Impaired IL-7 / IL-7Rα signaling in HIV infection: Role of the transcriptional repressor GFI1 in suppressing IL-7Rα expression and driving the proliferation of human CD8 T lymphocytes

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ABSTRACT

Cytotoxic CD8 T lymphocytes kill virus-infected cells and are critical for viral clearance from the body. Cytokines, particularly those sharing the common γ receptor chain (γc), play a key role in this cytotoxic function as well as in the growth, differentiation and homeostasis of CD8 T lymphocytes. In order to exert these biological effects, cytokine-dependent signal transduction via the Janus kinase (Jak) / Signal Transducers and Activators of Transcription (STAT) pathway, the phosphoinositide 3-kinase (PI3-K) and mitogen-activated protein kinase (MAPK) pathways is required. In HIV infection however, the CD8 T lymphocytes become defective and are characterized by impaired cytotoxicity, altered differentiation patterns, and increased susceptibility to apoptosis. I hypothesized that impaired cytokine responsiveness resulting from defects in cytokine-dependent signal transduction contributes to the CD8 T cell impairment observed in HIV+ patients. I investigated the activation of the Jak/STAT signaling pathway to cytokines in CD8 T cells from HIV+ patients. Interestingly, these cells were responsive to IL-2, IL-4, IL-10, IL-15, and IL-21 at the level of their respective STAT activation. However, impairment of the IL-7 / IL-7Rα signaling axis was identified and characterized by a defect in STAT5 signaling. The impaired STAT5 activation correlated with a low IL-7Rα surface expression. The expanded population of IL-7Rα^{low}-expressing CD8 T cells, found particularly in viremic HIV+ patients, expressed higher levels of the transcriptional repressor Growth Factor Independent-1 (GFI1) compared to their IL-7Rα^{high} counterparts. This prompted further investigations into the role of GFI1 in IL-7Rα regulation in primary human CD8 T cells as a model. Though silencing of GFI1 did not modulate basal IL-7Rα expression, exogenous overexpression negatively regulated IL-7Rα surface levels. The γc cytokines, IL-2, IL-4, IL-7, and IL-15, but not IL-21, were found to efficiently suppress IL-7Rα expression however, only IL-4 simultaneously upregulated GFI1 expression. RNA interference studies targeting GFI1 in IL-4 stimulated CD8 T cells established a specific role for GFI1 in sustaining the suppression of IL-7Rα expression. Furthermore, transient downregulation of GFI1 in CD8 T cells subjected to IL-4-dependent proliferation reduced their proliferative capacity. Other functions identified for GFI1 were in the suppression of CXCR4 and Bax expression in CD8 T cells. Studies aimed at identifying the signal transduction pathways responsible for regulating GFI1 and IL-7Rα expression revealed that IL-4-mediated downregulation of IL-7Rα expression required activation of the Jak/STAT and the PI3K pathways. On the other hand, IL-4-induced upregulation of GFI1 expression was mediated via the PI3K pathway. The JNK and P38 MAPK pathways appeared to be important as regulators of basal IL-7Rα expression levels, but had no statistically significant effects on GFI1 expression. To conclude, these studies have clarified the important biological effects of GFI1 in mature human CD8 T lymphocytes. Furthermore, exposure to IL-4 may generate CD8 T cell populations with an exhausted phenotype similar to those found in chronically-infected HIV+ patients, characterized by reduced cytotoxic activity and increased IL-4 production. Thus, the IL-4 study model may prove valuable for investigating the activity of human CD8 T cells in such chronic diseases and those characterized by a type 2 cytokine profile.

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AICD	Activation induced cell death
AIDS	Acquired immune deficiency syndrome
APC	Antigen-presenting cell
ART	Antiretroviral therapy
ARV	Antiretroviral
BSA	Bovine serum albumine
Cdc	Cell division cycle
Cdk	Cyclin-dependent kinase
CFSE	Carboxyfluorescein succinimidyl ester
CTL	Cytotoxic T lymphocyte
ERK	Extracellular signal-regulated kinases
ETO	Eight Twenty-One
ETS	E Twenty-Six transformation-specific
FBS	Fetal bovine serum
GABPα	Guanine adenine binding protein alpha
G-CSF	Granulocyte colony-stimulating factor
GFI1	Growth factor independent-1
GFI1B	Growth factor independent-1B
GRE	Glucocorticoid response element
HAART	Highly active antiretroviral therapy
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus

HRP	Horse radish peroxidase
IFNγ	Interferon gamma
IL-7Rα	Interleukin-7 receptor alpha
IL-'X'	Interleukin-'X'
IMDM	Iscove modified Dulbecco's medium
ISRE	Interferon-stimulated response element
Jak	Janus kinase
LB	Luria broth
LCMV	Lymphocytic choriomeningitis virus
LNT	Lymph node T cell
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MTG8 / MTG16	Myeloid translocation gene on chromosome 8 or 16
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-1	Programmed death-1
PDK1	Phosphoinositide-dependent protein kinase 1
PFA	Paraformaldehyde
PHA-M	Phytohemagglutinin – mucoprotein form
Phospho- or p-	Phosphorylated

PI	Phosphatidyl-inositide or phosphatidylinositol
PI	Propidium iodide
PIP	Phosphatidyl-inositide or phosphatidylinositol phosphate
PI3K	Phosphoinositide 3-kinase
PMSF	Phenylmethanesulfonylfluoride
PVDF	Polyvinylidene fluoride
RTI	Reverse transcriptase inhibitor
RIPA	Radioimmunoprecipitation assay
SD	Standard Deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser-	Serine
siRNA	Small-interfering ribonucleic acid
STAT	Signal transducers and activators of transcription
Tat	Trans-activator of transcription
TBST	Tris-buffered saline Tween-20
TCE	T cell clonal expansion
TCR	T cell receptor
Th1	Type 1 helper
Th2	Type 2 helper
Thr-	Threonine
TNFα	Tumor necrosis factor alpha
Tyr-	Tyrosine
UPS	Ubiquitin proteasome system

CHAPTER 1
Background

Innate and Adaptive Immunity

The body's first response against infections is to activate the innate arm of the immune system composed of the physical and chemical barriers of the body's epithelia and cells and molecules critical in preventing and containing infections when the epithelial barrier is breached [1-3]. This rapid response is characterized by pathogen pattern recognition receptors and proteins of the complement system which enhance phagocytosis, lysis, and chemotaxis [4-9]. Dendritic cells, macrophages, and granulocytes respond to foreign pathogens by phagocytosis, receptor recognition, and cytokine and chemokine secretions [10-14]. Cytokines and chemokines are cellular proteins and growth factors, which affect the behavior of cells by binding to their cognate receptors or act as chemoattractants recruiting cells to sites of infection and inflammation, respectively. Some of these cytokines include tumor necrosis factor α (TNFα) and the chemokine IL-8 [15,16]. IL-8 recruit neutrophils and TNFα contain local infections by mediating its signaling through the TNF receptor 1 which contain the death domain to induce apoptotic signals [13,16,17]. Interferons -α and -β (IFNα/β) induced by viral infections activate NK cells and increase the expression of major histocompatibility complex (MHC) class I molecules on infected cells in order to facilitate their killing [18-24]. Activated NK cells secrete IFNγ to inhibit viral replication and release lytic granules such as granzymes and perforin for the lysis of virus-infected cells [19-22,25,26].

The adaptive immune response on the other hand establish immunological memory and long-term protection through the production of antigen-specific antibodies and the development of memory CD4 and CD8 T cells as well as their cytokine secretion pattern [27-30]. Naïve CD4 T cells recognize antigens presented by the MHC class II

molecules of antigen-presenting cells (APCs) such as dendritic cells and macrophages, whereas CD8 T cells recognized viral peptides displayed by MHC class I molecules [31-34]. The activated, naïve T cells differentiate into armed effector cells to clear virus infections and upon viral clearance such cells are eliminated by activation-induced cell death (AICD) for example. AICD is a form of programmed cell death triggered by Fas-FasL cell surface protein interactions and plays a part in normal lymphocyte development and function [35]. Through incompletely understood mechanisms, responding lymphocytes are able to differentiate into long-lived memory cells, which can mobilize quickly upon re-exposure to foreign antigens [30,36-38]. Classically, CD4 T cells have been subdivided into type 1 helper T (Th1) cells and type 2 helper T (Th2) cells [39-41]. Th1 cells secrete TNF, IFNγ and IL-2, which are involved in activating macrophages, and mobilizing CD4 or CD8 T cell cytotoxicity. Th2 cells secrete IL-4, IL-6, and IL-10 for example, and activate B cells to produce antigen-specific antibodies [38,39,41-44]. A more thorough description of the killing mechanisms mediated by cytotoxic CD8 T cells will be described in the sections to follow as well as the role of some of the cytokines mentioned above, which are particularly relevant to this thesis.

CD8 T Lymphocytes and Virus Infections

CD8 T lymphocyte differentiation

It has been suggested that CD8 T lymphocytes undergo linear differentiation to become effector T cells and subsequently antigen-specific memory CD8 T cells (Fig. 1.1) [45-49]. More specifically, in the thymus, cells mature primarily through signaling by the T cell receptor (TCR) complex and the activity of IL-7 signaling via the IL-7Rα / gamma

c (γc) chain complex [30,50]. The γc chain is shared among other receptors and the cytokines binding these receptors are described as γc cytokines, which will be described in greater detail below, as will the IL-7R complex. Following maturation in the thymus, naïve CD8 T cells are released into the periphery and IL-7 signaling continues to be important in their homeostatic maintenance [30,50,51]. The role of IL-15 in ontogeny and the maintenance of peripheral naïve CD8 T cells has also been suggested although to a lesser extent than that of IL-7 signaling [30]. Naïve CD8 T cells become activated by APCs which present to the TCR of the CD8 T cell processed viral peptide epitopes bound to MHC class I molecules [29,30,34,38,52,53]. In addition, naïve cells are activated via interaction of their cell-surface co-stimulatory molecules (e.g. CD28) with their respective ligands (e.g. CD80/CD86) on APCs [29,34,52,53]. Activation and differentiation of the CD8 T cell may also require help from CD4 T cells to further increase the expression of co-stimulatory molecules and for their production of cytokines such as IL-2 [29,32,33,36,54]. The activated CD8 T cells proliferate in the presence of IL-2, IL-15, and IL-21 into a pool of antigen-specific CD8 T cells that differentiate into effector cytotoxic CD8 T cells capable of lysing virus-infected cells [30-33,54]. Following massive expansion of the effector CD8 T cells that serves to clear the infection, contraction of a large proportion of the effector CD8 T cells occurs except for a small fraction that remains responsive to IL-7 and IL-15 [30,51,52]. These cells are proposed to be the precursor population to the long-lived memory CD8 T cells whose homeostatic maintenance are primarily dependent on IL-15 and to a lesser extent, IL-7 [30,36,52,55]. Here, CD4 T cells are proposed to maintain the expression of the cognate

Figure 1.1 Regulation of CD8 T lymphocyte differentiation by γc cytokines.

Following the maturation of thymocytes, naïve CD8 T cells leave the thymus and are maintained primarily by IL-7, and secondarily by IL-15. The naïve cells circulate into the periphery migrating in and out of secondary lymphoid organs patrolling the lymph nodes, the spleen, and the mucosal lymphoid tissues for foreign pathogens. Antigen-presenting cells present viral peptides via the MHC class I molecules to the CD3 / TCR of naïve CD8 T cells. The interactions between co-stimulatory molecules (CD28, CD80/CD86) are also important in determining the strength of the response of naïve T cells. Activated naïve CD8 T cells undergo clonal expansion and differentiate into antigen-specific effector CD8 T cells. At this stage, the CD8 T cells gain cytototoxic activity, IL-7Rα expression becomes suppressed and their maintenance and function is dependent on IL-2, IL-15, and IL-21. Upon viral clearance, a subset of effector cells expressing high levels of IL-7Rα, which depend primarily on IL-7 for maintenance escape cell death and form the long-lived memory T cell populations. Memory cells expressing high IL-7Rα and IL-15R are largely dependent on the presence of IL-15 for their survival. Upon re-emergence of the virus, the memory CD8 T cells can respond quickly to activate the T cell-mediated cytotoxic immune response.

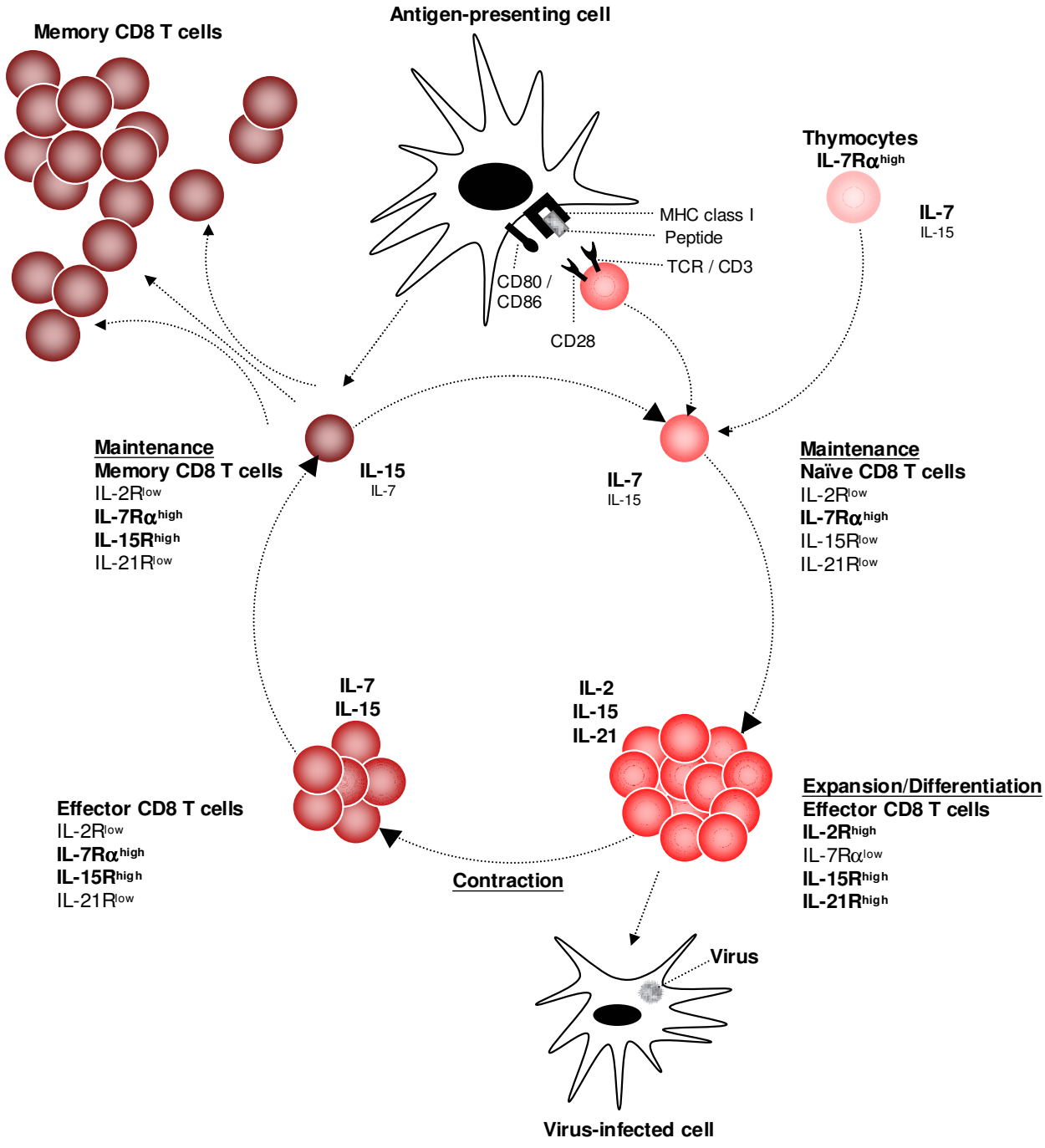

Memory CD8 T cells
Antigen-presenting cell
Thymocytes
IL-7Rα high
IL-7
IL-15
MHC class I
Peptide
TCR / CD3
CD80 /
CD86
CD28
Maintenance
Memory CD8 T cells
IL-2Rlow
IL-7Rα high
IL-15Rhigh
IL-21Rlow
IL-15
IL-7
IL-7
IL-15
Maintenance
Naïve CD8 T cells
IL-2Rlow
IL-7Rα high
IL-15Rlow
IL-21Rlow
IL-7
IL-15
IL-2
IL-15
IL-21
Effector CD8 T cells
IL-2Rlow
IL-7Rα high
IL-15Rhigh
IL-21Rlow
Contraction
Expansion/Differentiation
Effector CD8 T cells
IL-2Rhigh
IL-7Rαlow
IL-15Rhigh
IL-21Rhigh
Virus
Virus-infected cell

FIGURE 1.1

receptors for IL-7 and IL-15 on long-lived memory CD8 T cells for homeostatic maintenance and survival [36]. Memory CD8 T cells are able to respond quickly to generate a rapid cytolytic response limiting the effects of the cytopathic effects of renewed infection with a viral pathogen (Fig. 1.1) [37,56].

Role of CD8 T lymphocytes in controlling viral infections

Cytotoxic CD8 T cells are of particular importance in the clearance of virus-infected target cells [31-33]. Virus-infected cells are recognized by cytotoxic CD8 T cells by the viral peptides displayed in conjunction with MHC class I molecules on their surface [31-34]. Target cells are killed through cytolysis by pore-forming proteins, granulysin and perforin, and entry of serine proteases including granzymes into target cells released from the cytolytic granules of cytotoxic CD8 T cells [57-59]. More specifically, apoptosis occurs via caspase-dependent and -independent mechanisms mediated by granzymes or granule-bound serine proteases [57-59]. Other mechanisms by which cytotoxic CD8 T cells control virus infections include the release of cytokines such as IFNγ, TNFα and TNFβ that serve to prevent successful infection of target cells [60]. IFNγ interferes with viral replication, increases MHC class I expression, and activates macrophages [21,22,24,38,39,41,44]. TNFα/β synergizes with IFNγ to activate macrophages and targets virus-infected cells expressing TNF receptor I [20-24]. The adaptive immune response may appear to be a simplistic stepwise mechanism capable of resolving most acute infections, but fails to contain other viruses, which as a result can develop into chronic infections which such is the case for the human immunodeficiency virus (HIV). The actual complex nature of the immune system is exploited by HIV.

Human Immunodefiency Virus (HIV)

HIV is one of the most devastating viruses currently disseminated worldwide with an estimated 31.1-35.8 million people infected at the end of 2008 [61]. It is transmitted within bodily fluids such as blood, vaginal and seminal fluids, and breast milk [62]. Upon transmission, HIV infects a variety of immune cells, particularly CD4 T cells, but also macrophages, dendritic cells, stem cells, and even epithelial cells, for example [62-64]. In contrast to resting cells, HIV replicates upon cell activation effectively disseminating the virus. Infected resting cells on the other hand form viral reservoirs enabling HIV to persist in the body indefinitely [65-67]. Although treatments exist to contain the virus, there is no effective vaccine to prevent infection. The immune system is in a constant state of activation, trying unsuccessfully in most patients to eliminate the virus from the body. It eventually becomes exhausted, fails to contain HIV, and becomes susceptible to opportunistic infections that characterize acquired immune deficiency syndrome (AIDS).

HIV life cycle

Entry of HIV into the cell begins by interaction of the viral glycoprotein, gp160, with cell surface receptors CD4 and a chemokine co-receptor, CXCR4 or CCR5, in particular, but is not limited to these co-receptors [62,65,66]. The viral glycoprotein gp160 is composed of gp120 and a buried component, gp41, such that interaction of gp120 with CD4 leads to a conformational change exposing gp41 and an area of gp120 free to interact with the co-receptors [68-71]. Fusion of the viral and host membrane mediated by gp41 causes the release of the viral capsid containing two copies of positive single-stranded RNA into the cytoplasm along with integrase, reverse transcriptase, and proteases thus beginning the infectious cycle [65,66,68-71]. Reverse transcriptase

generates, from the viral RNA, a double-stranded DNA sequence which translocates into the nucleus to integrate into the host genome using integrase [72-74]. Here, the integrated DNA can lie dormant in the resting cell representing the latent phase of HIV infection or alternatively, utilize the host replication system to replicate proviral DNA upon activation of the infected cell [65,66]. The proviral DNA is transcribed into mRNA where unspliced and spliced mRNA migrate to the cytoplasm for translation into viral proteins, and during maturation, HIV proteases cleave the polyproteins into individual functional HIV proteins and enzymes [66,67]. The viral RNA genome consist of structural (e.g. *gag*, *pol*, *env*) and regulatory genes (e.g. *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*) which encode for proteins involved in producing new virus particles, infecting cells or causing cell death [66,67]. The various structural components assemble and bud out at the host cell membrane producing mature and infectious virions [65-67].

Stages of HIV disease

Primary HIV infection is characterized as the stage of disease prior to the detection of HIV antibodies with high viral RNA levels which can typically last weeks to several months [75,76]. During this time, CD4 T cell numbers decline in the peripheral blood, but experience an even more drastic reduction in the mucosa [75,76]. Cytotoxic CD8 T cells critical in viral clearance are induced and peak in numbers to kill-virus infected cells, which is then accompanied by a rebound in CD4 T cell counts [76,77]. However, the virus is not completely cleared and can remain latent in reservoir cells [63,78]. It is noteworthy to mention that slower disease progression is accompanied by a sustained cytotoxic CD8 T cell response throughout the course of infection[79,80].

In the chronic phase of the disease, the immune system becomes dysregulated as a result of chronic immune activation and the progressive loss of CD4 T cells [81-83].

Chronic immune activation results from the combination of a number of specific events, which include viral persistence and chronic presentation of constantly evolving viral antigens to T cells [84]. Seroconversion occurs, but the effectiveness of HIV-specific antibodies are also limited by a continuously evolving virus and due to its glycosylated antigenic sites [77,85]. As a result only a small fraction of HIV-specific antibodies are neutralizing, and therefore provide little to no protection against the virus. Similarly, HIV-specific T cell responses generated towards the virus largely represent effector cells instead of memory cells [86]. Furthermore, despite steady CD8 T cell numbers, CD4 and CD8 T cell-mediated cytotoxicity becomes ineffective [84], and will be described in greater detail in sections to follow.

During the chronic stage of the disease, the infected individual can remain largely asymptomatic after seroconversion from several years to decades [75,76]. However, the rate of disease progression varies between individuals, and the CD4 T cell count and plasma viral load are the guiding markers for beginning treatment with highly active antiretroviral therapy (HAART) which usually consist of a combination of three or four antiretroviral (ARV) drugs [87,88]. ARVs consist of different classes of drugs including nucleoside and non-nucleoside reverse transcriptase inhibitors, as well as protease, integrase, maturation, and entry inhibitors. The initiation of HAART delays the onset of AIDS, inhibits viral replication, and is accompanied by a steady increase in CD4 T cell numbers, the emergence of polyfunctional HIV-specific CD8 T cells as well as partial restoration of immune functions [89-91]. Nevertheless, despite sustained and effective HAART, the virus still develops resistance to the drugs and the impact of chronic immune activation is not entirely alleviated, thus contributing to the onset of AIDS.

Impaired CD8 T cell functions in HIV infection

The inability of the immune system to overcome HIV infection has been widely reported [77,81,84,86,89,92-94]. Impairement of the cytotoxic CD8 T cell response, decreased CD4 T cell count, and increased virus load aid in the progression to AIDS, which is characterized by an increased occurrence of malignancies and opportunistic infections [83]. More specifically, the ineffectiveness of both the innate and adaptive immune responses are involved in the ongoing immune activation and eventual immune deficiency [95]. HIV exerts devastating effects on CD4 T cell numbers and establishes virus reservoirs within macrophages and stem cells for example, whereas CD8 T cells are not similarly targetted [63,64].

Of particular interest to this study are the CD8 T lymphocytes, which have proven critical in controlling HIV replication [37,89-91,96]. The regulation of CD8 T cells becomes altered throughout HIV disease progression as indicated by their expression of cell surface activation and differentiation markers [86,97,98]. HIV-specific CD8 T cells are most often characterized by a $CD45RA^{-}CCR7^{+}CD27^{+}$ and $CD28^{-}$ phenotype, which is in contrast to that of antigen-specific effector CD8 T cells that effectively clear viruses, characterized as $CD45RA^{-}CCR7^{+}CD27^{+}$ and $CD28^{+}$ [31,99,100]. In addition, HIV-infected patients exhibit dysfunctional IL-7Rα expression in both CD4 and CD8 T lymphocytes [89,96,101,102], which is believed to contribute to reduced CD4 count as well as the altered functionality of CD8 T cells. HIV-infected individuals experience an expansion of effector-like memory cells and memory CD8 T cells which persist. These memory cells possess low IL-7Rα expression similar to effector CD8 T cells [102]. During chronic HIV infection, the transition from effector to memory CD8 T cells

appears to be halted mid-transition [103]. Interestingly, activated effector-like IL-7Rα^{low} CD8 T cells in HIV infection are prone to spontaneous and activation-induced apoptosis [86]. Increased plasma IL-7 levels positively correlating with increased HIV disease severity has also been implicated as a contributing factor to apoptosis [104,105]. In this case, chronic IL-7 production and increased exposure to HIV proteins enhanced Fas expression on naïve and memory CD8 T cells *in vitro*, promoting Fas-induced apoptosis of naïve T cells and to a greater extent memory cells [106,107]. It is important to note that *ex vivo* studies implicated the involvement of IL-7 in reducing apoptosis of circulating CD8 T cells with functional IL-7 / IL-7Rα signaling during the early stages of HIV disease [108]. Although the number of cytotoxic CD8 T cells was diminished by Fas-mediated apoptosis, the surviving CD8 T cells in circulation exhibited poor cytolytic function [78,86,92,108]. HIV-specific CD8 T cells have poor *ex vivo* killing activity characterized by low perforin expression [90,92,109,110]. Migueles et al. showed reduced perforin expression and proliferative capacity in CD8 T cells of patients lacking immunological control of the HIV virus [108]. In contrast, polyfunctional HIV-specific CD8 T cells were described in long-term non-progressors and individuals with successful suppression of HIV replication by antiretroviral therapy (ART) [90,108].

In HIV infection, an altered cytokine secretion pattern has been observed switching from a predominantly type 1 cytokine profile to a type 2 cytokine environment [92,97,105,111,112]. For example, increased production of the type 2 cytokines, IL-4 and IL-10, was observed along with increased IL-7 levels, whereas reduced production of IL-2, IFNγ, and TNFα have been measured [92,97,105,111-115]. The functional roles for some of these cytokines will be described more extensively in later sections. Since IL-7

is important in the homeostasis of the T cell compartment, its increased production following HIV infection has been proposed to accelerate thymic output to counter the CD4 T cell loss [105,116,117]. CD8 T cells not only succumbed to cell death through IL-7-induced Fas-mediated apoptosis, but also suffered a loss of their cytolytic activity as well as a decline in CD8 expression and increased IL-4 production [112,118-120]. The involvement of IL-4 in promoting the development of non-cytolytic CD8 T cells with low CD8 levels as well as contributing to reduced perforin and granzyme expression has also been established [121-123]. Low cytolytic activity by CD8 T cells was also reflected in their reduced production of IFNγ and TNFα [90,113,124]. Bulk and HIV-specific CD8 T cells from viremic patients produced significantly lower levels of IFNγ, and TNFα in comparison to CMV-specific stimulation [90,109,125-127]. Following ART-induced suppression of HIV replication cytokine production could be restored. Impaired IL-2 production by bulk and HIV-specific CD8 T cells was also detected, but its primary role to stimulate the proliferation of activated cells was similarly restored following suppression of HIV replication by ART [90]. Finally, increased production of IL-10 in HIV infection was observed and may also contribute to the altered cytokine profile observed since a major role for IL-10 is to suppress the synthesis of Th1 cytokines such as IL-2 and IFNγ [128].

Signal Transduction

CD8 T lymphocyte impairment in HIV infection may be reflected in their ability to respond weakly or not at all to the survival and differentiation signals which may have dire consequences, leading to cell dysfunction and ultimately cell death. The γc cytokines

play a critical role in the proper maintenance and function of CD8 T lymphocytes by activating signal transduction pathways that regulate their survival, proliferation, and differentiation [30,52,129,130]. Further description of the functional roles of the γc cytokines: IL-2, IL-4, IL-7, IL-15, and IL-21 and the main signal transduction pathways they induce are provided in the sections below.

Janus kinase (Jak) / Signal Transducers and Activators of Transcription (STAT) pathway

Interleukins bind to their specific receptor to activate Jak tyrosine (Tyr) kinases, thus initiating a cascade of intracellular signaling events [131-133]. The increased kinase activity allows for the phosphorylation of tyrosine residues on the receptor creating sites for interaction with STAT proteins through their phosphotyrosine-binding SH2 domain. STAT proteins are recruited to the phosphorylated receptors and are themselves phosphorylated. These phosphotyrosines act as docking sites for interaction with other STAT SH2 domains enabling STAT dimerization. Phosphorylated STAT dimers translocate to the nucleus to act on a specific promoter sequence. In mammals, there are seven STAT genes, each binding to gene promoter sequences that may vary from a consensus sequence by individual base pairs or spacing between the half-sites [131-133]. STAT specificity also arises from the interaction between STAT SH2 domains and receptor phosphotyrosine motifs as well as through the potential induction of multiple STAT molecules [131]. The γc cytokines IL-2, IL-7, and IL-15 largely activate STAT5, whereas IL-4 induces phosphorylation of STAT6 [134]. Activation of the Jak/STAT pathways leads to many of the downstream events elicited by γc cytokines. STAT5a and STAT5b are essential for the expression of genes that promote hematopoietic survival

[135-137], whereas induction of STAT6 by IL-4 is important in gene regulation, cell growth, and differentiation depending on the cell type [138].

Phosphoinositide 3- Kinase (PI3K) pathway

Growth and survival factors including cytokines bind receptor tyrosine kinases or G protein-coupled receptors to activate the pathway. PI3Ks constitute four different families of enzymes which contain serine/threonine (Ser/Thr) kinase activity, however, three families constitute lipid kinases and the fourth consists of Ser/Thr protein kinases [139,140]. Class I PI3Ks phosphorylate phosphatidyl-inositide lipids (PI) to produce lipid second messengers (PIPs) which are recruited to various cellular membranes [141]. A specific downstream effect is for example the rising levels of PIP_3 which leads to the activation of phosphoinositide-dependent protein kinase 1 (PDK1) and phosphorylation of the most well known member of the PI3K pathway, AKT [141-143]. They have a specific role in mitogenesis, apoptosis, vesicular trafficking, and cytoskeleton rearrangement [108,142,144,145]. Class II and III contain enzymes that are able to phosphorylate PIs and Class IV contains PI3K-related enzymes involved in signal transduction and DNA damage response [145,146]. Other cellular functions mediated by PI3Ks include cell growth, proliferation, differentiation, motility, survival, and intracellular trafficking [147].

Mitogen-Activated Protein Kinase (MAPK) pathway

External stimuli are interpreted by activating members of the MAPK pathway called the extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases [JNKs / SAPKs (stress-activated protein kinase)], and P38 [148,149]. The ERK cascasde transmits mostly mitogenic signals from stimuli such as growth factors,

neurotransmitters, lipopolysaccharides (LPS), and cytokines [150,151]. Growth factors and pro-inflammatory cytokines as well as ultraviolet, LPS, and environmental stressors are involved in activating the P38 and JNK cascades, conveying mostly stress signals whereas ERK5 transmits both mitogenic and stress signals [151-156]. The functional responses to these stimuli are mediated through their interaction with multiple receptors such as chemoattractant receptors, Toll-like receptors, G-protein coupled receptors, receptor tyrosine kinases, and cytokine receptors [157]. Activation of the MAPK pathway induces a three-tiered cascade beginning with the MAP4K to MAP2K levels which transmit signals to the MAPK level; ERK, JNK1/2, and/or P38 proteins. The MAPKs become phosphorylated at their threonine and tyrosine residues then phosphorylate target proteins such as transcription factors or activate subordinate MAPK-activated protein kinases which then enter the nucleus to transactivate genes [151,157]. Activation of ERK, JNK, and/or P38 represents a source of functional diversity for the MAPK pathway in many fundamental cellular processes such as proliferation, survival, differentiation, apoptosis, motility, and metabolism. More specifically, P38 activation is involved in regulating cytokine expression, and similar to ERK phosphorylation cell survival and proliferation in CD8 and CD4 T cells as well as induction of transcriptional factors involved in controlling the cell cycle [148-150,157-159]. Finally, activation of JNK1 appears to be involved in the negative regulation of Th2 development and JNK2 has a role in IFNγ production and Th1 differentiation whereas a balance between JNK1 and JNK2 expression is involved in CD8 T cell activation [159,160].

Cytokines

The γc cytokines described below play a key role in the growth, differentiation, and maintenance of lymphocytes. As mentioned above particular attention will be dedicated to IL-10 and the γc cytokines: IL-2, IL-4, IL-7, IL-15, and IL-21. Although a fundamental role for these cytokines has been dedicated to lymphocyte function they also have critical functional roles in cells of the myeloid lineage.

IL-2

IL-2 is secreted by activated T lymphocytes and binds to a receptor complex comprised of three polypeptide subunits: IL-2Rα, which increases IL-2 binding affinity as well as IL-2Rβ, and the shared γc chain, which are required for signaling [161]. IL-2R is expressed at low levels on naïve and memory T cells and at high levels on effector T cells [130] marking the importance of IL-2 signaling in activated T cells. The functions of IL-2 / IL-2R signaling are mediated through activation of the Ras/MAPK, Jak/STAT5, and PI3K-AKT signaling pathways [162]. IL-2 signaling is involved in the production of immunoglobulins by B cells and inducing the differentiation and proliferation of natural killer (NK) cells [163,164]. More importantly, IL-2 plays a role in the proliferation of B and T lymphocytes. Interestingly, IL-2-IL-2Rα-deficient mice have normal T cell development, show an expansion of polyclonal T and B cells, but exhibit features associated with autoimmune diseases, and have defects in the regulatory T cell functions [30,130,165,166]. This defect leads to the accumulation of activated T cells emphasizing the importance of IL-2 in early activation for the transition of activated T cells into effective memory cells [167].

In *in vitro* and *in vivo* studies, IL-2 production was significantly decreased after HIV infection of peripheral blood mononuclear cells (PMBCs) and CD4 T cells [124,168]. The effects of HIV infection on IL-2 production was even more prominent with increased disease severity [86,124,169]. Furthermore, there exists contradictory findings with regards to the expression of IL-2R on CD8 T cells in HIV-infected individuals [97,170-172], as such a more comprehensive study was conducted by David et al. on chains of the IL-2R [173]. Increased surface expression of IL-2Rα, IL-2Rβ, and γc were observed on CD8 T cells of patients with high viral load treated with either one or two reverse transcriptase inhibitors (RTI) compared to CD8 T cells from HIV-negative controls [173]. However, in HAART-treated patients with successful viral suppression and increased CD4 T cell numbers, each chain of the IL-2R was decreased on CD8 T cells compared to patients receiving RTI only, and moreover the IL-2Rβ and γc chain were expressed at levels similar to those in HIV-negative donors [173]. Despite increased expression of IL-2R components in patients receiving RTIs compared to HAART-treated patients, CD8 T cells were less responsive to IL-2 as measured by their entry into the cell cycle S and G_2/M phase [173]. Indeed, defective IL-2 signaling of the Jak/STAT5 pathway was demonstrated in CD8 T cells of HIV-positive individuals naïve to ART with high viral load [98]. Interestingly, this dysregulation occurred downstream of IL-2R expression and involved the Jak3 kinase [98]. HIV infection appears to flip the "off" switch on the immune system at different cellular levels and kinetics. Importantly, HAART turns this switch back "on", at least partially, such as restoring Jak/STAT5 signaling in response to IL-2 [98].

IL-4

IL-4 is secreted by T cells and mast cells and binds to its heterodimeric receptor, the type I or type II IL-4R. However, hematopoietic cells such as T and B cells only express the type I IL-4R composed of the IL-4Rα and γc chain to induce the Jak/STAT6 pathway, the PI3K pathway, and the MAPK pathway [174,175]. The type II IL-4R expressed on the surface of myeloid cells such as monocytes, macrophages and fibroblasts, is composed of IL-4Rα and IL-13Rα, and both IL-4 and IL-13 can bind the receptor [138,174,176,177]. IL-4 is primarily recognized to have a role in B cell activation and promoting Th2 differentiation [178-180]. Although exposure to IL-4 during differentiation results in a type 2 polarization, the literature suggests a more complex and multifunctional role in CD8 T lymphocytes [121-123,181-184].

Increased, decreased, or unaltered effects of IL-4 have been reported on CTLs and their antigen/pathogen clearance capacity [121-123,182]. The main function of CD8 T cells is reputed to be the MHC class I restricted lysis of infected cells and the production of IFNγ and TNF [185]. However, findings in the last decade have demonstrated that short-term IL-4 exposure following TCR stimulation of naïve CD8 T cells generated functional long term memory CD8 T cells producing both IFNγ and IL-4 [182]. Furthermore, activation of naïve CD8 T cells in type 2 polarizing conditions (IL-2, IL-4, and anti-IFNγ antibody) led to the production of type 2 polarized CD8 T cell clones [123]. The CD8 T cell clones produced IFNγ and IL-4 and displayed variable levels of CD8 expression and cytolytic activity [121-123]. Low CD8 expression was generally associated with moderate levels of IFNγ production, high levels of IL-4 production, reduced cytolytic activity and perforin and granzyme expression [121-123]. The

described signature of type 2 polarized CD8 T cells producing IL-4 has been identified in CD8 T cells of HIV-infected persons [114,119,120,186]. In fact *in vivo*, elevated IL-4 levels were observed following HIV infection at various disease stages and initiation of HAART were shown to reduce IL-4 levels [114,119,120,186]. Conversely, *in vitro* studies demonstrated that in the supernatant of cultured PBMCs and CD4 T cells from HIV-infected individuals reduced or unaltered concentrations of IL-4 were reported [187,188]. *In vitro*, the effects of elevated IL-4 levels were shown to increase IL-4Rα levels [189]. Similarly, elevated IL-4 levels in HIV-infected individuals were shown to increase IL-4Rα expression as were HIV proteins [190].

IL-10

IL-10 was first described as a cytokine produced by Th2 murine cells with the ability to inhibit the activation and cytokine production of Th1 cells [191]. However, it is now known that IL-10 is a potent anti-inflammatory cytokine produced by T and B cells, monocytes, macrophages, and dendritic cells which binds the IL-10Rα and IL-10Rβ complex [128,192,193]. IL-10Rα is expressed at low levels on most hematopoietic cells, but is generally induced at higher levels upon activation except in the case of T cells [128]. Interestingly, IL-10Rα is expressed on the surface of activated CD8 T cells. In contrast, IL-10Rβ is constitutively expressed on most cells and tissues [128]. IL-10 activates the Jak/STAT signaling pathway to mediate numerous functions. The key mediator of IL-10 induced functions is STAT3 [128]. IL-10 could also induce STAT5 and STAT1 activation however their roles were unclear, as the dominant negative expression of the two did not negatively affect IL-10 responsiveness of monocytes, macrophages, T cells, and B cells [194].

IL-10 is a potent suppressor of pro-inflammatory cytokine production by macrophage and dendritic cells as well as suppressor of monocyte function by inhibiting CD80 and CD86 expression and the expression of MHC class II antigens [195,196]. In contrast, IL-10 has a stimulatory role on B cells by increasing MHC class II antigens and promoting their differentiation, survival, and proliferation [197]. On the other hand, IL-10 inhibited the growth and proliferative capacity of CD4 T cells and their capacity to produce IL-2. However, positive and negative roles for IL-10 on the cytotoxicity and proliferative capacity of CD8 T cells have been demonstrated [198-201]. IL-10 has been described to enhance IL-2/IL-4-mediated proliferation and cytotoxicity of CD8 T cells [202]. Other studies have reported that the cytolytic activity of activated CD8 T cells against bacterial pathogens is dimished in the presence of IL-10 [193], and the observations extend to the inhibitory effect of increased IL-10 production during chronic viral infections [203-205]. Finally, a study demonstrated that IL-10 enhanced CD8 T cell expansion during priming, but reduced proliferation upon re-exposure to the antigen [206]. The role of IL-10 in the immune system is further complicated following HIV infection. In *in vitro* and *in vivo* studies, the production of IL-10 was increased in PBMCs and monocytes following HIV infection [113,114,207-209].

IL-15

IL-15 is secreted primarily by dendritic cells and monocytes [165,210]. IL-15 has many similarities with IL-2 in part due its shared receptor components [165,211]. IL-15 binds IL-15Rα on activated monocytes and dendritic cells to present it *in trans* to NK cells and memory CD8 T cells expressing IL-2/IL-15Rβ, and γc [212,213]. Binding of IL-15 to its receptor induces activation of Jak kinases as well as the phosphorylation and

activation of STAT5. IL-15, like IL-2, is important in antibody production by B cells as well as in the proliferation of NK cells [163,164]. IL-15 is involved in the survival and expansion of naïve CD8 T cells, but it is its role in the generation of memory CD8 T cells that is quite vital [55,164,210]. It enhances the functional activity of CD8 T cells via induction of IFNγ and key cytolytic mediators such as perforin and granzyme B [214]. IL-15 serum levels were reportedly increased during acute HIV infection and were associated with high viral loads [215]. Similar findings were reported in HIV-infected patients at different stages of disease [216]. However, other reports suggest that despite high IL-15 mRNA levels in peripheral blood mononuclear cells (PBMCs), in HIV-infected patients at the onset of AIDS development, IL-15 serum levels were reduced and PBMCs were compromised in their ability to produce IL-15 in response to a viral stimulus, namely the human herpesvirus [217]. Interestingly, HIV-specific CTLs were responsive to IL-15 *ex vivo* and *in vitro*, where additional studies demonstrated that IL-15 exposure promoted cytotoxicity functions and prevented spontaneous and Fas-induced apoptosis [214,218]. White et al. showed that responsiveness of patient CTLs to IL-15 was even greater than CD8 T cells from uninfected controls [219]. Moreover, IL-15 stimulation led to the upregulation of IL-15Rβ in proliferating CTLs of HAART-treated HIV+ patients without CD4 recovery [220]. IL-15Rα was enhanced in PBMCs isolated from acute HIV-infected individuals compared to uninfected controls [219,221,222], whereas another study demonstrated that IL-15Rα was not upregulated in early or chronic HIV infection [223].

IL-21

IL-21 is secreted by natural killer T (NKT) cells, and the Th1, Th2, and Th17 subsets of CD4 T cells once activated. IL-21 binds to IL-21Rα and the partnered γc chain which are present on B cells, NK cells, dendritic cells, macrophages, and activated T lymphocytes, to name a few [224-227]. IL-21 / IL-21R signaling leads to the activation of the Jak/STAT pathway to weakly induce STAT1 and STAT5 activation and strongly induce STAT3 phosphorylation in CD8 T lymphocytes [224,228]. Activation of STAT1 is believed to be involved in promoting apoptosis whereas STAT3 activation may be implicated in oncogenesis [229,230]. The synergistic activity of IL-21 with IL-7 or IL-15 translated to increased activation of STAT5 in CD8 T cells [228]. Furthermore, IL-21 alone did not induce proliferation, but acting in synergy with IL-7 and IL-15 induced antigen-independent proliferation of naïve and memory CD8 T cells. In addition, IL-21 activation of the MAPK and the PI3K pathway were shown to be critical for IL-21-mediated proliferation [228]. Moreover, IL-21 stimulation increased survival and cytotoxic functions of CD8 T cells such as IFNγ production [231]. Until recently, the role of IL-21 in HIV was not well known. However, recent findings suggest that IL-21 production is drastically reduced in CD4 T cells and correlates with reduced CD4 T cell numbers and thus may be a biomarker for HIV disease progression [232,233].

IL-7

First discovered in 1988, IL-7 binds to the heterodimeric IL-7 receptor composed of an α and γc chain and is secreted by stromal cells in the thymus and lymphoid tissues [116,234]. Binding of IL-7 to its receptor leads to the activation of the Jak/STAT5, the PI3K-AKT, and the MAPK JNK and P38 pathways [235,236]. The effects of IL-7 / IL-

7Rα signaling are quite diverse. In the thymus, the early stem cells require IL-7 for their survival and proliferation, and rearrangement of TCR genes, whereas at the later stages of development IL-7 is important for the selection of CD8 T cells [235]. Following their exit from the thymus, IL-7 is involved in the maintenance and survival of naïve CD8 T cells that express high IL-7Rα in contrast to the expansion and differentiation phase, where the effector T cells express low IL-7Rα [130]. Following an acute infection, during the expansion phase, a subset of antigen-specific CD8 T cells with sustained expression of IL-7Rα represent IL-7 responsive T cells that will constitute the long lasting memory CD8 T cells [130,237]. Therefore in HIV infection, dysregulation of IL-7 levels may have a serious impact on these cells. During HIV infection, plasma IL-7 levels are increased and correlate with disease progression [105]. Furthermore, IL-7Rα expression levels are decreased on the surface of CD4 and CD8 T cells [82,89,96,238]. HAART improves overall immune function and has exerted a tremendous beneficial impact on the health and lifespan of infected individuals [89,96,238]. Importantly here, IL-7Rα downregulation is reversed under HAART, albeit in part [89,96,101,239].

Interleukin-7 Receptor

IL-7 is an essential γc cytokines for the development and maintenance of lymphocytes, playing a critical role in both T and B cells in mice and T cells in humans [235]. In order to mediate its biological functions, IL-7 binds a heterodimeric receptor called the interleukin -7 receptor (IL-7R), which is composed of two chains, an α subunit (IL-7Rα), and the γc chain [240-242]. IL-7Rα is expressed on bone-marrow derived macrophages as well as various cells of the lymphoid lineage such as precursor,

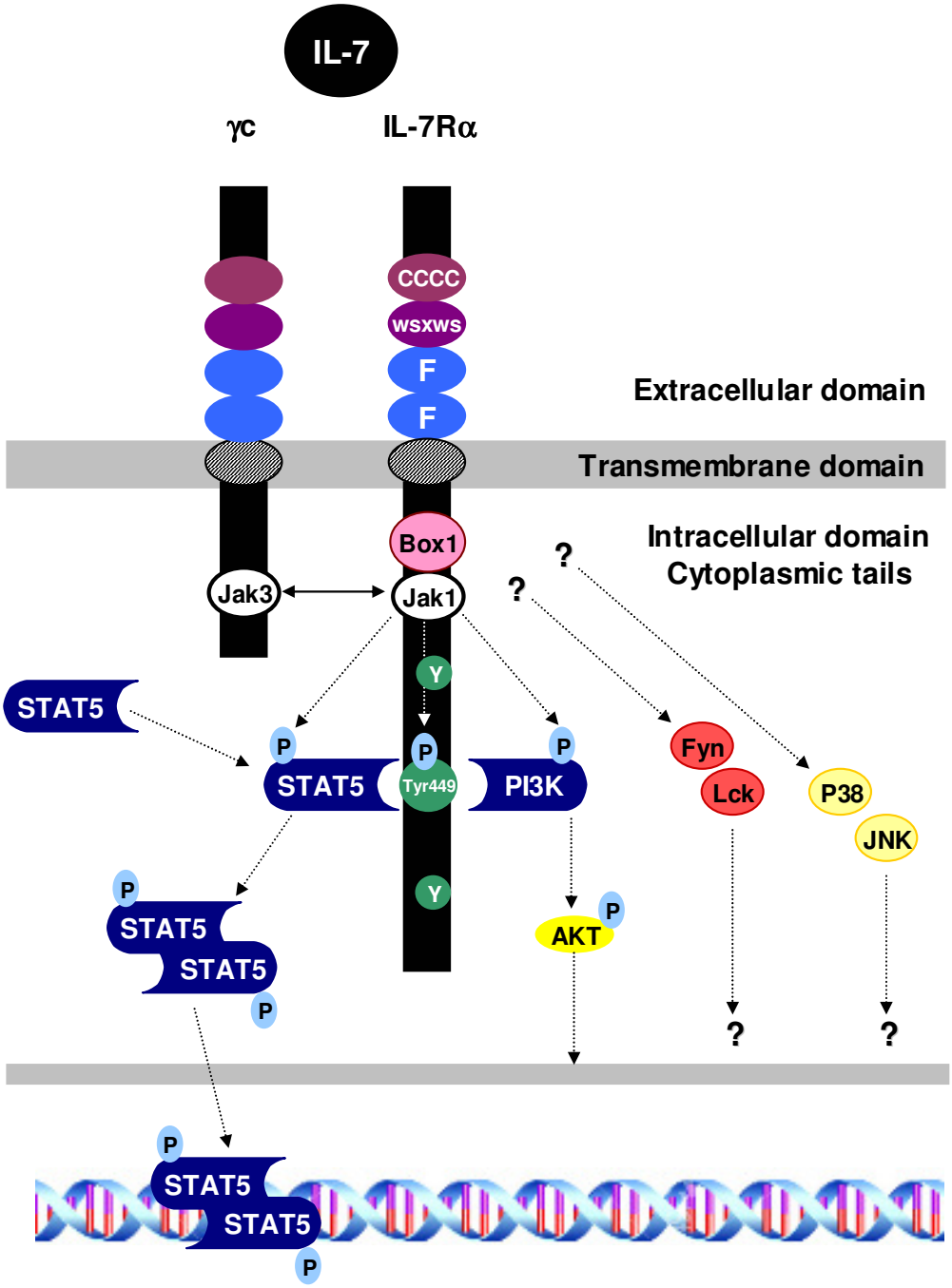
developing, and mature lymphocytes. In addition, IL-7Rα is expressed on the surface of healthy epithelial, endothelial, and stromal cells as well as cancer cells and cell lines [235,243-246].

IL-7Rα Genomic and Protein Expression

IL-7Rα is a protein of 49.5 kDa consisting of 439 amino acids and the gene is located on human chromosome 5 and murine chromosome 15 both genes spanning eight exons of a combined size of 19.79 kb and seven introns of 22.17 kb [247,248]. IL-7Rα is a member of the type I cytokine receptor family composed of an extracellular domain, a transmembrane region, and an intracellular domain (Fig. 1.2). The extracellular domain is composed of two fibronectin-like domains, four conserved cysteine residues, and a WS motif [116,235]. The IL-7Rα membrane glycoprotein has a 25 amino acid transmembrane domain and an intracellular domain of 195 amino acids called the cytoplasmic tail. The cytoplasmic tail possesses a region rich in acidic residues, a region rich in serine residues, and a region containing highly conserved tyrosine residues (Tyr401, Tyr449, Tyr456) as well as a small membrane-proximal domain of 8 amino acids termed "Box1" [116,249]. Box1 and the predominant regions of the cytoplasmic tail provide various binding opportunities for protein kinases and adaptors to activate multiple signaling pathways [116].

Figure 1.2 Schematic representation of the IL-7Rα protein and signaling pathways.

IL-7R is composed of an extracellular domain, a transmembrane and an intracellular domain. IL-7Rα is part of the type 1 cytokine receptor family and is composed of two fibronectin-like domains (F), a WS motif (wsxws), and four conserved cysteine residues (CCCC) in the extracellular domain. The intracellular domain contains a small membrane proximal domain called Box1 and a conserved tyrosine residues including Tyr449 within the cytoplasmic tail. IL-7 / IL-7Rα binding leads to phosphorylation of Jak1, which is dependent on Box1, and IL-7 signaling brings into proximity the γc chain resulting in mutual tyrosine (trans)phosphorylation of the Jak proteins. Phosphorylation of Jak1 leads to phosphorylation of Tyr449, which is critical for STAT5 phosphorylation. Tyr449 also serves as an activation initiation site for the PI3K pathway and JAK3 has also been shown to associate wth the p85 subunit. Activation of the Jak/STAT5 pathway plays a critical role in cell survival for example by increasing Bcl-2 production while the PI3K pathway has a role in proliferation. IL-7 signaling also induces activation of the Src family kinases ($p56^{lck}$ and $p59^{fyn}$) which bind IL-7Rα however the exact mechanism and their importance remains unclear, and the ensuing effects on mature T cells have not been well described (Fig. 1.2). Similarly, IL-7 signaling induces activation of MAPK signaling proteins: P38 and JNK which have a role in T cell proliferation, but the mechanisms for this have not been well elucidated.


IL-7
γc
IL-7Rα
CCCC
WSXWS
F
F
Extracellular domain
Transmembrane domain
Box1
Intracellular domain
Cytoplasmic tails
Jak3
Jak1
?
?
Y
STAT5
P
P
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STAT5
Tyr449
PI3K
Fyn
Lck
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FIGURE 1.2

Interleukin-7 Receptor α Signaling

The IL-7 / IL-7Rα signaling axis rapidly induces tyrosine phosphorylation of Jak1 and Jak3, and DNA binding activity of STAT5a/b (Fig. 1.2) [235,250]. Jak3 is pre-associated with the intracellular domain of the γc chain, whereas Jak1 is pre-associated with IL-7Rα. Upon IL-7 / IL-7Rα interaction, the γc chain is recruited, bringing into proximity their intracellular domains bearing Jak1 and Jak3. These two kinases have low intrinsic enzymatic activity which becomes increased after mutual (trans)phosphorylation. The Jak kinases phosphorylate the critical Tyr449 site on IL-7Rα which binds STAT5 as well as other signaling or adaptor molecules, which in turn become phosphorylated by Jak1 and/or Jak3. Following phosphorylation, STAT molecules dimerize, translocate to the nucleus, and trigger the transcriptional events of their target genes. Tyr449 also serves as an activation initiation site for the PI3K pathway by binding the p85 subunit and in T cells Jak3 associates with the p85 subunit [116,235,251-253]. Phosphorylation of Jak1 is dependent on the small membrane-proximal domain in the intracellular region of IL-7Rα, Box1, and mutation of Tyr449 eliminated STAT5 phosphorylation, suggesting critical roles for Box1 and Tyr449 in IL-7Rα signaling [251,254]. The array of genes induced in an IL-7-responsive manner include members of the Bcl-2 family: upregulation of Bcl-2 and Bcl-X_L expression and inhibition of Bad and Bax expression as well as increasing Fas expression [107,251]. The Bcl-2 family consists of anti-apoptotic (i.e. Bcl-2, Bcl-X_L, Mcl-1) as well as pro-apoptotic (i.e. Bad, Bax, Bad) molecules [235,255,256]. Additional genes induced by IL-7 were c-jun / c-fos and c-myc also involved in cell growth, differentiation, and programmed cell death. IL-7-induced AKT is involved in cell proliferation [257-259].

Finally, IL-7 is also involved in controlling recombination genes of immune receptors by regulating accessibility of specific loci for V(D)J recombination [235]. Furthermore, other signaling pathways involving the Src family Tyr kinases ($p56^{lck}$ and $p59^{fyn}$), MAPK signaling proteins, and STAT3 are mobilized following IL-7 binding to IL-7Rα[235,260,261]. Although a limited amount of information has been gathered regarding the role of the MAPK signaling proteins in CD8 T cells, P38 and JNK, but not ERK have been implicated in IL-7-induced T cell proliferation [262,263]. Members of the Src family kinases have been proposed to be induced by IL-7 stimulation and interact with IL-7Rα in activated and resting T cells [264]. However, the function of the Src family members have been proposed to be more important in TCR-mediated responses and IL-7-induced B cell proliferation instead of IL-7-mediated proliferation of T cells [116].

Biological Functions of IL-7 / IL-7Rα signaling

T lymphocyte development and differentiation

The biological effect of IL-7Rα signaling are quite expansive since it is expressed on many hematopoietic cells, but its expression on the lymphoid lineage is of particular relevance in my study of its role in CD8 T cells. Under normal physiological conditions in the CD8 T cell life span, IL-7Rα expression is elevated in the naïve state and low in the effector CD8 T cell subset (Fig. 1.1). This is thought to maximize the effects of IL-7 signaling in yet to be signaled naïve T cells. IL-7Rα rises in the memory subset which is critical for their long term maintenance via signals from IL-7 [45,56,265,266]. IL-7 signaling induces the expansion of naïve CD8 T lymphocytes as well as memory cells.

The expansion and survival of the effector cytotoxic CD8 T lymphocytes (CTLs) becomes strongly dependent on the other γc cytokines such as IL-2, IL-15, and IL-21 whose cognate receptors become upregulated [237]. Through the cytotoxic functions of the CTLs, which include degranulation, IFNγ and TNFα production, viruses are cleared. This is followed by the death of antigen-specific effector cells which are no longer needed [46,90,267,268]. However, a subset of antigen-specific CTLs maintain IL-7Rα expression, and these cells survive and represent the long lasting memory T cell pool where IL-7 / IL-7Rα signaling is essential to establish the stable memory population [36,45]. Thus, the IL7Rα gene is expressed in the immune system in a developmental stage-specific manner and is strictly regulated rising and falling not only at various stages in lymphoid development, but during activation and differentiation.

IL-7 has emerged to be a non-redundant upstream key regulator in the survival of immature and mature T lymphocytes through its triggering of the IL-7R complex [269,270]. Deletion of IL-7 or IL-7Rα in mice leads to dramatic reductions in thymocytes, bone marrow B cell precursors, and peripheral B cells, CD4 and CD8 T cells. Importantly, the IL-7Rα findings in mice regarding T lymphocytes were recapitulated in humans [129,271]. The other γc cytokines IL-2, IL-15, and IL-21 have specific functions throughout the CD8 T cell life span in T cell homeostasis and differentiation with some redundancy between their roles. Interplay between IL-7 / IL-7Rα signaling and responsiveness to IL-2, IL-15, and IL-21 appears necessary for proper maintenance of CD8 T cells [237].

T lymphocyte survival

Clearly, IL-7Rα and its modulation, as described above, are important for the survival of CD8 T cells. For well over a decade, one of the very first genes identified as an IL-7 target induced upon binding to IL-7Rα has been Bcl-2 [255,272]. The Bcl-2 protein has a biphasic expression pattern in lymphocyte progenitor cells concordant with that of IL-7Rα expression and thus suggestive of a role in the survival and selection of early precursors [235,273,274]. It is important to note that the Bcl-2 protein is not the sole survival factor targeted by IL-7 since it is possible for Bcl-2-deficient cells to survive in response to IL-7 [235,275]. IL-7 is also able to induce Bcl-X_L protein expression [251]. It is also important to consider that IL-7 may target pro-apoptotic factors by repressing their activity [251].

In fact, IL-7 / IL-7Rα signaling is required to carefully regulate the balance between the anti-apoptotic and pro-apoptotic members of the Bcl-2 family [116,235,276]. Distinct regions of the intracellular domain of IL-7Rα are involved in recruiting the signaling pathways that regulate three important members of the Bcl-2 family: Bcl-2, Bax, and Bad [116]. The IL-7 / IL-7Rα signaling axis leads to Bcl-2 synthesis for cell survival, cytosolic retention of Bax, and regulation of Bad phosphorylation, which are necessary to suppress the activities of these death proteins [116,276]. Tyr449 and Box1 of IL-7Rα are essential for bringing about Bcl-2 synthesis and the retention of Bax in the cytosol, whereas Box1 is also necessary for phosphorylation of Bad [251]. IL-7 withdrawal can reverse these survival activities [276]. However, residual Bcl-2 expression can continue to protect the cells from Bax-induced damage resulting from its release from the cytosol. In addition, activated Bad can translocate to the mitochondria to

inactivate Bcl-2 and eventually Bad levels may surpass those of Bcl-2, causing cell death. Once membrane integrity is lost, factors such as cytochrome c are released, and Bax can more freely translocate to the mitochondria to promote cell death [235,277-279]. Jiang et al. (2004) suggest that the survival function can largely be attributed to the activation of Jak1 followed by its binding to Box1, eventual phosphorylation of Tyr449, and finally STAT5 phosphorylation [116].

In addition to Jak1, IL-7-induced the association of IL-7Rα with the PI3K-AKT survival pathway and the entailing activity has been ascribed to the phosphorylation of Tyr449. Phosphorylation of Tyr449 was essential for PI3K-dependent cell cycle entry and proliferation of B lymphocytes [280,281]. IL-7 / IL-7Rα signaling has been shown to be critical in the development of human thymocytes affecting primarily αβ T cells and to a lesser extent γδ T cells [135]. The intracellular domain of IL-7Rα played a critical role in signaling specifically Tyr449. Tyr449 is associated with two major signaling pathways: the Jak/STAT and PI3K pathways in human thymocytes to phosphorylate STAT5 and AKT, respectively [254,280]. Finally, IL-7-induced PI3K-AKT was critical for the proliferation and survival of human thymocyte precursors, but not in T cell differentiation, where STAT5 seemed to be of greater importance [135,252,253,282,283].

Other signal transduction pathways may be required for the survival and biological functions of IL-7R, but have yet to be fully investigated. The Src family kinases are non-receptor tyrosine kinases guided to the plasma membrane by myristoylation or palmitoylation signals [116,235,284]. Their function is dependent on three critical SRC Homology (SH) domains: one of which contains the catalytic site and two others mediate interactions with proline-rich polypeptides and phosphorylate

residues such as those present on activated IL-7Rα [116,235,264,284,285]. Src kinases possess 9 members including $p56^{lck}$ and $p59^{fyn}$ which have the potential to act on T cells through IL-7Rα and its neighbouring signaling proteins from the PI3K, MAPK, and Jak/STAT pathways [116,235,264,284-286]. In mature human T cells, $p56^{lck}$ was shown to be activated by IL-7 and IL-7Rα was shown to physically associate with both $p59^{fyn}$ and $p56^{lck}$ [264,287]. However, the consequence of the interactions between $p56^{lck}$, $p59^{fyn}$, and IL-7Rα in cell growth, survival, and maturation have not been clearly elucidated. The expression of $p56^{lck}$ throughout thymocyte development is important for the development and expansion of CD4/CD8 double positive thymocytes at the TCR signaling stage and not the IL-7-dependent phase [92,264,288]. Similarly, $p59^{fyn}$-deficient mice have defective thymocytes, but experience normal peripheral T lymphocyte expansion and development [289]. Although, $p59^{fyn}$ and $p56^{lck}$ appear independent of IL-7 signaling they are essential for the cooperative effects of low affinity TCR signaling and IL-7 signaling on the expansion of the naïve peripheral T cell pool [290].

IL-7 / IL-7Rα signaling has also been proposed to play a role in cell cycle progression. In the earliest phases of thymocytes development, IL-7 deficiency resulted in an increase in cells arrested in the G_0 / G_1 phase relative to the cells cycling into the S / G_2 / M phase [291]. IL-7 signaling appeared to be involved in regulating Cdc25 and $p27^{kip1}$, both negative cell cycle regulators, which were inversely correlated with IL-7Rα expression [116,292]. Furthermore, Cdc25A and $p27^{kip1}$ reduction was accompanied by an increase in cdk2 activity, which is involved in promoting the transition from the G_1 to the S phase.

Immunosenescence

A common belief regarding the immune system of the elderly is that it suffers from deterioration and progressive impairments. This however is somewhat of a misnomer since the changes can be interpreted as a series of remodeling events where some functions are reduced or increased and other functions remain unchanged [293].

IL-7Rα is carefully modulated during the CD8 T cell life span, but during immunosenescence a shift in the proportion of specific CD8 T cell subsets expressing IL-7Rα occurs in the aging population [293-295]. Immunosencescence is characterized by a high memory to naïve ratio due to the curtailing of naïve T cell production and the conversion of naïve T cells into memory cells resulting from encounters with pathogens incurred over the years. Among the most radical changes with aging is the emergence of CD8 T cell clonal expansions (TCE) observed in rodents and humans [295-297]. Messaoudi et al. showed that in mice, these cells represent a distinct subset of central memory CD8 T cells which express higher than normal levels of CD122 (IL-2Rβ / IL-15Rβ chain) and IL-7Rα in comparison to other memory cells [295]. These cells proliferate slowly, but continuously and do so oblivious to environmental cues and potentially out-compete other CD8 T cells for survival signals. In humans, these cells were mostly identified as antigen responding TCEs having had previous encounters with persistent viruses. It is important to note that a subset of antigen-experienced IL-7Rα^{low}–expressing replicatively senescent CD8 T cells has also been identified at increased proportions in elderly humans [294]. In contrast to the TCEs, the antigen-experienced IL-7Rα^{low}–expressing replicatively senescent CD8 T cells are mainly a subset of CD45RA+ effector memory CD8 T cells with a limited TCR repertoire. Despite their

reduced responsiveness to IL-7 and the physiological impact of reduced IL-7Rα expression causing reduced Bcl-2 synthesis, the IL-7Rα^{low} CD8 T cells were maintained *in vivo*.

Regulation of IL-7Rα expression

The mechanisms involved in the regulation of IL-7Rα have not been clearly described especially in human T lymphocytes. However, a wealth of information has been gathered from murine studies [189,286,298]. Several studies have investigated the regulation of IL-7Rα expression with respect to the biological mediators involved (e.g. cytokines, antigens, and mitogens) as well as the molecular mechanisms that these mediators recruit. Mainly, transcriptional mechanisms, but also post-transcriptional IL-7Rα regulation such as receptor shedding, have been proposed [189,299-305]. To further complicate matters, IL-7Rα expression during chronic virus infections is altered. The prime example is HIV wreaking havoc on the immune system, which includes the dysregulation of IL-7Rα, and alterations in the cytokine environment [82,111,118].

Biological Mediators of IL-7Rα Regulation

Gamma c (γc) cytokines

Suppression of IL-7Rα expression by IL-6 and the γc cytokines: IL-2, IL-4, IL-7, and 15 has been described in mouse and human studies [189,286,302]. In the presence of these cytokines, IL-7Rα protein and mRNA expression levels are reduced and upon cytokine withdrawal *de novo* transcription and protein synthesis were necessary to restore IL-7Rα expression [189,237]. IL-2 mediated downregulation of IL-7Rα expression

levels was shown to be dependent on the PI3K-AKT pathway in murine T cells [286]. The ability of IL-2 to decrease IL-7Rα expression may reduce its competition with IL-7 since they both share the γc chain of their respective receptors, highlighting the cross-talk between IL-2 and IL-7 / IL-7Rα signaling [161,286]. IL-4 and IL-15 are also able to quickly downregulate IL-7Rα protein and mRNA expression levels, but the mechanism has not yet been described [189]. Interestingly, in humans IL-7Rα^{low} CCR7^{-} memory CD8 T cells were unable to activate the PI3K pathway in response to TCR-mediated stimulation, but in the presence of IL-15, signaling via the PI3K pathway was restored leading to sucessful proliferation [306]. Enhanced IL-15 signaling in IL-7Rα^{low} CCR7^{-} memory CD8 T cells was in part due to high expression levels of its cognate receptor, IL-2/IL-15Rβ, in these cells [306]. The functional similarities and shared receptors by IL-2 and IL-15 raise the possibility that the mechanism for IL-7Rα regulation by IL-15 may also be through the PI3K-AKT pathway.

IL-7 has a role in the regulation of IL-7Rα expression, which has been proposed to be a mechanism to maximize IL-7 signaling to previously unsignaled cells [189,237,307-309]. One study compared downregulation of IL-7Rα protein and mRNA levels by IL-7 and TCR signaling in naïve human T cells [237]. As suspected, different signaling strengths provided by IL-7 and TCR stimulation correlated with their suppressive activity on IL-7Rα gene expression levels and the restoration time required for surface receptor expression was suggestive of differential regulation of IL-7Rα expression. IL-7 weakly suppressed IL-7Rα expression correlating with a more rapid IL-7Rα recovery, which was in contrast to the strong suppressive activity provided by TCR

stimulation. Further experiments showed that T cells activated by cytomegalovirus (CMV) re-expressed IL7Rα over the same time period as TCR stimulation [237].

TNFα and IFNα/β

Activation of the immune system results in the release of cytokines and chemokines which along with invading pathogens can have regulatory effects on IL-7Rα expression. Amongst these factors, TNFα, IFNα, and IFNβ play important roles in the innate and adaptive immune response and are involved in the positive regulation of IL-7Rα (Fig. 1.3) [7,19,23,27]. TNFα has been shown to significantly upregulate IL-7Rα expression at the protein and mRNA levels in murine T cells where the mechanism was partially dependent on the transcription factor NF-κB [189,310]. Upstream of the transcription site of the IL-7Rα promoter, the interferon-stimulated response element (ISRE) has been shown to be involved in the positive regulation of IL-7Rα expression by IFNα and IFNβ [189,310,311]. The molecular mechanisms involved in regulating IL-7Rα expression will be described in more detail below.

HIV Tat-mediated downregulation

Chronic stimulation by HIV virus particles and viral proteins has been identified as a potential pathogenic mechanism to downregulate IL-7Rα expression and importantly this could be reversed by effective ART [89,96,239,312]. The HIV Tat protein is an activator of viral gene transcription and is secreted by HIV-infected cells, which can then be endocytosed by binding to the cell surface through heparin sulfate glycosaminoglycans [313-315]. In fact, secreted HIV Tat proteins specifically downregulated IL-7Rα expression on naïve and memory human CD8 T cells without affecting its partnered receptor the γc chain [312]. Moreover, downregulation of IL-7Rα

Figure 1.3 Schematic representation of the IL-7Rα promoter based on findings from murine T cells.

Constitutive expression of IL-7Rα is regulated by the transcriptional regulators GABPα, PU.1, and GFI1 in lymphocytes at different developmental stages. GABPα and PU.1 bind the same *GGAA* motif on the IL-7Rα promoter to positively regulate IL-7Rα expression on mature lymphocytes: T and B cells and progenitor B cells, respectively. IL-7-mediated increase in GFI1 is involved in negative regulation of IL-7Rα expression. Inducible expression of IL-7Rα is regulated by glucocorticoids which bind to their cognate promoter sequence and regulate IL-7Rα by binding the glucocorticoid response element (GRE). IFNα/β enhances IL-7Rα expression through an interferon-stimulated response element (ISRE), and TNF induced IL-7Rα expression in part through NF-κB. The schematic representation is modified from Mazzucchelli et al. [316].

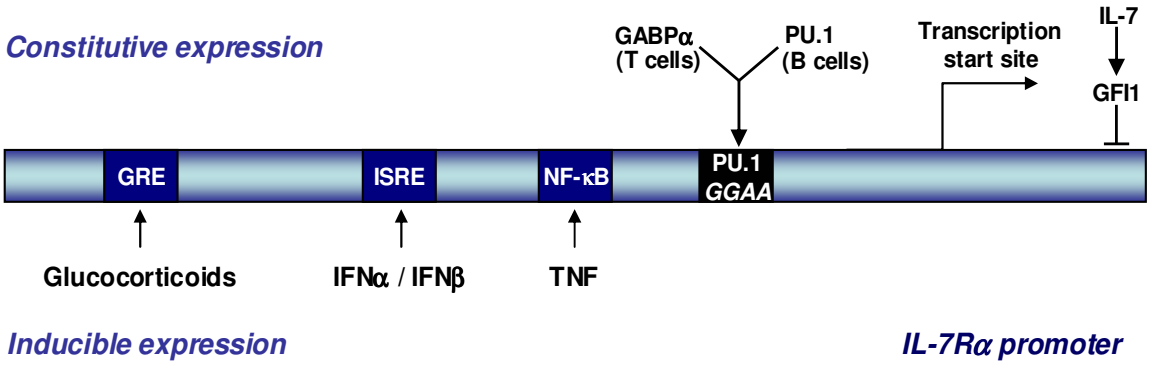

Constitutive expression
GABPα
(T cells)
PU.1
(B cells)
Transcription
start site
IL-7
GFI1
GRE
ISRE
NF-κB
PU.1
GGAA
Glucocorticoids
IFNα / IFNβ
TNF
Inducible expression
IL-7Rα promoter

FIGURE 1.3

expression by Tat exposure resulted in reduced responsiveness and proliferation to IL-7. Importantly, the effects of Tat exposure were reversible as would be predicted since the use of ART, which reduces viral load, is accompanied by partial restoration of IL-7Rα expression.

Molecular Mechanisms Involved in IL-7Rα Regulation

GABPα and PU.1

A great deal of work has been conducted to understand IL-7Rα regulation at the transcriptional level however a large proportion of this work has been conducted in murine lymphocytes. Two transcriptional factors have been implicated in the positive regulation of IL-7Rα, PU.1 and Guanine Adenine Binding Protein alpha (GABPα) (Fig. 1.3) [299-301,303]. GABPα and PU.1 are ETS (E-twenty six) transcription factors. All ETS family members are characterized by a domain involved in protein-protein interaction and a highly conserved DNA binding domain which binds to sites with a central *GGAA* DNA sequence [299,300,303,316]. Numerous normal physiological functions have been assigned to ETS family members which includes the regulation of cellular differentiation, cell cycle control, migration, proliferation, apoptosis, and angiogenesis as well as a role in the progression of cancer when dysregulated [299,300,303,317]. GABPα and PU.1 act as transcriptional activators in the case of IL-7Rα regulation [299,300,303,316].

Positive regulation of IL-7Rα occurs in murine cells of the lymphoid lineage by recruiting different transcription factors to the same *GGAA* motif. Regulation of IL-7Rα in murine B cells and T cells is also dependent on the developmental stage of the cell

[299,300]. In mouse progenitor B cells, PU.1 regulates IL-7Rα expression via a *GGAA* motif whereas the same sequence was bound by GABPα in mature T cells, performing a similar task [300]. Thus it was concluded that GABPα was involved in the regulation of IL-7Rα in T cells while PU.1 was irreplaceable in IL-7Rα regulation in pro-B cells. However, after further scrutiny of the role of GABPα and PU.1 in the regulation of IL-7Rα, it was discovered that GABPα was not strictly expressed in T cells, but was sufficient to regulate IL-7Rα expression in committed B lineage cells in the absence of PU.1 [299]. Amplification of the activity of PU.1 was also visualized in murine T cells. The glucocorticoid response element located upstream in the noncoding sequence of the IL-7Rα promoter when bound led to increased IL-7Rα mRNA and protein expression in mouse T cells, which was partly dependent on the *GGAA* motif of PU.1 [318].

DNA methylation

In addition to the positive regulatory mechanisms described above, there exist molecular mechanisms that negatively regulate IL-7Rα such as DNA methylation and transcriptional repression by the Growth Factor Independent-1 (GFI1) protein. Kim et al. observed that IL-7Rα^{high} CD8 T cells in humans had decreased methylation in the IL-7Rα gene promoter thus higher levels of promoter activity in comparison to IL-7Rα^{low} CD8 T cells [319]. Furthermore, these findings were also observed in the Jurkat T cell line. Jurkat T cells express low levels of IL-7Rα and similar to the human CD8 T cell, IL-7Rα promoter methylation was localized to CpG sites. These findings suggest that DNA methylation of the IL-7Rα gene promoter was involved in the negative regulation of IL-7Rα expression in T cells.

Growth Factor Independent -1 (GFI1)

The GFI1 gene was first discovered in 1993 [320] in studies of Moloney murine leukemia virus (Mo-MuLV)-induced rat T cell lymphomas. Rat lymphoma cell lines carried integrated provirus at a locus containing the GFI1 gene. Dysregulation of the GFI1 gene by provirus integrations rendered T cell growth independent of IL-2 and has since been recognized as one of the most frequent sites for retroviral integration [321]. Closely related to GFI1 is GFI1B also a transcriptional repressor with similar functions and structural features as GFI1 as well as a high degree of sequence similarity [322].

GFI1 cellular distribution

GFI1 is a nuclear zinc finger protein with transcriptional repressor activity detected at different expression levels in precursor thymocytes, mature thymocytes, peripheral naïve and activated T lymphocytes [323,324]. GFI1 is not solely restricted to T cells. Its expression ranges in cells from the hematopoietic to the lymphoid system to sensory epithelia, lung, and parts of the central nervous system [323,325,326]. More specifically, GFI1 is also expressed in neutrophils and early B lymphocytes, but not in erythroid or megakaryocytic cells [325,327,328]. Conversely, the related nuclear protein GFI1B is expressed in erythroid and megakaryotic cells, but at very low to undetectable levels in cells of the lymphoid and myeloid lineage. It is in part because of this wide range of expression in different cell types that GFI1 has such an expansive impact on cellular functions from early cell development to fully differentiated cells.

GFI1 structure

The GFI1 gene encodes a nuclear protein with a molecular weight between 47 and 55 kDa flanked by six carboxy-terminal zinc-finger domains and an amino-terminal domain of 20 amino acids called SNAG (Fig. 1.4) [329,330]. The term SNAG is derived

Figure 1.4 Schematic representation of the GFI1 protein.

(a) The GFI1 protein possesses a 20 amino acid SNAG domain at the N-terminus, an intermediate region between amino acids 20-257, and six carboxy-terminal zinc finger domains. The SNAG domain is required for transcriptional repressor activity whereas the third, fourth, and fifth zinc finger domains are involved in DNA binding. A role for the remaining zinc fingers and the intermediate domain has not been elucidated, but they may play a role in co-factor binding or protein-protein interaction. (b) Representative diagram of the structure of a zinc finger ring showing cysteine and histidine surrounding a zinc atom, and each black circle represents an amino acid residue. The diagrams were modified from Moroy et al. [324,331].

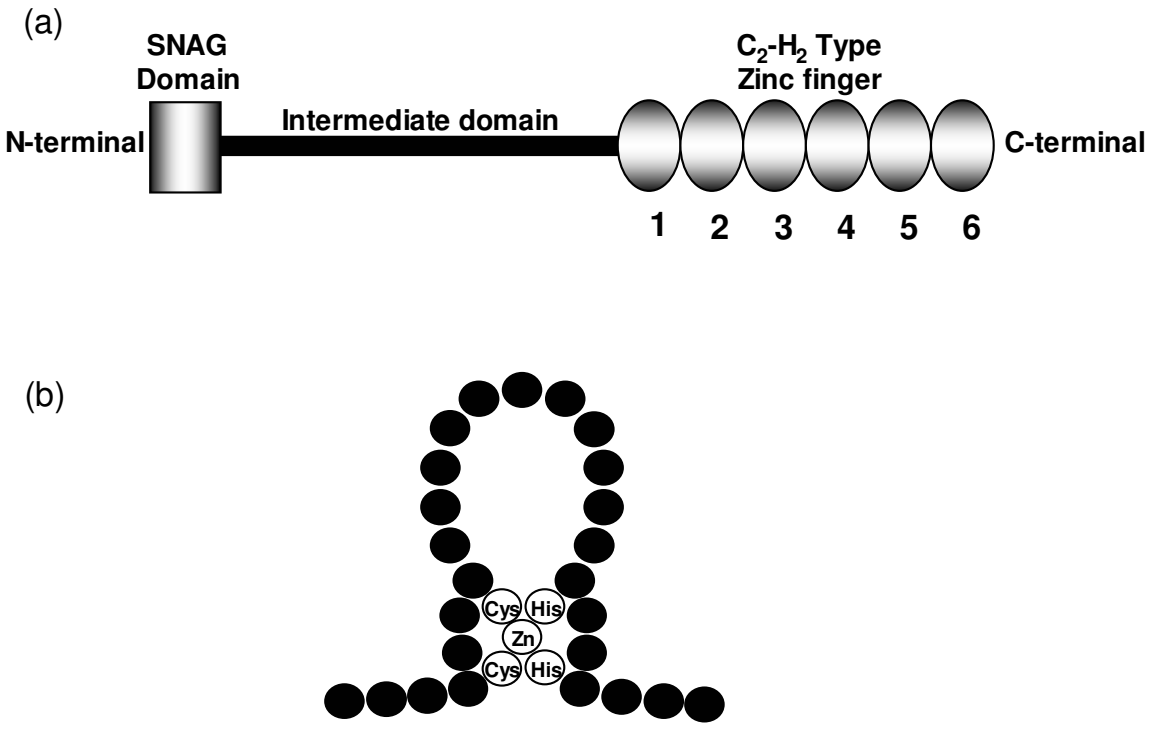

(a)
SNAG
Domain
C_2-H_2 Type
Zinc finger
N-terminal
Intermediate domain
C-terminal
1
2
3
4
5
6
(b)
Cys
His
Zn
Cys
His

FIGURE 1.4

from the Snail and Slug proteins and possesses transcriptional repression activity [329]. DNA binding has been assigned to 3 of the 6 zinc fingers in the C-terminal end whereas the remaining zinc fingers have been hypothesized to serve as protein/protein interaction domains [324]. Although GFI1 is a relatively small protein, the C_2H_2 zinc finger region, the flanked co- factor binding region (amino acids 20-257) and its minimal sequence

domains [324]. Although GFI1 is a relatively small protein, the C_2H_2 zinc finger region, the flanked co- factor binding region (amino acids 20-257) and its minimal sequencerequirements for binding (*AATC*) [330] permits GFI1 to act cooperatively with numerous other proteins [301,317,332-337]. It is the flanked co-factor binding region which distinguishes GFI1 from GFI1B [324]. Thus, GFI1 is able to have an impact on the activity and expression of proteins involved in various cellular functions. In humans, it is interesting to note that GFI1 is located on chromosome 1p22 with the surrounding area garnering much attention as a locus involved in malignant transformations and several genetic diseases [338-342].

Molecular basis for the transcriptional repressive functions of GFI1

There is considerable information regarding how GFI1 exerts its transcriptional repressor activity. Most studies have implied a simple mechanism whereby GFI1 binds to the promoter of the target to act alone repressing transcription through its SNAG domain. However, the co-factor binding region flanked by the SNAG domain and the zinc finger region has been shown to sequester the protein inhibitor of activated STAT3 (PIAS3) away from STAT3 to enhance the latter's activity [332]. In addition, more sophisticated mechanisms have been described in a limited number of studies which suggest cooperation with co-repressors by binding to the GFI1 zinc finger domain [335,336].

GFI1 is a nuclear protein which migrates to the nuclear matrix following an activation signal. The complexity of the particular response that ensues has been described by McGhee et al. in mammalian cells [336]. Once GFI1 is attached to the nuclear matrix it forms complexes with domains I and II of a co-repressor ETO (MTG8) and histone deacetylase (HDAC1, -2, -3) proteins, resulting in chromatin modification of target genes. HDAC enzymes direct deacetylation of histones resulting in a repressed state as visualized by closed chromatin conformations [343-345]. However, in the presence of a HDAC inhibitor such as trichostatin A, GFI1-mediated transcriptional repression was dramatically reduced even when GFI1 expressed the SNAG domain, suggesting that HDACs are important in mediating the repressor activity of GFI1 [335,336]. The SNAG domain and the activity by the SNAG-independent mechanism described above may not be the only negative regulatory mechanisms. GFI1 attachment to another member of the ETO family, MTG16, suggests yet another undescribed mechanism by which GFI1 may exerts its activity. A similar SNAG-independent mechanism was used by GFI1 to coordinate epigenetic repression of the cell cycle regulator $p21^{cip/WAF1}$ [335]. In order to modify the chromatin of $p21^{Cip/WAF1}$, GFI1 recruited histone lysine methyltransferase G9a and HDAC1 to form a complex at the $p21^{cip/WAF1}$ promoter. Normally, G9a methylates H3 lysine 9 (H3-K9) [86]. However, following complex formation with GFI1 and HDAC, an increase in H3-K9 methylation occurs, resulting in transcriptional repression of $p21^{cip/WAF1}$ [335]. Notably, transcriptional repression is lost upon silencing of GFI1 [335].

Regulation of GFI1 expression

GFI1 protein and mRNA levels are strictly regulated in developing precursor thymocytes, and the absence of a linear correlation between protein and mRNA levels is

also suggestive of a post-transcriptional regulatory component [346]. On the other hand, constitutively high GFI1 levels are measured in mature thymocytes [324] and low levels in mature peripheral T cells, but GFI1 becomes upregulated upon TCR activation [323]. A regulatory mechanism for GFI1 expression in monocytes and granulocytes has been described, but regulation of GFI1 expression in T cells remains unclear [298].

The Ubiquitin Proteasome System (UPS) is a regulatory mechanism for GFI1 identified in granulocytes and monocytes [347,348]. Prolonged expression of GFI1 in monocytic cell lines is required to bind the promoter of granulocyte-specific genes in monocytes to inhibit granulocytic differentiation. Ubiquitin ligase Triad1 interacts with the DNA binding domain of GFI1 in monocytes to prolong its half-life [347,349]. In the U937 promonocytic cell line, GFI1 mRNA levels diminish rapidly, but protein levels become increased due to decreased proteasomal degradation influenced by Triad 1 activity. However, in granulocytes GFI1 mRNA levels are high, but the protein is rapidly degraded by the UPS [347,348]. Interestingly, it has been proposed that Triad1 may compete for GFI1 binding with an unidentified E3 ubiquitin ligase involved in the proteasomal degradation of GFI1.

Steps towards clarifying the regulation of forced GFI1 expression in lymphocytes has been investigated in primary mouse thymocytes and human T cell lines [298]. Ectopic expression of GFI1B similar to GFI1 was involved in transcriptional repression through the same DNA sequence [298,330,350]. Autoregulation of endogenous GFI1 by GFI1 and GFI1B was specific to T cells since similar observations were not made in myeloid cell lines [298]. The activity of GFI1B and GFI1 in autoregulation is proposed to play a role in the development of T cells and the functional aspects of developing T cells to maturity [351].

Negative regulation of IL-7Rα

The first studies supporting a role for GFI1 in the regulation of IL-7Rα were conducted in murine T cells [189,301]. A novel mechanism was described by Park et al. for the suppression of IL-7Rα via IL-7 and other pro-survival cytokines (IL2, IL4, IL-6, and IL-15) in murine lymph node T (LNT) cells [189]. The authors showed that IL-7Rα expression required continued transcription and *de novo* protein synthesis. A physiological mechanism was proposed in which the downregulation of IL-7Rα expression in cells already exposed to IL-7 would maximize IL-7 availability for unsignaled T cells which require IL-7 to survive. The study demonstrated that IL-7-mediated suppression of IL7Rα transcription utilized the transcriptional repressor GFI1 in CD8 T cells while CD4 T cells did not [189].

In response to virus infection of the host, mature CD8 T cell differentiation begins in which IL-7Rα expression is dynamically regulated [32,36,37]. Chandele et al. investigated the regulation of IL-7Rα expression on murine virus-specific CD8 T cells of the effector and memory phenotype which express low and high IL-7Rα expression, respectively [301]. During lymphocytic choriomeningitis virus (LCMV) infection, GFI1 was required for stable IL-7Rα repression in effector CD8 T cells and acted by antagonizing GABPα binding and recruiting HDAC1 to the IL-7Rα promoter. In contrast, GABPα positively regulated IL-7Rα expression in memory precursor effector cells, correlating with hyperacetylation of the IL-7Rα promoter [301].

As mentioned, GFI1B is a nuclear oncoprotein closely related to GFI1. Normally, GFI1 and GFI1B are differentially expressed in lymphoid cells where GFI1B levels are low during T cell development, and undetectable in terminally differentiated thymocytes

which instead express GFI1 upon activation [320,332]. Doan et al. (2003) investigated the impact of transgenic expression of GFI1 and GFI1B in mature murine thymocytes [302]. Overexpression of GFI1B resulted in impaired T cell activation in contrast to the transgenic expression of GFI1. More interestingly, transgenic GFI1B expression displayed a similar suppressive effect on IL-7Rα expression as GFI1 in murine CD8 LNT cells [189]. Furthermore, forced GFI1B expression in mature thymocytes had an impact on T cell lineage commitment by reducing IL-7Rα expression and single positive CD4-CD8+ T cells, which could be alleviated by transgenic expression of Bcl-2 or GFI1 to encourage commitment to the CD8 T cell lineage [302].

IL-7Rα Regulation in Primary Human CD8 T Lymphocytes

The IL-7Rα regulatory mechanisms discussed above largely identified in murine lymphocytes, were cytokine-mediated, and also occurred at the transcriptional level [189,286,299-303,312,316,318,319,352]. To my knowledge the molecular mechanisms for IL-7Rα regulation in primary human CD8 T cells have not been reported although several γc cytokines are known to suppress IL-7Rα expression in these cells [93,189,238,286,312,316]. In addition, modulation of IL-7Rα expression during CD8 T cell differentiation is largely mediated by γc cytokines which are also responsible for their function, maintenance, and cytotoxicity [30,52]. As described above, impairment of CD8 T cells following HIV infection occurs at various levels which include disruption of signal transduction pathways and downregulation of IL-7Rα expression. Therefore, a greater understanding of IL-7Rα regulation is imperative since it could potentially be a new marker in monitoring HIV disease progression. Therefore, the objectives for my

thesis to explore IL-7Rα regulation in primary human CD8 T lymphocytes are listed below.

Hypotheses and Objectives

Infection with HIV results in chronic activation of the immune system and extensive alterations in cell signaling in T lymphocytes which are in part due to disruption of the cytokine secretion profile [98,353,354]. Responsiveness to IL-7 and other crucial γc cytokines are necessary for the growth, differentiation, survival, function, and maintenance of CD8 T cells [30,52,130]. However, particularly in viremic HIV+ patients, a higher proportion of effector-like memory IL-7Rα^{low} CD8 T cells characterized by an exhausted phenotype has been observed [86]. This may be due to altered Jak/STAT signaling in these cells, a pathway critical to the cytokine-mediated biological functions mentioned above.

Hypothesis 1: Defective cytokine responsiveness via the Jak/STAT pathway exists in CD8 T cells from HIV-infected individuals, thus contributing to the observed impairments in their differentiation and function.

In order to investigate hypothesis 1 my objectives were as follows.

Objective 1: Evaluate the capacity of CD8 T cells in HIV-infected individuals to signal through the Jak/STAT pathway in response to cytokines important in their cytotoxicity, maintenance, survival, and growth.

Objective 2: Identify the potential molecular mechanism involved in the defective IL-7-dependent STAT5 activation and reduced IL-7Rα expression that I observed in patient CD8 T cells.

Importantly, results from objective 2 showed that high levels of GFI1 expression correlated with reduced IL-7Rα expression in human CD8 T cells. Considerable information has also accumulated concerning the functional roles of GFI1 in murine

lymphocytes [298,317,323,330,332-335,337,355-359]. Furthermore, IL-2, IL-4, and IL-7 have been shown to downregulate IL-7Rα in human and murine lymphocytes [189,237,286,316]. Moreover, GFI1 expression has also been linked to the downregulation of apoptotic factors as well as enhanced proliferation of cells of the lymphoid lineage in murine lymphocytes [317,323,334,335,356,357]. However, a comprehensive study of the role of GFI1 in primary human CD8 T lymphocytes has not been conducted.

Hypothesis 2: GFI1 is involved in suppressing IL-7Rα expression and in the survival and proliferation of human CD8 T cells.

The following objectives were designed to evaluate the second hypothesis.

Objective 3: Evaluate the regulation of IL-7Rα expression and the role of GFI1 in resting and cytokine-stimulated human CD8 T lymphocytes.

Objective 4: Investigate the role of GFI1 in the proliferation and survival of CD8 T cells.

Objective 5: Identify the signaling pathways implicated in the regulation of IL-7Rα and GFI1 expression in human CD8 T cells.

CHAPTER 2
Materials and Methods

Study volunteer characteristics

Chronically-infected HIV+ volunteers were from the Immunodeficiency Clinic, Ottawa Hospital – General Campus, and included patients undergoing ART (>1 year; n=17) and those off-therapy (>6 months; n=17) (Table 2.1). Plasma viral loads were 10(1-500) x 10^3 [mean (range)] and <50 copies/ml while CD4 counts were 267(46-733) and 566(288-1002) cells/μl in off-therapy and ART patients, respectively. HIV-negative adult volunteers (n=22 male; n=18 female) were recruited from the Children's Hospital of Eastern Ontario, Ottawa.

Antibodies, cytokines, mitogens and pharmaceutical inhibitors

The anti-human monoclonal antibodies used for flow cytometry were: PE-labeled anti-IL-7Rα [R&D Systems (R&D), Minneapolis, MN, US], anti-CD3, anti-phospho-tyr-STAT (pSTAT)-6 [BD Biosciences (BD), Mississauga, ON, CAN]; Alexa488-conjugated anti-pSTAT5, anti-pSTAT3; FITC-labeled anti-Programmed Death (PD)-1, PECy-7-labeled anti-CD8, PE-labeled anti-human CD28, anti-human CXCR4 (BD), and a secondary antibody for GFI1 (N-20) bovine anti-goat IgG FITC (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, US).

CD8 T cells were stimulated with recombinant human interleukin (IL)-2, IL-4, IL-7, IL-10, IL-15, IL-21 (R&D), or dexamethasone (Sigma, Oakville, ON, CAN) as well as recombinant human TNFα (Biosource Invitrogen, Burlington, ON, CAN) and IFNγ (Pierce Endogen, Thermo Scientific, Rockford, IL, US). Phytohemagglutinin mucoprotein-form (PHA-M) in liquid was purchased from Invitrogen. The pharmaceutical inhibitors tested were SP 600125 (BIOMOL Int., Cedarlane Laboratories,

Table 2.1. Characteristics of HIV-positive volunteers on and off antiretroviral therapy.

On-therapy > 1 year			**Off-therapy > 6 months**		
Study Particpant	*Viral load (copies/mm^3)*	*CD4 (cells/ mm^3)*	*Study Particpant*	*Viral load (copies/mm^3)*	*CD4 (cells/ mm^3)*
V6	<50	369	*V1*	9 172	327
V7	<50	703	*V2*	31 722	733
V8	<50	942	*V3*	39 226	284
V9	<50	793	*V4*	5 993	362
V10	<50	1 002	*V5*	790 075	289
V11	<50	326	*P13*	172 139	84
V12	n/d	n/d	*P28*	n/d	n/d
P40	<50	536	*P41*	86 964	337
P42	<50	520	*P43*	498 000	46
P46	<50	288	*P47*	29 841	63
P52	<50	395	*P49*	274 767	604
P60	<50	758	*P50*	300 108	297
P61	<50	395	*P56*	1 043	157
P66	<50	299	*P57*	46 949	186
P68	<50	643	*P62*	188 073	51
P69	<50	707	*P63*	102 873	168
P70	<50	821	*P64*	9 849	451
P73	<50	548	*P65*	10 927	437
P75	<50	747	*P67*	249 611	42

Burlington, ON, CAN), SB 203580, PD 98059, LY 294002, and JAK Inhibitor 1 (Calbiochem/EMD Chemicals Inc., Gibbstown, NJ, US).

The anti-human antibodies used for western blotting were: βactin (Sigma), P38α, JNK (FL), phospho(p)-AKT1/2/3 (Ser473)-R, GFI1 (N-20) (Santa Cruz), Bax, Bcl-2, pP38 MAP Kinase (Thr180/Tyr182), pSAPK/JNK (Thr183/Tyr185), Akt, STAT6, and pSTAT6 (Tyr641) (Cell Signaling Technology/ New England Biolabs Ltd, Pickering, ON, CAN). The secondary antibodies were: donkey anti-goat IgG-Horse Radish Peroxidase (HRP) conjugate (Santa Cruz), goat anti-rabbit IgG (H+L)-HRP conjugate, and goat anti-mouse IgG (H+L)-HRP conjugate (BIO-RAD, Mississauga, ON, CAN).

Cell purification

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Baie d'Urfe, QC, CAN) at 300 x g for 30 minutes (no brake). PBMCs were washed 3 times in 1X Phosphate-Buffered Saline (PBS) (Wisent Inc. St. Bruno, QC, CAN) prior to CD8 T cell purification. CD8+ T lymphocytes were purified from PBMCs by negative isolation using the CD8+ T Cell Isolation Kit II (Miltenyi Biotec, Auburn, CA, US) as instructed in the provided manual. Further purification of CD8 T lymphocytes into CD8+/IL-7Rα-positive and CD8+/IL-7Rα-negative lymphocytes were performed as indicated by negative selection for CD8 T cells followed by positive selection using PE-labeled anti-IL-7Rα antibodies and anti-PE-Microbeads (Miltenyi Biotec). The instrument used for purification was the AutoMACS (Miltenyi Biotec).

Cytokine stimulation and inhibition of induced signaling pathways

In order to investigate the effect of cytokines on IL-7Rα and GFI1 expression levels, CD8 T lymphocytes were stimulated where indicated with the specified cytokine at 37°C / 5% CO_2 in 500 µl of Iscove modified Dulbecco's medium (Wisent Inc.) supplemented with fetal bovine serum (MEDICORP Inc. Mtl, QC, CAN) and penicillin / gentamycin (complete IMDM) for a final concentration of 100 IU/ml and 4.8 µg/ml, respectively. CD8 T lymphocytes were incubated with the cytokines at the concentrations indicated. At 24 and 48 hour intervals, the modulation of surface proteins specifically anti-IL-7Rα was visualized by flow cytometry using $2x10^5$ cells / tube whereas GFI1 mRNA expression levels were analyzed by real time PCR (RT-PCR) using $4x10^5$ cells / condition. In order to identify a role for signaling pathways in the regulation of these proteins, the CD8 T lymphocytes were pretreated for 2 hours at 37°C / 5% CO_2 with pharmaceuticals inhibitors at the indicated concentrations prior to stimulation then measured by immunoblotting, real time PCR, or flow cytometric analysis.

The phosphorylation of STAT proteins, P38, JNK, AKT, and ERK in CD8 T lymphocytes was studied. Here, $4x10^5$ CD8 T lymphocytes were resuspended in 500 µl of complete IMDM and pretreated where relevant with pharmaceutical inhibitors for 2 hours at 37°C / 5% CO_2 and followed by cytokine stimulation for 15 minutes or in a time course study. Phosphorylation of STAT proteins was visualized by flow cytometry or western blotting, whereas other protein phosphorylation was visualized by western blotting.

Surface and intracellular staining

For surface staining, $2x10^5$ CD8 T lymphocytes were added to 5 ml polystyrene round bottom FACS tubes washed 2 times in 2 ml of 0.1% bovine serum albumin (BSA) / 1X PBS for 5 minutes at 600 x g. The supernatant was decanted and the cells were resuspended in 100 µl of 0.1% BSA/PBS. The cells were incubated with the desired antibodies including anti-CD3 and/or anti-CD8 along with anti-CXCR4, anti-CD28, anti-IL-7Rα or anti-PD-1 antibodies at room temperature for 15 minutes then washed as described above, the supernatant was decanted, and cell surface expression markers were analyzed by flow cytometry. The antibody concentrations used were empirically determined (data not shown).

Intracellular staining was conducted on $4x10^5$ stimulated CD8 T lymphocytes to detect GFI1 protein expression and phosphorylation of STAT proteins in the presence and absence of pharmaceutical inhibitors. Following stimulation, CD8 T lymphocytes were fixed with 750 µl of 2% paraformaldehyde (PFA) / 1X PBS for 10 minutes at room temperature. Cells were washed as previously indicated and permeabilized with 500 µl of cold absolute methanol for 10 minutes at 4^oC. Lastly, cells were washed 2 times then resuspended in 200 µl of 0.1% BSA / 1X PBS and stained with anti-pSTAT antibodies for 15 minutes at room temperature or anti-GFI1 antibody for 25 minutes. Cells were washed and analyzed by flow cytometry for pSTAT expression. A secondary anti-goat antibody was used to detect GFI1 protein expression by staining for 15 minutes at room temperature in the dark then washed and analyzed by flow cytometry. Flow cytometry was conducted using the BD FACSCanto and data was recorded using the BD FACSDiva software v5.0.3, and analyzed using the WinMDI v2.8 Joseph Trotter.

Immunoblotting

For intracytoplasmic and surface proteins, cells were lysed in a Tris buffer (50mM HEPES pH7.5, 150mM NaCl, 10% Glycerol, 1% Triton X-100, 1.5mM $MgCl_2$, 100mM NaF, 1mM EGTA pH7.7) containing the Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN, US) and the following phosphatase inhibitors: 1mM sodium orthovanadate and 1mM sodium fluoride. For nuclear proteins, cells were lysed in a radioimmunoprecipitation (RIPA) buffer prepared with 50mM Tris-HCl pH7.5, 50mM sodium chloride, 1% Nonidet P40 Substitute, 0.5% sodium deoxycholate, and 0.1% sodium lauryl sulfate containing the Complete Protease Inhibitor Cocktail and 1 mM of sodium orthovanadate. Samples were incubated in the lysis buffer for 45 minutes then centrifuged at 16 652 x g for 20 minutes at 4°C. Samples were mixed at a 1:1 dilution with protein loading dye and boiled for 5 minutes. The proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) then transferred to a PDVF membrane. After blocking [5% skim milk in 1X tris buffered saline Tween-20 (TBST)], membranes were washed for 1 hour at 10 minute intervals in 1X TBST and probed overnight with primary antibodies. The membranes were washed prior to probing with secondary antibodies. Bound antibodies were detected by chemiluminescence (GE Healthcare, Life Sciences, Baie d'Urfe, QC, CAN) using the GeneSnap software on the ChemiGenius^2 (Syngene BioImaging System, Synoptics Ltd, Frederick, MD, US). Membranes were stripped by incubating with 10 ml of stripping buffer containing 7.1 µl 0.1X DTT for 30 minutes in a rotational incubator at 50°C. As described above, the membrane was washed and following blocking re-probed as above with other primary antibodies. Densitometry of developed membranes was measured using the GeneTools

software (Syngene, Synoptics Ltd) to evaluate changes in protein expression levels. Relative fold change in protein expression levels for GFI1 and Bax were normalized to housekeeping genes, βactin, or GAPDH, and the ratio of phosphorylated signaling proteins to their respective total proteins was determined.

Reverse Transcription and Real Time-PCR (RT-PCR)

Total cellular RNA was extracted (RNeasy Mini kit; Qiagen, Mississauga, ON, Canada) and cDNA was reverse transcribed (High Capacity cDNA Archive kit; Applied Biosystems (ABI), Streetsville, ON, Canada) using the GeneAmp PCR System 2700 from Applied Biosystems at 25^{o}C for 10 minutes and 37^{o}C for 2 hours. cDNA was amplified using TaqMan Gene Expression Assays (ABI) (IL-7Rα [Hs00902334_m1], GFI1 [Hs00382207_m1], GABPα [Hs00417506_m1], and βactin [Hs99999903_m1]) and the Universal Master Mix (ABI). To conduct Real Time PCR (RT-PCR) assays the instrument used was the 7500 Real Time PCR (ABI) (1 cycle at 50^{o}C – 2 minutes / 1 cycle at 95^{o}C for 10 minutes / 45 cycles at 95^{o}C for 15 seconds; 60^{o}C – 1 minute), and the results were recorded and analyzed with the 7500 System software v1.3.0. Relative mRNA expression levels were determined by the comparative Ct method [360]. For RT-PCR constituents see appendix.

Small-interfering RNA and plasmid transfection

GFI1 silencing was investigated in CD8 T lymphocytes using small-interfering (si) RNA targeting GFI1 (siGFI1) (Qiagen, Mississauga, ON, CAN). CD8 T lymphocytes were transfected using the transfection reagent Metafectene^{TM} Pro (Biontex

Laboratories GmbH, Germany). CD8 T lymphocytes were seeded at 2 or $4x10^5$ cells per 500 µl of complete IMDM overnight in a 24 well-plate. The next day, the media was replaced with IMDM without serum and antibiotics (incomplete IMDM) prior to the addition of the transfection complex. The transfection complex comprised of 0.4 µg siGFI1 diluted in 30 µl 1X PBS was added to 1.6 µl of Metafectene Pro in 30 µl 1X PBS dilution and incubated for 15 minutes at room temperature. The transfection mixture was added to the lymphocytes dropwise, incubated at 37°C / 5% CO_2 for 4 or 8 hours then the media was replaced with complete IMDM and incubated for an additional 24, 48, and 72 hours to assay cell extracts by western blotting or RT-PCR analysis.

Exogenous overexpression of GFI1 was studied in human CD8 T lymphocytes using pCMV-SPORT6 vector containing GFI1 (pGFI1) (Thermo Scientific Open Biosystems). Purified CD8 T cells were transfected using the Metafectene® EASY transfection reagent (Biontex Laboratories). CD8 T lymphocytes were seeded at $2x10^5$ cells/ 100 µl of complete IMDM overnight in a 96 well-plate. Prior to transfection, 24 hours following seeding the media was replaced with incomplete IMDM. The transfection complex was obtained by gently mixing 1 µl of Metafectene® EASY into 20 µl of 1X EASY buffer followed by the addition of 0.5 µg of pGFI1 or the vector backbone. The transfection mixture was incubated for 15 minutes at room temperature then added to the cells and incubated for 4 hours at 37°C / 5% CO_2. After 4 hours, the media was replaced with complete IMDM and incubated for 24 and 48 hours to conduct flow cytometry and western blotting analysis.

Preparation of GFI1 cDNA clone: pCMV-SPORT6 and empty vector

Full length cDNA GFI1 clone in the pCMV-SPORT6 vector was purchased from Thermo Scientific Open Biosystems (Atlanta, GA, US) for transfection into primary human CD8 T lymphocytes. The clone was collected from the vial using a sterilized loop then streaked unto a Luria Broth (LB) agar plate containing a final concentration of 100 μg/ml of ampicillin and incubated at 37°C overnight. Single colonies were collected from the plate and cultured in 20 ml of LB (Ampicillin [100 μg/ml]) and incubated on a shaker at 37°C at 210 rpm for 16 hours (VWR Int. model#9121017). After incubation, the cultures were centrifuged at 7740 x g for 5 minutes at room temperature. Large batches (20 ml) of the plasmid were extracted using the following protocols modified from the third edition of 'Molecular Cloning: A Laboratory Manual' [361].

The cultures were centrifuged for 5 minutes at 7740 x g and the supernatant was decanted. The pellet was resuspended with 400 μl of solution I then 800 μl of solution II was added, and rapidly inverted to mix and stored on ice. The suspension was incubated with 600 μl of solution III, gently inverted, vortexed 10 seconds, and stored on ice for 5 minutes before centrifuging at 12 000 x g for 5 minutes at 4°C. The supernatants were transferred to fresh tubes to which 500 μl of phenol / chloroform (1:1) was added and vortexed for 10 seconds. The mixture was centrifuged (12 000 x g at 4°C) for 5 minutes and then the top layer of the supernatant (~1.5 ml) was collected without disturbing the white interface and transferred to fresh tubes. Two volumes of room temperature 95% ethanol was mixed with the top layer by vortexing and incubated at room temperature for 2 minutes. The precipitate of plasmid DNA was centrifuged for 5 minutes at 12 000 x g at 4°C. The supernatant and fluids adhering to the tube walls were carefully removed and

the tubes were left standing in the inverted positions to ensure all the fluid was removed. The dried pellet was resuspended in 1 ml of cold 70% ethanol, centrifuged at 12 000 x g / 4°C and the supernatant was gently discarded. The tube was inverted position to drain all the fluid and the pellet was dried. Lastly, the plasmid was resuspended in 150 µl of distilled water and quantified using the NanoDrop 2000 spectrophotometer (Thermo Scientific).

Empty pCMV-SPORT6 vector was generated by digestion under the following conditions: 10 µg plasmid DNA, 5 µl 10X NEBuffer 3, 0.5 µl 100X BSA, 2 µl Sal1 and 2 µl Xho1 and sterile dH_2O for a final volume of 50 µl at 37°C for 2 hours using the Perkin Elmer DNA Thermal Cycler 480. The digested product (50 µl) was mixed with 2 µl of 6X loading dye was resolved on a 1% agarose gel prepared in 1X TAE at 140 volts. Comparison with a 1 kb ladder was used to assess the size of the 2 bands resulting from the digestion. The vector was ~ 4.4 kb and the insert size of GFI1 ~2.5 kb. The vector was purified from the gel using The QIAquick Gel Extraction kit (Qiagen) as instructed in the QIAquick gel extraction microcentrifuge protocol. The DNA was eluted in 35 µl, and 2 µl of the product was migrated on a 1% agarose gel (1X TAE) to assess the purity of the final product for ligation.

The ligation was conducted under the following conditions: 4 µl 5X buffer ligase, 1 µl 5X T4 ligase, 5 µg purified DNA, and sterile dH_2O for a total volume of 20 µl at 16°C overnight. For transformation, the ligated product (2 µl) was added to 50 µl DH5α cells (*E. coli* chemically competent cells), mixed gently and incubated for 30 minutes on ice. Next the cells were heat-shocked for 30 seconds at 42°C without shaking in a waterbath. The tubes were immediately placed on ice, 250 µl of room temperature LB

was added to the cells then incubated at 37°C while being agitated horizontally at a speed of 210 rpm for 1 hour. Following the incubation period, 50 µl of the transformed cells were added to an agar plate containing a final concentration of 100 µg/ml of ampicillin and incubated overnight at 37°C. Single colonies selected 24 hours later were added to 3 ml of LB supplemented with a final concentration of 100 µg/ml of ampicillin and cultured overnight. Aliquots of the culture were collected for plasmid extraction as described above and for increased production of empty vector in larger batches. A 1/500 and 1/1000 dilution of the culture was grown in 20 ml of LB (with ampicillin 100 µg/ml) and 24 hours later the plasmid was purified using QIAprep Spin Miniprep Kit (Qiagen) following the "Bench Protocol: QIAprep Spin Miniprep kit" using a microcentrifuge. The plasmid was eluted in 50 µl of sterile distilled water, and quantified on the Nanodrop 2000 spectrophotometer (Thermo Scientific). The empty vector plasmid and the plasmid containing GFI1 were resolved on an agarose gel to confirm their sizes (Fig. 2.1).

Cell cycle analysis and proliferation of GFI1-silenced CD8 T lymphocytes

Proliferation of GFI1$^{-/-}$ CD8 T lymphocytes in response to IL-4 and a suboptimal concentration of PHA-M was measured by CFSE staining. CD8 T lymphocytes were resuspended in 5% FCS / 1X PBS at concentrations lower or equal to 25x10^6 lymphocytes / 500 µl. To the cells was added an equal volumes of 5% FCS in 1X PBS containing CFSE to obtain a final concentration of 1 or 2.5 µM CFSE (25x10^6 lymphocytes – 2.5 µM / 10x10^6 lymphocytes -1 µM) followed by a 10 minute incubation at 37°C. Next, staining was quenched by adding ice cold complete IMDM and incubated on ice for 5 minutes. The CFSE-stained cells were washed 3 times in room temperature

Figure 2.1 The pCMV-SPORT6 vector containing GFI1 cDNA clone.

The average GFI1 insert size is 2.5 kb and the vector backbone is approximately 4.4 kb. The empty vector was generated as described in the materials and methods, by digesting the full length vector with Xho1 and Sal1 which creates compatible overhangs. Ligation of the empty vector was conducted, followed by transformation after which the transformed bacterial cells were streaked on an agar plate. Single colonies were collected and grown in LB broth followed by plasmid extraction. The purified vector backbone and the vector containing GFI1 (pGFI1) were digested and resolved on a 1% agarose gel to estimate the size of the products by comparing with a 1 kb ladder.

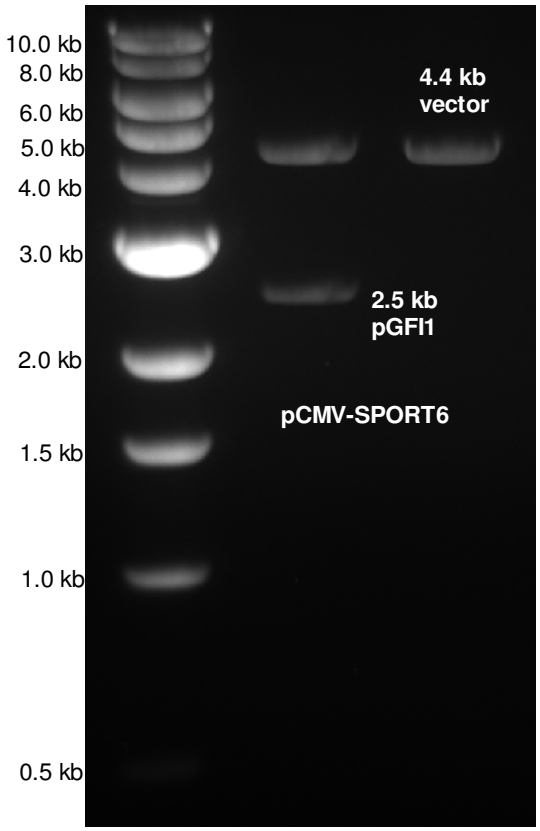

10.0 kb
8.0 kb
6.0 kb
5.0 kb
4.0 kb
3.0 kb
2.0 kb
1.5 kb
1.0 kb
0.5 kb
4.4 kb
vector
2.5 kb
pGFl1
pCMV-SPORT6

FIGURE 2.1

complete IMDM and resuspended at a concentration of $8x10^5$ cells / ml in complete media. The cells were seeded at a concentration of $4x10^5$ cells / 500 µl and stimulated with IL-4 (50 ng/ml) and PHA-M (2 µg/ml). The cells were transfected as previously described, but in complete IMDM with siGFI1 on day 2 and harvested at daily intervals beginning on day 2 to conduct flow cytometry analysis. Cell cycle entry and cell death was studied by propidium iodide (PI) staining on $1x10^5$ harvested cells from the proliferation assay. The cells were fixed with 100 µl of cold absolute methanol, vortexed, and incubated for 15 minutes at 4^oC. Following 2 washes in 2 ml of 1X PBS at 1130 x g at room temperature, the cells were vortexed with 25 µl of PI (1 mg/ml) and 25 µl of RNase (10 µg/ml) and incubated for 1 hour at 4^oC. Flow cytometry analysis was conducted after one wash in 1X PBS to analyze cell cycle entry or cell death.

Statistical analysis

Significance for IL–7Rα^{high} to IL-7Rα^{low} CD8 T lymphocyte comparisons in each donor was established by calculating 95% confidence intervals. The T-test and Bonferroni test were used for between group and multiple column comparisons, respectively. Pearson's *r* (two-tailed) was used to determine correlations between continuous variables. $p \leq 0.05$ was considered significant.

CHAPTER 3

Inverse Association of Repressor GFI1 with CD8 T Cell IL-7Rα Expression and Limited STAT Signaling in Response to IL-7 among γc Cytokines in HIV+ Patients

Introduction

Cytotoxic CD8 T lymphocyte (CTL) responses are critical to control HIV replication and progression to AIDS. However, despite seemingly vigorous responses in HIV-infected patients, CD8 T cells fail to prevent establishment of chronic infection, and exhibit altered differentiation patterns, impaired lytic capacity, and increased susceptibility to apoptosis [86,89,96,125,220,362]. The proliferation, differentiation, function, and maintenance of these cells is largely controlled by cytokines, particularly those sharing the common γ chain (γc) receptor subunit, including Interleukin (IL)-2, IL-7, and IL-15 through cytokine-induced activation of the Signal Transducer and Activator of Transcription (STAT) intracellular signalling pathway [30,52,130].

Kryworuchko et al. have previously shown that in a subset of HIV-infected volunteers naïve to HAART stimulation of CD8 T cells with IL-2 did not lead to STAT5 activation despite substantial expression of the intermediate affinity IL-2R (IL-2Rβ and γc) [98]. Defective STAT5 activation was associated with impaired activation of the upstream Jak3 kinase, but was restored under HAART [98]. Therefore, CTL alterations observed in HIV+ patients may be related to STAT signaling defects in response to IL-2 and other cytokines known to be critical in the growth, differentiation, and maintenance of these cells. Though the mechanism remains unclear, IL-7Rα is downregulated in CD8 T cells from viremic HIV+ patients, a phenomenon associated with an increased proportion of effector-like, low-IL-7Rα-expressing (IL-7Rα^{low}) memory CD8 T cells that is partially restored under ART [86,89,96,238]. The literature has also demonstrated a role for GFI1 in repressing IL-7Rα expression in murine T cells [189,301]. Furthermore, the sequence required for GFI1 to bind the IL-7Rα promoter in murine T cells also exists

in human CD8 T cells [189,329,330]. The exhausted phenotype exhibited by IL-7Rα^{low} CD8 T cells has also been observed in T cells expressing PD-1 which appears to be selectively regulated in HIV-infected persons [362-367]. PD-1 becomes downregulated on infected cells likely to maximize cell survival by protecting it from early apoptosis and thus contributing to the spread of the virus, but this protein is increased on HIV-specific T cells thus rendering these cells sensitive to apoptosis [362,366].

I hypothesized that altered differentiation patterns and defects in CD8 T cell function may be due to impaired activation of the Jak/STAT pathway by cytokines that are critical to drive these processes. In order to investigate this hypothesis, I evaluated the capacity of CD8 T cells from HIV+ volunteers to signal through the Jak/STAT pathway in response to cytokines *ex vivo* including IL-2, IL-4, IL-7, IL-15 as well as IL-10, all reputed to impact CD8 T cell growth [52,116,122,129,130,193,211,368]. The patients in this study were off-therapy for greater than 6 months and those on ART for greater than 1 year (Table 2.1), and were selected based on previous work from this laboratory [98]. Furthermore, I investigated the potential molecular mechanism responsible for the alterations in Jak/STAT observed. I established that STAT5 activation was not impaired in response to IL-2 and IL-15 as well as STAT6 in response to IL-4 in the HIV+ individuals studied when compared to HIV-negative donors. However, in response to IL-7, STAT5 phosphorylation was severely disrupted in CD8 T cells from viremic HIV-positive individuals off-therapy. Moreover, this disruption correlated with reduced IL-7Rα expression.

The second objective aimed to provide insight into the mechanism mediating this IL-7Rα repression in CD8 T cells from HIV-infected patients. As previously mentioned,

the IL-7Rα^{low} phenotype has been shown to be regulated by GFI1 in mice, and it is currently the only transcriptional modulator identified as an IL-7Rα repressor. Thus studies were conducted in IL-7Rα^{low} and IL-7Rα^{high} expressing CD8 T cell subsets isolated from HIV+ and HIV-negative volunteers. Results showed that IL-7Rα mRNA levels were higher in the subsets identified as IL-7Rα^{high} CD8 T cells versus their IL-7Rα^{low} counterparts. Interestingly, my findings showed that the IL7Rα^{low} phenotype in CD8 T cells may indeed be regulated at the transcriptional level since GFI1 mRNA expression was increased in IL7Rα^{low} CD8 T cells, compared to IL7Rα^{high} CD8 T cells. Expression of the transcriptional activator GABPα did not exclude this protein in IL-7Rα regulation, but failed to support its role. Furthermore, increased PD-1 levels were observed in CD8 T cells from viremic HIV-positives donors, but did not distinguish the IL-7Rα^{low} from the IL-7Rα^{high} subset of cells.

Results

Objective 1. Evaluate the capacity of CD8 T cells in HIV-infected individuals to signal through the Jak/STAT pathway in response to cytokines important in their cytotoxicity, maintenance, survival, and growth.

IL-7Rα^{low} CD8 T cells fail to activate STAT5 in response to IL-7, but maintain Jak/STAT signaling following IL-2, IL-4, IL-15, IL-21, and IL-10 stimulation

I evaluated the capacity of CD8 T cell populations (IL-7Rα^{high} and IL-7Rα^{low}) found in chronically-infected HIV+ patients to recruit the STAT signaling pathway in response to a panel of cytokines implicated in their growth and differentiation. The focus was on γc-sharing cytokines (IL-2, IL-4, IL-7, IL-15, IL-21) and IL-10 which exhibits both stimulatory and inhibitory effects on CD8 T cells [52,128]. Interestingly, IL-7 stimulation revealed a substantial % of CD8 T cells from HIV+ patients that failed to activate (tyr-phosphorylate) STAT5 (pSTAT5-negative) by flow cytometry (Fig. 3.1a). In contrast, pSTAT5 was induced by IL-7 in the majority of CD8 T cells from HIV-negative controls, but pSTAT5-negative cells were detectable, albeit at a significantly lower frequency compared to HIV+ individuals (Fig. 3.1a). Moreover, impaired pSTAT5 activation was apparently unique to IL-7, as stimulation with IL-2, and IL-15 did not reveal the same pSTAT5-negative population (Fig. 3.1a). Furthermore, pSTAT6 and pSTAT3 induction by IL-4, and IL-10 and IL-21, respectively was not impaired (Fig. 3.1a). Overall, pSTAT5-negative CD8 T cells in response to IL-7 represented on average 15.6 $\pm$ 7.1 % in HIV-negative controls, increased in off-therapy patients (56.0 ± 14.2 %),

and in patients undergoing ART (34.2 ± 13.5 %), values remained higher, but returned towards that of HIV-negative controls (Fig. 3.1b). In addition, HIV-positive individuals had a population of CD8 T cells that expressed substantially low levels of IL-7Rα compared to HIV-negative controls (Fig. 3.2a). Furthermore, the capacity of CD8 T cells to activate STAT5 in response to IL-7 strongly correlated ($r=0.97$, $p<0.001$) with cell surface IL-7Rα expression (Fig. 3.2b).

Figure 3.1 Impaired IL-7-dependent STAT5 activation in CD8+ T cells isolated from HIV+ individuals.

PBMCs from (a) HIV-positive individuals (top panel) and HIV-negative controls (bottom panel) were stimulated for 15 minutes at 37^{o}C with IL-7 (1.5 ng/ml), IL-2 (34 ng/ml), IL-15 (2 ng/ml), IL-10 (1 ng/ml), IL-4 (2 ng/ml), and IL-21 (40 ng/ml). The indicated STAT activation (pSTAT) was analyzed in CD8 T cells by flow cytometry using a BD FACS Canto analyzer, and data was analyzed using WinMDI 2.8 software (Joe Trotter, Scripps Institute, San Diego). The histograms shown are representative of results in gated CD8 T cells from one HIV-positive individual off-therapy and one HIV-negative control. Dashed box indicates pSTAT5-negative CD8 T cells following IL-7 stimulation. (b) Comparison of % pSTAT5-negative CD8 T cells following IL-7 stimulation in CD8 T cells from HIV-negative controls and HIV+ patients off-therapy (-) and on ART (+). Each symbol represents data from one study participant and mean values for each patient group are indicated by a horizontal dash and plotted along with the SD (vertical error bars). The Bonferroni test for multiple comparisons was used to generate *P* values where $P<0.001$ (***) and $P<0.01$ (**).

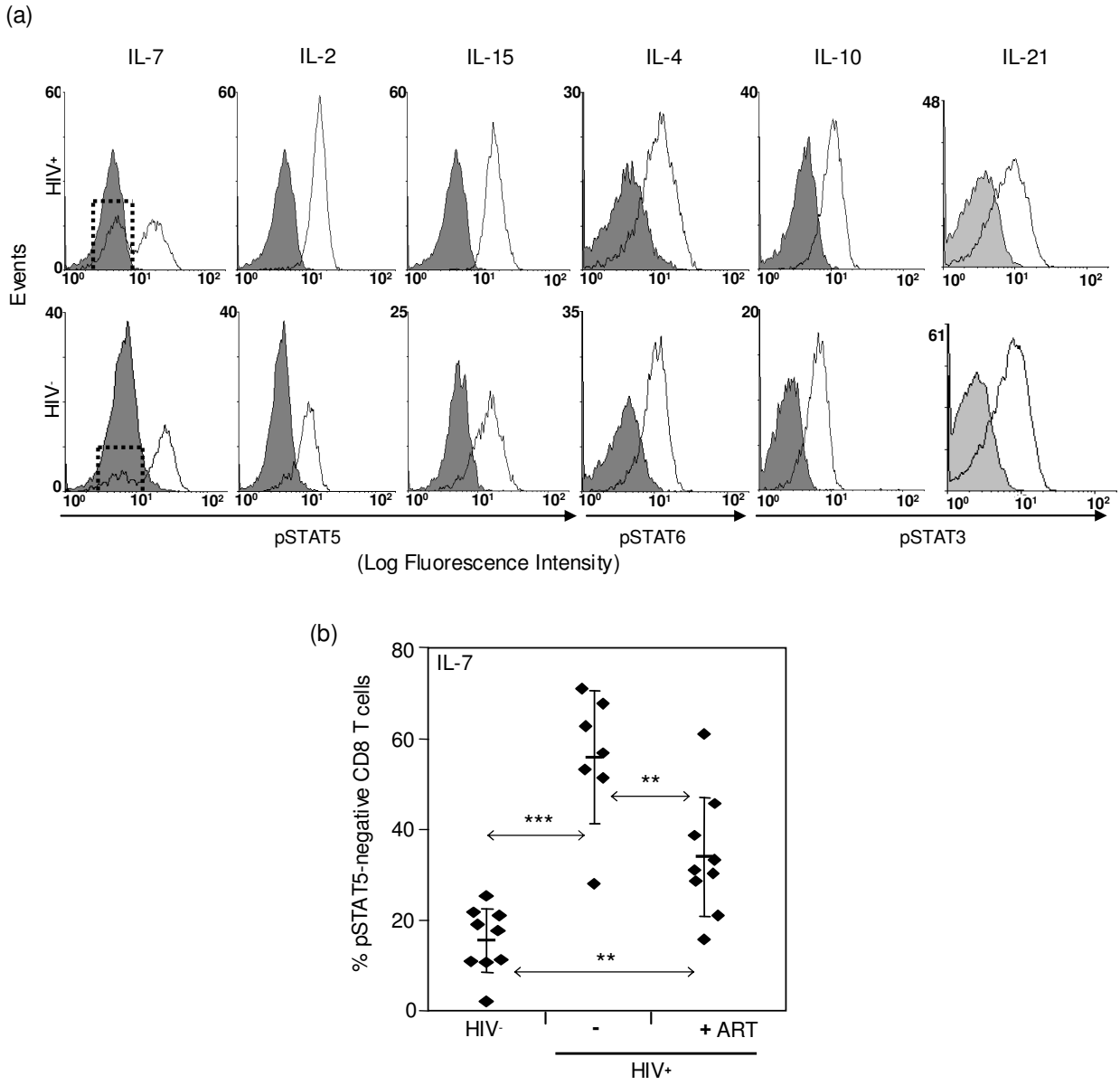

(a)
IL-7
IL-2
IL-15
IL-4
IL-10
IL-21
HIV+
HIV-
Events
pSTAT5
pSTAT6
pSTAT3
(Log Fluorescence Intensity)
(b)
IL-7
% pSTAT5-negative CD8 T cells
***
**
**
HIV-
-
+ ART
HIV+

FIGURE 3.1

Figure 3.2 Positive correlation between pSTAT5 negative CD8 T cells and low IL-7Rα expression in human CD8 T cells.

Flow cytometry analysis was conducted to measure IL-7Rα surface expression using the PE anti-IL-7Rα antibody and STAT5 phosphorylation was visualized by intracellular staining using Alexa-fluor 488-conjugated anti-STAT5 antibody (a) IL-7Rα and CD8 expression in PBMCs from one representative HIV-negative donor (left plot) and one HIV+ patient off-therapy (middle plot) is shown. (b) The % pSTAT5-positive CD8 T cells in response to IL-7 stimulation vs % IL-7Rα^{high} CD8 T cells in HIV-positive patients [on ART (◊) and off-therapy (♦)] was compared (right scatter plot). Pearson's r and P values are also shown.

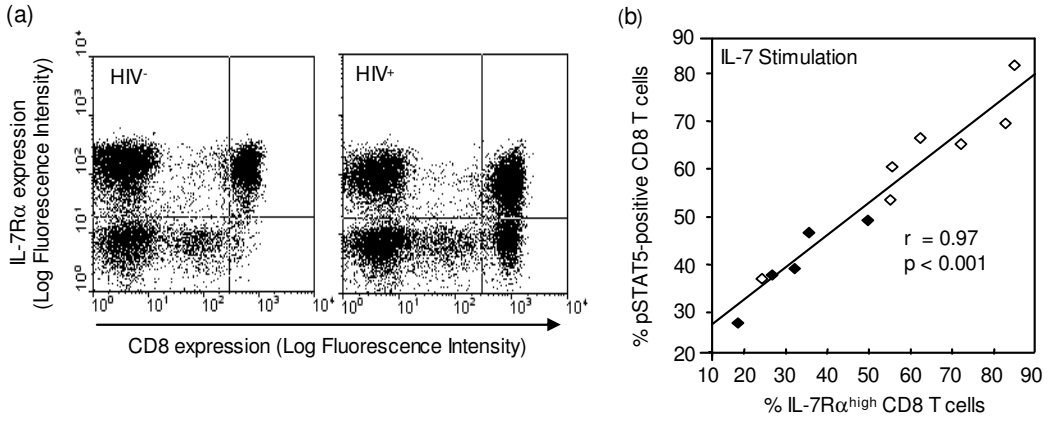

(a)
HIV-
HIV+
IL-7Rα expression
(Log Fluorescence Intensity)
CD8 expression (Log Fluorescence Intensity)
(b)
IL-7 Stimulation
r = 0.97
p < 0.001
% pSTAT5-positive CD8 T cells
% IL-7Rα high CD8 T cells

FIGURE 3.2

Objective 2. Identify the potential molecular mechanism that may be involved in the defective IL-7-dependent STAT5 activation and reduced IL-7Rα expression observed in patient CD8 T cells.

Reduced levels of GFI1in IL-7Rα^{high} *vs.* IL-7Rα^{low} CD8 T cells

To investigate the molecular mechanism by which IL-7Rα expression may be downregulated in CD8 T cells from HIV+ patients, mRNA expression of IL-7Rα and a number of reputed transcriptional regulators of the IL-7Rα gene was studied by RT-PCR. In Fig. 3.3a, relative mRNA expression of these genes is presented as fold change, comparing purified IL-7Rα^{high} to IL-7Rα^{low} CD8 T cells from HIV+ patients. GFI1 mRNA expression was significantly reduced (95% confidence) in IL-7Rα^{high} *vs.* IL-7Rα^{low} CD8 T cells of HIV+ patients and this was paralleled by an increase in IL-7Rα mRNA expression. Expression of the transcription factor GABPα was not significantly altered in IL-7Rα^{high} *vs.* IL-7Rα^{low} CD8 T cells. GFI1B, the other GFI1 repressor family member, was undetectable in these two CD8 T cell sub-populations (data not shown). As the IL-7Rα^{low} subset was also detectable, albeit at dramatically reduced proportions, similar experiments were performed in HIV-negative controls and yielded comparable results (Fig. 3.3b).

Recent studies [362-364] suggesting a role for inhibitory receptor PD-1 in HIV-specific CD8 T cells dysfunction, prompted the evaluation of PD-1 expression in IL-7Rα^{low} and IL-7Rα^{high} CD8 T cell populations. Although the %PD-1+ CD8 T cells was increased in HIV+ patients compared to HIV-negative controls, there was no significant

difference in %PD-1+ cells between IL-7Rα^{low} and IL-7Rα^{high} CD8 T cell sub-populations (Fig. 3.4).

Figure 3.3 Reduced GFI1 mRNA levels in IL-7Rα^{high} compared to IL-7Rα^{low} CD8 T cells.

IL-7Rα^{high} and IL-7Rα^{low} CD8 T cells were purified from (a) HIV-positive donors and (b) HIV-negative controls. RNA extracts from purified CD8 T cell populations were reverse transcribed and used in Real Time PCR experiments to evaluate the expression of IL-7Rα, GFI1, GFI1B, GABPα and βactin. Relative gene expression levels were determined by the comparative Ct method [360]. Results are plotted as fold change comparing IL-7Rα^{high} vs. IL-7Rα^{low} CD8 T cells from each patient for the indicated gene.

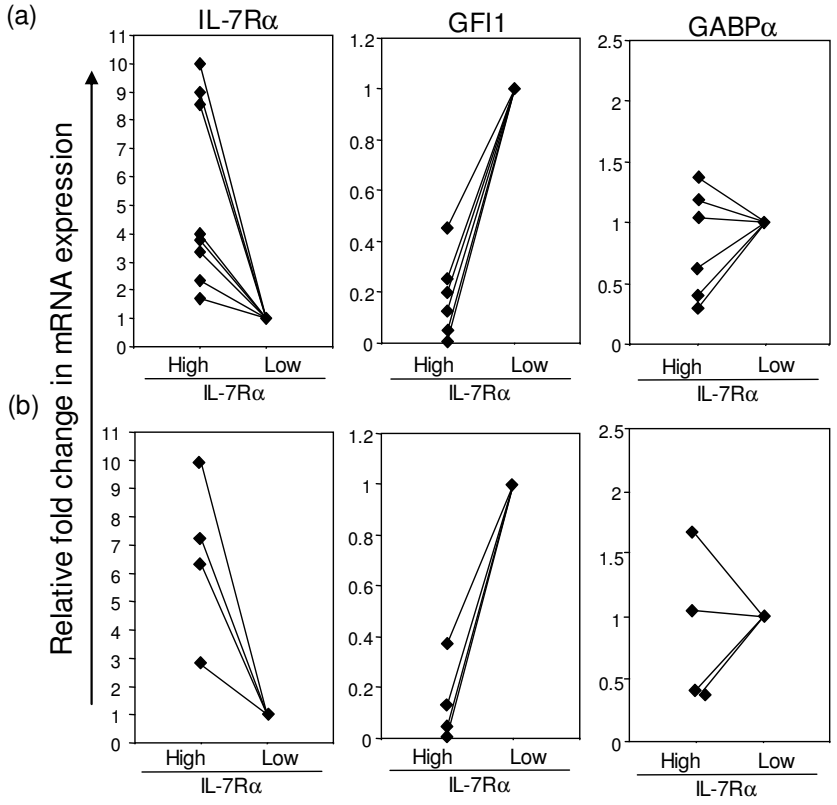

(a)
(b)
IL-7Rα
GFI1
GABPα
Relative fold change in mRNA expression
High
Low
IL-7Rα

FIGURE 3.3

Figure 3.4 Enhanced PD-1 expression in CD8 T cell populations of HIV-positive individuals on and off ART.

PD-1 surface expression on IL-7Rα^{high} and IL-7Rα^{low} CD8 T cells of HIV-negative controls and HIV-infected donors [on ART (+), and off-therapy (-)] was determined by flow cytometry and plotted as % PD-1-postive cells. Similar results were obtained when PD-1 expression was compared by mean fluorescence intensity (MFI). For all data, each symbol represents results from one patient. Mean values for each patient group are indicated by a horizontal dash and plotted along with the SD (vertical error bars). The Bonferroni test was applied for multiple group comparisons. *P* values are indicated only where significant where $P<0.001$ (***) and $P<0.05$ (*). Due to limitations in the number of cells obtained from some patients, it was not possible to conduct all experiments on all patient samples and this is reflected in the number of data points plotted.

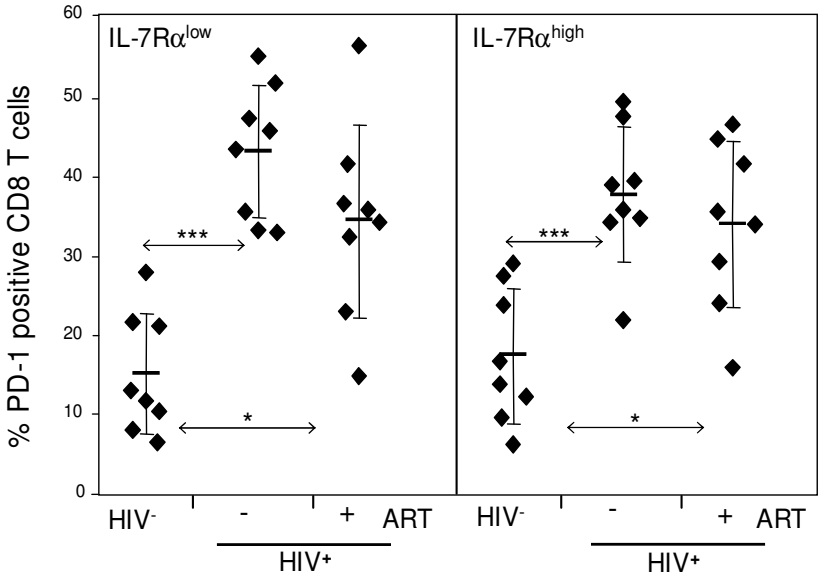

% PD-1 positive CD8 T cells
60
50
40
30
20
10
0
IL-7Rα^low
IL-7Rα^high
***
*
***
*
HIV-
-
+
ART
HIV+
HIV-
-
+
ART
HIV+

FIGURE 3.4

CHAPTER 4

Functional Role of Growth Factor Independent -1 (GFI1) in Primary Human CD8 T Lymphocytes: Repressor Activity, Survival and Proliferation

Introduction

GFI1 may impact many different biological processes and disease states including the development of immunity as well as HIV/AIDS progression [331,334,338,369,370]. Consistent with this is the fact that GFI1 has numerous transcriptional targets including IL-7Rα, including genes such as the pro-apoptotic protein Bax, and the chemokine receptor CXCR4, a co-receptor for HIV [317,333,371]. In addition, GFI1 binding sites have been localized to genes with functionally distinct roles including cell cycle regulators and various transcription factors [298,317,356,358]. However, none of the functional roles described for GFI1 have been substantiated in mature human CD8 T lymphocytes.

A comparative genomics study of mammalian CXCR4 orthologs identified highly conserved double GFI1 binding sites among human, chimpanzee, mouse, and rat CXCR4 orthologs [372]. CXCR4 is a transmembrane G protein-coupled receptor for the chemokine CXCL12 and is expressed on T and B cells, monocytes as well as dendritic cells and endothelial cells. In humans, the double GFI1 binding sites are located within the proximal enhancer region of the CXCR4 gene. GFI1 has also been shown to associate with the IL-7Rα promoter to suppress the receptor in murine CD8 T lymphocytes, but not in CD4 T cells and the mechanism implicated is IL-7 driven [189]. Findings described in chapter 3 suggested a role for GFI1 in the repression of IL-7Rα expression in human CD8 T cells [373]. The molecular mechanism involved in IL-7Rα downregulation in human CD8 T cells under normal physiological conditions and in the context of disease such as chronic HIV infection remains unclear.

Aberrant GFI1 expression has also been implicated in altering cell proliferation and survival [320,323,356,357]. For example GFI1 expression is high in developing cells, but increases in mature T cells upon activation [346,355]. Furthermore, GFI1 has been implicated in restricting proliferation in hematopoietic stem cells [374,375] whereas in lymphoid cells, GFI1 has a role in promoting cellular expansion [320,323,329,334,356,357]. Interestingly, IL-4 upregulates GFI1 expression in murine Th2 CD4 T lymphocytes thereby driving their proliferation [356]. In other reports, this cytokine downregulated IL-7Rα expression both in human and murine CD8 T lymphocytes [189,376]. However, the molecular mechanism for this and the role of GFI1 in human CD8 T cells has not been previously described.

Increased GFI1 expression levels in human IL-7Rα^{low} CD8 T cells may have a role in the persistence of effector-like memory IL-7Rα^{low} CD8 T cells observed during chronic HIV infection. Reduced Bcl-2 expression and increased expression of apoptotic factors render low IL-7Rα-expressing CD8 T cells in HIV-positive individuals more sensitive to apoptosis *ex vivo*, but *in vivo* these cells are maintained at high levels [45,51,86,101,235,306,377]. Interesting parallels exist between effector-like IL-7Rα^{low} CD8 T cells, murine lymphocytes and cell lines with enhanced GFI1 expression which may partially explain the increased proportions of IL-7Rα^{low} CD8 T cells *in vivo* in viremic HIV-positive patients. Furthermore, a cooperative interaction between GFI1 and the ETS1 transcription factor in the Bax promoter occurs to repress this pro-apoptotic factor [317]. Similar phenomenons involving GFI1 may enhance the persistence of IL-7Rα^{low} CD8 T cells *in vivo*.

The aim of this part of the thesis was to more directly explore the hypothesis that GFI1 is involved in repressing IL-7Rα expression in human CD8 T cells, but also to determine whether it plays a role in their survival and proliferation. The first objective was to investigate the role of GFI1 in regulating IL-7Rα expression. I observed that silencing GFI1 did not have the effect of further increasing basal IL-7Rα expression, but exogenous overexpression downregulated receptor expression. In light of previous publications [189,237,286,306] showing IL-7Rα regulation was cytokine driven, I evaluated the effect of various cytokines with known activities on CD8 T cells to downregulate IL-7Rα expression. Amongst the cytokines tested only IL-4 stimulation mediated suppression of IL-7Rα expression that was accompanied by GFI1 upregulation. Consistent with this, I found that IL-4-induced repression of IL-7Rα expression could be curtailed by GFI1 silencing. Overexpression of GFI1 was also able to inhibit CXCR4 and Bax expression levels. The second objective focused on exploring whether GFI1 influenced human CD8 T cell proliferation and survival. Interestingly, I demonstrated that GFI1 may accelerate IL-4-dependent entry into the cell cycle.

Results

Objective 3. Evaluate the regulation of IL-7Rα expression and the role of GFI1 in resting and cytokine-stimulated human CD8 T lymphocytes.

Effect of GFI1 on basal expression levels of IL-7Rα in primary human CD8 T lymphocytes

I detected elevated levels of the transcriptional repressor GFI1 in IL-7Rα^{low} human CD8 T cells compared to their IL-7Rα^{high} counterparts [373]. Therefore in this section, I evaluated more directly whether GFI1 negatively regulates basal IL-7Rα expression levels in primary human CD8 T lymphocytes. As described in the materials and methods, CD8 T cells were purified by negative selection from the peripheral blood of healthy volunteers and exhibited a predominantly high IL-7Rα expression level. To investigate whether GFI1 impacts basal IL-7Rα expression levels, silencing of GFI1 was performed using two GFI1 siRNAs targeting opposite ends of the gene. In brief, the purified human CD8 T cells were transfected with the siRNAs. Aliquots of the cells were analyzed 24 hours later for IL-7Rα expression by flow cytometry as well as RT-PCR analysis and western blotting. Interestingly, GFI1 silencing did not have any significant effect on basal IL-7Rα surface expression levels (Fig. 4.1a). Reduction in GFI1 expression by specific siRNAs was confirmed (Fig. 4.1b) and assessment of knockdown efficiency indicated that GFI1 mRNA levels were reduced to 23% and 38% of their initial levels (Fig. 4.1c). It is possible that GFI1 expression may not have been sufficiently suppressed for its effect on IL-7Rα expression to be observed. Therefore,

Figure 4.1 Reduced GFI1 expression has no effect on basal IL-7Rα expression levels.

Purified CD8 T cells were transfected with one of two distinct siRNA sequences targeting the GFI1 gene or with a non-silencing control. (a) An aliquot of the CD8 T cells was collected at 24 hours post-transfection and IL-7Rα surface expression was visualized by staining with a PE-conjugated anti-IL-7Rα antibody and flow cytometry analysis. The histogram is representative of 3 independent experiments. (b) GFI1 silencing was determined by flow cytometric analysis of intracellular protein expression. (c) RNA was extracted from an aliquot of transfected CD8 T cells, reverse transcribed and RT-PCR analysis was conducted to measure GFI1, IL-7Rα, and GABPα mRNA expression levels. Relative gene expression levels were determined by the comparative Ct method [360]. The bar graph is a compilation of 3 independent experiments showing the relative mRNA expression levels, and the vertical bars represent the SD. The Bonferroni test for multiple comparisons was used to generate *P* values where $P<0.01$ (**) and $P<0.05$ (*).

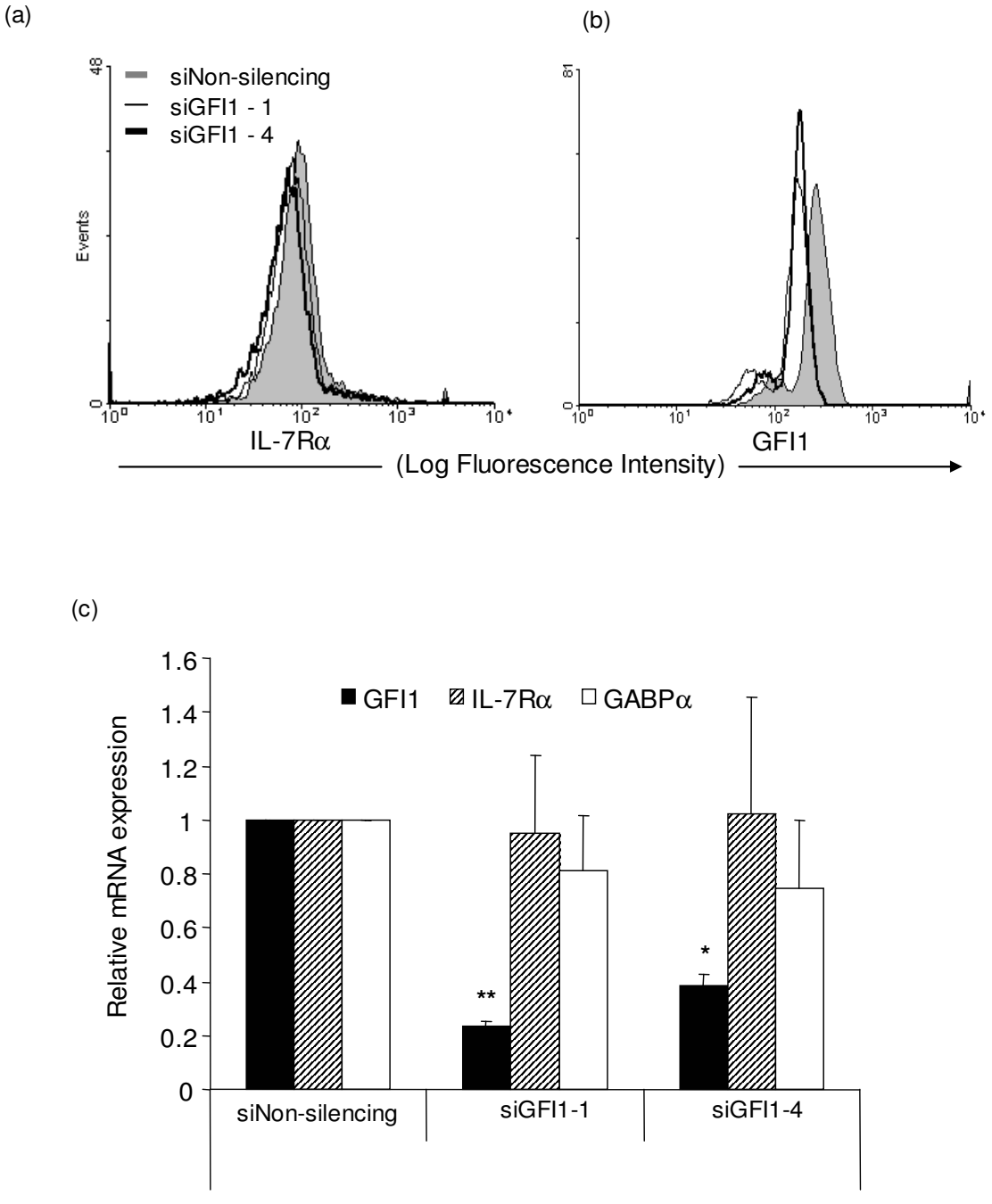

(a)
(b)
siNon-silencing
siGFI1 - 1
siGFI1 - 4
Events
IL-7Rα
GFI1
(Log Fluorescence Intensity)
(c)
GFI1
IL-7Rα
GABPα
Relative mRNA expression
siNon-silencing
siGFI1-1
siGFI1-4

FIGURE 4.1

overexpression of GFI1 was conducted by transient transfection of primary CD8 T cells with a plasmid expressing a full length GFI1 cDNA. Interestingly, IL-7Rα expression was reduced by 45% at 24 hours on average in GFI1-transfected cells compared to controls (Fig. 4.2). These results demonstrate that GFI1 was indeed capable of negatively regulating IL-7Rα expression in primary human CD8 T lymphocytes.

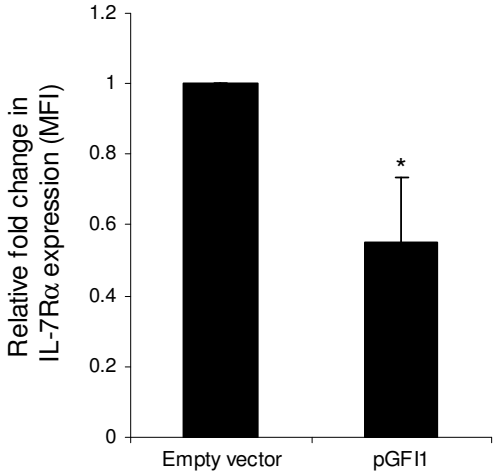
IL-4 stimulation of primary human CD8 T lymphocytes increases GFI1 expression levels while suppressing IL-7Rα expression

Since the above siRNA experiments yielded negligible effects on IL-7Rα expression, I hypothesized that GFI1 may be more important for regulating cytokine-mediated alterations in IL-7Rα expression rather than basal levels. In support of this, is the fact that GFI1 expression is low in resting T cells, but increases upon activation and cytokine stimulation [346,355]. In mice, IL-7 induced GFI1 appears to be involved in IL-7Rα downregulation whereas IL-4-induced GFI1 expression has so far not been associated with downregulating the receptor [189]. Therefore, I aimed to establish a model study system where cytokine-induced downregulation of IL-7Rα via the action of GFI1 could be evaluated. I tested the effect of a series of cytokines with known effects on CD8 T cells, many of which (γc cytokines in particular) having been found to modulate IL-7Rα and/or GFI1 expression in other systems. The glucocorticoid dexamethasone was used (10^{-8} M) as a positive control having been previously shown to upregulate IL-7Rα expression (Fig. 4.3a) [318,352].

CD8 T cells were incubated with cytokines at concentrations sufficient to induce signaling of their respective Jak/STAT pathways and analyzed after 24 and 48 hours by

Figure 4.2 Exogenous overexpression of GFI1 negatively regulates IL-7Rα expression. CD8 T cells were transfected with pGFI1 or an empty vector and incubated for 24 hours. CD8 T cell aliquots were stained with PE-conjugated anti-IL-7Rα antibody followed by flow cytometry analysis. (a) The bar graphs depicts 3 independent experiments showing the relative MFI fold change in IL-7Rα expression between CD8 T cells transfected with an empty vector compared to cells overexpressing GFI1. The vertical bars represent the SD. The T- test was used to generate *P* values where $P < 0.05$ is indicated by (*). (b) The histogram is a representative figure showing downregulation of IL-7Rα in CD8 T cells transfected with pGFI1 compared to the empty vector control.

(a)


1.2
1
0.8
0.6
0.4
0.2
0
Relative fold change in IL-7Rα expression (MFI)
*
Empty vector
pGFI1

(b)

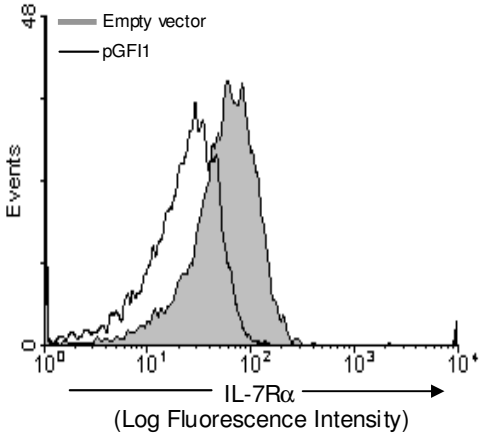

48
0
Events
Empty vector
pGFI1
10⁰
10¹
10²
10³
10⁴
IL-7Rα
(Log Fluorescence Intensity)

FIGURE 4.2

Figure 4.3 Downregulation of IL-7Rα expression by IL-2, IL-4, IL-7, and IL-15.

Isolated human CD8 T lymphocytes were incubated with IL-2 (34 ng/ml), IL-4 (2 ng/ml), IL-7 (1.5 ng/ml), IL-15 (2 ng/ml), IL-21 (20 ng/ml), and dexamethasone (Dex, 10^{-8} M) for 24 and 48 hours. (a) CD8 T cells were collected for flow cytometry analysis of surface IL-7Rα expression by staining with a PE-conjugated anti-IL-7Rα antibody. The relative fold change in IL-7Rα expression was calculated by comparing the MFI of receptor expression in cytokine stimulated cells divided by levels in unstimulated cells. (b) RNA extracts were reverse transcribed and RT-PCR was conducted to determine IL-7Rα mRNA expression levels. Relative gene expression levels were determined by the comparative Ct method [360]. Relative IL-7Rα protein and mRNA expression was represented by bar graphs where n=6 for IL-2, n=7 for IL-4 and IL-7, and n=3 for IL-15, IL-21 and Dex for 24 hour time points. Similarly for 48 hours, relative IL-7Rα protein and mRNA expression was represented by bar graphs where n=3 for IL-2, n=5 for IL-4 and IL-7, and n=3 for IL-15, IL-21 and Dex. The T- test was used to generate *P* values comparing cytokine treatment with the unstimulated (uns) group where $P < 0.001$ (***), $P < 0.01$ (**), and $P < 0.05$ (*), and the SD are represented by vertical bars.

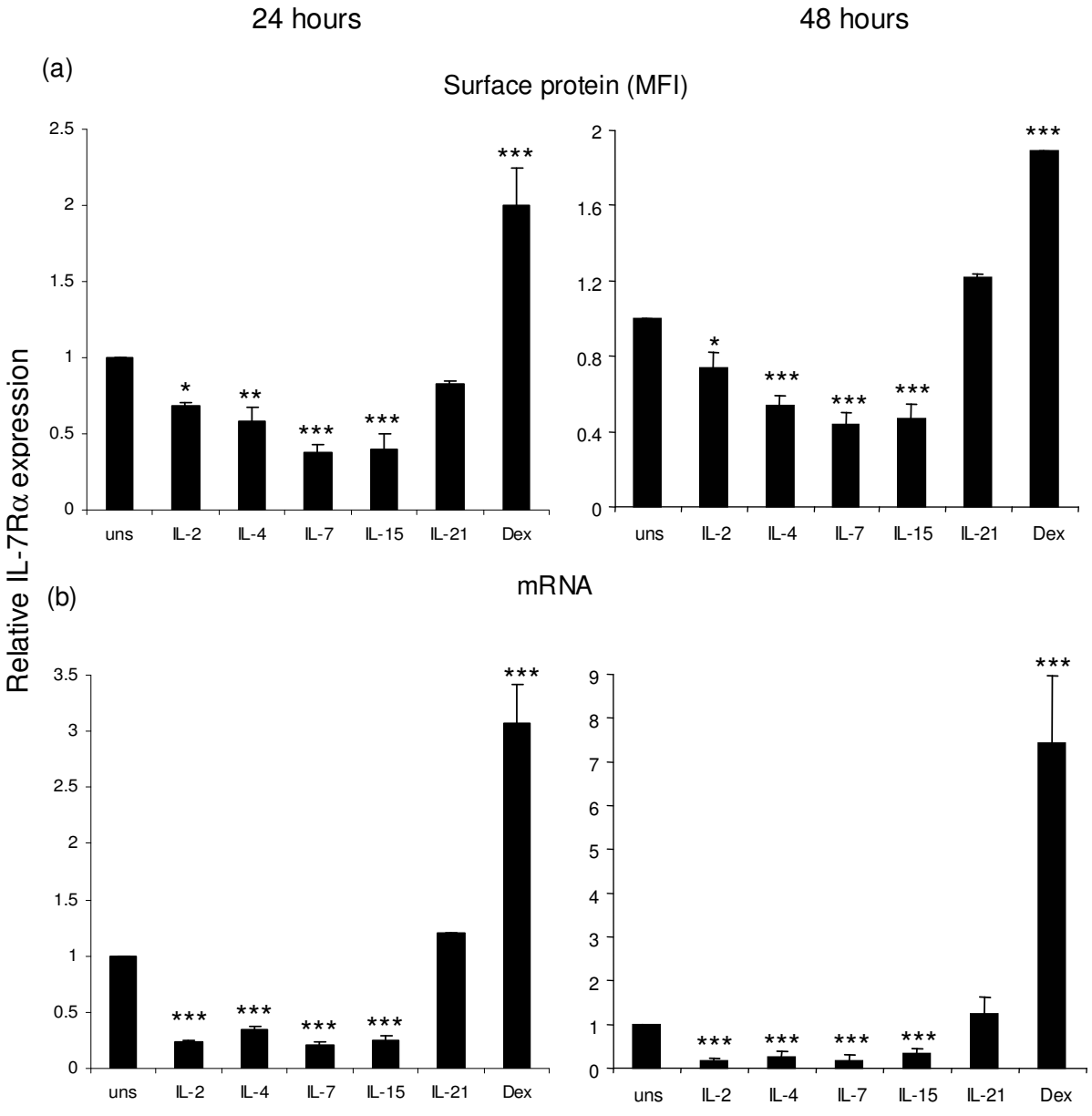

24 hours
48 hours
(a)
Surface protein (MFI)
Relative IL-7Rα expression
(b)
mRNA
uns
IL-2
IL-4
IL-7
IL-15
IL-21
Dex

FIGURE 4.3

flow cytometry for IL-7Rα expression and by RT-PCR for GFI1 expression levels. Among the γc cytokines tested IL-2, IL-4, IL-7, and IL-15 all suppressed IL-7Rα expression at the protein (Fig. 4.3a) and mRNA (Fig. 4.3b) expression levels, while IL-21 had no significant effect. IFNγ, TNFα, and IL-10 did not exhibit significant effects on IL-7Rα protein expression levels at 24 or 48 hours (Fig. 4.4a). The statistically significant effect for TNFα on IL-7Rα mRNA levels observed at 48 hours did not emerge at the protein expression levels (Fig. 4.4b). I showed that IL-2 (34 ng/ml) and IL-7 (1.5 ng/ml) stimulation reduced IL-7Rα protein and mRNA expression levels by 24 and 48 hours (Fig. 4.3a, b), but did not have any significant effect on GFI1 mRNA expression (Fig. 4.5a). Uniquely, IL-4 (2 ng/ml) upregulated GFI1 expression levels by 24 hours (Fig. 4.5b) while suppressing IL-7Rα at the protein and mRNA levels (Fig. 4.3a, b). Moreover, the suppressive effect of IL-4 on IL-7Rα expression was studied in comparison to IL-7, and with distinct kinetics being observed. Following IL-4 and IL-7 withdrawal at 24 hours post-stimulation, IL-7Rα expression was restored by 72 hours for IL-4 (Fig. 4.6a) and 48 hours for IL-7 (Fig. 4.6b). Therefore, IL-4-mediated downregulation of IL-7Rα appears to be mechanistically distinct from that of IL-7.

Delayed recovery of IL-4-induced downregulation of IL-7Rα is partially reversed upon silencing GFI1 expression

Following these findings, it was then postulated that enhanced GFI1 expression in response to IL-4 may be required to downregulate and/or sustain suppression of IL-7Rα expression in human CD8 T lymphocytes. Therefore, small-interfering RNA studies

Figure 4.4 TNFα, IFNγ, and IL-10 do not have significant effects on IL-7Rα expression.

Isolated human CD8 T lymphocytes were incubated with TNFα (10 ng/ml), IFNγ (10 ng/ml), IL-10 (1.0 ng/ml), and dexamethasone (Dex, 10^{-8} M) for 24 and 48 hours. (a) CD8 T cells were collected for flow cytometry analysis of surface IL-7Rα expression by staining with a PE-conjugated anti-IL-7Rα antibody. The relative fold change in IL-7Rα expression was calculated by comparing receptor expression MFI in stimulated cells divided by levels in unstimulated cells. (b) RNA extracts were reverse transcribed and RT-PCR was conducted to determine IL-7Rα mRNA expression levels. Relative gene expression levels were determined by the comparative Ct method [360]. Relative IL-7Rα protein and mRNA expression was represented by bar graphs where n=4 for IFNγ, n=4 for TNFα and n=3 for IL-10 and Dex for 24 hour time points. Similarly for 48 hours, relative IL-7Rα protein and mRNA expression was represented by bar graphs where n=2 for IFNγ, TNFα, and IL-10 and n=3 for Dex. The T- test was used to generate *P* values comparing cytokine treatment with the unstimulated (uns) group where $P < 0.001$ (***), and SD are represented by vertical bars.

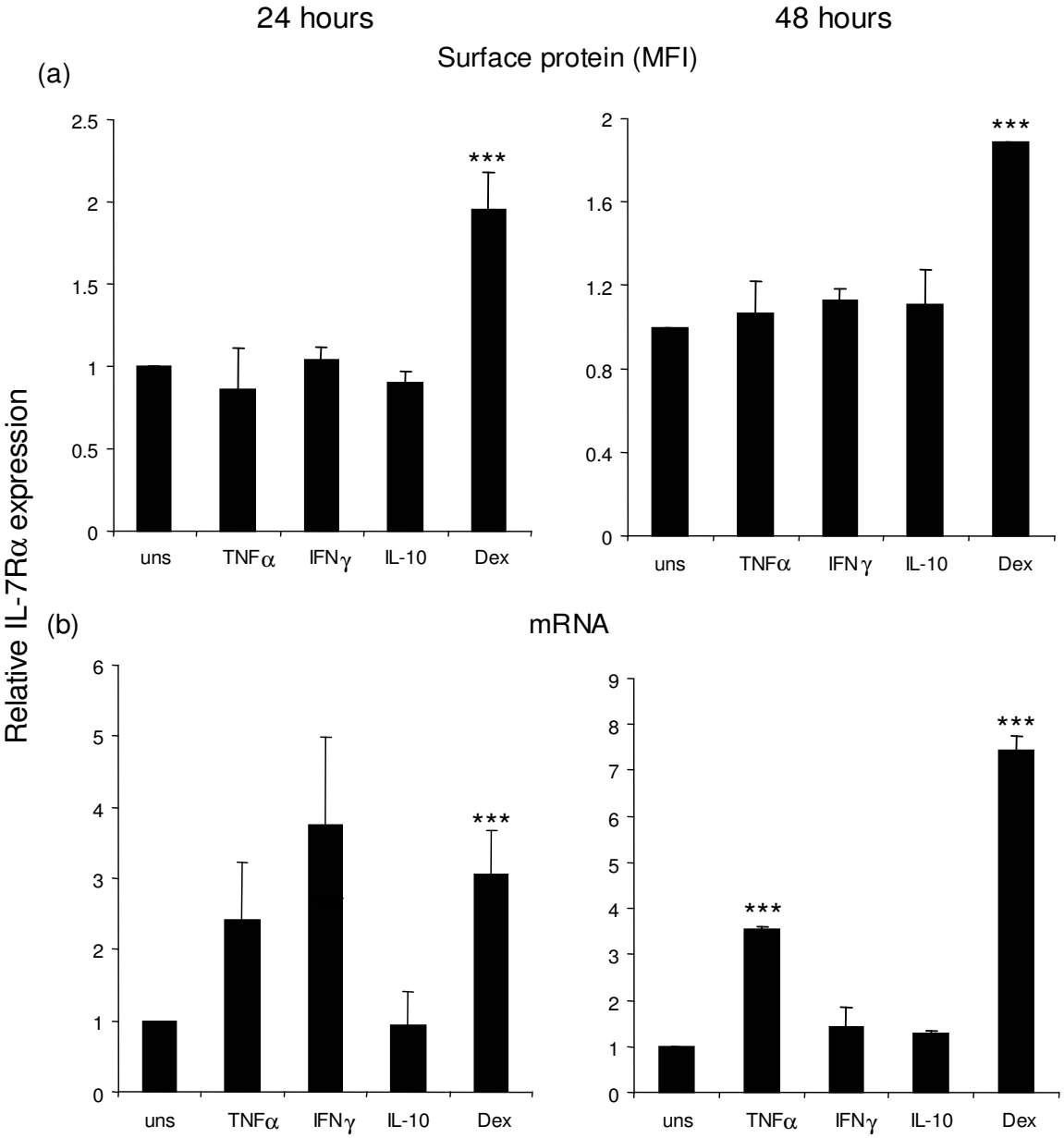

24 hours
48 hours
(a)
Surface protein (MFI)
Relative IL-7Rα expression
2.5
2
1.5
1
0.5
0
1.6
1.2
0.8
0.4
***
uns
TNFα
IFNγ
IL-10
Dex
(b)
mRNA
6
5
4
3
9
8
7

FIGURE 4.4

Figure 4.5 Upregulation of GFI1 expression by IL-4 stimulation.

Isolated human CD8 T lymphocytes were incubated with IL-2 (34 ng/ml), IL-4 (2 ng/ml), and IL-7 (1.5 ng/ml) for 24 hours. (a) GFI1 mRNA expression levels in response to IL-2, IL-4, and IL-7 stimulation were determined by RT-PCR analysis and values were used to generate a dot plot where each symbol represents an independent sample. The horizontal bar represents the average. Relative gene expression levels were determined by the comparative Ct method [360]. The T- test was used to generate *P* values comparing cytokine treatment with the unstimulated (uns) group where $P < 0.01$ (**). (b) Protein extracts (35 μg) from IL-4 (50 ng/ml) stimulated CD8 T cells were resolved on a 10% SDS-PAGE and subjected to western blotting analysis. The membranes were probed with anti-GFI1 antibodies then stripped and reprobed for βactin expression. The immunoblot is representative of at least 3 independent experiments and densitometry values were used to determine relative fold increases in GFI1 protein expression compared to βactin protein levels.

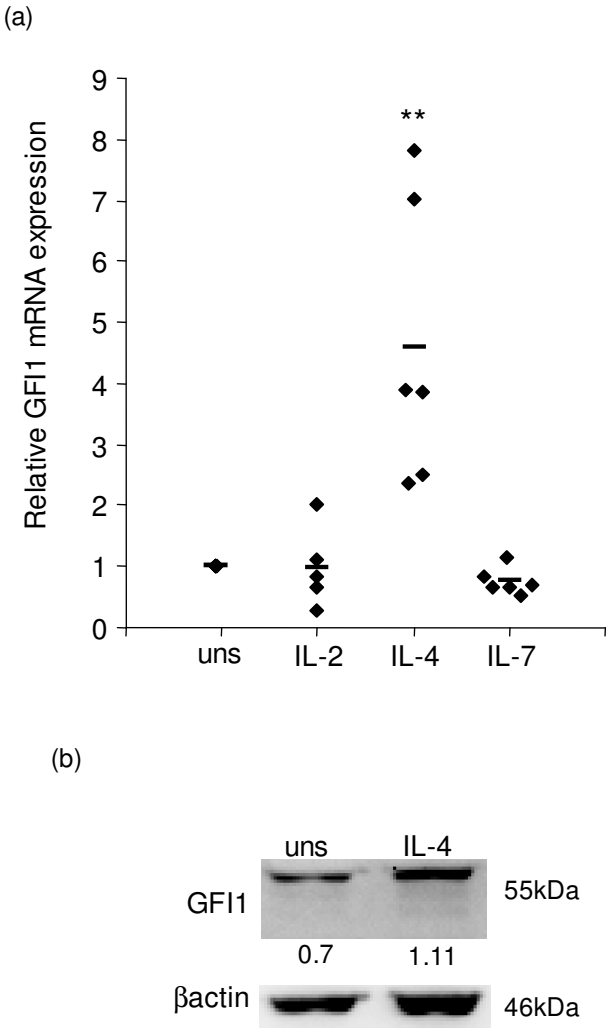

(a)
Relative GFI1 mRNA expression
9
8
7
6
5
4
3
2
1
0
**
uns
IL-2
IL-4
IL-7
(b)
uns
IL-4
GFI1
55kDa
0.7
1.11
βactin
46kDa

FIGURE 4.5

Figure 4.6 Extended suppression of IL-7Rα expression following withdrawal of IL-4 compared to IL-7Rα expression following IL-7 removal.

Purified human CD8 T cells were stimulated with (a) IL-4 (2 ng/ml) and (b) IL-7 (1.5 ng/ml) and 24 hours post-stimulation the cytokines were withdrawn by extensive washing indicated by a dashed box. IL-7Rα expression was assessed by surface staining with a PE-conjugated anti-IL-7Rα antibody and visualized by flow cytometry at 24 and 48 hours post-cytokine removal. The relative fold change in IL-7Rα expression was calculated by normalizing receptor expression MFI for each time point to the MFI at 0 hours. Each symbol represents the mean from 3 independent experiments and the vertical bars are the SD.

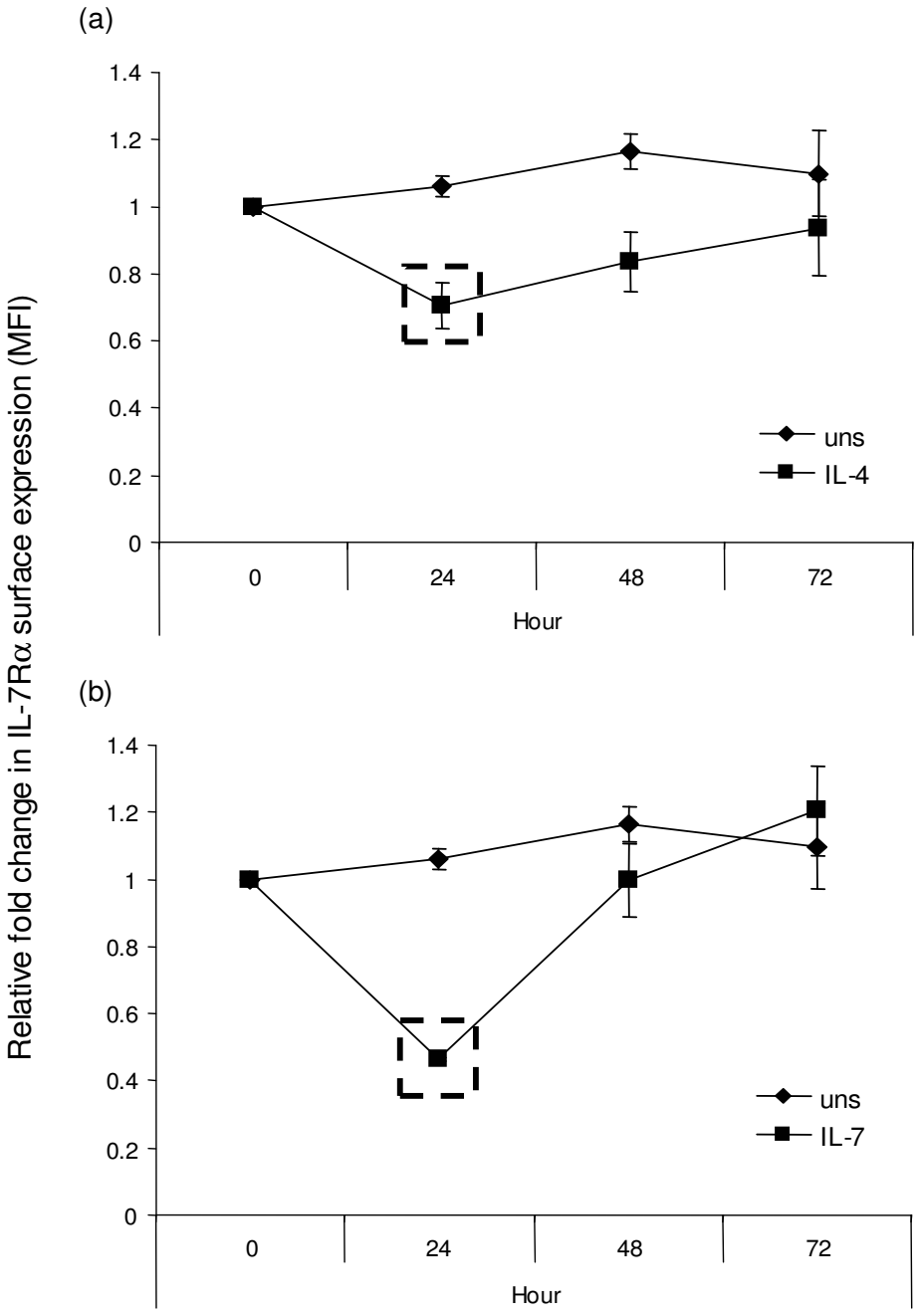

(a)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
24
48
72
Hour
uns
IL-4
(b)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
24
48
72
Hour
uns
IL-7
Relative fold change in IL-7Rα surface expression (MFI)

FIGURE 4.6

targeting GFI1 were conducted to assess this. To evaluate the role of GFI1 in IL-4-mediated downregulation of IL-7Rα, CD8 T cells were incubated with a siRNA control or GFI1-specific siRNAs for 8 hours then stimulated with IL-4 (2 ng/ml) for 24 hours. Reduced levels of GFI1 mRNA expression were confirmed in GFI1-silenced CD8 T cells by RT-PCR analysis (Fig. 4.7b). RT-PCR analysis was also used to measure IL-7Rα mRNA expression levels and surface levels by flow cytometry analysis. In CD8 T cells transfected with GFI1-specific and control siRNA, IL-7Rα expression were similarly reduced post-IL-4 stimulation at both the surface and mRNA levels (Fig. 4.7a).

To determine the role of GFI1 in sustaining suppression of IL-7Rα expression, CD8 T cells were stimulated with IL-4 (2 ng/ml) for 24 hours. Subsequently, IL-4 was withdrawn by washing, and cells were transfected with GFI1–specific and non-silencing siRNA. IL-7Rα surface expression was then measured by flow cytometry. As seen in figure 4.8a, a 20% greater recovery in IL-7Rα recovery was consistently observed after 48 hours in CD8 T cells transfected with siGFI1 compared to cells transfected with non-silencing control siRNA (Fig. 4.8a). GFI1 silencing under these conditions was confirmed at 48 hours by western blotting (Fig. 4.8b). Taken together, it appeared that GFI1 is involved in sustaining suppression of IL-7Rα expression in response to IL-4 stimulation, rather than in the process of downregulating the receptor.

Figure 4.7 IL-4-mediated downregulation of IL-7Rα expression was not affected by GFI1 silencing post-stimulation in primary human CD8 T lymphocytes.

Primary human CD8 T lymphocytes were transfected with GFI1-specific siRNAs: siGFI1-1 or siGFI1-4, or a non-silencing siRNA control (siNS), and 8 hours post-transfection CD8 T cells were stimulated with IL-4 (2 ng/ml) for 24 hours. (a) IL-7Rα mRNA expression levels (top panel) in untreated and IL-4-treated transfected CD8 T cells were measured by RT-PCR analysis, and relative gene expression levels were determined by the comparative Ct method [360]. Aliquots of CD8 T cells were collected for flow cytometry analysis of surface IL-7Rα expression (bottom panel) by staining with a PE-conjugated anti-IL-7Rα antibody. The relative fold change in IL-7Rα expression was calculated by dividing receptor expression MFI for each condition by that of unstimulated siNS-transfected CD8 T cells. (b) Relative GFI1 mRNA expression levels were also measured in IL-4-stimulated CD8 T cells transfected with GFI1-specific and control siRNA as described above. Bar graphs for IL-7Rα expression were generated where vertical bars represents the SD for 3 independent experiments for flow cytometry and 2 experiments for RT-PCR analysis, and 2 independent experiments for GFI1 mRNA levels. The Bonferroni multiple comparisons test was used to generate *P* values comparing stimulated to unstimulated transfected CD8 T cells where $P<0.001$ (***), and $P<0.01$ (**).

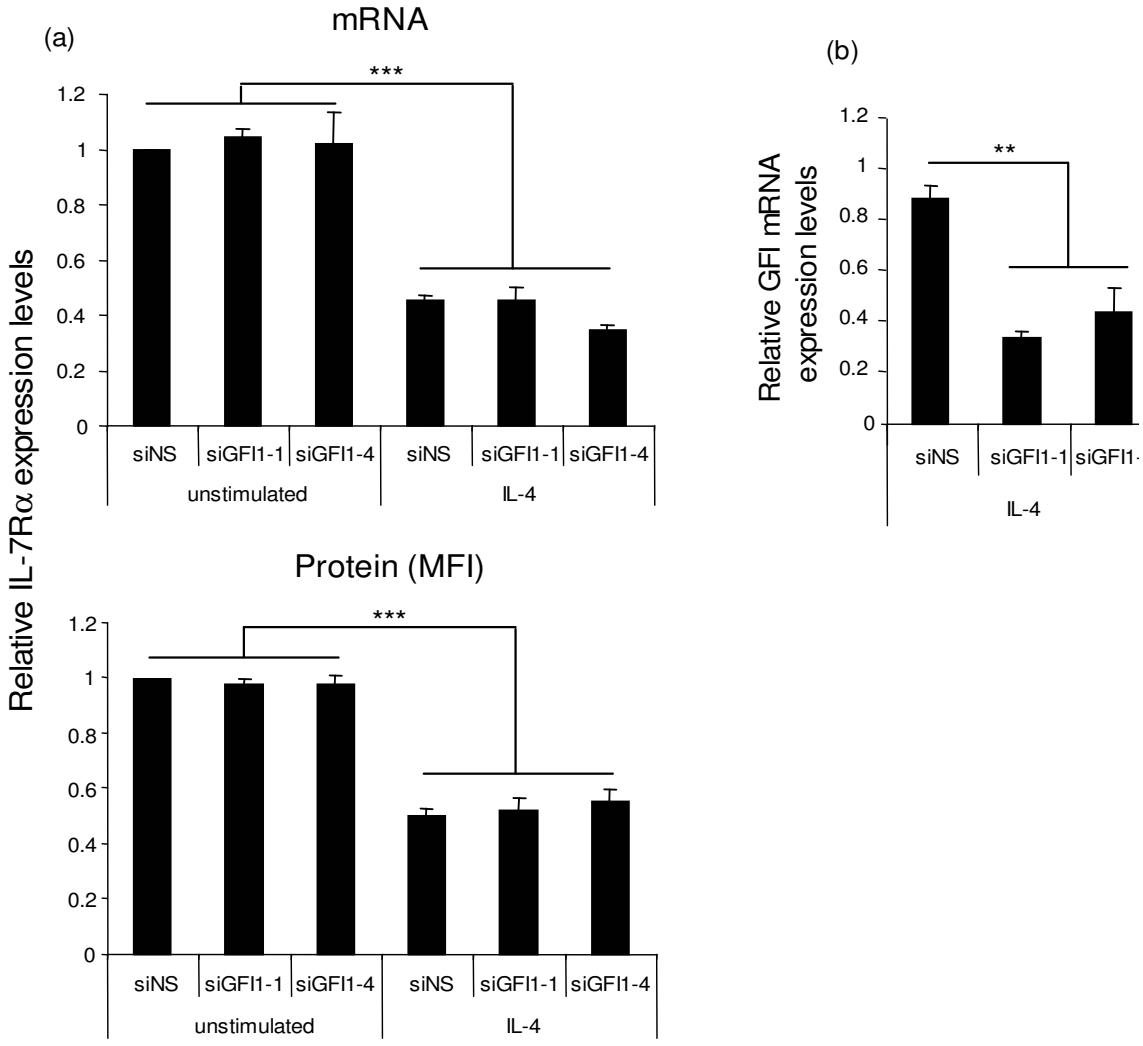

(a)
mRNA
***
1.2
1
0.8
0.6
0.4
0.2
0
siNS
siGFI1-1
siGFI1-4
siNS
siGFI1-1
siGFI1-4
unstimulated
IL-4
Relative IL-7Rα expression levels
Protein (MFI)
***
1.2
1
0.8
0.6
0.4
0.2
0
siNS
siGFI1-1
siGFI1-4
siNS
siGFI1-1
siGFI1-4
unstimulated
IL-4
(b)
**
1.2
1
0.8
0.6
0.4
0.2
0
siNS
siGFI1-1
siGFI1-
IL-4
Relative GFI mRNA expression levels

FIGURE 4.7

Figure 4.8 IL-7Rα downregulation mediated by IL-4 stimulation is restored more readily in CD8 T cells with GFI1 knockdown.

CD8 T cells were stimulated with IL-4 (2 ng/ml) and 24 hours post-stimulation, the cytokine was withdrawn by extensive washing and the cells were transfected with siRNA targeting GFI1 or a non-silencing siRNA control for 48 hours. (a) Aliquots of transfected CD8 T cells were surface stained with PE-conjugated anti-IL-7Rα antibody and analyzed by flow cytometry. The bar graph shows relative fold change in MFI of IL-7Rα expression in stimulated and unstimulated (uns) cells of 4 independent experiments. (b) Protein extracts of the CD8 T cells were subjected to western blotting analysis and the membranes were probed using an anti-GFI1 antibody then stripped and reprobed with anti-βactin as a loading control. The densitometry is representative of three independent experiments where the fold change in GFI1 protein expression is normalized to βactin protein levels. The vertical bars represent the SD. The T-test was used to generate *P* values where $P<0.05$ (*) was considered significant.

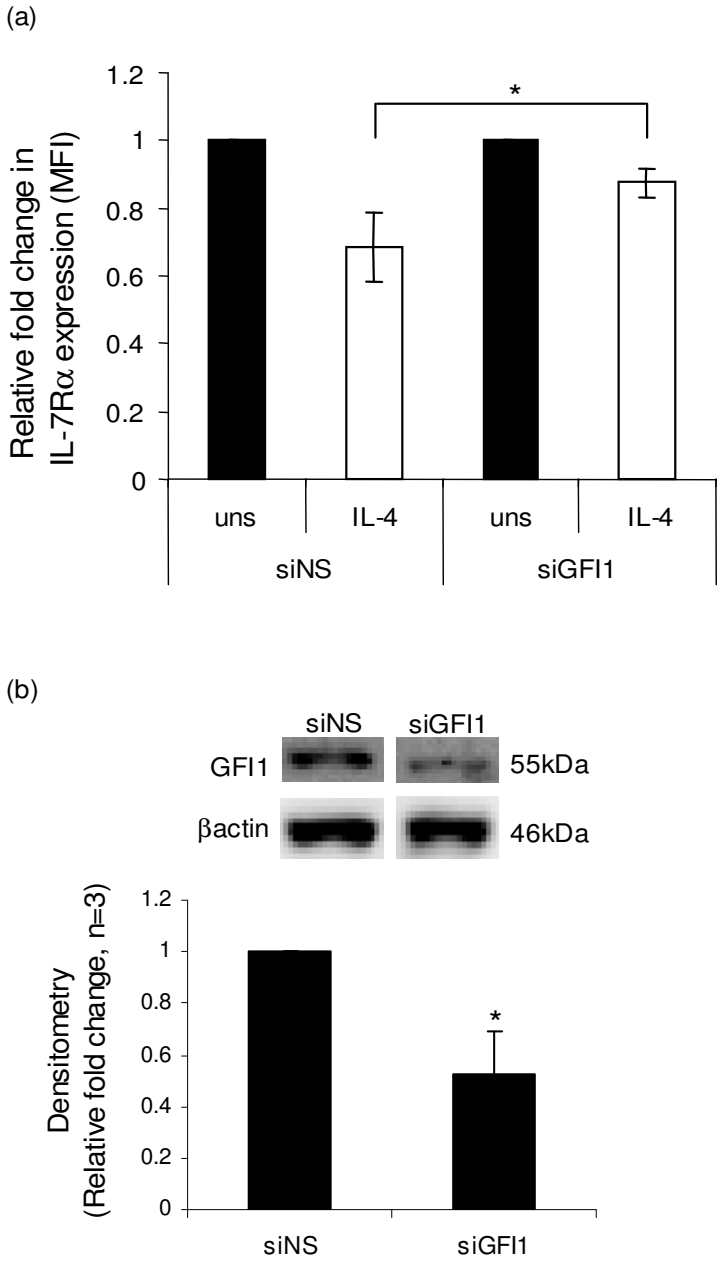

(a)
Relative fold change in IL-7Rα expression (MFI)
1.2
1
0.8
0.6
0.4
0.2
0
*
uns
IL-4
uns
IL-4
siNS
siGFI1
(b)
siNS
siGFI1
GFI1
55kDa
βactin
46kDa
Densitometry (Relative fold change, n=3)
1.2
1
0.8
0.6
0.4
0.2
0
*
siNS
siGFI1

FIGURE 4.8

Objective 4. Investigate the role of GFI1 in the proliferation and survival of CD8 T cells.

GFI1 promotes IL-4-induced proliferation in primary human CD8 T lymphocytes

In murine studies, GFI1 promotes the proliferation of CD4 T cells in response to IL-4. I investigated whether GFI1 was involved in human CD8 T cell proliferation. In order to detect IL-4-dependent proliferation, CFSE-labelled CD8 T cells were stimulated with IL-4 (50 ng/ml) along with PHA-M (2 μg/ml), determined through optimization experiments (Fig. 4.9; 4.10). On day 3 of proliferation, the CD8 T cells were transfected with siGFI1 and cell division was subsequently analyzed at 24 hour intervals by flow cytometric analysis of CFSE staining intensity (Fig. 4.11a) and intracellular PI staining (Fig. 4.11b). GFI1 protein expression was reduced at 24 hours and 48 hours post-transfection that is, on day 5 and day 6 of proliferation, respectively (Fig. 4.11c). Proliferation was visibly affected on day 5 of proliferation where 59.07% of CD8 T cells transfected with siGFI1 did not move into the first division (M1) compared to 44.74% of CD8 T cells transfected with a non-silencing siRNA (Fig. 4.11a). Concordantly, a higher percentage of cells were detected in successive cell divisions in cells transfected with the control siRNA compared to GFI1-specific siRNA. Furthermore, cell cycle analysis demonstrated a potential retention in the G_1 phase (M1) with 49.9% of CD8 T cells transfected with siGFI1 compared to 39.7% of CD8 T cells transfected with control siRNA (Fig. 4.11b).

Therefore, silencing GFI1 expression resulted in an increase in the percentage of CD8 T cells that had not entered into the cell cycle, and the first cell division, which coincided with an increase in the proportion of cells remaining in the G_1 phase, as

Figure 4.9 Proliferation of human CD8 T cells stimulated with increasing concentrations of IL-4 and PHA-M alone.

Purified human CD8 T cells were CFSE-labelled and incubated with different concentrations of PHA-M or IL-4 for 4 and 5 days. The stained CD8 T cells were collected for flow cytometric analysis. Histograms of PHA-M - or IL-4 - treated CD8 T cells were overlayed on histograms of untreated CFSE-labelled CD8 T cells, and each peak represents one cell division. (a) On day 4 and (b) day 5, CD8 T cells incubated with 50 and 100 ng/ml of IL-4 alone did not proliferate significantly. (a) On day 4, CD8 T cells were treated with increasing concentrations of PHA-M (1, 2, 4, 8, 16 or 32 μg/ml). At concentrations greater than 8 μg/ml histogram plots were suggestive of a toxic effect. (b) On day 5, one cell division was observed for CD8 T cells treated with 1, 2 and 4 μg/ml of PHA-M, whereas 4 μg/ml of PHA-M alone resulted in a second cell division.

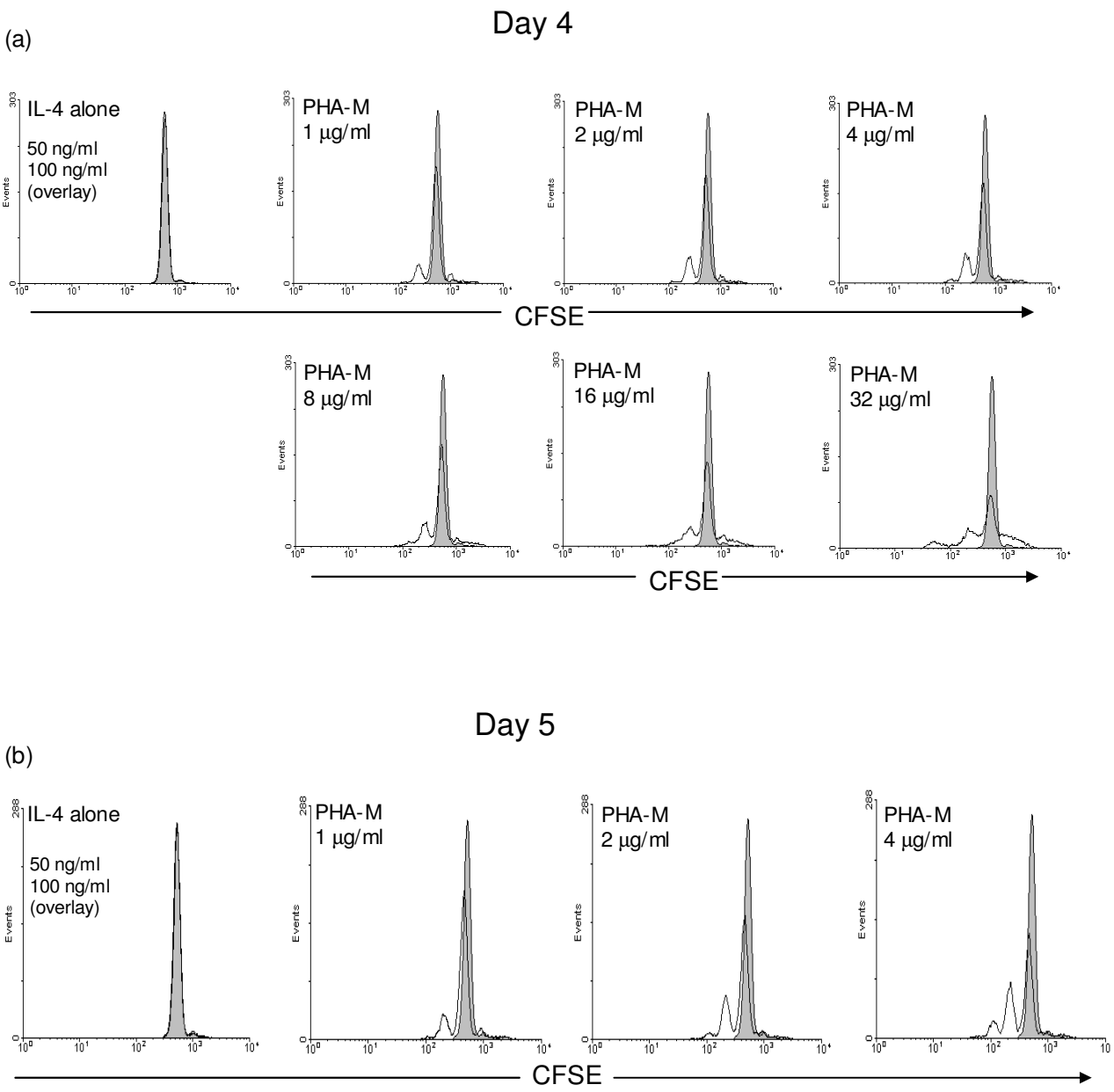

Day 4
(a)
IL-4 alone
50 ng/ml
100 ng/ml
(overlay)
PHA-M
1 µg/ml
PHA-M
2 µg/ml
PHA-M
4 µg/ml
PHA-M
8 µg/ml
PHA-M
16 µg/ml
PHA-M
32 µg/ml
Events
CFSE
Day 5
(b)
IL-4 alone
50 ng/ml
100 ng/ml
(overlay)
PHA-M
1 µg/ml
PHA-M
2 µg/ml
PHA-M
4 µg/ml
Events
CFSE

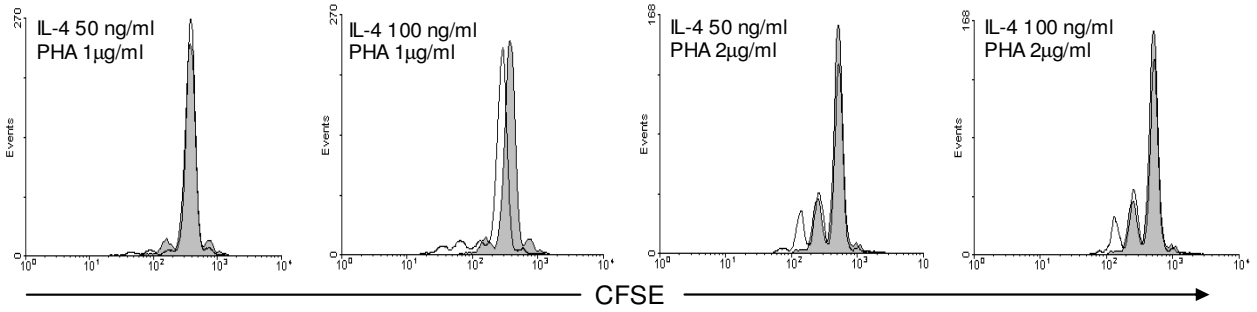
FIGURE 4.9

Figure 4.10 Proliferation of human CD8 T cells with a suboptimal dose of PHA-M and IL-4.

Purified human CD8 T cells were labelled with CFSE and incubated with IL-4 and suboptimal doses of PHA-M for 4 and 5 days. The CD8 T cells were collected for flow cytometric analysis of cell division. The histograms generated show cells treated with PHA-M and IL-4 overlayed on histograms of cells treated with the same concentrations of PHA-M-alone. Each peak represents one cell division. CD8 T cells were incubated with combinations of 1 or 2 μg/ml of PHA-M with 50 or 100 ng/ml of IL-4. (a) On day 4 and (b) day 5, the IL-4 concentrations (50 or 100 ng/ml) tested were incubated with 1 μg/ml of PHA-M, and resulted in marginally detectable levels of IL-4-dependent proliferation. At 2 μg/ml of PHA-M along with IL-4, IL-4-dependent proliferation was clearly observed on both day 4 and 5 with an additional cell division detected on day 5. Therefore, further experiments were conducted over 5 days with 2 μg/ml of PHA-M and 50 ng/ml of IL-4.

Day 4

(a)



Day 5

(b)

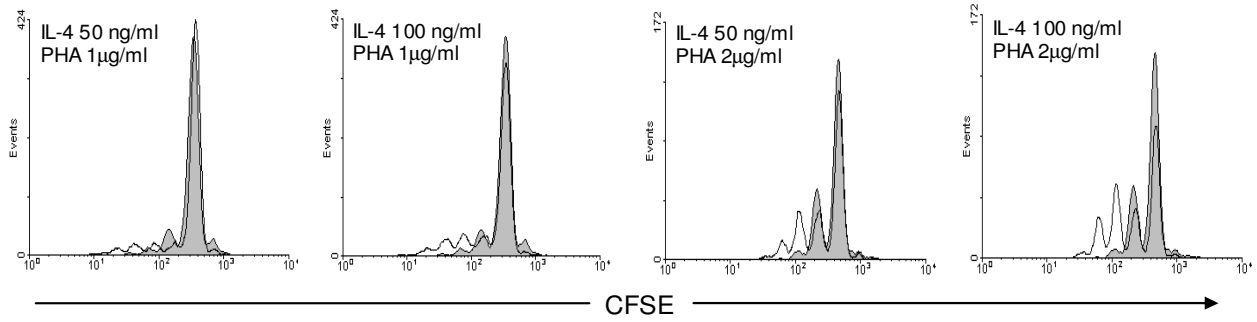


FIGURE 4.10

Figure 4.11 Reduced GFI1 expression in CD8 T cells subjected to IL-4-mediated proliferation exhibit delayed proliferation and increased proportion of cells remaining in the G_1 phase.

(a) Purified human CD8 T cells were stained with CFSE to measure IL-4-dependent proliferation resulting from stimulation with IL-4 (50 ng/ml) in the presence of PHA-M (2 μg/ml). On day 3 (*T3*) of proliferation the CD8 T cells were transfected with siRNA targeting GFI1 and a control siRNA. On day 5 (*D5*) of proliferation, 2 days post-transfection, an aliquot of the CD8 T cells was collected for analysis by flow cytometry. The percentage of CD8 T cells in each cell division was determined by gating on each peak representing one cell division as indicated by markers (M1-M4). (b) Cell cycle analysis on *D5* was determined by collecting an aliquot of the proliferating CD8 T cells, staining with PI. This was followed by flow cytometric analysis using markers for gating to determine the percentage of cells in the G_1 phase, indicated by 'M1', and the G_2 / S / M phase indicated by 'M2'. (c) Protein extracts (30 μg) collected from the CD8 T cells on day 4 (*D4*) and *D5* of proliferation, one and two days post-transfection, respectively, were resolved by SDS-PAGE and subjected to western blotting analysis. The membranes were probed with an anti-GFI1 antibody then stripped and reprobed for the loading control GAPDH. Changes in protein expression levels were assessed by densitometry and normalization of the GFI1 signal to that of GAPDH, where n=2.

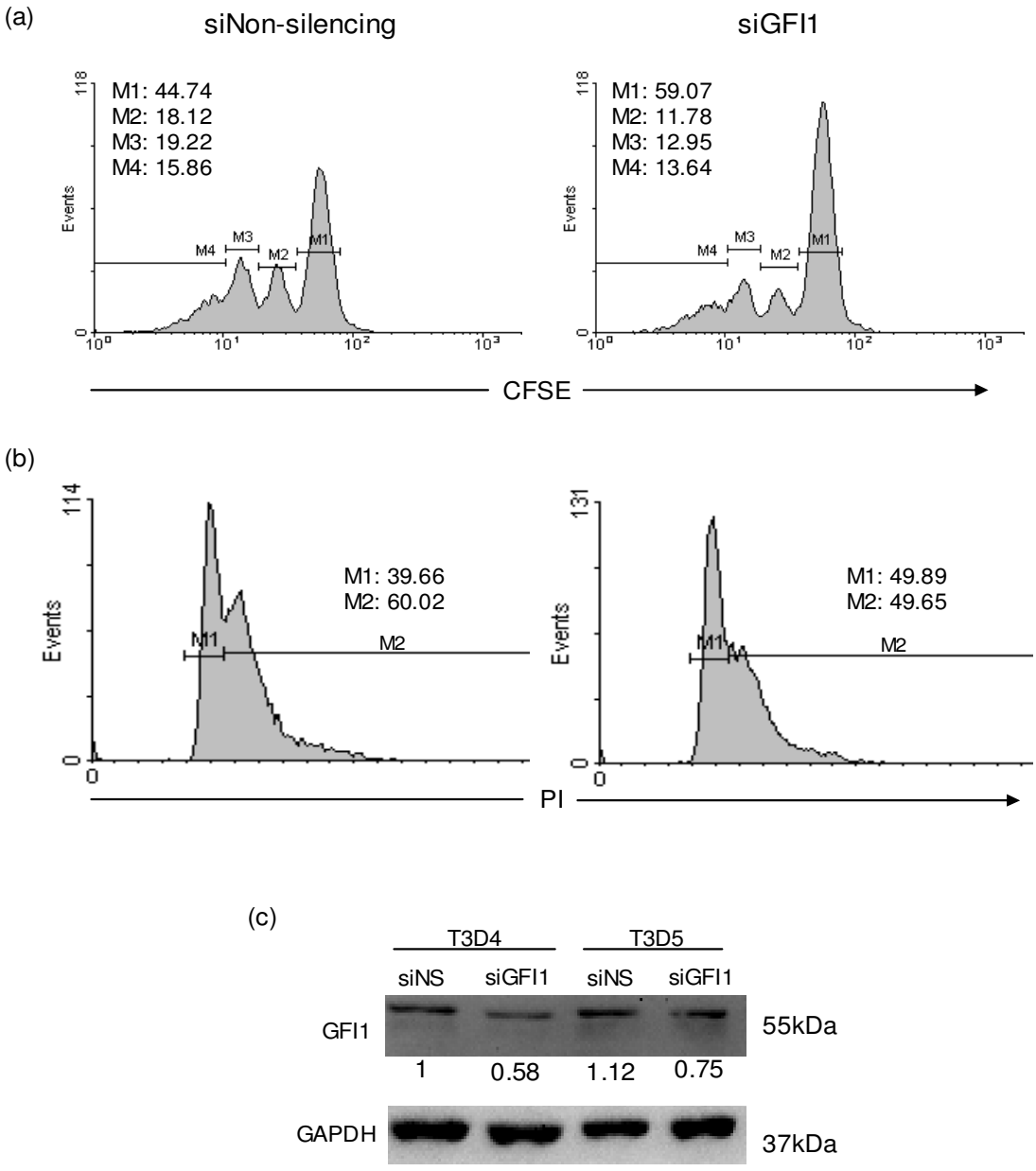

(a)
siNon-silencing
siGFI1
M1: 44.74
M2: 18.12
M3: 19.22
M4: 15.86
M1: 59.07
M2: 11.78
M3: 12.95
M4: 13.64
Events
CFSE
(b)
M1: 39.66
M2: 60.02
M1: 49.89
M2: 49.65
PI
(c)
T3D4
T3D5
siNS
siGFI1
siNS
siGFI1
GFI1
55kDa
1
0.58
1.12
0.75
GAPDH
37kDa

FIGURE 4.11

compared to control siRNA transfected CD8 T cells.

GFI1 negatively regulates Bax and CXCR4 protein expression levels in human CD8 T lymphocytes

GFI1 has also been identified in various study models as a repressor of CXCR4 expression in various study models including cancer stem cells and cells of the granulocytic lineage, as well as a repressor of Bax in lymphocytes [317,333,371]. This was investigated by transfecting CD8 T lymphocytes with the GFI1-expressing plasmid described above, and results were analyzed at 24 hour intervals. To analyze CXCR4 expression, flow cytometry was conducted and Bax expression levels were measured by subjecting cellular lysates to western blotting. In human CD8 T cells, surface CXCR4 expression was reduced by 46% at 48 hours (Fig. 4.12) while Bax expression was reduced by 49% (Left) and 56% (right) at 24 hours in 2 independent experiments (Fig. 4.13). Surface CD28 expression was also evaluated in parallel at 24 and 48 hours by flow cytometry as an additional control for specificity of GFI1 over-expression. As expected, GFI1 over-expression had no significant effect on CD28 protein levels in CD8 T cells (Fig. 4.14).

Figure 4.12 Exogenous overexpression of GFI1 negatively regulates CXCR4 expression.

CD8 T cells were transfected with pGFI1 or an empty vector and incubated for 48 hours. CD8 T cell aliquots were stained with PE-conjugated anti-CXCR4 antibody followed by flow cytometry analysis. (a) The bar graph depicts 3 independent experiments showing the relative MFI fold change in CXCR4 expression between CD8 T cells transfected with an empty vector compared to cells overexpressing GFI1 where SD is represented by vertical bars. The T- test was used to generate *P* values where $P < 0.05$ are indicated by (*). (b) The histogram overlay shows a representative experiment in which the downregulation of CXCR4 in CD8 T cells was observed in cells transfected with the GFI1-expressing plasmid compared to the empty vector.

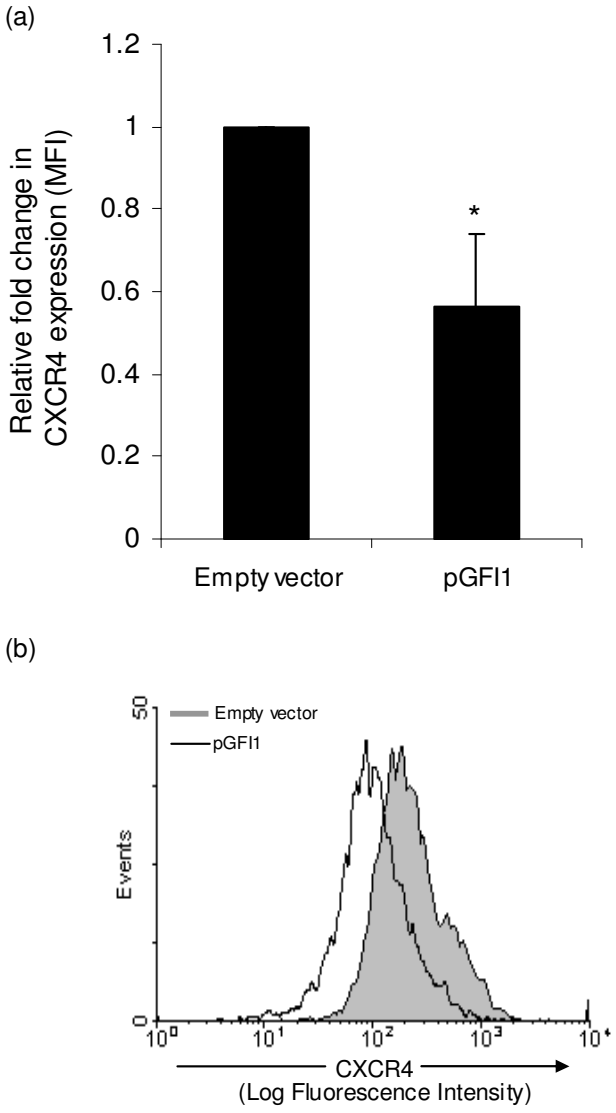

(a)
Relative fold change in CXCR4 expression (MFI)
1.2
1
0.8
0.6
0.4
0.2
0
*
Empty vector
pGFI1
(b)
Empty vector
pGFI1
Events
50
0
10^0
10^1
10^2
10^3
10^4
CXCR4
(Log Fluorescence Intensity)

FIGURE 4.12

Figure 4.13 Exogenous GFI1 overexpression negatively regulates Bax protein expression.

Purified human CD8 T cells were transfected with pGFI1 or empty vector, as described in the materials and methods. Cell pellets were collected 24 hours post-transfection for protein extraction. Protein extracts (35 μg) were subjected to SDS-PAGE followed by western blotting analysis. (a) Membranes were probed with the anti-Bax antibody then stripped and reprobed with the anti-GFI1 antibody. To control for loading the membranes were again stripped and re-probed for βactin expression. (b) Densitometry of the signals obtained in the two representative membranes shown in (a) was performed. Data was plotted as the fold change for Bax and GFI1 protein expression levels after normalization to βactin protein levels in cells transfected with an empty vector and the vector containing the GFI1 insert.

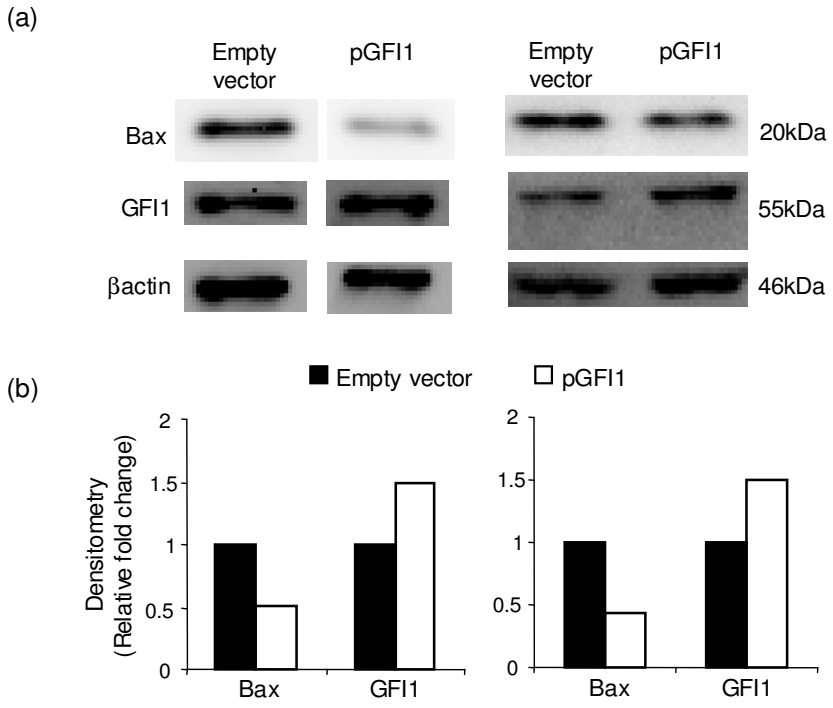

(a)
Empty vector
pGFI1
Empty vector
pGFI1
Bax
GFI1
βactin
20kDa
55kDa
46kDa
(b)
Empty vector
pGFI1
Densitometry (Relative fold change)
2
1.5
1
0.5
0
Bax
GFI1
2
1.5
1
0.5
0
Bax
GFI1

FIGURE 4.13

Figure 4.14 Exogenous GFI1 overexpression had no significant effect on CD28 expression in primary human CD8 T lymphocytes.

Purified CD8 T cells were transfected with pGFI1 or empty vector for (a) 24 and (b) 48 hours. Aliquots of transfected cells were surface stained for CD28 expression using a PE-conjugated anti-CD28 antibody followed by flow cytometry analysis. The bar graph depicts CD28 expression of pGFI1-transfected CD8 T cells relative to cells transfected with the empty vector using MFI measurements where the vertical bars represent the SD. The bar graph shows a compilation of 3 independent experiments for each time point (upper panels) along with a histogram overlay plotting CD28 expression in one representative experiment.

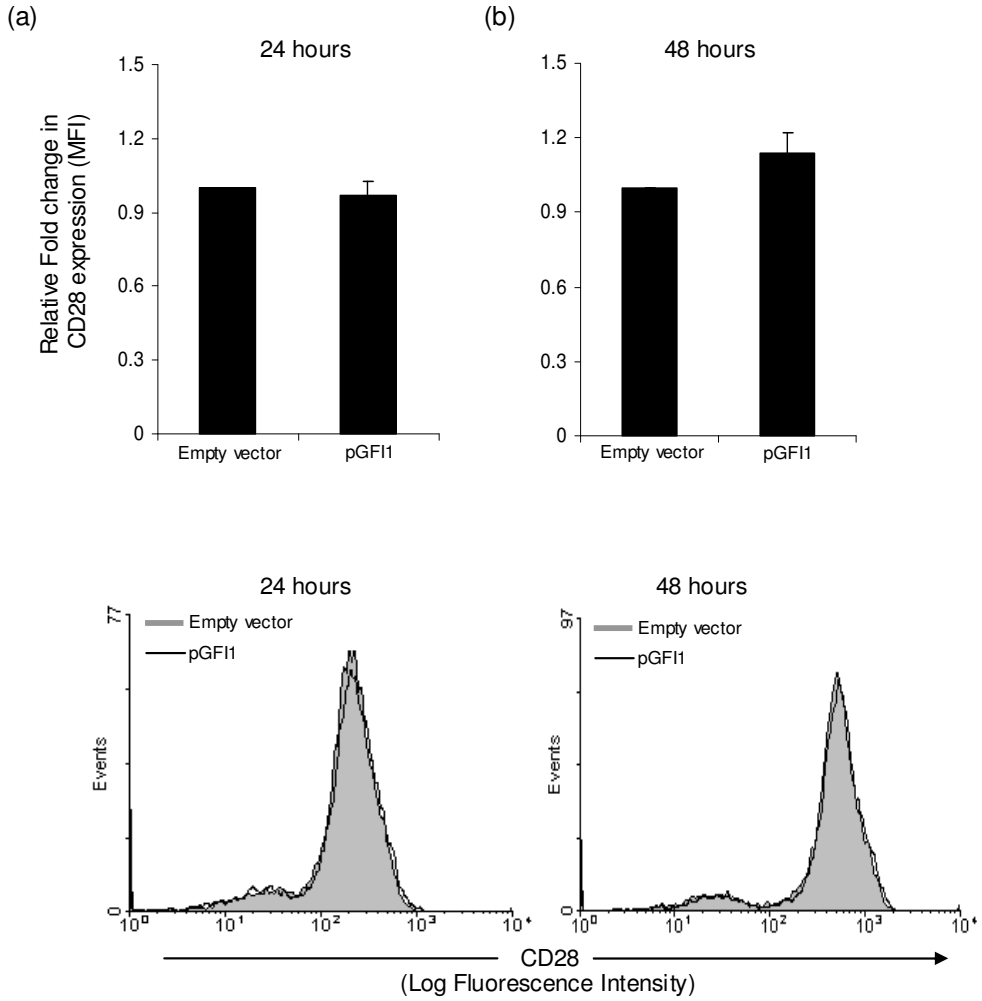

(a)
24 hours
Relative Fold change in CD28 expression (MFI)
1.5
1.2
0.9
0.6
0.3
0
Empty vector
pGFI1
(b)
48 hours
1.5
1.2
0.9
0.6
0.3
0
Empty vector
pGFI1
24 hours
Empty vector
pGFI1
77
Events
0
10^0
10^1
10^2
10^3
10^4
48 hours
Empty vector
pGFI1
97
Events
0
10^0
10^1
10^2
10^3
10^4
CD28
(Log Fluorescence Intensity)

FIGURE 4.14

CHAPTER 5

Signal Transduction Pathways Regulating IL-7Rα and GFI1 Expression at the Basal Level and in Response to IL-4 Stimulation in Human CD8 T cells

Introduction

The essential γc cytokines IL-2, -7, -15, and -21 are required during different phases of the CD8 T cell life span, and IL-7Rα and GFI1 are differentially modulated during this time [237]. In chapter 4, I showed that within the γc cytokine family only IL-4 was able to suppress IL-7Rα expression via GFI1. In lymphocytes, IL-4 binds the type I receptor complex of IL-4Rα to induce heterodimerization with the γc chain, which leads to phosphorylation of their respective JAKs and initiates the phosphorylation cascade depicted in Fig. 5.1 [175,378]. Exposure to IL-4 during primary differentiation of CD4 T cells results in type 2 polarization, the literature suggests a more versatile cytokine with contradictory effects on the transition of naïve CD8 T cells to effector and memory cells as well as on CTL activity and antigen clearance capacity [121-123,182]. More specifically, IL-4 exposure has been shown to lead to the differentiation of a subset of CD8 T cells characterized by reduced cytolytic activity compared to CD8 T cells cultured in neutral conditions (IL-2 alone) [122]. The mechanisms by which naïve CD8 T cells become type 2 or type 1 cytotoxic T cells may provide insight into the acquired phenotypes of CD8 T cells during chronic infections like for example in the case of HIV infection. Interestingly, CD8 T cells have been reported to secrete IL-4, and have low cytotoxic capabilities in HIV-infected patients [119,183]. More recent HIV studies describe increased production of IL-4 with increased disease severity and more rapid disease progression [111,112,379,380]. On the other hand, in the absence of CD4 T cell help, early IL-4 production and IL-4Rα signaling was shown to be important for CD8 T cell cytotoxicity, but in an acute infection such as with the influenza virus [181,381].

Figure 5.1 Schematic diagram of IL-4 signaling by the Type I IL-4 receptor.

IL-4Rα is composed of 12 exons and 11 introns and is characterized by an extracellular, transmembrane, and intracellular domain [138,382]. Exons 1 to 8 encode a 5'untranslated (5'UT) region and the extracellular domain composed of the signal peptide (Sig), 4 cysteine residues, and a WS motif [138,382]. The binding of IL-4 to IL-4Rα induces heterodimerization with the γc chain [174,176]. The dimerization activates the Jak proteins, which initiates the phosphorylation cascade involving specific tyrosine residues [174,176]. The tyrosine residues in the cytoplasmic tail of the IL-4R becomes phosphorylated and acts as docking sites for signaling molecules which in turn become tyrosine phosphorylated [174,176]. The first cytoplasmic tyrosine residue (Y1=Tyr497) is in a sequence motif called the I4R motif that interacts with protein tyrosine-binding domains (PTB) contained for example in the insulin receptor substrate family (IRS1/2) [138,174,180,383]. Tyrosine residues 2–4 (Y2=Tyr575, Y3=Tyr603, Y4=Tyr631) interact with the SH2-domain of STAT6 and the fifth cytoplasmic residue (Y5=Tyr713) lies in the consensus ITIM motif that acts as a docking site for SH2-domains of tyrosine phosphatases such as Shp-1, which is involved in negative regulation by dephosphorylating STAT6 [174,176,384]. Phosphorylated STAT6 dimerizes, translocates to the nucleus, and binds to the promoter of IL-4 responsive genes [174,176]. Phosphorylated IRS binds to the p85 subunit of PI3 kinase and to growth factor-receptor-bound protein 2 (Grb2), which may lead to the activation of transcription factors such NF-κB [174,176]. Grb2, complexed to the guanine nucleotide exchange protein SOS, catalyzes the activation of Ras [148,149]. Ras in turn activates the Raf kinase to initate signaling of the MAPK pathway through a cascade of kinase activation of MEK and ERK

proteins where ERK proteins translocate to the nucleus to activate genes suh as c-Fos and other transcription factors like Elk1 [148,149]. In murine CD4 T cells, initiation of the kinase cascade by Ras can also lead to activation of JNK which results in IL-4-mediated proliferation and differentiation, which represents a cascade of events instigated by the nuclear phosphorylation of c-Jun [383]. IL-4 signaling also leads to the activation of P38 signaling in murine T and B cell lines [385]. In primary human CD8 T cells, IL-4-induced activation or upregulation of MEK, JNK and P38 has not been described. Activation of these pathways regulates cellular differentiation, growth, and survival factors as well as expression of various cell surface markers [174,176].

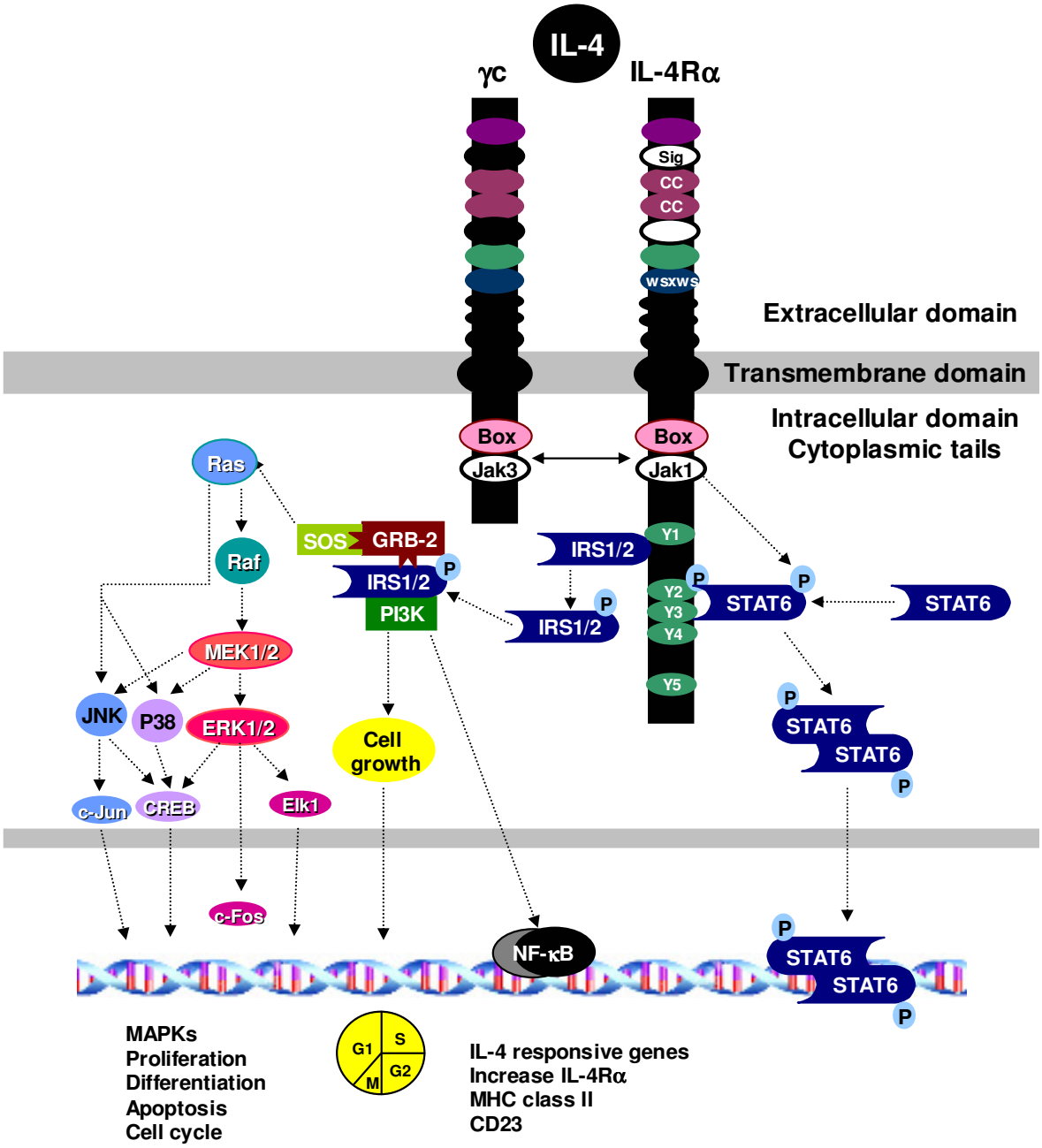

IL-4
γc
IL-4Rα
Sig
CC
CC
WSXWS
Extracellular domain
Transmembrane domain
Intracellular domain
Cytoplasmic tails
Box
Jak3
Box
Jak1
Ras
Raf
SOS
GRB-2
IRS1/2
P
PI3K
IRS1/2
P
IRS1/2
Y1
Y2
Y3
Y4
Y5
P
STAT6
P
STAT6
MEK1/2
JNK
P38
ERK1/2
Cell growth
P
STAT6
STAT6
P
c-Jun
CREB
Elk1
c-Fos
NF-κB
P
STAT6
STAT6
P
MAPKs
Proliferation
Differentiation
Apoptosis
Cell cycle
G1
S
G2
M
IL-4 responsive genes
Increase IL-4Rα
MHC class II
CD23

FIGURE 5.1

It has been reported and confirmed here in primary human CD8 T cells that γc cytokines including IL-2, IL-4, IL-7, and IL-15, are able to downregulate IL-7Rα expression. The effects of these cytokines are mediated by activation of specific signaling pathways, described above. However, in terms of IL-7Rα regulation, only the mechanism induced by IL-2 has been explored and shown to involve the PI3K pathway in murine T cells [286]. IL-2, IL-4, and IL-7 are among the few γc cytokines described to induce GFI1 expression [189,333,356,371]. G-CSF has been reported to induce GFI1 production in cells of the granulocytic lineage [333], as well as the tumor suppressor protein p53 in cancer stem cells [371]. The signaling pathways responsible for basal and IL-4-dependent regulation of IL-7Rα and GFI1 have not been described previously in CD8 T cells and will be the focus of this chapter of the thesis.

IL-4 stimulation of CD8 T cells leads to the downregulation of IL-7Rα and I demonstrate here, through the use of pharmaceutical inhibitors targeting the Jak/STAT and the PI3K pathways, that these pathways are involved in IL-4-induced IL-7Rα downregulation. In contrast, inhibition of P38 and JNK phosphorylation suggested that these distinct MAP kinases may be involved in maintaining basal expression levels. Interestingly, the ERK MAP kinase pathway did not appear to have a role in modulating basal IL-7Rα expression levels or the regulatory effects of IL-4 on the receptor. My results further revealed that the modulation of GFI1 by IL-4 relied on PI3K pathway activation, but not the Jak/STAT pathway. Furthermore, results did not support a role for the MAP kinase signaling pathways (P38, JNK, and MEK/ERK) in the regulation of GFI1 by IL-4.

The results from this chapter will be presented in two sections. The first examines the pathways involved in basal and IL-4-dependent regulation of IL-7Rα expression while the second section describes the signaling pathways critical in mediating the upregulation of GFI1 expression in response to IL-4.

Results

Objective 5. Identify the signaling pathways implicated in the regulation of IL-7Rα and GFI1 expression in human CD8 T cells.

The Jak/STAT and PI3K pathway are involved in IL-4 mediated downregulation of IL-7Rα in primary human CD8 T lymphocytes

IL-4 stimulation of human CD8 T lymphocytes results in the downregulation of IL-7Rα expression similar to the majority of the other γc cytokines evaluated other than IL-21 [189]. I also showed that these suppressive effects of IL-4 depended, at least in part, on the action of the transcriptional repressor, GFI1. However, the signaling pathways involved in modulating IL-7Rα and GFI1 expression particularly in response to IL-4, have not been investigated. Two major signaling pathways activated by IL-4 that were investigated include the Jak/STAT6 and the PI3K pathway [174,176]. Primary human CD8 T cells were pre-incubated for 2 hours with pharmaceutical inhibitors JAK inhibitor 1 targeting Jak/STAT and LY 294002 targeting PI3 kinase. This was followed by IL-4 [2 ng/ml] stimulation for 24 hours. Cellular protein lysates were subjected to western blot analysis in order to confirm the biological activity of the inhibitors used, as indicated by a reduction in the phosphorylation of the relevant signaling proteins. In addition, analysis of cell viability by PI staining at 24 hours post-stimulation was conducted to ensure that the inhibitor concentrations used did not exert overtly toxic effects on the cells (Fig. 5.2). In CD8 T cells, toxicity was observed, but this was marginal and occurred particularly at high concentrations of the JNK inhibitor SP 600125.

Figure 5.2 Cell death as a measure of toxicity due to signal transduction inhibitors.

Lymphocytes and purified human CD8 T lymphocytes were incubated with increasing concentrations of the pharmaceutical inhibitors: JAK inhibitor 1 (10, 25, 50, 100 nM), and LY 294002 (LY), SB 203580 (SB), SP 600125 (SP), and PD 98059 (PD) (10, 25, 50, 100 μM) for 24 hours at 37^{o}C / 5% CO_2 followed by PI staining and flow cytometric analysis to determine the percentage of dead cells (% PI-positive). (a) PBMCs were incubated with SB, JAK and PD and with (b) LY and SP (top panel) followed by PI staining, and results obtained on gated lymphocytes are shown. (b, lower panel) Purified CD8 T cells were incubated with SP and LY, followed by PI staining, and the percentage of dead cells is represented by the % PI-positive cells.

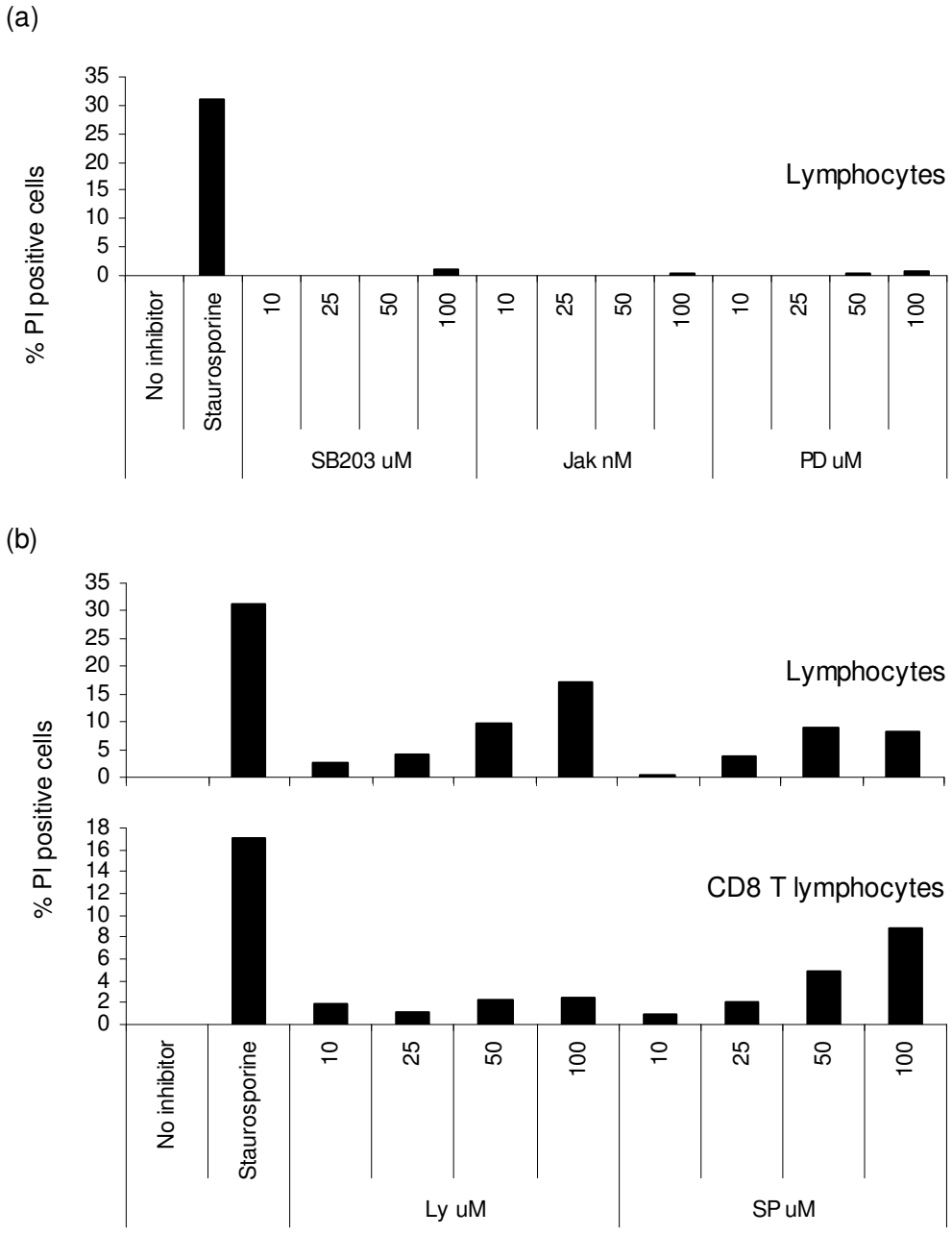

(a)
% PI positive cells
35
30
25
20
15
10
5
0
Lymphocytes
No inhibitor
Staurosporine
10
25
50
100
10
25
50
100
10
25
50
100
SB203 uM
Jak nM
PD uM
(b)
% PI positive cells
35
30
25
20
15
10
5
0
Lymphocytes
18
16
14
12
10
8
6
4
2
0
CD8 T lymphocytes
No inhibitor
Staurosporine
10
25
50
100
10
25
50
100
Ly uM
SP uM

FIGURE 5.2

Incubation of CD8 T cells with JAK inhibitor 1 followed by IL-4 stimulation for 15 minutes resulted in a dramatic reduction in IL-4-mediated STAT6 phosphorylation (Fig. 5.3a). Evaluation of the results at 24 hours by flow cytometry and RT-PCR demonstrated that pre-incubation of CD8 T cells with JAK inhibitor 1 reversed IL-4 induced downregulation of the receptor at both the protein and mRNA levels (Fig. 5.3b, c). Statistically significant differences were noted at doses of 10 nM and 25 nM ($n = 6$; $p<0.05$; Fig. 5.3b). A representative histogram shows the clear reversal of the suppressive effects of IL-4 on IL-7Rα expression obtained at 25 nM and 50 nM (Fig. 5.3c). Treatment of CD8 T cells with LY 294002, a PI3 kinase inhibitor, was also evaluated and the biological activity of the inhibitor was confirmed by visualizing AKT phosphorylation on western blots (Fig. 5.4a). Inhibition of the PI3 kinase pathway similar to that of Jak/STAT, lead to the reversal of IL-4-mediated suppression of IL-7Rα at both the protein and mRNA levels (Fig. 5.4b, c). Concentrations of 25 and 50 μM of LY 294002 showed statistically significant increases in IL-7Rα surface expression ($p<0.05$) compared to IL-4 alone whereas all three concentrations tested significantly restored IL-7Rα mRNA levels ($n = 6$; Fig. 5.4b).

<u>The P38 and JNK MAPK pathways are not implicated in IL-4-mediated suppression of IL-7Rα, but may play a role in its basal expression levels.</u>

The role of MAPK signaling pathways were investigated by targeting the signaling proteins P38, JNK, and MEK using SB 203580, SP 600125, and PD 98059 inhibitors, respectively. CD8 T cells were pre-incubated with the inhibitors for 2 hours

Figure 5.3 Reversal of IL-4-induced IL-7Rα downregulation by blockade of Jak/STAT signaling.

Purified human CD8 T cells were incubated with increasing concentrations (10, 25, 50 nM) of JAK inhibitor 1 for 2 hours at 37^{o}C followed by IL-4 (2 ng/ml) stimulation. (a) Cellular protein extracts (30 μg) from unstimulated (uns) CD8 T cells and cells stimulated with IL-4 for 15 minutes following incubation with JAK inhibitor 1 were resolved by SDS-PAGE, transferred to a PVDF membrane, and probed for pSTAT6. The membrane was stripped two consecutive times first to visualize total STAT6 then βactin. (b) Surface IL-7Rα expression on CD8 T lymphocytes incubated with JAK inhibitor 1 prior to 24 hour stimulation with IL-4 was analyzed by flow cytometry in the left panel. The fold change in IL-7Rα expression MFI measurements of treated cells with or without IL-4 relative to untreated unstimulated cells is shown. RNA extracts from the CD8 T cells were also reverse transcribed and IL-7Rα mRNA expression levels shown in the right panel were determined by RT-PCR analysis. Relative gene expression levels were determined by the comparative Ct method [360]. Bar graphs represent 6 independent experiments and vertical bars represent the SD. (c) Representative histogram overlays illustrating the effects of JAK inhibitor 1 on IL-7Rα surface expression on unstimulated CD8 T cells (left panel) and IL-4-stimulated CD8 T cells (right panel). The Bonferroni test for multiple comparisons was used to generate *P* values where $P < 0.001$ (***), $P<0.01$ (**), and $P<0.05$ (*).

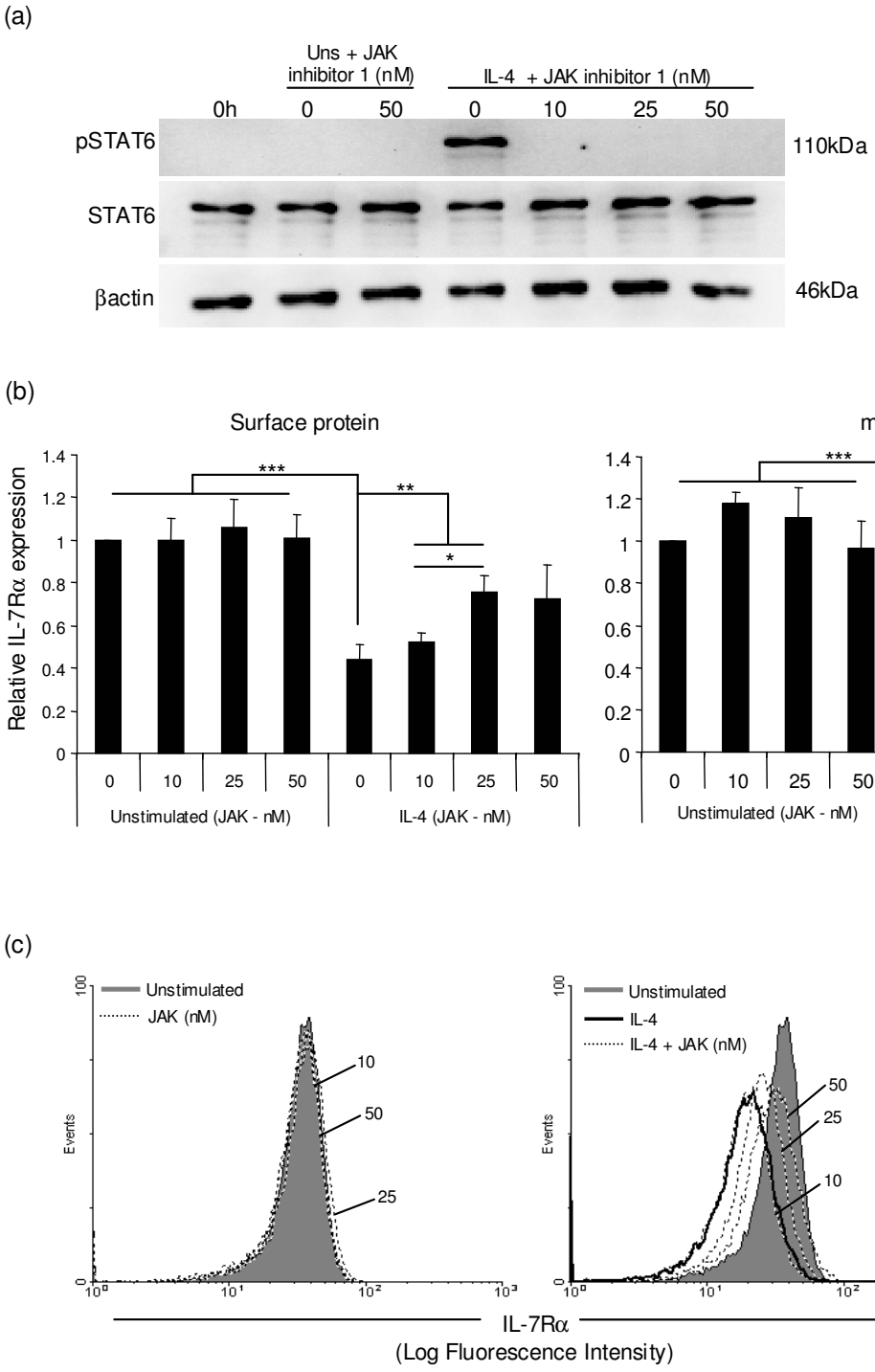

(a)
Uns + JAK inhibitor 1 (nM)
IL-4 + JAK inhibitor 1 (nM)
0h
0
50
0
10
25
50
pSTAT6
110kDa
STAT6
βactin
46kDa
(b)
Surface protein
mRNA
Relative IL-7Rα expression
***
**
*
Unstimulated (JAK - nM)
IL-4 (JAK - nM)
(c)
Unstimulated
JAK (nM)
IL-4
IL-4 + JAK (nM)
Events
IL-7Rα
(Log Fluorescence Intensity)

FIGURE 5.3

Figure 5.4 Reversal of the suppressive effects of IL-4 on IL-7Rα expression by blockade of the PI3K signaling pathway.

Purified human CD8 T cells were incubated with increasing concentrations (10, 25, 50 μM) of LY 294002 (LY) for 2 hours at 37^{o}C followed by IL-4 (2 ng/ml) stimulation. (a) Protein extracts (30 μg) from unstimulated (uns) CD8 T cells and cells stimulated with IL-4 for 15 minutes following incubation with LY were resolved by SDS-PAGE, transferred to a PVDF membrane, and successively probed for pAKT, total AKT and βactin. (b) Surface IL-7Rα expression shown in the left panel was measured by flow cytometry on CD8 T lymphocytes incubated with LY and stimulated with IL-4 for 24 hours. The fold change in MFI measurements of IL-7Rα expression of treated cells with or without IL-4 relative to untreated unstimulated cells was calculated. RNA extracts from the CD8 T cells were also reverse transcribed and GFI1 mRNA expression levels shown in the right panel was determined by RT-PCR analysis. Relative gene expression levels were determined by the comparative Ct method [360]. Bar graphs represent 6 independent experiments where SD is respresented by vertical bars. (c) Representative histogram overlays illustrating the effects of LY on IL-7Rα surface expression on unstimulated CD8 T cells (left panel) and IL-4-stimulated CD8 T cells (right panel). The Bonferroni test for multiple comparisons was used to generate *P* values where $P < 0.001$ (***), $P<0.01$ (**), and $P<0.05$ (*).

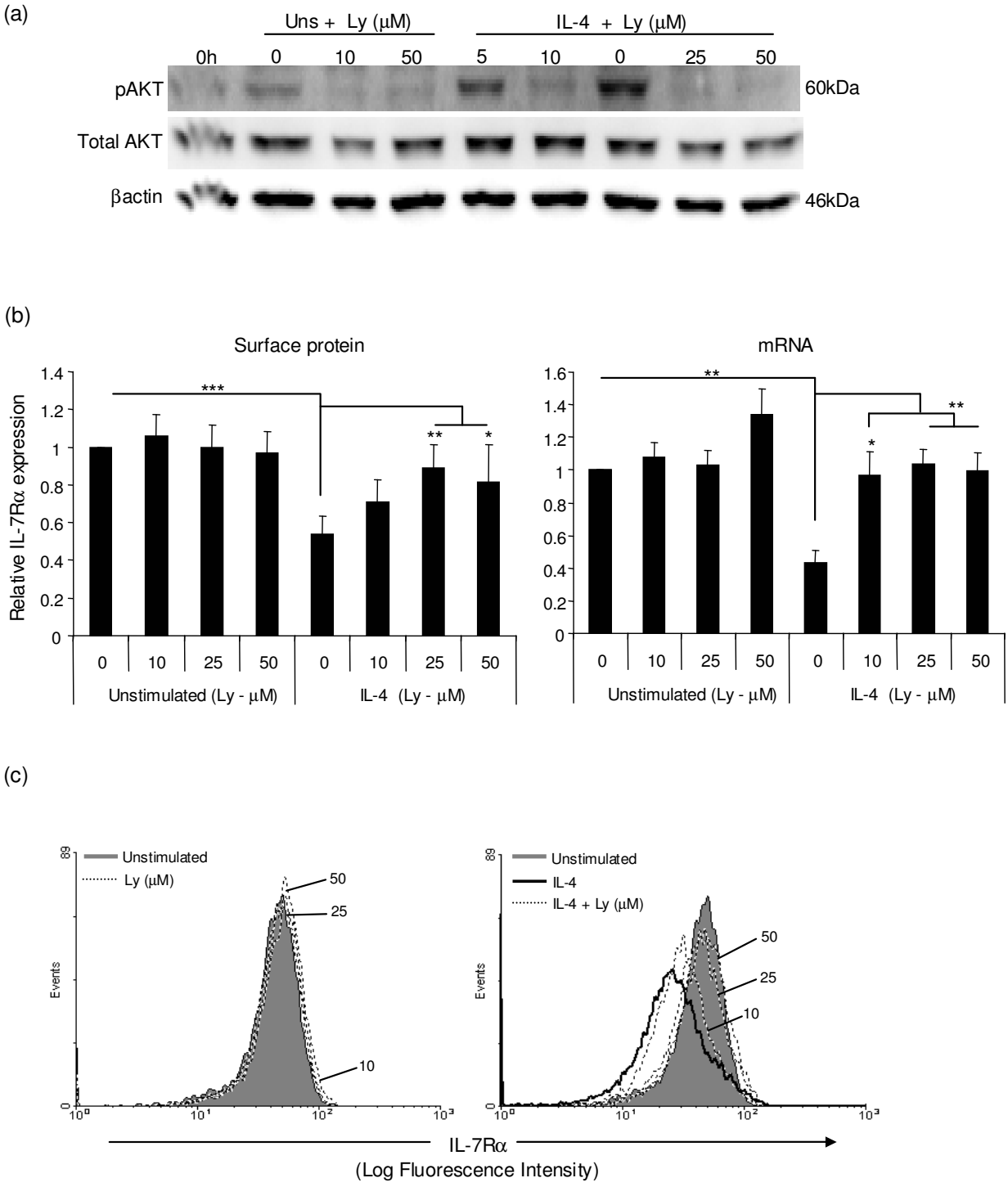

(a)
Uns + Ly (μM)
IL-4 + Ly (μM)
0h
0
10
50
5
10
0
25
50
pAKT
60kDa
Total AKT
βactin
46kDa
(b)
Surface protein
mRNA
Relative IL-7Rα expression
Unstimulated (Ly - μM)
IL-4 (Ly - μM)
(c)
Unstimulated
Ly (μM)
IL-4
IL-4 + Ly (μM)
Events
IL-7Rα
(Log Fluorescence Intensity)

FIGURE 5.4

followed by IL-4 [2 ng/ml] stimulation for 24 hours and measurement of IL-7Rα expression by flow cytometry. The inhibition of the MAPK P38 pathway with SB 203580 had no significant effect on IL-4-induced downregulation of IL-7Rα, but rather inhibited basal expression levels (Fig. 5.5a, b). P38 inhibition led to a statistically significant dose-dependent downregulation of IL-7Rα basal expression at the protein ($n = 6$; $p<0.001$) and mRNA ($n= 6$; $p<0.001$) levels (Fig. 5.5a). Interestingly, the SP 600125 inhibitor used to investigate the role of JNK in IL-4-mediated suppression of IL-7Rα yielded similar results. Here, pre-incubation of CD8 T cells with SP 600125 led to a reduction in basal IL-7Rα expression levels, but had no effect on the downregulation of IL-7Rα by IL-4 (Fig. 5.6a, b). Statistically significant and dose dependent reductions of basal IL-7Rα expression at the protein ($n = 6$; $p<0.001$) and mRNA levels ($n = 6$; $p<0.001$) were observed (Fig. 5.6a).

In order to confirm MAPK inhibitor biological activities, after 15 minutes of IL-4 stimulation and culture, CD8 T cell lysates were prepared and subjected to western blot analysis. Consistent with the effects on basal IL-7Rα expression, substantial constitutive P38 phosphorylation was noted, such that no further pP38 upregulation was detected in response to IL-4 stimulation (Fig.5.7a). Nevertheless, SB treatment reduced phosphorylated P38 levels in unstimulated cells and in the presence of IL-4 (Fig.5.7a). In the case of the JNK pathway, constitutive JNK phosphorylation was also detected and was upregulated minimally in response to IL-4. Thus, it was difficult to demonstrate the effect of the inhibitor (Fig. 5.7b, left panels). Additional experiments were therefore conducted with the mitogen PHA-M [10 μg/ml] in an attempt to more strongly induce JNK phosphorylation. Under these conditions, it was possible to more clearly show

Figure 5.5 Downregulation of basal IL-7Rα expression by blockade of the MAPK P38 signaling pathway.

Purified human CD8 T cells were incubated with increasing concentrations (10, 25, 50 μM) of SB 203580 (SB) for 2 hours at 37^{o}C followed by IL-4 (2 ng/ml) stimulation. (a) Surface IL-7Rα expression was measured on CD8 T lymphocytes incubated with SB prior to a 24 hour culture in the presence and absence of IL-4, and then analyzed by flow cytometry in the left panel. The fold change in MFI measurements of IL-7Rα expression of treated cells with or without IL-4 relative to untreated unstimulated cells is depicted. RNA extracts from the CD8 T cells were also reverse transcribed and IL-7Rα mRNA expression levels shown in the right panel was determined by RT-PCR analysis. Relative gene expression levels were determined by the comparative Ct method [360]. Bar graphs represent 6 independent experiments plotted along with SD (vertical bars). (b) Representative histogram overlays illustrating the effects of SB on IL-7Rα surface expression in unstimulated CD8 T cells (left panel) and IL-4-stimulated CD8 T cells (right panel). The Bonferroni test for multiple comparisons was used to generate *P* values where $P < 0.001$ (***) and $P<0.05$ (*).

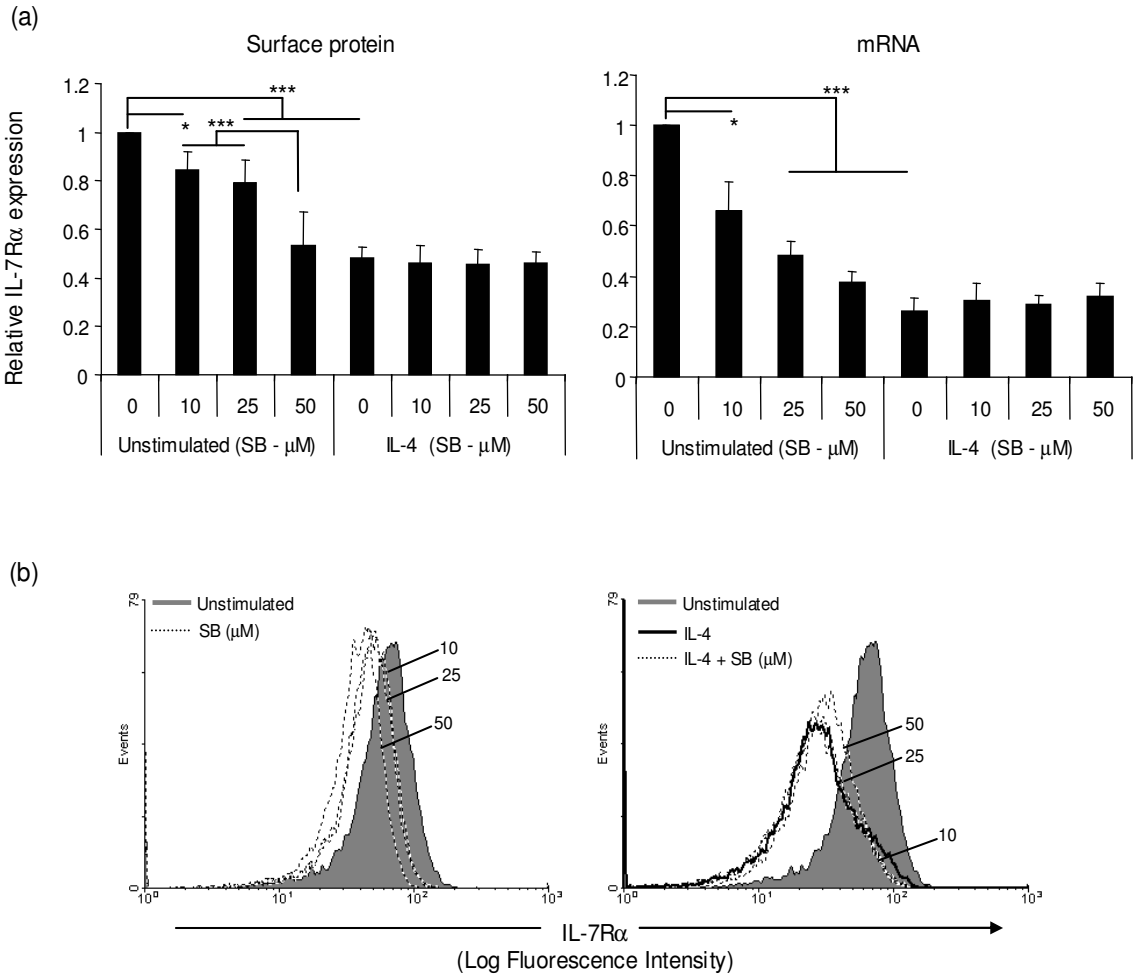

(a)
Surface protein
mRNA
Relative IL-7Rα expression
1.2
1
0.8
0.6
0.4
0.2
0
***
*
***
0
10
25
50
Unstimulated (SB - μM)
IL-4 (SB - μM)
(b)
Unstimulated
SB (μM)
IL-4
IL-4 + SB (μM)
Events
79
10
25
50
IL-7Rα
(Log Fluorescence Intensity)

FIGURE 5.5

Figure 5.6 Downregulation of basal IL-7Rα expression by blockade of the MAPK JNK signaling pathway.

Purified human CD8 T cells were incubated with increasing concentrations (10, 25, 50 μM) of SP 600125 (SP) for 2 hours at 37^{o}C followed by IL-4 (2 ng/ml) stimulation. (a) Surface IL-7Rα expression was measured on CD8 T lymphocytes incubated with SP prior to a 24 hour culture in the presence or absence of IL-4 and then analyzed by flow cytometry in the left panel. The fold change in MFI measurements of IL-7Rα expression of treated cells with or without IL-4 relative to untreated unstimulated cells was displayed. RNA extracts from the CD8 T cells were also reverse transcribed and IL-7Rα mRNA expression levels shown in the right panel was determined by RT-PCR analysis. The relative gene expression levels were determined by the comparative Ct method [360]. Bar graphs represent 6 independent experiments plotted along with SD (vertical bars). (b) Representative histogram overlay illustrate the effects of SP on IL-7Rα surface expression in unstimulated CD8 T cells (left panel) and IL-4-stimulated CD8 T cells (right panel). The Bonferroni test for multiple comparisons was used to generate *P* values where $P < 0.001$ (***) and $P<0.05$ (*).

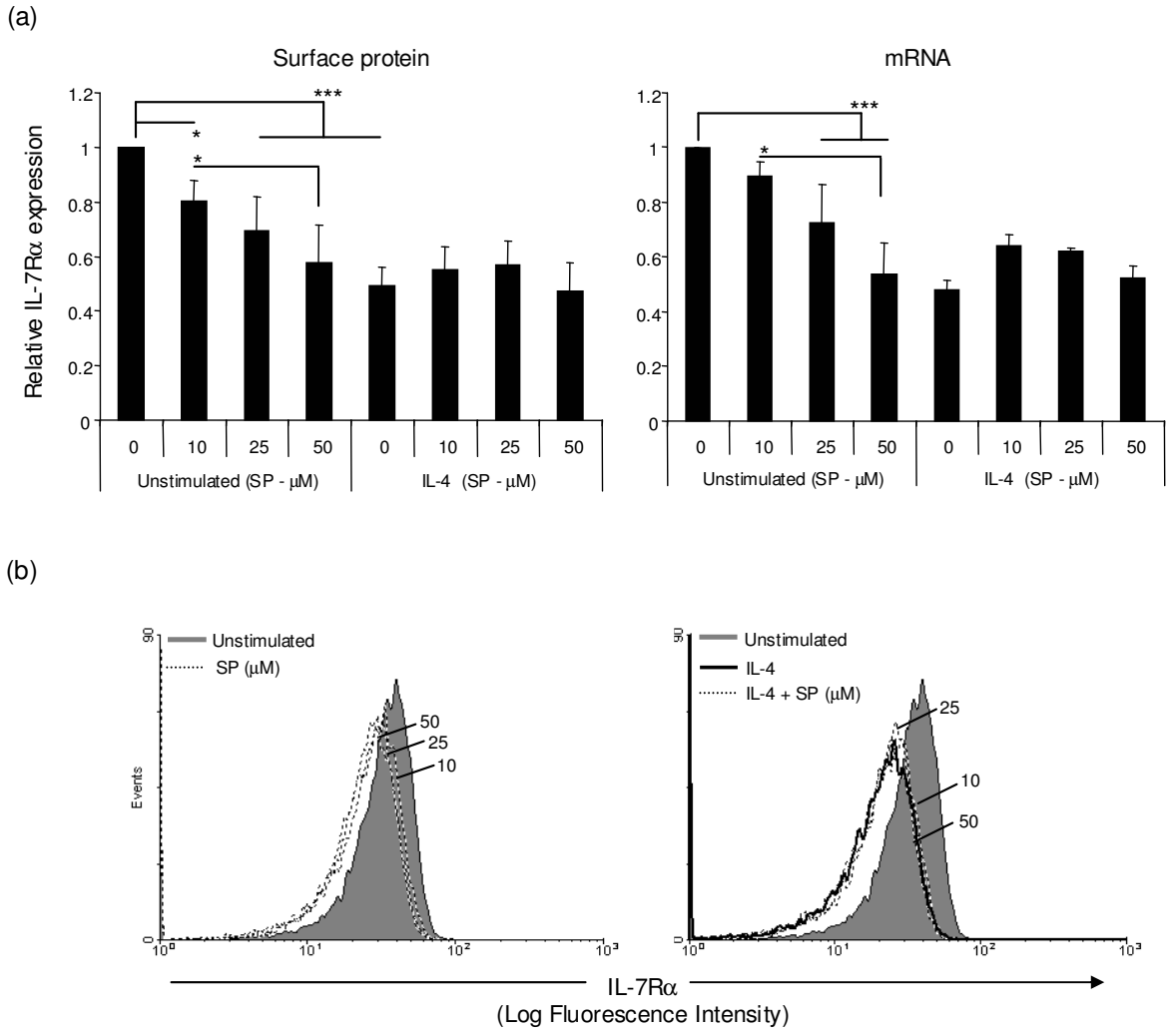
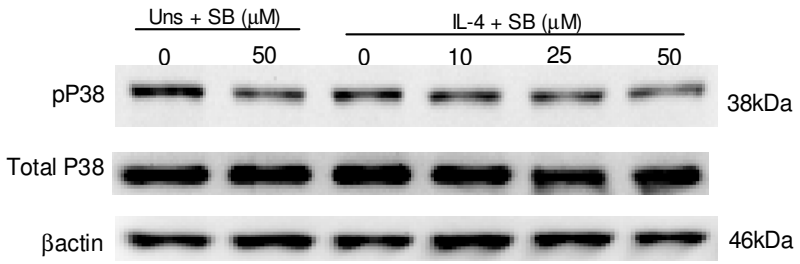

(a)
Surface protein
mRNA
Relative IL-7Rα expression
1.2
1
0.8
0.6
0.4
0.2
0
***
*
*
0
10
25
50
Unstimulated (SP - μM)
IL-4 (SP - μM)
(b)
Unstimulated
SP (μM)
IL-4
IL-4 + SP (μM)
Events
50
25
10
IL-7Rα
(Log Fluorescence Intensity)

FIGURE 5.6

Figure 5.7 Pharmacological activity of SB 203580 and SP 600125 on IL-4-induced phosphorylation of P38 and JNK expression, respectively.

Purified human CD8 T cells were incubated with increasing concentrations (10, 25, 50 μM) of SB 203580 (SB) or SP 600125 for 2 hours at 37^{o}C followed by IL-4 (2 ng/ml) or PHA-M (10 μg/ml) stimulation. Cellular protein extracts (30 μg) from unstimulated (uns) CD8 T cells and cells stimulated with IL-4 following incubation with pharmacological inhibitors were resolved by SDS-PAGE, transferred to a PVDF membrane. (a) Protein extracts from unstimulated cells and cells stimulated with IL-4 for 15 minutes followed by SB treatment were successively probed for pP38, total P38 then βactin. (b) Cellular protein extracts from unstimulated CD8 T cells and cells stimulated with IL-4 for 15 minutes or PHA-M for 24 hours following incubation with SP were probed successively for pJNK, total JNK, and βactin. Densitometry of phosphorylated proteins and total proteins are normalized to βactin, and the ratio of phosphorylated protein expression is compared to total protein levels.

(a)


Uns + SB (μM)
IL-4 + SB (μM)
0
50
0
10
25
50
pP38
38kDa
Total P38
βactin
46kDa

(b)

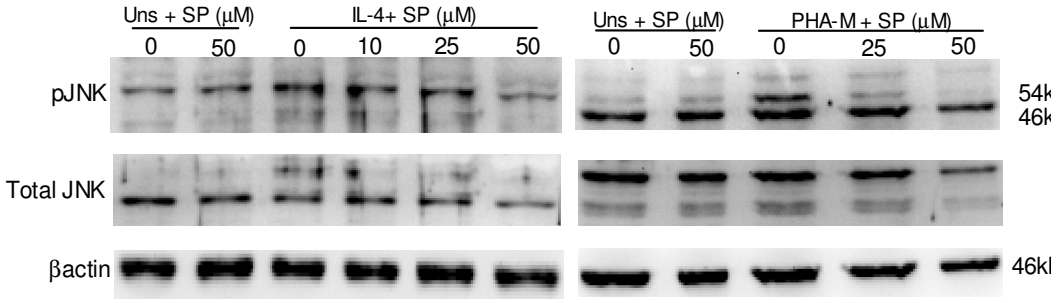

Uns + SP (μM)
IL-4+ SP (μM)
Uns + SP (μM)
PHA-M + SP (μM)
0
50
0
10
25
50
0
50
0
25
50
pJNK
54k
46k
Total JNK
βactin
46kl

FIGURE 5.7

inhibition of JNK phosphorylation at 25 μM, confirming the activity of the SP 600125 JNK inhibitor (Fig. 5.7b, right panels). The final inhibitor tested was PD 98059, a MEK inhibitor. Similarly, elevated levels of constitutive ERK phosphorylation were observed and IL-4 stimulation did not further upregulate this in CD8 T cells. Nevertheless, ERK phosphorylation was strongly inhibited by the MEK inhibitor PD 98059 (Fig. 5.8a), confirming its biological activity. PD 98059 however, did not have an impact on basal IL-7Rα expression levels or IL-4-mediated downregulation of IL-7Rα expression (Fig. 5.8b, c; n= 6, 24 hours; n=3, 48 hours). Although the inhibitor appeared to have an effect on IL-7Rα mRNA levels at 24 hours, the effects were lost at 48 hours. Moreover, no significant effects were observed at the protein level. Therefore, the MAPK ERK pathway appears not to be significantly involved in the regulation of CD8 T cell IL-7Rα expression.

The PI3K pathway is involved in IL-4 induced upregulation of GFI1 expression

The signaling pathways involved in GFI1 regulation were also investigated. In brief, CD8 T cells were pre-treated for 2 hours with the inhibitors, as described in the preceding section followed by IL-4 stimulation for 6 and 24 hours and RT-PCR analysis of GFI1 mRNA expression. Results did not support a role for the Jak/STAT pathway (Fig. 5.9a), as JAK inhibitor 1 failed to significantly affect IL-4-induced upregulation of GFI1 expression. Conversely, the PI3 kinase inhibitor at 50 μM significantly attenuated (n=6; $P<0.05$) the capacity of IL-4 to upregulate GFI1 expression (Fig. 5.9b). Similar experiments were conducted to evaluate the role of the MAPK pathway. Results did not

suggest a statistically significant role for the P38 (Fig. 5.10a), JNK (Fig. 5.10b), or MEK/ERK (Fig. 5.10c) signaling pathway in IL-4-induced GFI1 expression.

Figure 5.8 The effect of the ERK/MEK pathway of CD8 T cell IL-7Rα expression.

Purified human CD8 T cells were incubated with increasing concentrations (10, 25, 50 μM) of PD 98059 (PD) for 2 hours at 37°C followed by IL-4 (2 ng/ml) stimulation. (a) Cellular protein extracts (30 μg) from unstimulated (uns) CD8 T cells and cells stimulated with IL-4 for 15 minutes following incubation with PD were resolved by SDS-PAGE, transferred to a PVDF membrane, and successively probed for pERK and total ERK expression. (b, c) Surface IL-7Rα expression was measured on CD8 T lymphocytes incubated with PD prior to a (b) 24 or (c) 48 hour culture in the presence or absence of IL-4, and then analyzed by flow cytometry in the left panel. The relative fold change in MFI measurements of IL-7Rα expression of stimulated and unstimulated PD-treated cells compared to untreated cells in the absence of IL-4 stimulation. RNA extracts from the CD8 T cells were also reverse transcribed and IL-7Rα mRNA expression levels are shown in the right panel as determined by RT-PCR analysis. Relative gene expression levels were determined by the comparative Ct method [360]. Bar graphs represent 6 independent experiments where vertical bars represent the SD. The Bonferroni test for multiple comparisons was used to generate *P* values where $P < 0.001$ (***).

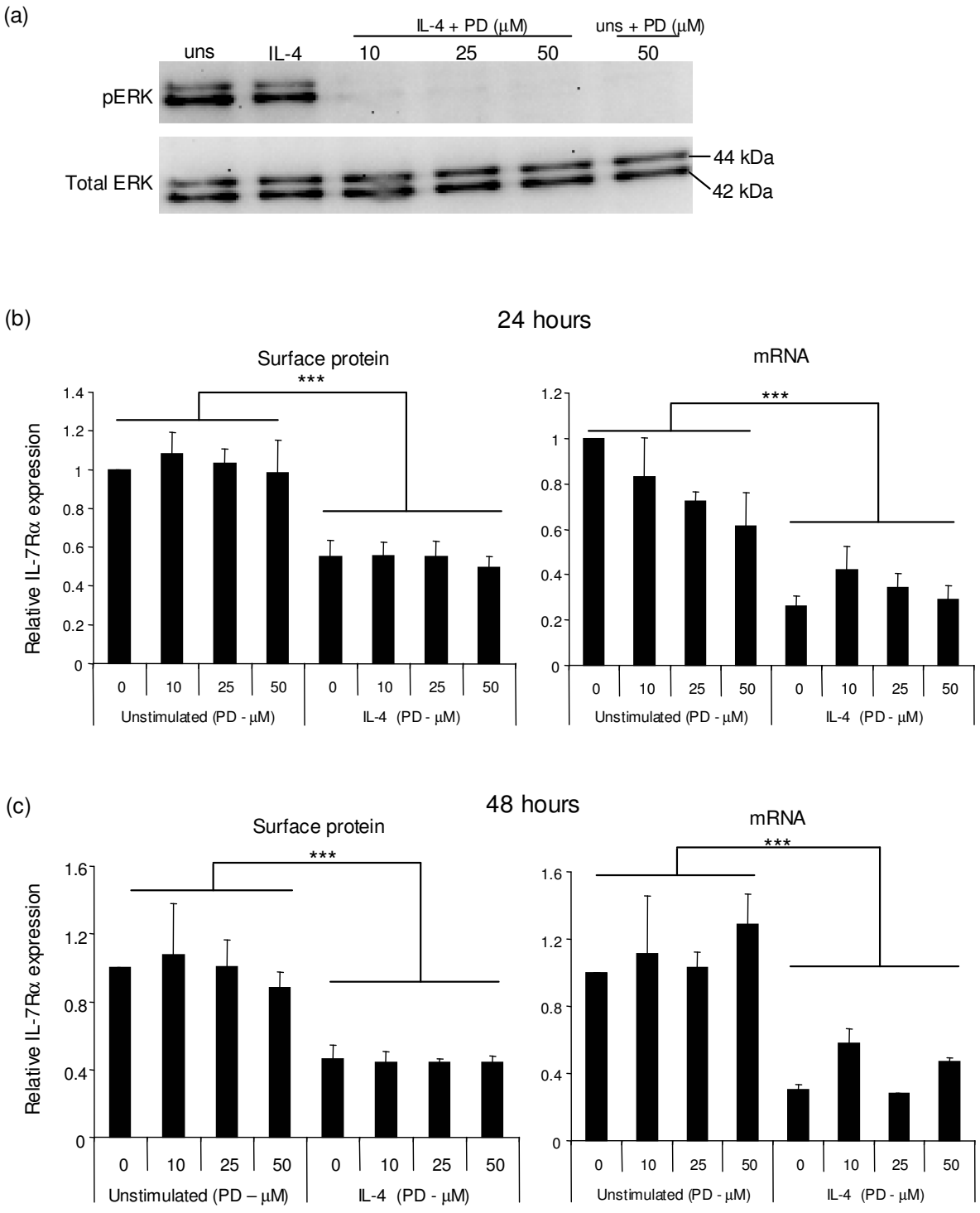

(a)
IL-4 + PD (μM)
uns + PD (μM)
uns
IL-4
10
25
50
50
pERK
Total ERK
44 kDa
42 kDa
(b)
24 hours
Surface protein
mRNA
***
Relative IL-7Rα expression
Unstimulated (PD - μM)
IL-4 (PD - μM)
(c)
48 hours
Surface protein
mRNA
***
Relative IL-7Rα expression
Unstimulated (PD – μM)
Unstimulated (PD - μM)
IL-4 (PD - μM)

FIGURE 5.8

Figure 5.9 Blockade of the PI3K pathway in CD8 T cells resulted in the suppression of GFI1 mRNA expression levels.

CD8 T lymphocytes were incubated with pharmaceutical inhibitors for 2 hours at 37^{o}C prior to incubation with IL-4 (2 ng/ml) for 6 and 24 hours. RNA extracts from the CD8 T cells were reverse transcribed followed by RT-PCR analysis to measure GFI1 mRNA expression levels. Relative GFI1 expression levels in CD8 T cells treated with (a) JAK inhibitor 1 or (b) LY 294002 (LY) and then stimulated with IL-4 for 6 hours (left panels) and 24 hours (right panels) are shown. Relative gene expression levels were determined by the comparative Ct method [360]. Bar graphs represent a mean of 6 independent experiments plotted along with the SD (vertical bars). The Bonferroni test for multiple comparisons was used to generate *P* values where $P < 0.001$ (***), $P<0.01$ (**), and $P<0.05$ (*).

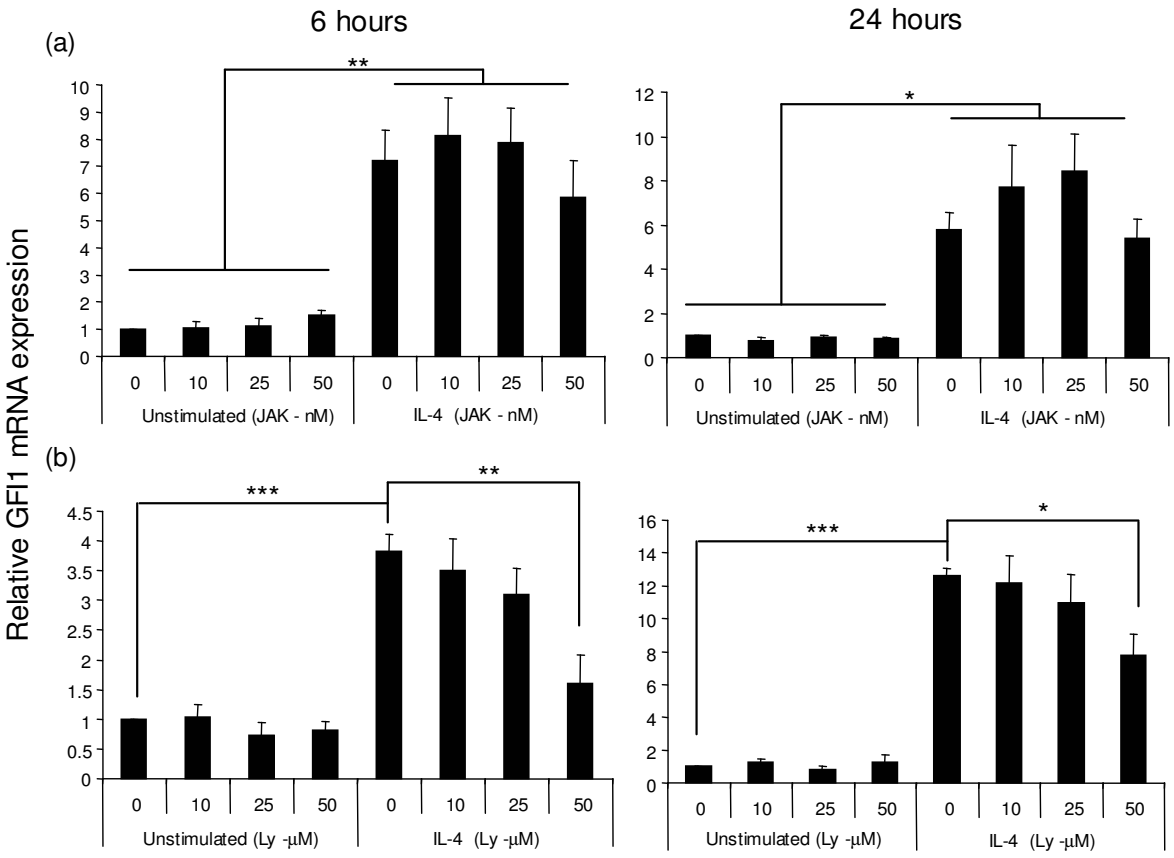

6 hours
24 hours
(a)
(b)
Relative GFI1 mRNA expression
Unstimulated (JAK - nM)
IL-4 (JAK - nM)
Unstimulated (Ly -μM)
IL-4 (Ly -μM)
0
10
25
50
**
*
***

FIGURE 5.9

Figure 5.10 The effect of MAP kinases P38, JNK, and ERK on GFI1 mRNA expression levels in CD8 T lymphocytes.

Purified CD8 T cells were incubated with (a) SB 203580 (SB), (b) SP 600125 (SP), and (c) PD 98059 (PD) for 2 hours to inhibit the phosphorylation of P38, JNK, and ERK, respectively, followed by IL-4 (2 ng/ml) stimulation. RNA extracts from CD8 T cells were reverse transcribed then analyzed by RT-PCR to measure GFI1 mRNA expression levels at 6 (left panels) and 24 hours (right panels) of IL-4 stimulation. Relative gene expression levels were determined by the comparative Ct method [360]. Bar graphs represent a mean of 6 independent experiments, and SD is represented by vertical bars. The Bonferroni test for multiple comparisons was used to generate *P* values where $P < 0.001$ (***), $P<0.01$ (**) and $P<0.05$ (*), and vertical bars represent the standard deviation.

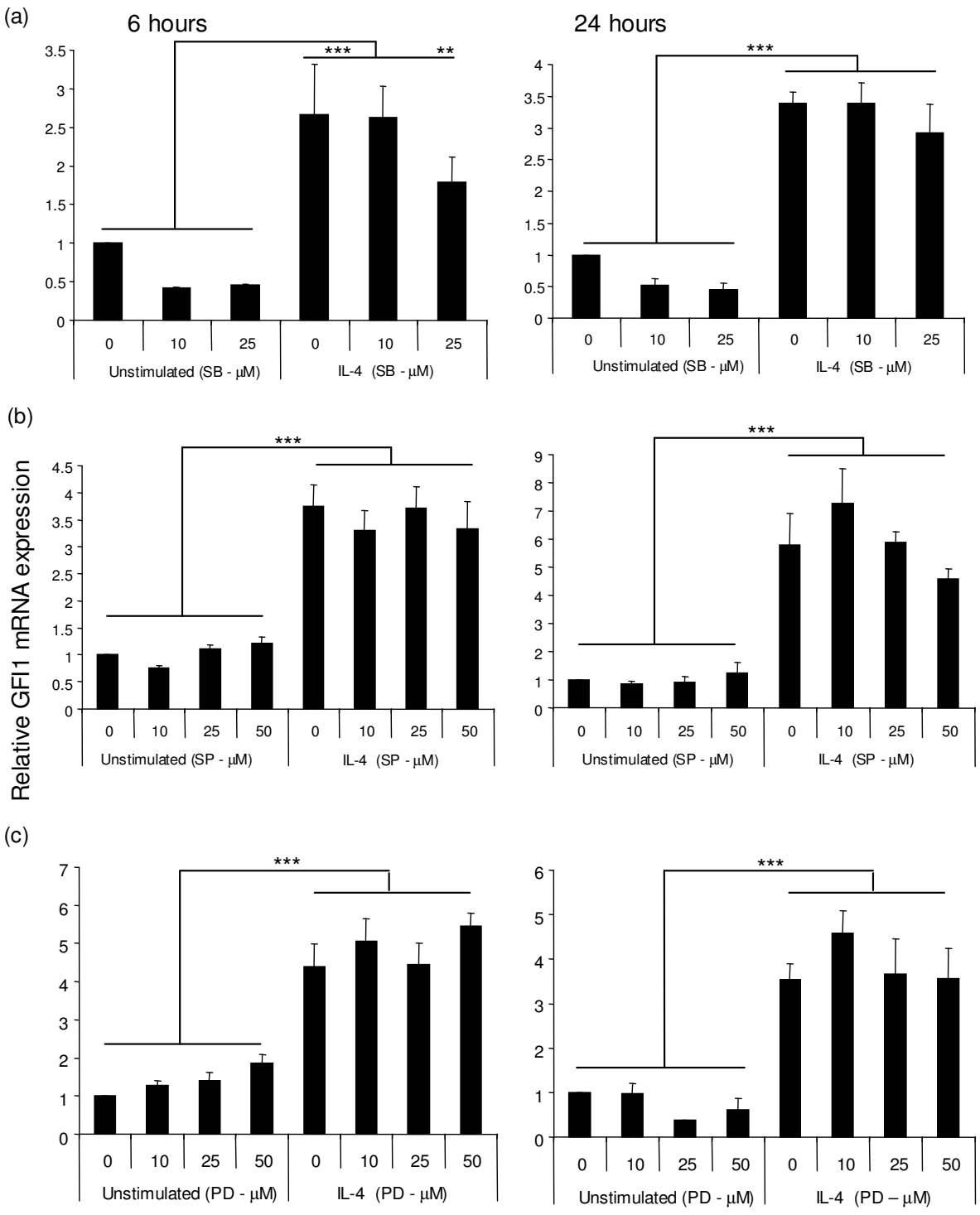

(a)
6 hours
24 hours
***
**
***
Unstimulated (SB - μM)
IL-4 (SB - μM)
(b)
Relative GFI1 mRNA expression
***
***
Unstimulated (SP - μM)
IL-4 (SP - μM)
(c)
***
***
Unstimulated (PD - μM)
IL-4 (PD - μM)
IL-4 (PD – μM)

FIGURE 5.10

CHAPTER 6
Discussion

Inverse Association of Repressor GFI1 with CD8 T Cell IL-7Rα Expression and Limited STAT Signaling in Response to IL-7 among γc Cytokines in HIV+ Patients

It is well established that IL-7Rα expression is reduced in CD8 T cells from HIV+ *vs.* HIV-negative controls [86,93,97,101,238,272,386-388]. As observed in this study (Fig. 3.2a) and by others, this is particularly evident in untreated viremic patients, with ART exerting at least partial IL-7Rα restoration [86,238,272]. In HIV+ patients, IL-7Rα^{low} CD8 T cells have been suggested to represent T effector-like cells, as defined by cell surface phenotype (CCR7-, CD62L-, CD45RA+ or RA-) and diverse functional features *ex vivo* including enhanced susceptibility to apoptosis, inferior proliferative capacity, increased IFN-γ, but reduced IL-2 production, compared to their IL-7Rα^{high}-expressing counterparts [86]. Furthermore, elevated PD-1 expression in HIV-specific CD8 T cells has been associated with functional impairment, reduced survival and an activated, early/intermediate differentiation phenotype [362,363,365,389] with lower proportions of IL-7Rα-positivity [363], as compared to cytomegalovirus-specific CD8 T cells in viremic patients. I also noted increased PD-1 expression on bulk CD8 T cells from HIV+ *vs.* HIV-negative patients, but in both IL-7Rα^{high} and IL-7Rα^{low} subsets, suggesting that IL-7Rα expression may be related to CD8 T cell activation/differentiation stage rather than PD-1 levels.

<u>IL-7Rα^{low} CD8 T cells fail to activate STAT5 in response to IL-7, but maintain Jak/STAT signaling following IL-2, IL-4, IL-15, IL-21, and IL-10 stimulation</u>

The responsiveness of IL-7Rα^{low}-expressing CD8 T cells to cytokines involved in regulating CD8 T cell growth and differentiation has remained largely unexplored until recently. My results suggest that impaired responsiveness of CD8 T cells to IL-7, as

indicated here by attenuated pSTAT5 induction, was due to low/absent IL-7Rα cell surface expression, observations consistent with other studies showing reduced function and survival of T cells from HIV+ patients in response to IL-7 [56,89,101,272]. However, it is interesting to note here that these cells retained the capacity to activate the STAT pathway in response to IL-2, IL-15, IL-4, IL-10, and IL-21. Notably, the level of cytokine-dependent STAT activation in the remaining IL-7Rα^{high} CD8 T cell subset was not significantly affected between study groups either. The intact IL-2-induced STAT5 signaling in CD8 T cells from patients off-therapy for >6 months is in contrast to previous findings in bulk CD8 T cells from patients completely naïve to therapy and may reflect a more progressive disease in the patient cohort studied previously [98]. This is consistent with a recent report extending findings of defective IL-2-induced STAT5 activation to all CD8 T cell subsets (naïve, memory, effector) from patients with progressive disease compared to long-term nonprogressors and those responsive to ART [353].

Reduced levels of GFI1in IL-7Rα^{high} *vs.* IL-7Rα^{low} CD8 T cells

IL-7Rα^{low} CD8 T cells in HIV+ individuals may arise as a result of chronic antigen exposure and/or signals received from elevated IL-7 serum levels or other cytokines (e.g. IL-4) known to be altered through the course of chronic HIV infection [86,113,118,124,388,390]. I speculate that amidst chronic stimulation and reduced IL-7Rα expression, responsiveness to other critical CD8 T cell cytokines, reportedly dysregulated in HIV+ patients [4,118,216,386,391], may thus predominate and contribute to the altered functionality, expansion and/or maintenance of IL-7Rα^{low} CD8 T cells in the periphery of HIV+ patients. The molecular mechanisms regulating IL-7Rα expression in CD8 T cells, particularly from HIV+ patients, is not well understood.

However, the role of transcriptional regulators, including the transcriptional repressor GFI1 and the Ets family transcription factor GABPα has been studied in T cells [189,294,300,301]. GFI1 and GABPα are negative and positive regulators of IL-7Rα expression in mouse T cells, respectively, and were recently implicated in the formation of IL-7Rα^{high} and IL-7Rα^{low} CD8 T cells in response to LCMV infection [189,300,301]. In humans, effector/effector-memory CD8 T cells comprise the majority of IL-7Rα^{low} CD8 T cells while naïve and memory subsets are IL-7Rα^{high} [294]. In contrast to mice, no significant differences were found in GABPα and GFI1 mRNA expression when comparing human naïve, IL-7Rα^{high} and IL-7Rα^{low} effector memory CD8 T cells, as defined by their CD45RA+ CCR7- phenotype [294]. This differed with my findings of increased GFI1 mRNA expression in IL-7Rα^{low} *vs.* IL-7Rα^{high} CD8 T cells, data concordant with IL-7Rα mRNA and protein expression in these subsets. The source for this discrepancy may be that I purified CD8 T cells based solely on their expression of IL-7Rα rather than also sub-stratifying based on effector-memory phenotype. Importantly, I confirmed my TaqMan RT-PCR results using a SybrGreen-based RT-PCR assay (Fig. 6.1) [294].

My mRNA expression data does not exclude GFI1B and GABPα, but failed to support a role for these reputed IL-7R transcriptional regulators in distinguishing IL-7Rα^{low} from IL-7Rα^{high} CD8 T cells [300,302]. GABPα mRNA expression did not vary significantly between IL-7Rα^{high} and IL-7Rα^{low} CD8 T cells, consistent with the previous report in humans [294]. Expression of GFI1B was not detected consistently in human CD8 T cells and may reflect its predominant role in the regulation of human erythroid development [392,393].

Figure 6.1 Comparison of IL-7Rα expression levels by TaqMan and Sybr Green-based real time analysis in CD8 T cells.

Purified CD8 T cells from two HIV-positive individuals were separated based on IL-7Rα expression into IL-7Rα^{low} CD8 T cells and IL-7Rα^{high} CD8 T cells. RNA extracts from the purified CD8 T cell populations were reverse transcribed and used in RT-PCR experiments to evaluate the expression of GFI1 and βactin. Relative gene expression levels were determined by the comparative Ct method [360]. Results were plotted as fold change comparing IL-7Rα^{high} vs. IL-7Rα^{low} CD8 T cells in two representative study participants.

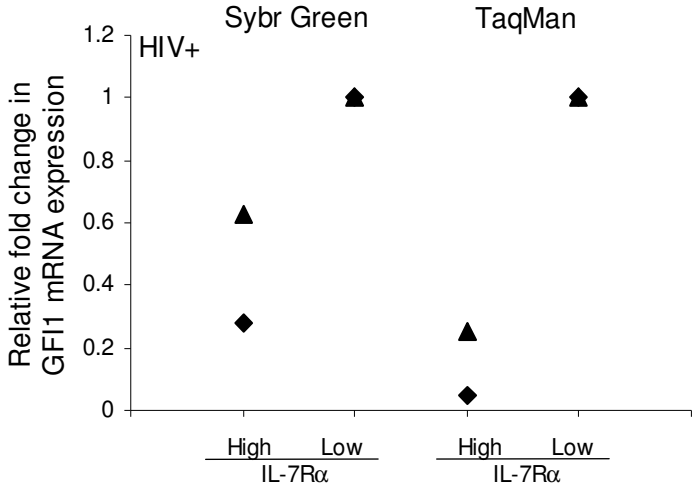

Sybr Green
TaqMan
HIV+
Relative fold change in GFI1 mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
High
Low
IL-7Rα
High
Low
IL-7Rα

FIGURE 6.1

In conclusion, I demonstrated that the IL-7Rα^{low} effector-like CD8 T cells found at increased proportions in HIV+ patients failed to activate STAT5 in response to IL-7, which strongly correlated with their lack of IL-7Rα. Interestingly, these cells, like their IL-7Rα^{high} counterparts, were capable of activating the STAT pathway in response to other cytokines involved in CD8 T cell growth and differentiation including IL-2, IL-15, IL-4, and IL-10. My results suggested for the first time that a mechanism involving GFI1 may be operative in human CD8 T cells for the transcriptional repression of IL-7Rα, but a mechanism that is not restricted to CD8 T cells from HIV+ patients alone. However, more direct functional evidence was required to substantiate this and was pursued in the next section of the thesis. Figure 6.2 illustrates the potential role of GFI1 as a transcriptional repressor of IL-7Rα expression in human CD8 T cells, and the accompanied impaired STAT5 phosphorylation resulting from lack of responsiveness to IL-7 (Fig. 6.2).

Figure 6.2 Schematic summarizing findings of cytokine responsiveness in CD8 T cells from HIV-positive and HIV-negative individuals.

IL-7 / IL-7Rα signaling was investigated in CD8 T cells purified from PBMCs of HIV-negative and HIV-positive study participants. Particularly in viremic HIV-positive individuals, the responsiveness of CD8 T cells to IL-7 was impaired as indicated by reduced activation of STAT5. In contrast, respective STAT activation in response to other cytokines tested (IL-2, IL-7, IL-10, IL-15, and IL-21) remained intact in CD8 T cells isolated from HIV-positive and HIV-negative volunteers. The increase in the IL-7-dependent pSTAT5 negative population in HIV-positive individuals correlated with an increased proportion of IL-7Rα^{low} human CD8 T cells. GFI1 expression was elevated in IL-7Rα^{low} cells compared to their IL-7Rα^{high}-expressing counterparts.

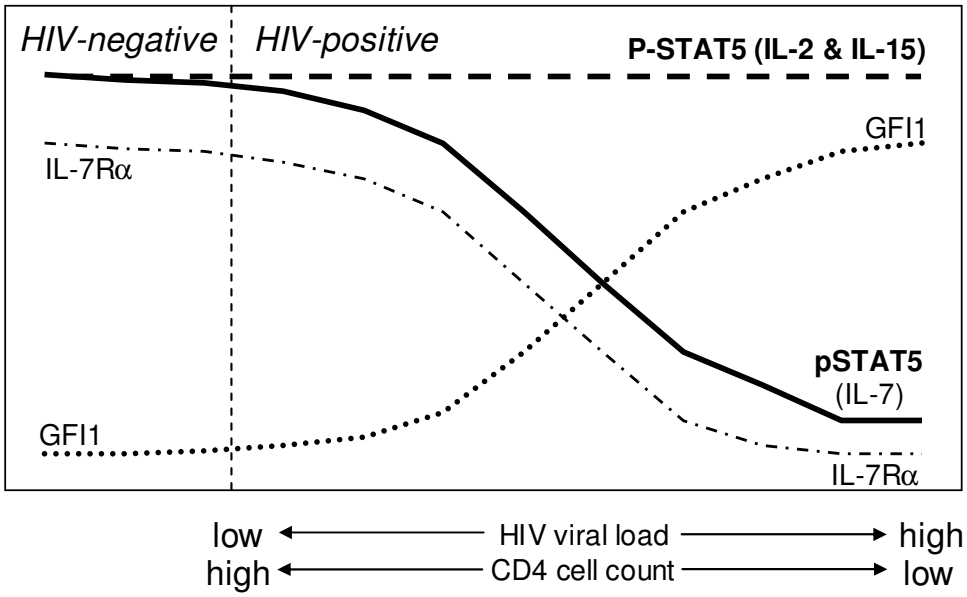

HIV-negative
HIV-positive
P-STAT5 (IL-2 & IL-15)
GFI1
IL-7Rα
pSTAT5
(IL-7)
GFI1
IL-7Rα
low
HIV viral load
high
high
CD4 cell count
low

FIGURE 6.2

Functional Role of Growth Factor Independent – 1 (GFI1) in Primary Human CD8 T Lymphocytes: Repressor Activity, Survival and Proliferation

Multiple roles for GFI1 have been well described using human cell lines as well as murine lymphocytes [301,317,333,335,358]. In these models, GFI1 has been assigned the function of a transcriptional repressor targeting genes such as CXCR4, Bax, and the protein to which much of this thesis has focused on, IL-7Rα [317,333,371]. Since enhanced GFI1 expression has been linked to increased proliferation in cells of the lymphoid lineage, it can also be reasoned that GFI1, through transcriptional repression of genes negatively regulating this process, may also function as an "activator" [323,356,357]. One of the main objectives of this chapter was to more directly investigate the reputed functional roles of GFI1 in primary human CD8 T lymphocytes. I showed that IL-4 was the only γc cytokine amongst the series of cytokines tested that increased GFI1 expression levels which correlated with its capacity to downregulate IL-7Rα expression. Interestingly, IL-4 stimulation exerted a sustained inhibitory effect on IL-7Rα expression of longer duration than IL-7. Considering this information, I showed that silencing GFI1 expression prior to IL-4 stimulation had no effect on IL-4-mediated downregulation of IL-7Rα expression. However, I further showed that reduced GFI1 expression levels post-IL-4-stimulation, resulted in a quicker recovery of surface IL-7Rα expression upon removal of IL-4 from culture. On the other hand, reduced GFI1 levels in the absence of exogenous stimulation did not impact basal IL-7Rα expression. It was interesting to note that exogenous overexpression of GFI1 did however, result in downregulation of IL-7Rα as well as repression of other previously identified GFI1 targets including CXCR4 and Bax. I also provided evidence for the involvement of GFI1

in IL-4-mediated CD8 T cell proliferation. Transient downregulation of GFI1 in the early stages of proliferation appeared to block cells from moving into the first cell division which coincided with an increase in the retention of cells in the G_1 phase. Taken together, the results clearly demonstrate a functional role for GFI1 in primary human CD8 T cells. The results from chapter 4 will be further discussed in three separate parts. The first will discuss the role of GFI1 in the regulation of IL-7Rα in resting and IL-4-activated human CD8 T cells. The second part will address the role of GFI1 in CD8 T cell proliferation. Lastly, the functional role of GFI1 as a negative transcriptional regulator of CXCR4 and Bax expression will be discussed.

Effect of GFI1 on basal expression levels of IL-7Rα in primary human CD8 T lymphocytes

My initial findings suggested a role for GFI1 in negatively regulating IL-7Rα expression, as discussed in chapter 3. Subsequent experiments attempted to establish more direct evidence to support this hypothesis. Initially silencing GFI1 expression with siRNA in primary resting human CD8 T cells was attempted, but had no significant effects on IL-7Rα expression. Hypothetically, reducing the transcriptional repressor activity of GFI1 on the IL-7Rα promoter would be expected to increase IL-7Rα expression. However, CD8 T cells at the resting stage already express high IL-7Rα levels. Furthermore, GFI1 is expressed at relatively low levels during this resting stage and is upregulated in activated T cells and following IL-4 stimulation in this case [323]. These factors may have precluded the capacity to detect any further increases in IL-7Rα as a result of GFI1 knockdown by siRNA. It is also possible that GFI1 may require a trigger to initiate its transcriptional repressor activity. Alternatively, the maintenance of

basal IL-7Rα expression levels may invoke a regulatory mechanism that does not include GFI1 or requires cooperative interaction with other factor(s) [317,334,335]. Indeed, Chandele et al. determined that IL-7Rα expression was modulated by the activity of GFI1 and GABPα in murine effector and memory virus-specific CD8 T cells [301]. Positive regulation of IL-7Rα by GABPα in memory precursor cells was correlated with hyperacetylation of the IL-7Rα promoter whereas GFI1 acted by antagonizing GABPα binding and recruiting HDAC1 to the IL-7Rα promoter in effector cells [301]. Nevertheless, my observation that exogenous overexpression of GFI1 downregulated receptor expression indicated that GFI1 is functional as an IL-7Rα transcriptional repressor in human CD8 T cells. Clearly, further study is required to better understand the control of IL-7Rα expression in resting CD8 T lymphocytes.

Resting cells may require an IL-7Rα regulatory mechanism that is distinct from that invoked in activated T cells. Indeed, I observed that amongst the γc cytokines that downregulated IL-7Rα expression, only IL-4 was able to concomitantly upregulate GFI1 expression. Thus, the role of GFI1 in IL-4-mediated negative regulation of IL-7Rα expression was investigated further. Results indicated that GFI1 may not be involved in the process of downregulating the receptor. Therefore, I hypothesized that GFI1 may serve to sustain IL-7Rα suppression in response to IL-4 similar to a phenomenon observed by Chandele et al. [301]. To test this, IL-7Rα was dowregulated in response to IL-4 stimulation. Subsequently the cytokine was removed and GFI1 was silenced by RNA interference. In contrast to cells transfected with a control siRNA, IL-7Rα recovered more readily in the absence of GFI1, supporting a role for GFI1 in sustaining low IL-7Rα expression in CD8 T cells. Although IL-7Rα recovery was statistically

significant, it was somewhat modest and likely reflective of the partial (48%) reduction in GFI1 expression in siRNA knockdown experiments. The role of GFI1 in sustaining IL-4-mediated downregulation of IL-7Rα expression in primary human CD8 T cells has never been reported.

Several γc cytokines namely IL-2, IL-4, IL-7, and IL-15 investigated in my thesis effectively downregulated IL-7Rα expression on human CD8 T cells which is consistent with reports showing the impact of these cytokines on the receptor of murine T cells [189,286,394]. In contrast, my results in human CD8 T cells showed that GFI1 was upregulated solely in response to IL-4. Furthermore, the effect of IL-21 on IL-7Rα has not been previously reported and was distinct compared to the other γc cytokines investigated as it did not significantly affect IL-7Rα expression. The literature has also implicated a role for IL-10, IFNα/β, and TNFα on increasing IL-7Rα expression in murine T cells [310,311,395]. My findings in human CD8 T cells are distinct as these factors had no significant effects on IL-7Rα protein expression under the conditions tested and may reflect species-related differences. However, more variable effects were observed at the mRNA level. Detection of consistent effects on IL-7Rα protein expression, particularly in the case of TNFα, may require sampling over a longer timeline. Several notable differences exist between the regulation of IL-7Rα expression in murine T cells and my findings in human CD8 T lymphocytes, suggesting that a different mechanism is operative in human lymphocytes. Nevertheless, I showed for the first time that IL-4-induced GFI1 expression was involved in sustaining the downregulation of IL-7Rα expression in primary human CD8 T cells. It is noteworthy

that IL-4 stimulation increases expression of IL-4Rα thus potentially sustaining its effect on GFI1 and IL-7Rα, in contrast to the suppressive effects of IL-7 on IL-7Rα [189].

The suppression of IL-7Rα expression by IL-7 has been hypothesized to maximize signaling through IL-7Rα in unsignaled T cells [189,237,307-309]. Similarly the γc cytokines in this study (IL-2, IL-4, and IL-15 in addition to IL-7) also downregulated IL-7Rα expression on human CD8 T cells. Upon exposure of the human CD8 T cells to these cytokines, IL-7Rα^{low}-expressing CD8 T cells and their responsiveness to IL-2, IL-4, IL-15 as well as IL-21 may thus predominate [30,52].

GFI1 promotes IL-4-induced proliferation in primary human CD8 T lymphocytes

I investigated the role of GFI1 in IL-4-dependent CD8 T cell proliferation. My results showed a reduced proliferative capacity in response to IL-4 when GFI1 expression was inhibited. A relative 24.26% increase in cells that had not transitioned into the cell cycle, remaining in G_1, was found for siGFI1 transfected CD8 T cells compared to controls. These effects, albeit moderate, were consistent and reproducible and may have been more dramatic if the transfection was stable and more effective instead of transient in nature. If a human CD8 T cell line could have been generated in which GFI1 expression was stably silenced, the effects of GFI1 may have been more pronounced on IL-4-induced proliferation. In fact, lymphomagenesis in a murine cytotoxic T cell line stably overexpressing GFI1 acted cooperatively with the MYC and PIM-1 oncogenes to promote proliferation and did so in the absence of IL-2 compared to untransfected cells [334]. In the past, the role of GFI1 in proliferation has been evaluated in T lymphocytes isolated from GFI1-deficient mice or through constitutive GFI1 expression in lymphocytes [189,323,334,356,357]. Constitutive GFI1 expression through stable

transfection of a Jurkat T cell line promoted lymphomagenesis by accelerating proliferation [323]. Increased proliferation is likely attributed to rapid transition through the cell cycle where resting cells in G_0 move into G_1 then S, followed by entry into G_2 and finally the M (mitosis) phase, where protein synthesis and cell growth occurs in G_1 / G_2 and DNA synthesis in the S phase [396]. The G_1 and G_2 phases represent the critical checkpoints where cells verify their readiness to transition through the cell cycle [396-398]. Constitutive GFI1 expression pushes the cell passed the G_1 cell cycle checkpoint, a regulator for AICD and S phase entry [323]. GFI1 has also been shown to repress the $p21^{Cip/WAF1}$ cell cycle regulator in a Jurkat T cell line [335]. Importantly, mature CD4 and CD8 T cells from GFI1-deficient mice experience delayed cell cycle entry [355]. In murine CD4 T cells, IL-2 utilized GFI1 and STAT5 signaling to promote the expansion of Th2 cells while IL-4-induced proliferation was mediated by GFI1 and required STAT6 signaling [356,357]. As briefly described in the introduction of chapter 4, GFI1 was involved in enhancing the proliferative capacity of lymphocytes in contrast to hematopoeitic stem cells [335,356,374,375], which supports my findings regarding the effect of GFI1 on the proliferation of human CD8 T lymphocytes.

GFI1 negatively regulates Bax and CXCR4 protein expression levels in human CD8 T lymphocytes

Enhanced GFI1 expression was associated with reduced CXCR4 expression in primary human CD8 T cells. As previously mentioned, the capacity of GFI1 to suppress CXCR4 is not unexpected since human CXCR4 possesses double GFI1 binding sites [372]. However, my findings further support current knowledge regarding the suppressive effect of GFI1 on CXCR4 has only been reported for granulocytic cells and hematopoietic stem cells. GFI1 induction by the p53 tumor suppressor reduces CXCL12-

CXCR4 signaling in cancer stem cells inhibiting activation of the PI3K pathway [371]. In another model system, G-CSF signaling induced GFI1 expression thus inhibiting CXCL12-CXCR4 signaling and enabled mobilization of granulocytic and hematopoietic stem cells from the bone marrow into the peripheral blood [333]. CXCL12-CXCR4 signaling is critical in the proliferation, survival, migration, and homing of cancer cells, granulocytic, and hemapoeitic stem cells [333,371,372]. These functions may extend to lymphocytes since modulation of CXCR4 has been reported on human CD8 T cells [399]. More specifically, CXCR4 was reportedly expressed at high levels on naïve and central memory human CD8 T cells, but was reduced on effector and effector memory CD8 T cells where CXCL12-CXCR4 signaling was important in CD8 T cell migration and its cytolytic activity [399-403]. Importantly, a mechanism implicating GFI1 in the regulation of human CD8 T cell CXCR4 expression is proposed in this study.

Interestingly, Bax was also negatively regulated by exogenous overexpression of GFI1 in human CD8 T cells. This is consistent with the literature documenting that GFI1 acts cooperatively with the proto-oncoprotein ETS1 to repress Bax expression in a human kidney cell line, 293T [317]. Moreover, enhanced GFI1 expression in the Jurkat T cell line was associated with inhibition of AICD and apoptosis in proliferating T cells [323,329,356]. Early findings also showed that GFI1 in IL-2-dependent T cells or murine thymocytes repressed Bax and Bad expression without influencing Bcl-2 levels, indicating that apoptotic cell death could be delayed without affecting Bcl-2 [329]. The repression of Bax in human CD8 T cells would be expected to contribute to their increased survival. However, further investigations are required to evaluate this and it is likely that multiple anti-apoptotic or pro-apoptotic factors may influence CD8 T cell survival under these conditions.

In summary my findings have conclusively shown diverse functional roles for GFI1 using small interfering RNA targeting GFI1 and exogenous overexpression of GFI1 in primary human CD8 T lymphocytes. In human CD8 T cells, GFI1 acts as a transcriptional repressor targeting IL-7Rα, CXCR4, and Bax and has a role in promoting the proliferation of CD8 T cells in response to IL-4 stimulation (Fig. 6.3).

Figure 6.3 Schematic representation of the functional effects of IL-4 inducible and exogenous GFI1 expression in primary human CD8 T lymphocytes: Suppression of IL-7Rα, CXCR4, and Bax expression as well as regulation of proliferation.

The effects of modulating GFI1 expression using small-interfering RNA targeting GFI1 (siGFI1) and GFI1-expressing plasmid (pGFI1) was investigated in resting and IL-4-stimulated human CD8 T lymphocytes. Although reducing GFI1 expression levels in IL-7Rα^{high} resting CD8 T lymphocytes has no significant effect on basal IL-7Rα levels, exogenous overexpression of GFI1 resulted in suppression of IL-7Rα expression in addition to CXCR4 and Bax expression levels. IL-4 stimulation induced downregulation of IL-7Rα expression and enhanced GFI1 expression. Following IL-4 withdrawal, IL-7Rα expression recovered more readily if GFI1 expression was silenced. IL-4-induced proliferation was detected using a sub-optimal dose of PHA-M. Silencing of GFI1 expression under these conditions reduced IL-4-dependent proliferation of primary human CD8 T lymphocytes. Symbols (****) to (*) represents 'high' expression to 'low' expression and (↓) represents a reduction in protein expression.

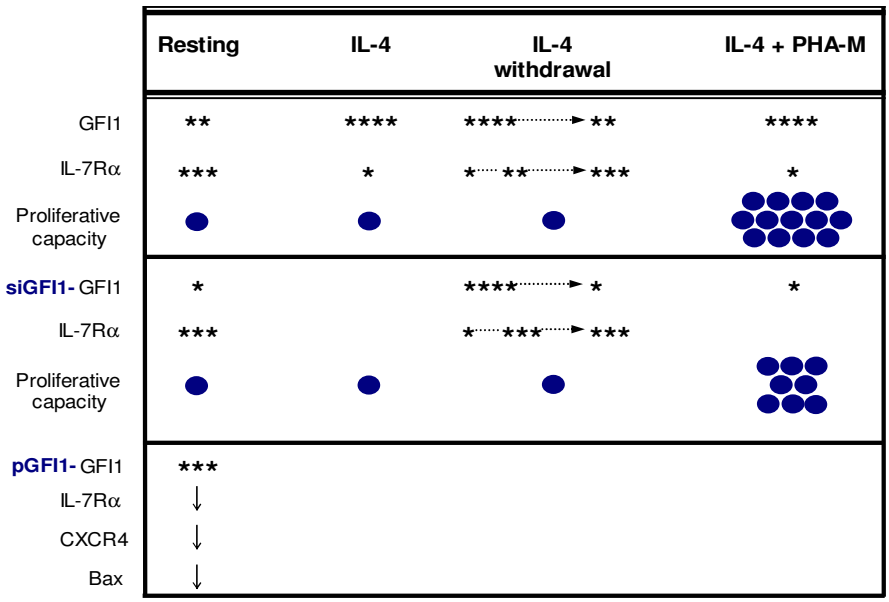

Resting
IL-4
IL-4 withdrawal
IL-4 + PHA-M
GFI1
IL-7Rα
Proliferative capacity
siGFI1- GFI1
IL-7Rα
Proliferative capacity
pGFI1- GFI1
IL-7Rα
CXCR4
Bax

FIGURE 6.3

Signal Transduction Pathways Regulating IL-7Rα and GFI1 Expression at the Basal Level and in Response to IL-4 Stimulation in Human CD8 T cells

Cytokine stimulation has been found to regulate IL-7Rα expression at the mRNA level [286,299,300,303]. In chapter 4 and consistent with this, I identified a mechanism involving the transcriptional repressor GFI1 in IL-4-induced suppression of IL-7Rα expression in human CD8 T cells. However, the signaling cascade required for IL-4-induced upregulation of GFI1 and the negative regulation of IL-7Rα expression are not known. Therefore, the objective of this chapter was to identify these signaling pathways in primary human CD8 T lymphocytes. This was accomplished through the use of pharmaceutical inhibitors targeting pivotal signaling proteins of the Jak/STAT, PI3K as well as the MAPK (P38, JNK, MEK/ERK) pathways, respectively. IL-4 mainly induces the Jak/STAT6 and PI3K pathways [174]. I showed that the effects of IL-4-mediated downregulation of IL-7Rα are abrogated by Jak and PI3K inhibitors in a dose-dependent manner. Interestingly, inhibition of JNK and P38 signaling suggested that these pathways may be involved in maintaining basal IL-7Rα expression levels. ERK signaling had no significant effects on IL-4-mediated IL-7Rα downregulation or basal receptor expression. At the level of GFI expression, inhibition of the PI3 kinase pathway resulted in the suppression of IL-4-mediated upregulation of this repressor whereas the Jak/STAT6 and MAPK pathways appeared not to be involved. Basal expression levels of GFI1 were not significantly modulated by inhibiting the MAPK P38, ERK/MEK, and JNK signaling pathways investigated.

IL-4 is known to mediate many of its effects by signaling through the Jak/STAT6 pathway including proliferation and survival [138,180,384,385,404,405]. Indeed,

STAT6-deficient murine lymphocytes were shown to be compromised in their ability to respond to IL-4 mitogenically reflected by reduced transition from G_1 to the S phase of the cell cycle and dysregulation of the Cdk inhibitor $p27^{kip1}$, for example [406,406]. I used a broad-spectrum Jak inhibitor to implicate the Jak/STAT6 pathway in IL-4-induced upregulation of GFI1 and the downregulation of IL-7Rα expression. The observation that IL-4-induced STAT6 activation was involved in the negative regulation of IL-7Rα was a novel finding. Thus far, activation of the PI3K pathway by IL-2 was implicated in the downregulation of IL-7Rα expression in murine splenocytes [286]. Since then little progress had been made to fully characterize which signaling pathways are necessary for the negative regulation of IL-7Rα expression following cytokine stimulation in human CD8 T cells or other cell types. In order to investigate the role of the PI3K pathway in IL-4-mediated regulation of IL-7Rα expression in human CD8 T cells, the PI3K inhibitor, LY 294002, was used. I showed for the first time that the PI3K pathway was involved in IL-4 suppression of IL-7Rα expression. In parallel, I demonstrated that IL-4-mediated upregulation of GFI1 expression in human CD8 T cells did not require the Jak/STAT6 pathway, findings which were not consistent with a previous report in STAT6-deficient murine T cells [356]. In contrast and findings that have not been previously reported, was my observation that the PI3K pathway was involved in IL-4-mediated upregulation of GFI1 expression in human CD8 T cells. It appears that there may be species-related and/or cell type (CD4 vs CD8) associated differences in the signaling pathways required for the upregulation of GFI1 expression by IL-4. The fact that IL-4-induced Jak/STAT6 did not appear to be required for GFI1 upregulation while important for IL-7Rα downregulation, was not entirely surprising

since I implicated GFI1 in sustaining suppression of IL-7Rα rather than in the downregulation process itself. Conducting siRNA studies targeting STAT6 and AKT proteins are necessary to confirm the role of these pathways in IL-4-mediated regulation of IL-7Rα and GFI1 expression.

Activation of the MAPK JNK and P38 pathway are associated with cell death, differentiation, and proliferation of CD8 T cells [148,149,157,159,160,251,385,407]. I demonstrated through the use of a P38 inhibitior, SB 203580, and a JNK inhibitor, SP 600125, that these signaling pathways were not found to have a statistically significant impact on GFI1 expression, but have a role in maintaining basal IL-7Rα expression on human CD8 T cells. This has not been previously reported. Phosphorylation of c-Jun by JNK enables the transcription factor nuclear activating protein-1 (AP-1) to form transcriptionaly active complexes composed of dimers of the Fos and Jun family of proteins which bind the promoters of IL-2Rα and IL-7Rα to negatively regulate these receptors [159,311]. Reduced levels of c-Jun translated into fewer AP-1 complexes interacting with the AP-1 binding sites on the IL-2Rα promoter of CD8 T cells, and the IL-7Rα promoter region of immature thymic T cells [159,311]. In addition, during the progression of murine thymocyte development where IL-7Rα becomes downregulated, c-Jun-deficiency resulted in increased IL-7Rα surface and mRNA expression [408]. Intriguingly, inhibition of DNA binding was linked to phosphorylation of c-Jun at a cluster of sites located next to its basic region whereas phosphorylation at sites within the N-terminal transactivation domain potentiated its ability to activate transcription [409]. These c-Jun N-terminal sites could be phosphorylated by the JNK and P38 signaling proteins in human CD4 and CD8 T cells. Therefore, this would require more direct

investigation through mutational analysis of the promoter in normal human CD8 T cells. In addition, siRNA experiments targeting P38 and JNK proteins to confirm the role of these signaling pathways in regulating basal IL-7Rα expression are necessary.

Furthermore, findings associated with pharmaceutical inhibitors in general need to be interpreted with caution since their use can lead to non-specific inhibitory effects and toxicity [410-419]. In order to control for any inherent cytotoxicity mediated by the inhibitors used in my studies, cytotoxicity was evaluated by PI staining and showed minimal cell death, particularly at the concentrations exhibiting a biological effect. In an attempt to minimize the potential non-specific effect of these inhibitors, a range of concentrations, commonly found in the literature [410-413,415-420] were used and their biological activity was demonstrated by western blotting. Notably for the JNK pathway, a previous study [190] showed that the SP 600125 used only at doses less than 10 μM was specific for the JNK MAP kinase family. As a result, findings with the JNK inhibitor may certainly have a non-specific component. Taken together, these results clearly require confirmation through the use of small interfering RNAs targeting these pathways in the future.

In summary, I have successfully identified the signaling pathways involved in regulating basal and IL-4-dependent IL-7Rα and GFI1 expression levels in human CD8 T cells (Fig. 6.4) and thus more specifically implicate how these pathways may influence human CD8 T cell growth and differentiation.

Figure 6.4 The Jak/STAT and the PI3K pathway are involved in IL-4-mediated negative regulation of IL-7Rα expression, but only the PI3K pathway is involved in IL-4-induced GFI1 expression.

Regulation of basal IL-7Rα expression was mediated by the MAPK P38 and JNK signaling proteins. The involvement of the Jak/STAT pathway and the PI3K pathway in IL-7Rα and GFI1 regulation were analyzed using JAK inhibitor 1 and LY 294002, respectively. SB 203580, SP 600125, and PD 98059 were used to determine the role of the MAPK P38, JNK, and MEK signaling proteins, respectively. The pharmaceutical inhibitors were incubated with the human CD8 T lymphocytes for 2 hours prior to stimulation with IL-4 for 6 and 24 hours to investigate GFI1 regulation by real time PCR analysis and for 24 hours to visualize modulations in IL-7Rα expression by flow cytometry. IL-4-mediated positive regulation is represented by (→) and negative regulation by (⊣). Basal IL-7Rα expression regulation is indicated by (→) and a pink box indicates constitutive activation of MAPK JNK, P38, and MEK signaling proteins.

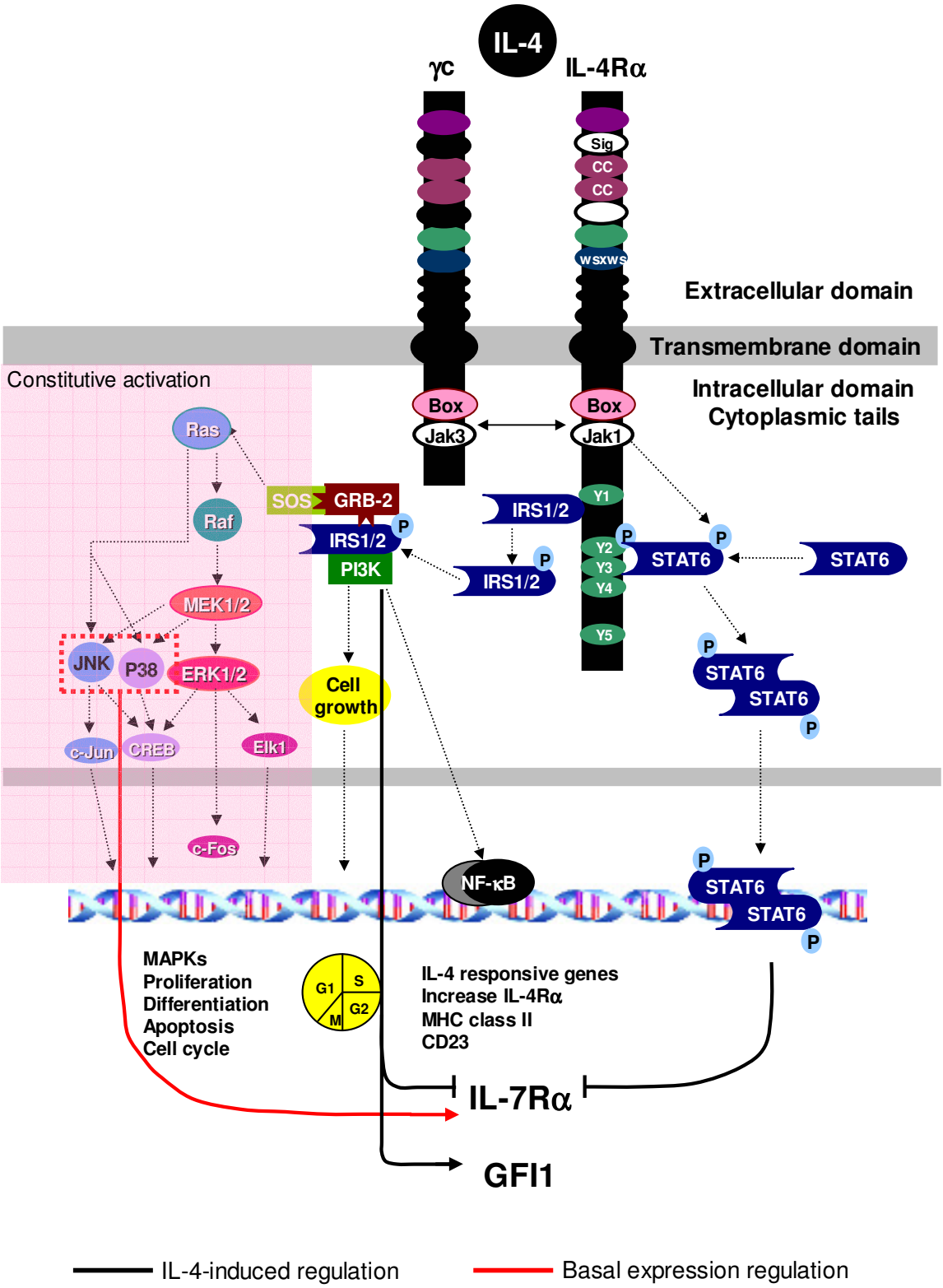

IL-4
γc
IL-4Rα
Sig
CC
CC
WSXWS
Extracellular domain
Transmembrane domain
Intracellular domain
Cytoplasmic tails
Constitutive activation
Box
Jak3
Box
Jak1
Ras
Raf
SOS
GRB-2
IRS1/2
P
PI3K
IRS1/2
P
IRS1/2
Y1
Y2
Y3
Y4
Y5
P
STAT6
P
STAT6
MEK1/2
JNK
P38
ERK1/2
Cell growth
P
STAT6
STAT6
P
c-Jun
CREB
Elk1
c-Fos
NF-κB
P
STAT6
STAT6
P
MAPKs
Proliferation
Differentiation
Apoptosis
Cell cycle
G1
S
G2
M
IL-4 responsive genes
Increase IL-4Rα
MHC class II
CD23
IL-7Rα
GFI1
IL-4-induced regulation
Basal expression regulation

FIGURE 6.4

CHAPTER 7

Concluding Remarks, Significance and Future Directions

My thesis is the first study to evaluate the functional roles for the transcriptional repressor GFI1 in primary human CD8 T lymphocytes. Initial findings in CD8 T cells from chronically-infected HIV+ patients, provided evidence for an impaired IL-7 / IL-7Rα signaling axis [373]. This was manifested in an attenuated IL-7-induced STAT5 activation that correlated with an increased proportion of IL-7Rα^{low}-expressing CD8 T cells, particularly in viremic patients. In contrast, IL-7Rα^{low} CD8 T cells were quite capable of mobilizing the Jak/STAT pathway in response to the other cytokines tested. Furthermore, IL-7Rα^{low} cells exhibited an increased expression of GFI1 compared to their IL-7Rα^{high}-expressing counterparts. The notable decrease in IL-7Rα expression and its association with enhanced GFI1 expression layed the foundation for further investigations into the mechanisms responsible for the negative regulation of CD8 T cell IL-7Rα expression.

Though basal IL-7Rα expression was not significantly altered by silencing GFI1 expression in resting CD8 T cells, exogenous overexpression of GFI1 did result in decreased IL-7Rα expression, confirming the functionality of the repressor in human CD8 T cells. Thus, the impact of GFI1 expression was further studied in cytokine-stimulated CD8 T cells and in a cytokine-mediated proliferation model. IL-4-mediated downregulation of IL-7Rα expression appeared to be distinct from that mediated by IL-7 or other γc cytokines tested. I found that IL-4 stimulation upregulated GFI1 expression and GFI1, at least in part, was involved in sustaining IL-7Rα suppression rather than in the process of downregulating the receptor. It would also be interesting to investigate whether GFI1 has a role in the negative regulation of IL-7Rα in response to other γc cytokines. Although GFI1 expression was found not to be affected by these cytokines,

unlike IL-4, this doesn't necessarily reflect the potential modulatory effect of these cytokines on the functional activity of GFI1.

Another key finding of the thesis was that the expansion of primary human CD8 T lymphocytes in response to IL-4 was promoted by GFI1. Transient suppression of GFI1 expression during IL-4 mediated proliferation resulted in an accumulation of CD8 T cells in the G_1 phase of the cell cycle and a concomitant reduction in the proportion of cells entering into the first division. For the same reason as above, it would be exciting to investigate the effect of GFI1 on the the proliferative capacity of CD8 T lymphocytes in response to other γc cytokines. In addition, investigating the effects of the GFI1 repressor on cell cycle regulators in IL-4-mediated CD8 T cell proliferation is of great interest.

Though experiments using PI staining shown in chapter 4 did not reveal any noticeable effects on cell death by silencing GFI1 expression, it would be important to more systematically explore the role of GFI1 in CD8 T cell survival. Enhanced GFI1 expression showed the potential to influence cell survival in view of the observation that increased GFI1 expression suppressed the expression of the pro-apoptotic protein, Bax. On the other hand, GFI1 has not been reported to regulate Bcl-2, and preliminary evidence in human CD8 T cells confirmed the lack of any significant effect on Bcl-2 protein expression (data not shown). In the future, it would be interesting to determine whether other Bcl-2 family members are modulated by IL-7 stimulation via GFI1.

The biological effects mediated by GFI1 in human CD8 T cells include the repression of a number of important cell surface (IL-7Rα, CXCR4), and intracellular (Bax) proteins, thereby potentially regulating their survival and proliferative capacity *in*

vivo. In order to begin to delineate the molecular mechanism regulating the expression of this transcriptional repressor and one of its critical downstream targets (IL-7Rα) in human CD8 T cells, I attempted to define the signaling pathways that were required for this process in response to IL-4. I found that the PI3K pathway was involved in the positive regulation of GFI1 while both the PI3K and the Jak/STAT pathways were involved in the negative regulation of IL-7Rα by IL-4. Future experiments to confirm the effects of the broad pharmaceutical inhibitors used for these pathways would involve RNA interference studies where initial experiments would focus on silencing AKT and STAT6 molecules specifically. An interesting question to ask for example would be whether silencing AKT in CD8 T cells would attenuate IL-7Rα downregulation in response to IL-4 or lead to a more rapid recovery of IL-7Rα via reduced GFI1 expression levels. In addition, the implication of the MAPK JNK and P38 signaling pathways in regulating basal IL-7Rα expression levels in human CD8 T cells represents a novel mechanism identified in the thesis. Similarly, and in view of the limitations encountered with the use of broad pharmaceutical inhibitors, further experiments targeting these pathways with specific RNAi are required.

In summary, the work conducted in this thesis and the new questions that arise provide a number of research opportunities. The significance of the findings could contribute not only towards increasing our knowledge of normal human CD8 T cell growth and differentiation, but also towards the understanding of how these phenomena may be dysregulated during chronic HIV infection (and potentially tumorigenesis). The IL-4 study model may mimic some of the effects that ensue from the physiological increase in IL-4 production reported to occur during chronic HIV infection [111-

113,118,186,188]. This model may highlight how the IL-4 increase could affect CD8 T cell activity, and through which signaling pathways it would mediate changes to cell function. Deviation of cytolytic T cell function, induced by type 2 cytokines like IL-4, has been an attractive concept to explain the failure of the immune system in certain diseases, notably chronic HIV infection. However, the ramifications of IL-4 stimulation on CD8 T cells have proven to be quite confusing. An increase in circulating IL-4 in HIV infection supports a type 1 to type 2 cytokine switch in both CD4 and CD8 T cells of the immune system [114]. In fact, clinical progressors lack functional cytotoxic CD8 T lymphocytes that produce IL-4 which in turn exerts its effect on the circulating lymphocyte population [129]. In contrast, clinical nonprogressors experience more balanced type 1 to type 2 cytokine responses as well as produce IL-4, but only in response to certain HIV peptides [114]. On the other hand, IL-4 / IL-4Rα signaling has been shown to be necessary for cytotoxic CD8 T cell responses against the influenza virus in the absence of CD4 T cell help. In theory, the increase in IL-4 production during HIV infection could be due to the skewing of the immune response, but also to compensate for the lack of CD4 T cell help due to their declining numbers. Clearly, further investigation is required to fully understand the impact of IL-4 on human CD8 T cells in clinically healthy individuals as well as in virus-infected individuals.

Finally, developing an HIV vaccine to elicit a protective antiviral immune response at mucosal surfaces has been suggested to be critical since these are generally the first sites to encounter the HIV virus [421,422]. Ranasinghe et al. showed in mice that systemic immunization using heterologous poxvirus containing modified Gag and Pol genes yielded higher levels of IL-4 and IL-13 secretion in comparison to mucosal

immunization [423]. Systemic immunization also generated low avidity HIV-specific CTLs. Unfortunately, high avidity CTLs instead of low avidity CTLs have been associated with increased functional capacity to clear infections [424]. The study also showed that depletion of IL-13 increased CTL avidity as did the absence of IL-4 and STAT6 activation. Intriguingly though, the complete absence of IL-4 was detrimental since IL-4 was necessary for long-term maintenance of Th1 / Th2 cytokine balance in CTLs. Such studies emphasize the importance of further investigating the effects of IL-4 concentration levels and the duration of IL-4 exposure on primary human CD8 T cells.

In conclusion, the work from my thesis provides insight as to the nature of GFI1's functional role in normal human CD8 T cell growth and differentiation, but also how this repressor may be acting in the context of chronic HIV infection and other diseases such as cancer since GFI1 is an oncoprotein. Chronic HIV infection is associated with a dramatic decrease in IL-7Rα expression. Therefore, a greater understanding of the regulation of IL7Rα may provide potential targets, possibly GFI1, for therapeutic purposes. Investigating the outcome of increased IL-4 production and its effects on human CD8 T cells may also contribute to our understanding of and potentially reversing the development of an exhausted phenotype in CD8 T cells during the course of chronic HIV infection.

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Appendix

Reagent formulations

10X Phosphate-buffered saline (PBS) – pH 7.4

NaCl	80 g
KCl	2 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g
Distilled H_2O (dH_2O)	1 L - Final volume

Sterilize by autoclaving.

Cell purification solutions – AutoMACS

Rinsing buffer – pH 7.2

EDTA	1.6 g
1X PBS	2 L - Final volume

Store at 4°C.

Running buffer – pH 7.2

EDTA	1.6 g
Fetal calf serum (FCS)	10 ml
1X PBS	2 L - Final volume

Store at 4°C.

Cleaning solutions

70% w/v reagent grade ethanol

Western Blotting

10 X Running Buffer – pH 8.3

Tris base	30 g
Glycine	144 g
Sodium dodecyl sulfate (SDS)	10 g
Add last	
dH_2O	1 L - Final volume

10X Transfer Buffer – pH 8.3

Tris base	30 g
Glycine	144 g
dH_2O	1 L - Final volume

1X Transfer buffer
100 ml 10X Transfer buffer
700 ml dH_2O
200 ml methanol

Solution components for Tris-glycine SDS-polyacrylamide Gel Electrophoresis	*Volumes (ml) per lower gel mold:*
8%	
dH_2O	4.6
30% Acrylamide	2.7
1.5M Tris-base - pH 8.8	2.5
10% SDS	0.1
10% Ammonium persulfate	0.1
TEMED	0.006
10%	
dH_2O	4
30% Acrylamide	3.3
1.5M Tris-base – pH 8.8	2.5
10% SDS	0.1
10% Ammonium persulfate	0.15
TEMED	0.020

Solution components for Tris-glycine SDS-polyacrylamide Gel Electrophoresis	*Volumes (ml) per 5% stacking gel mold*
dH_2O	2.7
30% Acrylamide	0.67
1.0M Tris-base – pH 6.8	0.5
10% SDS	0.04
10% Ammonium persulfate	0.04
TEMED	0.004

Modified from Harlow and Lane (1988)

Stripping buffer – pH 6.7

2 M Tris-HCl	15.6 ml
β-mercaptoethanol	3.65 ml
SDS	10 g
dH_2O	500 ml

1 M 1,4-dithio-DL-threitol (DTT)

DTT	3.085 g
10 mM sodium acetate – pH 5.2	20 ml - Final volume

Filter sterilize, freeze and store in 1ml aliquots.

10X Tris-buffered saline with Tween (TBST) pH 7.6

Tris-HCl	24.2 g
NaCl	80 g
Tween-20	10 ml
Add last	
dH_2O	1 L - Final volume
Store at 4^oC.	

Reverse Transcription PCR

	Quantities for 1 reaction (µl)
10X RT	5
25 mM dNTPs	2
10X Random primer	5
RT	2.5
ddH_2O (RNAse free)	10.5
RNA	25
Total	50

Real Time PCR

	Quantities for 1 reaction (µl)
Universal Master mix	12.5
Primers	1.25
ddH_2O (RNAse free)	8.75
cDNA	2.5
Total	25

Gene	*TaqMan Gene Expression Assays RefSeq*	*Sybr Green Assays*
βactin	NM_001101.3	
GABPα	NM_144618.2	forward, 5'-AGCATCAGTGCAATCTGCTA-3' reverse, 5'-TTCCCAGGTGAGCTTCTATC-3'
GFI1	NM_001127215.1 NM_001127216.5 NM_005263.3	forward, 5'-TGACTTGGGGAAGGAATTTA-3' reverse, 5'-CCAGTGATGAGGTTTTCACA-3'
GFI1B (Hs01062469_m1)	NM_001135031.1 NM_004188.4	
IL-7Rα	NM_002185.2	forward, 5'-TGGACGCATGTGAATTTATC-3' reverse, 5'-CATTCACTCCAGAAGCCTTT-3'

Luria Broth (LB) – pH 7.0

Bactotryptone	10 g
Bactoyeast extract	5 g
NaCl	10 g
dH_2O	1 L - Final volume

Sterilize by autoclaving and store at room temperature
Cool to 50°C and add desired antibiotic: 100 µg/ml of Ampicillin or Kanamycin.

LB agar

Bactotryptone	10 g
Bactoyeast extract	5 g
NaCl	10 g
Bacto Agar	10 g
dH_2O	1 L - Final volume

Prepare LB and pH to 7.0. Add BactoAgar, autoclave to dissolve and sterilize. Let cool down to 50°C and add desired antibiotic. Gently mix by swirling and aliquot LB agar to Petri plates. Let solidify, seal with parafilm and store at 4°C in the inverted position.

10X Tris-acetate (TAE) – pH 7.6 to 7.8

Tris-base	48.4 g
0.5 M Na_2EDTA – pH8.0	20 ml
Glacial acetic acid	11.42 ml
dH_2O	1 L - Final volume

Store at room temperature.

Solutions for plasmid extraction large scale ~ 20 ml
Solution I
50 mM glucose
25 mM Tris-HCl – pH 8.0
10 mM EDTA – pH 8.0
Prepared in batches of 100 ml in dH_2O, autoclave for 15 minutes at 10 lb/sq. in. on liquid cycle, and store at 4°C. Add RNAse.

Solution II
0.2 N NaOH (Dilute fresh from a
10 N stock)
1% SDS
Buffer is prepared in dH_2O and must be used rapidly.

Solution III

5 M potassium acetate	60 ml
Glacial acetic acid	11.5 ml
dH_2O	28.5 ml

Store at 4°C.

Author Permissions

Scott Durum, PhD

Figure 1.3 Schematic representation of the IL-7Rα promoter based on findings from murine T cells.

From: "Durum, Scott (NIH/NCI) [E]"
Subject: RE: IL-7R promoter & Request for permission
Date: Wed, 18 August, 2010 4:06 pm
To: Anita C. Benoit

Anita,

You are welcome to use the figure and I look forward to reading your future publications on the subject of IL-7R expression.

Best, Scott

-----Original Message-----
From: [mailto:Anita C. Benoit]
Sent: Wednesday, August 18, 2010 1:34 PM
To: Durum, Scott (NIH/NCI) [E]
Subject: IL-7R promoter & Request for permission

Dear Dr Durum,

I am writing to request permission to use one of your figures published in:
Interleukin-7 receptor expression: intelligent design. Nature. 2007.7:144.
I have enjoyed reading your review as well as your publications centered on IL-7 and T cells.

I am currently in the progress of completing my thesis which consist of investigating some of the mechanisms involved in regulating the receptor. I have used your figure in my introduction discussing the mechanisms regulating IL-7Ralpha expression. The figure is attached as well as the figure legend.

Thank you,
Anita

Anita C. Benoit, PhD candidate
University of Ottawa, Ontario Canada

Tarik Möröy, PhD

Figure 1.4 Schematic representation of the GFI1 protein.

From: Möröy Tarik
Subject: Re: Gfi1 figure & request for permission
Date: Wed, 18 August, 2010 1:31 pm
To: Anita Benoit

Dear Ms Benoit

You have my permission to use the figures in your thesis.

Sincerely

--
Tarik Möröy, PhD
President and Scientific Director
Institut de recherches cliniques de Montreal, I R C M
Canada Research Chair in Hematopoiesis and immune cell differentiation
Professor, Dept de microbiologie et immunologie
Université de Montréal
110, Avenue des Pins West
Montreal, QC, H2W 1R7
Canada
www.ircm.qc.ca

On 18/08/10 1:19 PM, wrote:

Dear Dr Möröy,

I am writing to request permission to use diagrams of the GFI1 structure in my thesis that you have depicted in two of your publications.

1- The zinc finger protein and transcriptional repressor Gfi1 as a regulator of the innate immune response. Immunobiology. 213 (2008) 341-352
2- The zinc finger transcription factor Growth factor independence 1 (Gfi1). The International Journal of Biochemistry & Cell Biology 37 (2005) 541-546.l

My thesis consist of examining the functional roles of Gfi1 in primary human CD8 T lymphocytes. I have found your reviews as well as publications of your own work to be very valuable in understanding the transcriptional repressor.

I have attached my figure along with the figure legend.

Thank you,

Anita

Anita C. Benoit, PhD candidate
University of Ottawa, Ontario Canada