THERAPEUTIC IMPLICATIONS OF THE 4-1BB COSTIMULATORY PATHWAY ON CD8 T CELLS DURING CHRONIC HIV INFECTION

By

Chao Wang

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Immunology
University of Toronto

© Copyright by Chao Wang, 2011
Therapeutic implications of the 4-1BB costimulatory pathway on CD8 T cells during chronic HIV infection

Chao Wang

Degree of Doctor of Philosophy

Department of Immunology
University of Toronto

2011

ABSTRACT

A hallmark of chronic human immunodeficiency virus (HIV) infection is the impairment of CD8 T cell survival and effector functions, which likely contributes to HIV pathogenesis. A number of factors could be attributed to this impairment, including the declining number of CD4 T cells, progressive destruction of secondary lymphoid tissues and an increasingly inhibitory environment. As highly active antiretroviral therapy shows limited efficacy in improving CD8 T cell functions, this thesis explores the therapeutic application of costimulatory molecules in directly stimulating non-functional HIV-specific CD8 T cells and ultimately their relevance to the control of chronic HIV infection. Costimulatory molecules are adjuvants for functional activation of T cells that act in concert with the antigen-specific signal. The Tumor Necrosis Factor (TNF) family member, 4-1BBL, emerges as the most effective costimulatory molecule in the antigenspecific expansion of human memory CD8 T cells as compared to the related TNF family members CD70 and LIGHT. As well, 4-1BBL improves the cytolytic function of T lymphocytes on a per cell basis. Furthermore, 4-1BBL is identified as a key component
in the therapeutic rescue of CD8 T cell function and its effect is at least partially dependent on its signaling adaptor TNF receptor associated factor 1 (TRAF1), both *in vitro* and *in vivo*. This thesis also identifies the loss of TRAF1 as a new mechanism of immune dysregulation of HIV-specific CD8 T cells during the chronic phase of HIV infection and offers a means to correct it. The loss of TRAF1 has functional relevance in HIV suppression and HIV-specific CD8 T cell responses. Finally, a combination therapy involving agonistic anti-4-1BB antibody is shown to be successful in a proof of concept treatment of chronic lymphocytic choriomeningitis virus (LCMV) infection in mice, resulting in sustained reduction in viral load. A new model of HIV-specific CD8 T cell dysfunction is constructed based on these findings.
ACKNOWLEDGEMENTS

衷心感谢爸爸,妈妈,哥哥,陈功和麦康乃尔先生及夫人多年以来的支持

Special Thanks to my parents, brother, Drs. Fleming and Aileen McConnell, Gung Chen and many friends who have supported me throughout my Ph.D. training.

I thank Dr. Carl Ware for providing recombinant HVEM-Fc and LTβR-Fc, Dr. Mario Ostrowski for provision of BL7 target cells, Birinder Ghumman for purification and plaque assays of the recombinant adenoviruses, Benoit Bessette (University of Montreal) for production of the influenza-M1/MHC I tetramer. I thank Cytheris S.A. for providing recombinant human IL-7 and advice in its use; Dr. Robert A. Mittler for provision of the 3H3, anti-4-1BB producing, hybridoma; Gabor Gyenes for HIV donor information mining and Dionne White for help with flow cytometry. I thank Dr. Tania Watts, Dr. Mario Ostrowski, Dr. Yuanqing Liu, Dr. Pamela Ohashi and Dr. Jennifer Gommerman for critical discussions. Last, but not the least, I thank Dr. Darina Frieder and Ann J. McPherson for proof reading.
CHAPTER I
INTRODUCTION

Background 2

The role of CD8 T cells during chronic HIV infection 3

HIV, a virus that’s difficult to eradicate 5

Mutation and viral load 5

Viral latency and the hunt for latent viral reservoir 7

Tropism 8

Factors contributing to disease progression - lessons from nature’s experiment 10

The Natural Host of SIV 10

HIV controllers 10

1. Virological factors 11

2. Host factors 12
HIV progressors

1. Viral Persistence
2. Immune hyper-activation
3. Immune dysfunction

The inhibitory environment for HIV-specific CD8 T cells during chronic infection

LCMV

Expression of inhibitory molecules

Increased level of Inhibitory cytokines during chronic viral infection

The costimulatory pathways

B7/CD28 pathway

1. Expression
2. Functions

4-1BB/4-1BBL pathway

1. Expression
2. Signaling
3. Function during chronic viral infection

Conclusion

CHAPTER II: THE SCREENING

“Qualitative difference of the LIGHT/CD70/4-1BBL gene cluster in direct costimulation of human memory CD8 T cell responses”
Abstract 33

Introduction 33

Results 35

Expression of 4-1BBL, CD70, LIGHT and their respective receptors 35

4-1BBL and CD70, but not LIGHT, are strong stimulators of influenza-specific CD8 T cells, with CD70 being less potent 36

Differential effect of 4-1BBL and CD70 on driving effector functions 41

CD70 preferentially costimulates T cells from donors with a more differentiated influenza-specific memory CD8 T cell pool 43

4-1BBL rescues the response of ineffective donors to CD70 43

Soluble LIGHT may contribute to the ineffectiveness of LIGHT 45

Generation of human ICOSL adenovirus and its functional analysis 48

Discussion 50

Materials and Methods 54

Recombinant adenoviruses 54

Donors and consent 54

Costimulation assays 54

Flow cytometry 55

CTL assays 56

ELISA 56

Statistical analysis 56

CHAPTER III: THE IN VITRO RESCUE

“4-1BBL induces TRAF1-dependent Bim modulation in human T cells and is
a critical component in the costimulation-dependent rescue of
functionally impaired HIV-specific CD8 T cells”

<table>
<thead>
<tr>
<th>Abstract</th>
<th>58</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>58</td>
</tr>
<tr>
<td>Results</td>
<td>60</td>
</tr>
</tbody>
</table>

*Functional status of HIV-specific CD8 T cells* 60

*Effect of 4-1BBL and CD80 costimulation on expansion of HIV-specific CD8 T cells* 60

*Following costimulation, expanded HIV-specific CD8 T cells express multiple markers of effector function upon restimulation* 63

*Activation of HIV-specific CD8 T cells by 4-1BBL and CD70* 65

*Analysis of PD-1 expression in the presence and absence of costimulation* 67

*4-1BBL induces Bim down-regulation in activated virus-specific CD8 T cells* 67

*4-1BBL-induced Bim modulation is TRAF1 dependent* 70

*Analysis of division and survival of costimulated HIV-specific CD8 T cells* 70

<table>
<thead>
<tr>
<th>Discussion</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials and Methods</td>
<td>77</td>
</tr>
</tbody>
</table>

*Donors and consent* 77

*Tetramers and peptides* 78

*Costimulation assays* 78

*CTL functional analysis by ⁵¹Cr-release assay* 79

*Flow cytometry* 80

*RNA interference to decrease TRAF1 levels in T cells* 80
Chapter IV: THE IN VIVO THERAPY

“Loss of TRAF1 during chronic viral infection desensitizes the 4-1BB costimulatory pathway: implications for therapy”
Chapter V

MODELS AND FUTURE DIRECTIONS

Survival of HIV-specific CD8 T cells: a key to targeting the viral reservoir? 114

Hypothesis part I: the selective deletion of functional cells 114

Hypothesis part II: mechanism of deletion 116

Effector functions of HIV-specific CD8 T cells, a matter of translation? 121

The relevance of PI3K pathway 123

Therapeutic implications of the 4-1BB costimulatory pathway 125

When and how to engage the 4-1BB pathway? 129

Other points of interest 131

The requirement of costimulation during the chronic phase of HIV infection 131

The therapeutic implication of TRAF1 131

Chapter VI

REFERENCES 134
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1</td>
<td>Unfractionated PBMC</td>
<td>61</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>HIV sample information</td>
<td>85</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

CHAPTER I

Figure 1.1  Schematics of HIV genomic organization  

Figure 1.2  Signal transduction by 4-1BB in T cells  

CHAPTER II

Figure 2.1  Ectopic expression of 4-1BBL, CD70 and LIGHT on monocytes  

Figure 2.2  Expression of costimulatory receptors on Ag-specific CD8 T cells  

Figure 2.3  Schematics of costimulation assay  

Figure 2.4  Differential expansion of influenza-specific CD8 T cells in response to 4-1BBL, CD70 and LIGHT  

Figure 2.5  Effector functions of influenza-specific CD8 T cells stimulated with 4-1BBL, CD70 or LIGHT  

Figure 2.6  CD70 preferentially stimulates CD8 T cells from donors with more differentiated influenza-specific effector/memory pool  

Figure 2.7  4-1BBL rescues CD70 non-responders  

Figure 2.8  Exploring the ineffectiveness of LIGHT  

Figure 2.9  Generation of human ICOSL-adenovirus and functional analysis  

CHAPTER III

Figure 3.1  HIV-specific CD8 T cells show a limited repertoire of effector functions regardless of PD-1 expression  

xii
Figure 3.2  Expansion and functional rescue of HIV-specific CTL by costimulation

Figure 3.3  Differential effects of costimulatory combinations in rescuing functionally impaired HIV-specific CTL

Figure 3.4  Kinetics of PD-L1 and PD-1 expression

Figure 3.5  Role of 4-1BBL and CD70 in cell division and survival of influenza specific CD8 T cells

Figure 3.6  Decreasing TRAF1 by siRNA results in increased levels of Bim during 4-1BBL mediated costimulation

Figure 3.7  4-1BBL-mediated costimulation results in lower Bim expression in HIV-specific CTL

CHAPTER IV

Figure 4.1  Flow cytometry gating strategy and representative FACS staining

Figure 4.2  Loss of TRAF1 protein expression during chronic HIV infection

Figure 4.3  Activation, TRAF1 expression and HIV viral load

Figure 4.4  Requirement for TRAF1 in HIV-specific CD8 T cell responses

Figure 4.5  4-1BB expression on LCMV-specific CD8 T cells

Figure 4.6  TRAF1 expression in LCMV-specific CD8 T cells during acute and chronic infection

Figure 4.7  TRAF1 levels do not differ in APC in Armstrong versus clone 13 infected mice

Figure 4.8  Cytokine regulation of TRAF1 levels in T cells

Figure 4.9  The effect of TGFβ on TRAF1 expression at the protein and mRNA level

Figure 4.10  Combined treatment with IL-7 and agonistic anti-4-1BB therapy rescues exhausted CD8 T cells and decreases viral load, in a TRAF1 dependent manner
Figure 4.11  *LCMV* clone 13 infection induced similar responses in wildtype and *TRAF1/-/-* mice

CHAPTER V

Figure 5.1  *Survival impairment of HIV-specific CD8 T cells* 120

Figure 5.2  The hypothetical *role of PI3K pathway in the impairment of effector functions in HIV-specific CD8 T cells* 126

Figure 5.3  *SNP annotation of the human TRAF1 at the protein level* 133
LIST OF PUBLICATIONS


ABBREVIATIONS

AIDS – Acquired Immune Deficiency Syndrome
Ag - Antigen
ARV - AIDS associated viruses
APC – Antigen Presenting Cell
CTL – Cytotoxic T Lymphocyte
DC – Dendritic Cell
ELISA – Enzyme-Linked Immunosorbent Assay
ERK – extracellular signal regulated kinase
FACS – Fluorescence Activated Cell Sorting
FDC – Follicular Dendritic Cells
FRC – Fibroblastic Reticular Cells
GALT – Gut Associated Lymphoid Tissue
GM-CSF – Granulocyte Macrophage Colony Stimulating Factor
HAART – Highly Active Antiretroviral Therapy
HIV – Human Immunodeficiency Virus
HTLV - Human T Lymphotropic retroviruses
IFNγ - Interferon γ
JNK – c-Jun N-terminal kinase
LAV - Lymphadenopathy Associated Virus
LCMV – Lymphocytic Choriomeningitis Virus
LPS - Lypopolysaccharides
LTR – Long Terminal Repeat
MAP kinase – Mitogen-Associated Protein kinase

MHC – Major Histocompatibility Complex

mTOR – mammalian Target Of Rapamycin

NFκB – Nuclear Factor κB

NK cells – Natural Killer cells

PBMC – Peripheral Blood Mononuclear Cells

PCR – Polymerase Chain Reaction

PI3K – Phosphoinositide 3-Kinase

PTEN – Phosphatase and tensin homologue deleted on chromosome 10

SIV – Simian Immunodeficiency Virus

SIVagm – Simian Immunodeficiency Virus for African Green Monkey

SIVcpz – Simian Immunodeficiency Virus for chimpanzee

SIVsm – Simian Immunodeficiency Virus for Sooty Mangabey

TCR – T cell Receptor

TGFβ – Transforming Growth Factor β

TNF – Tumor Necrosis Factor

TRAF – TNF Receptor Associated Factor
CHAPTER I

INTRODUCTION
Background

Human Immunodeficiency Virus (HIV), previously known as LAV (Lymphadenopathy Associated Virus), HTLV (Human T Lymphotropic retroviruses)-III and ARV (AIDS associated viruses), was first isolated from patients in 1983 and later confirmed to be the etiological agent of Acquired Immunodeficiency Syndrome (AIDS) (1-4).

New incidence of HIV infection dropped 17% in 2008 as compared to 8 years ago, perhaps reflective of joint scientific, social and political effort (the AIDS epidemic update 2009). The AIDS epidemic continues, however, as the number of individuals living with HIV continues to grow with no effective cure (33.4 million as of December, 2008). In 2008 alone, 2.7 million adults and children became newly infected and 2 million died of AIDS. In most cases of HIV infection, patients are not diagnosed until sero-conversion, by which time the chronicity of HIV has been established. While early infection can arguably be treated with more success, chronic HIV infection presents unique challenges to therapeutic interventions.

There are a few dozen antiretroviral drugs currently available for treating HIV infection. All antiretroviral medications directly target HIV life cycle and transmission, including reverse transcriptase inhibitors, protease inhibitors, integrase inhibitors and fusion/entry inhibitors. Since the introduction of highly active antiretroviral therapy (HAART), a combination of at least three antiretroviral drugs, the quality of life and longevity of people living with HIV have greatly improved. However, the toxicity of HAART is high and not all patients can tolerate it. For those that have been treated, withdrawal of HAART results in viral rebound, suggesting that HIV is not eradicated and continuous treatment is required. In light of this challenge, studies investigating other aspects of HIV pathogenesis are emerging, particularly those that cannot be corrected by HAART at a population level, including but not limited to the extent of HIV viral reservoir, low CD4 counts and immune dysregulation in both blood and mucosa (5-7).

Immune dysfunctions in cellular immunity, among other viral and host factors, likely contribute to the difficulties in eradicating HIV during chronic infection. The
mechanisms of CD8 dysfunction over time have begun to be understood, including factors such as the disruption of secondary lymphoid tissue, the lack of CD4 help and increasing T cell inhibition, which to a certain extent may be antagonized by costimulation. Thus, directly stimulating antigen-specific CD8 T cells could be beneficial to chronically infected HIV\(^+\) individuals.

This thesis explores the roles of several costimulatory molecules in improving dysfunctional CD8 T cell responses during chronic HIV infection and discusses their therapeutic relevance. Costimulatory molecules are a group of molecules that function in concert with antigen specific signals to modulate T cell activation. I prescreened several relevant costimulatory molecules for their ability to directly stimulate influenza-specific CD8 T cells independently of CD4 lymphocytes in Chapter II, then analyzed selected costimulatory molecules in rescuing dysfunctional HIV-specific CD8 T cells \textit{in vitro} in Chapter III. One particular molecule, 4-1BB, and its costimulatory pathway, has emerged as a potent and unique player. I provided evidence in Chapter IV that the 4-1BB costimulatory pathway is desensitized via the loss of TRAF1 during chronic HIV infection. I explored means to correct this newly identified immune dysfunction \textit{in vivo} and suggested a novel therapy.

\textbf{The role of CD8 T cells during chronic HIV infection}

CD8 T cells are potent effectors of the adaptive immune response and function either to directly kill virus-infected cells or to produce cytokines that can affect surrounding cells (8). The importance of CD8 T cells during early HIV control has been well recognized (9-13). The most direct evidence comes from CD8 depletion studies carried out in SIV-infected rhesus monkeys which showed diminished control of SIV replication and faster disease progression (14, 15). Furthermore, a T-cell based vaccine that induced a stronger and broader CD8 T cell response was able to reduce the SIV viral set point by 2.4 log and reduced AIDS-related mortality in rhesus monkeys (16). Interestingly, a recent study argued the initial SIV control by CD8 T cells is through the non-cytopathic effect of CTL rather than direct killing (17). Viral clearance, however, has been shown to require MHC-
class I in other persistent viral infection models (18). Regardless of the mechanism of control, CD8 T cells and perhaps other components of the immune system (notably NK cells (19, 20)) are responsible for the initial viral control. During chronic HIV infection, however, the protective effects of CD8 T cells are limited and most infected and untreated individuals have $10^3 - 10^6$ HIV RNA copies/ml in plasma which gradually increases with disease progression.

Many different mechanisms have been proposed to explain the inability of CD8 T cells to control HIV during the chronic phase, including intrinsic CD8 factors and the extrinsic microenvironment. Intrinsic CD8 factors could include the available quantity and quality of T cells specific to HIV antigen. As a large quantity of HIV-specific CD8 T cells (up to 18% total CD8 T cells) were found in HIV progressors (21), qualitative differences could potentially be important. Indeed, HIV-specific CD8 T cells during chronic infection were found to have a reduced ability to produce TNFα (22), IFNγ (23, 24), decreased cytolytic functions as measured by perforin (22, 25), and lower proliferative capacity (26). Furthermore, HIV-specific memory CD8 T cells from patients with poor viral control were characterized by having single effector functions, in contrast to the poly-functional cells in both blood and mucosa (6) of HIV controllers (27-29) or other memory cells generated during acute infections (30). In addition to effector functions, the migration of HIV-specific CD8 T cells was shown to be impaired at high viral loads (31), posing another challenge to viral control.

The defect in CD8 T cell function during chronic HIV infection must be considered together with the unique microenvironment in which the T cells are immersed, much of which is determined during the early phase of infection. In this regard, two questions are particularly relevant: 1. Does the microenvironment contribute to CD8 T cell dysfunction, and as such would any CD8 T cell focused therapy be sustainable in vivo? 2. Does correcting CD8 T cell dysfunction in turn improve the microenvironment, and thus other factors, that are important for HIV disease progression? I discuss some of the characteristics of this microenvironment in the next three sections. It is likely that direct stimulation of CD8 T cells with costimulatory molecules may overcome some but not all
of these obstacles. As such, control of HIV disease progression to AIDS will require a multifaceted approach in addition to the work presented in this thesis.

**HIV, a virus that’s difficult to eradicate**

HIV is a lentivirus belonging to a larger family of retroviruses. Its genome encodes Gag (consisting four proteins: matrix, capsid, nucleocapsid and p6), Env (consisting gp120 and gp41), Pol (consisting protease, reverse transcriptase and integrase) and six other accessory proteins (Vif, Vpr, Nef, Tat, Rev and Vpu) (32) in approximately 9kb RNA (see Fig. 1.1). Based on current understanding, at least three viral properties contribute to the challenges facing therapeutic interventions during chronic HIV infection: its rapid mutation rate, its latency and its tropism. HIV can also evade CD8 T cell surveillance by directly downregulating MHC class I via Tat, Nef or Vpu (33).

**Mutation and viral load**

The error prone reverse transcriptase of HIV provides the biochemical basis for frequent emergence of viral mutants that escape host immune surveillance (34). Approximately one base substitution can be introduced per viral genome per generation, thus nearly every virus in a population differs from each other (35). The extent of HIV genomic variation is partly reflected in the current characterization of 3 HIV-1 groups, Major (M), Outlier (O) and nonmajor & nonoutlier (N); as well the M group is divided into many subgroups: Clade A1-4, B, C, D, F1-2, G, H and K (reviewed in (36)). It has been estimated that for an individual with $2 \times 10^5$ HIV RNA copy/ml in their plasma, $10^{10}$ virus can be produced in a day, creating millions of viral mutants (37). What about the chronic phase of HIV infection, where many individuals are asymptomatic and have minimal detectable viral load in the plasma? It has been well recognized that a viral dichotomy exists between blood and lymphoid tissues before the onset of AIDS (38). Strikingly, as much as 25% of CD4 T cells in the germinal center can harbor HIV DNA in untreated or non-ideally treated HIV+ individuals throughout different stages of HIV infection, although not all the viral genomes are necessarily replication competent (39). Thus,
Figure 1.1 Schematics of HIV genomic organization. Each gene that first gives rise to a polypeptide is indicated, with arrows pointing to the individual gene products after processing. LTR = Long Terminal Repeat.
active viral replication and continuous emergence of viral escape mutants are also features of chronic HIV infection.

**Viral latency and the hunt for the latent viral reservoir**

The HIV life cycle can be divided into two phases: 1. host cell entry and genomic integration; 2. activation of the integrated pro-virus and viral replication. As such, two types of viral latency can be differentiated: pre and post integration. It is the latter, stable post-integration latency, that is most relevant to HIV persistence (reviewed in (40)). A recent report attributed the HIV rebound after interruption of HAART to latently infected cells, rather than continued low-level replication (41), suggesting the clinical relevance of the latent HIV reservoir.

The presence of replication-competent HIV in resting CD4 T cells was initially demonstrated in untreated HIV+ individuals (42). During the asymptomatic phase, as few as $10^7$ resting CD4 T cells were estimated to harbor replication competent HIV DNA in a host, a minute fraction of CD4 T cells with detectible HIV RNA (43). However, despite the success of HAART in reducing HIV viral load and clearing viruses from secondary lymphoid tissue (44), latently infected CD4 T cells carrying replication-competent HIV-1 were still detectible (45-47), and were found as early as 10 days post initial infection (48). IL-2 has been shown to reduce the size of the latent resting CD4 T cell pool in HIV+ individuals receiving HAART (49). However, viral relapse occurred in all patients after cessation of HAART with or without intermittent IL-2 therapy (50), suggesting the existence of other HIV reservoir(s). In support of this view, Chun et al. showed that the genetic makeup of rebound plasma HIV after HAART in the majority of patients was distinct from those found in the latently infected resting CD4 T cell pool (51). As well, during HAART, the predominant plasma clones from residual viremia represented a homogeneous population that remained genetically stable for years and distinct from those integrated into the latent resting CD4 T cells, suggesting the existence of other viral reservoir(s) that were likely not in circulation (52, 53).
In addition to resting CD4 T cells, macrophages are susceptible to HIV infection and can be productively infected (54, 55). HIV has also been found in monocytes (56), tissue mast cells (57), natural killer (NK) cells (58) and microglial cells in the brain (54) directly \textit{ex vivo}. Peripheral blood dendritic cells (DC) infected with HIV have been found \textit{ex vivo}, however the role of DC as an HIV reservoir remains controversial (59). In sum, whether any of these cell types can serve as a latent HIV reservoir contributing to \textit{in vivo} viral rebound remains to be determined. The recent development of an SIV-infected macaque model (60) may be important for systematic investigation of HIV sanctuaries including not only the cell types but also the tissue microcompartment, such as the central nervous system, reproductive tract and the gut associated lymphoid tissue.

\textit{Tropism}
CD4 was identified as the cell-surface receptor for HIV entry in 1986, beginning the journey of understanding host and HIV interactions (61, 62). Ten years later, two more co-receptors were identified. Edward Berger’s team cloned CXCR4 (then termed Fusin) as a co-receptor for the T cell tropic strain (X4) of HIV using plasmid libraries (63). The same group, as well as two additional teams led by John Moore/Richard Koup and Dan Littman respectively, independently identified CCR5 (64-66) as the co-receptor for the macrophage-tropic strain (R5) of HIV. The identification of co-receptor CCR5 was likely inspired by Cocchi F. et al.’s work identifying RANTES, MIP-1α and MIP-1β, all ligands to CCR5, to be the major HIV-suppressive factors produced by CD8 T cells (67). The more virulent X4 viruses appear late during the course of HIV infection (68); whereas the founder virus(es) in infected humans are CCR5 tropic (69-71). The identification of co-receptors as well as individuals with the mutant form of CCR5, naturally resistant to HIV infection, began a wave of therapeutic studies (for a review, see (72)).

The targeting of CD4 T cells, a central component of adaptive immunity, contributes to the complexity of HIV-host interactions and favors viral pathogenesis in a number of ways. Firstly, HIV gains better access to targets whenever an immune response is mounted against viral particles or virus infected cells. Activated CD4 T cells facilitate
viral production by having increased CCR5 expression and suppressed anti-viral APOBEC3G inhibition (73). Indeed, most of the HIV viral load is supported through the infection-production cycle in activated CD4 T cells (37). Thus, with the help of the responding immune system, HIV or HIV infected cells travel from the initial viral focus established from a single virus (70), to the draining lymph node where the virus meets large quantities of activated T cells, and finally the whole body, particularly in the GALT (gut-associated lymphoid tissue) where most activated CD4^+CCR5^+ memory T cells reside (74). This initial viral dissemination largely determines the HIV latent reservoir as well as the viral set point that are characteristics of the chronic phase.

Secondly, HIV infection results in CD4 T cell decline which presumably limits the strength and breadth of the antibody and CD8 T cell responses during primary and perhaps chronic infection as discussed in a separate section. It should be noted that the direct cytopathicity of HIV is probably not a major contributor. Roughly one quarter of CD4 T cell death during the acute infection can be attributed to direct killing (75), whereas the rest are likely killed through apoptosis due to immune activation.

In conclusion, HIV out-runs the host immune system by its mutation speed and choice of sanctuary, making it extremely difficult to eradicate. In fact, no human has been shown to clear HIV once it has been established during the acute phase, whether naturally or therapeutically, with perhaps the exception of one rare case where an HIV^+ patient received a bone marrow transplant from a CCR5 mutant donor as discussed below (76). However, not all hosts of immunodeficiency viruses develop AIDS, providing a framework for analysis of protective host factors. Whether increasing CD8 T cell function could eradicate the virus remains to be determined. There is currently no evidence that CD8 T cells can impact the size of the HIV viral reservoir although such analysis cannot be completed without knowledge of what constitutes the reservoir in the first place. In some HIV controllers, as discussed below, replication defective HIV mutant strains have been isolated, perhaps driven by protective immunity of unknown nature. The studies presented in this thesis focus on directly stimulating CD8 T cells,
thereby bypassing the need for CD4 help, presumably in the form of CD40L (discussed in the last section).

**Factors contributing to disease progression - lessons from nature’s experiment**

Arguably, the biggest challenge for preventing AIDS progression in already infected individuals lies in the lack of complete understanding of HIV pathogenesis and the factors that cause disease progression. Current hypotheses include factors such as high viral load, chronic immune activation and increasing immune dysfunction and pathology. To understand the contributions of each of these viral and host factors, it is important to compare responses between infected hosts that do and do not progress to AIDS.

*The Natural Host of SIV*

HIV-1 and HIV-2, the two types of HIV, were likely introduced into humans during the early 20\textsuperscript{th} century through zoonotic transmission. HIV-2 has evolved from a strain of simian immunodeficiency virus in sooty mangabeys (SIV\textsubscript{SM}); whereas HIV-1, which gave rise to the global AIDS pandemic, originated from SIV\textsubscript{cpz} of a subspecies of the chimpanzee (reviewed and discussed in (77)). While HIV causes AIDS in humans, many SIV infected non-human primates, including SIV\textsubscript{SM} infected sooty mangabeys and SIV\textsubscript{cpz} infected chimpanzees, are natural hosts of the respective viruses and interestingly show no sign of disease progression. It is clear that the SIV that infects the natural host can be highly pathogenic: SIV\textsubscript{SM}, which naturally infects sooty mangabey, can cause progressive infection in rhesus macaques (78); and similarly, SIV\textsubscript{agm} (for African Green Monkey) can cause simian AIDS in pigtail macaques (79). Furthermore, a substantial reduction of CD4 T cells have been reported in SIV infected natural hosts (80, 81). As well, high viremia is common (82, 83). Thus far, only immune activation has been identified as the fundamental difference between SIV infection of its natural hosts and progressive HIV infection in humans (reviewed in (84)), which is further discussed in a later section.

*HIV controllers*
The rate of disease progression in HIV infected individuals varies significantly. HIV controllers were initially termed Long term non-progressors (LTNP) which describes a small fraction of untreated HIV infected individuals that maintain stable CD4 T cell counts and show no sign of disease progression for a long period of time (85, 86). The definition of LTNP is empirical and thus it represents a heterogeneous group of individuals. Since the initial description, some of the LTNP have now gone on to progressive disease. A smaller subgroup, however, are still AIDS free since their infection almost 30 years ago. Recently, the HIV Controller Consortium defined controllers into Elite (or aviremic) and Viremic, with a particular emphasis on viral load which marks their most striking difference from HIV progressors. Elite HIV controllers were defined as individuals who have maintained HIV RNA levels below 50 copies/ml for at least 1 year in the absence of antiretroviral therapy, whereas Viremic HIV controllers are those who maintained RNA level between 50 and 2000 copies/ml. Episodes of viremia are acceptable as long as they represent the minority of all available determinations.

The mechanisms of protection against HIV in controllers have been under intensive investigation. While virologic, genetic and host-immune-response factors have all been associated with protection, no one factor can account for protection in all the HIV controllers. Importantly, some but not all of these findings may shed light on therapeutics for chronically infected HIV progressors.

1. Virologic factors

Early evidence suggests that infection with attenuated HIV mutant may result in slowed progression. Indeed, HIV with unusual polymorphisms have been isolated from HIV controllers (87-92). It is unclear however, whether these mutant strains were responsible for the initial infection or were evolutionary products of protective immunity. In several cases, such as blood transfusion or vertical transmission, it is possible to trace the initial viral strain. Data from those studies suggest that a small fraction of HIV controllers are indeed infected with weakened HIV strains (93-95). However, replication defective HIV mutant likely represents the exception rather than the norm as the cause for slowed
progression in HIV controllers. Many Elite controllers were shown to be infected with pathogenic viruses (90, 96, 97), some of which have caused AIDS in donor individuals (98, 99). These data argue that host immune factors can contribute significantly to long term viral control.

2. Host factors
It is now clear that host genetics, such as polymorphisms in HLA types, NK cell receptors as well as mutations in the HIV co-receptor CCR5, are associated with the rate of HIV disease progression. Interestingly, Hütter et al. showed that an HIV+ recipient remained aviremic in the 20-month follow up after bone marrow transplantation from a donor that is not only compatible, but also homozygous for mutations in the CCR5 gene that results in HIV resistance (76). Not withstanding the contributions of these studies to the field of HIV research, direct applications on therapeutics for HIV progressors are limited, and thus not the focus of this discussion (for a review of genetic factors, see (100)).

Many HIV controllers do not harbor one or more of these favorable genetic factors, nor were they infected with non-pathogenic virus (96), suggesting that other host immune factors must be at play. Indeed, broadly neutralizing antibodies have been isolated in HIV controllers (101-106). Passive antibody infusion therapy with these broadly neutralizing antibodies to date has achieved some, but limited, success in treating patients chronically infected with HIV (107). As well, protective and fully functional cellular immunity has often been described for viral controllers (6, 26-28, 108-110). It is unclear whether functional cellular immunity is the cause of disease control or the product of low viral load during the chronic phase of infection.

In conclusion, studies of natural hosts of SIV and HIV controllers presented two scenarios where AIDS progression is not observed: 1. high viral load and CD4 T cell depletion* (*qualitative differences may exist between the CD4 T cell depletion in natural hosts versus HIV progressors as discussed later) with low level of chronic immune activation; 2. low viral load, normal level of CD4, intermediate level of
activation (see later section) and protective/effective immunity. The contrasting characteristics between natural hosts of SIV and HIV controllers may represent different adaptations to the immunodeficiency viruses. It is also possible that yet unknown factors contribute to protection or progression.

**HIV progressors**
Most individuals infected with HIV progress to AIDS. In contrast to HIV controllers and natural hosts of SIV, HIV progressors are characterized by high HIV load, chronic immune activation and immune dysfunction. Many of these factors are largely determined during the acute phase, contributing to the increased difficulty in treating HIV infection at the chronic stage.

1. **Viral persistence**
The relationship between HIV viral load and disease progression has been studied extensively. Mellors et al. showed that viral load correlates with progression to AIDS and death (111, 112). Furthermore, HAART, which reduces viral replication, has significantly prolonged the life expectancy of infected individuals, suggesting that high viral load can cause disease progression. Interestingly, the level and kinetics of viral replications in both the acute and chronic phase in pathogenic SIV/HIV infections were remarkably similar to those observed in SIV infection of the natural hosts (80, 113-115). This observation argues that high viremia is perhaps necessary but not sufficient to cause AIDS. In support of this view, Rodriguez et al. showed viremia only minimally predicted disease outcome (116). This study, however, used CD4 T cell decline as readout. In a separate report, Mellors et al. showed mean HIV viral load could account for 51% and 58% of the variability in AIDS and death respectively (117), suggesting viremia is a strong but not complete predictor. It should be noted that plasma viremia was measured in all of these studies, which is not equivalent to viral load in the tissues.

What is the impact of viral persistence to CD8 T cell dysfunction? Taking advantage of a bone marrow (BM) chimera where MHC-class I is lacking in non-BM derived cells, and thus virus cannot be cleared from non-hematopoietic cells, Mueller et al. showed that
CD8 T cell dysfunction persisted during LCMV clone 13 infection (18). The authors interpreted the result to mean that persistent antigen can cause CD8 T cell dysfunction. It is also possible however, that CD8 T cell dysfunction requires interaction with non-BM derived cells. In a separate study, Bucks and colleagues took a more direct approach where they repeatedly infected mice with live influenza virus twice a week for four weeks (118). This study showed that in the absence of high viral titer, sustained proinflammatory cytokine production and infection of CD4 T cells, persistent antigen alone was sufficient to drive CD8 T cell dysfunction. Furthermore, such dysfunction was reversible once antigen was withdrawn. In light of this finding, studies of therapeutic reinvigoration of CD8 T cells in the absence of persistent antigen should be undertaken with caution. In this thesis, the role of costimulatory molecules on impaired CD8 T cells was studied both in vitro and in vivo.

2. Immune hyper-activation

Chronic systemic immune activation is associated with pathogenic HIV infection and the idea that it may contribute to HIV disease progression was first put forward by Ascher and Sheppard (119) and later by Grossman et al. (120) from a different angle. What is immune hyper-activation? While a complete quantitative characterization of immune activation is lacking, the phenotype has been frequently described. Hyperactive spontaneous B cell responses were shown ex vivo in the early 80’s (121). As well, increased T cell turnover was reported in vivo in HIV infected individuals (122). Of relevance to CD8 T cells, susceptibility to activation induced apoptosis was found to be high in uninfected T cells (123-127). Moreover, the serum levels of proinflammatory cytokines and chemokines were elevated during HIV infection (for a review, see (128)). Most of these studies did not distinguish between activation due to HIV-specific response versus bystander activation. In fact, the hyperactive response could constitute a ‘normal’ but ineffective immune response against continuous HIV replication (129). To date, direct evidence is lacking as to whether immune activation is the cause or contributes significantly to disease progression.
There is some indirect evidence that immune activation is important. The activation of CD8 T cells, as measured by CD38 and/or HLA-DR expression, has been correlated with AIDS/death in HIV infected individuals in both early and chronic infection (130, 131), and in some cases, is a stronger predictor than viral load (132). Furthermore, as mentioned above, chronic immune activation is not observed in SIV infected natural hosts, and is thus thought to contribute to disease progression in HIV progressors (133, 134). Memory T cells (measured by CD45RA/CD27) from HIV controllers were also shown to have a lower activation level (measured by CD38^{+}CD8^{+}) than those from progressors (135), albeit significantly higher than those from healthy donors (136). The latter is not surprising, as some form of inflammation is necessary for appropriate activation of the adaptive immune response.

Several factors may contribute to the immune hyper-activation in HIV infected individuals. Ott et al. showed that the HIV Tat protein resulted in the superinduction of IL-2 at the transcriptional level in T cells in response to TCR signaling (137). Recent studies revealed microbial translocation as a cause of immune activation during the chronic phase of progressive infection (138-140). In contrast, there is little evidence of circulating microbial products in the SIV infected natural hosts (138). Microbial translocation refers to circulating microbial products, likely from the lumen of the gastrointestinal tract. Indeed, enteropathy was reported many years ago in late stage AIDS patients (141) and altered small intestine integrity was found in symptomatic HIV\(^+\) individuals (142). It is unclear what may cause the gastrointestinal leakage. Profound CD4 T cell depletion in the mucosa is not sufficient to cause microbial translocation (80, 81). A recent study reported selective depletion of CD4 T cells producing IL-17 (Th17 cells) in HIV infected humans (143) and SIV infected rhesus macaques (144), shedding light on potential qualitative differences in CD4 T cell depletion that may contribute to immune activation and disease progression.

Interestingly, the level of serum LPS, an indicator of microbial translocation, in HIV controllers is also significantly higher than in healthy donors (136) and not significantly different from HIV progressors (138). Several interpretations are possible. One
hypothesis could be that while microbial translocation occurs in HIV controllers, they are capable of mounting a protective immune response and thus limiting the damage from chronic immune activation. This argues that pathology caused by immune activation is secondary to immune dysfunction. I now discuss the relevance of immune dysfunction during HIV infection.

3. Immune dysfunction

Dysfunction of virtually every component of the immune system can be demonstrated during the course of HIV infection. While there is no direct evidence that immune dysfunction causes HIV disease progression, it is conceivable that loss of immune functions can result in further loss of viral control. Of particular relevance to CD8 T cell activation and effector function are the destruction of secondary lymphoid tissue and loss of helper CD4 T cells.

Secondary lymphoid tissue histopathology was readily reported in lymph node biopsies performed for routine diagnostics (145). Follicular involution and destruction of the follicular dendritic cell (FDC) network were the most represented patterns replacing follicular hyperplasia during the intermediate stage of HIV infection (i.e. absolute CD4 counts: 200-500/µl). As the expansion of FDC serves as a predominant mechanical mechanism in trapping HIV virions extracellularly (146), the destruction of this network resulted in loss of containment of HIV replication and a rise of viral load in the plasma. In contrast, such disruption was not observed in HIV controllers (145). Other structural cells such as fibroblastic reticular cells (FRC) in the lymph node could also be disrupted (147). Both B cell affinity maturation and T cell activation can be affected by the loss of FDC and FRC networks respectively. The loss of CD4 T cells, particularly T follicular helper cells, may lead to withdrawal of trophic factors necessary for FDC survival thus contributing to their disruption.

CD4 T cells play a central role in normal immune responses and it is likely that many of the immune defects observed during HIV infection are secondary to CD4 T cell depletion. Surprisingly, a recent clinical trial using IL-2 in combination with HAART resulted in
sustained increase in CD4 T cell counts, but yielded no clinical benefits (148). IL-2 may have induced factors that were not measured in this study which contributed negatively to the clinical outcome. Other studies aimed at increasing CD4 T cell counts (e.g. IL-7 trials) are currently underway and may shed light on this matter. It is also possible that the measurement of CD4 T cell counts in the plasma is not representative of the systemic picture. Indeed, during acute HIV infection, severe CD4 T cell depletion, mostly memory cells, occurs in the gut-associated lymphoid tissue (GALT) (149-153). The number of CD4 T cells in the gut was never fully recovered even in the presence of HAART unlike that in the plasma (154). The biological consequence of this CD4 T cell loss in the gut is not fully understood. A recent study showed selective depletion of CD4 T cells producing IL-17 (Th17 cells) in HIV infected humans (143) and SIV infected rhesus macaques (144). Th17 cells have important functions in mounting mucosal defense against bacterial pathogen in GALT and their depletion may be responsible for some of the gut associated dysfunctions observed during HIV infection (155). While direct evidence is lacking as to whether therapeutic reconstitution of the gut T cell (Th17) response can contribute to the delay of disease progression in HIV progressors, gene array analysis has revealed that mucosal T cell responses correlate with protection against disease progression in HIV controllers (156).

Loss of CD4 T cells has a direct impact on naïve CD8 T cell activation, mostly through the loss of provision of CD40L, a member of the TNF superfamily. One paradigm for T cell help proposes that CD4 T cells prime antigen presenting cells (APC), inducing upregulation of costimulatory molecules and production of cytokines, which in turn efficiently activate naïve CD8 T cells (157-159). It has also been proposed that CD4+ lymphocytes can help CD8 T cells directly through CD40/CD40L interactions (160), however in an influenza model, CD40 deficient CD8 T cells had normal responses, arguing for a bridge function of APC (161). As continuous activation of naïve T cells contributes to the heterogeneity of anti-viral CD8 T cell responses during persistent infection (162), CD4 T cell help is relevant even during the chronic phase of HIV infection. In contrast, the dependence of memory CD8 lymphocytes on CD4 T cell help is controversial. In favor of the dependence on help, CD4 T cells are a major producer of
IL-2, which is required for the generation of an effective CD8 T cell memory response (163). Recent studies revealed that IL-21, likely produced by CD4 T cells, is important in driving CD8 T cell effector functions and is an important regulator of CD8 T cell exhaustion in a chronic LCMV model (164-166). As well, HIV-specific IL-21 producing CD4 T cells can be found during both acute and chronic HIV infections and the relative levels of such CD4 T cells correlated with viral control (167). Furthermore, memory CD8 T cells generated without CD4 T cells were shown to upregulate the coinhibitory molecule PD-1 which could further restrict CD8 T cell function (168). I next discuss the inhibitory environment of chronic HIV infection.

**The inhibitory environment for HIV-specific CD8 T cells during chronic infection**

In addition to the loss of secondary lymphoid structure and T helper cells, which are important in optimal activation of CD8 T cells, the recent discovery of abnormal expression of inhibitory molecules offers new insight to CD8 T cell dysfunction at the molecular level. This emerging field of study began with Barber and colleagues’ work on reinvigorating dysfunctional CD8 T cells via PD-L1 blockade during persistent LCMV infection (169). In light of the recent contributions of the LCMV model to HIV research, I first provide some background on the relevance of LCMV to HIV infection, and then discuss the growing inhibitory network for CD8 T cells during HIV infection, which has likely evolved to prevent the exacerbation of immune pathology.

**LCMV**

Lymphocytic choriomeningitis virus (LCMV) is a natural rodent virus belonging to the Arenaviridae family. LCMV has been isolated in several species of animals used in experimental research in the early 20th century including white mice, grey house mice, monkeys, guinea pigs and dogs (reviewed in (170)). Since their isolation, many of the LCMV strains have become one of the most widely used to study cellular immunity. Of relevance to this thesis is the Armstrong strain and one of its variants: clone 13 (selected by (171)). LCMV infection of mice can result in different outcomes, depending on the strain of virus used, the age of the host and the route of infection. For example, when
delivered intracerebrally to newborn mice, Armstrong can cause sustained infection; in contrast, only clone 13 can cause chronic infection in adult mice when delivered intravenously (172). Strikingly, there are only two amino acid differences between clone 13 and its Armstrong parental strain (173).

LCMV differs from HIV infection in a number of ways. LCMV causes systemic infection whereas HIV is primarily a mucosal infection with development of systemic failure relatively later during disease progression. Most importantly, LCMV does not result in latency, nor does it have the same capacity to mutate. There is some overlap in viral tropism, but LCMV does not preferentially target CD4 T cells. Both LCMV Armstrong and clone 13 can infect neurons (174). Clone 13 has a preferential tropism for macrophages (175) and dendritic cells (176) as compared to the parental Armstrong strain. As well, clone 13 can directly infect fibroblastic reticular cells, and result in destruction of the stromal network within secondary lymphoid organs (177).

It is perhaps not surprising that features of CD8 T cell responses to LCMV and HIV share striking similarities, given the importance of DC/FRC and CD4 T cells, the preferential targets of the two infections respectively, in shaping cytotoxic T cell responses. As well, both infections are characterized by persistent antigen. Indeed, LCMV-specific CD8 T cells from chronic infection were also shown to have limited effector functions (178) and the term functional exhaustion was first used to describe the disappearance of functional CTL during chronic LCMV Docile infection (179) which was later extended to describe other infections such as HIV. CD8 T cell dysregulation in LCMV occurs in a staged fashion. Cells lose proliferative capacity and cytolytic functions first, followed by the loss of their ability to produce cytokines such as TNFα and finally IFNγ (180). Furthermore, PD-1 upregulation was identified as a molecular mechanism of T cell exhaustion in both LCMV and HIV infections (169, 181-183). Since then, there has been much cross-fertilization of the chronic viral infection field in general. Thus LCMV is an appropriate model for studying CD8 T cell dysfunction with the caveat that not all of the factors leading to HIV disease progression are present.
Expression of inhibitory molecules

HIV-specific CD8 T cells during chronic infection are characterized by expression of several inhibitory molecules such as PD-1 (181-183), Tim-3 (184), CD160 and LAG-3 (185). Recent studies suggest that the expression of inhibitory molecules is regulated by the transcription factor Blimp-1 (186-188). Perhaps counter-intuitively, Blimp-1 is also required for CD8 T cell cytotoxic functions including the upregulation of granzyme B production (189). To reconcile these findings with the lack of effector functions in exhausted CD8 T cells, it is possible that other mechanisms are at play in preventing expressions of effector molecules at the protein level. Indeed, granzyme B mRNA, but not protein, remains expressed in exhausted CD8 T cells during chronic LCMV infection (190).

PD-1 is an inhibitory member of the CD28 superfamily found on activated T cells, B cells and myeloid cells. PD-1 can interact with two ligands: PD-L1 and PD-L2. While PD-L1 is expressed broadly on both hematopoietic and non-hematopoietic cells, PD-L2 expression is limited to antigen presenting cells (APC) (191). A recent report showed that PD-L1 can interact with CD80, another member of the same family, through bidirectional signaling, revealing another dimension of the PD-1 pathway (192). The exact inhibitory mechanism of PD-1 remains to be uncovered, however, PD-1 ligation can inhibit membrane-proximal T cell signaling events and downstream T cell proliferation and effector functions (191). Recent transcriptional analysis of HIV-specific CD8 T cells revealed that PD-1 may exert its inhibitory functions by upregulating the transcriptional factor BATF of the AP-1 family, which in turn limits T cell proliferation and function (193).

The PD-1 pathway is vital in regulating CD8 T cell dysfunction. During HIV infection, in vitro blockade of PD-L1 resulted in enhanced cytokine production of CD8 T cells in response to HIV antigen (181-183), likely a secondary effect to T cell survival and proliferation (181). Most strikingly, in vivo blockade of PD-L1 led to viral clearance in several organs during clone 13 infection (169). To date, it remains the only therapy targeting CD8 T cells (apart from the work presented in chapter IV) that achieved viral clearance.
control when administered during the chronic phase of infection. It remains uncertain, however, whether the viral control was caused directly by the reinvigoration of dysfunctional CD8 T cells via PD-L1 blockade. In fact, the recovery of CD8 T cell dysfunction and viral control can be two separate events. Using bone marrow chimeric mice, Mueller and colleagues showed that PD-L1 on hematopoietic cells was responsible for inhibiting CD8 T cell proliferation and function, whereas deficiency of PD-L1 on non-hematopoietic cells resulted in viral clearance and affected pathology (194). This study argues that improving CD8 T cell function alone is not sufficient for viral clearance of LCMV infection. Regardless of the mechanism of action, the PD-1 pathway has shown great therapeutic potential. Blocking PD-1 in SIV-infected rhesus macaques improved T cell immunity in the plasma and gut and resulted in prolonged host survival (195). Interestingly, PD-1 was shown to be a predictor of HIV disease progression using fresh blood samples but not those cryopreserved (196).

Expression of the glycoprotein Tim-3 on dysfunctional CD8 T cells also showed functional consequences. Tim-3 belongs to the Tim (T cell/transmembrane, immunoglobulin, and mucin) family consisting of pattern recognition receptors specialized in recognizing phosphatidylserine (reviewed in (197)). Tim-3 is found to bind galectin-9 which results in the selective death of IFNγ producing T cells (198). Expression of Tim-3 has been positively correlated with HIV viral load and blocking the Tim-3 signaling pathway resulted in restoration of HIV-specific T cell function (184). Furthermore, co-blockade of the Tim-3 and PD-1 pathway in vivo synergistically improved CD8 T cell function and viral control (199). The mechanism of inhibition by Tim-3, however, remains unclear.

*Increased levels of inhibitory cytokines during chronic viral infection*

IL-10 is an immune regulatory cytokine that has critical and indispensable anti-inflammatory properties (200). Indeed, IL-10 deficiency renders mice susceptible to a 20-fold lower dose of LPS and injection of IL-10 has protective effects (200). IL-10 can function to suppress T cell cytokine production and proliferation and alter functions of APC (200). Of note, IL-10 can block upregulation of costimulatory ligands (CD80 and
CD86) on macrophages (201). Cells that can produce IL-10 include T cells, APC and recently B-10 B cells were identified as a source of IL-10 in both human and mice (202-204). During chronic LCMV infection, significant upregulation of serum IL-10 was observed (205, 206), produced mainly by CD8α(neg) DC (206). These two studies went on to show, using IL-10 and IL-10R knockout mice respectively, that deficiency in IL-10 signal was sufficient for resolution of persistent viral infection. Similar effects were achieved using blocking antibody to IL-10 during early infection, whereas delayed treatment to day 12 resulted in significant but smaller effects, arguing for a relatively early role of IL-10 during LCMV persistence. This is in contrast to the PD-1/PD-L1 pathway, which resulted in viral clearance when blocked later in the infection, but caused pathology/death when absent from the beginning of the infection (169).

HIV progressors were shown to have elevated levels of IL-10 regardless of HAART, which could potentially suppress CD8 T cell function (207). Indeed, blocking IL-10 in vitro has resulted in increased proliferation of both CD4 and CD8 T cells from HIV+ individuals (208). The role of IL-10 in HIV pathogenesis, however, remains controversial (209). Polymorphism in the IL-10 gene promoter that leads to increased IL-10 production is associated with poor viral control during early infection, but delays HIV disease progression during the chronic phase, suggesting the anti-inflammatory role of IL-10 could be essential in protection against chronic immune activation as disease progresses (210).

TGFβ is another suppressive cytokine that has been implicated in chronic viral infection. TGFβ has pleiotropic functions in wound healing, embryogenesis, carcinogenesis and the immune system (Reviewed in (211, 212)). It can impact virtually all cells of the leukocyte lineages. The central role of TGFβ in the immune system is to maintain tolerance and to contain and resolve inflammation. Specifically TGFβ can suppress T cell proliferation, survival, effector functions and trafficking. TGFβ is secreted in a latent form complex that’s targeted to the extracellular matrix (213). Upon engagement of the receptor complex, TGFβ induces phosphorylation of Smad family proteins whose nuclear translocation results in target gene transcription (211). Of relevance, costimulatory
molecules such as CD40, CD28 and 4-1BB (214) can counteract the suppressive ability of TGFβ (211).

Sustained TGFβ expression in LCMV-specific CD8 T cells was reported for clone 13 infection (215). Tinoco et al. showed that TGFβ receptor deficiency on T cells resulted in improved LCMV-specific CD8 T cell survival which was dependent on the lack of the pro-apoptotic molecule Bim. As well, clone 13 infection was rapidly resolved when TGFβ signaling was disrupted specifically in T cells at the onset of infection (215). It is unclear what the role of TGFβ may be during the chronic stage of persistent infection. TGFβ is also implicated in HIV pathogenesis. HIV Tat can upregulate TGFβ in antigen stimulated PBMC, monocytes, T cells and astrocytes in vitro, which may in turn exert suppressive effects (216). TGFβ can also increase the susceptibility of macrophages to HIV infection by increasing expression of CXCR4 (217). Analysis of intestinal tissue from SIV infected rhesus macaques revealed elevated levels of TGFβ which is positively associated with rapid disease progression (218). In vitro analysis in the same study revealed a role of TGFβ in SIV-specific CD8 T cell survival and proliferation.

In conclusion, CD8 T cell dysfunction specific to HIV could be driven and maintained by an inhibitory environment through elevated suppressive cytokines such as IL-10 and TGFβ in the local environment and increasing numbers of inhibitory molecules expressed on T cells respectively. Direct blocking of some of these pathways has shown promise in viral control with the caveat of increasing immune pathology. The nature of much of the inhibition is to antagonize stimulatory signals. I next discuss members of the costimulatory families that are the subject of exploration in the work presented in this thesis.

**The costimulatory pathways**

Effective T cell activation requires at least two signals: an antigen specific signal from engagement of T cell receptor via MHC/peptide complex (signal 1) and a noncognate costimulatory signal (signal 2) provided by antigen presenting cells (APC).
Costimulatory receptors are tightly regulated and often upregulated on T cells receiving signal 1. Thus in principal, engagement of costimulatory receptors often results in an antigen specific impact. The two signal concept was first proposed for discriminating B cell activation from anergy (219, 220) and later extended to T cells (221, 222). The current understanding of costimulation is more complex and it should be noted that the qualitative requirement of signal 2 (costimulation) varies and may depend on the avidity, and thus duration, of TCR-MHC/peptide interaction, the type of T cells (naïve or memory) that have been activated and the inflammatory microenvironment (223).

A large number of molecules have been implicated in costimulation, including both stimulatory and inhibitory members, many of which fall into two superfamilies: the CD28/B7 superfamily and TNFR/TNF superfamily. The costimulatory network is growing with increasing complexity attributed to discoveries of new family members, expression patterns on many cell types, cross-member interactions as well as bidirectional signaling. Some family members can be found on non-hematopoietic cells and thus have functional roles beyond T cell activation. Here I focus on several relevant family members that have been shown to impact CD8 T cell activation and effector functions during chronic viral infection.

**B7/CD28 pathway**

The B7/CD28 pathway is well characterized and plays a pivotal role in immune regulation. B7.1(CD80) and B7.2(CD86) interact with CD28 to provide a major costimulatory signal for augmenting and sustaining T cell responses (224-226) and provide an inhibitory signal when interacting with a different receptor, CTLA-4 (227, 228), of much higher affinity (229, 230). A recent report showed that B7.1 can interact with another B7 family member, PD-L1, and deliver an inhibitory signal to T cells (192), further complicating the B7/CD28 pathway.

1. **Expression**

CD28 and CTLA-4 are both type I transmembrane glycoproteins with a MYPPPY motif within the IgV-like domain that is required for binding CD80 and CD86 (231). CTLA-4
is induced upon cell activation. In contrast, CD28 is constitutively expressed on almost all T cells in mice (232), 50% of human CD8 T cells and 95% of human CD4 T cells (233). CD28 can also be found on some plasma cells and natural killer cells. During chronic infection, however, most (70-95%) HIV-specific CD8 T cells were shown to be CD28 negative, perhaps reflective of their differentiation state (234). It is unclear whether these CD28 negative HIV-specific T cells can still respond to B7.

B7.1 and B7.2 are also type I transmembrane proteins mostly found on APC, but also T cells. B7.2 is constitutively expressed at low levels and rapidly upregulated upon APC activation, whereas B7.1 is expressed on APC 3-4 days post-activation (201, 235). Linsley et al. showed that B7.1, while sharing similar costimulatory properties with B7.2, binds to CTLA-4 with a different kinetics, which may account for its different functional role (229).

2. Functions

CD28 signaling, in the presence of TCR ligation, decreases the threshold for T-cell activation (236), enhances survival by upregulating Bcl-xL (237), increases cell proliferation, increases production of multiple cytokines (e.g., IL-2) (225, 238), induces cell-cycle progression (226) and regulates glucose metabolism (239). Despite the importance of the pathway, CD28/B7 is indispensable for optimum cytotoxic CD8 T cell responses in some (240-242) but not all (243-245) acute viral infections, suggesting other costimulatory molecules may play partially redundant roles.

The role of the B7/CD28 pathway during chronic viral infection also depends on the type of infection studied. During murine gammaherpesvirus 68 (MHV-68), a natural murine pathogen that causes latent infection, both CD28<sup>−/−</sup> and wildtype mice maintained effective long term viral control for up to 50 days (246), suggesting that CD28 is not important in the infection outcome. Interestingly, a separate study using CD80/CD86<sup>−/−</sup> mice showed reactivation of MHV-68 virus in the lung at day 42 and viral persistence for over 100 days (247). The contradictory results could be attributed to other binding partners of CD80/CD86 or significantly different virus dose used to challenge the mice.
(20000 pfu/mouse in (246) and 400 pfu/mouse in (247)). While the role of CD28 may be less clear during chronic latent infection, CD28 was shown to be essential in the LCMV clone 13 model. CD28-/- mice showed significantly reduced LCMV-specific CD8 T cell responses 4-5 months post clone 13 infection, and most notably, resulted in a 2-4 log increase in viral load in multiple organs (244). Similarly, during polyoma virus infection, a low-level persistent viral infection model, knocking out CD28 resulted in severely impaired primary and secondary antigen-specific CD8 T cell responses as well as poor viral control (248).

A recent study showed that the B7 pathway is also important in affecting the quality of memory CD8 T cells made during persistent viral infection. Using persistent LCMV Traub as a model, Grujic et al. showed that CD80/86-/- mice generated dysfunctional CD8 T cells 6-8 months post infection compared to wildtype mice: Ag-specific CD8 T cells produced significantly lower levels of IFNγ and less IL-2 upon in vitro restimulation and had reduced ability to expand in response to antigen in vivo (249). This phenotype shows striking similarity to those of HIV-specific CD8 T cells during the chronic phase.

In conclusion, the B7/CD28 pathway can play a significant role during chronic infection. It is still unclear, however, whether costimulation is required once the chronic phase of an infection is already established. Taking advantage of CTLA-4Ig, Kemball et al. showed that blocking CD28 and CD40 (using blocking antibodies) pathways at the same time during the chronic phase did not affect polyoma viral control (248). It is possible that costimulation is dispensable during the chronic phase of persistent infection or that other costimulatory molecules may play overlapping roles. In support of the latter hypothesis, in the same study, blocking CD27 and CD28 during the chronic phase resulted in significant reduction in the number of viral-specific CD8 T cells (248). I discuss the role of another costimulatory molecule, 4-1BB, next.

4-1BB/4-1BBL pathway

4-1BB (CD137) was first identified by Kwon and colleagues (250) and its human homologue ILA was later cloned by Schwarz et al. independently (251). 4-1BB interacts
with 4-1BBL (mouse (252), human (253)), its sole binding partner identified to date. This pathway has emerged as playing a key role independent of CD28 in driving CD8 T cell survival and effector functions (reviewed in (254)). Importantly, DeBenedette et al. showed that CD28-/- T cells can respond to the 4-1BBL costimulatory signal (255). In light of the fact that majority of HIV-specific CD8 T cells lack CD28 expression during chronic infection, 4-1BBL is an attractive candidate in our search for a therapy in improving dysfunctional CD8 T cells during chronic HIV infection.

1. Expression

4-1BBL can be found on a variety of cell types including activated APC (256, 257), human neurons and astrocytes (258), myeloid progenitors and hematopoietic stem cells (259-261). While in vivo detection of 4-1BBL is difficult during an ongoing infection (262) perhaps a result of stringent regulation, 4-1BBL can be readily found in chronic or inflammatory environments on fibroblast from patients with chronic beryllium disease (263), cardiac myocytes during acute myocarditis in mice (264) and aortic tissue from individuals with takayasu's arteritis (265). CD40 is a major regulator of 4-1BBL expression on B cells and DC (255, 257, 266). As well, 4-1BBL message can be induced in macrophage upon LPS treatment (267).

In contrast to CD28, 4-1BB is not expressed on resting T cells but can be induced on activated T cells (268). In response to non-replicating antigens in vivo, 4-1BB expression is transient and can be found within 24 hours, coinciding with the early activation marker CD69 (269, 270). When antigen persists, however, 4-1BB expression can be prolonged (262, 271-273). Interestingly, direct ex vivo detection of 4-1BB has not been observed on HIV-specific CD8 T cells from chronically infected individuals (unpublished), but can be induced (274). As such, it is feasible to exploit the therapeutic application of this pathway for chronic HIV infection. In addition to T cells, 4-1BB is found on many other cell types such as monocytes (275), follicular dendritic cells (276), DC (257, 277), activated NKcells (278, 279), differentiating myeloid-lineage cells (259), Ig-E stimulated mast cells (280), eosinophils (281) and neutrophils (282, 283).
2. Signaling

Like other prosurvival members of the TNFR family, 4-1BB signals through recruitment of specific TNFR-associated factors (TRAFs). Upon receptor clustering, 4-1BB can recruit TRAF1 and TRAF2 (284, 285)(human 4-1BB can also recruit TRAF3 (284)), resulting in activation of NF-κB, c-Jun N-terminus kinase (JNK), extracellular signal regulated kinase (ERK) and p38 mitogen associated protein (MAP) kinase signaling cascade (284-289). 4-1BB induces T cell survival through TRAF1 and ERK dependent downregulation of the pro-apoptotic molecule Bim (274, 288) and NF-κB dependent upregulation of the pro-survival members of the Bcl-2 family such as Bfl-1 and Bcl-xL (286) (see Fig. 1.2, reprinted from (290) with permission).

TRAF1 and TRAF2 were originally identified in immunoprecipitates of TNFR2 by Rothe et al. (291). TRAF2 is constitutively expressed and its transcript can be found in almost all tissues (291). In contrast, TRAF1 is an inducible protein and has more limited expression (291). In response to 4-1BB signaling, TRAF2 deficiency or overexpression of non-functional TRAF2 results in impaired NF-κB and p38 activation and consequentially poor cell proliferation and cytokine production (284, 285, 287). The role of TRAF1 in 4-1BB signaling is less defined. Our laboratory recently showed that TRAF1 is a critical survival factor for activated and memory mouse CD8 T cells in a Bim dependent manner (292). Furthermore, Sabbagh and Pulle et al. showed the TRAF1 regulation of Bim is downstream of 4-1BB and is ERK dependent (288).

3. Function during chronic viral infection

Similar to CD28, the 4-1BB/4-1BBL pathway was found to be indispensable for optimal primary response in some (262, 293-296) but not all (242, 297, 298) acute viral infections. The role of 4-1BB/4-1BBL during persistent infection is poorly understood. During MHV-68 infection, 4-1BBL deficient mice were shown to have significantly increased viral load (299). The increased viral load does not appear to be due to a defect in the quantity of memory CD8 T cells, but rather their decreased function in degranulation in the latent phase. The ability of the 4-1BBL pathway to induce cytotoxic functions is
Figure 1.2 Signal transduction by 4-1BB in T cells. Upon aggregation of 4-1BB in T cells tumor necrosis factor receptor-associated factor 1 (TRAF1) and TRAF2 are recruited, leading to activation of nuclear factor κB (NF-κB) and the extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen-associated protein (MAP) kinase cascades. TRAF2 dominant negative or TRAF2−/− mice show that TRAF2 is essential for p38 and NF-κB signaling downstream of 4-1BB. In the absence of TRAF1, 4-1BB-dependent ERK activation is impaired and Bim levels increase in the cell. (Figure reprinted from (287) with permission).
particularly relevant to their therapeutic implication to dysfunctional CD8 T cells specific to HIV.

In summary, the 4-1BB/4-1BBL pathway plays a vital role during viral infection. Its importance during memory maintenance and in particular the ability to stimulate CD28 negative CD8 T cells (reviewed in (290)) prompted us to investigate the therapeutic application of this pathway during chronic HIV infection. As well, 4-1BBL is found in a gene cluster that contains two other TNF family members, CD70 and LIGHT. Both molecules have been implicated in costimulation of CD8 T cells. Of particular relevance, the importance of CD27, the receptor for CD70, was shown in an *in vivo* study of an HIV patient where CD27⁺ clones derived from HIV-specific CD8 T cells survived much better than those that do not express CD27 (300). Given the potency of B7 as a costimulatory molecule, we also investigated the role of ICOSL, the remaining stimulatory member of the B7 family. An introduction of these molecules is given in Chapter II.

**Conclusion**

CD8 T cells play an important role in viral control during chronic HIV infection. However, CD8 T cell function is increasingly impaired as disease progresses, perhaps a result of the increasing inhibitory cytokine environment, the loss of CD4 help, the expression of inhibitory molecules and the disruption of secondary lymphoid structure. As well, constant appearance of escape mutants, the hidden/latent viral reservoir which provides a source of persistent antigen and chronic immune activations are but some of the challenges facing control of HIV via CD8 T cells or other arms of the immune system.

Different costimulatory molecules may be important for antigen-dependent activation of different T cells at different times during the course of an infection. In the case of chronic HIV infection, both naïve and effector memory CD8 T cells are activated in response to persistent viral antigen. While it is conceivable that costimulation is required in such cases, it is unclear which specific costimulatory molecules are of relevance at the later stage of HIV infection independently of priming. The lack of CD28 expression on the
majority of HIV-specific CD8 T cells suggests that there might be an insufficient or altered costimulatory requirement during the chronic phase of the response. Furthermore, the loss of CD4 T cells may impact the expression of costimulatory ligand, as CD40/CD40L interactions are known to upregulate B7 and 4-1BB-L. On the other hand, inflammatory mediators such as lipopolysaccharides (LPS) were also shown to upregulate B7.1/B7.2; as well B7.1 expression can be enhanced by IL-1, GM-CSF and TNF-α (301). Given the chronic inflammatory environment of HIV infection, further studies are required to analyze the availability of costimulatory ligands.

In this study, I investigated the therapeutic application of several costimulatory molecules in their ability to directly stimulate CD8 T cells independent of CD4 lymphocytes. Successful candidates were then used to analyze their impact on dysfunctional HIV-specific CD8 T cells. I provided evidence in this work that costimulatory pathways could be impaired during chronic HIV infection and suggested a solution for repairing such a defect.
CHAPTER II

- THE SCREENING -

“Qualitative difference of the LIGHT/CD70/4-1BBL gene cluster in direct costimulation of human memory CD8 T cell responses”

Figure 2.4 B and E, and Figure 2.5 were published in:


T. Wen generated Adenoviruses expressing 4-1BBL, CD70 and LIGHT. I conducted all experiments myself.
Abstract
The factors that control the quality and quantity of CD8 memory T cell responses remain incompletely defined. 4-1BBL, CD70 and LIGHT, members of the TNF family, have been implicated in the CD28-independent costimulation of T cells, and thus are candidates for manipulation of HIV-specific memory CD8 T cells. Their relative importance, however, in direct CD8 T cell activation independent of CD4 lymphocytes is unknown. In this study we used replication defective adenovirus vectors to deliver 4-1BBL, CD70 or LIGHT to healthy donor monocytes and used the modified APC to stimulate autologous influenza-specific memory CD8 T cell responses. At limiting antigen dose, 4-1BBL was the most potent of the three TNF ligands for expanding influenza-specific CD8 T cells and augmenting their cytolytic activity on a per cell basis. LIGHT had minimal activity in these assays. Although less potent than 4-1BBL as a function of antigen dose, CD70 was a strong costimulator for about half the donors tested and was particularly good in inducing a high frequency of cytokine producing cells. Furthermore, CD70 was most effective in costimulating CD8 T cells from donors with a more differentiated CD8 effector/memory T cell pool. Combining CD70 and 4-1BBL resulted in the rescue of influenza-specific expansion from donors with a poor response to CD70 alone. Thus, 4-1BBL and CD70 represent two promising candidates for boosting human CD8 T cell responses.

Introduction
The mechanisms that regulate the magnitude and quality of memory CD8 T cell responses are incompletely understood. Costimulatory molecules, which may be integrated by T cells to act at different times, in different places (lymphoid organ, extralymphoid tissue) and on different T cell subsets (CD4, CD8, naïve, effector, memory, regulatory) (254, 302), are considered important candidates for manipulation of T cell memory. Teasing apart the qualitative difference between the plethora of costimulatory molecules is of benefit for selection of the best candidates for further studies presented in this thesis.
Some CD8 T cell responses and particularly CD8 recall responses are relatively independent of CD28/B7 costimulation, leading to the suggestion that CD8 T cells are less costimulation dependent (223, 303-305). However, there is accumulating evidence that other costimulatory molecules, such as 4-1BB and ICOS, can play a role in CD28-independent T cell responses which are of relevance to HIV-specific CTL for reasons discussed in chapter I (255, 306-308). In particular, 4-1BB ligand (4-1BBL) is emerging as a key player in CD8 T cell memory (242, 295, 309).

The 4-1BBL gene lies on human chromosome 19p13 and mouse chromosome 17 in close proximity with two other TNF ligand genes encoding CD70 and LIGHT (310). A fairly extensive literature suggests that all three TNF family ligands have costimulatory activity for T cells (254). As discussed in Chapter I, mice lacking 4-1BB or 4-1BBL show defective memory responses to viruses (242, 295, 298, 309). 4-1BBL is also a potent adjuvant for memory anti-viral responses of human CD8 T cells \textit{ex vivo} (311, 312), with direct effects on CD8 T cells in contrast to a closely linked family member, OX40L, which affect CD8 T cell response indirectly through CD4s (313).

Similarly to 4-1BB, CD27, the receptor to CD70, provides survival signals to CD8 T cells subsequent to the effects of CD28 (314). CD27 deficiency impaired both CD4 and CD8 primary and memory responses in an influenza mouse model (315). Whether the impairment in memory is secondary to a poor primary response is unknown. Similarly, transgenic overexpression of CD70 as well as therapeutic injection of soluble CD70 caused antigen-specific CD8 T cell expansion (316, 317). It is unclear in these studies, however, whether the effect of CD27/CD70 pathway on CD8 T cells is through direct effects. Of note, constitutive expression of CD70 in uninfected healthy mice results in B cell depletion (318), T cell-driven chronic immune activation and premature death due to immune deficiency (319), thus therapeutic application of CD70 should be taken with caution.

Evidence that LIGHT functions as a costimulatory ligand comes from several different \textit{in vitro} models (320-322). Furthermore, overexpression of LIGHT in T cells \textit{in vivo} results
in expanded populations of CD4 and CD8 T cells and an autoimmune disorder in the mouse gut (323-325). Studies with LIGHT-deficient mice have also revealed a role for LIGHT in some (326-328), but not all (329) CD8 T cell responses.

It is difficult to compare the effects of these three costimulators based on the literature, as they have been tested in separate studies in a variety of different models. Given the potential importance of these ligands as adjuvants for enhancing CD8 T cell responses, it was of interest to compare their efficacy in human T cell stimulation in a side-by-side comparison. Here, we describe the generation of recombinant adenoviruses expressing CD70 and LIGHT in comparison to the previously generated 4-1BBL vector. Using a reductionist approach, we compared the costimulatory activity of 4-1BBL, CD70 and LIGHT when expressed on APC. We find that 4-1BBL and CD70 have significant and complementary effects on human CD8 recall responses, whereas LIGHT has minimal efficacy in this model. Thus, we identified two candidate molecules for rescuing functionally impaired HIV-specific CD8 T cells in chapter III.

ICOSL (Inducible costimulatory ligand), a member of the B7 superfamily, is vital for T helper cell responses and antibody class switch (302). At the time of the 4-1BBL / CD70 / LIGHT comparative study, mouse work from our lab had shown that ICOSL can function synergistically with 4-1BBL to activate CD28- CD8 T cells. I have subsequently generated the human ICOSL-adenovirus vector and begun to investigate the role of ICOSL in directly stimulating influenza-specific memory CD8 T cells. Analysis up to now is also included in this chapter for comparative purposes.

**Results**

**Expression of 4-1BBL, CD70, LIGHT and their respective receptors**

The ectopic expressions of costimulatory ligands on primary human monocytes were analyzed. Forty-eight hours post-infection with the respective replication-defective adenoviruses, monocytes expressed CD70, LIGHT or 4-1BBL in a dose dependent manner, whereas control adenovirus (cAdV) induced little expression of either ligand.
At saturation, approximately 40-50% of monocytes expressed the three ligands and the expression patterns of CD70, LIGHT and 4-1BBL as a function of MOI were comparable for each of the recombinant vectors. An MOI of 100 was used for all subsequent experiments as this resulted in optimal expression with good monocyte viability. We next asked whether receptors to 4-1BBL, CD70 and LIGHT are present on CD8 T cells early in the culture period. CD27 was found on majority of resting CD8 T cells (Fig. 2.2A). In contrast, both HVEM (LIGHT receptor) and 4-1BB were inducible on antigen-specific CD8 lymphocytes on day 1 and day 3 respectively (Fig. 2.2 C, D).

**4-1BBL and CD70, but not LIGHT, are strong stimulators of influenza-specific CD8 T cells, with CD70 being less potent**

We compared the efficacy of 4-1BBL, CD70 and LIGHT, when expressed on APC, in the direct costimulation of human memory CD8 T cells. Purified CD8 T cells were cultured with influenza peptide-pulsed autologous monocytes infected with 4-1BBL-, CD70-, LIGHT- or control adenovirus (see Fig. 2.3 for schematics). The expansion of influenza-specific CD8 T cells was measured seven to nine days after coculture (Fig. 2.4A). The costimulatory effect of each TNF ligand can be affected by antigen load and time. We first asked whether there is a differential requirement for antigen dose in response to different costimulation conditions. The dose response curve (Fig. 2.4B) revealed that LIGHT is a poor costimulator in all donors within the range of antigen dose tested (0.004-25 µM). As well, 6 out of 12 donors did not respond to CD70 (named CD70 non-responders), whereas all donors responded to 4-1BBL (Fig. 2.4B). Figure 2.3C summarizes the minimum antigen dose required for each successful costimulation (defined by > 3 fold tetramer^+ CD8 T cell expansion over response to cAdV background). Interestingly, 4-1BBL required a ten-fold-lower antigen dose on average (p = 0.035) for costimulation as compared to CD70 (Fig. 2.4C). The lack of costimulatory activity for LIGHT or CD70 in some donors is not due to the time of analysis on the cultured cells. Figure 2.3D shows that limited expansion of influenza-specific CD8 T cells was observed in response to LIGHT or CD70 between 5 and 9 days post coculture in CD70 non-responders, in contrast to the 4-1BBL stimulated cultures (Fig. 2.4D). Of note, in CD70
Figure 2.1 Over-expression of 4-1BBL, CD70 and LIGHT on monocytes. Monocytes from healthy donors were enriched by plate adherence and infected for 16 hours with 0, 10, 50, 100 or 200 MOI of adenoviruses carrying either the human 4-1BBL, CD70, LIGHT gene or with an empty vector. The monocytes were allowed to rest for a day after infection and the expression of 4-1BBL, CD70 and LIGHT on monocytes were measured by flow cytometry. Representative plots are shown gated on live monocytes (n=4).
Figure 2.2

A. CD27 and CD28 expression was measured on tetramer$^+$ CTL from unfractionated PBMC. C. HVEM expression was measured one day after coculture with modified autologous monocytes. Representative FACs plot was shown. D. The expression of 4-1BB was measured on tetramer$^+$ CD8 T cells after coculture with modified autologous monocytes for 1, 2 and 3 days. Representative FACs plots are shown for tetramer$^+$ CD8 T cells stimulated with either (a) 4-1BBL or (b) cAdV modified monocytes (n=4).
Figure 2.3  
**Schematics of costimulation assay.** As described in methods.
Figure 2.4 Differential expansion of influenza-specific CD8 T cells in response to 4-1BB, CD70 and LIGHT. CD8 T cells were co-cultured with autologous monocytes infected with adenoviruses carrying costimulatory molecules and analyzed for influenza-tetramer+ CD8 T cells at day 7, 8 or 9, as described in the methods. A. Representative flow cytometry data gated on CD8 T cells. B. Representative Ag-dose response curves. A2-restricted influenza M1 peptide was titrated within the range of 0.004 µM to 25 µM (n=12). C. Extraction of results in B, the lowest peptide concentration required for successful costimulation (> 3 fold expansion: calculated as the number of tetramer+ CD8 T cells in response to costimulatory molecules over that to control adenovirus. This also defines CD70 responder vs. non-responder). D. Representative kinetics curve (n = 4). E. The highest fold expansion (as defined in C) from dose response curve is shown for responses to 4-1BB, CD70 or LIGHT. Experiments were repeated 1 or 2 times for each of the 12 donors depending on sample availability. The duplicate dose response curves were similar.
responders, peak response to CD70 occurred earlier than that to 4-1BBL, consistent with the earlier expression of the CD70 receptor (Fig. 2.4D right panel).

We next analyzed the relative potency of 4-1BBL and CD70 when antigen dose is optimal for each TNF ligand. Figure 2.3E summarized the peak response for each ligand in every donor, as measured by the largest number of tetramer\(^+\) CD8 T cells induced in the dose response curve. Ectopic expression of CD70 resulted in significantly higher recovery of tetramer\(^+\) cells compared to that of LIGHT (Fig. 2.4E). The response to CD70, however, was lower than that to 4-1BBL (Fig. 2.4E), which had a peak expansion ranging from 3 to as high as 49 fold above the response to Ag plus control adenovirus modified monocytes. Thus 4-1BBL and CD70, but not LIGHT, are each capable of expanding human influenza-specific memory CD8 T cells, with 4-1BBL inducing a higher proportion of donor responses at a lower peptide dose.

**Differential effect of 4-1BBL and CD70 on driving effector functions**

To compare the effector functions of influenza-specific CD8 T cells generated in response to LIGHT, CD70 or 4-1BBL, we harvested T cells from day 8 culture and measured cytokine production upon peptide restimulation and their cytotoxicity. For these studies, we focused on a subset of donors whose T cells showed a significant response to CD70 costimulation (CD70-responders). Both 4-1BBL and CD70 induced a significantly higher frequency of IFN-\(\gamma^+\) TNF-\(\alpha^+\) CD8 T cells, consistent with their expanded tetramer\(^+\) T cell population (Fig. 2.5A). Interestingly, a higher proportion of CD70-induced influenza-specific CD8 T cells produced both IFN-\(\gamma\) and TNF-\(\alpha\) as compared to that induced by 4-1BBL (Fig. 2.5A, \(p = 0.03\)). In contrast, 4-1BBL was superior to CD70 in inducing perforin expression (Fig. 2.5B) and target cell killing (Fig. 2.5C right panel) under conditions where cultures had similar frequencies of Ag-specific T cells (Fig. 2.5C left panel). Thus 4-1BBL improves the level of cytolytic function per T cell, whereas CD70 is a more potent inducer of cytokines such as IFN-\(\gamma\) and TNF-\(\alpha\). LIGHT had negligible activity in any of the effector functions tested (Fig. 2.5).
Figure 2.5

A. T cells from 8-day cultures of CD70 responders from figure 2.3E were restimulated with A2/M1 influenza peptide as described in Materials and Methods. Intracellular IFNγ/TNFα coproduction was measured in tetramer+ CD8 T cells. B. Analysis of Perforin expression of CD70-responders from figure 2.3 E. C. Cytolytic effector function of T cells from CD70 and 4-1BBL-stimulated cultures. Left panel, The frequency of tetramer-positive cells in the cultures used for the 51Cr-release assay in the right panel (n = 3). For details, see Materials and Methods. Three of the four donors were repeated in separate experiments with similar results.
CD70 preferentially costimulates T cells from donors with a more differentiated influenza-specific memory CD8 T cell pool

As discussed above, isolated CD8 T cells from half of healthy donors tested did not respond significantly to CD70 as a function of antigen dose (Fig. 2.4B). It was possible that the poor response to CD70 in some donors was due to a generalized defect in CD8 T cell memory. However, Figure 2.5A shows that the donors that failed to respond to CD70 exhibited a wide range of responses to 4-1BBL, arguing against a weaker overall response being responsible for the difference (Fig. 2.6A).

To further analyze the differential response to CD70 among donors, we examined the starting populations of influenza-specific memory CD8 T cells in more detail. We found that CD70 responders and non-responders did not differ in the percent of tetramer+ CD8 T cells in the starting population (Fig. 2.6B). The limited response to CD70 in some donors was also not due to lower expression of its receptor, CD27, as over 90% of the antigen-specific memory CD8 T cells from non-responders expressed CD27 (Fig. 2.6C). CCR7 has been used to distinguish central memory cells that can re-enter lymph nodes from effector memory cells which circulate outside the lymphoid organs (330). We observed a trend towards a higher frequency of CCR7 expressing cells in CD70 non-responders, although this was not statistically significant perhaps due to limited sample size (Fig. 2.6D).

The loss of CD28 and CD27 from T cells has been shown to correlate with their exposure to different viral infections, with the acquisition of effector function, and with the age of the immune system (234, 331-335). Interestingly, the donors that respond to CD70 have a higher frequency (P = 0.0087) of CD28− T cells in their starting population of tetramer+ cells, whereas those of the non-responders were almost all CD28+ (Fig. 2.6E). Thus, CD70 may influence CD8 T cells from donors that have a starting Ag-specific T cell pool with a more differentiated or recently antigen exposed effector/memory phenotype.

4-1BBL rescues the response of ineffective donors to CD70
Figure 2.6 CD70 preferentially stimulates CD8 T cells from donors with more differentiated influenza-specific effector/memory pool. A. Comparison of CD70 responders and non-responders for responses to 4-1BBL. Data from figure 2.4E are replotted with CD70 responders shown with empty symbols and CD70 non-responders as filled symbols. B. C. D. and E. Donor PBMC were analyzed directly ex vivo for B. % tetramer+ CD8 T cells and the proportion of tetramer+ CD8 T cells that express C. CD27, D. CCR7 and E. CD28. E. Representative FACS plots for the summary data in the left panel are shown in the right panel from one CD70 responder (a) and one CD70 non-responder (b). Histograms of CD28 expression are gated on influenza-specific CD8 T cells.
To test the hypothesis that CD70 may be a potent stimulator of more differentiated cells, we reasoned that additional costimulatory help, such as CD4 T cells or 4-1BBL, would improve the responses from CD70 non-responders. As CD70 has been implicated in both CD8 and CD4 responses, we first asked whether addition of CD4 T cells to the culture increases the expansion of influenza memory CD8 response of CD70 non-responders. Figure 2.7A showed that in the presence of CD4 T cells, three out of six CD70 non-responders showed improved tetramer+ CD8 T cell expansion as compared to pure CD8 culture (Fig. 2.7A), albeit at the population level, this improvement is not significant (p = 0.11). Of note, the presence of CD4 T cells also did not improve the response to LIGHT or 4-1BBL (Fig. 2.7A).

We next asked whether 4-1BBL can reduce the threshold required to trigger enhanced CD8 T cell expansion in donors with weak response to CD70. Monocytes were infected with CD70, 4-1BBL and LIGHT-expressing adenoviruses alone or in combinations and incubated with different doses of the influenza M1 peptide. Results are reported as fold-expansion at the optimal peptide dose in Figure 2.6B. In several donors that had minimal responses to CD70 alone, there was clear enhancement of T cell expansion in response to CD70 plus 4-1BBL, suggesting additive effects. Furthermore, the dose response curve and kinetics curve (Fig. 2.7 C and D) showed that 4-1BBL and CD70 together can induce potent expansion of influenza-specific CD8 T cells at a lower antigen dose and an earlier time point than either ligand alone in some donors. In contrast, LIGHT had no effect on T cell expansion even in the presence of 4-1BBL (Fig. 2.7B).

Soluble LIGHT may contribute to the ineffectiveness of LIGHT

We next explored possible underlying reasons for the limited costimulatory activity of LIGHT observed when expressed on APC. The ineffectiveness is unlikely due to improper folding and expression of the recombinant LIGHT molecule, as cells infected with LIGHT-adenovirus, in comparison to cAdV, showed dosage-dependent binding to anti-LIGHT antibody as well as to the soluble forms of its two receptors, HVEM-Fc and LTβR-Fc (Fig. 2.8A, B and C).
Figure 2.7

A. CD70

B. LIGHT

C. Ag dose response

D. Kinetics

Figure 2.7 4-1BBL rescues CD70 non-responders. A. As in figure 2.3, the number of influenza-specific CD8 T cells was measured after 8 days of co-culture with or without CD4 T cells. B. Fold expansion at optimal antigen dose in response to 4-1BBL, CD70, LIGHT, alone or in combination. C. Representative dose response curves as in figure 2.3B. (n = 4). D. Representative kinetics as in figure 2.3D. (n = 4).
Exploring the ineffectiveness of LIGHT. 

**A.B.C.** Ectopically expressed LIGHT on human monocytes is recognized by its two receptors, HVEM and lymphotoxin β receptor. Human monocytes were infected with recombinant adenovirus expressing LIGHT or with control empty vector. Monocytes were analyzed for binding of **A.** anti-LIGHT mAb, **B.** LTβR-Fc or **C.** HVEM-Fc as indicated in the figures. 

**D.** Detection of soluble LIGHT in culture of costimulation assay. Influenza/M1 peptide-pulsed monocytes were infected with LIGHT-AdV or cAdV, followed by incubation with or without purified autologous CD8 T cells. Supernatant was harvest five days after co-culture and analyzed for soluble LIGHT by ELISA. Optical density is reported as an indication of the relative quantity of the LIGHT protein. Data are representative of two independent experiments. 

**E.** BTLA, a second binding partner to HVEM, is also expressed on CD8 T cells in the culture, as indicated by the differential percent of CD8 T cells binding to anti-LIGHT antibody and those to HVEM-Fc. Purified CD8 T cells and infected monocytes were cocultured as described in D. Cells were analyzed on day 3 and 4 after coculture. Representative data are shown for day 3.
Soluble LIGHT has been shown to block the costimulatory activity of membrane-bound LIGHT (336). We next asked whether soluble LIGHT can be detected in our system. Monocytes were infected with LIGHT-adenovirus or cAdV with or without CD8 T cells. Culture supernatant was harvested for relative quantification of LIGHT using ELISA. We observed substantial increase in soluble LIGHT from the coculture of LIGHT-AdV infected monocytes and T cells as compared to LIGHT-AdV infected monocytes alone or cAdV infected monocytes with T cells (Fig. 2.8D). Thus, production of soluble LIGHT may inhibit the costimulatory activity of membrane bound LIGHT in our system.

Recent study showed that HVEM, the receptor for LIGHT, could also function as a ligand for the immunoreceptor tyrosine inhibitory motif-containing receptor, B and T lymphocyte attenuator (BTLA) (345, 346). We asked whether BTLA could be detected on T cells in our system. Figure 2.8E showed that a higher proportion of CD8 T cells bound to HVEM-Fc as compared to those bound to anti-LIGHT antibody on day 3 of culture (Fig. 2.8E), suggesting the presence of a second binding partner to HVEM.

**Generation of human ICOSL adenovirus and its functional analysis**

To generate the ICOSL adenovirus, human ICOSL was cloned from the human acute monocytic leukemia cell line (THP-1) (Fig. 2.9A). Plaques containing positive clones were then identified using flow cytometry (Fig. 2.9A). Expression of ICOSL on primary human monocytes infected with of ICOSL-adenovirus showed saturation (approximately 60% of the monocytes) at 100MOI (Fig. 2.9B), similar to cells infected with the other adenoviruses (Fig. 2.1). We next analyzed the expression of the ICOSL receptor (ICOS). In contrast to 4-1BB, ICOS is not induced on activated CD8 T cells when co-cultured with monocytes infected by cAdV alone (Fig. 2.9C, a). Furthermore, ICOS can be induced on activated CD8 T cells (CFSE-) when 4-1BBL is overexpressed on monocytes, albeit at day 5 post coculture, much later than the induction of 4-1BB on day 3 (Fig. 2.8C, b). Preliminary functional analysis of ICOSL revealed little expansion of influenza-specific CD8 T cells in response to ICOSL alone as compared to CD80 (Fig. 2.9D). Further analysis is required to assess the long term effect of ICOSL, perhaps in
Figure 2.9  Generation of human ICOSL-adenovirus and functional analysis. A. Generation of human ICOSL-adenovirus. Left panel: ICOSL expression on the source of cloning (THP-1) was detected by flow cytometry. Right panel: Adenovirus-containing supernatant was used to infect 293N3S cells, and expression of human ICOSL detected by flow cytometry. Positive plaques were used for large scale production of replication deficient hICOSL-AdV. B. Ectopic expression of ICOSL on primary human monocytes after infection by increasing dose of ICOSL-AdV as described in Fig. 2.1 (n = 2). C. Purified CD8 T cells were CFSE labeled and incubated with 4-1BBL-AdV or cAdV infected monocytes as described in Fig. 2.3. Cells were harvested on day 1, 2, 3, 4, 5 and 6 for analysis of ICOS expression. Representative FACS plots from day 4-6 are shown, gated on CFSE-CD8 T cells at different conditions: a) cAdV infected culture; b) 4-1BBL-AdV infected culture (n=3). D. ICOSL-AdV and CD80-AdV were compared in a costimulation assay as in Fig. 2.3. Percent of influenza-specific CD8 T cell expansion is reported. Top panel: representative FACS plots gated on CD8 T cells; Bottom panel: summary of fold expansion.
combination with 4-1BBL, given its impact on induction of ICOS expression on CD8 T cells.

Discussion
This chapter has identified 4-1BBL and CD70, but not LIGHT, as potential candidates for manipulating human memory CD8 T cell responses. Of the three TNF ligands, 4-1BBL is the most potent and consistent inducer of recall CD8 T cell expansion and increased lytic functions on a per cell basis. Although less effective than 4-1BBL, CD70 was a strong costimulator for 6 out of 12 donors and gave additive effects when combined with 4-1BBL. CD70 costimulation also led to a greater proportion of dual IFNγ/TNFα producing effector cells. The additive effects of 4-1BBL and CD70 and particularly their complementary effect on driving different aspects of effector function are relevant to chronic HIV infection. As discussed in chapter I, the ability of CD8 T cells to produce multiple cytokines such as IFNγ, TNFα and IL-2 is characteristic of an effective immune response (337), whereas impaired HIV-specific CD8 T cells are characterized by poor cytotoxic functions and reduced ability in producing multiple cytokines (22-25). Thus, 4-1BBL and CD70 costimulation together may serve as attractive candidates for immunotherapy during chronic HIV infection.

In addition to driving differential effector functions, 4-1BBL and CD70 are set apart by other qualitative differences, some of which are worth considering when designing therapies. Consistent with literature, this study showed that 4-1BB is an inducible receptor and its expression is transient whereas CD27 is constitutively expressed on memory CD8 T cells. Similarly during HIV infection, CD27 is expressed on the majority of HIV-specific CD8 T cells (234), whereas little 4-1BB could found on a range of HIV-tetramer+ cells directly ex vivo (unpublished). With regard to therapy, the more stringent regulation of 4-1BB expression may limit its costimulatory effect to cells that has been recently exposed to antigen and thus reduce potential pathology. In support of this view, transgenic expression of CD70 in uninfected mice led to B cell depletion (318), T cell-driven chronic immune activation and premature death due to immune deficiency (319).
Although more limited data is available for 4-1BBL-transgenic mice, constitutive expression of 4-1BBL under the control of an MHC class II promoter also results in B cell depletion, but with a much slower kinetics (338). Furthermore, 4-1BBL was found to be a potent stimulator at low antigen dose, more than 10 fold lower than that was required of CD70 in our system (Fig. 2.4C). As such, 4-1BBL may be particularly important for CTL responses against subdominant epitopes, which in turn maintains the breadth of CD8 T cell responses and exerts more pressure against immune escape. The effects of 4-1BBL on CD8 T cells were similar in the presence or absence of CD4 T cells, suggesting that 4-1BBL acts mainly on the CD8 T cells in this model. For CD70, there was a trend toward higher responses of CD8 T cells in the presence of CD4 help in some donors. Thus CD70 can influence CD8 T cells directly and may have additional effects via CD4 T cells. As emphasized in chapter I, the ability to directly stimulate CD8 T cells is of particular relevance to chronic HIV infection where there are declining CD4 T cells.

The finding that only half of the donors responded to CD70 costimulation in an Ag-dependent manner, whereas 12 out of 12 responded to 4-1BBL costimulation, suggests that CD70 may act on a subpopulation of T cells. The failure to respond to CD70 was not due to a weaker response overall, as some of the CD70 non-responders had high responses to 4-1BBL. Furthermore, the CD70 non-responders did not have a smaller starting population of influenza M1-specific CD8 memory T cells. Rather, we found that the influenza-specific memory CD8 pools from the CD70-responders contained a significantly lower frequency of cells expressing CD28 and a trend towards a lower frequency of cells expressing CD27 and CCR7. The loss of CD28 and CD27 has been associated with the stable accumulation of T effector function (334, 339). Based on examination of the surface phenotype of memory T cells specific for different viruses, it has been proposed that human CD8 T cells undergo a linear differentiation pathway from naive cells to memory and effector cells (340). In particular, memory cells (CD45RO+) have been subdivided into early memory (CCR7+CD27+CD28+) that have high proliferative capacity but low cytolytic activity; intermediate memory (CCR7-CD27+CD28-) and effector memory (CCR7-CD27-CD28-) that have the lowest proliferative capacity but the highest cytolytic function (341). The cell surface phenotype
of influenza-specific memory CD8 T cells from CD70 responders suggests that a greater proportion of their CD8 influenza-specific memory population exhibited this effector/memory phenotype. This might reflect a more recent or a higher exposure to influenza virus. Thus we hypothesize that CD70 stimulates donors whose memory pool is reflective of a more differentiated effector/memory phenotype. The finding that CD70 is particularly good at inducing TNFα and IFNγ producing cells is consistent with it acting on the more differentiated effector memory subset. Furthermore, CD27-deficient mice show minimal defects in the spleen, but substantial defects on CD4 and CD8 effector T cell numbers in the lung (342), also suggestive that the CD70-CD27 interaction is important for the maintenance of effector T cells. It is also of interest that CD70 costimulation becomes more effective when some form of help either via CD4 T cells or 4-1BBL is present.

HIV-specific CD8 T cells are largely CD28 negative (234). The effective CD70 costimulation of T cells from a memory pool with higher frequency of CD28- phenotype may be explained by direct stimulation of the CD28- T cell subset. However, when we purified CD28- T cells by depletion of the CD28+ subset from the CD8 or the total T cell pool, we were unable to demonstrate any Ag-dependent expansion of the T cells (unpublished). Thus although the CD70 responding donors have on average 30% of their T cells lacking CD28, the overall response of all the donors requires the presence of the CD28+ CD8 T cells. The finding that a higher frequency of CD28- T cells predicts a good response to CD70 may be more reflective of the overall phenotype of the memory population than evidence of a direct effect on CD28- T cells.

In contrast to CD70, 4-1BBL has been implicated in direct stimulation of CD28- T cells (255). The rescue of CD8 T cell responses in CD28- mice by stimulatory 4-1BB antibody (343, 344), however, requires the presence of ICOS (307). This observation prompted us to investigate the role of ICOSL on CD8 T cells. Preliminary evidence in this study showed that human ICOSL alone, when expressed on activated monocytes, has limited costimulatory effect on purified CD8 T cells in comparison to CD80. This lack of costimulatory activity may be due to delayed expression of ICOS on T cells in
comparison to receptors to other costimulatory ligands tested (Fig. 2.9C). Further analysis is required to analyze the role of ICOSL, particularly in combination with 4-1BBL, on the direct stimulation of CD28- T cells. Future experiments are discussed in Chapter V.

In contrast to our findings with 4-1BBL and CD70, the present studies suggest a minimal role for LIGHT on APC as a costimulatory molecule for activation of T cells, even though LIGHT was highly expressed on recombinant AdV modified-monocytes and capable of binding anti-LIGHT as well as its known receptors. Recent evidence shows that in addition to acting as a receptor for LIGHT, HVEM can function as a ligand for the immunoreceptor tyrosine inhibitory motif-containing receptor, B and T lymphocyte attenuator (BTLA) (345, 346). Thus, it is possible that HVEM-BTLA interaction predominates during T cell activation resulting in minimal impact of LIGHT-HVEM signaling in T cells. Consistently with this hypothesis, a second binding partner to HVEM can be induced in T cells in our culture (Fig. 2.8E). Soluble LIGHT is known to block the costimulatory activity of membrane-bound LIGHT (336). Indeed, soluble LIGHT was detectable by ELISA in the cultures containing T cells and LIGHT-modified monocytes, but not in the cultures lacking either T cells or LIGHT overexpression. The presence of soluble LIGHT in the cultures may be another reason why monocyte expressed LIGHT has minimal efficacy in T cell costimulation in this model.

In summary, results from this chapter as well as two previous studies on which I am a co-author (312, 313), proposed two candidate TNF ligands for further studies with HIV samples in chapter III: 4-1BBL and CD70, but not OX40L or LIGHT. 4-1BBL is the most potent and consistent stimulator of human memory CD8 T cells, increases cytolytic function per cell and requires a low dose of antigen. CD70, on the other hand, improves IFNγ/ TNFα coproduction and functions on CD8 T cells with a more differentiated effector/memory phenotype. Thus, the two qualitatively different molecules have complementary effects and serves as attractive candidates for manipulating human anti-viral memory response.
**Materials and Methods**

**Recombinant adenoviruses**

Replication defective adenovirus 5 expressing 4-1BBL (4-1BBL-AdV) or no transgene as a control (cAdV) were generated using the two-plasmid rescue method (347) and were reported previously (311). Following the same strategy, human CD70, LIGHT or ICOSL genes were cloned into the pDC315 vector independently using BamHI and EcoRI restriction sites. Vector fidelity was confirmed by sequencing. Replication defective adenovirus was generated and passaged through 293 cells. Large scale virus purification was done using virus-infected 293N3S cells. Following cell lysis, viruses were purified by two rounds of cesium chloride gradient ultracentrifugation. Virus titers were measured by plaque assay. The expressions of CD70, LIGHT and ICOSL protein were confirmed using specific monoclonal antibodies by FACS analysis of adenovirus-infected human monocytes and 293 cells. In addition, the integrity of the LIGHT molecule was confirmed using HVEM-Fc and LTβR-Fc binding (reagents kindly provided by Carl Ware, La Jolla Institute for Allergy and Immunology). Of note, cAdV or 4-1BBL-Adv treatment of the monocytes led to increases in the levels of MHC class I and II on the monocytes and in the transient induction of CD86 expression. Adenovirus infection did not influence the endogenous levels of CD80 or 4-1BBL (312).

**Donors and consent**

Healthy donors were between the age of 20 and 55, and 50% of donors were female. All donors gave informed consent, as approved by the University of Toronto human subjects review board. PBMC were purified by Ficoll-PaquePlus gradient centrifugation and frozen with 10% DMSO and 50% FBS in media at -150°C. Donors were screened for the presence of HLA-A2 and influenza M1/A2 tetramer binding cells by FACS analysis. Samples used in this study had an original influenza-tetramer positive population ranging from 0.08% to 0.6%.

**Costimulation assays**

Freshly-thawed PBMC were suspended at 8 X 10^6 cells/ml in media and plated at 0.1ml/well of a 96-well-plate. Adherent monocytes were enriched at 37°C for 1 hour and
then infected with adenoviruses overnight. Non-adherent cells were removed and stored overnight at 37°C, to be used as a source of T cells (see below). 4-1BBL-AdV, CD70-AdV, LIGHT-AdV alone or their combinations, or cAdV were added to the adherent monocytes at a multiplicity of infection (MOI) of 100, followed by centrifugation at 3000rpm at 37°C for 1 hour. The M1 influenza peptide (influenza HLA-A2-restricted GILGFVFTL, M1, 58-66) was added to the monocytes post-centrifugation at a concentration ranging from 4nM to 25µM for titration experiments. For subsequent experiments, the peptide dose that gave the highest influenza-specific CD8 T cell expansion over background for each donor was used. Following overnight incubation, the activated monocytes were washed to remove excess adenovirus and peptide. CD8 T cells or total T cells were purified from the non-adherent fraction using the negative selection kit from Miltenyi Biotec (MACS). CD8 T cell purity was approximately 92% routinely, whereas total T cell purity is approximately 99.5%. Purified CD8 or total T cells were added to activated monocytes at a concentration of 1 X 10^5 cells/well or 2 X 10^5 cells/well respectively. A small amount (0.04Unit/ml) of exogenous IL-2 (eBiosciences) was added to the pure CD8 T cell cultures, whereas no exogenous cytokines were added to the total T cell cultures. The low dose of IL-2 added to the CD8 T cells had been predetermined by titration on a subset of healthy donors as the minimal amount required for Ag and costimulation dependent CD8 T cell expansion. T cells and activated monocytes were co-incubated at 37°C for 7 to 9 days. T cell expansion was closely monitored by microscopy every day from day 6 of co-culture to avoid overgrowth.

**Flow cytometry**
Influenza M1/A2 tetramers were prepared at the CANVAC network for vaccine and immunotherapeutics (Montreal, Quebec) according to previously described methods (311, 348). Monoclonal antibodies used include: anti-h4-1BBL and -hCD70 from BD Biosciences, anti-hLIGHT (R&D Systems), anti-CD8α, -CD28, -CD27, -CCR7, -IFN-γ, -TNF-α, -IL-2 and -perforin from eBiosciences. For intracellular cytokine staining, samples were restimulated with 5µM M1 peptide for 6 hours in the presence of GolgiPlug (BD Biosciences). Restimulated cells were first stained with surface markers,
fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences), and followed by intracellular staining with monoclonal Abs. In all figures, gating of populations was based on staining with isotype controls.

**CTL assays**
The HLA-A2-expressing T2 cell line was used as target. T2 cells were pulsed with 5μM M1 peptides or irrelevant HLA-A2-restricted melanoma peptides overnight. The next day, targets were labelled with 200μCi of Na251CrO4 (Amersham) and incubated with effector T cells from day 8 of costimulation assay for 5 hours. Supernatant was collected for analysis of radioactive chromium release.

**ELISA**
ELISA plates were coated with 5ug/well of anti-human Ig (Fc specific, Sigma) in PBS at 37 deg for 90 min, and then blocked with 2%BSA for 60min. HVEM-Fc (1ug/well) in 1%BSA, 0.5%Tween PBS was then added for 1h at 37°C. Supernatant and cells from day-5 costimulation assay infected with LIGHT-AdV or cAdV, with or without purified CD8 T cells was harvested. Various dilutions of supernatant were added to the ELISA plates for a 2-hour incubation at 37deg. Lysed monocytes (with 1% NP40, equivalent of 20% cells from one well) infected with LIGHT-AdV was used as a positive control for ELISA. Soluble LIGHT was then detected with anti-human LIGHT antibody (1ug/well) followed by anti-mouse HRP (1:1000). ELISA was developed using ABTS as a substrate.

**Statistical analysis**
The unpaired two-tail Student’s t test with 95% confidence intervals was used for all statistical comparisons except figure 2.4C and E, where paired two-tail Student’s t test was used. Means +/- standard deviations were used to represent data from CTL assays.
CHAPTER III

- THE IN VITRO RESCUE -

“4-1BBL induces TRAF1-dependent Bim modulation in human T cells and is a critical component in the costimulation-dependent rescue of functionally impaired HIV-specific CD8 T cells”

The work presented in this chapter was published in:


T. Wen generated the adenovirus vectors; JP Routy, RP Sekaly provided access to PBMC from HIV infected donors and Nicole Bernard’s lab did HLA typing and ELiSpot analysis of the donors. I conducted all experiments myself.
Abstract

During chronic infection, HIV-specific CD8 T cells exhibit progressive signs of functional impairment, attributed to persistent antigenic stimulation, up-regulation of the inhibitory receptor PD-1, and declining T cell help. Strategies that directly improve CD8 T cell function offer the potential of restoring immune control of HIV. Although PD-1 expression has been identified as a cause of functional impairment in HIV, in this study, PD-1 expression was observed on only a subfraction of HIV-specific CD8 T cells in a subfraction of donors, whereas HIV-specific CTL from all donors exhibited a limited repertoire of effector functions. CD137L (4-1BBL) is emerging as an important stimulator of antiviral CD8 T cell responses. Regardless of the PD-1 status of the donors, here we show that 4-1BBL, when combined with CD80 or CD70, expands a population of Ag-specific CD8 T cells expressing multiple markers of effector function, from the functionally impaired starting population. In contrast, CD70 in combination with CD80 was insufficient for these effects. The unique contribution of 4-1BBL correlated with down-regulation of the proapoptotic molecule Bim in activated CD8 T cells. Decreasing the level of TRAF1 in T cells using siRNA resulted in increased levels of Bim in the 4-1BBL-stimulated T cells. Thus, costimulation via 4-1BBL leads to TRAF1-dependent Bim down-modulation in T cells, resulting in increased T cell expansion. These studies identify 4-1BBL as a critical component in therapeutic strategies aimed at improving CD8 T cell function.

Introduction

Cytotoxic T lymphocytes are important mediators of immune control of viral infection (9, 14, 15, 349-352). However, HIV persists and disease progresses in most infected individuals. As discussed earlier, the inability to control chronic viral infections is associated with the functional impairment of virus-specific CTL, which is exacerbated by the progressive loss of CD4 T cell help (180, 353-357). In support of a role for CD8 T cells in viral control, the function of HIV-specific CD8 T cells is intact in long-term nonprogressors (26, 358). Although it is arguable whether the loss of CTL function is the
cause or the effect of viral persistence, recent evidence shows that restoration of CTL function through the blockade of PD-L1, the ligand for the coinhibitory receptor PD-1, results in significant viral load reduction during chronic LCMV infection in mice, even in the absence of CD4 T cells (169). PD-1 is also up-regulated on HIV-specific CTL and its expression has been positively correlated with viral load (181-183, 359). Indeed, PD-L1 blockade can restore CTL function of HIV-specific T cells \textit{ex vivo} (181-183). However, LCMV challenge is lethal in PD-L1-deficient mice (169) and there are concerns that systemic PD-L1 blockade might lead to autoimmunity (360). Thus, Ag-specific strategies for improvement of CTL function during chronic viral infection in the context of high PD-1 expression remain an important therapeutic target for HIV.

CD137L (4-1BBL) is emerging as a prominent candidate for enhancing CD8 T cell function in HIV. A wealth of studies in mice have shown that 4-1BBL or anti-4-1BB antibodies improve anti-viral and anti-tumor CD8 T cell responses (254, 361, 362). 4-1BBL stimulation can prolong the survival of CD8 T cells following anti-CD3/CD28 stimulation (286, 363). 4-1BBL is also a potent adjuvant for memory antiviral responses of human CD8 T cells \textit{ex vivo} (311, 312), with more potent effects than CD80 (B7.1), OX40L (313), CD70, LIGHT or ICOSL as shown in Chapter II. However, to date, the functional capabilities of HIV-specific CD8 T cells expanded by costimulation in the absence of T cell help have not been evaluated. Recent studies have shown that control of viremia correlates with the ability of CD8 T cells to produce multiple cytokines (28, 337, 357). CD8 T cells which coproduce IL-2 and IFN-\(\gamma\) can function in the absence of CD4 help, whereas the presence of CD8 T cells that produce only IFN-\(\gamma\) is associated with functional impairment (180). In this study, we report the ability of costimulation with 4-1BBL in combination with CD80 or CD70 to induce an expanded population of CTL with multiple effector functions from a functionally impaired starting population of memory T cells in the absence of CD4 T cells. The critical role of 4-1BBL in the rescue of HIV-specific CTL function is explained at least in part by the ability of 4-1BBL-stimulated cells to maintain low levels of the proapoptotic molecule Bim via TRAF1 signaling.
Results

Functional status of HIV-specific CD8 T cells

To assess the effect of costimulation on the expansion and function of HIV-specific CTL, we first examined the surface phenotype and functionality of the starting population of HIV-specific CD8 T cells from a cohort of HIV-infected individuals. Consistent with literature (234) for most donors, the majority of HIV-specific CD8 T cells were CD27^+CD28^− (Table 3.1). After a brief restimulation of donor PBMC with relevant HIV-peptide, we found that, without exception, the HIV-specific CTL from this cohort are very poor producers of IL-2, and few cells express the degranulation marker CD107a (Fig. 3.1, A and B). As expected (364), T cells from most donors are capable of producing IFNγ. However, none of the HIV-specific T cells coexpressed IL-2 or CD107a with IFNγ and only one donor (HPI4, see Table 3.1) showed coexpression of TNFα and IFNγ by 26% of their HIV-specific T cells. The failure to detect coexpression of IFNγ with CD107a or IL-2 is not due to technical limitations as such populations are detected following costimulation (see Fig. 3.2). Thus, HIV-specific CD8 T cells from this HIV-infected cohort show a limited repertoire of effector molecules, consistent with the functional impairment of the vast majority of the HIV-specific CD8 cells.

We next examined the levels of PD-1 on the HIV-specific (tetramer^+) population of T cells from the same donors. Despite the functional impairment observed for virtually all the HIV-specific CTL in all donors analyzed, PD-1 was detected on HIV-specific CD8 T cells from only a subset of donors (Fig. 3.1C, right panel). For those PD-1^+ donors with detectable PD-1 expression, PD-1 was detected on only a subset of HIV-specific CTL, ranging from 11.9 to 30.6% of tetramer^+ T cells (Fig. 3.1C, left panel, Table 3.1). Overall, as previously observed (181), the presence or absence of PD-1 did not correlate with the immediate peptide-specific recall responses of the T cells (Fig. 3.1B).

Effect of 4-1BBL and CD80 costimulation on expansion of HIV-specific CD8 T cells

To test the role of costimulation in expanding HIV-specific CD8 T cells, we took advantage of the same model described in Chapter II in which autologous donor monocytes are pulsed with antigenic peptides and modified with replication defective
### Table 3.1

Unfractionated PBMC

<table>
<thead>
<tr>
<th>Patient ID (epitope)</th>
<th>CD4 counts</th>
<th>tetramer%</th>
<th>% of HIV-tetramer+ CD8 T cells that are</th>
<th>PD-1+</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPI1 (A2Gag)</td>
<td>343</td>
<td>0.86</td>
<td>1.61, 4.02, 81.8, 18.3</td>
<td>18.3</td>
</tr>
<tr>
<td>HPI2 (B8Nef)</td>
<td>470</td>
<td>2.5</td>
<td>14, 26.6, 47.3, 11.9</td>
<td>11.9</td>
</tr>
<tr>
<td>HPI3 (A2Gag)</td>
<td>630</td>
<td>0.7</td>
<td>11.3, 60.1, 25.5, 14.4</td>
<td>14.4</td>
</tr>
<tr>
<td>HPI4 (A2Gag)</td>
<td>1056</td>
<td>0.11</td>
<td>43.9, 30.6, 22, 8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>HPI5 (B8Nef)</td>
<td>594</td>
<td>5.33</td>
<td>6.54, 92.4, 0.95, 7.8</td>
<td>7.8</td>
</tr>
<tr>
<td>HPI6 (A2Gag)</td>
<td>991</td>
<td>1.39</td>
<td>7.58, 72.9, 18.6, 1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>HPI7 (B8Nef)</td>
<td>529</td>
<td>0.34</td>
<td>22.7, 56.8, 20.5, 3.2</td>
<td>3.2</td>
</tr>
<tr>
<td>HPI8 (B7p17)</td>
<td>1025</td>
<td>0.26</td>
<td>10.3, 60.4, 28.3, 22.1</td>
<td>22.1</td>
</tr>
<tr>
<td>HPI9 (A2Pol)</td>
<td>325</td>
<td>0.29</td>
<td>20.7, 55.2, 20.7, 5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>HPI9 (B7Nef)</td>
<td>325</td>
<td>0.31</td>
<td>1.21, 80.9, 17.4, 30.6</td>
<td>30.6</td>
</tr>
<tr>
<td>HPI9 (A2p15)</td>
<td>325</td>
<td>0.75</td>
<td>2.03, 74.1, 23.5, 2.7</td>
<td>2.7</td>
</tr>
</tbody>
</table>

**Table 3.1** *Unfractionated PBMC* were thawed, stained with HIV-tetramer, and analyzed for expression of PD-1, CD8, CD27, and CD28, as detected by flow cytometry, by gating on the tetramer-positive cells. The CD4 T cell counts in the starting population, prior to purification of CD8 T cells are also reported.
Figure 3.1 *HIV-specific CD8 T cells show a limited repertoire of effector functions regardless of PD-1 expression.*

**A.** LEFT: representative intracellular staining for cytokine production by CD8 T cells from an HIV-infected donor after peptide restimulation (see methods). RIGHT: HIV-tetramer staining on CD8 T cells before peptide restimulation.

**B.** LEFT: summary of responses of 9 donors. RIGHT: Cytokine production per tetramer+ CD8 T cell was obtained by dividing % cytokine+ of CD8 by % tetramer+ of CD8.

**C.** PD-1 expression on HIV-specific CTL was measured by staining unfractionated PBMC and gating on the HIV tetramer+ populations. LEFT: summary of percent PD-1 expressing tetramer+ cells. The five donors with more than 10% PD-1+ tetramer+ cells were classified as PD-1exp and those with less than 10% as PD-1nil. RIGHT: representative FACs analysis of PD-1 expression from one PD-1exp and one PD-1nil donor (gated on HIV-tetramer+ CD8 T cells).
recombinant adenoviruses (AdV) to express costimulatory ligands (see Materials and Methods (311)). Costimulation of isolated CD8 T cells with monocytes overexpressing 4-1BB increased the expansion of HIV-specific CD8 T cells for most donors (Fig. 3.2, Ai and Bi). In contrast, CD80 had a relatively minor effect on CD8 T cell expansion, with only two donors showing a significant effect (Fig. 3.2, Ai and Bi). Not surprisingly, the two CD80-responsive donors were those with the highest frequency of CD28^+ tetramer^+ CD8 T cells (Table 3.1, HPI4 and HPI7). However, the combination of CD80 and 4-1BB resulted in enhanced CD8 T cell expansion as compared with controls, for all donors tested (Fig. 3.2Bi). Thus, dual costimulation with 4-1BB and CD80 increases the proportion of donors that respond to costimulation and increases the overall level of HIV-specific CTL expansion.

**Following costimulation, expanded HIV-specific CD8 T cells express multiple markers of effector function upon restimulation**

The surface expression of CD107a upon peptide stimulation is indicative of degranulation and cytotoxic activity (365). The starting population of HIV-specific CD8 T cells expressed little or no CD107a, and a negligible population coexpressed CD107a and IFN-γ following peptide stimulation (Fig. 3.1B). In contrast, brief restimulation of the 4-1BBL-expanded cultures with specific HIV peptide revealed a significant IFNγ/CD107a coexpressing CD8 T cell population in most donors and the response was increased in all donors when both 4-1BBL and CD80 were included in the initial cultures (Fig. 3.2, Aii and Bii). Not surprisingly, the CD80-costimulated cultures, which contained few Ag-specific CD8 T cells, also contained a much lower frequency of peptide-specific CD107a^+/IFN-γ^+ CD8 T cells. Furthermore, the levels of cytokine production per cell were increased with 4-1BBL/CD80 costimulation in most donors, as demonstrated by a higher median fluorescence intensity (MFI) for IFN-γ staining (see Fig. 3.2 Aii). Analysis of CTL killing in a ^51^Cr-release assay confirmed the correlation between CD107a expression and CTL effector function and showed that 4-1BBL overexpression alone could induce some CTL function, with enhanced effects when combined with CD80 (Fig. 3.2Av).
Figure 3.2 Expansion and functional rescue of HIV-specific CTL by costimulation. Purified (at least 92%) CD8 T cells from HIV-infected donors were cultured with peptide-pulsed or control treated autologous monocytes that had been modified with respective adenoviruses as indicated. Controls included Ag with cAdV and costimulation without Ag for each donor. Cells were harvested and analyzed as in methods. A. Representative data from donor HPI1 at day 8. i, MHC I tetramer analysis. ii, Analysis of CD107α and IFNγ expression on CD8 T cells. iii, Analysis of IL-2 and IFNγ expression on CD8 T cells. iv, Isotype controls for ii and iii. v, Cytolytic effector function of T cells (reported as average SEM of triplicate cultures for donor HPI1 and representative of two donors). B, Summary of HIV-specific CTL expansion and function for each donor (n=11 epitopes from nine donors). i, Fold-expansion is calculated by dividing the number of HIV-specific live T cells recovered after costimulation by the number of Ag-specific cells recovered in the cAdV+Ag-stimulated cultures. ii, CD107α/IFNγ coexpression. iii, IL-2/IFNγ coexpression. The line marked Bg indicates the frequency of IL-2-producing cells below which the results cannot be distinguished from background. Statistical significance was analyzed relative to the control culture containing no Ag, but including costimulation (see Materials and Methods). All flow cytometry data are gated on CD8 T cells.
Although the IL-2-expressing populations observed are quite small, costimulation with 4-1BBL alone, and even more so 4-1BBL together with CD80 resulted in a population of CD8 T cells capable of producing both IL-2 and IFN-γ upon peptide restimulation (Fig. 3.2, Aiii and Biii). Overall, these data show that independently of CD4 T cells, HIV-specific CD8 T cells capable of expressing multiple markers of effector function upon restimulation can be generated from a functionally impaired starting population of CD8 T cells. 4-1BBL is a potent mediator of these effects, with enhanced effects when combined with CD80 costimulation.

**Activation of HIV-specific CD8 T cells by 4-1BBL and CD70**

The finding in Chapter II that CD70, although not as potent as 4-1BBL, could stimulate influenza-specific T cell responses from donors with a high proportion of CD28 tetramer+ CD8 T cells suggested it might be a good candidate for restoring functions of HIV-specific CD8 T cells, which are largely CD28 negative (Table 3.1). However, CD70 alone was found to have marginal activity in stimulating HIV-specific CD8 T cells for all donors tested (unpublished). We therefore tested CD70 in combination with CD80 or 4-1BBL (Fig. 3.3). The results show that the combination of 4-1BBL with CD70 is as least as good, if not better than 4-1BBL plus CD80 in generating HIV-specific CD8 T cells capable of producing multiple cytokines and degranulating upon restimulation (Fig. 3.3). Again, the increased expression of CD107a correlated with increased CTL activity in a 51Cr-release assay, with 4-1BBL/CD70 or 4-1BBL/CD80 costimulated cultures showing improved CTL function compared with control cultures. In contrast, the combination of CD80 and CD70 showed much weaker activity in these assays.

As shown in Fig. 3.1, a subset of donors exhibited PD-1 expression on a proportion of the starting population of HIV-specific CD8 T cells. To determine whether the presence of PD-1 in the cultures was indicative of greater functional impairment of the CTL, we identified the donors in Fig. 3.3 by the PD-1 status of their starting population of HIV-specific T cells (<10% PD-1+ = PD-1nil or >10% PD-1+ = PD-1exp). There was a trend to decreased expansion (Fig. 3.3A) and decreased production of IL-2 (Fig. 3.3D) in response to suboptimal costimulation with CD80 plus CD70 in the PD-1exp cultures, although this
**Figure 3.3** Differential effects of costimulatory combinations in rescuing functionally impaired HIV-specific CTL. Purified CD8 T cells were stimulated with the indicated costimulatory molecule combinations, expressed on autologous monocytes as described in figure 3.2. P values above a single set of data indicate significance relative to control with no Ag plus costimulation; those between two groups indicate significance between the different costimulatory conditions plus Ag. The filled symbols indicate T cells from donors in which there was a significant population (> 10%) of PD-1 expressing HIV-specific CTL in the starting population, whereas PD-1\textsuperscript{nil} represents donors with minimal PD-1 expression on the starting T cell population. The P values next to the vertical bars are a comparison between the PD-1\textsuperscript{exp} and PD-1\textsuperscript{nil} donors. A. Fold expansion of Ag-specific T cells. B. Frequencies of IFN\textgamma/CD107a co-producers and C. IFN\textgamma/IL-2 co-producers from the cultures in A, were determined as in figure 3.2.
did not reach statistical significance (p = 0.08 and p = 0.07, respectively). Cultures which included 4-1BBL showed no evidence of segregation between the PD-1\textsuperscript{nil} and PD-1\textsuperscript{exp} cultures.

Analysis of PD-1 expression in the presence and absence of costimulation

To determine whether 4-1BBL could influence the expression of PD-1 or its ligand directly, cultures from PD-1\textsuperscript{exp} donors were monitored for the expression of PD-1 and PD-L1 kinetically, before the onset of cell division, which begins at about day 4 (Fig. 3.4). Consistent with the literature (181), PD-L1 is expressed at low levels on unstimulated monocytes from HIV\textsuperscript{+} donors (Fig. 3.4A). By day 1, PD-L1 expression was further up-regulated on the monocytes which had been modified with 4-1BBL, CD80, and to a lesser extent cAdV (Fig. 3.4A). PD-L1 expression persists through day 3 of culture (Fig. 3.4A). PD-1 expression on PD-1\textsuperscript{+} HIV-specific CTL was maintained up to and including day 3 of coculture with AdV-modified monocytes regardless of whether the AdV expressed additional costimulatory ligands (Fig. 3.4B). Thus, PD-1 expression is independent of overexpression of costimulatory molecules in the cultures.

4-1BBL induces Bim down-regulation in activated virus-specific CD8 T cells

Data from HIV-specific and influenza-specific (chapter II) CD8 T cell responses identify 4-1BBL as a key costimulator for induction of CD8 T cells with multiple effector functions, even from a starting population of functionally impaired memory T cells. The effects of 4-1BBL on expansion of this population could be due to effects on cell division or survival or both. I measured the rate of cell division of influenza-specific CD8 T cells from healthy donors using CFSE. Analysis of the CFSE profiles shows that by day 4 of culture few cells have divided, whereas by day 8, based on MFI of undivided vs divided populations it appears that the cells have divided about four times (Fig. 3.5A). CD70-stimulated cells showed a slight delay in the kinetics of cell division compared with 4-1BBL, but by day 8, 4-1BBL- and CD70-stimulated T cells showed a similar proportion of divided cells (Fig. 3.5A). Both CD70- and 4-1BBL-stimulated T cells up-regulated the anti-apoptotic Bcl-2 family member Bcl-xL, with CD70-stimulated T cells showing, if anything, marginally higher levels of Bcl-xL (Fig. 3.5B, left panel). However, by day 8 of
Figure 3.4 Kinetics of PD-L1 and PD-1 expression. Purified CD8 T cells from four PD-1exp HIV donors were CFSE labeled and stimulated with modified autologous monocytes each loaded with a specific HIV peptide as in figure 3.2. Uninfected monocytes and unstimulated purified CD8s were kept in media and harvested at day 1. 4-1BBL-AdV, CD80-AdV or cAdV modified monocytes and the cocultured CD8s were harvested at day 1 and 3 for FACS analysis. Representative FACS plots from one donor (HPI9) are shown in A and B. A. FACS plots are gated on monocytes based on forward and side scatter. PD-L1 expression from 4-1BBL-AdV modified monocytes is shown in black line, uninfected monocytes in grey line and isotype control is shown in solid grey. B. Expression of PD-1 on HIV-tetramer+ CD8 T cells. The 4-1BBL-stimulated compared to the unstimulated tetramer+ population is shown.
Figure 3.5 Role of 4-1BBL and CD70 in cell division and survival of influenza specific CD8 T cells. Isolated CD8 T cells from A2-positive healthy donors were stimulated for 4, 6 or 8 days with costimulatory molecule modified autologous monocytes and influenza M1 peptide. A. The CD8 T cells were first labeled with CFSE, and after culture, cells were stained with influenza-tetramer and CD8, and analyzed by flow cytometry. Representative FACS plots are shown gated on influenza-tetramer+ CD8 T cells (n = 6). B. Left panel: Representative Bcl-x<sub>L</sub> expression on influenza-specific CTL (n = 4). Right panel: number of tetramer+ cells recovered from the same cultures. Error bars are based on standard deviation for the same donor in two separate experiments. Similar results were obtained for three other donors. C. Bim expression in influenza-specific CTL. Left panel shows representative FACS plots. Right panel reports the summary of differential Bim expression in response to 4-1BBL and CD70 costimulation after both 6 and 8 days of culture with data for each donor linked.
culture, the 4-1BBL-stimulated cultures had accumulated 2- to 3-fold more Ag-specific T cells (Fig. 3.5B, right panel), suggesting a survival advantage not explained by Bcl-xL expression.

Bim is a proapoptotic BH3-only member of the Bcl-2 family that contributes to the death of activated (366) and memory (292) T cells. Previous studies (292) have shown that in activated primary mouse T cells Bim is maintained at lower levels in the presence of TRAF1. Because 4-1BB recruits TRAF1 during signaling in T cells (285), we compared the levels of Bim in tetramer+ T cells stimulated by CD70 vs 4-1BBL. At both days 6 and 8 of coculture, the expression of Bim in influenza-specific CTL is lower (in both percent and MFI) for all donors examined in response to 4-1BBL compared with CD70 costimulation (Fig. 3.5C). These data suggest that a unique aspect of 4-1BBL-mediated costimulation is its effectiveness in maintaining low levels of Bim in the activated T cells.

**4-1BBL-induced Bim modulation is TRAF1 dependent**

To determine whether the Bim modulation observed is directly linked to 4-1BBL-induced effects on the CD8 T cells, we used siRNA to knockdown TRAF1 expression in the CD8 T cells (Fig. 3.6A). As compared with transfection with scrambled RNA, the level of Bim expression is significantly higher in 4-1BBL-expanded influenza-specific CD8 T cells treated with siRNA to reduce TRAF1 expression (Fig. 3.6B). In contrast, TRAF1 siRNA had little effect on the level of Bim expression in CD70-induced tetramer+ cells (Fig. 3.6B). Furthermore, 4-1BBL-induced expansion of influenza-specific CD8s was significantly reduced in TRAF1 siRNA-transfected compared with control (scrambled siRNA) transfected T cells (Fig. 3.6C) for 4-1BBL-stimulated cultures. In contrast, the effect of TRAF1 siRNA on CD70-dependent T cell expansion was marginal (Fig. 3.6C).

**Analysis of division and survival of costimulated HIV-specific CD8 T cells**

As was the case for healthy donor influenza-specific CD8 T cells, HIV-specific CD8 T cells showed no evidence of cell division for the first 4 days of culture (data not shown). For HIV-specific T cells, recovery of cells was lower with individual costimulatory ligands (Fig. 3.7A). The combinations of 4-1BBL/CD80 and 4-1BBL/CD70 each induced
**Figure 3.6** Decreasing **TRAF1** by siRNA results in increased levels of **Bim** during 4-1BBL mediated costimulation. CD8 T cells from healthy donors were purified and transfected with either scrambled RNA (scRNA) or siRNA specific for TRAF1. After resting overnight, transfected T cells were coincubated with modified monocytes for 8 days. **A.** Representative Western blot showing TRAF1 and β-actin expression in CD8 T cells stimulated with 4-1BBL-modified monocytes, harvested at day 8 of coculture. A total of 100 nM (1) or 300 nM (3) of either scRNA or siRNA were used in this experiment. 300nM of siRNA was used for transfection in all other experiments. **B.** Upper panel, Representative Bim expression gated on influenza-tetramer+ CD8 T cells harvested from 8 days of coculture. Lower panel, Summary of Bim expression in influenza-specific CD8 T cells stimulated with 4-1BBL-modified monocytes (n=5, data are representative of two separate experiments). **C.** Upper panel, Representative FACS plots from one donor, gated on CD8 T cells. Lower panel, Summary of the percent of influenza-tetramer CD8 T cells (n=5, data are representative of two separate experiments).
comparable cell division (about two to four divisions between days 4 and 8, Fig. 7A and data not shown). In contrast, few cells were recovered from the CD70/CD80 cultures, although those CFSE-labeled cells that were recovered showed evidence of division (Fig. 3.7A). The absence of CFSE-negative tetramer+ T cells and the finding that the bulk of the costimulated cells showed evidence of two to four divisions (Fig. 3.7A and data not shown) argue that cells expanded only 4- to 16-fold in the cultures. This limited number of divisions makes it unlikely that the functional T cells observed in Figure 3.2 arose from a rare functional cell that outgrew the nonfunctional starting population.

Ag-specific T cells from 4-1BBL/CD80-stimulated cultures showed lower levels of Bim expression than T cells stimulated by CD70/CD80, consistent with increased T cell survival in these cultures (Fig. 3.7B). Furthermore, there was an inverse correlation between Bim levels and the recovery of Ag-specific cells that had divided in culture (Fig. 3.7C, p < 0.0001). These data suggest that 4-1BBL contributes to CD8 T cell survival at least in part through maintaining lower levels of Bim in the activated T cells.

Discussion

In the face of viral persistence, CD8 T cell effector function deteriorates over time (9, 14, 15, 26, 180, 349-358). This functional impairment may reflect the delicate balance that the host must maintain between controlling viral infection and avoiding long-term tissue damage; improving CD8 T cell function, however, can reduce viral load (169). In this chapter, we provide evidence that functional impairment in HIV-specific CD8 T cells can be overcome by costimulation. We found that overexpression of 4-1BBL in combination with CD70 or CD80 results in a population of HIV-specific CD8 T cells exhibiting key features of functional restoration, from a functionally impaired HIV-specific memory CD8 T cell population. In contrast, CD70 in combination with CD80 was much less effective than either combination involving 4-1BBL, identifying 4-1BBL as a key player in rescuing HIV-specific CTL from functionally impaired precursors.
4-1BBL-mediated costimulation results in lower Bim expression in HIV-specific CTL. CD8 T cells from HIV-infected subjects were CFSE labeled and cocultured with AdV-modified autologous monocytes for 8 days. Cells were stained and analyzed as in figure 5. A. Representative CFSE labeling results for HIV-specific CD8 T cells. B. Upper panel, representative histograms of differential Bim expression on dividing (CFSElo) HIV-specific CTL from two donors in response to 4-1BBL/CD80 and CD70/CD80 dual costimulation. Lower panel summarizes data from 7 HIV+ donors. C. The number of dividing HIV-specific CTL was plotted against the percent of Bim+ CFSElo HIV-specific CTL. Only results from dual costimulation were included since single costimulation did not allow recovery of sufficient cells for analysis, as reflected in A. Statistics was calculated using SPSS software (negative correlation, r = -0.7909, p < 0.0001).
The 4-1BBL mediated expansion of functional CD8 T cells from a functionally limited starting population could be due to recruitment of naive T cells, the outgrowth of a small population of functional T cells that were below the limits of detection at the start of the culture, or could represent a gain of function. By gating on the tetramer-positive CD8 T cells combined with CFSE labeling, we observed that there was minimal cell division during the first 4-5 days in cultures with T cells from either healthy donors (Fig. 3.5) or HIV-infected donors (data not shown). By day 8 of culture, a substantial portion of the CFSE-labeled tetramer-positive cells in the costimulated cultures showed evidence of two to four divisions (Figs. 3.5 and 3.7, and data not shown), with very few cells observed in the undivided population and no evidence that there is a subpopulation of highly divided (CFSE negative) tetramer+ T cells. Thus, it is unlikely that the functional T cells recovered after costimulation in HIV infected donor T cell cultures arise from either naive precursors or from a rare subpopulation of functional memory T cells in the starting population, as such a population should have been detected in the starting tetramer+ population to account for its presence after only four divisions. Furthermore, culture of HLA-A2-positive healthy donor T cells with syngeneic monocytes expressing 4-1BBL and CD80 together with A2-restricted HIV-peptides for 8 days failed to induce any Ag-specific T cells (data not shown). Together, the evidence suggests that following division in the presence of overexpressed costimulators, the functionally impaired HIV-specific CD8 T cells gain multiple effector functions.

Although PD-1 has been shown to correlate with functional impairment during chronic viral infections and blockade of PD-1/PD-L1 interaction can rescue CTL from PD-1-expressing nonfunctional precursors (367), in this study, we observed functional impairment of CD8 T cells even in the absence of detectable PD-1 expression. This suggests there may be other mechanisms causing functional impairment in HIV-specific CD8 T cells, and certainly one of these conditions may be the absence of CD4 T cells (178), which are progressively lost during HIV infection. A number of studies have shown that CD8 T cells primed or maintained in the absence of CD4 T cell help are impaired in their ability to respond to Ag and control infections (368-374). IFNγ/IL-2-secreting CD8 T cells have been reported to support proliferation of HIV-specific CTL
independently of CD4 (337). The finding that 4-1BBL can stimulate isolated CD8 T cells to expand and acquire multiple effector functions argues that it may overcome deficits in CD8 T cells by contributing to IL-2 production by the T cells themselves, perhaps overcoming the absence of CD4 T cells.

Despite the limited expression of PD-1 in these donors, the data in Fig. 3.3, showed a trend toward greater functional impairment of T cells from donors with higher PD-1 expression: after suboptimal costimulation with CD70 plus CD80, donors with >10% PD-1+ Ag-specific T cells showed decreased expansion (p = 0.08) and a lower frequency of multifunctional T cells (p = 0.07), compared with T cells from donors with minimal PD-1 expression. In contrast, 4-1BBL-containing cultures showed enhanced expansion of T cells regardless of the PD-1 status of the starting population (Fig. 3.3). Because of the relatively low proportion of PD-1-expressing cells in the cultures, it is difficult to determine whether 4-1BBL can directly impact this population of cells. It should be noted that since the publication of this study, we became aware that the commercial antibody used in this study underestimates the level of PD-1 expression as compared to the antibody generated by Freeman et al. (R. Ahmed, personal communication). Thus, it is quite possible the PD-1 levels on HIV-specific CD8 T cells in this study are much higher than those measured, supporting the idea that 4-1BBL costimulation can function independent of the presence of PD-1 on T cells.

As shown in Chapter II, CD70 was efficacious in stimulating T cells from a subset of healthy donors with a higher frequency of CD28 T cells in the starting memory population, perhaps reflecting a more recent Ag exposure or a more differentiated state of the T cells. Despite its efficacy in stimulating influenza-specific CTL, CD70 was unable to rescue the expansion of HIV-specific CD8 T cells from any of the HIV-infected donors. However, in combination with 4-1BBL, CD70 was at least as good as CD80 in increasing the effects of costimulation through 4-1BBL (Fig. 3.3).

Why is 4-1BBL more effective than CD70 in rescuing HIV specific CD8 T cells? Both 4-1BB and CD27 function by recruiting TRAFs, which link them to NF-κB activation, Bel-
4-1BB induction, and the survival of T cells (254). 4-1BB has been identified as an important survival factor for mouse and human CD8 T cells (269, 375). Similarly, CD27 provides survival signals to CD8 T cells subsequent to the effects of CD28 (314). In humans, CD70 can promote the proliferation and resistance to apoptosis of CD8 T cells and when adoptively transferred back into an HIV-positive donor, CD27+ HIV-specific CD8 T cells survived longer compared with CD27- HIV-specific CD8 T cells (300).

The survival of activated T cells is greatly affected by the balance between the pro- and antiapoptotic Bcl-2 family members (376). In particular, Bcl-2 and Bcl-xl function to oppose the effects of the upstream proapoptotic Bcl-2 family member Bim (376). Previous results have shown that 4-1BBL stimulation of human CD8 T cells results in Bcl-xl up-regulation, under conditions where Bcl-2 remains constant (311). Consistent with these findings, we found that T cells expanded using both 4-1BBL and CD70 showed evidence of Bcl-xl up-regulation. However, we observed greater recovery of cells from 4-1BBL-stimulated cultures, correlating with decreased levels of Bim for both HIV and influenza-specific CD8 T cells (Figs. 3.5 and 3.7). Recent work from our laboratory has shown that the presence of TRAF1 in activated and memory T cells plays a critical role in their survival, through maintaining low levels of Bim. Bim is transcriptionally activated by the FOXO3A transcription factor in cells following cytokine withdrawal (377). In addition, Bim can be down-regulated in cells by ERK phosphorylation, leading to its proteasome-dependent degradation (378). TRAF1 is recruited to the 4-1BB signaling complex in T cells (285) and 4-1BB signaling has been linked to ERK activation (286). Thus, a plausible model for the effects of 4-1BBL on Bim levels in activated T cells is that 4-1BB signaling leads to TRAF1 recruitment and Bim down-modulation via an ERK-dependent mechanism. Indeed, decreasing the levels of TRAF1 in CD8 T cells using siRNA resulted in increased levels of Bim in the T cells and decreased T cell expansion following 4-1BBL costimulation, compared with T cells treated with scrambled RNA (Fig. 3.6). This result suggests that TRAF1-dependent Bim down-modulation enhances T cell survival following 4-1BB signaling in T cells. CD27 is known to recruit TRAF2 and 5 in T cells (379-381) both of which are required for CD27 signaling (380, 381) (382), but to date, evidence that CD27 signaling recruits TRAF1 in
T cells is lacking. Down-regulation of TRAF1 in T cells had minimal effects on CD70-mediated stimulation (Fig. 3.6). Thus, differential recruitment of TRAF1 may explain the different efficacy of 4-1BBL and CD70 in Bim down-regulation.

In addition to its effects on rescuing CTL via survival effects leading to a greater number of T cells, it is likely that 4-1BBL contributes more directly to increased effector functions as shown in Chapter II. T cells from 4-1BBL-stimulated cultures show increased levels of perforin per cell (Ref. (311) and chapter II Fig. 2.5B) and, on a per cell basis, 4-1BBL-stimulated cells were found to be more efficacious in killing target cells than CD70-stimulated T cells (Fig. 2.5C). Thus, the effects of 4-1BBL appear to be more than just promoting survival of functional cells, rather 4-1BBL can improve the effector function of epitope-specific CTL on a per cell basis.

In sum, the data in this report show that costimulation by 4-1BBL in combination with other costimulatory ligands can expand fully functional HIV-specific CTLs from a starting population with limited functional capabilities. Although the effects of 4-1BBL were enhanced when combined with other costimulatory ligands such as CD80 or CD70, 4-1BBL was an essential component of the costimulation, at least in part through its effects on TRAF1-dependent Bim down-modulation in activated T cells. The finding that these effects are independent of the presence of CD4 T cells or PD-1+ T cells in the starting cultures raises the possibility of a therapeutic application for 4-1BBL to generate functional HIV-specific T cells. One caveat of this study is that the ex vivo cultures lack the persistent antigen which drives functional impairment in vivo. Moreover, the in vitro cultures include IL-2, albeit at low levels, which may also contribute to the recovery of the T cells in response to 4-1BBL. I next examine the in vivo effect of the 4-1BB pathway in chapter IV.

**Materials and Methods**

*Donors and consent*
PBMC were obtained from HIV+ and HIV− volunteers and cryopreserved until use. For HIV+ donors, PBMC were obtained by leukapheresis. All donors gave informed consent as approved by the research ethics boards of all participating institutions. HIV+ donors were HLA typed and ELISPOT analysis was used to determine their CD8 T cell specificities using a panel of overlapping HIV peptides as previously described (312) and the dominant epitopes selected for further study. Donor HPI9 had three dominant reactivities and all three were studied (Table 3.1). Table 3.1 summarizes the properties of the starting population of HIV-specific CD8 T cells. Healthy donors were identified as HLA-A2 positive by flow cytometry and studied using the immunodominant influenza matrix M1 peptide.

**Tetramers and peptides**

Biotinylated MHC I/peptide monomers were prepared as previously described (311, 348). Monomers were conjugated with extravidin-PE (Sigma-Aldrich). The HIV tetramers used include: A2-Gag (SLYNTVATYL), A2-Pol (ILKEPVHGV), A2-p15 (FLGKIWPSYK), B7-p17 (RPGGKKKYKL), B7-Nef (TPGPGVRYPL), and B8-Nef (FLKEKGGL), and the influenza tetramer is A2-M1 (GILGFVFTL). Peptides were obtained from the Sheldon Biotechnology Center (McGill University, Montreal, Quebec, Canada) or from the Alberta Peptide Institute (Edmonton, Alberta, Canada).

**Costimulation assays**

PBMC were suspended at 8 X 10^6 cells/ml and plated at 0.1 ml/well of a 96-well plate. Adherent monocytes were enriched at 37°C for 1 h and then infected with adenovirus overnight. Non-adherent cells were removed and stored overnight at 37°C, to be used as a source of T cells. 4-1BBL-AdV, CD80-AdV, CD70-AdV, LIGHT-AdV alone or their combinations, or control AdV (cAdV) were added to the adherent monocytes at a multiplicity of infection of 100, as determined by titration experiments (chapter II, Fig. 2.1), followed by centrifugation at 3000 rpm at 37°C for 1 h. Of note, infection of monocytes with a mixture of AdV carrying either the 4-1BBL or the CD80 gene resulted in substantial coexpression of the two costimulatory molecules (312). Similar results were observed for 4-1BBL and CD70 coexpression (data not shown). The influenza M1
peptide was added to the monocytes at an optimal concentration as determined in Chapter II for each donor. For HIV experiments, peptide titrations were performed between 0.5 and 3µM (data not shown) and the peptide concentration that gave the highest response for each donor was used for additional experiments. An irrelevant HLA-A2-restricted melanoma peptide was used for all A2 donors or no peptide was added for other HLA types. The “no Ag control” cultures included monocytes modified by both 4-1BBL- and CD80-AdV. Following overnight incubation, the activated monocytes were washed to remove excess AdV and peptide. CD8 T cells were purified from the nonadherent fraction using the negative selection kit from Miltenyi Biotec (MACS). CD8 T cell purity was routinely ≥ 92%. For CFSE experiments, purified CD8 T cells were labeled with 0.5 µM CFSE. CD8 T cells were added to modified monocytes at a concentration of 7.5 X 10^5 cells/ml. A small amount (0.04 U/ml) of exogenous IL-2 (eBioscience) was added to the pure CD8 T cell cultures. The low dose of IL-2 added to the CD8 T cells had been predetermined by titration on a subset of healthy donors as the minimal amount required for Ag- and costimulation-dependent CD8 T cell expansion. For several HIV donors, the addition of exogenous IL-2 was not required to achieve functional expansion in response to dual costimulation including a PD-1exp donor (data not shown); however, for consistency we added 0.04 U IL-2/ml for all CD8 T cell cultures, both healthy and HIV, a dose that is far below the level required to overcome T cell anergy (10, 16, 21, 35, 36). T cells and activated monocytes were coincubated at 37°C for 7–9 days. Kinetic studies were done to identify the peak of expansion of Ag-specific CD8 T cells for every HIV donor reported (data not shown).

**CTL functional analysis by 51Cr-release assay**

The T2 cell line and the EBV-transformed BL7 cell lines (provided by Dr. M. Ostrowski, University of Toronto, Toronto, Canada) were used as target. For HLA-A2-restricted donors, T2 cells were pulsed with 5 µM of either relevant HIV peptides, or irrelevant HLA-A2-restricted melanoma peptides overnight. For HLA-B8-restricted donors, BL7 cells were pulsed with 5 µM relevant HIV peptides overnight. The next day, targets were labeled with 200 µCi of Na_2^{51}CrO_4 (Amersham) and incubated with effector T cells for 5 h. Supernatant was collected for analysis of radioactive chromium release.
**Flow cytometry**

Cell fluorescence was measured using a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star). The following mAbs were used (human (h) specific): anti-h4-1BBL, -hCD70, -hCD107a, -h4-1BB, and their respective isotype controls (BD Biosciences); anti-hLIGHT and -hHVEM (R&D Systems), anti-hCD8α, -hCD28, -hCD27, -hPD-L1, -hPDL2, -hIFN-γ, -hTNFα, -hIL-2, -hBcl-xL, and -perforin (eBioscience), anti-Bim (Alexis Biochemicals). For intracellular cytokine staining, samples were restimulated with 5 µM peptide for 6 h in the presence of GolgiPlug (BD Biosciences). Restimulated cells were first stained with surface markers, fixed, and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences), followed by intracellular staining. In all figures, gates were drawn based on staining with isotype controls.

**RNA interference to decrease TRAF1 levels in T cells**

Purified CD8 T cells were transfected with 300 nM of either TRAF1 siRNA or scrambled RNA (Integrated DNA Technologies) using the Amaxa Human T cell Nucleofector kit. Transfected cells were rested overnight at 37°C and stimulated with modified monocytes as described in the costimulation assays. Cells were harvested at day 8 of coculture for both Western blot and FACS analysis. TRAF1 knockdown was verified by Western blot using anti-TRAF1 Ab (Cell Signaling Technology). Five of the eight donors showed significant knockdown (50%) and were followed by FACS analysis for Bim expression and expansion of tetramer-positive cells.

**Statistical analysis**

Statistics were performed using paired the Student t test for parameters measured in the same donor and the unpaired Student t test otherwise. Values of p < 0.05 were considered significant. Correlation was analyzed using the two-tailed bivariate test with Spearman coefficients.
Chapter IV

- THE IN VIVO THERAPY -

“Loss of TRAF1 during chronic viral infection desensitizes the 4-1BB costimulatory pathway: implications for therapy”

The work presented in this chapter has been submitted:


A.J. McPherson helped with western blots and mice dissections; K.S. Kawamura and A.R. Elford helped with LCMV infections; R.B. Jones and N. Aidarus helped with HIV suppression assays; G.H.Y. Lin helped with mice dissections and initial pilot experiments; P.A. Lang and T. Ambagala helped with LCMV viral titer assays; M. Pellegrini and T. Calzascia helped with initial pilot experiments. F.Y. Yue processed and stored some HIV specimen; E. Kremmer provided the anti-human-TRAF1 antibody; C.M. Kovacs, E. Benko, C. Tremblay, J.P. Routy and N.F. Bernard provided HIV patient samples.
Abstract
Chronic infection places an enormous burden on human health. Regulatory mechanisms have evolved to limit immune pathology, but can impair viral control during persistent infection. Here we show that the signaling adaptor TRAF1 is lost from virus specific CD8 T cells during chronic infection with HIV in humans or LCMV clone 13 in mice. In contrast, TRAF1 is maintained at a higher level in virus-specific T cells of HIV controllers or during acute LCMV infection. TRAF1 expression negatively correlates with HIV load during the chronic phase of infection and knocking down TRAF1 in CD8 T cells from viral controllers results in decreased HIV suppression and 4-1BBL-dependent HIV-specific T cell responses. Transforming growth factor β can cause loss of TRAF1, whereas IL-7 can correct the TRAF1 expression defect during chronic infection. Combined therapy with IL-7 and agonist antibody to the TNFR family member 4-1BB results in TRAF1-dependent expansion of virus specific CD8 T cells and improved clearance of established clone 13 infection. These findings describe a novel mechanism of immune dysfunction during chronic infection and a means to correct it.

Introduction
Immune dysregulation is a hallmark of chronic viral infection (383). Chronic infection with HIV or Hepatitis C virus (HCV) in humans or with LCMV clone 13 in mice results in upregulation of inhibitory receptors, such as programmed death 1 (PD-1) and TIM-3 on the effector T cells, as well as the sustained production of immune regulatory cytokines such as TGFβ and IL-10 (169, 181-184, 199, 205, 215, 384). It is thought that these regulatory mechanisms, while minimizing immune pathology, contribute to the inability of the immune system to control viral load during progressive HIV infection. Although highly active antiretroviral therapy (HAART) has significantly reduced AIDS-related morbidity and mortality, antiretroviral drugs do not fully restore immune dysregulation and patients often fail multi-drug treatment (5, 7). Thus, there is a need for additional therapies. Interfering with IL-10 or TGFβ signaling early in disease or blockade of PD-L1 and TIM-3 later in chronic infection, results in expansion of functional effector T cells and improved viral clearance (169, 199, 205, 215). Moreover,
blockade of the PD-1 inhibitory pathway has shown promise in an SIV infection model in primates (195). Notwithstanding these promising results, it is likely that control of chronic HIV infection will require a multi-pronged approach.

4-1BB is a costimulatory member of the tumor necrosis factor receptor (TNFR) family that is transiently induced upon T cell receptor signaling (290). The study presented in Chapter III revealed that overexpression of its ligand, 4-1BBL, can reinvigorate exhausted HIV-specific CD8 T cells from chronically infected donors *ex vivo* (274). It was unclear, however, why the endogenous 4-1BB pathway failed to protect CD8 T cells from exhaustion during HIV infection *in vivo*. It is possible that costimulation was insufficient to overcome the extensive inhibitory mechanisms at play, or that the ligand was simply limiting *in vivo*. Here, we provide evidence that the 4-1BB costimulatory pathway is actually desensitized during chronic infection and suggest a means of overcoming this limitation to provide a novel immunotherapy for HIV.

4-1BB signals by recruiting two TNFR associated factors, TRAF1 and TRAF2 (284, 285, 385). TRAF2 is a ubiquitously expressed protein that is required for NF-κB and MAPK activation downstream of several TNFR family members, including 4-1BB (386). TRAF1 is an NF-κB inducible protein, whose expression is low in resting cells and limited to cells of the immune system (387). In T cells, overexpression of TRAF1 results in delayed contraction of LCMV-specific CD8 T cells (388) and deficiency of TRAF1 impairs the survival of activated and memory CD8 T cells, due at least in part to increased levels of the proapoptotic molecule BIM (292). Moreover, knockdown of TRAF1 in primary human influenza-specific T cells results in increased levels of BIM and decreased T cell expansion in response to 4-1BBL as shown earlier (274).

In this study, we provide evidence that TRAF1 levels are significantly lower in HIV-specific CD8 T cells from chronically infected as compared to recently infected donors or viral controllers. Similarly, during chronic infection of mice with LCMV clone 13, TRAF1 is lost from virus-specific T cells between day 7 and 21 of infection. In contrast, TRAF1 levels are maintained at higher levels in memory T cells following acute
infection with the Armstrong strain of LCMV. This defect in TRAF1 expression can be overcome by treating mice with IL-7. Moreover, a combined therapy with IL-7 and anti-4-1BB administered 21 days after clone 13 infection results in TRAF1-dependent expansion of virus specific T cells and decreased viral load. This study identifies a new mechanism of immune dysfunction during chronic infection and a therapy to overcome it.

**Results**

*Defective TRAF1 expression during chronic HIV infection*

As TRAF1 is critical for 4-1BB-induced survival signaling (274, 288), we examined TRAF1 expression in HIV-specific T cells from recently and chronically infected donors (Table 4.1) by flow cytometry (Fig. 4.1). The proportion of HIV-tetramer+ T cells expressing TRAF1 was significantly lower in individuals at the chronic as compared to the early stage of the infection, whereas viral controllers showed an intermediate phenotype (Fig. 4.2A). As TRAF1 is inducible upon cell activation, the level of activation of the T cells could be a confounding factor in comparing the different groups. To address this issue, we measured expression of the activation marker CD38 on the HIV-specific (tetramer+) T cells (Fig. 4.3A). The early and chronic infection groups showed similar levels of CD38 expression and viral load, whereas the viral controllers showed lower CD38 expression, consistent with their lower viral load (Fig. 4.3B). Overall, the frequency of TRAF1 expressing cells correlated with the frequency of activated T cells, as measured by CD38 staining (Fig. 4.3C). Analysis of the frequency of TRAF1 positive versus CD38 positive HIV-specific CD8 T cells reveals that HIV-specific T cells in viral controllers have higher levels of TRAF1 for a given level of activation than T cells from chronic progressors (p=0.009, Fig. 4.2B). We also followed TRAF1 levels longitudinally in three donors for whom consecutive samples were available (Fig. 4.2C). We observed a 20-83% loss of TRAF1 over time for the five HIV epitopes examined (Fig. 4.2C), despite a similar level of activation as measured by CD38 levels (data not shown). These results demonstrate that TRAF1 levels decrease in HIV-specific CD8 T cells over time.
### Table 4.1

**HIV sample information**

<table>
<thead>
<tr>
<th>Subjects #</th>
<th>Status</th>
<th>CD4 count</th>
<th>CD8 count</th>
<th>Viral Load (RNA copies/ml)</th>
<th>Duration of HIV infection (month)</th>
<th>treatment status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Early</td>
<td>740</td>
<td>770</td>
<td>170463</td>
<td>2</td>
<td>none</td>
</tr>
<tr>
<td>2</td>
<td>Early</td>
<td>580</td>
<td>1450</td>
<td>23099</td>
<td>5</td>
<td>none</td>
</tr>
<tr>
<td>3</td>
<td>Early</td>
<td>600</td>
<td>1460</td>
<td>349556</td>
<td>3</td>
<td>none</td>
</tr>
<tr>
<td>4</td>
<td>Chronic</td>
<td>670</td>
<td>1080</td>
<td>16196</td>
<td>16</td>
<td>none</td>
</tr>
<tr>
<td>5</td>
<td>Early</td>
<td>550</td>
<td>870</td>
<td>50</td>
<td>4</td>
<td>none</td>
</tr>
<tr>
<td>6</td>
<td>Chronic</td>
<td>420</td>
<td>1080</td>
<td>98386</td>
<td>24</td>
<td>none</td>
</tr>
<tr>
<td>7</td>
<td>Early</td>
<td>560</td>
<td></td>
<td>500000</td>
<td>5</td>
<td>none</td>
</tr>
<tr>
<td>8</td>
<td>Early</td>
<td>710</td>
<td>1230</td>
<td>8459</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td>9</td>
<td>Early</td>
<td>802</td>
<td></td>
<td>49852</td>
<td>&lt; 1</td>
<td>none</td>
</tr>
<tr>
<td>10</td>
<td>Early</td>
<td>632</td>
<td>355</td>
<td>&lt; 50</td>
<td>5</td>
<td>treated</td>
</tr>
<tr>
<td>11</td>
<td>Early</td>
<td>247</td>
<td>730</td>
<td>153367</td>
<td>11</td>
<td>none</td>
</tr>
<tr>
<td>12</td>
<td>Chronic</td>
<td>600</td>
<td>1220</td>
<td>211598</td>
<td>31</td>
<td>none</td>
</tr>
<tr>
<td>13</td>
<td>Chronic</td>
<td>400</td>
<td>1320</td>
<td>4140</td>
<td>25</td>
<td>none</td>
</tr>
<tr>
<td>14</td>
<td>Chronic</td>
<td>247</td>
<td>707</td>
<td>15010</td>
<td>48</td>
<td>none</td>
</tr>
<tr>
<td>15</td>
<td>Chronic</td>
<td>270</td>
<td>860</td>
<td>296381</td>
<td>23</td>
<td>none</td>
</tr>
<tr>
<td>16</td>
<td>Chronic</td>
<td>470</td>
<td>690</td>
<td>21926</td>
<td>29</td>
<td>none</td>
</tr>
<tr>
<td>17</td>
<td>Chronic</td>
<td>170</td>
<td>670</td>
<td>78120</td>
<td>27</td>
<td>none</td>
</tr>
<tr>
<td>18</td>
<td>Chronic</td>
<td>330</td>
<td>580</td>
<td>68760</td>
<td>24</td>
<td>none</td>
</tr>
<tr>
<td>19</td>
<td>Chronic</td>
<td>457</td>
<td>720</td>
<td>23606</td>
<td>24</td>
<td>none</td>
</tr>
<tr>
<td>20</td>
<td>Chronic</td>
<td>132</td>
<td></td>
<td>22418</td>
<td>&gt; 24</td>
<td>none</td>
</tr>
<tr>
<td>21</td>
<td>Chronic</td>
<td>400</td>
<td></td>
<td>373631</td>
<td>24</td>
<td>none</td>
</tr>
<tr>
<td>22</td>
<td>Chronic</td>
<td>410</td>
<td></td>
<td>40021</td>
<td>&lt; 1</td>
<td>none</td>
</tr>
<tr>
<td>23</td>
<td>Chronic</td>
<td>390</td>
<td>416</td>
<td>218113</td>
<td>24</td>
<td>none</td>
</tr>
<tr>
<td>24</td>
<td>Chronic</td>
<td>217</td>
<td>241</td>
<td>245239</td>
<td>72</td>
<td>none</td>
</tr>
<tr>
<td>25</td>
<td>Viral Controllers</td>
<td>1040</td>
<td>1090</td>
<td>1417</td>
<td>96</td>
<td>none</td>
</tr>
<tr>
<td>26</td>
<td>Viral Controllers</td>
<td>1487</td>
<td></td>
<td>5749</td>
<td>120</td>
<td>none</td>
</tr>
<tr>
<td>27</td>
<td>Viral Controllers</td>
<td>720</td>
<td>2840</td>
<td>1769</td>
<td>216</td>
<td>none</td>
</tr>
<tr>
<td>28</td>
<td>Viral Controllers</td>
<td>510</td>
<td>680</td>
<td>160</td>
<td>144</td>
<td>none</td>
</tr>
<tr>
<td>29</td>
<td>Viral Controllers</td>
<td>830</td>
<td>1700</td>
<td>8207</td>
<td>120</td>
<td>none</td>
</tr>
<tr>
<td>30</td>
<td>Viral Controllers</td>
<td>480</td>
<td>690</td>
<td>3419</td>
<td>36</td>
<td>none</td>
</tr>
<tr>
<td>31</td>
<td>Viral Controllers</td>
<td>1040</td>
<td>1090</td>
<td>50</td>
<td>108</td>
<td>none</td>
</tr>
<tr>
<td>32</td>
<td>Viral Controllers</td>
<td>670</td>
<td>740</td>
<td>1942</td>
<td>96</td>
<td>none</td>
</tr>
<tr>
<td>33</td>
<td>Viral Controllers</td>
<td>1080</td>
<td>1320</td>
<td>50</td>
<td>132</td>
<td>none</td>
</tr>
<tr>
<td>34</td>
<td>Early</td>
<td>630</td>
<td></td>
<td>103493</td>
<td>1.5</td>
<td>treated</td>
</tr>
<tr>
<td>35</td>
<td>Viral Controllers</td>
<td>470</td>
<td>600</td>
<td>2399</td>
<td>144</td>
<td>none</td>
</tr>
<tr>
<td>36</td>
<td>Viral Controllers</td>
<td>325</td>
<td>941</td>
<td>5370</td>
<td>180</td>
<td>none</td>
</tr>
<tr>
<td>37</td>
<td>Viral Controllers</td>
<td>800</td>
<td></td>
<td>49</td>
<td>&gt; 120</td>
<td>none</td>
</tr>
<tr>
<td>38</td>
<td>Viral Controllers</td>
<td>570</td>
<td></td>
<td>2169</td>
<td>&gt; 120</td>
<td>none</td>
</tr>
</tbody>
</table>

**Table 4.1 HIV sample information.** The Early group refers to donors infected with HIV-1 within the previous 12 months; Chronic progressors are defined as individuals infected with HIV-1 for > 1 year with a CD4 count decline of >50 cells/mm3/year, and viral controllers are defined to be infected with HIV-1 for > 1 year with no evidence of CD4 T cell count decline and a viral load of < 10000 copies/ml.
Figure 4.1 Flow cytometry gating strategy and representative FACS staining. A. Gating strategy used for analysis of the frequency of TRAF1 positive cells. Cells were stained with CD3, CD8, Tetramer, CD38 and TRAF1. Note that donors showed two kinds of staining profiles for their HIV-specific T cells: some donors showed a biphasic distribution with both TRAF1 positive and negative T cells specific for the same epitope within a single donor, whereas others showed a uniform population, for this reason we used % positive for our analysis in figure 4.2. B. TRAF1 levels are low in resting T cells and increase upon CD3/CD28 stimulation. CD8+CD45RA+ T cells with 0, 1 or 3 days of activation with anti-CD3 and anti-CD28 were stained for TRAF1 with 1F3 antibody. Shaded histograms indicate FMO controls.
Figure 4.2  Loss of TRAF1 protein expression during chronic HIV infection. See next page for legend.
Figure 4.2  Loss of TRAF1 protein expression during chronic HIV infection.  A. Frequency of TRAF1-positive HIV-specific CD8 T cells in recently or chronically infected donors or viral controllers, as defined in table 4.1. TRAF1 and/or CD38 protein expression was measured directly ex vivo in HIV-specific (tetramer positive) CD8 T cells in PBMC. Top panel: summary of all donors analyzed, lower panels show representative histograms from each group, with shaded panels indicating FMO controls, open histograms indicating TRAF1 staining on CD3+CD8+tetramer+ T cells (gating strategy is shown in figure 4.1). Statistical analysis was performed by one-way anova.  B. Correlation between frequency of TRAF1+ and CD38+ HIV-specific T cells as determined on CD8+HIV- tetramer+ cells from viral controllers and chronic progressors. Statistical analysis was performed using linear regression (p = 0.009).  C. TRAF1 expression in HIV-specific T cells using longitudinal samples from three donors with 5 HIV-specific epitopes. Top panels: representative TRAF1 staining during early and later time point within the same donor shown for two donors; bottom panel: summary plot with each donor represented by a different symbol and filled and empty symbols distinguishing two epitopes within the same donor. FMO controls are indicated in the shaded histograms.  D. TRAF1 expression in total CD8+CD45RA- T cells before and after activation. PBMC were stimulated with 1μg/ml of anti-CD3 and 10μg/ml of anti-CD28 for 6 days. Difference in median fluorescence intensity against FMO of TRAF1 is reported.  E. % TRAF1 expression in HIV-specific CD8 T cells from the later stage of HIV infection, including both chronically infected donors and viral controllers, is plotted against log viral load as indicated.
Figure 4.3 Activation, TRAF1 expression and HIV viral load. PBMC from HIV infected donors were stained directly ex vivo for CD38, CD8 and HIV-tetramer expression. **A.** Frequency of CD38+ cells among the HIV tetramer+ CD8 T cells for each group of donors. **B.** HIV viral load for the different donor groups; **C.** Frequency of TRAF1+ HIV tetramer+ CD8 T cells plotted against CD38+ HIV-tetramer+ CD8s for all three groups of donors; **D.** Frequency of TRAF1+ T cells of total CD8 T cells for the three groups of donors. **E.** Frequency of TRAF1+ HIV-specific CD8 T cells of total HIV+ T cells, were plotted against viral load from patients during early stage of HIV infection.
To determine whether the differences in TRAF1 expression were attributable to the effect of chronic infection or were innately present, we measured the TRAF1 expression in total CD8 T cells directly \textit{ex vivo}. We observed no differences in the overall frequency of TRAF1$^+$ CD8$^+$CD45RA$^-$ T cells between the groups (Fig. 4.3D), in contrast to the differences observed when the HIV-specific (tetramer$^+$) CD8 T cells were examined (Fig 4.2A). We next examined the potential of T cells in donors from each group to upregulate TRAF1 in response to T cell receptor signaling. All donors examined showed similar upregulation of TRAF1 in their total CD45RA$^-$ memory population in response to anti-CD3/anti-CD28 signaling, regardless of their stage or control of HIV infection (Fig. 4.2D). We conclude from these results that TRAF1 is lost specifically from the HIV-specific T cells of chronically infected donors over time of infection, but is maintained in a higher frequency of cells and at higher levels in viral controllers.

We next asked whether the presence of TRAF1$^+$ HIV-specific T cells is predictive of control of HIV viral load. While there is no correlation during the early stage of HIV infection (Fig. 4.3E), TRAF1 expression showed a weak, albeit significant, negative correlation with viral load during the later stage (Fig. 4.2E).

\textit{Requirement of TRAF1 for HIV viral suppression and 4-1BBL-dependent HIV-specific T cell responses}

To determine whether the defective TRAF1 expression could play a role in HIV control, we knocked down TRAF1 in CD8 T cells from viral controllers and assessed T cell function in a viral suppression assay. Purified CD8 T cells from three viral controllers and an uninfected donor were transfected with siRNA for TRAF1 or control RNA and then rested overnight before incubating with autologous HIV-infected CD4 T cells and autologous irradiated PBMC, as a source of APC. The ability of CD8 T cells to control HIV infected CD4 T cells was determined by measuring the frequency of Gag$^+$ CD4 T cells via flow cytometry (Fig. 4.4A) following 5 to 7 days coculture. For HIV viral controllers, TRAF1 knockdown resulted in significant increase in the frequency of HIV infected (Gag$^+$) CD4 T cells compared to cultures given control-treated CD8 T cells (Fig. 4.4A). In contrast, as expected, there was no significant control of Gag$^+$CD4 T cells in
Figure 4.4 Requirement for TRAF1 in HIV-specific CD8 T cell responses. See next page for figure legend.
Figure 4.4  

**Requirement for TRAF1 in HIV-specific CD8 T cell responses.**  

**A.** Viral suppression assay. CD8 and CD4 T cells were purified from HIV-positive or negative donors. CD4 T cells were infected with a primary isolate of HIV and at 48 hours, cocultured with autologous CD8 T cells that had been transfected with either TRAF1-specific siRNA or a control scrambled RNA (ctrl). Irradiated autologous PBMC were added as a source of APC. The frequency of Gag⁺ T cells was measured by flow cytometry 5-7 days later to assess the proportion of infected CD4 T cells. Both Gag⁺CD4⁺ (early infected) and Gag⁺CD4⁻ (late infected) T cells were included for analysis of frequency of HIV-infected CD4 T cells. i) Representative suppression curves (based on 3-5 replicates at each effector to target ratio for each donor and representative of three viral controllers and a healthy uninfected control). Statistical significance was determined by linear regression of %Gag⁺ T cells against log (effector:target) ratio using Graphpad (Prism) software. ii) Representative western blot analysis of TRAF1 levels after knockdown. RNAi transfected CD8 T cells from each donor for every experiment were activated with anti-CD3/CD28 antibodies in separate wells for 48 hours. Cells were then lysed and subjected to western blot. iii) Representative FACS plots, gated on CD8 negative cells.  

**B.** Costimulation assay. Purified CD8 T cells from viral controllers were nucleofected with either control RNA or TRAF1 siRNA and incubated with autologous monocytes that had been pulsed with control or HIV peptide and pre-treated with replication defective adenovirus expressing 4-1BBL or CD80 at an MOI of 100 as previously described. 8 days later the cells were harvested for FACS analysis or western blot. i) Western blot analysis of TRAF1 levels at the end of the 8-day culture. ii) summary plot for three experiments with cells from two different donors, shown as the number of HIV-tetramer⁺ CD8 T cells recovered in the TRAF1 siRNA transfected population over those transfected with control RNA for stimulation with HIV peptide pulsed 4-1BBL or CD80 expressing monocytes. iii) representative FACS plots.
cultures of T cells from an HIV uninfected donor, with or without TRAF1 knockdown. These data provide proof of principal that decreasing TRAF1 levels in CD8 T cells could result in decreased control of HIV.

We next went on to assess whether the loss of TRAF1 could affect 4-1BBL-dependent CD8 T cell responses to HIV. We knocked down TRAF1 in CD8 T cells from viral controllers with siRNA (Fig. 4.4B) and then stimulated them with autologous monocytes that had been pulsed with HIV peptide antigens and modified with replication defective recombinant adenoviruses to express CD80 or 4-1BBL. When TRAF1 was knocked down at the onset of culture, we observed substantial impaired expansion of HIV-specific CD8 T cells in response to overexpressed 4-1BBL, with lesser effects on the response to overexpressed CD80 (Fig. 4.4B). As CD28, the receptor for CD80, does not use TRAF1 in its signaling pathway, these results show that loss of TRAF1 can desensitize the 4-1BB costimulatory pathway in HIV-specific T cells.

Selective downregulation of TRAF1 during chronic LCMV infection in mice

In order to assess TRAF1 expression during a chronic infection in vivo, we moved to a mouse model. Infection of mice with clone 13 of LCMV results in viral persistence and CD8 T cell exhaustion, whereas the Armstrong strain of LCMV causes an acute infection that is readily cleared (169, 173, 178). Of note, 4-1BB is expressed on 20-60% of LCMV-specific CD8 T cells from both spleen and liver during the chronic phase of clone 13 infection, likely reflective of recent TCR engagement (Fig. 4.5). We infected 5-week old mice with either Armstrong or clone 13 and sorted LCMV-tetramer+ CD8 T cells from spleens on day 7 and day 21 post-infection. Purified LCMV specific T cells were analyzed for TRAF1 expression by western blot. Similarly to our findings with HIV-specific T cells, LCMV-specific CD8 T cells from mice chronically infected with clone 13 had reduced TRAF1 protein expression on day 21 compared to the LCMV-specific T cells from the acute Armstrong infection, whereas at day 7 post-infection no such decrease was observed (Fig. 4.6A). In contrast, TRAF2 did not show differential expression with acute or chronic infection over time (Fig. 4.6A). Moreover, the loss of TRAF1 was specific to the LCMV-specific cells, as TRAF1 levels in PD-1 negative cells
Figure 4.5 4-1BB expression on LCMV-specific CD8 T cells. 4-1BB expression is measured on tetramer+ CD8 T cells in the liver and spleen from mice infected with LCMV clone 13 for 29 days. Representative FACS plots are shown, gated on LCMV-tetramer+ CD8 T cells as indicated. Data are representative of 3 similar experiments.
(see Fig. 4.8E), or in B cells and MHC-II^B220^- APC were not changed over time of infection (Fig. 4.7). We next asked whether the loss of TRAF1 is a transcriptional or post-transcriptional event. Mice were infected and tetramer^+ cells sorted as above. Using semi-quantitative RT-PCR, we observed similar TRAF1 mRNA levels in LCMV-specific T cells from Armstrong and clone 13 infections at day 21 (Fig. 4.6B). These results suggest that TRAF1 protein loss during chronic infection occurs post-transcriptionally.

_TGFβ and IL-7 have opposing effects on TRAF1 expression in T cells_

Recent evidence has shown that production of the regulatory cytokine TGFβ is sustained during chronic as compared to acute LCMV infection, resulting in increased expression of BIM by the T cells and their loss by apoptosis (215). As TRAF1 can maintain lower levels of BIM in antigen-activated T cells (274, 292), we hypothesized that TGFβ might influence TRAF1 levels. To test this hypothesis we incubated TCR transgenic CD8 T cells with peptide antigen with or without recombinant TGFβ for 36 hours and observed that TRAF1, but not TRAF2, protein was significantly reduced upon TGFβ treatment (Fig. 4.8A). We next tested whether TGFβ can regulate TRAF1 at the post-translational level. We blocked protein translation using cycloheximide treatment of pre-activated CD8 T cells and observed that TRAF1 protein disappears faster in TGFβ treated T cells than in those left untreated (Fig. 4.8B). It should be noted that TGFβ can also regulate TRAF1 mRNA levels when a higher dose is used (Fig. 4.9), suggesting a more complicated mechanism could be at play in TRAF1 dysregulation during chronic viral infection in vivo.

IL-7 was previously reported to antagonize the effect of TGFβ (389). IL-7 is also in clinical trials for treating HIV patients (390). We therefore tested whether IL-7 can up-regulate TRAF1 expression. Indeed, CD45RA^- memory CD8 T cells from both healthy and HIV^+ infected individuals exhibit an antigen-independent increase in TRAF1 expression in response to IL-7 as well as in response to several other members of the common-γ-chain cytokine family (Fig. 4.8C, D).
**Figure 4.6**

TRAF1 expression in LCMV-specific CD8 T cells during acute and chronic infection. Mice were infected with either acutely infecting LCMV Armstrong (A) or chronically infecting clone 13 (C). Tetramer+ (gp33, gp276 and NP396) CD8 T cells were FACS sorted on day 7 and 21 post infection and the purified tetramer+ T cells were subjected to either western blotting analysis or semi-quantitative RT-PCR analysis. **A.** Representative blots of TRAF1, TRAF2 and Actin protein levels (left panel) and summary of TRAF1 and TRAF2 levels normalized to Actin for tetramer+ T cells isolated from individual mice at the times indicated for the two different viral infections (right panels). Total CD8 T cells were sorted from uninfected mice (N0) as a control. **B.** mRNA levels of TRAF1 relative to Actin on the sorted T cells for the indicated viruses and times of infection. Data are representative of 3 (for protein) or 2 (for mRNA) independent mouse experiments. Right side of the gel in b) indicates a titration of the template, indicating non-saturation of the signal.
Figure 4.7 TRAF1 levels do not differ in APC in Armstrong versus clone 13 infected mice. A. PD-1+ T cells; B. PD-1 negative T cells; C. MHCII+B220+ B cells or D. MHCII+B220- APC were sorted from mice at either day 7 or day 21 of Armstrong or clone 13 infection, and subject to Western blot for TRAF1 and Actin staining. At day 21 post-Armstrong infection, as there were no PD-1+ T cells, CD44+/- T cells were sorted instead.
Figure 4.8  Cytokine regulation of TRAF1 levels in T cells.
see next page for figure legend.
Figure 4.8  Cytokine regulation of TRAF1 levels in T cells. A. Splenocytes from OT-1 mice were stimulated with SIINFEKL peptide for 36 hours with or without TGFβ. Live CD8 T cells were purified and lysed for western blotting for TRAF1, TRAF2 and Actin. Data are representative of 3 experiments. B. Splenocytes from OT-1 mice were stimulated with SIINFEKL peptide for 20 hours with or without TGFβ, followed by 1µg/ml of cycloheximide treatment (CHX). Cells were harvested at 0, 1, 2, 3 and 4 hours post CHX treatment, live CD8 T cells were purified and then subjected to western blot. Data are representative of 2 experiments. C. Purified human PBMC were stimulated with either anti-CD3 (1µg/ml)/CD28 (10µg/ml) as a positive control or 20ng/ml of IL-2, IL-7, IL-15 or IL-21, or media only for 6 days. At day 6, cells were analyzed by flow cytometry. Bottom graph shows summary data, gated on CD45RA-CD8+ T cells, reported as the difference in median fluorescence intensity of TRAF1 compared to FMO control. Statistical analysis was performed using one-way anova. D. PBMC from HIV infected donors were CFSE-labeled and incubated with cytokines as described in B. Data were gated on CD45RA-CFSElow CD8 T cells except for unstimulated samples, which were gated on CD45RA-CD8 T cells as they did not undergo division. Statistical analysis was performed using one-way anova within each group of HIV donors (early, viral controller (VC) and chronic). E. Mice were infected with LCMV clone 13. At day 21-post infection, mice were treated with 10µg/mouse of IL-7 or PBS. Tetramer+ and PD-1- CD8 T cells were sorted on day 23 as described in Fig. 4.6 and lysed for western blot. CD8 T cells from uninfected TRAF1−/− and WT mice were used as controls.
We next asked whether IL-7 could restore TRAF1 expression in T cells *in vivo* during the chronic phase of LCMV infection. We treated mice with IL-7 on day 21 post clone 13 infection, harvested and then sorted LCMV-specific CD8 T cells from the splenocytes on day 23. Although this brief IL-7 treatment did not reduce viral load (data not shown), we observed a significant increase in TRAF1 expression in tetramer$^+$ CD8 T cells (Fig. 4.8E), consistent with the *in vitro* data.

**IL-7 combined with agonistic anti-4-1BB therapy rescues exhausted CD8 T cells and decreases viral load in a TRAF1-dependent manner**

As TRAF1 is required for maximal signaling through 4-1BB (274, 288), and IL-7 restores TRAF1 levels in T cells of chronically infected mice *in vivo*, we reasoned that a combined therapy involving 4-1BB and IL-7 might be of benefit. To this end, we treated clone-13 infected mice at 25 days post infection with an agonistic anti-4-1BB antibody with or without recombinant IL-7 (delivered at day 21, 23 and 25) and sacrificed mice 5 days later for analysis of effects on T cells. IL-7 alone had little or no effect, whereas stimulatory anti-4-1BB antibody alone induced a limited increase in LCMV-specific lymphocyte numbers (one out of four epitopes, Fig. 4.10A). However, the combination of IL-7 and anti-4-1BB increased the number of T cells specific to four different LCMV epitopes (Fig. 4.10A), and resulted in a significant increase in the % of multi-functional T cells that can produce IFN$\gamma$ and express the degranulation marker CD107a in response to LCMV antigen (Fig. 4.10B).

We next analyzed the impact of the therapy on viral load. Mice were infected and treated on day 25 as above and 12 days later organs were harvested for analysis of viral load. The combination of anti-4-1BB and IL-7, but neither treatment alone, resulted in viral clearance in the liver and a significant decrease in viral load in the lung and spleen (Fig. 4.10C). The effect on viral load in the kidney was more modest, but significant. It should be noted that when we looked earlier, at 5 days after treatment, there was a larger reduction of viral load in the kidney (Fig. 4.10C), suggesting that there had been a transient effect in that organ. These data show that 4-1BB and IL-7 can cooperate to improve T cell function and viral clearance in chronic LCMV infection of mice.
Figure 4.9 The effect of TGFβ on TRAF1 expression at the A. protein; B. mRNA level. Splenocytes from OT-1 mice were activated with SIINFEKL peptide for 36 hours with 0nM, 2nM or 10nM of TGFβ. Live CD8 T cells were purified and subjected to either semi-quantitative RT-PCR or western blotting.
Figure 4.10 Combined treatment with IL-7 and agonistic anti-4-1BB therapy rescues exhausted CD8 T cells and decreases viral load in a TRAF1 dependent manner. See next page for figure legend.
Combined treatment with IL-7 and agonistic anti-4-1BB therapy rescues exhausted CD8 T cells and decreases viral load, in a TRAF1 dependent manner. Mice were infected with LCMV clone 13 for 21 days and treated with either IL-7 alone, agonistic anti-4-1BB (3H3) alone, or in combination as indicated, using the following treatment regime: 10µg/mouse of IL-7 on day 21, 23 and 25; 100µg/mouse of 3H3 on day 25. Mice were sacrificed on d30 (A, B) or day 37 (C). A. The number of tetramer positive CD8 T cells specific for LCMV epitopes was examined for each treatment group, with each data point representing a single mouse. Statistical analysis by one-way anova. B. Splenocytes from mice in each group were subjected to LCMV peptide restimulation with brefeldin A, monensin and anti-CD107a or isotype control for 6 hours, cells were harvested for surface and intracellular staining and FACS analysis. Statistical analysis by one-way anova. C. Organs were harvested at day 30 and day 37 and viral titers were measured. Data are representative of 3 similar experiments for tetramer analysis and viral clearance, the latter presented as the median of 7 or 8 individual mice. The dotted line indicates the limit of detection of the assay. D. WT and TRAF1−/− mice were treated with IL-7/anti-4-1BB or left untreated as described in panel a). At day 37 (12 days post-treatment), the number of gp33-specific CD8 T cells were enumerated in the spleen (left panel). Viral titers were evaluated in spleen and liver (right panels). Data in D are pooled from two independent experiments with seven TRAF1−/− and ten wildtype mice per group.
To test whether TRAF1 was important for the effects of anti-4-1BB/IL-7 therapy, we infected WT or TRAF1−/− mice with LCMV clone 13 and treated as in Fig. 4.10A (Fig. 4.10D). The IL-7 and anti-4-1BB treated TRAF1−/− mice did not respond to therapy, exhibiting T cell numbers equivalent to the untreated controls (Fig. 4.10D). The lack of increase in the number of gp33-specific T cells in the spleen following therapy of TRAF1−/− mice correlated with increased viral load in the spleen and liver (Fig. 4.10D right panels), but not in the kidney or lung (data not shown). The increased viral load in TRAF1−/− treated mice was not due to higher viral load in TRAF1−/− mice before treatment, as in the absence of treatment, WT or TRAF1−/− mice infected with clone 13 showed a similar viral load at day 30 post-infection (Fig. 4.11). These results show that IL-7 plus anti-4-1BB therapy can improve T cell expansion and viral control in a TRAF1-dependent manner.

Discussion
This chapter has revealed a novel mechanism by which T cells can become dysfunctional during chronic viral infection, through the downregulation of a signaling adaptor, TRAF1. In contrast to other members of the TRAF family, TRAF1 lacks the RING domain required for NF-κB activation (391). However, structural analysis of the TRAF1/2 coiled coil domains suggests that the TRAF1 (TRAF2)2 heterotrimer is a better recruiter of cellular inhibitor of apoptosis proteins than a TRAF2 homotrimer (392), providing a structural explanation for enhanced NF-κB activation in the presence of TRAF1 (393). Although TRAF1 is more of a modulator than an absolutely required mediator of TRAF2-dependent TNFR signaling (288, 393-396), it can play a substantial role in the survival of activated and memory T cells (292), and there are several hints that TRAF1 is important in human disease. TRAF1 is overexpressed in more than 50% of human cancers of B cell origin (397) and single nucleotide polymorphisms in TRAF1 or in the TRAF1/C5 region have been linked to non-Hodgkin’s lymphoma (398) and rheumatic disease (399, 400), respectively. In this study we showed that TRAF1 is selectively lost from antigen-specific T cells with progression of chronic infection for both HIV infection in humans and LCMV clone 13 infection in mice and that there is a negative correlation
Figure 4.11 *LCMV* clone 13 infection induced similar responses in wildtype and *TRAFT<sup>1/-</sup>* mice. Mice were infected as in Fig. 4.10. A. The frequency of LCMV-specific (GP33-tetramer<sup>+</sup>) CD8 T cells were measured on both day 7 and day 21 post LCMV clone 13 infection. B. Viral load was measured in the kidney at day 30 following infection.
of the frequency of TRAF1+ cells with viral load during the later stage of HIV infection. These findings suggest that TRAF1 may be a useful indicator of the state of the HIV-specific T cells during the chronic stage of infection, as TRAF1 levels are high in activated cells but low in dysfunctional T cells. This is in contrast to PD-1, whose expression is high on both activated as well as on dysfunctional chronically stimulated cells.

The decreased frequency of TRAF1+ T cells with disease progression does not necessarily reveal whether loss of TRAF1 is a cause or an effect of chronic infection. However, the correlation of viral load with TRAF1 late but not early in infection is consistent with the loss of TRAF1 being a secondary rather than an initiating event. Our results show that the TRAF1 expression defect is at the protein, but not the mRNA level (Fig 4.6), a finding that has been made for other molecules in exhausted CD8 T cells, including IFNγ (190). We showed that TGFβ can act post-translationally to limit TRAF1 expression in CD8 T cells in response to peptide antigen. As TGFβ is produced early in chronic LCMV and SIV infection (215, 218), it is possible that TGFβ contributes to loss of TRAF1 in vivo. It is also possible that inhibitory receptors, such as PD-1, which block the PI3K/AKT pathway could also result in decreased TRAF1 due to the effect of AKT on mTOR and protein translation, although this was not examined here. Thus, a likely scenario is that early during infection, excessive viral replication leads to sustained expression of IL-10, PD-1 and TGFβ, which in turn results in loss of TRAF1 from the antigen-specific T cells.

We showed in this study that knocking down TRAF1 in CD8 T cells from viral controllers resulted in reduced viral suppression as measured by Gag+ CD4 T cells. This result provides proof of principal that the partial loss of TRAF1 during chronic HIV infection could have functional consequences, thereby contributing further to progressive immune dysfunction. We also showed that TRAF1 knockdown specifically impairs the ability of HIV-specific CD8 T cells from viral controllers to expand in response to 4-1BBL and that TRAF1 was critical for anti-4-1BB induced therapy of chronic infection in vivo. In addition to the effect of TRAF1 on 4-1BB signaling, it is possible that TRAF1
contributes to T cell responses through other TNFRs. TNFR2, CD30, HVEM and GITR can also be found on activated CD8 T cells and can bind TRAF1 (386), and as such, these TNFRs as well as 4-1BB, could be contributing to the TRAF1-dependent effects observed in the viral suppression assay and \textit{in vivo}. However, recent evidence shows that GITR uses TRAF2/TRAF5 rather than TRAF1 for NFκB activation in primary T cells (401).

Given their importance in T cell homeostasis, we tested IL-7 as well as other members of the common γ chain family of cytokines for restoration of TRAF1 levels. We found that IL-2, IL-7 and IL-15, but not IL-21 upregulated TRAF1 protein in CD8 T cells in an antigen-independent manner. IL-7, in particular, has been shown to antagonize the TGFβ signaling pathway (389) and was selected for further study. One caveat of using these cytokines as a therapy for chronic viral infection is the suboptimal expression of receptors for both IL-7 and IL-15 on chronically stimulated T cells (402, 403). However, both IL-7R\textsubscript{low} and IL-15R\textsubscript{low} LCMV-specific CD8 T cells can still divide in response to IL-7 \textit{in vitro} and IL-15 \textit{in vivo}, albeit to a lesser extent than their respective counterparts (404). Importantly, we showed that purified T cells from HIV-infected donors respond to these cytokines by upregulating TRAF1 \textit{in vitro}, despite their reduced proliferation compared to T cells from donors at early stage of infection (data not shown). Moreover, IL-7 increased the level of TRAF1 protein in LCMV-specific CD8 T cells during clone 13 infection \textit{in vivo}, although indirect effects of IL-7 cannot be ruled out.

The combined therapy of IL-7 and 4-1BB showed significant improvement in the number of functional virus specific T cells and decreased viral load in the clone 13 infection model in mice. Although it is likely that IL-7 has additional effects on T cells besides restoring TRAF1 (389), the IL-7 plus anti-4-1BB therapy was at least partially dependent on TRAF1. The absence of TRAF1 impaired the ability of IL-7 and anti-4-1BB to expand gp33-specific T cells and this result correlated with increased viral load in the spleen and liver of the TRAF1\textsuperscript{-/-} IL-7/anti-4-1BB-treated mice. This finding is consistent with our studies of T cells from HIV infected donors, where knocking down TRAF1 resulted in impaired 4-1BBL-dependent CD8 T cell responses to HIV.
The transient IL-7 and anti-4-1BB therapy of chronically infected mice was tested under the demanding conditions of the chronic phase of the response (day 21-25) when chronicity and loss of T cell function has been well established in the clone 13 infection model. Moreover, we monitored the effects on viral load 12 days after cessation of treatment. Other studies have shown improved T cell responses and reduced viral load in chronic LCMV by providing IL-2 or by interfering with IL-10, TGFβ, PD-1 or TIM-3 (215, 405-407). However, with the exception of PD-L1 blockade, those treatments were given relatively early and were sustained for several weeks. In the PD-1 studies, treatment was given in the chronic phase from day 23-37, but the effect was monitored immediately or shortly thereafter (169, 406, 407). Thus the finding that a single dose of anti-4-1BB agonist after a brief IL-7 therapy given late in disease course can have significant and sustained effects after the cessation of treatment is notable and suggests that a combined therapy with IL-7 and anti-4-1BB may be useful for more established chronic infection in humans. Although anti-4-1BB agonistic antibodies at doses of only 200µg/mouse can cause immune system anomalies when given to healthy mice (408), the ability to achieve effects with a single 100µg dose of anti-4-1BB in combination with IL-7 therapy suggests that transient use of low dose anti-41BB in conjunction with other therapies may be tolerable.

A recent study showed that HAART is unable to restore CD8 T cell effector function to the level of those from HIV controllers, despite reducing the viral load of the chronically infected donors (5). Therefore, treatment that directly improves CD8 T cell function may be beneficial to viral control. In addition to its effects on T cell survival, 4-1BB signaling increases the level of effector function per CD8 T cell (274, 312, 409), likely contributing to its therapeutic effect. Our studies suggest that a combined anti-4-1BB/IL-7 therapy will be relevant to HIV and may also be applicable to other chronic infections, such as HCV or HBV.

Methods
Reagents
Biotinylated MHC/HIV peptide monomers: A2-SLYNTVATL, A2-ILKEPVHGV, A2-FLGKIWPSYK, B7-TPGPGVRYPL, B7-IPRRIRQGL, B8-FLKEKGGL, B27-KRWIILGLNK and B57-KAFSPEVIPMF were generated at the National Immune Monitoring Laboratory St., Laurent, Quebec (http://www.niml.org/) or at the NIH tetramer facility, Emory University, Atlanta, GA and mixed with PE or APC-streptavidin. LCMV tetramers H-2K\(^{b}\)-GP34: AVYNFATM; H-2D\(^{b}\)-GP33: KAVYNFATM; H-2D\(^{b}\)-GP276: SGVENPGGYCL and H-2D\(^{b}\)-NP396: FQPQNGQFI were from the MHC tetramer core laboratory, Baylor College of Medicine. Human TRAF1 specific antibody 1F3(410) (Serviceeinheit Monoklonale Antikörper; Institut für Molekulare Immunologie, Munich, Germany) was labeled with Alexa 430 (Molecular Probes) for flow cytometry analysis of human TRAF1. Anti-4-1BB (3H3) (411) was purified from the hybridoma, kindly provided by Robert Mittler, Emory University, Atlanta. All other antibodies for flow cytometry were purchased from ebioscience. The following western blot antibodies were used: anti-mTRAF1(Santa Cruz Biotechnology), anti-mTRAF2 (Cell Signaling), anti-hTRAF1 (Cell Signaling) and anti-Actin (Sigma).

**HIV infected donors**

PBMC from HIV\(^{+}\) donors were obtained by leukapheresis and cryopreserved until use. Healthy donor samples were obtained by venipuncture. All donors gave informed consent as approved by the research ethics boards of all participating institutions. Donors were HLA typed and ELISPOT analysis was used to determine their CD8 T cell specificities as previously described (312) and the dominant epitopes were selected for further study. Human donor information is summarized in Table 4.1.

**Mice and lymphocytic choriomeningitis virus**

5-week old C57BL/6 mice (Charles River) were infected by intravenous infusion of LCMV Armstrong strain (5000 plaque forming units (pfu) per mouse) or LCMV Clone 13 strain (2 X \(10^6\) pfu/mouse). For quantification of TRAF1/2 levels, splenocytes were harvested from day 7 and day 21 post Armstrong or clone 13 infections. Pooled (GP33-, GP276- and NP396-) tetramer\(^{+}\) CD8 T cells, PD-1\(^{low}\) CD8 T cells, B220\(^{+}\)MHCII\(^{+}\) B cells or B220\(^{-}\)MHCII\(^{+}\) cells were sorted and subject to western blots or semi-quantitative RT-
PCR. To test induction of TRAF1 by IL-7 in vivo, 10 µg of recombinant human IL-7 (Cytheris) or PBS were injected intraperitoneally (i.p.) on day 21 and day 22 post Clone 13 infection. For IL-7 therapy, 10µg of IL-7 or PBS were given i.p. on day 21, 23 and 25 with 100 µg of agonistic anti-4-1BB or rat Ig (Sigma) on day 25. Mice were sacrificed on day 30 or day 37 for flow cytometry analysis and viral titer assays. TRAF1−/− mice(412) (now available from Jackson laboratories), were obtained from Erdinyi Tsitsikov, Harvard Medical School and were bred in our facility. All animal experiments were approved by the University of Toronto and/or University Health Network biosafety committee according to the guidelines of the Canadian Council on animal care.

**Intracellular cytokine and degranulation assay**

Splenocytes were incubated for 6 hours with 100nM LCMV-specific peptide, brefeldin, monensin and anti-CD107a antibody. Cells were first stained with anti-CD3 and anti-CD8 antibody (ebioscience), fixed, permeabilized (BD biosciences), stained with anti-IFNγ antibody and followed by flow cytometry analysis.

**Costimulation assay**

Autologous monocytes were infected with replication-deficient adenoviruses expressing either 4-1BBL, CD80 or control as previously described (312, 409). CD8 T cells were purified and transfected (Amaxa) with 600nM TRAF1 siRNA or scrambled control RNAi (Integrated DNA technology), rested overnight and incubated with activated monocytes for 8 days. T cells were then harvested for FACS analysis.

**HIV suppression assay**

CD4 T cells were enriched from PBMC using negative selection (Stemcell), activated with 1µg/ml each of anti-CD3 (OKT3), anti-CD28 (28.2) antibodies (ebioscience) and PHA (Sigma) for 24 hours and washed extensively. The primary isolate of HIV 91_US4 was obtained from the NIH AIDS reagent program and expanded on activated primary CD4 T cells. Virus was purified through a 20% sucrose cushion and used to magnetofect CD4 T cells using Viromag beads following manufacturer's instructions (Ozbiosciences). CD8 T cells were enriched from PBMC using negative selection (Miltenyi) and
transfected (Amaxa) with 600nM of TRAF1 siRNA or control RNAi (Integrated DNA technology), rested overnight and coincubated with 25,000 CD4 T cells/well at a serial dilution with effector CD8 to target CD4 ratio of 8:1 (or 6:1, 5:1 depend on sample size availability), 1:1, 1:4, 1:16 and no effector in three to five replicates. PBMC were enriched for APC (Stemcell), irradiated, and plated at 10,000/well with the CD8 and infected CD4 T cells in the presence of 20U/ml IL-2 (Hoffmann-La Roche). At day 5-7 of coculture, cells were stained for surface markers and intracellular Gag, and subjected to flow cytometry for analysis of the frequency of Gag$^+$ T cells.

**TGFβ assay**
Splenocytes from OT-1 mice were incubated with $10^{-7}$M SIINFEKL peptide at 2.5 million/ml with or without TGFβ (5nM). CD8 T cells were purified at 36 hours post coincubation, and subjected to western blots or semi-quantitative RT-PCR. For blocking protein translation, 1µg/ml cycloheximide (CHX) was added to the culture at 20 hours post coincubation, and CD8 T cells harvested and purified at 0, 1, 2, 3 and 4 hours post addition of CHX.

**Western blots**
Equal numbers of sorted cells were loaded in each well. Anti-TRAF1 antibody (Cell signaling) was used at 1:1000 dilution; anti-TRAF2 antibody (Cell signaling) was used at 1:500 and anti-Actin (Sigma) was used at 1:1000.

**Semi-quantitative RT-PCR**
The following mouse TRAF1 primer pair was used: 5’-CAT GCA GGA GCA TGA GGC TAC C –3’; 5’ – CCA CCA CCC TCT GCT CCA AGC – 3’.

**Viral immuno-plaque assay**
To determine LCMV titers, organs were homogenized and supernatant dilutions used to infect a monolayer of MC57 cells under an overlay of Methyl Cellulose in DMEM. 48 hours later, cells were fixed and stained with VL-4 rat anti-LCMV mAb and a color reaction of Ortho-Phenylenediamine were used to determine the Focus Forming Unit.
Statistics

All statistical analysis was performed using Graphpad software (Prism). Except where otherwise indicated, statistics were calculated using student t test with p values indicated on the figures.
Chapter V

MODELS AND FUTURE DIRECTIONS
This thesis set out to investigate the therapeutic implications of costimulation on CD8 T cell dysfunction during chronic HIV infection. Studies presented in Chapters II, III and IV have highlighted three major findings: 4-1BBL is a superior costimulatory molecule in driving cytolytic function and direct expansion of memory CD8 T cells, as compared to CD70 and LIGHT; 4-1BBL is an essential therapeutic component, relying on TRAF1, in the costimulation dependent rescue of dysfunctional CD8 T cells both in vitro and in vivo, with implications in viral clearance; as well, the evanescence of TRAF1 is found to be a distinct later-stage immune dysfunction during persistent viral infection, likely resulting in further loss of viral control. I first discuss the contributions of these findings to the construction of several models intended to clarify the nature and consequences of HIV-specific CD8 T cell dysfunction. I then discuss the therapeutic relevance of 4-1BB based on the models generated.

**Survival of HIV-specific CD8 T cells: a key to targeting the viral reservoir?**

The work presented in this thesis showed that one unique property of 4-1BBL in rescuing dysfunctional CD8 T cells in vitro, as compared to other costimulatory molecules, is the ability to downregulate the pro-apoptotic molecule Bim (Fig. 3.7 B). This finding highlights the importance of HIV-specific CD8 T cell survival, and raises the question whether the overall functional impairment during chronic HIV infection could partly be explained by the selective depletion of functional cells; and furthermore, is Bim involved in this process of selective depletion?

*Hypothesis part I: the selective depletion of functional cells*
There is some evidence supporting the depletion of functional cells during HIV infection. A previous study (312) showed that functional expansion of HIV-specific CD8 T cells required more costimulation than influenza-specific CD8 T cells, likely reflecting the need for more survival or proliferative factors. Work presented in this thesis argues for the lack of survival factors as a similar number of cell divisions was observed in HIV-specific CD8 T cells in response to any combination of costimulatory molecules (Fig. 3.7), whereas cell numbers were unable to accumulate in response to CD80/CD70 dual costimulation, especially those with higher PD-1 expression (Fig. 3.3). When taken directly *ex vivo*, HIV-specific CD8 T cells of the effector memory phenotype (CCR7−CD62L− and CD45RA+−), but not of the central memory phenotype (CCR7+CD62L+CD45RA−), have increased susceptibility to spontaneous apoptosis and FASL induced apoptosis as compared to their CMV-specific CD8 T cell counterparts (413). Most importantly, purified CD8 T cells from these patients, a heterogeneous population with varying disease progression, showed similar cytotoxicity and the ability to produce IFNγ against HIV or CMV targets. As such, depletion of functional CD8 T cells specific to a particular epitope could contribute to the overall phenotype of functional impairment and thus lack of CTL control during HIV infection.

An extreme example of the selective depletion is the loss of CD8 T cells specific to the LCMV NP396 epitope during clone 13 infection (180). This depletion is of disease relevance as the NP-antigen persists in large quantity in clone-13 infected mice as measured by the ability of the T cell-depleted APC from clone 13 infected mice to induce responses from NP-specific memory T cells purified from acutely infected mice (180).
Furthermore, Bim deficiency resulted in a striking 10-fold increase in the proportion of functional NP396-specific spleen CD8 T cells and this increase corresponds to a faster viral clearance from the spleen kinetically, arguing for a preferential depletion of functional cells via a Bim dependent mechanism (reanalysis of (414)). Of note, similar increase of functional over non-functional cells is not observed with the GP33- or GP276-specific cells. It is likely that similar loss of functional clones exists during chronic HIV infection and contributes to HIV pathogenesis, although further studies are required to explore this idea. In this regard, studies presented in this thesis have shown that agonistic anti-4-1BB antibody alone resulted in a 3-fold expansion of the number of NP396-specific, but not of GP33- or GP276-specific CD8 T cells in the spleen (Fig. 4.10 A), highlighting the therapeutic potential of the 4-1BB pathway in preventing depletion of functional clones. This restoration of numbers of functional virus-specific T cells alone did not translate into a sustained reduction in viral load (only transiently as shown in Fig. 4.11) probably due to the reduced effect of agonistic anti-4-1BB under the condition of limited TRAF1 expression.

**Hypothesis part II: mechanism of depletion**

The second part of the hypothesis addresses the underlying mechanism(s) of the selective depletion. Upon HIV infection, monocyte-derived macrophages from healthy donors express elevated levels of FASL in HIV p24 positive cells (415, 416). As well, in HIV-infected as compared to healthy donors, FASL protein expression is found on an increased percent of macrophages (as measured by CD68\(^+\)S100\(^-\)) in the interfollicular and paracortical regions of secondary lymphoid tissues (416). Together, these studies provide
a framework supporting the possibility of depletion of antigen-specific CD8 T cells through FAS/FASL interaction when engaged with an HIV-infected macrophage, and thus limiting cytolytic activity of CD8 T cells on cells that are potential HIV reservoir. However, deliberately delivering FASL signal via antibodies resulted in differential levels of apoptosis from HIV-specific CD8 T cells and CMV-specific T cells from the same donors despite similar levels of activation and expression of FAS (413). This observation argues that cell intrinsic factors contribute to FASL susceptibility of HIV-specific CTL. Furthermore, blocking FASL/FAS interaction did not interfere with spontaneous apoptosis of bulk CD8 T cells from SIV-infected macaques, which was found to involve the loss of mitochondria potential in a caspase independent manner (417). As the Bcl-2 family is involved in maintaining mitochondria membrane potential, it is reasonable to speculate that Bim could contribute to the increased apoptosis and thus depletion of functional HIV-specific CD8 T cells. Consistent with this idea, analysis of bulk CD8 T cells from SIV-infected macaques showed elevated levels of Bim and another pro-apoptotic molecule Bak, but not Bax (417). Bim is also partially responsible for the HIV Tat protein induced T cell apoptosis (418). As the Tat protein can be secreted by infected cells and taken up by uninfected cells (419), the Tat/Bim finding could be relevant to CD8 T cell survival in vivo.

Prior to the studies presented in this thesis, the relevance of Bim in HIV-specific CD8 T cells was unclear. Figure 3.7 C showed that the frequency of HIV-specific CD8 T cells that express Bim negatively correlated with the resulting expansion of functional CD8 T cells in response to costimulation (p < 0.0001, R = 0.79). As well, Bim levels are elevated
in dividing HIV-specific CD8 T cells in response to CD80/CD70 costimulation in an antigen-dependent manner (Fig. 3.7B). These findings highlight the relevance of Bim expression in the survival of HIV-specific CD8 T cells, but do not show whether Bim is more prone to upregulation during HIV infection. Indeed, when analyzed directly ex vivo, Bim expression in HIV-specific CTL from PBMC of HIV infected donors is not significantly different between viral controllers and HIV progressors (data not shown), in contrast to the finding in bulk CD8 T cells from SIV infected macaques (417). Thus, perturbation in other pro- or anti-apoptotic factors might be important for memory T cells at resting state, and Bim could function to counteract or amplify this/these factor(s). In support of this view, Petrovas et al. showed that HIV-specific CD8 T cells have reduced levels of Bcl-2, but not Bcl-xL, as compared to total CD8 T cells (420), suggesting that Bcl-2 might be the limiting factor for the survival of HIV-specific CTL. Interestingly, the loss of even one Bim allele rescued Bcl-2−/− mice from death (421). As well, a recent study using Bim+/−Bcl-2−/− and Bim−/−Bcl-2−/− mice has revealed a critical role of the Bim/Bcl-2 balance in maintaining both naïve and memory T cell homeostasis (422). Thus, Bim could play a critical role in balancing Bcl-2 in the survival of HIV-specific CD8 T cells.

A second, non-mutually exclusive, hypothesis is that Bim is only dysregulated in recently activated HIV-specific CD8 T cells. Furthermore, this thesis offered one potential mechanism of Bim dysregulation: via loss of TRAF1. Figure 3.6 showed that upon antigen-specific activation, TRAF1 knockdown resulted in elevated Bim expression in response to 4-1BBL costimulation. As TRAF1 is selectively lost during chronic HIV
infection (Fig. 4.2), it might result in Bim dysregulation in vivo when HIV-specific CD8 T cells are activated in the presence of pathways that require TRAF1 as a signaling molecule. Over time, Bim$^{\text{high}}$ HIV-specific CD8 T cells which are IL-7R$^{\text{low}}$ could be rapidly cleared (423, 424), and thus no longer detected. This hypothesis is of clinical relevance as knocking down TRAF1 in viral controllers resulted in the loss of HIV suppression (Fig. 4.4). It is unclear whether this loss of suppression could be attributed to increasing Bim level and thus apoptosis of HIV-specific CD8 T cells. Further study is required to analyze whether Bim upregulation is indeed more profound in individuals chronically infected with HIV as compared to HIV controllers when equivalent antigen specific activation signal is provided.

Overall, a possible model (Fig. 5.1) for increased apoptosis of HIV-specific CD8 T cells is as follows: at resting state, the abnormally low Bcl-2 levels render cells susceptible to apoptosis induced by environmental stress, likely a result of low levels of receptors to IL-7 and IL-15 which usually maintain the steadystate Bcl-2 expression (425, 426); when activated, unchecked Bim upregulation, probably a result of loss of TRAF1 and other unexplored factors as discussed in a later section, leads to depletion of HIV-specific CD8 T cell clones; the depletion process is further enhanced by FASL expression especially on HIV infected cells/HIV reservoir. Craig Thompson’s group has shown that Bcl-xL, another Bcl2 family member, but not Bcl-2, is inducible by CD28 costimulation (237). As well, 4-1BB signaling also does not affect Bcl-2 expression (311). Taken together, this model highlights the importance of combination therapies that would target both Bcl-2 and Bim which counterbalance each other. Results in Chapter IV highlight the success
Survival impairment of HIV-specific CD8 T cells. At resting state, the abnormal low Bcl-2 levels renders cells susceptible to apoptosis induced by environmental stress, likely a result of low levels of receptors to IL-7 and IL-15 which usually maintain the steady state Bcl-2 expression (420, 421); when activated, unchecked Bim upregulation, probably a result of loss of TRAF1 and other unexplored factors, leads to depletion of HIV-specific CD8 T cell clones; the depletion process is further enhanced by FASL expression especially on HIV infected cells/HIV reservoir.
of one such combination: IL-7 plus anti-4-1BB, where either therapy alone has met limited success when delivered at the chronic phase of infection. Of note, this model explains the susceptibility of HIV-specific CD8 T cells to death, but does not address why functional cells are selectively killed. IL-10 has been shown to prevent spontaneous apoptosis of bulk CD8, but not CD4, T cells from SIV-infected macaques (417). As elevated levels of IL-10 have been detected during chronic HIV infection (207), there could be differential responses to IL-10 between functionally impaired CD8 T cells and fully functional effectors specific to HIV, although this idea is not explored in this thesis.

Effector functions of HIV-specific CD8 T cells, a matter of translation?

The susceptibility to apoptosis, at the very least, provides a confounding factor in analysis of effector functions of HIV-specific CD8 T cells. In fact, any analysis of cytokine production or cytolytic functions of HIV-specific CD8 T cells requires in vitro culture for several hours or even days, providing sufficient time for spontaneous apoptosis of functional cells prior to readout. As well, in some cytotoxicity assays, EBV transformed cells lines were used as target cells (427, 428), which may have upregulated FASL expression (429) and thus induced death of functional cells that are prone to apoptosis.

Other evidence suggests effector function impairment of surviving cells is an integral part of pathogenesis during chronic HIV infection. Gene chip analysis showed that exhausted LCMV-specific CD8 T cells have reduced mRNA expression of perforin as compared to memory CD8 T cells generated from acute LCMV infection (190). As well, although
Bim deficiency resulted in a 10-fold increase in the frequency of functional NP396-specific cells, over 80% of NP396-tetramer+ CD8 T cells are still incapable of producing both IFNγ and TNFα (414). With the caveat that Bim may not be a critical apoptotic factor for all functional cells, this study suggests that effector function impairment is a characteristic of CD8 T cells during persistent infection, independent of cell death.

The finding that TRAF1 deficiency during persistent viral infection occurs at the protein, but not mRNA level is of particular interest (Fig. 4.6). A similar, but often overlooked, finding is that the defect for IFNγ and granzyme B in exhausted CD8 T cells is also at the protein, but not mRNA expression level (190). These findings highlighted a possible defect in the protein translation or protein degradation machinery during persistent viral infection. In support of a translational defect in exhausted CD8 T cells, gene chip analysis showed downregulation of a large number of ribosomal subunits as well as several elongation and initiation factors (190). The mTOR (mammalian target of rapamycin) pathway regulates protein translation and plays a central role in promoting effector CD8 T cell generation by facilitating the anabolic processes (reviewed in (430)). As the mTOR pathway can be activated by the PI3K pathway which is likely inhibited during the chronic phase of persistent infection (discussed in a later section), there could be selective prevention of protein translation in an mTOR dependent manner. Further, the dysregulation of the mTOR pathway, if true, may also reflect a defect in energy metabolism in exhausted CD8 T cells (190) as the mTOR pathway regulates lipid biosynthesis and is implicated in glucose and amino acid metabolism (431, 432). This idea is explored further in a later section.
In summary, HIV-specific CD8 T cells from the chronic phase of HIV infection are characterized with a survival defect at the level of Bcl-2 impairment and Bim elevation and effector function defects, some of which are at the post transcriptional level. What has caused such impairment? Next, I'll present a model at the signaling pathway level that may determine or contribute to these outcomes.

**The relevance of PI3K pathway**

Identifying key signaling molecules that contribute to the impairment of HIV-specific CD8 T cells is of pivotal importance for drug design. The phosphotidylinositol-3-kinase (PI3K) signaling pathway plays a critical role in T cell activation and homeostasis (433). The recruitment and activation of the PI3K pathway is essential for CD28 costimulation (434), whereas PD-1 suppresses PI3K/Akt activation (435). As many HIV-specific CD8 T cells lack CD28 expression and have elevated PD-1 levels, it is possible that the PI3K pathway is inhibited in impaired CD8 T cells at the later stage of persistent viral infection. As well, HIV-specific CD8 T cells have a reduced response to common-γ-chain cytokines, also PI3K activators, thus removing another source for PI3K activation (436).

What is the consequence of PI3K inhibition or reduced activation? The activation of PI3K pathway has been implicated in promoting T cell survival, proliferation, effector function and trafficking to peripheral tissues (reviewed in (433, 437)). Thus, reduction or inhibition of the PI3K signaling could result in the phenotypes observed in HIV-specific CD8 T cells during chronic HIV infection. For example, as PI3K positively regulates the mTOR pathway (437), the reduced PI3K activity may result in a defect in the protein
translation machinery which could explain the discrepancy between protein and mRNA expression of TRAF1 (Fig. 4.6), IFNγ or granzyme B (190). As well, PI3K drives T cell survival via its effect on FOXO3a (437), and thus limited PI3K activation could result in reduced Bcl-2 and increased Bim expression. The latter effect of the lack of PI3K activation could be further exacerbated by HIV Tat, which is shown to induce FOXO3a in uninfected primary CD4 T cells (438), and thus could affect uninfected CD8 T cells in a similar manner.

There is some controversy as to whether the PI3K pathway is indeed inhibited during persistent viral infections. Overexpression of TGFβ in vivo (439) and supplementing TGFβ in vitro (440) resulted in downregulation of PTEN, an inhibitor of the PI3K pathway. The observation that TGFβ expression is sustained in Ag-specific CD8 T cells during persistent viral infection argues for the higher activation of PI3K. As well, there is a large proportion of CD62L-CCR7- HIV-specific CD8 T cells in circulation, reflective of PI3K activation which is responsible for transcriptional downregulation of CD62L, CCR7 and S1P1, collectively controlling T cell entry and egress from lymph nodes (437). PTEN deficient mice, which result in constitutive activation of the PI3K pathway, resulted in sustained IL-10 production (441), consistent with elevated levels of IL-10 observed during chronic HIV infection, albeit the source of IL-10 is likely not CD8 T cells. Is, then, the PI3K pathway over-activated or inhibited during HIV infection; and what is its relevance to HIV pathogenesis?
The contrary observations discussed above could be reconciled with a temporal two-stage model (Fig. 5.2). During the acute phase of persistent viral infection, the PI3K pathway in antigen-specific CD8 T cells is over-activated, fueled by the large availability of antigen and perhaps sustained levels of TGFβ. As a result, there is a large proportion of CD62L−CCR7− HIV-specific CD8 T cells in circulation, which are not yet unfunctional. During the chronic phase of persistent viral infection, the PI3K pathway is less activated or even inhibited, as insufficient common-γ-chain cytokine, increased coinhibition and reduced costimulation become predominant and impact the circulating memory/effector memory HIV-specific CD8 T cells. To test this hypothesis, we are in the process of generating mice with inducible PTEN deficiency. We plan to investigate the impact of PTEN knockout on CD8 T cell survival and effector function impairment and viral clearance during early and late clone 13 infections. Exactly when and how the switch of PI3K activation occurs are intriguing questions relevant to the timing of potential treatment and worth further exploration. Of note, analysis of the expression and function of the mTOR pathway in impaired CD8 T cells may be of particular interest as a downstream target of PI3K.

**Therapeutic implications of the 4-1BB costimulatory pathway**

Studies presented in this thesis as well as discussions thus far have highlighted the relevance of 4-1BB to CD8 T cell focused therapy for chronic HIV infection in a number of ways. 4-1BB upregulates Bcl-xL (Fig. 3.5B and (286)) which, although not impaired in its expression in HIV-specific memory CD8 T cells, could replace Bcl-2 when overexpressed in sustaining the survival of IL-3-dependent cells upon cytokine
The hypothetical role of PI3K pathway in the impairment of effector functions in HIV-specific CD8 T cells. During the acute phase of persistent viral infection, PI3K pathway in antigen-specific CD8 T cells is over-activated, fueled by large availability of antigen (and costimulation) and perhaps sustained levels of TGFβ. As a result, there are a large proportion of CD62L-CCR7- HIV-specific CD8 T cells in circulation, which are not yet unfunctional. During the chronic phase of persistent viral infection, PI3K pathway is less activated or even inhibited, as insufficient common-γ-chain cytokine, increasing coinhibition and reduced costimulation become predominant and impact the circulating memory/effector memory HIV-specific CD8 T cells.
withdrawal (442). Furthermore, overexpression of Bcl-x₅ prevented T cell apoptosis induced by FAS or IL-2 withdrawal in vitro (237). Enhancing Bcl-x₅ alone, however, is insufficient in rescuing the expansion of HIV-specific CD8 T cells, particularly in a PD-1<sup>high</sup> environment, reflected in the poor T cell recovery in response to CD80 and CD70, both of which are inducers of Bcl-x₅ (Fig. 3.3 A). In contrast, when Bim downregulation is coupled with Bcl-x₅ upregulation, via 4-1BBL costimulation alone, significant expansion of HIV-specific CD8 T cells can be achieved (Fig. 3.2Bi). Furthermore, engaging the 4-1BB pathway in vivo resulted in the expansion of an otherwise lost functional clone specific to LCMV NP396 (Fig. 4.10A). Thus 4-1BBL is a powerful driver of memory CD8 T cell survival with effects on a number of Bcl-2 family members. This characteristic of the 4-1BB pathway may provide critical help when targeting the HIV reservoir.

Another property of 4-1BBL is its ability to antagonize inhibitory signals. The effects of 4-1BBL on functional expansion of HIV-specific CTL appears to be in a PD-1 independent manner (Fig. 3.3), perhaps reflecting overlapping effector functions of 4-1BB engagement and PD-1 pathway blockade, such as through the PI3K pathway. Further studies are required to clarify this point. This ability of 4-1BB appears to be unique, as the combination of two other costimulatory molecules, CD80 and CD70, resulted in a trend toward preferential expansion of PD-1<sup>low</sup> HIV-specific CTL (p = 0.08, Fig. 3.3). Of note, the PD-1 pathway is also implicated in CD8 T cell survival during HIV infection (181), albeit through unclear molecular mechanisms. It has also been shown that 4-1BB costimulation, but not CD28, can reverse TGFβ suppression of human cord blood
CD8 T cell differentiation (214). This effect of 4-1BB is at the level of TGFβ signaling through suppression of TGFβ induced phosphorylation of Smad2 (214).

Survival signals are insufficient for control of chronic viral infection, partly reflected in the limited viral load reduction during the clone 13 infection of Bim deficient mice (414). 4-1BBL, when coupled with another costimulatory molecule, can result in significant increase in functional cells from a non-functional starting population (Fig. 3.3). It is unclear from this experiment, however, whether 4-1BBL has the ability to enhance effector function beyond its rescue of the survival defect of HIV-specific CD8 T cells. Using an influenza model, we were able to demonstrate that 4-1BBL induced stronger cytolytic function on a per cell basis as compared to CD70 (Fig. 2.5). As well, when engaged in vivo, 4-1BB increased the proportion of IFNγ+TNFα+Tetramer+CD8 T cells during chronic LCMV infection (Fig. 4.10B and data not shown). Thus, engaging the 4-1BB pathway can increase effector functions of exhausted CD8 T cells. A study from Lin and Liu showed that engagement of 4-1BB has a much larger impact in granzyme production on in vitro generated memory as compared to naïve transgenic T cells (443), further highlighting the ability of the 4-1BB pathway to increase effector functions preferentially from a memory-like T cell population.

Despite the favorable characteristics of 4-1BB, the potency of a stand alone therapy is modest, perhaps reflective of the loss of TRAF1 during chronic viral infection. As discussed earlier, some defects in impaired CD8 T cells are likely a result of limited cytokine signaling such as those of IL-7. Importantly, this thesis presented a successful
combination therapy, agonistic anti-4-1BB antibody plus IL-7, for treating persistent viral infection when administered during the chronic phase. The combination, but not either therapy alone, resulted in sustained viral reduction or viral clearance in multiple organs (Fig. 4.10). The addition of IL-7 likely complements the anti-4-1BB therapy by increasing the TRAF1 expression in antigen-specific CD8 T cells (Fig. 4.8) and antagonizing the effect of TGFβ (389). As well, IL-7 is a potent inducer of Bcl-2, additive to the effect of 4-1BB on Bcl-xL and Bim. In reverse, 4-1BB therapy may provide unique enhancement on effector functions. Interestingly, double knockout of CD80 and CD86 resulted in significantly lower fraction of LCMV-specific CD8s (4 epitopes) expressing CD127 (IL-7Rα) during LCMV Traub infection, raising the possibility of regulation of CD127 by costimulation (249). Further study is required to analyze whether the reduced CD127 expression could be recovered via agonistic anti-4-1BB antibody.

**When and how to engage the 4-1BB pathway?**

Important questions remain to be addressed as to when and how best to deliver the 4-1BB signal, and how much to deliver to avoid pathology. As well, should the 4-1BB therapy be coupled with HAART or IL-7 or both or with other available approaches? Answers to these questions depend on the goal of the therapy and the toxicity of the chosen approach. If the purpose is to help HIV infected individuals that are either intolerant to the toxicity of HAART therapy or responded poorly to HAART, a transient low dose agonistic anti-4-1BB antibody therapy might be beneficial when coupled with other therapies to reduce viral load. If the goal of the therapy is to reduce the HIV reservoir, one could foresee the
best T cell clones are those that are able to migrate to the site of the reservoir, recognize the infected cells, survive long enough, and are functional so as to kill the target cell quickly before new viruses are made. Although 4-1BB activation may enhance both HIV-specific CD8 T cell survival and function, the time lapse between 4-1BB engagement and the activation of the latent cell/reservoir before new virus production becomes critical. For this reason, an antibody approach is likely less effective as it adds a compounding factor to when and where the antibodies would go in the patient. Furthermore, anti-4-1BB antibody treatment generates considerable toxicity when delivered continuously (444) or in naïve mice (408). It should be noted that toxicity tested in rodent models is not necessarily equivalent to that in primates. For example, while PD-1 deficiency resulted in the death of mice infected with LCMV clone 13 (169), repeated injection of PD-1 blocking antibodies in SIV infected macaques from day 1 showed no sign of pathology and resulted in alleviation of disease progression (195). Given the undesirable toxicity of high dose 4-1BB antibody therapy in recent clinical trials for treating melanoma, however, toxicity tests should be given priority when analyzing the effect of agonistic anti-4-1BB antibody in treating SIV infected macaques.

Alternative means to deliver the 4-1BB signal have been explored. Schabowsky et al. showed that a soluble form of 4-1BBL when linked to straptavdin (SA-4-1BBL) has better immunomodulatory activity and less toxicity than agonistic antibody (445). Furthermore, SA-4-1BBL can be used together with biotinylated antigen and results in an efficient antigen delivery system with enhanced anti-tumor effect when coupled with DC therapy (446). Another alternative to antibody therapy is the use of Aptamers, oligonucleotide-based-ligands, that are more cost-effective. Anti-4-1BB aptamers were generated and showed to mediate tumor rejection in mice (447). A recent study showed
the efficacy of tumor clearance by T cells expressing costimulatory molecules (448). Delivering 4-1BBL on T cell clones may improve efficiency of the therapy as neighbouring T cells that may or may not be specific to the antigen presented on the HIV reservoir cells could provide a 4-1BB signal. Further studies are required to analyze the feasibility of this idea.

**Other points of interest**

*The requirement of costimulation during the chronic phase of HIV infection*

While this thesis has explored the therapeutic application of the 4-1BB pathway, it is unclear whether costimulation is required during chronic HIV infection. And if so, which molecule is of particular relevance. As discussed in chapter I, limited reports have explored the roles of costimulatory molecules during persistent viral infection. Furthermore, none of these studies analyzed the impact of deficiency in costimulation at the chronic stage of infection independent of the acute phase. The latter point is of vital importance as it has become increasingly clear that the chronic phase of persistent viral infection bears unique and sometimes opposite characteristics compared to that of the acute phase. I showed in a preliminary study that 4-1BBL deficient mice had no impact on serum viral load in clone 13 infected mice from day 7 to day 42 (data not shown). Inducible 4-1BBL knockout mice or blocking antibodies used at the later stage are necessary to determine whether the 4-1BB pathway is required during the later stage of chronic viral infection.

*The therapeutic implication of TRAF1*

This thesis has identified the loss of TRAF1 in CD8 T cells as a novel immune dysregulation of chronic HIV infection. Importantly, this defect is of functional relevance as knocking down TRAF1 in viral controllers resulted in a striking defect in HIV suppression (Fig. 4.4). While we discussed the relevance of the loss of TRAF1 to the 4-1BB costimulatory pathway, TRAF1 is downstream of several other costimulatory molecules. Thus, identifying the means to correct the TRAF1 defect may help improve signals from multiple pathways. It is therefore of interest to investigate whether HAART could improve TRAF1 expression. As well, although we have shown that IL-7 can
increase TRAF1 levels during chronic LCMV infection *in vivo* (Fig. 4.8), it is unclear whether a similar effect could be observed in humans. We are currently in collaboration with the IL-7 clinical trial underway in Montreal. We plan to analyze the effect of HAART with or without IL-7 therapy on TRAF1 expression in HIV-specific CD8 T cells. In addition to a functional role, we entertain the idea that TRAF1 could be a prognostic marker for the success of HAART/IL-7 therapy. Longer term studies are required to test the predictive value of TRAF1 expression for HIV disease outcome.

The rising interest in TRAF1 and human disease has also prompted us to study single nucleotide polymorphism (SNP) in the TRAF1 coding region. Several SNPs that result in amino acid changes are summarized (Fig. 5.3). I have designed a PCR approach to identify specific SNP in donor PBMC and studies are underway analyzing potential consequence of SNP in TRAF1 expression in human and potential disease correlation.

In sum, this thesis offered a spectrum of analysis on the ability of the 4-1BB pathway, as compared to other costimulatory molecules, to counteract the impairment of HIV-specific CD8 T cells during chronic infection. As well, new insight on dysregulation of the 4-1BB costimulatory pathway, via loss of TRAF1, is revealed during persistent viral infection. Finally, combination therapy of agonistic 4-1BB and IL-7 is successful in treating chronic LCMV infection, offering proof-of-concept results for the future design of a 4-1BB therapy in treating individuals chronically living with HIV.
Figure 5.3  SNP annotation of the human TRAF1 at the protein level. All SNP were annotated based on dbSNP from NCBI as of May 19, 2009. Not annotated here, but there are 5 synonymous SNP in the coding region; 61 SNP in the introns and 8 SNP in the 3’UTR.
Chapter VI

REFERENCES


specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. The Journal of experimental medicine 192:63-75.


genes in a human immunodeficiency virus type 1-infected asymptomatic individual. *Journal of virology* 69:5743-5753.


94. Calugi, G., F. Montella, C. Favalli, and A. Benedetto. 2006. Entire genome of a strain of human immunodeficiency virus type 1 with a deletion of nef that was recovered 20 years after primary infection: large pool of proviruses with deletions of env. *Journal of virology* 80:11892-11896.


with nonprogressive human immunodeficiency virus type 1 infection. *Journal of virology* 70:7752-7764.


immunodeficiency virus-1 entry in human monocyte-derived macrophages. *Immunology* 114:565-574.


cells mediates intestinal inflammation and contributes to IgA nephropathy. *The Journal of clinical investigation* 113:826-835.


specific cytotoxic T lymphocytes by artificial APCs expressing ligands for the T-cell receptor, CD28 and 4-1BB. *Nature biotechnology* 20:143-148.


