uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Juzer Kakal

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Microbilogy and Immunology)

GRADE / DEGREE

Department of Biochemistry, Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Transcriptional Regulation of the CD127 Gene in Primary Human CD8 T-Cells

TITRE DE LA THÈSE / TITLE OF THESIS

Dr. Paul MacPherson

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Dr. Kathryn Wright

Dr. Marko Kryworuchko

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

TRANSCRIPTIONAL REGULATION OF THE CD127 GENE IN PRIMARY HUMAN CD8 T-CELLS

by

Juzer Kakal

A thesis submitted in partial fulfillment of the
requirements for the degree of Masters in Science (M.Sc.),
in Microbiology and Immunology.

University of Ottawa

Faculty of Medicine

January 16, 2009

Your file Votre référence
ISBN: 978-0-494-51839-7
Our file Notre référence
ISBN: 978-0-494-51839-7

TRANSCRIPTIONAL REGULATION OF THE CD127 GENE IN PRIMARY HUMAN CD8 T-CELLS

ABSTRACT

Factors regulating expression of the IL-7 receptor throughout the CD8 T-cell life-span have not been fully characterized but IL-7 has been shown to down regulate the IL-7 receptor α-chain (CD127) at the cell surface. Here we show that IL-7 induced a decline in the level of total CD127 transcripts in CD8 T-cells. Also, IL-7 had no effect on CD127 mRNA stability, or alternative splicing. We also find that down regulation of CD127 by IL-7 requires *de novo* transcript and protein synthesis. Reporter assay analysis of the CD127 promoter showed that that sequences up to 3kb upstream of the TATA box are required for basal CD127 gene expression. Within this region a Glucocorticoid Response Element near -2.2kb was identified as an important regulator of CD127 transcription. Conversely, IL-7 did not down regulate the ~3kb promoter construct suggesting that the IL-7 responsive elements may lie outside this region.

for pappa

ACKNOWLEDGEMENTS

This work is incomplete without recognizing those who have had a tremendous influence that has helped me arrive to this point. To all of you, I express my deepest gratitude for your timely advice, wisdom, patience and sympathetic ear, which have allowed me to see beyond times of confusion, doubt and sometimes despair.

I am grateful to you, Paul, for your faith in me and for providing an environment that has fostered a lot more than the learning of mere scientific techniques and presentation skills. Working under your supervision has truly been an eye-opening experience in terms of helping me identify my future goals.

My experience would be incomplete without Patty's patience and understanding, Huzefa's dilemmas, Maha's spontaneity, Scott's 'logical compass', Mark's passion and loyalty, Denny's laid-back attitude, Jessica's 'moments of wonder in science', Alex's idealism, Elliott's relentless ideas, Alison's positive outlook, Andrea's sympathetic ear, Angela's wealth of experience, Subreena's cheerful demeanour, Peter's discussions over breakfast, Darren's generosity and grand stories; all your attributes and friendships have truly enriched my life.

I would also like to thank everyone else at the Angel labs at the OHRI and the CHEO Virology group, especially my committee members Dr Angel and Dr Kumar for all your advice and feedback that has allowed this project come to fruition.

Last but not least, I would like to thank all my friends and families both here in Canada and in East Africa. Your unwavering faith, support and belief in me have always been and will continue to be a positive influence in my life.

"I don't think necessity is the mother of invention - invention, in my opinion, arises directly from idleness, possibly also from laziness. To save oneself trouble."

Agatha Christie (1890 - 1976),

An Autobiography, 1977

"It is easier to get forgiveness than permission."

Stewart's Law of Retroaction,

Murphy's Law, Book Two

TABLE OF CONTENTS

1 CHAPTER 1: INTRODUCTION 11

1.1 INTERLEUKIN-7: AN IMPORTANT REGULATOR OF CYTOLYTIC FUNCTION 12
1.2 THE GAMMA CHAIN CYTOKINE RECEPTOR FAMILY 13
1.3 IL-7 AND IL-7 RECEPTOR 14
1.4 DYSREGULATION OF IL-7/IL-7R SIGNALING 16
1.5 REGULATION OF CD127 GENE EXPRESSION 18
1.5.1 REGULATION OF CD127 BY INTERLEUKIN-7 20
1.5.2 REGULATION OF CD127 BY DEXAMETHASONE 20

2 CHAPTER 2: MATERIALS AND METHODS 23

2.1 CD8 T-CELL PURIFICATION 24
2.2 CELL CULTURE 25
2.3 FLOW CYTOMETRY 25
2.4 TOTAL RNA ISOLATION 25
2.5 CDNA SYNTHESIS 26
2.6 REAL-TIME PCR 26
2.7 CLONING OF THE CD127 PROMOTER INSERTS INTO THE PGL4B VECTOR 29
2.8 NUCLEOFECTION 38
2.9 LUCIFERASE ASSAY 41
2.10 PROTEIN ASSAY 42
2.11 WESTERN BLOTTING 42
2.12 DATA AND STATISTICAL ANALYSIS 44

3 CHAPTER 3: RESULTS 45

3.1 INTERLEUKIN (IL)-7 DOWN REGULATES CD127 EXPRESSION IN PURIFIED HUMAN CD8 T-CELLS AT THE LEVEL OF TRANSCRIPTION 46
3.1.1 IL-7 DOWN REGULATES CD127 PROTEIN EXPRESSION ON THE SURFACE OF PURIFIED CD8 T-CELLS 46

3.1.2 IL-7 DOWN REGULATES CD127 AT THE LEVEL OF TRANSCRIPTION IN PURIFIED CD8 T-CELLS: ANALYSIS BY REAL TIME PCR 47
3.1.3 TREATMENT OF PURIFIED CD8 T-CELLS WITH IL-7 DOES NOT BIAS ALTERNATIVE SPLICING OF CD127 TRANSCRIPTS IN PURIFIED CD8 T-CELLS 53
3.1.4 IL-7 DOWN REGULATES CD127 AT THE LEVEL OF TRANSCRIPTION IN PURIFIED CD8 T-CELLS: ANALYSIS BY CONVENTIONAL PCR. 55
3.1.5 DE NOVO TRANSCRIPT SYNTHESIS IS REQUIRED FOR IL-7 TO DOWN REGULATE CD127 TRANSCRIPTS IN CD8 T-CELLS. 65
3.1.6 SUMMARY: 68
3.2 CHARACTERIZATION OF THE CD127 GENE PROMOTER IN PURIFIED CD8 T-CELLS 69
3.2.1 WEB-BASED ANALYSIS OF THE UPSTREAM CD127 PROMOTER REGION. 69
3.2.2 CLONING THE UPSTREAM REGULATORY REGION OF THE CD127 GENE. 76
3.2.3 TRANSFECTION OF PUTATIVE CD127 PROMOTER CONTAINING PLASMIDS INTO PURIFIED CD8 T-CELLS 78
3.2.4 CD127 GENE PROMOTER ANALYSIS IN CD8 T-CELLS 79
3.2.5 SUMMARY: 98
3.3 REGULATION OF THE CD127 PROMOTER 99
3.3.1 REGULATION OF THE CD127 PROMOTER BY DEXAMETHASONE 99
3.3.2 REGULATION OF THE CD127 PROMOTER BY IL-7 109
3.3.3 SUMMARY: 117

4 CHAPTER 4: DISCUSSION 118

4.1 REGULATION OF CD127 MRNA EXPRESSION 119
4.2 REGULATION OF THE CD127 PROMOTER 123

5 CONCLUSION 126

6 REFERENCES 127

7 CURRICULUM VITAE 136

LIST OF FIGURES AND TABLES

FIGURES

Figure 2-1: Schematic showing the various CD127 promoter constructs that were transfected into primary CD8 T-cells. 39

Figure 3-1: IL-7 causes a down regulation of CD127 surface protein expression on purified CD8 T-cells. 48

Figure 3-2: IL-7 down regulates CD127 expression in CD8 T-cells at the level of transcription....51

Figure 3-3: The CD127 mRNA transcript. 54

Figure 3-4: IL-7 down regulates mRNAs encoding both the transmembrane and secreted isoforms of CD127 protein....56

Figure 3-5: The CD127 transcript showing primer binding sites. The CD127 mRNA consists of 8 exons. 59

Figure 3-6: There are two predominant species of CD127 mRNA in CD8 T-cells....60

Figure 3-7: Transcripts encoding both the full-length and secreted isoforms of CD127 are down regulated by IL-7 in CD8 T-cells. 63

Figure 3-8: *De novo* transcript synthesis is required for IL-7 to down regulate CD127 transcripts in CD8 T-cells. 66

Figure 3-9: *cis*-regulatory regions of the CD127 gene....71

Figure 3-10: The CD127 upstream regulatory region and putative transcription factor binding sites....74

Figure 3-11: Nucleofected CD8 T-cells express similar levels of CD127 surface protein compared to untransfected cells. 80

Figure 3-12: Luciferase gene expression from constructs up to -1200 bp from the TATA box. 83

Figure 3-13: Sequences from the CD127 TATA box to the ATG do not significantly affect luciferase gene expression....85

Figure 3-14: The -2900 to TATA promoter construct increases luciferase gene expression 13 fold above background. 88

Figure 3-15: Relative luciferase expression of theCD127 promoter constructs. 91

Figure 3-16: Relative luciferase expression of the -2900-1760^1200-TATA construct..... 93

Figure 3-17: Relative Luciferase Activity of the -2900 to -478 promoter construct. 96

Figure 3-18: Dexamethasone up regulates CD127 mRNA in CD8 T-cells. 100

Figure 3-19: Dexamethasone up regulates transcription from the CD127 gene promoter. .. 104

Figure 3-20: Dexamethasone up regulates CD127 protein expression. 107

Figure 3-21: IL-7 does not down regulate luciferase gene expression from the cloned CD127 promoter in CD8 T-cells. 110

Figure 3-22: *De novo* protein synthesis is required for IL-7 to down regulate CD127 gene transcription..... 113

Figure 3-23: IL-7 did not induce a change in Gfi-1 protein expression in CD8 T-cells over 24 hour incubation with IL-7. 115

TABLES

Table 2-1: Primers used for the amplification/quantitative analysis of CD127 and RPS18 transcripts 28

Table 2-2: Primer sequences used in the amplification of the CD127 promoter inserts..... 31

Table 2-3: Primers used for site directed deletion of the GR responsive element at -2269 to -2255 upstream of the TATA box within the CD127 promoter..... 37

Table 3-1: List of CD127 promoter constructs cloned into pGL4B. 77

LIST OF ABBREVIATIONS

Act D – Actinomycin D
AIDS – Acquired Immuodeficiency Syndrome
ATP – Adenosine Triphosphate
CD – Cluster of differentiation
Cdk – cyclin dependent kinase
CHX – Cycloheximide
Dex - Dexamethasone
DNA - Deoxyribonucleic acid
ECL – Enhanced Chemiluminescence
FoxP3 - forkhead box P3
Gfi-1 – Growth Factor Independent 1
GFP – Green Fluorescent Protein
GR – Glucocorticoid Receptor
GRE – Glucocorticoid Response Element
HAART – Highly Active Antiretroviral Therapy
HIV – Human Immunodeficiency Virus
HRP – Horseradish Peroxidase
IFN – Interferon
IL – Interleukin
ISRE – Interferon Sensitive Response Element
Jak – Janus Kinase
MCL-1 - Myeloid cell leukemia sequence 1
MHC – Major Histocompatibility Complex
mRNA – messenger RNA or transcript
NCBI – National Centre of Biotechnology Information
NF-κB- nuclear factor-kappa B
NK – Natural Killer
PBMC – Peripheral Blood Mononuclear Cells
PCR – Polymerase Chain Reaction
PE – Phycoerythrin
PI3K - Phosphoinositide 3-kinase
PID – Primary Immunodeficiency
RLU – Relative Light Units
RNA – Ribonucleic Acid
RPS18 – Ribosomal protein of 18 kDa
SCID – Severe Combined Immunodeficiency
SMAD - class of proteins that modulate the activity of TGF-β ligands
Src – kinase from the family of proto-oncogenic tyrosine kinases
STAT – Signal Transducer and Activator of Transcription
TATA – TATAA signal or TATA box
TCR – T-cell Receptor
TGF – Transforming Growth Factor
TSLP – Thymic Stromal Lymphopoietin
TSS – Transcription Start Site
X-SCID – X-linked Severe Combined Immunodeficiency

CHAPTER 1: INTRODUCTION

1.1 Interleukin-7: an Important Regulator of Cytolytic Function

Interleukin (IL)-7 plays an important role in the homeostasis, proliferation, differentiation and survival of CD8 T-cells. Specifically, IL-7 enhances CD8 T-cell expansion (3, 76), up regulates telomerase (64), induces production of perforin leading to increased anti-viral and anti-tumor activity (6, 31, 63) and reduces the apoptotic potential of these cells by up regulating the expression of pro-survival molecules (1, 43). CD8 T-cells are responsible for immune surveillance and cell-mediated immunity through antigen sampling on MHC Class I molecules (28). These cells seek and destroy cells that display internal abnormalities due to infection or tumor-like activity through release of cytolytic molecules and engagement of death inducing receptors on the surface of target cells (28). More specifically, antigen presenting cells (APCs) such as dendritic cells process and present foreign antigen to Cytotoxic (CD8) T-cells which recognize a specific antigen on a MHC class I complex and in turn become activated and begin to proliferate. The activation process also involves the synthesis of cytolytic molecules like perforin and granzymes. Upon contacting infected cells that present the same foreign antigen, the CD8 T-cells release the cytolytic molecules and engage death receptors on the surface to induce apoptosis in the infected cell. This report will aim to contribute some understanding of the regulation of IL-7 receptor in primary human CD8 T-cells and thereby augment our understanding of the role of IL-7 as a key regulator of human cell-mediated immunity.

1.2 The gamma chain cytokine receptor family

The gamma-chain (γc) cytokine receptor family is a group of cytokines whose receptors share the gamma subunit of the interleukin (IL) -2 receptor complex, otherwise called CD132. Together with IL-7, this family of cytokines comprises of IL-2, IL-4, IL-9, IL-15, and IL-21. While two of the cytokines in this family, IL-2 and IL-15, bind to heterotrimeric receptor complexes, the remaining cytokines (IL-4, IL-7, IL-9 and IL-21), bind to receptor complexes that are heterodimeric in nature. Another characteristic of these receptor subunits, is that they are all single-pass fibronectin type I molecules (46). While the alpha chain primarily provides specificity of ligand binding, the CD132 or gamma chain possesses essential tyrosine residues located on the intracellular side of the plasma membrane that can be phosphorylated by several kinases such as PI3K, Jak and Src (1, 30, 48). Due to the ubiquitous expression of the gamma-chain on virtually all lymphocytes, the responsiveness of the receptor complexes is controlled by the presence or absence of the alpha chain providing for specificity of signal transduction (1).

The role of gamma-chain cytokines have been extensively studied in mice where $\gamma c^{-/-}$ mice show a significant reduction in the numbers of NK, B and T-cells (reviewed in (1)). In the context of viral infections, it becomes important to identify factors that lead to a dysfunction especially in the homeostasis of the T-cell pool since these cells of the adaptive immune system play an important role in the clearance of intracellular pathogens (28). Among the gamma chain cytokine family members, IL-2, IL-15 and IL-7 have been implicated in lymphoid homeostasis and T-cell ontogeny (39). Studies from IL-2 and IL-2Rα knockout mice show an initially normal T-cell subset phenotype, which over time become enriched in an activated phenotype leading to autoimmunity due to dysregulation of FoxP3

expressing $CD4^+$ regulatory T-cells (55, 56, 77). Conversely, IL-15 and IL-15Rα knockout in mice has been shown to cause a reduction in intraepithelial lymphocytes, Natural Killer (NK) T-cells and CD8 memory cells (58).

1.3 IL-7 and IL-7 receptor

A member of the gamma chain cytokine receptor family, the IL-7 receptor is composed of two chains: a unique alpha chain (CD127) and a common gamma chain (CD132) (30). Similar to what has been observed for the other members of the gamma chain cytokine receptors family members, the alpha chain of the IL-7 receptor complex, (CD127) provides ligand binding specificity while the gamma chain (CD132) participates in signal transduction. Binding of IL-7 to CD127 induces a conformational change and results in dimerization with CD132 (30, 43). Furthermore, the alpha chain of the IL-7 receptor (CD127) also forms a heterodimer with thymic stromal lymphopoietin (TSLP) receptor that binds TSLP (30, 48). TSLP is a cytokine produced by skin and smooth muscle cells and TSLP/TSLPR signaling is primarily involved in antigen-independent maturation of dendritic cells in the thymus, activation of mast cells during allergic response and differentiation of Regulatory T-cells. Furthermore, TSLP receptor is homologous to CD132 in its structure and signaling (reviewed in (75)).

According to microarray studies of the human transcriptome, IL-7 is produced by virtually all cell types and the highest producers of IL-7 are B-lymphocytes and $CD34^+$ human hematopoietic progenitor cells (67). Interestingly, IL-7 mRNA production in stromal cells is unaffected despite changes in either the size of the lymphocyte pool or amount of circulating IL-7 (17).

As previously mentioned, IL-7 plays a non-redundant role in the survival, differentiation and homeostasis of both mature and progenitor lymphocytes (32). Studies from mice that lacking the IL-7 gene, showed a phenotype of severe lymphopenia with a particular loss in T-cells (51). Mice lacking IL-7 were also unable to maintain homeostasis of their naïve or memory T-cells compared to those lacking other cytokines of the gamma chain family (58, 69). Moreover, the lack of IL-7 led to increased apoptosis in these cells and this was partly due to dysregulation of anti-apoptotic proteins such as Bcl-2, Bcl-X_L, MCL-1 and pro-apoptotic molecules like Bad, Bax and Bim proteins (32, 42). Beyond protection from apoptosis, IL-7 is also responsible for promoting proliferation of resting T-cells in mice (58, 69). IL-7 has been shown in mice to regulate the G1 to S phase checkpoint via the breakdown of $p27^{kip1}$, an inhibitor if G_1 cyclins and cyclin-dependent kinases (CDKs) (15, 34, 82). In our lab, we have shown that primary human CD8 T-cells incubated with IL-7 proliferated to a greater extent following enagagement of the TCR (13). Furthermore, IL-7 signaling has been shown to play a key role in regulation of glucose uptake of T-cells and maintenance of intracellular pH of growing cells (reviewed in (30)). Furthermore, IL-7 or IL-7Rα knockout mice show a severe reduction in the number of all lymphocytes and their precursors (51, 73). This is slightly different in humans in that IL-7 and IL-7Rα do not seem to play a significant role in B-cell development although patients lacking in IL-7/IL-7Rα do still exhibit a significant reduction in all T-cells including Natural Killer (NK) T-cells (53). Interestingly, when these findings are examined in combination with those of IL-2R and IL-15R knockout mice, it becomes apparent that in resting naïve CD8 T-cells, the expression of IL-2 and IL-15 receptor subunits is low compared to that of IL-7R suggesting that IL-7 and IL-7 receptor signaling plays a more significant role in the maintenance of the resting CD8

T-cell pool than IL-2 or IL-15, whose roles may be important in other stages of CD8 T-cell differentiation (39, 43).

Unlike the near ubiquitous expression of IL-7, the expression of the IL-7 receptor alpha-chain (CD127) is fairly restricted. CD127 has been shown to be expressed in monocytes, B-cells and T-cells including Natural Killer T-cells (27, 43). Upon examination of CD127 expression in developing T-cells in the thymus, the triple negative ($CD3^-$, $CD4^-$, $CD8^-$) common lymphoid progenitor begins by expressing CD127 on its surface to allow these cells to proliferate and provide them with protection from apoptosis and most importantly, promote the IL-7/IL-7R signaling dependent T-cell Receptor γ - chain rearrangement (30, 36, 57). The $CD3^+$, $CD4^+$, $CD8^+$ cells which undergo positive and negative selection do not express CD127. Re-expression of CD127 in CD8 or CD4 positive T-cells allows for these cells to receive IL-7 mediated homeostatic signals (30, 43, 81). Using murine models expressing chimeric IL-7 receptor molecules conjugated to the intracellular tails of other gamma chain cytokine receptors, Jiang et al and Maki et al. were able to show that although IL-9 signaling may somewhat allow normal development of alpha-beta (αβ) T-cells, signaling through this cytokine (IL-9) was unable to reconstitute rearrangement of the TCR gamma locus or allow development of gamma-delta (γδ) T-cells (29, 41). Taken together, these studies underscore the important and non-redundant role of IL-7/IL-7R signaling in proper T-cell development and function.

1.4 Dysregulation of IL-7/IL-7R signaling

There are several conditions that appear to lead to impairments in adaptive immunity or primary immunodeficiencies (PID). Severe combined immunodeficiency (SCID) is a type

of PID that encompasses several different genotypes, which ultimately result in a common phenotype of deficient lymphocyte development leading to adaptive immune system dysfunction (5, 21, 28). Of particular interest is X-linked SCID (X-SCID), which results in a complete loss of NK and T-cells in patients with this condition (5, 54). The molecular basis of X-SCID has been described as the loss of signaling through IL-7 receptor due to mutations leading to loss of function of CD132 (5, 62). Moreover, findings by Roifman et al. have shown that in patients bearing mutations in CD127 have significant reductions in circulating NK and T-cells resulting in severe immunodeficiency. This indicates that IL-7/ IL-7R signaling plays a critical role in NK and T-cell development and homeostasis (54).

Another type of Immunodeficiency is the Acquired Immune Deficiency Syndrome (AIDS). This disease, caused by the Human Immunodeficiency Virus (HIV), leads to a breakdown in cell-mediated immunity through the destruction of CD4 T-cells (28). Interestingly, during the course of HIV infection, while CD4 T-cell numbers decline, the number of CD8 T-cells remain relatively unchanged (19, 44, 66). Despite the persistence of CD8 T-cells, these cells are unable to respond to antigen and fail to demonstrate cytolytic activity due to poor expression of perforin, granzymes or interferon (IFN)-γ (4, 24, 44, 60, 80). Our lab has shown that during the course of an HIV infection, CD127 is down regulated in HIV positive patients and this receptor is restored to relatively normal levels in patients who are on successful HAART treatment (40). Furthermore, expression of this receptor has been shown to correlate inversely with virologic suppression (40). Additionally, we have also shown that this virus-mediated down regulation of CD127 leads to defects in CD8 T-cell proliferation and function – further highlighting the importance of IL-7/ IL-7R signaling in T-cell homeostasis and function (13).

1.5 Regulation of CD127 Gene Expression

As previously described, signaling through IL-7R is integral to CD8 T-cell survival, development, homeostasis and function (reviewed in (17, 30, 34, 43, 48, 58, 63, 69)). Owing to its important role, expression of this receptor is tightly regulated throughout T-cell development. Regulation of CD127 gene expression remains poorly understood, particularly in resting human CD8 T-cells. This project aims to contribute to our understanding of how this essential gene is regulated in primary human resting CD8 T-cells.

The human CD127 gene promoter shares a 75% homology to its murine equivalent (37). In the murine promoter, several upstream *cis*-regulatory elements have been identified to influence the expression of the CD127 gene (43). The core promoter region is located within the first 197bp upstream of the transcription start site (TSS). Moreover, this region has been shown to contain sites for RUNX, Ikaros, GABP and NF-κB (43). Additionally, an interferon-stimulated response element (ISRE) was identified approximately 1.1kb upstream of the TSS (52). A conserved region approximately 3kb upstream of the TSS was also identified as responsive to glucocorticoid receptor (GR) (37). It is interesting to note that all of these identified upstream *cis*-regulatory elements are positive regulators of the murine CD127 gene expression (37, 43, 52). Conversely, a suppressor of CD127 gene expression (10) Growth Factor Independent (Gfi)-1, has been shown to bind within introns 2 and 4 of the CD127 gene (50). DNA methylation has classically been associated with gene silencing. Findings in one study have found that high expression of CD127 in human CD8 T-cells correlated with hypomethylation of the CD127 promoter thereby resulting in increased transcription of CD127 – thereby adding promoter methylation as a regulatory factor in CD127 gene transcription (33). It is important to note that although the role of each of the

previously mentioned regulatory elements has been evaluated in a reductionist manner, recent evidence shows interplay between the binding of GABP and Gfi-1 in the regulation of the CD127 gene expression suggesting co-operation and crosstalk between the various regulatory elements and thereby adding a further level of complexity in the regulation of the CD127 gene (7).

The ~1.6kb mRNA encoding for CD127 can be alternatively spliced to produce two major transcripts that encode for either transmembrane or secreted isoforms of CD127 protein (71). In children with acute lymphoblastic leukemia, Korte et al describe the presence of several other isoforms of CD127 mRNA (35). However, most of these isoforms result in out-of-frame splice variants that lead to premature stop codons or unproductive transcripts. This study and others have also found that the transcript encoding for the secreted isoform of CD127 protein, is a result of exon 6 skipping, causing a shorter transcript that encodes an mRNA that is ~100bp shorter than the full length isoform (22, 35). Moreover, they identified that in healthy mature T-cells, the secreted and transmembrane isoforms of CD127 protein are considered to be natural variations and that the relative abundance of the transmembrane protein was significantly higher than that of secreted CD127 protein (35). Furthermore, a study in multiple sclerosis patients has shown that an imbalance in the ratio of transmembrane versus secreted isoforms of CD127, which is a result of a disrupted exon splicing silencer, can be a risk factor for multiple sclerosis (22). This study identifies yet another level of regulation of CD127 – one that causes a down regulation of CD127 surface protein through a reduction in transcripts encoding the transmembrane isoform while simultaneously quenching any additional IL-7 mediated signals through sequestration of the ligand by secreted IL-7R. In view of the natural occurrence of these CD127 isoforms, it

becomes important to examine whether CD127 expression is regulated at this level in healthy individuals.

1.5.1 Regulation of CD127 by Interleukin-7

Members of the gamma chain (γc) cytokine family of cytokines (IL-2, 4, 7 and 15) have been shown to down regulate CD127 mRNA expression in murine models. In particular, IL-7 has been shown to down regulate surface expression of its own receptor CD127, in purified CD8 T-cells (50). Moreover, studies looking at CD127 surface protein expression in human primary CD8 T-cells have shown that the magnitude of IL-7 mediated down regulation at 24 hours post-treatment is the greatest (74).

1.5.2 Regulation of CD127 by Dexamethasone

Glucocorticoids are known anti-inflammatory molecules that have been shown to induce CD127 expression in murine B and T lymphocytes. Although reports have shown that exposure of T-cells to glucocorticoids can lead to enhanced apoptosis in these cells, the same reports have found that treatment with IL-7 leads to a rescue from apoptosis through up regulation of anti-apoptotic proteins such as GILZ and Bcl-2 (16, 25). Moreover, Franchimont et al have shown a positive role of glucocorticoids in T-cell survival and function (16). Although known as immunosuppressants, glucocorticoids have been shown to enhance IL-7 mediated T-cell response, memory cell development, T-cell activation and survival of both mature T-cells and thymocytes (16).

The following chapters will show that IL-7 and glucocorticoids differentially regulate CD127 gene transcription in primary human CD8 T-cells. We will examine hypotheses of

whether the down regulation by IL-7 occurs through an imbalance in the ratios of secreted versus transmembrane isoforms of CD127 and whether IL-7 accelerates the degradation of CD127 transcripts. Additionally, we will examine the regulation of the human CD127 promoter in resting CD8 T-cells and compare its regulation to that identified in murine models. Lastly we will examine the possibility of whether IL-7 or GR influence the regulation of the human CD127 promoter in CD8 T-cells via influencing the binding of upstream *cis*-regulatory elements in the CD127 promoter.

Hypothesis:

Interleukin -7 and glucocorticoids differentially regulate CD127 gene expression in primary human CD8 T-cells.

Objectives:

1. To test the hypothesis that IL-7 down regulates CD127 transcripts and surface protein expression in primary human CD8 T-cells.

2. To identify whether IL-7 mediated down regulation of CD127 surface protein occurs due to alternative splicing of CD127 transcripts.

3. To establish whether *de novo* protein synthesis is required for IL-7 mediated down regulation of CD127.

4. To test the hypothesis that glucocorticoid treatment up regulates CD127 transcript and protein expression through interaction at a *cis*-regulatory glucocorticoid response element (GRE) located between -2255 and -2269bp upstream of the TATA box in the human CD127 promoter.

5. To identify the CD127 promoter region and regulatory elements required for basal CD127 gene expression in resting CD8 T-cells

CHAPTER 2: MATERIALS AND METHODS

2.1 CD8 T-cell purification

Whole blood was collected by venipuncture from healthy volunteers into 60mL syringes (BD Biosciences) containing 1mL of 100 USP U/mL of Heparin Lock flush solution (Hospira). The blood was mixed by inversion, overlaid on Ficoll-Hypaque (GE Healthcare) in a 2:1 (blood: ficoll) ratio and centrifuged in a Megafuge 1.0 centrifuge (Heraeus Instruments) at 750*g* for 20 min with the brake turned off. Following centrifugation, the plasma layer was discarded and the buffy coat containing the peripheral blood mononuclear cells (PBMC) was transferred into a sterile tube and washed twice with 50mL sterile Phosphate Buffered Saline (PBS) followed by centrifugation at 443*g* for 10 min for each wash. Cells were counted on a hemacytometer prior to the second wash. CD8 T-cells were isolated using Miltenyi's CD8 T-cell purification protocol. In brief, 1E7 PBMCs were incubated with 20µl of anti-CD8 antibody conjugated ferromagnetic beads for 20min at 4°C. The cells were then washed with PBS and the cell pellet was resuspended in MACS buffer (5% BSA, 2mM EDTA in PBS) and CD8 T-cells were separated using the AutoMACS cell separator (Miltenyi Biotech). Purified CD8 T-cells were counted on a hemacytometer and resuspended at 1E6 cells/mL in RPMI 1640 medium (Hyclone) supplemented with 20% fetal calf serum (FCS; Cansera) plus 100U/mL penicillin and 100µg/mL streptomycin (RPMI-20). Purified CD8 T-cells were allowed to recover overnight in a 37°C, 5% CO_2 jacketed incubator.

2.2 Cell Culture

Where indicated, purified CD8 T-cells were cultured for 24 hours either in medium alone or in the presence of 10ng/mL Interleukin (IL)-7 (BioSource) or 20μM Dexamethasone (BioVision). In experiments where inhibitors for transcription or translation were used, purified CD8 T-cells were pre-incubated for 1 hour with 2μg/mL Actinomycin-D (BioVision) or 100μM Cycloheximide (Sigma-Aldrich) then treated with 10ng/mL IL-7 for an additional 24 hours.

2.3 Flow Cytometry

Treated cells were labeled for 30 min in the dark at room temperature with 2μL/1_E5 cells of anti-human CD8-PC5 (clone B9.11,) and 4μL/1_E5 cells of anti-CD127-Phycoerythrin (PE) (clone R34.34) monoclonal antibodies (Immunotech Beckman Coulter). Flow cytometry was performed on an Epics ALTRA flow cytometer instrument. Live lymphocytes were gated based on forward and side scatter and 10,000 events were counted per condition. Data analysis was performed using the Summit v.4 analysis software (Dako Cytomation).

2.4 Total RNA Isolation

For each experimental condition ~1E6 cells were treated. Total RNA was isolated from CD8 T-cells according to the manufacturer's instructions using the Ambion RNaqueous Total RNA isolation Kit. This protocol involves isolation of RNA using a silica matrix column and preferential precipitaton of RNA. The manufacturer's protocol was followed with the following modifications: Cells were lysed in 300μL of lysis solution and the final

elution was done in two consecutive steps in a total volume of 50μL (35μL + 15μL) of elution solution heated to 70°C. Total RNA was quantified on a nanodrop ND-1000 instrument (Nanodrop) using the elution solution as a blank. cDNA synthesis was performed immediately after RNA isolation and remaining RNA samples were stored at -80°C.

2.5 cDNA synthesis

cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. This method uses a combination of oligo dTs and random decamers for the priming of the reverse transcription reaction. Each reaction contained an equivalent mass of RNA resuspended in 15μL of nuclease-free water, plus 4μL of the iScript Reverse Transcription Buffer and 1μL of the iScript Reverse Transcriptase enzyme. The reaction was performed on the Bio-Rad iCycler instrument where samples were incubated first for 5 min at 25°C to allow primer binding, followed by 30 min at 42°C for reverse transcription and then 5 min at 85°C for enzyme inactivation.

2.6 Real-Time PCR

For relative quantification of CD127 transcripts encoding for the secreted and transmembrane isoforms, PCR was performed as described previously, in parallel using the CD127 exon 4/5 Fwd with CD127 exon 5 Rev, CD127 exon5/7 Fwd with CD127 exon 8 Rev, and RPS18 exon 2/3 Fwd with RPS18 exon3 Rev primers (Table 2-1). Data from these three experiments was analyzed for IL-7 mediated down regulation of total CD127 versus transcripts encoding for the secreted isoform of CD127. In a separate experiment, CD127 Exon 4/5 Fwd primer was used in combination with CD127 Exon 8 Rev primer to measure

the change in transcripts encoding for secreted versus transmembrane isoforms of CD127 protein (Table 2-1).

CD127 and RPS18 transcripts were quantified in triplicate by real-time PCR using primers shown in Table 2-1. The 2X SYBR Green Supermix containing PCR Buffer, iScript thermostable polymerase, dNTPs, and $MgCl_2$ from Bio-Rad was used. Supermix, primers (Table 2-1) and cDNA were combined according to the manufacturer's recommendations. Samples were incubated first at 95°C for 3 min for enzyme activation. This was followed by 50 cycles as follows: 95°C for 30s to denature DNA secondary structure, 57°C for 20s for primer annealing, and then 72°C for 20s for polymerization. At the end of each cycle, samples were held at 85°C for 5s for fluorescence acquisition. Following amplification, samples were denatured for 1 min at 95°C followed by final extension at 72°C for 1 min. Melt curve analysis was performed by measuring fluorescence for 5s from 65°C with 0.5°C increments for 60 cycles in a Bio-Rad iCycler instrument (Bio-Rad). In order to quantify the relative abundance of CD127 transcripts by agarose gel electrophoresis, the PCR reaction was terminated at the logarithmic phase of 33 cycles then resolved on a 2% agarose gel (Fisher Scientific) containing TAE buffer (40mM Tris-acetate, 1mM EDTA) (Sigma-Aldrich). Ethidium bromide (Sigma-Aldrich) at a concentration of ~0.5μg/mL was added to the gel to allow visualization of DNA bands.

Table 2-1: Primers used for the amplification/quantitative analysis of CD127 and RPS18 transcripts

Primer Name	Sequence (5' to 3')
CD127 exon 4/5-Fwd	ATGGACGCATGTGAATTTATC
CD127 exon 5-Rev	TTATTGATCTCTGGAGTTCTGAAG
CD127exon 5/7-Fwd	TAGCTCAGGATTAAGCCTAT
CD127 exon 8-Rev	CTGGAGTGATGACTACATC
RPS18 exon 2/3-Fwd	CTGCCATTAAGGGTGTGG
RPS18 exon 3-Rev	TCCATCCTTTACATCCTTCTG

2.7 Cloning of the CD127 promoter inserts into the pGL4B vector

Genomic DNA isolation

One frozen (-80°C) aliquot containing 1E7 Jurkat T-cells (ATCC), a lympohoblastic T-cell line suspended in DMSO supplemented with 10% FCS was washed twice in sterile PBS then lysed for genomic DNA extraction using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). This protocol involves the isolation of high molecular weight DNA after lysis in a chaotropic, salt-containing lysis buffer and binding of DNA onto a silica-matrix spin-column. After washing cell debris and other macromolecules from the column with wash solution, the DNA was eluted in 250µL nuclease free H_2O.

The following CD127 promoter constructs were generated: -2900 to TATA, -1468 to TATA, -1200 to TATA, -626 to TATA, -626 to ATG, -262 to TATA and -262 to ATG.

Polymerase Chain Reaction (PCR) amplification of CD127 promoter fragments

Isolated genomic DNA was used as a template to generate CD127 promoter fragments by PCR using the primers (300nM) indicated in Table 2-2. The amplification reactions were carried out according to the manufacturer's instructions using the Expand Long PCR Kit (Roche). This kit uses a proprietary blend of thermostable polymerases, allowing for high fidelity amplification of long and/or difficult to amplify fragments. The amplification was performed as follows: 93°C for 2 min for initial activation, then 7 cycles consisting of 93°C for 10s, Ta_1 (Table 2-2) for 30s for annealing, and 72°C for 3 min for extension. These 7 cycles were run at the lower temperature in order to allow for the formation of enough templates containing the restriction enzyme cut sites at the tails of the PCR amplified inserts. Following the 7 cycles, 28 cycles were carried out using the same parameters except the annealing temperature was increased to Ta_2 (Table 2-2). This was done to increase the

annealing stringency of the primers and reduce non-specific binding of the primers. Lastly a final extension step at 72°C was carried out for 8 min to finish the amplification of any incomplete products. The amplification reactions were carried out using the Mastercycler EPs thermal cycler instrument (Eppendorf).

Gel Electrophoresis and extraction of amplified promoter fragments

To remove primers and unwanted amplification products, the amplified DNA was separated on a 1% agarose gel containing TAE buffer (40mM Tris-acetate, 1mM EDTA). Ethidium Bromide (Sigma-Aldrich) at a concentration of ~0.5μg/mL was added to the gel to allow visualization of DNA bands. Following electrophoresis, the gel was exposed briefly (<15 seconds) to long wave ultraviolet (UV) light (320nm) and the prominent band corresponding to the correct fragment size compared to a 1kb molecular weight ladder (Fermentas) was excised and collected in a 2mL microcentrifuge tube. The DNA contained in the excised gel band was recovered using the Qiagen gel extraction kit which employs silica-matrix spin column technology to purify DNA fragments.

Restriction digests of vector and inserts DNA

Purified insert DNA and the pGL4B Vector were double digested with KpnI (10U) and NheI (10U) for 1 hour at 37°C in a 50μL reaction containing 5μL of 10X buffer I (10 mM Bis-Tris-Propane-HCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.0), 0.5μL of 100X BSA (10mg/mL), and 42.5μL insert DNA (measured at <1μg/μL) and 1μL of each enzyme (New England Biolabs). The digested DNA was then purified of small digested fragments using a spin column purification kit from Qiagen according to the manufacturer's protocol (PCR Cleanup Kit - Qiagen).

Table 2-2: Primer sequences used in the amplification of the CD127 promoter inserts.

Sequences shown in bold letters indicate artificially introduced restriction enzyme digest sites for KpnI (GGTACC), NheI (GCTAGC) and NcoI (CCATGG). In order to generate an artificial NcoI cut site, two base pairs, GA were changed to CC (underlined). Ta_1 and Ta_2 indicate the different annealing temperatures used during PCR cycles. For each primer combination, the lowest annealing temperature of the two primers was used for the amplification of each individual insert.

Primer Name	Sequence 5' to 3'	Ta_1	Ta_2
Forward			
2900 KpnI	**GGGGTACC**ACTACATCATACAAACTCATC	51°C	60°C
1468 KpnI	**GGGTACC**ACTGTGAATCAAGCATAGAGTTTTATG	57°C	62°C
1200 KpnI	**GGGGTACC**TAGGCACTAATTTAGTTCATATGTAC	56°C	62°C
626 KpnI	**GGGGTACC**GATTCACTTTGTAAGTTTGAATTTTTGTCTTC	55°C	63°C
262 KpnI	**GGGGTACC**TCTAGCCTAAGATAGCTTGAGC	57°C	63°C
100 KpnI	**CGGGGGTACC**TCAGACTTCCTGTTTCTG	50°C	64°C
Reverse			
TATA rev NheI	**CTAGCTAGC**TTAGATCTAGGGCTTTATACTTATCTAAC	58°C	62°C
ATG rev NcoI	**CATGCCATGG**AGAGAGATAGAGAGAGAGAGTAGATG	56°C	66°C

Phosphatase treatment of vector DNA

Digested pGL4B vector was then subjected to phosphatase treatment in order to remove 5' and 3'phosphate groups to prevent re-circularization of the vector. This was done using Antarctic Phosphatase (New England Biolabs), where up to 1μg of vector DNA in 1X Antarctic Phosphatase Buffer (50 mM Bis Tris-Propane, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ pH 6.0) with 5U of Antarctic Phosphatase enzyme was incubated for 1 hour at 37°C. Following dephosphorylation, the enzyme was inactivated by heating the reaction mixture to 65°C for 15 min. This vector was then purified using Qiagen's PCR purification kit according to the manufacturer's instructions.

Ligation of CD127 promoter inserts into the pGL4 vector at the KpnI and NheI/NcoI sites

Ligations were performed in a 50μL reaction volume using a DNA insert: vector molar ratio of 5:1. Each reaction contained 10μL of 5X ligase buffer (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000) with 1μL T4 DNA ligase enzyme (5U/μL; Invitrogen). Ligations were carried out at room temperature for 1 hour.

Transformation of ligated products into DH5α E.Coli

Following ligation, DNA was transformed into competent *E.coli* cells (DH5α strain; Invitrogen). Alkaline phosphatase treated vector alone and unmodified pGL4B plasmid were also transformed in parallel as negative and positive transformation controls. After frozen transformation competent bacteria were thawed on ice and gently resuspended by occasional inversion, 50μL were aliquoted into pre-chilled 2mL microcentrifuge tubes. Next, 1μL of ligation mixture was added and mixed by gently tapping. The resulting mixture was incubated for 5 min on ice, heat shocked for 45 seconds in a 42°C water bath, and then cold shocked for 2 min on ice. Following this, 250μL of S.O.C growth medium (2% Tryptone,

0.5% Yeast Extract, 0.4% Glucose, 10mM NaCl, 2.5mM KCl, 5mM $MgCl_2$, 5mM $MgSO_4$) were added to the mixture, which was then incubated in a 37°C shaking incubator for 1 hour. The transformed bacteria were then plated onto 1.5% Agar, Luria Broth (LB) (Sigma-Aldrich) plates containing 100μg/mL Ampicillin (Fisher Scientific). Plates were incubated inverted in a 37°C incubator for ~16 hours.

Amplification of positive bacterial colonies and isolation/characterization of plasmid DNA

Luria Broth (2mL) containing 100μL/mL Ampicillin was inoculated with an individual colony and allowed to shake at 37°C for 6-8 hours until an OD of ~0.5 was reached. Following this, 1mL of the bacterial culture was used for a miniprep plasmid extraction using Qiagen's miniprep kit. This protocol uses silica-matrix spin column technology to isolate purified plasmid DNA from bacterial cultures. DNA was eluted in 50μL of nuclease free H_2O and quantified spectrophotometrically by measuring absorbance at 260 and 280 nm on a Nanodrop ND-1000 instrument.

The purified plasmid was then double digested using KpnI and NheI enzymes as previously described, and the resulting mixture was electrophoresed on a 1% agarose gel containing 1X TBE buffer (89 mM Tris-base, 89 mM Boric Acid, 2 mM EDTA). Digested products demonstrating two bands following digestion corresponding to the empty vector and insert were then sequenced at the Ottawa Genomics Innovation Center. Cloned CD127 promoter fragments were aligned against the Ensembl database human IL7r sequence using the BLAST alignment tool from NCBI.

Generation of the 2406 and 1760 to TATA and 2900 to 478 constructs using restriction digest

The 2406 to TATA and 1760 to TATA constructs were generated using the 2900 to TATA construct as a template. First, the 2900 to TATA plasmid was linearized at the unique KpnI cut site at the 5' end of the CD127 promoter insert. This was carried out in a 50μL reaction with 10U KpnI in Buffer I and BSA at 37°C for 1 hour, after which the plasmid was re-isolated using Qiagen's PCR purification Kit. To generate the 2406 to TATA construct, the KpnI digested plasmid was next digested at the BstAPI site at nucleotide 2406 within the promoter insert. This was done using 5U BstAPI at 60°C for 1 hour in 1X SEBuffer W (10 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 8.5). To generate the 1760 to TATA construct, the KpnI digested plasmid was cut at the BaeI site at nucleotide 1760 within the promoter insert. This was done using 5U of BaeI enzyme at 37°C for 1 hour in a 50μL reaction containing Buffer 2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9) supplemented with 100 μg/mL bovine serum albumin and 20 μM S-adenosylmethionine (NEB). The -2900 to -478 construct was generated by digesting with KpnI and BsaXI as previously described. Following each restriction digest, the DNA was re-isolated using Qiagen's PCR purification Kit. The final constructs were then generated by blunt end ligation. To achieve this, the single stranded overhangs at each restriction site were filled in using 3U T4 DNA polymerase (NEB) in 1X NEBuffer 2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9) supplemented with 100 μg/mL bovine serum albumin and 100 μM dNTPs (Invitrogen) and incubating for 5 min at 37°C. The enzyme was then inactivated by addition of 2μL of 0.25M EDTA. The DNA was again re-isolated using Qiagen's PCR purification kit. The final 2406 to TATA and the 1760 to TATA constructs were then generated by an intra-molecular re-ligation. This was done using 5U of T4 DNA ligase in a 50μL reaction with 1μL of 5X ligase buffer (250 mM

Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000) at 14°C for ~16 hours.

Generation of the delta 1760-1200 construct

The unique BaeI and BsaXI restriction sites within the 2900 to TATA CD127 promoter fragment were used to generate the delta 1760-1200 construct. The 2900 to TATA plasmid was digested in consecutive 50µL reactions, first using 5U of BaeI enzyme in 1X Buffer 2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9) supplemented with 100µg/mL bovine serum albumin and 20 µM S-adenosylmethionine (NEB) and incubated at 37°C for 1 hour, followed by 2U of BsaXI in a 50ul reaction in 1X Buffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 1 mM Dithiothreitol, pH 7.9) at 25°C for 1 hour. Following digestion, the DNA was re-isolated and the single strand overhangs filled in using T4 DNA polymerase as previously described. The resultant ~6 kb linearized plasmid was then separated from the deleted fragment on a 1% agarose gel in 1X TAE buffer and purified as previously described. Intra-molecular blunt end ligation as described above provided the final construct.

Generation of the 2900-2269^2255-TATA construct

This 13 base pair deletion was carried out using the Stratagene Site Directed Mutagenesis Kit XL. This kit uses a modification of the megaprimer amplification strategy to introduce site directed deletions in target plasmids. The megaprimers are complimentary to each other and designed here to cause a specific deletion of 13bp located between -2269 to -2255 upstream of the CD127 TATA box by formation of a hairpin loop in the template plasmid. The reaction uses an ultra high fidelity polymerase (Pfu Ultra) that uses the primers to amplify the template plasmid. After the PCR amplification, the template is digested with DpnI, which specifically digests methylated DNA. Since the starting plasmid was isolated

from the DH5-α strain of E. coli, a dam+ strain that yields methylated plasmid DNA, DpnI digestion destroys the original template leaving only the amplified plasmid carrying the desired deletion.

The 2900 to TATA plasmid was used as a template to amplify the 2900-2269^2255-TATA construct. The primers were designed using the Stratagene Site directed Mutagenesis primer design program (Table 2-3). The PCR amplification conditions used were as follows: 95°C for 30s for initial enzyme activation, then 20 cycles of 95°C for 30s, 55°C for 1 min, then 72°C for 7 min. The completed reaction was cooled on ice and then digested with DpnI at 37°C for 1 hour before transforming into competent bacteria.

Table 2-3: Primers used for site directed deletion of the GR responsive element at -2269 to -2255 upstream of the TATA box within the CD127 promoter.

^ denotes the location of the 13bp deletion.

Primer Name	Sequence 5' to 3'
del 2269 - 2255 sense	TACCAAAATATTGTGTCTTGGCTTTT^TCACAACTAAAGGAAAGAGATAC
del 2269 - 2255 antisense	GTATCTCTTTCCTTTAGTTGTGAAAA^AGCCAAGACACAATATTTTGGTA

2.8 Nucleofection

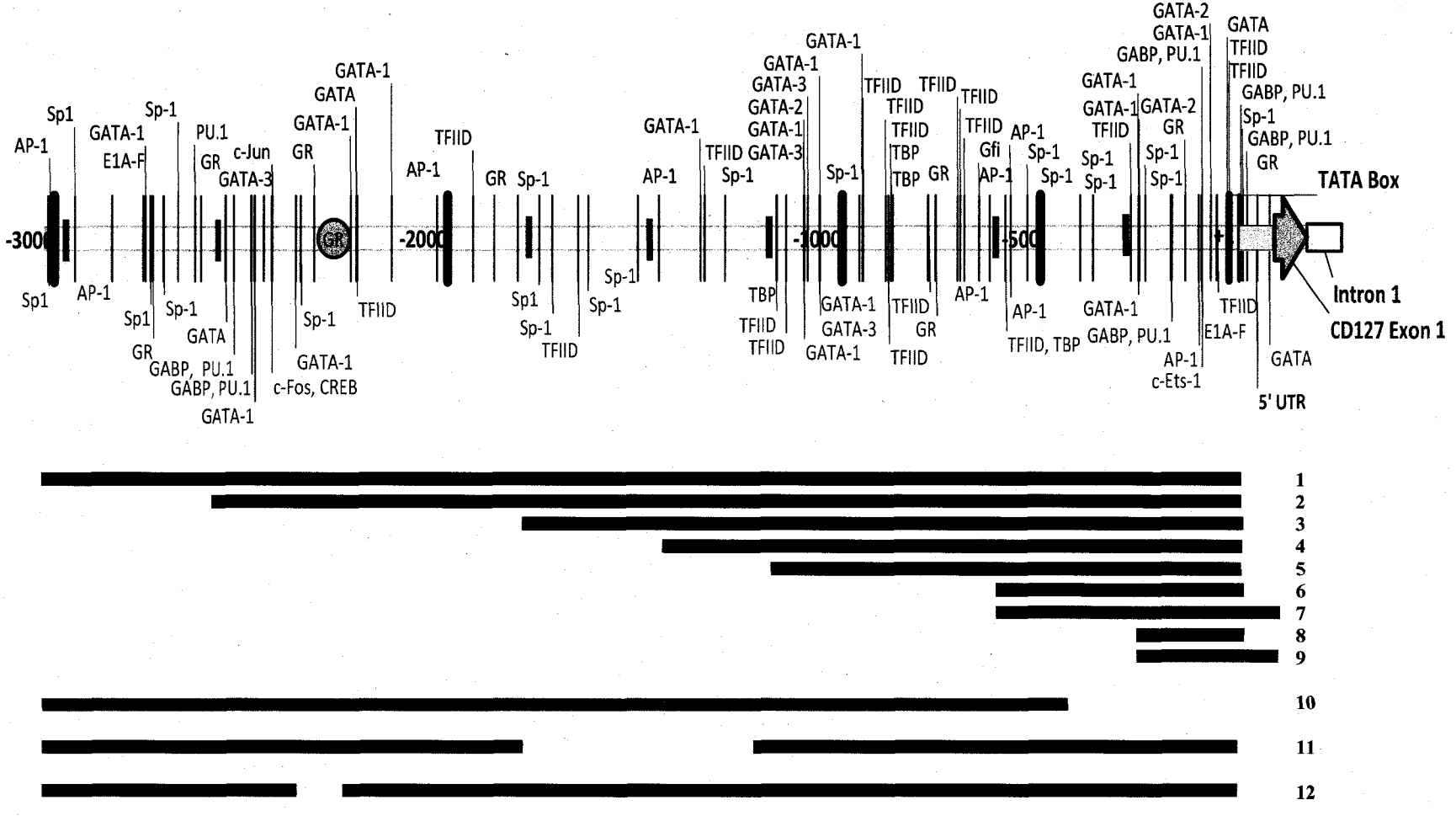
Figure 2-1 summarizes the constructs that were cloned into the pGL4B plasmid for the study of CD127 promoter regulation in CD8 T-cells.

Plasmids containing the various CD127 promoter fragments shown in figure 2-1 were co-transfected into primary CD8 T-cells using the nucleofection protocol. Each of these plasmids contained the Firefly luciferase gene driven off the CD127 promoter. A second plasmid phRTK, which carries the Renilla luciferase gene was driven off the herpes simplex virus thymidine kinase promoter was co-transfected with the CD127 promoter constructs to correct for transfection efficiencies. A plasmid ratio of 8:1 (w/w) of CD127/Firefly: Renilla was used for all nucleofection experiments where 4μg of CD127 promoter containing Firefly plasmid was mixed with 0.5μg of Renilla expressing plasmid. The Human T-cell Nucleofector Kit (Amaxa) was used for all nucleofection experiments. This kit uses a combination of electroporation and lipofection in order to deliver nucleic acids into target cells.

Purified CD8 T-cells (1E7 cells/ condition) were pelleted by centrifugation at 210*g* for 10 min and washed once with PBS. Supernatants were removed completely by aspiration and the cell pellet was then resuspended in 100μL/condition of pre warmed Nucleofector solution. The cell suspension was then transferred to nucleofector cuvettes containing pre-aliquoted plasmids and mixed by tapping gently. Electroporation was carried out using the pre-set Nucleofector U-24 program (Amaxa). Immediately after the pulse, the nucleofected cells were resuspended in pre-warmed (37°C) RPMI-20 medium and allowed to recover in a 37°C, 5% CO_2 jacketed incubator for approximately 6 hours.

Figure 2-1: Schematic showing the various CD127 promoter constructs that were transfected into primary CD8 T-cells.

Twelve constructs were used and constructs 1, 2, 3, 4, 5, 6 and 8 spanned from -2900, -2406, -1760, -1468, -1200, -626 and -262 upstream of the TATA box respectively. Constructs 7 and 9 contained the 5'UTR of the CD127 promoter (-626 to ATG and -262 to ATG respectively). Construct 10 deleted the core promoter elements located within the 0.5kb upstream of the TATA box (-2900 to -478). Construct 11 was generated to determine the regulatory capacity of the -1760 to -1200 region (2900-1760^1200-TATA). Construct 12, deleted a 15bp conserved glucocorticoid receptor (GR) responsive site located between -2269 and -2255 upstream of the TATA box.



The cells were then pelleted and resuspended at a concentration of 1E6 cells/mL in fresh RPMI-20 medium and incubated either in media alone or treated with IL-7 or dexamethasone for 24 hours as described.

2.9 Luciferase Assay

Following incubation, cells were harvested into 2mL microcentrifuge tubes and pelleted by centrifugation at 750g for 10 min in a Fisher Scientific microcentrifuge. Supernatants were aspirated, and the cells lysed with 100μL of 1X Passive Lysis Buffer (Promega). Tubes were vortexed for 20-30s and lysates were incubated on ice for 20 min. Lysates were then centrifuged at 17,000g for 10 min to pellet cell debris. The resulting supernatants were transferred into fresh 0.5mL microcentrifuge tubes.

Luciferase assays were performed in triplicate as described in the Dual-Luciferase Assay Kit protocol (Promega). The assay involves two substrates added sequentially. The first substrate reacts specifically with the Firefly luciferase enzyme resulting in a chemiluminescent reaction detected at 560nm. The second solution quenches the Firefly luciferase reaction and provides a second substrate for the Renilla luciferase enzyme. This chemiluminescent reaction is detected at 480nm. Assays were performed in duplicate in a Lumat luminometer (Berthold). The program used was as follows: inject luciferase assay reagent II (100μL), 2s delay, 15s measurement for Firefly luciferase activity, inject Stop and Glo reagent (100μL), 2s delay with a final 15s measurement of Renilla luciferase activity. Luciferase luminescence data was represented as relative light units (RLU). The Normalized RLU values were calculated by dividing the mean firefly luciferase RLU by the renilla luciferase RLU, that was used to correct for transfection efficiency and then by the protein

absorbance value. The resulting data were then represented as a mean change from the empty vector (pGL4B) control.

2.10 Protein Assay

Protein quantification was performed according to the Pierce BCA Assay Kit protocol (Fisher Scientific). The assay involves a reduction of a copper substrate by proteins. The reduced copper ion then forms a complex with bicinchoninic acid (BCA), which produces a colorimetric change which can be read at 562nm. The absorbance values were measured on a SpectraMAX-190 plate reader (Fisher Scienfitic) and protein concentrations were determined from a standard curve using bovine serum albumin (Pierce).

2.11 Western Blotting

Purified CD8 T-cells (1E6 cells per sample) were lysed for 45min on ice in 50μL RIPA buffer (50 mM TrisHCl pH7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS). Cell debris was then pelleted by centrifugation at 17,000g for 10 min. Following this, 18μL of the resulting supernatant was mixed with 6μL of 3X Laemmli buffer (187.5 mM Tris-HCl pH 6.8, 6% (w/v) SDS, 30% glycerol and 0.03% (w/v) bromophenol blue supplemented with 5% β-mercaptoethanol (Sigma-Aldrich)). The samples were boiled for 5-10 min and then resolved by SDS PAGE on a 10% resolving gel with a 6% stacking gel. The samples were run beside a Precision Plus Protein™ Dual Color ladder (Bio-Rad) at 160V for ~1 hour. After electrophoresis, the gel was washed briefly in ddH2O, and then equilibrated in 1X Bjerrum and Schafer-Nielsen transfer buffer (48 mM Tris base, 39 mM glycine) supplemented with 20% methanol and 0.00375% SDS) for 15 min. An Immobilon

polyvinylidene fluoride (PVDF) membrane (Millipore) was first activated in methanol for 5 min, and then equilibrated in 1X transfer buffer for 15 min. Next, the gel and membrane were assembled as per the manufacturer's instructions (Bio-Rad). Protein transfer was performed in a Bio-Rad semi-dry transfer apparatus for 30 min at 15V (Bio-Rad). Following transfer, the PVDF membrane was blocked overnight at 4°C with gentle rocking using a 2% blocking reagent (ECL Advance Chemilumiescence Kit: GE Healthcare) dissolved in 1X Tris-Buffered Saline with Tween-20 (TBST) (0.05M Tris/HCl, 0.30M NaCl, 0.1% Tween-20, pH 7.6). Following the blocking step, the membrane was incubated with a 1:500 dilution of polyclonal goat anti-human CD127 antibody (R&D Biosciences) in 2% blocking solution for 1 hour at room temperature with constant agitation. The membrane was then washed three times for 10 minutes each with 1X TBST. Next, the membrane was incubated with a 1:5000 dilution of horseradish peroxidase (HRP) conjugated-donkey anti-goat IgG antibody in 2% blocking solution for 1 hour again at room temperature with constant agitation. The membrane was again washed three times in TBST and CD127 protein was visualized by chemiluminescence as follows. Equal parts of reagent A and reagent B from the ECL Advance Chemiluminescence Kit were mixed by inversion in a 2mL microcentrifuge tube. The resulting mixture was then applied to the membrane ensuring the entire surface was uniformly coated in liquid. The chemiluminescent bands were photographed using the Alpha Innotech Imager and analyzed for densitometry using the Alpha Innotech AlphaEase software (Alpha Innotech). Densitometry analysis was performed using an unsaturated image of the gel captured on a medium speed and high resolution setting and calculating the band pixel intensity adjusted to background levels within a defined area. The pixel intensity was then multiplied by a normalization factor derived from the control bands to adjust for any errors in sample loading.

2.12 Data and Statistical Analysis

The real-time PCR and luciferase analyses were carried out in triplicate for each sample, and then values were averaged to minimize intra-experimental variability; the resulting averaged values were then used for further analysis. Furthermore, all data is represented as a mean change from controls where the control is set to a value of 1 in order to minimize the biological variability between samples. All statistical analyses were performed using the GraphPad Prism v.5.0 software (Graph Pad Software). A paired student's t-test was used and values with a p-value cutoff of less than or equal to 0.05 were considered significant. Data that is statistically significant has been indicated with an asterisk (*). Error bars indicate standard error of the mean (SEM). Where indicated, the n value represents biological replicates.

CHAPTER 3: RESULTS

3.1 Interleukin (IL)-7 down regulates CD127 expression in purified human CD8 T-cells at the level of transcription

3.1.1 IL-7 down regulates CD127 protein expression on the surface of purified CD8 T-cells

IL-7 has been reported to down regulate the expression of CD127 on the surface of CD8 T-cells in both humans and murine models (50, 74). Vrankovic et al have shown that the effect in vitro is greatest and sustained at high levels of IL-7 (10ng/mL) (74).

To confirm these findings, blood was obtained from healthy volunteers with no identified risk factors for HIV infection and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density centrifugation. CD8 T-cells were then separated from PBMC by magnetic bead isolation and allowed to recover overnight at a concentration of 1E6 cells/mL in RPMI-20 at 37°C. The following day, cells were plated at a density of 1E6 cells/mL in either media alone or media supplemented with 10ng/mL of IL-7 and incubated for an additional 24 hours. We chose to use this concentration (10ng/mL) of IL-7 based on findings in our lab and those published by Vranjkovic et al (74) that showed a sustained down regulation of CD127 by IL-7 at these concentrations in primary human CD8 T-cells past 24 hours. To determine the level of CD127 expression on the cell surface, Phycoerythrin (PE) conjugated anti-CD127 and PE-Cy5 conjugated anti-CD8 antibodies were added to the cells and incubated for 20 min and then surface CD127 expression on CD8 positive cells was examined by flow cytometry. Figure 3-1 shows the expression of CD127 on CD8 T-cells. Consistent with previous reports, IL-7 induced a significant down regulation of CD127 on the cell surface. Indeed, 80% ± 5% of CD8 T-cells maintained in media alone were positive

for CD127 while only 1.35% ± 1% of cells incubated with 10ng/mL IL-7 were CD127+ after 24 hours (n=4, $p<0.0001$).

3.1.2 IL-7 down regulates CD127 at the level of transcription in purified CD8 T-cells: Analysis by Real Time PCR

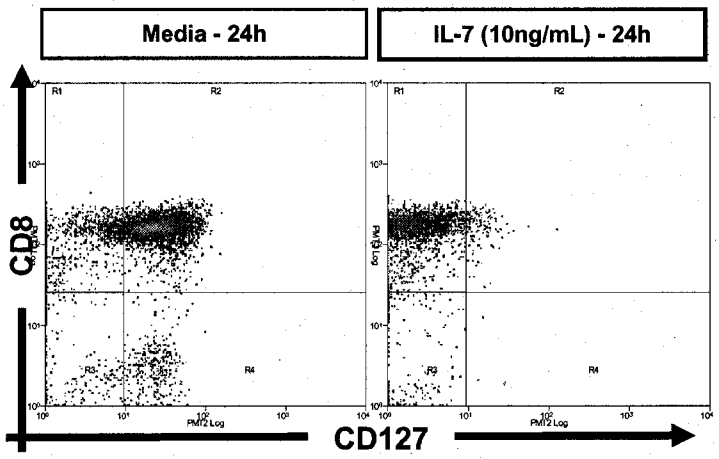
Surface expression of CD127 protein could be down regulated by IL-7 at any of a number of levels including transcription, translation, protein degradation, or protein shedding. Indeed, none of these are mutually exclusive and IL-7 may well act at more than one level.

The CD127 mRNA transcript is 1640 bases consisting of 8 exons. The polypeptide encoded from exons 1-5 makes up the extracellular ligand-binding domain of CD127. Exon 6, encodes for the transmembrane domain of the CD127 protein and the remaining two exons encode for the intracellular tail of the CD127 polypeptide. In order to identify CD127 transcripts by Real-Time PCR, a forward primer spanning the boundary of exons 4 and 5 was used along with a reverse primer binding within exon 5. The region contained in this amplicon is known to be present in all known isoforms of CD127 transcripts. The forward, exon-boundary spanning primer was designed as such in order to avoid spurious amplification of any contaminating genomic DNA. As a reference for total cellular transcripts, mRNA encoding Ribosomal Protein Subunit of 18kDa (RPS18) was also amplified for each sample. A standard curve was plotted in order to determine the amplification efficiency of each reaction and allow for correction of intraexperimental variations. The fold change was calculated using the $2^{-\Delta\Delta Ct}$ method as described by Vandesompele (72) and Livak (38) where the threshold cycle (Ct) is the point where the amounts of fluorescent amplicons begin to increase at a logarithmic rate.

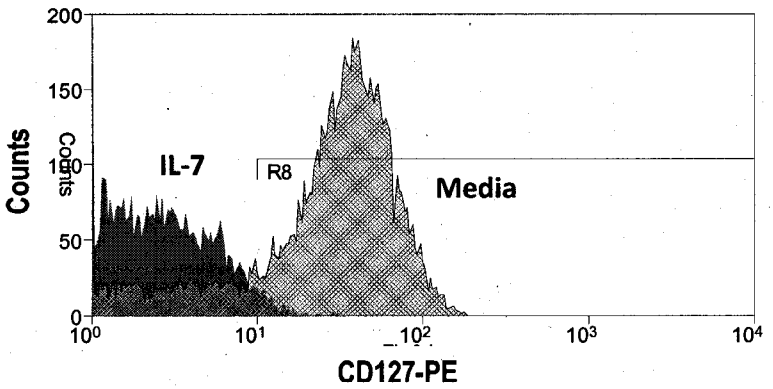
Figure 3-1: IL-7 causes a down regulation of CD127 surface protein expression on purified CD8 T-cells.

Purified CD8 T-cells were incubated in media alone or in the presence of 10ng/mL of IL-7. (A) Representative flow cytometric dot plot from one individual showing CD127 expression on CD8 T-cells. (B) Flow cytometric histogram overlay from the same individual showing the distribution of CD127 on CD8 T-cells (C) Pooled data from four independent experiments showing IL-7 treatment for 24 hours significantly down regulates CD127 surface expression on CD8 T-cells ($p<0.0001$).

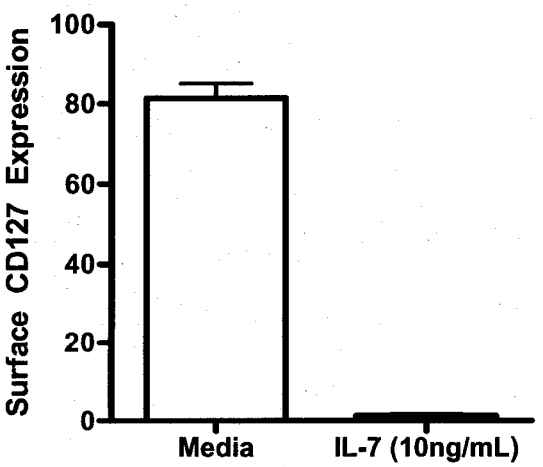
A



B



C



This formula provides the expression of a gene of interest (CD127) as a measure of how its threshold cycle changes relative to that of a reference gene (RPS18) – a gene that remains unaffected by the treatment in question (IL-7). This equation is derived from the general exponential function $y=2^x$ that represents the exponential increase in fluorescence intensity due to exponential increase in PCR amplicon number. The $-\Delta\Delta Ct$ value represents the difference in the change in threshold cycle (Ct) between two samples (ΔCt of CD127 samples versus ΔCt of S18 samples).

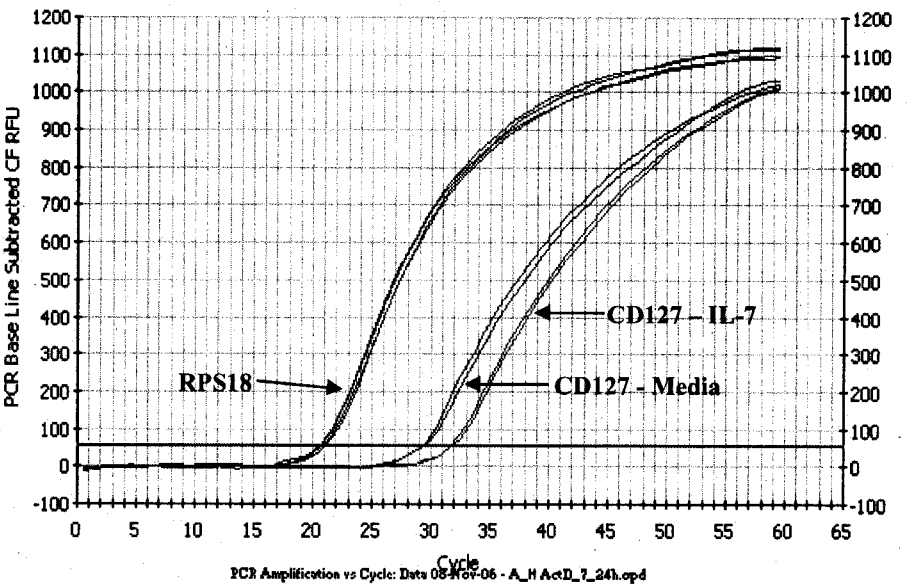
To investigate if IL-7 down regulated CD127 expression at the level of transcription, CD8 T-cells were isolated and incubated in either media alone of in the presence of 10 ng/mL of IL-7. After 24 hours, cells were harvested and RNA isolated and quantified by Real Time PCR.

Figure 3-2A shows a representative Real Time amplification curve for RPS18 and CD127 mRNA from CD8 T-cells of one individual. For cells maintained in media alone and cells treated with IL-7 for 24 hours, amplification products of RPS18 crossed threshold at the same cycle indicating both samples contained the same amount of cellular RNA. In contrast, CD127 amplification products from cells maintained in media alone crossed threshold in fewer cycles compared to cells treated with IL-7. This indicates cells in media contained more CD127 mRNA compared to cells treated with IL-7. In total, CD127 mRNA was quantified from cells maintained in media or treated with IL-7 (10 ng/mL) for 12 individuals (Figure 3-2B). As shown, IL-7 induces a 66% ± 2% decrease in CD127 mRNA in resting CD8 T-cells ($n=12$, $p < 0.0001$).

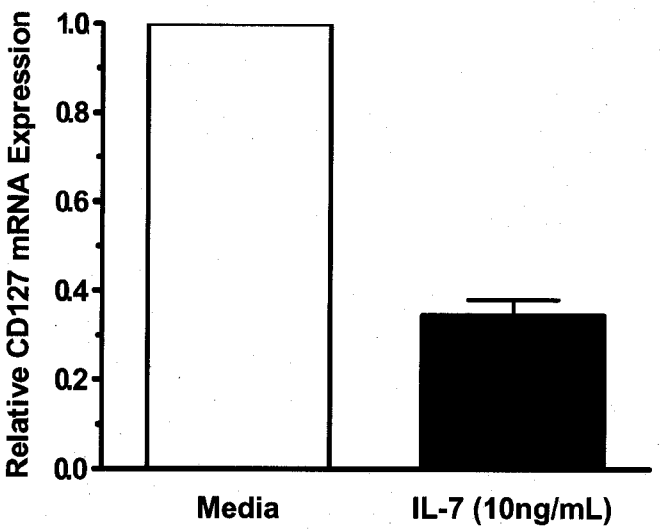
Figure 3-2: IL-7 down regulates CD127 expression in CD8 T-cells at the level of transcription.

(A) Representative Real-Time PCR amplification curve showing a lower abundance of CD127 transcripts in IL-7 treated samples compared to cells maintained in media alone. (B) Pooled data from twelve individual experiments demonstrating purified CD8 T-cells treated with IL-7 for 24 hours contain less CD127 mRNA compared to cells maintained in media ($p<0.0001$). The transcripts were measured by Real-Time PCR and normalized to RPS18 reference gene expression. Data is represented as a fold change from media control and error bars show Standard Error of the Mean (SEM).

A



B

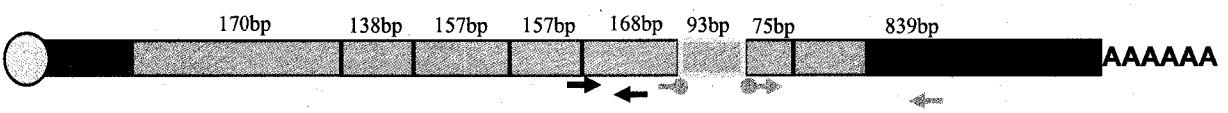


3.1.3 Treatment of purified CD8 T-cells with IL-7 does not bias alternative splicing of CD127 transcripts in purified CD8 T-cells

The CD127 transcript is composed of 8 exons that are alternatively spliced to encode for a single-pass type I transmembrane isoform of CD127 protein, or a secreted isoform of CD127 lacking the transmembrane domain encoded by exon 6 (35, 52). It is therefore conceivable that IL-7 could down regulate CD127 protein at the cell surface by biasing CD127 mRNA splicing towards the secreted isoform of the protein. To investigate this possibility, a set of primers was designed to measure changes in mRNA encoding the secreted isoform. As shown in figure 3-3, we designed a forward primer that spanned the boundary of exons 5 and 7 with a corresponding reverse primer binding within exon 7. Due to the nature of the forward primer, only transcripts encoding for the secreted isoform of CD127 could be amplified.

Figure 3-3: The CD127 mRNA transcript.

The CD127 mRNA consists of 8 exons (1640bp) whereby exon 6 encodes the transmembrane region of the CD127 protein (yellow border). This figure shows the size of each exon, the 5' and 3' untranslated regions (UTRs) depicted by the dark rectangles, the 5' cap (circle) and poly (A) tail. The arrows show the primer binding sites for transcripts encoding total CD127 protein (green arrows) and secreted CD127 protein (yellow arrows).



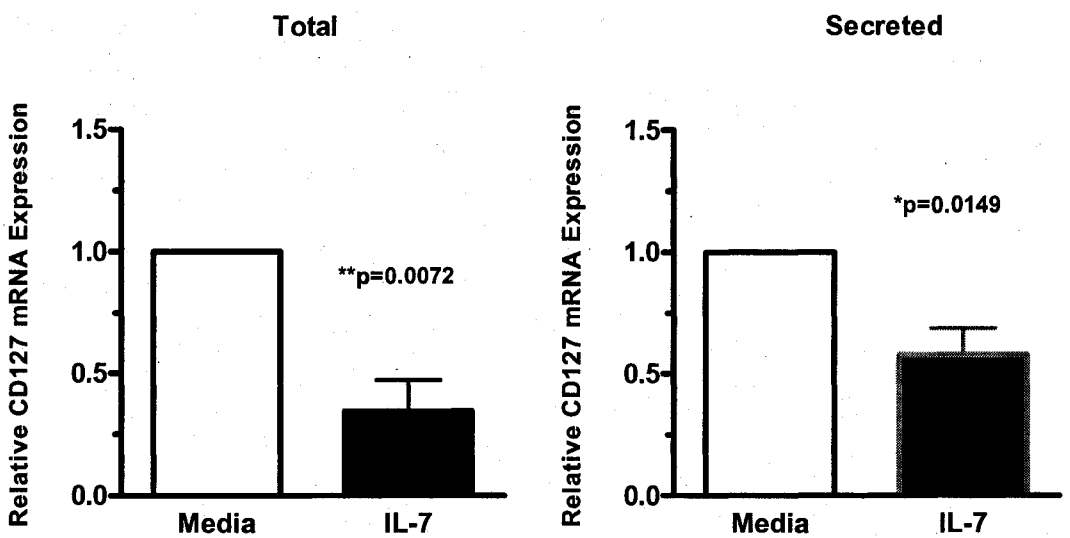
Purified CD8 T-cells from 4 individuals were incubated in either media alone or were treated with 10ng/mL of IL-7 for 24 hours. Following treatment, total RNA was isolated and reverse transcribed. Transcripts encoding the secreted isoform of CD127 were quantified by Real-Time PCR using the forward primer spanning the boundary of exons 5 and 7. Total CD127 transcripts were quantified in parallel using the forward primer spanning the boundary of exons 4 and 5. As above, RPS18 was used as a reference gene. As shown in figure 3-4, IL-7 treatment caused a 43% ± 9% reduction in transcripts encoding the secreted isoform of CD127 protein (n=4, p=0.0149) and a 66% ± 12% reduction in total CD127 mRNAs was observed (n=4, p=0.0072). Furthermore, upon analysis of the difference in mean fold changed observed between the down regulation by IL-7 of CD127 total mRNAs versus those encoding for secreted CD127, we found that the mRNAs encoding for the secreted isoform of CD127 were down regulated to a lesser degree than those encoding for all CD127 transcripts (p=0.0244). This suggests that IL-7 mediated down regulation of CD127 mRNA may actively target the down regulation of membrane bound CD127 protein

3.1.4 IL-7 down regulates CD127 at the level of transcription in purified CD8 T-Cells: Analysis by Conventional PCR.

In order to confirm our Real Time PCR data, CD127 mRNA transcripts were also measured by conventional PCR. This method allows for the amplification of larger amplicons at the cost of copy number resolution. The advantage of this method is that it allows for the quantitative analysis of CD127 mRNAs encoding both the transmembrane and the secreted isoforms of CD127 in the same reaction.

Figure 3-4: IL-7 down regulates mRNAs encoding both the transmembrane and secreted isoforms of CD127 protein.

Pooled data showing that IL-7 significantly down regulates both CD127 mRNAs (n=4, p=0.0072) encoding the full-length and secreted isoforms of CD127 protein (n=4, p=0.0149). The transcripts were measured by real-time PCR and normalized to RPS18 reference gene expression. Data is represented as a fold change from media control and error bars show SEM.



Conventional PCR eliminates the need for correction of different amplification efficiencies arising from the use of two different primer sets as required in Real-Time PCR. As shown in figure 3-5, we used a forward primer binding to the boundary of exons 4 and 5 and a reverse primer binding within exon 8. This allows for the amplification of mRNAs encoding for both the transmembrane and secreted isoforms of CD127 protein in the same reaction. Since these products would differ in size by 93bp – the size of exon 6 – they can be separated by gel electrophoresis and quantified by densitometry.

To determine if the two PCR products representing transcripts encoding full-length and secreted CD127 protein could be successfully identified, RNA was isolated from untreated CD8 T-cells and subjected to PCR. Figure 3-6 shows a representative agarose gel of the amplification products from one representative individual. Consistent with the literature (22, 35, 71), we identified two products representing the predominant CD127 transcripts. The larger band, at 790bp is consistent in size with an amplicon arising from a transcript encoding the transmembrane isoform of the CD127 while the smaller band, at 697bp is the expected size of an amplicon arising from a transcript encoding the secreted isoform of the protein. Both of these isoforms have been previously identified by Korte et al (35). This method also allows us to determine the relative proportions of mRNAs encoding the transmembrane and secreted isoforms of CD127. As shown here and consistent with previous reports (35), transcripts encoding the transmembrane isoform of CD127 protein are significantly more abundant compared to those encoding the secreted isoform.

Figure 3-5: The CD127 transcript showing primer binding sites. The CD127 mRNA consists of 8 exons.

Arrows show the primer binding sites used in conventional PCR. The forward primer binds at the boundary of exons 4 and 5, and the reverse primer binds within the 3'UTR that is a part of exon 8.

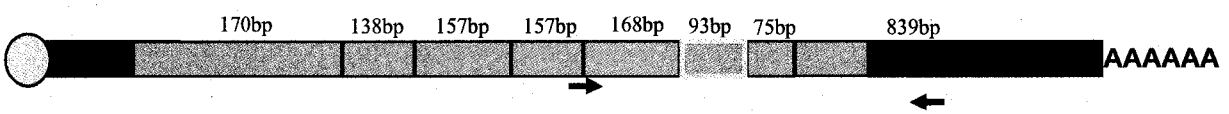
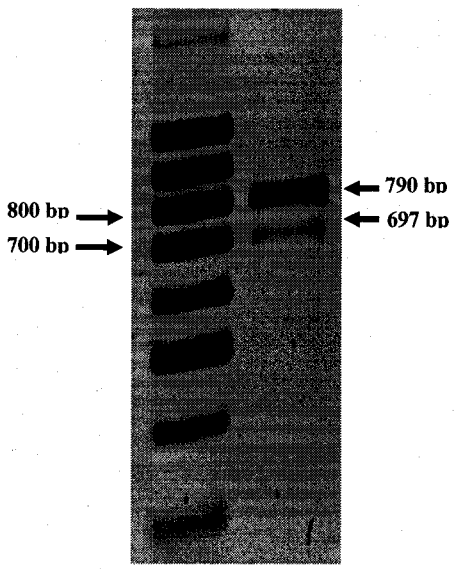


Figure 3-6: There are two predominant species of CD127 mRNA in CD8 T-cells.

Agarose gel demonstrating two predominant CD127 transcripts. The 790bp amplicon corresponds to the full-length transcript while the 697bp amplicon corresponds to a transcript encoding for the secreted isoform of CD127.

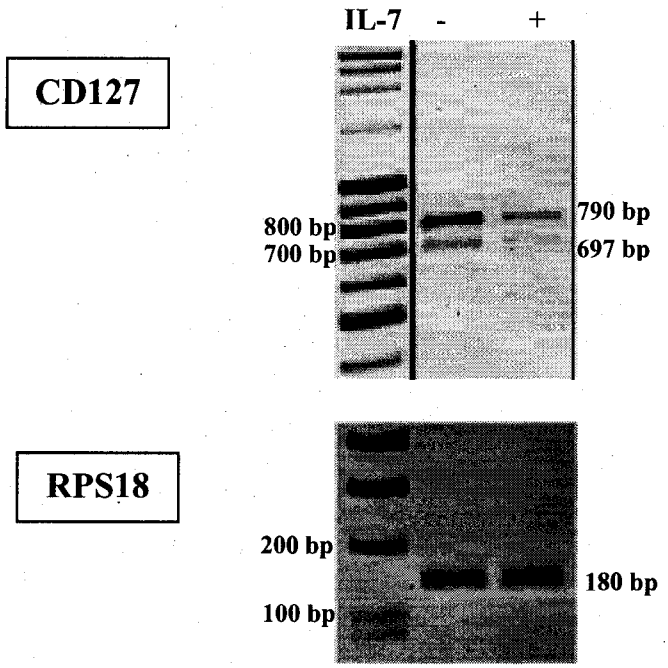


PCR amplification was then used to measure the relative levels of CD127 transcripts encoding transmembrane and secreted isoforms of protein in CD8 T-cells maintained in either media alone or treated with IL-7 for 24 hours. To do this, an equal mass of RNA in treated and untreated samples was reverse transcribed and amplified by PCR. The RPS18 gene was amplified in parallel as a control. The PCR reaction was stopped as soon as the amplification cycle reached the middle of the logarithmic phase, and the resulting amplicons were resolved by gel electrophoresis. After staining with Ethidium Bromide, the band intensities were measured by densitometry. Figure 3-7A shows a representative semi-quantitative PCR gel of CD127 and RPS18 transcripts from CD8 T-cells of one individual. CD127 transcripts (top gel), seen as two bands at 790bp and 697bp are more intense in the untreated sample (-) compared to those in the sample treated with IL-7 (+). In contrast, the band intensities for RPS18 in the same samples are equivalent (bottom gel). Figure 3-7B shows pooled data from nine individuals where samples treated with IL-7 contained 46 ± 1.5% ($p=0.0001$) less amplicons representing transmembrane CD127 compared to a modest 16 ± 1% ($p=0.02$) decline observed in the amplicons representing secreted CD127. Furthermore, the difference in the ratio of decline in the two amplicons was also found to be statistically different ($p=0.0183$) further suggesting that IL-7 not only causes a down regulation of all CD127 mRNAs but the down regulation is more pronounced in the transmembrane CD127 mRNA pool than in the secreted CD127 transcripts.

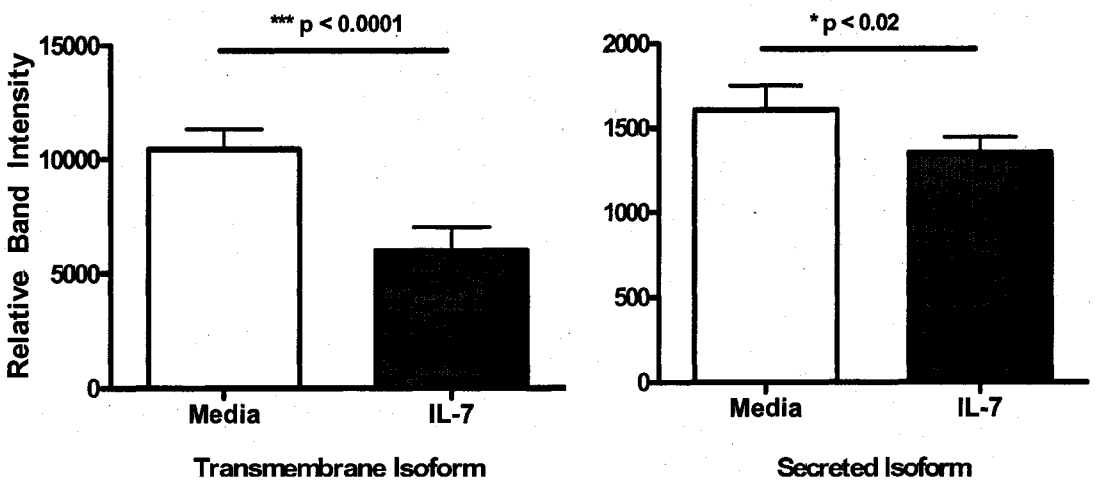
Figure 3-7: Transcripts encoding both the full-length and secreted isoforms of CD127 are down regulated by IL-7 in CD8 T-cells.

(A) Representative agarose gels showing a decrease in both CD127 transcripts (top) in media (-) versus IL-7(+) treated samples. RPS18 transcript levels remained the same in both media and IL-7 treated samples (bottom). (B) Relative band intensities by densitometry demonstrating reduced CD127 transcripts in CD8 T-cells treated with IL-7. Data is pooled from 9 individual experiments. Statistically significant results are indicated by an asterisk (*).

A



B

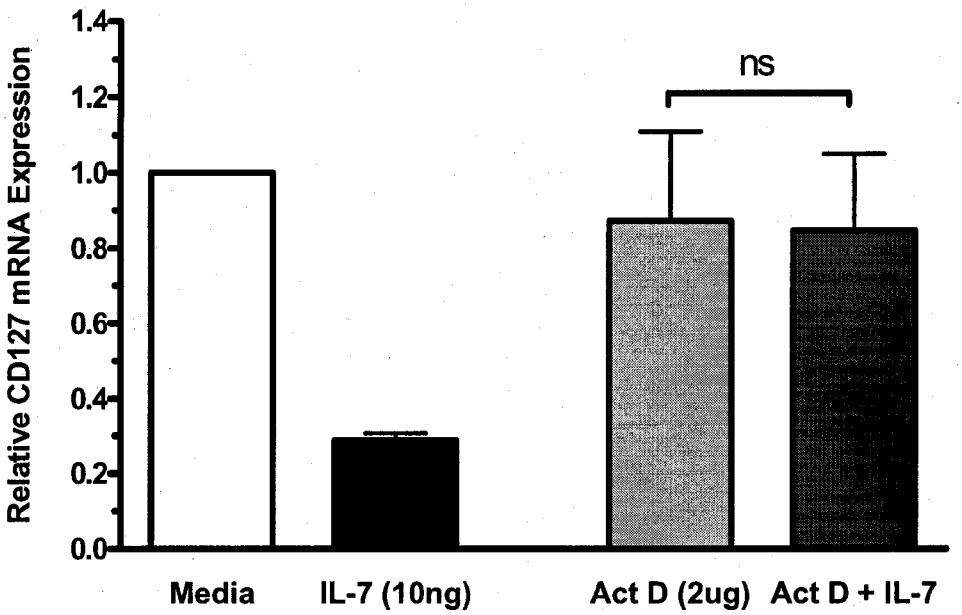


3.1.5 *De novo transcript synthesis is required for IL-7 to down regulate CD127 transcripts in CD8 T-cells.*

It is possible that IL-7 decreases the amount of CD127 mRNA detected in cells by promoting its degradation. To determine if this was the case, we isolated CD8 T-cells and arrested *de novo* transcript production by treating with 2μg/mL Actinomycin (Act) D. Actinomycin D binds DNA at the transcription initiation complex and prevents the elongation of RNA polymerase (65). In the absence of transcriptional regulation, any decline in RNA levels may be a result of enhanced degradation. As demonstrated in figure 3-8, CD8 T-cells treated with IL-7 (10ng/mL) alone for 24 hours showed a 72% ± 0.25% reduction in CD127 transcripts. In contrast, CD8 T-cells treated with Actinomycin D (2μg/mL) or Actinomycin D (2μg/mL) plus IL-7 (10ng/mL) contained similar levels of CD127 mRNA and not significantly different from cells maintained in media alone (0.84 ± 0.15 fold and 0.87 ± 0.2 fold respectively relative to media). Given that cells treated with Actinomycin D alone for 24 hours did not show a significant reduction in CD127 mRNAs suggests CD127 transcripts have a half-life longer than 24 hours. This data also suggests that that IL-7 mediated down regulation of CD127 transcripts requires the synthesis of *de novo* transcripts that are required to mediate the down regulation of CD127 mRNA by IL-7. Additionally, this data suggests that down regulation of CD127 by IL-7 does not directly utilize existing cellular machinery to promote mRNA degradation although this does not rule out the possibility that IL-7 promotes the production of a factor that ultimately leads to CD127 transcript degradation.

Figure 3-8: *De novo* transcript synthesis is required for IL-7 to down regulate CD127 transcripts in CD8 T-cells.

Cells treated with IL-7 in the presence of Act D contained the same level of CD127 transcripts as cells treated with Act D alone. In contrast, cells treated with IL-7 alone as a control showed a 72% ± 0.25% reduction in CD127 mRNA. Transcripts were measured by real-time PCR and normalized to RPS18 reference gene expression. Pooled data from five independent experiments is represented as a fold change from media control. Error bars show SEM.



3.1.6 Summary:

Purified CD8 T-cells treated with IL-7 (10ng/mL) down regulated CD127 surface protein and transcripts within 24 hours. Although IL-7 did not bias alternative splicing of transcripts to promote the production of mRNA encoding the secreted isoform of CD127, IL-7 did appear to cause an increase in the down regulation of CD127 transcripts encoding for the membrane bound protein compared to its secreted counterpart. Transcriptionally arrested CD8 T-cells demonstrated that CD127 mRNA has a half life longer than 24 hours. Also, when these transcriptionally arrested cells were incubated in the presence of IL-7, there was no additional decline in CD127 mRNA levels suggesting that IL-7 mediated down regulation of CD127 transcripts does not utilize existing cellular machinery to degrade CD127 transcripts. Moreover, this data indicated that *de novo* transcript synthesis is critical in order to mediate IL-7-induced down regulation of CD127 mRNA. Furthermore, we hypothesize that that the newly synthesized protein eventually causes the decline in CD127 either through mRNA degradation or transcription initiation.

3.2 Characterization of the CD127 Gene Promoter in purified CD8 T-cells

3.2.1 Web-based analysis of the upstream CD127 promoter region.

The human and mouse CD127 gene promoters share 75% homology (43). Although the regulation of the mouse CD127 promoter has been studied by several groups in murine thymocytes and peripheral B and T-cells (10, 16, 18, 20, 37, 42, 45, 50, 52, 61, 70, 78, 79), the human promoter has yet to be fully characterized. The differential regulation of CD127 in mice has been shown to occur through expression of different constellations of transcription factors in different cell types (43). Of note is the binding at the GGAA motif, of the Ets family of transcription factors such as GABP, in murine thymocytes and T-cells. This same motif also serves as the binding site for PU.1 in mouse B-cells (78). Since the expression of these two factors is strictly restricted to cell type, and their expression and translocation to their binding sites may be regulated by different signaling cascades, it is possible to have simultaneous, yet differential regulation of CD127 in different cell types. In view of this, it becomes important to study the regulation of the CD127 gene promoter in resting, primary human CD8 T-cells as it may be regulated differently compared to other cell types.

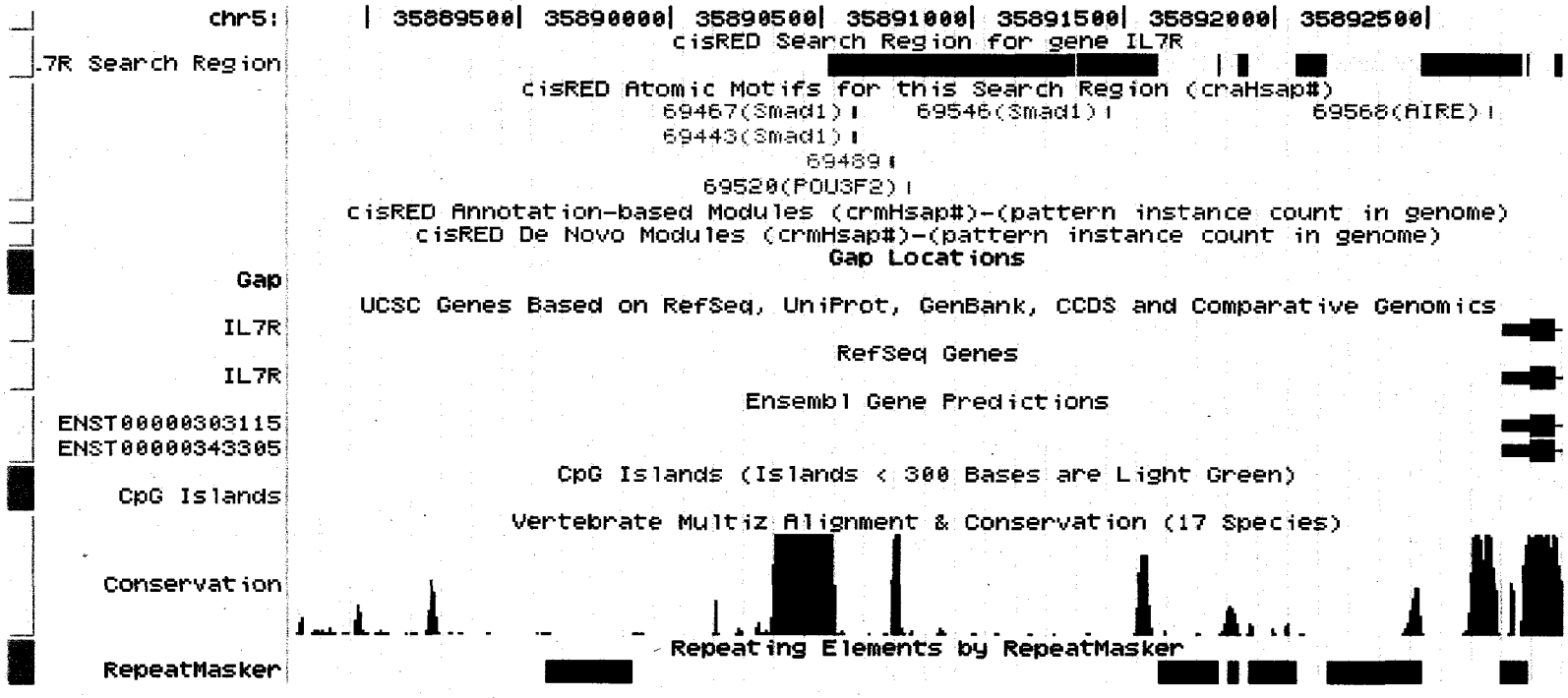
The human CD127 promoter is located on chromosome 5 at the 5p13 locus between position 35,892,748-35,912,678 (12). In order to identify the CD127 promoter region and location of important regulatory elements, we first analyzed a 4kb region upstream of the first exon of the CD127 gene to look for conserved non coding regions in the human, mouse, rat and nematode promoters. This analysis was performed using cisRED, a web-based genome-wide regulatory module and element prediction software. More specifically, this software uses a combination of atomic motif analyses where known consensus sequences for

transcription factor binding sites, hierarchial clustering of known transcription factors and predicted putative regulatory modules based on these clusters to identify regulatory regions (8). Figure 3-9, shows the alignment of the 4kb region upstream of exon 1 in the human CD127 promoter using cisRED analysis (8), where several conserved regions were found near -2.5kb, -2kb, -1.2kb, -0.2kb and +0.2kb relative to the beginning of exon 1. Our cisRED atomic motif analysis of conserved DNA sequence motifs revealed binding sites for AIRE, POU3F2 and several SMAD1 elements. AIRE has been shown to recruit positive transcription elongation factor b (p-TEFb) to the RNA polymerase II complex and promote proper elongation of nascent transcripts in thymic medullary epithelial cells (47). SMAD1 has been shown to act as an adapter module that is responsible for the linking of specific DNA-binding transcription factors to the RNA polymerase holocomplex thereby allowing for specific DNA binding factors to regulate RNA polymerase activity. SMADs bind to phosphorylated receptors and become phosphorylated themselves in order to be active. Active SMADS form multimers consisting of SMAD dimers and a co-SMAD. This active complex translocates to the nucleus where it exerts its effects via interaction with other DNA binding factors and the RNA polymerase holocomplex. (reviewed in (14)).

Altogether, the cisRED analysis revealed that the binding sites for factors that are important for transcription initiation and elongation are evolutionarily conserved across the mouse, rat, nematode and human CD127 promoters.

Figure 3-9: *cis*-regulatory regions of the CD127 gene.

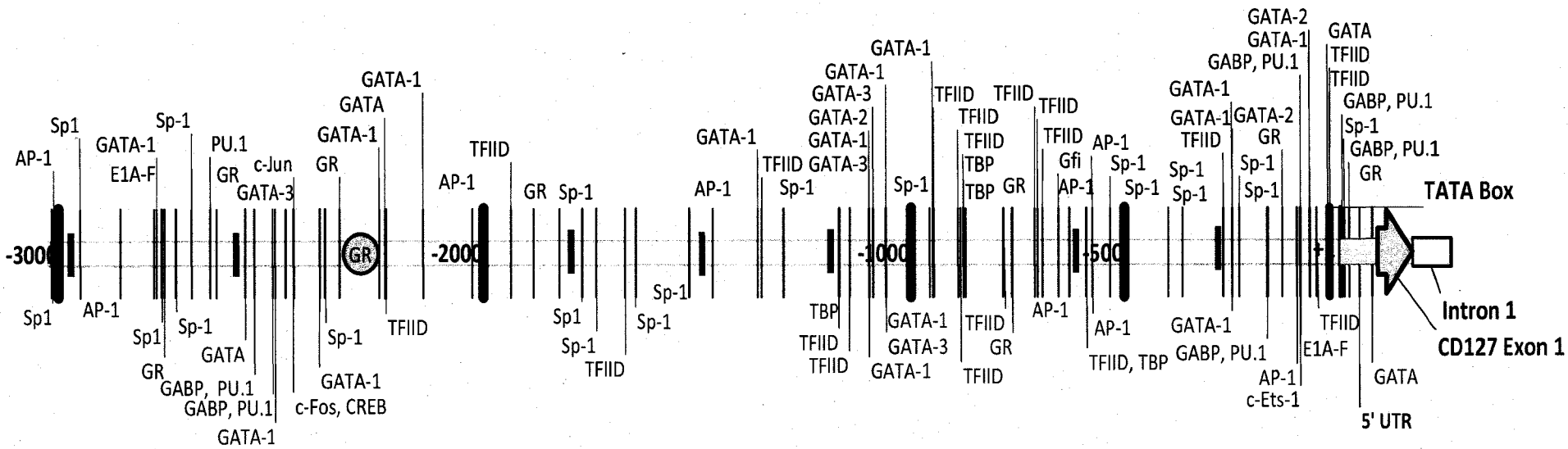
Schematic showing upstream *cis*-regulatory regions and conserved non-coding sequences within the putative CD127 promoter. Red bars (top) denote the putative promoter region based on density of transcription factor binding sites and distance from the first exon. Gray bars denote excluded areas that are known repeat regions. cisRED atomic motifs show the binding of transcription factors (SMAD, AIRE and POU3F2) to the upstream regulatory regions (brown/beige). Dark blue (bottom) show conserved regions in the non-coding sequences upstream of exon 1 (light purple – middle). +1 is located at the beginning of exon 1, conserved regions from left to right are located near -2.5kb (200bp), -2kb (20bp), -1.2kb (50bp) and within the first -0.5kb (100bp) upstream of the TATA box. Black bars at the very bottom are repeat regions that are located within the -4000bp to exon 1 region.



In addition to the cisRED regulatory module analysis, we also performed a putative transcription factor binding analysis using another web-based program called Transcription Element Search System (TESS) (59). The output from this analysis was then cross referenced with analysis using a third transcription factor prediction program called Footer version 2.0 (9). This program uses evolutionary information about *cis*-regulatory regions in murine and human promoters comparing putative transcription factor binding sites and assigns scoring based on their relative position in relation to known models of binding preferences for a particular factor (9). This program also allows the user to select sets of transcription factors and enter a value of the weighted average p-value so as to reduce false positives. From the 229 identified putative transcription factor binding sites that met the weighted average p-value cutoff of 0.0005, three categories of transcription factor binding sites were identified in the region spanning ~3kb upstream of the CD127 gene: (1) factors previously shown to regulate CD127 in mice (GATA, GAPB, PU.1, GR and Gfi); (2) factors important for basal transcription (TBP and TFIID); and (3) known regulators of gamma-chain cytokine receptors (AP-1, c-Ets, c-Fos, c-Jun and Sp-1) (figure 3-10).

Figure 3-10: The CD127 upstream regulatory region and putative transcription factor binding sites.

The human CD127 genomic sequence from the Ensembl database was analyzed using web-based transcription factor binding site identification software (Footer v2.0). Factors were mapped based on their ability to bind to the sense strand (red) or antisense strand (blue). 5' truncation mutants from the TATA box to individual orange bars were generated in order to characterize regulatory regions.



Legend:

(|) Factors binding to the sense strand

(|) Factors binding to the antisense strand

GR Distal glucocorticoid-sensitive CNS element

(|) Truncation sites

3.2.2 Cloning the upstream regulatory region of the CD127 gene.

Following identification of putative transcription factor binding sites, a DNA fragment containing sequences from the TATA box to -2900 base pairs upstream of the CD127 gene was cloned from Jurkat T-cells. In order to provide directionality, unique cut sites (KpnI - Forward and NheI – Reverse) were inserted into the primers and these were used to amplify the selected region. The 2900 base pair fragment was then cloned into the pGL4B plasmid, a luciferase reporter gene expressing basic vector. Next, a series of deletion mutants were constructed. These constructs were designed based to the binding position and densities of putative transcription factors shown in figures 3-9 and 3-10. Table 3-1 gives a detailed description of the characteristics of each construct. The aim was to generate a uniform (~500bp interval) map of upstream regulatory elements in the CD127 promoter. Regions upstream of -2900bp were not cloned as DNA analysis from the Ensembl database revealed few transcription factor binding sites beyond this point, or the regions were identified as part of the neighboring SPEF2 gene by BLAST analysis.

Table 3-1: List of CD127 promoter constructs cloned into pGL4B.

Firefly luciferase expressed from the pGL4B was used as a measure of CD127 promoter activity. This table shows the various constructs that were made in order to study the regulation of the CD127 promoter in CD8 T-cells.

Promoter Constructs	Description
2900 to TATA	Whole construct
2406 to TATA	Includes conserved noncoding region between -2269 and -2255
1760 to TATA	Does not include GR sensitive region between -2269 and -2255
1468 to TATA	Contains additional promoter elements between -1200 and -1468
1200 to TATA	Contains additional elements between -626 and -1200
626 to TATA	Contains the core promoter elements and generic 5' UTR from pGL4B plasmid
626 to ATG	Contains core promoter elements and 5' UTR of the CD127 promoter
262 to TATA	Contains the TSS, TATA box of the CD127 promoter and 5' UTR from pGL4B plasmid
262 to ATG	Contains the transcription start site, TATA box and 5' UTR of the CD127 promoter
100 to ATG	Contains only the TATA box and 5'UTR elements of the CD127 promoter
2900-1760^1200-TATA	Deleted the region between -1760 and -1200
2900-2269^2255-TATA	Specifically deleted the 11bp conserved GR sensitive positive regulatory element
2900 to -468	Does not include essential TATA box and transcription start site elements
pGL4B	Empty vector control
phRTK	Nucleofection control

3.2.3 *Transfection of putative CD127 promoter containing plasmids into purified CD8 T-cells*

One of the major challenges in studying gene expression in primary cells is the low efficiency associated with most transfection methods used for introduction of foreign genetic material. Although viral transduction is a common method used for transgene delivery into primary cells, it generally requires cell activation and division. Since activation of primary CD8 T-cells has been shown to down regulate CD127 gene expression (13) (50), this method was avoided here.

After investigating several transfection methods, Amaxa's nucleofection protocol was found to be the most efficient means of transfecting primary CD8 T-cells. This method uses a combination of electroporation and lipofection and provided nucleofection efficiencies of up to 50%.

Using this protocol, isolated CD8 T-cells nucleofected with a CMV promoter-driven luciferase gene expressed high levels of luciferase within 6 hours post-transfection (data not shown). To ensure nucleofection itself did not result in down regulation of CD127, we compared surface CD127 expression by flow cytometry in isolated CD8 T-cells that were either maintained in media alone or nucleofected with a plasmid expressing Green Fluorescent Protein (GFP). Cells within the live lymphocyte gate were then examined for CD127 surface protein expression. As shown in figure 3-11, in the untransfected samples, 81% ± 2% of the cells expressed CD127 surface protein. Similarly, in the samples that were nucleofected, 87.4% ± 2% of the cells were CD127 positive indicating nucleofection does not in and of itself affect CD127 expression. As expected, nucleofection did result in some

degree of cell death. Whereas 85% ± 0.6% of untransfected cells were found within the live gate, only 58% ± 1% ($p<0.0001$) of transfected cells were also in this gate.

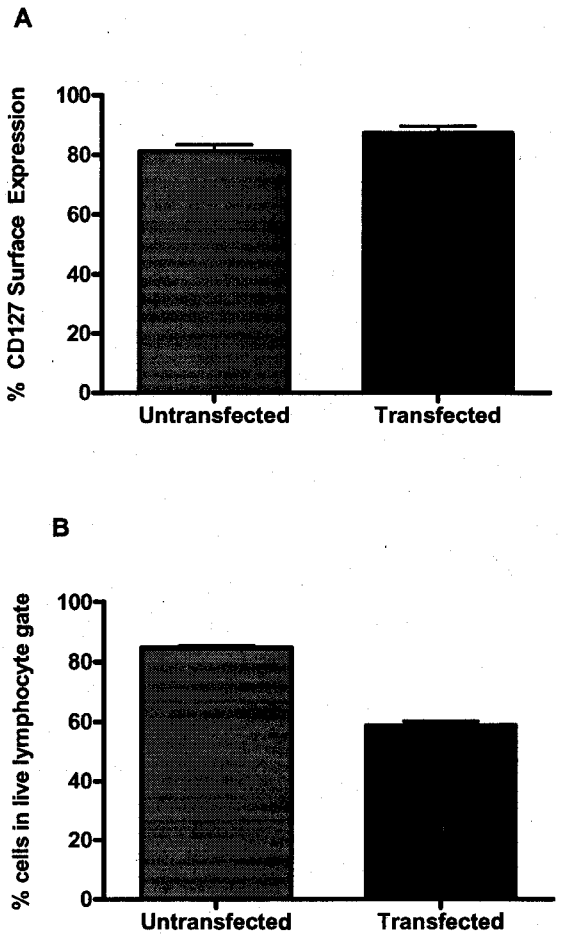
3.2.4 CD127 gene promoter analysis in CD8 T-cells

For all nucleofection experiments described below, purified CD8 T-cells were co-transfected with a CD127 promoter-containing reporter plasmid expressing firefly luciferase and a second plasmid expressing renilla luciferase expressed from a constitutively active, herpes virus thymidine kinase promoter (phRTK).

The cells were transfected at ~1_E7 cells/mL, allowed to recover for 6 hours and then resuspended in fresh medium at a density of 1_E6 cells/mL. At this point, cells were either treated as described or allowed to remain in media for an additional 24 hours. Cells were then lysed and assayed for luciferase gene expression and total protein. Luciferase gene expression was used as a measure of CD127 promoter activity. The luciferase activity was normalized to renilla luciferase expression to correct for transfection efficiency and to total protein in each individual sample. All experiments contained the empty vector (pGL4B) as control and thus all values are expressed as a fold change compared to pGL4B.

Figure 3-11: Nucleofected CD8 T-cells express similar levels of CD127 surface protein compared to untransfected cells.

(A) Pooled data (n=8) showing that transfected CD8 T-cells express similar levels of CD127 surface protein expression as compared to untransfected cells maintained in media for 24 hours. (B) Pooled data showing that there are significantly fewer cells present in the live lymphocyte gate in transfected CD8 T-cells compared to untransfected cells (n=8, $p<0.0001$). Error bars show Standard Error of the Mean (SEM).



3.2.4.1 The first 262 base pairs contain the core promoter element.

Promoter constructs extending from the TATA box to -262, -626 and -1200 base pairs each expressed luciferase at about four-fold over empty vector control (Figure 3-12). This suggests that the core promoter elements for the CD127 gene are contained within the first 262bp upstream of the TATA box. Addition of sequences from -262 to -1200bp did not lead to further increases in luciferase gene expression.

The CD127 transcript contains an untranslated 5' region 90bp in length. We questioned whether this region played a role in enhancing ribosomal binding and thus gene expression. To investigate this hypothesis, we compared luciferase gene expression from constructs that either contained the core promoter sequences up to the TATA box or containing the additional 90bp spanning from the TATA box to the ATG site. As seen in figure 3-13, the relative luciferase gene expression in clones whose 3' border ended at the TATA box (short) compared to clones whose 3' end extended to the CD127 ATG (long), the luciferase gene expression was comparable (3 fold ± 0.5 versus 2.9 fold ± 0.7 in the 262 short and long constructs respectively; 4.5 fold ± 1.5 versus 2.9 fold ± 0.5 in the 626 short and long constructs respectively $p>0.1$). From these findings we conclude the untranslated 5' leader sequences do not enhance gene expression.

Figure 3-12: Luciferase gene expression from constructs up to -1200 bp from the TATA box.

Relative luciferase gene expression measured as Relative Light Units (RLU) from the TATA box to -262, -626 and -1200bp. Data is from four individual experiments; error bars show Standard Error of the Mean (SEM).

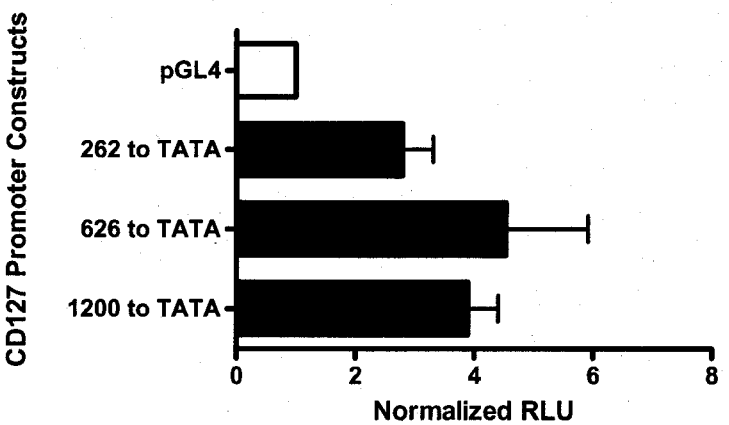
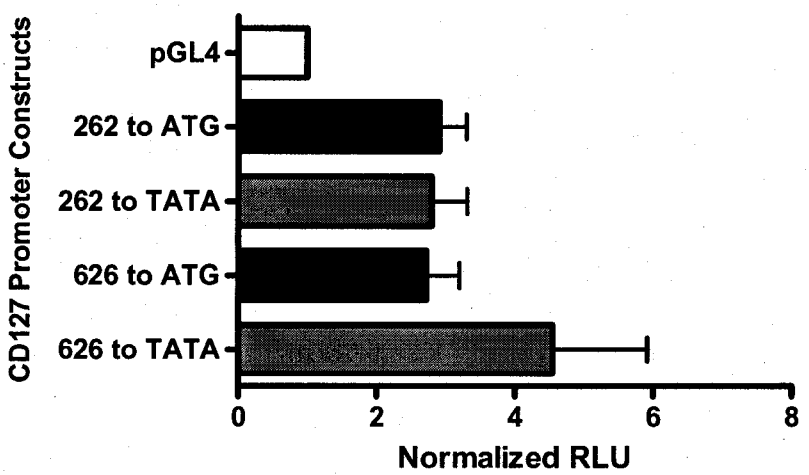


Figure 3-13: Sequences from the CD127 TATA box to the ATG do not significantly affect luciferase gene expression.

Pooled data (n=4) showing normalized luciferase gene expression for core promoter fragments whose 3' was at the CD127 TATA (short; gray bars) compared to the same core promoter elements whose 3' end spanned to the ATG of the CD127 gene (long; black bars). Luciferase gene expression was not significantly different between the long and short fragments. Error bars show Standard Error of the Mean (SEM).



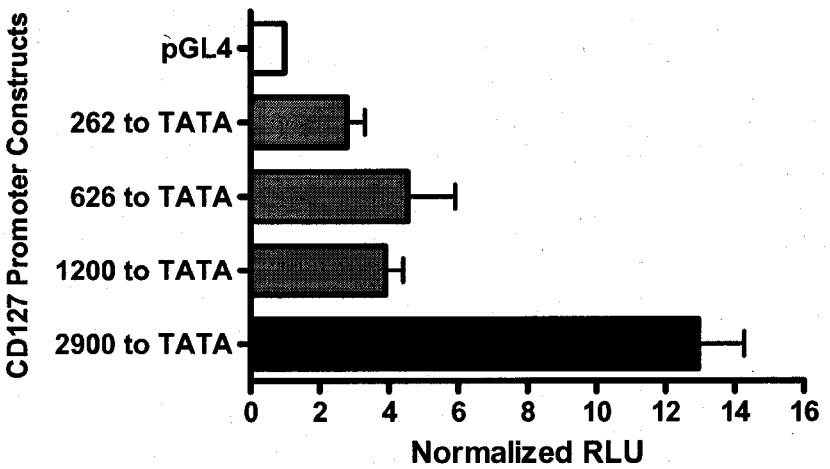
3.2.4.2 Sequences between -1200 and -2900 base pairs upstream of the TATA element increase CD127 promoter activity

To further characterize the CD127 gene promoter, sequences from the TATA element to -2900bp were cloned into the pGL4B plasmid upstream of the luciferase gene and transfected into primary CD8 T-cells. As seen in figure 3-14, sequences from TATA to -2900bp increased luciferase gene expression 13 ± 1 fold over pGL4B alone ($n=4$, $p\leq0.0001$), and approximately 3-fold over the core promoter (TATA to -262, -626 and -1200; $n=4$, $p\leq0.005$). These data then demonstrate positive regulatory sequences between -1200 and -2900bp upstream of the TATA box capable of increasing CD127 basal gene expression.

In order to characterize the region between -1200 and -2900bp upstream of the CD127 TATA box, a series of 5' deletion mutants were constructed. The first, -2406 to TATA construct, eliminates a series of GATA transcription factor binding sites but contains a conserved regulatory region described by Lee et al (37) located between -2269 and -2255bp. The second promoter construct extending from -1760 to TATA removes this conserved region. The third construct extending from -1468 to TATA removes an additional 300bp from -1760bp upstream of the TATA box. As shown in figure 3-15, the -2406 to TATA fragment expresses luciferase activity similar to that seen with the -2900 to TATA construct (14 ± 1.2 fold and 13 ± 1 fold respectively, $p>0.1$). This then indicates that the basal promoter required for optimal CD127 expression in primary CD8 T-cells is contained from the TATA box to -2406 and that the 0.5kb between -2406 and -2900 do not provide additional positive effect on transcription.

Figure 3-14: The -2900 to TATA promoter construct increases luciferase gene expression 13 fold above background.

Pooled data showing normalized luciferase gene expression for the -2900 to TATA (black) compared to constructs containing only the core promoter (gray bars) ($n=4$, $p \leq 0.005$). Error bars show Standard Error of the Mean (SEM).



Furthermore, luciferase activity from the -1760 to TATA construct was significantly lower than that of the -2900 to TATA construct (10 ± 1 fold and 13 ± 1 fold respectively, $p>0.0005$). This indicates that the conserved region described by Lee et al (37) between -2269 and -2255bp may play an important role in regulation of basal promoter activity in primary resting CD8 T-cells. Additionally, the -1468 to TATA construct provided luciferase expression at 6.5 ± 1.5 fold above empty vector, significantly greater than the -1200 to TATA construct ($p<0.0274$) but significantly less compared to the -2900 to TATA construct ($p=0.0459$) (figure 3-15). Taken together, these data suggest that there are positive regulatory elements located between -1200 and -1468 bp, between -1468 and -1760 bp, and between -1760 and -2406 bp.

To further explore the importance of sequences between -1760 and -1200bp, we constructed a deletion mutant removing this region from the -2900 to TATA region. As shown in figure 3-16, deletion of these sequences did significantly diminish luciferase gene expression when compared to the complete -2900 to TATA ($n=4$, $p=0.0239$). Since deletion of this region did not result in complete abrogation of luciferase activity, there may be some compensatory regions that lie between -1760 and -2900bp, although these regions do not completely compensate for the loss of this region in the CD127 promoter.

Figure 3-15: Relative luciferase expression of theCD127 promoter constructs.

Pooled data showing normalized luciferase gene expression for each of the fragments (n=4). Statistically significant differences are indicated by a (*). Error bars show Standard Error of the Mean (SEM).

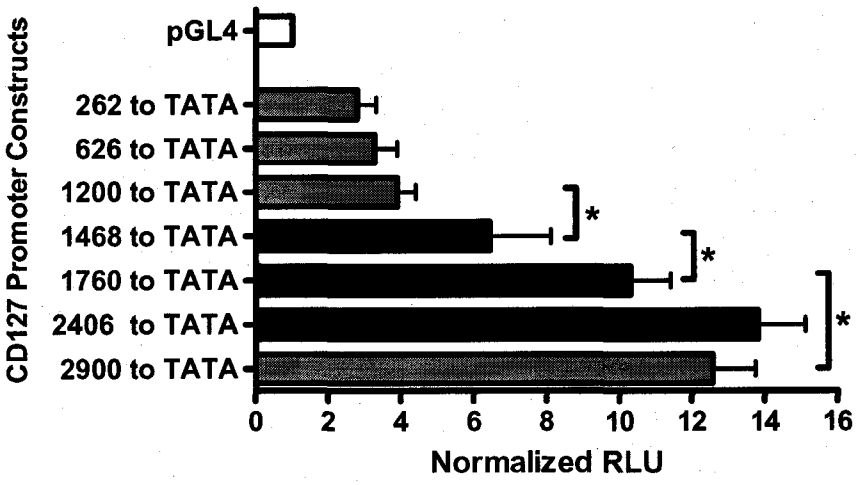
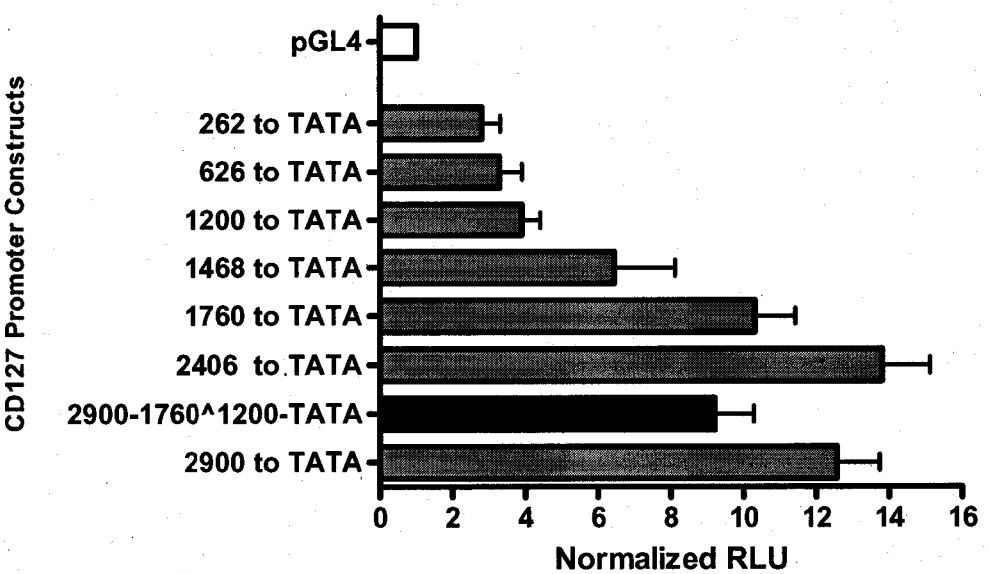


Figure 3-16: Relative luciferase expression of the -2900-1760^1200-TATA construct.

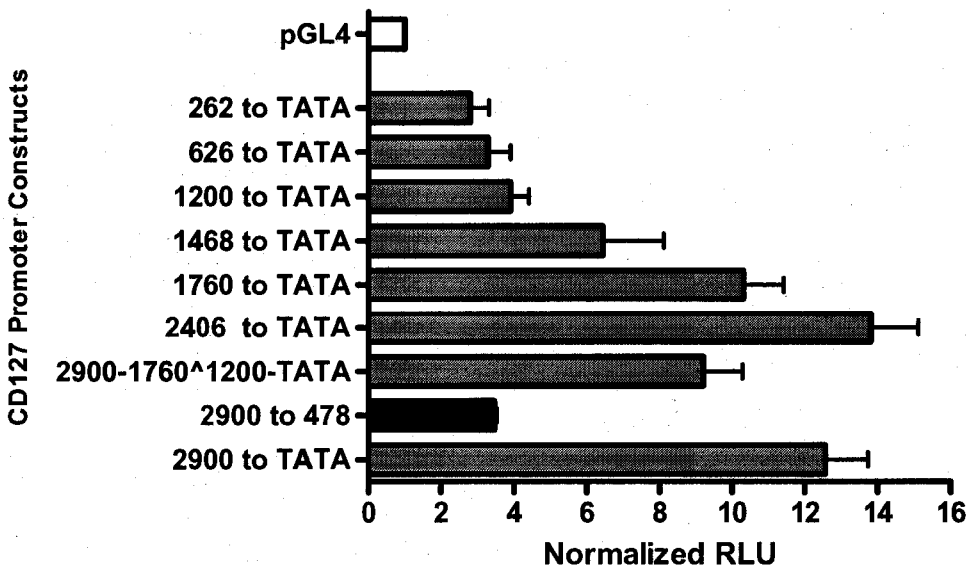
Pooled data showing normalized luciferase gene expression for the -2900-1760^1200-TATA construct was significantly less than that of the -2900 to TATA construct (n=4, p=0.0239). Error bars show Standard Error of the Mean (SEM).



Our web-based analysis of the CD127 gene promoter indicated the highest density of transcription factor binding sites within the first 500bp upstream of the TATA box. In order to assess the importance of this region, we generated a 3' deletion mutant removing the first 478bp of the CD127 promoter and maintaining sequences up to -2900bp (-2900 to -478). Following transfection into primary resting CD8 T-cells, this 3' deletion mutant provided luciferase gene expression only 4-fold over empty vector ($n=4$, $p<0.005$), significantly lower than the complete 2900 to TATA construct ($n=4$, $p<0.0001$; figure 3-17). These data indicate the *cis*-regulatory elements contained within the first 478bp upstream of the TATA box form and important part of the core CD127 promoter and removal of these elements severely impairs promoter activity. Although these elements do not by themselves cause a significant induction of the CD127 promoter as seen in the constructs spanning up to -1200bp from the TATA box, these elements are non-redundant and contribute to optimal basal promoter activity.

Figure 3-17: Relative Luciferase Activity of the -2900 to -478 promoter construct.

Pooled data showing normalized luciferase activity of the -2900 to -478 promoter construct (black) relative to other constructs (gray) and empty vector (white). Luciferase expression in the -2900 to -478 construct is significantly diminished in relation to that of the -2900 to TATA construct ($n=4$, $p<0.0001$) Error bars show Standard Error of the Mean (SEM).



3.2.5 Summary:

The upstream *cis*-regulatory elements of the CD127 promoter were cloned into a luciferase reporter vector and analyzed so as to understand the regulation of the CD127 gene in resting CD8 T-cells. To this end, we took advantage of the Nucleofection method of transfection, achieving reasonably high transfection efficiencies of approximately ~50% in primary CD8 T-cells. Since this method does not require activation or proliferation of CD8-T cells, it did not in and of itself cause a down regulation in CD127 surface expression. Using different CD127 promoter constructs, we found that the first 262bp upstream of the TATA box contain the core promoter elements. Including sequences up to -1200bp did not further increase gene expression. We also established that sequences from -1200 to -2406 upstream of the TATA box, significantly increased luciferase gene expression from 4-fold to 14-fold above empty vector suggesting this region contains important *cis*-regulatory elements important for CD127 promoter activity. We also found that there are partial-compensatory positive regulatory elements for the region between -1200 and -1760bp that are located between -1760 and -2900bp. We also noted that the conserved non-coding region from -2255 to -2269 identified by Lee et al (37) may play a role in regulation of basal CD127 promoter activity in resting CD8 T-cells. Taken together, these data indicate that the core CD127 promoter elements necessary for efficient basal transcription are contained within the first 262bp upstream of the TATA box and that additional positive regulatory elements are located between -1200 and -1468bp, -1468 and -1760bp and -1760 and -2406bp.

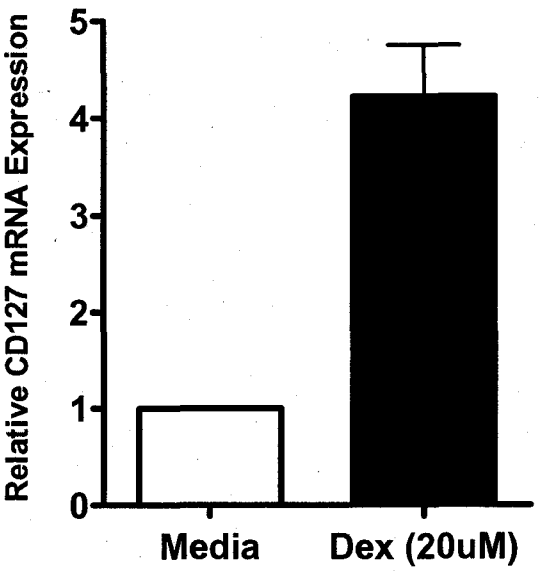
3.3 Regulation of the CD127 Promoter

3.3.1 Regulation of the CD127 Promoter by Dexamethasone

Several groups have shown glucocorticoids increase transcription from the CD127 promoter in murine B and T-cells (37, 45) as well as in human thymocytes, NK T-cells and peripheral T-cells (16, 18). To determine if glucocorticoids play a role in regulating CD127 gene expression specifically in human CD8 T-cells, we began by examining RNA levels in the presence or absence of Dexamethasone (Dex). CD8 T-cells were isolated from healthy donors and incubated either in media alone or in media plus 20μM Dexamethasone for 24 hours. Total RNA was then isolated and CD127 transcripts were quantified by Real-Time PCR. As demonstrated in figure 3-18, CD8 T-cells treated with Dexamethasone for 24 hours showed a 4.2 ± 0.5 fold increase in CD127 mRNA relative to media control (n=3, p=0.0125). This indicates that the CD127 gene promoter is indeed either directly or indirectly responsive to glucocorticoid stimulation.

Figure 3-18: Dexamethasone up regulates CD127 mRNA in CD8 T-cells.

Pooled Real-Time PCR data showing that Dexamethasone (20μM) induces a 4.2 ± 0.5 fold increase in CD127 mRNA levels in purified CD8 T-cells after 24 hours (n=3, p=0.0125). Error bars indicate SEM.



In order to confirm the Real-Time PCR data and to identify potential glucocorticoid responsive elements within the CD127 promoter, CD8 T-cells were transfected with the same series of CD127 promoter constructs described in section 3.2 and analyzed for their response to Dexamethasone. As shown in figure 3-19, Dex (20μM) increased luciferase expression from the -2900 to TATA construct by 2.4 ± 0.2 fold over the same construct maintained in media alone (n=3, $p<0.0001$). This then confirms the CD127 promoter is either directly or indirectly up regulated by glucocorticoids. To identify potential glucocorticoid receptor (GR) responsive elements within the human CD127 promoter and the role they play in CD127 gene expression in CD8 T-cells, we examined the responsiveness of our various promoter constructs to Dexamethasone. Interestingly, Lee et al (37) identified a conserved GR responsive element between -2255 and -2269 upstream of the TATA box. To address the importance of this element in human CD8 T-cells, a 13 base pair deletion from -2255 to -2269 was generated within the TATA to -2900 promoter fragment (-2900-2269^2255-TATA) specifically deleting the GR responsive element. Figure 3-19 shows that the -2900-2269^2255-TATA construct has similar levels of luciferase gene expression (11.2 ± 2.9 fold) compared to that observed in the 2900 to TATA construct (13 ± 1 fold). These data therefore suggest that the 13bp GRE element does not play a role in regulating basal CD127 gene expression.

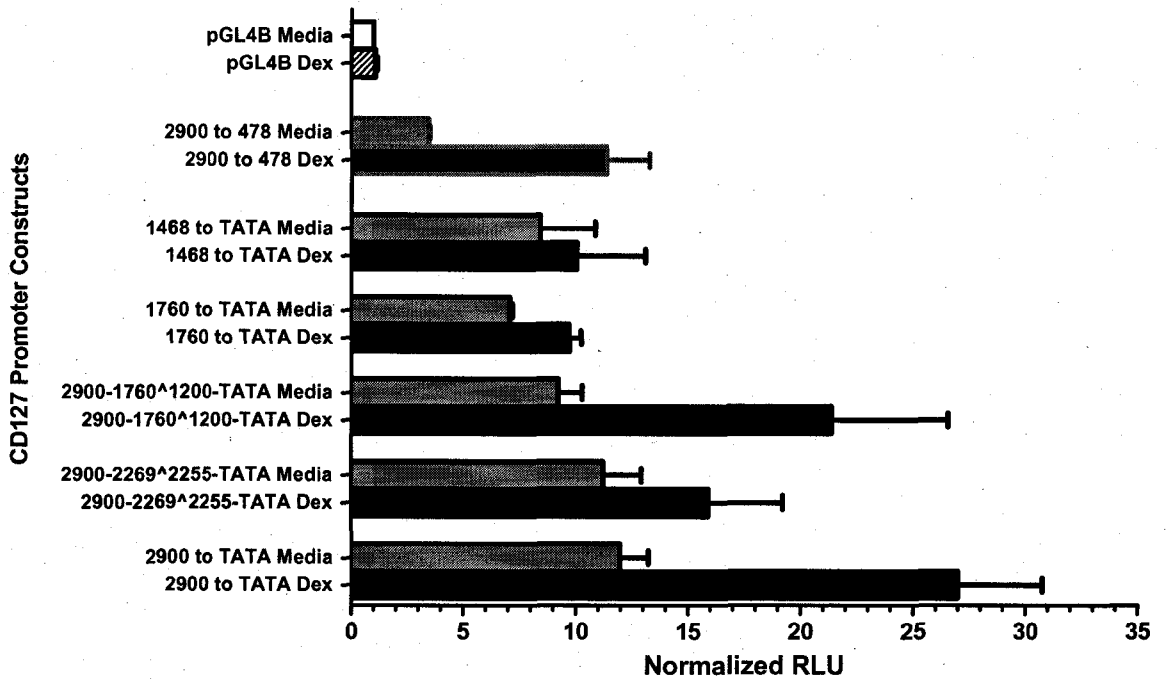
As shown in figure 3-19, the promoter constructs containing the GR responsive element located from -2255 to -2269 showed increased luciferase expression following treatment with Dexamethasone. Indeed, luciferase expression increased by 2.4 ± 0.2 fold for the -2900 to TATA fragment and by 2.2 ± 0.3 fold for the -2900-1760^1200-TATA construct in the presence of Dexamethasone compared to the same promoter constructs in cells

maintained in media alone. Promoter fragments extending from the TATA to -1460 or to -1760 did not respond to Dex (n=3, 1.3 ± 0.2 fold and 1.2 ± 0.05 fold increase in luciferase expression respectively) As expected, deletion of the 13 base pair GR responsive element located between -2255 and -2269 from the CD127 promoter resulted in a loss of responsiveness to Dexamethasone (1.52 ± 0.25 fold). These data then demonstrate that the human CD127 gene promoter is indeed up regulated by glucocorticoids in resting CD8 T-cells and that the GR responsive element is located between -2255 and -2269bp upstream of the TATA box. Interestingly, the -2900 to -478 promoter construct which lacks core promoter activity was also up regulated by Dexamethasone by 3.32 ± 0.75 fold over the same construct in cells maintained in media alone. Taken together these data suggest the GR responsive element within the CD127 promoter plays an important role in regulating the expression of this gene in human CD8 T-cells. Moreover, the activity of this element is neither affected by its distance from the TATA box as demonstrated by the activity of the 2900-1760^1200-TATA construct nor the presence of core promoter elements within the first 0.5kb upstream of the TATA box as demonstrated by the activity of the -2900 to -478 construct.

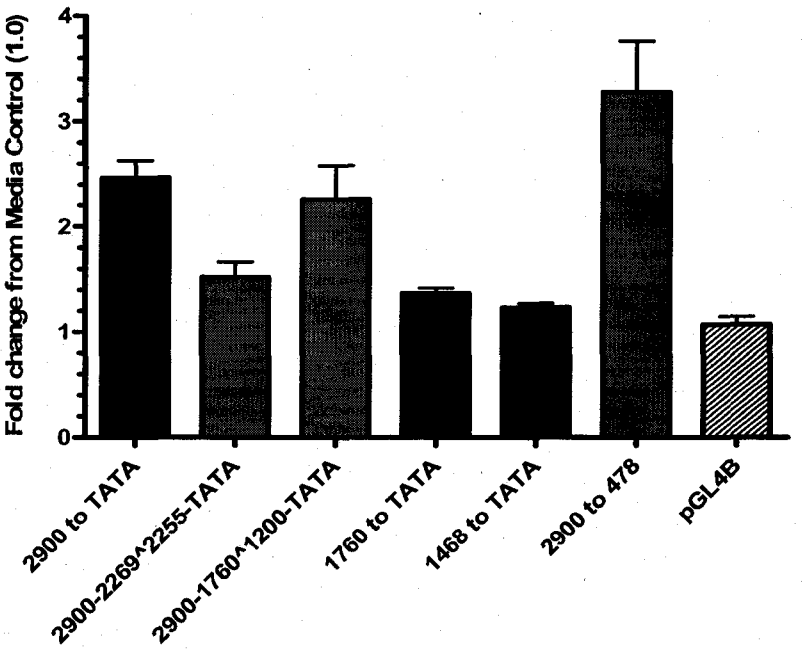
Figure 3-19: Dexamethasone up regulates transcription from the CD127 gene promoter.

(A) Pooled data showing the effect of Dex treatment for 24 hours on CD8 T-cells transfected with different CD127 promoter constructs. (B) Bar graph showing mean fold change in luciferase gene expression in CD8 T-cells treated with Dex for 24 hours normalized to media control. Error bars show Standard Error of the Mean (SEM) (n=3).

A



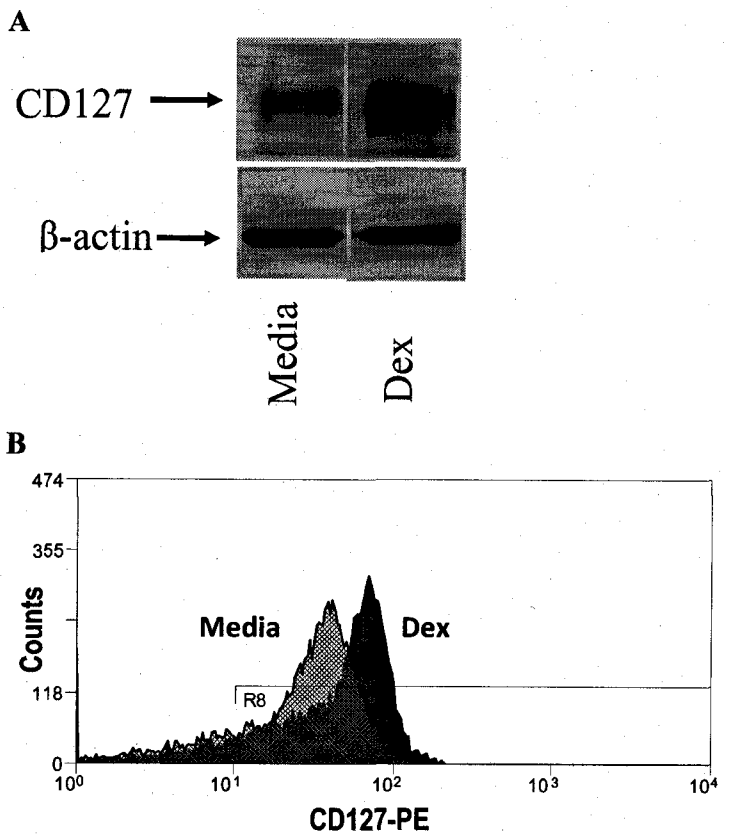
B



In order to confirm that the increase in promoter activity and CD127 mRNA seen in the presence of Dexamethasone translated into increased CD127 protein, we measured CD127 surface expression by flow cytometry and total protein expression by Western Blot in the presence and absence of Dex. Purified CD8 T-cells were incubated in media alone or with 20μM Dexamethasone for 24 hours and then analyzed by flow cytometry and Western Blot. As shown in figure 3-20, CD8 T-cells treated with Dex showed an increase both in total (3.8 ± 0.7 fold) and surface CD127 protein expression (9.3 ± 2%) over a 24 hour period (n=2). Consistent with reports in literature, these data indicate Dexamethasone increases *de novo* CD127 protein synthesis by up regulating transcription from the CD127 promoter.

Figure 3-20: Dexamethasone up regulates CD127 protein expression.

(A) Western Blot showing Dexamethasone up regulates total CD127 protein in CD8 T-cells after 24 hours. (B) Representative flow cytometry histogram overlay showing Dexamethasone up regulates CD127 surface protein expression.



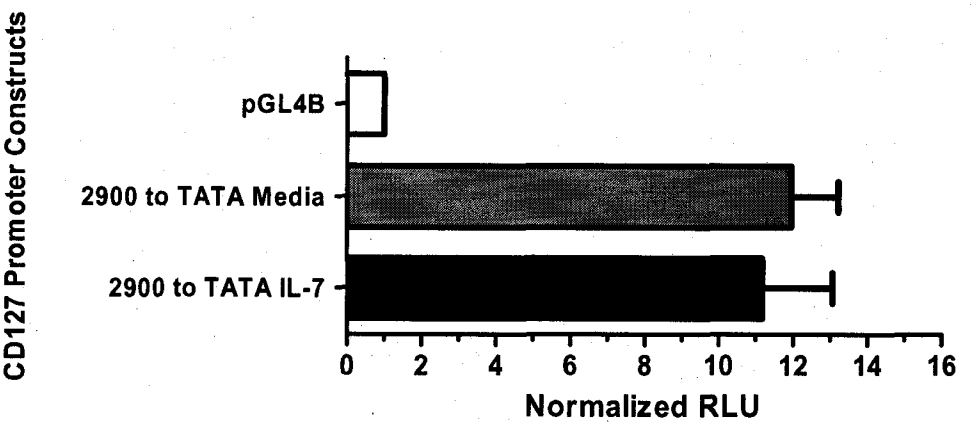
3.3.2 Regulation of the CD127 promoter by IL-7

We have previously demonstrated that IL-7 induces a decrease in CD127 transcripts in CD8 T-cells. In view of this data, we hypothesized that the down regulation of CD127 by IL-7 may occur at the level of the promoter. To address this hypothesis, purified CD8 T-cells were transfected with the -2900 to TATA CD127 promoter construct and then incubated either in media alone or in the presence of 10ng/mL IL-7 for 24 hours. As shown in figure 3-21, IL-7 did not down regulate luciferase gene expression from the cloned CD127 promoter in CD8 T-cells. It is possible then the IL-7 responsive elements may not lie within the -2900 to TATA fragment used in these experiments.

The transcriptional repressor Growth Factor Independent (Gfi)-1 has been implicated in the down regulation of CD127 gene expression in murine CD4 and CD8 T-cells (7, 50). Indeed, Park et al found a Gfi-1 site located in the second intron of the murine CD127 gene (50) plays a role in the IL-7 mediated down regulation of the CD127 promoter (7, 50). To investigate whether IL-7 down regulated the CD127 promoter in human CD8 T-cells by inducing expression of a transcription repressor, purified CD8 T-cells were pre-treated with 100μM of the translation inhibitor, Cycloheximide (CHX). This molecule inhibits the elongation of *de novo* polypeptide chains by causing a block in the translocation step of tRNA molecules in the large ribosomal subunit (11). After pre-treating with CHX for 1 hour, CD8 T-cells were incubated with 10ng/mL IL-7 for an additional 24 hours.

Figure 3-21: IL-7 does not down regulate luciferase gene expression from the cloned CD127 promoter in CD8 T-cells.

Pooled data (n=4) using the -2900 to TATA CD127 promoter construct. Error bars indicate SEM.



CD127 transcripts were then quantified by Real-Time PCR relative to RPS18 reference gene expression. As shown in figure 3-22, we found that CD8 T-cells treated with IL-7 alone for 24 hours down regulated CD127 mRNA expression by 65 ± 5 %, while cells treated with IL-7 in the presence of CHX down regulated CD127 mRNA by only 20 ± 15% relative to media controls (n=3 p=0.0002). These data indicate IL-7 up regulates expression of a repressor which in turn suppresses CD127 gene expression.

In view of studies in mice that have shown the involvement of Growth Factor Independence (Gfi)-1 in IL-7 mediated down regulation of the mouse promoter (7, 50) and data showing that IL-7 appears to require *de novo* protein synthesis to cause the down regulation of CD127 transcripts (figure 3-21), we asked whether IL-7 causes the up regulation of Gfi-1 in CD8 T-cells. Purified CD8 T-cells were incubated in media alone or treated with either 1ng/mL or 10 ng/mL of IL-7 for 24 hours. Cells were lysed and CD127, Gfi-1 and β-actin proteins were assayed by Western blotting. Also, the lysate from THP-1 cells, a monocytic cell-line known for high expression of Gfi-1 was used as a positive control. Figure 3-23 shows a representative blot from one individual where only the highest dose of IL-7 treatment (10ng/mL) caused a down regulation in total CD127 protein in CD8 T-cells over 24 hours. This down regulation in CD127 protein did not however correlate with an increase in Gfi-1 protein levels suggesting that although IL-7 treatment requires *de novo* protein synthesis for the down regulation of CD127 gene expression at the 24 hour time point, the newly synthesized protein was not Gfi-1.

Figure 3-22: *De novo* protein synthesis is required for IL-7 to down regulate CD127 gene transcription.

Pooled data showing CD8 T-cells treated with Cycloheximide (CHX) in the presence or absence of IL-7. Cells treated with IL-7 in the presence of CHX did not down regulate CD127 mRNA expression compared to cells treated with IL-7 alone ($n=5$ $p<0.005$). Statistical significance is indicated by an *. Error bars indicate SEM.

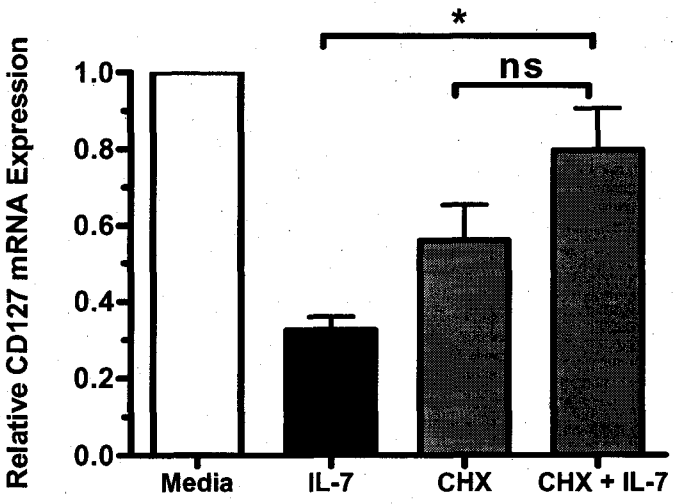
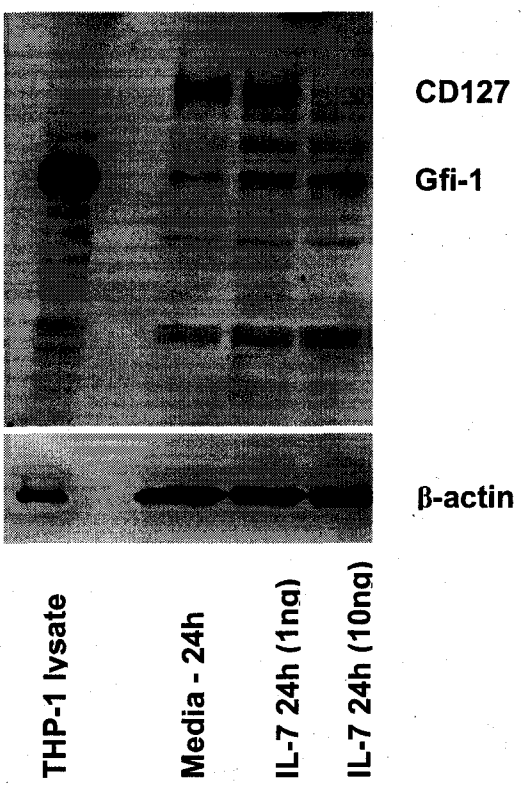


Figure 3-23: IL-7 did not induce a change in Gfi-1 protein expression in CD8 T-cells over 24 hour incubation with IL-7.

Representative Western Blot showing CD8 T-cells treated with IL-7 for 24 hours. Blot was probed simultaneously for Gfi-1 and CD127 then for β-actin. THP-1 monocytic cell-line lysate was used as a positive control for Gfi-1 protein expression.



3.3.3 Summary:

As reported previously in mice, dexamethasone up regulates CD127 transcripts and protein in human CD8 T-cells. Promoter constructs containing the GR responsive element between -2255 and -2269bp showed a ≥ 2 fold increase in luciferase gene expression when incubated in the presence of dexamethasone compared to media controls. IL-7 did not down regulate the cloned CD127 promoter construct from the TATA to -2900bp suggesting the IL-7 responsive regulatory elements may lie outside of this ~3kb upstream region. Interestingly, IL-7 was unable to down regulate endogenous CD127 expression in the presence of cycloheximide indicating *de novo* protein synthesis is required for IL-7 to influence CD127 gene expression. Whether IL-7 induces a transcriptional repressor or other protein that inhibits gene expression is currently not known. Over a 24 hour incubation period, we find that IL-7 causes a decrease in total CD127 protein content in CD8 T-cells and this decrease does not correlate with an increase in a known repressor, Gfi-1 over this incubation period.

CHAPTER 4: DISCUSSION

4.1 Regulation of CD127 mRNA Expression

Studies have shown that treating murine lymph node T (LNT) –cells with IL-7 causes a specific down regulation of IL-7's cognate receptor (CD127) as early as 6 hours post-treatment (50). These findings have also been corroborated in studies involving human thymocytes (10) and human CD4 and CD8 T-cells where the down regulation of CD127 has been shown to occur as early as 2 hours and persisting up to 72 hours in the presence of IL-7 (2, 68). Data from our lab corroborates these findings by showing that IL-7 leads to a similar level of down regulation both at the protein and transcript levels.

A recent study showed that an imbalance in the ratio of transmembrane versus secreted isoforms of CD127 protein could lead to an apparent down regulation of surface CD127 expression, suggesting a new mode of regulation of the CD127 gene (22). In view of these findings, we investigated the ratio of transcripts encoding for the transmembrane versus secreted isoforms of CD127 protein in resting primary human CD8 T-cells. We found that the abundance of transcripts encoding for the transmembrane isoform of CD127 protein was noticeably higher than that of the secreted isoform. This data is in line with findings by Korte et al in all mature T-cells (35). Altogether, these findings suggest that the bulk of CD127 transcriptional activity is directed towards the synthesis of the membrane bound CD127, which is involved in transmission of IL-7/IL-7R signaling events. Moreover, due to the lower relative abundance of transcripts encoding for secreted CD127, these data suggest that secreted CD127 exists at a much lower concentration in the extracellular milieu and therefore may play a role in fine tuning rather than fundamentally influencing IL-7 signal transduction in CD8 T-cells.

To address the hypothesis of whether IL-7 leads to a dysregulation of the ratios of transcripts encoding for secreted (secCD127) versus transmembrane (tmCD127) isoforms of CD127 protein, CD127 transcripts were quantified by two different PCR based methods. In our real-time PCR strategy, we compared in parallel, the levels of secCD127 to all CD127 transcripts (totCD127) in CD8 T-cells incubated in the presence or absence of IL-7. Our data from the real-time PCR experiment indicated that IL-7 treatment caused a down regulation of both totCD127 and secCD127 – although the degree of down regulation of the secCD127 isoform was significantly less than that seen for totCD127. In view of this data, we used a conventional PCR strategy for the relative quantification of CD127 transcripts encoding for both secCD127 and tmCD127. As previously described, using this method allowed for the simultaneous measurement of both transcripts and the measurement of the ratios of the two transcripts in untreated versus treated samples. Similar to data obtained by real-time PCR, we found that IL-7 treatment caused a down regulation in all CD127 transcripts although the magnitude of down regulation was significantly larger in the tmCD127 compartment compared to that seen for secCD127. Taken together, these data indicate that IL-7 does down regulate all CD127 mRNAs although the magnitude of down regulation of secCD127 transcripts is significantly lower than that seen in tmCD127. It is unclear whether the difference in magnitude of down regulation between the two transcripts encoding for CD127 protein is a result of instrument sensitivity or due to selective down regulation of tmCD127 over secCD127. For instance, instrument sensitivity may play a role in these observations as these effects become apparent when the relative magnitude of down regulation of secCD127 is compared between the real-time PCR method and the semi-quantitative PCR methods. Indeed, the down regulation of secCD127 measured by the semi-quantitative method is only 15% compared to 40% seen by the real-time PCR method further underscoring that the

differences in assay sensitivity may make it difficult to resolve changes that occur at different orders of magnitude (i.e. between high abundance to low abundance versus low abundance to lower abundance). Conversely, tmCD127 could be down regulated actively due to dysregulation of alternative splicing machinery or selective degradation of the tmCD127 over the secCD127 transcripts. The differences in assay sensitivity notwithstanding, our data indicate that IL-7 not only down regulates global CD127 mRNA expression, but also alters the ratio of tmCD127: secCD127 thereby providing a highly efficient and dynamic mode for down regulating CD127.

Regulation of gene expression has also been shown to occur at the level of mRNA stability whereby an external stimulus leads to the destabilization and subsequent degradation of existing mRNA pools (23). In order to establish whether IL-7 causes a destabilization and subsequent decrease in CD127 mRNA half life, we examined the persistence of CD127 transcripts in the presence of IL-7. Our data indicates that CD8 T-cells treated with IL-7 in the presence of Actinomycin (Act) D, did not down regulate CD127 transcripts – indicating that IL-7 by itself does not decrease CD127 mRNA stability. Equally, consistent with findings in literature (2, 50), our data may also be interpreted as showing that the down regulation of CD127 transcripts in response to IL-7 treatment requires *de novo* transcription of a yet unidentified factor that ultimately mediates the down regulation of CD127 mRNA. In line with this, we find that CD8 T-cells pre-treated with Cycloheximide (CHX) are also unable to down regulate CD127 mRNA in response to IL-7; further underscoring the requirement for new gene expression in order to down regulate the CD127 gene in human CD8 T-cells. Studies in murine models have shown the involvement of Growth Factor Independence (Gfi)-1 as a suppressor of CD127 gene expression by binding

to DNA regulatory elements in the murine CD127 promoter (7, 50). On the contrary, recent findings in human T-cells have shown that suppression of CD127 occurs independently of Gfi-1 expression (2, 49). Our data also indicates that IL-7 treatment does down regulate CD127 total protein expression, but this down regulation is independent of increased Gfi-1 protein expression over a 24 hour incubation period. Altogether, our data along with findings by Alves et al (2) indicate that IL-7 induced down regulation of CD127 requires *de novo* synthesis of factor(s). It is conceivable that Gfi-1 may still play a role in down regulating CD127 gene expression as shown by Chandele et al (7) and Park et al (50). IL-7 could either cause an increase in Gfi-1 protein production past 24 hours or IL-7 may cause an increase in Gfi-1 protein translocation into the nucleus and ultimately to the CD127 promoter consistent with the findings by Park et al (50) using ChIP analysis.

Furthermore, we also investigated the possibility that the down regulation of CD127 gene expression in CD8 T-cells by IL-7 could occur at the level of transcription initiation. Using a CD127 promoter driven reporter gene expression model, we found that IL-7 did not cause a down regulation at the level of the CD127 promoter. This data is consistent with recent findings by Alves et al, which found that IL-7 induced down regulation of CD127 surface protein expression occurred irrespective of the promoter driving CD127 gene expression in CD4 and CD8 T-cells (2). On the other hand, IL-7 starvation of lymphocytes has been shown to cause hypoacetylation of the V(D)J locus suggesting that IL-7 signaling may cause an increase in global acetylation activity (26). Preliminary data from our lab has shown that incubation of CD8 T-cells with a histone deacetylase (HDAC) inhibitor leads to a dose dependent down regulation of CD127 (data not shown). Although studies investigating the effect of IL-7 in the presence of deacetylase inhibitors are ongoing, it is conceivable that

IL-7 mediated hyperacetylation of CD8 T-cell histones may lead to increased chromatin accessibility thereby allowing for expression of potential suppressors of CD127 gene expression as shown by our IL-7 plus inhibitor experiments.

4.2 Regulation of the CD127 promoter

In order to understand the regulation of the CD127 promoter in resting human CD8 T-cells, we used a series CD127 promoter driven reporter constructs to identify important *cis*-regulatory elements upstream of the TATA box. Based on analysis of the putative transcription binding sites and information from the NCBI database, the CD127 promoter region has been predicted to lie within 1.1kb upstream of the TATA box (8, 46). Further analyses of the CD127 promoter region revealed a 100bp highly conserved region located within 0.5kb immediately upstream of the TATA box (8). In view of this data, we examined the expression of a reporter construct spanning from the TATA box up to -1200 upstream. Truncations of this region spanning from the TATA box up to -262 and -626 were generated in order to better identify the core promoter region. Our data indicates, consistent with literature that the core promoter elements did indeed lie within the 1.1kb identified region upstream of the TATA box. Additionally, our data more precisely identified the core promoter region and indicated that the regions between -1200 and -262 did not impart any positive regulatory function in the basal expression of the CD127 gene.

Alignment of the region upstream of exon 1 revealed a highly conserved 200bp region (across 17 species) located approximately 2.5kb upstream of the TATA box (8). A study by Lee et al identified a conserved non-coding region located near -2.2kb upstream of the TATA box that is responsive to glucocorticoid treatment (37). Consistent with these

findings, our data found that this region not only contributes to glucocorticoid-induced CD127 promoter up regulation due to the presence of a glucocorticoid responsive element (GRE), but also contain sequences which enhance basal promoter activity by >2 fold above that of the core promoter. Upon exclusion of this region as depicted by the -1760 to TATA construct, we found a decrease in promoter activity indicating the importance of this additional element in basal CD127 gene expression in CD8 T-cells. Consistent with reports in literature in murine models and human PBMCs (16, 18, 37), our data indicate that CD8 T-cells treated with glucocorticoids, up regulate CD127 transcription and protein expression within 24 hours of treatment. The biological implications of up regulating CD127 by a CD8 T-cell upon exposure to glucocorticoids could be hypothesized to be due to a rescue of these cells from inflammation-induced apoptosis (16).

A cluster of transcription factor binding sites (c-Fos, AP1, CTF-2, DBP, Pit-1B), were mapped around -1468 upstream of the TATA box. This indicates that this region may play an important role in the regulation of the CD127 promoter. Although these transcription factors have not been shown to influence CD127 gene expression, based on reporter gene expression data from the plasmid carrying the -1468 to TATA region relative to that of the -1200 to TATA region of the CD127 promoter, we found that the inclusion of the region between -1468 and -1200 led to a significant increase in promoter activity. Therefore, this data suggests that the binding of transcription factors near -1468 upstream of the TATA box may play a role in the optimal basal expression of the CD127 gene.

Altogether, the data from the CD127 promoter-driven reporter constructs outline several upstream *cis*-regulatory regions involved in basal CD127 promoter activity in CD8 T-cells. The core promoter elements are shown to lie within the first -0.5kb that lie upstream

of the TATA box. Additionally, the regions between the TATA box and the ATG did not play a significant role in the regulation of basal CD127 promoter activity. Moreover, regions between -1.8kb and -1.2kb, including the putative positive regulatory elements near -1.5kb, can be deleted without a significant decline in basal promoter activity indicating the presence of redundant positive regulatory elements located upstream of -1.8kb. Furthermore, a conserved positive regulatory region located approximately 2.2kb upstream of the TATA box plays an important role in up regulation of basal CD127 promoter activity. Additionally, this conserved region contains a glucocorticoid-sensitive element that plays an important role in the induction of glucocorticoid-mediated up regulation of the CD127 promoter.

CONCLUSION

Membrane bound receptors play an important role in downstream signal transduction and therefore their expression is often tightly regulated. As in the case of CD127, the loss of receptor expression can lead to improper development of the adaptive immune system and immunodeficiency. On the other hand, over expression of this receptor has been implicated in higher instances of lymphomas. In CD8 T-cells, the regulation of CD127 in one part occurs at the level of transcription. Although the mechanism of this IL-7 mediated regulation of CD127 remains a question for future investigation in our lab, our data indicates that the regulation of CD127 by IL-7 and glucocorticoids occurs differentially; where glucocorticoids induce the expression of CD127 through a *cis*-regulatory element, while IL-7 acts indirectly through a yet unidentified protein.

REFERENCES

1. **Alves, N. L., F. A. Arosa, and R. A. van Lier.** 2007. Common gamma chain cytokines: dissidence in the details. Immunol Lett **108:**113-20.

2. **Alves, N. L., E. M. van Leeuwen, I. A. Derks, and R. A. van Lier.** 2008. Differential regulation of human IL-7 receptor alpha expression by IL-7 and TCR signaling. J Immunol **180:**5201-10.

3. **Armitage, R. J., A. E. Namen, H. M. Sassenfeld, and K. H. Grabstein.** 1990. Regulation of human T cell proliferation by IL-7. J Immunol **144:**938-41.

4. **Boutboul, F., D. Puthier, V. Appay, O. Pelle, H. Ait-Mohand, B. Combadiere, G. Carcelain, C. Katlama, S. L. Rowland-Jones, P. Debre, C. Nguyen, and B. Autran.** 2005. Modulation of interleukin-7 receptor expression characterizes differentiation of CD8 T cells specific for HIV, EBV and CMV. Aids **19:**1981-6.

5. **Buckley, R. H., R. I. Schiff, S. E. Schiff, M. L. Markert, L. W. Williams, T. O. Harville, J. L. Roberts, and J. M. Puck.** 1997. Human severe combined immunodeficiency: genetic, phenotypic, and functional diversity in one hundred eight infants. J Pediatr **130:**378-87.

6. **Carini, C., and M. Essex.** 1994. Interleukin 2-independent interleukin 7 activity enhances cytotoxic immune response of HIV-1-infected individuals. AIDS Res Hum Retroviruses **10:**121-30.

7. **Chandele, A., N. S. Joshi, J. Zhu, W. E. Paul, W. J. Leonard, and S. M. Kaech.** 2008. Formation of IL-7Ralphahigh and IL-7Ralphalow CD8 T cells during infection is regulated by the opposing functions of GABPalpha and Gfi-1. J Immunol **180:**5309-19.

8. **cisRED.** 2006. cis-regulatory element database - Region for Gene ENSG00000168685 (HGNC Symbol: IL7R). UCSC.

9. **Corcoran, D. L., E. Feingold, J. Dominick, M. Wright, J. Harnaha, M. Trucco, N. Giannoukakis, and P. V. Benos.** 2005. Footer: a quantitative comparative genomics method for efficient recognition of cis-regulatory elements. Genome Res **15:**840-7.

10. **Doan, L. L., M. K. Kitay, Q. Yu, A. Singer, S. Herblot, T. Hoang, S. E. Bear, H. C. Morse, 3rd, P. N. Tsichlis, and H. L. Grimes.** 2003. Growth factor independence-1B expression leads to defects in T cell activation, IL-7 receptor alpha expression, and T cell lineage commitment. J Immunol **170:**2356-66.

11. **Ennis, H. L., and M. Lubin.** 1964. Cycloheximide: Aspects of Inhibition of Protein Synthesis in Mammalian Cells. Science **146:**1474-6.

12. **Ensembl.** 2008. Ensembl Gene Report for ENSG00000168685 (IL7R) - Human. Sanger Institute.

13. **Faller, E. M., McVey, Mark J., Kakal, Juzer A.and MacPherson, Paul A.** 2006. Interleukin-7 Receptor Expression is Down Regulated by the HIV-1 Tat Protein J Acquir Immune Defic Syndr **43:**257-69.

14. **Feng, X. H., and R. Derynck.** 2005. Specificity and versatility in tgf-beta signaling through Smads. Annu Rev Cell Dev Biol **21:**659-93.

15. **Fero, M. L., M. Rivkin, M. Tasch, P. Porter, C. E. Carow, E. Firpo, K. Polyak, L. H. Tsai, V. Broudy, R. M. Perlmutter, K. Kaushansky, and J. M. Roberts.** 1996. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. Cell **85:**733-44.

16. **Franchimont, D., J. Galon, M. S. Vacchio, S. Fan, R. Visconti, D. M. Frucht, V. Geenen, G. P. Chrousos, J. D. Ashwell, and J. J. O'Shea.** 2002. Positive effects of glucocorticoids on T cell function by up-regulation of IL-7 receptor alpha. J Immunol **168:**2212-8.

17. **Fry, T. J., and C. L. Mackall.** 2005. The many faces of IL-7: from lymphopoiesis to peripheral T cell maintenance. J Immunol **174:**6571-6.

18. **Galon, J., D. Franchimont, N. Hiroi, G. Frey, A. Boettner, M. Ehrhart-Bornstein, J. J. O'Shea, G. P. Chrousos, and S. R. Bornstein.** 2002. Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. Faseb J **16:**61-71.

19. **Gea-Banacloche, J. C., S. A. Migueles, L. Martino, W. L. Shupert, A. C. McNeil, M. S. Sabbaghian, L. Ehler, C. Prussin, R. Stevens, L. Lambert, J. Altman, C. W. Hallahan, J. C. de Quiros, and M. Connors.** 2000. Maintenance of large numbers of virus-specific CD8+ T cells in HIV-infected progressors and long-term nonprogressors. J Immunol **165:**1082-92.

20. **Goodwin, R. G., D. Friend, S. F. Ziegler, R. Jerzy, B. A. Falk, S. Gimpel, D. Cosman, S. K. Dower, C. J. March, A. E. Namen, and et al.** 1990. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. Cell **60:**941-51.

21. **Gosseye, S., N. Diebold, C. Griscelli, and C. Nezelof.** 1983. Severe combined immunodeficiency disease: a pathological analysis of 26 cases. Clin Immunol Immunopathol **29:**58-77.

22. **Gregory, S. G., S. Schmidt, P. Seth, J. R. Oksenberg, J. Hart, A. Prokop, S. J. Caillier, M. Ban, A. Goris, L. F. Barcellos, R. Lincoln, J. L. McCauley, S. J. Sawcer, D. A. Compston, B. Dubois, S. L. Hauser, M. A. Garcia-Blanco, M. A. Pericak-Vance, and J. L. Haines.** 2007. Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis. Nat Genet **39:**1083-91.

23. **Hanke, J. H., L. N. Nichols, and M. E. Coon.** 1992. FK506 and rapamycin selectively enhance degradation of IL-2 and GM-CSF mRNA. Lymphokine Cytokine Res **11:**221-31.

24. **Heintel, T., M. Sester, M. M. Rodriguez, C. Krieg, U. Sester, R. Wagner, H. W. Pees, B. Gartner, R. Maier, and A. Meyerhans.** 2002. The fraction of perforin-expressing HIV-specific CD8 T cells is a marker for disease progression in HIV infection. Aids **16:**1497-501.

25. **Hernandez-Caselles, T., M. Martinez-Esparza, D. Sancho, G. Rubio, and P. Aparicio.** 1995. Interleukin-7 rescues human activated T lymphocytes from apoptosis induced by glucocorticoesteroids and regulates bcl-2 and CD25 expression. Hum Immunol **43:**181-9.

26. **Huang, J., and K. Muegge.** 2001. Control of chromatin accessibility for V(D)J recombination by interleukin-7. J Leukoc Biol **69:**907-11.

27. **Ibelgaufts, H.** April 2007 2007, posting date. COPE Cytokines and Cells Pathfinder Encyclopedia 19.7. [Online.]

28. **Janeway, C. A., Travers, P, Walport, Mark, Schlomchik, Mark.** 2001. immuno biology: The Immune System in Health and Disease, 5th edition ed. Garland Publishing, a member of Taylor and Francis Group, 29 West 35th St, New York, NY, 10001-2299.

29. **Jiang, Q., J. Huang, W. Q. Li, T. Cavinato, J. R. Keller, and S. K. Durum.** 2007. Role of the intracellular domain of IL-7 receptor in T cell development. J Immunol **178:**228-34.

30. **Jiang, Q., W. Q. Li, F. B. Aiello, R. Mazzucchelli, B. Asefa, A. R. Khaled, and S. K. Durum.** 2005. Cell biology of IL-7, a key lymphotrophin. Cytokine Growth Factor Rev **16:**513-33.

31. **Jicha, D. L., J. J. Mule, and S. A. Rosenberg.** 1991. Interleukin 7 generates antitumor cytotoxic T lymphocytes against murine sarcomas with efficacy in cellular adoptive immunotherapy. J Exp Med **174:**1511-5.

32. **Khaled, A. R., and S. K. Durum.** 2002. Lymphocide: cytokines and the control of lymphoid homeostasis. Nat Rev Immunol **2:**817-30.

33. **Kim, H. R., K. A. Hwang, K. C. Kim, and I. Kang.** 2007. Down-regulation of IL-7Ralpha expression in human T cells via DNA methylation. J Immunol **178:**5473-9.

34. **Kittipatarin, C., and A. R. Khaled.** 2007. Interlinking interleukin-7. Cytokine **39:**75-83.

35. **Korte, A., J. Kochling, L. Badiali, C. Eckert, J. Andreae, W. Geilen, C. Kebelmann-Betzing, T. Taube, S. Wu, G. Henze, and K. Seeger.** 2000. Expression analysis and characterization of alternatively spliced transcripts of human IL-7Ralpha chain encoding two truncated receptor proteins in relapsed childhood all. Cytokine **12:**1597-608.

36. **Lantelme, E., L. Orlando, P. Porcedda, V. Turinetto, M. De Marchi, A. Amoroso, S. Mantovani, and C. Giachino.** 2008. An in vitro model of T cell receptor revision in mature human CD8+ T cells. Mol Immunol **45:**328-37.

37. **Lee, H. C., H. Shibata, S. Ogawa, K. Maki, and K. Ikuta.** 2005. Transcriptional regulation of the mouse IL-7 receptor alpha promoter by glucocorticoid receptor. J Immunol **174:**7800-6.

38. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25:**402-8.

39. **Ma, A., R. Koka, and P. Burkett.** 2006. Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis. Annu Rev Immunol **24:**657-79.

40. **MacPherson, P. A., C. Fex, J. Sanchez-Dardon, N. Hawley-Foss, and J. B. Angel.** 2001. Interleukin-7 receptor expression on CD8(+) T cells is reduced in HIV infection and partially restored with effective antiretroviral therapy. J Acquir Immune Defic Syndr **28:**454-7.

41. **Maki, K., and K. Ikuta.** 2008. MEK1/2 induces STAT5-mediated germline transcription of the TCRgamma locus in response to IL-7R signaling. J Immunol **181:**494-502.

42. **Matsui, H., H. Asou, and T. Inaba.** 2007. Cytokines direct the regulation of Bim mRNA stability by heat-shock cognate protein 70. Mol Cell **25:**99-112.

43. **Mazzucchelli, R., and S. K. Durum.** 2007. Interleukin-7 receptor expression: intelligent design. Nat Rev Immunol **7:**144-54.

44. **Migueles, S. A., A. C. Laborico, W. L. Shupert, M. S. Sabbaghian, R. Rabin, C. W. Hallahan, D. Van Baarle, S. Kostense, F. Miedema, M. McLaughlin, L. Ehler, J. Metcalf, S. Liu, and M. Connors.** 2002. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. Nat Immunol **3:**1061-8.

45. **Mittelstadt, P. R., and J. D. Ashwell.** 2003. Disruption of glucocorticoid receptor exon 2 yields a ligand-responsive C-terminal fragment that regulates gene expression. Mol Endocrinol **17:**1534-42.

46. **NCBI, N. C. o. B. I.-.** July 23 2008 1988, posting date. U.S National Library of Medicine. [Online.]

47. **Oven, I., N. Brdickova, J. Kohoutek, T. Vaupotic, M. Narat, and B. M. Peterlin.** 2007. AIRE recruits P-TEFb for transcriptional elongation of target genes in medullary thymic epithelial cells. Mol Cell Biol **27:**8815-23.

48. **Palmer, M. J., V. S. Mahajan, L. C. Trajman, D. J. Irvine, D. A. Lauffenburger, and J. Chen.** 2008. Interleukin-7 receptor signaling network: an integrated systems perspective. Cell Mol Immunol **5:**79-89.

49. **Pargmann, D., R. Yucel, C. Kosan, I. Saba, L. Klein-Hitpass, S. Schimmer, F. Heyd, U. Dittmer, and T. Moroy.** 2007. Differential impact of the transcriptional repressor Gfi1 on mature CD4+ and CD8+ T lymphocyte function. Eur J Immunol **37:**3551-63.

50. **Park, J. H., Q. Yu, B. Erman, J. S. Appelbaum, D. Montoya-Durango, H. L. Grimes, and A. Singer.** 2004. Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival. Immunity **21:**289-302.

51. **Peschon, J. J., P. J. Morrissey, K. H. Grabstein, F. J. Ramsdell, E. Maraskovsky, B. C. Gliniak, L. S. Park, S. F. Ziegler, D. E. Williams, C. B. Ware, J. D. Meyer, and B. L. Davison.** 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. J Exp Med **180:**1955-60.

52. **Pleiman, C. M., S. D. Gimpel, L. S. Park, H. Harada, T. Taniguchi, and S. F. Ziegler.** 1991. Organization of the murine and human interleukin-7 receptor genes:

two mRNAs generated by differential splicing and presence of a type I-interferon-inducible promoter. Mol Cell Biol **11:**3052-9.

53. **Puel, A., S. F. Ziegler, R. H. Buckley, and W. J. Leonard.** 1998. Defective IL7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. Nat Genet **20:**394-7.

54. **Roifman, C. M., J. Zhang, D. Chitayat, and N. Sharfe.** 2000. A partial deficiency of interleukin-7R alpha is sufficient to abrogate T-cell development and cause severe combined immunodeficiency. Blood **96:**2803-7.

55. **Sadlack, B., H. Merz, H. Schorle, A. Schimpl, A. C. Feller, and I. Horak.** 1993. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell **75:**253-61.

56. **Sakaguchi, S.** 2000. Regulatory T cells: key controllers of immunologic self-tolerance. Cell **101:**455-8.

57. **Schlissel, M. S., S. D. Durum, and K. Muegge.** 2000. The interleukin 7 receptor is required for T cell receptor gamma locus accessibility to the V(D)J recombinase. J Exp Med **191:**1045-50.

58. **Schluns, K. S., W. C. Kieper, S. C. Jameson, and L. Lefrancois.** 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat Immunol **1:**426-32.

59. **Schung, J.** 2003, posting date. Using TESS to Predict Transcription Factor Binding Sites in DNA Sequence. J. Wiley and Sons. [Online.]

60. **Shankar, P., M. Russo, B. Harnisch, M. Patterson, P. Skolnik, and J. Lieberman.** 2000. Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection. Blood **96:**3094-101.

61. **Singh, H., K. L. Medina, and J. M. Pongubala.** 2005. Contingent gene regulatory networks and B cell fate specification. Proc Natl Acad Sci U S A **102:**4949-53.

62. **Smyth, C. M., S. L. Ginn, C. T. Deakin, G. J. Logan, and I. E. Alexander.** 2007. Limiting {gamma}c expression differentially affects signaling via the interleukin (IL)-7 and IL-15 receptors. Blood **110:**91-8.

63. **Smyth, M. J., Y. Norihisa, J. R. Gerard, H. A. Young, and J. R. Ortaldo.** 1991. IL-7 regulation of cytotoxic lymphocytes: pore-forming protein gene expression,

interferon-gamma production, and cytotoxicity of human peripheral blood lymphocytes subsets. Cell Immunol **138:**390-403.

64. **Soares, M. V., N. J. Borthwick, M. K. Maini, G. Janossy, M. Salmon, and A. N. Akbar.** 1998. IL-7-dependent extrathymic expansion of CD45RA+ T cells enables preservation of a naive repertoire. J Immunol **161:**5909-17.

65. **Sobell, H. M.** 1985. Actinomycin and DNA transcription. Proc Natl Acad Sci U S A **82:**5328-31.

66. **Spiegel, H. M., G. S. Ogg, E. DeFalcon, M. E. Sheehy, S. Monard, P. A. Haslett, G. Gillespie, S. M. Donahoe, H. Pollack, W. Borkowsky, A. J. McMichael, and D. F. Nixon.** 2000. Human immunodeficiency virus type 1- and cytomegalovirus-specific cytotoxic T lymphocytes can persist at high frequency for prolonged periods in the absence of circulating peripheral CD4(+) T cells. J Virol **74:**1018-22.

67. **Su, A. I., T. Wiltshire, S. Batalov, H. Lapp, K. A. Ching, D. Block, J. Zhang, R. Soden, M. Hayakawa, G. Kreiman, M. P. Cooke, J. R. Walker, and J. B. Hogenesch.** 2004. A gene atlas of the mouse and human protein-encoding transcriptomes. Proc Natl Acad Sci U S A **101:**6062-7.

68. **Swainson, L., E. Verhoeyen, F. L. Cosset, and N. Taylor.** 2006. IL-7R alpha gene expression is inversely correlated with cell cycle progression in IL-7-stimulated T lymphocytes. J Immunol **176:**6702-8.

69. **Tan, J. T., E. Dudl, E. LeRoy, R. Murray, J. Sprent, K. I. Weinberg, and C. D. Surh.** 2001. IL-7 is critical for homeostatic proliferation and survival of naive T cells. Proc Natl Acad Sci U S A **98:**8732-7.

70. **Tian, B., D. E. Nowak, M. Jamaluddin, S. Wang, and A. R. Brasier.** 2005. Identification of direct genomic targets downstream of the nuclear factor-kappaB transcription factor mediating tumor necrosis factor signaling. J Biol Chem **280:**17435-48.

71. **UniprotKB.** 2002 - 2008. Reviewed, UniProtKB/Swiss-Prot P16871 (IL7RA_HUMAN). Protein Knowledgebase, UniProt Consortium I.

72. **Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman.** 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol **3:**RESEARCH0034.

73. **von Freeden-Jeffry, U., P. Vieira, L. A. Lucian, T. McNeil, S. E. Burdach, and R. Murray.** 1995. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J Exp Med **181:**1519-26.

74. **Vranjkovic, A., A. M. Crawley, K. Gee, A. Kumar, and J. B. Angel.** 2007. IL-7 decreases IL-7 receptor alpha (CD127) expression and induces the shedding of CD127 by human CD8+ T cells. Int Immunol **19:**1329-39.

75. **Wang, J., and F. Xing.** 2008. Human TSLP-educated DCs. Cell Mol Immunol **5:**99-106.

76. **Welch, P. A., A. E. Namen, R. G. Goodwin, R. Armitage, and M. D. Cooper.** 1989. Human IL-7: a novel T cell growth factor. J Immunol **143:**3562-7.

77. **Willerford, D. M., J. Chen, J. A. Ferry, L. Davidson, A. Ma, and F. W. Alt.** 1995. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. Immunity **3:**521-30.

78. **Xue, H. H., J. Bollenbacher, V. Rovella, R. Tripuraneni, Y. B. Du, C. Y. Liu, A. Williams, J. P. McCoy, and W. J. Leonard.** 2004. GA binding protein regulates interleukin 7 receptor alpha-chain gene expression in T cells. Nat Immunol **5:**1036-44.

79. **Xue, H. H., P. E. Kovanen, C. A. Pise-Masison, M. Berg, M. F. Radovich, J. N. Brady, and W. J. Leonard.** 2002. IL-2 negatively regulates IL-7 receptor alpha chain expression in activated T lymphocytes. Proc Natl Acad Sci U S A **99:**13759-64.

80. **Yang, O. O., H. Lin, M. Dagarag, H. L. Ng, R. B. Effros, and C. H. Uittenbogaart.** 2005. Decreased perforin and granzyme B expression in senescent HIV-1-specific cytotoxic T lymphocytes. Virology **332:**16-9.

81. **Young, C. D., and J. B. Angel.** 2006. Optimization of culture and storage conditions for an in vitro system to evaluate thymocyte phenotype and function. J Immunol Methods **312:**157-66.

82. **Zhang, S., V. A. Lawless, and M. H. Kaplan.** 2000. Cytokine-stimulated T lymphocyte proliferation is regulated by p27Kip1. J Immunol **165:**6270-7.

CONTRIBUTIONS OF COLLABORATORS

I would like to acknowledge Elliott Faller, Charlene Young and Andrea Patey for all the help with flow cytometry and Chathura Prematunga for all the help in optimization of transfection protocols. I would also like to recognize Dr. Alan Mears for help with the optimization of real-time PCR experiments and Dr. Angela Crawley for help in statistical analysis. I would also like to sincerely thank Scott Sugden for his industrious contributions for the cloning, optimization of the nucleofection protocol, luciferase assays and western blotting experiments.

I would also like to acknowledge the Wallace lab and in particular Shawn Beug and Dana Wall for the reporter plasmids and advice on cloning and luciferase assays. Additionally, I would like to thank the Parks, Bulman, Kothary, Gray, Griffith and especially the Angel, Kumar and Picketts labs for use of instruments, protocols and reagents.

The idea for this project was conceived in large part by Dr Paul MacPherson and his advice in conjunction with that of my committee members (Drs. Angel and Kumar), my lab mates and my peers has contributed greatly to keeping this project focused and help it come as far as it has.

The characterization of the CD127 gene expression in humans would not be possible were it not for all of our blood donors and funding agencies (OHTN, CFI, IOF and CIHR) that believed in this and other projects ongoing in our lab.

CURRICULUM VITAE

Juzer Kakal

Tel: (
Cell:
Fax:
e-mail:

Education

University of Ottawa, Ottawa, ON
M.Sc. in Microbiology and Immunology *05/2006 – 12/2008*
Major Research Project – HIV Immunobiology: Characterization of the mechanism of regulation of the interleukin-7 receptor alpha chain (CD127) in primary human cytotoxic T-cells under the supervision of Dr. Paul MacPherson at the Ottawa Health Research Institute – Department of Chronic Diseases.

University of Ottawa, Ottawa, ON
B.Sc. (Hons.) in Biochemistry *09/2000 - 04/2004*
Major Research Project – Organic Synthesis: Carried out synthesis of bioactive, D-Glucose derived macrocycles as part of honours research project under the supervision of Dr. Prabhat Arya at the National Research Council of Canada - Steacie Institute for Molecular Sciences.

Work Experience

Ottawa Health Research Institute GMP Facility, Ottawa, ON
Standard Operating Procedure Writer *01/2008 - Present*
Consulting part-time on writing, revision and implementation of standard operating procedures (SOPs) for equipment, materials and manufacturing control programs for the Ottawa Health Research Institute's Good Manufacturing Practices facility.

Ottawa Health Research Institute, Ottawa, ON
Research Technician *09/2004 - 04/2006*
Conducted independent research in areas of molecular biology and immunology, presented data at various conferences and seminars and carried out other related duties.

University of Ottawa – Department of Chemistry, Ottawa, ON
Technician *09/2002 - 04/2004*
Provided support in the set-up and management of undergraduate chemistry laboratories. Also carried out various duties like inventory, restocking, sales of safety equipment and proper disposal of hazardous chemical waste.

University of Ottawa – CORE DNA Sequencing and Synthesis Facility, Ottawa, ON
Technician *05/2003 - 08/2003*
Managed custom DNA oligonucleotide synthesis, quality control and purification operations and managed the Invitrogen and GE Healthcare BioBars.

University of Ottawa – Faculty of Science, Ottawa, ON
Inventory Technician *05/2002 - 08/2002*
Performed chemical and instrument inventory of all labs in the faculty of science.

Other Experience

Demonstrator *09/2008 - Present*
Performing chemistry and biology demonstrations for elementary and high school students for the "Let's Talk Science" and "Science Travels" programs organized by the University of Ottawa.

Judge *04/2007, 04/2008*
Provided evaluation, scoring and comments for the Ottawa Regional Science Fair.

Mentor *09/2005- 05/2007*
Provided supervision and training in experimental techniques and scientific method, hypothesis generation, data analysis and presentation to honours project and summer students. Also provided feedback, evaluation and progress reports.

Teaching Assistant – to Professor William Ogilvie for CHM1320 *01/2004 - 04/2004*
Supervised students, graded lab reports and evaluated in lab performance of students.

Tutor *01/2002 – 04/2004*
Provided one-on-one tutoring for Organic Chemistry, Biochemistry and Molecular Biology courses.

Awards

University of Ottawa – Excellence Scholarship	*09/2006 - 09/2008*
Ontario HIV Treatment Network - Masters Studentship	*09/2006 - 09/2008*
Keystone Symposia – HIV Immunopathogenesis – Conference Scholarship	*03/2008*
University of Ottawa Faculty of Graduate & Postdoc Studies – Travel Scholarship	*03/2008*
University of Ottawa Dept. of Microbiology and Immunology – Travel Scholarship	*03/2008*
Graduate Student Association of University of Ottawa – Academic Projects Fund	*03/2008*
XVI International AIDS Conference – Delegate Scholarship	*08/2006*
Ontario HIV Treatment Network - Conference Scholarship	*11/06, 11/07, 11/2008*
Ottawa Health Research Inst. Research Day – Best Poster Award	*11/2006, 11/2007*
University of Ottawa – Dept of Microbiology and Immunology – Best Poster Award	*05/2006*
University of Ottawa, Facult of Science - Dean's Honours List	*09/2003 - 04/2004*

Publications

1. "Transcriptional Regulation of the CD127 gene in Primary Human CD8 T-cells" *12/2008*
   Juzer A. Kakal and Paul A. MacPherson
   MSc. Thesis

2. "Interleukin-7 and the HIV Tat protein act synergistically to down regulate CD127 expression on CD8 T-cells" *12/2008*
   Elliott M. Faller, **Juzer A. Kakal**, Ritesh Kumar and Paul A. MacPherson
   International Immunology 2009; doi: 10.1093/intimm/dxn140

3. "Interleukin-7 Receptor Expression on CD8 T-Cells is Down Regulated by the HIV Tat Protein" *11/2006*
   Elliott M. Faller, Mark J. McVey, **Juzer A. Kakal** and Paul A. MacPherson
   Journal of Acquired Immunodeficiency Syndrome, 2006 Nov 1; 43(3): 257-69.

4. "Towards the Synthesis of D-Glucose Derived Macrocycles by the Ring-Closing Metathesis *04/2004*
   Juzer A. Kakal and Prabhat Arya
   BSc. Thesis

Abstracts

1. *"Transcriptional Regulation of the CD127 Gene in CD8 T-cells"*
   Juzer Kakal, Scott Sugden, Elliott Faller and Paul MacPherson
   Ontario HIV Treatment Network Annual Conference - Toronto *11/2008*
   Ontario Health Research Institute Research Day - Ottawa *11/2008*

2. *"Regulation of CD127 Gene Expression in CD8 T-cells"*
   Juzer Kakal, Scott Sugden, Elliott Faller and Paul MacPherson
   Ontario HIV Treatment Network Annual Conference - Toronto *11/2007*
   University of Ottawa Department of Microbiology and Immunology - Ottawa *02/2008*
   Keystone Symposia: HIV Pathogenesis - Banff *03/2008*
   Canadian Association of HIV Research - Montreal *05/2008*

3. *"HIV Impairs CD8 T-cell function by removing the IL-7 Receptor alpha-chain from the cell surface and targeting it for degradation"*
   Elliott Faller, **Juzer Kakal,** Mark McVey, Scott Sugden and Paul MacPherson
   Keystone Symposia: HIV Pathogenesis - Banff *03/2008*
   Canadian Association of HIV Research - Montreal *05/2008*

4. *"Regulation of the Human CD127 Gene Promoter by Interleukin-7"*
   Scott Sugden, **Juzer Kakal,** Elliott Faller and Paul MacPherson
   Association of Medical Microbiology and Infectious Diseases of Canada Annual Conference - Halifax *03/2006*

5. *"Transcription of the CD127 gene is down regulated by IL-7 in CD8 T-cells"*
   Juzer Kakal, Elliott Faller, Ritesh Kumar and Paul MacPherson
   Ontario HIV Treatment Network Annual Conference - Toronto *11/2006*
   Ontario Health Research Institute Research Day - Ottawa *11/2006*

6. *"HIV-1 Tat Protein Impairs CD8 T Cell Function By Down Regulating Transcription Of The IL-7r Alpha Chain (CD127)"*
   Elliott Faller, Mark McVey, **Juzer Kakal** and Paul MacPherson
   XVI International AIDS Conference – Toronto *08/2006*
   Canadian Association of HIV Research Conference – Quebec City *05/2006*

7. *"Transcriptional Regulation of the Human IL-7 Receptor Alpha-Chain Gene in CD8 T-Cells."*
   J. Kakal, E. Faller, C. Prematunga, and P. MacPherson
   Canadian Association of HIV Research Conference – Quebec City *05/2006*

8. *"HIV-1 Tat Protein Induces a Decrease in Both Surface and Intracellular CD127 Protein Expression in CD8 T-Cells with a Concomitant Decline in CD127 mRNA Transcripts. Implications for CD8 T-cell Function in HIV Infection."*
   Elliott Faller, **Juzer Kakal** and Paul MacPherson
   International Society for Analytical Cytology Conference – Quebec City *05/2006*

9. *"Analysis of the Putative CD127 Promoter"*
   Chathura B. Prematunga, **Juzer A. Kakal,** Elliott M. Faller and Paul A. MacPherson
   University of Ottawa Undergraduate Research Symposium – Ottawa *03/2006*

10. *"HIV-1 Tat Protein Induces Downregulation Of CD127 Transcripts In CD8 T-Cells"*
    J Kakal, E Faller and P MacPherson
    Ontario HIV Treatment Network Annual Conference - Toronto *11/2005*

11. *"HIV Tat Protein Impairs CD8 T-Cell Function By Down Regulating Transcription Of The IL-7R α Chain (CD127)"*
E Faller, **J Kakal**, M McVey and P MacPherson
Ontario Health Research Institute Research Day - Ottawa *11/2005*

12. *"Towards the synthesis of medium size ring derivatives from carbohydrates."*
Juzer Kakal and Prabhat Arya
University of Ottawa Undergraduate Research Symposium – Ottawa *03/2004*