UNDERSTANDING THE MECHANISMS BY WHICH INTERLEUKIN (IL)-7 DOWN-REGULATES EXPRESSION OF THE IL-7 RECEPTOR ALPHA-CHAIN (CD127) IN HUMAN CD8 T CELLS

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Thesis submitted to the

Faculty of Graduate and Postdoctoral Studies

in partial fulfillment of the requirements

for a doctoral degree in Microbiology and Immunology

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Abstract

Interleukin (IL)-7 is an essential non-redundant cytokine and throughout the life-span of a T cell signaling via the IL-7 receptor influences cell survival, proliferation and function. It is therefore no surprise that expression of the IL-7 receptor alpha-chain (CD127) is tightly regulated. In this study I establish IL-7 down regulates CD127 gene transcription and surface protein expression in primary human CD8 T cells through two mechanisms.

Upon binding IL-7, surface CD127 is rapidly internalized and phosphorylated at the critical tyrosine residue Y449. Concurrent activation of the JAK/STAT5 pathway stimulates expression of CIS, a member of the SOCS family of proteins. CIS protein already expressed at basal levels and induced by IL-7 bind directly to CD127 as demonstrated by Co-immunoprecipitation assays and colocalize with both CD127 and the early endosomal marker EEA1. Subsequent proteasomal degradation of CD127 and CIS is dependent on an E3 ligase. Through siRNA-mediated knockdowns I confirm CIS plays a predominant role in the IL-7 mediated degradation of CD127.

The mechanism by which IL-7 suppresses CD127 transcripts in primary human CD8 T cells was also examined. Through qPCR and nuclear run-on assays I illustrate that IL-7 suppresses CD127 gene transcription in a time- and dose-dependent manner. The IL-7 mediated suppression of CD127 transcripts is dependent on JAK/STAT5 signaling. Notably, cycloheximide blocked IL-7's ability to down-regulate CD127 transcripts suggesting IL-7 stimulates the *de novo* synthesis of a transcriptional repressor of the CD127 gene. Through PCR arrays, qPCR and Western blot analysis the IL-7 inducible transcription factor c-Myb was identified as a candidate repressor. The region within the CD127 gene promoter required for IL-7 mediated transcriptional suppression was identified through progressive truncations using firefly luciferase as a reporter gene and is located from -1760 to -2406 bp upstream of the TATA box and contains three putative c-Myb binding sites. Using siRNA-mediated knockdown and transient over-expression, I illustrate c-Myb suppresses CD127 gene transcription in primary human CD8 T cells. A thorough understanding of the mechanisms by which IL-7 regulates CD127 expression is imperative and may reveal novel insights into the contribution of abnormal IL-7 signaling to diseases affecting immune function.

Acknowledgments

I would like to express my sincere gratitude to my supervisor Dr. Paul MacPherson for guidance, encouragement, advice and for providing excellent working environment for his students. Dr. MacPherson has helped me to mature and grow as a young scientist and provided me with many opportunities to travel and present my research findings at national and international meetings and supported me tremendously throughout my PhD studentship.

I would like to thank my thesis advisory committee: Dr. Valerie Wallace and Dr. Robin Parks for helpful discussion and valuable advice during the course of my research project.

I would also like to thank laboratory colleagues: Elliott Faller, Parmvir Parmar, Scott Sugden, Hafsa Cherid and Abdulkareem El Salfiti for their support, technical assistance and helpful discussions throughout my research progress. Thank you Dr. Cecilia Costiniuk, Nebras Ghazawi and Dr. Rami Darwich for your encouragement, help and motivation!

I want to acknowledge the Canadian Institutes of Health Research (CIHR) for a PhD Doctoral Research Scholarship and the University of Ottawa for Admission Scholarships as well as travel bursaries.

I also want to thank all volunteers who willingly donated blood samples for this study and Nurses at the General Hospital for their kind help in drawing blood specimens.

Finally I dedicate this thesis to my parents Zainah and Mustafa as well as my siblings Nebras, Ahmed, Wesam, Sawsan, Mohammed and Renad.

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List of Abbreviations

AIDS - Acquired Immunodeficiency Syndrome
APC - Antigen Presenting Cell
Bad - Bcl-2-associated agonist of cell death
Bax - Bcl-2–associated X protein
Bcl-2 - B-cell CLL/lymphoma 2
BMT - Bone Marrow Transplant
BSA - Bovine Serum Albumin
CD - Cluster of differentiation
Cdk - Cyclin Dependent Kinase
CHX - Cycloheximide
CIS - Cytokine-Inducible SH2-Containing Protein
CMV - Cytomegalovirus
CoIP - Co-Immunoprecipitation
CTL - Cytotoxic T Lymphocyte
DEPC - Diethylpyrocarbonate
Dex - Dexamethasone
DMSO - Dimethyl sulfoxide
DRB - 5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazole
EBV- Epstein-Barr Virus
ECL - Enhanced Chemiluminescence
EDTA - Ethylenediaminetetraacetic Acid
EGF - Epidermal growth factor
erbB-2 - Epidermal Growth Factor Receptor 2
FasL - Fas Ligand
FCS - Fetal Calf Serum
FITC - Fluorescein Isothiocyanate
FoxP3 - Forkhead box P3
G-CSF - Granulocyte Colony-Stimulating Factor
GH - Growth hormone
GLUT-1 - Glucose transporter 1
GM-CSF - Granulocyte Macrophage Colony-Stimulating Factor
Gfi-1 - Growth Factor Independent 1
GFP - Green fluorescent protein
GR - Glucocorticoid Receptor
GVHD - Graft-Versus-Host-Disease
HAART - Highly Active Antiretroviral Therapy
HCl - Hydrochloric acid
HIV - Human Immunodeficiency Virus
HRP - Horseradish Peroxidase
Hsp - Heat shock protein
HSV - Herpes Simplex Virus
IFN - Interferon
IFNR - Interferon receptor

IL - Interleukin
IL-7R - Interleukin-7 receptor
IL-7Rα - Interleukin-7 receptor alpha chain
ISRE - Interferon-Stimulated Response Element
JAK - Janus kinase
JAKi - Inhibitor of Janus kinase activity
K_D - Dissociation constant
Kda - Kilodalton
KIR - Kinase Inhibitory Region
LB - Luria broth
Leu - Leupeptin
LPS - Lipopolysaccharide
MACS - Magnetic-Activated Cell Sorting
MCL-1 - Myeloid cell leukemia factor 1, an anti-apoptotic gene
MHC - Major Histocompatibility Complex
MS - Multiple Sclerosis
NF-κB - Nuclear Factor Kappa B
NK - Natural killer cells
NOD - Nucleotide Oligomerization Domain
NP-40 - Nonidet P-40
NCBI - National Centre of Biotechnology Information
OSM - Oncostatin M
PAMP - Pathogen Associated Molecular Pattern
PBMC - Peripheral Blood Mononuclear Cells
PBS - Phosphate Buffered Saline
PC5 - Phycoerythrin-Cy5
PCD - Programmed Cell Death
PCR - Polymerase Chain Reaction
PDGF - Platelet-derived growth factor
PE - Phycoerythrin
PI - Propidium Iodide
PI3K - Phosphoinositide 3-kinase
PI3Ki - Inhibitor of phosphoinositide 3-kinase
PMCA-1 - Plasma Membrane $Ca2^{+}$ ATPase-1
PMSF - Phenylmethylsulfonyl Fluoride
PRR - Pattern-Recognition Receptor
PU.1 - Purine-rich box binding protein 1
PVDF - Polyvinylidene Fluoride
qPCR - Quantitative Polymerase Chain Reaction
RA - Rheumatoid arthritis
RLU - Relative light units
RNA -Ribonucleic acid
RPMI - Roswell Park Memorial Institute (Cell growth media)
RPS18 - 40S Ribosomal Protein S18
RT - Reverse Transcription
SCID - Severe Combined Immunodeficiency
SDM -Site-directed mutagenesis

SDS - Sodium dodecyl sulfate
SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SH2 - Src homology domain 2
siRNA - Small interfering RNA
SMER3 - Small Molecule Enhancer of Rapamycin 3
SOCS - Suppressor of Cytokine Signaling
STAM - Signal-Transducing Adapter Molecule
STAT - Signal Transducer and Activator of Transcription
STAT5i - Inhibitor of STAT5 phosphorylation
T-ALL - T-cell acute lymphoblastic leukemia
Tat - Transactivator of transcription
TBST - Tris-Buffered Saline with Tween-20
TCR - T-cell receptor
TGF-β - Transforming growth factor beta
TLR - Toll-like receptor
TNF - Tumor necrosis factor
TNFR - Tumor necrosis factor receptor
TSLP - Thymic stromal lymphopoietin
TSS - Transcription Start Site
Ub - Ubiquitin
WCL - Whole cell lysis

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1. Chapter 1. Introduction

1.1 Innate and Adaptive Immune Responses

The immune system is highly regulated and provides effective host defense against pathogens. There are two aspects of immunity: innate and adaptive. The innate immune system provides an immediate response to pathogens and also sets in motion a more specific, complex and flexible adaptive response. The skin and mucous membranes provide physical and chemical barriers against invading microbes. If a pathogen successfully overcomes this first line of defense, components of the innate system including phagocytic cells (neutrophils, macrophages, eosinophils, basophils, dendritic cells, natural killer cells and mast cells) and the complement system recognize highly conserved elements from the pathogen (1). These common structures are composed of carbohydrates, lipopolysaccharides or lipoproteins, among other forms, and are commonly referred to as Pathogen Associated Molecular Patterns (PAMPs) (2). The patterns are recognized by the pathogen recognition receptors (PRRs) found on the surface of all cells of the innate immune system (2). Examples of PRRs are Mannose-binding lectins, lipopolysaccharide (LPS)-binding proteins and Toll-like receptors (TLRs). The recognition of a pathogenic pattern by PRRs initiates specific signaling cascades which lead to the production and release of pro-inflammatory cytokines and chemokines (3). For example, the activation of the Toll-like receptors in dendritic cells and macrophages activates the transcription factor NF-κB (4). NF-κB in turn up regulates the production and secretion of a number of cytokines such as TNF-α, IL-1, IL-6, IL-12, G-CSF, interferons and co-stimulatory proteins CD80 and CD86 which play important roles in macrophage activation and dendritic cell maturation (4, 5). Further, the activation of NF-κB

also up regulates the expression of adhesion molecules such as endothelial leukocyte adhesion molecules and vascular cell adhesion molecules that are important for the ability of innate cells to migrate to sites of infection (4, 5). Dendritic cells and macrophages function as antigen presenting cells (APCs) which play key roles in linking innate to adaptive immunity. APCs activate the adaptive immune system by presenting foreign polypeptides to T lymphocytes (1).In addition to augmenting the innate immune response, cytokines also contribute to the adaptive immune response by supporting the differentiation and proliferation of effector T cells and activation and proliferation of B cells (1, 6).

The adaptive immune system is composed of highly specialized cells that target pathogens with great specificity. The two 'arms' of the adaptive immune response are the cell-mediated and humoral (antibody-mediated) immune responses, which are mediated by T and B lymphocytes, respectively. Flexibility, specificity, clonal expansion and immunological memory are all hallmarks of adaptive immunity (7). Lymphocyte antigen receptors are generated with great diversity to enable the recognition of a wide spectrum of different pathogens. The generation of diversity for the receptors of the adaptive immune response involves recombination events at the genetic level. The pathogen recognition molecules of the adaptive immune system are the membrane-bound immunoglobulin on the B-cell surface (B-cell receptor, BCR) and the T-cell receptor (TCR) (8). The generation of BCRs and TCRs is characterized by somatic gene rearrangements which generates a diverse repertoire of these pathogen recognition molecules (7). A pathogen can directly bind to and be recognized by membrane-bound immunoglobulins on the B-cell surface that are specific to it, while processed pathogenic polypeptides presented on major histocompatibility

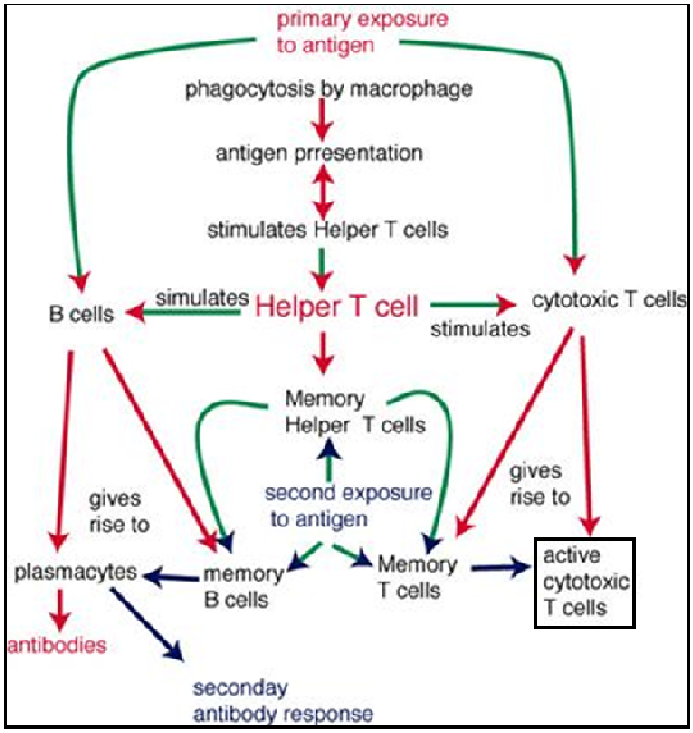
complex (MHC) molecules by antigen presenting cells (APCs) are recognized by the TCR of naïve T cells in lymph nodes (8). The binding of pathogenic molecules to these pathogen recognition receptors results in lymphocytic activation and their rapid expansion by mitotic cell division to provide a large number of plasma cells, which secrete specific antibodies, and cytotoxic as well as helper T cells. This process is commonly referred to as clonal selection and expansion.

Expanded pathogen-specific T cells target infected cells and kill them either by inducing apoptosis or by lysing the infected cell via release of cytotoxic molecules. After the infection is cleared, an important contraction phase takes place in which the vast majority of effector T cells die by apoptosis and only a small proportion of these cells stay in circulation as memory lymphocytes. Immunological memory is a fundamental aspect of adaptive immunity. Secondary exposure to the same antigen results in a rapid and effective immunological response against the pathogen.

Thus, the immune response against a pathogen is elicited through a highly regulated process which involves many cell types and molecules of both the innate and adaptive systems (summarized in figure 1). It is modulated by important co-stimulatory mechanisms. For example, cells of the innate immune system release cytokines and chemokines which not only further augment the innate immune response but also help activate the adaptive immune response. At the same time immune system is also regulated by very important intrinsic negative feedback mechanisms to prevent over-activation of immunity (9, 10). The highly co-operative process involving both positive and negative feedback pathways ensure an effective response to pathogens.

Figure 1. Adaptive immunity. Cell mediated responses and antibody responses allow for an intricate, flexible and specific immunity against pathogens. These responses are mediated through a highly organized cooperative system involving T and B lymphocytes as well as antigen presenting cells (APCs). Figures were obtained from Dr. Nicole Okazaki class notes of human physiology course. Chapter: The Lymphatic System.
URL: http://faculty.weber.edu/nokazaki/Human_Physiology/Class%20notes/251LYMPH.htm

A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simulates
Helper T cell
cytotoxic T cells
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Memory
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second exposure
to antigen
gives
rise to
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plasmacytes
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Memory
T cells
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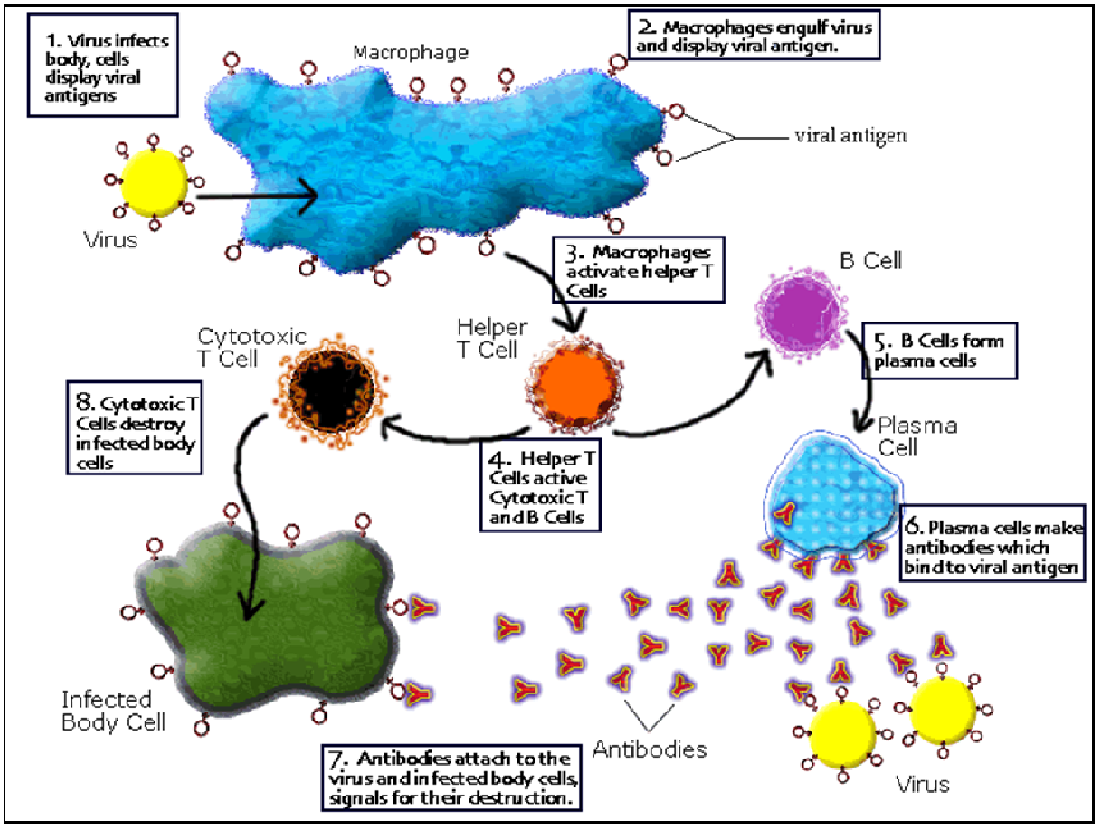

1. Virus infects body, cells display viral antigens
Macrophage
2. Macrophages engulf virus and display viral antigen.
viral antigen
Virus
3. Macrophages activate helper T Cells
B Cell
Cytotoxic
T Cell
Helper
T Cell
5. B Cells form plasma cells
8. Cytotoxic T Cells destroy infected body cells
Plasma
Cell
4. Helper T Cells active Cytotoxic T and B Cells
6. Plasma cells make antibodies which bind to viral antigen
Infected
Body Cell
Antibodies
7. Antibodies attach to the virus and infected body cells, signals for their destruction.
Virus

Figure 1.

To fully and safely manipulate immunologic therapies, it is important to understand the intrinsic pathways by which the immune system ensures an appropriate balance between immune activation and immune suppression.

1.2 Cytotoxic CD8 T Cells

$CD8^+$ T lymphocytes (or CD8 T cells) are the major effectors of cell-mediated immunity (11). Upon viral infection, these cells become activated and travel to sites of infection where they recognize and destroy infected cells. Naïve CD8 T cells are first activated in lymph nodes through contact with antigen presenting cells (APCs) expressing peptide-loaded MHC class I molecules. Peptide antigens displayed by MHC class I molecules are derived from the protein components of intracellular pathogens (such as viruses). These antigens (typically 8-10 amino acids) are produced by proteasomal degradation of their parental foreign proteins in the APC's cytosol. They are subsequently transported to the endoplasmic reticulum, where they are loaded onto MHC class I molecules. MHC class I-peptide complexes then move through the Golgi network to the cell surface where they are recognized by the TCR on specific CD8 T cells (12). Co-stimulation through the CD28 surface molecule, recognizing CD80 and CD86 on the surface of activated APCs, is also required for CD8 T cell activation. In addition to TCR/CD28 receptor engagement, a variety of biochemical signals -many of which are released by APCs and neighboring activated CD4 T cells- also contribute to activation (13, 14). In particular, a wide range of cytokines including IL-2, -4, -6, -7, -12, -15, -21, IFN-α and IFN-γ have been shown to promote CD8 T cell responses to antigens (15).

Once activated by encountering a foreign antigen, CD8 T cells undergo rapid differentiation and proliferation (clonal expansion) to develop an 'effector' phenotype (16). These cytolytic effector $CD8^+$ T lymphocytes (CTLs) traffic to the periphery and up regulate the production of cytolytic effector molecules. Infected cells are identified through the same foreign antigens presented on MHC class I molecules on the infected cells' surfaces as were presented initially by the APC. CD8 T cells act rapidly to induce target cell apoptosis, illustrated in figure 2 (17). The destruction of target cells is mediated by the directional release of preformed lytic granules, containing perforin, granulysin and granzymes onto the target cell surface. Perforin is able to form homomultimeric channels through the plasma membrane of target cells, while granzymes, entering the target cell through perforin-created pores, are able to enzymatically induce DNA damage and stimulate apoptotic cascades (18-21). Granulysin also enters the cell through perforin channels. It is a cationic peptide that in low concentrations has anti-microbial activity through the disruption of bacterial membranes (22), and at high concentrations directly induces target cell apoptosis through both induction of caspase-3 (23) and through its ability to disrupt target cell mitochondrial membranes (24). In addition to granule release, activated CD8 T cells also up regulate surface expression of FasL. This molecule interacts with Fas expressed on target cells, which in turn initiates protease-dependent apoptosis in target cells (25). After the infection is cleared, most virus-specific CD8 T cells undergo apoptosis themselves as they are no longer required (26). A subset of these CD8 T cells remain in circulation however, as memory cells and can quickly expand to large numbers of activated effector T cells in response to a secondary exposure to the same pathogen (27).

Figure 2. Cytotoxic T-lymphocyte (CTL) mediated targeting of infected cells. CD8+ T cells recognize antigenic peptides displayed by antigen-presenting cells (APCs) and become activated through binding of peptide-MHC Class I complexes. The activation of CD8 T cells triggers their CTL-mediated cytotoxicity which ultimately kills target cells by one of three mechanisms (A) indirectly, by releasing tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), (B) directly, by inducing apoptosis of target cells upon encountering death receptor on the infected cell and (C) directly, by lysing target cells via directed release of cytotoxic proteins (perforin and granzymes) stored in lytic granules. Figure was obtained with permission from Nature publishing group (Permission from corresponding author and Nature publishing group are depicted in the appendix) (17).

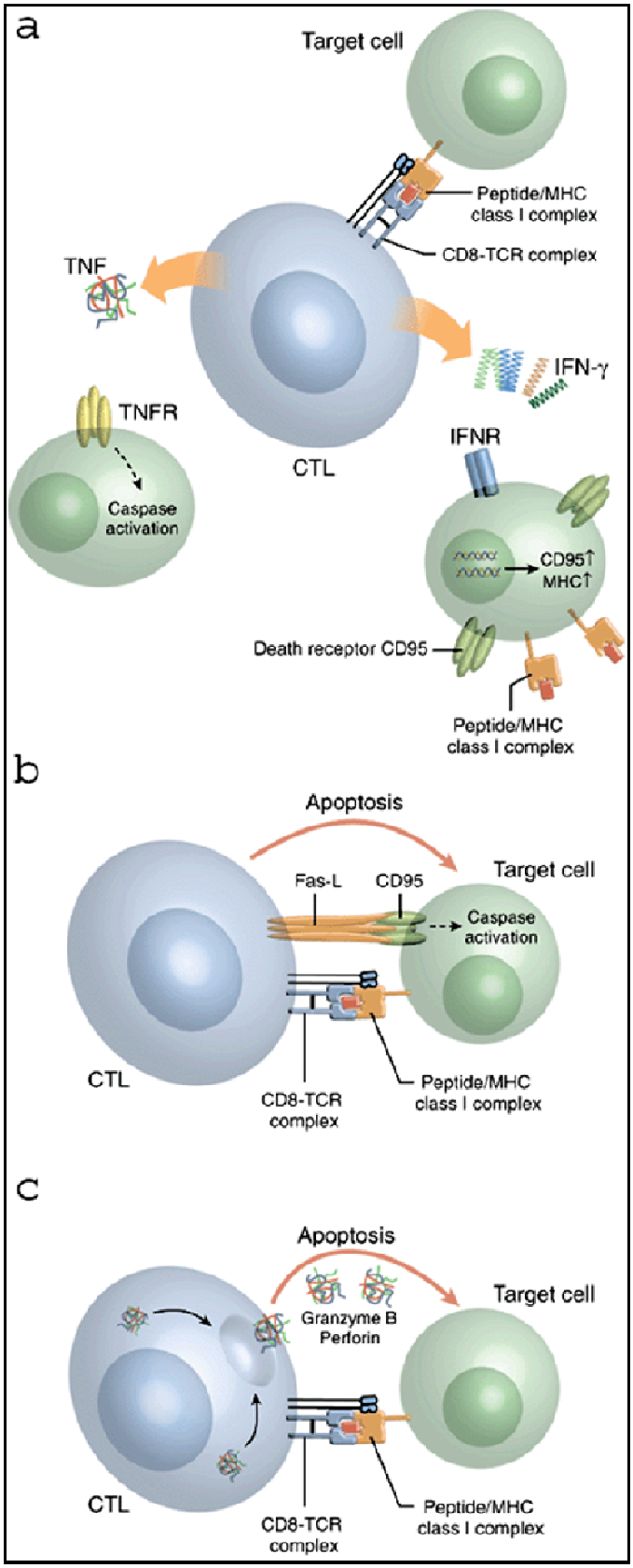

a
Target cell
Peptide/MHC
class I complex
CD8-TCR complex
TNF
IFN-γ
TNFR
CTL
IFNR
Caspase
activation
CD95↑
MHC↑
Death receptor CD95
Peptide/MHC
class I complex
b
Apoptosis
Target cell
Fas-L
CD95
Caspase
activation
CTL
CD8-TCR
complex
Peptide/MHC
class I complex
c
Apoptosis
Target cell
Granzyme B
Perforin
CTL
CD8-TCR
complex
Peptide/MHC
class I complex

Figure 2.

Activation of CD8 T cells must be tightly regulated both in terms of population size and activation state. Hypo-responsive CD8 T cells such as those found in HIV-infected individuals with acquired immunodeficiency syndrome (AIDS) leave the host susceptible to a wide variety of pathogens such as *Pneumocystis jirovecii* and mycobacterium avium complex. Conversely, inappropriate or exaggerated activation of CD8 T cell have been implicated in a diverse array of autoimmune diseases such as multiple sclerosis (MS) (28), ulcerative colitis (29), type I diabetes (30) among many others.

1.3 Gamma-chain Cytokines and T-cell Homeostasis

1.3.1 The Gamma-chain (γc) Cytokine Family

Cytokines are small cell-signaling proteins which mediate inter-cellular communication between different components of the immune system and between the immune system and the host. The gamma-chain (γc) cytokine family consists of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, each with important roles in T cell development, survival and function, illustrated in figure 3 (31). These cytokines share the common gamma chain (CD132) as part of their respective receptor complexes. The receptors of IL-2 and IL-15 are heterotrimeric and are composed of a specific alpha chain (IL-2Rα and IL-15Rα), a shared beta chain (IL-2/IL-15Rβ (CD122)) as well as the shared gamma chain (CD132). In contrast, the receptors of IL-4, IL-7, IL-9 and IL-21 are heterodimeric in nature where in addition to the shared gamma chain, their receptors are composed of only one other unique subunit or alpha chain (IL-4Rα, IL-7Rα (CD127), IL-9Rα and IL-21Rα chains). The alpha chains possess the specific binding sites for their respective cytokines (32).

Figure 3. The common γ-chain cytokine receptor family. Common γ-chain cytokine receptors (IL-2, -4, -7, -9, -15 and -21) are associated with JAK1 and JAK3 and upon binding of cytokine receptor complex formation brings the receptor-associated JAK1 and JAK3 into juxtaposition allowing subsequent trans-phosphorylation and activation. The phosphorylation and activation of JAK1 and JAK3 initiate a number of signaling pathways that are pivotal for T lymphocyte development, survival and function. Figure was obtained with permission from Nature publishing group (Permission from corresponding author and Nature publishing group are depicted in the appendix) (33).

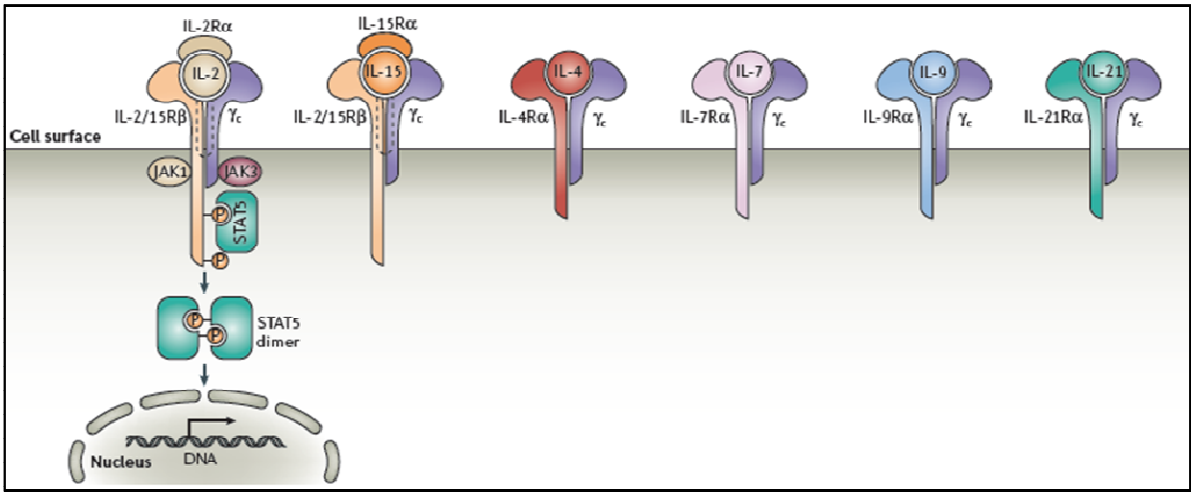

IL-2Rα
IL-2
IL-2/15Rβ
γc
IL-15Rα
IL-15
IL-2/15Rβ
γc
IL-4
IL-4Rα
γc
IL-7
IL-7Rα
γc
IL-9
IL-9Rα
γc
IL-21
IL-21Rα
γc
Cell surface
JAK1
JAK2
STAT5
STAT5
dimer
Nucleus
DNA

Figure 3.

Binding of the common γ-chain cytokines to their high affinity binding site on the α chain promotes formation of the receptor complex, that is the γ-chain and α-chain in IL-4, -7, -9 and -21 or the γ, α and β-chains in IL-2 and -15. All gamma-chain cytokine receptors signal via activation of several kinases such as Janus kinases (JAK) and Phosphoinositide 3-kinases (PI3K). Receptor complex formation brings JAK3, which associates primarily with the γ-chain (34), and JAK1 (on the α- or β-chain) into juxtaposition and allows their subsequent trans-phosphorylation and activation (33, 35). Activation of JAK1 and JAK3 results in the phosphorylation of both receptor subunit cytoplasmic domains and initiates a number of signaling cascades that in turn mediate important cellular functions (31, 34-37).

1.3.2 Signaling through Receptors of Gamma-chain Cytokines Receptors

Signaling by γ-chain cytokines is mediated primarily by the Janus kinase (JAK) family of receptor-associated tyrosine kinases which plays critical roles in the transduction of the cytokine signal to the nucleus and in the regulation of receptor trafficking (38-42). There are four members of the JAK family in mammals: JAK1, JAK2, JAK3 and Tyk2 that interact with the cytoplasmic domains of cytokine receptors (38-42). Activated JAK proteins phosphorylate several substrates in the cell chief among these are the Signal Transducers and Activators of Transcription (STAT) proteins (35). There are 7 different STAT proteins in mammals: STAT1 (43), STAT2 (43), STAT3 (44, 45), STAT4 (45, 46), STAT5A (47-51), STAT5B (49-52) and STAT6 (53). The cytokine-dependent phosphorylation of specific STAT proteins by JAK kinases results in their homodimerization or heterodimerization and consequent translocation to the nucleus where they bind directly to specific DNA sequences

within promoter regions of target genes and regulate their expression (35). Activated JAK kinases also phosphorylate tyrosine residues in several other substrates in the cell (39, 54). Phosphorylation of tyrosine residues in the cytoplasmic tails of cytokine receptors themselves establishes docking sites for STAT molecules (35, 55) as well as for a number of other signaling molecules such as Shc (52), Grb2 (56), SHP-2 (57), Vav (58), the p85 subunit of the phosphoinositide 3-kinase (PI3K) (59) and for the signal-transducing adapter molecule (STAM) (60).

The essential roles common γ-chain cytokines play in T cell differentiation, maturation and proliferation have been extensively studied in mice where γ-chain knockout mice demonstrate a complete lack of or at least significant reduction in the number of NK, B and T-cells, reviewed in (31). In fact, mutations in CD127 (61), CD132 (62, 63) and JAK3 (64) genes cause severe combined immune deficiency syndrome (SCID) in human and in mice.

Figure 4 summarizes differential requirements of gamma-chain cytokines during the life-span of a T cell. IL-7 in particular is important for thymocyte generation and development. Once naïve T cells travel to peripheral secondary lymphoid organs, their survival is dependent on IL-7 and IL-15 (31). When a resting mature T cell is activated by encountering an antigen, it expresses the receptor subunits for IL-2, IL-15 and IL-21, down regulates IL-7 receptor expression and differentiates into effector T cells with cytotoxic properties. Upon clearance of the foreign pathogen, most of the activated T cells undergo apoptosis except for a small number of activated cells expressing high levels of the receptors

Figure 4. Different cellular requirements of gamma-chain cytokines throughout the life of a T cell. IL-7 signaling plays essential roles during early stages of T cell development as well as in maintaining homeostasis and survival of naïve T cells. Upon encountering a foreign antigen and subsequent activation, T cells depend on IL-2, IL-15 and IL-21 to differentiate into effector T cells which can target infected cells. IL-7 also contributes to the effector cell phenotype and mediates the production and release of cytotoxic molecules such as perforin and granzymes. Upon clearance of the pathogen, a small number of effector T cells that maintained high expression of receptors of IL-7 and IL-15 survive and constitute the pool of long-lived memory cells. Figure was *adapted and was obtained with permission from Elsevier B.V. (Permission from the publishing group is depicted in the appendix) (31).

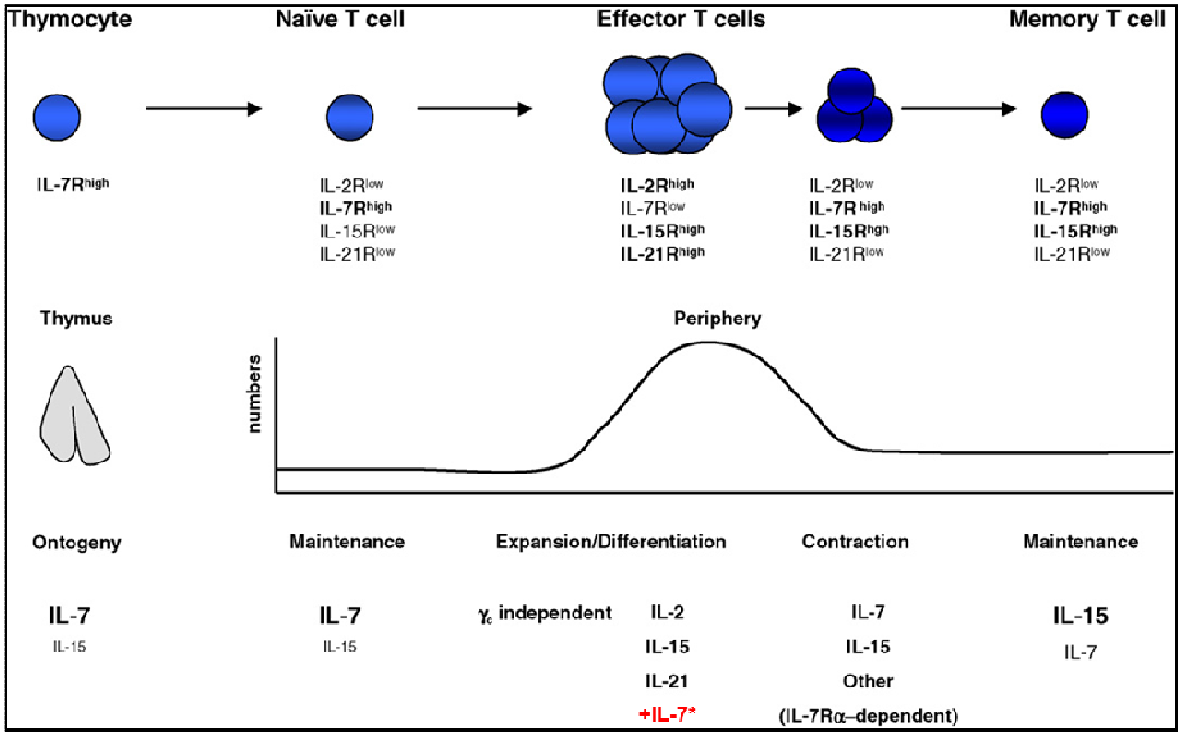

Thymocyte
Naïve T cell
Effector T cells
Memory T cell
IL-7Rhigh
IL-2Rlow
IL-7Rhigh
IL-15Rlow
IL-21Rlow
IL-2Rhigh
IL-7Rlow
IL-15Rhigh
IL-21Rhigh
IL-2Rlow
IL-7Rhigh
IL-15Rhigh
IL-21Rlow
IL-2Rlow
IL-7Rhigh
IL-15Rhigh
IL-21Rlow
Thymus
Periphery
numbers
Ontogeny
Maintenance
Expansion/Differentiation
Contraction
Maintenance
IL-7
IL-15
IL-7
IL-15
γc independent
IL-2
IL-15
IL-21
+IL-7*
IL-7
IL-15
Other
(IL-7Rα–dependent)
IL-15
IL-7

Figure 4.

for IL-7 and IL-15 which are spared from cell death and form a pool of long-lived memory cells. The maintenance of these memory cells depend on IL-7 and IL-15 signaling (31).

1.3.3 Intrinsic Inhibitors of Cytokine Signaling

Cytokine signaling is negatively regulated by multiple mechanisms (65). The three main protein families involved are suppressors of cytokine signaling (SOCS) proteins, protein tyrosine phosphatases, and the protein inhibitor of activated STAT (PIAS) family. Suppressor of cytokine signaling (SOCS) proteins, including SOCS1-7 and the Cytokine-inducible SH2-containing (CIS) protein, are expressed at low levels in T cells and are induced by signaling through some cytokine receptors. They provide negative feedback and inhibit signaling by binding to the receptor and blocking STAT binding or by forming a complex with the receptor and recruiting ubiquitin ligases (65). Once ubiquitinated, the SOCS-cytokine receptor complex is targeted to the proteasome for degradation. This mechanism is discussed in detail in Chapter 4. The Protein tyrosine phosphatases (PTPs) PTPRC and PTPN1 are expressed in T cells at constant levels and together with tyrosine kinases regulate the phosphorylation of STAT molecules (66). The protein inhibitor of activated STAT (PIAS) protein family also regulates cytokine signaling via several mechanisms, including blocking the DNA-binding activity of transcription factors such as STAT3 and by recruiting transcriptional co-repressors to and promoting sumoylation of transcription factors such as STAT1 (67).

1.4 Interleukin-7 and its Receptor

1.4.1 Interleukin-7

Interleukin-7, was identified in the late 1980s as an important cytokine that promotes the growth of murine B cell precursors (68) and enhances the proliferation of mitogen-activated murine and human T cells (69). It is now known that IL-7 plays essential, non-redundant roles in the development of T and B cells in mice (61, 70) and of T cells in humans (71) also reviewed in (72-74). It promotes the survival and differentiation of immature T cells within the thymus (75), enhances the response of cytotoxic T-cells to foreign antigen, and is critical for immune cell homeostasis (76) and for establishment of memory (77). In humans, IL-7 is a single-chain 25 kDa glycoprotein (35). It is encoded by the IL-7 gene located on chromosome 8q12-13 (68), and is produced by thymic stromal cells (78, 79), intestinal epithelial cells (78), microvascular endothelial cells (80), hepatocytes (81, 82), follicular (83) and peripheral blood dendritic cells (35, 84), among others (85-87). Circulating levels of IL-7 in sera of healthy individuals are relatively low ranging from 0.3-8 pg/ml (75, 88). IL-7 may also be sequestered at the cell surface by heparan sulfate and fibronectin which may protect it from proteolytic cleavage and increase its availability to neighboring target cells (89, 90). It was thought the production of IL-7 was constitutive and that T cells competed for limiting amounts of this essential cytokine (91). This, however, has turned out not to be the case and IL-7 is now recognized as an acute-phase cell-signaling molecule and this will be discussed in later sections (82). IL-7 thus appears to play a role in the response to pathogens and may be important in the transition from innate to adaptive

immunity. As will be discussed, IL-7 plays essential roles in thymopoiesis and homeostasis of naïve and memory T cells and in the activation and cytolytic function of CD8 T cells.

1.4.2 The IL-7 receptor alpha-chain

1.4.2.1 Expression The IL-7 receptor is a heterodimer composed of a unique α-chain (CD127) and the common γ-chain (CD132) that is shared with the receptors for IL-2, -4, -9, -15 and -21 (92). The α- and γ-chains are expressed independently on the cell surface and are constitutively associated through their cytoplasmic tails with JAK1 and JAK3 respectively (93-96). CD127 is expressed at high levels on the surface of thymocytes, mature naïve and memory T cells, natural killer T cells and monocytes (91). CD127 is also expressed at low levels on dendritic cells and bone-marrow derived macrophages (91, 97). The IL-7 receptor α-chain also forms a part of the Thymic stromal lymphopoietin (TSLP) receptor expressed on dendritic cells and T cells (98). While TSLP signaling appears to play a role in the maturation of dendritic cells (99), TSLP and TSLP receptor knockout mice display normal T cell development (100, 101) and recent studies demonstrated TSLP signaling does not play a significant role in the T cell response to viral infections including influenza (102).

1.4.2.2 Structure As illustrated in figure 5, CD127 is a type I transmembrane protein which is composed of an extracellular domain, a transmembrane domain as well as an intracellular domain (92). The extracellular domain is composed of two fibronectin type III sub-domains joined by a 3_{10}-helical linker (103) and is extensively glycosylated *in vivo* (104). Although the glycosylation does not occur directly within the IL-7 binding site, it significantly enhances IL-7 binding to CD127 by up to 300-fold and stabilizes the subsequent dimerization to CD132 (104). The intracellular tail of CD127 consists of

Figure 5. Major IL-7 signaling pathways in T cells. IL-7 binding to CD127 induces dimerization of CD127 and CD132 which subsequently leads to the phosphorylation and activation of JAK1 and JAK3. This results in the activation of the JAK/STAT as well as PI3K signaling pathways. CD127 is a type I transmembrane protein consisting of an extracellular domain, a single transmembrane domain and a cytoplasmic tail which is composed of a Box1 motif which is found most proximal to the membrane, a region rich in acidic residues (A region), a serine rich region (S region) and a distal region (D region) which contains three critical tyrosine residues Y401, Y449 and Y456.

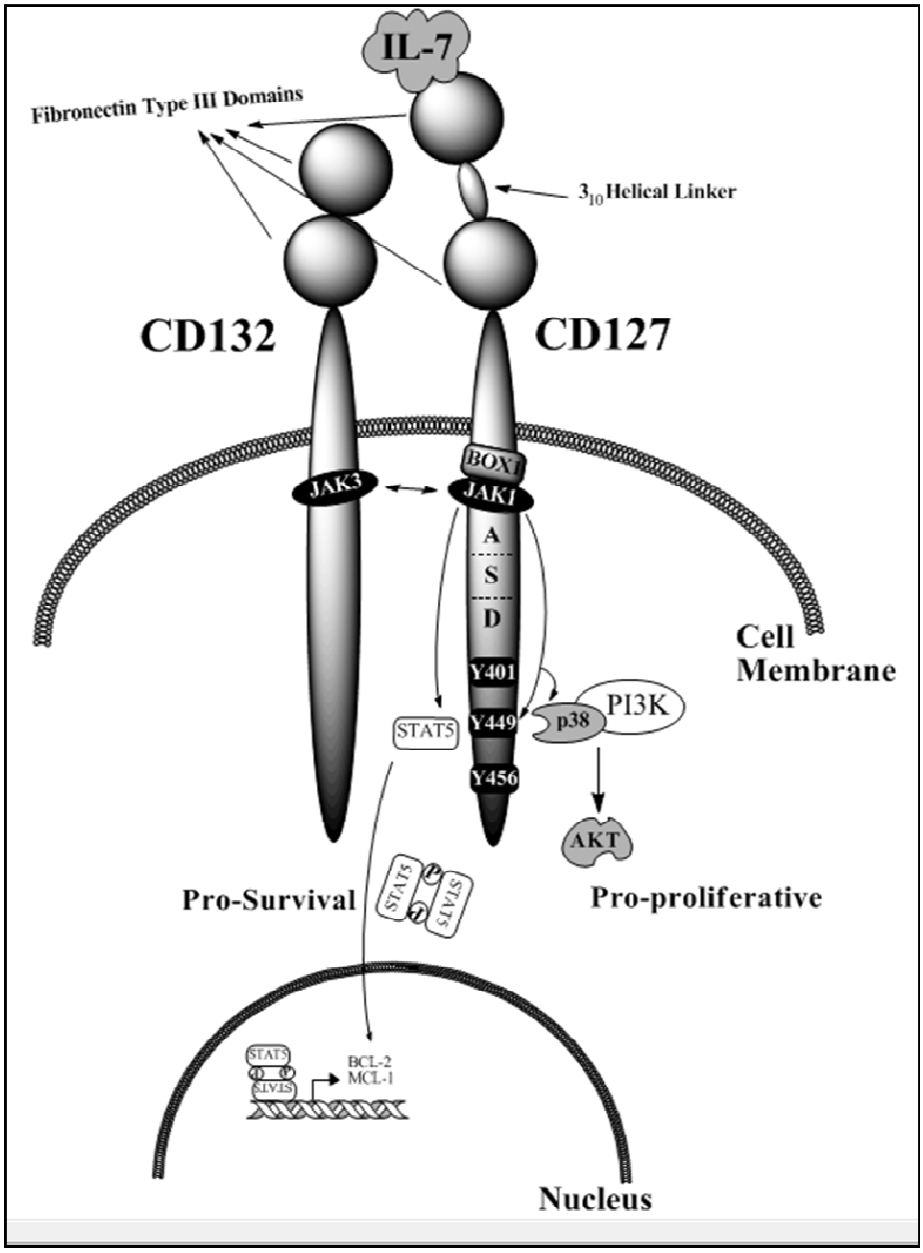

IL-7
Fibronectin Type III Domains
3_{10} Helical Linker
CD132
CD127
JAK3
BOX1
JAK1
A
S
D
Y401
Y449
Y456
STAT5
p38
PI3K
AKT
Cell Membrane
Pro-Survival
Pro-proliferative
STAT5
SOCS
BCL-2
MCL-1
Nucleus

Figure 5.

a Box 1 motif, pivotal for cytokine signal transduction through association with JAK kinases (105), as well as an acidic region (A), a serine-rich region (S) and a distal region (D). Within the distal region, three critical tyrosine residues (Y401, Y449, Y456) are conserved between mouse and humans and are essential for IL-7 signaling. Tyrosine 449 is phosphorylated by JAK3 and functions as docking sites for target proteins (35, 92, 93).

1.4.2.3 Alternative/Soluble form of CD127 protein

Nascent CD127 mRNA can be alternatively spliced to remove exon 6 (encoding the transmembrane domain) and thus encodes a shorter secreted isoform of CD127 (sCD127) (9, 106, 107). The precise function of sCD127 is incompletely understood, although a correlation between abnormal distributions of sCD127 may be linked with Multiple sclerosis disease progression (108). The role of sCD127 in HIV infection is controversial with conflicting data on whether sCD127 contributes to HIV pathogenesis (28, 109, 110).

1.4.2.4 Signaling Through the IL-7 Receptor

IL-7 signaling occurs through the interaction of IL-7 with the specific IL-7-binding site on the IL-7 receptor α-chain (CD127) (111). Interaction of IL-7 with its high affinity binding site on CD127 promotes dimerization of the α- and γ- receptor subunits. The interaction is stabilized upon complex formation with CD132. Subunit dimerization brings JAK1 and JAK3 into juxtaposition allowing their subsequent trans-phosphorylation and activation (35). Activation of JAK1 and JAK3 results in the phosphorylation of both CD127 and CD132 and initiates a number of signaling cascades. Activation of JAK-STAT and PI3 kinase are considered the major signaling pathways in T cells (35). Although IL-7 has been

shown to stimulate a variety of other signaling molecules including $p56^{lck}$, $p59^{fyn}$, p38 and members of the Src family of kinases in T lymphoblastic cell lines, this has not been described in primary T cells (112).

Phosphorylation of CD127 proteins at tyrosine Y449 by JAK3 allows docking via their SH2 domains and subsequent phosphorylation of STAT5A and STAT5B (35, 55). Phosphorylated STAT molecules dimerize and translocate to the nucleus to up regulate expression of several genes. For examples STAT5 phosphorylation has been shown to play a role in V(D)J recombination at the TCR γ-locus in murine thymocytes (23) as well as in the differentiation of αβ thymocytes in humans (113).

IL-7 also activates PI3 kinase by JAK dependent phosphorylation and activation of the catalytic p85 subunit (113-115). AKT/protein kinase B (PKB) is a downstream target of the PI3 kinase and plays important roles in glucose metabolism, cell proliferation and apoptosis (116).

1.5 Pleiotropic Effects of IL-7 on T-cells

IL-7 plays a pivotal role throughout the lifespan of a T-cell. The roles of IL-7 in T cell development, homeostasis, cytolytic function and establishment of immunologic memory will be reviewed in the following sections and is illustrated and summarized in figure 6.

Figure 6. Role of IL-7 in mature T cell biology. IL-7 is essential for CD8 T cell development, homeostasis, proliferation, and function. **Top panel,** IL-7 is essential for T cell homeostasis and proliferation. IL-7 stimulates both entry into the cell cycle through increased cyclin-dependent kinase 2 (cdk2) activity and up regulation of telomerase. IL-7 also reduces the apoptotic potential of CD8 T cells by up regulating the expression of pro-survival proteins such as BCL-2 and MCL1 and redistributing the cell-death proteins BAX and BAD. **Second panel**, IL-7 mediates antigen-specific CTL responses in CD8 T cells. IL-7 plays key roles in CD8 T cell activation and clonal expansion, up regulates the expression of cytolytic effector molecules such as perforin, granzyme B and gransulin and modulates the survival and proliferation of memory CD8 T cells.

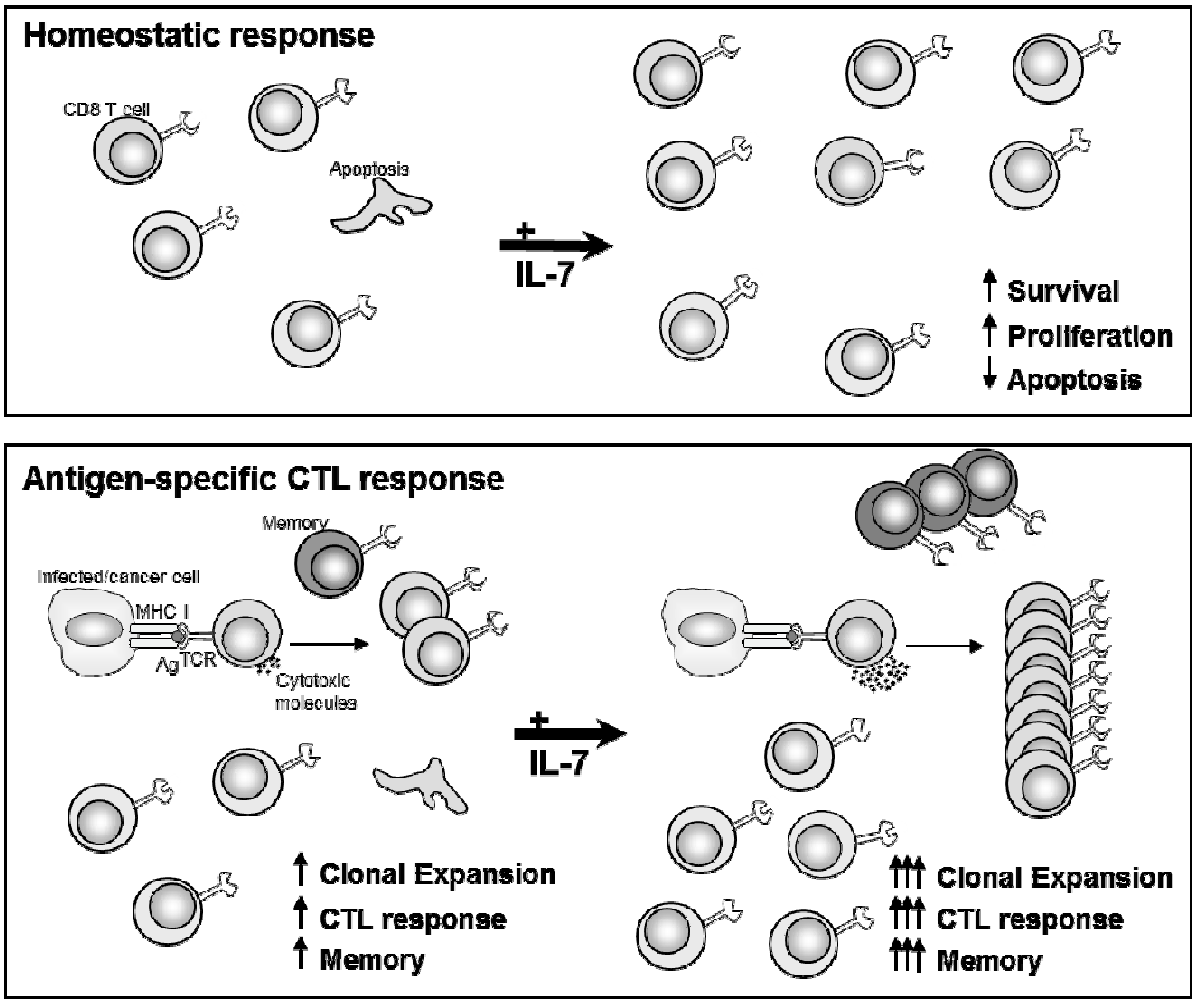

Homeostatic response
CD8 T cell
Apoptosis
+
IL-7
Survival
Proliferation
Apoptosis
Antigen-specific CTL response
Memory
Infected/cancer cell
MHC I
AgTCR
Cytotoxic molecules
+
IL-7
Clonal Expansion
CTL response
Memory
Clonal Expansion
CTL response
Memory

Figure 6.

1.5.1 IL-7 and Thymopoiesis

IL-7 signaling plays a very clear role in thymopoiesis (91). One of the first lines of evidence suggesting the prominent role of IL-7 in T cell development was the treatment of mice with antibodies against IL-7 (117) or the IL-7R (118) both of which inhibited T cell development and resulted in severe lymphopenia. It is now well established that IL-7 plays an essential role in the development of T cells in humans (71) (also reviewed in (72-74)). Homozygous mutations in the CD127 gene preventing production of functional IL-7Rα proteins are directly linked to severe combined immunodeficiency syndrome (SCID) with a complete block in T cell development (71). IL-7-induced STAT5 phosphorylation has been shown to play an important role in V(D)J recombination in the differentiation of TCRαβ thymocytes in humans (113). In fact, IL-7 contributes to thymopoiesis by maintaining survival of immature $CD3^{-}CD4^{-}CD8^{-}$ (triple negative) thymocytes during the first stage of development by up regulating the expression of the pro-survival BCL-2 proteins (72).

1.5.2 IL-7 and T-cell Proliferation and Homeostasis

IL-7 plays important roles in T cell homeostasis by maintaining constant numbers of naïve and memory T cells in the peripheral circulation. Constitutive low levels of IL-7 are believed to promote basal quiescent T cell division (119). IL-7 reduces the apoptotic potential of CD8 T cells by up regulating the expression of the pro-survival proteins BCL-2 and MCL1 and redistributing the cell-death protein BAX (23, 72, 75, 77, 88, 91, 120-123). Further, IL-7 induced PI3K-dependent phosphorylation and activation of AKT has been shown to phosphorylate and inactivate both the pro-apoptotic protein BAD and the cell-cycle

inhibitor $p27^{kip1}$ suggesting IL-7 signaling through PI3 kinase sustains cell survival and proliferation (124, 125). IL-7 signal transduction via PI3 kinase appears to promote cell proliferation and its associated high metabolic rate by up regulating expression of the glucose transporter GLUT1 and the transferrin receptor (CD71) in thymocytes and T-lymphocytic leukemia cells (126, 127). Thus, IL-7 signaling modulates PI3 kinase activity to maintain cell proliferation and its associated high metabolic rate.

IL-7 stimulates proliferations of CD8 T cells in a time- and dose-dependent manner both in humans and in murine models by stimulating both entry into the cell cycle, through increased cyclin-dependent kinase 2 (cdk2) (69, 121, 128, 129), and up regulation of telomerase (121). Mice treated with IL-7 for as little as 2 days showed a significant increase in the basal level of CD8 T cell proliferation and a smaller increase in CD4 T cell proliferation in response to *ex vivo* stimuli without inducing polyclonal T cell activation (130). Indeed, under lymphopenic conditions, IL-7 promotes T cell proliferation and survival in murine and primate models, as well as in humans (131-134). In SIV-infected monkeys, IL-7 treatment for three weeks increased the number of circulating naïve and memory T cells (134). Consistent with this, Levy and colleagues (2009) reported when IL-7 was administered to AIDS patients on antiretroviral therapy, a significant dose dependent increase of naïve and memory CD4 and CD8 T cells was observed (135). Further, IL-7 had been shown to play important roles in immune reconstitution by inducing proliferation of immature thymocytes in mice that underwent bone marrow transplantation (BMT) (136). Whereas BMT recipient mice had profound thymic hypoplasia at day 28 after transplantation, BMT recipients that were pre-injected with IL-7 showed normal levels of T

lymphocytes and accelerated bone marrow engraftment (136). In fact, a number of studies have highlighted the potential therapeutic application of IL-7 in increasing T cell numbers under lymphopenic conditions (75, 124, 135, 137-140).

1.5.3 The Effects of IL-7 on the Establishment of CD8 T Cell Memory Populations

IL-7 has been shown to play a key role in the establishment of memory CD8 T cells. A number of studies demonstrated that IL-7 alone or in combination with IL-2 and IL-15 regulates the proliferation of memory CD8 T cells (26, 141-143) (also reviewed in(144)). Evidence for the specific requirement for IL-7 in regulating the differentiation of effector CD8 T cells into memory cells came from a seminal study by Kaech *et al* (77). It was shown in this study that a small percentage (5-15%) of LCMV-specific effector CD8 T cells in mice maintained high expression of IL-7Rα (CD127) following infection with the lymphocytic choriomeningitis virus (LCMV) (77). When these IL-7R^{hi} effector cells were isolated and transferred into naïve uninfected mice, they established a memory cell pool capable of mounting a recall response to subsequent challenge with LCMV antigen. This suggests that effector CD8 T cells expressing high levels of IL-7Rα can give rise to a long-term memory population. Consistent with this, Huster *et al.* also demonstrated long term memory cell precursors are characterized by high levels of IL-7Rα surface expression (120). Further, Sawa *et al.* found numbers of antigen-specific memory CD8 T cells in mice were significantly increased following LPS-induced IL-7 production (82). Thus, IL-7 plays key roles in establishing memory T cells.

1.5.4 Interleukin-7 and the Cytotoxic Activities of CD8 T cells

IL-7 not only plays a pivotal role in T cell developmental and homeostatic regulation, but also plays an important role in the activation, differentiation, proliferation and cytolytic function of cytotoxic CD8 T lymphocytes (CTLs) (145-147). Naïve CD8 T cells activated in response to a specific antigen undergo proliferation and differentiation to the effector phenotype and begin to express cytotoxic molecules that enable them target infected cells. Quiescent naïve CD8 T cells are $CD45RA^{+}CD27^{+}CD28^{+}CD62L^{+}CCR7^{+}CD127^{+}$ and express neither the neural cell adhesion molecule CD56 nor perforin (148). Once a specific antigen is presented to naïve CD8 T cells in lymph nodes by antigen presenting cells via MHC class I molecules, naïve T cells proliferate and differentiate to produce an effector T cell population with a characteristic phenotype and functions. The cells initially down regulate both CD28 and CD45RA and up regulate CD45RO (149). Further, the cells proliferate in an IL-2-, IL-7- and IL-15-dependent manner and maintain CD27 expression (149-152). In the later stage of differentiation, the expression of a number of cell surface molecules is down regulated including CD127 (120, 153-156), CD62L, CCR7 as well as CD27 as the cells progress to an effector phenotype (157). Terminally differentiated effector cells re-express CD45RA, and up regulate CD56 as well as synthesize cytotoxic molecules including perforin and granzymes (149, 158-160). As these effector cells are low in CD127 they are susceptible to apoptosis and therefore their numbers decrease as the infection clears (149, 158-160). A small proportion of these cells however stay in circulation and differentiate into memory cells, marked by the expression of $CD45RO^{+}CD27^{+}CD28^{+}CD127^{high}$ (77, 161).

1.5.4.1 Levels of IL-7 increase in acute infection and play important roles in the control of and protection from infections

While it was previously believed that the production of IL-7 was constitutive and that T cells competed for limiting amounts of this essential cytokine (162), Sawa and colleagues (2009) established IL-7 is in fact an acute-phase cell-signaling molecule (82). Here, they demonstrated lipopolysaccharide (LPS) binding to Toll-like receptor 4 (TLR4) on non-lymphoid cells induces production of interferon (IFN)-β which in turn up regulates expression of IL-7 in hepatocytes (82). This up regulation of IL-7 enhances T cell survival and function. In fact, the roles of IL-7 in improving host immunity were very recently demonstrated in several disease models such sepsis and influenza (102, 163). In one study, cecal puncture and consequent translocation of gut bacteria into the blood stream in mice was utilized as an experimental model for sepsis (163). Here, mice with induced sepsis were either injected with saline or with daily doses of 2.5 μg of recombinant IL-7 subcutaneously for 5 days. While all mice in the saline treated group died, nearly half (40%) of the mice receiving IL-7 survived (163). Survival in the IL-7 treated group was associated with increased T cell counts as well as enhanced expression of the activation marker CD69 on T cells. Plumb *et al.* recently illustrated IL-7 plays a key role in the T cell response to influenza A virus (102). Here they compared the severity of the disease between wild type mice and mice carrying a mutated version of CD127 unable to transduce IL-7 signaling (tyrosine 449 to phenylalanine) (164). Mice with impaired IL-7 signaling lost significantly more weight than wild type with significantly higher viral titers in the lungs compared to control mice. In addition, a significant reduction in both the numbers and function of

influenza A specific T cells was observed in the mice with impaired IL-7 signaling. These results clearly illustrate a role for IL-7 in the CTL response to infections.

1.5.4.2 Upon activation, IL-7 contributes to naïve CD8 T cells differentiation to an effector phenotype

Upon activation, IL-7 enhances differentiation of naïve CD8 T cells to the effector phenotype. IL-7 alone up regulates the expression of both the activation inducer molecule CD69 as well as CD44 (165) and lowers the expression of CD62L (166). IL-7 also augments the expression of the IL-2R alpha chain, CD25 on activated T cells (166, 167). IL-7 does not mediate its pro-activation/expansion effects exclusively through CD25 however, as IL-7 can rescue the cytotoxic and proliferative capacity of alloreactive CD8 T cells in the presence of monoclonal antibodies against this molecule (168).

1.5.4.3 IL-7 plays pivotal roles in the proliferation of activated T cells

IL-7 has been shown to enhance proliferation of IFN-γ^+ perforin$^+$ cytotoxic T cells as well as increase the numbers of effector memory T cells ($CD45RA^+CD62L^-$) (82, 169, 170). IL-7's role in proliferation of activated T cells *in vivo* was illustrated in a recent study by Saini *et al*. Here, absence of IL-7 or IL-7R expression resulted in a considerable deficiency in antigen-mediated T cell proliferation in mice (171). IL-7 has been shown to mediate T cell proliferation through a number of mechanisms including up regulation of cyclin-dependent kinase 2 (cdk2) (130), essential for cell cycle progression from G1 to the S phase (172). IL-7 had also been shown to down regulate the expression of the Cyclin-dependent

kinase inhibitor 1B ($p27^{Kip1}$) (122). As telomere length is reduced with cell division, the activity of telomerase is essential for T cell clonal expansion (173). IL-7 alone up regulates the activity of telomerase in PBMCs and also significantly augments telomerase activity in cells activated by anti-CD3 monoclonal antibodies (121). Our lab has also confirmed IL-7's ability to induce proliferation of primary human CD8 T cells as measured by both [^{3}H]-TdR incorporation and Ki-67 protein expression (166, 174).

1.5.4.4 IL-7 up regulates the expression of cytolytic effector molecules

In addition to mediating CD8 T cell differentiation and proliferation, IL-7 up regulates the expression of cytolytic effector molecules including perforin and granzyme B. IL-7's role in augmenting synthesis of perforin in human CD8 T cells was characterized shortly after its discovery (175). Our lab has also demonstrated that IL-7 alone when incubated with purified human CD8 T cells *in vitro* induces perforin synthesis (174). IL-7 also induces granzyme B production in activated CD8 T cells (165) and enhances IFN-γ production following TCR stimulation (82, 169).

1.5.4.5 IL-7 mediates the cytotoxic/killing activities of CD8 T cells

Consistent with its role in up regulating the expression of cytotoxic molecules, IL-7 mediates the cytotoxic activities of CD8 T cells. In fact, the role of IL-7 in inducing a pathogen-specific CTL response to kill infected cells was evaluated shortly following its identification (176, 177). Kasper *et al*. found that mice infected with *Toxoplasma gondii* had a much higher survival rate when concurrently treated with IL-7 (176). Survival was

correlated with increased CD8 T cell IFN-γ production and augmented CTL responses as demonstrated ex-vivo by incubating $CD8^+$ T cells isolated either from IL-7 treated mice or controls with Toxoplasma-infected macrophages (176).

The importance of IL-7 in mediating the cytotoxic activity of CD8 T cells was also demonstrated in influenza A infection. Kos *et al*. examined naïve CD8 T cells stimulated with a potent immunogenic short synthetic peptide derived from the influenza A virus nucleoprotein *in vitro* in the presence or absence of IL-7 (178). Compared to untreated cells, cells that were incubated with IL-7 generated significantly higher influenza A-specific anti-viral activity with a dose-dependent increase in the lysis of infected cells (178). As discussed, Plumb *et al.* recently reported a significant increase in influenza viral titers in mice with defective IL-7 signaling as a result of a mutation in the critical tyrosine Y449 on CD127 challenged with influenza A virus, indicating the requirement of IL-7 signaling (102). The mutation in CD127 was correlated with a significant reduction in specific CD8 T cell numbers and with defects in influenza A specific T cell response (102).

IL-7 has also been shown to enhance antigen-specific CTL activity in many other viral infections. Pellegrini *et al*. reported IL-7 administration to mice that are chronically infected with LCMV resulted in viral clearance (179). This was shown to occur through an increase in virus-specific CD8 T cell counts and enhancement of cytolytic function as measured by their *ex vivo* capacity to produce IFN-γ in response to cognate peptides. Similarly, Wiryana *et al*. showed mice treated with IL-7 successfully cleared herpes simplex virus (HSV) in comparison to control mice (180). Virus clearance was dependent on CD8 T cell activity as depletion of CD8+ T cells abolished the ability to clear the viral infection

(180). The role of IL-7 in CTL clearance of HSV infected cells was further examined by Sin *et al.* using another mouse model in which mice were immunized with a DNA vaccine encoding HSV and were co-injected with IL-7-encoding plasmids (181). Here, mice with augmented IL-7 expression displayed higher protection against HSV infection compared to control mice. A similar effect has been observed with hepatitis C virus (HCV) (182). Cynomolgus monkeys vaccinated with a DNA vaccine against HCV and co-injected with human IL-7-encoding DNA showed higher HCV-specific CTL compared to controls as demonstrated by increased CD8 T cell IFN-γ production in response to HCV peptides.

A number of studies have also illustrated IL-7 is able to stimulate CTL responses against cancerous tumors (146, 183). IL-7 induces production of both CTL and lymphokine-activated killer (LAK) cells in human peripheral blood mononuclear cells (183) and was shown to be effective in treating metastasized pulmonary sarcoma in mice (146). In that study, IL-7 was used to treat cells isolated from the popliteal draining lymph nodes of cancerous mice *ex vivo*. The re-introduction of IL-7 treated cells resulted in a significant decrease in the number of pulmonary metastases when compared to controls (146).

1.6 IL-7 and Diseases Affecting Immune Function

Upon CD8 T cell activation, IL-7 contributes to the differentiation of naïve cells to the effector phenotype and plays pivotal roles in the proliferation of activated T cells as well as in up regulating the expression of cytolytic effector molecules which mediate the cytotoxic activity. Due to the central role IL-7 plays in this process, it is no surprise

aberrations in CD127 expression are associated with a number of disease processes including autoimmune diseases and leukemogenesis.

1.6.1 IL-7 and Multiple Sclerosis

Multiple sclerosis (MS) is a chronic autoimmune disorder characterized by self-reactive T cells and demyelination within the central nervous system (184). While the causes of MS remain largely unknown, several HLA alleles have been associated with increased risk of MS such as the HLA-DRB1*15:01 (185). In addition, a number of single nucleotide polymorphisms (SNPs) linked with MS have been reported (186). Whole-genome linkage screens in MS patients revealed the major risk SNP rs6897932 is located within exon 6 of the CD127 gene (187).

How mutations in CD127 contribute to MS remains unknown but the frequency of naïve, memory and effector memory CD8 T cells and levels of CD127 on their surface in MS patients are significantly higher than in healthy individuals (188). The high expression of the IL-7 receptor is also accompanied by augmentation of STAT5 phosphorylation in response to IL-7 signaling and increased granzymes A and B synthesis (188). The increase in granzyme A is relevant to the onset of MS as it has been shown to play a role in myelin sheath destruction (189). The role of CD127 in MS has also been highlighted in murine experimental autoimmune encephalomyelitis. In this model of MS, IL-7R$\alpha^{-/-}$ mice showed a significant reduction in both inflammatory cytokine levels and in demyelination with the CNS compared to wild type mice (190).

1.6.2 IL-7 and Rheumatoid Arthritis

The expression of CD127 on T cells is also increased in rheumatoid arthritis (RA) reviewed in (191). T cells isolated from the joints of patients with RA express significantly higher levels of CD127 compared to healthy controls (192). In addition, in a mouse model for RA, treatment with antibodies against IL-7 eliminated arthritis (193) clearly indicating a specific contribution of aberrant signaling by IL-7 to the development of RA. It is thought that elevated IL-7 signaling contributes to the development of RA by inducing expression of tumor necrosis factor- (TNF)-α, which was shown to induce inflammation and joint damage in most RA patients (192).

1.6.3 IL-7 and Cancer

While overexpression of CD127 and IL-7 signaling are associated with autoimmune diseases such as MS and RA, reduced expression of CD127 has been associated with poor immunity and cancer. This was highlighted in a study by Vudattu *et al.* where lower levels of CD127 protein was found on T cells isolated from breast cancer patients compared to healthy donors (194). Perhaps not surprisingly, T cells from these patients also failed to phosphorylate STAT5 in response to IL-7 stimulation *in vitro* and produced significantly less IL-2 and IFN-γ in response to stimulation with PMA/Ionomycin. Although the mechanism by which CD127 is down regulated in breast cancer remains unknown, it is likely that low of CD127 expression on CD8 T cells contribute to a reduced CTL response and ineffective protection against cancer.

Defects in IL-7 signaling are also described in leukemogenesis. A study reported somatic gain-of-function mutations in the CD127 gene in childhood T-cell acute lymphoblastic leukemia (T-ALL) (195). Here, it was shown that mutations in exon 6 introduce a cysteine residue in the transmembrane domain of CD127 which allows homodimerization between mutant CD127 proteins via disulfide bonding (195). This was shown to stimulate constitutive JAK/STAT signaling without IL-7, leading to cell transformation and tumor formation. The constant IL-7 signaling by this mutation leads to leukemia by enhancing cell viability, inducing cell-cycle progression and promoting growth factor independence (195).

Thus, while signaling through the IL-7 receptor significantly enhances T cell responses to foreign antigens, fine-tuned negative regulatory mechanisms must take place to establish a balance between immune activation and suppression. Over-expression of CD127 has been associated with autoimmune diseases including MS and RA while decreased CD127 expression appears to increase susceptibility to cancer and opportunistic infections as in HIV infection.

1.7 Regulation of IL-7 Receptor Expression

As discussed above, the adaptive immune response is complex and is modulated by tightly regulated intrinsic feedback mechanisms. IL-7 signaling is pivotal for T cell development, survival and activation and thus it is not surprising signaling through the IL-7 receptor is tightly regulated. In fact, IL-7 stimulation down regulates expression of its own receptor alpha subunit (CD127) in a negative feedback mechanism. This phenomenon had

been shown in several T cell lines (196), murine (154) and human primary T cells where IL-7 down regulates the expression of both surface CD127 protein and transcription of the CD127 gene (154, 167, 197). However, the mechanisms governing this regulatory process have been only partially characterized (36, 154, 166, 167, 196-199). A thorough understanding of these mechanisms is imperative and may reveal novel insights into the contribution of abnormal IL-7 signaling to diseases affecting immune function.

1.8 Hypothesis

Given the pivotal roles IL-7 plays in T cells development, survival and function, I hypothesize expression of the IL-7 receptor alpha chain (CD127) mRNA transcripts and surface protein in primary human CD8 T cells is tightly regulated in response to IL-7 stimulation and occurs through distinct mechanisms.

1.9 Objectives

1. To systematically define the pathways and kinetics by which IL-7 down regulates CD127 surface protein expression and gene transcription in resting human CD8 T cells.

2. To elucidate the mechanism by which IL-7 down regulates CD127 protein from the surface of CD8 T cells and targets it for degradation.

3. To elucidate the mechanism by which IL-7 suppresses transcription of the CD127 gene in primary human CD8 T cells.

Chapter 2. Materials and Methods

2.1 Reagents

2.1.1 Cytokines

Interleukin-7 and interleukin-10, obtained from Invitrogen Biosource (Carlsbad, CA, USA), were resuspended in phosphate buffered saline (PBS) plus 0.1% BSA at a stock concentration of 10 ng/μl and stored at -80^{o} C.

2.1.2 Inhibitors

STAT5 inhibitor (573108; 100 mM), JAK Inhibitor 1 (420097; 10 mM) and PI3K inhibitor (LY294002; 10 mM) were purchased from EMD Biosciences (San Diego, CA, USA) and were dissolved in DMSO at the indicated stock concentrations. Cycloheximide solution (100 mg/ml in DMSO), the transcriptional inhibitor 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB, 21 mM in anhydrous 100% ethanol), Dynasore (100 mM in DMSO), Filipin (1.5 mM in DMSO), lysosomal inhibitors Leupeptin and E64 (both 1 mM in DMSO) and the proteasomal inhibitor MG132 (500 mM in DMSO) were obtained from Sigma-Aldrich (St Louis, MO, USA). Dexamethasone was obtained from BioVision (San Francisco, CA, USA) and was resuspended in DMSO at a stock concentration of 10 mM. Small Molecule Enhancer of Rapamycin 3 (SMER3), a specific inhibitor of E3 ubiquitin ligase, was purchased from R&D Systems (Minneapolis, MN, USA) was was resuspended in DMSO at 75 mM. All the inhibitors, once re-suspended at the indicated stock concentrations were stored at -20^{o} C. CD8 T cells were incubated with these inhibitors at the indicated final concentrations for 1 hour prior to the addition of IL-7.

2.1.3 Antibodies

2.1.3.1 Antibodies for Flow Cytometry

Anti-CD8-phycoerythrin-Cy5 (PC5) (B9.11), and anti-CD127-phycoerythrin (PE) (R34.34) fluorochrome-labeled monoclonal antibodies were purchased from Immunotech Beckman Coulter (Marseille, France). Monoclonal anti-phospho-STAT5-fluorescein isothiocyanate (FITC) (Y694), anti-phospho-STAT3-FITC (Y705) and anti-Notch 1- PE (mN1A) antibodies were purchased from BD Biosciences (San Jose, CA, USA). All fluorochrome labeled antibodies used for flow cytometry were titrated and used at saturating concentrations.

2.1.3.2 Antibodies for Western blot and CoIP Assays

Polyclonal goat anti-human CD127 antibodies were obtained from R&D Systems (1µg/ml). Polyclonal rabbit anti-human c-Myb, polyclonal goat anti-human Gfi-1 and monoclonal mouse anti-human β actin antibodies (C4) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and were used at a final concentration of 1 µg/ml. The following antibodies were obtained from Abcam (Cambridge, MA, USA) and were used at a concentration of 1µg/ml for Western blot and Co-IP assays: polyclonal mouse anti-CIS (ab88383) , polyclonal rabbit anti-SOCS 1 (ab3691), polyclonal rabbit anti-SOCS 2 (ab3692), polyclonal rabbit anti-SOCS 3 (ab3693), polyclonal rabbit anti-SOCS4 (ab3694), polyclonal rabbit anti-SOCS5 (ab125265), polyclonal rabbit anti-SOCS6 (ab13950), polyclonal rabbit anti-SOCS7 (ab13951).

2.2 Cell Purification and Culture

2.1.1 Primary Human CD8 T Cell Purification and Culture

Primary human CD8 T cells were isolated from blood of healthy volunteers with consent. Blood was drawn into syringes containing sodium heparin (20 units/ml), and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque density centrifugation. In brief, 30 mL of blood was slowly layered above 12.5 mL of Ficoll-Paque™ PLUS (GE Healthcare) and was centrifuged for 30 minutes at 16000 rpm. The PBMC fraction was transferred to fresh 50 mL tubes and PBMCs were pelleted and washed twice with PBS before resuspension in 800 μL of MACS buffer (PBS with 0.5% BSA and 2 mM EDTA) per 10^8 cells. CD8 MicroBeads (Miltenyi Biotec, Auburn, CA, USA) were added to the cells in MACS buffer at a ratio of 1:4 and the mixture was incubated on a rotator at 4°C for 25 minutes. The excess beads were then washed out by cell pelleting. After washing with PBS and resuspending the labeled cells with 2 mLs of magnetic-activated cell sorting (MACS) buffer, CD8 T cell purification was performed using the AutoMACS Isolation System (Miltenyi Biotech) using the cell sorter program 'possel_s' and CD8+ fractions were collected at the 'positive' fractions output. Purified CD8 T cells were cultured at a density of 1 x 10^6 cells/ml at 37°C in media comprised of RPMI 1640 (Hyclone, Logan, UT, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 0.2 M L-glutamine (GIBCO, Grand Island, NY, USA) and 20% fetal calf serum (FCS; Cansera, Rexdale, ON, Canada). Cell purity was consistently >95% $CD8^+$ as assessed by flow cytometric analysis with only 2.6±0.9% $CD8^+CD3^-CD16^+CD56^+$ NK cells. All cultures were maintained in a humidified incubator at 37°C in the presence of 5% CO_2. All research

conducted using human blood was reviewed and approved by the Ottawa Hospital Research Ethics Board. The ethical consent forms are attached in the appendix.

2.1.2 Culture of Cell-lines

The Jurkat-CD127 cell line was a kind gift from Dr René van Lier (Academic Medical Centre, Amsterdam, The Netherlands). These cells contain the retrovirally transduced CD127-IRES-GFP sequence and express the human CD127 cDNA from the cytomegalovirus (CMV) promoter (197). The human lymphoblastic CEM, A3, Jurkat and SupT cell lines were obtained from the American Type Culture Collection (ATCC, USA).

T cell lines were cultured at a density of 1 x 10^6 cells/ml in media comprised of RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 0.2 M L-glutamine and 10% FCS. All cultures were maintained in a humidified incubator at 37^{o}C in the presence of 5% CO_2.

2.3 Quantitative Real Time-PCR (qPCR)

Total RNA was harvested from 5 x 10^5 CD8 T cells using the RNAqueous®-4PCR kit for isolation of DNA-free RNA (Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA was quantified using a Nanodrop ND-1000 instrument (Nanodrop, Wilmington, DE) and reverse transcribed using the iScript cDNA Synthesis Kit from Bio-Rad (Hercules, CA, USA). Equal volumes of cDNA from each sample were then mixed with IQ SYBR Green Supermix (Bio-Rad) and used to quantify CD127 transcripts relative to the RPS18 reference gene. Both reverse transcription (RT) and qPCR were carried out on the iCycler thermal cycler from Bio-Rad. Samples were incubated at 95^{o}C for 3

minutes and then amplified over 40 cycles as follows: 95^{o}C for 30 seconds, 57^{o}C for 20 seconds and 72^{o}C for 20 seconds. At the end of each cycle, samples were held at 85^{o}C for 5 seconds for fluorescence acquisition. Following amplification, samples were denatured for 1 minute at 95^{o}C followed by final extension at 72^{o}C for 1 minute. Primers used to amplify various genes are depicted in Table 1. Tm of all primers ranged between 57.5-61^{o}C. Data analysis was carried out using the $2^{\Delta\Delta Ct}$ principle with the Bio-Rad Gene expression analysis macro program.

2.4 PCR Array

The mRNA expression profiles of 84 transcription factor genes were analyzed using a PCR array specifically the human transcription factors reverse transcriptase RT² profiler from SABiosciences (Frederick, MD, USA). In brief, purified CD8 T cells were incubated in media alone, with IL-7 (10 ng/ml) for 3 hours, or pre-incubated with the STAT5 inhibitor at a final concentration of 500 μM for 1 hour followed by addition of IL-7 (10 ng/ml) for additional 3 hours. Similarly, the expression of 84 genes related to JAK- and STAT-mediated signaling was analyzed using the human JAK/STAT signaling PCR array (SABiosciences, Frederick, MD, USA) where purified CD8 T cells were either incubated in media alone or with IL-7 (10 ng/ml) for 3 hours. cDNA was generated from 1 μg of total RNA using the RT² First Strand Kit and mixed with RT² SYBR® Green qPCR Mastermix (SABiosciences). For data analysis, the online RT² Profiler PCR Array Data Analysis software was used (http://www.sabiosciences.com/pcrarraydataanalysis.php). To confirm the PCR array results, quantitative Real-time PCR was performed as described above.

Table 1. Primers used for qPCR assays.

Gene	Forward primers (5'-3')	Reverse primers (5'-3')
RPS18	CTGCCATTAAGGGTGTGG	TCCATCCTTTACATCCTTCTG
CD127	ATGGACGCATGTGAATTTATC	TTATTGATCTCTGGAGTTCTGA
β actin	GAAACTACCTTCAACTCCATC	CGAGGCCAGGATGGAGCCGCC
Gfi-1	CTAGGGCTCTACGGCGACTT	GCGTGGAAAACACCTTGCTG
c-Myb	GAAGGTCGAACAGGAAGGTTATCT	GTAACGCTACAGGGTATGGAACA
ARNT	GTGGCAGTAGCTCTGTGGACC	AGCCAAGTCCATTCCTGCAT
CEBPB	TGTCCAAACCAACCGCACAT	AGCAACAAGCCCGTAGGAAC
CEBPG	CGGTTGAAAAGCAAGCAGAAAGCA	GATCCCAGAAAATAGCCTCCAATG
ESR1	GGAGGGCAGGGGTGAA	GGCCAGGCTGTTCTTCTTAG
ETS1	AAACTTGCTACCATCCCGTACGT	ATGGTGAGAGTCGGCTTGAGAT
GATA3	CTGCTTCATGGATCCCTACC	GATGGACGTCTTGGAGAAGG
IRF1	AAAAGGAGCCAGATCCCAAGA	CATCCGGTACACTCGCACAG
STAT4	GCTGAGAGCTGTAGTGTTTACCGA	AATAAAGGCCGGTTGTCTGCT
STAT5A	GACCTTACCAAACCCCTTGG	CCGTTCACCCAGCCTAGTTA
Notch-1	TCAGCGGGATCCACTGTGAG	ACACAGGCAGGTGAACGAGTTG
CIS	GATCTGCTGTGCATAGCCAA	ACAAAGGGCTGCACCAGTTT
SOCS1	TTTTTCGCCCTTAGCGTGAA	GCCATCCAGGTGAAAGCG
SOCS2	CAGGGAATGGCAGAGACACT	TGGCAGAGAGAGAAGGGATG
SOCS3	GAAGATCCCCCTGGTGTTGA	TTCCGACAGAGATGCTGAAGA
SOCS4	CTTAGATCATTCCTGTGGGC	ATGCCACCTAAAGGCTAAATC
SOCS5	AATTGTGCCACAGAAATCCCT	AGCATCCAATGAACTCTGGG
SOCS6	GGACTCACTGGCACAGAAGC	TTCAGAGTCCCTGATTGAATGC
SOCS7	AAATATAGTTCCCCGTCCCC	CAGGAATGACAAATGATCCG

2.5 Nuclear Run-on Assay

To directly measure the rate of CD127 gene transcription, nuclear run-on assays were conducted using established protocols (154, 200, 201). For nuclei preparation, CD8 T cells (2×10^7) were first washed in ice cold wash buffer (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and 150 mM Sucrose) and then incubated in 0.5 ml of cell lysis buffer (0.5% Nonidet P-40 [NP-40], 10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and 150 mM sucrose) at 4°C for 5 min. Nuclei were released by Dounce homogenization (Wheaton Science, Millville, NJ USA), collected by centrifugation (4°C, 900 g for 9 minutes) and gently washed with ice cold wash buffer. RNA synthesis was allowed to continue by suspending the nuclei in 200 µl of transcription buffer (100 mM KCl, 10 mM Tris-HCl, pH 8.0, 2.5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 0.5 mM each of ATP, GTP, CTP, 100 mM sucrose and 10% glycerol) on ice and either 8 µl biotin-16-UTP (Roche Molecular Biochemicals, Mannheim, Germany) to label the nascent mRNA or with 0.5 mM UTP to generate negative control unlabeled mRNA. The reactions were incubated for 30 min at 29°C and then stopped by adding 6 µl 250 mM $CaCl_2$ plus 6 µl RNase-free DNase I (10 U/mL stock; Roche Molecular Biochemicals, Basel, Switzerland) and incubating for 10 min at 29°C. RNA purification of both biotin-labeled and total RNA was performed using the RNAqueous®-4PCR kit for isolation of DNA-free RNA (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Purified RNA was resuspended in 50 µL diethylpyrocarbonate (DEPC)-treated water. Biotin-labeled RNA was then bound to streptavidin-coupled magnetic Dynabeads M-280 (Dynal-Invitrogen, Carlsbad, CA, USA) suspended in 500 µL binding buffer (10 mM Tris-HCl, pH7.5, 1 mM EDTA and 2 M NaCl) and incubated for 20 min at 42°C and then for 2 hours at room temperature. Beads were separated from total RNA by a magnetic field

(apparatus supplied by Dynal) and beads were washed by incubation in a rotary shaker for 15 minutes once in 500 μL 15% formamide solution -in water- and twice in 2X standard saline citrate (0.3M NaCl, 0.03M trisodium citrate, pH 7.0). Beads were then resuspended in 30 μL DEPC-treated water and stored at -20°C. Reverse transcription and qPCR using RPS18 and CD127 primers were performed as already described.

2.6 Flow Cytometry

Surface CD127 protein expression was analyzed by incubating cells with anti-CD127-PE antibodies for 30 minutes in the dark at room temperature followed by analysis on a Coulter Epics ALTRA flow cytometer (Fullerton, CA, USA). The protocol was modified slightly in figure 8A. To prevent further internalization of CD127 while staining with the antibody, CD8 T-cells were treated with IL-7 for the times indicated and then incubated with anti-CD127-PE antibodies for 30 minutes on ice. Intracellular phospho-STAT3 and phospho-STAT5 staining was performed as follows: isolated CD8 T cells were fixed in 2% paraformaldehyde in PBS for 10 min at room temperature and then permeabilized by incubating in cold 100% methanol for an additional 10 min at 4^{o}C. After washing in phosphate buffered saline, cells were incubated with anti-phospho-STAT3-FITC or anti-phospho-STAT5-FITC antibodies in the dark for 30 min and then analyzed by flow cytometry. Live cells were gated on the basis of side and forward scatter and at least 10,000 events were recorded for each sample. Isotype controls were performed for each fluorochrome-conjugated antibody. Resulting profiles were analyzed with FCS Express 2 software (De Novo, Los Angeles, CA, USA) and statistical analysis was done using a two-tailed, paired student t-test with 95% confidence intervals.

2.7 Confocal Microscopy

Purified primary human CD8 T cells (5 x 10^5 cells per condition) were incubated in medium alone or with IL-7 (10 ng/ml) for the times indicated. Cells were fixed and permeabilized using the Fix & Perm® Kit from Life Technologies (Burlington, ON, Canada) and then washed with PBS containing 5% BSA. Unless otherwise noted, sequential antibody labeling was performed in 30 minute steps in PBS plus 5% BSA at 4°C. After each labeling step, cells were washed with PBS plus 5% BSA. SOCS proteins were stained with either rabbit anti-SOCS1 (ab109245, Abcam), rabbit anti-SOCS2 (epr2588(2), Abcam) or goat anti-CIS antibodies (AF3194, R&D Systems). SOCS primary antibodies were then detected with the appropriate secondary Alexa 647-conjugated goat anti-rabbit IgG (A21245, Life Technologies) or donkey anti-goat IgG antibodies (A21447, Life Technologies). CD127 molecules were then stained with a mouse anti-human CD127 monoclonal antibody (40131, R&D Systems) followed by Alexa 555-conjugated goat anti-mouse IGg1 (A21127, Life Technologies) in PBS plus 5% goat serum (Sigma-Aldrich). Cells were then stained with fluorescein isothiocyanate (FITC)-conjugated mouse anti-EEA1 (14/EEA1, BD Biosciences) or mouse anti-LAMP1 (H4A3, BD Biosciences), or with unconjugated mouse anti-MCP20 antibodies (MCP20, Abcam). Cells stained with FITC-conjugated anti-EEA1 or anti-LAMP1 antibodies were blocked with 40 μg/ml goat anti-rabbit IgG (A10533, Life Technologies) overnight at 4°C and then further amplified using the FITC Amplification Kit (A11053) from Life Technologies. Cells stained with the anti-MCP20 antibodies were incubated with 10 μg/ml nonspecific goat IgG (I5256, Sigma-Aldrich) followed by Alexa 488-conjugated goat anti-mouse antibodies (Life Technologies). Finally, cells were washed in PBS, stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 5 minutes and then fixed to

coated microscope slides (Scientific Device Laboratory, Des Plaines, IL, USA) using the Thermo Cytospin 4 (Pittsburgh, PA, USA). Cells were visualized using a Zeiss LSM 510 confocal microscope (Zeiss Canada, Toronto, ON, Canada) at 630X magnification using a Pln Apo 63X/1.4 oil DIC II objective. Image capture, pixel density quantification and analysis were performed with Zen 2008 software (Carl Zeiss MicroImaging Inc., Jena, Germany) and image processing was completed with Adobe Photoshop CS4 software (Adobe Systems Inc., Mountain View, California). **The confocal microscopy work was performed by Dr. E Faller**.

2.8 SDS-PAGE and Western Blot Analysis

CD8 T cells (1-2 x 10^6 cells per condition) were collected at the indicated time points by centrifugation (6000 g for 10 minutes). Whole cell lysis was carried out in 50 μL RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS plus 1X Halt Protease Inhibitor Cocktail [Thermo Scientific, Rockford, IL, USA]) at 4^{o}C for 45 minutes. For figure 19 (dynasore and filipin inhibitors), cells were lysed in 50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 10% glycerol, 2% SDS plus 1X Halt Protease Inhibitor Cocktail and sonicated on ice with two pulses of 25-30 seconds each at AMP=25% (Vibra-Cell™, Sonics & Materials Inc., Newtown, CT, USA). Protein concentration was quantified using the Bradford protein assay (Sigma-Aldrich). Equal amounts of protein were added to 2x Laemmli loading buffer (25 % glycerol, 125 mM Tris-HCl pH 6.8, 4 % SDS, 10% β-mercaptoethanol, 0.01% bromophenol blue), boiled for 10 minutes and loaded onto an 8% SDS-polyacrylamide gel. Proteins were transferred to Immun-Blot PVDF membranes (Millipore, Billerica, MA, USA) using a semi-dry transfer apparatus (Bio Rad, Hercules, CA,

USA) at 20 volts for 25 minutes. Membranes are then blocked with 5% nonfat dry milk in Tris-Buffered Saline plus 0.1% Tween-20 (TBST) for at least 1 hour. To detect CD127, membranes were incubated with a 1:500 dilution of polyclonal goat anti-human CD127 antibody (R&D Systems) in 2% nonfat dry milk in TBST overnight at 4˚C with constant agitation. The next day, membranes were washed three times for 10 minutes each in TBST and then incubated with a 1:5000 dilution of horseradish peroxidase (HRP) conjugated-donkey anti-goat secondary antibody (R&D Systems) in 2% nonfat dry milk in TBST for 1 hour at room temperature with constant agitation. Membranes were again washed three times in TBST and CD127 proteins were visualized by chemiluminescence using the ECL Advance Western Blotting Detection Kit (GE Healthcare, Chalfont St. Giles, UK) according to the manufacturer's instructions. For re-probing loading controls, membranes were stripped for 30 minutes at 50 ^{o}C in stripping buffer (2% SDS, 62.5 mM Tris-HCl pH 6.8, 100 mM β-mercaptoethanol), washed 5 times with TBST, blocked with 5% nonfat milk in TBST for 1 h and then re-probed with a monoclonal mouse anti-human β actin antibody followed by an HRP conjugated goat anti-mouse antibody (R&D Systems).

2.9 Co-Immunoprecipitation

Purified CD8 T cells (5 x 10^6 cells per condition) were incubated in media alone or with IL-7 for 3 hours. All Co-IP buffers were supplemented with 1X Halt Protease Inhibitor Cocktail (Thermo Scientific). Cells were centrifuged at 6000 g for 10 minutes and lysed in 100 µl of Lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10% glycerol, 2 mM DTT) on ice for 15 minutes. Lysates were diluted with 400 µl of Dilution buffer (10% glycerol, 20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 1 mM DTT, 1 mM

PMSF, and 1 mg/ml BSA) and were pre-cleared to minimize background by incubating with 25 µL of Protein G Agarose beads (Pierce-Thermo Fisher Scientific, Waltham, MA, USA) on a rotary shaker for 30 minutes at 4^{o}C. Pre-cleared supernatants were then incubated with 2.5 µg of polyclonal goat anti-human CD127 antibody or isotype control goat antibody (R&D Systems) and 40µl of Protein G agarose beads on a rotary shaker for 8 hours at 4^{o}C. The beads were then washed three times with wash buffer (10 % glycerol, 100 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1 % NP-40) for 20 minutes at 4^{o}C and proteins were then eluted off the beads by adding 60 µL of 3X Laemmli loading buffer, boiled for 5 minutes, band loaded onto an 8% SDS PAGE. Immunobloting for SOCS 1-7 and CIS was then performed as described above.

2.10 siRNA-mediated Gene Knock-down Assays

Four pairs of siRNA sequences targeting either human c-Myb (003910-00-0005), SOCS1 (E-011511-00-0005), SOCS2 (017604-00-0005) or CIS (017381-00-0005) were designed and synthesized by Dharmacon (Accell SMARTpool siRNA, Thermo Fisher Scientific). In parallel, a pool of four non-targeting (scrambled) siRNAs (001950-01-05) was used as a negative control. All siRNAs were resuspended in RNase-free water at a stock concentration of 20 µM. The knock-down assays were conducted as per manufacturer instructions. In brief, purified CD8 T cells were washed three times in PBS and resuspended at 2×10^{6} cells/ml in Accell siRNA delivery media (Dharmacon) containing the indicated siRNA at a final concentration of 0.3 µM. The media was supplemented with 100 unit/ml penicillin and 100 µg/ml streptomycin (GIBCO). Cells were incubated with the siRNA for 4

days. Silencing efficiency of the different siRNAs was monitored by quantitative RT-PCR and Western blot analysis.

2.11 Promoter Mapping Bioinformatic Analysis

The human CD127 genomic sequence from -3kb to +0.5kb with respect to transcription start site was analyzed using the web-based putative transcription factor binding site identification software programs: Transcription Element Search System (TESS; http://www.cbil.upenn.edu/tess/) as well as Genomatix MatInspector (http://www. genomatix.de). Transcription factor binding sites were mapped to both sense and antisense strands.

2.12 Promoter Constructs with Progressive Truncations

To identify the region within the CD127 gene promoter required for IL-7 mediated transcriptional suppression, existing constructs in our lab were used. The upstream noncoding region from -2900 bp to the TATA box and progressive truncations thereof were cloned upstream of the firefly luciferase reporter gene, listed in Table 2. These clones were generated by Juzer Kakal, a former Master's student in our lab. In brief, a DNA fragment containing sequences from the TATA box to -2900 bp upstream of the CD127 gene was cloned from Jurkat T cells. This fragment was then cloned into the pGL4B plasmid upstream of the luciferase reporter gene (Invitrogen Biosource). Deletion mutants were constructed based on the locations of putative transcription factor binding sites. The details are provided in J. Kakal's thesis (202).

Table 2. List of the CD127 promoter constructs cloned into pGL4B.

Promoter Constructs	Description
2900 to TATA	Whole construct
2406 to TATA	Truncated construct 1
1760 to TATA	Truncated construct 2
1468 to TATA	Truncated construct 3
626 to TATA	Truncated construct 4
pGL4B	Empty vector control
phRTK	Nucleofection control

2.13 Site-Directed Mutagenesis

To examine the requirement of c-Myb binding within the CD127 promoter for the IL-7 mediated suppression of CD127 gene transcription, new constructs were generated using the full length plasmid (TATA to -2900) as a template in which c-Myb-binding sites, identified by the putative transcription factor binding site bioinformatics analysis described above, were mutated by site-directed-mutagenesis (SDM) protocol. Mutations in c-Myb binding sites on the CD127 promoter were achieved using the Stratagene Site Directed Mutagenesis Kit XL (Stratagene, La Jolla, CA, USA) which uses the ultra-high fidelity polymerase (Pfu-Ultra) for DNA amplification. The full length (2900 to TATA) plasmid was used as a template to generate plasmids with mutations in c-Myb binding sites. To introduce the site specific mutations, megaprimers, obtained from Invitrogen and summarized in table 3, that contain the mutations in the c-Myb binding sites in the middle of the primers were used as starting template to generate copies of the plasmid with the mutations. The primers were designed using the Stratagene Site directed Mutagenesis primer design program (Table 3). The sample reactions were prepared using the following: 50 ng of template DNA, 250 ng of forward and reverse primers, 5 µl of 10X reaction buffer, 1 µl of dNTP mix, 1 µl of Pfu ultra enzyme and completed to 50 µl of nuclease-free water. The PCR amplification conditions used were as follows: 95°C for 30s for initial enzyme activation, then 18 cycles of 95°C for 30s, 55°C for 1 min, then 68°C for 14 minutes. Since the non-mutant plasmid template was originally generated in E. coli, it is methylated. Therefore, to get rid of the non-mutant DNA template after the SDM reaction, the PCR product is digested with DpnI which eliminate methylated DNA. The digestion took place overnight at 37 ^{0}C. The DNA was then

Table 3. Primers used to mutate c-Myb binding sites within the CD127 gene promoter. The primers were designed using the Stratagene Site directed Mutagenesis primer designer program. URL: https://www.genomics.agilent.com.

Primer name	Mutation site w.r.t TSS	Mutation achieved	Nucleotide sequence (5'-3')
Site 1-forward	2724-2729	CAGTTG to ACTGCA	ATGGGTGAAGAGGAGGAGAAGAAATATGGCCAAGTACTGCACCACGCCAAACTGCTTGC
Site 1-reverse	2724-2729	CAGTTG to ACTGCA	GCAAGCAGTTTGGCGTGGTGCAGTACTTGGCCATATTTCTTCTCCTCCTCTTCACCCAT
Site 2-forward	2160-2165	CAGTTT to ACTACC	CCAGTTACTCACCCATGAAGTGACAATCTGTTAGACTACCAGGAGATTTGCATTGGTAGATAGATATAATTTGG
Site 2-reverse	2160-2165	CAGTTT to ACTACC	CCAAATTATATCTATCTACCAATGCAAATCTCCTGGTAGTCTAACAGATTGTCACTTCATGGGTGAGTAACTGG
Site 3-forward	1858-1863	CAGTTG to ACTGCA	CAAATGGAGAAGATGGAATATGTCCCCTTACTGCAGATTCTTGCTCCGAAGTATTCCTTGAGCC
Site 3-reverse	1858-1863	CAGTTG to ACTGCA	GGCTCAAGGAATACTTCGGAGCAAGAATCTGCAGTAAGGGGACATATTCCATCTTCTCCATTTG
Site 4-forward	1801-1806	TAACTG to CGCACA	GTATTCCTTGAGCCTATACTCCAAAGTGTGGCATTACGCACAGTGCAGACGGAGTCTGTCT
Site 4-reverse	1801-1806	TAACTG to CGCACA	AGACAGACTCCGTCTGCACTGTGCGTAATGCCACACTTTGGAGTATAGGCTCAAGGAATAC
Site 5-forward	1715-1720	TCGTTA to GTTGCC	ACTGGTACAAATGTCCCACCTCCTTTTTTTGTTACTGTTGCCTTGTCAAATAATTGTAATGATGGGACAAGCTAAATC
Site 5-reverse	1715-1720	TCGTTA to GTTGCC	GATTTAGCTTGTCCCATCATTACAATTATTTGACAAGGCAACAGTAACAAAAAAAGGAGGTGGGACATTTGTACCAGT

transformed into competent bacteria and was amplified using the NucleoSpin® Plasmid QuickPure kit (miniprep) or the NucleoBond® Xtra Maxi kit (maxiprep) both from Macherey-Nagel (Düren, Germany). Both kits use silica columns to purify DNA. For generating double, triple and quadruple mutations, plasmid DNA that underwent single, double and triple mutations were used as template and the same protocol was followed to generate additional mutations. The DNA sequences of all plasmids used in this study were confirmed by sequencing by the Stemcore facility at the Ottawa Hospital Research Institute (OHRI) using the Sanger method.

2.14 Cell Transfection

2.14.1 Transfection of Human CD8 T cells with CD127 Promoter Constructs

To identify the region within the CD127 gene promoter required for IL-7 mediated transcriptional suppression, the promoter constructs depicted in Table 2 were transfected into primary human CD8 T cells. In brief, plasmid DNA used for transfection was isolated from Dh5α competent E. Coli using the NucleoSpin® Plasmid QuickPure kit (miniprep) or the NucleoBond® Xtra Maxi kit (maxiprep) both from Macherey-Nagel. Primary human CD8 T cells were isolated as described above and cultured overnight in RPMI-20. The next day cells were incubated for 6 hours with 10 ng/ml of IL-7 to induce the transcriptional repressor or incubated in media alone and then transfected at 5 million cells per condition with 2.5 μg of plasmid containing the CD127-promoter driving expression of the Firefly luciferase gene. Cells were co-transfected with a second plasmid phRTK to account for transfection efficiencies. The phRTK plasmid carries the Renilla luciferase gene driven by the herpes simplex virus thymidine kinase promoter and was transfected into primary CD8 T cells at a

plasmid ratio of 8:1 (CD127/Firefly: Renilla). Plasmids as well as CD8 T cells were suspended in 100 μL of Nucleofector® solution and cell transfection was achieved using the Nucleofector II device with the U-014 program setup. Post-nucleofection, cells were allowed to recover in pre-warmed serum-free RPMI-1640 at a concentration of 2.5 $x10^6$ cells/ml for 6 hours before being transferred into RPMI plus 20% FCS at a concentration of $1x10^6$ cells/ml. For cells treated with IL-7, every time the media was changed or added IL-7 (10 ng/ml) was included.

2.14.2 Transfection of Human CD8 T cells with c-Myb-Encoding Plasmid

The pEF1neo-3FLAG-hcM-HA plasmid encoding the full-length human c-Myb gene under the control of the human EEF1A1 promoter (203) was a kind gift from Dr. Thomas Sæther, Oslo, Norway. To achieve high transfection efficiency, primary human CD8 T cells were activated and then transfected with the indicated plasmids. Primary human CD8 T cells were isolated as described above and cultured overnight in RPMI-20. The next day cells were treated with anti-CD3/anti-CD28 antibodies conjugated to magnetic beads using the T cell activation/expansion kit from Miltenyi Biotech. After 24 hours stimulation, the beads were removed using magnetic separation and CD8 T cells were transfected by nucleofection as described above. As a negative control, cells were transfected with the empty vector pcDNA3.1$^{(-)}$ plasmid containing a similar backbone to the c-Myb plasmid. Transfected CD8 T cells were then incubated in fresh pre-warmed RPMI-20 and levels of CD127 transcripts and c-Myb proteins were measured at the indicated time points by qPCR and Western respectively.

2.14.3 Transfection of Jurkat cells with the CD127 Promoter Constructs

Jurkat cells were seeded at a concentration of 0.9 million cells/ml and were cultured overnight in RPMI-1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 0.2 M L-glutamine and 10% FCS. The next day cells were transfected with the CD127 promoter constructs in table 2 by nucleofection as described above at $2.5x10^6$ cells per condition using the X-005 program. Post-nucleofection, cells were allowed to recover at a concentration of 2.5 $x10^6$ cells/ml for 4 hours in serum-free RPMI-1640 before being transferred into RPMI-10 at a concentration of $1x10^6$ cells/ml. CD127-promoter activity was measured after 24 hours using the by luciferase assay described below.

2.15 Luciferase Assay

To measure the luciferase expression of the different promoter constructs in primary human CD8 T cells as well as in Jurkat T cells, the Dual-Luciferase Assay Kit was used (Promega, Madison, WI, USA). Briefly, cells were collected at the indicated time points by centrifugation (6000 g for 10 minutes). Whole cell lysis was carried out in 200 μl of 1X Passive Lysis Buffer, diluted from a 5X stock (Promega). The tubes were thoroughly mixed and were incubated for 20 minutes with agitation at room temperature. To get rid of debris, tubes were centrifugated (6000 g for 10 minutes) and supernatents were transferred to new tubes. The luciferase assay was carried out according to Dual-Luciferase® Reporter Assay System Protocol from promega. Two substrates were added sequentially to measure the activities of firefly (CD127 promoter constructs) and Renilla (control plasmid to account for transfection efficiencies). The activity of firefly was measured by adding the substrate 1 (Luciferase Assay Reagent II, LAR II) generating a luminescent signal that's detected at 560

nm. The reaction is then quenched by adding a 1X 'Stop & Glo' reagent, prepared from a 50X stock, generating another luminescent signal that's detected at 480 nm. Luminescent signals (in relative light units (RLU)) were detected by GloMax 20/20 Luminometer (Promega). The mean values of firefly luciferase RLU were divided by the renilla luciferase RLU to correct for transfection efficiency and the data were then represented as mean fold change in luciferase gene expression compared to control vector (pGL4B).

2.16 Data and Statistical Analysis

Statistical analyses were performed using Microsoft Excel and the GraphPad Prism v.5.0 software (Graph Pad Software). Two-tailed, paired student t-test with 95% confidence intervals was used and values with a p-value cutoff of less than or equal to 0.05 were considered significant.

3. Chapter 3[†] - IL-7 Down Regulates IL-7Rα Expression in Human CD8 T Cells by Two Independent Mechanisms

3.1 Rationale

Despite the central importance of IL-7 in the development, maintenance and function of T cells, the mechanisms regulating expression of the IL-7 receptor in response to IL-7 signaling have been only partially characterized (154, 167, 197, 198). Our lab and others have previously shown IL-7 down regulates expression of the CD127 receptor subunit on the surface of T cells (154, 166, 167, 196-199). Although some groups have reported transcriptional suppression of the CD127 gene in response to IL-7 (154, 197), others have found IL-7 does not affect CD127 mRNA levels in human T cells (198). Further, where IL-7 has been shown to suppress CD127 gene transcription it has not been established whether the loss of CD127 protein from the cell surface is the result of transcriptional down regulation and reduced protein synthesis or the result of a second mechanism acting at the cell membrane (154, 197). Indeed, it was recently reported IL-7 down regulates surface CD127 protein in the T-leukemia cell lines HPB-ALL and TAIL7 through receptor internalization (196). Whether this applies to primary human CD8 T cells has yet to be determined. In addition, the detailed kinetics and roles of PI3K and JAK as well as downstream phosphorylation of STAT proteins in regulating the amount of CD127 on the cell membrane or CD127 gene transcription or both have never been delineated.

In view of this I set out to clearly and systematically define the mechanisms by which IL-7 down regulates CD127 expression on the surface of resting human CD8 T cells and to

[†] A version of this chapter has been published (204). Ghazawi *et al*. IL-7 Down Regulates IL-7Rα Expression in Human CD8 T Cells by Two Independent Mechanisms. Immunology and Cell Biology. **2013**. 91: 149-158.

determine to what extent the early down regulation of surface CD127 protein is dependent on transcriptional suppression. In this chapter I illustrate that IL-7 suppresses expression of its own receptor in primary human CD8 T-cells by two independent mechanisms, one transcriptional and one at the level of surface protein. Through these pathways IL-7 provides negative feed-back on its own signaling cascades allowing for fine-tuned immune responses in human CD8 T cells in different microenvironments and in response to different immunological challenges.

3.2 Results

3.2.1 IL-7 down regulates CD127 mRNA transcripts and surface protein in a time- and dose-dependent manner.

I first set out to elucidate the kinetics of IL-7-mediated suppression of CD127 surface protein and mRNA transcripts in primary human CD8 T cells. To do this, purified CD8 T cells were incubated with increasing concentrations of IL-7 (100-10,000 pg/ml) and levels of CD127 mRNA transcripts were measured by qPCR and compared to untreated cells normalizing to RPS18 expression. Surface CD127 protein expression was monitored by flow cytometry. As shown in figure 7A, IL-7 suppresses the level of CD127 mRNA transcripts in CD8 T cells in a dose dependent manner. At least 100 pg/ml IL-7 was required to see a decrease in the level of CD127 mRNA (34±4% reduction) with 10,000 pg/ml inducing a 78±8% decline in transcripts. Maximal suppression at all concentrations of IL-7 occurred at 3 hours. At lower concentrations of IL-7 (100-1000 pg/ml) the reduction in CD127 transcripts was transient with a dose response in time to recovery while 10,000 pg/ml IL-7 maintain transcriptional suppression for over 72 hours. Similarly, the effects of IL-7 on CD127 surface protein are also dose dependent both in the extent of the down regulation and in the duration of suppression (figure 7B). A transient (17±3%) decrease in surface CD127 protein could be detected on CD8 T cells with as little as 100 pg/ml of IL-7. This effect became more marked with increasing concentrations of IL-7, where 5000 pg/ml induced a 41±2% reduction in CD127 surface protein within 3 hours with a maximal suppression of 90±2% at 12 hours. Recovery of CD127 on the cell surface took progressively longer with increasing doses of IL-7 but concentrations of 5000-10,000 pg/ml maintained suppression for over 72 hours. Thus we show the effects of IL-7 on suppressing CD127 mRNA transcripts

Figure 7. IL-7 down regulates CD127 mRNA transcripts and surface protein in CD8 T cells in a time and dose-dependent manner. Purified CD8 T cells were incubated in media alone or in the presence of increasing concentrations of IL-7 (100-10,000 pg/ml) for up to 72 hours. (A) CD127 mRNA transcript levels were measured by qPCR normalizing to RPS18. (B) Surface CD127 protein expression was analyzed by flow cytometry. Values represent relative CD127 mRNA or surface protein expression (percent positive cells) compared to media controls. Error bars represent standard error of the mean (SEM) of four independent experiments. (C) Representative flow cytometry histograms from one individual in (B) showing CD127 expression on CD8 T cells incubated in media alone or with IL-7 (250 or 10,000 pg/ml) for 3-12 hours.

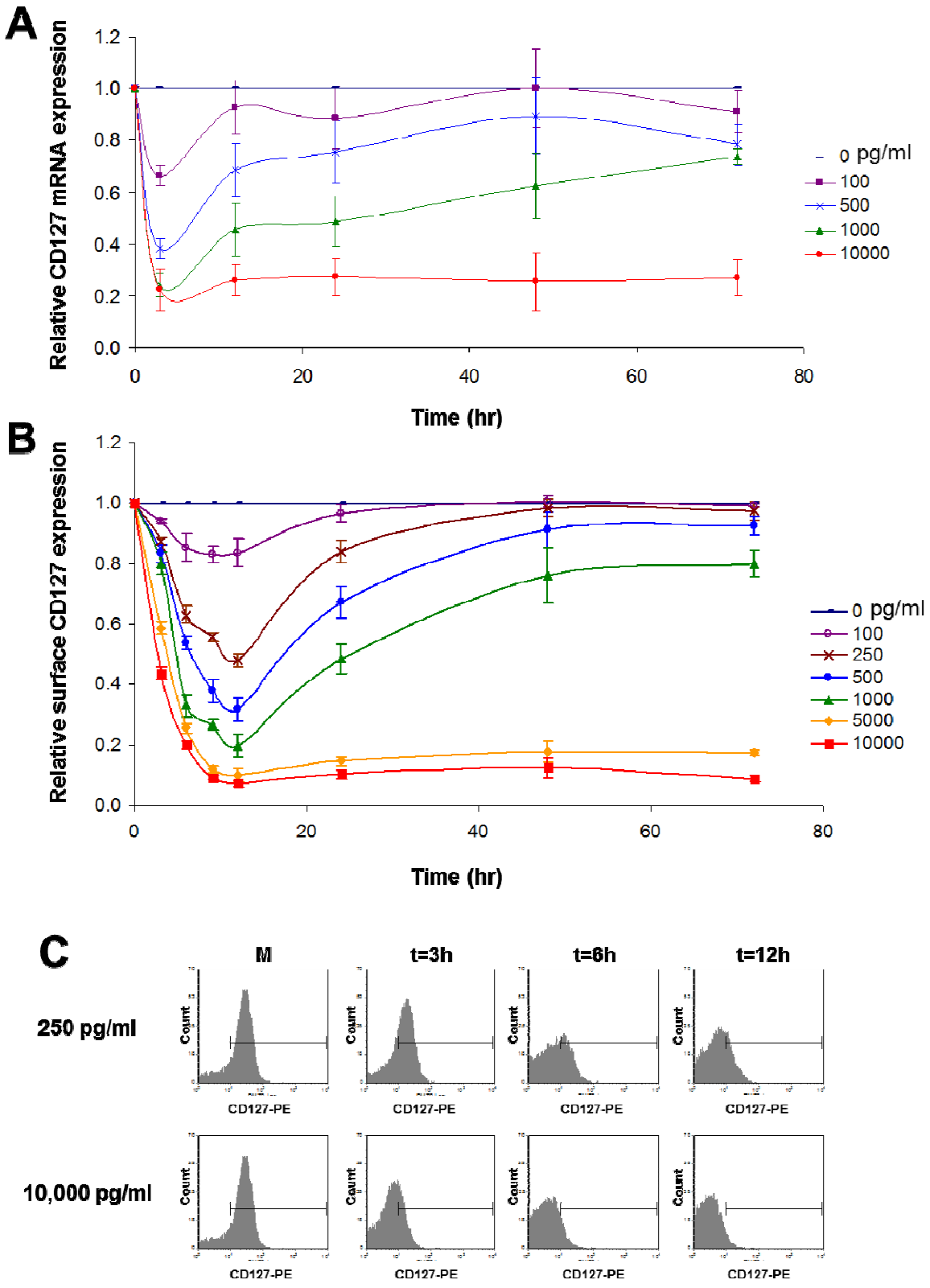

A
Relative CD127 mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
Time (hr)
0 pg/ml
100
500
1000
10000
B
Relative surface CD127 expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
Time (hr)
0 pg/ml
100
250
500
1000
5000
10000
C
M
t=3h
t=6h
t=12h
250 pg/ml
10,000 pg/ml
Count
CD127-PE

Figure 7.

and surface protein are both dose- and time-dependent with transient reductions in expression at lower IL-7 concentrations (100-1000 pg/ml) and sustained suppression at higher concentrations (10,000 pg/ml). The maximal reductions in CD127 mRNA and surface protein occur at 3 hours and 12 hours respectively at all concentrations of IL-7.

3.2.2 IL-7 down regulates CD127 protein at the cell surface independent of transcriptional suppression.

As shown in figure 7, the maximal effect of IL-7 on surface CD127 protein expression was observed several hours following maximal transcriptional suppression. This could suggest loss of CD127 protein from the cell membrane is entirely dependent on transcriptional suppression of the CD127 gene and natural turnover of surface protein. We felt this was unlikely, however, since IL-7 induced a maximal reduction in surface CD127 protein within 12 hours while the natural half-life of CD127 at the cell surface is approximately 55 hours (205). To determine to what extent the down regulation of surface CD127 protein is dependent on transcriptional suppression we treated CD8 T cells with IL-7 (10 ng/ml) and measured CD127 mRNA transcripts and surface protein at 20 minute intervals by qPCR and flow cytometry respectively. Interestingly, the down regulation of CD127 protein at the cell surface occurred within 20 minutes following addition of IL-7 and leveled off at 54±4% pre-treatment levels over the next 2 hours (figure 8A). In contrast, IL-7 induced suppression of CD127 mRNA transcripts was delayed by 40-60 minutes post-treatment (figure 8B). These data then demonstrate the initial down regulation of CD127 protein at the cell surface actually precedes and is therefore likely independent of transcriptional suppression.

Figure 8. IL-7 induces the down regulation of surface CD127 protein within 20 minutes while suppression of CD127 mRNA is delayed by 40-60 minutes. Purified CD8 T cells were incubated in media alone or in the presence of IL-7 (10 ng/ml) and analyzed at 20 minute intervals. (A) Surface CD127 expression was measured by flow cytometry. (* $p<0.05$ compared to media). (B) CD127 mRNA transcript levels were measured by qPCR normalizing to RPS18 (* $p<0.05$ compared to media). Values represent relative CD127 mRNA or surface protein (mean fluorescence intensities) compared to media controls. Error bars represent SEM of four independent experiments.

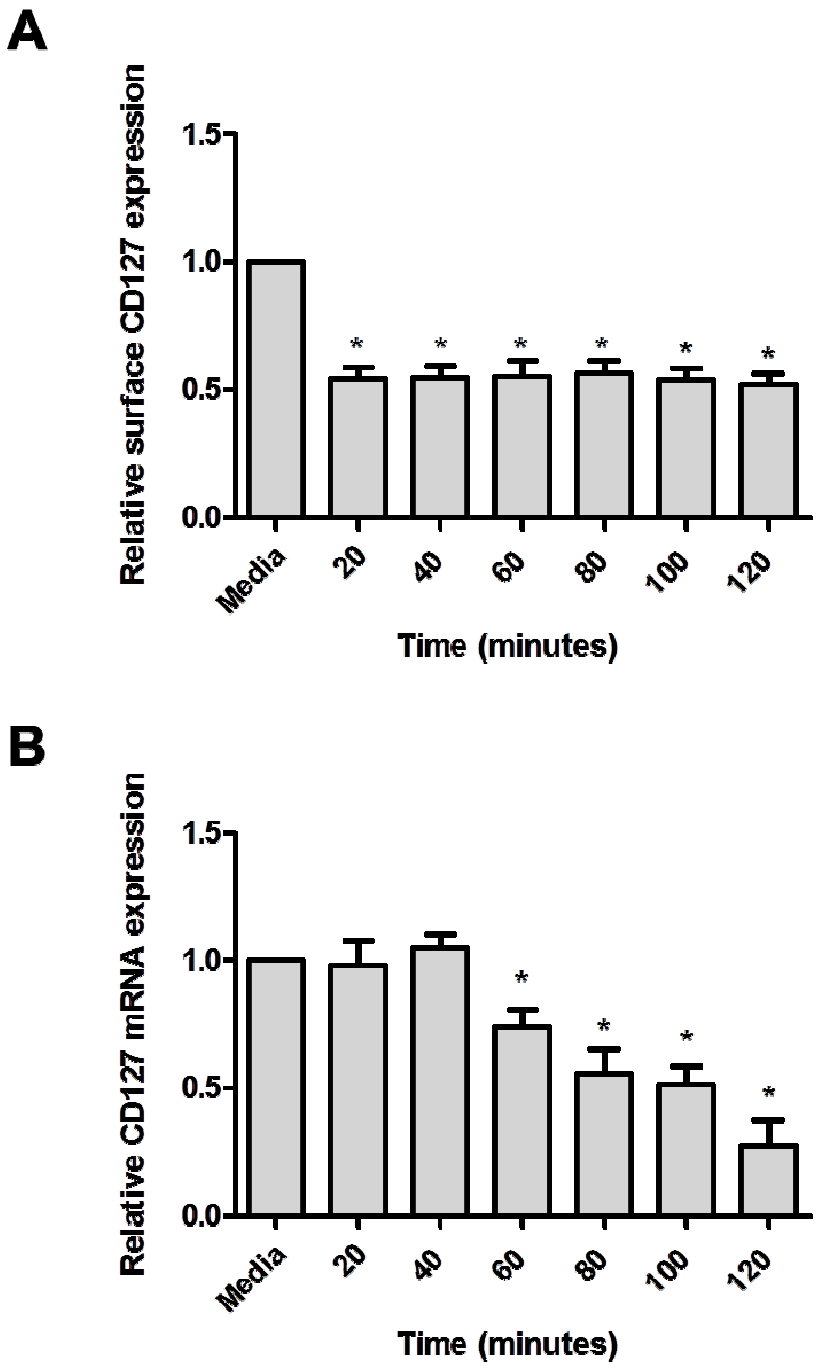

A
Relative surface CD127 expression
1.5
1.0
0.5
0.0
*
*
*
*
*
*
Media
20
40
60
80
100
120
Time (minutes)
B
Relative CD127 mRNA expression
1.5
1.0
0.5
0.0
*
*
*
*
Media
20
40
60
80
100
120
Time (minutes)

Figure 8.

To further distinguish down regulation of surface CD127 protein from transcriptional suppression we treated CD8 T cells simultaneously with IL-7 and Dexamethasone (Dex), a glucocorticoid previously shown to up regulate CD127 transcripts in murine B and T cells.(206, 207) We reasoned if IL-7 is able to down regulate CD127 surface protein independent of transcriptional suppression we would see this reduction at the cell surface even when levels of CD127 transcripts are augmented by the simultaneous addition of Dex. As expected (Figure 9A), treatment with Dex alone (20 μM) increased CD127 mRNA transcript levels in human CD8 T-cells some 2.6-fold within 1 hour while IL-7 alone (10 ng/ml) reduced CD127 transcripts by 64±5% at 4 hours reaching 80±4% suppression at 24 hours. In contrast, the combination of IL-7 plus Dex together maintained CD127 mRNA transcripts at levels comparable to that in untreated cells. Interestingly when added in combination with IL-7, Dex appeared to induce an initial increase in CD127 transcripts within 1 hour presumably due to the rapid effect of Dex binding to the glucocorticoid receptor in the cytoplasm and its translocation to the cell nucleus. This was followed by an apparent decline in CD127 transcripts by 4 hours likely due to the delayed suppressive effects of IL-7. Although these variations were consistent the changes were not statistically significant compared to cells maintained in medium alone and ultimately CD127 mRNA leveled off to pre-treatment levels at 24 hours. Despite the initial increase and overall lack of change in the level of CD127 transcripts, IL-7 still down regulated CD127 protein at the cell surface by 55±8% in the presence of Dex (figure 9B). These data then confirm IL-7 down regulates surface CD127 protein independent of transcription. Notably, at 24 hours the level of surface CD127 protein was significantly higher in the presence of Dex plus IL-7 over IL-7 alone. This is most likely due to synthesis and replenishment of new CD127 protein in the

Figure 9. IL-7 down regulates the expression of surface CD127 protein even when CD127 mRNA levels are augmented by Dexamethasone. Purified CD8 T cells were incubated in media alone or in the presence of IL-7 (10 ng/ml), Dexamethasone (Dex; 20 μM), or IL-7 plus Dex for 1, 4 and 24 hours. (A) CD127 mRNA transcript levels were measured by qPCR normalizing to RPS18. (B) Surface CD127 expression was analyzed by flow cytometry (* $p<0.05$ and # $p>0.05$ compared to media). Values represent relative CD127 mRNA or surface protein expression (percent positive cells) compared to media control. Error bars represent SEM of four independent experiments.

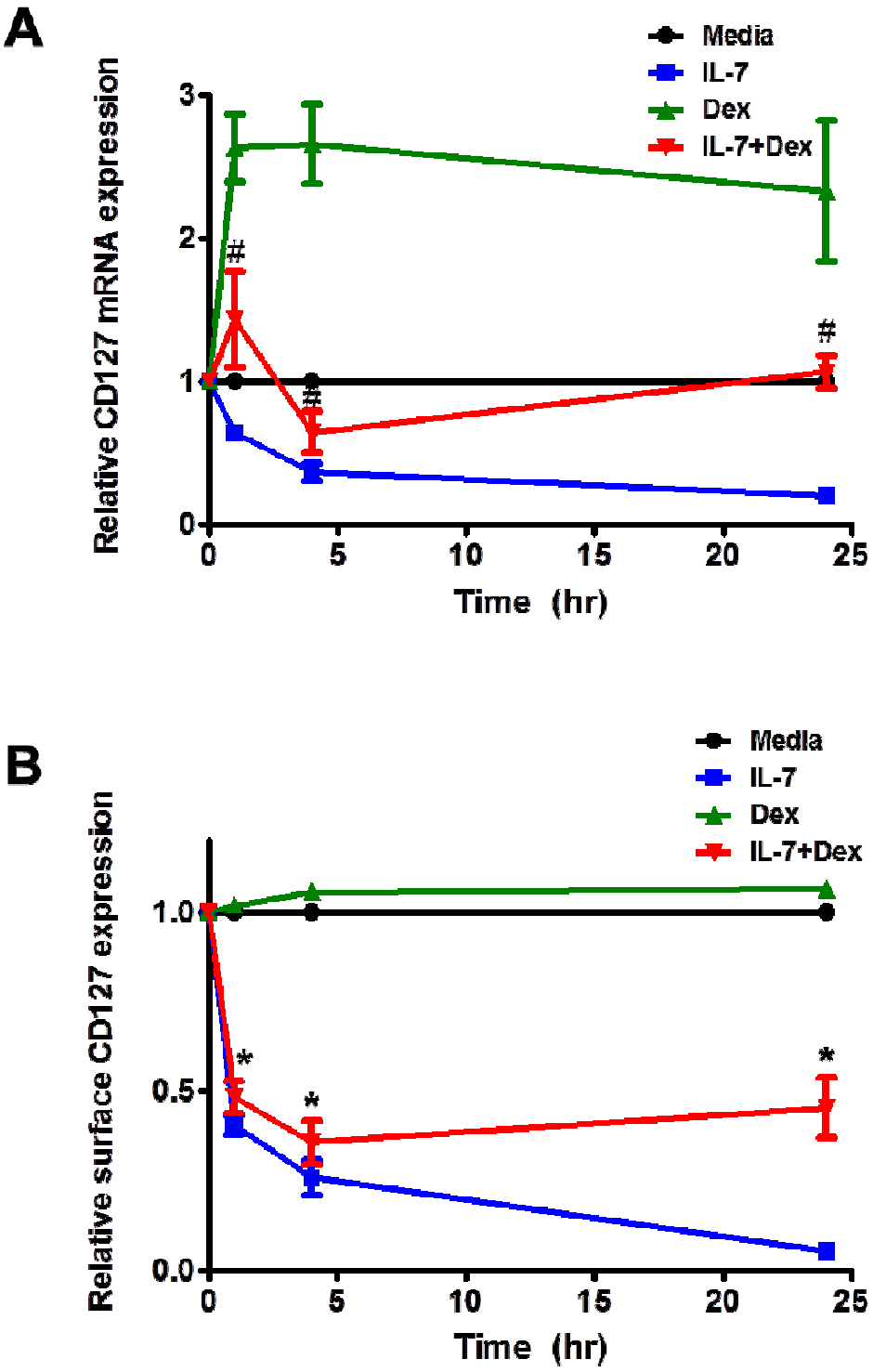

A
Media
IL-7
Dex
IL-7+Dex
Relative CD127 mRNA expression
3
2
1
0
#
#
#
0
5
10
15
20
25
Time (hr)
B
Media
IL-7
Dex
IL-7+Dex
Relative surface CD127 expression
1.0
0.5
0.0
*
*
*
0
5
10
15
20
25
Time (hr)

Figure 9.

presence of normal transcript levels and highlights the contribution of IL-7 induced transcriptional suppression in further limiting CD127 surface expression at later time points.

Finally, to fully separate IL-7 induced down regulation of CD127 protein at the cell surface from transcriptional suppression, we utilized a Jurkat-CD127 cell line engineered to expresses the CD127 cDNA from the CMV promoter (197). Since IL-7 is not known to attenuate transcription from the CMV promoter, we expected CD127 transcripts would remain unchanged in the presence of IL-7 while surface CD127 protein would be down regulated. As shown in figure 10, this is exactly what we found. While treatment with IL-7 did not suppress CD127 mRNA transcripts in the Jurkat-CD127 cell line (figure 10A) over 6 hours, IL-7 did significantly down regulate CD127 protein at the cell surface (figure 10B). This down regulation of surface CD127 protein in the absence of changes in CD127 mRNA levels confirms IL-7 induces the loss of CD127 protein from the cell surface independent of mRNA suppression.

3.2.3 IL-7-mediated down regulation of CD127 mRNA transcripts is dependent on JAK/pSTAT5.

JAK/STAT and PI3K are considered the major IL-7-mediated signaling pathways in T cells (35, 72) and we therefore set out to determine which if either of these pathways is involved in IL-7 induced down regulation of CD127 transcripts and surface protein. The role of IL-7 in activating JAK3/STAT5 signaling is well established (110, 166, 167, 197) and we confirm here IL-7 induces phosphorylation of STAT5 in primary human CD8 T cells (figure 11A). Phosphorylation of STAT3 in response to other γ chain cytokines such as IL-2 (208), IL-4 (208), IL-9 (209), IL-15 (208), and IL-21(210) has been previously reported but to our knowledge induction of STAT3 phosphorylation by IL-7 in human CD8 T cells has not been

Figure 10. IL-7 down regulates the expression of surface CD127 protein in the Jurkat-CD127 cell line without affecting CD127 mRNA levels. Jurkat-CD127 cells were incubated in media alone or with IL-7 (10 ng/ml) for 1, 3 and 6 hours. (A) CD127 mRNA transcript levels were measured by qRT-PCR normalizing to RPS18 ($p=0.126$ at 1 hour, 0.821 at 3 hours and 0.328 at 6 hours relative to media). (B) Surface CD127 expression was analyzed by flow cytometry (* $p<0.006$ percent positive cells compared to media). Data are represented as relative changes compared to media controls. Error bars represent SEM of three independent experiments.

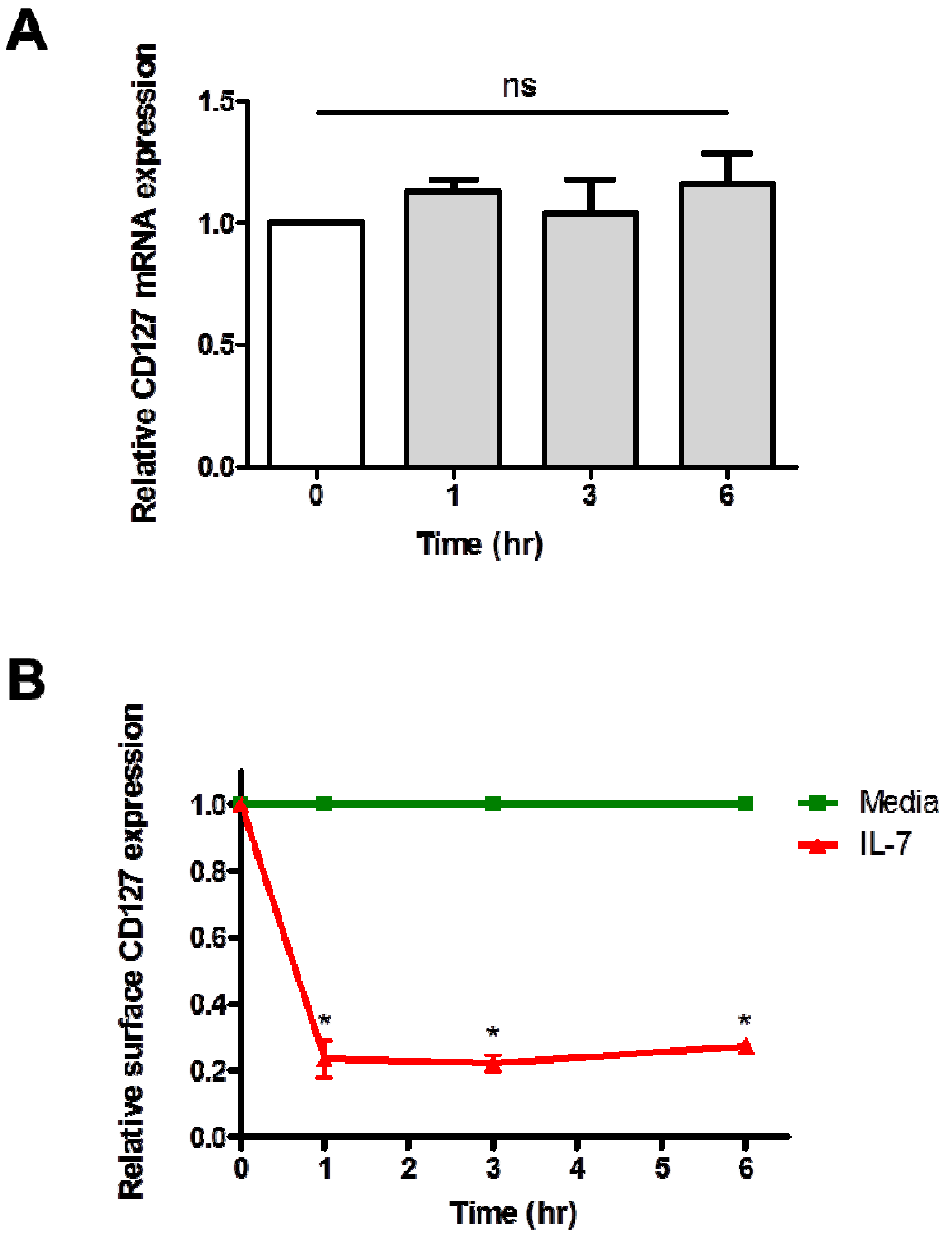

A
ns
Relative CD127 mRNA expression
1.5
1.0
0.5
0.0
0
1
3
6
Time (hr)
B
Relative surface CD127 expression
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
Time (hr)
Media
IL-7
*
*
*

Figure 10.

investigated. To examine this, we treated CD8 T cells with IL-7 (10 ng/ml) or IL-10 (10 ng/ml) as a positive control (211-213) and measured intracellular phospho-STAT3 by flow cytometry. As shown in figure 11B, IL-10 induces STAT3 phosphorylation whereas IL-7 does not. Finally, we used a synthetic STAT5 inhibitor which specifically targets the SH2 domain of STAT5 to block its phosphorylation in the presence of IL-7. Figure 11C shows a dose titration and complete block of STAT5 phosphorylation in the presence of IL-7 plus 500 μM inhibitor.

To examine the role of the JAK/STAT5 and PI3K signaling pathways in IL-7 induced down regulation of CD127 transcripts, we pre-treated CD8 T cells with inhibitors of either JAK or PI3K for 1 hour followed by IL-7 (10 ng/ml) for 80 minutes. The PI3K inhibitor (LY294002; 10 μM) successfully blocked phosphorylation of Akt following IL-7 stimulation in primary human CD8 T-cells while the JAK inhibitor (1.5 μM) blocked IL-7-induced STAT5 phosphorylation (appendix figure 1). Further, the effects of the inhibitors' solvent (DMSO) on CD127 transcripts or surface expression as well as on IL-7's repressive effects are negligible (appendix figure 2). As shown in figure 12A, pre-incubating cells with JAK inhibitor completely blocked IL-7 mediated down regulation of CD127 transcripts whereas inhibition of PI3K signaling had no effect. These results clearly indicate a requirement for JAK activity but not PI3K in the down regulation of CD127 mRNA by IL-7. We next questioned whether STAT5 phosphorylation played a role in the suppression of CD127 transcripts by IL-7. To investigate this, CD8 T cells were pre-incubated for 1 hour with 500 μM STAT5 inhibitor followed by IL-7 (10 ng/ml) for 60 minutes and levels of CD127 mRNA transcripts were measured by qPCR. As shown in figure 12B, inhibition of STAT5

Figure 11. IL-7 induces the phosphorylation of STAT5 but not STAT3 in human CD8 T cells. Purified CD8 T cells were maintained in media alone or treated with IL-7 (10 ng/ml) or IL-10 (10 ng/ml) for 45 minutes and then fixed and permeabilized and stained with FITC-labelled anti-phospho-STAT3 or anti-phospho-STAT5 antibodies. (A) Representative flow cytometry histogram showing phospho-STAT5 in untreated cells (gray fill) and cells stimulated with IL-7 (black line). (B) Representative flow cytometry histogram showing phospho-STAT3 in untreated cells (gray fill), and cells stimulated with IL-7 (black line) or IL-10 (dashed line). (C) Purified CD8 T cells were maintained in media alone, treated with IL-7 (10 ng/ml) for 8 hours, or pre-incubated with increasing concentrations of STAT5 inhibitor (100-500 μM) for 1 hour and then treated with IL-7 (10 ng/ml) for additional 8 hours. Cells were then fixed, permeabilized and stained with FITC-labelled anti-phospho-STAT5 antibodies and analyzed by flow cytometry. Data show percent positive cells for each condition (* $p<0.03$ and # $p=0.49$ compared to media). Error bars represent SEM of four independent experiments.

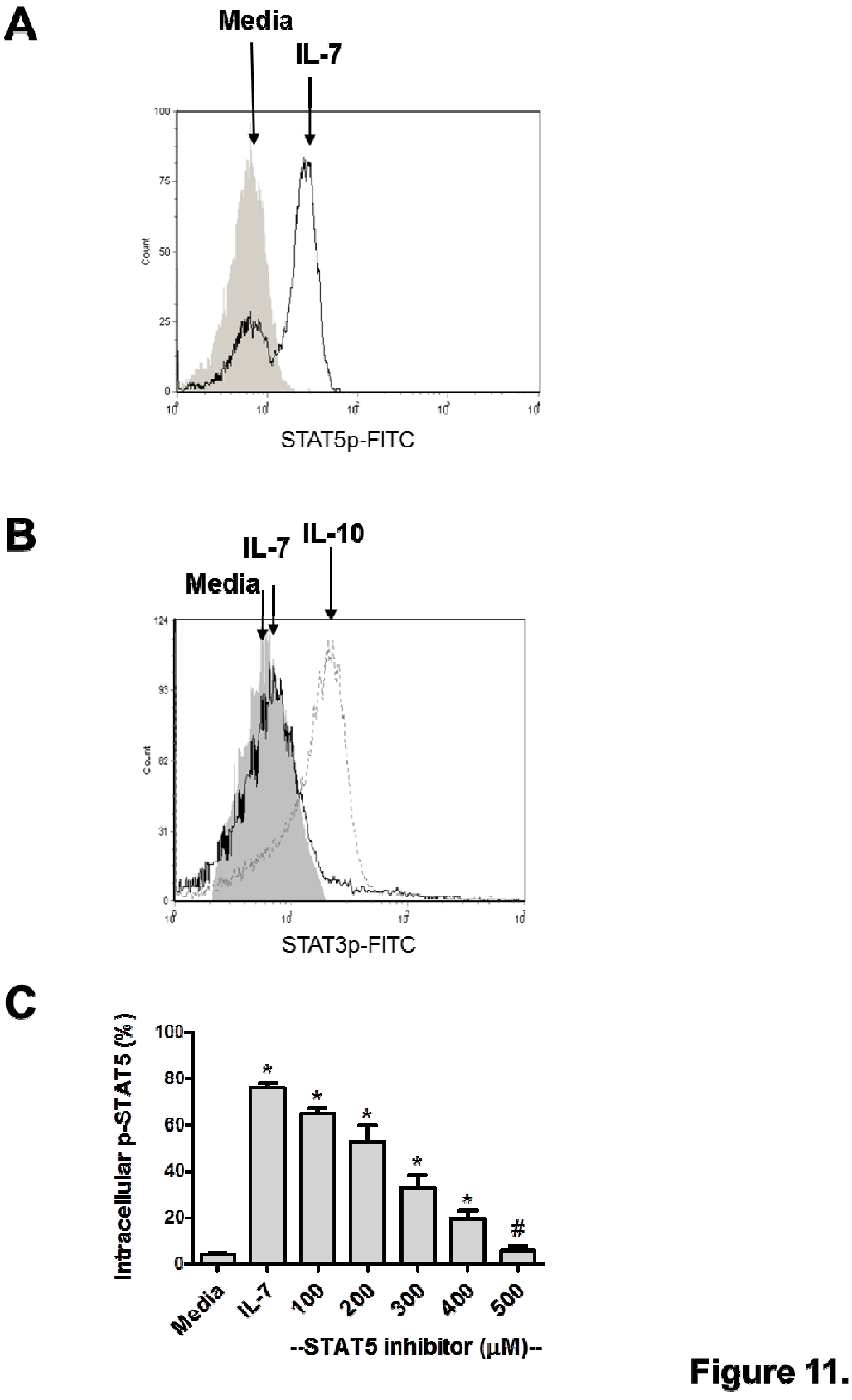

A
Media
IL-7
Count
STAT5p-FITC
B
IL-7
IL-10
Media
Count
STAT3p-FITC
C
Intracellular p-STAT5 (%)
Media
IL-7
100
200
300
400
500
--STAT5 inhibitor (μM)--

Figure 11.

Figure 12. IL-7 mediated suppression of CD127 transcripts is dependent on JAK activity and STAT5 phosphorylation but not on PI3K. Purified CD8 T cells were maintained in media alone or pre-incubated with inhibitors of JAK (1.5 μM), PI3K (10 μM) or STAT5 phosphorylation (500μM) for 1 hour followed by treatment with IL-7 (10 ng/ml) for 80 minutes in the case of the JAK and PI3K inhibitors (A) and for 60 minutes in the case of the STAT5 inhibitor (B). CD127 transcript levels were measured by qPCR normalizing to RPS18. Data are represented as relative changes compared to media control. (A) *$p<0.005$ relative to media. (B) $p=0.02$ IL-7 compared to media, $p=0.37$ STAT5i+IL-7 compared to STAT5i, $p=0.01$ STAT5i+IL-7 compared to IL-7. Error bars represent SEM of four independent experiments.

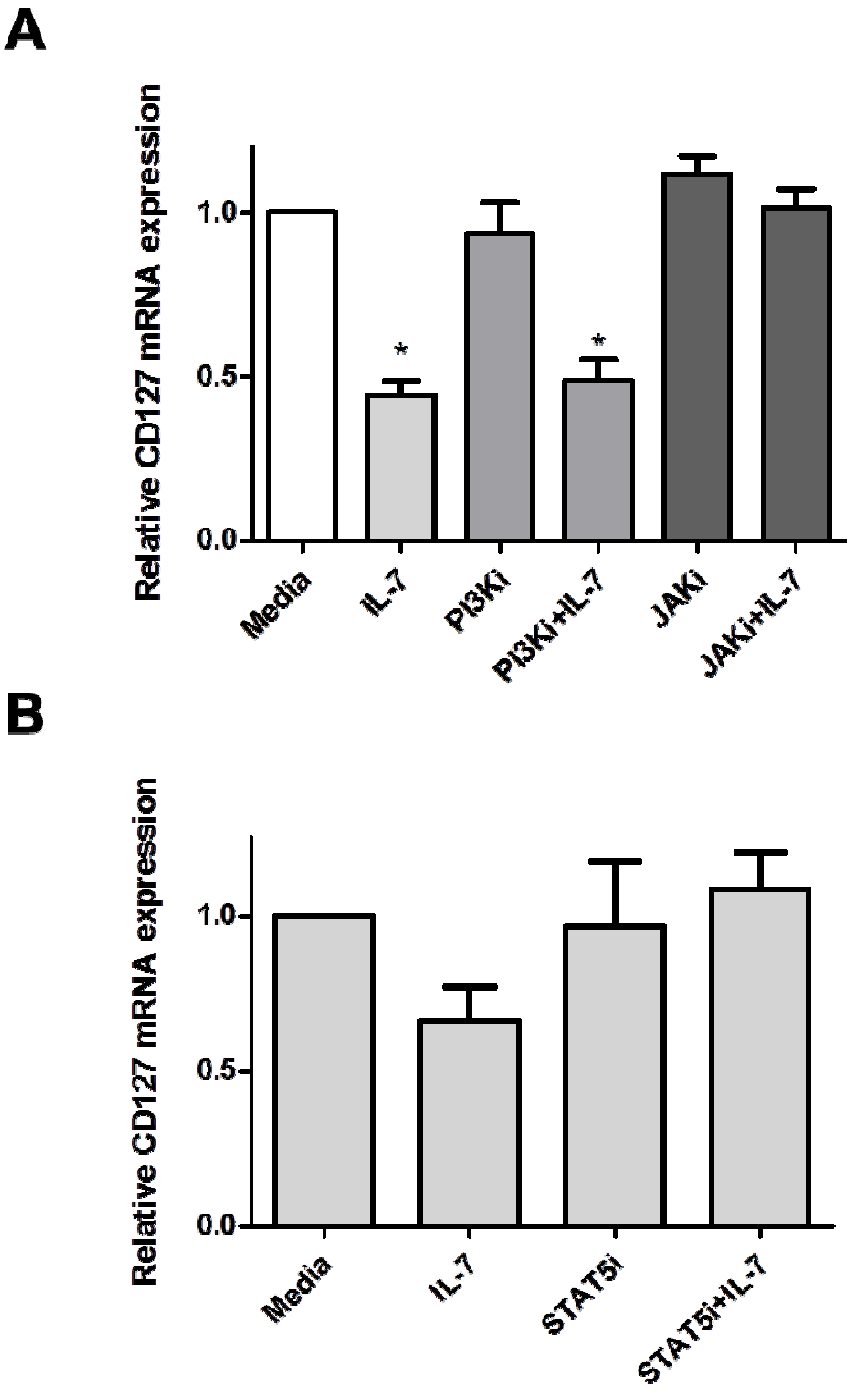

A
Relative CD127 mRNA expression
1.0
0.5
0.0
*
*
Media
IL-7
PI3Ki
PI3Ki+IL-7
JAKi
JAKi+IL-7
B
Relative CD127 mRNA expression
1.0
0.5
0.0
Media
IL-7
STAT5i
STAT5i+IL-7

Figure 12.

phosphorylation blocked IL-7's effect confirming transcriptional suppression of CD127 by IL-7 is dependent on the JAK/STAT5 signaling pathway.

3.2.4 Although IL-7-mediated down regulation of CD127 protein at the cell surface occurs independently of JAK/STAT5 and PI3K, IL-7 induced degradation of CD127 is JAK dependent.

To examine the requirement of the JAK/STAT5 and PI3K signaling pathways in IL-7 stimulated down regulation of CD127 protein at the cell surface, CD8 T cells were we pre-treated with inhibitors of either JAK, PI3K or STAT5 for 1 hour followed by IL-7 (10 ng/ml) for 80 minutes in the case of JAK and PI3K inhibitors or for 60 minutes in the case of the STAT5 inhibitor. Levels of surface CD127 protein were then measured by flow cytometry. As shown in figures 13A and 13B, inhibition of JAK, PI3K, and STAT5 phosphorylation had no effect on IL-7 mediated removal of CD127 from the cell surface. In all cases, IL-7 stimulated a reduction in surface CD127 protein equally in the presence and absence of inhibitors. As expected, IL-7 induced the phosphorylation of tyrosine 449 (Y449) in the cytoplasmic tail of CD127 within 15 minutes and inhibition of JAK prevented this from occurring (figure 13C). Taken together these data indicate phosphorylation of Y449 by JAK is not required to mark CD127 for removal from the cell membrane. Interestingly, inhibition of JAK did prevent subsequent degradation of CD127 protein. When examined by Western blot of whole cell extracts, CD127 was markedly reduced in CD8 T cells treated with IL-7 for 6 hours but in the presence of JAK inhibitor CD127 remained at levels equivalent to media control following IL-7 stimulation (figure 13D). Thus, while IL-7 mediated removal of CD127 from the cell surface is independent of JAK, JAK activity is required to target CD127 for degradation likely through phosphorylation of Y449. Finally, when CD8 T cells

Figure 13. While IL-7 induced down regulation of CD127 protein at the cell surface is independent of JAK activity and phosphorylation of tyrosine 449, subsequent degradation of CD127 is dependent on JAK. Purified CD8 T cells were pre-incubated with inhibitors of JAK, PI3K (A) or STAT5 phosphorylation (B) followed by IL-7 (10 ng/ml) as described in figure 12 and surface CD127 expression was analyzed by flow cytometry. Data are represented as relative changes compared to media control (* $p<0.05$ compared to media). Error bars represent SEM of at least four independent experiments. (C) Western blot showing phosphorylation of tyrosine 449 on the cytoplasmic tail of CD127 is induced by IL-7 and requires JAK. Purified CD8 T cells were maintained in media alone, treated with IL-7 (10 ng/ml) for 15 minutes, JAK inhibitor (1.5 μM) for 1 hour and 15 minutes, or JAK inhibitor for 1 hour followed by the addition of IL-7 (10 ng/ml) for 15 minutes. Whole cell lysates were then analyzed by Western blot probing for phospho-Y449 using a polyclonal rabbit anti-human CD127 phospho-Tyr449 antibody or probed for total CD127 as control using a polyclonal goat anti-human CD127 antibody. (D) Western blot showing IL-7 induced degradation of CD127 is JAK dependent. Purified CD8 T cells were maintained in media, treated with IL-7 (10 ng/ml) for 6 hours, JAK inhibitor (1.5 μM) for 6 hours, or JAK inhibitor for 1 hour followed by the addition of IL-7 (10 ng/ml) for 6 hours. Whole cell lysates were then analyzed by Western blot probing for total CD127 with a polyclonal goat anti-human CD127 antibody. Blots were then stripped and re-probed for β-actin as loading control. (E) CD127 re-accumulates on the cell surface in the presence of IL-7 and JAK inhibitor. Purified CD8 T cells were maintained in media, pre-treated with JAK inhibitor (1.5 μM) for 1 hour followed by the addition of IL-7 (10 ng/ml), or treated with IL-7 alone and CD127 surface expression was monitored by flow cytometry at the times indicated. Data are represented as relative changes compared to media controls. Error bars represent SEM of four independent experiments.

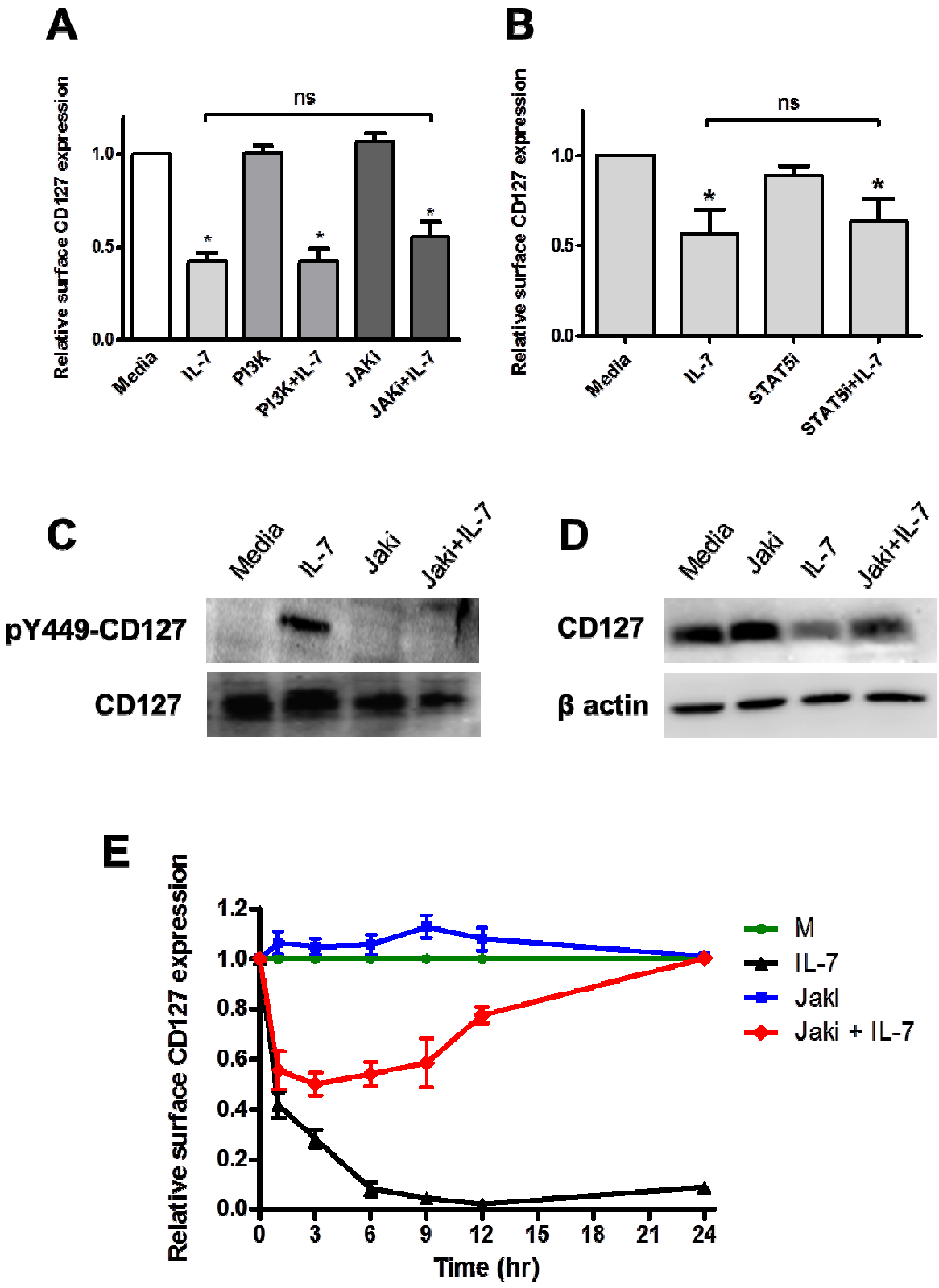

A
Relative surface CD127 expression
ns
1.0
0.5
0.0
Media
IL-7
PI3K
PI3K+IL-7
JAKi
JAKi+IL-7
B
Relative surface CD127 expression
ns
1.0
0.5
0.0
Media
IL-7
STAT5i
STAT5i+IL-7
C
Media
IL-7
Jaki
Jaki+IL-7
pY449-CD127
CD127
D
Media
Jaki
IL-7
Jaki+IL-7
CD127
β actin
E
Relative surface CD127 expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
3
6
9
12
15
18
21
24
Time (hr)
M
IL-7
Jaki
Jaki + IL-7

Figure 13.

were stimulated with IL-7 (10 ng/ml) in the presence of JAK inhibitor, CD127 protein was rapidly removed from the cell surface but then recovered over several hours (figure 13E). While we cannot entirely exclude recovery due to the absence of transcriptional suppression, this is not the pattern seen when CD8 T cells were treated with IL-7 plus Dex and CD127 transcripts were maintained at pretreatment levels. Here, inhibition of JAK allowed re-accumulation of CD127 on the cell membrane. These data taken together suggest IL-7 binding to CD127 directly triggers receptor internalization perhaps by inducing a conformational shift in the protein or presumably by dimerization with CD132. Once in the early endosome, CD127 may then be sorted such that CD127 phosphorylated at Y449 by JAK is targeted for degradation while unphosphorylated CD127 is recycled back to the cell surface.

3.3 Discussion

While IL-7 signaling plays pivotal roles in the development and function of T cells, the mechanisms regulating expression of the IL-7 receptor have only been partially characterized. Although several studies have already reported IL-7 down regulates the expression of CD127 mRNA transcripts and surface protein in human and murine T cells (154, 197), conflicting data have also been provided suggesting IL-7 does not affect CD127 gene transcription (198). Whether these discordant results are due to the source of T cells used in each study (eg umbilical cord versus adult peripheral blood), culture conditions, or the single time point of analysis is unclear. Further it has not been determined whether IL-7 mediated down regulation of CD127 protein at the cell surface is the result of transcriptional suppression and reduced protein synthesis or due to a second pathway acting at the cell surface as has been reported for T-leukemic cell lines (196). Here, we address a number of these questions and delineate the detailed kinetics and separate contributions of IL-7 mediated suppression of CD127 gene transcription and the initial down regulation of CD127 surface protein in primary human CD8 T cells, and provide evidence with respect to the mechanisms and pathways by which these occur.

The effects of IL-7 on reducing CD127 transcripts and surface protein are dose dependent both in the extent of the down regulation and in the duration of suppression with maximal effects of IL-7 on CD127 mRNA and surface protein at 3 and 12 hours respectively. Low concentrations of IL-7 (100-1000 pg/ml) induced transient declines in CD127 mRNA and protein while higher concentrations (10,000 pg/ml) sustained suppression for over 72 hours. While this may be due to either a bona fide concentration effect or due to sustained signaling at higher IL-7 concentrations, either way this allows flexibility in

regulating CD127 expression in different microenvironments with different levels of IL-7. Whereas decreased transcription of the CD127 gene was viewed as the primary mechanism by which IL-7 down regulates surface CD127 protein (154, 167, 197, 214), we now show transcriptional suppression contributes only at later time points and serves to maintain low CD127 levels after the receptor has been removed from the cell membrane by IL-7. We demonstrate by several methods that IL-7 reduces CD127 transcripts and surface protein through separate pathways. In both the Jurkat-CD127 cell line and when added to primary human CD8 T cells in combination with dexamethasone, IL-7 removed CD127 protein from the cell surface in the absence of changes in CD127 mRNA levels. We also show IL-7 mediated down regulation of CD127 protein at the cell surface is rapid and occurs within 20 minutes before there is any reduction in CD127 transcripts. This results in an initial new equilibrium in protein turnover maintaining lower but relatively constant CD127 expression on the cell surface over the next 2 hours until CD127 transcriptional suppression kicks in to prevent protein replenishment and further down regulate CD127 expression. The contribution of transcriptional suppression in reducing CD127 expression at later time points is evident in CD8 T cells treated with IL-7 and dexamethasone where CD127 mRNA and surface protein are higher at 24 hours compared to cells treated with IL-7 alone. This effect is less evident in the Jurkat-CD127 cells because protein turn over on the surface of this cell line is much more rapid compared to resting primary cells (appendix figure 3). These data together suggest brief exposure to IL-7 may allow rapid transient decreases in surface CD127 protein while sustained exposure to IL-7 results in transcriptional suppression and further reductions in receptor expression over time. This would provide a finely-tuned mechanism by which CD8 T cells could respond to small changes in IL-7 concentrations in the microenvironment and to different immunological challenges.

Suppression of CD127 mRNA transcripts by IL-7 is dependent on the JAK/STAT5 signaling pathway. It is already established that IL-7 stimulation leads to phosphorylation of CD127 at Y449 by JAK3 (35), allowing docking via their SH2 domains and subsequent phosphorylation of STAT5 proteins (35, 55, 215). Phospho-STAT5 then dimerizes and translocates to the nucleus where it may either directly or indirectly suppress CD127 transcription. Given the delay in transcriptional suppression by 40-60 minutes following stimulation with IL-7, we hypothesize IL-7 signaling via STAT5 results in the up regulation of a transcriptional repressor or an epigenetic modifier protein that in turn suppresses CD127 gene expression. This will be examined in more detail in Chapter 5.

The IL-7 mediated down regulation of CD127 protein on the surface of human CD8 T cells is rapid and occurs within 20 minutes. Similarly, Swainson *et al* (167) reported surface CD127 protein is reduced on human CD4 T cells within 30 minutes of IL-7 stimulation (10 ng/ml) and Henriques *et al* (196) documented the rapid internalization of CD127 protein from the surface of the leukemic cell line HPB-ALL following addition of IL-7 (50 ng/ml). We show here that as expected IL-7 induces the phosphorylation of tyrosine 449 within the cytoplasmic tail of CD127 by JAK but that this phosphorylation is not required for the initial removal of CD127 protein from the cell membrane. Indeed, IL-7 still down regulates surface CD127 protein in the presence of JAK inhibitor. However, inhibition of JAK does prevent subsequent degradation of CD127 and allows the re-accumulation of receptor on the cell surface over 12-24 hours. Taken together the data suggest IL-7 binding to CD127 directly triggers receptor internalization perhaps through dimerization with CD132 or by inducing a conformational shift in the protein. Once in the early endosome, CD127 may then be sorted such that CD127 phosphorylated at Y449 by JAK is targeted for

degradation while unphosphorylated CD127 is recycled back to the cell surface. This pathway will be examined in more detail in the next chapter (Chapter 4).

4. Chapter 4. Roles of Suppressor of Cytokine Signaling (SOCS) Proteins in the IL-7 Mediated Degradation of the CD127 Protein in Human CD8 T Cells

4.1 Rationale

In the previous chapter (Chapter 3) I illustrated IL-7 down-regulates CD127 transcripts and surface protein in human CD8 T cells through two separate pathways. In resting human CD8 T cells, CD127 is continuously recycled on and off the cell membrane with basal receptor turnover and degradation by the lysosome (196, 205). In contrast, IL-7 binding to CD127 triggers rapid receptor internalization and phosphorylation at tyrosine 449 by JAK leading to subsequent receptor degradation. The mechanism by which internalized CD127 is targeted for degradation is investigated in this chapter.

Suppressors of cytokine signaling (SOCS) proteins, which consist of SOCS1-7 and the Cytokine-inducible SH2-containing (CIS) protein, are induced by JAK-STAT signaling. SOCS proteins are composed of an SH2 domain, a SOCS box and a variable amino terminal region, and they inhibit receptor signaling by several mechanisms (Figure 14) (216, 217). SOCS1 and SOCS3 bind directly via their SH2 domain to phosphotyrosine residues within the activation loop of JAK kinases and occlude the catalytic site with their kinase inhibitory region (KIR) acting as a pseudosubstrate blocking further phosphorylation of STAT molecules (65). On the other hand, CIS and SOCS2 proteins bind via their SH2 domains to phosphotyrosine residues on the cytoplasmic tail of the cytokine receptor preventing further interactions with STAT and displacing JAK kinase from the complex (65). SOCS proteins also bind directly to Elongin C via their SOCS box and thus recruit the multi-protein E3 ubiquitin-ligase complex, resulting in the ubiquitination and subsequent proteasomal

Figure 14. The SOCS family of proteins. Suppressor of cytokine signaling (SOCS) proteins consist of SOCS1-7 and the Cytokine-inducible SH2-containing (CIS) protein and are induced by JAK-STAT signaling. (A) SOCS proteins are composed of an SH2 domain, a SOCS box and a variable amino terminal region. (B) The SOCS family of proteins, although homologous, are evolutionarily distinct. (C) Crystal structure of SOCS2. (D) Illustration of the interactions of SOCS with target proteins and recruitment of E3 ubiquitin ligases, which ultimately target the complex to the proteasome for degradation. Figure was obtained with permission from Elsevier B.V. (Permission from the publishing group is depicted in the appendix) (218).

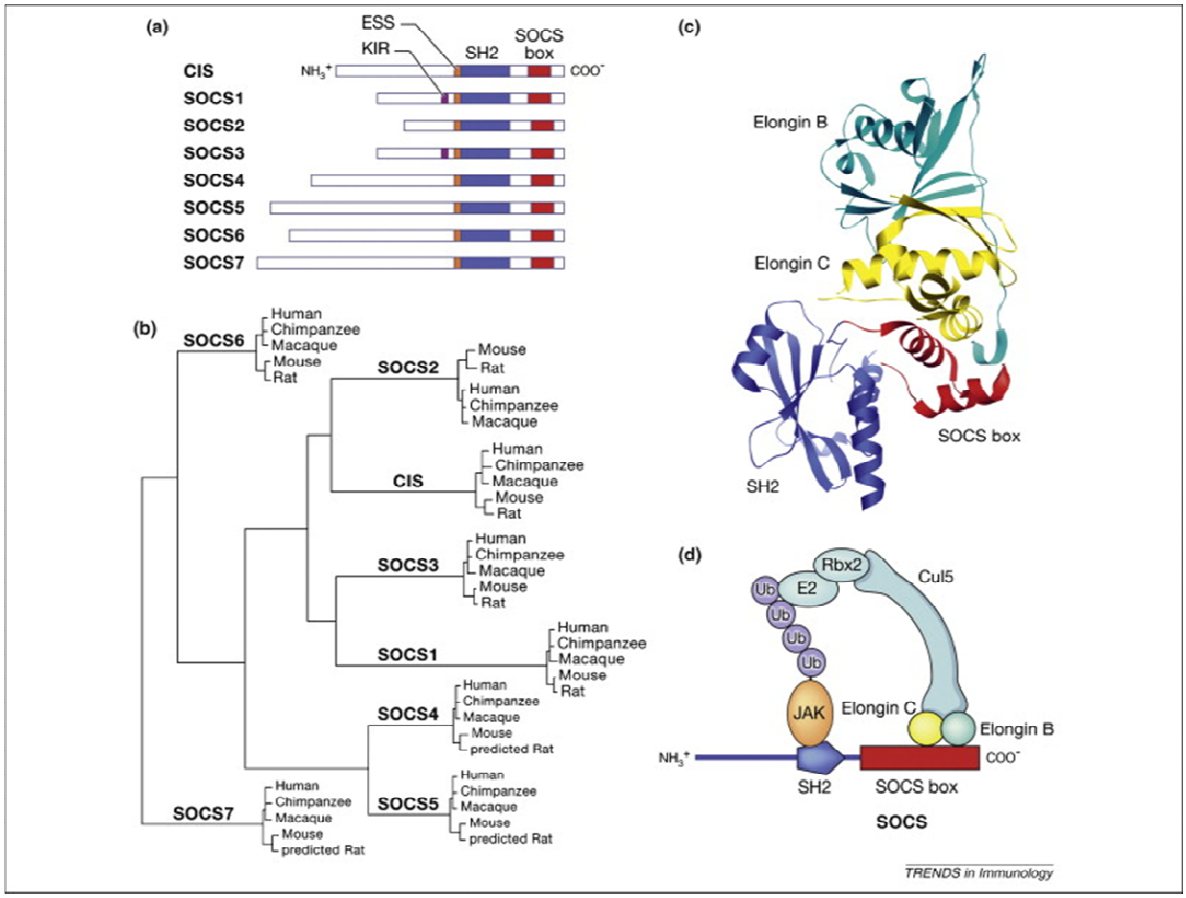

(a)
ESS
KIR
SH2
SOCS box
CIS
SOCS1
SOCS2
SOCS3
SOCS4
SOCS5
SOCS6
SOCS7
(b)
SOCS6
Human
Chimpanzee
Macaque
Mouse
Rat
SOCS2
Mouse
Rat
Human
Chimpanzee
Macaque
CIS
Human
Chimpanzee
Macaque
Mouse
Rat
SOCS3
Human
Chimpanzee
Macaque
Mouse
Rat
SOCS1
Human
Chimpanzee
Macaque
Mouse
Rat
SOCS4
Human
Chimpanzee
Macaque
Mouse
predicted Rat
SOCS5
Human
Chimpanzee
Macaque
Mouse
predicted Rat
SOCS7
Human
Chimpanzee
Macaque
Mouse
predicted Rat
(c)
Elongin B
Elongin C
SOCS box
SH2
(d)
Rbx2
Cul5
Ub
E2
Ub
Ub
Ub
JAK
Elongin C
Elongin B
SH2
SOCS box
SOCS
TRENDS in Immunology

Figure 14.

degradation of JAK kinase and the associated cytokine receptor (Figure 15) (65, 216-219). Factors that induce SOCS family proteins as well as phenotypes of mice with SOCS knockouts and the changes that occur upon ectopic overexpression of SOCS proteins in mice are listed in Table 4.

There is some evidence that IL-7 induces the expression of SOCS proteins in mice, specifically SOCS1 in murine thymocytes and peripheral T cells (220) and both SOCS1 and SOCS3 in the murine B62.1 IND B cell line (221). However, the induction of SOCS proteins by IL-7 and the role these proteins play in regulating IL-7 signaling have never been studied in humans. In this chapter I examine the expression of the eight members of the SOCS family of proteins in primary human CD8 T cells in response to IL-7 stimulation and show that IL-7 specifically induces the expression of CIS, SOCS1 and SOCS2. I further show that this induction occurs via the JAK/STAT-5 pathway and delineate the detailed kinetics of the IL-7 mediated induction of CIS, SOCS1 and SOCS2 transcripts and protein. Finally I demonstrate for the first time that CIS and SOCS2 interact directly with CD127 following stimulation with IL-7 and direct the receptor to the proteasome for degradation.

Figure 15. SOCS proteins negatively regulate JAK/STAT signaling via a number of mechanisms. Upon cytokine stimulation, JAK/STAT signaling induces the expression of one or more members of the SOCS family of protein. The SOCS proteins in turn negatively regulate JAK/STAT via a number of mechanisms. SOCS1 and SOCS3 bind directly to phosphotyrosine residues within the activation loop of JAK via their SH2 domain and occlude the catalytic site with thier kinase inhibitory region (KIR) acting as a pseudosubstrate blocking further phosphorylation of STAT. On the other hand, CIS and SOCS2 proteins bind via their SH2 domains to phosphotyrosine residues on the cytoplasmic tail of the cytokine receptor preventing further interactions with STAT and displacing JAK kinase from the complex. SOCS proteins also bind directly to Elongin C via their SOCS box and thus recruit the multi-protein E3 ubiquitin-ligase complex, resulting in the ubiquitination and subsequent proteasomal degradation of JAK kinase and the associated cytokine receptor. Figure was obtained with permission from the the American Society for Microbiology (Permission from the corresponding author as well as the publishing group are depicted in the appendix) (65).

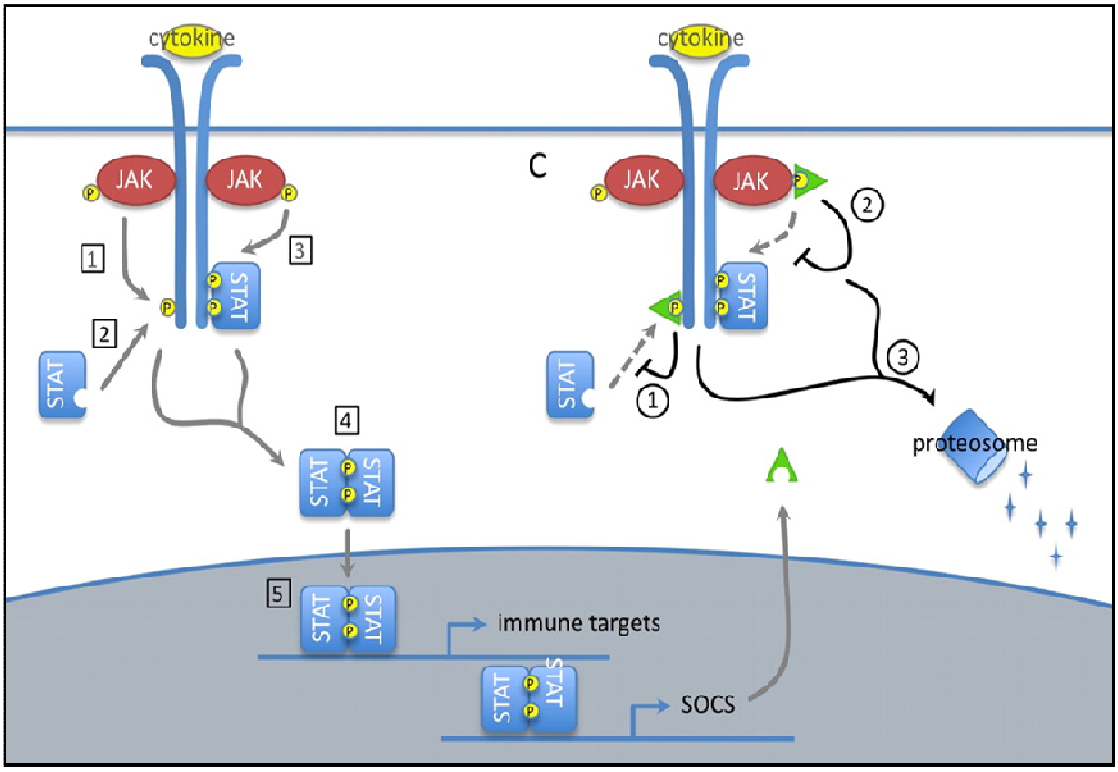

cytokine
JAK
JAK
STAT
1
2
3
4
5
STAT
STAT
STAT
STAT
immune targets
STAT
STAT
SOCS
C
cytokine
JAK
JAK
STAT
2
1
3
STAT
proteosome
A

Figure 15.

Table 4. A summary of known factors that induce the expression of SOCS proteins as well as the phenotypes of mice with knockouts of SOCS genes, and the reported changes that occur upon ectopic overexpression SOCS proteins in mice. Table was obtained with permission from Elsevier B.V. (Permission from the publishing group is depicted in the appendix) (218).

SOCS	Inducing factors	Knockout	Transgenic expression
CIS	IL-2, IL-3, IL-6, IL-9, IL-10, CNTF, EGF, EPO, GH, GM-CSF, Leptin, PRL, TPO, TSLP	No reported phenotype	↓ Lactation; ↓NK and ↓ γδ T cells;↓ IL-2 signaling, similar to STAT5 KO mice
SOCS1	IL-2, IL-4, IL-6, IL-9, IL-10, IL-21, CNTF, CT1, EPO, G-CSF, GH, IFN-α/β, IFN-γ, Insulin, LIF, PRL, SCF, TNF-α, TSH	Neonatal lethality; ↑IFNγ production and responsiveness; ↑lymphopenia and multiorgan haematopoietic infiltrate	↓$CD8^+$/↑$CD4^+$ development; ↓ γδ T cells; spontaneous activation and increased apoptosis of peripheral T cells
SOCS2	IL-2, IL-6, CNTF, EPO, GH, Insulin, PRL	Gigantism	Gigantism
SOCS3	IL-1, IL-2, IL-3, IL-4, IL-6, IL-9, IL-10, IL-11, IL-12, IL-22, IL-23, IL-27, CNTF, CT1, EGF, EPO, GH, IFN-α/β, IFN-γ, Insulin, Leptin, LIF, OSM, PDGF, PRL, TGF-β, TNF-α, TPO, TSH	Embryonic lethality due to placental deficiency; ↑ erythrocytosis	Embryonic lethality with ↑ anemia.
SOCS4	EGF	Not reported	Not reported
SOCS5	EGF	No reported phenotype	↑Th1/↓Th2
SOCS6	SCF, Insulin	Slight decrease in growth	Not reported
SOCS7	Simvastin	↑ Hydrocephalus; Slight decrease in growth; ↑glucose clearance; ↑ hypoglycemia	Not reported

4.2 Results

4.2.1 IL-7 induces endocytosis of surface CD127 protein.

In Chapter 3, I established IL-7 induces rapid down regulation (<20 minutes) of CD127 protein from the cell surface as measured by flow cytometry (figure 8A) and observed a significant decrease in the level of total CD127 protein within the cell (10 ng/ml, 6 hrs, figure 13D). I also found removal of membrane associated CD127 in response to IL-7 was independent of JAK activity but subsequent degradation was dependent on this kinase presumably by phosphorylation of tyrosine Y449 in the cytoplasmic tail (figure 13C).

To first examine the kinetics of loss of total CD127 protein in response to IL-7 stimulation, a time course was performed where CD8 T cells were either incubated in media alone or were treated with IL-7 for 3-24 hours and levels of total CD127 protein were measured by Western blot analysis. As shown in Figure 16, following IL-7 treatment total CD127 protein decreased over 3-12 hours. Given surface levels of CD127 decrease within 20 minutes of IL-7 stimulation (figures 8A) levels of total protein do not change in the first 3 hours (figure 16) suggest the receptor may be internalized and then degraded.

To examine the role of endocytosis in loss of CD127 from the cell membrane following IL-7 stimulation, two endocytic inhibitors were used. Filipin is a lipid rafts disruptor (222) and Dynasore is a cell-permeable inhibitor of dynamin (223). CD8 T cells were incubated in media alone, with IL-7 (10 ng/ml) or were pre-treated with Filipin or with Dynasore followed by IL-7 and levels of CD127 proteins were measured by flow cytometry and Western blot analysis. When endocytosis was blocked by either inhibitor, CD127 remained on the cell surface and total CD127 protein remained unchanged (figure 17). Thus, it appears binding to IL-7 to the IL-7 receptor induces CD127 internalization via endocytic

Figure 16. IL-7 down regulates total CD127 protein in human CD8 T cells. (A) Total CD127 protein expression as measured by Western blot analysis in purified human CD8 T cells treated with IL-7 (10 ng/ml) for 3-24 hours using a polyclonal goat anti-human CD127 antibody. Hsp70 was used as an internal loading control. (B) Quantification of protein band intensities from six independent experiments using unsaturated images and normalizing band pixel intensity to Hsp70 in each lane. Values represent fold change relative to media control and error bars represent standard error of the mean. (*$p<0.05$ relative to media control).

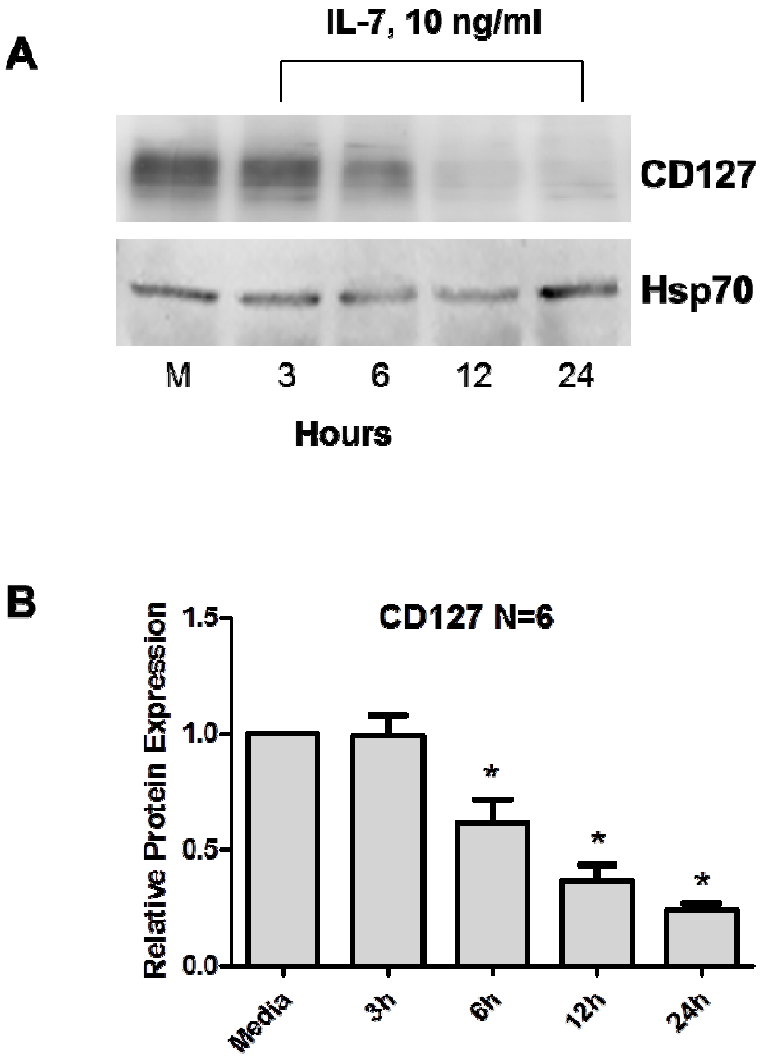

A
IL-7, 10 ng/ml
CD127
Hsp70
M
3
6
12
24
Hours
B
CD127 N=6
Relative Protein Expression
1.5
1.0
0.5
0.0
*
*
*
Media
3h
6h
12h
24h

Figure 16.

Figure 17. IL-7-mediated degradation of CD127 protein is dependent on cellular endocytic machinery in human CD8 T-cells. (A+B) Purified human CD8 T cells were either incubated in media alone, with IL-7 (10 ng/ml) for 6 hours, with the dynamin I inhibitor Dynasore (100 μM) or with the lipid raft disruptor Filipin (5 μg/ml) for 6 hours, or with either inhibitors for 1 hour followed by the addition of IL-7 (10 ng/ml) for 6 hours. Total CD127 protein was detected by Western blot analysis using a polyclonal goat anti-human CD127 antibody to detect whole CD127 protein. Blots were stripped and re-probed for β-actin as a loading control. (C) A similar experimental design as in (A+B) but surface CD127 protein expression was measured by flow cytometry (n=5). Error bars represent mean ± standard error of mean of at least three individual experiments. (*$p < 0.05$ compared to media control).

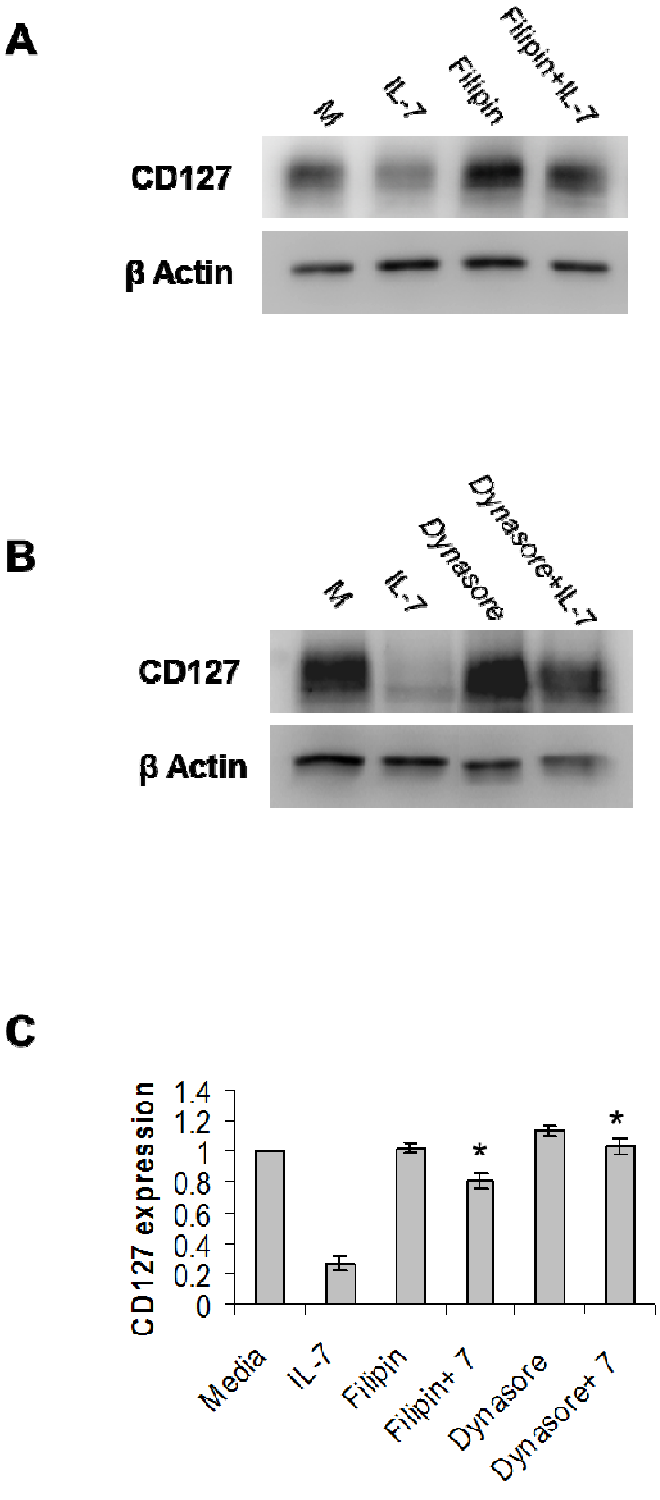

A
M
IL-7
Filipin
Filipin+IL-7
CD127
β Actin
B
M
IL-7
Dynasore
Dynasore+IL-7
CD127
β Actin
C
CD127 expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Media
IL-7
Filipin
Filipin+ 7
Dynasore
Dynasore+ 7

Figure 17.

vesicles and subsequent receptor degradation. This pathway was further delineated by **Dr. E. Faller** using confocal microscopy.

Receptor internalization by endocytosis occurs by clathrin-dependent or clathrin-independent mechanisms. Since CD8 T cells are devoid of caveolin (224, 225) and IL-7 has been previously shown to induce the phosphorylation of clathrin heavy chain (226), we hypothesized that the IL-7 dependent internalization of CD127 proteins is mediated by clathrin-dependent endocytosis. To test the hypothesis, we visualized the colocalization of CD127 with clathrin by confocal microscopy. After 5 minutes of IL-7 treatment, we observed a significant increase in the colocalization of clathrin with CD127 proteins, and the magnitude of the colocalization remained constant for 30 minutes (figure 18A). Interestingly, there is a large amount of CD127 that is colocalized with clathrin in media controls, suggesting that CD127 is being regularly recycled through clathrin mediated endocytosis under homeostatic conditions. However, there is a significant increase in the colocalization of CD127 and clathrin in IL-7 stimulation. Together with the endocytic inhibitors data, it is apparent IL-7 signaling accelerates a natural recycling process of surface CD127 proteins through clathrin-mediated endocytosis which is dependent on dynamin.

Protein internalization by endocytosis is characterized by transport from the cell surface to early and late endosomes. Early and Late endosomes can be identified by specific markers including Early Endosome Antigen 1 (EEA1) and Rab7, respectively. While colocalization with EEA1 can be associated with protein recycling on and off the cell membrane, proteins localized to Rab7 in late endosomes progress to lysosomal-dependent (227) or proteasomal-dependent (228-230) degradation pathways. As shown in figures 18B, CD8 T cells showed increased colocalization with early EEA1 compared to media controls after 30 minutes of IL-7 treatment compared to cells incubated in media alone. Similarly, we

Figure 18. IL-7 induces internalization of CD127 by clathrin mediated endocytosis and transport of the receptor via early and late endosomes to the proteasome. Following IL-7 stimulation, CD127 colocalizes with (A) clathrin (5 minutes), then with (B) the early endosomal marker (EEA1, 30 minutes), then with (C) the late endosomal marker (RAB7, 30 minutes) and is ultimately (D) targeted to the proteasome (proteasomal marker MCP20, 90 minutes). There is no colocalization between CD127 and (E) the lysosome (LAMP1, 90 minutes) in the presence of IL-7. Colocalization of CD127 and the indicated protein markers was determined by confocal microscopy in CD8 T cells incubated in media alone or with IL-7 (10 ng/ml). Representative images show overlay images of CD8 T cells stained with DAPI (blue), CD127 (red), marker proteins (green). Colocalized pixels appear yellow. (F) Schematic diagram of the internalization and compartmentalization of CD127 protein following IL-7 stimulation.

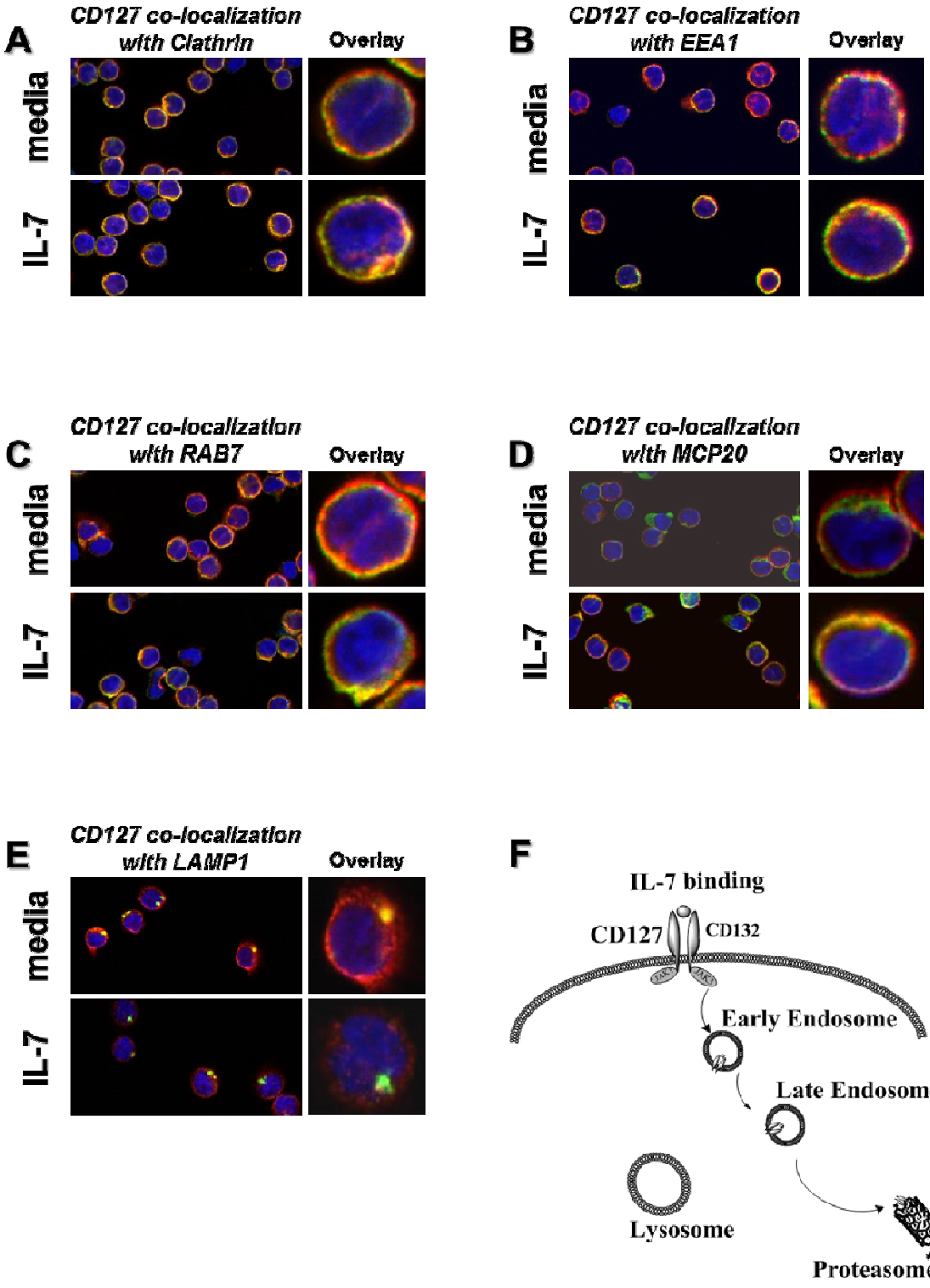

A
CD127 co-localization with Clathrin
Overlay
media
IL-7
B
CD127 co-localization with EEA1
Overlay
media
IL-7
C
CD127 co-localization with RAB7
Overlay
media
IL-7
D
CD127 co-localization with MCP20
Overlay
media
IL-7
E
CD127 co-localization with LAMP1
Overlay
media
IL-7
F
IL-7 binding
CD127
CD132
Early Endosome
Late Endosome
Lysosome
Proteasome

Figure 18.

observed a significant increase in colocalization of CD127 with the late endosomal marker Rab7 (figure 18C). These results implicate the internalized CD127 proteins by IL-7 signaling once are taken off the surface of human CD8 T cells by clathrin-mediated endocytosis are then transported to early and late endosomes and may then be targeted to degradation pathways.

4.2.2 IL-7 induced degradation of CD127 proteins is dependent on the proteasome

IL-7 induces rapid clathrin-dependent internalization of CD127 proteins off the surface of human CD8 T cells within 20 minutes of IL-7 stimulation (figure 8A). The internalized proteins are then transported to early and late endosomes are targeted for degradation, which is observed 3-6 hour after IL-7 stimulation (figure 16). It is likely that these proteins are transported to lysosomes or to the proteasome for degradation. To investigate this, we first measured the colocalization of CD127 with the 20S proteasome subunit (MCP20) by confocal microscopy in cells incubated in media alone and in IL-7 stimulation. While there was no significant colocalization between CD127 and MCP20 in cells incubated in media alone, we observed a significant increase in the colocalization of CD127 with MCP20 within 1.5 hours of treatment with IL-7 (figure 18D). On the other hand, we did not observe any increase in colocalization of CD127 and LAMP1 even after 3 hours of IL-7 stimulation (figure 18E).

IL-7 induced degradation of CD127 via the proteasome was confirmed using specific inhibitors. As shown in figure 19, when CD8 T cells were treated with IL-7 (10 ng/ml) for 6 hours, levels of total CD127 proteins decreased by 41+/-6% compared to media alone ($p<0.01$). However, when cells were pre-incubated with the 26S proteasome inhibitor

Figure 19. IL-7 mediated degradation of CD127 protein is dependent on the proteasome. (A) Purified human CD8 T cells were incubated in media alone, with IL-7 (10 ng/ml) for 6 hours, or were pre-incubated with the proteasome inhibitor MG132 (5 μM) for 1 hour followed by the addition of IL-7 (10 ng/ml) for an additional 6 hours. CD127 protein was detected by Western blot analysis using a polyclonal goat anti-human CD127 antibody. Blots were stripped and re-probed for β-actin as a loading control. (B) Quantification of CD127 protein band intensities from eight independent experiments using unsaturated images and normalizing band pixel intensity to β-actin in each lane. Values represent fold change relative to media control and error bars represent standard error of the mean. (*$p<0.05$ relative to media control).

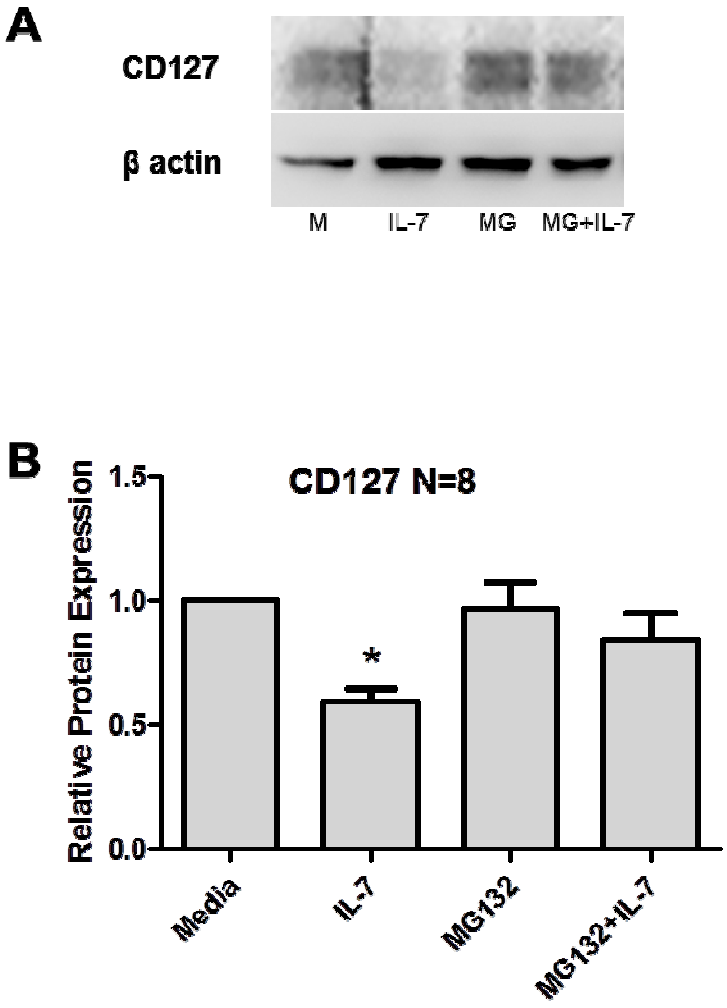

A
CD127
β actin
M
IL-7
MG
MG+IL-7
B
CD127 N=8
Relative Protein Expression
1.5
1.0
0.5
0.0
*
Media
IL-7
MG132
MG132+IL-7

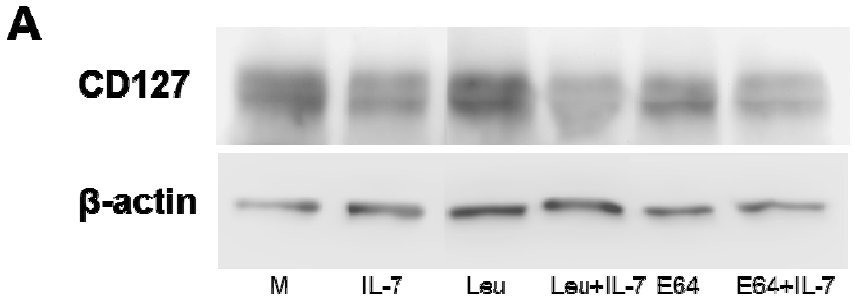
Figure 19.

MG132 then IL-7 was added for additional 6 hours, total CD127 proteins only decreased by 16+/-11% (p=0.03, compared to IL-7 treated cells). Thus, IL-7 was no longer able to effectively induce CD127 degradation when the activity of the proteasome was blocked. Also, when CD8 T cells were incubated with lysosomal inhibitors Leupeptin or E64 (231), IL-7 still induced the degradation of CD127 further confirming the degradation may be independent on the lysosomal pathway (figure 20). These data are consistent with the confocal images and illustrate CD127 is degraded by the proteasome following IL-7 stimulation.

4.2.3 Identifying factors that are implicated in targeting CD127 to the proteasome

As already illustrated in chapter 3, IL-7 mediated degradation of the CD127 protein is dependent on JAK activity (figure 13D). Cytokine receptor signaling has been shown to be negatively regulated by multiple protein families including the suppressor of cytokine signaling (SOCS) proteins, protein tyrosine phosphatases (PTPs) and protein inhibitor of activated STAT (PIAS) protein family. To investigate whether any of these proteins were up regulated by IL-7, I utilized a PCR array examining 84 genes including those known to negatively regulate JAK/STAT signaling. The array was carried out in CD8 T cells incubated in media alone or treated with IL-7 (10 ng/ml) for 3 hours. As shown in Table 5, IL-7 does not affect the expression of PIAS1, PIAS2, PTPRC, PTPN1 or PTPRC but does appear to induce the expression of SOCS1 and 2 gene expression. These data were consistent with unpublished microarray data from our lab which examined changes in global gene expression in CD8 T cells treated with IL-7 (10 ng/ml, 24 hours, Table 6) and showed IL-7 up regulated the expression of SOCS1 (4 fold), SOCS2 (200 fold) and CIS (30 fold).

Figure 20. IL-7-mediated degradation of CD127 protein is independent of the lysosome in human CD8 T cells. (A) Purified human CD8 T cells were incubated in media alone, with IL-7 (10 ng/ml) for 6 hours, or were pre-incubated with the lysosomal protease inhibitors Leupeptin (Leu, 10 μM) or E64 (10 μM) for 1 hour followed by the addition of IL-7 for an additional 6 hours. CD127 protein was detected by Western blot analysis using a polyclonal goat anti-human CD127 antibody. Blots were stripped and re-probed for β-actin as a loading control. (B) Quantification of CD127 protein band intensities from three independent experiments using unsaturated images and normalizing band pixel intensity to β-actin in each lane. Values represent fold change relative to media control and error bars represent standard error of the mean. (*$p<0.05$ relative to media control).


A
CD127
β-actin
M
IL-7
Leu
Leu+IL-7
E64
E64+IL-7

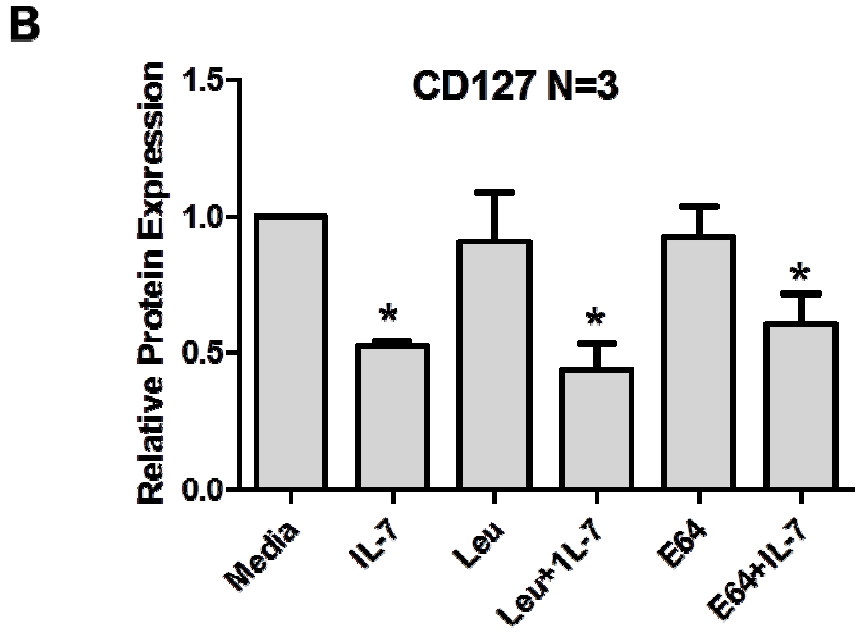

B
CD127 N=3
Relative Protein Expression
1.5
1.0
0.5
0.0
*
*
*
Media
IL-7
Leu
Leu+IL-7
E64
E64+IL-7

Figure 20.

Table 5. Expression of genes involved in the JAK/STAT pathway in human CD8 T cells following IL-7 stimulation compared to media control. Purified CD8 T cells were incubated in either media alone or with IL-7 (10 ng/ml) for 3 hours. Cells were then lysed and mRNA transcripts of 84 genes involved in JAK/STAT pathway were measured using PCR array plates (SABiosciences) normalizing to GAPDH and RPL13A expression. N=1.

Gene Symbol	Description	ΔFold relative to media
OSM	Oncostatin M	121.1
SOCS1	Suppressor of cytokine signaling 1	4.3
SOCS2	Suppressor of cytokine signaling 2	7.2
SOCS3	Suppressor of cytokine signaling 3	2.4
SOCS4	Suppressor of cytokine signaling 4	1.1
SOCS5	Suppressor of cytokine signaling 5	0.8
ISG15	ISG15 ubiquitin-like modifier	2.2
PIAS1	Protein inhibitor of activated STAT, 1	0.99
PIAS2	Protein inhibitor of activated STAT, 2	1.10
PPP2R1A	Protein phosphatase 2, regulatory subunit A, alpha isoform	1.36
PTPN1	Protein tyrosine phosphatase, non-receptor type 1	1.93
PTPRC	Protein tyrosine phosphatase, receptor type, C	0.91
SIT1	Signaling threshold regulating transmembrane adaptor 1	0.55
STAM	Signal transducing adaptor molecule (SH3 domain and ITAM motif) 1	0.66
JAK1	Janus kinase 1	0.50
JAK2	Janus kinase 2	1.30
JAK3	Janus kinase 3	1.30

Table 6. Analysis of changes in global gene expression in human CD8 T cells treated with IL-7 as measured by DNA microarray. Purified CD8 T cells were incubated in either media alone or with IL-7 (10 ng/ml) for 24 hours and global gene expression was quantified by a DNA microarray by Mr. J. Kakal. N=1. Key entries relevant to this study were summarized.

Gene Symbol	Description	ΔFold
CIS	Cytokine-inducible SH2-domain-containing protein	+31.2
SOCS1	Suppressor of cytokine signaling 1	+4.3
SOCS2	Suppressor of cytokine signaling 2	+222.1
c-Myb	V-myb avian myeloblastosis viral oncogene homolog transcription factor	+11.7

Together, these preliminary data suggest the SOCS proteins are likely candidates to target CD127 to the proteasome for degradation.

4.2.4 IL-7 up-regulates CIS, SOCS1, SOCS2 and SOCS3 mRNA transcripts in primary human CD8 T cells

To determine the basal expression of the eight SOCS genes in resting primary human CD8 T cells, levels of SOCS1-7 and CIS transcripts were measured by qPCR normalizing to RPS18 expression. The level of SOCS1 transcripts was arbitrarily set at 1 and expression of the other seven genes is shown relative to this. As shown in figure 21, all eight SOCS genes are expressed in human CD8 T cells. While CIS, SOCS1, SOCS3 and particularly SOCS2 are expressed at lower levels, basal mRNA expression for SOCS4-7 is much higher ranging 10 to nearly 30-fold greater than SOCS1. To determine which SOCS transcripts, if any, are induced by IL-7, purified CD8 T cells were incubated with increasing doses of IL-7 (0.1, 1 and 10 ng/ml) for 3 to 48 hours and transcript levels for all eight SOCS genes were measured by qPCR and compared to untreated cells normalizing to RPS18 expression. As shown in figure 22A, IL-7 induces the expression of CIS, SOCS1, 2 and 3 mRNA transcripts in a time- and dose-dependent manner. Up-regulation was maximal within 3 hours and declined thereafter. In cells stimulated with 10 ng/ml IL-7, transcripts encoding CIS, SOCS1 and SOCS2 could still be detected above baseline at 48 hours while at lower concentrations of IL-7 and for SOCS3 at all IL-7 concentrations transcripts returned to basal levels within 24 hours. SOCS2 transcripts showed the greatest induction increasing nearly 300-fold with 10 ng/ml IL-7 compared to unstimulated cells. CIS and SOCS1 transcripts increased approximately 10 to 18-fold while SOCS3 mRNA increased a more modest 3-fold over baseline in cells stimulated with the highest concentration of IL-7. In contrast, IL-7 even at

Figure 21. Basal expression of SOCS mRNA in resting primary human CD8 T cells. Basal levels of SOCS1-7 and CIS mRNA transcripts were measured in purified resting CD8 T cells by qPCR and normalized to RPS18 expression. Data are represented as fold changes relative to SOCS1 gene expression arbitrarily set at 1. Error bars represent standard error of the mean of six independent experiments.

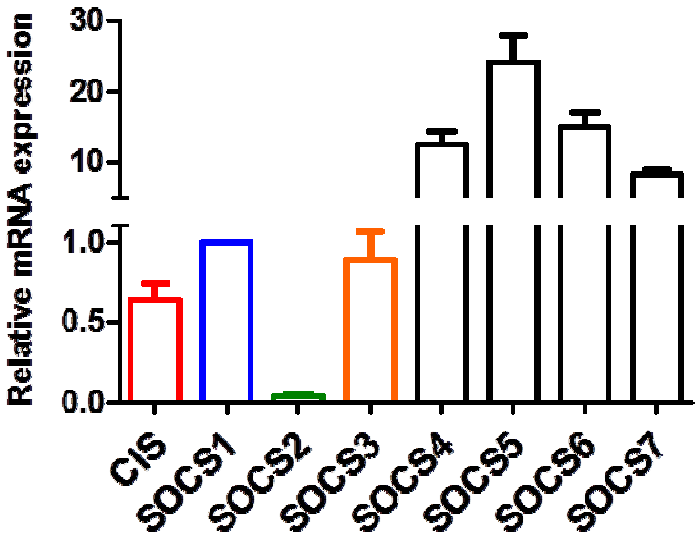

Relative mRNA expression
30
20
10
1.0
0.5
0.0
CIS
SOCS1
SOCS2
SOCS3
SOCS4
SOCS5
SOCS6
SOCS7

Figure 21.

Figure 22. IL-7 up-regulates CIS and SOCS1-3 mRNA transcripts in a dose and time dependent manner but does not affect the expression of SOCS4-7 in human CD8 T cells. Purified CD8 T cells were incubated for the indicated times in media alone or in the presence of (A) 0.1, 1, or 10 ng/ml IL-7, or (B) 10 ng/ml IL-7. CIS and SOCS1-7 mRNA transcript levels were measured by qPCR normalizing to RPS18 expression. (C) Abundance of CIS and SOCS1-3 mRNA, relative to each other, following stimulation of CD8 T cells with 10 ng/ml IL-7. In all panels error bars represent standard error of the mean of at least three independent experiments.

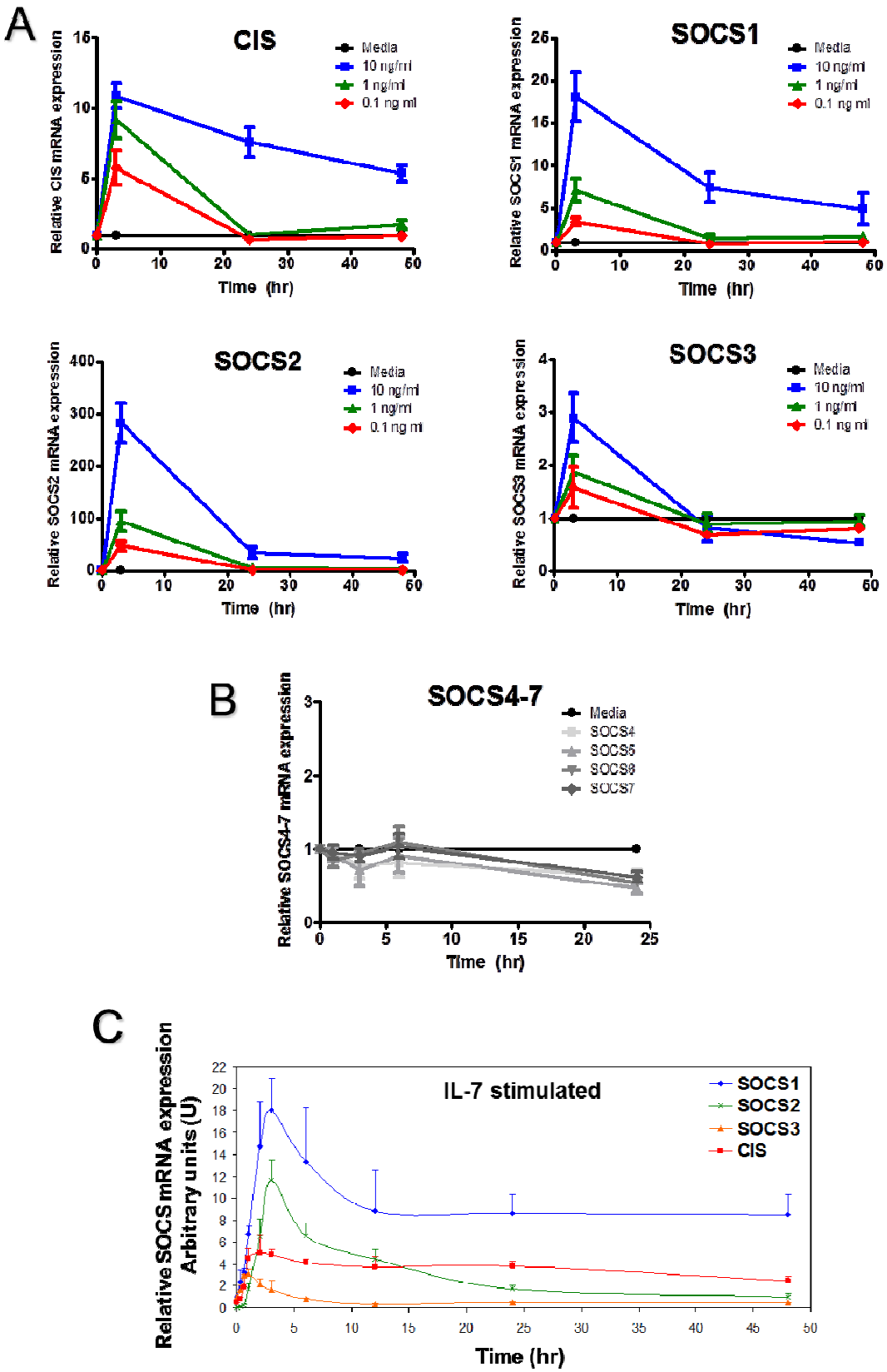

A
CIS
Media
10 ng/ml
1 ng/ml
0.1 ng ml
Relative CIS mRNA expression
Time (hr)
SOCS1
Relative SOCS1 mRNA expression
SOCS2
Relative SOCS2 mRNA expression
SOCS3
Relative SOCS3 mRNA expression
B
SOCS4-7
Media
SOCS4
SOCS5
SOCS6
SOCS7
Relative SOCS4-7 mRNA expression
C
IL-7 stimulated
SOCS1
SOCS2
SOCS3
CIS
Relative SOCS mRNA expression
Arbitrary units (U)

Figure 22.

10 ng/ml did not induce the expression of SOCS4-7 (figure 22B). Finally given the relative differences in basal expression we also compared the levels of the four IL-7 inducible SOCS genes relative to each other following stimulation with IL-7 (10 ng/ml). As seen in figure 22C, SOCS1 and SOCS2 transcripts predominate at 3 hours while elevated SOCS1 and CIS mRNA levels persist over 24-48 hours following IL-7 stimulation. These data then demonstrate that while SOCS4-7 have relatively higher basal transcript levels in resting primary human CD8 T-cells, IL-7 induces the expression of CIS, SOCS1 and SOCS2 mRNA and to a lesser extent that of SOCS3.

4.2.5 IL-7 induces CIS and SOCS1-3 transcripts via the JAK/STAT5 signaling pathway

JAK/STAT5 and PI3K are considered the major IL-7 induced signaling pathways in T cells (35, 72). To determine which of these pathways mediate the up-regulation of CIS and SOCS1-3 transcripts following IL-7 stimulation, we pre-treated CD8 T cells with inhibitors of JAK, PI3K or STAT5 phosphorylation for 1 hour followed by IL-7 (10 ng/ml) for 3 hours and examined the levels of CIS and SOCS1-3 transcripts by qPCR normalizing to RPS18. As shown in figure 23, inhibition of PI3K signaling did not affect the ability of IL-7 to up-regulate CIS and SOCS1-3 mRNA. However, pre-incubating cells with either JAK inhibitor 1 or with the STAT5 phosphorylation inhibitor completely blocked IL-7 mediated up-regulation of CIS, SOCS1, SOCS2 and SOCS3 transcripts (figures 23A and 23B). These data clearly demonstrate IL-7 induction of CIS and SOCS1-3 transcripts occurs via the JAK/STAT5 pathway.

To determine whether STAT5 phosphorylation plays a direct or indirect role in the IL-7 mediated induction of SOCS transcripts, we pre-incubated CD8 T cells with the protein translation inhibitor cycloheximide for 1 hour followed by IL-7 (10 ng/ml) for 1 hour and

Figure 23. Induction of SOCS mRNA by IL-7 is dependent on JAK and phosphorylation of STAT5 but is independent of PI3K and new protein synthesis. Purified CD8 T cells were pre-incubated for 1 hour with inhibitors of (A) JAK (JAK inhibitor 1, 1.5 μM), PI3K (LY294002, 50 μM), (B) STAT5 phosphorylation (500μM), or (C) protein synthesis (cycloheximide, 100 nM) followed by 10 ng/ml IL-7 for 3 hrs (A and B) or 1 hr (C). Transcripts levels of the IL-7 inducible SOCS genes were then measured by qPCR and normalized to RPS18. Error bars represent standard error of the mean of at least three independent experiments. (*$p<0.05$ and **$p>0.05$ relative to IL-7)

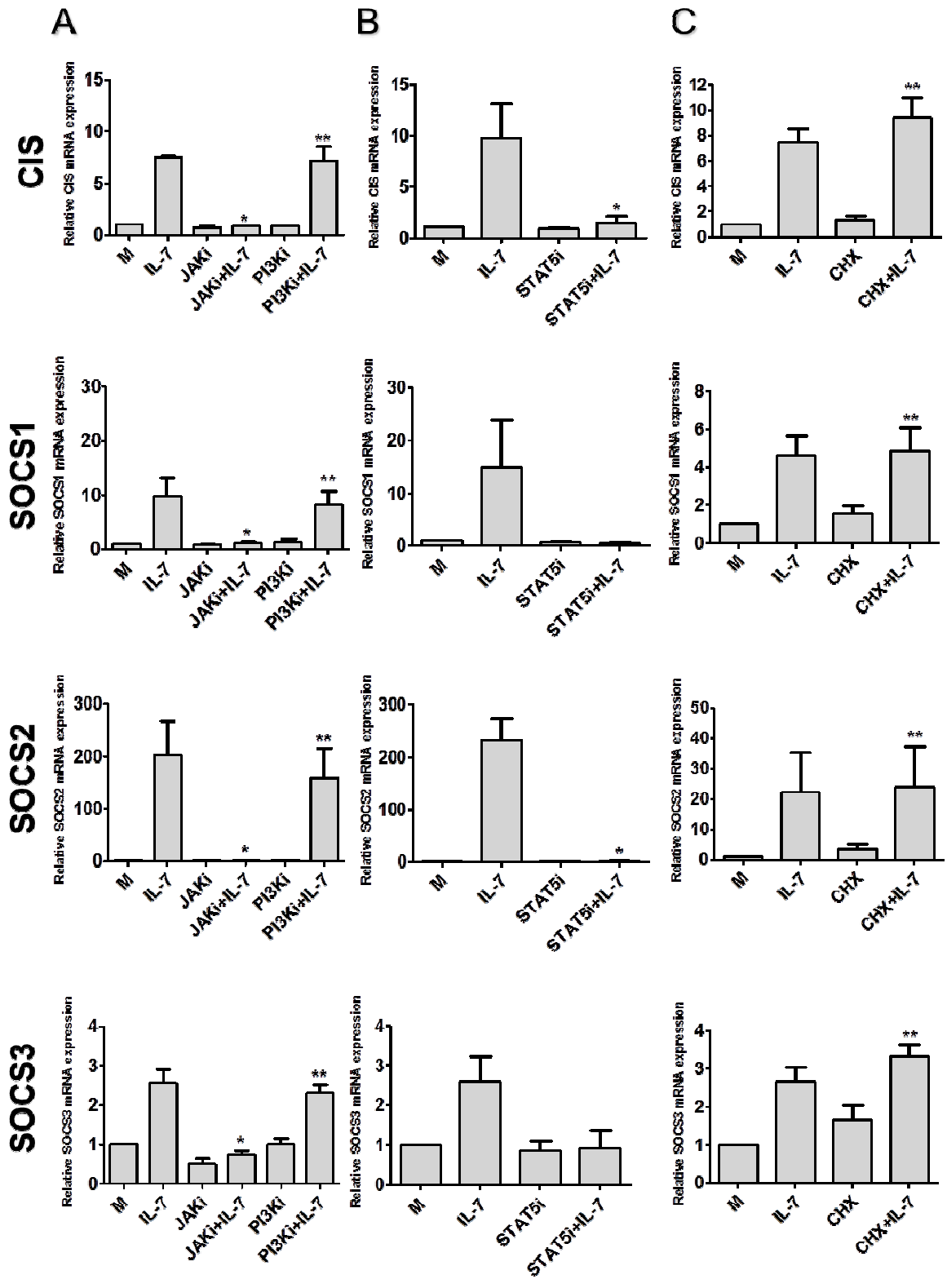

A
B
C
CIS
SOCS1
SOCS2
SOCS3
Relative CIS mRNA expression
Relative SOCS1 mRNA expression
Relative SOCS2 mRNA expression
Relative SOCS3 mRNA expression
M
IL-7
JAKi
JAKi+IL-7
PI3Ki
PI3Ki+IL-7
STAT5i
STAT5i+IL-7
CHX
CHX+IL-7

Figure 23.

measured CIS and SOCS1-3 transcripts by qPCR. The duration of IL-7 stimulation was abbreviated in these experiments to reduce the exposure to and therefore toxicity of cycloheximide, and as a result induction of SOCS transcripts was lower compared to 3 hours. As shown in figure 23C, IL-7 was still able to up-regulate CIS and SOCS1-3 mRNA when new protein synthesis was blocked indicating JAK/STAT5 signaling directly induces transcription of these SOCS genes. This is not surprising given at least the CIS gene promoter is known to contain STAT5 binding sites and STAT5 phosphorylation has previously been shown to up regulate CIS expression in both mouse and human cell lines (232, 233).

4.2.6 IL-7 induction of CIS, SOCS1 and SOCS2 mRNA transcripts is matched by increases in protein expression

To determine if the IL-7 induced increases in CIS and SOCS1-3 transcripts was reflected at the protein level, purified CD8 T cells were treated with IL-7 (10 ng/ml) and protein expression was determined by Western blot analysis. As anticipated from the transcript data, IL-7 stimulation resulted in time-dependent increases in the levels of the inducible SOCS proteins. CIS and SOCS1 proteins increased 2-fold within 1 hour of IL-7 stimulation with a maximal 3 to 4-fold induction by 3 hours compared to untreated controls (figure 24). SOCS2 protein expression increased approximately 2-fold and peaked between 1-2 hours after IL-7 stimulation. Consistent with the low induction of SOCS3 transcripts, SOCS3 protein showed no significant increase following treatment with IL-7. Interestingly, although an increase in CIS mRNA transcripts appears to be sustained over 24-48 hours following IL-7 treatment, CIS protein levels dropped almost back to baseline between 3 and

Figure 24. Induction of CIS and SOCS1-3 transcripts by IL-7 is reflected at the protein level. (A) Purified CD8 T cells were incubated in media alone or with IL-7 (10 ng/ml) for the times indicated. Expression of CIS and SOCS1-3 proteins in whole cell lysates were then analyzed by Western blot using polyclonal antibodies. Blots were then stripped and re-probed for β-actin as loading control. (B) Quantification of respective SOCS protein band intensities from three independent experiments using unsaturated images and normalizing band pixel intensity to β-actin in each lane. Values represent fold change relative to media control and error bars represent standard error of the mean. (*$p<0.05$ relative to media control).

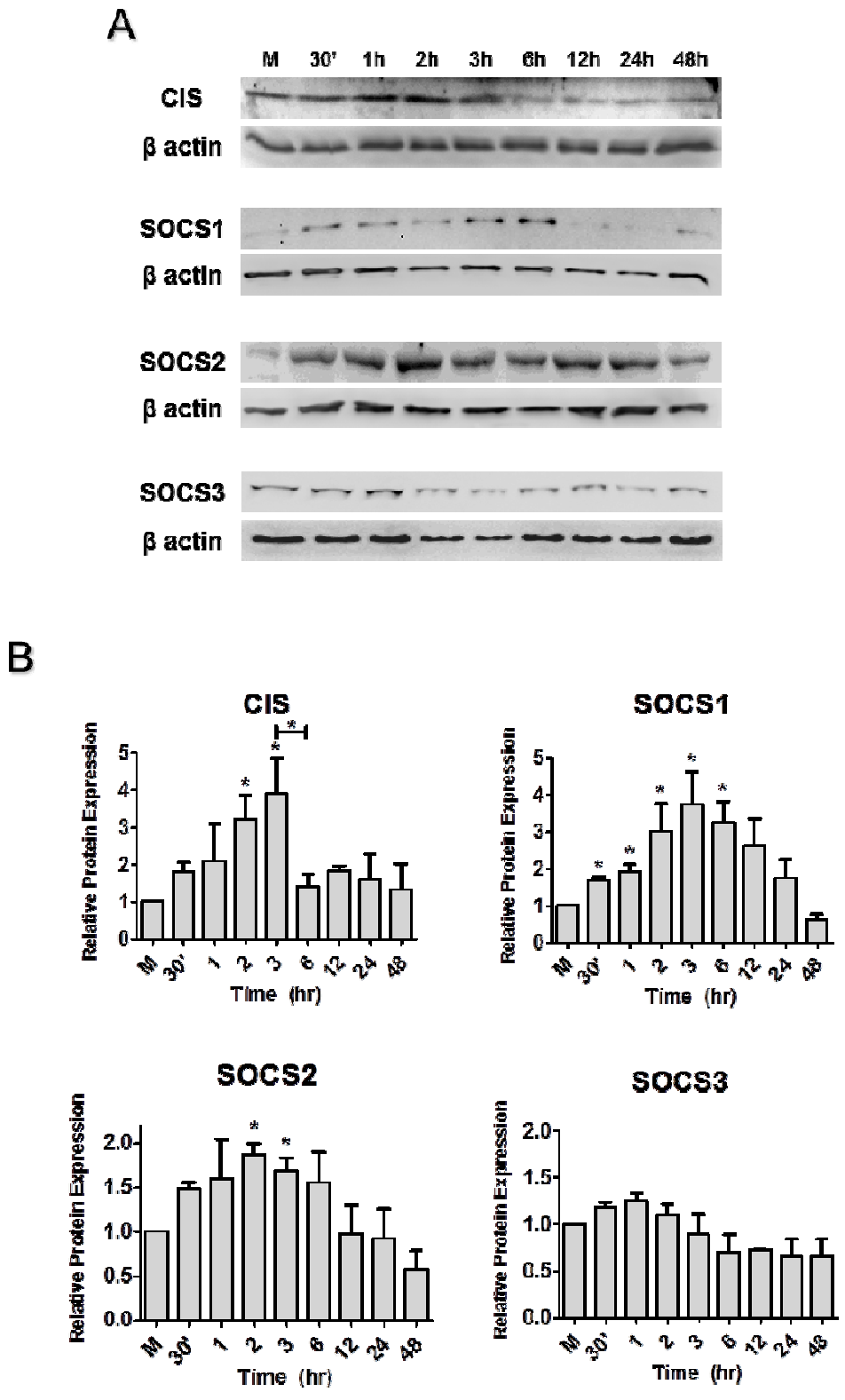

A
M 30' 1h 2h 3h 6h 12h 24h 48h
CIS
β actin
SOCS1
β actin
SOCS2
β actin
SOCS3
β actin
B
CIS
SOCS1
SOCS2
SOCS3
Relative Protein Expression
Time (hr)
M 30' 1 2 3 6 12 24 48

Figure 24.

6 hours post stimulation suggesting consumption of CIS protein. In contrast SOCS1 and 2 proteins decreased back to baseline over 12-24 hours.

4.2.7 CIS and SOCS2 proteins physically interact with CD127 and are involved in targeting the receptor to the proteasome for degradation.

In view of their established roles, we next asked whether any of the SOCS proteins physically interact with CD127. To answer this, co-immunoprecipitation experiments were performed. Primary human CD8 T cell were incubated in media alone or with IL-7 (10 ng/ml) for 3 hours, when SOCS proteins were maximally induced, after which CD127 was immunoprecipitated from cell lysates and immune complexes probed for CIS and SOCS1 through 7. As shown in figure 25A, CIS, SOCS1 and SOCS2 in fact co-precipitate with CD127. While very low levels of SOCS2 and essentially no CIS protein associate with CD127 when CD8 T cells are maintained in medium alone, stimulation with IL-7 induces a substantial increase in CIS and SOCS2 binding to CD127. In contrast, SOCS1 associates with CD127 in resting cells and this interaction is not increased in the presence of IL-7. With respect to the remainder of the SOCS family of proteins, we were not able to detect any interaction between CD127 and SOCS3-7 in either the presence or absence of IL-7 (Figure 25B).

Since we have already shown IL-7 induces the proteasomal degradation of CD127 (figure 19), these data suggested to us that CIS and SOCS2 might be involved in targeting CD127 to the 26S proteasome following IL-7 stimulation. To address this possibility, we used the E3 ubiquitin ligase inhibitor SMER3. Purified CD8 T cells were incubated in media alone, with IL-7 (10 ng/ml) for 6 hours when CD127 is degraded, or pre-treated with SMER3

Figure 25. CIS, SOCS1 and SOCS2 physically interact with CD127. Purified CD8 T cells incubated in the presence or absence of IL-7 (10 ng/ml) for 3 hours were lysed and CD127 was immunoprecipitated with polyclonal goat anti-human CD127 antibodies or goat isotype as control. Immune complexes were then probed by Western for **(A)** CIS, SOCS 1-3 or **(B)** SOCS4-7. Whole CD8 T cell lysate was used as detection control in (B). One representative blot of 5 is shown.

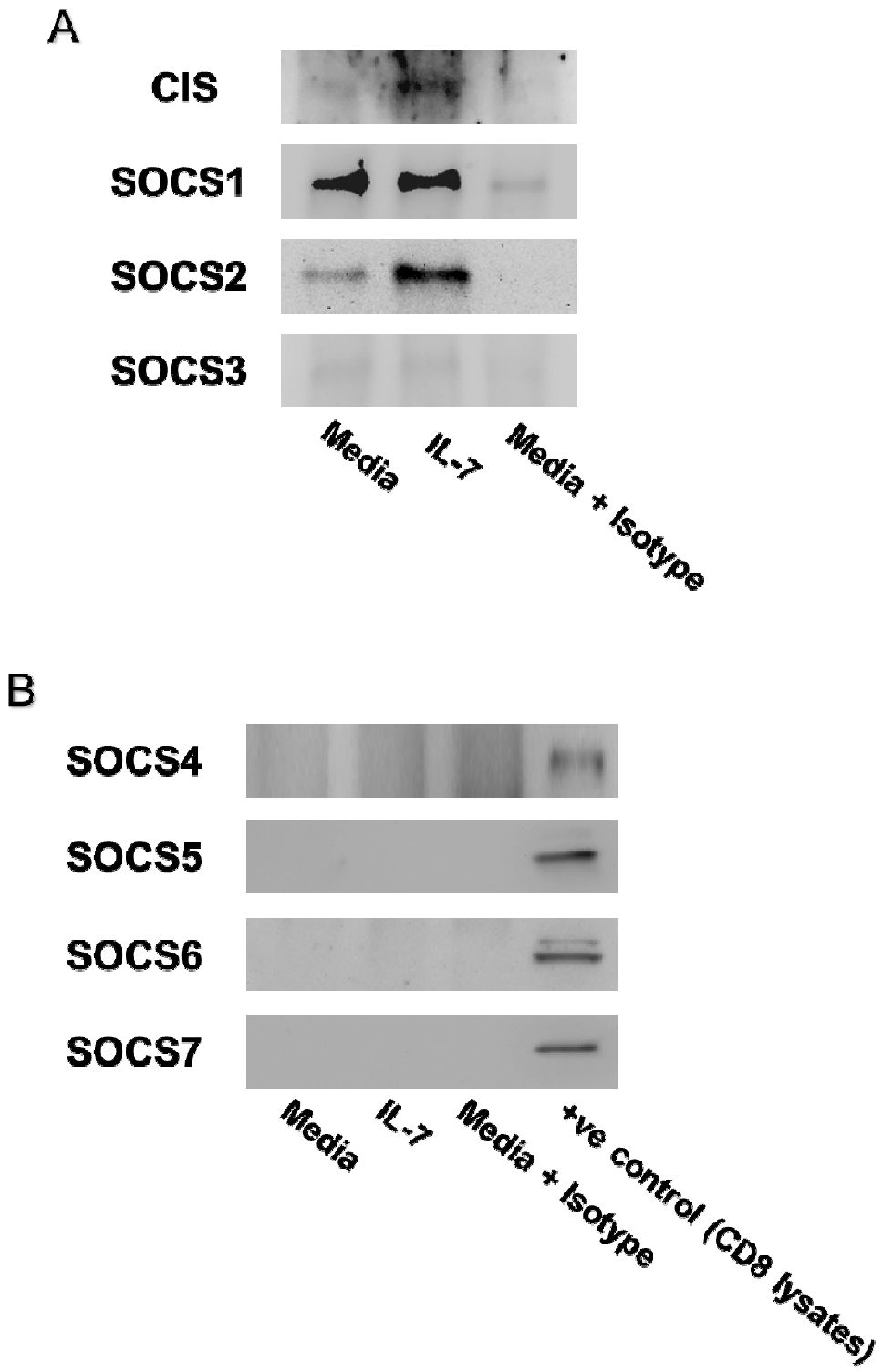

A
CIS
SOCS1
SOCS2
SOCS3
Media
IL-7
Media + Isotype
B
SOCS4
SOCS5
SOCS6
SOCS7
Media
IL-7
Media + Isotype
+ve control (CD8 lysates)

Figure 25.

for 1 hour followed by the addition of IL-7 (10 ng/ml) for 6 hours. Levels of CD127, CIS and SOCS2 were then measured by Western blot from whole cell extracts. As shown in figure 26, levels of CIS and SOCS2 increased in the presence of SMER3 alone indicating ubiquitin ligase likely plays a role in the normal turnover of these proteins. Further, following IL-7 stimulation in the presence of SMER3, CIS and SOCS2 remained elevated and CD127 degradation was blocked suggesting ubiquitin ligase is indeed required to target the receptor complex to the proteasome (figure 26). Given (a) the established role of SOCS proteins (65, 216-219), (b) the direct association of CD127 with CIS and SOCS2, and (c) the accumulation of all three proteins in the presence of SMER3 following treatment with IL-7, these data suggest upon stimulation of CD8 T cells with IL-7, CIS and/or SOCS2 bind CD127 and likely recruit an E3 ubiquitin ligase which in turn targets the receptor complex to the proteasome for degradation.

4.2.8 Following IL-7 stimulation, CIS colocalizes with CD127 as wells as with EEA1 in early endosomes and with MCP20 at the proteasome.

We have already shown IL-7 induces the rapid internalization of CD127 from the surface of CD8 T cells through endocytic vesicles with CD127 passing from early to late endosomes and ultimately to the proteasome for degradation (figure 18). To further explore the interactions between CD127 and SOCS1, SOCS2 and CIS in this pathway, purified human CD8 T cells were incubated in media alone or with IL-7 (10 ng/ml) for 1 hour and colocalization of CD127 with SOCS proteins was determined by confocal microscopy (**Dr. E Faller**). As shown in figure 27A and consistent with the immunoprecipitation data, in unstimulated CD8 T cells little to no CIS protein is associated with CD127 while low levels of SOCS1 and SOCS2 colocalize with the receptor. Following IL-7 stimulation, however,

Figure 26. Degradation of CD127 and SOCS proteins is dependent on E3 ubiquitin ligase actvities. Purified CD8 T cells were incubated in media alone, with IL-7 (10 ng/ml) for 6 hours, or pre-incubated with the E3 ligase inhibitor SMER3 (30 μM) for 1 hour followed by the addition of IL-7 (10 ng/ml) for 6 hours. Cells were then lysed and whole cell extracts were analyzed for CD127, CIS and SOCS2 expression by Western blot using β-actin as loading control. One representative blot of 2 is shown.

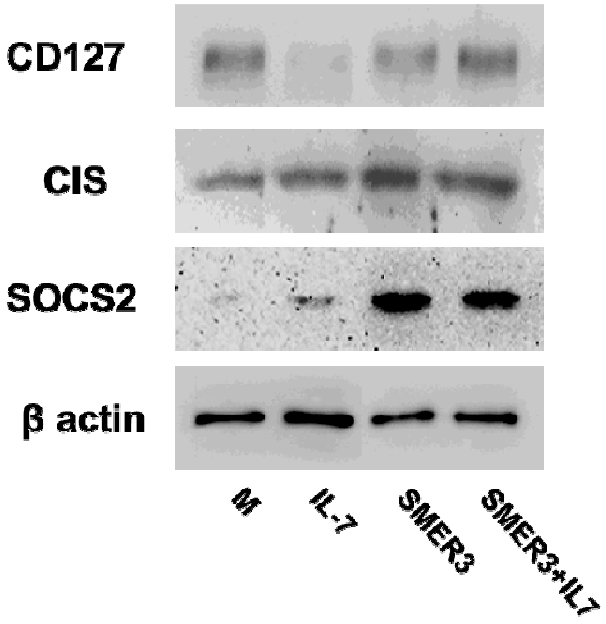


Figure 26.

Figure 27. IL-7 induces colocalization of CIS and SOCS2 with CD127 and with the early endosome marker EEA1; and CIS and CD127 with the proteasomal marker MCP20. Purified human CD8 T cells were incubated in media alone or with IL-7 (10 ng/ml) for 1 hr (A-C) or 3 hrs (D). Colocalization of SOCS1, SOCS2 and CIS proteins with (A) CD127, (B) the early endosome marker EEA1, (C) the proteasomal marker MCP20, or (D) the lysosomal marker LAMP1 as measured by confocal microscopy. Representative images are presented showing CD8 T cells stained with DAPI (blue) where SOCS proteins are green and CD127, EEA1, MCP20 and LAMP1 are red. Colocalized pixels appear yellow. Colocalized pixels are also shown in grayscale images where colocalization appears white.

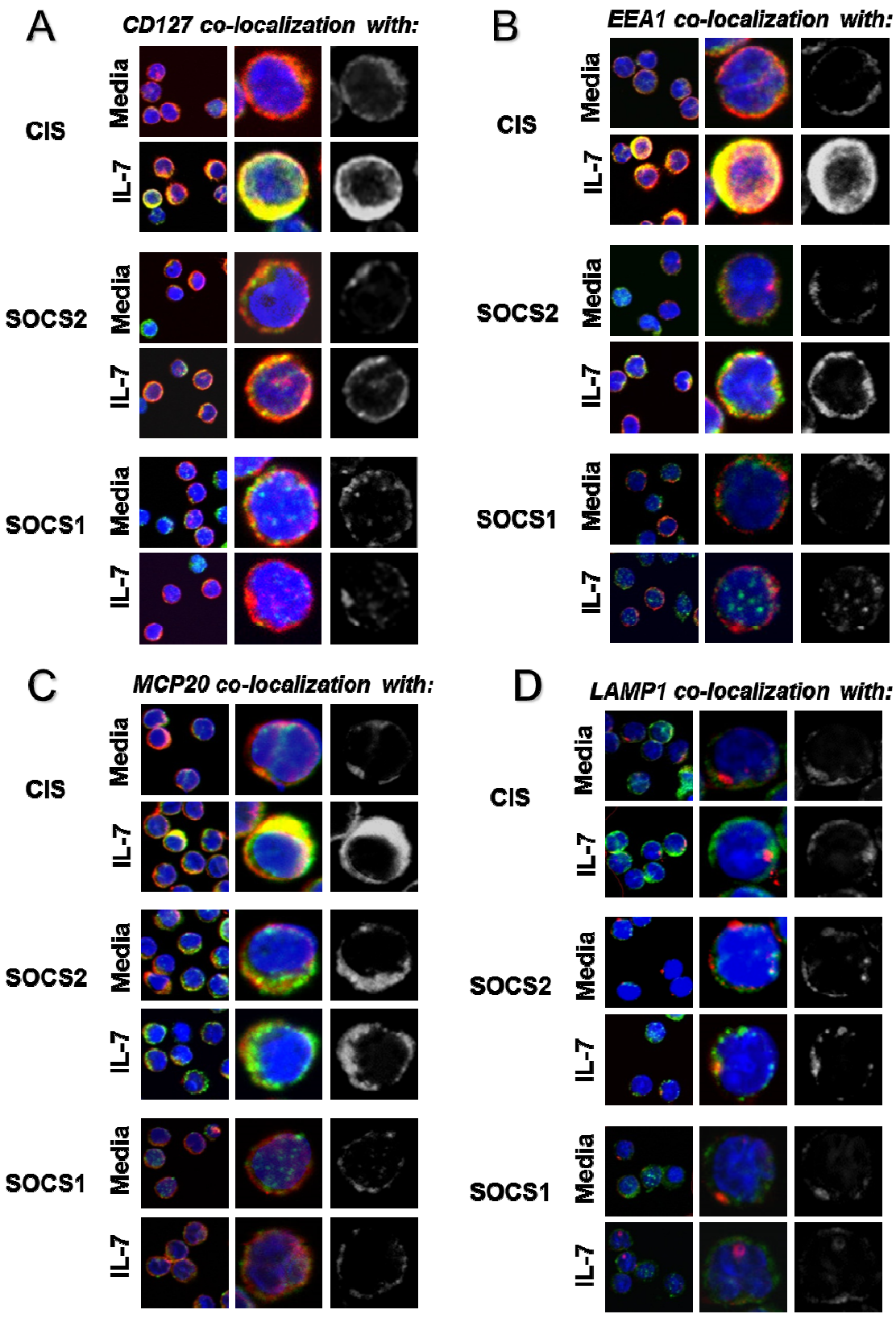

A
CD127 co-localization with:
CIS
SOCS2
SOCS1
Media
IL-7
B
EEA1 co-localization with:
C
MCP20 co-localization with:
D
LAMP1 co-localization with:

Figure 27.

and again consistent with the co-immunoprecipitation data, colocalization of CD127 with CIS and SOCS2 was clearly evident by quantification overlapping pixels for the overall cell population which increases on average 2.0+/-0.03 fold (p=0.0002) and 1.54+/-0.05 fold (p=0.03) for CIS and SOCS2 respectively while colocalization with SOCS1 does not change (p=0.46).

As shown in figure 27B, following stimulation of CD8 T cells with IL-7, CD127 is internalized and travels through early endosomes associating with EEA1 and then to late endosomes colocalizing with RAB7 before moving to the 26S proteasome where CD127 colocalizes with MCP20. Here we find following treatment with IL-7, CIS in particular follows the same path. As shown in figure 27B, stimulation of CD8 T cells with IL-7 leads to increased colocalization of CIS and SOCS2 with the early endosomal marker EEA1 by 1.54+/-0.04 and 1.43+/-0.04 fold respectively ($p<0.02$) compared to media controls. Similarly, following treatment with IL-7 CIS also colocalizes with MCP20 (figure 27C) with the association increasing 2.05+/-0.02 fold compared to media alone (figure 28C; $p=2x10^{-06}$). Interestingly, neither SOCS1, SOCS2 nor CIS colocalize with lysosomal-associated membrane protein 1 (LAMP1) either in the absence or presence of IL-7 (figure 27D). In summary then, IL-7 stimulation of CD8 T cells results in increased binding and colocalization of CD127 with CIS and SOCS2, increased colocalization of CD127, CIS and SOCS2 with EEA1, and increased colocalization of CD127 and CIS with MCP20. The data taken together suggest that following IL-7 stimulation, CIS and possibly SOCS2 bind CD127 and likely through recruitment of an E3 ubiquitin ligase shuttle the receptor through early to late endosomes and ultimately to the proteasome for degradation.

4.2.9 Following IL-7 stimulation, CIS plays a predominant role in targeting CD127 for degradation.

To further delineate the roles of CIS and SOCS2 in targeting CD127 for degradation, we carried out siRNA-mediated knock-down assays to inhibit expression of SOCS2 and CIS in human CD8 T cells. The Accell siRNA system was utilized as this has proved successful in primary human T cells which are inherently difficult to transfect (234, 235). Because IL-7 at 10 ng/ml so profoundly increases the level of CIS and SOCS2 mRNA (10-fold for CIS and nearly 300-fold for SOCS2; figure 22), we used lower amounts of IL-7 (0.5 ng/ml) in these experiments to allow more effective block of the induction by siRNA. At this concentration, IL-7 induces the degradation of CD127 by 38.6+/-3% within 6 hours (figure 16). To test the efficiency of the knock downs, purified CD8 T cells were transfected with the respective siRNA and then treated with IL-7 for 3 hours, the point of maximal CIS and SOCS2 protein induction (figure 24B). As shown in figure 28, the targeted siRNA successfully blocked IL-7 induction of CIS (figure 28A) and SOCS2 (figure 28B) expression without affecting the non-targeted SOCS protein. Scrambled siRNA had no effect. Next, to determine if preventing the induction of CIS and/or SOCS2 had any effect on IL-7 induced CD127 degradation, CD8 T cells were transfected with the corresponding siRNA alone or in combination and were then maintained in medium alone or treated with IL-7 (0.5 ng/ml) for 6 hours. As shown in figures 28C and 28D, in resting CD8 T cells maintained in medium only knock down of CIS or SOCS2 either alone or in combination had no effect on CD127 expression. As noted, addition of IL-7 (0.5 ng/ml) alone induced a 38.6+/-3% reduction in CD127. However, when CD8 T cells were treated with IL-7 in the presence of CIS siRNA, CIS protein induction was blocked (figure 28A) and CD127 was not degraded (figures 28C and 28D). Knock down of SOCS2 induction did not appear to affect IL-7 induced CD127 degradation compared to

Figure 28. siRNA-mediated knock-down of CIS induction by IL-7 prevents CD127 degradation. Purified CD8 T cells were transfected with siRNA targeting CIS or SOCS2 or with non-targeting scrambled siRNA and then incubated with IL-7 (0.5 ng/ml) for 3 hours. Untransfected cells were maintained in medium alone or treated with IL-7 in parallel. Knock-down efficiency was monitored by Western blot of whole cell extracts probing for (A) CIS protein or (B) SOCS2 protein with β-actin as loading control. (C) Purified CD8 T cells were transfected with siRNA targeting CIS or SOCS2, with both siRNA or with non-targeting scrambled siRNA and then maintained in medium alone or incubated with IL-7 (0.5 ng/ml) for 6 hours. Untransfected cells were maintained in medium alone or treated with IL-7 in parallel. CD127 protein levels were monitored by Western blot of whole cell extracts with β-actin as loading control. (D) Quantification of CD127 protein band intensities from four independent experiments (n=2 for scrambled siRNA+IL-7) using unsaturated images and normalizing band pixel intensity to β-actin in each lane. Values represent fold change relative to media control and error bars represent standard error of the mean. (*p=0.02 and **p=0.23 relative to IL-7).

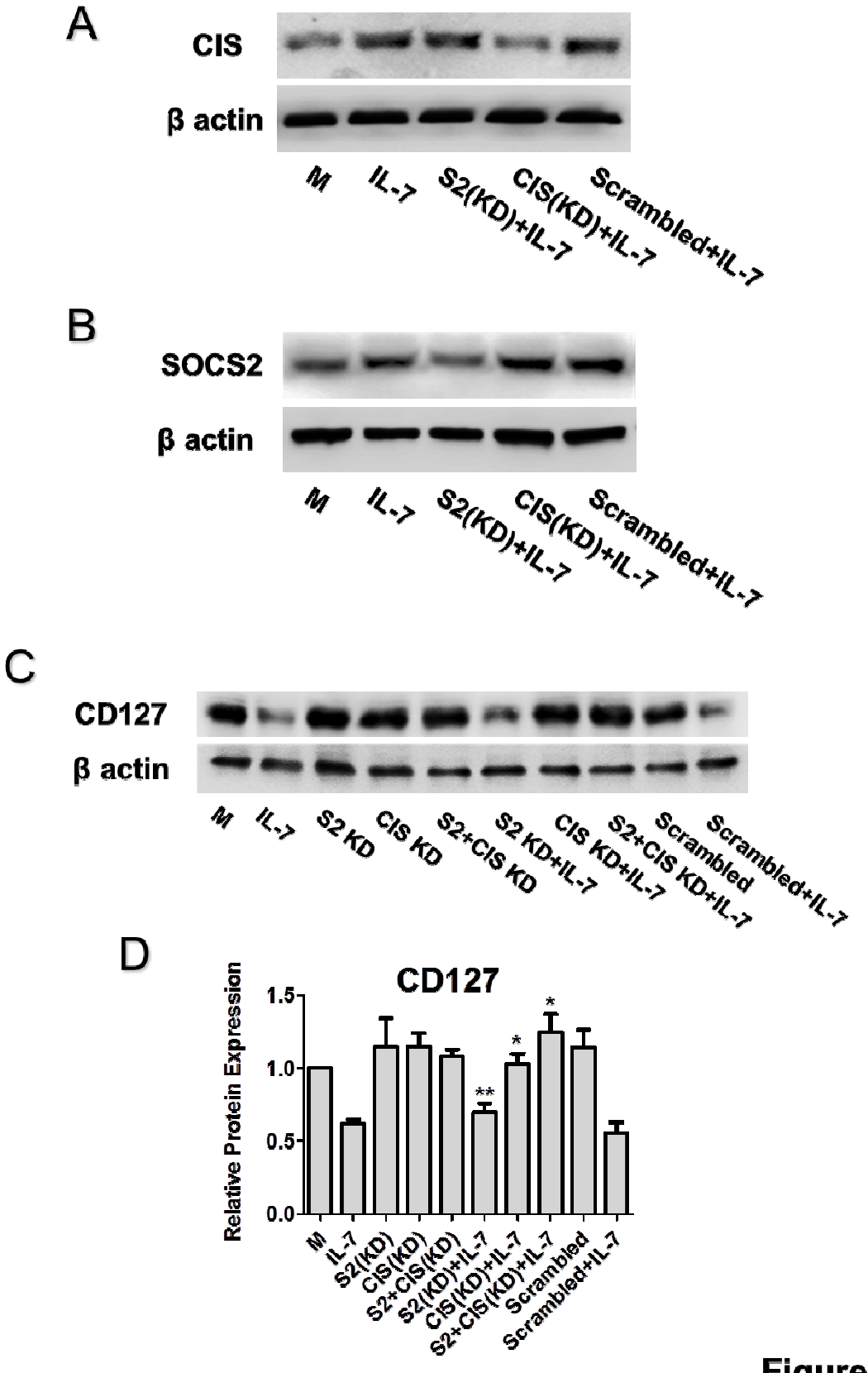

A
CIS
β actin
M
IL-7
S2(KD)+IL-7
CIS(KD)+IL-7
Scrambled+IL-7
B
SOCS2
β actin
M
IL-7
S2(KD)+IL-7
CIS(KD)+IL-7
Scrambled+IL-7
C
CD127
β actin
M
IL-7
S2 KD
CIS KD
S2+CIS KD
S2 KD+IL-7
CIS KD+IL-7
S2+CIS KD+IL-7
Scrambled
Scrambled+IL-7
D
CD127
Relative Protein Expression
1.5
1.0
0.5
0.0
*
*
**
M
IL-7
S2(KD)
CIS(KD)
S2+CIS(KD)
S2(KD)+IL-7
CIS(KD)+IL-7
S2+CIS(KD)+IL-7
Scrambled
Scrambled+IL-7

Figure 28.

knockdown of SOCS2 plus CIS together had only a marginally greater effect on CD127 degradation than knock down of CIS alone. Scrambled siRNA had no effect on CD127 expression or its degradation following treatment with IL-7. These data support the images on confocal microscopy and taken together suggest IL-7 stimulation of CD8 T cells results in the up regulation of CIS protein which binds CD127 in early endosomes and through recruitment of an E3 ligase shuttles the receptor to the proteasome for degradation. SOCS2 may play a more minor role in CD127 turn over.

4.3 Discussion

Although a few studies have reported induction of SOCS1 and SOCS3 proteins in response to IL-7 in murine T cells (220), this is the first comprehensive report examining the expression of all eight members of the SOCS family of proteins in response to IL-7 in human T cells. This is also the first time CIS and perhaps SOCS2 have been implicated in directing CD127 to the proteasome following IL-7 stimulation.

We have shown here that IL-7 induces the expression of a specific subset of SOCS genes in primary human CD8 T cells, specifically those encoding CIS and SOCS1, 2, and 3. While the induction was greatest for SOCS2 transcripts and weakest for SOCS3, the effect was dose dependent and maximal within 3 hours in all cases. We have also shown the induction of CIS and SOCS1, 2, and 3 transcripts by IL-7 is dependent on JAK/STAT5 signaling. This is consistent with previous studies illustrating SOCS proteins are generally induced through the JAK/STAT pathway following cytokine stimulation (65, 236). It is already established that IL-7 signaling leads to phosphorylation of CD127 at tyrosine 449 (Y449) by JAK3 (figure 17 and (35)), allowing docking via their SH2 domains and subsequent phosphorylation of STAT5 proteins (35, 55, 215). Phospho-STAT5 molecules then dimerize and translocate to the nucleus where they up regulate the expression target genes. Phospho-STAT5 dimers likely act directly on the gene promoters for CIS and SOCS1-3 as pre-treating CD8 T cells with cycloheximide did not block transcript up regulation. In fact, SOCS gene promoters possess STAT5 binding sites (232, 237-239) and pSTAT5 have been shown to bind within CIS (232, 240) and SOCS2 (239) gene promoters.

The more robust induction of CIS, SOCS1 and SOCS2 transcripts by IL-7 was matched by increases in the corresponding proteins. CIS and SOCS1 proteins increased 3 to

4-fold within 3 hours compared to untreated controls and SOCS2 protein increased approximately 2-fold between 1-2 hours after IL-7 stimulation. Consistent with the weak induction of SOCS3 transcripts, SOCS3 protein showed no significant increase following treatment with IL-7. This is somewhat in contrast to what was reported in mice where IL-7 has been shown to down regulate expression of SOCS3 in splenic T cells following infection with the lymphocytic choriomeningitis virus (LCMV) (179, 241). This difference between mouse and human T cells may suggest differences in the roles SOCS proteins play across different species or in the context of specific infections.

Following peak induction by IL-7, CIS protein levels drop back to baseline by 6 hours while SOCS1 and SOCS2 proteins decline over 24 hours. This rapid decrease in CIS protein while CIS mRNA transcripts remain elevated suggests possible protein consumption. Interestingly, the decrease in CIS protein is coincident with the degradation of CD127 which begins between 3-6 hours following IL-7 stimulation (figure 24B). Also of note is the difference in magnitude in SOCS2 transcript and protein induction in response to IL-7. SOCS2 mRNA is induced almost 300 fold whereas SOCS2 protein is induced only 2 fold three hours following treatment with IL-7 at 10 ng/ml. This may reflect the half-lives of SOCS proteins. Indeed, as seen in figure 26 treatment of CD8 T cells with the E3 ubiquitin ligase inhibitor SMER3 lead to a significant accumulation of SOCS2 and CIS proteins compared to media controls. Consistent with this Siewert *et al* determined the half-lives of SOCS1, SOCS2 and SOCS3 in COS-7 cells and found them to be very short-lived ($t_{1/2}$ <100 minutes) (242).

The SOCS family of proteins has been shown to down regulate expression of many cytokine receptors. CIS and SOCS2 in particular bind via their SH2 domains to phosphotyrosine residues on the cytoplasmic tail of activated cytokine receptors and recruit

via their SOCS box the multi-protein E3 ubiquitin-ligase complex resulting in the ubiquitination and subsequent proteasomal degradation of the associated cytokine receptor (for reviews: (236, 243)). Here we show CIS and SOCS2 physically interact with CD127 in human CD8 T cells following treatment with IL-7 and that inhibition of the E3 ligase with SMER3 prevents subsequent degradation of the protein complex. SOCS1 also binds CD127 but this interaction does not change in the presence of IL-7 suggesting SOCS1 has no role in regulating CD127 expression in response to cognate cytokine signaling. We currently do not know the relevance of SOCS1 binding to CD127 in human CD8 T cells. Of note, one study did find high levels of SOCS1 in double positive thymocytes in mice which do not express CD127, and when CD127 was transgenically expressed SOCS1 interfered with IL-7 mediated STAT5 phosphorylation. Thus SOCS1 may have a role in modulating IL-7 signaling at different T cell developmental stages (244). Further investigations are required to understand the significance of SOCS1 binding to CD127 in mature resting human CD8 T cells.

In contrast to SOCS1, both CIS and SOCS2 binding to CD127 is significantly increased in the presence of IL-7. As discussed in chapter 3, the internalization of CD127 from the surface of CD8 T cells in response to IL-7 is rapid and does not require JAK activity but phosphorylation of tyrosine 449 by JAK is required for CD127 degradation (figure 13C). We have also shown that following IL-7 induced internalization, CD127 moves from early to late endosomes and ultimately to the proteasome where it is degraded (figure 18). Here we find CIS and to some extent SOCS2 follow the same path (figure 27). Following treatment with IL-7, CIS and SOCS2 colocalization with CD127 and CIS, SOCS2 and CD127 all colocalize with EEA1 in early endosomes while CD127 and CIS also

colocalize with MCP20 at the proteasome. Consistent with this when induction of CIS protein by IL-7 is blocked with siRNA, degradation of CD127 does not occur.

While the role of CIS in IL-7 induced degradation of CD127 is clear, the role of SOCS2 is less evident. The involvement of more than one member of the SOCS family of proteins in regulating a single cytokine receptor has been described including for growth hormone receptor as well as for the erythropoietin and prolactin receptors (245). CIS and SOCS2 negatively regulate cytokine signaling in a similar manner by binding to phosphorylated receptors and targeting them for degradation (236). That said, distinct roles for CIS and SOCS2 have been reported. Lavens *et al*, observed differential binding of CIS and SOCS2 with the leptin receptor and demonstrated that CIS interacts with two phosphorylated sites on the leptin receptor (Y985 and Y1077) while SOCS2 binds only to Y1077 (245). Interestingly, SOCS2 can block the access of CIS to Y985 on the same receptor through interaction with the SOCS box of CIS (245, 246). Given the cytoplasmic tail of CD127 possesses three tyrosine residues as potential phosphorylation sites (Y449, Y401 and Y456) (35) it is possible that CIS and SOCS2 have differential binding patterns to the activated receptor and thus contribute differently to its regulation. This requires further investigation.

From the data presented here a picture emerges describing how expression of the IL-7 receptor alpha-chain on the surface of human CD8 T cells is regulated by IL-7 (figure 29). IL-7 binding to CD127 triggers receptor internalization perhaps through dimerization with CD132 or by inducing a conformational shift in the protein. Internalization occurs through clathrin coated pits and the receptor initially accumulates in early endosomes. IL-7 binding to its receptor also activates JAK3 which phosphorylates Y449 (figure 13C) in the cytoplasmic tail of CD127 as well as STAT5 molecules which in turn translocate to the

Figure 29. Model showing homeostatic recycling as well as IL-7 induced down regulation of CD127 protein on CD8 T cells. In resting CD8 T cells, CD127 recycles on and off the cell membrane likely through early endosomes with basal protein turn over in the lysosome. IL-7 binding to CD127 initiates rapid internalization of surface CD127 protein and activation of JAK which in turn phosphorylates Y449 in the cytoplasmic tail of CD127 as well as STAT5. Phospho-STAT5 molecules dimerize and translocate to the cell nucleus where they induce expression of CIS, SOCS1 and SOCS2 proteins. CIS and SOCS2 then bind to the phosphorylated tail of CD127 in early endosomes and recruit an E3 ubiquitin ligase shuttling the complex through late endosomes to the proteasome for degradation.

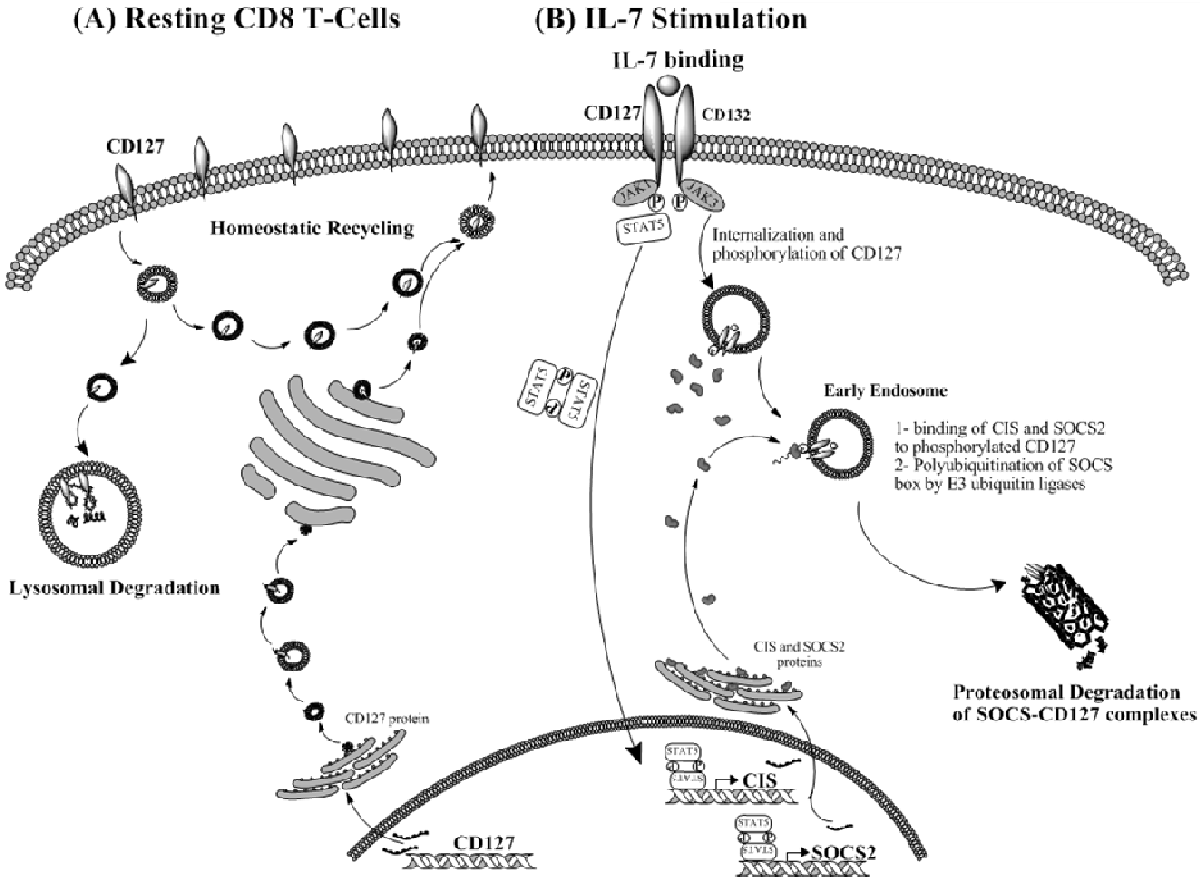

(A) Resting CD8 T-Cells
(B) IL-7 Stimulation
IL-7 binding
CD127
CD132
CD127
JAK1
JAK2
STAT5
Homeostatic Recycling
Internalization and
phosphorylation of CD127
Early Endosome
1- binding of CIS and SOCS2
to phosphorylated CD127
2- Polyubiquitination of SOCS
box by E3 ubiquitin ligases
Lysosomal Degradation
CIS and SOCS2
proteins
Proteosomal Degradation
of SOCS-CD127 complexes
CD127 protein
CIS
SOCS2
CD127

Figure 29.

nucleus and induce the expression of several proteins including CIS and SOCS2. Within the early endosomes CD127 is then be sorted such that unphosphorylated receptor is recycled back to the cell surface while phosphorylated CD127 is bound by CIS and SOCS2 via their SH2 domains. CIS and perhaps SOCS2 next recruit an E3 ubiquitin ligase which in turn targets the receptor complex through late endosomes and to the proteasome for degradation. Through this mechanism IL-7 provides negative feedback on its own signaling allowing CD8 T cells to regulate their proliferation and cytolytic function in response to different immunological challenges in different microenvironments.

5. Chapter 5. Interleukin-7 Suppresses Transcription of the CD127 Gene by Inducing Expression of c-Myb

5.1 Rationale

In Chapter 3 I illustrated IL-7 down-regulates CD127 gene transcription in a time and dose dependent manner via the JAK/STAT5 pathway and that IL-7 induced suppression of CD127 mRNA transcripts was delayed by 40-60 minutes post-treatment. This time delay suggests IL-7 may induce the expression of a transcriptional repressor of the CD127 gene.

The human CD127 gene promoter is 75% homologous to its equivalent in mice (206). The differential regulation of CD127 in mice has been shown to occur through expression of different constellations of transcription factors in different cell types (162). The core promoter region is located within the first 200 bps upstream of the transcription start site (TSS) and contains sites for RUNX, Ikaros, GABP and NF-κB (162). This region also possesses a binding site for the transcription factor ETS-1 which has been shown in a recent study to also maintain CD127 receptor expression in peripheral human T cells (247). Further, an interferon-stimulated response element (ISRE) was identified approximately 1.1 kb upstream of the TSS (248). Glucocorticoids (GCs) have also been shown to up regulate the transcription of CD127 in murine B and T lymphocytes (206, 207) as well as in human T cells (249, 250). This induction by glucocorticoids occurs through binding to a conserved GC binding site approximately 3kb upstream of the transcription start site (206). In addition, Notch-1 is an important signaling pathway which modulates multiple cell differentiation pathways during embryonic and adult life (251). It has been shown in recent studies that Notch-1 signaling activates CD127 gene expression in thymocytes (252, 253). When Notch-1 signaling is initiated, a metalloprotease is activated which cleaves Notch-1 receptor and

this cleaved intracellular Notch-1 receptor protein translocate to the nucleus where it forms a multi-protein coactivator complex along with p300 and Mastermind-like protein 1 (MAML1). This complex then up regulates transcription of CD127 (251-253). Whether Notch-1 signaling is important for regulating CD127 expression in mature human CD8 T cells is unknown. Collectively, the majority of the described transcription factors positively regulate CD127 gene transcription.

Members of the gamma chain (γc) cytokine family of cytokines have been shown to down regulate CD127 mRNA expression in human as well as in murine T cells (154). While the detailed mechanism by which gamma cytokines, particularly IL-7, down regulate CD127 expression is incompletely understood, Xue and colleagues (2002) illustrated that IL-2 down regulates levels of CD127 transcripts in human PBMCs via a phosphatidylinositol 3-kinase/Akt-dependent mechanism as demonstrated by utilizing the PI3-kinase inhibitors wortmannin and LY294002 which blocked IL-2 mediated down regulation of CD127 transcripts (156). Further, Park and colleagues reported IL-7 induces the expression of the transcriptional repressor Gfi-1 in mouse CD8 T cells. Gfi-1 proteins bind within introns 2 and 4 of the CD127 gene and appear to block transcription (154, 254). Despite the central role of IL-7 signaling in human T cell development and function, transcriptional regulation of the CD127 gene in response to IL-7 in human CD8 T cells remains incompletely understood.

The present chapter examines the regulatory mechanism by which IL-7 suppresses transcription of the CD127 gene in primary human CD8 T cells.

5.2 Results

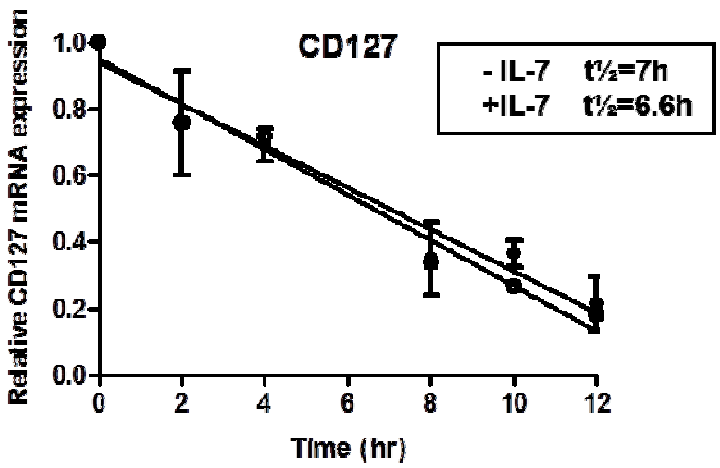
5.2.1 IL-7 down-regulates levels of CD127 mRNA transcripts in human CD8 T cells directly by attenuating the rate of CD127 gene transcription

As previously discussed in Chapter 3, IL-7 down regulates CD127 gene transcripts in a time and dose dependent manner (figure 7A). This could occur through one of two possibilities, either by reducing the half-life of CD127 mRNA or by attenuating the rate of CD127 gene transcription. To determine if IL-7 reduces the stability of CD127 transcripts, the RNA polymerase II inhibitor 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) was used. This inhibitor blocks *de novo* transcription by inhibiting P-TEFb kinase activity (255, 256) and preventing transcriptional elongation. Purified human CD8 T cells were incubated with DRB for 2 hours to halt transcription followed by the addition of IL-7 (10 ng/ml). Levels of CD127 mRNA were then measured at 2 hour intervals by qPCR. Comparing cells maintained in DRB alone and those treated with both DRB and IL-7 (figure 30A), the decay rate of CD127 transcripts remains the same. IL-7 does not alter the half-life of CD127 mRNA (7 hours in DRB alone and 6.6 hours with DRB+IL-7). As a control, the half-life of β actin mRNA was also measured and found to be 5.8-5.9 hours in the presence and absence of IL-7 (Figure 30B). This is consistent with the published half-life of β actin mRNA in human PBMCs of 5.6 hours (257).

To examine the rate of CD127 gene transcription, nuclear run-on assays were used. Here, biotinylated uracil was used to label nascent mRNAs which were then captured by streptavidin-coupled magnetic beads. CD8 T cells were incubated in media alone or with IL-7 (10 ng/ml) for 3 hours after which nuclei were isolated and *in vitro* transcription was allowed to occur with biotinylated uracil for 30 minutes. Nascent transcripts were captured

Figure 30. IL-7 does not affect CD127 mRNA stability in CD8 T cells. CD8 T cells were either pre-incubated with the transcription inhibitor DRB (500 μM) for 2 hr followed by the addition of IL-7 (10 ng/ml) or treated with DRB alone and cells were collected every 2 hrs. CD127 (panel A) and β actin (panel B) mRNA transcripts were measured by qPCR. Data are represented as relative change from media control and error bars show SEM (n=4).

A


CD127
- IL-7 t½=7h
+IL-7 t½=6.6h
Relative CD127 mRNA expression
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
Time (hr)

B

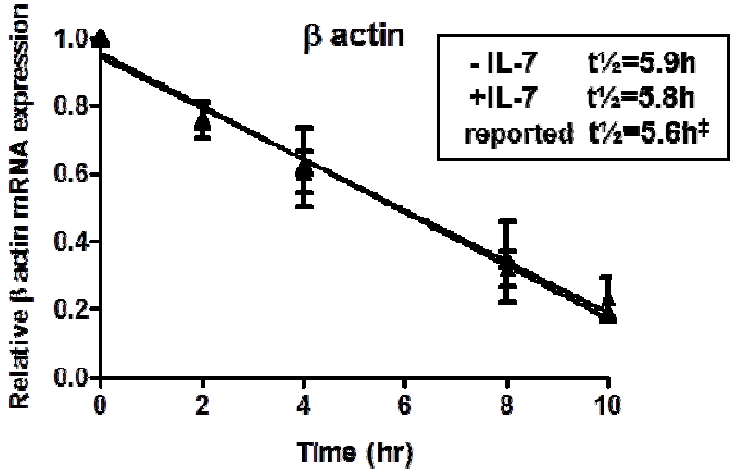

β actin
- IL-7 t½=5.9h
+IL-7 t½=5.8h
reported t½=5.6h‡
Relative β actin mRNA expression
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Time (hr)

Figure 30.

on streptavidin-coupled beads and CD127 mRNA was quantified by qPCR. As shown in figure 31, the rate of CD127 gene transcription in human CD8 T cells was significantly attenuated following treatment with IL-7.

5.2.2 IL-7 down-regulates CD127 gene transcription by inducing a STAT5-dependent protein

Interaction of IL-7 with its high affinity binding site on CD127 initiates JAK/STAT5 and PI3K signaling pathways (35, 72). Confirming the data presented in chapter 3, CD8 T cells were incubated with inhibitors of JAK, PI3K and STAT5 phosphorylation followed by the addition of IL-7 at a concentration of 10 ng/ml for 3 hours. As shown in figure 32, inhibition of JAK activity and inhibition of STAT5 phosphorylation blocked IL-7 mediated suppression of CD127 transcripts.

STAT5 proteins once phosphorylated either homodimerize or heterodimerize and translocate to the nucleus where they regulate the expression of target genes (35). Given the 40-60 minute delay in IL-7 mediated suppression of CD127 gene transcription (figure 8B), I hypothesized IL-7 signaling through JAK/STAT5 results in the up regulation of a transcriptional repressor. To investigate this, CD8 T cells were incubated in media alone, with IL-7 (10 ng/ml) for 1 hour or with the translational inhibitor cycloheximide for 1 hour followed by IL-7 treatment for an additional hour. As anticipated, cycloheximide blocked IL-7's ability to down regulate CD127 mRNA (figure 33), confirming new protein synthesis is required for CD127 transcriptional suppression.

Figure 31. IL-7 attenuates the rate of CD127 gene transcription in CD8 T cells. Nuclear run-on assays were conducted where purified CD8 T cells were first incubated in media alone or in the presence of IL-7 (10 ng/ml) for 3 hours and then nuclei were isolated and in vitro transcription was conducted. Biotinylated Uracil was used to label nascent mRNAs which were then captured by streptavidin-coupled Dynabeads and CD127 mRNA was quantified by qPCR normalizing to RPS18. Data are represented as a relative change from media control and error bars show SEM (n=4) and * denotes $p<0.001$.

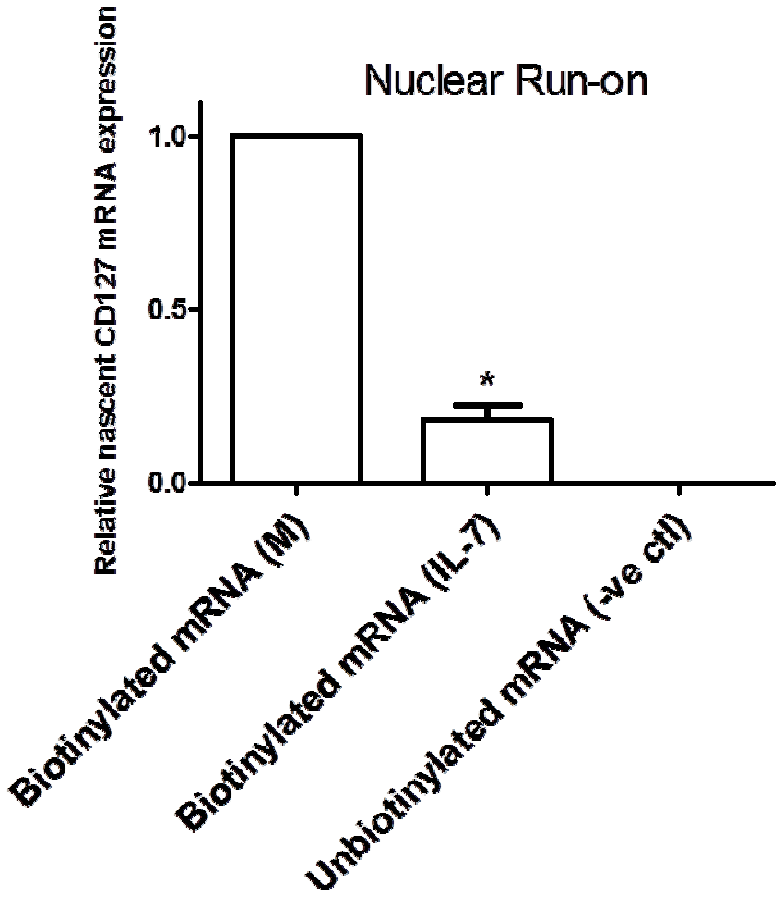
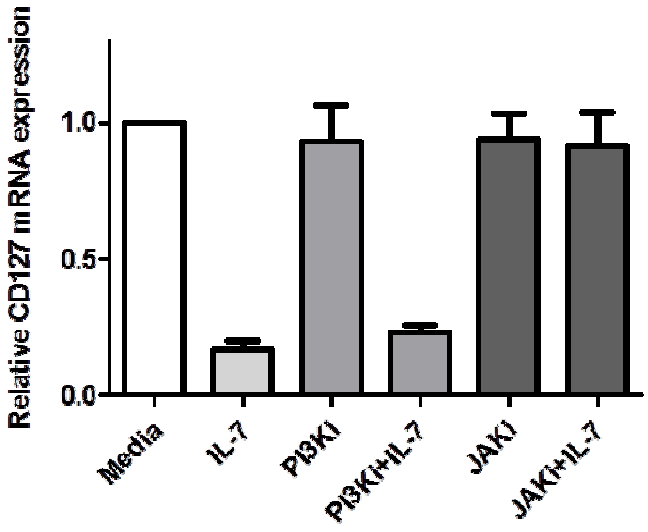

Nuclear Run-on
Relative nascent CD127 mRNA expression
1.0
0.5
0.0
*
Biotinylated mRNA (M)
Biotinylated mRNA (IL-7)
Unbiotinylated mRNA (-ve ctl)

Figure 31.

Figure 32. IL-7-induced transcriptional suppression of the CD127 gene is dependent on JAK activity and STAT5 phosphorylation but not on PI3K. Purified CD8 T cells were pre-incubated with inhibitors of JAK (JAK inhibitor 1, 1.5 μM), PI3K (LY294002, 50 μM) and STAT5 (100-500μM) for 1 hour followed by the addition of IL -7 (10 ng/ml). (A) CD127 transcripts in cells pre-treated with inhibitors of JAK or PI3K (A) or inhibitor of STAT5 (B) followed by IL-7 for 3 hrs. Transcripts were measured by qPCR and normalized to RPS18. Data are represented as fold change relative to media control. Error bars represent SEM of four independent experiments. (* $p<0.05$ compared to media).

A


Relative CD127 mRNA expression
1.0
0.5
0.0
Media
IL-7
PI3Ki
PI3Ki+IL-7
JAKi
JAKi+IL-7

B

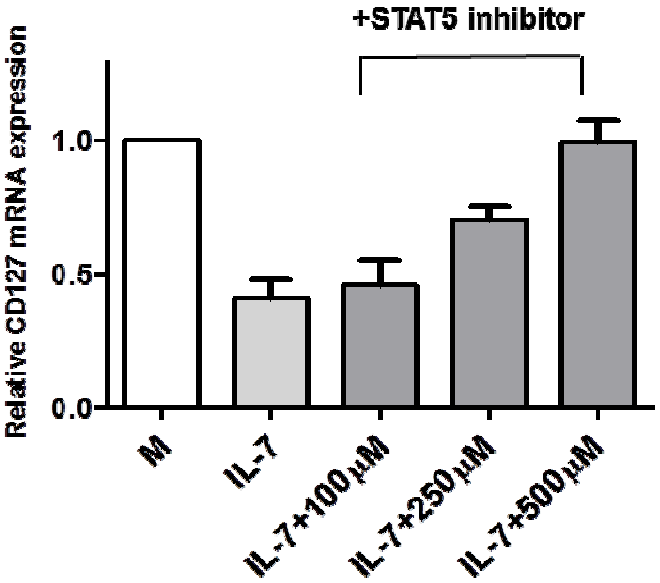

+STAT5 inhibitor
Relative CD127 mRNA expression
1.0
0.5
0.0
M
IL-7
IL-7+100μM
IL-7+250μM
IL-7+500μM

Figure 32.

Figure 33. IL-7 mediated down regulation of CD127 gene transcription is dependent on new protein synthesis. Purified CD8 T-cells were pre-treated with Cycloheximide (CHX, 100 nM) for 1 hr followed by the addition of IL-7 (10 ng/ml) for 1 hr. Transcripts were measured by qPCR and normalized to RPS18. Data are represented as fold change relative to media control. Error bars represent SEM of five independent experiments. (* $p<0.05$ compared to media).

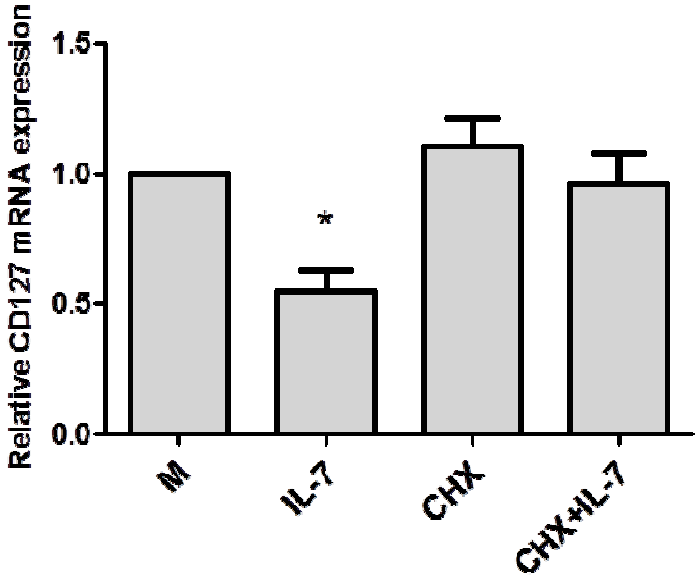


Figure 33.

5.2.3 IL-7 mediated suppression of CD127 transcription is independent of Gfi-1 expression and of Notch-1 signaling

Park *et al.* have previously shown IL-7 induced Gfi-1 down regulates CD127 gene expression in murine CD8 T cells (154, 254). To investigate the potential role of Gfi-1 in human T cells, Gfi-1 levels were measured following IL-7 treatment. Purified CD8 T cells were incubated in media alone or were treated with 10 ng/ml of IL-7 from 30 minutes to 72 hours. Cells were lysed and Gfi-1 protein expression was measured by Western blotting. As shown in figure 34A, IL-7 treatment did not up-regulate the expression of Gfi-1 protein at any time point. Given that new protein synthesis is required for IL-7 mediated suppression of CD127 transcription, these data indicate Gfi-1 is likely not involved in IL-7 mediated down regulation of CD127 gene transcription in mature resting human CD8 T cells.

Notch-1 has been shown to activate CD127 transcription in human thymocytes (252, 253). It was possible then that IL-7 down regulates CD127 gene expression by suppressing this transcriptional activator. To determine if IL-7 caused a decrease in Notch-1 expression, we measured Notch-1 mRNA transcripts by qPCR and protein expression by intracellular flow cytometry in primary human CD8 T cells. After 24 hours of IL-7 stimulation we did not see any change in Notch-1 mRNA or protein expression (figures 34B-D). There is therefore no evidence implicating Notch-1 in the regulation of CD127 expression in mature human CD8 T cells in response to IL-7.

Figure 34. Neither Gfi-1 nor Notch-1 expression changes in human CD8 T cells following treatment with IL-7. (A) Purified CD8 T cells were treated with IL-7 (10 ng/ml) for 0.5-72 hours and Gfi-1 protein expression was measured by Western blot analysis in while cell lysates. Blot were stripped and re-probed with β-actin as loading control. (B) CD8 T cells were incubated in media alone or treated with IL-7 (10 ng/ml) for 3, 12 or 24 hours after which RNA was isolated and CD127 transcripts were quantified measured by qPCR, normalizing to RPS18. To examine the level of Notch protein, CD8 T cells were incubated in media or treated with IL-7 (10 ng) for 24 hours. Cells were then fixed and permeabilized and stained for Notch-1 using a PE-labeled anti-Notch-1 monoclonal antibody. (C) Representative flow cytometry histogram. Gray fill represents IgG isotype control. Notch-1 expression in cells maintained in media is shown in red while expression in cells treated with IL-7 is shown in blue. (D) Quantification of flow data (mean channel florescence). Error bars represent SEM of three independent experiments.

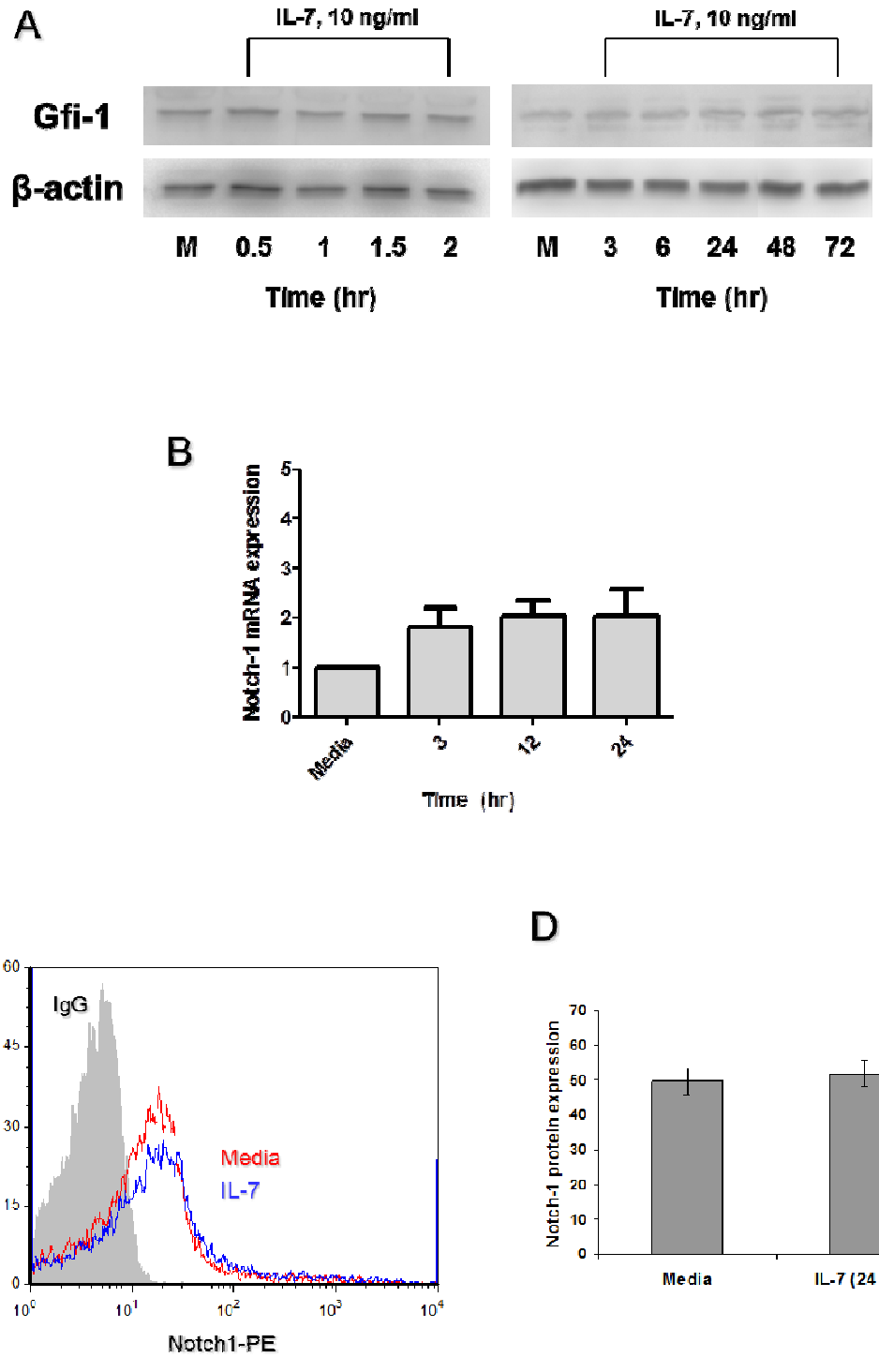

A
IL-7, 10 ng/ml
IL-7, 10 ng/ml
Gfi-1
β-actin
M 0.5 1 1.5 2
M 3 6 24 48 72
Time (hr)
Time (hr)
B
Notch-1 mRNA expression
Media
3
12
24
Time (hr)
C
IgG
Media
IL-7
Cell Counts
Notch1-PE
D
Notch-1 protein expression
Media
IL-7 (24 hrs)

Figure 34.

5.2.4 c-Myb is up-regulated by IL-7 signaling

To identify the transcriptional repressor induced by IL-7 and responsible for down regulating CD127 gene expression in human CD8 T cells, I utilized a PCR array examining the expression of 84 transcription factor genes. I first carried out two PCR arrays where CD8 T cells were incubated in media alone or treated with IL-7 (10 ng/ml) for 3 hours. To then limit the transcription factors to those induced by STAT5 phosphorylation I performed another set of arrays comparing CD8 T cells treated with IL-7 (10 ng/ml) to those treated with IL-7 in the presence of the STAT5 phosphorylation inhibitor. Nine transcription factors were identified that were induced at least 2-fold by IL-7 and required STAT5 phosphorylation (Table 7). The array data were then confirmed by qPCR analysis examining the induction of each of the nine genes in response to IL-7 at 0.5, 1.5 and 3 hours in the presence of IL-7 or IL-7 plus the STAT5 phosphorylation inhibitor. As seen in figure 35, the qPCR analysis indicated most of the transcription factor genes identified by the array were only modestly induced (<3 fold) by IL-7. c-Myb, a transcription factor with recognized repressor functions (258-262), stood out being induced >25 fold by IL-7 (Table 7 and confirmed in figure 35). This finding was consistent with the previous affymetrix microarray carried out in our lab. Here, c-Myb expression in human CD8 T cells was up-regulated 11.7 fold in response to IL-7 (10 ng/ml) at 24 hours (Table 6).

5.2.5 Induction of c-Myb expression by IL-7 mirrors CD127 transcriptional suppression

To examine the kinetics and signaling pathways involved in the IL-7 mediated induction of c-Myb, I utilized inhibitors of JAK, PI3K or STAT5 phosphorylation. CD8 T cells were incubated in media alone, with IL-7 (10 ng/ml) for 3 hours, or pre-incubated with

Table 7. Transcription factors up-regulated in human CD8 T cells in response to IL-7 and dependent on STAT5 phosphorylation. In the first analysis (IL-7 to M), purified CD8 T cells were incubated in either media alone or with IL-7 (10 ng/ml) for 3 hours. In the second analysis (S5i+IL-7 to IL-7), purified CD8 T cells were incubated either with the inhibitor of STAT5 phosphorylation (S5i) plus IL-7 (10 ng/ml) or IL-7 alone. Cells were then lysed and mRNA transcripts of 84 transcription factors were measured using PCR array plates (SABiosciences) normalizing to GAPDH and RPL13A expression. N=1.

Symbol	Description	Gene name	IL7 to M (fold)	S5i+IL-7 to IL-7 (fold)
c-Myb	V-myb myeloblastosis viral oncogene homolog (avian)	Cmyb/c-myb/c-myb_CDS/efg	27.6	-60.3
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	C/EBP-beta/CRP2/ IL6DBP/LAP	5.5	-3.4
STAT4	Signal transducer and activator of transcription 4	SLEB11	3.4	-2.4
CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma	GPE1BP/IG/EBP-1	3.3	-2.3
IRF1	Interferon regulatory factor 1	IRF-1/MAR	3.2	-3.4
ETS1	V-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	ETS-1/EWSR2/ FLJ10768	2.4	-2.7
ARNT	Aryl hydrocarbon receptor nuclear translocator	HIF-1beta/ HIF1B/ HIF1BETA/TANGO	2.4	-2.5
ESR1	Estrogen receptor 1	DKFZp686N23123/ER/ESR/ESRA/Era/NR3A1	2.2	-3.7
GATA3	GATA binding protein 3	HDR/MGC2346/MGC5199/MGC5445	2.1	-2.4

Figure 35. Confirming the PCR array by qPCR. Purified CD8 T cells were incubated in media alone or pre-incubated with an inhibitor of STAT5 phosphorylation (500μM) for 1 hour followed by the addition of IL-7 (10 ng/ml) for 3hrs, or with IL-7 alone for 0.5, 1.5 or 3hrs and the indicated transcripts were measured by qPCR and normalized to RPS18. PCR primers are shown in table 1. Data are represented as fold change relative to media control. Error bars represent standard error of mean of three independent experiments.

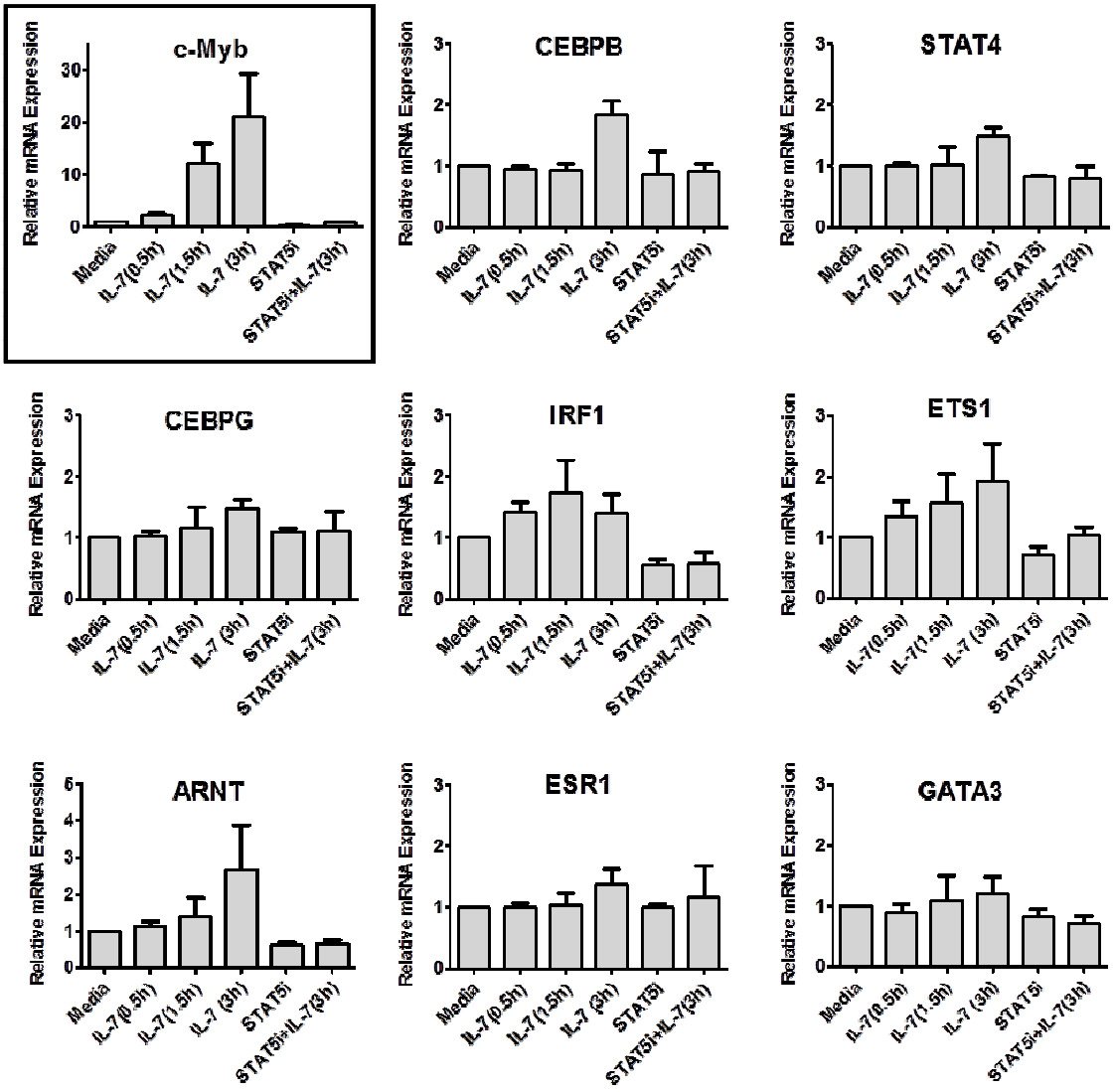

c-Myb
CEBPB
STAT4
CEBPG
IRF1
ETS1
ARNT
ESR1
GATA3
Relative mRNA Expression
Media
IL-7(0.5h)
IL-7(1.5h)
IL-7 (3h)
STAT5i
STAT5i+IL-7(3h)

Figure 35.

each of the inhibitors for 1 hour followed by addition of IL-7 (10 ng/ml) for an additional 3 hours. As expected the induction of c-Myb transcripts is, like CD127 suppression, dependent on JAK/STAT5 signaling and is independent on PI3K (Figure 36A). Next, to elucidate the kinetics of c-Myb transcript induction, purified CD8 T cells were incubated with increasing doses of IL-7 (0.1-10 ng/ml) for 3-48 hours and simultaneous levels of c-Myb and CD127 transcripts were measured by qPCR analysis. As shown in figure 36B, IL-7 up-regulates c-Myb mRNA transcripts in a time- and dose-dependent manner. Interestingly, the kinetics of c-Myb induction and dose response to IL-7 mirrors IL-7 mediated suppression of CD127 gene transcription. Although only correlative, these data are consistent with c-Myb acting as a transcriptional repressor to down regulate CD127 gene expression.

To confirm induction of c-Myb transcripts by IL-7 is reflected at the protein level, CD8 T cells were incubated in either media or with IL-7 (10 ng/ml) for up to 24 hours and c-Myb protein was examined by Western blotting. As seen in figure 36C, c-Myb protein is significantly induced within 1 hour and was maintained for greater than 24 hours following IL-7 stimulation. It is worth noting the appearance of c-Myb protein between 30-60 minutes following addition of IL-7 coincides exactly with the 40-60 minute delay in CD127 transcriptional suppression noted in figure 8B.

The correlation between STAT5 phosphorylation, c-Myb induction and CD127 transcriptional suppression was further examined in an IL-7 washout experiment. Here, CD8 T cells were incubated with IL-7 (10 ng/ml) for 24 hours and then either maintained in IL-7 for an additional 24 hours or were washed 3 times with PBS and resuspended in fresh media in the absence of IL-7. Levels of intracellular phospho-STAT5 as well as c-Myb and CD127 mRNA were measured by flow cytometry and qPCR respectively. As seen in figure 37, in the ongoing presence of IL-7, STAT5 phosphorylation was maintained along with persistent

Figure 36. IL-7 up regulates c-Myb mRNA transcripts in human CD8 T cells in a time and dose-dependent manner via JAK/STAT5 signaling, mirroring IL-7 suppression of CD127 gene transcription. (A) CD8 T cells were pre-incubated with inhibitors of JAK (JAK inhibitor 1, 1.5 μM), PI3K (LY294002, 50 μM) and STAT5 phosphorylation (500 μM) for 1 hour followed by the addition of IL-7 (10 ng/ml) for 3 hours or with IL-7 alone and c-Myb transcripts were measured by qPCR normalizing to RPS18. Data are represented as fold change relative to media control. (B) IL-7 induces the up regulation of c-Myb transcripts in human CD8 T cells in a time and dose-dependent manner, mirroring the down regulation of CD127 transcripts. Purified CD8 T cells were incubated in media alone or in the presence of 100, 1000 and 10,000 pg/ml of IL-7 for 3, 12, 24 and 48 hours. c-Myb mRNA transcripts were measured by qPCR and normalized to RPS18 expression. Data are represented as fold change relative to media control. Error bars represent standard error of mean of three independent experiments. (C) IL-7 also induces c-Myb protein expression in human CD8 T cells. Purified CD8 T cells were treated with IL-7 (10 ng/ml) for 0.5-24 hours and c-Myb protein expression was measured by Western blot analysis using a polyclonal anti-human c-Myb antibody. Blots were then stripped and re-probed for β-actin as loading control.

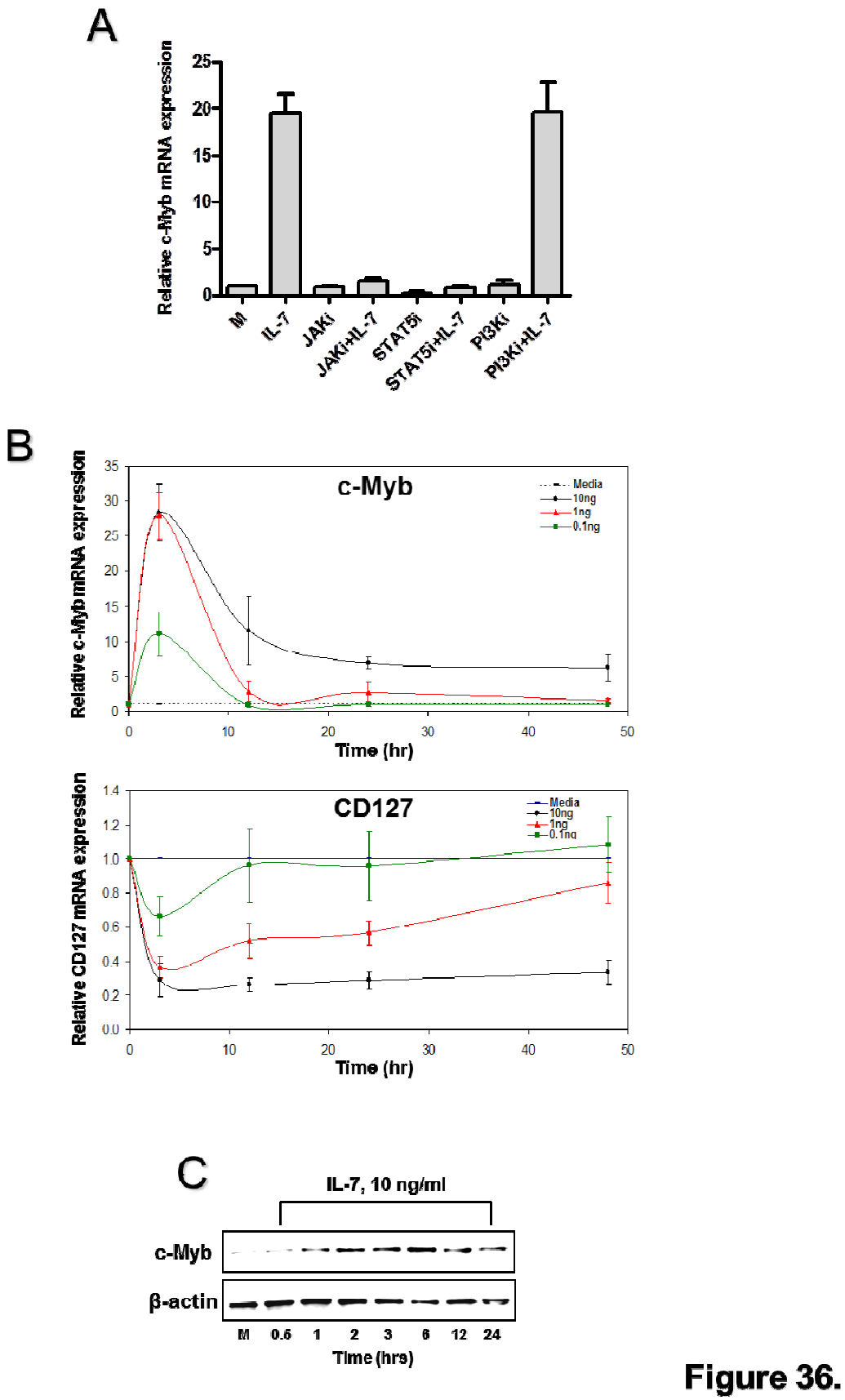

A
Relative c-Myb mRNA expression
25
20
15
10
5
0
M
IL-7
JAKi
JAKi+IL-7
STAT5i
STAT5i+IL-7
PI3Ki
PI3Ki+IL-7
B
c-Myb
Media
10ng
1ng
0.1ng
Relative c-Myb mRNA expression
Time (hr)
CD127
Relative CD127 mRNA expression
Time (hr)
C
IL-7, 10 ng/ml
c-Myb
β-actin
M
0.5
1
2
3
6
12
24
Time (hrs)

Figure 36.

Figure 37. Constant IL-7 signaling is required to maintain STAT5 phosphorylation, c-Myb induction and CD127 suppression. Purified CD8 T cells were treated with IL-7 (10 ng/ml) for 24 hrs and then either maintained in IL-7 for additional 24 hrs or washed 3 times with PBS and incubated in IL-7-free fresh media for 24 hours. To examine the level of phosphor-STAT5 proteins, cells were fixed and permeabilized and stained for pSTAT5 using a FITC-labeled monoclonal anti-phospho-STAT5 antibody. (A) Levels of intracellular phospho-STAT5 were measured by flow cytometry. To measured c-Myb and CD127 mRNA transcripts, RNA was isolated and c-Myb (B) and CD127 (C) transcripts were quantified by qPCR, normalizing to RPS18. Data are represented as a fold change from media control and error bars represent standard error of the mean of three independent experiments.

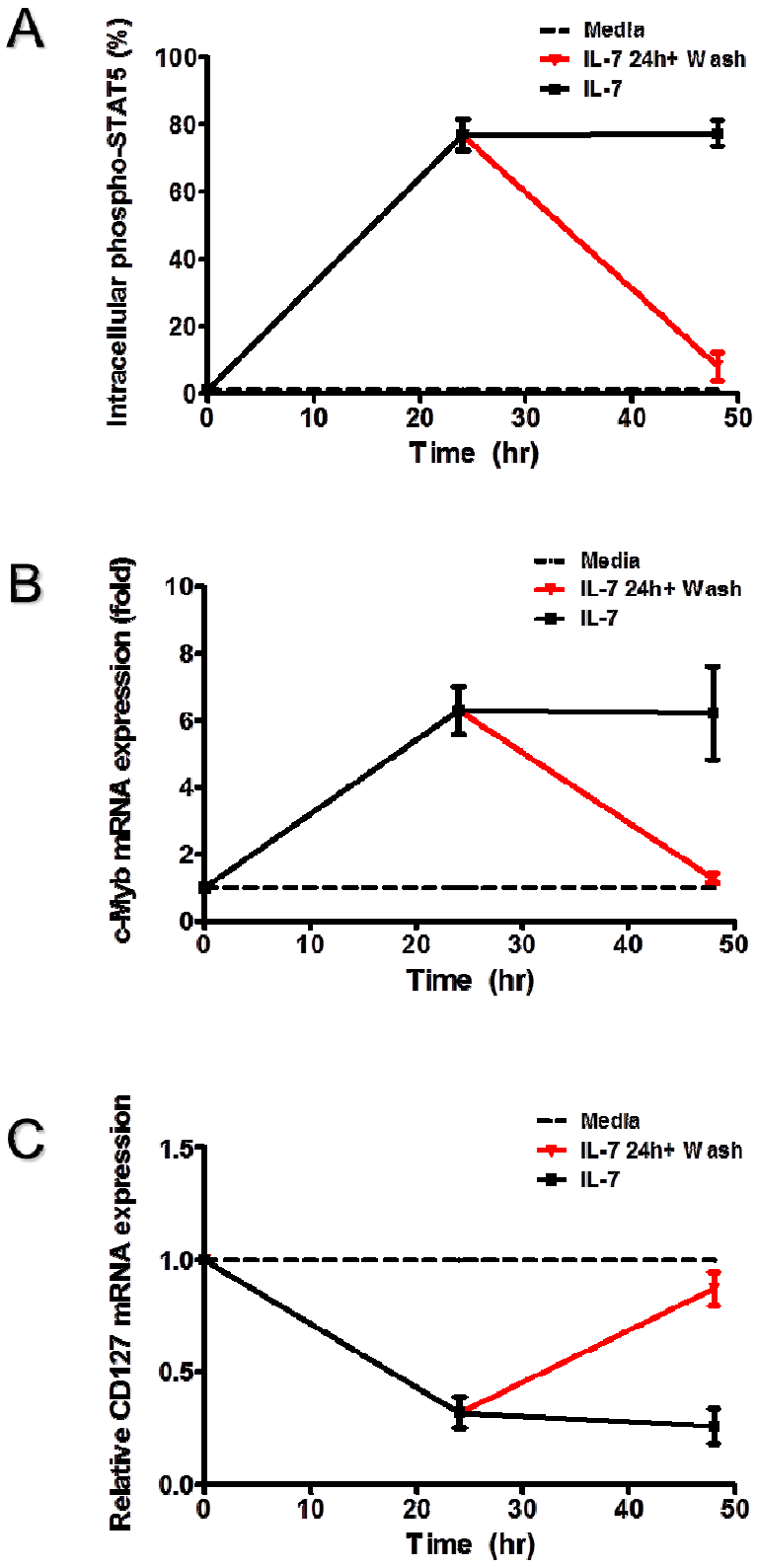

A
Intracellular phospho-STAT5 (%)
Media
IL-7 24h+ Wash
IL-7
Time (hr)
B
c-Myb mRNA expression (fold)
Media
IL-7 24h+ Wash
IL-7
Time (hr)
C
Relative CD127 mRNA expression
Media
IL-7 24h+ Wash
IL-7
Time (hr)

Figure 37.

c-Myb expression and CD127 transcript suppression. When IL-7 was removed from the media, the decrease in STAT5 phosphorylation correlated with a drop in c-Myb expression and an increase in CD127 transcripts.

Lastly, the inverse correlation between expression of c-Myb and CD127 was examined in several lymphoblastic T cell lines including CEM, A3, SupT and Jurkat T cells. In all four T cell lines and in primary human CD8 T cells c-Myb and CD127 mRNA and protein were measured by qPCR and Western respectively. Whereas CD8 T cells express high levels of CD127 and low levels of c-Myb mRNA and protein, the association is reversed in all four T cell lines (figure 38).

This consistent inverse correlation between c-Myb and CD127 expression strongly implicates c-Myb as a possible transcriptional repressor regulating CD127 gene expression in response to IL-7.

5.2.6 siRNA mediated knock down of c-Myb relieves IL-7 mediated CD127 transcriptional suppression

siRNA-mediated knock-down assays of c-Myb gene expression in primary human CD8 T cells were carried out using the Accell siRNA system (234, 235). Because IL-7 at 10 ng/ml so significantly increases the level of c-Myb mRNA (20-30 fold) lower amounts of IL-7 (0.2 ng/ml) were used in these experiments to allow more effective block of the induction by siRNA. Four pairs of siRNA sequences targeting human c-Myb were utilized to specifically knock-down c-Myb gene expression along with a scrambled non-targeting siRNA as control. CD8 T cells were washed three times in PBS and resuspended at 2×10^6 cells/ml in Accell siRNA delivery media (Dharmacon) containing siRNA targeting c-Myb or scrambled control at a final concentration of 0.3 μM and incubated at 37^0C for four days.

Figure 38. Expression levels of c-Myb and CD127 transcripts and protein are inversely correlated in several T lymphoblastic cell lines. (A) Purified CD8 T cells and four human T-lymphoblastic T-cell lines (1.0×10^6 cells per condition) were examined for expression of CD127 and c-Myb transcripts by qPCR, normalizing to RPS18. The level of transcripts in CD8 T cells was set at 1 and the cell lines are shown as relative fold change. **(B)** As above, 1.0×10^6 cells were harvested and whole cell lysates were analyzed by Western using anti-human CD127 and anti-human c-Myb antibodies. Blots were then stripped and re-probed for β-actin as loading control. (B) Quantification of respective protein band intensities from two independent experiments using unsaturated images and normalizing band pixel intensity to β-actin in each lane. Values represent fold change relative to media control and error bars represent standard error of the mean.

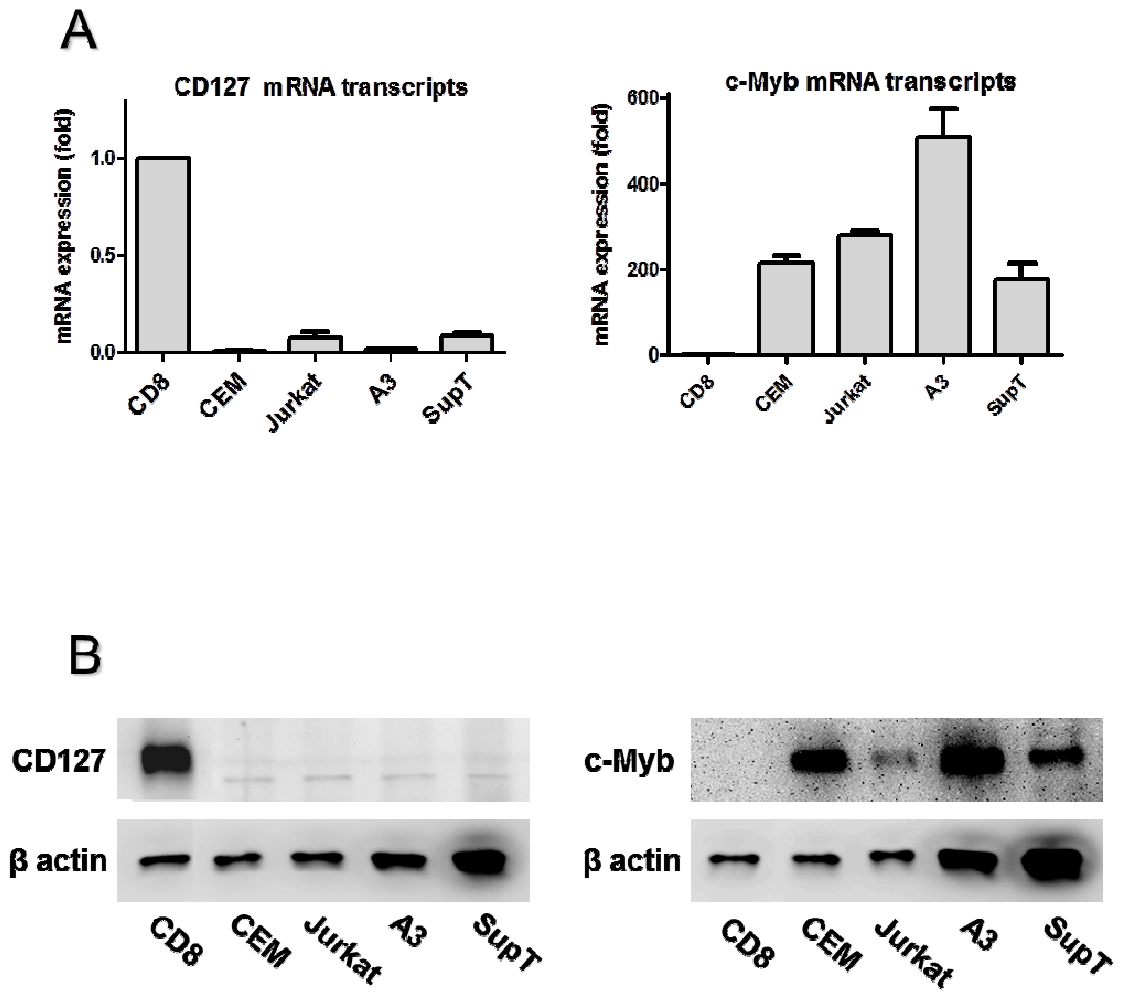

A
CD127 mRNA transcripts
mRNA expression (fold)
1.0
0.5
0.0
CD8
CEM
Jurkat
A3
SupT
c-Myb mRNA transcripts
mRNA expression (fold)
600
400
200
0
CD8
CEM
Jurkat
A3
SupT
B
CD127
β actin
CD8
CEM
Jurkat
A3
SupT
c-Myb
β actin
CD8
CEM
Jurkat
A3
SupT

Figure 38.

Cells then either remained incubated in media or were treated with IL-7 (0.2 ng/ml) for 1 hour and cells were collected, lysed and CD127 and c-Myb mRNA was quantified by qPCR. The non-targeting siRNA did not affect the basal levels of c-Myb or CD127 mRNA or the IL-7 mediated induction of c-Myb or suppression of CD127 transcripts (figure 39A and 40C). Using the siRNA targeting c-Myb, we were able to partially attenuate the induction of c-Myb mRNA by IL-7 by approximately 40% (figure 39B). This was confirmed by Western blotting showing lower levels of c-Myb protein in CD8 T cells transfected with the c-Myb siRNA and treated with IL-7 (0.2 ng/ml) for 1 hour (figure 39E). Under these conditions where induction of c-Myb was attenuated, transcriptional suppression of the CD127 gene by IL-7 was partially relieved showing higher levels of CD127 mRNA ($p<0.05$).

5.2.7 Ectopic expression of c-Myb prevents recovery of CD127 mRNA following TCR stimulation

We next asked whether over-expressing c-Myb protein in primary human CD8 T cells would affect CD127 gene transcription. We reasoned if c-Myb is implicated in the repression of CD127 gene transcription as indicated by the knock-down assays then over-expressing c-Myb would result in down-regulation of CD127 mRNA expression. As primary human CD8 T cells are inherently difficult to transfect, we utilized an alternative strategy whereby the cells were stimulated via the TCR with anti-CD3/anti-CD28 antibodies. This greatly increases transfection efficiency. TCR stimulation also results in significant down regulation of surface CD127 proteins and transcripts (approximately 80%) (167, 263). Following stimulation, levels of CD127 recover completely within 48 hours (263). We therefore examined whether c-Myb would prevent the recovery of CD127 transcripts after TCR stimulation. Here, CD8 T cells were isolated as described above and cultured overnight

Figure 39. siRNA-mediated c-Myb knock down relieves IL-7 mediated suppression of the CD127 gene. Purified CD8 T cells were incubated with siRNA targeting c-Myb or with a non-specific scrambled siRNA control for 4 days followed by the addition of IL-7 (200 pg/ml) for one hour and (A-B) c-Myb or (C-D) CD127 mRNA transcripts were measured by qPCR normalizing to RPS18. Error bars represent standard error of the mean of seven independent experiments and * denotes $p<0.05$. Data are represented as fold changes relative to media control. Knock-down efficiency was also monitored by Western blot analysis. CD8 T cells (1×10^6 cells per condition) were treated as above and whole cell lysates were examined by Western using a polyclonal antibody against human c-Myb. Blots were stripped and re-probed for β-actin as a loading control.

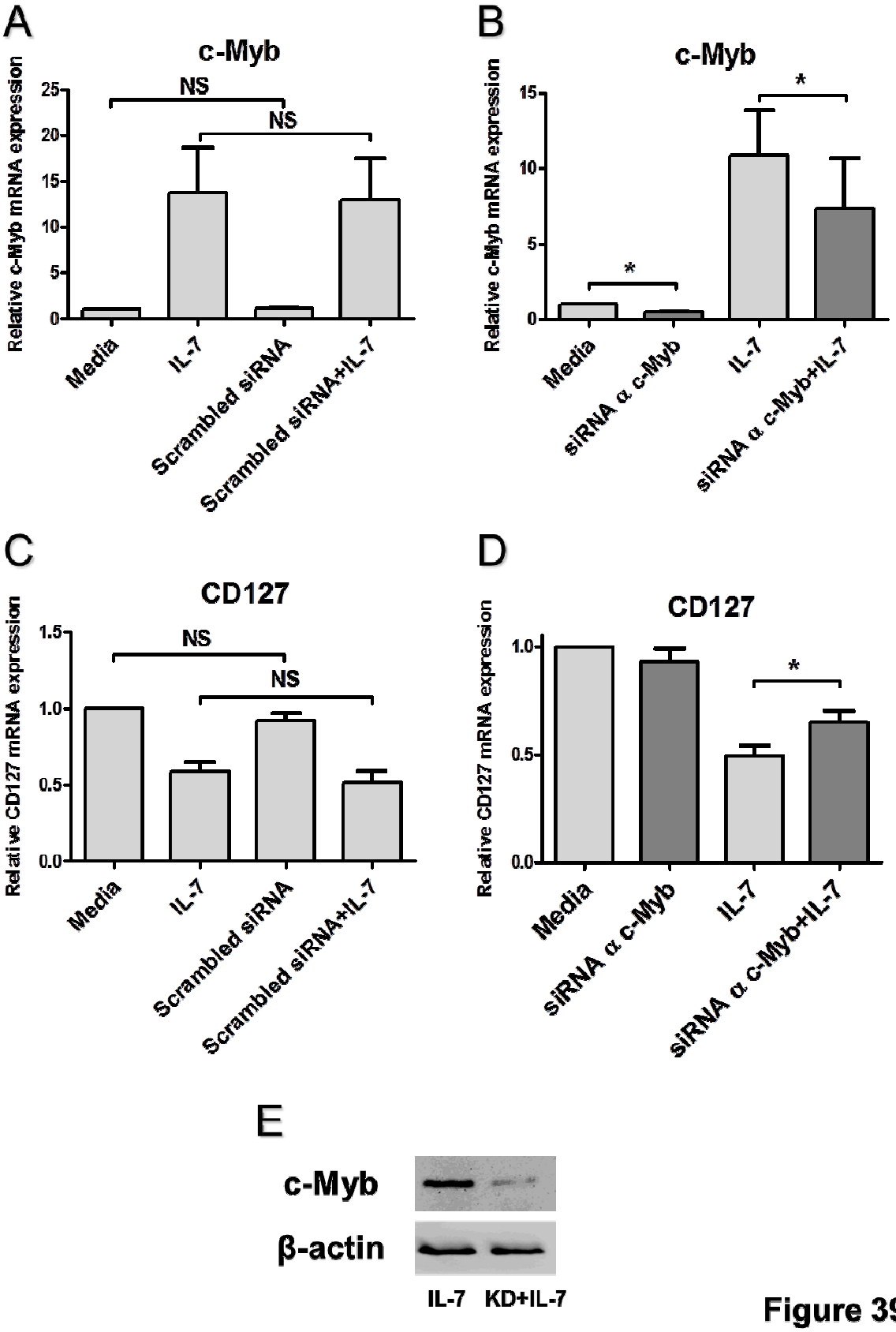

A
c-Myb
Relative c-Myb mRNA expression
NS
NS
25
20
15
10
5
0
Media
IL-7
Scrambled siRNA
Scrambled siRNA+IL-7
B
c-Myb
Relative c-Myb mRNA expression
*
*
15
10
5
0
Media
siRNA α c-Myb
IL-7
siRNA α c-Myb+IL-7
C
CD127
Relative CD127 mRNA expression
NS
NS
1.5
1.0
0.5
0.0
Media
IL-7
Scrambled siRNA
Scrambled siRNA+IL-7
D
CD127
Relative CD127 mRNA expression
*
1.0
0.5
0.0
Media
siRNA α c-Myb
IL-7
siRNA α c-Myb+IL-7
E
c-Myb
β-actin
IL-7
KD+IL-7

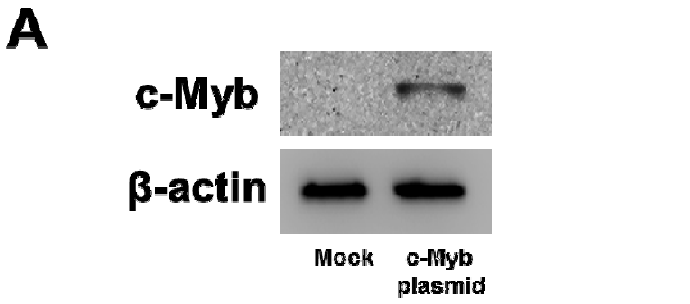
Figure 39.

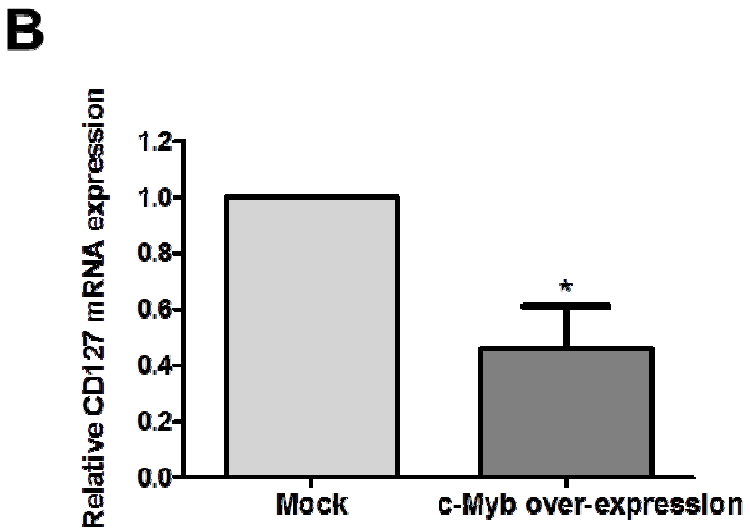
in RPMI-20. The next day cells were stimulated by treating with anti-CD3/anti-CD28 antibodies conjugated to magnetic beads. After 24 hours, the beads were removed using magnetic separation and CD8 T cells were transfected with an expression plasmid coding for c-Myb or with an empty plasmid control by nucleofection. Forty eight hours later, levels of CD127 transcripts and surface protein were measured by qPCR and flow cytometry respectively and levels of c-Myb protein were measured by Western blotting. As shown in figure 40A, c-Myb protein was effectively expressed in primary CD8 T cells using the c-Myb encoding plasmid. As anticipated, when c-Myb was transiently over-expressed in primary CD8 T cells, levels of CD127 transcripts were significantly suppressed compared to cells transfected with the control plasmid. Thus, c-Myb is able to prevent the recovery of CD127 transcripts (figure 40B). The effect was also observed at the level of surface CD127 protein (figure 40C). c-Myb then does act as a suppressor of CD127 gene expression.

5.2.8 The IL-7 responsive region within the CD127 gene promoter is located between -1760 and -2406 bp upstream of TATA and contains three putative c-Myb binding sites

Finally, to identify the region within the CD127 gene promoter required for IL-7 mediated transcriptional suppression, existing constructs from our lab were used. Here the CD127 gene promoter from the TATA box to -2900 bp and progressive truncations thereof were cloned and placed upstream of the firefly luciferase reporter gene (clones generated by J. Kakal (202), figure 41). To identify the IL-7 repressive region, CD8 T cells were incubated either in media alone or in presence of 10 ng/ml IL-7 for 6 hours to up regulate the transcriptional repressor (presumably c-Myb) and were then transfected with the different CD127 gene promoter constructs. After transfection, the cells were maintained in media containing IL-7 (10 ng/ml) for an additional 24 hours and were then lysed and luciferase

Figure 40. Ectopic expression of c-Myb prevents recovery of CD127 mRNA following TCR stimulation. Purified human CD8 T cells were activated by TCR stimulation with anti-CD3 and anti-CD28 antibodies for 24 hours and then transfected with a plasmid encoding c-Myb or with control plasmid (mock). Following transfection, activation beads (anti-CD3/anti-CD28) were removed and cells were allowed to recover for 48 hours. (A) Expression of c-Myb protein was examined by Western blotting using a polyclonal antibody against human c-Myb. Blots were stripped and re-probed for β-actin as a loading control. CD127 mRNA transcripts (B) and surface proteins (C) were measured by qPCR normalizing to RPS18, and flow cytometry respectfully. Data are represented as fold change relative to mock. Error bars represent standard error of the mean of four independent experiments and * denotes $p<0.05$.


A
c-Myb
β-actin
Mock
c-Myb plasmid


B
Relative CD127 mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Mock
c-Myb over-expression

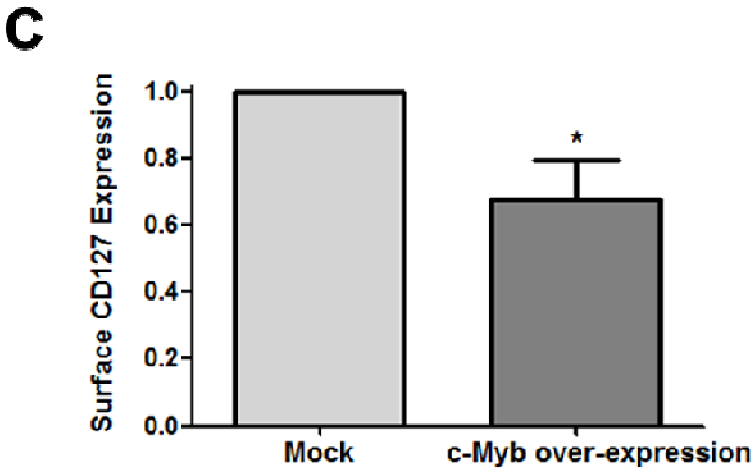

C
Surface CD127 Expression
1.0
0.8
0.6
0.4
0.2
0.0
*
Mock
c-Myb over-expression

Figure 40.

Figure 41. Schematic illustration of the different CD127 gene promoter truncation constructs. Schematic diagrams showing the various CD127 promoter constructs that were transfected into human CD8 T cells. Five constructs were used spanning from -626, -1468, -1760, -2406 and -2900 to the TATA box.

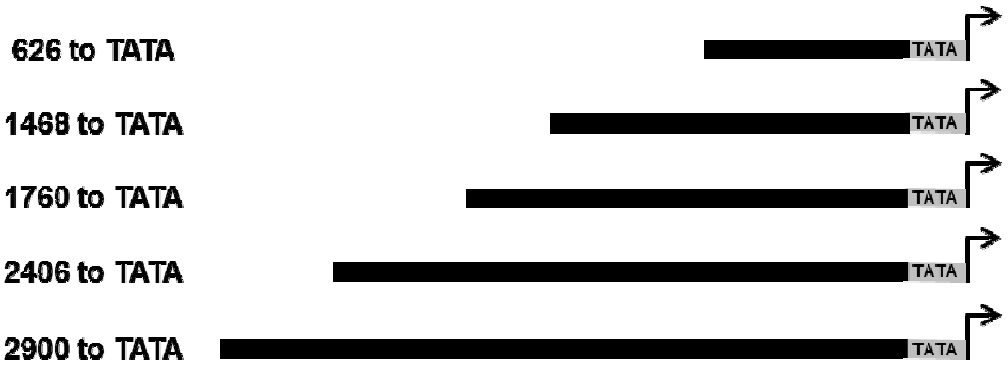

626 to TATA
TATA
1468 to TATA
TATA
1760 to TATA
TATA
2406 to TATA
TATA
2900 to TATA
TATA

Figure 41.

activity was measured and compared to cells maintained in media alone. As shown in figure 42, promoter constructs up to 1760 bp upstream of TATA were not responsive to IL-7 and failed to down regulate luciferase activity. However, the constructs extending to -2406 and -2900 bp were significantly suppressed by IL-7. These data then suggest the promoter element involved in CD127 transcriptional suppression by IL-7 lies within the region of -1760 bp to -2406 from TATA.

To further implicate c-Myb in the suppression of CD127 gene transcription, we examined the activity of these promoter constructs in Jurkat cells which express high levels of endogenous c-Myb protein. As expected, promoter truncations from TATA to -1760 have the higher luciferase activity (figure 43). In contrast, promoter constructs extending to -2406 and -2900 were significantly suppressed, confirming a repressor region lies within the region -1760 to -2406 bp upstream of TATA. Of particular note, the CD127 gene promoter region from -1760 to -2406 contains three putative c-Myb binding sites with the consensus sequence of C/TAACG/TG, one on the sense strand (-1806 to -1811 [TAACTG]) and two on the anti-sense strand (-1863 to -1868 [CAACTG]) and (-2165 to -2170 [AAACTG]). There is an additional c-Myb binding site located -2729 to -2734 bp upstream of TATA [CAACTG] (figure 44). Three of these sites are identical to the Myb consensus sequence (-1806, -1863 and -2729) while one is 83% homologous to the consensus sequence (-2165).

To finally confirm a role for c-Myb in suppressing CD127 gene transcription, the four identified c-Myb binding sites were mutated within the full length CD127 gene promoter construct. All 6 nucleotides in each of the c-Myb binding sites were mutated to scrambled sequences by site-directed mutagenesis. Each of the four sites (-1806, -1863, -2165 and -2729) was mutated one at a time and in combination. As control, one additional putative

Figure 42. The region within the CD127 gene promoter required for IL-7 mediated suppression of CD127 gene transcription in human CD8 T cells is located between -1760 to -2406 upstream of TATA. IL-7 mediated transcriptional suppression from truncated CD127 promoter constructs using luciferase as reporter. CD8 T cells ($5x10^6$ cells per condition) were incubated in IL-7 (10 ng/ml) for 6 hours and then transfected with CD127 promoter constructs for 24 hours by nucleofection and then promoter activity was measured by Luciferase assay. The region from -1760 to -2406 is required for suppression with IL-7 (10 ng/ml, 6 hours). Bar graph shows mean fold change in luciferase expression in CD8 T cells treated with IL-7 normalized to pGL4B control. Error bars show SEM (n=3). * denotes p value <0.05 compared to vector control.

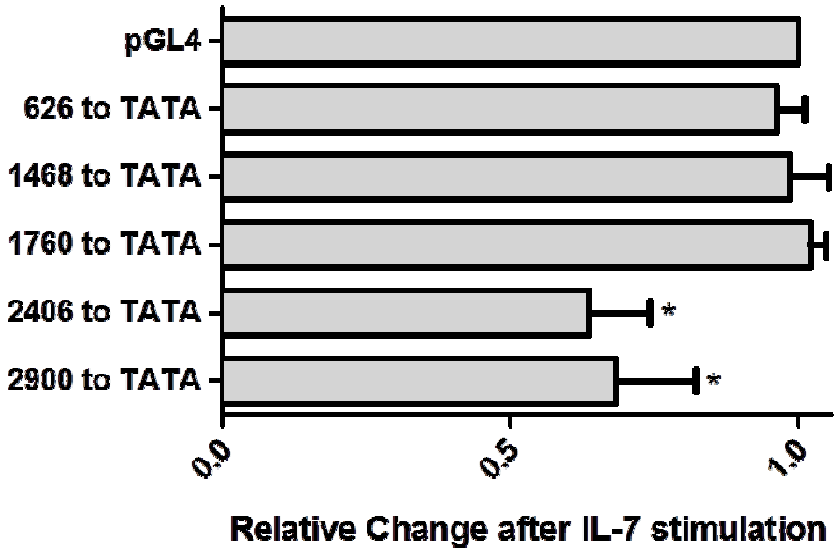

pGL4
626 to TATA
1468 to TATA
1760 to TATA
2406 to TATA
2900 to TATA
*
*
0.0
0.5
1.0
Relative Change after IL-7 stimulation

Figure 42.

Figure 43. Luciferase expression driven by D127 promoter constructs beyond -1760 is suppressed in Jurkat cells expressing high levels of c-Myb protein. Jurkat cells ($5x10^6$ cells per condition) were transfected with the different promoter truncation constructs by nucleofection and luciferase expression was measured 24 hours later and was normalized to vector control (pGL4B). Bar graph show mean fold change in luciferase gene expression in Jurkat cells. Error bars show Standard Error of the Mean (SEM) (n=3). * denotes p value <0.05 compared to vector (pGL4B) control.

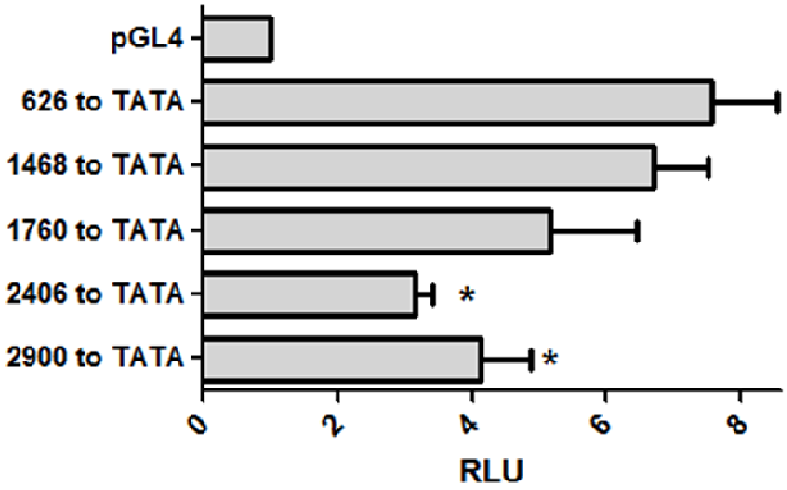

pGL4
626 to TATA
1468 to TATA
1760 to TATA
2406 to TATA
2900 to TATA
*
*
0
2
4
6
8
RLU

Figure 43.

Figure 44. The region within the CD127 gene promoter responsive to IL-7 suppression contains three putative c-Myb binding sites. The human CD127 genomic sequence was analyzed using the web-based putative transcription factor binding site identification software programs: Transcription Element Search System (TESS; http://www.cbil.upenn.edu/tess/) as well as Genomatix MatInspector program (http://www. genomatix.de) and putative c-Myb binding sites were identified. Factors were mapped based on their ability to bind to the sense (in green) strand or antisense strand (in red). Putative c-Myb binding sites are shown at -1806, -1863, -2165 and -2729.


TSS
2729
2165
1863
1806
TATA Box
-3000
-2000
-1000
Myb binding sites
On sense strand
On antisense strand

Figure 44.

c-Myb binding site that is not located within the suppressive region (located at -1720 bp upstream of TATA) was also mutated. A summary of all the plasmids harboring mutations of the c-Myb binding sites within the CD127 gene promoter as well as the control plasmids is depicted in figure 45. To test the activity of the promoter containing the mutations, the plasmids were transfected into Jurkat cells that express high levels of c-Myb. The promoter construct from TATA to -1468 was used as positive control. As expected, the promoter construct from TATA to -2900 generated lower luciferase activity compared to the construct containing TATA to -1468. The mutation at -1720 downstream of the suppressive region did not relieve the suppression (figure 46A). In addition, mutating each of the three individual sites (-1806, -1863 and -2165) within the -1760 to -2406 region did not relieve the suppression. However, when double mutants of the c-Myb binding sites within the -1760 to -2406 region were generated, the combinations of (-1806 and -1863) and (-1863 and -2165) showed higher luciferase expression compared to the full length promoter ($p<0.05$, figure 46B). Interestingly, mutating the site (-2729) on its own partially blocked the suppression of the promoter, although not statistically significant, suggesting a role for the most upstream c-Myb binding site (figure 46A). Two additional constructs were then generated containing triple mutations that are likely relevant for the suppression (-1806, -1863 and -2729) and (-1806, -1863 and -2165) as well as one construct with all four c-Myb sites mutated (-1806, -1863, -2165 and -2729). Both plasmids with three mutations showed significant relief of suppression compared to full length plasmid ($p<0.05$, figure 46C).

While the quadruple mutation showed the highest luciferase expression, notably the difference between the activity of the construct with mutations at sites -1806, -1863 and -2729 and the quadruple mutant is not significant ($p>0.05$), suggesting the c-Myb site at –

Figure 45. Schematic illustration of the various constructs containing mutations in c-Myb binding sites within the CD127 gene promoter. Schematic diagrams showing the various promoter constructs containing mutations in c-Myb binding sites within the human CD127 gene promoter. These sites are detailed in table 3. In black: putative c-Myb binding sites and in red: sites that were mutated by site directed mutagenesis.

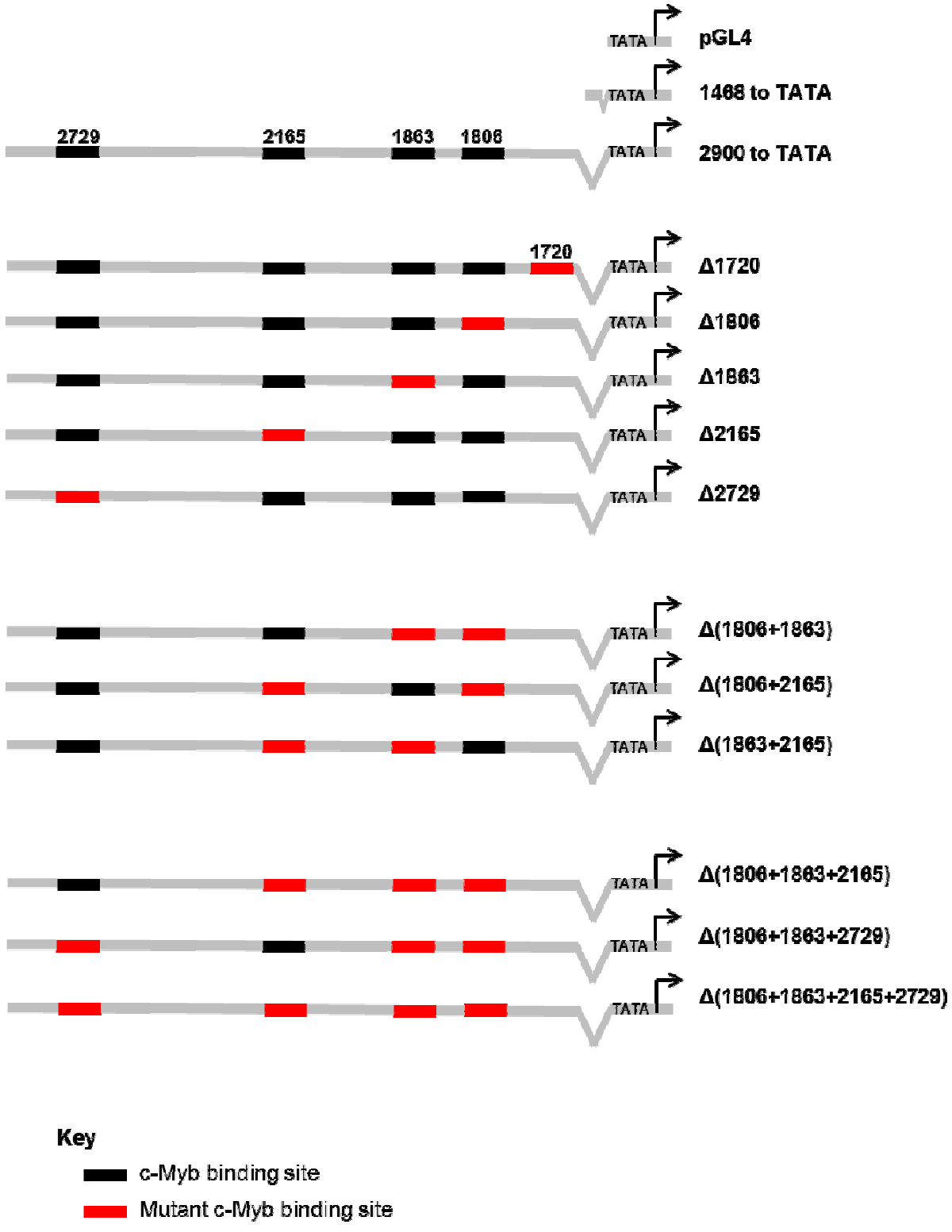

TATA
pGL4
1468 to TATA
2729
2165
1863
1806
2900 to TATA
1720
Δ1720
Δ1806
Δ1863
Δ2165
Δ2729
Δ(1806+1863)
Δ(1806+2165)
Δ(1863+2165)
Δ(1806+1863+2165)
Δ(1806+1863+2729)
Δ(1806+1863+2165+2729)
Key
c-Myb binding site
Mutant c-Myb binding site

Figure 45.

Figure 46. Mutation of c-Myb binding sites within the region on CD127 promoter responsive to IL-7 suppression relives the suppression of the promoter activity. Identified c-Myb binding sites within the region -1760 to -2900 from TATA which is responsive to IL-7 suppression were mutated by site directed mutagenesis. Jurkat cells ($5x10^6$ cells per condition) were transfected with the promoter truncation constructs by nucleofection and luciferase expression of the different promoter constructs containing (A) single (B) double or (C) triple mutations in the c-Myb binding sites illustrated in figure 46 was measured 24 hours later and was normalized to vector control (pGL4B). Bar graph show mean fold change in luciferase gene expression in Jurkat cells. Error bars show Standard Error of the Mean (SEM) (n=3). * denotes p value <0.05 compared to vector control.

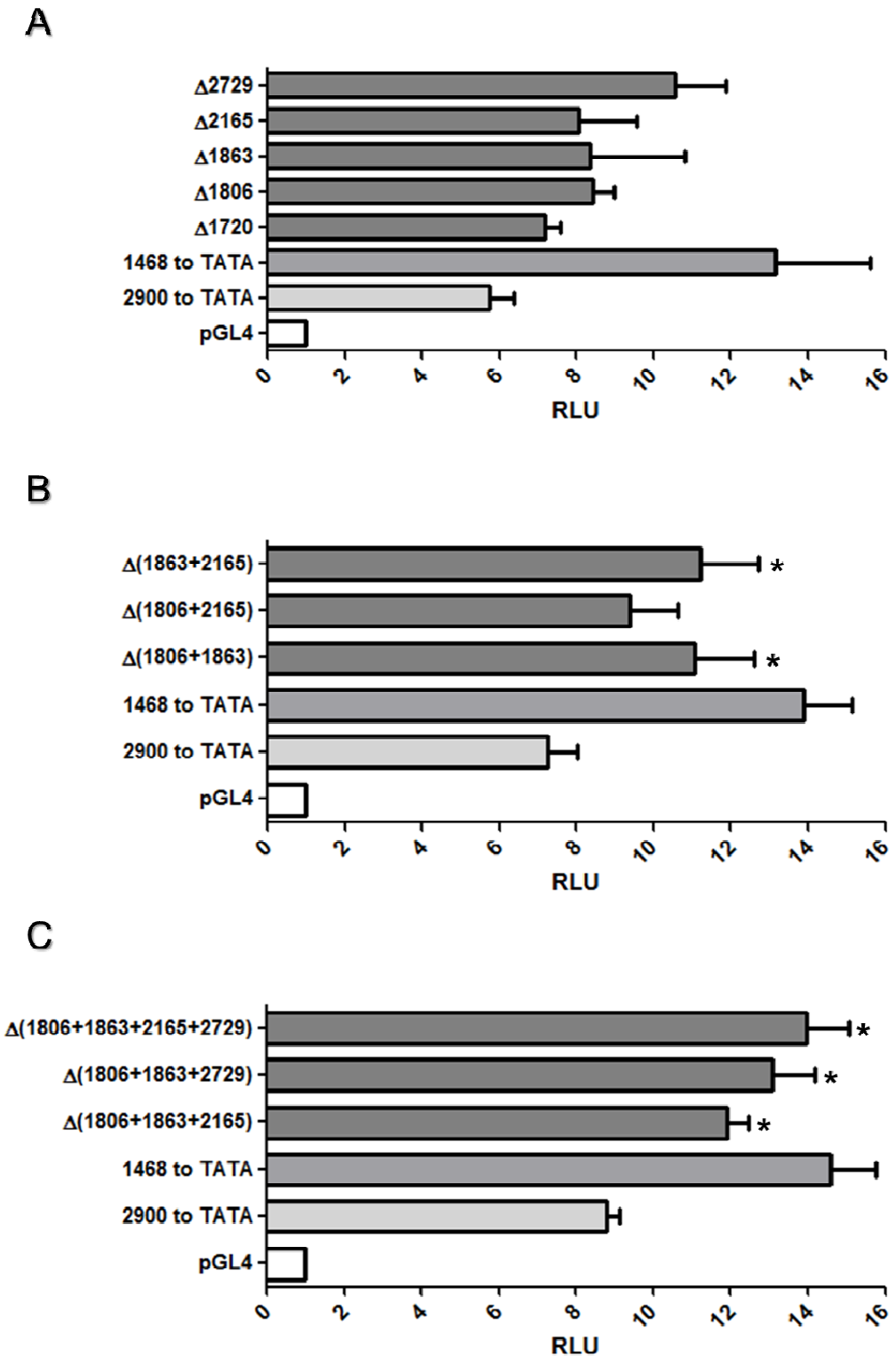

A
Δ2729
Δ2165
Δ1863
Δ1806
Δ1720
1468 to TATA
2900 to TATA
pGL4
0
2
4
6
8
10
12
14
16
RLU
B
Δ(1863+2165)
Δ(1806+2165)
Δ(1806+1863)
1468 to TATA
2900 to TATA
pGL4
*
*
0
2
4
6
8
10
12
14
16
RLU
C
Δ(1806+1863+2165+2729)
Δ(1806+1863+2729)
Δ(1806+1863+2165)
1468 to TATA
2900 to TATA
pGL4
*
*
*
0
2
4
6
8
10
12
14
16
RLU

Figure 46.

2165 may play a minor role -if any- in the transcriptional suppression. Collectively, these data identify the repressive region within the CD127 gene promoter and demonstrate a role for the c-Myb binding sites in suppressing CD127 gene transcription.

5.3 Discussion

Given the different roles IL-7 plays at different stages of the T cell life span, it is anticipated expression of the IL-7 receptor will be regulated by different constellations of transcription factors in different settings. While IL-7 was shown to up regulate the expression of the transcriptional repressor Gfi-1 which down-regulates CD127 transcription in mice (154, 254, 264), it has already been suggested that the mechanism of suppression in human T cells may be different and independent of Gfi-1 (197, 244). In fact, I demonstrated here the lack of induction of Gfi-1 proteins in response to IL-7 stimulation in primary human CD8 T cells (figure 34A). Indeed, here I implicate c-Myb in IL-7 mediated suppression of CD127 gene transcription in mature human CD8 T cells.

In this chapter I have shown that while IL-7 does not affect the half-life of CD127 mRNA (figure 30), it does suppress the rate of CD127 gene transcription as measured by nuclear run-on assays (figure 31). JAK/STAT5 signaling is required for this down regulation and appears to up regulate a repressor as inhibition of *de novo* protein synthesis prevents IL-7 mediated suppression of CD127 transcripts. Using PCR arrays, DNA microarrays and qPCR, c-Myb was identified as an IL-7 inducible gene. The IL-7 mediated induction of c-Myb was time and dose dependent and required JAK/STAT5 signaling. Further, the induction profile of c-Myb mirrored the kinetics of CD127 suppression (figure 36C) and the appearance of c-Myb protein between 30-60 minutes following addition of IL-7 coincides exactly with the 40-60 minute delay in CD127 transcriptional suppression. In addition, T lymphoblastic cell lines expressing high levels of c-Myb also express low levels of CD127. Using siRNA gene knock down, partial reduction in c-Myb expression relieved in part CD127 transcriptional suppression in CD8 T cells treated with IL-7. In addition, transient

ectopic over-expression of c-Myb prevented the recovery of CD127 following CD8 T cell stimulation via the TCR (figures 40 and 41). Taken together, these data suggest c-Myb induced by IL-7 via the JAK/STAT5 pathway represses CD127 transcription in primary human CD8 T cells.

The c-Myb proto-oncogene is a 75 kDa DNA-binding transcription factor (265) which is highly expressed in immature hematopoietic cells and is essential for their cellular proliferation, lineage fate selection, and the differentiation of myeloid as well as B- and T-lymphoid progenitor cells (266, 267). It binds to the consensus sequence C/TAACG/TG within various gene promoters (268). As illustrated in figure 47, the c-Myb protein is composed of an N-terminal DNA binding domain (DBD), a transactivation domain (TAD) and a C-terminal negative regulatory domain (NRD) (268, 269). Through its three domains, c-Myb has been shown to complex with several transcription factors such as ETS, C/EBPs, PU.1 and Pax-5, to modulate the expression of many genes (266, 270-274). C-Myb may act as either an activator (275-277) or repressor (258-261) of gene expression. In fact, Zhao *et al,* illustrated that Myb represses more than half of its direct targets in ERMYB myeloid progenitor cells (278). c-Myb represses the human epidermal growth factor receptor 2 (HER2 or c-erbB-2) and the plasma membrane Ca^{2+} ATPase-1 (PMCA-1) by direct binding to these gene promoters (260, 261). In addition, the highly homologous v-Myb oncoprotein represses the transcription of several genes, including Ets-2 through binding to the promoter of the Ets-2 gene (279). Further, the homologous B-Myb had been shown to repress collagen gene transcription in vascular smooth muscle cells (SMCs) *in vitro* and *in vivo* (93, 280). It has been postulated that the Myb-mediated repression occurs through a number of mechanisms. Myb may bind directly to binding sites within gene promoters and displace trans-activators. Alternatively, Myb may physically interact with other transcription factors

Figure 47. Structure of the c-Myb protein. c-Myb possesses three domains that modulate in turn DNA binding, transcriptional activation, and transcriptional suppression. The DNA binding domain binds directly to the c-Myb consensus sequence within target promoters. The activation domain recruits transcriptional coactivators such as the CREB-binding protein (CBP). The negative regulatory domain (NRD) suppresses c-Myb activity by intramolecular interactions with the Myb DNA binding domain. Figure was obtained with permission from Frontiers in Biosciences (Permission from the corresponding author as well as the publishing group are depicted in the appendix) (269).

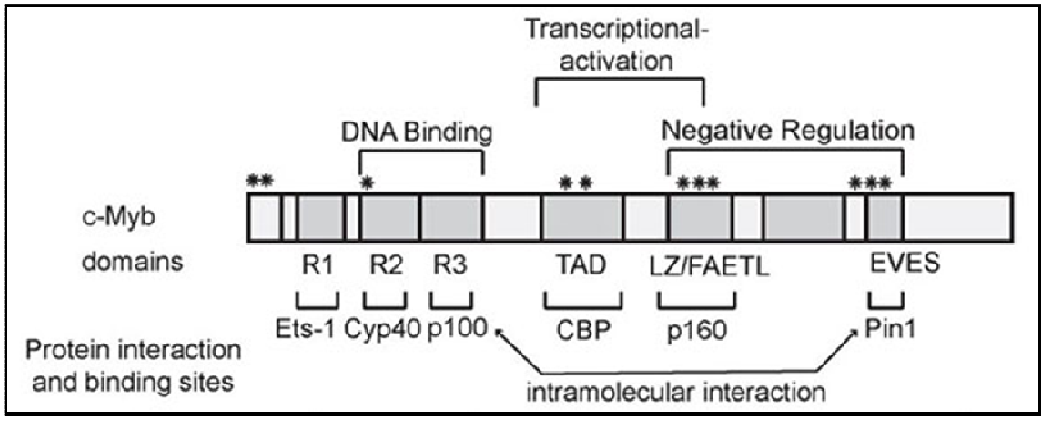

Transcriptional-
activation
DNA Binding
Negative Regulation
c-Myb
domains
R1
R2
R3
TAD
LZ/FAETL
EVES
Ets-1
Cyp40
p100
CBP
p160
Pin1
Protein interaction
and binding sites
intramolecular interaction

Figure 47.

or positive regulators and block their transactivation activity (259, 266). For example, Myb has been shown to contribute to a transcriptional repressor complex which suppresses CD4 gene expression (281).

One of the approaches by which the role of c-Myb in suppressing CD127 transcription in human CD8 T cells was illustrated in this study was siRNA knockdowns. Since the blocking effects of the siRNA mediated Myb knock down were relatively low – although consistent and statistically significant– it is possible that other isoforms of Myb (A-Myb, B-Myb and v-Myb) not affected by the siRNA limited the effect on CD127 expression via functional redundancies. Interestingly, a recent study examined the functional specificities of two isoforms of Myb (c-Myb and v-Myb) (282). In this study, each isoform was over-expressed in primary human monocytes known to express CD127 (283). In each case, the global gene expression profiles were examined using DNA microarrays. By analyzing the microarray data from this study, it was noted over-expression of either form of Myb resulted in suppression of CD127 transcripts. Thus, both isoforms of Myb may be redundant in regulating CD127 gene expression in primary human monocytes (microarray entry of that study was depicted in appendix figure 4).

The region within the CD127 gene promoter necessary for IL-7 mediated suppression of transcription in human CD8 T cells was also identified. This region is located from -1760 to -2406 bp upstream of the TATA box (figure 42). In addition, through bioinformatics and mutational analysis I implicated four c-Myb binding sites within the CD127 gene promoter (-1806, -1863, -2165 and -2729) in the suppression of CD127 gene expression. Mutating at least three specific c-Myb binding sites (-1806, -1863 and -2729) is required for maximal relief of suppression. To further confirm the requirement of these c-Myb binding sites and to directly examine the physical recruitment of c-Myb to the CD127 gene promoter in response

to IL-7 stimulation in primary human CD8 T cells, we will carry out Chromatin Immunoprecipitation (ChIP) assays.

Collectively, I reveal for the first time the pathway by which IL-7 suppresses CD127 gene transcription in human CD8 T cells and describe a role for c-Myb in this process (model in figure 48). Here, IL-7 signaling via JAK/STAT5 induces c-Myb protein expression which binds in turn within the suppressive region of the CD127 gene promoter to suppress transcription.

As already discussed, dysregulation of IL-7 signaling has been implicated in a number of diseases such as multiple sclerosis (9, 188), arthritis (284) as well as in cancer (194). The expression of CD127 is also dysregulated in various infections including EBV (285-287), CMV (285, 287), HCV (288) and HIV infections (153, 199, 289-294). It is possible that abnormalities in c-Myb levels contribute to some of these diseases by dysregulating CD127 expression. For instance, a disorder that results in up regulation of c-Myb may compromise CD8 T cell function as a result of reduced CD127 expression and impaired IL-7 signaling. Thus, understanding the detailed mechanisms by which IL-7 regulates expression of its own gene transcription may help in characterizing how the IL-7 receptor is dysregulated in chronic viral infections and in several autoimmune diseases. This in turn may well lead to novel therapeutic strategies.

Figure 48. Model showing IL-7 induced down regulation of CD127 gene transcription in human CD8 T cells. IL-7 binding to CD127 initiates activation of JAK which in turn phosphorylates STAT5. Phospho-STAT5 dimers translocate to the cell nucleus where they induce expression of c-Myb. c-Myb in turn binds to its consensus sites within the CD127 gene promoter and suppresses CD127 gene transcription.

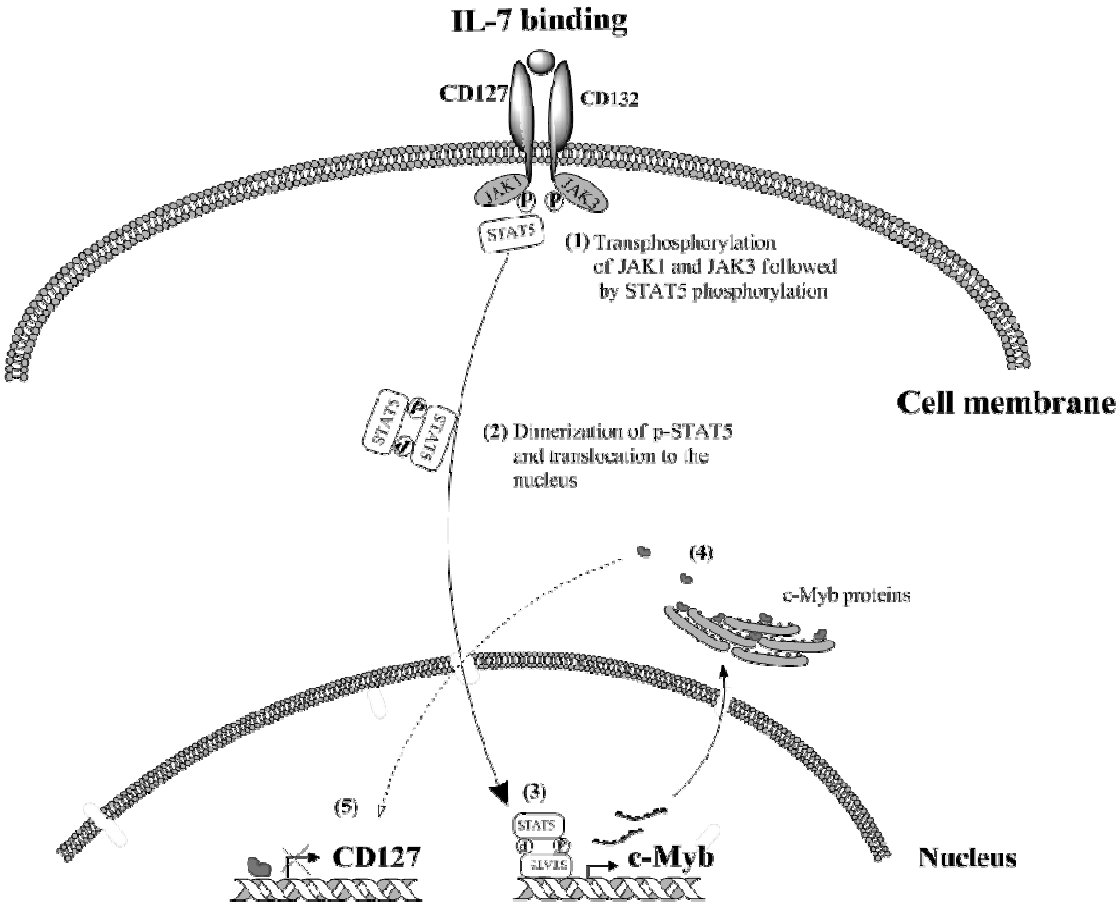

IL-7 binding
CD127
CD132
JAK1
JAK3
P
P
STAT5
(1) Transphosphorylation of JAK1 and JAK3 followed by STAT5 phosphorylation
Cell membrane
STAT5
STAT5
(2) Dimerization of p-STAT5 and translocation to the nucleus
(4)
c-Myb proteins
(5)
(3)
STAT5
STAT5
CD127
c-Myb
Nucleus

Figure 48.

6. Chapter 6. Conclusion

Interleukin-7 signaling plays pivotal roles in the development, survival and function of T cells. Given the fundamental roles of IL-7, it is no surprise dysregulated expression of the IL-7 receptor is associated with a number of chronic infections including HIV and hepatitis C as well as to a number of chronic inflammatory autoimmune diseases such as multiple sclerosis and rheumatoid arthritis (9, 153, 188, 194, 199, 284, 288-294). IL-7 has already entered clinical trials in the treatment of lymphopenia (135) and IL-7 receptor inhibition has been suggested as a treatment for both multiple sclerosis and rheumatoid arthritis (9, 10). In order to further develop these novel therapeutic strategies and avoid unwanted side effects, it is critical that we understand how expression of the IL-7 receptor alpha-chain (CD127) is regulated.

In this study, I have shown IL-7 down regulates the expression of CD127 in human CD8 T cells by two independent mechanisms, one at the level of surface protein expression and one at the level of transcription. I delineated these two mechanisms by examining the kinetics of suppression and by identifying the signaling pathways involved in both processes. IL-7 binding to CD127 initiates rapid internalization of surface CD127 protein. This may occur by conformational change or perhaps through dimerization with CD132, but the initial internalization is independent of JAK activity. Activation of JAK in turn phosphorylates Y449 in the cytoplasmic tail of CD127 which allows docking and phosphorylation of STAT5. Phospho-STAT5 dimers translocate to the cell nucleus where they induce expression of CIS, SOCS1 and SOCS2 proteins. CIS and SOCS2 then bind to the phosphorylated tail of CD127 in early endosomes and recruit an E3 ubiquitin ligase shuttling the complex through late endosomes to the proteasome for degradation. IL-7 also suppresses

CD127 gene transcription and I have shown that IL-7 signaling via STAT5 phosphorylation induces expression of c-Myb protein which in turn suppress CD127 gene transcription. The transcriptional suppression contributes only at later time points and serves to maintain low CD127 levels after the receptor has been removed from the cell membrane by IL-7 stimulation. IL-7 thus down regulates its own receptor expression via a highly regulated multi-level process with rapid initial removal of the receptor from the cell surface followed by transcriptional suppression of the CD127 gene (figure 49). These pathways ensure i) a rapid response to changing IL-7 concentrations in the extracellular milieu, ii) allows flexibility in regulating CD127 expression in different microenvironments with different levels of IL-7 and iii) maintains suppression of CD127 when IL-7 signaling is sustained.

A disruption in this dynamicity could lead to poor or inappropriate CD8 T cell responses. Indeed, dysregulation of IL-7 signaling has been implicated in a number of diseases such as multiple sclerosis (9, 188), rheumatoid arthritis and juvenile idiopathic arthritis as well as in breast cancer (194, 284). The expression of CD127 is also dysregulated in EBV (285-287), CMV (285, 287), and HCV (288) infections. HIV infection in particular is associated with decreased global expression of CD127 on CD4 and CD8 T cells (153, 199, 289-294). In fact, our lab was among the first to establish decreased CD127 expression on the surface of CD8 T cells in HIV-1 infected individuals (292). This has since been confirmed by many other groups (153, 290, 295-297). Since then, our lab have examined the effects of soluble HIV proteins on the expression of surface CD127 and have shown that the HIV-1 Tat protein down regulates CD127 on CD8 T cells isolated form healthy individuals (174). Soluble HIV Tat protein secreted from infected CD4 T cells enters neighboring cells by endocytosis and then physically interacts with CD127 proteins at the cell membrane and induces internalization and proteasomal degradation of the receptor (205). In this study, the

Figure 49. Cumulative model illustrating homeostatic recycling of CD127 at the cell surface and down regulation of CD127 surface protein and gene transcription following IL-7 stimulation. In resting CD8 T cells, CD127 recycles on and off the cell membrane likely through early endosomes with basal protein turn over in the lysosome. IL-7 binding to CD127 initiates rapid internalization of surface CD127 protein and activation of JAK which in turn phosphorylates Y449 in the cytoplasmic tail of CD127 as well as STAT5. Phospho-STAT5 dimers translocate to the cell nucleus where they up regulate the expression of CIS, SOCS1 and SOCS2 proteins. CIS and SOCS2 then bind to the phosphorylated tail of CD127 in early endosomes and likely recruit an E3 ubiquitin ligase shuttling the complex through late endosomes to the proteasome for degradation. In parallel, activated STAT5 dimers induce c-Myb expression which in turn binds to the CD127 gene promoter and suppresses CD127 transcription.

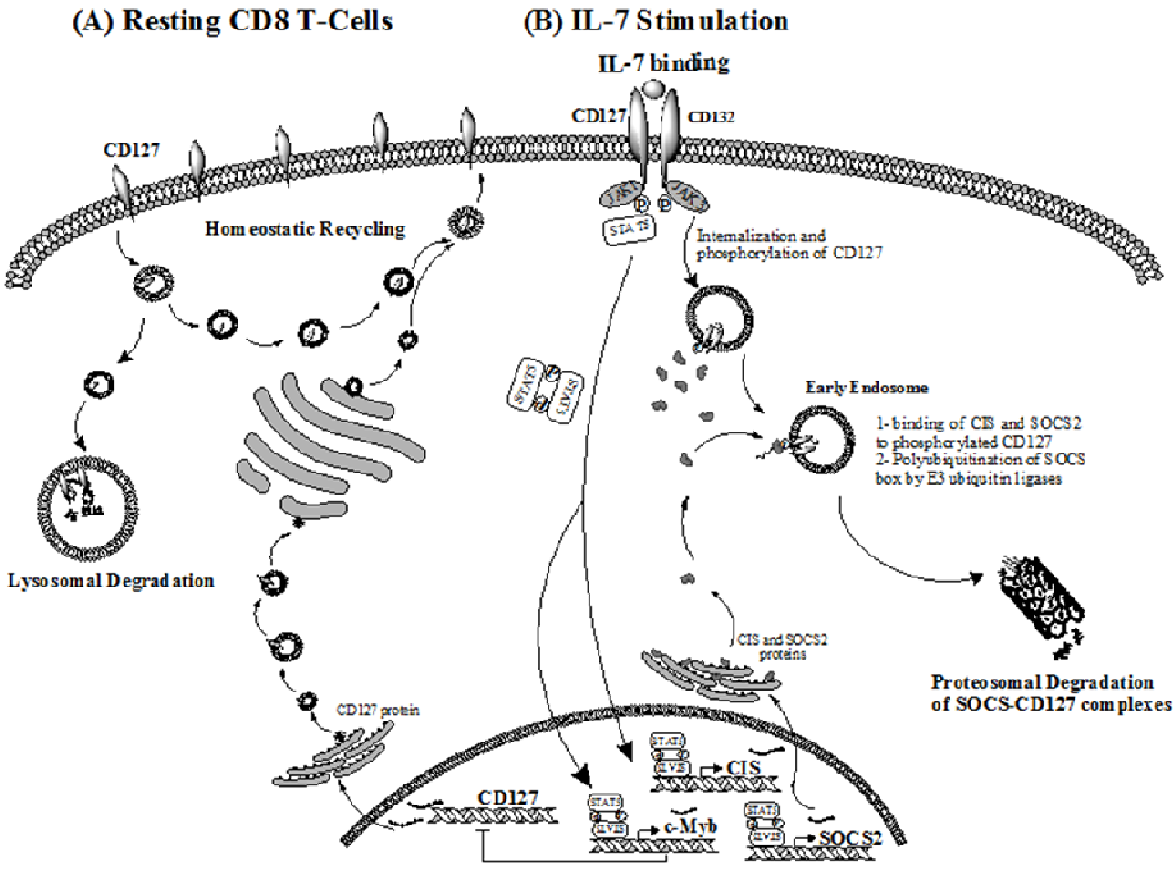

(A) Resting CD8 T-Cells
(B) IL-7 Stimulation
IL-7 binding
CD127
CD132
JAK1
JAK3
STAT5
Homeostatic Recycling
Internalization and
phosphorylation of CD127
Early Endosome
1- binding of CIS and SOCS2
to phosphorylated CD127
2- Polyubiquitination of SOCS
box by E3 ubiquitin ligases
Lysosomal Degradation
CIS and SOCS2
proteins
Proteosomal Degradation
of SOCS-CD127 complexes
CD127 protein
CD127
CIS
c-Myb
SOCS2

Figure 49.

mechanism by which IL-7 down regulates CD127 proteins from the cell surface of CD8 T cells and these important findings opened avenue to examine how factors such as the HIV Tat protein down regulate CD127 likely by usurping IL-7 mediated pathways. Our lab has now recently shown the HIV Tat protein binds directly to CD127 via its N-terminal domain and recruits CIS protein to the complex through its basic domain (Sugden *et al*, 2013 unpublished observations). Tat thus acts as an adaptor protein bypassing IL-7 signaling to recruit CIS and direct the receptor to the proteasome for degradation.

IL-7's therapeutic potential has been examined in various *in vivo* and *in vitro* models, as well as in human clinical trials (74). As already discussed, IL-7 increases both T cell numbers and functions under lymphopenic conditions. It therefore holds promise as a treatment in patients with impaired immunity due to lymphocyte depletion from factors such as infectious diseases, age and cytotoxic chemotherapy. A number of studies highlighted the potential therapeutic application of IL-7 in regenerating T cells following bone marrow transplantation (BMT) (75, 137). Common complications of BMT include subsequent immune deficiency characterized by abnormal proportions of CD4 and CD8 T cells, severely impaired proliferation of T cells in response to mitogens (298), absence of antigen-specific clonal expansion, and the emergence of graft-versus-host-disease (GVHD) (299). Since IL-7 induces thymic-dependent and thymic-independent T cell proliferation, IL-7 may be used to overcome immunodeficiencies by promoting lymphocyte reconstitution following BMT. Indeed, IL-7 has been shown to significantly enhance the survival of mice that were infected with influenza virus following syngeneic BMT, with a remarkable increase in differentiation and proliferation of immature thymocytes observed in the surviving mice (138). Lymphopenia which results from cytotoxic chemotherapy in cancer patients is another

promising target for IL-7 therapy. Following chemotherapy, there is commonly a decrease in T cell counts. In fact, two human phase I clinical trials were carried out at the US National Cancer Institute using patients with refractory malignancies including metastatic melanoma and metastatic sarcoma (139, 140). In these trials, various doses of recombinant human IL-7 (3 to 60 μg/Kg) were administered subcutaneously for 14-28 days. IL-7 was shown to increase CD4 as well as CD8 T cell counts in a concentration dependent manner. These trials suggest an important role for IL-7 in the treatment of patients with chemotherapy-induced lymphopenia.

It is evident IL-7 emerges as a candidate with great potential for therapeutic applications in immunosuppressive disorders. Immunodeficiency-induced by HIV is characterized by a severe reduction in CD4 T cell populations. Due to the recognized roles of IL-7 in increasing T cell numbers, the therapeutic potential of IL-7 for AIDS patients was evaluated in a number of studies demonstrating both an increase in T cell counts and enhancement of T cell functions (124, 300-302). In fact, Levy *et al* (135) conducted a clinical trial that examined the outcomes of IL-7 administration in AIDS patients who were on antiretroviral therapy. In that trial IL-7 induced a significant, dose dependent increase in naïve and memory CD4 and CD8 T cells. CD8 T cells isolated from the patients on IL-7 therapy responded to HIV antigens *ex vivo* by releasing higher amounts of IFN-γ and IL-2 compared to untreated controls. These data are encouraging and indicate IL-7 at appropriate doses may be used in parallel to existing antiretroviral regimens in the treatment of HIV infection.

A major limitation in the treatment of immunological disorders associated with aberrant IL-7 signaling is a lack of understanding of IL-7/IL-7Rα regulation. In this study I

reveal for the first time the detailed pathways by which IL-7 down regulates the expression of its own receptor from the surface of human CD8 T cells and described the mechanism by which IL-7 suppresses CD127 gene transcription. As we have seen with the HIV Tat protein it is likely other diseases that disrupt regulated expression of the IL-7 receptor results from aberrations in these cellular pathways described here.

Patients with MS express higher levels of CD127 protein on their T cells with increased levels of cytolytic granzymes A and B (188). While the mechanism by which CD127 is up regulated in MS is not understood, disruption of the negative feedback process is one possibility that deserves attention (188). The SNP rs6897932 located within exon 6 of the CD127 gene encoding the transmembrane domain is described as a major risk factor for MS (9, 187, 303). Interestingly, this mutation was recently linked to other autoimmune disorders that are associated with abnormally high expression of CD127 including rheumatoid arthritis (284) as well as with type 1 diabetes (304). Further, mutations in exon 6 have in leukemic T-ALL cells been shown to cause homodimerization of CD127 proteins resulting in sustained JAK/STAT signaling independent of IL-7 (195). Currently, it is not known how mutations in exon 6 of the CD127 gene contribute to increased expression of CD127 in MS patients. Findings from this study however raise many questions and point to key molecules that can be investigated. As already discussed, once CD127 proteins are internalized by IL-7 stimulation, they are sorted via early and later endosomes where they physically interact with CIS protein. It is conceivable mutations in CD127 could prevent interaction with CIS, either directly by blocking binding of CIS or indirectly by preventing the compartmentalization and sorting of the receptor, leading to sustained CD127 expression at the cell surface.

Work presented in my thesis provides insights on the detailed mechanisms by which the expression of the IL-7 receptor alpha-chain (CD127) is regulated by IL-7 signaling and identified a number of 'key' proteins that are involved in the process. The findings open up new avenues in the investigation of how reported mutations within the CD127 gene that are associated with autoimmune diseases such as MS and RA fail to down regulate the CD127 protein to normal levels. This could be examined by cloning the CD127 gene harboring mutations and expressing the protein in cells that do not normally express the CD127 protein such as SupT, Jurkat and A3 T cell lines. Once the mutant proteins are expressed, the manners by which these proteins are down regulated in response to IL-7 signaling and their molecular interactions with the identified key proteins by this study such as CIS can provide critical insights on how mutations of the CD127 gene are implicated in autoimmune diseases. In fact, these mechanisms are currently being further delineated in our laboratory and may well provide new insights into the development of MS and RA.

Taken together, findings of this study may help better understand a number of autoimmune disorders and infectious diseases. Understanding the detailed mechanisms by which IL-7 regulates expression of its own receptor may well lead to novel therapeutic strategies aimed at modulating T cell numbers and functions in lymphopenic conditions as well as in chronic viral infections and in several autoimmune diseases.

7. References

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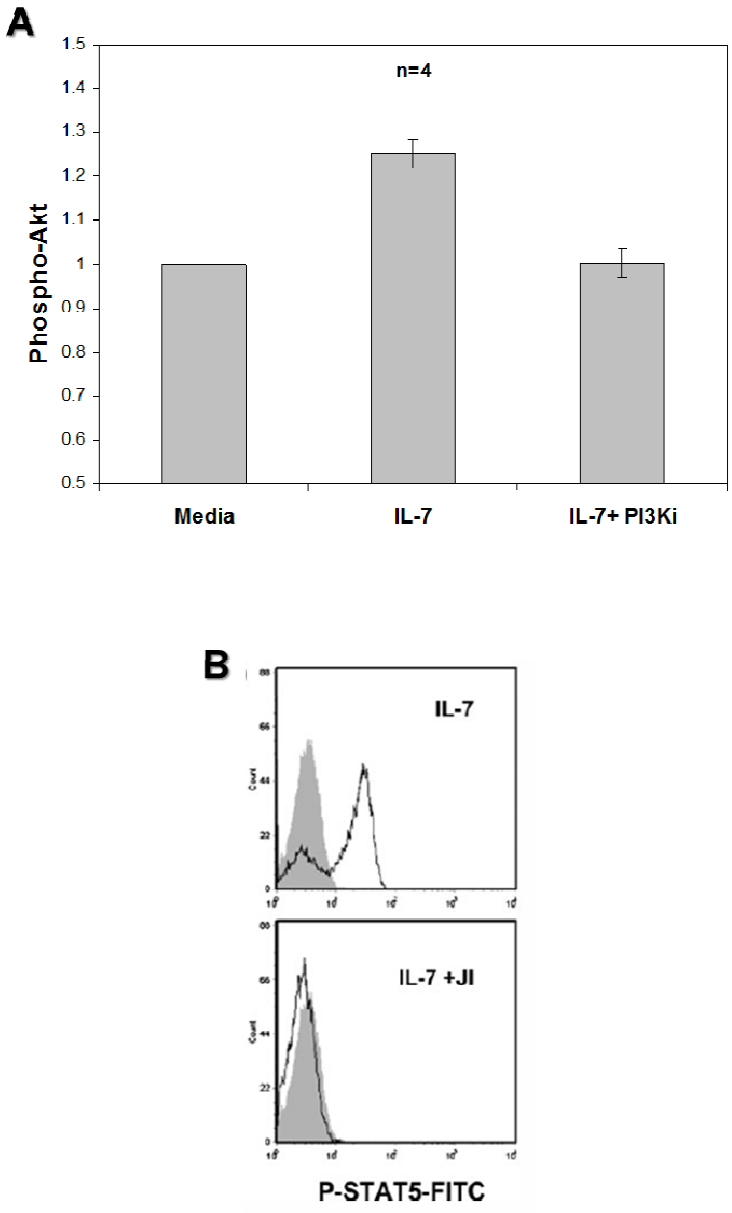
8. Contributions of Collaborators

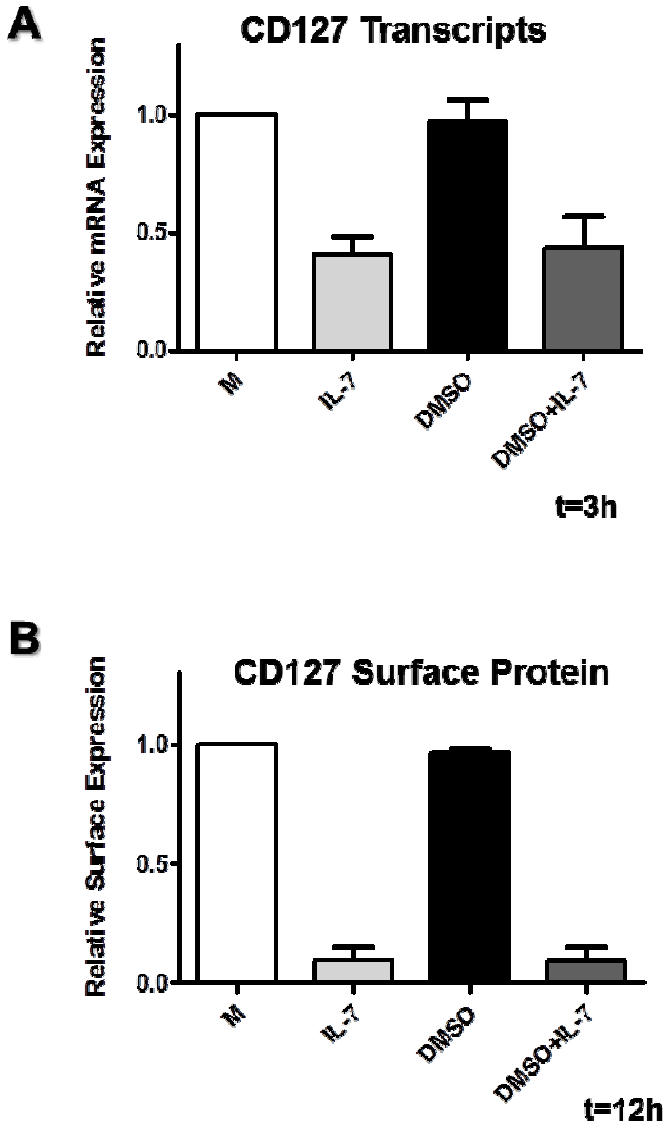
I would like to acknowledge several collaborators and also thank everyone who gave me advice. I particularly acknowledge Elliott Faller (Postdoc fellow): carried out work in figs 7B-C, 8A, 9B, 11, 17C, 18A-E, 27, 34C-D and 37A. Scott Sugden (PhD student): carried out work in figs 10B and 13A and 13C-D and helped in 13E and in transfection and flow work in fig 40. Parmvir Parmar (honors student) for assistance in figs 22-26, 31, 36, 40A, 42 and 43. Abdulkareem El-Salfiti (honors student) for assistance in figure 28. Hafsa Cherid (master's student) for assistance in lymphoblastic T cell lines work in figure 38B. I also used existing constructs in our lab (CD127 gene promoter cloned upstream of the firefly luciferase reporter gene, table 2) that were generated by a master's graduate, Mr. Juzer Kakal.

9. Appendices

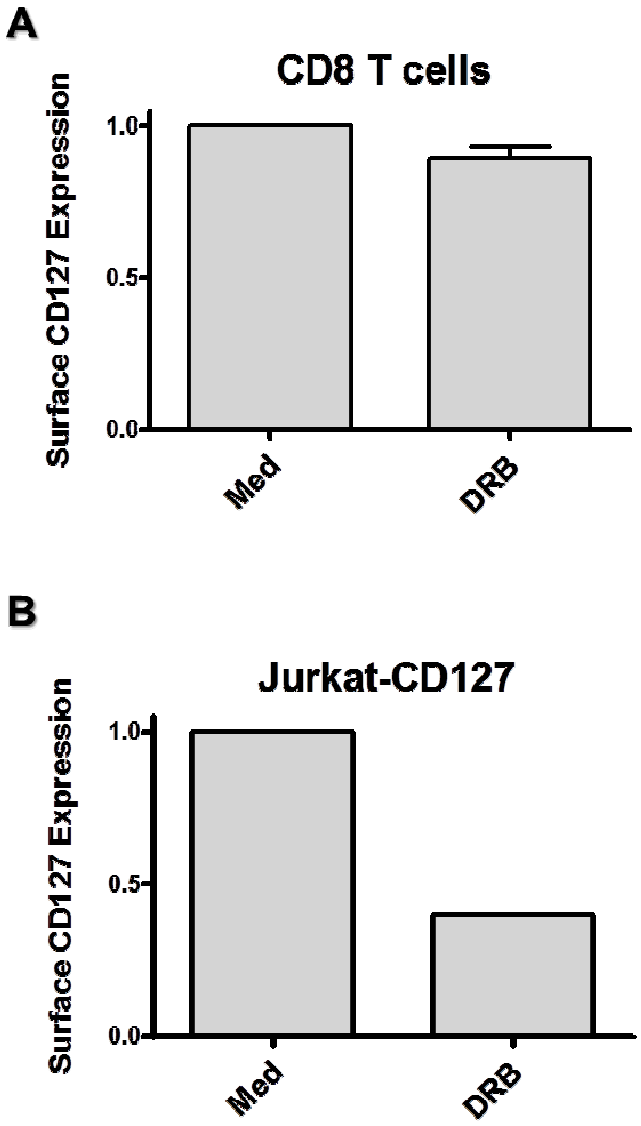
9.1 Appendix Figures.



Appendix Figure 1. Confirming the efficacy of the JAK and PI3K inhibitors. (A) The PI3K inhibitor (LY294002; 10 μM) successfully blocked phosphorylation of Akt following IL-7 stimulation in primary human CD8 T cells while **(B)** the JAK inhibitor (1.5 μM) blocked IL-7-induced STAT5 phosphorylation as measured by flow cytometry. Faller *et al*. 2009. Int Immunol 21:203-216. Work was carried out by Dr. E. Faller from our lab.



Appendix Figure 2. Confirming the effects of the inhibitors solvent (DMSO) are negligible on CD127 transcripts or surface expression as well as on IL-7's repressive effects. CD8 T cells were incubated in Media alone, IL-7 (10 ng/ml) alone, DMSO (5 µl/ml) alone or IL-7 and DMSO in combination and levels of **(A)** CD127 transcripts and **(B)** surface CD127 protein were measured by qPCR and flow cytometry respectively. Data are represented as relative changes compared to media controls. Error bars represent SEM of three independent experiments.



Appendix Figure 3. Jurkat-CD127 cells have a much faster rate of turnover/replacement of CD127 at the cell surface than CD8 T cells. Jurkat-CD127 and CD8 T cells were incubated in media alone or with the transciptional inhibitor DRB (100 mM) for 6 hours and Surface CD127 expression was analyzed by flow cytometry. The data illustrate that when transcription of the CD127 gene was shutoff by the transcriptional inhibitor, levels of surface CD127 proteins were sustained for much longer time in CD8 T cells than Jurkat-CD127 cells suggesting that where the receptor has a relatively long half-life on the surface of CD8 T cells, the receptor has a fast rate of turnover/replacement on the surface of Jurkat-CD127 cells as evident by the rapid drop in CD127 levels off the cell surface within only 6 hours of shutting off the CD127 gene.

Profile GDS1427 / 205798_at / IL7R
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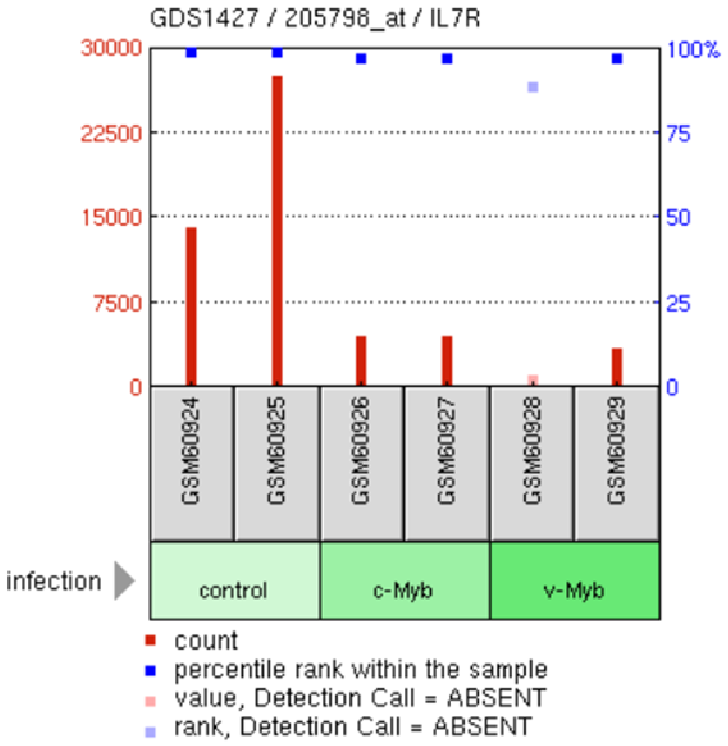
Organism Homo sapiens



Appendix Figure 4. Over-expression of c-Myb or its isoform v-Myb down-regulates CD127 in human monocytes. Adenovirus-mediated ectopic expression of c-Myb or its isoform v-Myb in human monocytes significantly suppress CD127 transcription. This is from supplementary microarray data from the following study: Liu F, Lei W, O'Rourke JP, Ness SA. Oncogenic mutations cause dramatic, qualitative changes in the transcriptional activity of c-Myb. Oncogene. 2006 Feb 2;25(5):795-805. URL: http://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS1427:205798_at

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Figure 2. Cytotoxic T-lymphocyte (CTL) mediated targeting of infected cells.

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Figure 3. The common γ-chain cytokine receptor family.

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Figure 4. Different cellular requirements of gamma-chain cytokines throughout the life of a T cell.

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Figure 14 and Table 4. The SOCS family of proteins.

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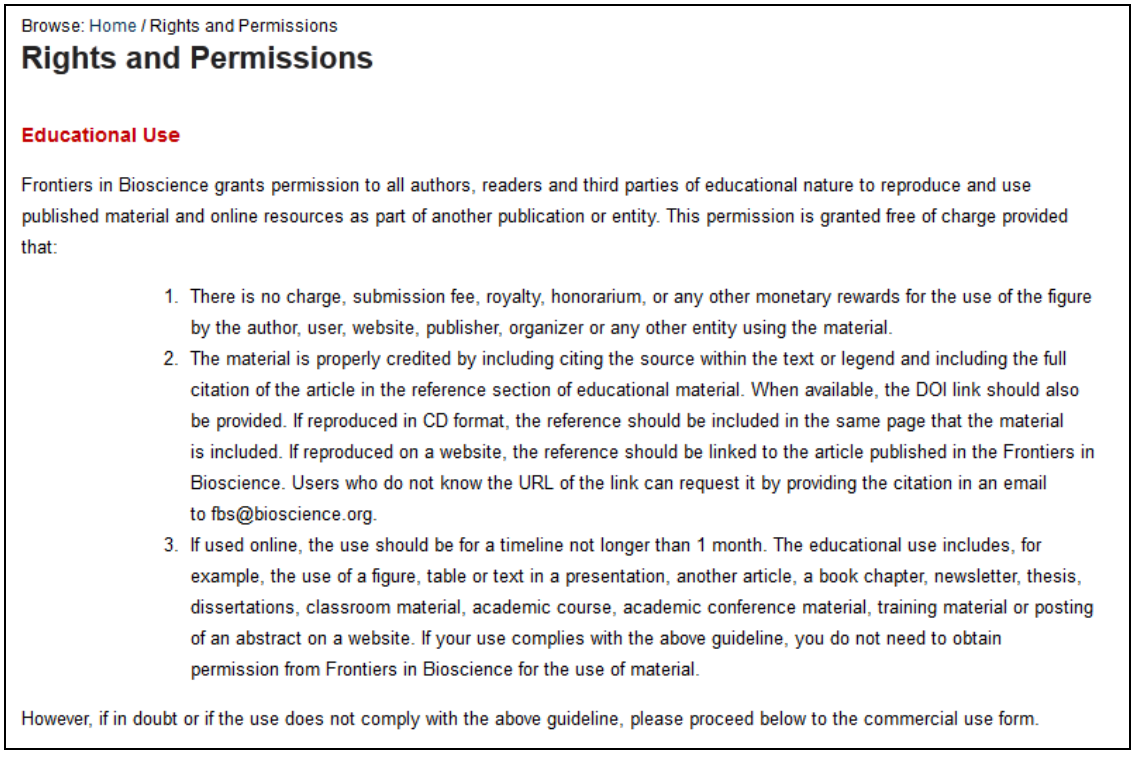
Figure 15. SOCS proteins negatively regulate JAK/STAT signaling via a number of mechanisms.

Figure 47. Structure of the c-Myb protein.



9.3 Ethical approval by the Ottawa Hospital Research Institute and blood consent forms

The Ottawa Hospital | L'Hôpital d'Ottawa

Information Sheet and Consent Form for Blood and/or Semen Collection and/or Colon Biopsies

Study: Impaired Immune Function during HIV Infection

Funding Agency: Canadian Institutes of Health Research

Principal Investigator: Dr. Paul MacPherson
613-737-8899 ext 73896

Introduction:
You are being asked to participate in a study focused on immune function in HIV infection. Before you decide to participate, it is important that you understand why this research is being conducted. Please take a moment to read the following information, and ask questions if there is anything that you find unclear.

Background:
HIV infection over time causes severe impairment of the immune system. HIV enters some immune cells such as CD4 T-cells and monocytes and either kills them or prevents them from functioning properly. Other immune cells such as CD8 T-cells are not infected by HIV but are still unable to function in the presence of HIV. By limiting the activity of these various immune cells, HIV weakens the entire immune system. When the immune system is weak, HIV is able to replicate freely and eventually people can become sick from opportunistic infections.
How HIV inactivates these different cells is not yet understood.

HIV infected cells travel through the blood to many sites in the body including the testicles and prostate. Once in these tissues, HIV may behave differently than when it is present in the blood or lymph nodes. Sometimes HIV can be detected in the semen even when it is undetectable in the blood. Control of HIV replication in the semen then may not perfectly mirror what occurs in the blood.

HIV can also infect the cells that line the colon, a part of the digestive tract, and the effect it has on the immune system in the gut may also not mirror what occurs in the blood or semen.

Purpose:
One of the goals of this research program is to determine how the activities of various immune cells change during HIV infection. This will be done by examining changes in the expression of molecules on the cell surface as well as changes in overall cell function. We will compare these changes between HIV negative individuals, HIV+ patients with active viral replication, HIV+ patients on antiretroviral therapy, and in long term nonprogressors.

Another goal of this research program is to determine how immunologic control of HIV differs between different tissue compartments, namely the blood, semen, and the digestive tract. This will be done by examining HIV viral loads and levels of immune modulators isolated from semen

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or gut. Cells of the immune system involved in controlling HIV replication may also be isolated from semen or gut in order to examine their functional abilities.

Study Procedures:
Participants will be typically asked to provide a sample of either blood **or** semen **or** gut tissue. Participants who provide semen or gut samples may be also asked to provide blood.

Blood Samples:
Participants will be asked to donate samples of blood ranging from 10 ml, less than 1 tablespoon ~~(one tube) to a maximum of 200ml, approximately 14 tablespoon (less than one half the volume~~ drawn by Canadian Blood Services for routine blood donation; one unit = 500ml, approximately 33 tablespoon). White cells will be isolated from the blood and analyzed in the laboratory for changes in the expression of different molecules, and for immune activity. Serum may also be examined for molecules which regulate the activity of the immune cells.

Semen Samples:
Participants may be asked to donate semen samples. Sterile containers will be provided. Samples will be collected privately by the participant but must be delivered to the clinic within two hours.

Gut Samples:
During your colonoscopy procedure small biopsies of the colon wall will be performed in order to gather gut-associated lymphoid tissue. Approximately 30 tissue biopsies will be obtained, each consisting of about 0.3mm of tissue (9 mm total).

Duration:
Your participation will be complete once the blood and/or semen and/or gut tissue sample is obtained.

Risks:

Blood Samples:
The risks of participating in this study are those associated with routine blood drawing and may include minor pain at the site where blood is drawn and minimal bruising. Some people may experience temporary lightheadedness after drawing blood. If this occurs, you may be asked to rest in the clinic for a short period of time until the lightheadedness passes.

Semen Samples:
There are no risks associated with semen donation. Semen samples will only be used to determine HIV viral loads and for immunologic studies. Under no circumstances will the semen be available to achieve fertilization under any conditions.

Gut Samples:
During the procedure, you may feel pressure in the rectum similar to the sensation you feel with the urge to have a bowel movement. You may also feel a small amount of cramping in your abdomen as well. The most serious risk of the procedure is perforation, poking a hole in the

lining of the intestine, but it is extremely uncommon, occurring once out of every 10,000 procedures. If this occurs the attending physician will be responsible for treatment. Some bleeding will likely occur at the point where the biopsies were done. If you should experience severe discomfort during colon mucosa biopsy the procedure will be stopped immediately; you will be seen by the physician and the research nurse will monitor you until you are ready to go home.

Due to the possible bleeding following the procedure, the risk of transmitting HIV after the biopsy procedure may be increased.

Benefits:
Participation in this study will provide no direct benefits to you. It will, however, help to advance our understanding of how HIV inactivates the immune system and may lead to new strategies for the development of immune based therapies to control HIV replication.

Voluntary Participation and Withdrawal:
You are under no obligation to participate in this study. If you choose to participate, you may change your mind at any time without providing a reason. You should inform the study doctor/study staff if you decide to withdraw so that your sample may be destroyed. You are not waiving your legal rights by agreeing to take part. Whether you choose to participate or not will have absolutely no effect on the medical care you receive, now or in the future.

Confidentiality:
All personal health information will be kept confidential, unless release is required by law. Representatives of the Ottawa Hospital Research Ethics Board, as well as the Ottawa Hospital Research Institute, may review your original medical records under the supervision of Dr. MacPherson's staff for audit purposes.

You will not be identifiable in any publications or presentations resulting from this study. No identifying information will leave the Ottawa Hospital.

The link between your name and the independent study number will only be accessible by Dr. MacPherson and/or his staff. The link and study files will be stored separately and securely. Both files will be kept for a period of 15 years after the study has been completed. All paper records will be stored in a locked file and/or office. All electronic records will be stored and protected by a user password, again only accessible by Dr. MacPherson and/or his staff. At the end of the retention period, all paper records will be disposed of in confidential waste or shredded, and all electronic records will be deleted.

Your chart will be used only to obtain CD4 counts, viral loads, and overall health status. Data will be compiled using a code. Your name will not be used and will not be known to laboratory personnel. The results of this study may be presented at meetings or in publications. Your identity will not be disclosed at any time in any of these presentations or publications.

Questions About The Study:

If during the course of this study you have questions concerning the study, you may contact the principle investigator Dr Paul MacPherson at 613-737-8899 ext 73896.

The Ottawa Hospital Research Ethics Board has approved this study. This committee considers the ethical aspects of all research projects involving people. If you have any questions about your rights with regard to participating in a research study, you may contact the Chair of the Ottawa Hospital Research Ethics Board at 613-798-5555, extension 14902. Do not sign the consent form unless you have had a chance to ask questions and have received satisfactory answers to all you questions. By signing the consent form you are not waiving your legal rights.

The Ottawa Hospital | L'Hôpital d'Ottawa

Consent Form
Impaired Immune Function during HIV Infection

Consent to Participate in Research

I understand that I am being asked to participate in a research study about immune function in HIV infection. This study has been explained to me.

I have read this, 5-page Participant Information Sheet and Consent Form (or have had this document read to me). All my questions have been answered to my satisfaction. If I decide at a later stage in the study that I would like to withdraw my consent, I may do so at any time.

I voluntarily agree to participate in this study and will provide a:

Please Initial:

_____Blood Sample

_____Semen Sample

_____Gut Sample

A copy of the signed Information Sheet and/or Consent Form will be provided to me.

Signatures

_____
Participant's Name (Please Print)

_____ _____
Participant's Signature Date

Investigator Statement (or Person Explaining the Consent)

I have carefully explained to the research participant the nature of the above research study. To the best of my knowledge, the research participant signing this consent form understands the nature, demands, risks and benefits involved in participating in this study. I acknowledge my responsibility for the care and well being of the above research participant, to respect the rights and wishes of the research participant, and to conduct the study according to applicable Good Clinical Practice guidelines and regulations.

_____
Name of Investigator/Delegate (Please Print)

_____ _____
Signature of Investigator/Delegate Date

Valid until MAR 2 8 2014

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10. Curriculum Vitae

Education

- PhD, Immunology and Microbiology, University of Ottawa. 2013.
- MSc, Cellular and Molecular Medicine, University of Ottawa. 2009.
- Honours BSc with Specialization in Biochemistry, University of Ottawa. 2006.
- High school diploma, St. Lawrence High School, Cornwall, Ontario. 2002.

Awards and Distinctions

1. American Association of Immunologists (AAI) Trainee Abstract Award – to present a talk as well as a poster presentation in Honolulu, USA (2013)
2. Young Investigator Award – to present at the Conference on Retroviruses and Opportunistic Infections (CROI) in Atlanta, USA (2013)
3. **XIX International AIDS Conference Scholarship to give two oral presentations in Washington D.C., USA** (2012)
4. Ottawa Hospital Research Institute - 10th Annual Research Day Poster Prize (2010)
5. Ontario HIV Treatment Network (OHTN) Research Conference Scholarship. **Awarded three times** (2009, 2010, 2011)
6. **Canadian Institutes of Health Research (CIHR) Doctoral Research Award** (2010-2013)
7. Department of Microbiology and Immunology Annual Poster Day Prize (2010)
8. Faculty of Graduate and Postdoctoral Studies (FGPS) Conference Grant (2010, 2011, 2013)
9. Department of Immunology and Microbiology Conference Grant (2010, 2011)
10. University of Ottawa Graduate Students' Association Conference Grant (2010, 2011)
11. University of Ottawa Admission Scholarship for PhD Studies (2009-2013)
12. Faculty of Graduate and Postdoctoral Studies Dean's Scholarship (2009)
13. Department of Pathology and Laboratory Medicine Annual Research Day Prize (2008)
14. University of Ottawa Admission Scholarship for Master's Studies (2007)
15. Plaque of Excellence by Ottawa Health Research Institute (OHRI) (2007)
16. University of Ottawa Excellence Scholarship for Master's Studies (2006)
17. **Canadian Institutes of Health Research (CIHR) CGS Master's Scholarship** (2006)
18. Queen Elizabeth II Aiming for the Top Award. **Awarded four consecutive times** (2002-2006)
19. University of Ottawa Entrance Scholarship. **Awarded four consecutive times** (2002-2006)
20. Dean's Honor List for all years of study (2002-2006)
21. Kaneb, SLHS and Hugh DeCourcy awards of academic excellence in high school (2002)
22. Ontario Scholar Award (2002)
23. Top 25th Percentile-National Biology Competition, University of Toronto (2002)
24. **Governor General's Academic Medal for Academic Excellence – Top high school graduate** (2002)

Published Refereed Papers (4)

1. **Feras Ghazawi**, Elliott Faller, Scott Sugden, Juzer Kakal and Paul MacPherson. IL-7 down regulates IL-7Rα expression in human CD8 T cells by two independent mechanisms. Immunology and Cell Biology. 2013. 91: 149-158.
2. Cecilia Costiniuk, Benjamin Hibbert, Trevor Simard, **Feras Ghazawi**, Jonathan Angel and Edward O'Brien. Circulating Endothelial Progenitor Cells in HIV Infection: A Systematic Review. Trends in Cardiovascular Medicine. 2013. 23:6. 192-200.
3. Jihong Chen, **Feras Ghazawi** and Qiao Li. Interplay of bromodomain and histone acetylation in the regulation of p300-dependent genes. Epigenetics. 2010. 5:6. 509-15
4. Jihong Chen, **Feras Ghazawi**, Wafae Bakkar and Qiao Li. Valproic acid and butyrate induce apoptosis in human cancer cells through inhibition of gene expression of Akt/protein kinase B. Molecular Cancer. 2006. 5:71

Submitted Refereed Papers (2)

1. **Feras Ghazawi**, Parmvir Parmar, Elliott Faller, Abdulkareem El Salfiti and Paul MacPherson. Suppressor of cytokine signaling (SOCS) proteins are induced by IL-7 and regulate CD127 expression in human CD8 T cells. Submitted to *Journal of Immunology* – Manuscript #12-03454-FL. **70% contribution.**
2. Cecilia Costiniuk, **Feras Ghazawi** and Stephen Kravcik. Myelopathy in HIV-Infected Individuals. Under review by *Journal of General Internal Medicine*.

Manuscripts in Preparation/Ready for Submission (5)

1. **Feras Ghazawi**, Elliott Faller, Parmvir Parmar and Paul MacPherson. IL-7 Suppresses CD127 Gene Transcription in CD8 T cells via Inducing Expression of the Transcription Factor c-Myb. Manuscript in preparation to be submitted to *Blood*. 2013. **70% contribution.**
2. Elliott Faller, **Feras Ghazawi** and Paul MacPherson. IL-7 induces clathrin mediated internalization and proteasomal degradation of the IL-7R alpha-chain in CD8 T-cells. Manuscript ready for submission to *PLOS*. **40% contribution.**
3. Juzer Kakal, **Feras Ghazawi**, Elliott Faller, Scott Sugden, Parmvir Parmar and Paul MacPherson. Transcriptional Regulation of the CD127 Gene by Dexamethasone in Primary Human CD8 T-cells. Manuscript ready for submission to *PLOS*. **30% contribution.**
4. Cecilia Costiniuk, **Feras Ghazawi**, Elliott Faller, Scott Sugden and Paul MacPherson. Regulation of CD8 T cell Functions by IL-7: Potential Vaccine Adjuvant. Review paper. Manuscript ready for submission to *Canadian Medical Association Journal.* **40% contribution.**
5. Scott Sugden, **Feras Ghazawi** and Paul MacPherson. HIV Tat Protein Binds the Cytoplasmic Tail of CD127 via Tat's Amino-Terminal Domain, Recruits CIS protein and Targets CD127 for Proteasomal Degradation via Tat's Basic Domain. Manuscript in preparation. **20% contribution.**

Oral/Invited Presentations (6)

1. **Feras Ghazawi**, Parmvir Parmar, Elliott Faller, Abdulkareem El Salfiti and Paul MacPherson. (2013). Suppressor of cytokine signaling proteins are induced by IL-7 and regulate CD127 expression in human CD8 T cells. *100th Annual Meeting, The American Association of Immunologists. Honolulu, Hawaii, USA.*
2. **Feras Ghazawi** and Paul MacPherson. (2012). Understanding the Mechanisms by which IL-7 Down-regulates Expression of the IL-7 Receptor Alpha-chain (CD127) in Primary Human CD8 T-Cells. *Invited presentation by Unité de Régulation des Infections Rétrovirales, Pasteur Institute, Paris, France.*
3. **Feras Ghazawi**, Parmvir Parmar, Elliott Faller, Scott Sugden, Nadia Sant and Paul MacPherson. (2012). SOCS proteins are induced by IL-7 and the interaction of SOCS proteins with the IL-7 receptor alpha (CD127) may play a role in regulating CD127 expression in human CD8 T cells. *XIX International AIDS Conference, Washington, District of Columbia, USA*
4. **Feras Ghazawi**, Elliott Faller, Parmvir Parmar and Paul MacPherson. (2012). IL-7 Suppresses CD127 Gene Transcription in Human CD8 T-Cells via a STAT5-Induced Repressor. *XIX International AIDS Conference, Washington D.C., USA*
5. **Feras Ghazawi**, Elliott Faller, Juzer Kakal and Paul MacPherson. (2011). IL-7 Suppresses Transcription of the CD127 Gene in Human CD8 T-Cells by Stimulating the Expression of a STAT5-Induced Repressor. *20th Annual Canadian Conference on HIV/AIDS Research. Toronto, Ontario, Canada*
6. **Feras Ghazawi**, Elliott Faller, Juzer Kakal and Paul MacPherson. (2010). Transcriptional Regulation of the CD127 Gene in CD8 T-Cells. *19th Annual Canadian Conference on HIV/AIDS Research. Saskatoon, Saskatchewan, Canada*

Published Abstracts (24)

1. **Feras Ghazawi**, Elliott Faller, Parmvir Parmar, Scott Sugden, Juzer Kakal, Hafsa Cherid and Paul MacPherson. (2013). IL-7 suppresses CD127 gene transcription in human CD8 T cells by inducing expression of the transcription factor c-Myb (poster presentation). *100th Annual Meeting - The American Association of Immunologists. Honolulu, Hawaii, USA*
2. Hafsa Cherid, **Feras Ghazawi** and Paul MacPherson. (2013). Regions of the CD127 cytoplasmic domain necessary for Tat-induced internalization (poster presentation). *22nd Annual Canadian Conference on HIV/AIDS Research. Vancouver, British Columbia, Canada*
3. Paul MacPherson, **Feras Ghazawi**, Elliott Faller, Scott Sugden, Hafsa Cherid, Juzer Kakal, Parmvir Parmar and Abdulkareem El-Salfiti. (2013). By usurping IL-7 mediated pathways, the HIV Tat protein targets CD127 for degradation and thus attenuates CD8 T-cell survival and function (oral presentation). *22nd Annual Canadian Conference on HIV/AIDS Research. Vancouver, British Columbia, Canada*
4. Cecilia Costiniuk, Sandra Côté, **Feras Ghazawi**, Lorna Carrasco-Medina, Charlene Young and Jonathan Angel. (2013). Oncolytic Viruses as a Potential Approach to Eliminate the HIV Reservoir (poster presentation). *22nd Annual Canadian Conference on HIV/AIDS Research. Vancouver, British Columbia, Canada*
5. **Feras Ghazawi**, Parmvir Parmar, Elliott Faller, Scott Sugden, Hafsa Cherid, Juzer Kakal, Abdulkareem El Salfiti and Paul MacPherson. (2013). Understanding the Mechanisms by which IL-7 Down-regulates Expression of the IL-7 Receptor Alpha-chain (CD127) in Primary Human CD8 T-Cells (poster presentation). *20th conference on Retroviruses and Opportunistic Infections (CROI). Atlanta, Georgia, USA*
6. Cecilia Costiniuk, Sandra Côté, **Feras Ghazawi**, Lorna Carrasco-Medina, Charlene Young and Jonathan Angel. (2013) Oncolytic Viruses as a Potential Approach to Eliminate the HIV

Reservoir. *Annual Conference of the Association of Medical Microbiology and Infectious Disease Canada. Quebec city, Quebec, Canada.*

7. **Feras Ghazawi**, Parmvir Parmar, Scott Sugden, Elliott Faller, Nadia Sant and Paul MacPherson. (2012). Suppressor of Cytokine Signalling (SOCS) Proteins are Induced by IL-7 and May Play a Role in Regulating CD127 Expression (poster presentation). *21st Annual Canadian Conference on HIV/AIDS Research. Montreal, Québec, Canada*
8. **Feras Ghazawi**, Elliott Faller, Parmvir Parmar, Juzer Kakal and Paul MacPherson. (2012). IL-7 Suppresses CD127 Gene Transcription in Human CD8 T-Cells via a STAT5-Induced Repressor (poster presentation). *21st Annual Canadian Conference on HIV/AIDS Research. Montreal, Quebec, Canada*
9. Elliott Faller, **Feras Ghazawi** and Paul MacPherson. (2012). IL-7 induces rapid internalization of CD127 proteins by clathrin mediated endocytosis, leading to subsequent degradation by the proteasome (oral presentations). *99th Annual Meeting - The American Association of Immunologists. Boston, Massachusetts, USA*
10. Elliott Faller, **Feras Ghazawi** and Paul MacPherson. (2012). IL-7 induces rapid internalization of CD127 proteins by clathrin mediated endocytosis, leading to subsequent degradation by the proteasome (oral presentation). *21st Annual Canadian Conference on HIV/AIDS Research. Montreal, Québec, Canada*
11. Cecilia Costiniuk, Sandra Côté, **Feras Ghazawi**, Lorna Carrasco-Medina, Charlene Young and Jonathan Angel. (2012) Oncolytic Viruses as a Potential Approach to Eliminate the HIV Reservoir (oral presentation). *The Ontario HIV Treatment Network 14th Annual Research Conference. Toronto, Ontario, Canada*
12. Elliott Faller, **Feras Ghazawi** and Paul MacPherson. (2012). IL-7 induces rapid internalization of CD127 proteins by clathrin mediated endocytosis, leading to subsequent degradation by the proteasome (poster presentation). *19th conference on Retroviruses and Opportunistic Infections (CROI). Seattle, Washington, USA*
13. **Feras Ghazawi**, Parmvir Parmar, Scott Sugden and Paul MacPherson. (2011). Suppressor of Cytokine Signalling (SOCS) Proteins are Induced by IL-7 and May Play a Role in Regulating CD127 Expression (poster presentation). *The Ontario HIV Treatment Network 13th Annual Research Conference. Toronto, Ontario, Canada*
14. **Feras Ghazawi**, Elliott Faller, Parmvir Parmar and Paul MacPherson. (2011). IL-7 Suppresses CD127 Gene Transcription in Human CD8 T-Cells via a STAT5-Induced Repressor (poster presentation). *The Ontario HIV Treatment Network 13th Annual Research Conference. Toronto, Ontario, Canada*
15. Scott Sugden, **Feras Ghazawi** and Paul MacPherson (2011). HIV TAT protein binds the cytoplasmic tail of CD127 via TAT's amino-terminal domain, resulting in down regulation of CD127 from the surface of CD127 of CD8 T cells (oral presentation). *The Ontario HIV Treatment Network 13th Annual Research Conference. Toronto, Ontario, Canada*
16. Elliott Faller, **Feras Ghazawi**, Scott Sugden, Juzer Kakal and Paul MacPherson. (2011). HIV Tat and IL-7 down regulate surface expression of the IL-7 receptor α-chain through overlapping pathways (oral presentation). *20th Annual Canadian Conference on HIV/AIDS Research. Toronto, Ontario, Canada*
17. Elliott Faller, **Feras Ghazawi** and Paul MacPherson. (2011). IL-7 induces rapid internalization of CD127 proteins by clathrin mediated endocytosis, leading to subsequent degradation by the proteasome (poster presentation). *The Ontario HIV Treatment Network 13th Annual Research Conference. Toronto, Ontario, Canada*
18. Elliott Faller, **Feras Ghazawi**, Scott Sugden, Juzer Kakal and Paul MacPherson. (2011). HIV Tat and IL-7 down regulate surface expression of the IL-7 receptor α-chain through overlapping pathways (poster presentation). *Keystone Symposia on HIV Evolution, Genomics and Pathogenesis (X7). Whistler, British Columbia, Canada*

19. **Feras Ghazawi**, Elliott Faller, Juzer Kakal and Paul MacPherson. (2010). IL-7 Suppresses Transcription of the CD127 Gene in CD8 T-Cells (poster presentation). *The Ontario HIV Treatment Network 12th Annual Research Conference. Toronto, Ontario, Canada*
20. Elliott Faller, **Feras Ghazawi**, Scott Sugden and Paul MacPherson. (2010). IL-7 induces clathrin mediated internalization and proteasomal degradation of the IL-7R alpha-chain in CD8 T-cells (poster presentation). *The Ontario HIV Treatment Network 12th Annual Research Conference. Toronto, Ontario, Canada*
21. **Feras Ghazawi**, Elliott Faller and Paul MacPherson. (2009). Effects of Interlukin-7 on Notch-1 Expression in CD8 T-Cells (poster presentation). *The Ontario HIV Treatment Network 11th Annual Research Conference. Toronto, ON*
22. **Feras Ghazawi**, Elliott Faller and Paul MacPherson. (2009). Effects of Interlukin-7 on Notch-1 Expression in CD8 T-Cells (poster presentation). *4th Annual Canadian Society for Life Science Research conference. Ottawa, Ontario, Canada*
23. Elliott Faller, **Feras Ghazawi** and Paul MacPherson. (2009). JAK/STAT signaling mediates regulation of IL-7R Expression on CD8 T cells by both transcriptional and non-transcriptional mechanisms (oral presentation). *The Ontario HIV Treatment Network 11th Annual Research Conference. Toronto, Ontario, Canada*
24. Jihong Chen, **Feras Ghazawi**, Wafaa Bakkar and Qiao Li. Histone deacetylase inhibitors induce apoptosis through inhibition of Akt expression (poster presentation). *14th Conference of the International Society of Differentiation* (*ISD*). *Innsbruck, Tyrol, Austria*

Conference Proceedings/Abstracts Published in Journals (13)

1. **Feras Ghazawi**, Parmvir Parmar, Elliott Faller, Abdulkareem El Salfiti and Paul MacPherson. (2013). Suppressor of cytokine signaling proteins are induced by IL-7 and regulate CD127 expression in human CD8 T cells. The Journal of Immunology, May 2013, Volume 190. Meeting Abstract Supplement 184.5.
2. **Feras Ghazawi**, Elliott Faller, Parmvir Parmar, Scott Sugden, Juzer Kakal, Hafsa Cherid and Paul MacPherson. (2013). IL-7 suppresses CD127 gene transcription in human CD8 T cells by inducing expression of the transcription factor c-Myb. The Journal of Immunology, May 2013, Volume 190. Meeting Abstract Supplement 184.11.
3. Paul MacPherson, **Feras Ghazawi**, Elliott Faller, Scott Sugden, Hafsa Cherid, Juzer Kakal, Parmvir Parmar and Abdulkareem El-Salfiti. (2013). By usurping IL-7 mediated pathways, the HIV Tat protein targets CD127 for degradation and thus attenuates CD8 T-cell survival and function. The Canadian Journal of Infectious Diseases and Medical Microbiology. Spring 2013, Volume 24. Supplement SB. Abstract O025.
4. Hafsa Cherid, **Feras Ghazawi** and Paul MacPherson. (2013). Regions of the CD127 cytoplasmic domain necessary for Tat-induced internalization. The Canadian Journal of Infectious Diseases and Medical Microbiology. Spring 2013, Volume 24. Supplement SB. Abstract P001.
5. Cecilia Costiniuk, Sandra Côté, **Feras Ghazawi**, Lorna Carrasco-Medina, Charlene Young and Jonathan Angel. (2013). Oncolytic Viruses as a Potential Approach to Eliminate the HIV Reservoir. The Canadian Journal of Infectious Diseases and Medical Microbiology. Spring 2013, Volume 24. Supplement SB. Abstract P021.
6. **Feras Ghazawi**, Parmvir Parmar, Scott Sugden, Elliott Faller, Nadia Sant and Paul MacPherson. Suppressor of Cytokine Signalling (SOCS) Proteins are Induced by IL-7 and May Play a Role in Regulating CD127 Expression. The Canadian Journal of Infectious Diseases and Medical Microbiology. Spring 2012, Volume 23. Supplement SB. Abstract P035.

7. **Feras Ghazawi**, Elliott Faller, Parmvir Parmar, Juzer Kakal and Paul MacPherson. IL-7 Suppresses CD127 Gene Transcription in Human CD8 T-Cells via a STAT5-Induced Repressor. The Canadian Journal of Infectious Diseases and Medical Microbiology. Spring 2012, Volume 23. Supplement SB. Abstract P036.
8. Elliott Faller, **Feras Ghazawi** and Paul MacPherson. IL-7 induces rapid internalization of CD127 by clathrin mediated endocytosis, leading to subsequent degradation by the proteasome. The Journal of Immunology, May 2012, Volume 188. Meeting Abstract Supplement 174.1.
9. Elliott Faller, **Feras Ghazawi** and Paul MacPherson. IL-7 induces rapid internalization of CD127 proteins by clathrin mediated endocytosis, leading to subsequent degradation by the proteasome. The Canadian Journal of Infectious Diseases and Medical Microbiology. Spring 2012, Vol. 23. Sup. SB. Abstract O085.
10. **Feras Ghazawi**, Elliott Faller, Juzer Kakal and Paul MacPherson. IL-7 suppresses transcription of the CD127 gene in human CD8 T cells by stimulating the expression of a STAT5-induced repressor. The Canadian Journal of Infectious Diseases and Medical Microbiology. Spring 2011, Volume 22. Supplement SB. Abstract O028.
11. Elliott Faller, **Feras Ghazawi**, Scott Sugden, Juzer Kakal and Paul MacPherson. (2011). HIV Tat and IL-7 down regulate surface expression of the IL-7 receptor α-chain through overlapping pathways. The Canadian Journal of Infectious Diseases and Medical Microbiology. Spring 2011, Vol. 22. Supplement SB. Abstract O082.
12. **Feras Ghazawi**, Elliott Faller, Juzer Kakal and Paul MacPherson. Transcriptional Regulation of the CD127 Gene in CD8 T-Cells. The Canadian Journal of Infectious Diseases and Medical Microbiology. Summer 2010, Volume 21. SB. O081.
13. Jihong Chen, **Feras Ghazawi**, Wafaa Bakkar and Qiao Li. Histone deacetylase inhibitors induce apoptosis through inhibition of Akt expression. Differentiation. October 2006. Volume 74, Issue 8, Pages 449–478. Abstract P72.

Lab. Skills/Experimental Techniques

Flow cytometry	Quantitative Real-Time PCR	Semi-quant. PCR	Western blotting
Co-IPs	ELISA	Luciferase assays	Multiplex assays
Basic cloning	HPLC	Nuclear run-on	PCR arrays
Bioinformatics	Site directed mutagenesis	Transfection assays	siRNA knockdown
Primary T cell isolation and culture and culturing of many cell lines including Jurkat, HeLa, SiHa, Hoc, CHO, Sup-T, A3, U9 and CEM			

Teaching Activities

Teaching Assistant at the University of Ottawa for the following courses:
BCH 2333: Introduction to biochemistry course (Discussion Groups Leader and Marker)
BCH 2333: Introduction to biochemistry laboratory (Lab Demonstrator)
BCH 3346: Molecular biology laboratory (Lab Demonstrator)
BCH 3170: Molecular biology course (Marker and Proctor)
BCH 4123: Pathological Chemistry (Marker)

Other Experience and Professional Memberships

2012-current. Member/Student, the American Association of Immunologists, Bethesda, USA
2012-current. Treasure, Scientists Without Borders Ottawa Chapter, Ottawa, Canada
2009-2010. Coordinator, Dignitas HIV/AIDS Student Chapter at the University of Ottawa.
2006-current. Member, Golden Key International Honor Society, Atlanta, Georgia, USA.
2005-2006. VP Executive of Microbio. and Virology Journal Club, University of Ottawa.
2004. Summer Volunteer in Dr. N. Tanphaichitr lab, Ottawa Hospital Research Institute.
2004-2007. Science Tutor. Part-time employment and volunteer to students with a financial need. University of Ottawa Peer Help Center.

Certificates/Licences

1. Biosafety Level 3-Laboratory Training, Radiation Safety Training, Workplace Hazardous Materials Information System (WHMIS) and Lab Biosafety Training certified by the Ottawa Hospital Research Institute (OHRI) – 2009-2013.
2. Standard First Aid and Cardiopulmonary resuscitation (CPR) training (level C) – Oct 08.
3. Canadian Council on Animal Care (CCAC) Institutional Animal User Training Program - Aug 2004.

Workshops

1. Inflammation and Mucosal Immunity in HIV Workshop. Montreal. May 2011.
2. Interleukin-7: Translating Laboratory Research into Clinical HIV Investigation Workshop. Montreal. April 2010.
3. New HIV Researcher Workshop Program by the Canadian Association for HIV Research. Saskatoon. May 2010.