidase-conjugated anti-sheep (Sigma), anti-mouse or anti-rabbit (Amersham) antibodies were respectively used. Proteins were visualised using the enhanced chemiluminiscense detection system (ECL) (Amersham). For sequential detection, membranes were stripped in 100 mM β-Mercaptoethanol, 2% SDS, 62.5 mM Tris (pH 6.8) for 45 minutes at 50 °C.

2.9 Densitometric analysis of bands obtained by Western blotting

The intensity of the bands obtained by Western blotting was electronically determined by densitometric analysis using the NIH image 1.61 PPC software on scanned Western blotting films. The band corresponding to the positive control or maximum activation was given the arbitrary value of 100% and relative percentages were calculated thereafter.

2.10 Plasmids, Transient transfections and CAT-assays

The GRR-CAT (chloramphenicol acetyl transferase) reporter plasmid contains five copies of the GRR (GTATTTCCCAGAAAAGGAAC) upstream of a thymidine kinase (tk) minimal promoter in the pBLCAT2 vector [184]. The E2A-CAT reporter comprises from -284bp to +62bp of the E2A promoter upstream of a CAT gene [561, 562]. The LexA-OP-tk CAT and the pEF-NLexA Elk-1C fusion protein reporter system [212] and the expression plasmid pEF-rCD2p110 [563] have been described. 48 hours IL-2 deprived Kit225 cells were transfected as previously described [240], and were cultured for 18 hours in the presence or absence of either human rIL-12 (20-60 ng/ ml) or human rIL-2 (20 ng/ ml). Lysates were obtained using 0.65% NP-40, 10 mM Tris (pH 8.0), 1 mM EDTA and 150 mM NaCl. Cleared lysates were heated at 68 °C for 10 minutes and assayed for CAT activity using 75 mM Tris pH 8.0, 0.05 μCi (^{14}C) acetyl coenzyme A (Amersham) and 1.6 mM chloramphenicol. After 18 hours at 37 °C, chloramphenicol was extracted with ethylacetate (Fisons), and the amount of radioactivity in the acetylated products and non-acetylated substrate of each reaction was determined by liquid scintillation counting of organic and aqueous phases, respectively. The amount of chloramphenicol acetylation was calculated as a percentage.

2.11 Staining of cell surface molecules for flow cytometry analysis

Antibodies were obtained conjugated to either fluorescent isothyocyanate (FITC) or phycoerythrin (PE). Non-conjugated antibodies were revealed using biotinylated anti-mouse, anti-rabbit or anti-rat antibodies respectively, followed by incubation with streptavidin-TRICOLOR or streptavidin-PE. For staining of primary T cells, Fc receptors were blocked with anti-FcγRII mAb (rat) or when staining with rat antibodies, an excess (20 μg/ ml) of rabbit IgG. For staining $2x10^6$ cells per sample were incubated with saturating concentrations of corresponding antibodies at 4 ℃ for 20 minutes. All incubations were in a 96 well V-bottom shape microtitre plate in 100 μl 1% BSA/ PBS. Cells were washed with 1% BSA/ PBS between incubations and prior to analysis.

The following antibodies were used: anti-human CD3-PE (mouse) (DAKO); anti-human CD4-FITC (mouse IgG1κ), anti-human CD8-PE (mouse IgG1κ), and anti-human CD25-PE (rabbit) (Pharmyngen). The anti-human IL-12Rβ1 (2B10) (rat IgG2a) and IL-12Rβ2 (2B6) (rat IgG2a), and the anti-human IFNAR1.1 (4B1) (mouse) and IFNAR2.2 (R2.2) (rabbit polyclonal) where kindly provided by Dr. Francesco Sinigaglia (Roche Products Ltd., Milano, Italy) and Dr. Ian Kerr (Biochemical Regulatory Mechanisms Laboratory, ICRF, London), respectively. Following antibodies of unknown specificity were used as isotype match controls: mouse IgG1κ-PE (for CD25) (Pharmingen); anti-rat IgG2aκ (for IL-12Rβ1/ β2), whole rabbit IgG (for IFNAR2.2) (Jackson Immunoresearch); and whole mouse IgG (for IFNAR1.1) (Sigma).

Stained cells were analysed on a FACS (flow activated cell sorting) calibur (Becton Dickinson). Events were collected and stored ungated in list-mode using CellQuest (Becton Dickinson) software. Live cells were gated according to their forward scatter (FSC)/ side scatter (SSC) profiles and by eliminating dead cells that

positively stained with the DNA binding dye TO-PRO3. Data were analysed using CellQuest software.

2.12 Cell viability analysis

To determine the effect of cytokines or inhibitors on cell viability, cells were incubated with propidium iodide (50 mg/ ml) to stain cellular DNA and analysed on a FACS calibur (Becton Dickinson). Data were analysed using CellQuest software.

2.13 Acid wash for cytokine removal

Binding of IL-12 to its receptor results in the masking of the IL-12Rβ2 epitope that is recognised by the anti-IL-12Rβ2 antibody (2B6) (data not shown). To analyse IL-12Rβ2 cell surface expression on IL-12-stimulated cells, acid wash was used to remove IL-12. Stimulated cells were washed twice in ice cold PBS before and after being re-suspended in ice cold RPMI-1640 medium (pH 4.0) for 1 minute. All spins were for 20 seconds at 10,000 X g at 4 °C. It is important to consider that this treatment is highly aggressive and significant cell death is produced. While other surface molecules can be affected by this treatment, the structure of IL-12R is well-preserved (data not shown).

CHAPTER 3

IL-12 selectively regulates STAT4 in human T lymphocytes

3.1 Introduction

As discussed in Chapter 1, IL-12 plays a key role in the onset of the cellular immune response by driving the differentiation of naive Th cells into Th1 cells. At the beginning of this project the biological activity of IL-12 had been widely studied, but the signal transduction cascade triggered by the interaction of IL-12 with its cognate receptor (IL-12R) was still poorly understood. It was known that binding of IL-12 to the IL-12R resulted in the activation of the Janus kinases Jak2 and Tyk2 [313, 564]. IL-12 had also been shown to induce the tyrosine phosphorylation and DNA binding of STAT4 [76, 490], an essential response for Th1 cell differentiation [7-9]. Finally, phosphoaminoacid analysis had demonstrated that IL-12 induces the serine phosphorylation of STAT4 [353].

The aim of the present chapter was to establish the system for the study of IL-12 activation of STAT4 in human T lymphocytes. As detailed in the prototypical model for Jak/ STAT activation (Chapter 1, section 1.7.4), STATs are phosphorylated on tyrosine residues in response to cytokine stimulation. Tyrosine phosphorylation allows STATs to dimerise through mutual SH2-pTyr interactions. Homo or heterodimerised STATs can then bind to specific DNA sequences, while in general non-tyrosine phosphorylated STAT monomers cannot. The majority of previously published data had made use of anti-phosphotyrosine immuno-precipitations followed by Western blot analysis with specific anti-STAT

antibodies or the converse experiment, to demonstrate the tyrosine phosphorylation of STATs. They have also employed electrophoretic mobility shift assays (EMSA) to show the induction of STAT/ DNA binding complexes. In our studies, we used a robust and sensitive alternative method that combines affinity precipitation using biotinylated double stranded oligonucleotides and Western blot analysis [171, 185]. Biotinylated, double stranded oligonucleotides bearing high affinity STAT binding sites can be coupled to streptavidin coated beads and used as an affinity matrix to purify DNA binding proteins from cell extracts. Proteins purified on these DNA affinity matrices can be further fractionated by SDS-PAGE and subjected to Western blot analysis with specific antisera.

3.2 Results

3.2.1 IL-12 activates STAT4 in both PBL-T cells and Kit225 cells

Tyrosine phosphorylation and DNA binding of STAT4 was monitored by its ability to bind to a matrix containing the high-affinity STAT4 DNA binding site GRR (GTATTTCCCAGAAAAGGAAC). GRR-affinity precipitated proteins were fractionated by SDS-PAGE and subjected to Western blot analysis with a specific anti-STAT4 antibody (anti-STAT4 C-). No STAT4 protein is detected in GRR complexes isolated from unstimulated PHA-activated peripheral blood (PBL) T cells or quiescent Kit225 cells, while STAT4 is present in the GRR complexes of both PBL-T cells and Kit225 cells stimulated with IL-12 (Figure 3.1 A). Two forms of STAT4 with distinct electrophoretic mobility, designated STAT4p1 and STAT4p2 can be seen in the IL-12 activated STAT4 complexes. Previous studies have characterised the molecular basis for this electrophoretic mobility change. The more rapidly migrating STAT4p1 is phosphorylated on tyrosine residues, while the more slowly migrating STAT4p2 is phosphorylated on both tyrosine and serine residues [353]. The presence of equivalent protein levels of STAT4, STAT3 and STAT5 in both unstimulated and IL-12 stimulated PBL-T cells was confirmed by Western blot analysis of the lysate remaining after GRR-affinity precipitation (Figure 3.1 B).

3.2.2 IL-12 only activates STAT4 in activated PBL-T cells

At the beginning of this project the IL-12/ STAT4 system was poorly understood. While the components of the IL-12R (IL-12Rβ1 and IL-12Rβ2) had been cloned and specific tissue expression of STAT4 had been reported, little was known about the regulation of their expression [63, 65, 488, 489]. Interestingly, T cells have been

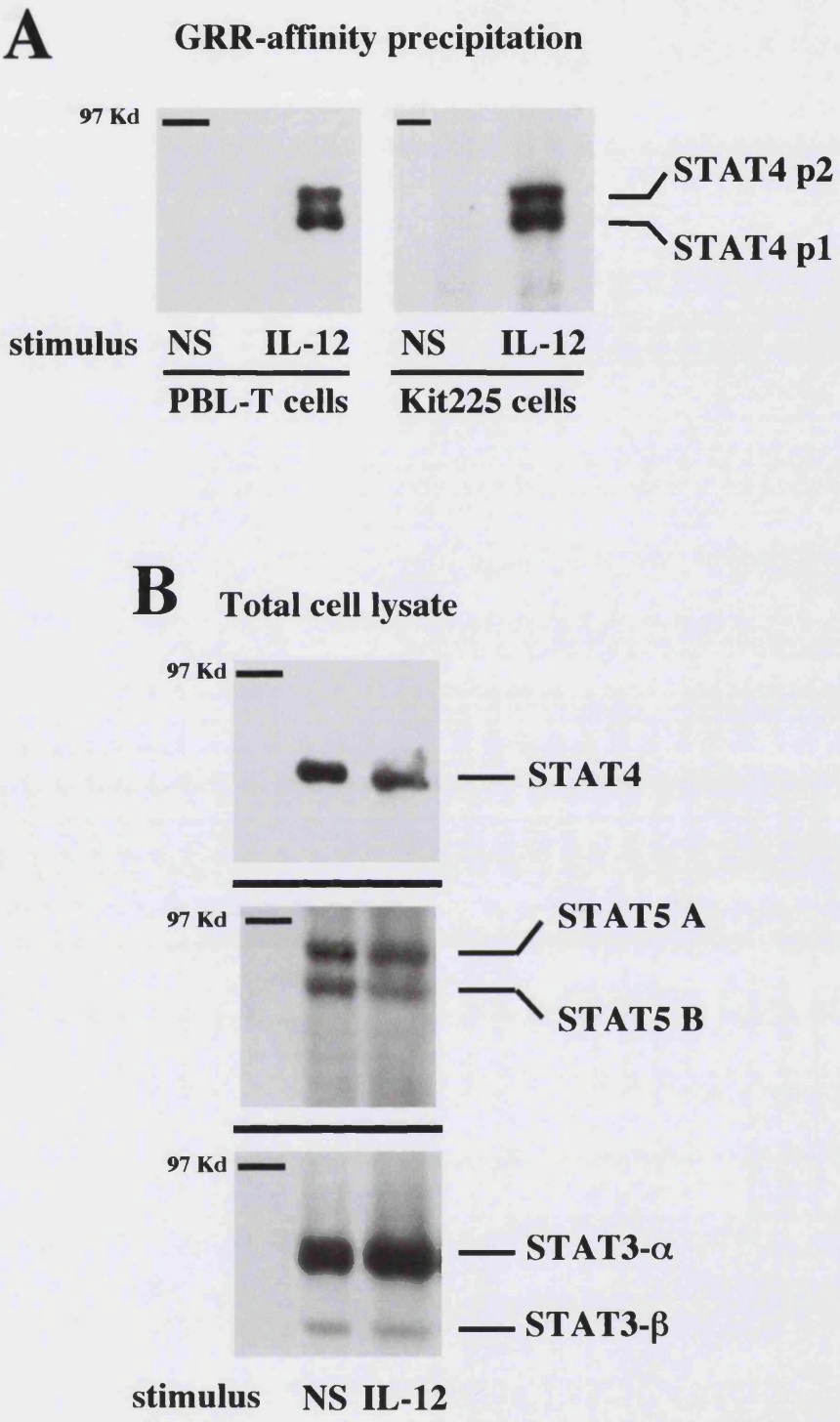


Figure 3.1) IL-12 activates STAT4 in both PBL-T cells and Kit225 cells.- **A)** IL-12-induced STAT4 DNA binding in PBL-T and Kit225 cells. **B)** STAT4, STAT5 and STAT3 are present in both unstimulated (NS) and IL-12 stimulated PBL-T cells. **A)** 20 x 10^6 cells were left unstimulated (NS) or stimulated with 20 ng/ ml of IL-12 for 18 minutes. Proteins were GRR-affinity precipitated, resolved on SDS-PAGE and immunoblotted with anti-STAT4 C-. **B)** An eight part of the total protein remaining in the lysate after GRR-affinity precipitation from PBL-T cells in (A) was acetone precipitated, resolved on SDS-PAGE and immunoblotted with anti-STAT4 C- followed by sequential reprobing with anti-STAT5 pan and anti-STAT3 pan.

shown to express IL-12R and STAT4 only following TcR triggering with anti-CD3 antibodies or by T cell stimulation with PHA [76, 95, 552, 565]. However, most studies concentrated on the regulation of IL-12 responsiveness during late stages of activation and Th cell differentiation, leaving the early kinetics of T cell activation induced IL-12R and STAT4 expression unexplored.

To study the regulation of IL-12 responsiveness in early kinetics of T cell activation, we compared the IL-12/ STAT4 response in primary peripheral blood derived resting T cells and T cells activated under different conditions. Primary T cells were left unstimulated or activated with PHA for different times prior to stimulation with IL-12 for 18 minutes (Figure 3.2 A upper panel) or incubated with the anti-CD3 mAb OKT-3, IL-12 or both stimuli for different times (Figure 3.2 A lower panel). All T cell activation experiments were done in the presence of macrophages. IL-12-induced STAT4 DNA binding was monitored using oligonucleotide affinity precipitation. Neither IL-12 or OKT-3 individually are capable of inducing the DNA binding of STAT4 in primary T cells (Figure 3.2 A). However, IL-12 induces STAT4/ DNA complexes in cells that were activated with OKT-3 or PHA for six and twelve hours, respectively (Figure 3.2 A). On longer exposure of the films, a weak IL-12/ STAT4 response was also seen in cells activated for three hours (data not shown). Both STAT4p1 and STAT4p2 complexes can be detected. Hence IL-12 is only able to activate STAT4 in pre-activated T cells and not in primary T cells.

We then compared STAT4 protein levels in primary T cells and cells that were activated with PHA for different times. As shown by STAT4 immunoblotting of total cell lysates, very low amounts of STAT4 can be detected in primary T cells (Figure 3.2 B). PHA activation induces a strong up-regulation of STAT4 protein levels, which can be detected as early as three to six hours following stimulation (Figure 3.2 B and data not shown). STAT4 protein levels increase steadily up to 24 hours and are maintained at similar level thereafter (data not shown). Interestingly,

STAT4 is not the only STAT protein whose expression is up-regulated by T cell activation. The protein expression levels of STAT1-α/ β, STAT6 and STAT5A but not of STAT3 and STAT5B, are up-regulated following PHA activation with similar kinetics to those of STAT4 (Figure 3.2 B and data not shown). Equal amounts of p85 (the regulatory subunit of PI3-K), which is constitutively expressed are shown as loading control. These data then show that PHA induces up-regulation of STAT4 protein levels and IL-12 responsiveness with parallel kinetics.

The surface expression levels of both chains (IL-12Rβ1 and IL-12Rβ2) of the IL-12R were compared in primary and PHA-activated T cells. Flow cytometry analysis on cells stained with antibodies that specifically recognise IL-12Rβ1 and IL-12Rβ2 was used in these analyses. Triple staining using anti-CD3 and anti-CD4 or anti-CD8 antibodies allowed monitoring of both $CD3^+$/ $CD4^+$ and $CD3^+$/ $CD8^+$ populations. We failed to detect the surface expression of either chain of the IL-12R in primary T cells when compared with an isotype match control antibody staining (data not shown). Up-regulation of both IL-12Rβ1 and IL-12Rβ2 is detectable in $CD4^+$ T cells stimulated with PHA for 24 hours, but not at earlier time points (Figure 3.3 and data not shown). $CD8^+$ T cells show a more delayed up-regulation of IL-12Rβ1 and IL-12Rβ2 by PHA, with initial surface expression detected at 48 hours. The discrepancy between the detection of IL-12R by flow cytometry analysis as compared to IL-12 induction of STAT4 DNA binding is probably a reflection of the relative sensitivity of these two techniques.

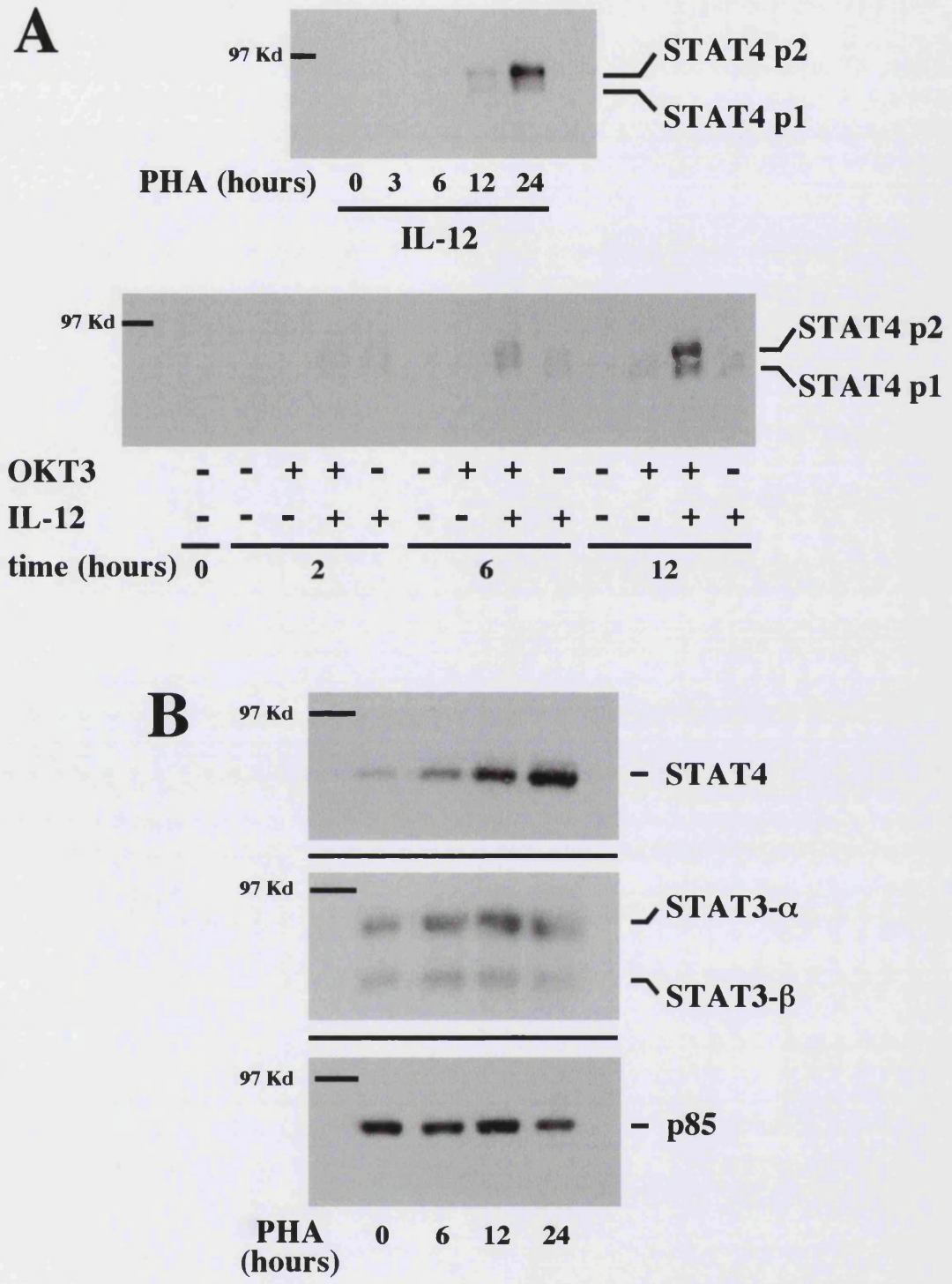


Figure 3.2) IL-12 only activates STAT4 in activated PBL-T cells.- **A)** IL-12-induced STAT4 DNA binding in primary and activated T cells. **B)** PHA induced STAT4 protein expression. **A)** Primary T cells were left unstimulated or activated with PHA for indicated times prior to stimulation with IL-12 for 18 minutes (upper panel) or incubated with the anti-CD3 mAb OKT-3, IL-12 or both stimuli for indicated times (lower panel). Proteins were GAS-STAT4-affinity precipitated, resolved on SDS-PAGE and immunoblotted with anti-STAT4 C-. **B)** Primary T cells were left unstimulated or activated with PHA for indicated times. 100 μg of total cell protein per sample were acetone precipitated, resolved on SDS-PAGE and immunoblotted with anti-STAT4 C- followed by sequential reprobing with anti-STAT3 pan and anti-p85.

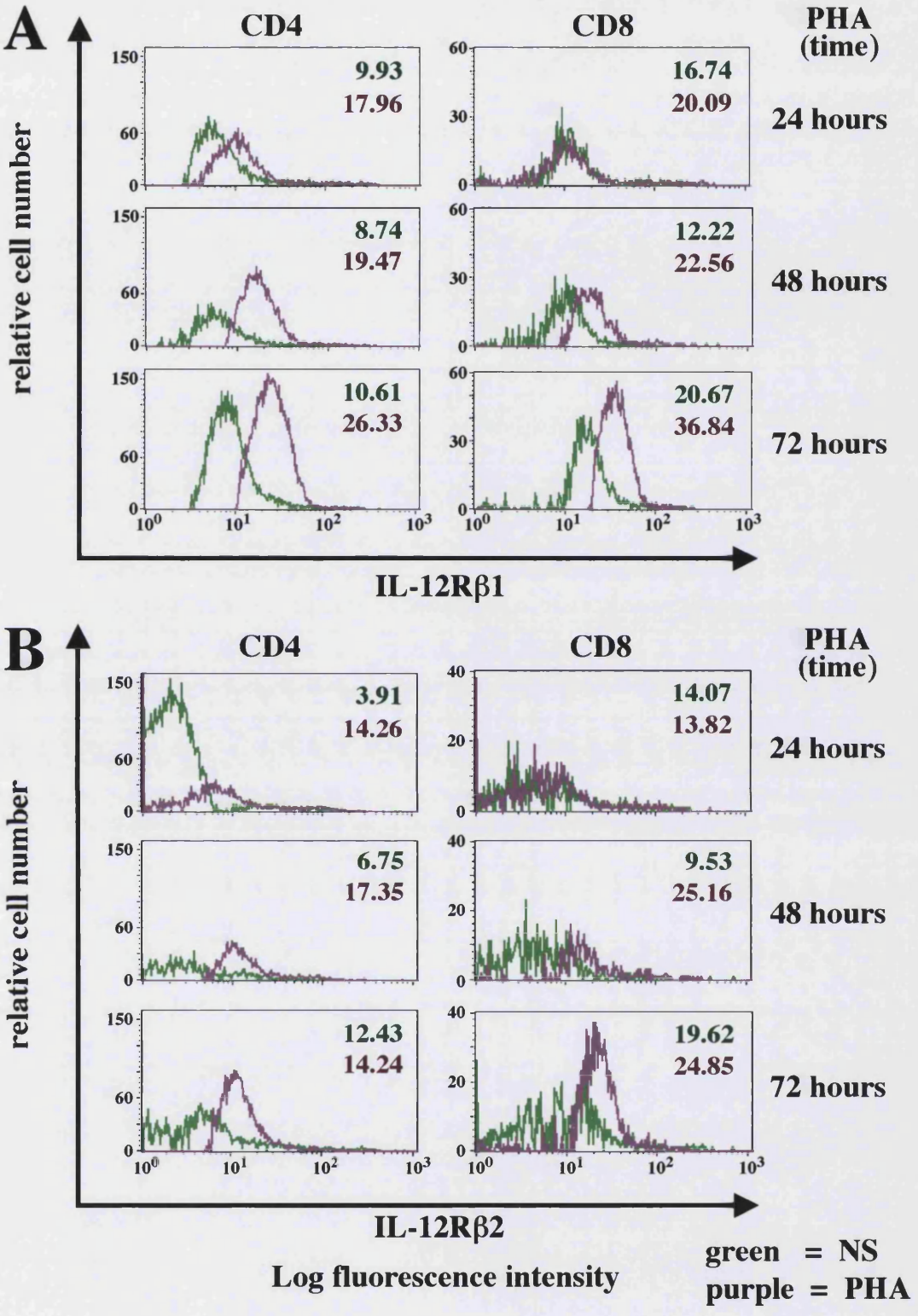


Figure 3.3) TcR triggering up-regulates the cell surface expression of IL-12Rβ1 and IL-12Rβ2.- Primary T cells were left unstimulated (green) or activated with PHA (purple) for indicated times. Cells were stained with anti-CD4, anti-CD8 and either an isotype match control (IMC), anti-IL-12Rβ1 (A) or anti-IL-12Rβ2 (B) antibodies as described in materials and methods. Stained cells were analysed by flow cytometry. The value of the corresponding IMC staining was subtracted from the value of the antibody staining in each case. Histograms represent IL-12Rβ1 (A) and IL-12Rβ2 (B) staining electronically gated on live $CD4^+$ (left column) or $CD8^+$ (right column) T cells.

3.2.3 IL-12 activation of STAT4: IL-12 dose response and comparison of the effectiveness of different oligonucleotides as reagents to affinity precipitate active STAT4

To establish the best conditions for IL-12 activation of STAT4 in our system an IL-12 dose response stimulation using the GRR-affinity precipitation was performed. Both STAT4p1 and STAT4p2 can be weakly detected in the DNA complexes of PBL-T cells stimulated with 2.5-10 ng/ ml of IL-12. Stronger activation is obtained with doses ranging from 15-25 ng/ ml (Figure 3.4 A). Therefore unless indicated, IL-12 was used at 20 ng/ ml for all our studies.

As mentioned in Chapter 1, each active STAT dimer has different binding preferences for specific DNA sequences. To determine the most effective oligonucleotide in binding IL-12 activated STAT4 in PBL-T cells, we carried out the DNA-affinity precipitation assay with a number of different oligonucleotides. These corresponded to sequences derived from the Interferon γ receptor response element (FcγR-GAS), GTATTTCCCAGAAAAGGAAC (GRR) [553]; the Sis-inducible element (SIE) of the c-fos promoter GTCGACATTTCCCGTCAATC [554] and the M67 variant of SIE (SIEM67) GTCGACATTTCCCGTAAATC [555]. The GRR oligonucleotide shows higher recovery of active STAT4p1 and STAT4p2 when compared to SIE and SIEM67 (Figure 3.4 B left panel). The optimal DNA binding sequence for STAT4 has been determined by immuno-precipitation of tagged-STAT4 and PCR amplification of bound oligonucleotides by two separate groups [344, 556]. An oligonucleotide bearing this sequence (GTGGCTTTCCGGGAATCCTTG) was synthesised and named GAS-STAT4. Comparison of the GAS-STAT4 and GRR oligonucleotides in IL-12 stimulated Kit225 cells show that GAS-STAT4 is more effective than GRR for the precipitation of both STAT4p1 and STAT4p2 (Figure 3.4 B right panel). GAS-STAT4 also binds higher amounts of STAT1, STAT3 and STAT5 in response to IFN-α and IL-2, respectively (data not shown). Therefore, beside initial studies

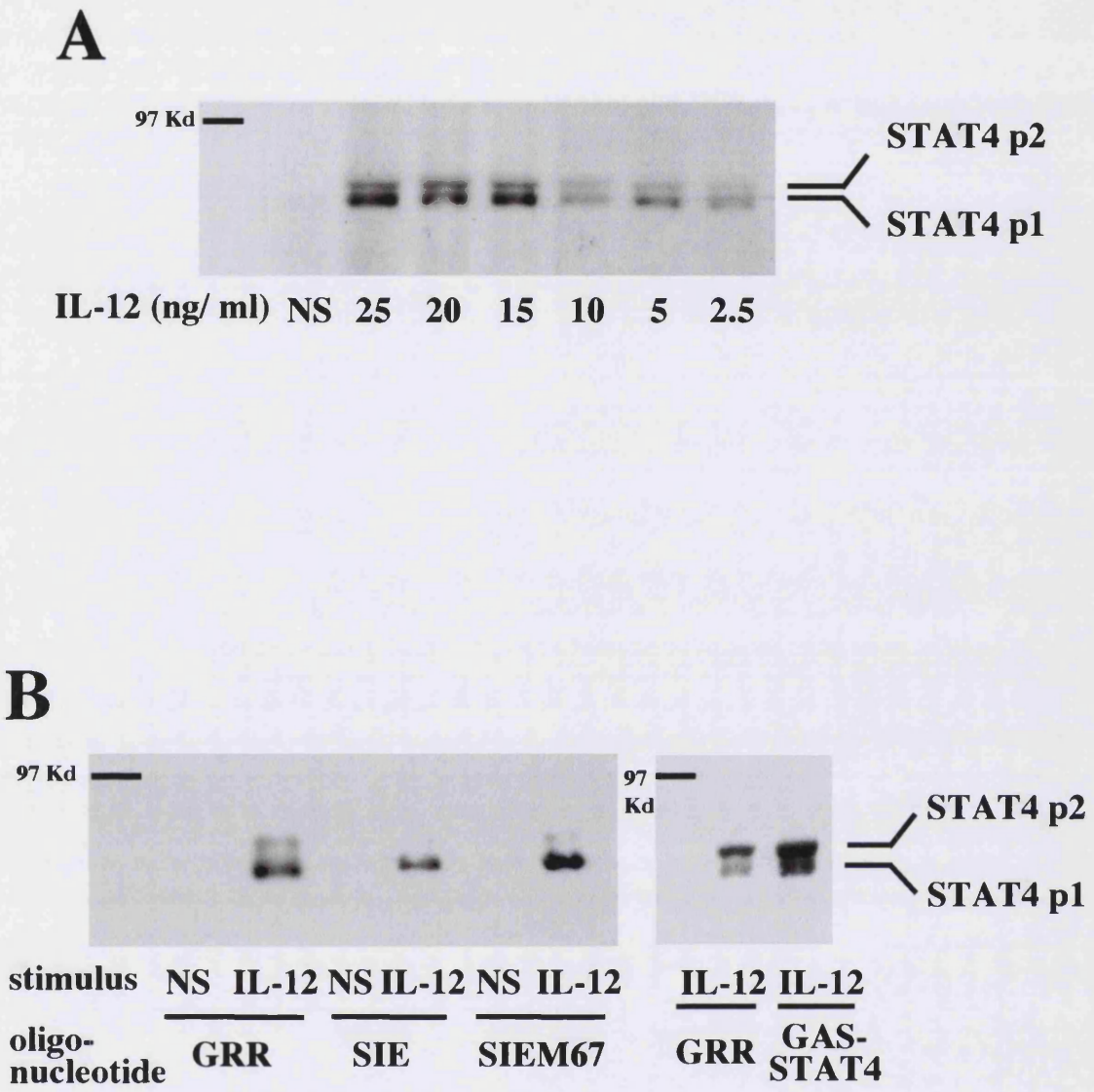


Figure 3.4) IL-12 activation of STAT4: Dose response and comparison of the effectiveness of different oligonucleotides as reagents to affinity precipitate active STAT4.- **A)** IL-12 dose response for STAT4 DNA binding. **B)** IL-12 induced STAT4 DNA binding comparing GRR, SIE, SIEM and GAS-STAT4 oligonucleotides. 20 x 10^6 PBL-T cells (A, B left panel) or Kit225 cells (B right panel) were left unstimulated (NS) or stimulated with 20 ng/ ml or indicated concentrations of IL-12 for 10 (A, B left panel) or 60 minutes (B right panel). Proteins were affinity precipitated using the GRR (A) or indicated oligonucleotide (B), resolved on SDS-PAGE and immunoblotted with anti-STAT4 C-.

that were performed using the GRR or SIEM67 oligonucleotides, the rest of the assays used the GAS-STAT4 oligonucleotide to affinity precipitate STAT molecules.

3.2.4 IL-12 does not induce DNA binding, tyrosine or serine phosphorylation of STAT1, STAT3 or STAT5 in human T lymphocytes

It has been shown that a single cytokine can activate more than one STAT and that the same STAT can be activated by more than one cytokine. Both the GRR and GAS-STAT4 oligonucleotides used to isolate STAT4 also bind to activated STAT1, STAT3 and STAT5. To examine the effect of IL-12 on STATs other than STAT4 in human T lymphocytes, we used GRR affinity precipitation in both PBL-T cells and Kit225 cells (Figure 3.5). IL-12 activation of STAT4 is selective since in these experiments we fail to detect significant levels of STAT1 (α and β), STAT5 (A and B) or indeed STAT3 (α and β) in the DNA binding complexes isolated from IL-12 stimulated T cells (Figure 3.5 A). However, active STAT1 (α and β), STAT5 (A and B) and STAT3 (α and β) can be readily seen in DNA complexes isolated from IFN-α or IL-2 activated T cells, respectively (Figure 3.5 A). The failure of IL-12 to induce tyrosine phosphorylation and consequent DNA binding of STAT3 and STAT5 was confirmed using anti-phosphotyrosine specific antibodies on total cell lysates of IL-12 stimulated Kit225 cells (Figure 3.5 B). IL-12 does not induce the tyrosine phosphorylation of STAT5 or STAT3 at any stimulation time, while robust phosphotyrosine bands are detected in cells stimulated with both IL-2 and IFN-α (Figure 3.5 B).

As will be discussed in Chapter 5 the IFN-α/ STAT4 and IL-12/ STAT4 responses in human T lymphocytes share a number of similarities. These raise the question of

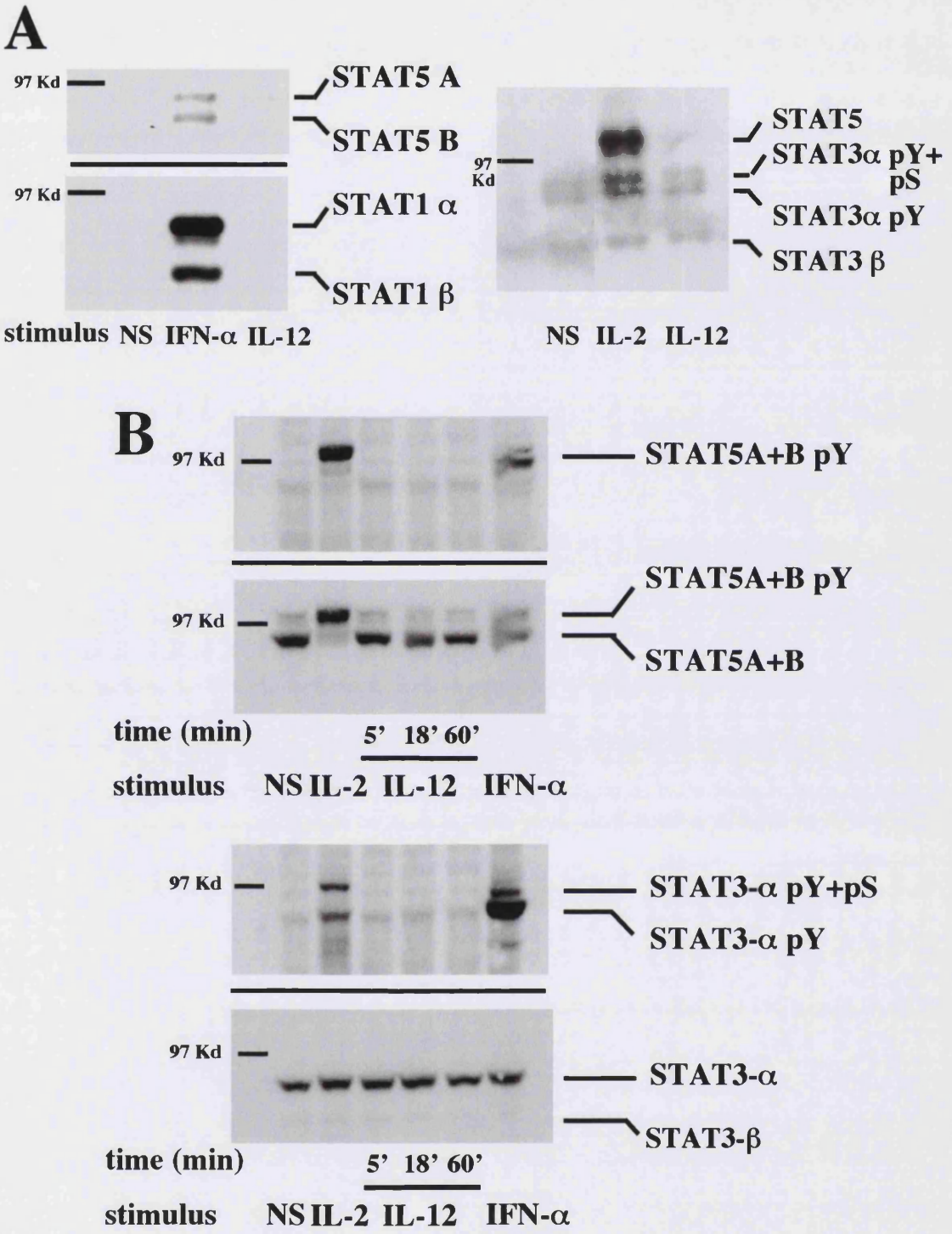


Figure 3.5) IL-12 does not induce tyrosine phosphorylation and DNA binding of STAT1, STAT3 or STAT5 in human T lymphocytes.- A) DNA binding of STAT1, STAT3 and STAT5 comparing IL-12 to IL-2 (right panel) or IFN-α (left panel) stimulation in PBL-T cells. **B)** Tyrosine phosphorylation of STAT5 (upper panel) and STAT3 (lower panel) comparing IL-12 to IL-2 and IFN-α stimulation in Kit225 cells. Cells (20 x 10^6 in A and 5 x 10^6 in B) were left unstimulated (NS) or stimulated with mentioned cytokines for 10-18 minutes or indicated times. Proteins were GRR-affinity precipitated (A) or acetone precipitated from total cell lysates (B), resolved on SDS-PAGE and immunoblotted as follows: A) anti-STAT5 pan, anti-STAT1 pan or anti-STAT3 pan; B) anti-pTyr694-STAT5 or anti-pTyr705-STAT3, followed by reprobing with anti-STAT5 pan or anti-STAT3 pan, respectively.

whether there is any reciprocal IL-12 regulation of the IFN-α activated transcription factors STAT1 and STAT3. We could not detect IL-12 regulation of STAT1 (α and β) and STAT3 (α and β) tyrosine phosphorylation and DNA binding (Figure 3.5). It is well established that cytokines can induce serine phosphorylation of inactive and non-tyrosine phosphorylated STAT1 and STAT3 molecules [185, 354, 356, 371]. A phosphopeptide containing the tyrosine-phosphorylation site (Y440) of the IFN-γRα-chain (IFN-γR-pY440) is an efficient and high affinity matrix for the precipitation of both active and inactive STAT1 and STAT3 molecules from cell extracts. Both splice variants (α and β) can be isolated, but only STAT1-α and STAT3-α can be phosphorylated on serine 727. The β splice variants lack the C-terminal sequence containing the serine 727 residue. Western blot analysis with antisera specifically reactive with either STAT1-α or STAT3-α molecules phosphorylated on serine 727 [185, 354], show that quiescent PBL-T cells contain low basal levels of serine phosphorylated STAT1-α (STAT1-αpSer727) and STAT3-α (STAT3-αpSer727) (Figure 3.6). These basal levels are strongly increased upon IFN-α treatment. However, no IL-12-induced increase in serine phosphorylation of either STAT1-α or STAT3-α is detected (Figure 3.6). Hence, IL-12 regulates tyrosine and serine phosphorylation of STAT4 but does not regulate serine phosphorylation of STAT1-α and STAT3-α.

3.2.5 The constant presence of IL-12 is required for sustained activation of STAT4

Each cytokine/ STAT system has different characteristics and kinetics. To deepen our understanding of the activation of STAT4 by IL-12, kinetics studies were performed. This showed to be very similar in PBL-T cells (data not shown) and Kit225 cells (Figure 3.7 A). IL-12-induced STAT4 tyrosine phosphorylation and

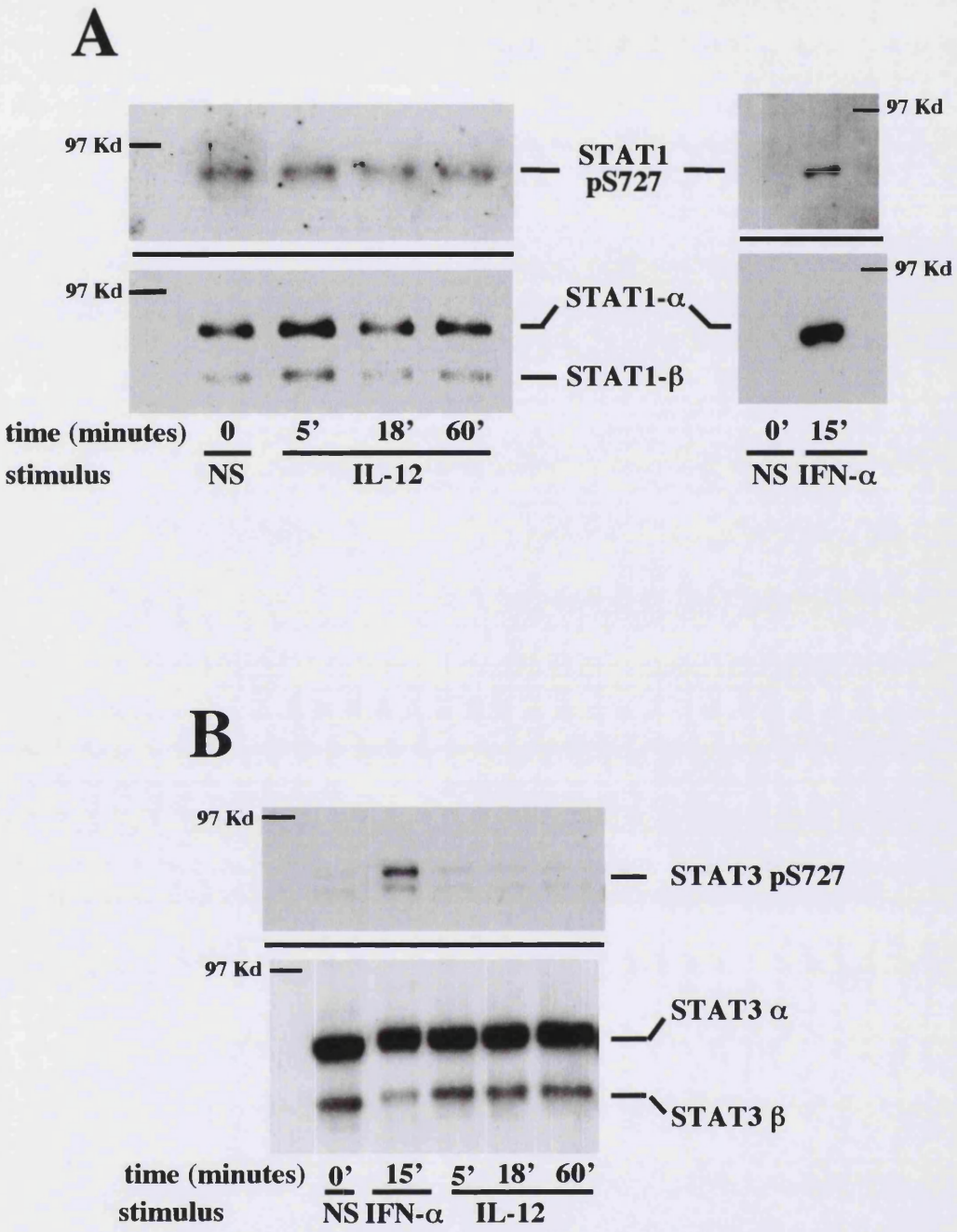


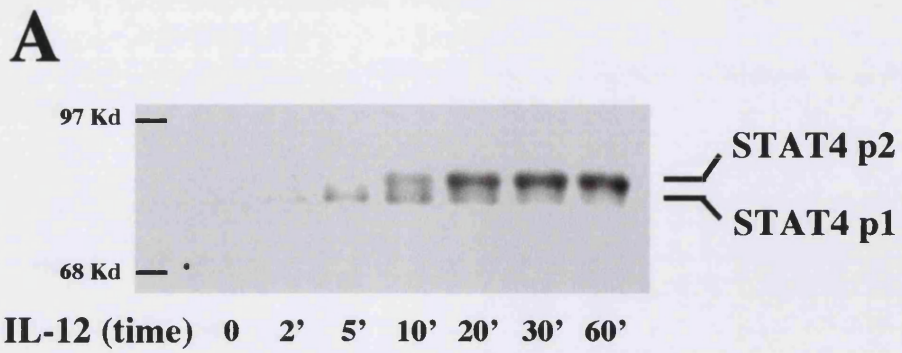
Figure 3.6) IL-12 does not induce the serine phosphorylation of STAT1 or STAT3 in human T lymphocytes.- IL-12-induced serine phosphorylation of STAT1-α **(A)** and STAT3-α **(B).** PBL-T cells (20 x 10^6 in A and 15 x 10^6 in B) were left unstimulated (NS) or stimulated with mentioned cytokines for 15 minutes or indicated times. Proteins were affinity precipitated using the IFN-γR-pY440 peptide (A left panels and B) or the GRR oligonucleotide (A right panels), resolved on SDS-PAGE and immunoblotted with anti-STAT1pSer727 (A upper panels) or with anti-STAT3pSer727 (B upper panel), followed by reprobing with anti-STAT1 pan or anti-STAT3 pan, respectively (A and B lower panels). Results are representative of at least three independent experiments.

consequent DNA binding is rapid and sustained (Figure 3.7 A). DNA binding of STAT4 can be detected after two minutes of stimulation with IL-12 and is still present in cells activated for one hour. The two different electrophoretic mobility forms of STAT4 are produced at specific stimulation times. The faster migrating band, corresponding to STAT4p1 is the only one present at early stages (2-5 minutes). The slower migrating band that corresponds to STAT4p2, appears at longer stimulation times. 10-20 minutes after addition of IL-12 equivalent amounts of the two different forms of STAT4 can be seen. At 30 minutes and later time points, STAT4p2 is the predominant form (Figure 3.7 A).

As will be discussed in Chapters 5 and 6, IL-12 activation of STAT4 can be sustained for several hours. To determine if IL-12 has to be constantly present to maintain STAT4 activation, we used an IL-12 pulse stimulation assay. After initial 60 minutes stimulation time, IL-12 was removed and GAS-STAT4-affinity precipitation performed on cells that were incubated in the presence or absence of IL-12 for further time points. In the constant presence of IL-12, active DNA bound STAT4 can be detected for at least 4 hours (Figure 3.7 B). Since the minimum stimulation time was 60 minutes, only the STAT4p2 is present. Upon cytokine removal, the amount of active STAT4 molecules is equivalent in the cells kept with and without IL-12 for the first hour. Later time points show that STAT4 activation reduces to approximately 50% at two hours and is almost completely lost at four hours after IL-12 withdrawal (Figure 3.7 B). Therefore, the constant presence of IL-12 is required for sustained activation of STAT4.

3.2.6 IL-12 selectively regulates the cellular localisation of STAT4

According to the prototypical model for Jak/ STAT activation (Chapter 1, section 1.7.4), latent STAT molecules are localised in the cytoplasm and upon activation, translocate to the nucleus where they bind to specific DNA sequences. The effects



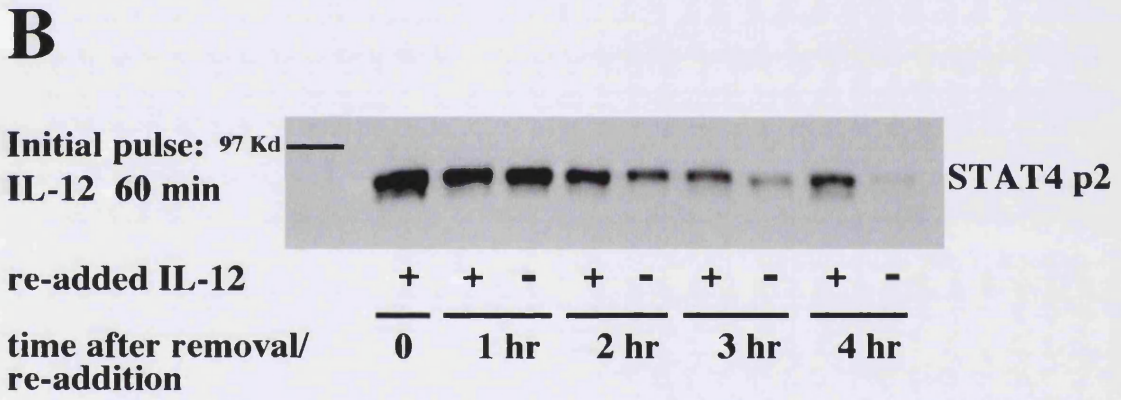


Figure 3.7) The constant presence of IL-12 is required for sustained activation of STAT4.- Kinetics (A) and pulse stimulation assay (B) of IL-12-induced STAT4 DNA binding. A) Kit225 cells were left unstimulated (NS) or stimulated with IL-12 for indicated times. B) PBL-T cells were pulse stimulated with IL-12 for 60 minutes, IL-12 was removed and cells were further incubated in the presence (+) or absence (-) of IL-12 for the indicated times. Proteins were affinity precipitated from 20 x 10^6 cells using the GRR (A) or GAS-STAT4 (B) oligonucleotides, resolved on SDS-PAGE and immunoblotted with anti-STAT4 C-. Results are representative of at least three independent experiments.

of IL-12 on STAT4 nuclear localisation had not been previously explored. To examine the cellular localisation of STAT4 in T lymphocytes, cytoplasmic and nuclear cell lysates were prepared from quiescent or IL-12 stimulated Kit225 cells (Figure 3.8 A) and PBL-T cells (data not shown). Western blot analysis shows STAT4 to be cytoplasmic in quiescent cells but present in the nucleus of IL-12 stimulated T cells. In the continued presence of IL-12, STAT4 accumulates in the nucleus so that within 60 minutes of IL-12R triggering approximately 50% of the total cellular pool of STAT4 is found in the nuclear compartment. Both STAT4p1 and STAT4p2 can be detected in the nuclei of T cells after 5 minutes of stimulation with IL-12. However, STAT4p2 is the predominant form detected in the nucleus in sustained (60 minutes) responses (Figure 3.8 A).

As shown in section 3.2.4, IL-12 is unable to induce tyrosine phosphorylation and DNA binding of STAT1, STAT3 and STAT5. Western blot analysis comparing cytoplasmic and nuclear lysates of IL-12 stimulated Kit225 cells shows that IL-12 also fails to induce nuclear translocation of these STATs (Figure 3.8 B). Low amounts of STAT1, STAT3 and STAT5 can be detected in the nucleus of unstimulated Kit225 cells but IL-12 does not induce further accumulation of either of these molecules.

1-(5-Isoquinolinylsolfonyl)-2-methyl-piperazine (H7) is a broad-spectrum serine/ threonine kinase inhibitor that has been shown to block the serine phosphorylation of STAT1-α, STAT3-α, STAT4 and STAT5 [184, 351, 353, 566]. To examine the role of the STAT4 post-translational modification in regulating the cellular localisation of STAT4, we assayed the effect of H7 on IL-12-induced STAT4 activation and nuclear translocation. IL-12-induced generation of STAT4p2 can be prevented by inhibition of cytokine regulated serine kinases with H7 (Figure 3.9). An H7 dose response experiment shows that approximately 40% of STAT4p2 is inhibited by concentrations of 50-100 μM, while 200 μM prevents around 60% of STAT4p2 (Figure 3.9 A). The effect of H7 was more pronounced at shorter time

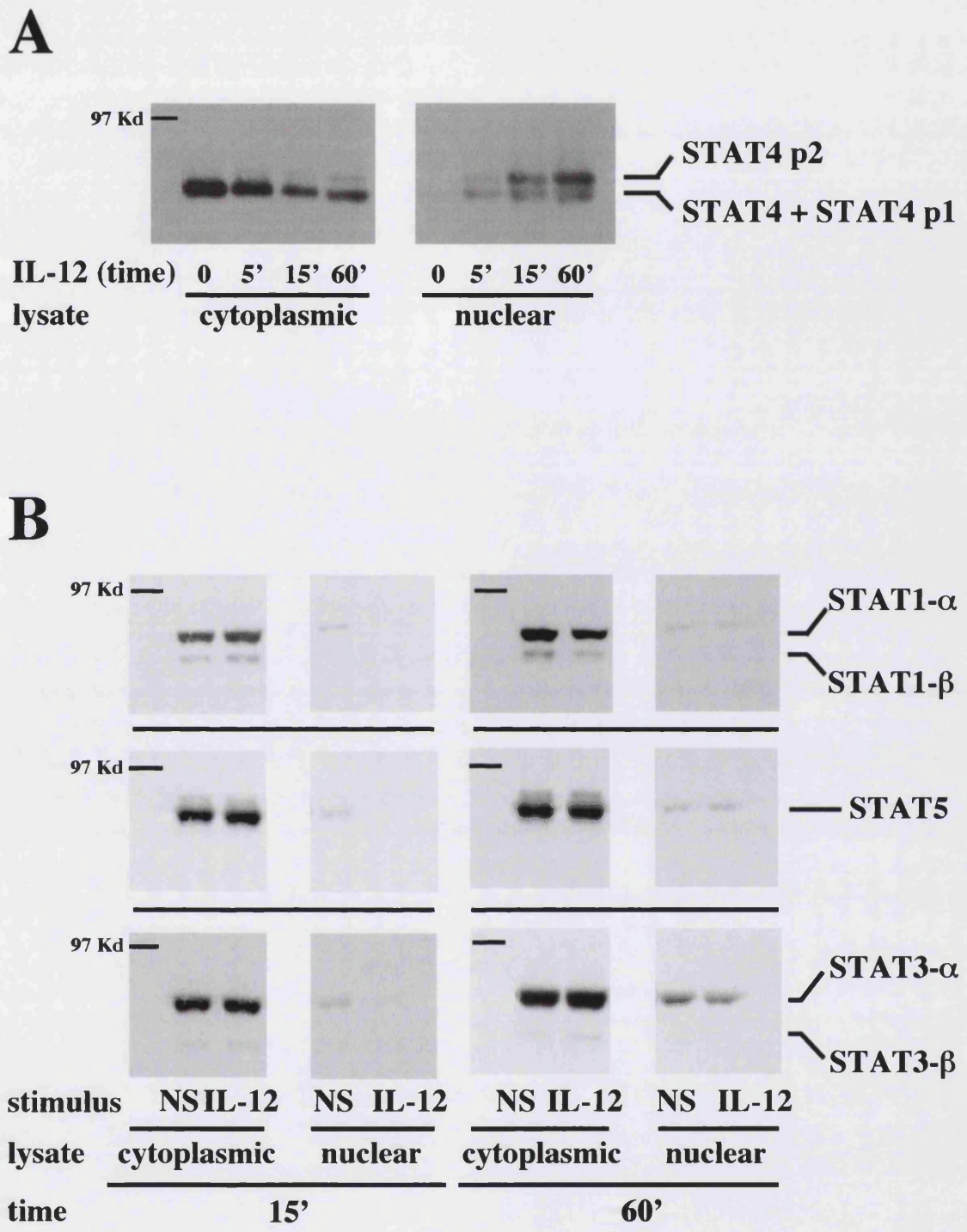


Figure 3.8) IL-12 selectively regulates the cellular localisation of STAT4.– IL-12-induced nuclear translocation of STAT4 (A) and STAT1, STAT5 and STAT3 (B). A, B) Kit225 cells were left unstimulated (NS) or stimulated with IL-12 for the indicated times. Acetone precipitated proteins from cytoplasmic (5 x 10^6 cells) or nuclear (10 x 10^6 cells) lysates were resolved on SDS-PAGE and immunoblotted with anti-STAT4 C- (A), anti-STAT1 pan, anti-STAT5 pan or anti-STAT3 pan, respectively (B). Results are representative of at least two independent experiments.

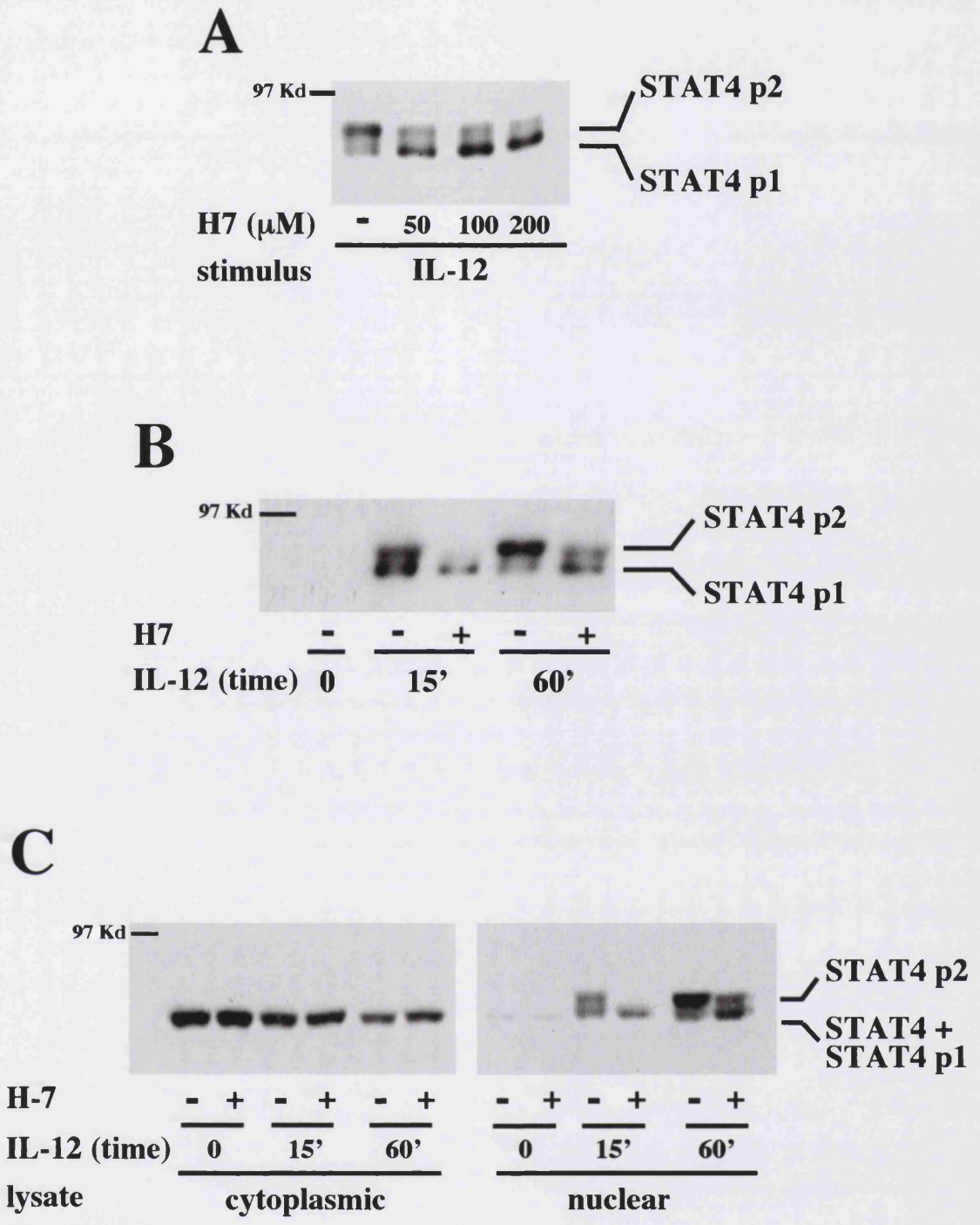


Figure 3.9) The H7 serine/ threonine kinase inhibitor prevents the accumulation of STAT4p2 without stopping the nuclear translocation of STAT4.- Effect of the H7 serine/ threonine kinase inhibitor on IL-12-induced STAT4 DNA binding (A, B) and nuclear translocation (C). PBL-T cells (A) and Kit225 cells (B, C) were incubated with 200 μM H-7 or the indicated concentrations for 30 minutes prior to stimulation with IL-12 for 18 minutes or the indicated times. Proteins were GRR-affinity precipitated from 20 x 10^6 cells (A, B) or acetone precipitated from cytoplasmic (5 x 10^6 cells) or nuclear (10 x 10^6 cells) lysates, resolved on SDS-PAGE and immunoblotted with anti-STAT4 C-. Results are representative of at least two independent experiments.

points. A kinetics assay reveals that H7 blocks around 80-90% of IL-12 induced STAT4p2 at 15 minutes stimulation time, while only 40-50% inhibition is seen at 60 minutes (Figure 3.9 B). However, the cellular fractionation experiments show that H7 prevents accumulation of STAT4p2 but does not stop the nuclear translocation of STAT4 (Figure 3.9 C). Thus, STAT4p2 molecules may accumulate in the nuclei of IL-12 activated cells but the generation of STAT4p2 is not obligatory for STAT4 nuclear translocation.

3.2.7 IL-12 induces the transcriptional activity of the GRR-CAT reporter gene

Once active STATs have translocated into the nucleus they bind to specific DNA sequences to turn on or off specific genes. To asses the transcriptional activity of IL-12 induced STAT4 a GRR-CAT (GRR-chloramphenicol acetyl transferase) reporter plasmid that has been shown before to monitor STATs transcriptional activity, was transfected into Kit225 cells [184, 240]. Lysates obtained from cells stimulated with different doses of IL-12, IL-2 or IFN-α for 18 hours were assayed for CAT-activity as described in materials and methods (Chapter 2). The IL-12 response is maximal at 20 ng/ ml inducing a three-fold increase when compared to unstimulated cells (Figure 3.10). The GRR-CAT reporter gene is not specific for STAT4. It is a well-established reporter gene for STAT1, STAT3 and STAT5 activity. In IL-12 activated cells however, the only GRR binding STAT is STAT4. IL-2 and IFN-α respectively induce STAT5 and STAT3 or STAT1 and STAT3 GRR binding and therefore both cytokines correspondingly induce activation of the GRR-CAT reporter gene (Figure 3.10).

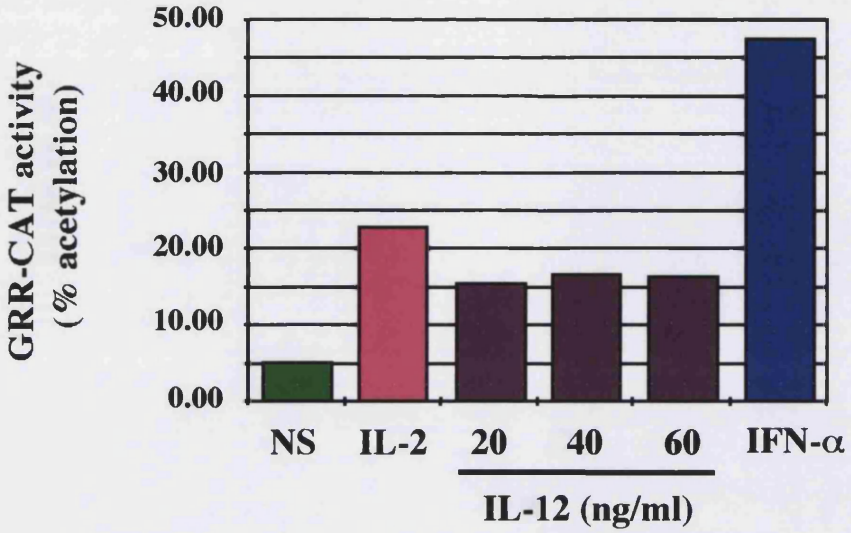


Figure 3.10) IL-12 induces the transcriptional activity of the GRR-CAT reporter gene.- Kit225 cells transfected with the GRR-CAT reporter plasmid, were left unstimulated (NS) or stimulated with 20 ng/ ml IL-2, 10^3 U/ ml IFN-α or the indicated concentrations of IL-12 for 18 hours. Lysates were obtained and assayed for CAT-activity as described in materials and methods. Values are given as percentage of acetylation. A representative assay from five independent experiments is shown.

3.3 Discussion

The data presented in this chapter show the basic characteristics of IL-12 activation of STAT4 in our system. IL-12 is able to activate STAT4 in both human PBL-T cells and the IL-2-depentent T cell line Kit225, in a very similar fashion. Two forms of active DNA bound STAT4 with distinct electrophoretic mobility, designated STAT4p1 and STAT4p2 can be seen in the IL-12 activated STAT4 complexes. STAT4p1 is generated rapidly within two minutes of IL-12R triggering. STAT4p2, which corresponds to tyrosine phosphorylated STAT4 molecules that have been further modified by serine phosphorylation [353], accumulates more slowly appearing 10-20 minutes following IL-12 exposure.

Generation of STAT4p2 is inhibited by the use of the broad-spectrum serine/ threonine kinase inhibitor H7. H7 blocks formation of STAT4p2 by approximately 80-90% at shorter time points (15-20 minutes) while the effect at longer time points (60 minutes) is around 40-50%. The reduced effectiveness of H-7 at the longer time points could reflect the instability of H-7 but this point was not examined in any detail. In repeated experiments it was seen that H-7 causes an approximate 20-35% reduction of total DNA bound STAT4 in IL-12 activated cells. The reduction on DNA binding that accompanies the H7 inhibition of STAT4p2 could suggest that the serine phosphorylation of STAT4 increases its DNA binding affinity. This is in agreement with the observation that it is the STAT4p2 fashion that remains active (DNA-bound) at longer time points and is also consistent with data from other STAT systems. H-7 has been shown to block IL-2 induced DNA binding of STAT3 by an unknown mechanism [185]. In the case of IL-12 and STAT4 we could not exclude that H-7 might also reduce the tyrosine phosphorylation of STAT4 possibly by partial inhibition of Jak2 and Tyk2.

Comparison of cytoplasmic and nuclear lysates of IL-12 stimulated human T lymphocytes demonstrates that the IL-12/ STAT4 response is accompanied by a rapid nuclear translocation of STAT4. During the sustained IL-12 response, it is STAT4p2 that accumulates in the nucleus. However, STAT4 serine phosphorylation is not required for nuclear translocation, because the inhibition of STAT4p2 by H7 does not impede STAT4p1 nuclear translocation. These studies of the post-translational modifications during the sustained response to IL-12 are a clear indication that IL-12 signal transduction involves the regulation of cytosolic serine kinases and/ or phosphatases.

Our present data show that IL-12 can only regulate STAT4 responses in activated PBL-T cells. This indicated that one or more of the components involved in the IL-12/ STAT4 response is absent in primary T cells. In agreement with a previous report, we found that primary T cells obtained from different donors express very low to undetectable levels of STAT4 protein [76]. Similarly, both chains of the IL-12R are absent in $CD4^+$ and $CD8^+$ primary T cells.

STAT4 protein and IL-12 responsiveness, as measured by IL-12-induced STAT4 DNA binding, can be detected as early as three hours following T cell activation. We were unable to detect up-regulation of the cell surface expression levels of IL-12Rβ1 and IL-12Rβ2 by flow cytometry analysis before 24 hours of T cell activation. Similarly, it has being reported that expression of IL-12Rβ1 and IL-12Rβ2 mRNAs could only be found at 24 hours following TcR triggering [77]. This apparent discrepancy just indicates that levels of IL-12R sufficient to mediate IL-12 activation of STAT4 early in T cell activation are below the detection limits of flow cytometry analysis and RNAse protection assays (Figure 3.3 and [77]).

Although most of the STATs are activated in cell culture by many different ligands, the phenotypes of various STAT knockout mice reveal an unexpected specificity in

the biological roles of these molecules (for review see [376]). Only the STAT4 knockout mice show defects in Th1 cell responses, the most important biological effect of IL-12 [7, 8]. The data on IL-12 activation of STATs other than STAT4 has been contradictory. STAT1 and STAT3 DNA binding, as well as STAT3 tyrosine phosphorylation, have been shown in mouse Th1 cells, in human NK cells and in a human T cell subpopulaiton ($CD8^{+}CD18^{bright}$ T cells) [490, 567, 568]. IL-12 has also been reported to induce both, tyrosine phosphorylation and DNA binding of STAT5 in PHA-activated T cells [569]. In contrast, in co-transfection of functional IL-12R into COS cells and immuno-precipitation assays in human PHA-activated T cells and γδ T cell lines, IL-12 failed to induce tyrosine phosphorylation or DNA-binding of STAT1, STAT2 or STAT3 [75, 76, 564]. Herein, we find no evidence that IL-12 can induce the tyrosine phosphorylation and DNA binding, or indeed the serine phosphorylation of STAT1, STAT3 and STAT5. Experiments employing oligonucleotide affinity precipitation techniques and/ or Western blot analysis with specific anti-phosphotyrosine antibodies have clearly shown that IL-12 is unable to induce the tyrosine phosphorylation and DNA binding of STAT1, STAT3 and STAT5. Both IFN-α and IL-2 activate STAT3 and STAT5. IFN-α is a strong activator of STAT3 and a weak activator of STAT5, while IL-2 has the opposite activation strengths. Our data show that STAT3 and STAT5 activation by IL-12 could not be detected even when it was compared with the respectively weakest stimulus. These data are further supported by the lack of IL-12-induced nuclear translocation of these molecules.

The present data indicate that STAT4 but not STAT1-α or STAT3-α are phosphorylated on serine residues in response to IL-12. This suggests that the STAT4 serine kinase/ phosphatase is not co-ordinately regulated with the STAT1-α and STAT3-α serine kinases. This could mean that different serine kinases phosphorylate STAT1-α, STAT3-α and STAT4. Alternatively, it is possible that there is a common STATs serine kinase and that cytokines control the specificity of

STATs serine phosphorylation by regulating the physical interaction of each particular STAT with the serine kinase. Cytokines and antigen receptors can induce serine phosphorylation of non-tyrosine phosphorylated STAT1-α and STAT3-α [185, 354, 356, 371]. It is not at all clear whether serine phosphorylation of STAT4 is dependent or independent of its tyrosine phosphorylation status.

At the beginning of this project, tyrosine and serine phosphorylation of STATs were recognised as important post-translational modifications. The tyrosine and serine phosphorylation sites of STAT1 and STAT3 were known and correspond to Tyr701 and Ser727 in STAT1, and Tyr705 and Ser727 in STAT3 [347, 353]. Homologue residues in STAT4 at positions Tyr693 and Ser721 were proposed as putative phosphorylation sites [353]. STAT4 constructs bearing mutations in either Tyr693 (STAT4pY693F) or Ser721 (STAT4S721A) have been recently reported [570]. The STAT4pY693F mutant was not phosphorylated on tyrosine residues in response to IL-12 when transient transfection and anti-phosphotyrosine immunoblotting were used in fibroblasts [570]. The transcription of an IL-12-inducible reporter gene was respectively abolished and reduced by co-transfection of the STAT4pY693F and STAT4S721A mutants in Jurkat cells [570]. These data indicate an important role for Tyr693 in IL-12-induced STAT4 activation and transcriptional activity. Although this study suggests a possible role for STAT4Ser721 in IL-12 induced STAT4 transcriptional activity, the phosphorylation of STAT4Ser721 in response to IL-12 was not determined [570]. The STAT4S721A mutant showed an irrelevant decrease in the IL-12-induced STAT4 mobility shift, indicating that this site is not the main STAT4 serine phosphorylation site [570]. The development of specific reagents could help to solve this question.

During the course of this project an attempt was made to raise specific antibodies for STAT4pSer721. Such antisera would have allowed us to study if IL-12 regulates the phosphorylation of STAT4Ser721 in activated T cells. Serum was

obtained from rabbits injected with a serine phosphorylated peptide corresponding to aa 719-722: Pro-Met-pSer-Pro from STAT4. This peptide was immunogenic and the rabbit successfully generated antisera reactive with the STAT4pSer721 peptide as judged by ELISA. However, it failed to detect IL-12-induced STAT4p2 or any other form of STAT4 when used in Western blot analysis. This could either indicate that Ser721 is not phosphorylated in response to IL-12 or could be a problem with the sensitivity of this technique. Similarly we tried to map the STAT4 serine phosphorylation sites in IL-12 activated T cells by mass spectrometry. This part of the project was time consuming but it ultimately failed. We perfected the technique of large-scale affinity precipitation of DNA-bound STAT4 from IL-12 activated T cells. Unfortunately, STAT4 protein levels in activated PBL-T cells are very low and the recovery of sufficient amounts of STAT4p2 for mass spectrometry analysis was not possible. The oligonucleotide precipitation technique was scaled up to process activated STAT4 from $1x10^9$ T cells, but this still provided insufficient material for analysis by mass spectrometry. It was impractical to scale up further and this approach was abandoned.

The lack of a specific STAT4 serine phosphorylation site makes it difficult to assess at this point the possible role of serine phosphorylation in STAT4 function. As mentioned in Chapter 1 (section 1.7.5), serine phosphorylation of STAT1-αS727 and STAT3-αS727 are required for maximal transcriptional activity in response to IFN–γ and IFN–α, respectively [347]. Constructs with mutations in the specific STAT4 serine phosphorylation site would be required to determine if IL-12-induced STAT4 serine phosphorylation has the same function as that shown for STAT1-αpS727 and STAT3-αpS727. An important role for serine phosphorylation in mediating STATs interaction with other proteins has been illustrated by the serine phosphorylation-dependent interaction of STAT1-α with MCM5, a member of the transcription machinery [365]. To date no STAT4-interacting protein has been reported.

In summary, this chapter shows that IL-12 selectively activates STAT4 in human T lymphocytes. IL-12/ STAT4 activation involves tyrosine and serine phosphorylation and is accompanied by nuclear translocation and transcriptional activity. Although the role of STAT4 serine phosphorylation remains elusive, it is clear that it is not required for STAT4 nuclear translocation. It might possibly have a role in STAT4 DNA binding but this needs to be explored further.

CHAPTER 4

IL-12 regulates STAT4 serine phosphorylation via Phosphatidylinositol 3-kinase, Protein Kinase C and Ras-independent signal transduction pathways

4.1 Introduction

IL-12 induces sustained activation and nuclear translocation of STAT4 and this regulatory process is coupled to both tyrosine and serine phosphorylation of this molecule (Chapter 3). IL-12 activated tyrosine kinases are the Janus kinases Jak2 and Tyk2 but little is known about IL-12 regulation of serine kinases. Manipulation of the IL-12/ STAT4 response could represent a useful tool to control altered Th1 cell responses involved in autoimmune diseases. The development of suitable manipulation approaches requires a better understanding of the pathways involved in the IL-12/ STAT4 response. The data shown in Chapter 3 suggest that the IL-12-induced STAT4 serine kinase may be specific for STAT4 and not for other STAT proteins, pointing to this serine kinase as a suitable target for therapeutic manipulation. Therefore, the object of the present Chapter was to test the possible link of IL-12 with known pathways that involve the activation of serine/ threonine kinases in T lymphocytes. This would allow us to either probe or discard the role of a novel serine kinase in IL-12/ STAT4 responses. We explored the role of the MAPKs Erk1,2, PI3-K and PKC in IL-12/ STAT4 regulation.

Cytokine stimulation induces two critical modifications to STAT proteins: tyrosine and serine phosphorylation. Tyrosine phosphorylation allows dimerisation and

consequent DNA binding. Serine phosphorylation of STATs has been described to be required for maximal transcriptional activity, to mediate interactions with other proteins and to increase STATs binding to certain low affinity DNA sequences (see Chapter 1, section 1.7.5 and [347, 365, 566]). For example, serine phosphorylation of STAT1-α (STAT1-αpSer727) regulates the interaction of tyrosine phosphorylated STAT1-α dimers with MCM5, a protein involved in DNA replication [365]. The phosphorylation of STAT3-α on serine 727 can be regulated via the Ras/ MAPK pathway, notably Erk1,2 [185, 357]. STAT3α-Ser727 resides within a consensus MAPK phosphorylation site located in the C-terminal transactivation domain (aa 725-728: Pro-Met-Ser-Pro) [347]. However, MAPK-independent pathways that couple cytokines to the regulated serine phosphorylation of STAT3-α have also been identified [185, 357]. The serine phosphorylation of STAT5 or STAT1-αSer727 is not regulated by the MAPKs Erk1,2 [184, 354, 371] and the identity of the STAT5 serine kinase is not known.

IL-12 induces STAT4 serine phosphorylation [353]. The carboxy-terminus of STAT4 has a putative MAPK phosphorylation site in its transactivation domain at position 721 (aa 719-722: Pro-Met-Ser-Pro), prompting the suggestion that IL-12 regulates STAT4 serine phosphorylation via MAPKs [353]. It has been described that IL-12 activates a kinase with similar ion exchange characteristics to the MAPK Erk1 [571], but the ability of IL-12 to regulate Erk1,2 MAPKs had never been directly shown in T lymphocytes.

4.2 Results

4.2.1 STAT4 is not regulated by the Ras/ MAPK pathway during IL-12 responses

It has been proposed that IL-12 regulates STAT4 serine phosphorylation via the MAPKs Erk1,2 [353]. In T lymphocytes the first step in the Ras/ MAPK pathway in response to IL-2 is the tyrosine phosphorylation of the adapter molecule Shc. This enables the recruitment of a second adapter protein Grb2 into the plasma membrane through its SH2 domain. Tyrosine phosphorylated Shc binds with high affinity to a GST-fusion protein containing the full length Grb2 (GST-Grb2). Western blot analysis using an anti-Shc antibody shows that GST-Grb2 does not affinity purify Shc molecules from quiescent or IL-12 stimulated Kit225 cells. However, Shc molecules can be readily precipitated from Kit225 cells stimulated with IL-2 (Figure 4.1 A upper panel). The SH3 domain of Grb2 binds to the guanine nucleotide exchange factor Sos in a stimulation-independent fashion. Equal protein loading in the GST-Grb2 complexes was confirmed by Western blot analysis using an anti-Sos antibody (Figure 4.1 A lower panel). Comparable results were obtained in PBL-T cells (data not shown). These data indicate that IL-12 does not induce the tyrosine phosphorylation of the adapter molecule Shc.

The Shc-Grb2 complex recruits the guanine nucleotide exchange factor Sos to the plasma membrane where it activates the Ras-GTPase. There are however, many alternative routes for Ras activation in T cells. Accordingly, we directly examined the ability of IL-12 to induce the accumulation of active Ras-GTP complexes. Active GTP-bound Ras interacts with the serine/ threonine kinase Raf-1 through its Ras binding domain (RBD). To assess Ras activation an affinity precipitation technique using a GST-fusion protein containing the RBD of Raf-1 (GST-RBD), can be employed. The GST-RBD fusion protein specifically binds to active Ras-

GTP complexes, but not to inactive Ras-GDP complexes [558]. Quiescent PBL-T cells were left unstimulated or stimulated with IL-12 or IL-2 for different times prior to affinity precipitation with the GST-RBD. Active Ras-GTP molecules are not detected in the RBD-GST complexes of quiescent or IL-12 stimulated PBL-T cells at any time from 10 to 60 minutes (Figure 4.1 B). In contrast, active Ras-GTP/ GST-RBD complexes can be readily seen in cells stimulated with IL-2. High amounts of active Ras-GTP can be detected at 10 minutes of IL-2 stimulation, remain at approximate levels at 15 minutes and decrease, but are still present after 1 hour. These data show that Ras activation in response to IL-2 is strong, rapid and sustained (Figure 4.1 B). In contrast, over a prolonged time course no increase in active Ras-GTP complexes is detected in IL-12 stimulated PBL-T cells (Figure 4.1 B).

One function for Ras is to regulate the activity of the MAPKs Erk1,2. The failure of IL-12 to activate Ras would predict that IL-12 could not activate Erk1,2. However, it is possible that an alternative route for MAPK activation could be triggered by IL-12. The presence of a putative MAPK phosphorylation site in the transactivation domain of STAT4 (aa 719-722: Pro-Met-Ser-Pro) prompted us to investigate more directly the effect of IL-12 on the MAPKs Erk1,2. The activation of Erk1,2 requires that both threonine and tyrosine residues within the molecule, specifically Thr183 and Tyr185, are phosphorylated. A sensitive method to follow activation of these MAPKs is to use Western blot analysis of T cell lysates with an anti-phospho Erk1,2 antibody that specifically recognises Erk1,2pThr183pTyr185 (active Erk1,2). Active Erk1,2 molecules can be readily detected in cell lysates prepared from IL-2 or Phorbol 12,13-dibutyrate (PdBu) stimulated T cells (Figure 4.1 C upper panel). In contrast, no active Erk1,2 is seen in quiescent cells or in PBL-T cells activated with IL-12 at any time from 2 to 60 minutes (Figure 4.1 C upper panel). Equivalent Erk1,2 total protein levels are shown as loading control (Figure 4.1 C lower panel). These data indicate that IL-12 does not activate Erk1,2 in PBL-T cells.

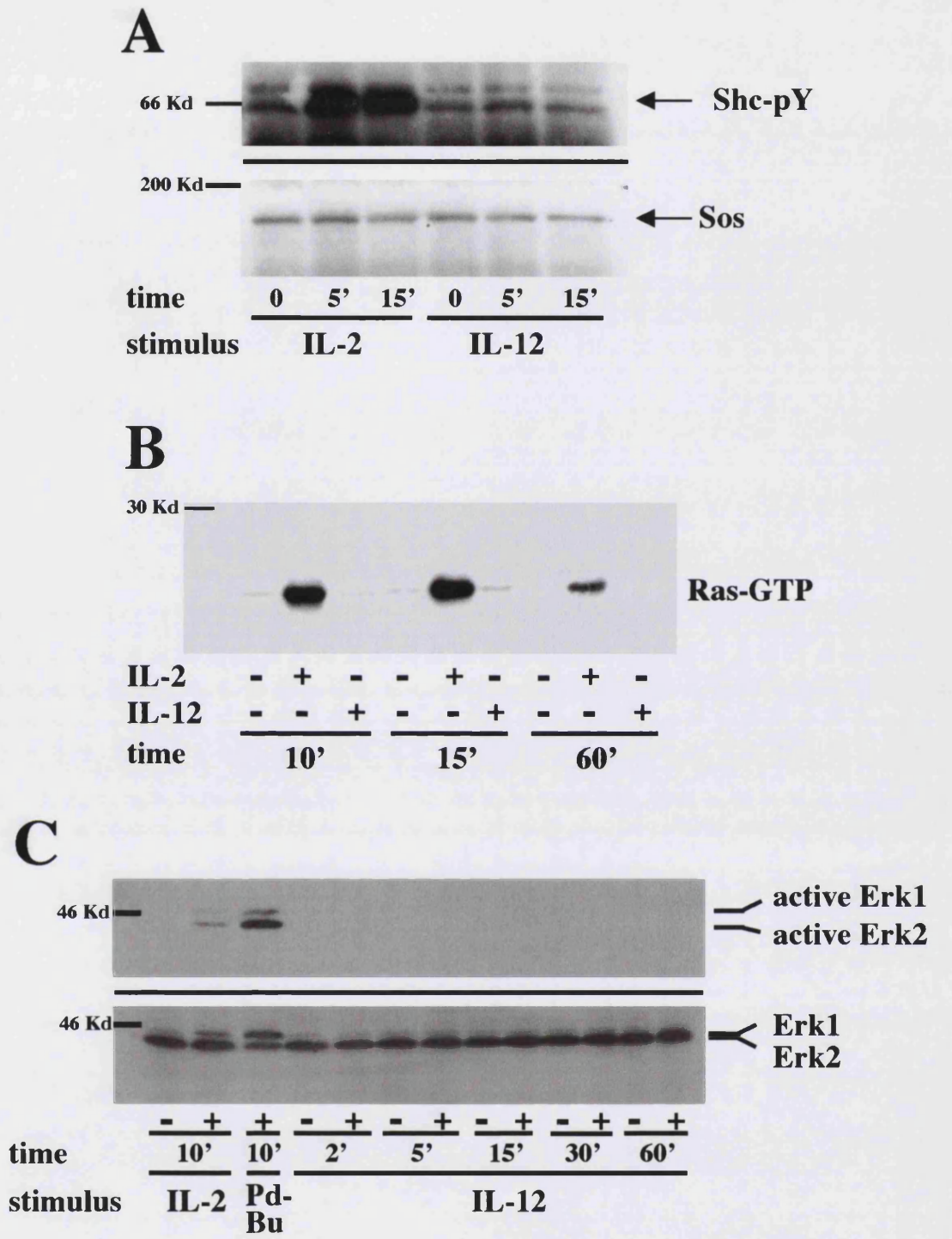


Figure 4.1) IL-12 does not regulate Shc tyrosine phosphorylation or activation of Ras and Erk1,2. IL-12-induced tyrosine phosphorylation of Shc (A) and activation of Ras (B) and Erk1,2 (C). Kit225 cells (A) and PBL-T cells (B, C) were left unstimulated (NS) or treated with indicated stimulus for 10 minutes or mentioned times. Proteins were either affinity precipitated from 20 x 10^6 cells using the GST-Grb2 fusion protein (A) or the GST-RBD fusion protein (B), or acetone precipitated from the total cell lysate of 5 x 10^6 cells (C). Proteins were resolved on SDS-PAGE and immunoblotted using: anti-Shc followed by reprobing with anti-Sos (A); anti-Ras pan (B) or anti-active Erk1,2 (Erk1,2pThr183pTyr185) followed by reprobing with anti-Erk1,2 pan (C). Results are representative of at least two independent experiments.

Active MAPKs Erk1,2 phosphorylate and activate the transcriptional activity of the ternary complex protein Elk-1 [212]. Accordingly, we examined the influence of IL-12 on Elk-1 transcriptional activity using a well-characterised reporter gene system [212]. This system employs two different plasmid constructs: a) a fusion protein of the DNA binding domain of LexA and the C-terminus of Elk-1 under the control of a constitutive promoter (pEF-NLexA-Elk-1C) and b) two copies of the LexA binding site upstream of a CAT reporter gene under the control of a thymidine kinase (tk) minimal promoter (LexA-OP-tkCAT). When these two plasmids are transfected into Kit225 cells, the LexA region of the fusion protein (NLexA-Elk-1C) binds constitutively to its site on the reporter gene construct (LexA-OP-tkCAT). Elk-1 activatory signals will induce the phosphorylation of the Elk-1 region of the fusion protein, leading to recruitment of the transcriptional machinery and consequent CAT gene induction. The data show that IL-2 induces a five-fold increase in CAT activity in Kit225 cells transfected with the pEF-NLexA-Elk-1C/ LexA-OP-tkCAT system, but no stimulatory effects of various doses (20-60 ng/ ml) of IL-12 on CAT reporter gene induction could be seen in repeated experiments (Figure 4.2 A). These data indicate that IL-12 does not induce the transcriptional activity of Elk-1.

Failure to detect activation of Erk1,2 and Elk-1 in response to IL-12 indicates that MAPKs are unlikely to be involved in IL-12-induced STAT4 serine phosphorylation. To further support these findings, we investigated the effect of the PD098059 compound (2'-Amino-3'-methoxyflavone) on IL-12-induced STAT4p2. The PD098059 compound specifically inhibits activation of the Erk1,2 kinases Mek1,2, and consequently blocks the Erk1,2 pathway [572]. PBL-T cells were left untreated or treated with either 10 μM or 50 μM PD098059 for 30 minutes prior to stimulation with IL-12. GRR-affinity precipitation followed by anti-STAT4 Western blot analysis were performed (Figure 4.2 B upper panel). Lysates remaining after the GRR-affinity precipitation were acetone precipitated and the inhibition of Erk1,2 activity by PD098059 was assayed using the anti-

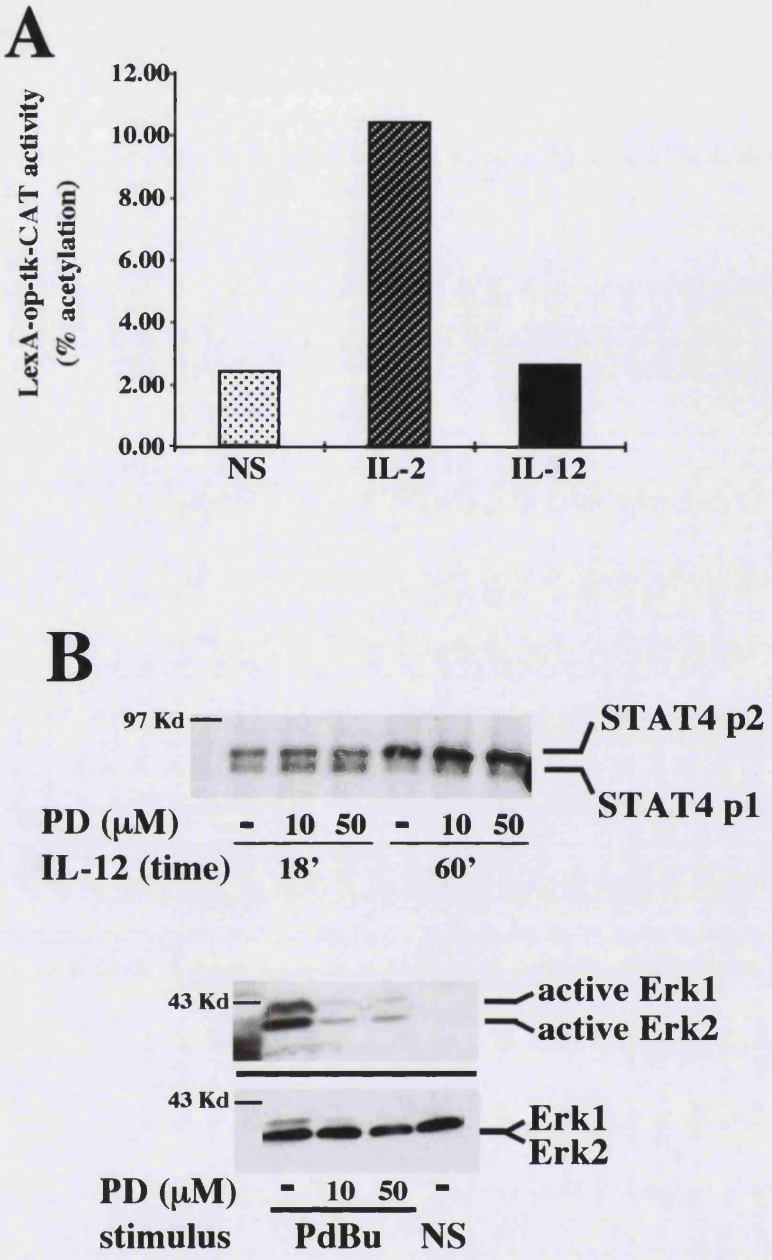


Figure 4.2) STAT4 is not regulated by the Ras/ MAPK pathway during IL-12 responses.- A) IL-12-induced activation of Elk-1. B) Effect of the Mek1,2 inhibitor (PD098059) on IL-12-induced STAT4 activation. A) Kit225 cells were transfected with expression plasmids for the LexA-OP-tk-CAT and pEF-NLexA-Elk-1C fusion protein reporter system (LexA/ Elk-1-CAT). Cells were cultured with 20 ng/ ml IL-2 or 60 ng/ ml IL-12 for 18 hours and lysates were assayed as indicated in materials and methods for CAT-activity. Values are given as percentage of acetylation. B) PBL-T cells were treated with 10 or 50 μM of the PD098059 compound as indicated (PD) prior to stimulation with IL-12 for indicated times. Proteins were either affinity precipitated using the GRR oligonucleotide (20 x 10^6 cells) (B upper panel) or acetone precipitated from the lysate remaining after the affinity precipitation in B upper panel (5 x 10^6 cells) (B lower panel). Proteins were resolved on SDS-PAGE and immunoblotted using: anti-STAT4 C- (B upper panel) or anti-active Erk1,2 (Erk1,2pThr183pTyr185) followed by reprobing with anti-Erk1,2 pan (B lower panel). Results are representative of at least two independent experiments.

Erk1,2pThr183pTyr185 antibody in Western blot analysis (Figure 4.2 B lower panel). The data show that both concentrations of PD098059 that effectively blocked Erk1,2pThr183pTyr185 in response to PdBu did not prevent the accumulation of STAT4p2 in response to IL-12 at any stimulation time (Figure 4.2 B). Hence, the Mek/ Erk pathway is not necessary for IL-12 regulation of STAT4. More important, there is no evidence that IL-12 activates the Ras/ MAPK pathway.

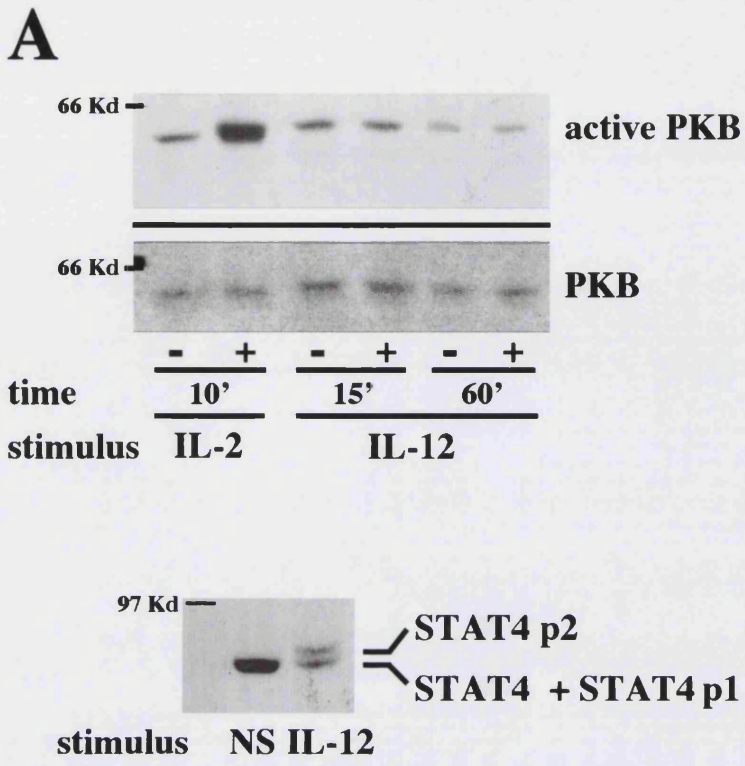
4.2.2 Role of PI3-K in the IL-12-induced STAT4 response

Many haematopoietic cytokines activate PI3-K that phosphorylates PI(4,5)-P_2 on the D-3 position of the inositol ring to produce PI(3,4,5)-P_3 (see Chapter 1, section 1.5.2.2). Recent studies have shown that antigen receptors in T and B cells regulate the serine phosphorylation of STAT1-α and STAT3-α via PI3-K mediated signals [356, 573]. The inhibitory effect of wortmanin (a PI3-K inhibitor) and rapamycin (a mTOR inhibitor) on STAT3-α serine phosphorylation in response to IFN-α and CNTF, respectively, also suggests a possible role for the PI3-K pathway in STATs serine phosphorylation. [574, 575]. Considering the lack of evidence for the involvement of the Ras/ MAPK pathway in the regulation of STAT4 by IL-12, we examined the role of PI3-K in IL-12 responses. One primary target for PI3-K in T cells is the serine/ threonine kinase PKB. Notably, activation of PI3-K is both necessary and sufficient for PKB activation in T cells [239]. To examine the association of PI3-K with IL-12/ STAT4 responses, we tested the ability of IL-12 to activate PKB. PKB activation is accompanied by serine phosphorylation on Ser473. A specific antibody that recognises active PKB molecules (anti-PKBpSer473) can be used in Western blot analysis to monitor PKB activity. PKBpSer473 molecules are readily detected in cells stimulated with IL-2 but no PKBpSer473 was seen in quiescent cells or in PBL-T cells stimulated with IL-12 at any time between 15 and 60 minutes (Figure 4.3 A upper panel). The full activity

of IL-12 in this experiment is demonstrated by the induction of STAT4p2 mobility shift in these cells (Figure 4.3 A lower panel). Therefore, over a prolonged time course IL-12 does not activate PKB in PBL-T cells.

The transcription factor E2F has been identified as a target of the PI3-K/ PKB pathway in T cells [240]. Assays of E2F transcriptional activity are thus a second sensitive way to monitor PI3-K/ PKB activation in T cells. E2A-CAT, which contains two E2F-binding sites upstream of the CAT gene is a well-characterised reporter gene that can be used to study the transactivation potential of E2F [561, 562]. 48 hours IL-2 deprived Kit225 cells were transfected with the E2A-CAT reporter plasmid and were cultured for 18 hours in the absence or presence of IL-12 (20, 40 and 60 ng/ ml) or were co-transfected with a constitutively active form of PI3-K (rCD2p110) [563]. Lysates were obtained and the percentage of CAT produced was determined as described in materials and methods (Chapter 2). The percentage of CAT produced from E2A-CAT is low in quiescent T cells and is not modified by IL-12 stimulation at any concentration from 20 to 60 ng/ ml (Figure 4.3 B and data not shown). However, E2A-CAT transcription is strongly induced by PI3-K signalling. Therefore, IL-12 does not induce E2F transcriptional activity over a wide cytokine dose.

Finally, we analysed the effect of the PI3-K inhibitor 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) on IL-12/ STAT4 responses. A GRR-affinity precipitation assay shows that LY2940002 does not inhibit IL-12-induced DNA binding of STAT4 or modulate the kinetics of STAT4p2 accumulation (Figure 4.4 upper panel). The activity of the LY2940002 inhibitor was verified by its blocking effect on IL-2-induced PKBpSer473 (Figure 4.4 lower panels). Collectively, these results suggest that IL-12 is not able to activate the PI3-K/ PKB pathway and we find no evidence for the involvement of PI3-K in IL-12 regulation of STAT4.



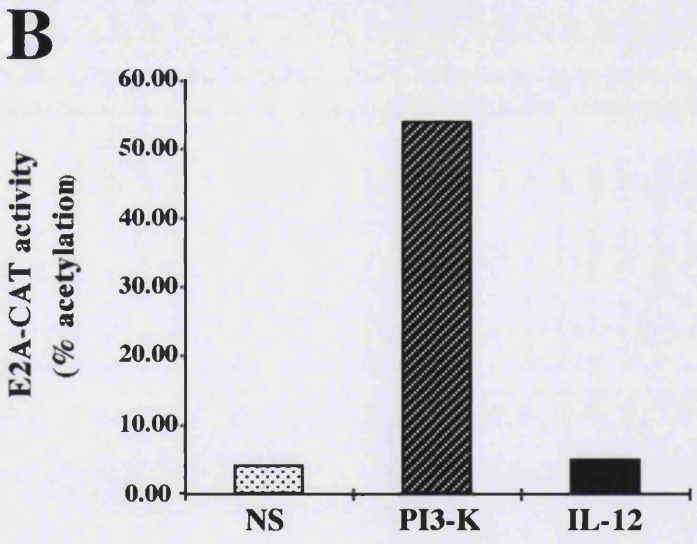


Figure 4.3) IL-12 does not activate PKB or E2F in human T cells.– IL-12-induced activation of PKB (A) and E2F (B). A) PBL-T cells were left unstimulated (NS) or stimulated with mentioned cytokines for 60 minutes or indicated times. Proteins were acetone precipitated from the total cell lysate of 5 x 10^6 cells, resolved on SDS-PAGE and immunoblotted with anti-active PKB (anti-PKBpSer473) followed by sequential reprobing with anti-PKB pan (A upper panel) and anti-STAT4 C- (A lower panel). B) Kit225 cells transfected with the E2A-CAT reporter plasmid were left unstimulated, stimulated with 60 ng/ ml IL-12 or co-transfected with rCD2p110, a constitutively active form of PI3-K (PI3-K). Cell lysates obtained after 18 hours were assayed for CAT-activity as described in materials and methods. Values are given as percentage of acetylation. Results are representative of at least three independent experiments.

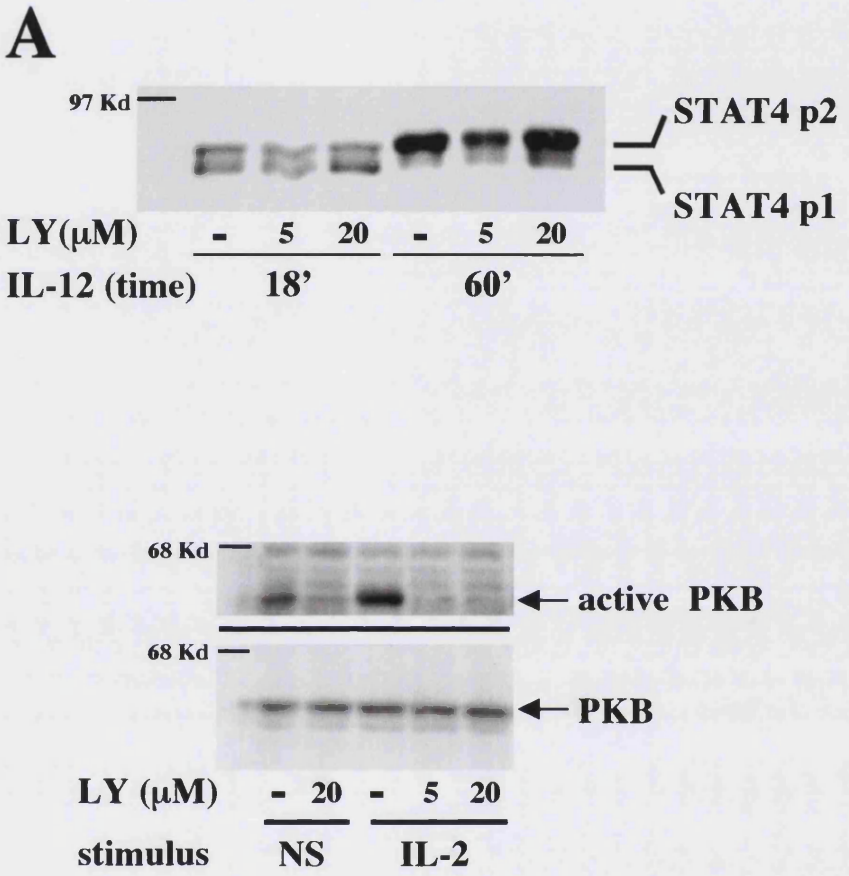


Figure 4.4) The IL-12/ STAT4 response is PI3-K-independent.– Effect of the PI3-K inhibitor LY294002 on IL-12-induced STAT4 activation. PBL-T cells were treated with 5 or 20 μM of the LY294002 compound (LY) as indicated, prior to be left unstimulated (NS) or stimulated with mentioned cytokines for 10 minutes or indicated times. Proteins were GRR-affinity precipitated (20 x 10^6 cells) (upper panel) or acetone precipitated from the lysate remaining after affinity precipitation in the upper panel (5 x 10^6 cells) (lower panels). Proteins resolved on SDS-PAGE were immunoblotted with anti-STAT4 C- (upper panel) or anti-active PKB (anti-PKBpSer473) followed by reprobing with anti-PKB pan (lower panels). Results are representative of at least three independent experiments.

4.2.3 The IL-12/ STAT4 response is PKC-independent

As illustrated above, IL-12 induction of STAT4 serine phosphorylation is not mediated by activation of the Ras/ MAPK or PI3-K pathways. These results prompted us to study the possible role of IL-12 in the regulation of another well-characterised signalling pathway that involves activation of serine/ threonine kinases in T lymphocytes. PKCs are a large family of related serine/ threonine kinases, which are subdivided according to their regulation by calcium, dyacilglycerol (DAG) and phospholipids. Two different sets of data indicate that PKCs can play a role in cytokine regulation of STATs serine phosphorylation. Pharmacological agents such as PdBu, PMA (phorbol-12-myristate-13-acetate) and ionomycin that respectively activate PKCs and calcium, have been shown to induce serine phosphorylation of STAT1-α and STAT3-α [185, 413]. Two PKC isoforms, PKC-α and PKC-δ have been implicated in the regulation of DNA binding of STAT5 and of serine phosphorylation of STAT3-α upon PRL stimulation in mammary gland [576-578]. We used an indirect approach to investigate the activation of PKCs in response to IL-12. The serine kinase protein kinase D (PKD, also known as PKC-μ) is activated in response to phorbol esters (such as PdBu) and TcR triggering by a PKC-dependent mechanism [579, 580]. In particular, the η and ε isoforms of PKC have been implicated in the regulation of PKD activity [579]. PKD possesses an autophosphorylation site at Ser916. The degree of Ser916 phosphorylation during lymphocyte activation exactly correlates with the activation status of PKD [560]. We explored the effect of IL-12 on PKD activation by using an antibody that specifically recognises PKD molecules phosphorylated on Ser916 (anti-PKDpSer916) [560] in Western blot analysis of IL-12 stimulated PBL-T cells. While PdBu treatment induces a strong phosphorylation of PKD on Ser916, we failed to detect any serine phosphorylated PKD in response to IL-12 (Figure 4.5 A).

Although above data show that PKD and therefore PKCη and PKCε are not activated by IL-12, it remained the possibility that other PKC isoforms could be involved in the IL-12/ STAT4 response. The inhibitor RO 31-8220 has been shown to block the activation of PKCs [581-583]. We assessed the effect of RO 31-8220 on IL-12 induced STAT4p2. An oligonucleotide affinity precipitation assay shows that RO 31-8220 does not have any effect on the IL-12 induced STAT4 DNA binding or STAT4p2 appearance over a broad inhibitor dose (0.25-5.0 μM) (Figure 4.5 B upper panel). The activity of RO 31-8220 was verified by inhibition of PdBu-induced Erk1,2pThr183pTyr185 (Figure 4.5 B lower panel). These data indicate that the PKC pathway is not implicated in IL-12 responses and therefore it does not mediate the serine phosphorylation of STAT4.

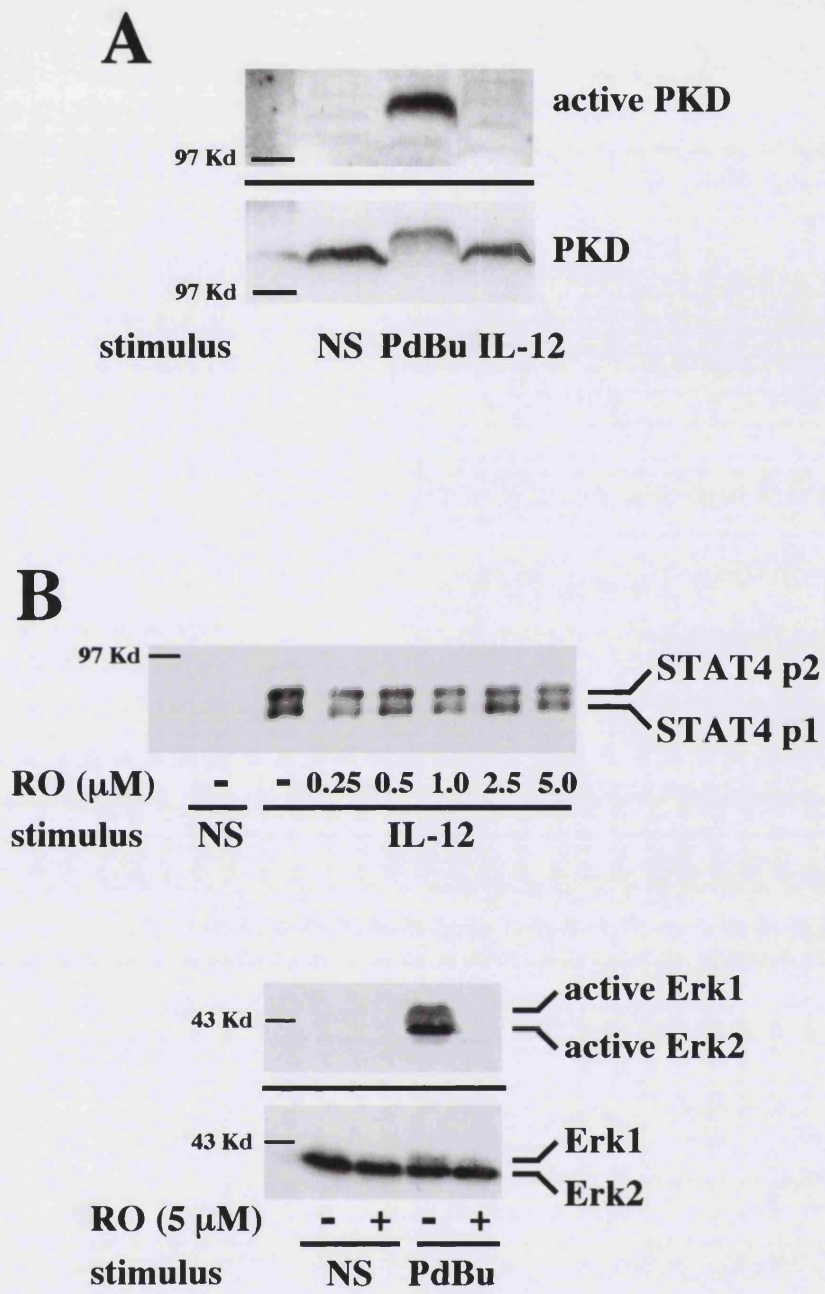


Figure 4.5) The IL-12/ STAT4 response is PKC-independent .– **A)** IL-12-induced activation of PKD. **B)** Effect of the PKC inhibitor RO31-8220 on IL-12-induced STAT4 activation. **A** and **B)** PBL-T cells were left unstimulated (NS) or stimulated with PdBu for 10 minutes or IL-12 for 18 minutes. Proteins were GRR-affinity precipitated from 20 x 10^6 cells (B upper panel) or acetone precipitated from total cell lysates (10 x 10^6 cells) (A) or from the lysate remaining after GRR-affinity precipitation in B upper panel (5 x 10^6 cells) (B lower panel). Proteins were resolved on SDS-PAGE and immunoblotted with anti-active PKD (anti-PKDpSer916) followed by reprobing with anti-PKD pan (A); anti-STAT4 C- (B upper panel); or anti-active Erk1,2 (Erk1,2pThr183pTyr185) followed by reprobing with anti-Erk1/Erk2 pan (B lower panel). Results are representative of at least three independent experiments.

4.3 Discussion

The studies of the post-transitional modifications of STAT4 during the IL-12 response are a clear indication that IL-12 signal transduction involves the regulation of cytosolic serine kinases and/ or phosphatases (Chapter 3). Previous studies have proposed that STAT4 serine phosphorylation is regulated by the MAPKs Erk1,2 [353]. The data presented in this Chapter refute this hypothesis: detailed analysis of IL-12 responses in the human T cell line Kit225 and in human PBL-T cells show that under conditions where IL-12 induces strong activation of STAT4, there is no evidence for IL-12 activation of the Ras/ MAPK pathway. Moreover, high concentrations of the PD098059 compound, which effectively block activation of Erk1,2 in response to PdBu, do not prevent IL-12 induction of STAT4p2. These data exclude that IL-12 regulates the serine phosphorylation of STAT4 via the Ras/ MAPK pathway.

The MAPKs p38 and JNK1,2 can phosphorylate and activate the transcriptional activity of the ternary complex protein Elk-1 [212-214]. Hence, assays of Elk-1 transcriptional activity can also be used to *in vivo* assay the cellular activation of p38 and JNK1,2 MAPKs. The failure to detect Elk-1 transcriptional activity by IL-12, indicates that p38 and JNK1,2 MAPKs are not activated in response to IL-12 in human PBL-T cells.

Further evidence comes from data presented in Chapter 3, showing that IL-12 induces the serine phosphorylation of STAT4 but not of STAT1-α or STAT3-α. Activation of MAPKs p38 and Erk1,2 induces STAT1-α and STAT3-α serine phosphorylation, respectively [185, 360]. Therefore, the lack of Elk-1 and Erk1,2 activation in response to IL-12 could explain the failure of IL-12 to induce STAT1-α and STAT3-α serine phosphorylation.

These data are in agreement with a previous report showing that IL-12 does not activate p38 and JNK1,2 MAPKs in human $CD8^{+}CD18^{bright}$ T cells [568]. In contrast, there is recent evidence showing activation of p38 MAPK in lysates of IL-12 stimulated NK3.3 cells, a mouse Th1 clone and mouse PBL-T cells [570, 584, 585]. These studies monitored p38 MAPK activity using *in vitro* kinase assays or Western blot analysis with anti-phospho p38 MAPK antibodies. It is difficult to assess the relative strength of IL-12 in inducing p38 MAPK phosphorylation from these reports, because it was compared to either basal or IL-2-induced (a weak activator of p38 MAPK [199]) phosphorylation of p38 MAPK. The ability of IL-12 to regulate the p38 MAPK was also assayed by transient transfection assays in fibroblast, in which the IL-12 effect was weak [570]. Beside the discrepancies on activation of p38 MAPK by IL-12, these studies confirmed that IL-12 does not activate the Erk1,2 or JNK1,2 MAPKs [570, 585].

One interesting fact about IL-12 and p38 MAPK, is that IL-12 activation of p38 MAPK seems more readily detectable in mouse T cells and NK cells than in human PBL-T cells [570, 584, 585]. The basis for the species and/ or cell lineage differences in IL-12 regulation of p38 MAPK is not known. There is precedent for this type of species specificity in STAT4 regulation. Clear evidence has shown that the signals involved in Th1 cell differentiation vary between the mouse and human systems. While IL-12 induces the activation of STAT4 in both mouse and human T cells, IFN-α can only activate STAT4 in human T cells (see Chapter 1, section 1.11.5 and [89, 519, 520]). There is also some evidence that both IL-12-induced signals and signals that regulate STAT4 are cell lineage specific. IL-12 activates NF-κB in DCs but not in NK or PBL-T cells [48]. Similarly IL-2 activates STAT4 in NK cells but not in T cells [526]. Therefore the activation of p38 MAPK in response to IL-12 in mouse systems and/ or different cell lineages does not necessarily mean that IL-12 will activate p38 MAPK in human PBL-T cells. Further studies in the human system are necessary to reconcile the discrepancies between the data presented in this Chapter and the data derived from the systems

discussed above.

One possible explanation for discrepancies in the conclusions about IL-12 and p38 MAPK is that the stoichiometry of this response seems to be very low [570, 584, 585]. Positive results may only be detected in cells that express high amounts of p38 MAPK or in systems in which p38 MAPK is overexpressed. For example, IL-12 is able to synergise with IL-2 to activate p38 MAPK in $CD8^{+}CD18^{bright}$ T cells but not in $CD8^{+}CD18^{dim}$ T cells [568]. $CD8^{+}CD18^{bright}$ T cells express much higher amounts of p38 MAPK compared to $CD8^{+}CD18^{dim}$ T cells [568]. It would be interesting to compare the levels of expression of p38 MAPK in the NK3.3 cell line with those in NK and PBL-T cells derived from healthy donors, as well as in mouse T cells compared to human T cells.

In the cellular systems where IL-12 activation of p38 MAPK has been reported, there were no direct analysis of the possible link between p38 MAPK activity and STAT4 serine phosphorylation. The direct effects of a p38 MAPK inhibitor on STAT4 serine phosphorylation were not explored by either changes in electrophoretic mobility (i.e. formation of STAT4p2) or inhibition of STAT4pSer721 in Western blot analysis with specific antibodies [570]. Similarly, indirect attempts with inhibitors and mutants of the p38 MAPK were used to explore the role of p38 MAPK in controlling the expression of STAT4 regulated genes. For example, the enhancement of transcriptional activity of a STAT4 reporter gene by overexpression of p38α MAPK and its upstream regulator MKK6, in transformed fibroblasts [570].

Despite of the lack of solid evidence for the direct involvement of p38 MAPK in STAT4 serine phosphorylation, there is not doubt that both STAT4 and p38 MAPK are required for Th1 cell differentiation [7-9, 233, 234]. As discussed in Chapter 1, while STAT4 is essential for IL-12-induced Th1 cell differentiation, p38 MAPK seems to play important roles in inducing up-regulation of IL-12 and IFN-γ

gene expression in macrophages and T cells, respectively (see Chapter 1, section 1.5.2.1 and [7-9, 233-236]). There is evidence that p38 MAPK inhibitors block IL-12 up-regulation of IFN-γ mRNA expression and secretion in mouse systems [585]. However, this effect is similar in wild type and STAT4-null cells, indicating that p38 MAPK activation may be involved in IL-12-induced IFN-γ gene regulation in a STAT4-independent manner [585]. Similarly, p38 MAPK was shown to be involved in IL-12-induced up-regulation of CD25 gene expression [584]. However, there is no direct evidence of the participation of STAT4 in the regulation of CD25 transcription [584, 586]. Taken together, these data indicate that STAT4 and p38 MAPK control specific Th1 cell differentiation signals through their participation in different signalling pathways.

Many haematopoietic cytokines activate PI3-K and stimulate the activity of the serine/ threonine kinase PKB. We investigated the activation of two recognised downstream targets of PI3-K in T cells: PKB and E2F [239, 240], in response to IL-12. We found no evidence that IL-12 activates PI3-K signalling pathways in human PBL-T cells. Moreover, PI3-K inhibitors do not influence IL-12 generation of STAT4p1 and STAT4p2. The failure of IL-12 to activate PI3-K pathways distinguishes IL-12 signalling from that of many other haematopoietic cytokines. For example, IL-2, IL-4 and IL-7 are all potent activators of the PI3-K/ PKB pathway. PI3-K signals are critical for proliferative responses in T cells. The failure of IL-12 to regulate this pathway could explain why this cytokine is a poor mitogen in T cells when compared to IL-2 and IL-4.

Some data have suggested a possible link between some of the isoforms of the PKC family of serine/ threonine kinases and STATs serine phosphorylation [185, 413, 576-578]. Data presented is this chapter show that PKD, which is regulated by PKCs is not activated in response to IL-12 in human PBL-T cells. This was confirmed by the use of the PKC inhibitor RO 31-8220, which shows that the block of PKC activation does not affect IL-12 induced STAT4p2. These results correlate

with two different sets of data. 1) It has long being recognised that the differentiation of naive T cells into the Th1 or Th2 phenotype not only depends on the cytokines present at the time of activation. Th cell differentiation is also regulated by the nature and intensity of the TcR-transduced stimulus [495, 497, 498, 587-589]. A recent report shows that T cell differentiation can be regulated by the balance of PKC and calcium signals induced by TcR triggering in both human and mouse T cells. Stimulation of calcium or inhibition of PKC favoured Th1 cell responses [590]. Therefore activation of PKC by IL-12 the major cytokine driving Th1 cell responses, would oppose the low TcR-induced PKC activation required for Th1 cell differentiation. 2) The activation of JNK MAPKs requires both PKC activation and calcium signals [198]. Our present data show that IL-12 does not activate PKC, indicating that IL-12 would not be able to activate JNK MAPKs. This is confirmed by the lack of IL-12 induced activation of Elk-1, a downstream effector of JNK MAPKs.

In summary, the data presented in this chapter show that the IL-12-induced STAT4 serine kinase is not sensitive to inhibitors of the PI3-K, PKC or MAPKs Erk1,2. Moreover, IL-12 activation of STAT4 in human PBL-T cells is not accompanied by stimulation of the Ras guanine nucleotide binding cycle or stimulation of MAPKs Erk1,2, activation of PKCs or initiation of the PI3-K signalling pathways. Therefore, we have eliminated the participation of these pathways in IL-12-induced STAT4 responses.

These data support the findings shown in Chapter 3 and indicate that the IL-12-induced STAT4 serine kinase is likely to be a novel enzyme not previously involved in T lymphocyte signalling. The specificity of this serine kinase on controlling STAT4 function may constitute a very useful and specific target for the manipulation of IL-12/ STAT4 regulated Th1 cell responses involved in autoimmune diseases. Therefore, defining the identity of this kinase remains of high importance. Determination of the STAT4 serine residue that is phosphorylated in

response to IL-12, followed by development of specific reagents for its study would be required to accomplish this task.

CHAPTER 5

The IFN-α/ STAT4 response in human T lymphocytes: Characteristics and comparison with the IL-12/ STAT4 response

5.1 Introduction

IFNs were originally discovered and characterised as soluble proteins that induce anti-viral activity in target cells. Intense research concentrated in understanding the mechanisms involved in the anti-viral and anti-proliferative responses induced by both types of IFNs (IFN-α/β and IFN-γ) [262]. The molecular characterisation of IFN-induced gene expression led to the discovery of the Jak/ STAT signalling pathway [294, 322, 345]. The essential role of STATs in IFN signalling was demonstrated by impaired IFN-induced anti-viral responses in the STAT1 knock out mice [377, 378].

The activation of STAT1 is common for both types of IFNs (for review see [422]), but only IFN-α/β have been reported to activate STAT2, STAT3, STAT4 and STAT5. The biological relevance of STAT1 and STAT2 in IFN-α/β-induced anti-viral responses has been established through knock out mice studies [377-379]. STAT3 and STAT5 are normally involved in promoting cell cycle progression and cellular transformation as well as preventing apoptosis in response to various stimuli (for review see [376, 406]). A recent paper has suggested the participation of STAT3 in IFN–α-induced anti-apoptotic signals in a lymphoblastoid cell line [591]. However, no role has been described for IFN-α/β-regulated STAT5 [592-

594].

The majority of IFN-α/β/ STAT studies has been conducted in non-lymphoid cells, but little is known about the control of IFN-α activation of STAT1, STAT2, STAT3 and STAT5 in T lymphocytes [171, 184, 595, 596]. Comprehensive studies of IFN-α/ STATs regulation in human T lymphocytes are therefore necessary to understand the involvement of these transcription factors in the immunomodulatory effects of IFN-α.

Activation of STAT4 is an essential response for Th1 cell differentiation [7, 8]. As discussed in Chapter 1 (section 1.11.5) it is now recognised that while IL-12 is able to activate STAT4 in both mouse and human systems, the activation of STAT4 in response to IFN-α is limited to humans [89, 519, 520]. Detailed studies on the characteristics of the IFN-α/ STAT4 response have not been previously reported.

Both IL-12 and IFN-α have been shown to drive Th1 cell differentiation in human *in vitro* systems [77, 89]. However, patients with impaired IL-12 responses and consequent Th1 cell differentiation deficiencies suffer from recurrent *Mycoplasma sp.* and *Salmonella sp.* infections [67-69]. This indicates that IFN-α is not able to fully compensate for the lack of IL-12 responses in human *in vivo* systems.

The present Chapter aimed to investigate the mechanism that impedes IFN-α to fully drive Th1 cell differentiation in human *in vivo* systems. We initially studied the characteristics of STAT4 activation in response to IFN-α in human T lymphocytes. This enabled us to perform a direct comparison between the IL-12/ STAT4 and IFN-α/ STAT4 responses. Some of the characteristics of the regulation of STAT1, STAT3 and STAT5 in response to IFN-α will also be shown and discussed.

5.2 Results

5.2.1 IFN-α activates STAT4 in both PBL-T cells and Kit225 cells

STAT4 has been shown to be fully activated in response to IFN-α in human T lymphocytes [89, 353]. Oligonucleotide affinity precipitation comparing IFN-α activation of PBL-T cells and the T cell line Kit225, was used to establish the conditions to study IFN-α/ STAT4 regulation in our system. Analogous to IL-12-induced STAT4 activation (Chapter 3, Figure 3.1 A), STAT4p1 and STAT4p2 can be isolated from both IFN-α-stimulated PBL-T cells and Kit225 cells but not from unstimulated cells (Figure 5.1 A). STAT4 shows the same pattern of oligonucleotide binding preference when induced in response to IL-12 or IFN-α (data not shown). The GAS-STAT4 oligonucleotide recovers the highest amount of STAT4p1 and STAT4p2, followed by GRR, SIEM67 and SIE (Chapter 3, Figure 3.4 B and data not shown).

5.2.2 IFN-α activates STAT1, STAT3 and STAT4 with different kinetics in human T lymphocytes

IFN-α has been shown to activate STAT1, STAT2, STAT3 and STAT5 in non-lymphoid cells [422, 575, 592-594, 597, 598]. However, little is know about the characteristics of IFN-α/ STATs regulation in human T lymphocytes. As shown in Chapter 3, IFN-α induces tyrosine phosphorylation and consequent DNA binding of STAT1, STAT3 and STAT5. IFN-α is a strong activator of STAT1 and by comparison with IL-2, is a strong activator of STAT3 and a weak activator of STAT5 in human T lymphocytes (Chapter 3, Figure 3.5).

The kinetics of activation of STAT1, STAT3 and STAT4 in response to IFN-α were compared in PBL-T cells. The initial kinetics of IFN-α activation of STAT4 is very similar to that induced by IL-12 (Chapter 3, Figure 3.7 A and Figure 5.1 B upper panel). DNA-bound STAT4 molecules can be detected at five minutes of IFN-α stimulation and are still present for at least 90 minutes. The appearance of the two distinct electrophoretic mobility forms of STAT4 is also comparable. STAT4p1 is present at early time points (5 minutes), while STAT4p2 appears at longer stimulation times. Equivalent amounts of STAT4p1 and STAT4p2 can be detected 15-30 minutes after addition of IFN-α. STAT4p2 is the predominant form at later time points (Figure 5.1 B upper panel).

IFN-α induces the rapid activation of STAT1, STAT3 and STAT4. DNA bound STAT1, STAT3 and STAT4 molecules are seen at five minutes of IFN-α stimulation. While IFN-α activation of STAT3 and STAT4 is sustained over 90 minutes, the IFN-α/ STAT1 response is more transient. The IFN-α/ STAT1 response is maximal at 15 minutes, is very low by 60 minutes and is no longer seen at 90 minutes (Figure 5.1 B middle and lower panels). The kinetics of STAT5 DNA-binding is very similar to that of STAT1 (data not shown). These data show that IFN-α induces the tyrosine phosphorylation and consequent DNA binding of STAT1, STAT3 and STAT4 with specific kinetics for each STAT. The IFN-α/ STAT1 response is much more transient than the IFN-α/ STAT3 or STAT4 responses.

5.2.3 IFN-α regulates the cellular localisation of STAT4

The initial kinetics of STAT4 activation, as well as the generation of STAT4p1 and STAT4p2 are very similar in response to IL-12 and IFN-α (Chapter 3, Figure 3.7 A

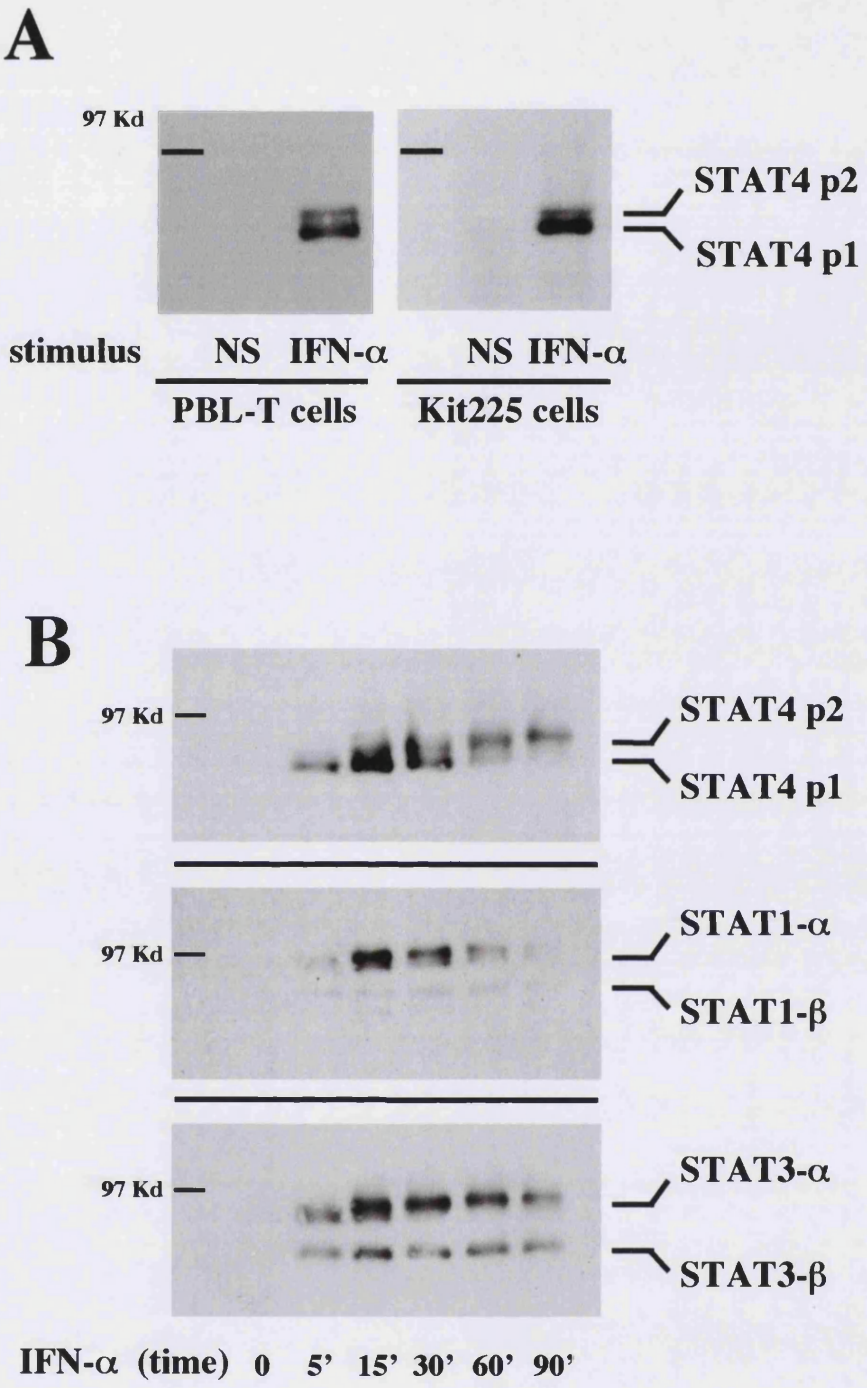


Figure 5.1) IFN-α activates STAT1, STAT3 and STAT4 with different kinetics in human T lymphocytes.- **A)** IFN-α-induced STAT4 DNA-binding in PBL-T and Kit225 cells. **B)** Kinetics of IFN-α-induced DNA-binding of STAT1, STAT3 and STAT4. 20 x 10^6 PBL-T cells (A, B) or Kit225 cells (A) were left unstimulated (NS) or stimulated with 10^3 U/ ml of IFN-α for 10 minutes or indicated times. Proteins were affinity precipitated using the GRR (A) or the GAS-STAT4 (B) oligonucleotide, resolved on SDS-PAGE and immunoblotted with anti-STAT4 C- (A) or anti-STAT4 C- followed by sequential reprobing with anti-STAT1 pan and anti-STAT3 pan.

and Figure 5.1 B upper panel). We have shown that STAT4 translocates to the nucleus in response to IL-12 in human T lymphocytes (Chapter 3, Figure 3.8 A). To examine if IFN-α regulates the cellular localisation of STAT4, cytoplasmic and nuclear cell lysates were prepared from quiescent and IFN-α stimulated Kit225 cells (Figure 5.2 A) and PBL-T cells (data not shown). Western blot analysis shows that the cellular localisation of STAT4 is regulated very similarly by IFN-α and IL-12 (Chapter 3, Figure 3.8 A and Figure 5.2 A). A low amount of STAT4 is present in the nucleus of unstimulated cells. Upon 18 minutes of IFN-α stimulation, the basal nuclear levels of STAT4 are clearly increased. The presence of both STAT4p1 and STAT4p2 can be noted. The amount of nuclear STAT4 is further increased with longer (60 minutes) IFN-α stimulation. STAT4p2 is detected predominantly in the nucleus of IFN-α stimulated T cells.

Studies of the post-translational modification of STAT4 in response to IL-12 indicated that STAT4 serine phosphorylation is not required for nuclear translocation (Chapter 3, Figure 3.9 C). To test if this is a general characteristic of STAT4 activation, we studied the effect of the H7 serine/ threonine kinase inhibitor on IFN-α-regulated STAT4 nuclear translocation. Pre-treatment of T cells with H7 blocks IFN-α-induced generation of STAT4p2 (Figure 5.2 B). The effect of H7 is more pronounced at shorter time points, in a similar way as for IL-12-induced STAT4p2. A kinetics assay reveals that H7 blocks approximately 90% of IFN-α-induced STAT4p2 at 15 minutes, while only around 60% inhibition is seen at 60 minutes stimulation time (Figure 5.2 B). However, the cellular fractionation experiment shows that H7 prevents accumulation of STAT4p2 but STAT4p1 molecules can still translocate into the nucleus of IFN-α-stimulated PBL-T cells (Figure 5.2 C). These data confirm that, independent of stimulus, STAT4 serine phosphorylation is not required for STAT4 nuclear translocation.

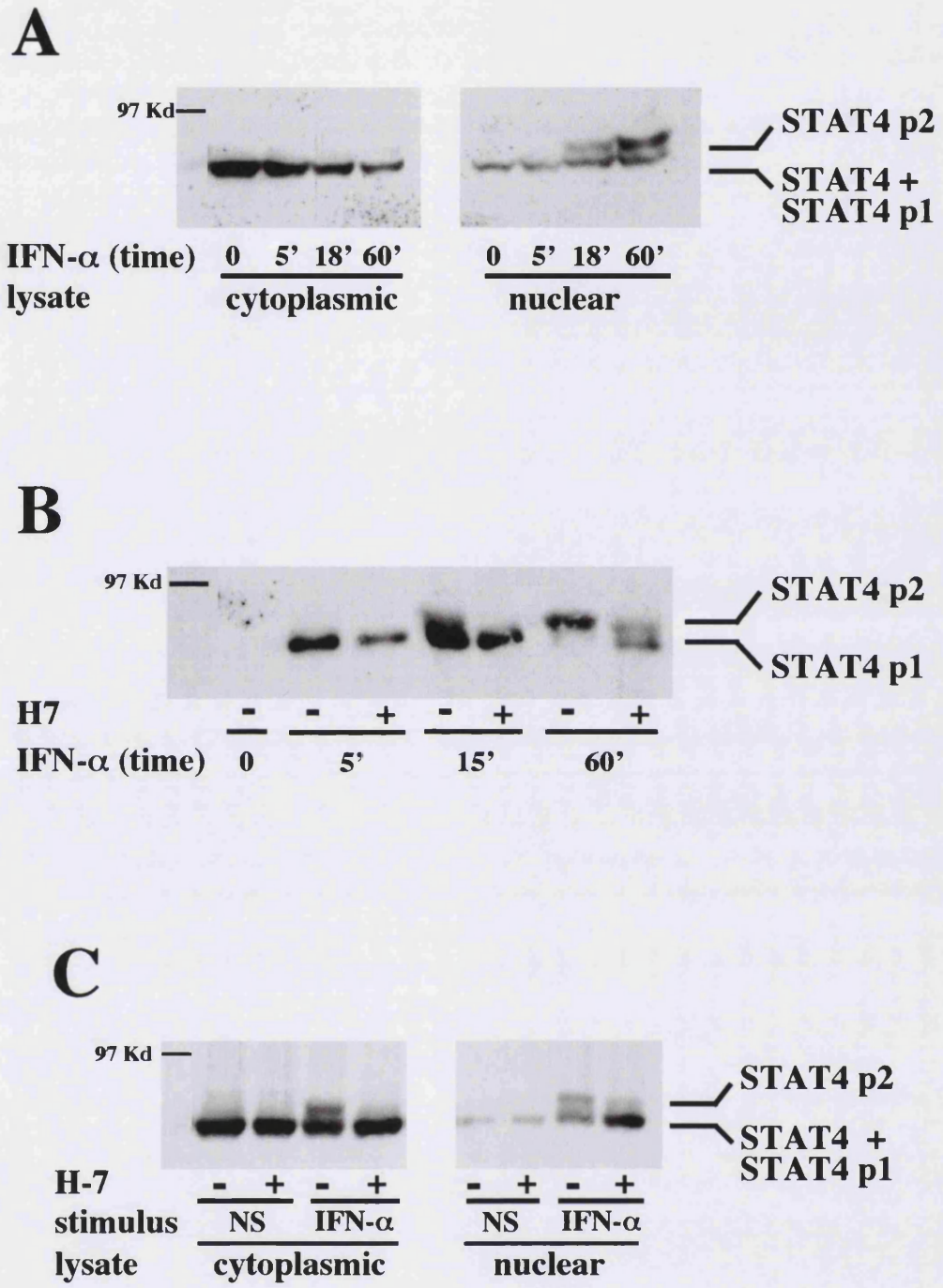


Figure 5.2) IFN-α regulates the cellular localisation of STAT4.- IFN-α-induced nuclear translocation of STAT4 (A). Effect of the H7 serine/ threonine kinase inhibitor on IFN-α-induced STAT4 DNA binding (B) and nuclear translocation (C). Kit225 cells (A, B) and PBL-T cells (C) were left unstimulated (NS) or stimulated with 10^3 U/ ml IFN-α for 18 minutes or the indicated times. Proteins were GRR-affinity precipitated from 20 x 10^6 cells (B) or acetone precipitated from cytoplasmic (5 x 10^6 cells) or nuclear (10 x 10^6 cells) lysates (A, C), resolved on SDS-PAGE and immunoblotted with anti-STAT4 C-. In B and C cells were incubated with 200 μM H-7 for 30 minutes prior to stimulation. Results are representative of at least two independent experiments.

5.2.4 The IFN-α/ STAT4 response is more transient than the IL-12/ STAT4 response

IL-12 and IFN-α have been shown to drive Th1 cell differentiation in human *in vitro* systems [77, 89]. However, patients with impaired IL-12 responses and consequent Th1 cell differentiation deficiencies suffer from recurrent *Mycoplasma sp.* and *Salmonella sp.* infections [67-69, 72]. Thus, IFN-α is not able to fully compensate for the lack of IL-12 responses in human *in vivo* systems. STAT4 activation is essential for Th1 cell differentiation [7, 8]. Differences in the regulation of STAT4 by IL-12 and IFN-α could explain the inability of IFN-α to induce full Th1 cell responses *in vivo*.

Using oligonucleotide affinity precipitation, we directly compared the early kinetics of activation of STAT4 by IL-12 and IFN-α (Figure 5.3 A). In agreement with the data shown in Chapter 3 and Figure 5.1 B, the initial kinetics of STAT4 activation as well as the generation of STAT4p1 and STAT4p2 are very similar in response to IL-12 and IFN-α (Figure 5.3 A). Both responses are rapid. DNA-bound STAT4 molecules appear at 2-5 minutes and remain present for at least 60 minutes upon stimulation. Equivalent amounts of STAT4p1 and STAT4p2 can be seen at 10-20 minutes and STAT4p2 is the predominant form at longer stimulation times in response to either cytokine. The IFN-α/ STAT4 response is slightly stronger as compared to the IL-12/ STAT4 response, but no major differences in the kinetics of STAT4 activation in response to IL-12 and IFN-α were seen.

Th cell differentiation is a long-term process. The constant presence of Th1 or Th2 driving cytokines and derived signals seems to be required to achieve full commitment to a specific Th phenotype. Therefore, the long-term kinetics of the IL-12/ STAT4 and IFN-α/ STAT4 responses where compared in PBL-T cells. An oligonucleotide affinity precipitation assay shows that IL-12 activation of STAT4

is more sustained than the IFN-α /STAT4 response (Figure 5.3 B). High levels of activated STAT4 are detected for at least nine hours after IL-12 stimulation. In contrast active STAT4 molecules are seen in cells that have been stimulated with IFN-α for three hours but not at longer time points (Figure 5.3 B). This transience of the IFN-α/ STAT4 response was consistent and was seen to last for only three to four hours (Figure 5.3 B and data not shown). These data show that the IFN-α/ STAT4 response is more transient than the IL-12/ STAT4 response in human PBL-T cells. The possible mechanisms involved in this differential regulation of STAT4 are the focus of the following sections.

5.2.5 IFN-α does not have a negative effect on PBL-T cell survival

The transient IFN-α/ STAT4 response in PBL-T cells could reflect a negative effect of IFN-α on T cell survival. To test this possibility we compared the viability of PBL-T cells incubated for six hours in the absence or presence of IFN-α, IL-2 and IL-12 (Figure 5.4). Propidium iodide staining and flow cytometry analysis were used to determine cell death. The percentage of dead cells is comparable (around 12 %) in IFN-α, IL-2 and IL-12 stimulated cells and is lower than in cells that were left unstimulated (~20 %) (Figure 5.4). Therefore, IFN-α does not have a negative effect on PBL-T cell survival, at least within the time in which the IFN-α/ STAT4 response decays. In fact, the reverse tendency was seen, T cells incubated with IFN-α had an enhanced survival when compared to unstimulated cells (Figure 5.4 and data not shown). These data indicate that the transient IFN-α/ STAT4 response is not caused by IFN-α induced cell death.

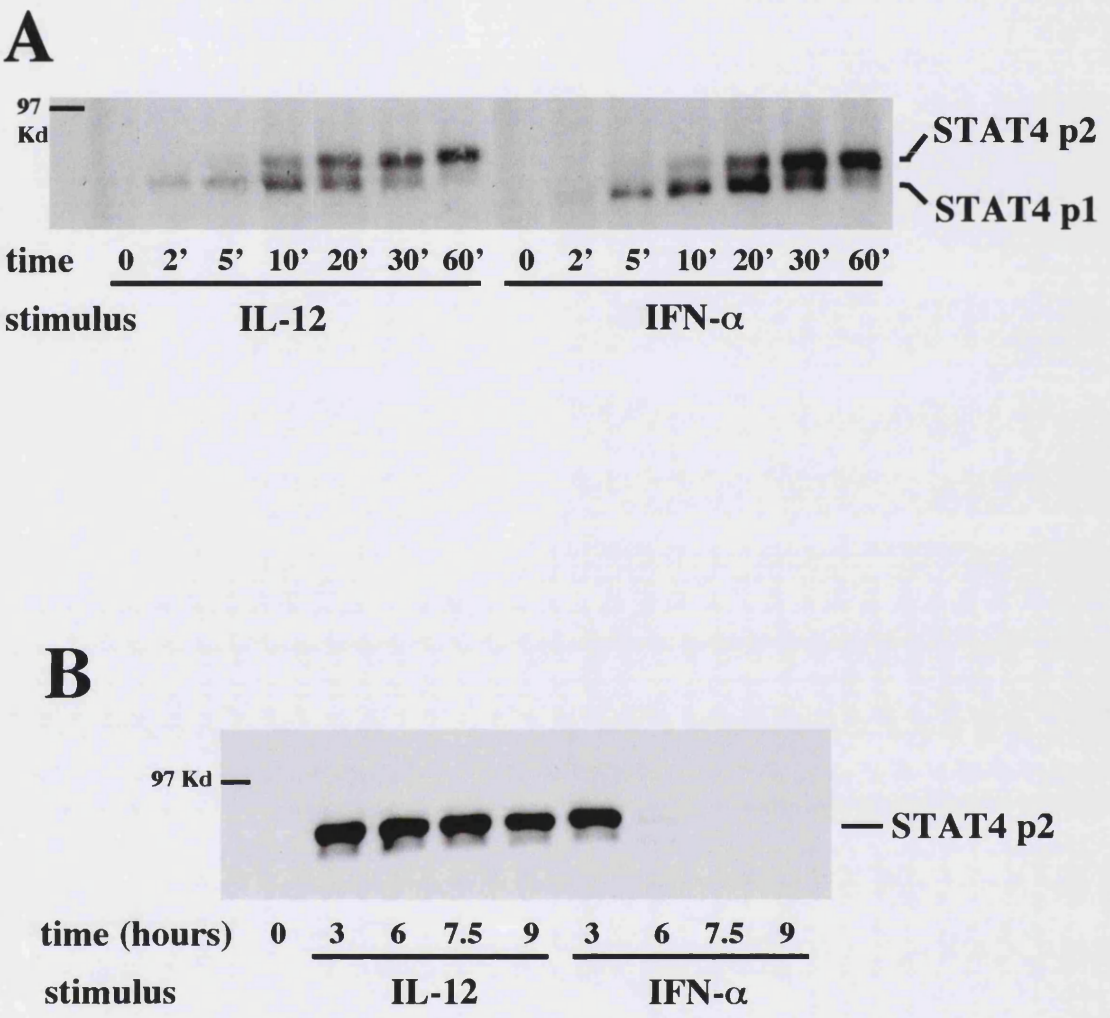


Figure 5.3) The IFN-α/ STAT4 response is more transient than the IL-12/ STAT4 response.- Comparison of short (A) and long-term (B) kinetics of IL-12/ STAT4 and IFN-α/ STAT4 responses. 20 x 10^6 PBL-T cells were left unstimulated (NS) or stimulated with IL-12 or IFN-α for indicated times. Proteins were GAS-STAT4-affinity precipitated, separated on SDS-PAGE and immunoblotted with anti-STAT4 C-. Results are representative of at least three independent experiments.

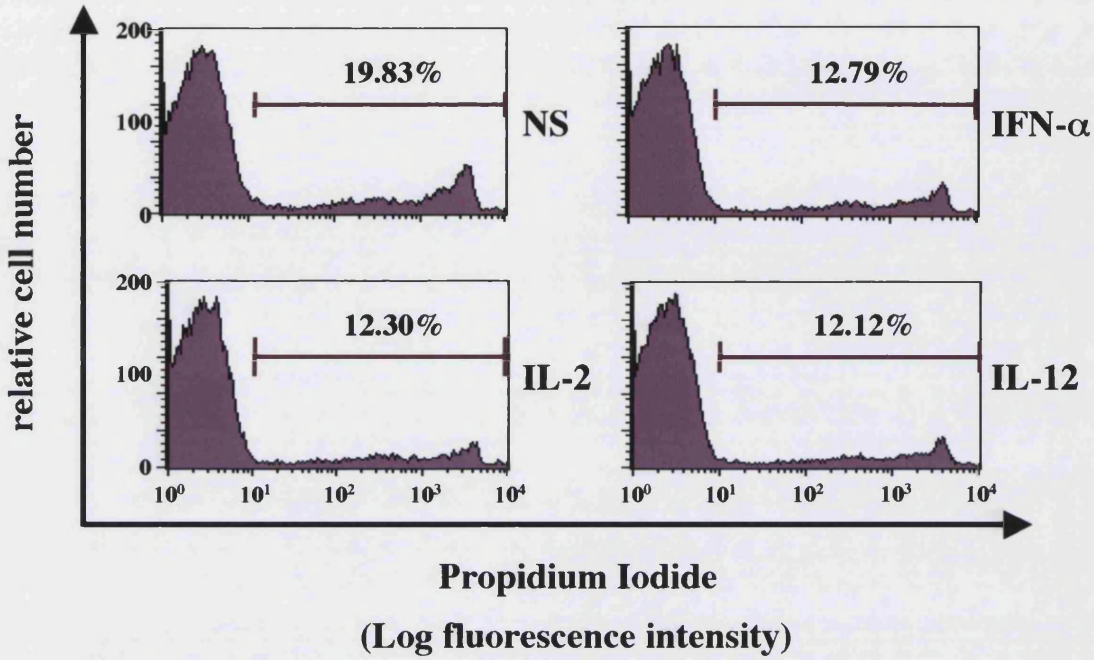


Figure 5.4) IFN-α does not have a negative effect on PBL-T cell survival .- Effect of IFN-α stimulation on PBL-T cell viability. PBL-T cells were left unstimulated (NS) or stimulated with indicated cytokines for 6 hours, prior to Propidium Iodide staining and flow cytometry analysis. The percentage of Propidium Iodide positive cells is indicated.

5.2.6 IFN-α negatively regulates its own STAT responses without altering the IL-12/ STAT4 response

Another explanation for transient cell responses to cytokines could be that during the course of the assay cytokines are depleted from the media after being internalised and degraded. To examine this possibility, PBL-T cells were incubated in the absence or presence of IFN-α for four hours (pre-incubation). Both of these populations were then re-stimulated or not with a fresh dose of IFN-α or further 18 minutes (second stimulation). The DNA-binding of STAT molecules was assayed using the oligonucleotide affinity precipitation technique. A small amount of DNA-bound STAT4, but not of STAT1, STAT3 or STAT5 can be detected in cells that have been incubated with IFN-α or four hours and did not receive a second stimulation (Figure 5.5 A). A second stimulation with IFN-α does not further induce the DNA-binding of STAT1, STAT3, STAT4 or STAT5. DNA-bound STAT molecules can only be induced in cells that have not previously encountered IFN-α (Figure 5.5 A). Thus, transient responses are not due to extinction of IFN-α from the media. These data indicate that IFN-α is inducing a negative regulatory mechanism that not only results in transient STAT responses but also impedes further IFN-α/ STAT responses.

A number of negative regulators of cytokine signalling have been described, including the PIAS proteins and the SOCS family of proteins (Chapter 1, section 1.7.9). PIAS proteins are constitutively expressed and associate with tyrosine phosphorylated STATs preventing their DNA binding and gene regulation [458-460]. SOCS expression is induced upon cytokine stimulation. SOCS negatively regulate the activation of STATs by the Jak tyrosine kinases acting in negative feedback loops but also in negative cross-talk regulation (Chapter 1, section 1.7.9). The induction of SOCS proteins or association of PIAS with active STATs in response to IFN-α in T cells have not been examined.

We considered the possibility that the IFN-α/ STAT4 response in T cells might be transient because of IFN-α-induced expression of a negative regulator of STAT4. In this case IFN-α treated T cells that could no longer activate STAT4 in response to IFN-α would also be unable to activate STAT4 in response to IL-12. To examine this possibility, a re-stimulation assay was performed. PBL-T cells were incubated in the absence or presence of IFN-α for four hours (pre-incubation). Both of these populations were then re-stimulated or not with a fresh dose of IFN-α or IL-12 for further 18 minutes (second stimulation) and the amount of DNA-bound STAT molecules was determined using the oligonucleotide affinity precipitation technique. As shown above, IFN-α can only induce the DNA binding of STAT4 in cells that have not been pre-incubated with IFN-α (Figure 5.5 B). However, equivalent amounts of DNA-bound STAT4 are induced in response to IL-12 in cells that have been pre-incubated or not with IFN-α (Figure 5.5 B). These data indicate that IFN-α negatively regulates its own STAT responses without altering the IL-12/ STAT4 response in PBL-T cells.

5.2.7 IFN-α down-regulates the cell surface expression of the IFN-α receptor

Receptor down-regulation following ligand occupancy is common in cytokine signalling. IFN-α-induced loss of IFN-α receptor (IFN-α-R) expression could explain the transience and specificity of the IFN-α/ STAT responses. To study this possibility, PBL-T cells were left unstimulated or stimulated with IFN-α for different times. The cell surface expression of both chains (IFNAR1.1 and IFNAR2.2) of the IFN-α-R, was determined by specific immuno-staining and flow cytometry analysis. These results show that IFN-α stimulation causes a clear decrease on the cell surface expression of the IFNAR1.1 chain (Figure 5.6). Cell

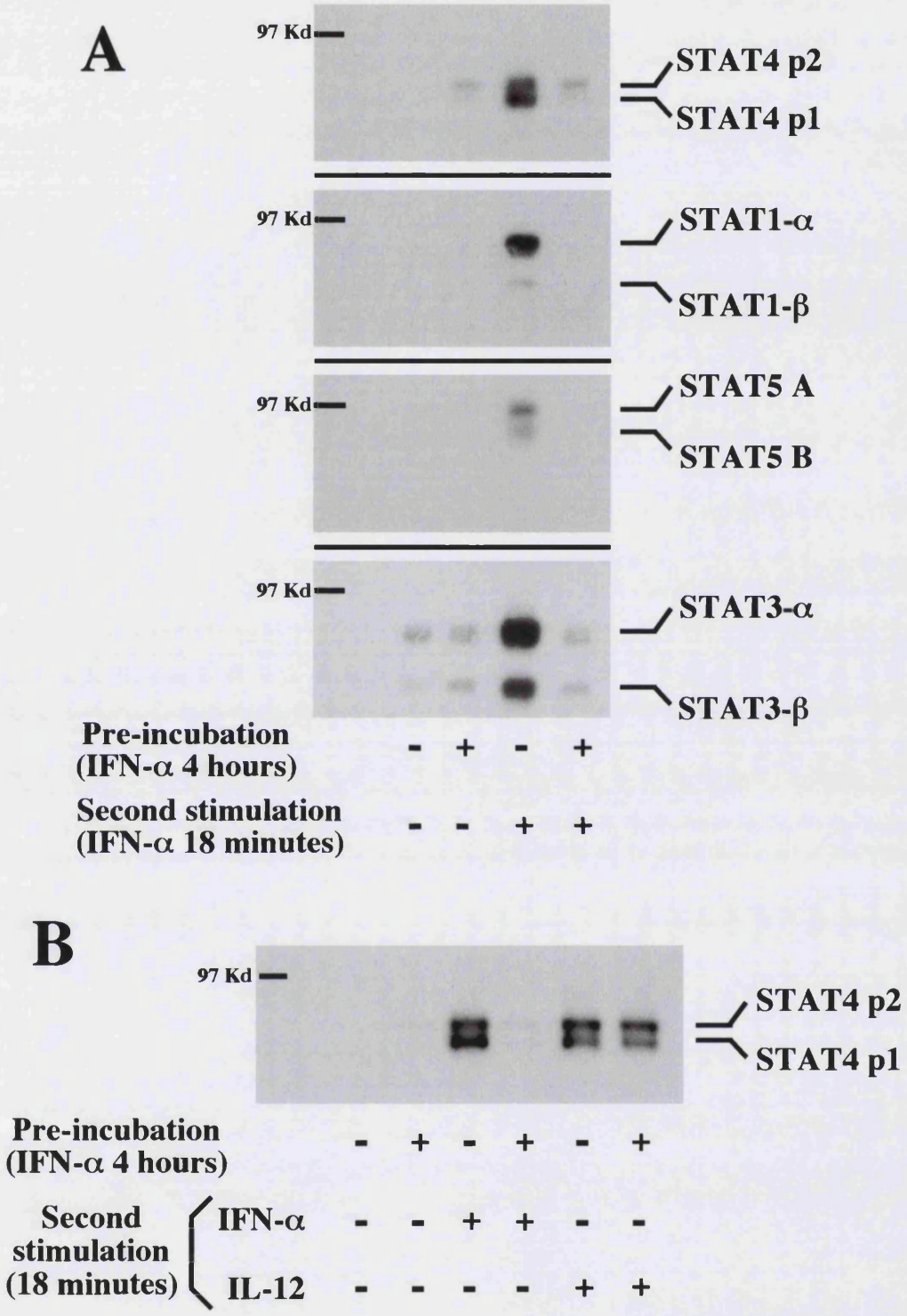


Figure 5.5) IFN-α negatively regulates its own STAT responses without altering the IL-12/ STAT4 response.- IFN-α/ STAT responses (A) and IL-12/ STAT4 response (B) in IFN-α pre-incubated cells. PBL-T cells were incubated in the absence (-) or presence (+) of IFN-α for 4 hours (pre-incubation), prior to a second stimulation with either IFN-α (A, B) or IL-12 (B) for 18 minutes. Proteins were GAS-STAT4-affinity precipitated from lysates containing equal amounts of total protein (870 μg in A and 880 μg in B), resolved on SDS-PAGE and immunoblotted with anti-STAT4 C- (B) or anti-STAT4 C- followed by sequential reprobing with anti-STAT1 pan, anti-STAT5 pan and anti-STAT3 pan (A).

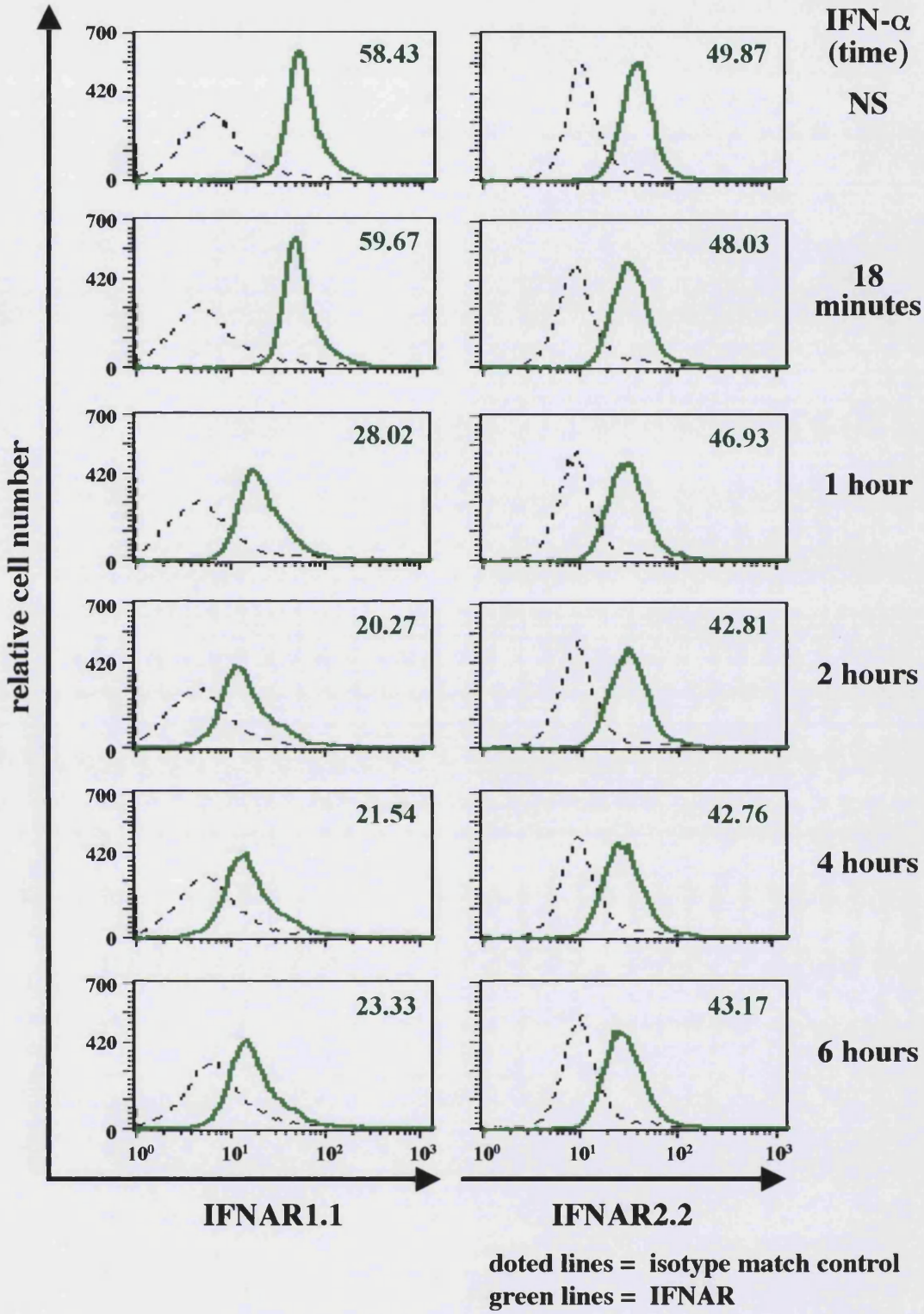


Figure 5.6) IFN-α down-regulates the cell surface expression of the IFN-α-Receptor.- Effect of IFN-α stimulation on IFNAR1.1 and IFNAR2.2 PBL-T cell surface expression. PBL-T cells were left unstimulated (NS) or stimulated with IFN-α for indicated times. The cell surface expression of IFNAR1.1 (left panel) and IFNAR2.2 (right panel) was determined by specific immuno-staining and flow cytometry analysis. Unspecific staining was determined with an isotype match control antibody (doted lines). Histograms represent IFNAR1.1 and IFNAR2.2 staining electronically gated on live PBL-T cells. The mean fluorescence intensity of each histogram is indicated in the upper right hand side corner. Results are representative of at least two independent experiments.

surface levels of the IFNAR1.1 chain suffer an approximate 70-80% reduction at one hour of IFN-α stimulation and later time points (Figure 5.6). The effect on the IFNAR2.2 chain cell surface levels is less striking but consistent, with a 10-15% reduction that appears at two hours of IFN-α stimulation and remains constant thereafter (Figure 5.6). The effect IFN-α on IFNAR1.1 and IFNAR2.2 was specific since IFN-α stimulation did not modify the cell surface expression of IL-12Rβ1 and induced the up-regulation of CD25 and IL-12Rβ2 (see Figure 6.8 A further below and data not shown). These data show that the cell surface levels of both chains of the IFN-α-R are down-regulated upon IFN-α stimulation, suggesting a mechanism for the transient IFN-α/ STAT responses.

5.2.8 IL-2 does not regulate the transient IFN-α/ STAT responses

The relevance of the differences between the IFN-α/ STAT4 and IL-12/ STAT4 responses resides in the capability of these cytokines to drive Th1 cell differentiation. The data presented above considers the presence of a single cytokine during the induction of STAT4 activation and consequent Th1 cell differentiation. However, under physiological conditions cytokines do not work in isolation. The process of Th1 cell differentiation occurs upon antigen presentation and TcR triggering when IL-12 is present. TcR triggering induces the production and secretion of the cytokine IL-2 by T cells. IL-2 acts in an autocrine and paracrine fashion to deliver the signals required for T cell development in the periphery. Therefore, IL-2 is present during Th1 cell differentiation and it may have an effect on other cytokine-induced signals.

The data shown above indicate that the transient IFN-α/ STAT responses are most likely due to IFN-α-induced down-regulation of the IFN-α-R. Receptor up-

regulation is a common mechanism of cytokine synergy within Th1 cell differentiation (see Chapter 1, Section 1.11.9). We therefore explored if IL-2 could regulate the IFN-α/ STAT responses by modulating the cell surface levels of the IFN-α-R. Immuno-staining and flow cytometry analysis were used to study the effect of IL-2 on the IFN-α-induced down-regulation of the IFN-α-R. The cell surface levels of both chains of the IFN-α-R decrease to the same extent upon four hours of IFN-α stimulation independently of the presence of IL-2 (Figure 5.7).

Although the presence of IL-2 did not stop the down-regulation of the IFN-α-R we tested whether or not IL-2 could regulate the transient IFN-α/ STAT responses. To study this possibility, we compared the induction of DNA-binding of STAT1, STAT3 and STAT4 in response to IFN-α in the absence and presence of IL-2 in PBL-T cells. IL-2 does not induce the DNA-binding of STAT4 or STAT1 and in comparison with IFN-α, is a weak activator of STAT3 in PBL-T cells (Figure 3.5 and data not shown). Both IL-2 and IFN-α induce the DNA binding of STAT5, but the effect of IFN-α is much weaker than that of IL-2 (Figure 3.5 and data not shown). An oligonucleotide affinity precipitation assay shows that the kinetics of activation of STAT1, STAT3 and STAT4 in response to IFN-α are not modified by the presence of IL-2 (Figure 5.8). The STAT4 response can be detected from 15 minutes and is still present at four hours of IFN-α stimulation. The STAT1 and STAT3 responses are more transient, being only detectable up to 1-1.5 hours upon IFN-α stimulation (Figures 5.1 and 5.8). Equivalent amounts of DNA-bound STAT1, STAT3 and STAT4 can be detected at each stimulation time when cells are stimulated with IFN-α or the combination of IFN-α and IL-2 (Figure 5.8). DNA binding of STAT5 is shown as a control for IL-2 activity. These data show that the presence of IL-2 does not affect the strength or the duration of the IFN-α/ STAT responses.

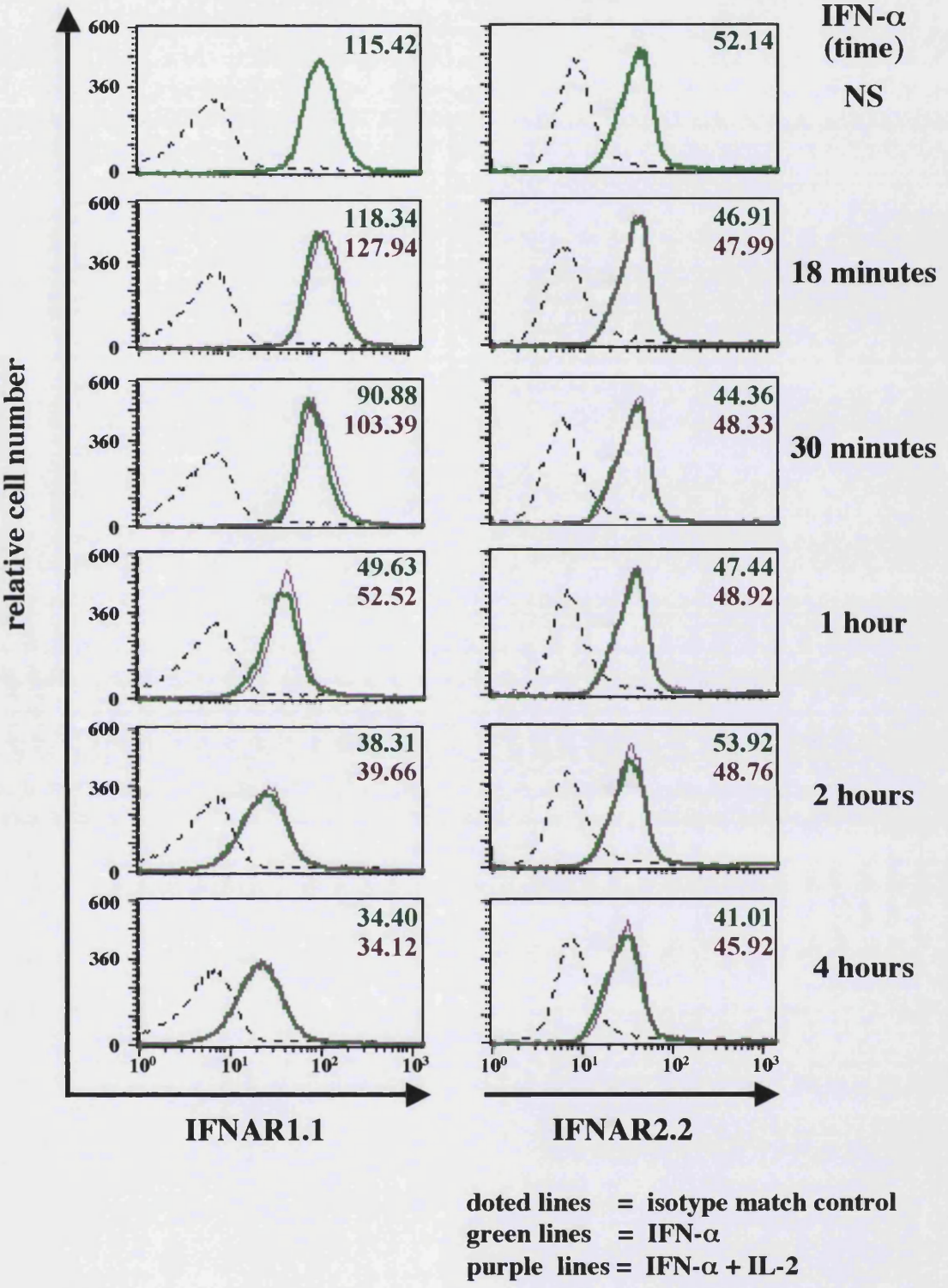


Figure 5.7) IL-2 does not regulate the IFN-α-induced down-regulation of the IFN-α-Receptor.- Effect of IL-2 on IFN-α-induced down-regulation of IFNAR1.1 and IFNAR2.2 PBL-T cell surface expression. PBL-T cells were left unstimulated (NS) or stimulated with IFN-α in the absence (green lines) or presence (purple lines) of IL-2 for the indicated times. The cell surface expression of IFNAR1.1 and IFNAR2.2 was determined by specific immuno-staining and flow cytometry analysis. Unspecific staining was determined with an isotype match control antibody (doted lines). Histograms represent IFNAR1.1 and IFAR2.2 staining electronically gated on live PBL-T cells. The mean fluorescence intensity of each histogram is indicated in the corresponding colour in the upper right hand side corner. Results are representative of at least two independent experiments.

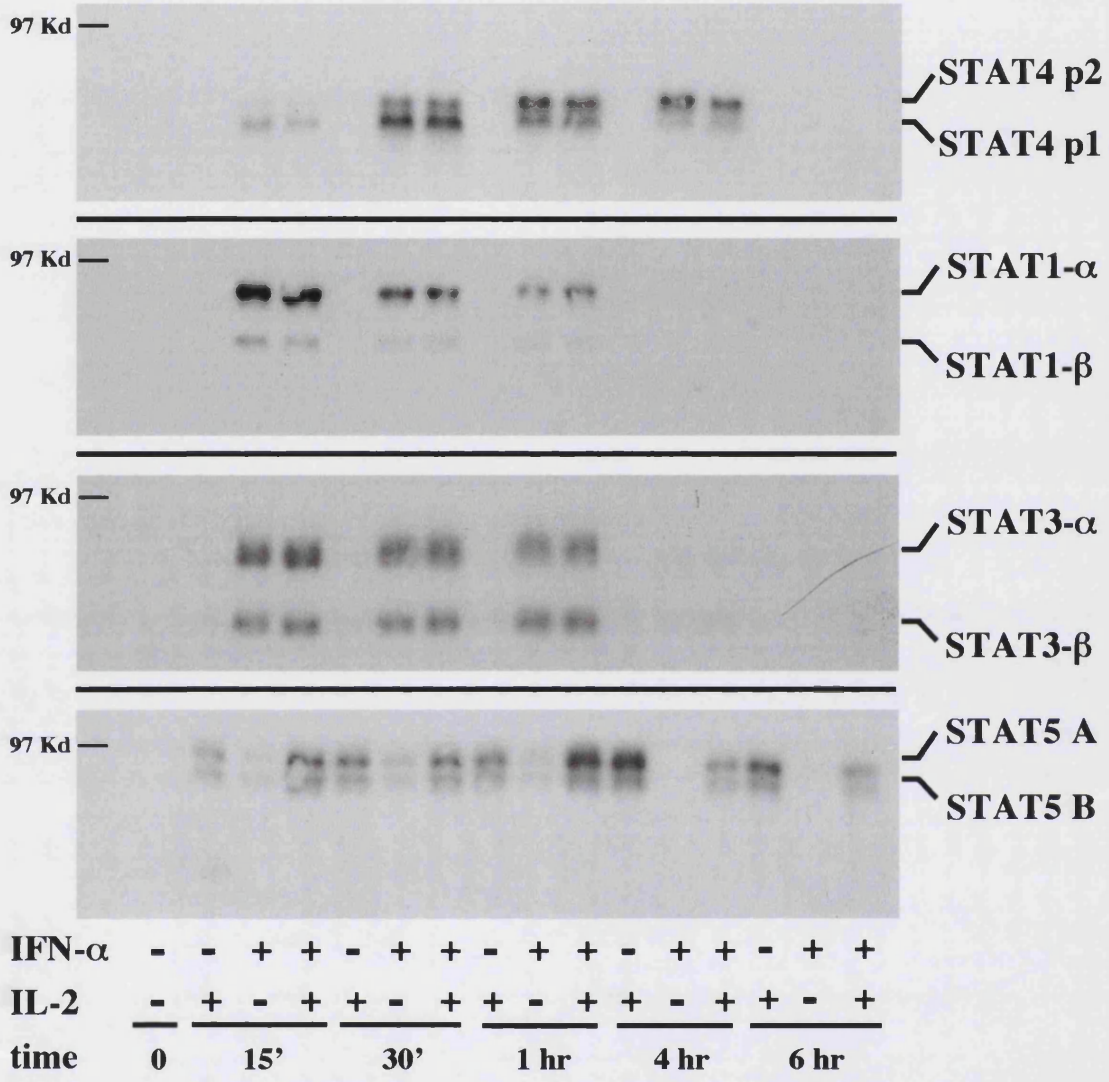


Figure 5.8) IL-2 does not regulate the IFN-α/ STAT responses.- Effect of IL-2 on IFN-α-induced STATs DNA-binding. PBL-T cells were left unstimulated or stimulated with IFN-α in the presence or absence of IL-2 for the indicated times. Proteins were GAS-STAT4-affinity precipitated from lysates containing equal amounts of total protein (500 μg), resolved on SDS-PAGE and immunoblotted with anti-STAT4 C- followed by sequential reprobing with anti-STAT1 pan, anti-STAT3 pan and anti-STAT5 pan. Results are representative of at least two independent experiments.

5.3 Discussion

The present data show that the initial kinetics and magnitude of the STAT4 response, as well as the generation of STAT4p1 and STAT4p2, are very similar in response to IL-12 and IFN-α. IFN-α also induces the nuclear translocation of STAT4. The inhibition of STAT4 serine phosphorylation by H7 confirms that this post-translational modification is not required for STAT4 nuclear translocation (Figure 5.2 C and Chapter 3, Figure 3.9 C).

Although activation of STAT4 in response to IL-12 and IFN-α share many similarities, the IL-12/ STAT4 response is significantly more sustained than the IFN-α/ STAT4 response (Figure 5.3 B). Moreover, in our system not only the activation of STAT4 in response to IFN-α is transient, but all IFN-α/ STAT responses are transient.

The transient IFN-α/ STAT responses could be due to the apoptotic properties described for IFNs in non-lymphoid cells [262]. Here we find that treatment of PBL-T cells with IFN-α for both short- (1-6 hours) and long-term (24-72 hours) periods has a survival rather than an anti-proliferative or apoptotic effect (Figure 5.4 and data not shown). These data are in agreement with recent findings showing that IFN-α promotes the survival of lymphoid cells, especially of memory T cells from the CD8+ lineage [281, 599]. Therefore, the transient IFN-α/ STAT responses are not caused by a negative effect of IFN-α on T cell survival.

Different mechanisms can be involved in the termination of cytokine-induced STATs activation. These include: 1) termination of the cytokine-derived signal by ligand/ receptor internalisation and degradation; 2) block of ligand/ receptor-derived signals by induction and/ or activation of inhibitory molecules such as receptor/ Jak

phosphatases and SOCS proteins; 3) inhibition of STATs-DNA binding by PIAS proteins; 4) inactivation of active STATs by dephosphorylation and/ or degradation; or 5) the combination of some or all of the above mechanisms. These possibilities are discussed in more detail.

IFN-α activates STAT1, STAT3, STAT4 and STAT5 in human T cells (Chapter 3, Figure 3.5 and Chapter 5, Figure 5.1). The kinetics of IFN-α-induced DNA binding is different for each particular STAT (Figure 5.1 B). Although the appearance of DNA-bound molecules is rapid for all STATs, the STAT4 and STAT3 responses are more sustained than the STAT1 and STAT5 responses. These data are in line with the common Jak1/ Tyk2 mediated tyrosine phosphorylation of all IFN-α-regulated STATs and suggest the existence of different inactivating mechanisms for each STAT.

The regulation of the mechanisms by which STATs are inactivated is still largely unknown. We observe a very transient activation of STAT1 in response to IFN-α in PBL-T cells (Figure 5.1 B). A similar response has been reported for IFN-γ-activated STAT1 in fibroblasts [434]. Using pulse-chase experiments one study showed that STAT1 protein levels remain constant following IFN-γ stimulation, with STAT1 being recycled into the cytoplasm following dephosphorylation [434]. The participation of a nuclear tyrosine phosphatase in the inactivation of STAT1 was proposed [434]. However, proteolytic degradation of IFN-γ-induced STAT1 and IL-3-induced STAT5 dimers following ubiquitinylation has also been described in fibroblasts [435, 436]. The participation of proteolytic degradation in inactivation of some STATs is supported by a recent study using the proteosome inhibitors MG132 and lactacystin [437]. Tyrosine-phosphorylated forms of STAT4, STAT5 and STAT6 respectively induced by IL-12, IL-3 and IL-4 were significantly stabilised upon addition of these inhibitors. However, IFN-α regulated STAT1 and STAT2 were not [437]. This is in agreement with our data indicating

that different mechanisms may be involved in the inactivation of each particular STAT. Moreover, a small amphipathic α-helical region has been proposed to specifically mediate the proteosomal degradation of STAT5 but not of other STATs [437].

Along with STATs inactivation, the transient IFN-α/ STAT responses may be due to a block in signal delivery either at the level of the cytokine or the cytokine receptor. It has been described that the tyrosine phosphatases SHP-1 and SHP-2 associate with the IFN-α-R in fibroblast and macrophages [438, 442, 443]. Activation of these phosphatases could be a possible mechanism for the termination of IFN-α/ STAT responses. Here we show that pre-incubation with IFN-α does not block the IL-12/ STAT4 response. If the activation of tyrosine phosphatases by IFN-α is implicated in the termination of IFN-α/ STAT responses, these phosphatases would have to be selective for IFN-α signalling. The implication of SHP-1 and SHP-2 activation in the signalling pathways of several cytokines, then suggests that these kinases are not likely to be responsible for the transient IFN-α/ STAT responses.

As explained in Chapter 1 (section 1.7.9), SOCS are inducible negative regulators of STAT activation [449-451]. The induction of SOCS proteins is not only involved in negative feedback loops but also in negative cross-talk regulation. The expression of SOCS proteins in response to IFN-α in T lymphocytes has not been reported and was not analysed in the present study. What is clear from the present data is that there is no cross-inhibition of IL-12/ STAT4 responses by IFN-α. Therefore, if any SOCS protein is induced by IFN-α this would have to be specific for IFN-α/ IFN-α-R-derived signals and not target any molecule that is commonly activated by IL-12 and IFN-α. A possible candidate is Jak1, since it is activated by IFN-α but not by IL-12. In this respect it is also noteworthy that the different mechanism of

STAT4 activation by IL-12 and IFN-α (see Chapter 1, section 1.11.5) makes it possible to envisage the potential for selectivity of action of a negative regulator. STAT4 is activated by IL-12 and IFN-α following its recruitment to the corresponding ligand/ receptor complexes. STAT4 directly interacts with the IL-12Rβ2 chain, but needs the presence of STAT2 to be recruited to the IFN-α-R complex [75, 519, 520]. The C- terminal domain of STAT2 is required for the STAT2/ STAT4 interaction, but it is not known if STAT4 directly associates with STAT2 or requires an intermediate adaptor protein [520]. Any protein that could prevent the STAT4/ STAT2 interaction required for STAT4 activation by IFN-α would block IFN-α responses without affecting the recruitment of STAT4 to the IL-12Rβ2 chain in response to IL-12.

It has been proposed that the transient IFN-α-induced DNA binding of STAT1 and STAT3 might be explained by the association of PIAS1 and PIAS3, respectively [458-460]. These proteins associate with tyrosine phosphorylated STATs preventing their DNA binding and it is thought that there is a specific PIAS protein for each STAT (Chapter 1, section 1.7.9). However, no specific PIAS protein for STAT4 or STAT5 have been reported and the systems in which PIAS proteins prevent STATs-DNA binding have not been defined [458-460]. The participation of a PIAS4 if it exists, will be excluded from our system by the data demonstrating that IFN–α does not negatively regulate the IL-12/ STAT4 response.

IFN-α stimulation of PBL-T cells induces the down-regulation of both IFNAR1.1 and IFAR2.2 (Figure 5.6). IFNAR1.1 cell surface levels decrease by approximately 70% at one hour of IFN-α stimulation, coinciding with the disappearance of DNA-bound STAT1 and STAT5 molecules. STAT3 and STAT4 remain DNA-bound for longer, being detected for 2 hours and 3-4 hours, respectively. When IFN-α-induced tyrosine kinase activity was inhibited by addition of staurosporine, the

amount of DNA-bound STAT1, STAT3 and STAT5 molecules was reduced as rapid as five minutes and completely disappeared by thirty minutes of the addition of the drug (data not shown). However, DNA-bound STAT4 is more stable upon termination of cytokine induced tyrosine kinase activity. It remains present for approximately four hours of addition of staurosporine or cytokine removal in IFN-α and IL-12 stimulated PBL-T cells, respectively (data not shown and Chapter 3, Figure 3.7 B). These data indicate that STAT1, STAT3 and STAT5 are more rapidly inactivated as compared to STAT4 and support the existence of different inactivation mechanisms for specific STATs. Therefore, the half-life of each particular DNA-bound STAT following termination of IFN-α-derived signals together with a strong reduction in IFN-α-R cell surface levels provide an explanation for the differential kinetics of each STAT DNA-binding in response to IFN-α.

IFN–α stimulation induces a more rapid down-regulation of IFNAR1.1 as compared to IFNAR2.2 (Figure 5.6). Since the IFNAR1.1 chain has a major role in signal transduction, the rapid reduction of this chain could explain the fast decrease in IFN–α-induced STATs DNA-binding. The stoichiometry of the IFNAR1.1 and IFNAR2.2 chains has not been determined. Therefore it is not possible to evaluate how the relative reduction in each chain contributes to the decrease in IFN–α-induced signals.

Earlier studies analysed the rate of internalisation of radiolabelled surface-bound IFN–α in lymphoid cell lines [600, 601]. These studies found that IFN–α was rapidly internalised and degraded [600, 601]. However, the fate of the IFN–α-R following internalisation has not been explored. Steady state studies in MDBK (Madin-Darby bovine kidney) cells found that the amount IFN–α occupied and unoccupied receptors were turn over at approximately the same rate, indicating that

IFN–α binding to its receptor does not increase the rate of IFN–αR internalisation [602]. We only found minor differences in the levels of IFNAR1.1 and IFNAR2.2 in cells incubated with media alone for different times. Our present data using antibodies that specifically recognise IFNAR1.1 and IFAR2.2 show that in human T cells binding of IFN–α to its receptor results in a decrease of cell surface levels, presumably by receptor internalisation. Receptor internalisation can be regulated in a ligand binding-dependent or independent manner. For example, while the IL-2Rβ chain is constitutively internalised and degraded, the IL-2Rα chain requires IL-2 binding for internalisation [603, 604]. Alternative mechanisms may regulate the internalisation of a cytokine receptor in different cell systems. Although we can not exclude that IFN-α-R may be internalised independently of the presence of IFN–α at a low rate, the present data clearly show that IFN–α interaction with the IFN-α-R induces the pronounced down-regulation of IFN-α-R. Taken together, these data indicate that IFN-α-R internalisation is regulated in a ligand-independent manner in MDBK cells but it is IFN–α-dependent in human T lymphocytes.

Since IFN-α-Rs are constitutively expressed on T cells, one would expect that upon internalisation, the cell surface levels of IFN-α-R would be eventually recovered through either recycling into the cell surface or *de novo* protein synthesis. The kinetics of IFN-α-R internalisation and recover of cell surface levels have not been studied. It is not known if the IFN-α-R chains get recycled back into the cell surface or if they are degraded along with IFN–α. We could not detect an increase in IFN–α-Rs or indeed any IFN–α responsiveness as assayed by IFN–α-induced STAT-DNA binding, in cells that were incubated with IFN–α for up to six hours (Figure 5.5 and data not shown). Thus IFNAR1.1 and IFNAR2.2 either require longer to be recycled/ synthesised or the rate of IFN–α-induced IFN–α-R internalisation is higher than the recovery of its cell surface levels. The second possibility would imply that the cell surface levels of IFN-α-R cannot be recovered

to signalling competent levels in the continuous presence of IFN–α.

The transient IFN-α/ STAT responses were not modified by the presence of IL-2. IL-2 did not prolong the STAT DNA binding or regulated the IFN-α-R down-regulation induced by IFN-α (Figure 5.7 and 5.8). However, when the cell surface expression of the IFN-α-R was analysed upon a long-term (48-96 hours) stimulation with IL-2, the levels of IFNAR1.1 chain increased by 20-30 % while those of IFNAR2.2 decreased by approximately 30 % (data not shown). These data indicate that although IL-2 can not modify the IFN-α-induced down-regulation of the IFN-α-R it does have an effect on the cell surface levels of the IFN-α-R. Further studies are necessary to determine the relevance of this observation.

The data discussed above have shown that the IFN-α/ STAT responses are transient. We proposed that IFN–α-induced down-regulation of the IFN-α-R together with a rapid inactivation mechanism of STAT1, STAT3 and STAT5 and a more delayed inactivation of STAT4, account for these transient responses. The mechanisms involved in IFN–α-induced STATs inactivation remain unknown.

The comparative kinetics of STAT4 DNA-binding show that the IL-12/ STAT4 response is considerably more sustained than the IFN-α/ STAT4 response. Different mechanism could account for this differential kinetics. We have shown that DNA-bound STAT4 is only detected for up to four hours upon removal of IL-12 or inhibition of IFN-α activated tyrosine kinases with the drug staurosporine. Therefore, STAT4 DNA binding requires the constant presence of either IL-12- or IFN–α-derived signals. This would imply that IL-12R levels, competent for IL-12-mediated signals are present in T lymphocytes for longer than those of IFN-α-R. The regulation of the IL-12R in response to IL-12 and IL-2 are the focus of Chapter 6. A second possibility is that during long-time stimulation, IL-12 induces the

activation/ expression of molecules that stabilise the DNA-binding of STAT4.

Although STAT4 remains DNA-bound for similar time after termination of IL-12- and IFN-α-derived signals, the fact that incubation with IFN–α does not prevent the IL-12/ STAT4 response indicates that different mechanisms are involved in the inactivation of STAT4 in response to each of these cytokines.

The IL-12/ STAT4 response is essential for the induction of Th1 cell differentiation in mouse and human systems [7, 8, 66, 100-102]. IFN–α also activates STAT4 but this response only occurs in the human system (section 1.11.5 and [89, 353, 517-520]). As mentioned above this is due to the differential recruitment of STAT4 into the IL-12R and IFN–α-R complexes [75, 519, 520]. IFN-α has been shown to drive Th1 cell differentiation in human *in vitro* systems, leading to the proposal that IFN–α could be an alternative cytokine for the induction of Th1 cell responses in humans [21, 286, 508]. IL-12Rβ1 deficient patients are unable to mount competent Th1 cell responses even when they have normal IFN–α responses [67-69, 72]. This indicates that *in vivo* IFN–α is not able to fully compensate for the lack of IL-12 responses. We propose that the differential kinetics of STAT4 activation in response to IL-12 and IFN–α are responsible for the inability of IFN-α to induce competent Th1 cell differentiation in human *in vivo* systems. This important biological implication will be discussed in more detail in Chapter 7.

CHAPTER 6

IL-2 potentiates the sustained IL-12/ STAT4 response in human T cells

6.1 Introduction

The presence and persistence of IL-12 and molecules required for IL-12 signalling are necessary for Th1 cell differentiation. For example, healing Th1 cell responses against *L. major* infection in IL-12p40 null mice require addition of exogenous IL-12 not only at the beginning but also throughout the course of infection [605]. Similarly, sustained expression of the IL-12Rβ2 chain is essential for developmental commitment to the Th1 phenotype in both mouse and humans [77, 86, 87, 523, 606].

The timing and duration of the IL-12/ STAT4 response depend on the functional expression of the molecules that participate in STAT4 activation. Therefore, it is important to understand the mechanisms involved in the regulation of IL-12, IL-12R and STAT4 expression. IL-12 is produced by antigen presenting cells, primarily monocytes and DCs by T cell-dependent and independent mechanisms. Bacteria, viruses, yeast, intracellular parasites and IFN-γ act in a T cell-independent manner, while activated T cells induce the production of IL-12 through CD40/ CD40L and MHCII-TcR interactions during antigen presentation (see Chapter 1, section 1.4.2).

STAT4 expression is also tightly regulated and restricted to activated T lymphocytes (Chapter 3, Figure 3.2 B and [76]). In monocytes and mature DCs, STAT4 expression is induced in response to LPS and IFN–γ and is negatively regulated by the Th2 cytokines IL-4 and IL-10 [73, 607]. STAT4 is constitutively expressed in freshly isolated NK cells but it has been suggested that IL-2 is necessary for sustained expression of STAT4 in cultured NK cells [85].

IL-12 signalling requires the functional expression of both IL-12Rβ1 and IL-12Rβ2 [66, 71, 75]. T cells only express IL-12Rβ1 and IL-12Rβ2 following antigen receptor mediated activation. Experimentally this is usually achieved by T cell activation with mitogens or following TcR triggering with anti-CD3 antibodies (Chapter 3, Figure 3.3 and [63, 95, 565]). TcR triggering alone is not sufficient for maximal expression of IL-12R, rather CD28/ B7 derived signals are also required for optimal induction apparently through the production of regulatory cytokines (see Chapter 1, section 1.4.4 and [78-81]). For example, it has been reported in murine T cells that the enhancement of IL-12Rβ1 and IL-12Rβ2 by CD28 co-stimulatory signals is likely to be mediated by induction of IL-2 [79-81]. After initial induction by antigen directed signals the magnitude and maintenance of functional IL-12R is then regulated by cytokines. Most studies have concentrated on the regulation of IL-12Rβ2, because its sustained expression proved to be essential for Th1 cell commitment [77, 86]. IFN-α, IL-12, IFN-γ and IL-18 induce IL-12Rβ2 expression in human and/ or mouse systems, respectively [9, 40, 77, 79, 86, 89, 516, 523, 539, 606, 608]. IL-12Rβ2 regulation by IL-12 and IFN–α has been proposed to be mediated through STAT4 [286]. However, little is know about the regulation of the IL-12Rβ1 expression.

Synergistic interactions between IL-12 and IL-2 are important in Th1 cell differentiation, the regulation of NK cell function and the maturation/ differentiation of cytotoxic $CD8^+$ T cells (see Chapter 1, section 1.5.3 and [104, 108, 122, 259,

260, 491]). In NK cells the regulatory effects of IL-2 on IL-12R expression may explain the role of IL-2 as a modulator of IL-12 responses. IL-2 up-regulates the constitutive expression of IL-12Rβ1 and IL-12Rβ2 in NK cells [85]. The experiments presented in this chapter were developed to investigate the influence of IL-2 on the IL-12/ STAT4 response in human T lymphocytes.

6.2 Results

6.2.1 Long term kinetics of the IL-12/ STAT4 response

We have shown that IL-12 activation of STAT4 is sustained for several hours (Chapter 5). However, T cell differentiation requires days. The kinetics of STAT4 activation over several days has not been studied. Accordingly, PBL-T cells were stimulated or not with IL-12 over a 24 hour period (Figure 6.1). Oligonucleotide affinity precipitation was performed using equal amounts of total protein from the different cell populations. Active DNA-bound STAT4 molecules can be detected at both 18 minutes and 24 hours after IL-12 stimulation, but not in unstimulated cells (Figure 6.1). Both electrophoretic mobility forms of STAT4 (STAT4p1 and STAT4p2) are present at 18 minutes, while only STAT4p2 is present at the 24 hours time point. However, the amount of DNA-bound STAT4 is strikingly reduced following 24 hours of IL-12 stimulation (Figure 6.1).

6.2.2 IL-12 stimulation down-regulates the cell surface levels of the IL-12R

A decrease in expression of the IL-12R could explain the termination of the IL-12/ STAT4 response over a period of 24 hours. To test this possibility, PBL-T cells were stimulated or not with IL-12 for up to 48 hours. The cell surface levels of both chains of the IL-12R were determined by specific immuno-staining and flow cytometry analysis. The pattern of expression of the IL-12Rβ1 chain and the IL-12Rβ2 chain are different in T cells maintained in IL-12 (Figure 6.2). IL-12 stimulation induces rapid down-regulation of IL-12Rβ2. The cell surface levels of IL-12Rβ2 are reduced after 18 minutes of exposure to IL-12 and decay even further by 50-60% between one and four hours of IL-12 stimulation. This down-regulation

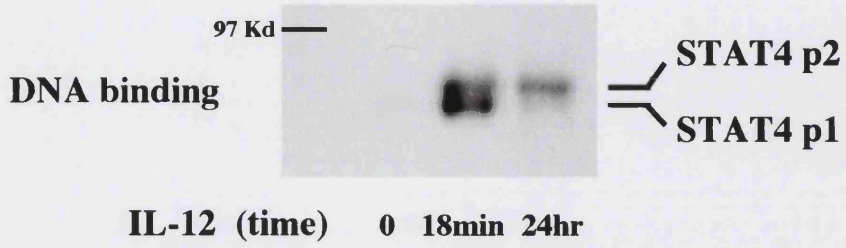


Figure 6.1) Long-term kinetics of the IL-12/ STAT4 response.- IL-12-induced STAT4 DNA binding over a 24 hour period. PBL-T cells were left unstimulated (0) or stimulated with IL-12 for indicated times. Proteins were affinity precipitated from lysates containing equal amounts of total protein (780 μg) using the GAS-STAT4 oligonucleotide, resolved on SDS-PAGE and immunoblotted with anti-STAT4 C-. Results are representative of at least two independent experiments.

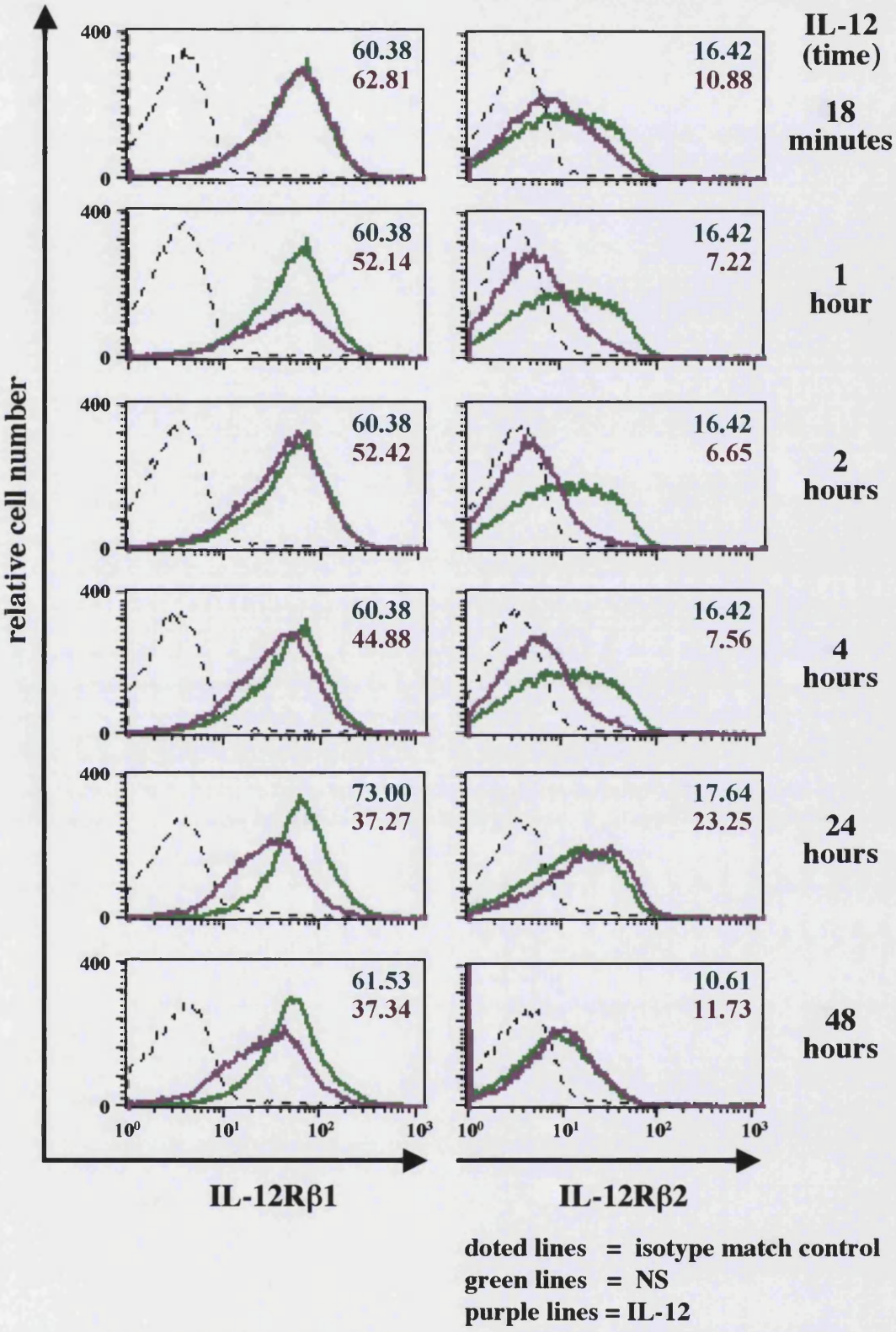


Figure 6.2) IL-12 down-regulates the cell surface levels of the IL-12 Receptor.- Effect of IL-12 stimulation on IL-12Rβ1 and IL-12Rβ2 PBL-T cell surface levels. PBL-T cells were left unstimulated (NS) (green lines) or stimulated with IL-12 (purple lines) for indicated times. Cells were stained with either an isotype match control (doted lines), anti-IL-12Rβ1 (left column) or anti-IL-12Rβ2 (right column) antibodies. Stained cells were analysed by flow cytometry. Histograms represent IL-12Rβ1 and IL-12Rβ2 staining electronically gated on live PBL-T cells. The mean fluorescence intensity of each histogram is indicated in the corresponding colour in the upper right hand side corner.

is transient and IL-12Rβ2 cell surface expression recovers and is then increased at 24 hours (~130%) and 48 hours (~110%) in the presence of IL-12 (Figure 6.2 right panels). In contrast there is a progressive decrease in IL-12Rβ1 cell surface levels in cells maintained in IL-12. IL-12Rβ1 levels start declining within one hour of exposure to IL-12 and continue decreasing over the time course being 40-50% lower at 24-48 hours of IL-12 stimulation, (Figure 6.2 left panels).

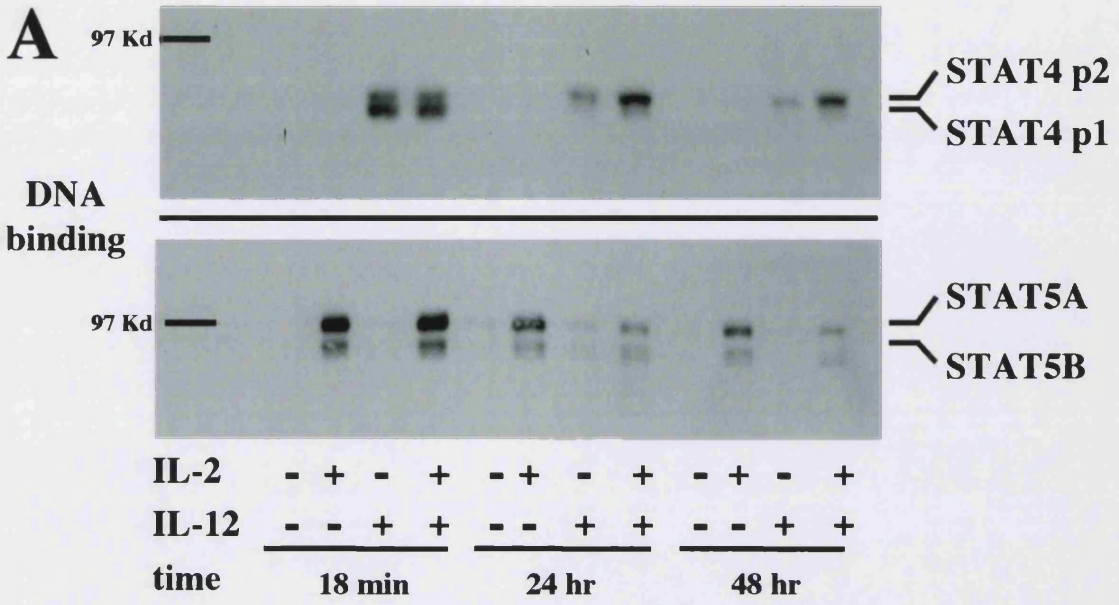
6.2.3 IL-2 potentiates the long term IL-12/ STAT4 response

IL-12 and IL-2 synergise *in vitro* for T cell and NK cell proliferation and cytotoxicity (see Chapter 1, section 1.5.3 and [104, 108, 122, 259, 260, 491]). IL-2 has been recently shown to regulate the expression of IL-12Rβ1, IL-12Rβ2 and STAT4 in NK cells suggesting a possible mechanism for IL-2/ IL-12 synergy in these cells [85]. To determine if IL-2 has any influence on IL-12 signalling in human PBL-T cells, we initially studied the role of IL-2 in IL-12-induced STAT4 DNA binding. PBL-T cells were left unstimulated or stimulated with IL-12 in the absence or presence of IL-2 for different periods of time. An oligonucleotide affinity precipitation assay shows that IL-12-induced DNA-bound STAT4 molecules can be detected from 18 minutes but this response is down-regulated at 24 and 48 hours (Figure 6.3 A upper panel). IL-2 itself does not induce the DNA binding of STAT4 at any time, but it potentiates the long-term IL-12/ STAT4 response. Equivalent amounts of DNA-bound STAT4 molecules can be seen in cells that have been stimulated with IL-12 for 18 minutes independently of the presence of IL-2. However, a marked increase in DNA-bound STAT4 molecules is seen at longer stimulation times when PBL-T cells are incubated with IL-12 and IL-2 (Figure 6.3 A upper panel). The induction of DNA-bound STAT5 molecules is shown as a control for IL-2 activity (Figure 6.3 lower panel). Interestingly, IL-12 seems to have a negative effect on the long-term IL-2-induced STAT5 DNA binding.

The overall effect of IL-2 on IL-12-induced DNA-bound STAT4 molecules is shown graphically. Parallel experiments to the one presented in Figure 6.3 A, were performed in PBL-T cells isolated from four different donors. The amount of DNA-bound STAT4 was determined using densitometric analysis as described in materials and methods (Chapter 2). The initial 18 minutes stimulation time was given the arbitrary value of 100% and individual percentage values were calculated thereafter. The graph shows that at 24 hours and later times the IL-12/ STAT4 response is sustained at higher levels when cells are stimulated in the presence of IL-2. The amount of DNA-bound STAT4 decreases by 60-75% at 24 hours and 80-85% at 48 hours of stimulation with IL-12 alone (Figure 6.3 B). IL-2 consistently potentiates the IL-12/ STAT4 response.

6.2.4 IL-2 up-regulates the cell surface expression of both chains of the IL-12R

The data presented above show that the cell surface levels of the IL-12Rβ1 chain are down-regulated following 24 hours of IL-12 stimulation (Figure 6.2). This correlates with a strong reduction of the IL-12/ STAT4 response (Figures 6.1 and 6.3). To examine how IL-2 could potentiate the IL-12/ STAT4 response we looked at the effects of IL-2 on cell surface levels of IL-12Rβ1 and IL-12Rβ2 in PBL-T cells. We first tested if addition of IL-2 would have an effect on the initial induction of IL-12R that occurs following primary T cell activation. Primary peripheral blood derived lymphocytes were stimulated with PHA for four days in the absence or presence of exogenous IL-2. The cell surface levels of IL-12Rβ1 and 12Rβ2 on T cells were then determined using specific immuno-staining and flow cytometry analysis. Cells that were stimulated with PHA and exogenous IL-2 show higher cell surface levels of both IL-12Rβ1 and IL-12Rβ2 compared to cells treated with PHA alone (Figure 6.4 A).



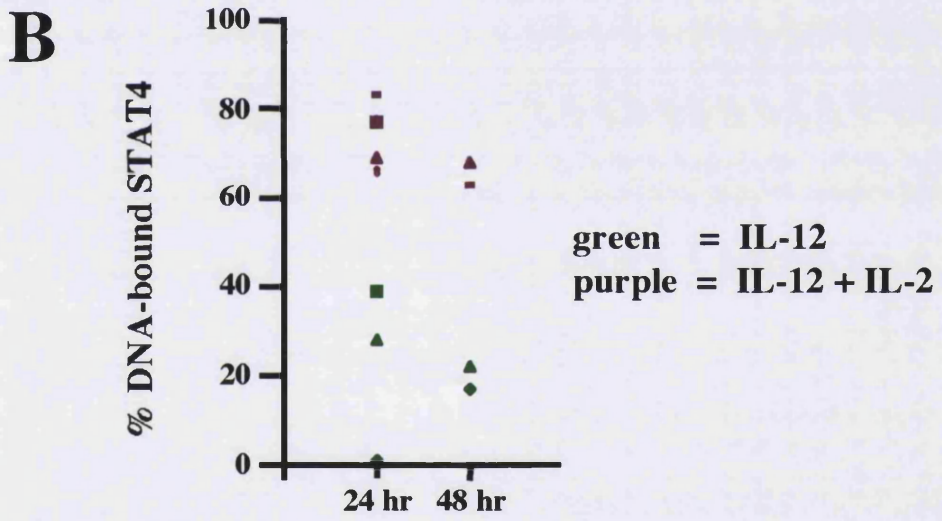


Figure 6.3) IL-2 potentiates the long-term IL-12/ STAT4 response.- **A)** Effect of IL-2 on the long term IL-12/ STAT4 DNA binding. **B)** Comparison between the long term IL-12/ STAT4 response in the absence and presence of IL-2. PBL-T cells were left unstimulated or stimulated with IL-2, IL-12 or both cytokines for indicated times. A) Proteins were GAS-STAT4-affinity precipitated from lysates containing equal amounts of total protein (970 μg), resolved on SDS-PAGE and immunoblotted with anti-STAT4 C- followed by sequential reprobing with anti-STAT5 pan. Results are representative of at least three independent experiments. B) The amount of DNA-bound STAT4 in response to IL-12 in the absence (green) or presence (purple) of IL-2 from four independent oligonucleotide affinity precipitation experiments, was determined by densitometric analysis as described in materials and methods. Values are given as the percentage of DNA-bound STAT4 when initial (18 minutes) stimulation time is given the arbitrary value of 100%.

We then tested the effects of IL-2 on IL-12R expression in PBL-T cells (i.e. T cells previously activated with PHA and polyclonally expanded in IL-2). Five-day-old PBL-T cells were quiesced by IL-2 deprivation for 48 hours and then stimulated with or without IL-2 for further 24 hours. As shown by flow cytometry analysis, IL-2 up-regulates the cell surface levels of IL-12Rβ1 and IL-12Rβ2 in PBL-T cells (Figure 6.4 B). This effect of IL-2 is selective. IL-2 does not up-regulate the cell surface expression of the IFNAR2.2 subunit of the IFN–α-R (Figure 5.7 and data not shown). These data show that IL-2 has a positive regulatory effect on the cell surface levels of IL-12Rβ1 and IL-12Rβ2. It can both potentiate the levels induced upon PHA-activation of primary T cells and have a direct effect on PBL-T cells.

6.2.5 IL-2 treated PBL-T cells show an enhanced IL-12/ STAT4 response

We compared the IL-12/ STAT4 response in PBL-T cells that have been pre-incubated or not with IL-2 for 24 hours (Figure 6.5). We first verified the levels of IL-12Rβ1, IL-12Rβ2 and STAT4 in both populations. As respectively determined by immuno-staining/ flow cytometry and Western blot analyses, PBL-T cells pre-incubated with IL-2 for 24 hours show higher cell surface expression of IL-12Rβ1 and IL-12Rβ2 than untreated cells (Figures 6.5 A). We also found an inconsistent increase in STAT4 protein levels in cells treated with IL-2 (Figures 6.5 B data not shown). We then stimulated each of these populations with IL-12 for one hour and analysed the amount of DNA-bound STAT4 using the oligonucleotide affinity precipitation technique (Figure 6.5 C). As expected, the amount of DNA-bound STAT4 is considerably higher in IL-12 activated T cells previously maintained in IL-2 for 24 hours. As a positive control for IL-2 activity, the activation of STAT5 is shown (Figure 6.5 C lower panel).

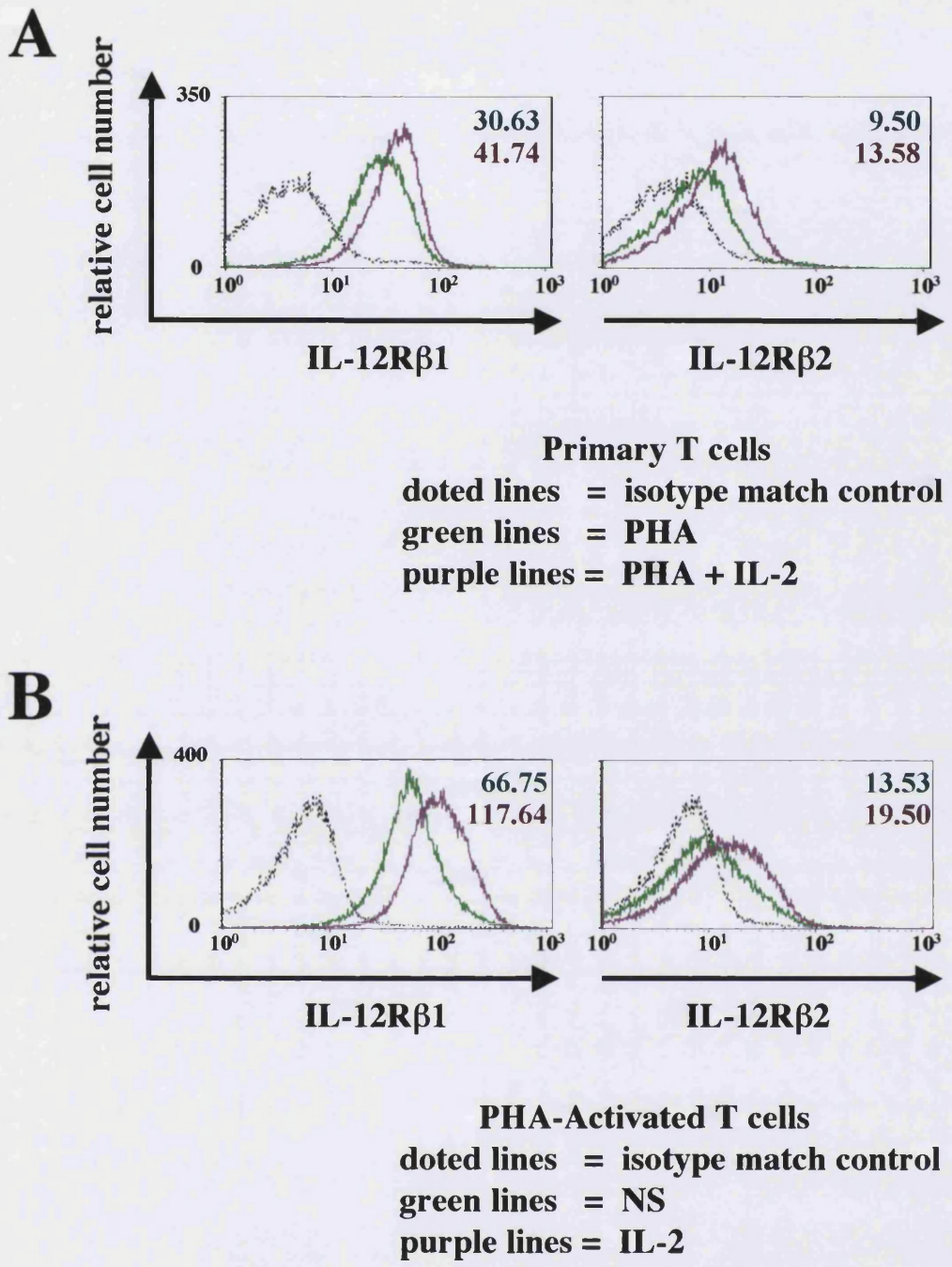


Figure 6.4) IL-2 up-regulates the cell surface expression of both chains of the IL-12R.- Effect of IL-2 stimulation on IL-12Rβ1 and IL-12Rβ2 surface expression on primary T cells (A) and PHA-activated PBL-T cells (B). A) Primary T cells were activated with PHA in the absence (green lines) or presence (purple lines) of IL-2 for four days. B) 5 days old PHA-activated PBL-T cells were rested for 48 hr and left unstimulated (green lines) or stimulated (purple lines) with IL-2 for 24 hours. Cells were stained with either an isotype match control (doted lines), anti-IL-12Rβ1 (left column) or anti-IL-12Rβ2 (right column) antibodies. Stained cells were analysed by flow cytometry. Histograms represent IL-12Rβ1 and IL-12Rβ2 staining, electronically gated on live PBL-T cells. The mean fluorescence intensity of each histogram is indicated in the corresponding colour in the upper right hand side corner. Results are representative of at least two independent experiments.

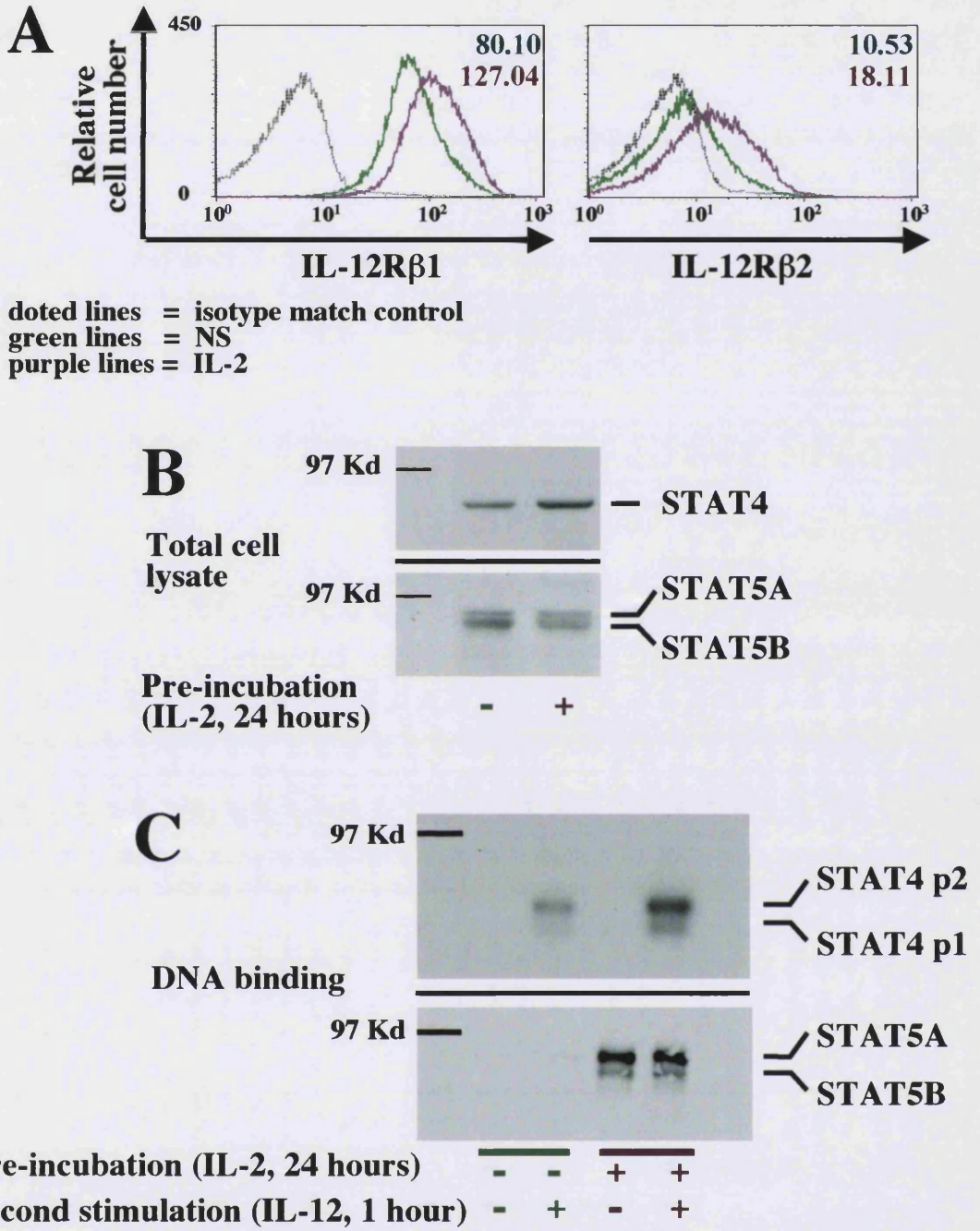


Figure 6.5) IL-2 treated PBL-T cells show an enhanced IL-12/ STAT4 response.- Effect of IL-2 treatment on IL-12Rβ1 and IL-12Rβ2 cell surface expression (**A**) and IL-12/ STAT4 response (**C**) in PBL-T cells. **B)** STAT4 protein content. Proliferating PBL-T cells were cultured in the absence (green) or presence (purple) of IL-2 (Pre-incubation) for 24 hours. A) Cells were immuno-stained using an isotype match control (doted lines), anti-IL-12Rβ1 (left column) or anti-IL-12Rβ2 (right column) antibodies and analysed by flow cytometry. Histograms represent IL-12Rβ1 and IL-12Rβ2 staining electronically gated on live PBL-T cells. The mean fluorescence intensity of each histogram is indicated in the corresponding colour. B) Cell lysates were obtained and 100 μg of total protein were acetone precipitated. C) Cells with either low (green histograms in A) or high (purple histograms in A) IL-12R cell surface expression were left unstimulated or stimulated with IL-12 for 1 hour. Cells were lysed and proteins were GAS-STAT4 affinity precipitated from 750 μg of total protein. B, C) Proteins were resolved on SDS-PAGE and immunoblotted with anti-STAT4 C- followed by reprobing with anti-STAT5 pan. Results are representative of at least two independent experiments.

6.2.6 IL-2 does not prolong the transient expression of IL-12Rβ2

Although IL-2 regulates the expression of IL-12Rβ1 and IL-12Rβ2 in activated T cells we had noted that PBL-T cells maintained in exponential growth in IL-2 did not maintain IL-12 responsiveness in terms of STAT4 activation. The typical growth protocol for PBL-T cells is to isolate T cells from peripheral blood followed by 3-4 days exposure to PHA to ensure induction of IL-2R. These activated T cells can then be maintained in exponential growth by addition of the cytokine IL-2. However, IL-2R expression is transient and 10-14 days after the removal of PHA IL-2R levels decline to a level that will no longer support IL-2 mediated proliferation. We noted that attempts to look at IL-12 induction of STAT4 DNA-binding at the end of this culture period in IL-2 were almost always unsuccessful (data not shown). This raised the possibility that expression of IL-12R on activated T cells was transient. Primary peripheral blood derived T cells were stimulated with PHA in the presence of IL-2 for four days. Cells were washed and then maintained in exponential growth in IL-2 for a further period. The cell surface levels of IL-12Rβ1 and IL-12Rβ2 were analysed by immuno-staining and flow cytometry at different times. Both IL-12Rβ1 and IL-12Rβ2 can be detected 18 hours after T cell activation with PHA (Figure 6.6). The representative experiment shows that the expression of the IL-12Rβ1 chain is maintained throughout 11 days of culture whereas IL-12Rβ2 expression is transient and declined to undetectable levels 11 days after T cell activation (Figure 6.6). IL−12Rβ1 levels reduce although expression of this receptor subunit is never completely lost (Figure 6.6 and data not shown).

IL-2 can up-regulate the cell surface expression of both IL-12Rβ1 and IL-12Rβ2 (Figure 6.4). However, the results in Figure 6.7 show that IL-12Rβ2 cell surface expression is transient even in the constant presence of IL-2. These data indicate that although IL-2 up-regulates the cell surface expression levels of IL-12Rβ1 and

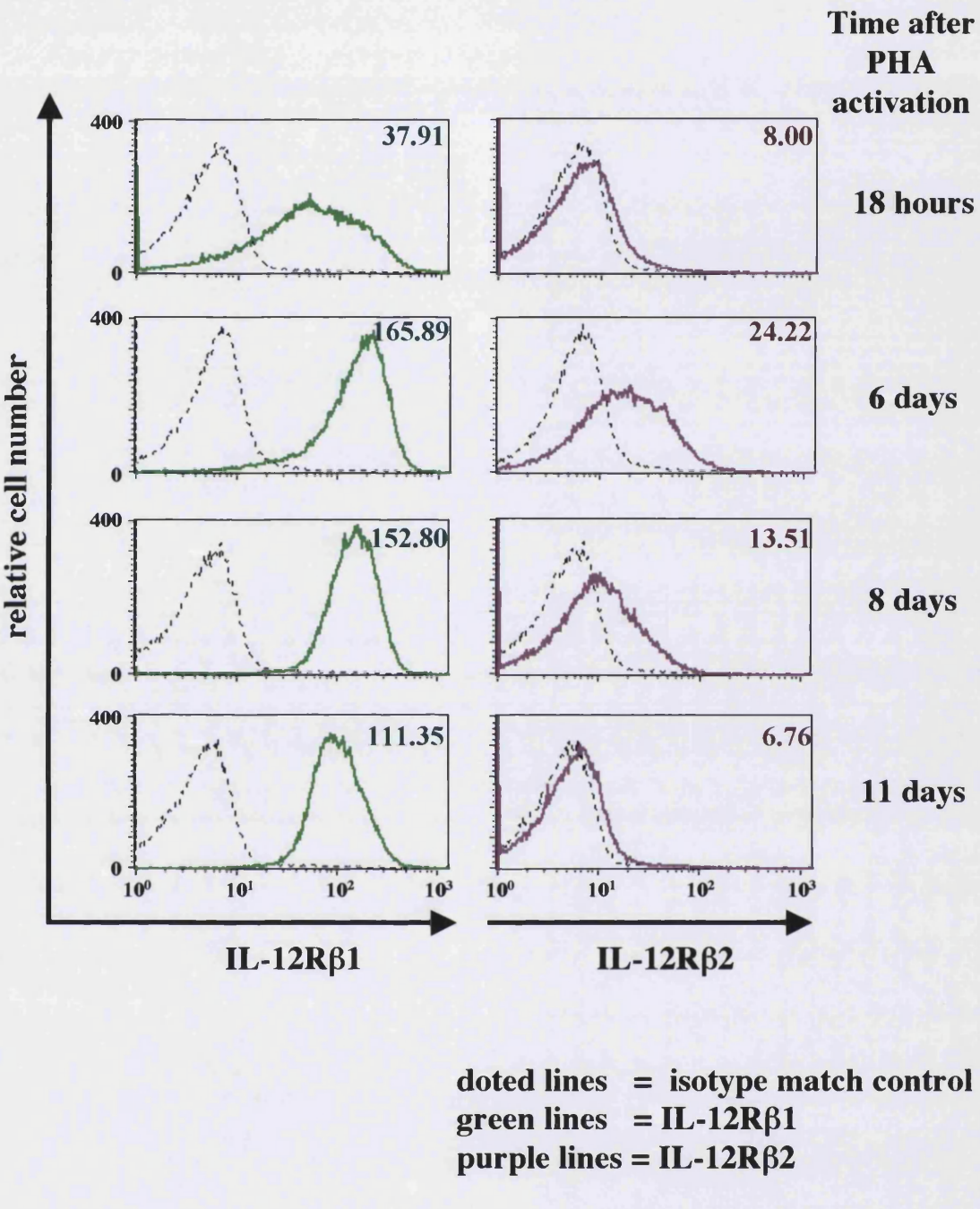


Figure 6.6) Kinetics of IL-12Rβ1 and IL-12Rβ2 expression following T cell activation.- **A)** Primary T cells were activated with PHA in the presence of IL-2. After four days, PHA was removed and the cells were cultured in the presence of IL-2. The cell surface expression of IL-12Rβ1 and IL-12Rβ2 was analysed at 18 hours and 6, 8 and 11 days after PHA activation. Cells were stained with either an isotype match control (doted lines), anti-IL-12Rβ1 (green lines) or anti-IL-12Rβ2 (purple lines) antibodies. Stained cells were analysed by flow cytometry. Histograms represent IL-12Rβ1 and IL-12Rβ2 staining electronically gated on live PBL-T cells. The mean fluorescence intensity of each histogram is indicated in the corresponding colour in the upper right hand side corner.

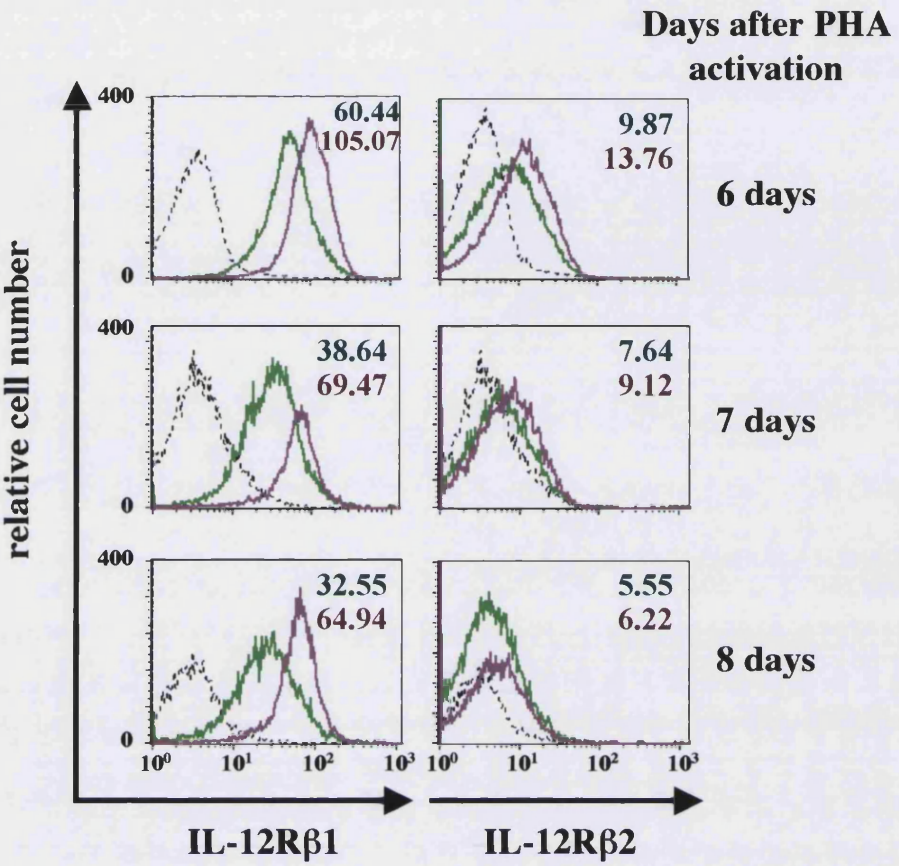


Figure 6.7) IL-2 does not prolong the transient expression of IL-12Rβ2 in PBL-T cells.- Effect of IL-2 on the kinetics of IL-12Rβ1 and IL-12Rβ2 cell surface expression in PBL-T cells. Primary T cells were activated with PHA in the presence of IL-2 for four days. PHA was removed and the cells were cultured in the absence (green lines) or presence (purple lines) of IL-2. Cells were stained at day 6, 7 and 8 after PHA activation with either an isotype match control (doted lines), anti-IL-12Rβ1 (left column) or anti-IL-12Rβ2 (right column) antibodies. Stained cells were analysed by flow cytometry. Histograms represent IL-12Rβ1 and IL-12Rβ2 staining electronically gated on live PBL-T cells. The mean fluorescence intensity of each histogram is indicated in the corresponding colour in the upper right hand side corner. A representative experiment of four is shown.

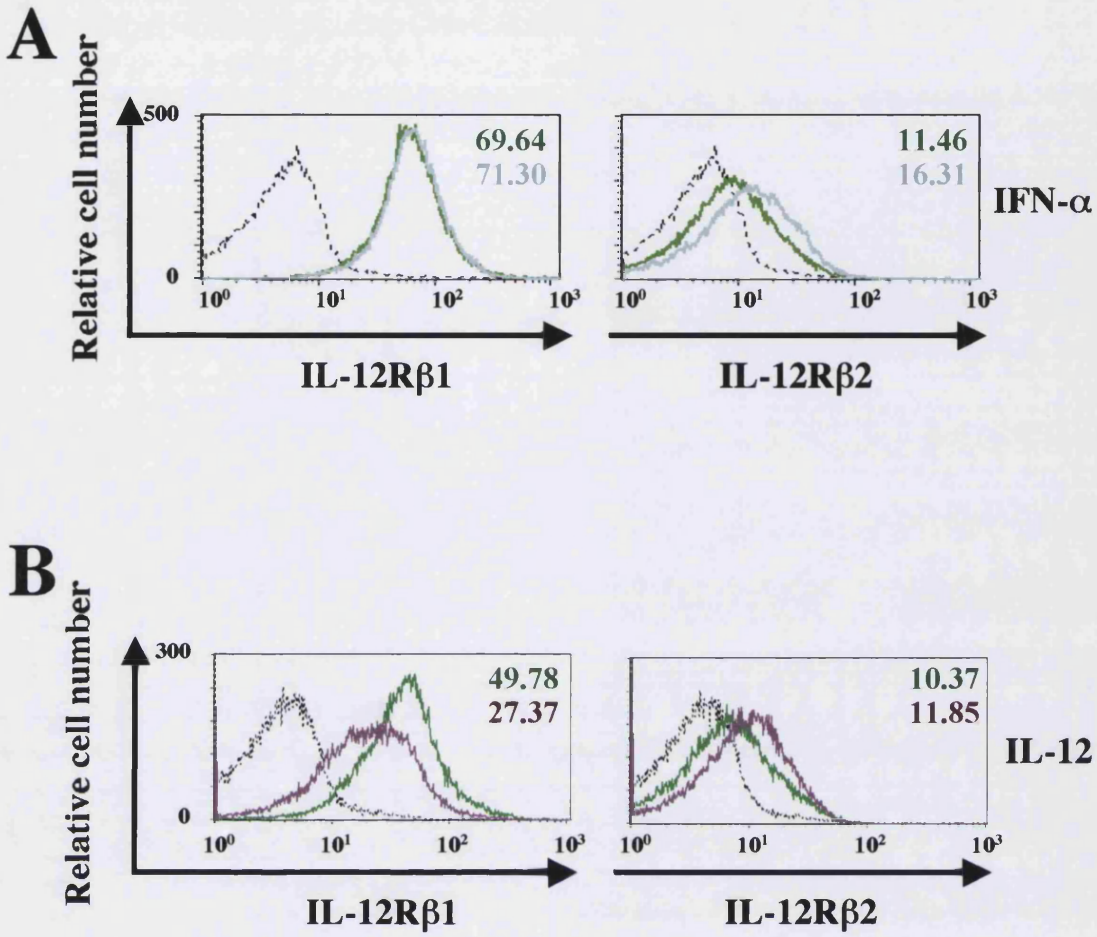
IL-12Rβ2 at various stages upon T cell activation, it does not prolong the transient expression of IL-12Rβ2.

6.2.7 IL-2 but not IL-12 or IFN–α, regulate the cell surface expression of IL-12Rβ1

To determine if the regulation of IL-12Rβ1 is specific for IL-2, we analysed the effect of IFN-α and IL-12 on the cell surface expression of IL-12Rβ1 and IL-12Rβ2 in PBL-T cells. Immuno-staining and flow cytometry analysis show a weak increase in IL-12Rβ2 levels upon four hours of IFN-α stimulation (Figure 6.8 A). This effect is transient, as it could not be detected at longer (24 hours) stimulation times (data not shown). However, IFN-α does not have any effect on IL-12Rβ1 at any stimulation time (Figure 6.8 A and data not shown). In accordance with the data presented in Figure 6.2, stimulation with IL-12 for 48 hours induces up-regulation of IL-12Rβ2 (~10-15% increase) but down-regulates the cell surface levels of IL-12Rβ1 (~50% decrease) (Figure 6.8 B). These data indicate that the up-regulation of IL-12Rβ1 cell surface expression is specific for IL-2, while IL-12Rβ2 can be up-regulated in response to IL-2, IL-12 or IFN-α.

6.2.8 IL-2-induced up-regulation of IL-12Rβ1 is LY294002 compound sensitive

An interesting question is the nature of the intracellular signals used by IL-2 to control IL-12R expression. IL-2 activates a diverse range of signal transduction pathways, including STAT3 and STAT5, Ras-MAPKs and the PI3-K/ PKB pathways. The involvement of MAPKs and PI3-K in IL-2 induced up-regulation of



doted lines = isotype match control
green lines = NS
grey lines = IFN-α
purple lines = IL-12

Figure 6.8) IL-2 but not IL-12 or IFN-α regulate the cell surface expression of IL-12Rβ1 .- Effect IFN-α **(A)** and IL-12 **(B)** on the cell surface expression levels of IL-12Rβ1 and IL-12Rβ2. A, B) 48 hour quiescent PBL-T cells were left unstimulated (green histograms) or stimulated with IFN-α for four hours (A: grey histograms) or IL-12 for 48 hours (B: purple histograms). Cells were stained with either an isotype match control (doted lines), anti-IL-12Rβ1 (left panels) or anti-IL-12Rβ2 (right panels) antibodies. Stained cells were analysed by flow cytometry. Histograms represent IL-12Rβ1 and IL-12Rβ2 staining electronically gated on live PBL-T cells. The mean fluorescence intensity of each histogram is indicated in the corresponding colour in the upper right hand side corner.

IL-12Rβ1 and IL-12Rβ2 can be studied with the aid of specific pharmacological inhibitors of these enzymes.

We initially analysed the effect of the LY294002 compound, a well characterised PI3-K inhibitor in the up-regulation of the cell surface levels of IL-12Rβ1 and IL-12Rβ2 by IL-2. PBL-T cells were incubated in the absence or presence of different concentrations of the LY294002 compound for one hour and then either left unstimulated or stimulated with IL-2 for 48 hours. The cell surface expression of both chains of the IL-12R was analysed by specific immuno-staining and flow cytometry (Figure 6.9). The cell surface expression of CD25 (IL-2Rα chain) was also analysed. In many cell systems inhibition of PI3-K activity is associated with the onset of apoptosis. However, inhibition of PI3-K with LY294002 causes cell cycle arrest without loss of viability in PBL-T cells (data not shown). The efficacy of the LY294002 compound was verified by its ability to prevent IL-2 activation of PKB. Inhibition of IL-2-induced PKB serine phosphorylation (PKBpSer473) by LY294002 was monitored using Western blot analysis (data not shown).

The LY294002 compound inhibits IL-2-induced up-regulation of IL-12Rβ1 in a dose-dependent manner (Figure 6.9). The lowest concentration of the inhibitor (5 μM) is sufficient to block the effect of IL-2, bringing the cell surface levels of IL-12Rβ1 to those seen in unstimulated cells (Figure 6.9). Higher concentrations (10-20 μM) of the LY294002 compound not only block the effect of IL-2 on IL-12Rβ1 cell surface expression, but reduce the basal levels seen on unstimulated cells by 20-40% (Figure 6.9). This effect can be clearly seen in cells that were treated with the LY294002 compound and were left unstimulated. The effect of the LY294002 compound on IL-12Rβ1 is specific, since the inhibitor does not block the IL-2-induced up-regulation of IL-12Rβ2 or CD25 (Figure 6.9). In contrast, the presence of the LY294002 compound causes a marked increase in the cell surface levels of

both IL-12Rβ2 and CD25. IL-12Rβ2 expression is higher when IL-2 and the LY294002 compound are present compared to IL-2 alone (Figure 6.9). This effect can also be seen in cells that were incubated with the LY294002 compound and were left unstimulated. There is also an "up-regulating" effect of the LY294002 compound on CD25 cell surface expression in both quiescent and IL-2 stimulated PBL-T cells.

6.2.9 IL-2-induced up-regulation of IL-12Rβ1 and IL-12Rβ2 is PD098059 compound insensitive

The PD098059 compound specifically inhibits activation of the Erk1,2 kinases Mek1,2, and consequently blocks the Ras-MAPK pathway at the level of Erk1,2 activation [572]. To test if the Ras-MAPK pathway is involved in the regulation of IL-12Rβ1 and/ or IL-12Rβ2 in response to IL-2, we used the PD098059 compound. An identical approach to that described for the LY294002 compound was used (see section 6.2.8). In this case, the efficacy of the PD098059 compound was verified by its ability to prevent IL-2 activation of Erk1,2. Western blot analysis was used to test inhibition of IL-2-induced phosphorylation of Erk1,2 (Erk1,2pThr183pTyr185) (data not shown). Flow cytometry analysis shows that the PD098059 compound does not have a negative effect on IL-2-induced up-regulation of IL-12Rβ1, IL-12Rβ2 or CD25 at any concentration (Figure 6.10). These results indicate that activation of the Ras-MAPK pathway in response to IL-2 is not involved in the up-regulation of IL-12Rβ1, IL-12Rβ2 or CD25.

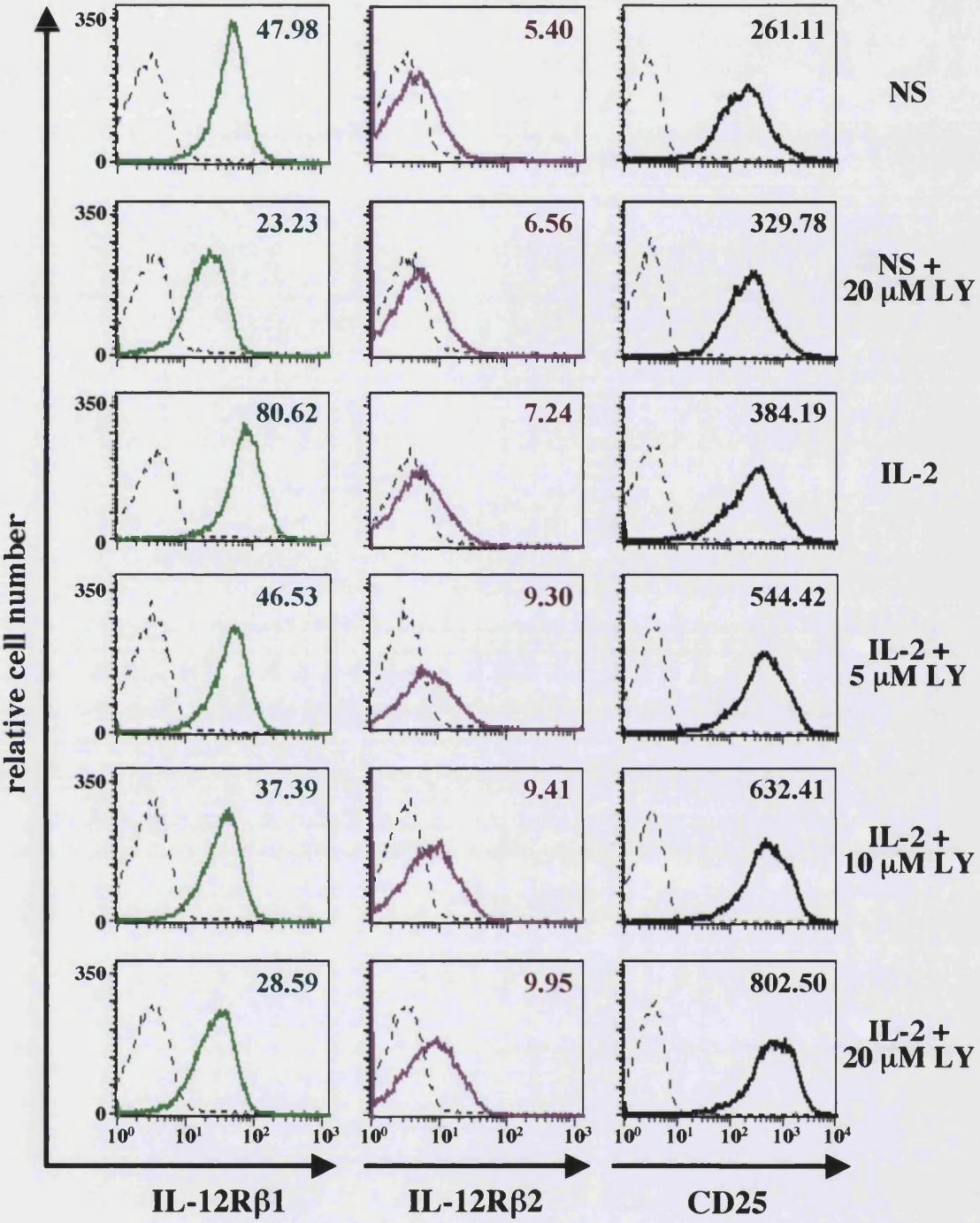


Figure 6.9) IL-2-induced up-regulation of IL-12Rβ1 is LY294002 compound sensitive.- Effect of the LY294002 compound on IL-2-induced up-regulation of IL-12Rβ1 and IL-12Rβ2 PBL-T cell surface expression. Proliferating PBL-T cells were incubated or not with indicated concentrations of the LY294002 compound (LY) for 1 hour prior to be left unstimulated (NS) or stimulated with IL-2 for 48 hours. Cells were stained with either an isotype match control (doted lines), anti-IL-12Rβ1 (left column), anti-IL-12Rβ2 (middle column) or anti-CD25 (right column) antibodies. Stained cells were analysed by flow cytometry. Histograms represent IL-12Rβ1, IL-12Rβ2 and CD25 staining electronically gated on live PBL-T cells. The mean fluorescence intensity of each histogram is indicated in the corresponding colour in the upper right hand side corner. A representative experiment of two is shown.

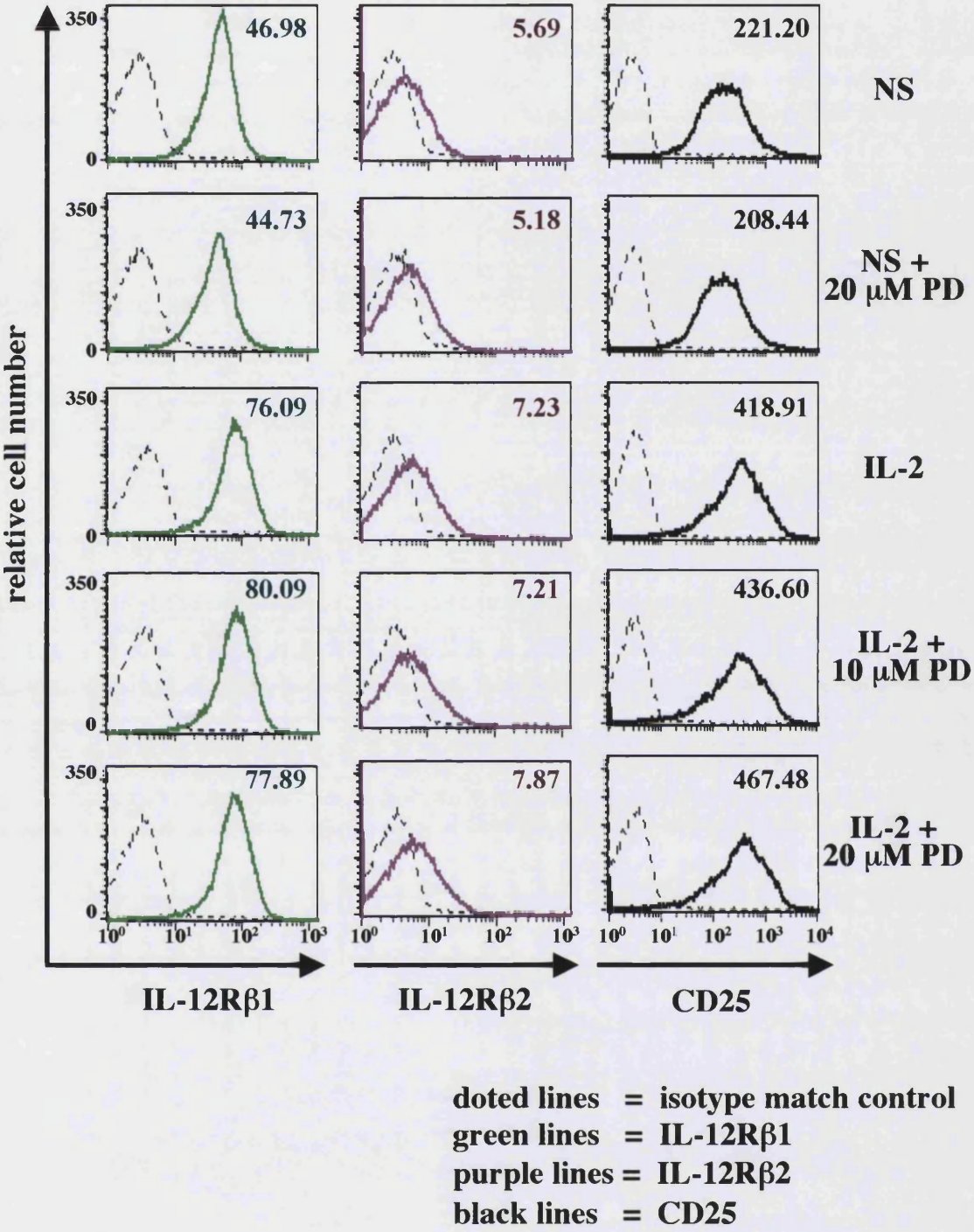


Figure 6.10) IL-2-induced up-regulation of IL-12Rβ1 and IL-12Rβ2 is PD098059 compound insensitive.- Effect of the PD098059 compound on IL-2-induced up-regulation of IL-12Rβ1 and IL-12Rβ2 PBL-T cell surface expression. Proliferating PBL-T cells were incubated or not with indicated concentrations of the PD098059 compound (PD) for 1 hour prior to be left unstimulated (NS) or stimulated with IL-2 for 48 hours. Cells were stained with either an isotype match control (doted lines), anti-IL-12Rβ1 (left column), anti-IL-12Rβ2 (middle column) or anti-CD25 (right column) antibodies. Stained cells were analysed by flow cytometry. Histograms represent IL-12Rβ1, IL-12Rβ2 and CD25 staining electronically gated on live PBL-T cells. The mean fluorescence intensity of each histogram is indicated in the corresponding colour in the upper right hand side corner. A representative experiment of two is shown.

6.3 Discussion

The data presented in this Chapter show that the IL-12/ STAT4 response is not sustained over prolonged periods unless IL-2 is also present. The down-regulation of the cell surface expression of IL-12Rβ1 during prolonged IL-12 exposure explains the transient IL-12/ STAT4 response. IL-2 up-regulates the cell surface expression of both chains of the IL-12R. Therefore, IL-2 is able to potentiate the IL-12/ STAT4 response by enhancing IL-12 responsiveness. In the combined presence of IL-2 and IL-12, the effects of IL-2 on the IL-12Rβ1 chain are likely to be most important, because IL-12 itself up-regulates the expression of IL-12Rβ2.

The decrease in the cell surface expression of IL-12Rβ1 and IL-12Rβ2 following IL-12 stimulation is most likely due to internalisation. Previous studies using radiolabelled IL-12 ($^{(125\text{-}I)}$IL-12) found maximal internalisation at one hour of stimulation, with approximately 50% of originally bound IL-12 left at 90 minutes [565]. These data are in agreement with our findings showing that IL-12Rβ2 levels decrease by 50-60% between one and four hours of IL-12 stimulation (Figure 6.2). The decrease in the levels of IL-12Rβ1 is slower when compared to IL-12Rβ2 (Figure 6.2). The higher density of IL-12Rβ1 compared to IL-12Rβ2 may explain the differences in this relative reduction [65, 565].

Although IL-12 was shown to be degraded following internalisation, the fate of IL-12Rβ1 and IL-12Rβ2 has not been determined [565]. It is not known if these chains are recycled back into the cell surface following dissociation from IL-12 or if they are degraded. We do not find major differences in the surface levels of IL-12Rβ1 or IL-12Rβ2 in PBL-T cells that have been incubated with media alone during a 48 hour period (Figure 6.2). We cannot exclude that both (or either) chains are internalised in a ligand-independent manner in such a rate that the overall cell surface levels remain constant. What is clear from the present data is that IL-12

binding induces the down-regulation of both chains of the IL-12R. The fact that the levels of IL-12Rβ1 are lower and not recovered at 24-48 hours of stimulation suggests that *de novo* protein synthesis is required to recuperate the levels of IL-12Rβ1.

The levels of IL-12Rβ2 are up-regulated by 24-48 hours of IL-12 stimulation (Figure 6.2). The up-regulation of IL-12Rβ2 by IL-12 has been reported before [77, 86, 89, 523]. In fact it has been shown that endogenous concentrations of IL-12 are sufficient to up-regulate IL-12Rβ2 expression in mouse *in vivo* systems [40]. This regulation appears to be at the transcriptional level and has been proposed to be mediated by STAT4 (see Chapter 5, section 5.3 and [9, 86, 89, 286, 516]).

The present data show that IL-2 up-regulates the cell surface expression of both IL-12Rβ1 and IL-12Rβ2. This is in agreement with previous reports suggesting a role for IL-2 in the regulation of the IL-12R. Primary T cells only express IL-12Rβ1 and IL-12Rβ2 following T cell activation (see Chapter 1, section 1.4.4; Chapter 3 (Figure 3.3) and [63, 95, 565]). It is now clear that this initial induction is only dependent on TcR signals. CD28 null T cells activated with anti-CD3 antibodies induce the expression of both IL-12Rβ1 and IL-12Rβ2 though at low levels [79, 81]. However, CD28 co-stimulatory signals are required for maximal IL-12Rβ1 and IL-12Rβ2 expression, apparently through the regulation of cytokine production. Induction of IL-12Rβ1 and IL-12Rβ2 by priming with anti-CD3 and anti-CD28 in mouse T cells, can be reduced by addition of anti-IL-2/ anti-IL-2R or anti-IFN–γ antibodies, respectively [80]. Similarly, the low levels of IL-12Rβ1 and IL-12Rβ2 induced in IFN-γ null and CD28 null T cells by anti-CD3 cross-linking can be respectively recovered by exogenous IFN-γ and IL-2 [79-81]. We find that IL-2 not only enhances the levels of IL-12Rβ1 and IL-12Rβ2 induced following T cell activation, but it also up-regulates both chains in already activated and clonally

expanded PBL-T cells (Figure 6.4). Thus, IL-2 has the same effect on the IL-12R expression levels at various stages upon T cell activation. These results extend previous findings showing that IL-2 is required for optimal expression of IL-12R following initial T cell priming.

We noted that IL-12/ STAT4 responses are lost between 10-14 days following T cell activation when cells are growing exponentially in the presence of IL-2 (data not shown). This can be explained by the transient expression of IL-12Rβ2, which reaches low to undetectable levels around day 11 after T cell activation (Figure 6.6). IL−12Rβ1 levels also decrease, but the expression of this receptor subunit is never completely lost (Figure 6.6 and data not shown). These data are in agreement with previous reports showing that even under Th1 cell conditions (i.e. IL-12 and anti-IL-4 antibodies), the expression of IL-12Rβ2 is transient, while that of IL-12Rβ1 is maintained although not at maximal levels [77, 80, 523]. The respective decrease and extinction of the cell surface levels of IL-12Rβ1 and IL-12Rβ2 occur independently of the presence of IL-2 (Figures 6.6 and 6.7). Therefore, although IL-2 up-regulates the cell surface expression levels of IL-12Rβ1 and IL-12Rβ2 at various stages upon T cell activation, it does not prolong the transient expression of IL-12Rβ2.

The transient expression of the IL-12Rβ2 may serve to avoid exaggerated and/ or unnecessary Th1 cell responses. The fact that re-stimulation through TcR triggering induces IL-12Rβ2 expression would then allow further Th1 cell responses in the persistent presence of a specific antigen. The continuous expression of IL-12Rβ1 may confer a selective advantage for IL-12 responses upon re-stimulation.

Different mechanisms could account for the transient expression of the IL-12Rβ2 chain. The expression of IL-12Rβ2 is lost at approximately the same time as the IL-2Rα chain. It could be possible that IL-2 may be able to sustain the expression of IL-12Rβ2 but the loss of the IL-2Rα chain and IL-2 responsiveness impedes this to occur. Although not completely extinguished, the levels of IL-2α chain at around day 11 following T cell activation are importantly reduced (data not shown). The induction of IL-12Rβ2 is consistently weaker in response to IL-2 when compared to IL-12 stimulation (data not shown). This could imply that IL-12Rβ2 induction requires a certain threshold of IL-2-derived signals that is no longer achieved in these long-term cultured cells.

As an alternative mechanism, it would be tempting to speculate that activated T cells have an "internal clock" dictating the timing of expression of IL-12Rβ2. This "clock" if it exists, is not modified by cytokine stimulation. IL-2 does not prolong the expression of the IL-12Rβ2 chain (Figure 6.7). Similarly, cells growing in Th1 conditions (i.e. IL-12 and anti-IL-4 antibodies) lose IL-12Rβ2 expression around day 8 following T cell activation [77, 86, 523]. However, T cell re-stimulation through TcR triggering induces IL-12Rβ2 expression. A possible explanation for this "internal clock" is that the accessibility of the IL-12Rβ2 locus may vary at different stages following T cell activation. It has been suggested that cytokine gene loci, including IL-2, IL-4, IL-12, IL-13, IFN–γ and GM-CSF, undergo changes in chromatin structure mediated in part through demethylation and histone acetylation, that allow access to gene-specific transcription factors. This process known as chromatin remodelling controls gene expression and has been suggested as an important mechanism regulating specific cytokine expression during Th1/ Th2 cell differentiation (for review see [506, 507]). It could be possible that TcR activated factors may induce the chromatin remodelling of the IL-12Rβ2 locus allowing the expression of this gene to be further controlled by cytokine-derived signals. Studies

on the accessibility of the IL-12Rβ2 gene would be required to test this hypothesis.

While expression of IL-2Rβ and IL-2Rγ chains is constitutive, long-term activated T cells lose expression of the IL-2Rα chain and are therefore no longer responsive to IL-2. However, these cells remain responsive to IL-15. Together with the IL-15Rα chain, IL-15 utilises the IL-2Rβ and IL-2Rγ chains and has many modulatory effects similar to those of IL-2. It is therefore possible that IL-15 could up-regulate IL-12R expression. The up-regulation of IL-12Rβ2 by IL-15 in long-term activated T cells would discriminate between the two mechanisms suggested above for the loss of IL-12Rβ2 expression.

While expression of IL-12Rβ2 is induced by various cytokines such as IL-12 itself, IL-18, IFN–α and IFN–γ, the expression of IL-12Rβ1 seems to be controlled only by IL-2 (Figures 6.4 and 6.8, and [9, 40, 77, 79, 85, 86, 89, 516, 523, 539, 606, 608]). In agreement with previous reports, we find that IFN–α and IL-12 can up-regulate the cell surface expression of IL-12Rβ2 but not of IL-12Rβ1 (Figure 6.8 and [77, 86, 89, 523]). It is interesting to note that IL-2 is the only cytokine that up-regulates both IL-12Rβ1 and IL-12Rβ2. This capacity has also been shown in NK cells [85]. The regulation of IL-12Rβ2 in response to IL-12 and IFN–α has been proposed to be mediated by STAT4 [286]. IL-2 activates STAT4 in NK cells but not in T cells [526]. Activation of STAT4 by IL-2 could then explained the induction of IL-12Rβ2 in NK cells [85, 526]. IL-2 activated STAT5 could interact with the STAT binding site present in the IL-12Rβ2 promoter. However, in long-term kinetics experiments where IL-2 up-regulates the expression of both chains of the IL-12R, the presence of IL-12 seems to down-regulate the IL-2/ STAT5 response (Figure 6.3 A). Therefore, IL-2/ STAT5 activation is not a likely mediator for the induction of IL-12Rβ2 or even IL-12Rβ1 by IL-2.

Preliminary attempts to uncover the signalling pathways involved in the regulation of IL-12Rβ1 and IL-12Rβ2 by IL-2 were performed using relatively selective inhibitors. The use of the LY294002 and PD098059 compounds that respectively inhibit PI3-K and Mek1,2 activity, shows that IL-2 up-regulates IL-12Rβ1 through a PI-3K-dependent and MAPK-independent mechanism (Figures 6.9 and 6.10). However, neither of these pathways is involved in the regulation of IL-12Rβ2.

A recent study showed that IL-2 increases the low levels of IL-12Rβ1 expressed in CD28 null T cells upon TcR triggering with anti-CD3 antibodies [81]. This response is insensitive to the mTor/ p70S6K inhibitor rapamycin [81]. Taken together these data indicate that IL-2 regulates IL-12Rβ1 expression through a PI3-K controlled pathway that is sensitive to LY294002 and insensitive to rapamycin. Previous studies in our laboratory have shown that IL-2-induced PI3-K controls the activation of PKB and p70S6K in human T cells [239, 241]. Activation of both kinases is LY294002 sensitive while rapamycin only inhibits p70S6K. Therefore, down-stream signals from PKB different from p70S6K seem suitable candidates for IL-2 regulated IL-12Rβ1 expression. Alternatively, tyrosine kinases from the Btk family such as Itk and Tec, which have been shown to be activated by IL-2 via PI3-K-derived signals, could also be implicated in the regulation of IL-12Rβ1 expression by IL-2 [245].

Other well-defined IL-2-regulated signalling pathways that could be involved in the regulation of IL-12Rβ1 and IL-12Rβ2 include the JNK and p38 MAPKs. These kinases have been shown to play important roles in the balance of Th1/ Th2 cell differentiation [230, 231, 233]. Activation of JNK2 seems to be required for IL-12Rβ2 expression, because JNK2 null mice show reduced Th1 cell responses due to decreased expression of IL-12Rβ2 [231]. The p38 MAPK has been reported to be important for the regulation of IL-12 production by macrophages and DCs, but it

does not seem to be implicated in IL-12Rβ2 expression in T cells [233, 234].

The induction of IL-12Rβ1 and IL-12Rβ2 in response to IL-2 may explain the biological synergy observed between IL-2 and IL-12 for responses such as T cell and NK cell proliferation and cytotoxicity [104, 108, 122, 259, 260, 491]. The idea that IL-15 could share the capacity of IL-2 for up-regulation of IL-12Rβ1 and/ or IL-12Rβ2 is supported by reports showing that IL-12 and IL-15 synergise in both NK cell-dependent and independent manners. In human NK cells, IL-12-induced IFN–γ production is enhanced by IL-15 [609]. Similarly, the anti-tumour effect of IL-12 plus IL-15 in a murine malignant pleurisy model in BALB/c mice is mediated by enhanced NK and $CD8^+$ T cells activities [610]. NK-depleted nude mice show complete rejection of small cell lung cancer cells engineered to secrete IL-12 and IL-15, while rejection of cells expressing either cytokine alone is only partial [611]. These data also suggest that IL-12Rβ1 and/ or IL-12Rβ2 may be regulated by cytokines that signal through the common gamma chain (γc). Interestingly, an earlier study showed that IL-7 synergises with IL-12 for cytotoxic activity and IFN–γ production in anti-CD3 activated human $CD8^+$ T cells, apparently through up-regulation of IL-12Rβ1 [612].

The IL-2/ IL-12 synergy is not only explained by up-regulation of IL-12Rβ1 and IL-12Rβ2 in response to IL-2, but could also involve the IL-12-induced up-regulation of the IL-2Rα chain (CD25). Although at lower levels than IL-2, IL-12 induces the up-regulation of IL-2Rα in both human and mouse T cells (data not shown and [584, 606, 613]. The mechanism involved in CD25 up-regulation by IL-12 is not known. Studies in mice suggested the participation of p38 MAPK [584]. However, the activation of p38 MAPK by IL-12 is human PBL-T cells is highly improbable (see Chapter 4, section 4.3). Although IL-2Rα up-regulation by IL-12 may participate in the IL-2/ IL-12 synergy, IL-12-derived signals are not essential

for IL-2Rα expression, because T cells lacking IL-12Rβ1 or IL-12Rβ2 present normal proliferative responses to IL-2 [66, 71].

In summary, the present Chapter shows that IL-2 potentiates the IL-12/ STAT4 response through up-regulation of IL-12Rβ1 and IL-12Rβ2. Although it has always been considered that regulation of IL-12Rβ2 is essential for maintenance of IL-12 responsiveness, the present data show that optimal expression levels of IL-12Rβ1 are also required for maximal IL-12/ STAT4 responses. The up-regulation of the IL-12R and consequent potentiation of IL-12/ STAT4 response by IL-2 is quite selective. IL-2 cannot prolong the IFN–α/ STAT responses because it is not able to oppose the down-regulation of the IFNAR1.1 and IFNAR2.2 chains induced by IFN–α stimulation.

CHAPTER 7

General Discussion

In the present work we have investigated some of the characteristics of the activation of STAT4 in response to IL-12 and IFN–α. We have found that while the IFN–α/ STAT4 response is transient, the IL-12/ STAT4 response is significantly more sustained, but can only be maintained for several days in the presence of IL-2. Interestingly, the respective transience and persistence of these responses are dependent on the regulation of the cell surface expression of the corresponding cytokine receptor. We propose that this differential kinetics may explain why IL-12 is competent for Th1 cell differentiation responses *in vivo* while IFN–α is not. Some of the general views arising from this work are discussed in more detail.

7.1 Serine phosphorylation of STAT4

While adequate Th1 and Th2 cell responses are protective from pathogen infections, their inappropriate regulation leads to autoimmune and allergic diseases, respectively (reviewed by [27, 614-618]). This implies that the ability to alter or reverse Th cell differentiation is a potential strategy for the treatment of such diseases. The essential role of STAT4 in Th1 cell differentiation and the lack of evidence for the participation of STAT4 in any other biological response point to STAT4 as a very specific target for therapeutic manipulation. A better understanding of the signalling pathways controlling STAT4 activation is required

to define the most adequate strategy for any therapy approaches. STAT4 activation in response to IL-12 requires activation of the Jak kinases Jak2 and Tyk2. Since Jak kinases are commonly activated by several cytokines their targeting would affect signalling pathways other than STAT4. An interesting possibility suggested by our present data, is that the IL-12-induced serine kinase may be specific for STAT4 and not for STAT1-α and STAT3-α. The data presented in Chapter 4 was developed to test this hypothesis and to explore the participation of well-known serine kinases in IL-12/ STAT4 responses. Here we found that none of the well-defined signalling pathways such as Ras/ MAPK, PI3-K and PKC that involve activation of serine/ threonine kinases are regulated by IL-12. Moreover, inhibition of these pathways with relatively specific pharmacological compounds does not affect the induction of STAT4 serine phosphorylation in response to IL-12. The participation of JNK kinases in IL-12 signalling pathways has been also eliminated by the present study and other reports [568, 570, 585]. Some studies have suggested that p38 MAPK may be regulated by IL-12 in NK cells and mouse T cells [570, 584, 585]. As extensively discussed in Chapter 4, to date there is no strong evidence of the participation of p38 MAPK or any other known serine kinase in STAT4 regulation. Some of the possible strategies that could be used to identify the STAT4 serine kinase will be discussed further below (see section 7.5).

7.2 IFN–α-regulated STATs Serine kinases

IFN-α induces tyrosine and serine phosphorylation of STAT1-α, STAT3-α and STAT4 (Chapters 3 and 5). One question is whether IFN-α uses a common serine kinase to phosphorylate these three STATs. IFN-α-induced STAT1-α and STAT3-α serine phosphorylation is almost coincident with tyrosine phosphorylation. In contrast, the kinetics of IFN-α-induced STAT4 tyrosine and serine phosphorylation are different. STAT4 tyrosine phosphorylation is as rapid

as STAT1-α and STAT3-α tyrosine phosphorylation, but there is a lag before serine-phosphorylated STAT4p2 appears. This is consistent with the existence of different serine kinases for STAT1-α, STAT3-α and STAT4. Further evidence comes from the observation that IL-12 induces the serine phosphorylation of STAT4 but not STAT1-α and STAT3-α (Chapter 3).

The p38 MAPK is involved in the phosphorylation of STAT1-αSer727 and STAT3-αSer727, while Erk1,2 is implicated in the serine phosphorylation of STAT3-αSer727 [185, 355, 357, 358, 360, 417, 619]. Recent studies have shown that IFN-α and IFN-β activate p38 MAPK in a lymphoblastoid cell line (Daudi cells) [620, 621]. However, the use of the p38 MAPK inhibitors SB203580 and SB202190 showed that this kinase is not involved in serine phosphorylation of STAT1-αSer727 in response to IFN-α [621]. Similarly, the SB203580 inhibitor or transfection of a double negative form of p38 MAPK did not have any effect on IFN-α-induced serine phosphorylation of STAT3-αSer727 [621]. Previous studies in our laboratory did not find any evidence of the activation of Erk1,2 in response to IFN-α in human T lymphocytes (Lafont V. and Cantrell D.A. unpublished results).

One IFN-α-regulated kinase that could be involved in the regulation of STATs serine phosphorylation is PKR. PKR is a ubiquitously expressed serine/ threonine kinase which best characterised role is the inhibition of translation through phosphorylation of eukaryotic initiation factor 2-α-subunit (eIF-2α) [464]. Recent studies have shown the physical interaction of PKR with STAT1 [622]. However, this interaction seems to work as a reciprocal negative regulatory mechanism and different data indicate that PKR is not involved in the serine phosphorylation of STAT1-α in response to IFN-α or IFN-γ [623]. For example, STAT1 protein levels and stability are increased in PKR null fibroblasts [624]. A STAT1 mutant, which lacks the capacity to interact with PKR, shows higher transcriptional

activity, anti-viral and anti-proliferative responses as well as normal levels of STAT1-αSer727 phosphorylation in response to IFNs [623]. *In vivo* and *in vitro* kinase assays show that incorporation of phosphate (^{32}P) into STAT1 molecules in response to IFNs is not regulated by wild type PKR or double negative PKR [622]. This indicates that PKR is unlikely to be involved in IFN-α-regulated STATs serine phosphorylation. Therefore, the identity of the STAT1-α, STAT3-α and STAT4 serine kinases is response to IFN-α remains elusive. Possible strategies to uncover these kinases are proposed in section 7.5.

7.3 Is activation of STAT4 in response to IFN-α sufficient for Th1 cell differentiation?

Some investigators have proposed IFN-α as the alternative cytokine to IL-12 for the induction of Th1 cell differentiation [21, 286, 508]. This is based on: 1) the capacity of IFN-α to activate STAT4 [353]; 2) the induction of Th1 cell differentiation by IFN-α in human *in vitro* systems [77, 89]; and 3) the constitutive expression of IFN-αR in both Th1 and Th2 cells which could provide IFN–α with a constant capacity for promoting Th1 cell responses [276, 277]. The data presented in Chapter 5 show that the initial IL-12/ STAT4 and IFN–α/ STAT4 responses share a number of similarities. These similarities could also suggest that IL-12 and IFN-α signals are redundant for Th1 cell differentiation. However, our data also show a very important difference: the IL-12/ STAT4 response is significantly more sustained than the IFN-α/ STAT4 response (Chapter 5). We propose that this differential kinetics may explain why IFN–α signals are not able to fully compensate for the lack of IL-12 signals in patients suffering from recurrent *Mycoplasma sp.* and *Salmonella sp.* infections [67-69, 72]. Some of the reports and ideas in favour and against the capacity of IFN–α to drive Th1 cell differentiation

are discussed.

Although IFN-α has been shown to drive Th1 cell differentiation in human *in vitro* systems, these responses are inefficient. IFN-α-induced Th1 cells express lower levels of IL-12Rβ2 chain and produce lower amounts of IFN-γ and LT, as compared to IL-12-induced Th1 cells [77, 89, 523, 625]. These could fit a model where sustained STAT4 activation is necessary for complete Th1 cell differentiation.

Studies with viral infections have also proposed IFN–α as an alternative cytokine to IL-12 for the induction of Th1 cell responses. This is based on the high levels of IFN–α/β that are produced during viral infections together with data showing that not every viral response is accompanied by production of IL-12. While IL-12 is produced during infections with murine cytomegalovirus (MCMV) and certain strains of influenza virus, IL-12 is not detectable during LCMV infection [626-628]. Beside the lack of IL-12, specific $CD8^+$ T cell responses are found during LCMV infection [629-631]. Similarly, high induction of IFN-α/β along with protective $CD8^+$ T cell responses and IFN-γ production have been reported during LCMV infection in IL-12p35 null mice [632]. Since it is well established that IFN-α/β does not activate STAT4 and is therefore unable to induce Th1 cell differentiation in mouse systems, the responses reported in those studies can only be explained by the participation of mechanisms different from IFN–α/ STAT4 responses. The existence of STAT4-independent pathways for Th1 cell development has also been found in the STAT4/ STAT6 double knock out mice [402].

A transient IFN–α/ STAT4 response would probably be insufficient to drive Th1 cell differentiation (Chapter 5). However, it is possible that viral infections in humans may induce additional factors that could prolong the IFN-α/ STAT4 response to drive competent Th1 cell differentiation. For example, human

macrophages infected with influenza A virus secrete IFN-α/β and IL-18, but not IL-12 [522]. Although IFN-α/β and IL-18 were shown to synergise for IFN-γ induction in T cells, the effect of this cytokine combination on Th1 cell differentiation was not assessed [522]. An important role for IL-18 in IFN–α responses is supported by data showing that influenza A-induced IFN–α regulates IL-18 production by macrophages [633]. It would be of interest to study the effect of IL-18 on the transient IFN-α/ STAT4 response.

There is evidence that *in vivo* administration of type I IFNs can favour the development of human Th1 and Tc1 cells [634, 635]. IFN-γ secreting cells temporarily increase in numbers when patients with multiple sclerosis, Kaposi's sarcoma and grass pollen-sensitivity are treated with IFN-β and IFN-α, respectively [634-636]. Since these patients do not have deficiencies in IL-12 signalling, IFN-α/β-induced up-regulation of IL-12Rβ2 and consequent potentiation of IL-12 signals may explain the increase in Th1 cell responses. Alternatively, other IFN–α-induced molecules as well as the regulation of IFN–α responses by other cytokines could also be involved. As shown in Chapter 6, the IL-12/ STAT4 response is only prolonged in the presence of IL-2, which up-regulates the IL-12R levels and therefore maintains IL-12 responsiveness. Although IL-2 itself does not prolong the IFN–α responses, another cytokine or factors *in vivo*, as proposed above for IL-18, could help to prolong the IFN–α/ STAT4 response.

Another alternative is that IFN–α may regulate Th cell differentiation by the induction of biological responses in other cells. It has been recently reported that macrophages express STAT4 following stimulation with IFN–γ or LPS [73]. Since these cells lack IL-12Rβ2 but express IFN–α-R, STAT4 is only activated by IFN–α [73]. The characteristics of the IFN–α/ STAT4 response in these cells as well as its biological relevance have not been addressed. It would be interesting to

compare the kinetics of STAT4 activation in response to IFN–α in T cells and macrophages. If the IFN–α/ STAT4 response was more sustained in macrophages than in T cells, the factors involved could shade light on alternative mechanisms to prolong the IFN–α/ STAT4 response.

The view that only sustained STAT4 activation is sufficient to induce Th1 cell differentiation is supported by reports indicating that not only the presence but also the persistence of IL-12-derived signals are necessary for this response. For example, sustained expression of IL-12Rβ2 and therefore IL-12 responsiveness, is essential for developmental commitment to the Th1 phenotype in both mouse and humans [77, 86, 87, 523, 606]. Similarly, healing Th1 cell responses against *L. major* infection in IL-12p40 null mice require addition of exogenous IL-12 not only at the beginning but also throughout the course of infection [605].

One interesting question is whether sustained activation of STAT4 alone explains the capacity of IL-12 to drive competent Th1 cell differentiation. There is of course the possibility that IL-12 stimulates other as yet unknown signalling mechanisms that explain its effects on Th1 cell differentiation. To date, no molecules beside Jak2, Tyk2 and STAT4 are known to be activated in response to IL-12. As discussed in section 7.1, the data presented in Chapter 4 has eliminated the participation of the Ras/ MAPK, PI3-K and PKC pathways in IL-12 signalling. A report suggested that IL-12 could activate Lck [637]. However, studies using Lck knock out mice as well as double negative Lck transgenic mice have shown that Lck signals are not required for Th1 cell differentiation [585, 638]. Although IL-12 activates NF-κB in DCs, no studies have concentrated on IL-12 activation of this transcription factor in T lymphocytes [48]. A recent report has shown that IFN–α induces the activation of NF-κB in a lymphoblastoid cell line [591, 639]. This eliminates NF-κB as a possible molecule differentially activated by IL-12 and IFN–α that could provide an explanation for the capacity of IL-12 to drive Th1 cell

responses *in vivo*. To date there is no evidence of any other IL-12-regulated molecule that could participate with active STAT4 for a prolonged IL-12/ STAT4 response. Some strategies for the search of downstream effectors of IL-12 will be proposed further below (section 7.5).

The induction of specific biological functions in cells other than T cells could also explain the capacity of IL-12 to drive Th1 cell differentiation *in vivo*. For example, IL-12 up-regulates its own responses by directly priming DCs for IL-12 production [48]. IL-12 also acts indirectly through the induction of the IL-12 promoting cytokine IFN–γ from T and NK cells [3, 41, 42, 104-108].

The present work shows that not only the IFN–α/ STAT4 response but all IFN–α/ STAT responses are transient. These data are supported by studies showing that expression of IL-12Rβ2, IL-18R, integrin-α6/β1 and CCR-1 is more sustained in response to IL-12 than to IFN-α [132, 513, 523, 524]. The biological relevance of this differential expression is illustrated by the migration of Th1 cells, which only occurs when integrin-α6/β1 and CCR-1 are induced in response to IL-12 but not to IFN-α [132]. Therefore, not only induced but also sustained expression of Th1 specific molecules appears to be a critical requisite for the achievement of Th1 cell functions.

Considering that both Th1 and Th2 cells respond to IFN-α, the short lasting effects of IFN-α may be a key mechanism to avoid the expression and function of Th1 specific proteins on Th2 cells. The fact that IL-2 that functions in both Th1 and Th2 cells, does not prolong the IFN–α responses supports this view. Therefore IFN–α could act as a priming cytokine for further IL-12 responses by up-regulating IL-12Rβ2, but does not seem to be sufficient to drive Th1 cell differentiation.

7.4 The regulated expression of IL-12Rβ1 may be as important as the regulation of IL-12Rβ2 expression for the IL-12/ STAT4 response

The present data show that the expression of IL-12Rβ1 is up-regulated by IL-2 and this correlates with the ability of IL-2 to prolong the IL-12/ STAT4 response (Chapter 6). Studies in Tyk2 null cells have shown that this kinase is essential for normal IL-12/ STAT4 responses [331, 332]. Since Tyk2 associates with IL-12Rβ1 in an IL-12-independent manner [314], then optimal expression levels of IL-12Rβ1 would ensure maximal presence of Tyk2 in the IL-12R complex. The decreased activation of STAT4 in Tyk2 null cells is biologically relevant. Tyk2 null splenocytes show decrease Th1 cell responses accompanied by low production of IFN–γ and Tyk2 null mice are unable to clear vaccinia virus and LCMV infections [331].

Apart from Tyk2, IL-12 induces the activation of Jak2 [313]. Although both Jak2 and Tyk2 knock out mice have been generated, IL-12 responses have only been tested in Tyk2 null T cells [324, 325, 331, 332]. As mentioned above, tyrosine phosphorylation of STAT4 in response to IL-12 was either completely abolished or diminished in Tyk2 null T cells [331, 332]. Although the protein levels of Jak2 and its activation in response to IFN–γ were normal in Tyk2 null fibroblasts, the activation of Jak2 in response to IL-12 was not addressed in Tyk2 null T cells [331, 332]. The order in which Jak2 and Tyk2 are activated in response to IL-12, as well as which of these two kinases is responsible for the phosphorylation of STAT4 and/ or its docking site have not been defined. What is clear from the Tyk2 null cells studies is that Tyk2 is required to mediate normal levels of IL-12-induced STAT4 tyrosine phosphorylation. Two possible mechanisms could be involved. If IL-12/ Jak2 responses prove to be normal in Tyk2 null T cells, then Jak2 activity would be responsible for the low levels of STAT4 tyrosine phosphorylation. This would

also suggest that Tyk2 is the major kinase that phosphorylates STAT4 and/ or its docking site. However, if IL-12/ Jak2 responses are reduced or missing in the Tyk2 null T cells, then Tyk2 is likely to be required for the adequate activation of Jak2. This second possibility would not resolve the issue of which of these two kinases regulates the tyrosine phosphorylation of STAT4 and/ or its docking site.

Although both of the associated Jak kinases of a particular cytokine receptor are required for adequate cytokine responses, it seems that certain down-stream signals are more dependent on the activation of a particular Jak. For example, while tyrosine phosphorylation of STAT3 in response to IFN–α is completely abolished in Tyk2 null fibroblasts, the IFN–α/ STAT2 response is normal [331, 332]. This would suggest that as Tyk2 appears to play the major role in STAT4 activation in response to IL-12, Jak2 might be more important for other pathways activated by this cytokine. A recent report studying IL-12 responses in T cells from patients with mutations in the IL-12Rβ1 has confirmed that this chain and therefore Tyk2 activation are essential for STAT4 activation in response to IL-12 [72]. This report has proposed that IL-12Rβ2 can mediate some IL-12 responses in the absence of IL-12Rβ1. This idea is difficult to reconcile with past data demonstrating that the presence of both chains is indispensable for IL-12-induced responses [66-71]. However, T cell clones from IL-12Rβ1 deficient patients are not bias towards the Th2 phenotype when re-stimulated *in vitro* by antigen or anti-CD3 mAb/ PMA. Some of these T cell clones produce low amounts of IFN–γ and show weak proliferative responses following IL-12 stimulation [72]. These responses become significant when the cytokine IL-18 is also present. Since IL-18 induces the expression of IL-12Rβ2, these results could indicate that in the absence of IL-12Rβ1 high expression levels of IL-12Rβ2 might mediate some IL-12 responses. Nevertheless, the activation of STAT4, which is essential for IL-12-induced Th1 cell differentiation requires IL-12Rβ1 and Tyk2.

Since it was shown that sustained expression of IL-12Rβ2 is required for Th1 cell differentiation, most studies concentrated on the mechanisms regulating the expression of this chain as well as its role in IL-12 responses [77, 86]. Our present results show that up-regulation of IL-12Rβ1 correlates with a prolonged IL-12/ STAT4 response (Chapter 6). These data together with studies indicating that Tyk2 activation is essential for STAT4 activation suggest that the regulation of IL-12Rβ1 expression may be as important as that of IL-12Rβ2 for the IL-12/ STAT4 response.

7.5 Future perspectives

The regulation of STAT4 remains of high interest due to the specific and important role of this molecule in Th1 cell differentiation. Not only the mechanisms involved in IL-12-induced Th1 cell differentiation, but many details from the IL-12/ STAT4 response are still unknown. Within these is the role of STAT4 serine phosphorylation in response to IL-12; the mechanism involved in STAT4 nuclear translocation; STAT4 co-factors and co-activators; other STAT4 interacting proteins; and the negative regulation of STAT4 activation. To date there is no knowledge about signalling pathways other than Jak2, Tyk2 and STAT4 that are activated by IL-12. Beside the clues provided by our present data, the signals involved in the regulation of IL-12R chains and STAT4 expression are also unknown. Similarly, the essential role of STAT1 in IFN–α-mediated anti-viral responses and the roles described for the serine phosphorylation of STAT1-αSer727 make it of great interest to uncover the IFN–α-induced STAT1-α as well as the STAT3-α serine kinases. It is also of high interest to define if the STAT4 serine kinase is common to IL-12 and IFN–α. Some of the possible approaches that could be used to address these points are discussed.

7.5.1 STAT4 serine phosphorylation site

The first step in unravelling the role of STAT4 serine phosphorylation will be to map the STAT4 serine phosphorylation site. This would then allow defining the role of STAT4 serine phosphorylation as well as the STAT4 serine kinase.

We intended to map the serine phosphorylation site of STAT4 by mass spectrometry analysis and also by rising specific phospho-antibodies for the putative STAT4 serine phosphorylation site (STAT4Ser721). As discussed in Chapter 3 (section 3.3) these attempts were unsuccessful. Although STAT4Ser721 was considered the most likely serine phosphorylation site of STAT4, a construct with a mutated STAT4Ser721 (STAT4S721A) still suffers an electrophoretic mobility shift (corresponding to STAT4p2) in response to IL-12 [570]. These data eliminate STAT4Ser721 as the major STAT4 serine phosphorylation site.

Beside Ser721, STAT4 has another 13 serine residues in its C-terminus [488, 489]. One strategy to identify the STAT4 serine phosphorylation site would be to make 13 different constructs, each with a mutation in one of these serine residues, followed by expression and functional studies of these mutants. Clearly a laborious and time-consuming task but may be the only way forward for the field. Once a serine phosphorylation site was examined the information could be used to try and isolate the STAT4 serine kinase.

7.5.2 Role of STAT4 serine phosphorylation

Once the STAT4 serine phosphorylation site is determined, the corresponding mutant construct could be used to address a possible role in STAT4 transactivation activity. Recently some STAT4 regulated genes have been described. These include Erm, IRF-1 and IL-18R [511-513, 524]. RNAse protection assays could be used to

compare the transcription of these endogenous genes in response to IL-12 in STAT4 null T cells infected with virus containing the wild type and serine mutant constructs of STAT4. The data presented in Chapter 3 suggested that STAT4 serine phosphorylation could be involved in enhancing STAT4 DNA binding. Comparing the DNA-binding of the wild type and serine mutant of STAT4 could test this possibility. Oligonucleotide affinity precipitation assays using not only the high affinity STAT4 oligonucleotide (GAS-STAT4) but also oligonucleotides with lower affinity for STAT4 (GRR, SIE and SIEM) could be used. The STAT4 serine mutant construct could also be used to test the participation of STAT4 serine phosphorylation in interactions of STAT4 with other proteins (see further below).

To date, the identity of STAT1-α, STAT3-α and STAT4 serine kinases in response to IFN–α and IL-12, respectively, remains elusive. Before searching for novel serine kinases it would be recommended to test the involvement of know possible serine kinases in IFN–α and IL-12 regulated STATs serine phosphorylation. Antigen receptors in T and B cells regulate the serine phosphorylation of STAT1-α and STAT3-α via PI3-K mediated signals [356, 573]. However, a link between IFN-α/ PI3-K-derived signals and serine phosphorylation of STAT1-α and/ or STAT3-α has not been established. The participation of PI3-K-derived signals in the IL-12/ STAT4 response was dismissed by the present work (Chapter 4). In a similar way, the LY294002 compound could be used to define the role of PI3-K derived signals in response to IFN–α in STAT1–α and STAT3–α serine phosphorylation. The induction of STAT1-αpSer727 and STAT3-αpSer727 by IFN–α could be compared between LY294002 treated and untreated T cells, using Western blot analysis with the specific anti-STAT1-αpSer727 and anti-STAT3-αpSer727 antibodies.

It is possible that STAT4 serine phosphorylation is regulated by the same or by different serine kinases in response to IFN–α and IL-12. For example, phosphorylation of STAT3-αSer727 in regulated by Erk1,2 in response to both TcR triggering and IL-2 stimulation [185]. However, serine phosphorylation of STAT1-αSer727 can be mediated by p38 MAPK in response to LPS and stress but not in response to IFN–γ [355]. PKR does not seem to be involved in the serine phosphorylation of STATs in response to IFN–α (section 7.2), but it could still be regulated by IL-12. This possibility is supported by studies in PKR null mice [640]. In these mice, sensitisation with the hapten 2,4-dinitro-1-fluorobenzene (DNFB) induces Th cell responses with higher IL-4 production by $CD4^+$ T cells and lower IFN–γ production by $CD8^+$ T cells as compared to wild type animals [640]. The activation of PKR in response to IL-12 could be addressed by immuno-precipitation of PKR from quiescent and IL-12 stimulated T cells followed by standard PKR *in vitro* auto-phosphorylation assays using ^{32}P-labelling techniques. Moreover, the role of PKR in IL-12-induced STAT4 serine phosphorylation could be tested using PKR null T lymphocytes.

If the identity of the IFN–α and IL-12 regulated STAT serine kinases still remained unknown after these approaches, then novel serine kinases would have to be explored. Having the serine phosphorylation sites of STAT1-α, STAT3-α and STAT4 defined, the same approaches could be used for the different kinases. Therefore I will generically refer to IL-12 and IFN–α as "cytokine" and to STAT1-α, STAT3-α and STAT4 as "STAT".

GST-fusion proteins or tagged constructs of the wild type and serine mutated STAT molecule would have to be made. Alternatively, peptides bearing the STAT serine phosphorylation site and its corresponding mutant could be used. These proteins would then be used as substrates for *in vitro* kinase assays using lysates of cytokine stimulated T cells. Lysates of non-stimulated T cells would be used as

negative controls. These lysates would have to be fractionated by either size exclusion or ion-exchanged columns and each of the fractions analysed for its capacity to phosphorylate the substrate. Once a fraction has been identified, the enzyme would have to be purified by serial rounds of column chromatography using different materials such as ion-exchange and hydrophobic interaction resins. The purification should be such that well separated protein bands should be detected after Silver staining and preferentially Coomasie staining on a SDS-PAGE. Each of these bands could be sequenced using mass spectrometry analysis. However, successful enzyme sequencing would depend on the abundance of the enzyme. Our experience tells us that independently of the high sensitivity of mass spectrometry, low abundant proteins are difficult to identify.

7.5.3 Signals involved in the induction of IL-12Rβ1 and IL-12Rβ2 by TcR

One fundamental event for Th1 cell differentiation is the cell surface expression of IL-12Rβ1 and IL-12Rβ2. As described in Chapter 1 (section 1.4.4) TcR-derived signals induce the initial expression of IL-12Rβ1 and IL-12Rβ2 following T cell activation. However, the mechanisms by which TcR triggering regulates the induction of these receptor chains have not been defined. It is possible that TcR signals are directly regulating the transcription of the IL-12Rβ1 and IL-12Rβ2 genes or that they induce chromatin remodelling of these genes, then allowing their up-regulation by cytokines such as IL-2 and IL-12. IL-12Rβ2 expression is reduced in JNK2 null mice, suggesting an important role for this kinase in the regulation of IL-12Rβ2 [231]. The direct effect of JNK2 activation in response to TcR in the expression of IL-12Rβ2 has not been studied.

An attempt was made during the course of this study to address the signalling pathways involved in the induction of IL-12Rβ1 and IL-12Rβ2 by TcR triggering.

Pharmacological agents with relative high specificity for the MAPKs Mek1,2 (PD098059), PI3-K (LY294002), p70S6K (rapamycin) and PKCs (RO 31-8220) as well as not yet commercially available inhibitors for Lck, JNK and p38 MAPK (provided by Sandy Orchard and Kate Hilyard, Roche Discovery Welwyn, Ltd., UK) were used. Unfortunately, the levels of expression of IL-12Rβ1 and IL-12Rβ2 that could be detected by specific immuno-staining and flow cytometry analysis at short periods after TcR triggering were very low and did not allow clear appreciation of the effects of the inhibitors. Analyses on mRNA expression using mRNAse protection or Northern blot were not performed. The use of such techniques may help dissecting the pathways involved in TcR-induction of IL-12Rβ1 and IL-12Rβ2. As suggested by our studies on the up-regulation of IL-12Rβ1 in response to IL-2, PI3-K derived signals different from p70S6K are good candidates for the regulation of IL-12Rβ1 expression. Similarly, JNK2 represents the putative IL-12Rβ2 inducer.

7.5.4 Inactivation of STAT4

Data presented in Chapter 5 suggests that STAT4 may be inactivated by different mechanisms in response to IL-12 and IFN–α. IFN–α does not negatively regulate the IL-12/ STAT4 response, however, it could be possible that IL-12 negatively regulates the IFN–α/ STAT4 response. This possibility was not tested but it could be easily addressed. Oligonucleotide affinity precipitation and Western blot analysis could be used to compare the IFN–α/ STAT4 response in T cells that have been previously stimulated or not with IL-12.

As discussed in Chapter 5 it is possible that induction of SOCS proteins could be involved in the inactivation of STAT4. The IL-12-induced SOCS protein would have to interact with the docking site of STAT4 of the IL-12Rβ2 or block activation

of Jak2. In the case of IFN–α, the SOCS protein should block Jak1 activation or prevent the recruitment of STAT4 into the IFN–α-R complex by interacting with the C-terminus of STAT2. The induction of SOCS proteins in response to IL-12 and IFN–α in T cells could be addressed using mRNAse protection assays. If any SOCS protein proves to be induced by IL-12 or IFN–α, its interaction with respectively Jak1, Jak2, the STAT4 docking site or the C-terminal domain of STAT2 could be studied in co-immuno-precipitation assays.

7.5.5 Putative proteins interacting with STAT4

Specific proteins could be interacting with STAT4 to either mediate its inactivation, nuclear translocation or modulating its DNA binding strength or specificity. GST-STAT4 fusion proteins could be used as a matrix to affinity precipitate proteins interacting with inactive monomeric STAT4. Proteins interacting with active DNA-bound STAT4 could be searched in GAS-STAT4 affinity precipitated STAT4. Alternatively, both inactive and active STAT4 could be immuno-precipitated using anti-STAT4 antibodies. Affinity- or immuno-precipitations will be performed with lysates from unstimulated or IFN–α and IL-12 stimulated T cells. Bound proteins would be resolved on SDS-PAGE. The binding of putative proteins as those involved in nuclear transport such as NPI-1 or co-activators such as CBP/ p300, could be investigated by Western blot analysis using specific antibodies. However, for novel interacting proteins sequencing by mass spectrometry would have to be used. Since these approaches imply certain level of protein purification, the use of purification with chromatographic techniques should not be necessary.

7.5.6 Other IL-12 regulated molecules

As mentioned above, to date IL-12 has only been shown to activate Jak2, Tyk2 and STAT4. One possible approach to define other molecules regulated by IL-12 would be to use peptides or GST-fusion proteins containing the IL-12Rβ2 cytoplasmic tyrosine residues (IL-12Rβ2Tyr678 and IL-12Rβ2Tyr767). These tyrosine residues are supposed to be phosphorylated following IL-12 stimulation. However, this has not been demonstrated. Therefore, the phosphorylation of these residues would have to be defined. One possible strategy would be to raise antibodies against the phosphorylated and non-phosphorylated forms of these tyrosine residues as tools to define which tyrosine residues within the IL-12Rβ2 are phosphorylated in IL-12 activated T cells. Once a key residue is identified then peptides with the sequence containing these residues both non-phosphorylated and tyrosine phosphorylated could be synthesised. These peptides could be used as affinity matrices to precipitate interacting molecules. Alternatively, GST-fusion proteins of the cytoplasmic tail of the IL-12Rβ2 could also been employed. As proposed for the STAT4 interacting proteins, mass spectrometry analysis would have to be used.

In summary, the data presented in this study has helped increasing our current knowledge of the characteristics of the IL-12/ STAT4 response and has also provided with important differences between the way in which STAT4 is regulated in response to IL-12 and to IFN-α. Many details from the IL-12/ STAT4 response are still missing and are required for the proposal of adequate strategies to target STAT4 as a means to control deregulated Th1 cell responses. Detailed studies on the function of STAT4 serine phosphorylation and STAT4 inactivation are required. Unravelling the mechanisms involved in the regulation of IL-12R expression is another future challenge. The finding of novel signalling pathways involved in IL-12 responses are also necessary to fully comprehend the way in

which this cytokine is able to trigger the complex process of Th1 cell differentiation.

CHAPTER 8

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