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An Investigation of Anergy As a Potential Mechanism of Human Immunodeficiency Virus Type

1 Escape from Control by Cytotoxic T Lymphocytes

A dissertation submitted in partial satisfaction of the  
requirements for the degree Doctor of Philosophy  
in Microbiology, Immunology, and Molecular Genetics

by

Mark Alexander Grossman

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## ABSTRACT OF THE DISSERTATION

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Professor Otto Orlean Yang, Chair

CD8<sup>+</sup> cytotoxic T lymphocytes (CTL) play an important role in HIV-1 infection. However, in most persons the HIV-1-specific CTL become dysfunctional. It has been suggested that anergy, a mechanism of T cell tolerance, may play a role in HIV-1-specific CTL dysfunction. Since HIV-1 is known to escape CTL recognition through mutation of epitopes to produce weak agonist altered peptide ligands (APL)—and anergy is thought to occur through suboptimal recognition—we hypothesized that weakly-recognized epitope variants may suboptimally stimulate the T-cell receptor (TCR) and trigger aberrant TCR signaling, leading to CTL anergy and enhanced viral persistence. We investigated weak agonist APL-mediated anergy as a potential mechanism of dysfunction in three HIV-1-specific CTL clones targeting three different epitopes. Clones were exposed to weakly-recognized epitope variants and screened for cytokine

secretion, gene expression of markers of anergy and other forms of T cell dysfunction, and subsequent capacity to kill index peptide-presenting target cells in secondary challenge. Under those conditions, although suboptimal stimulation dampened cytokine secretion, we observed no patterns in gene expression profile consistent with anergy. Additionally, pre-exposure of CTL to weak agonist epitope variants had no discernable effect on their subsequent capacity to kill index epitope-bearing target cells. These data suggest that while weak agonist epitope variants evade CTL pressure through reduced recognition, their weak agonism does not appear to drive dysfunction of HIV-1-specific CTL, arguing against a role of APL-induced anergy in HIV-1-specific CTL dysfunction. However, under different experimental conditions, one HIV-1-specific CTL clone appeared to demonstrate varying degrees of reduced recognition of index epitope after pre-treatment with weak agonist APL stimulation. To try to reconcile these seemingly contradictory results, additional literature on the role of costimulation in both anergy and CD28<sup>-</sup> effector CTL is incorporated into the discussion. In Chapter 7, I propose a speculative model of how HIV-1-derived weak agonist APL, in conjunction with blockade of CD28-independent costimulatory pathways, may be able to cause HIV-1-specific CTL anergy *in vivo*. In Chapter 8, alternative approaches are suggested for possible future studies to resolve the confounding issues that still surround the field of APL-induced T cell anergy.

The dissertation of Mark Alexander Grossman is approved.

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University of California, Los Angeles

2019

I wish to dedicate this thesis in honor of the memory of my close friend Rachael Lynn Marsik,  
who passed away in August 2011.

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1. Allevato M, Bolotin E, Grossman M, Mane-Padros D, Sladek FM, Martinez E (2017) Sequence-specific DNA binding by MYC/MAX to low-affinity non-E-box motifs. PLoS ONE 12(7): e0180147. <https://doi.org/10.1371/journal.pone.0180147>

**Chapter 1: HIV-1 Pathogenesis and the Role of CD8<sup>+</sup> Cytotoxic T Lymphocytes (CTL)**



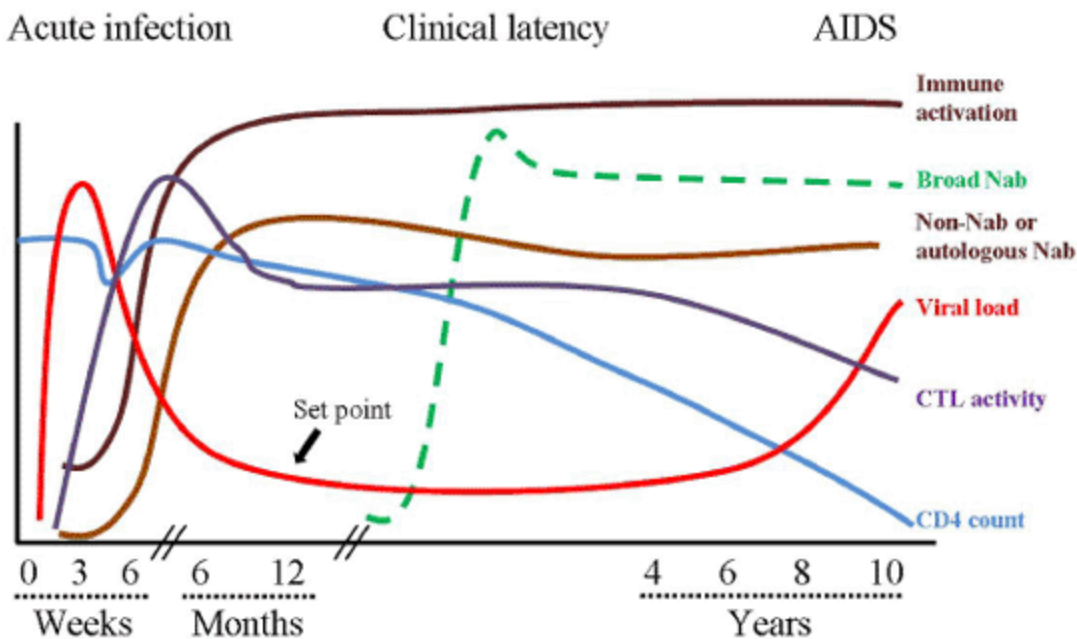
## **1.1 Overview of the HIV-1 Pandemic**

As of 2017, about 35 million people worldwide have died from HIV-1, and about 37 million people are currently living with HIV-1 infection [1]. Although the advent of antiretroviral therapy (ART) has reduced mortality and helped patients live much longer and healthier lives, to date no one has been cured of HIV-1 infection from ART. While ART regimens continue to be improved to reduce the severity of side effects, they nevertheless still have side effects. Additionally, the cost of lifetime ART is estimated to be almost \$380,000 per person in 2010 dollars [2], a prohibitively expensive treatment for many people, especially those living in third world countries. In addition to cost, besides administering treatment there is also the need for treatment management, which requires the infrastructure to measure viremia, CD4 T cell counts, and other parameters, something that's likely not available in such resource-limited areas. Even if the cost of ART were to drop at some point, it wouldn't change the fact that ART cannot cure HIV-1 infection by itself. Because of this, it's imperative that we develop better immunotherapy strategies to improve containment of viral replication.

## **1.2 Typical Clinical Course of HIV-1 Infection**

During acute infection there is rapid depletion of activated CD4<sup>+</sup> T cells in the gut. Viremia drops concurrent with the rise of HIV-1-specific CTLs, weeks before antibody responses come up. Despite CTL pressure, viremia does not completely clear and instead reaches a plateau called the “viral set point”, establishing a persistent chronic infection that varies in duration between different persons. The viral set point is inversely correlated with the rate of disease progression. Once the total CD4 count falls below a certain level, viremia increases again, and the person develops acquired immune deficiency syndrome (AIDS).

**Box 1.4** Schematic representation of clinical course of HIV-1 infection



The dashed line indicates that the broad Nabs have been found in a portion of HIV-1-infected patients.

**Figure 1.1** The relationship between plasma viremia, CTL responses, CD4 count, immune activation, and antibody responses over time in HIV-1 infection [3]. A rapid rise in viremia occurs in the first couple of weeks of infection, resulting in loss of CD4+ T cells. Around 3-6 weeks post-infection the induction of virus-specific CTL responses coincides with a reduction in viremia that plateaus and establishes a viral set point. General immune activation is rampant during the chronic phase of infection, and although antibody responses begin coming up a few weeks after CTLs, antibodies don't appear to have a dramatic effect on the set point viremia. After a variable duration of latency (which can last much longer than 10 years in some persons), once the CD4 count drops below a threshold of ~200 cells/uL, viral replication climbs once more and establishes the end stage, AIDS.

### **1.3 HIV-1**

HIV-1 is a single-stranded RNA virus that integrates into the human genome via the HIV-1 protein integrase [4-6]. The virion has two copies of viral RNA, each of which are converted into double-stranded DNA in a process called reverse transcription that is mediated by the enzyme reverse transcriptase [7]. Reverse transcriptase is highly error-prone, making a mutation about 1 in every  $10^4$  nucleotides. As HIV-1 has a genome of roughly 10 kb in length, this means that on average it will introduce 1 mutation every time a viral particle infects a cell. This high mutation rate, coupled with the fact that HIV-1 is very plastic (i.e. generally it can tolerate a lot of mutation without severely compromising replication), allows the virus to generate many possible sequence variants rather quickly [8]. Chapter 2 discusses how this high mutability allows HIV-1 to escape containment by CD8<sup>+</sup> cytotoxic T lymphocytes (CTL).

## **1.4 Importance of CD8<sup>+</sup> Cytotoxic T Lymphocytes (CTL) in HIV-1 Infection**

We know that CTL are important for the partial control of HIV-1 based on several lines of evidence. The drop in viremia that occurs during acute infection coincides with the rise of HIV-1 specific CTL responses [9-11] and the loss of HIV-1-specific CTL in part mediates the development of AIDS [12]. In rhesus macaques, animals that had their CD8<sup>+</sup> cells depleted experienced a dramatic rise in SIV viremia, which went back down after the CD8<sup>+</sup> cells returned [11, 13]. CTL can potently suppress viral replication *in vitro*, and the evolution of escape mutations within CTL-targeted epitopes during chronic infection *in vivo* is a clear demonstration that CTL exert pressure on HIV-1 [14-18]. The fact that an individual's HLA-I type can be predictive of the rate of disease progression is consistent with the idea of HIV-1 replication being determined, at least to some degree, by CTL targeting [19-25]. Lastly, a small percentage of HIV-1<sup>+</sup> persons are able to maintain virologic suppression (with a viral load < 50 copies/mL in blood) without being on ART [26]. These so-called 'elite controllers' and 'long-term nonprogressors' are living proof that CTL can be protective under the right circumstances, which provides hope for the future of T cell immunotherapy for HIV-1.

## **Chapter 2: HIV-1 Immune Escape from CTL**

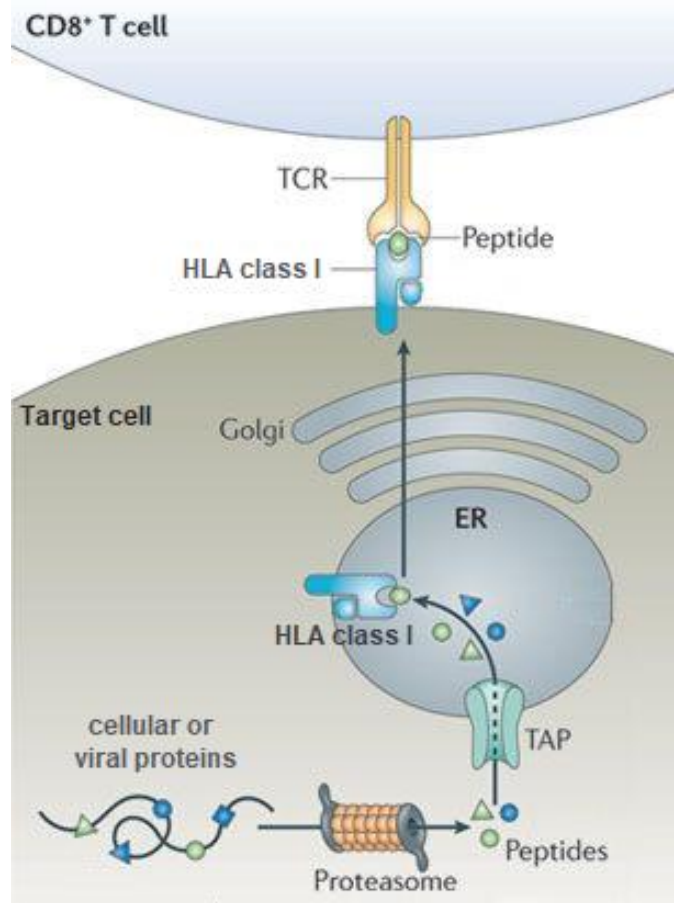
## 2.1 Preface

As explained in the previous chapter, CTL play an important role in HIV-1 infection. That being said, unfortunately in the vast majority of cases the virus-specific CTL are ultimately unsuccessful in controlling the infection, because HIV-1 has adapted mechanisms to escape from CTL. This chapter will discuss ways in which HIV-1 escapes recognition by the CTL to drive viral persistence *in vivo*.

## **2.2 CTL Are Targeted to the HLA Class I Presentation Pathway**

In order to understand how HIV-1 escapes from CTL recognition, we must first explore the basis by which CTL recognize HIV-1 infected cells. CTL recognize the presence of foreign peptide antigens that are presented by human leukocyte antigen class I (HLA-I) molecules. Every nucleated cell in the human body (that is, any cell type other than mature red blood cells) presents peptide antigens to the immune system via the HLA-I antigen presentation pathway (Figure 2.1). The HLA-I pathway has three major steps. First, as part of the normal housekeeping functions within cells, cytosolic proteins routinely get processed through an organelle called the proteasome, which is essentially a “protein shredder” that cleaves proteins into small pieces that are typically 8-11 amino acids in length. These 8- to 11-mer peptides then enter another organelle called the endoplasmic reticulum (ER) through the protein Transporter Associated with Processing (TAP). It is here, inside the ER, where newly synthesized HLA-I molecules reside, waiting for peptide antigens to bind to them. Binding of peptide to the HLA then causes a sequence of events that leads to the presentation of the peptide-HLA complex at the cell surface. The evolutionary purpose of having a system of presenting peptide antigens on the cell surface is to essentially show a ‘sampling’ of what the cell is making on the inside, to alert the immune system if there is something inside the cell that is not supposed to be there, as would be the case with a foreign antigen derived from an invading virus. Since the CTL cannot “see” what is inside the cell, the HLA-I pathway in effect allows them to scan pieces of the proteome on the cell surface, the only part of a target cell to which the CTL have access.

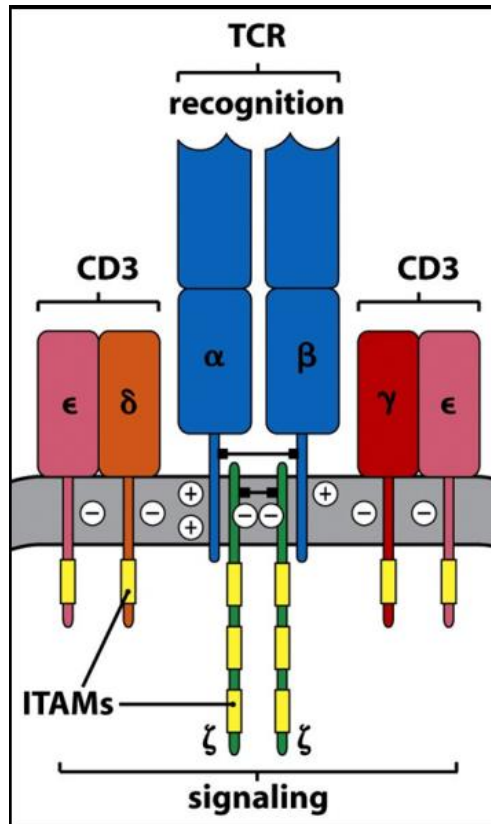




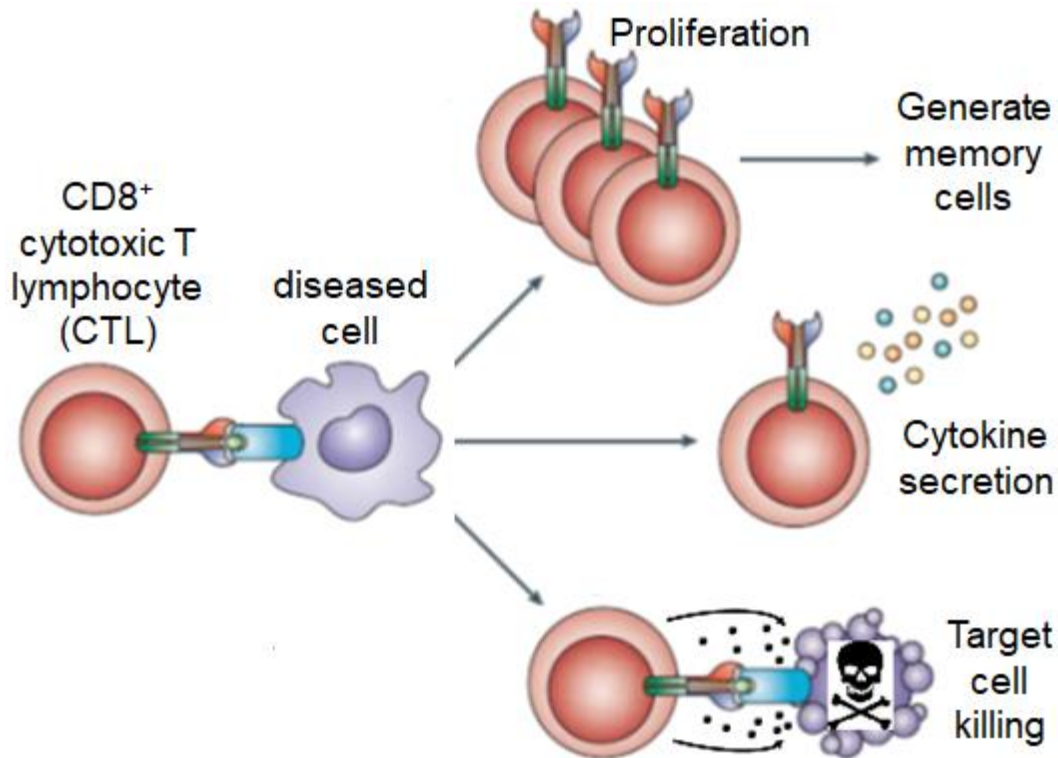
**Figure 2.1. The HLA-class I antigen presentation pathway (adapted from [27]).** Proteins derived from the host cell or an intracellular virus are cleaved by the proteasome into smaller polypeptides. These peptides then enter the endoplasmic reticulum through the TAP transporter protein and bind to HLA class I molecules. Upon peptide binding, the peptide-HLA complexes are sent through the secretory pathway to the cell surface, where they are scanned by CTL. This pathway allows CTL to “see” what the cell is making and helps them to distinguish a healthy cell from a diseased one.

### **2.3 CTL Recognize Foreign Antigens Via The T-Cell Receptor (TCR)-CD3 Complex**

Recognition of foreign antigen occurs through engagement of the peptide-HLA complexes on the surface of the target cell with the T-cell receptor (TCR) on the surface of the CTL. The TCR is a heterodimeric protein composed of an  $\alpha$  and a  $\beta$  chain, each of which have a variable domain and a constant domain. The TCR variable domains are responsible for conferring specificity to a particular peptide-HLA complex, while the constant domains are important for interaction with the different subunits of the CD3 complex; association with CD3 is a requirement for the TCR to be properly folded on the cell surface (Figure 2.2). When the specific, or “cognate”, antigen binds the TCR, signaling domains called ITAMs (immunoreceptor tyrosine-based activation motifs) on the cytoplasmic portion of CD3 are triggered to initiate downstream signaling (covered further in Section 3.2). If the amount of TCR triggering is strong enough to reach a certain threshold, it will lead to activation of the CTL and trigger effector function (Figure 2.3). The different effector functions of CTL have different requirements for triggering, but those details will be covered later in Section 3.5.



**Figure 2.2. The TCR/CD3 complex (from [28]).** The TCR is a heterodimeric protein composed of an  $\alpha$  and a  $\beta$  chain (blue), each of which have a variable domain and a constant domain. The variable domains are responsible for conferring specificity of binding to a particular peptide-HLA complex. The constant domains are important for interaction with the different subunits of the CD3 complex (red, pink, orange, and green), which contain ITAMs (yellow) that transduce downstream signaling when the receptor is triggered by antigen binding.

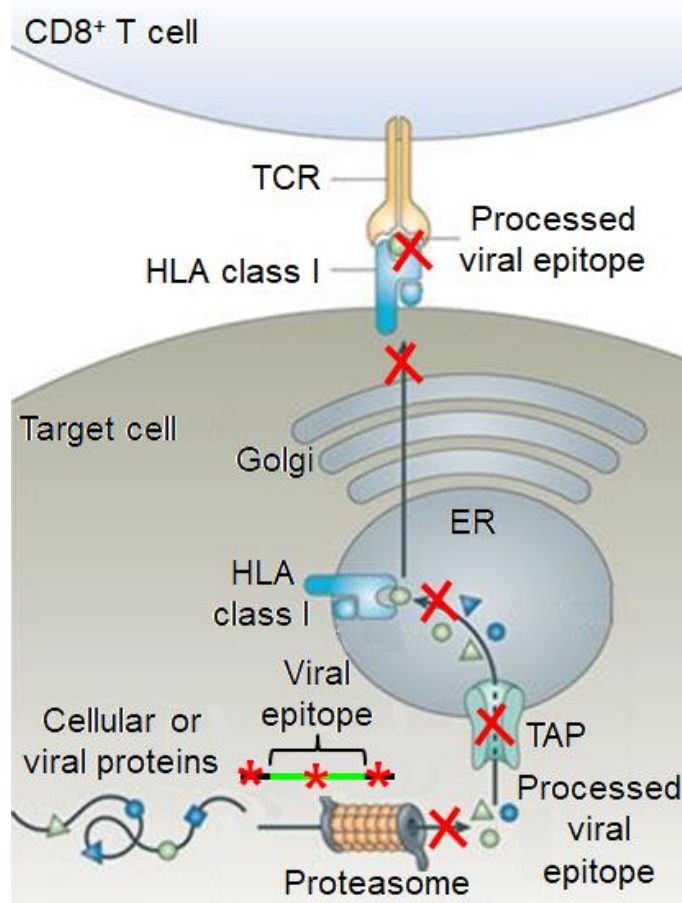


**Figure 2.3. Functions of CD8<sup>+</sup> cytotoxic T lymphocytes (adapted from [29]).** When the CTL recognizes a foreign antigen on the surface of a diseased cell, it can perform several effector functions. Release of cytotoxic granules directly at the target cell causes its death by apoptosis. CD8<sup>+</sup> T cells can make multiple cytokines, which include IFN- $\gamma$ , TNF- $\alpha$ , IL-2, MIP-1 $\alpha$ , and MIP-1 $\beta$  (discussed further in Chapter 6); these help with recruitment and activation of other immune cells at the site of infection or malignancy. Strongly activated CTL can also undergo rapid proliferation and generate long-lasting memory cells.

## 2.4 Mechanisms of HIV-1 Escape from CTL Pressure

The HIV-1 protein Nef downregulates the surface expression level of HLA-A and HLA-B molecules by causing them to be redirected from the trans-Golgi network to lysosomes for degradation [30]. The effect of this is reduced epitope/HLA-I complex density, which decreases the strength of TCR signaling and therefore the likelihood of recognition. As mentioned before, HIV-1 is highly mutable, and it can make mutations that interfere with every other step of the HLA class I presentation pathway that Nef cannot intervene in (Figure 2.4). Mutations within an epitope that cause epitope cleavage would result in reduced generation of that epitope.

Alternatively, mutations flanking the epitope sequence can inhibit processing; some mutations within the epitope can inhibit TAP-binding, HLA binding, or recognition by the TCR. Work by [14-18, 31-33] demonstrated that HIV-1 makes mutations in CTL-targeted epitopes *in vivo* in order to escape CTL responses.



**Figure 2.4. HIV-1 escapes CTL recognition by causing disruption in the HLA-I pathway (adapted from [27]).** HIV-1 can undergo mutations that inhibit either the generation of the epitope, the entry of it into the ER, binding to HLA-I molecules, or binding to the TCR. Additionally, it can redirect peptide-HLA complexes away from the surface via Nef-mediated downregulation. Red 'X's denote parts of the pathway that are affected by these evasion strategies.

## **2.5 Is avoidance of CTL recognition the only mode of viral escape?**

Current literature suggests that the main mechanism of HIV-1 escape from CTL is through reduced antigen recognition. We wanted to investigate whether there might be more to the story of escape. There is much interest in better elucidating the factors that control the functional potency and durability of modified T cells for adoptive T cell therapy. It is known that overstimulation of T cells can cause apoptosis [34] and functional exhaustion [35, 36], but what has not been well-studied is if/how suboptimal recognition of antigen has persistent effects on the T cells, particularly effector CTL in HIV-1 infection. Weak agonist altered peptide ligands (APL) have been suggested to play a role in escape from CTL recognition [37, 38] by antagonizing CTL responses to index epitope in a process termed “TCR antagonism”. However, due to the methods used in those studies, it appears to be more related to competitive TCR binding rather than bona fide inhibition of the normal CTL response (discussed further in Section 6.3). Nevertheless, these studies garnered interest in exploring effects of APL on CTL function.

**Chapter 3: Synapses, Scaffolds, and Signal Integration--The Molecular Basis of Antigen  
Recognition and Triggering of CTL Effector Functions**

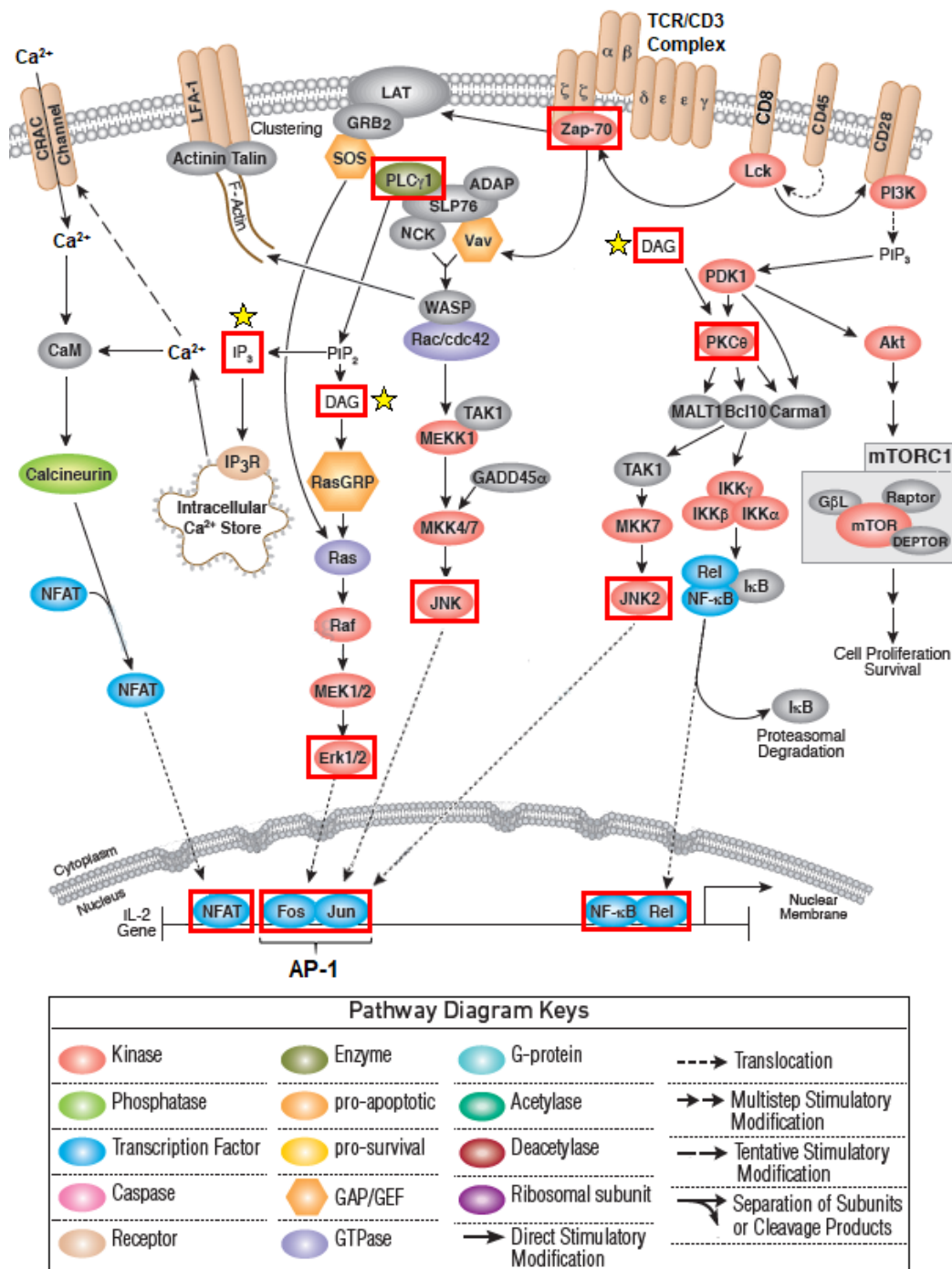


### **3.1 The immunological synapse**

The basis of antigen recognition by CTL relies on conversion of antigen binding at the cell surface into intracellular signals that determine the appropriate type and magnitude of CTL effector functions. These intracellular signals are generated downstream of the TCR (described in more detail in Section 3.2). However, before TCR signaling can begin, there is an important requisite step: the CTL and the antigen-presenting cell (APC) must first make physical contact with each other and form a cellular junction called the “immunological synapse”. The immunological synapse (IS) is formed when the actin cytoskeleton of the CTL pushes its membrane close enough to the membrane of the APC so that LFA-1, a cell adhesion molecule on the CTL, can bind to ICAM-1 on the APC. Binding of LFA-1 with ICAM-1 promotes T cell polarization and migration of TCR complexes towards the center of the IS. The pan-leukocyte marker and phosphatase CD45, a bulky molecule, is pushed toward the distal region of the IS. Naïve T cells have a version of CD45 called CD45RA, a bulkier version than the one expressed on effector T cells, called CD45RO. CD45RA must be pushed away from the center of the synapse because if it were not, its size would not allow the two cell membranes to be close enough for the TCR-CD3 complexes on the CTL to bind to peptide-HLA complexes on the APC. In the center of the IS, TCR-CD3 complexes form microclusters and participate in serial engagement with peptide-HLA ligands [39, 40]. Many peptide-HLA complexes will not result in downstream signaling, as is the case with self-peptide (due to tolerance mechanisms, which are discussed further in Section 4.2).

### 3.2 TCR Signaling Pathways (Signal 1)

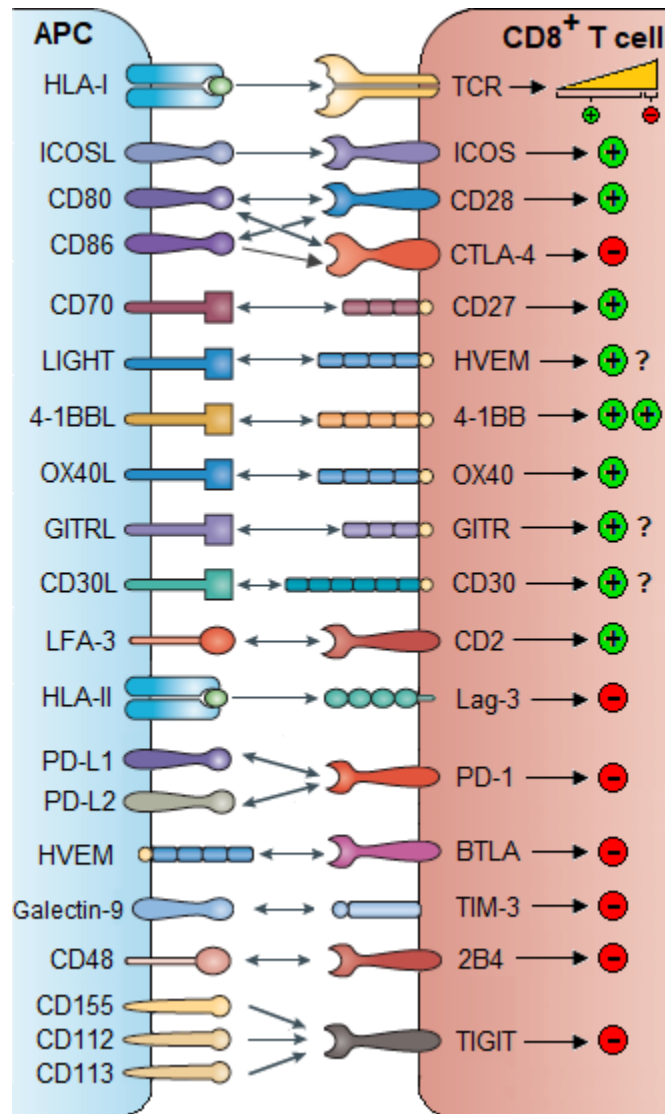
If a CD8<sup>+</sup> cytotoxic T lymphocyte (CTL) encounters its cognate antigen on the surface of an APC, the binding of antigen to the TCR will initiate downstream signaling through a series of signaling pathways generally referred to as “TCR signaling”. In the first step the T cell kinase Lck phosphorylates a subunit of the TCR-CD3 complex, called CD3 $\zeta$ , on domains called ITAMs (Immunoreceptor Tyrosine-based Activation Motifs). Phosphorylation of the ITAMs on CD3 $\zeta$  allows for the recruitment and activation of Zeta-Associated Protein of 70 kDa (ZAP-70), a kinase essential for transducing signals at the membrane further down into the cell. Active ZAP-70 then phosphorylates two important scaffold proteins, Linker of Activated T cells (LAT) and SH2 Domain-Containing Leukocyte Protein of 76kDa (SLP-76), on multiple residues. The phosphorylated sites on these scaffolds act as docking areas for the recruitment and activation of several factors, one of which is Phospholipase C-gamma 1 (PLC $\gamma$ 1). PLC $\gamma$ 1 is a key enzyme in the TCR signaling pathway, as it converts phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) into the critical second messengers inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> then leads to the activation of Nuclear Factor of Activated T cells (NFAT), an important transcription factor, via the Ca<sup>2+</sup>/calmodulin/calcineurin/NFAT pathway. DAG stimulates the activation of two important transcription factors: Nuclear Factor Kappa-B (NF $\kappa$ B) via the PKC $\theta$ /Bcl10/CARMA1 axis; and Activator Protein-1 (AP-1), a heterodimer of Fos and Jun family proteins, via the Ras/ERK and JNK pathways. When NFAT, AP-1 and NF $\kappa$ B are all active in the nucleus together, they initiate transcription of genes important for the CTL response, such as the cytokine Interleukin-2 (IL-2). More will be discussed about IL-2 in Section 3.4. The importance of NFAT, AP-1 and NF $\kappa$ B will be explored further in Section 3.5 and 4.3.4.



**Figure 3.1 The T cell receptor signaling pathway.** This diagram (adapted from [41]) does not reflect the full complexity of the signaling cross-talk that occurs *in vivo* and has been simplified for easier viewing. Key molecules are surrounded by red boxes, and the second messengers inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) are denoted with a gold star. The endpoint of strong TCR signaling is the activation of three critical transcription factors: NFAT, AP-1 (a heterodimer of Fos and Jun), and NFκB.

### 3.3 Costimulatory and Co-inhibitory Pathways (Signal 2)

Besides being signaled through the TCR, often referred to as “signal 1”, T cells also decode signals from a second source, or “signal 2”. Signal 2 comes from the engagement of co-stimulatory receptors with their ligands. Of critical importance in naïve T cells is the CD28 costimulatory pathway; CD28 binding to one of its two possible ligands, CD80 or CD86, is required to properly activate a naïve T cell and differentiate it into an effector cell. Because of this essential role of CD28 in priming T cells, the CD28 costimulatory pathway is probably the best characterized pathway in T cells (for both CD4 and CD8) aside from the TCR pathway. However, it is important to know that human CD8<sup>+</sup> T cells have many other surface receptors capable of signaling to either augment TCR signaling or inhibit it. These receptors and their ligands comprise pathways that are known as either ‘co-stimulatory’ or ‘co-inhibitory’. The better-known co-stimulatory receptor/ligand pairs are as follows: CD28-CD80/CD86, ICOS-ICOSL, CD27-CD70, HVEM-LIGHT, 4-1BB-4-1BBL, OX40-OX40L, GITR-GITRL, CD30-CD30L and CD2-CD58. In contrast, the receptor/ligand pairs with a co-inhibitory function are: CTLA-4-CD80/CD86, PD1-PD-L1/PD-L2, TIM-3-Galectin-9, Lag-3-HLA-II, BTLA-HVEM, 2B4-CD48, and TIGIT-CD112/CD113/CD155 [42, 43]. Not all receptor/ligand pairs are equal; some, like 4-1BB with its ligand, appear to be stronger costimulators than others like ICOS or GITR. The receptors that are classified as co-inhibitory associate with phosphatases like SHP-1, SHP-2, and PP2A, which interfere with the phosphorylation cascade that occurs in the TCR signaling pathways. Conversely, the co-stimulatory receptors are so named because they activate PI3K, which helps strengthen the distal parts of the TCR signaling pathway. Some of these co-stimulatory receptors will be discussed further in Section 7.4.



**Figure 3.2. Co-stimulatory and co-inhibitory receptor/ligand pairs in human CD8<sup>+</sup> T cells.** Adapted from [42], this shows an APC and a CD8<sup>+</sup> T cell interacting with each other through the receptor/ligand pairs that are better known (but not all that may exist) for having either a co-stimulatory (“+”), co-inhibitory (“-”), or questionable/weak (“?”) effect on human CD8<sup>+</sup> T cells. Despite this apparent simplicity, different co-stimulatory receptor/ligand pairs have different efficiencies of costimulating the various aspects of CTL functionality (cytotoxicity, cytokines, proliferation, survival, memory). In the case of TCR-pMHC, signals that aren’t too strong generally have a positive effect on function, but overstimulation can lead to activation-induced cell death (AICD), depicted with a “-” sign.

### **3.4 Role of IL-2 (Signal 3)**

IL-2 is a critical T-cell growth and survival factor. IL-2 signaling is necessary for maintaining expression of the pro-survival factor Bcl-2 and inhibition of the pro-apoptotic factor Bim during T cell activation [44]. HIV-1-specific CTL clones from our laboratory begin to die after 1-2 days of culture in medium without IL-2, and fail to proliferate when stimulated with anti-CD3 antibody and feeder PBMC in IL-2-deficient medium (our unpublished observations). At one point, we tested to see whether titrations of IL-7 alone, IL-15 alone, or both could maintain viability of our CTL clones in the absence of IL-2 without causing homeostatic proliferation. We observed that high concentrations of either IL-15 alone or both IL-7 and IL-15 could maintain CTL viability but also led to homeostatic proliferation in resting CTL (data not shown). This suggested that these three common  $\gamma$ -chain cytokines have overlapping yet distinct functions. IL-7 and IL-15 both contribute to T cell homeostasis in the blood [45], whereas IL-2 is predominantly involved in the expansion of antigen-specific T cells following antigen stimulation.

### 3.5 Signal Integration and Differential Requirements for Triggering Various CTL Effector Functions

For simplicity during teaching and to avoid confusion, students are taught that T cell activation and the T cell response is a “Yes/No”, “On/Off” phenomenon. In reality this is not the case. T cells have to give finely-tuned responses to antigen stimulation *in vivo*, because the quality and density of antigen are both important for ensuring a response with the appropriate magnitude and specificity. The requirement for a finely-tuned CTL response that can quickly adapt to changing circumstances (CTL are part of the *adaptive* immune system, after all) means that the different CTL functions cannot all be regulated in the same fashion. Differential requirements for triggering different functions allows T cells to tightly control the strength of the response to strike a good balance between immunity and preventing damage to healthy tissue.

T cells determine the appropriate type and magnitude of response by decoding a message that is comprised of multiple integrated signaling pathways. In previous sections of this chapter I described what those pathways were, but didn't go into detail about how they are differentially regulated nor how they control the quality of the downstream response. Lysis of target cells is dependent mainly on the release of cytotoxic granules containing perforin and granzymes. These lytic granules are pre-formed, and as such do not require *de novo* transcription. Degranulation is dependent on the cytoplasmic calcium flux, but not on a stable IS [46], so it happens rapidly—within 5 minutes of CTL-APC contact [28]. Depending on the combination of the CTL clone and the peptide-HLA complex, killing can occur with as few as 1-3 copies of pMHC per target [47], but such a low antigen density is likely inadequate for stable IS formation.

In contrast to cytolysis, cytokine production requires *de novo* transcription induced by the activation of NF $\kappa$ B. While IFN- $\gamma$  is believed to be the easiest cytokine for CTL to produce (in

terms of the amount of TCR signaling required for induction of its expression), IL-2 is the most difficult cytokine for CTL to make; its production requires concurrent NFAT, AP-1, and NF $\kappa$ B binding to the enhancer element of the IL-2 promoter [48].

Antigen-specific proliferation requires activation of all three transcription factors and sustained IL-2 signaling to maintain activity of STAT5. A stable IS may be necessary for the initiation of antigen-induced CTL proliferation, perhaps by increasing the time of CTL-target cell contact and thereby enhancing the duration of TCR signaling.

These separate CTL functions have different requirements for triggering because they have different functional avidities. Functional avidity is distinct from affinity, and is formally defined as the amount of peptide required to trigger 50% of maximal lysis, but there can be an even more general definition for it. Functional avidity is the output of three variables that come together: the affinity (strength of binding at the surface); the efficiency of signaling through multiple pathways (which is influenced by conditions in the cytoplasm), and the conditions in the nucleus that affect how the transcription factors will behave.



## **Chapter 4: T Cell Dysfunction**

#### **4.1 T Cell Exhaustion Limits the Magnitude of T Cell Responses to Foreign Antigen**

During the course of a chronic infection with a highly replicating pathogen, T cell responses can become so activated that they pose a risk of causing bystander damage to healthy tissue. One mechanism of T cell dysfunction known as “exhaustion” evolved to counter this potential problem. Exhaustion serves as a brake to keep the immune response to infection somewhat limited, so that the risk of damage to healthy tissues is minimized. While the mechanism of exhaustion and the signature of exhausted T cells remain unclear [36], at the very least a functional definition of exhaustion is that when T cells are repeatedly activated by antigen stimulation—as is the case during a chronic viral infection—the T cell functionality progressively wanes. As frequency and intensity of stimulation increase, so too does the degree of T cell exhaustion. The T cell inhibitory receptors CTLA-4 [36], Lag-3 [36], Tim-3 [36], PD-1 [36], and BTLA [36] are often cited as markers of exhausted T cells, although there is some proposed functional overlap with anergy for CTLA-4 and Lag-3 (further discussed in Section 4.3.4). As T cell exhaustion has been proposed to be a key mechanism of CTL dysfunction in HIV-1 infection [1], we also included these markers in our investigation, but the main focus was on a mode of T cell tolerance called anergy, which will be discussed in the next two sections.

## 4.2 T Cell Tolerance Minimizes the Risk of Developing T Cell Responses to Self-Antigen

As a consequence of the vast diversity of potential TCR sequences (estimated to be  $\sim 10^{15}$ ), there is an inherent risk that some of them will be reactive to self-antigens. If left unchecked, these self-reactive T cells would lead to autoimmunity. The immune system has evolved mechanisms of protecting the host from this danger. Hematopoietic CD34<sup>+</sup> stem cells released from the bone marrow develop in the thymus to mature into naïve T cells after they undergo rigorous selection processes including positive and negative selection. Precursor thymocytes pass through the cortex first, where positive selection allows the survival of only those thymocytes with receptors capable of binding to peptide-MHC with a certain low threshold of binding strength, or “affinity”; T cells with receptors that cannot bind to self-MHC well enough will die by neglect. Those cells that survive the journey through the cortex of the thymus then enter the medulla, where negative selection induces apoptosis in cells with receptors that have too high an affinity for self-peptides. This process is referred to as “central tolerance”. By the end of this stringent selection process, approximately 95% of all thymocytes do not survive. The T cells which survive thymic selection and enter the peripheral bloodstream have been essentially “vetted” as safe, as they are supposed not to recognize self-antigens after this selection process. No biological system is perfect, however; some self-reactive T cells inevitably escape thymic selection. This is presumably because not all self-antigens are expressed in the thymus, and even for self-antigens which are expressed, their expression level in the thymus may be lower than that found on peripheral tissues. Because of this, the “escapee” self-reactive T cells have the potential to enter the circulation and cause autoimmune pathology. The immune system has also evolved mechanisms of dealing with these peripheral self-reactive T cells, which are termed “peripheral tolerance mechanisms”. One is clonal deletion via apoptosis. This occurs when a

self-reactive T cell clone recognizes a self-peptide presented on a healthy tissue cell: the T cell receives a strong TCR signal from the antigen (signal 1), but in the context of inadequate costimulation from the CD28 ligands CD80 and CD86 (signal 2), which leads to the induction of apoptosis. The second mechanism is that encounter with its cognate self-antigen sends a signal that converts the self-reactive clone into a negative regulatory cell that has immunosuppressive function, although the precise biochemical mechanism of this is still unclear. The third mechanism of maintaining peripheral T cell tolerance to self-antigen is the induction of a dominant-negative program of hyporesponsiveness to antigen termed “anergy” [49], which will be the main focus of the remainder of this dissertation. A detailed summary of anergy is given in section 4.3.

## 4.3 T cell anergy

### 4.3.1 The Discovery of T Cell Anergy

T cell anergy is a dominant-negative state of antigen-induced hyporesponsiveness, the discovery of which was reported in the seminal 1987 publication in the *Journal of Experimental Medicine* by authors Marc Jenkins and Ron Schwartz [50]. In their model, murine CD4<sup>+</sup> Th1 clones specific to an epitope from pigeon cytochrome C (PCC) were rendered hyporesponsive (displayed defective proliferation and IL-2 production) to subsequent encounters with PCC when they were pre-exposed to PCC-pulsed target cells that had been fixed with the chemical crosslinker 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (ECDI), in the absence of IL-2 [50]. The authors surmised that the chemical crosslinking effectively created a costimulatory blockade. It was this realization that later led them (and other researchers) to believe that anergy occurs in naïve T cells by antigen encounter in the absence of costimulation through CD28—a seemingly ironic conclusion, given that it's unclear from the language used in that paper [50] whether the induced unresponsiveness in the *in vivo* experiments was describing bona fide naïve T cells. However, since naïve T cells are the primary targets of tolerance mechanisms and they require costimulation through CD28 in order to be primed properly, that conceptual model of anergy makes logical sense within that limited context.

### 4.3.2 Induction of Anergy in Naïve T Cells

Because of the finding from the aforementioned 1987 JEM paper [50], as well as the popular review of T cell anergy that Ron Schwartz wrote in 2003 [51], it remains a predominant belief that anergy is a phenomenon specific to naïve T cells, caused by antigen encounter (signal 1) in the absence of costimulation through CD28 (signal 2), resulting in defective proliferation and IL-2 production in response to subsequent antigen encounter. This definition of anergy has become so well-accepted that it is stated in the Janeway's Immunobiology textbook [28]. While this mechanism of anergy is very likely correct for self-reactive naïve T cells *in vivo*—as it is strongly supported by the discovery that T cells specific to the self-antigen MelanA are rendered hyporesponsive to antigen in healthy individuals *in vivo* [49]—the notion that anergy is characteristic of *only* naïve T cells is (to my knowledge and opinion) outdated, as anergy has been demonstrated also in effector and memory T cells, which will be discussed in Section 4.3.3.

### 4.3.3 Induction of Anergy in Effector and Memory T Cells

While many types of stimuli have been used to induce anergy or anergy-like phenotypes in effector or memory T cells (I say “anergy-like” for the fact that it’s difficult to distinguish bona fide anergy from other types of T cell dysfunction, a problem that is discussed further in Section 7.4.2), the most compelling methods for inducing anergy in antigen-experienced T cells are the following: ionomycin (a calcium ionophore) without PMA (an analog of diacylglycerol) [52-54]; low density of index (well-recognized) peptide [55, 56]; intermediate density of weak agonist (poorly-recognized) altered peptide ligand (APL) [56-60]; or high density of a highly unstable peptide-HLA complex where the peptide has a mutation(s) at HLA anchor residue(s) [58, 59]. While most demonstrations of anergy using these stimuli were performed in CD4<sup>+</sup> T cells, there is some evidence for it in the CD8<sup>+</sup> T cell system as well [61-64]. The signaling mechanism of how suboptimal stimulation with peptide leads to anergy in effector or memory T cells is unclear; one plausible explanation is that exposure to a transient, weak TCR signal may be enough to sustain NFAT transcriptional activity for a significant length of time without reaching the threshold needed to activate NFκB and AP-1, which are known to require stronger and more sustained TCR signals [65]. How the differential activation of NFAT in the absence of AP-1 connects to the affinity of the peptide for the TCR is still unclear.

The belief about anergy being a phenomenon specific to naïve T cells has persisted despite recent evidence for anergy in effector and memory T cells. New generations of students continue to be taught with the same Immunobiology textbook that appears not to have updated its section about T cell anergy beyond the ideas from the 2003 review by Schwartz [51]. Unless a faculty member is working in the field of T cell tolerance, they are unlikely to be familiar with

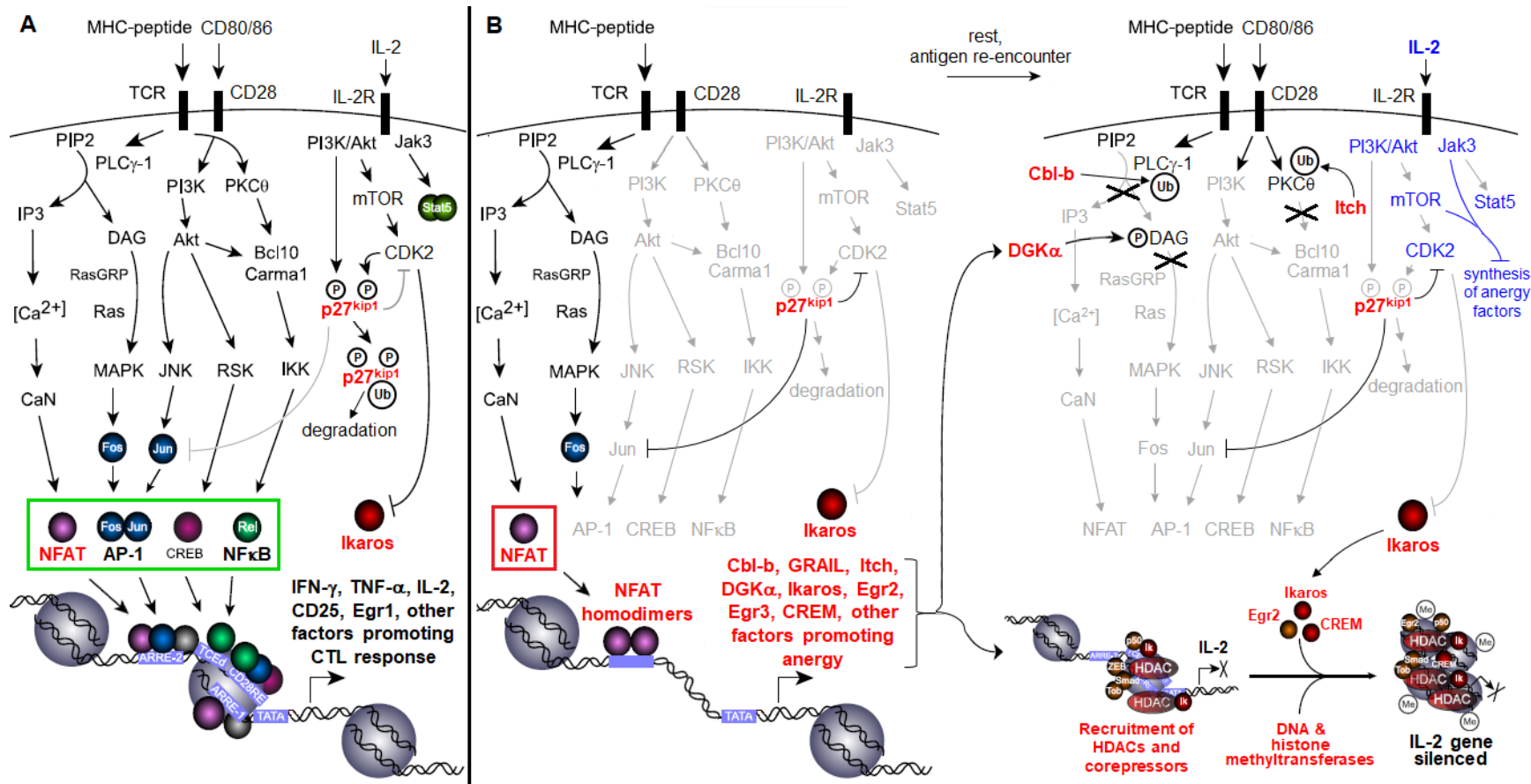
the more recent literature that supports anergy in effector or memory T cells. Additionally, searching the literature for an updated consensus model of anergy can be an immense challenge (see Section 7.4.2 for more discussion on the reasons for this).



#### 4.3.4 Molecular Signatures of Anergic T Cells

Evidence from literature suggests that self-tolerant/anergic T cells express elevated levels of CTLA-4 [49, 55], Lag-3 [64], Caspase-3 [54], Cbl-b [66], Deltex1 [67], DGK $\alpha$  [68], Egr2 [69], Egr3 [70], FoxP3 [70], GRAIL [52], Ikaros [48], Itch [52], p27<sup>kip1</sup> [48], and Tsc1 [71]; conversely, transferrin receptor (CD71) [72] and the heavy chain of the large neutral amino acid transporter (CD98HC) are downregulated in anergic cells [72]. For a majority of these markers, their association with anergy comes from evidence in murine CD4<sup>+</sup> T cells. Some papers mistakenly associate PD-1 as being a marker of anergy, but I believe this to be incorrect. Not only did a study by Maeda *et al.* [49] show a lack of PD-1 upregulation on anergic MelanA-specific human CD8<sup>+</sup> T cells (although they found CTLA-4 upregulation, intriguingly), it's also known that PD-1 expression increases in a gradual manner in T cells after repeated rounds of activation; this is inconsistent with the role of anergy in preventing the activation of a self-reactive T cell. Interestingly, IL-2 signaling through its receptor has been shown to transiently block or reverse established anergy, because it stimulates pathways that inhibit the synthesis of anergy-promoting factors [73]. This rescuing effect requires continuous IL-2 receptor signaling; if IL-2 is removed, the T cells return to their previous anergic status [64]. Additionally, ZAP-70-mediated phosphorylation of the scaffold protein LAT, an important molecule in the TCR signaling pathway, is also defective during anergy [74]. An excellent review by Andrew Wells [48] also discusses certain epigenetic patterns (repression of the IL-2 promoter, in particular) observed in T cells rendered anergic by immobilized anti-TCR antibody or by ionomycin without PMA, although it remains to be confirmed whether epigenetic signatures of anergy induced in murine CD4<sup>+</sup> helper T cells using those polyclonal stimuli translate into human CD8<sup>+</sup> cytotoxic T cells being anergized with suboptimal peptide stimulation. While the anergic state

does appear to be a long-lasting dominant-negative differentiation program [64], the molecular details of how the anergic state is maintained are still relatively unexplored, especially in human CD8<sup>+</sup> T cells.



**Figure 4.1. Comparison of antigen signaling during full activation versus suboptimal activation leading to anergy (adapted from [48]).** **A)** T cells stimulated with pMHC, CD80/86 and IL-2 have multiple signaling pathways engaged, leading to the concomitant activation of the transcription factors NFAT, AP-1 (Fos/Jun dimer), CREB, and NF $\kappa$ B and the induction of expression of cytokines and other factors important for the T cell response. **B)** In the absence of IL-2 and CD28 costimulation, TCR stimulation alone can only activate NFAT which, without its normal binding partner AP-1, binds DNA as a homodimer and induces expression of genes that promote the anergic state. The anergic state is enforced and maintained by two ways: 1) epigenetic remodeling, such as silencing of the IL-2 locus, and 2) active inhibition of TCR signaling pathways via E3 ubiquitin ligase-mediated degradation of the signaling molecules PLC- $\gamma$ 1 and PKC $\theta$ , DGK $\alpha$ -mediated inhibition of diacylglycerol (DAG)

signaling, and p27<sup>kip1</sup>-mediated inhibition of Jun. When IL-2 is present (panel **B**, right side), it stimulates the PI3K-Akt-mTOR and Jak3-STAT5 pathways (shown in blue text); besides helping to stimulate proliferation, mTOR and Jak3 cooperate to inhibit the expression of the factors promoting anergy (shown in red text), e.g. DGK $\alpha$ , Cbl-b, Itch, p27<sup>kip1</sup>, GRAIL (not shown). This inhibition of anergy factor expression effectively removes the 'roadblocks' to TCR signaling, allowing normal responsiveness to antigen. This IL-2 mediated rescue of responsiveness to antigen is only transient, however, and can only be maintained for as long as the IL-2 receptor continues to signal. Upon removal of IL-2, the cells reestablish the anergic state [64], presumably due to tight epigenetic control.

#### **4.4 Hypothesis of Weak Agonist HIV-1 Epitope Variants Triggering Suboptimal Signaling Leading to Anergy in HIV-1-Specific CTL**

Bringing these ideas full circle, our study investigated T cell anergy as a potential mechanism of dysfunction in HIV-1-specific CTL. Importantly, the definition of CTL dysfunction depends on which CTL function is being considered. Classic anergy was defined as having the hallmarks of defective proliferation and IL-2 production due to the focus on anergy in CD4<sup>+</sup> T cells, which have different functions than CTL. CTL functions have differential requirements for strength of TCR signaling, costimulation, IL-2 signaling, and stability of the immunological synapse. Additionally, the requirements for triggering various T cell functions are also influenced by differentiation state (naïve, lytic effector, effector memory, central memory) and the lineage (CD4 or CD8). Recognizing these distinctions matters because anergy was originally defined under a very narrow category: naïve CD4<sup>+</sup> helper T cells receiving TCR signal from peptide-MHCII ligands in the absence of costimulation through CD28. In this study we used a very different model system—effector/effector memory CD8<sup>+</sup> cytotoxic T cell clones specific to HIV-1 antigens presented on MHC-I. Because of this difference, our hypothesis is based primarily on findings of peptide-mediated anergy in antigen-experienced T cells from both murine and human systems. Given those data and prior findings that HIV-1 epitope variants yield a range of recognition by HIV-1-specific CTL clones reported by our group [75] and others [76, 77], we hypothesized that weakly recognized HIV-1 epitope variants might serve as weak agonists that suboptimally stimulate TCR signaling and drive anergy as a mechanism of CTL dysfunction. If correct, it would help explain how HIV-1 is able to persist and why HIV-1-specific CTL seem to become dysfunctional at an early stage of infection.

## **Chapter 5: Materials and Methods**

### 5.1 HIV-1-Specific CTL Clones

CTL clones S00001-SL9-3.23T, S42758-RL10-3.22, and S00076-KK10-10.37 recognize the epitopes SLYNTVATL (SL9, Gag 77-85, A\*02-restricted), RPAEPVPLQL (RL10, Rev 66-75, B\*07-restricted), and KRWIIMGLNK (KK10, Gag 263-272, B\*27-restricted), respectively. These were isolated at limiting dilution and maintained as previously described [75, 78].

### 5.2 Target Cell Lines

T1 (HLA-A\*02<sup>+</sup>), Jurkat (HLA-B\*07<sup>+</sup>), and EBV-immortalized B cells from Subject 00076 (HLA-B\*27<sup>+</sup>) were maintained in R10 medium (RPMI 1640 supplemented with HEPES, penicillin/streptomycin, L-glutamine, and 10% heat-inactivated fetal calf serum) as described previously [79]. These cell lines were used as HLA-matched antigen-presenting cells (APC) and target cells for the CTL clones S00001-SL9-3.23T, S42758-RL10-3.22, and S00076-KK10-10.37, respectively, in all chromium release killing assays.

### 5.3 Peptides

Peptides were purchased from Sigma and were >70% pure by standard desalting. Lyophilized peptides were reconstituted at 2 mg/mL in 10% DMSO and stored in aliquots at -80°C. These included: the SL9 index epitope, SLYNTVATL (Gag 77-85) and its corresponding variants SLFNTVAVL (“variant 1”) and SLFNTIATL (“variant 2”); the RL10 index epitope, RPAEPVPLQL (Rev 66-75) and its corresponding variants RPVEPVPLQL (“variant 1”) and RPTEPVPFHL (“variant 2”); and the KK10 index epitope, KRWIIMGLNK (Gag 263-272) and its corresponding variants KRWIILGLNK (“variant 1”) and KKWIILGLNK (“variant 2”).

#### **5.4 Assessment of Peptide-Specific Cytolytic Activity**

The cytolytic activity of the CTL clones was determined by standard  $^{51}\text{Cr}$ -release assay as previously described [75, 79]. Briefly, target cells were labeled with  $^{51}\text{Cr}$  and then seeded in a 96-well U-bottom plate at  $10^4$  cells per well for co-culture with or without  $5 \times 10^4$  CTL and the indicated peptides for 4 hours at  $37^\circ\text{C}$ , followed by measurement of released  $^{51}\text{Cr}$  by scintillation counting. Percent specific lysis was calculated by the formula:  $(\text{observed release} - \text{spontaneous release}) \div (\text{maximum release} - \text{spontaneous release}) \times 100$ .

#### **5.5 CTL Stimulation and Sample Preparation for Cytokine and Gene Expression Assays**

$2 \times 10^5$  CTL were seeded per well in a 96-well U-bottom plate. CTL were either left unstimulated (in R10 medium without peptide), or stimulated with serial 5-fold dilutions of the indicated peptides for 6 hours at  $37^\circ\text{C}$ . Following stimulation, the plate was centrifuged at  $1,000 \times g$  for 5 minutes at room temperature, and  $120 \mu\text{L}$  culture supernatant from each well was collected and frozen at  $-80^\circ\text{C}$  until analysis. CTL were lysed by adding  $40 \mu\text{L}$  of working lysis mixture supplemented with proteinase K (RNA QuantiGene 2.0 Plex kit, Affymetrix) to the remaining  $80 \mu\text{L}$  of culture and mixed by thorough pipetting. Lysates were transferred to sterile PCR strips and then incubated in a thermocycler at  $52.5^\circ\text{C}$  for 30 min to degrade the ribosomes. Lysates were mixed again by pipetting, then frozen at  $-80^\circ\text{C}$  until analysis.

#### **5.6 Analysis of Secreted Cytokines**

The concentration of secreted human IFN- $\gamma$ , TNF- $\alpha$ , IL-2, MIP-1 $\alpha$ , and MIP-1 $\beta$  in cell culture supernatant was measured by Luminex (R&D Systems) according to manufacturer's instructions. Wash steps were performed on a hand-held magnetic plate washer (Bio-Rad) according to



manufacturer's instructions. Data was acquired on a MAGPIX instrument using xPONENT 4.2 software.

### 5.7 Gene Expression Analysis

A custom RNA QuantiGene Plex 2.0 Luminex kit (Affymetrix) was used to quantitate the relative levels of mRNA transcripts for the following human genes: CD25, CD71, CD95, CD98HC, Tim-3, Lag-3, PD-1, CTLA-4, BTLA, TNFR1, TNFR2, Caspase-3, DGK-a, GRAIL, Itch, Cbl-b, p27<sup>kip1</sup>, Bcl-2, Bim, Egr1, Egr2, Egr3, FoxP3, Ikaros, Deltex1, Tsc1, HPRT1, and GAPDH. Each gene-specific probe was designed by the manufacturer to recognize all known isoforms of the mRNA. 80  $\mu$ L of undiluted CTL lysate was used as the input amount per sample for quantification. All steps for sample hybridization and detection were performed according to manufacturer's instructions. Wash steps were performed on a hand-held magnetic plate washer (Bio-Rad) according to manufacturer's instructions. Data was acquired on a MAGPIX instrument using xPONENT 4.2 software. With exception of the KK10-specific clone, both the cytokine and gene expression data were obtained from the same batch of CTL. Normalized expression levels compared to unstimulated controls were expressed as fold-change (Fold- $\Delta$ ) when baseline expression was readily detectable or change in median fluorescence intensity ( $\Delta$ MFI) when baseline expression was very low or undetectable using "per-well" normalization to the housekeeping genes HPRT1 and GAPDH by the following equations:

$$\text{Fold-}\Delta = (A_1 \times B_2) \div (A_2 \times B_1)$$

or

$$\Delta\text{MFI} = (A_1 \times B_2 \div B_1) - A_2$$

where

$A_1$  = signal of gene of interest in the stimulated sample

$B_1$  = geometric mean of the signals for HPRT1 and GAPDH in the stimulated sample

$A_2$  = signal of gene of interest in the unstimulated sample

$B_2$  = geometric mean of the signals for HPRT1 and GAPDH in the unstimulated sample

### **5.8 Peptide Pre-treatment Killing Assays**

Allogeneic feeder PBMC and antigen-presenting cells (APC) were irradiated with 3,000 and 10,000 rads respectively prior to culture. CTL were pretreated with feeder PBMC and peptide-pulsed APC at a 1:2:2 ratio ( $4 \times 10^4$  CTL,  $8 \times 10^4$  PBMC,  $8 \times 10^4$  APC per well) in 200  $\mu$ L R10 per well in a 96-well U-bottom plate for 48 hours at 37°C. The CTL then were assayed for killing activity by direct addition of  $5 \times 10^3$  target cells that had previously been  $^{51}\text{Cr}$ -labeled and index peptide-pulsed (SL9 at 10 ng/mL, RL10 at 1  $\mu$ g/mL, or KK10 at 100 ng/mL as appropriate) in 50  $\mu$ L per well. After 4 hours coculture at 37°C, lytic capacity against the index epitope was determined by measurement of released  $^{51}\text{Cr}$  as described above.

**Chapter 6: Suboptimal Stimulation by Weak Agonist Epitope Variants Does Not Drive  
Dysfunction of HIV-1-Specific CTL Clones**

## 6.1 Abstract

CD8<sup>+</sup> cytotoxic T lymphocytes (CTL) are important in the partial control of HIV-1 infection. However, in most patients the HIV-1-specific CTL become dysfunctional during early infection and fail to control viremia. There is increasing evidence for a role of T cell anergy—a mechanism of self-tolerance—in the regulation of T cell responses to a variety of pathogens, and we hypothesized that CTL anergy may be an important mechanism of CTL dysfunction in HIV-1 infection. Since HIV-1 is known to escape CTL pressure by mutating epitopes to reduce CTL recognition—and anergy is thought to occur through suboptimal recognition—we speculated that weakly-recognized epitope variants may suboptimally stimulate the T-cell receptor (TCR) and trigger aberrant TCR signaling, leading to CTL anergy and enhanced viral persistence. To investigate whether weakly recognized epitope variants induce anergy as a mechanism of dysfunction, three HIV-1-specific CTL clones targeting the epitopes SLYNTVATL (A\*02-restricted), RPAEPVPLQL (B\*07-restricted), and KRWIIMGLNK (B\*27-restricted) were exposed to suboptimally recognized epitope variants and screened for cytokine secretion, gene expression of anergy and other T cell dysfunction markers, and capacity to kill index peptide-presenting target cells in secondary challenge. While suboptimal recognition of epitope variants reduced cytokine production by CTL similarly to reduction in killing of target cells, gene expression profiles after suboptimal stimulation demonstrated no patterns consistent with T cell dysfunction due to anergy, exhaustion, or apoptosis. Additionally, pre-exposure of CTL to weak agonist epitope variants had no discernable impact on their subsequent capacity to kill index epitope-bearing target cells. These results suggest that while weak agonist epitope variants evade CTL pressure through reduced recognition, their weak agonism does not appear to drive dysfunction of HIV-1-specific CTL.

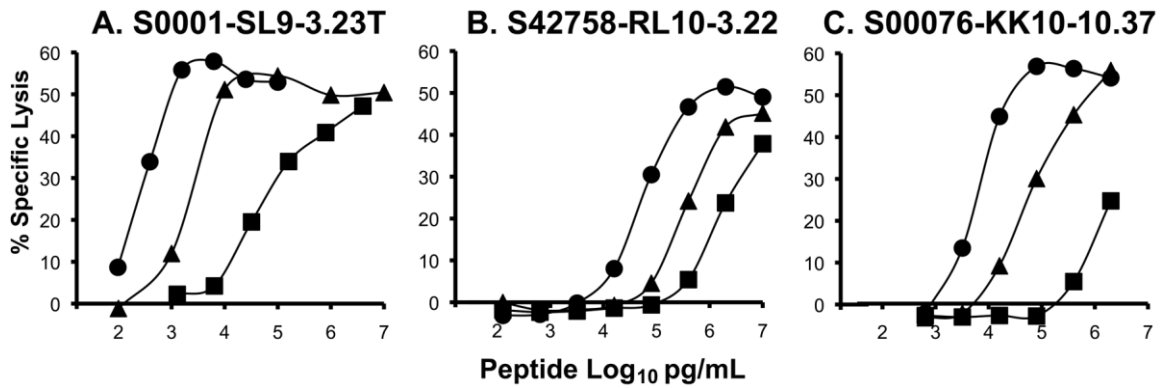
## 6.2 Results

### **Definition and selection of epitope variants that are suboptimally recognized by HIV-1-**

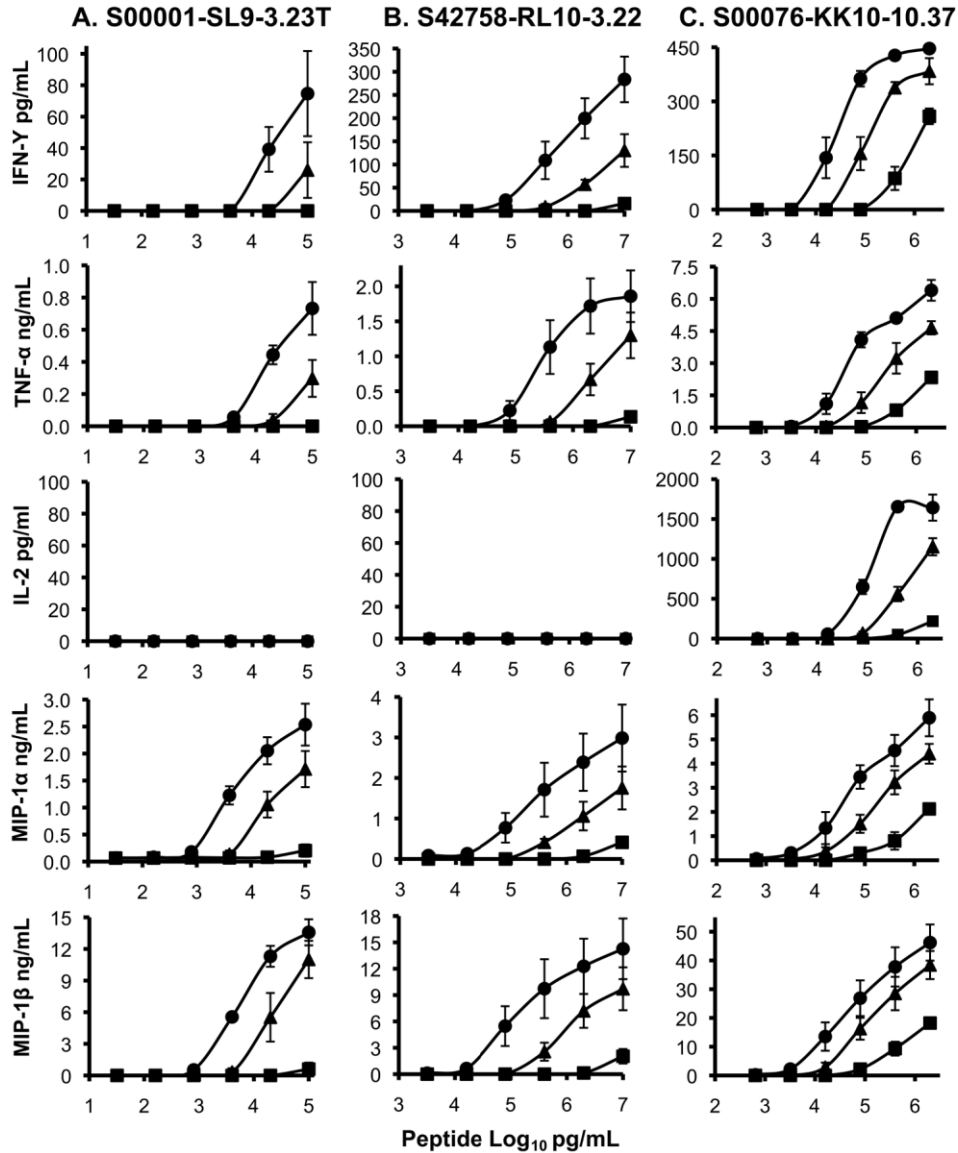
**specific CTL in killing assays.** HIV-1-specific CTL clones S00001-SL9-3.23T (recognizing the index epitope SLYNTVATL, Gag 77-85, A\*02-restricted), S42758-RL10-3.22 (recognizing the index epitope RPAEPVPLQL, Rev 66-75, B\*07-restricted), and S00076-KK10-10.37 (recognizing the index epitope KRWIIMGLNK, Gag 263-272, B\*27-restricted) were screened for their functional avidities against epitope variants in peptide titration killing assays (Figure 6.1). For each clone, epitope variants were selected to span approximately two orders of magnitude of weak agonism, with “variant 1” and “variant 2” selected as less recognized and least recognized variants respectively. For clone S00001-SL9-3.23T (Figure 6.1, A), these included SLFNTVAVL (variant 1) and SLFNTIATL (variant 2). For clone S42758-RL10-3.22 (Figure 6.1, B), these included RPVEPVPLQL (variant 1) and RPTEPVPFHL (variant 2). For clone S00076-KK10-10.37 (Figure 6.1, C), these included KRWIILGLNK (variant 1) and KKWIILGLNK (variant 2). These data identified weak agonist APL for each CTL clone, as defined by reduced functional avidity for triggering killing of target cells.

### **Cytokine release by HIV-1-specific CTL in response to varying levels of index and variant**

**epitope stimulation also reflects suboptimal triggering by weak agonists.** To assess the functional impact of suboptimal stimulation by index and variant epitopes in terms of cytokine production, secretion of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, MIP-1 $\alpha$ , and MIP-1 $\beta$  was measured in culture supernatant from CTL stimulated by titrations of index, variant 1, or variant 2 peptides (Figure 6.2). There was some heterogeneity in cytokine production between the CTL clones, most



**Figure 6.1. Identification of altered peptide ligand epitopes spanning a range of reduced agonism for triggering cytotoxicity by CTL clones.** For each CTL clone, two epitope variants were selected to span a broad range of reduced recognition as defined by triggering of target cell killing across varying peptide concentrations. For the remainder of this study these were termed index (●), variant 1 (▲), or variant 2 (■), where the index was the best recognized and variant 2 was the least recognized epitope sequence. A. For clone S0001-SL9-3.23T, these were SLYNTVATL (index), SLFNTVAVL (variant 1), and SLFNTIATL (variant 2); results are representative of four independent experiments. B. For clone S42758-RL10-3.22, these were RPAEPVPLQL (index), RPVEPVPLQL (variant 1), and RPT<sup>u</sup>EPVPFHL (variant 2); results are representative of three independent experiments. C. For clone S00076-KK10-10.37, these were KRWIIMGLNK (index), KRWIILGLNK (variant 1), and KKWIILGLNK (variant 2); results are representative of three independent experiments.

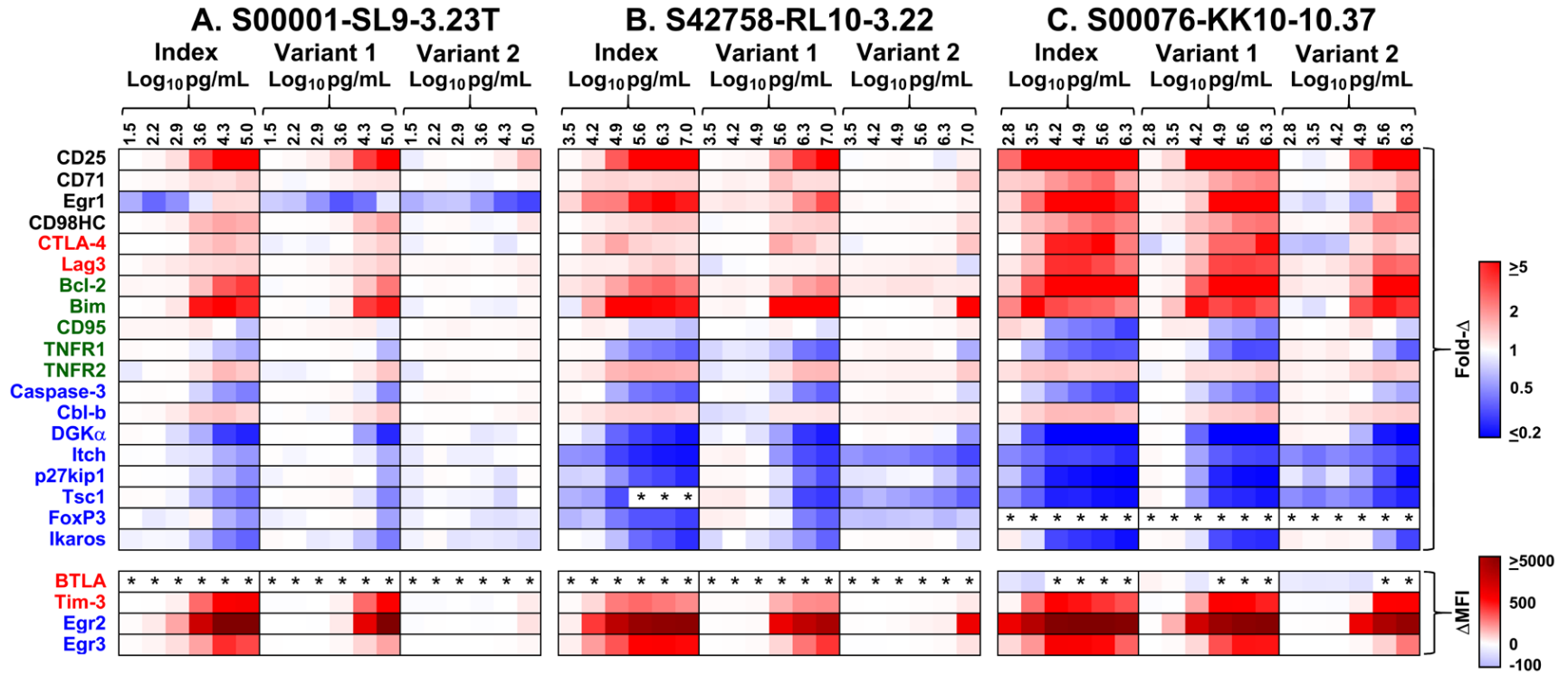


**Figure 6.2. CTL epitope variants that are weak agonists for triggering killing are similarly weak agonists for triggering cytokine release functions.** The concentrations of secreted IFN- $\gamma$ , TNF- $\alpha$ , IL-2, MIP-1 $\alpha$  and MIP-1 $\beta$  after six hours of stimulation with varying concentrations of index (●), variant 1 (▲), or variant 2 (■) peptide are shown for CTL clones S00001-SL9-3.23T (A), S42758-RL10-3.22 (B), and S00076-KK10-10.37 (C). The plots indicate average values with error bars representing one standard deviation for three independent experiments.

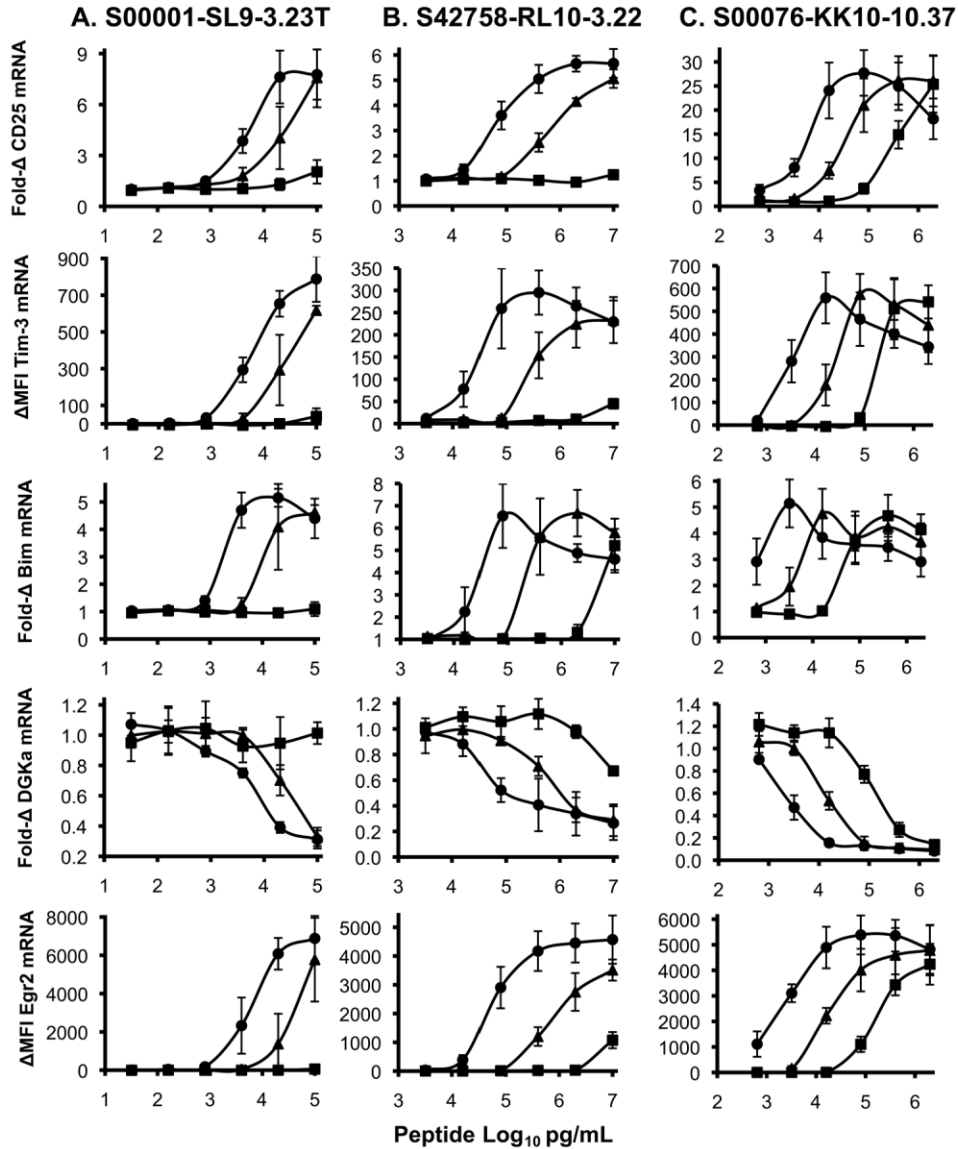
notably production of IL-2 by the KK10-specific clone (Figure 6.2, C) and not by the SL9- and RL10-specific clones (Figure 6.2, A and B). There also appeared to be small differences between clones in terms of the relationship of peptide doses required to trigger release of different cytokines; for example, SL9- and RL10-specific clones (Figure 6.2, A and B) seemed to require less peptide to produce MIP-1 $\alpha$  and MIP-1 $\beta$  compared to IFN- $\gamma$  and TNF- $\alpha$ , which was not observed for the KK10-specific clone (Figure 6.2, C). Overall, however, the relative efficiencies of CTL triggering of cytokine release by the index and variant peptides mirrored those for triggering target cell killing in Figure 6.1, further confirming that variants 1 and 2 functioned as APL with successively reduced CTL triggering.

**Weak agonism by APL stimulation also reduces CTL triggering in terms of gene expression, but does not induce selective expression of exhaustion, pro-apoptotic, or anergy markers.** To investigate whether suboptimal stimulation leads to expression of markers of dysfunction, RNA transcripts for proteins associated with T cell activation or dysfunction via exhaustion, apoptosis (which has also been linked to APL stimulation [44]), or anergy were quantified in CTL clones stimulated with the indicated peptides (Figures 6.3 and 6.4). Changes in expression relative to unstimulated controls were compared for the following genes: activation markers CD25 [70] and Egr1 [80]; metabolic regulators CD71 (transferrin receptor) [72] and CD98HC (heavy chain of CD98, or large neutral amino acid transporter, LAT1) [72]; exhaustion markers CTLA-4 [49, 55], Lag-3 [64], Tim-3 [36], PD-1 [36], and BTLA [36]; apoptosis regulators Bcl-2 [81], Bim [81], CD95 [82], TNFR1 [82], and TNFR2 [82]; and anergy factors Caspase-3 [54], Cbl-b [66], Deltex1 [67], DGK $\alpha$  [68], Egr2 [69], Egr3 [70], FoxP3 [70], GRAIL [52], Ikaros [48], Itch [52], p27<sup>kip1</sup> [48], and Tsc1 [71].





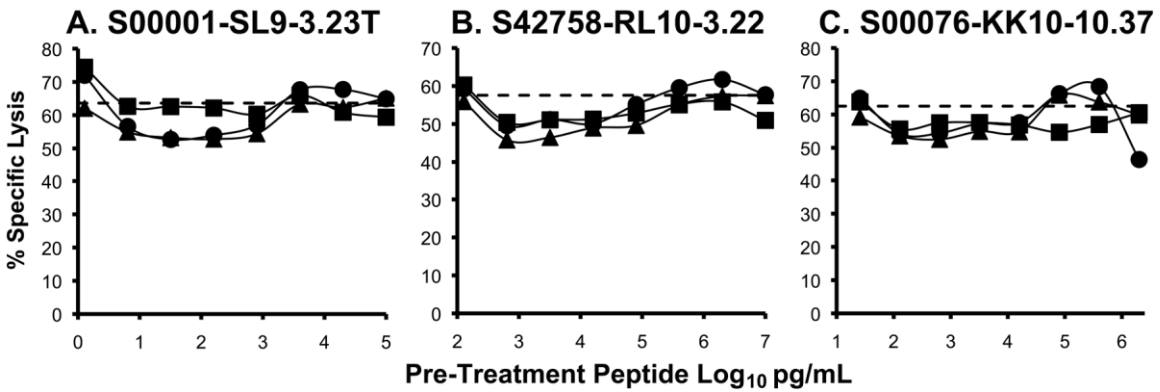
**Figure 6.3. Gene expression changes induced by suboptimal stimulation.** Stimulation-induced differential transcription is expressed as fold- $\Delta$  (when baseline expression was readily detectable) or  $\Delta$ MFI (when baseline expression was very low or undetectable) relative to unstimulated controls after 6 hours of stimulation with varying concentrations of index (◀), variant 1 (▶), or variant 2 (◀▶) peptide are shown for CTL clones S00001-SL9-3.23T (A), S42758-RL10-3.22 (B), and S00076-KK10-10.37 (C). The indicated relative levels of transcripts for proteins associated with activation/metabolism (black labels), exhaustion (red labels), apoptosis (green labels), and energy (blue labels) reflect average values from three independent experiments. \* indicates undetectable or minimally detectable expression.



**Figure 6.4. Suboptimal stimulation of CTL does not cause aberrant transcription of markers of T cell dysfunction.** Selected data from Figure 6.3 are plotted in detail for changes in mRNA levels (relative to unstimulated controls) for CD25, Tim-3, Bim, DGK- $\alpha$ , and Egr2 for CTL clones S00001-SL9-3.23T (A), S42758-RL10-3.22 (B), and S00076-KK10-10.37 (C) for index (●), variant 1 (▲), or variant 2 (■) peptides. Plots represent average values with error bars representing one standard deviation for three independent experiments.

Generally, changes in gene expression were similar between CTL clones, with reduced triggering of differential gene expression by variant epitopes compared to the index epitope that was dose-dependent. Activation- and metabolism -associated markers mostly increased with greater stimulation (Figure 6.3 and Figure 6.4 top row) except for Egr1 in the SL9-specific clone only (Figure 6.3, A). Most exhaustion markers increased with increasing stimulation (Figure 6.3 and Figure 6.4 second row), although PD-1 was undetectable (not shown) and BTLA was minimally detected (Figure 6.3). The apoptosis regulators Bcl-2, Bim (Figure 6.3 and Figure 6.4 third row), and TNFR2 (Figure 6.3) also increased with greater stimulation, while CD95 and TNFR1 decreased (Figure 6.3). Of note, weak stimuli did not increase anergy-related genes in comparison to the unstimulated controls. Most of the anergy factors decreased with greater stimulation (Figure 6.3 and Figure 6.4 fourth row) except for Egr2 (Figure 6.3 and Figure 6.4 last row) and Egr3 (Figure 6.3), which increased predominantly at higher peptide stimulation conditions; Deltex1 and GRAIL were undetectable across all conditions (not shown). Overall, weak agonism did not selectively trigger transcriptional profiles indicative of CTL dysfunction due to anergy, exhaustion, or apoptosis.

**Exposure of HIV-1-specific CTL to weak agonist APL does not ablate their subsequent ability to kill target cells.** To further evaluate whether suboptimal stimulation causes a dysfunctional phenotype, the CTL clones were pre-exposed to varying doses of index or variant epitopes and then challenged for lytic capacity against target cells labeled with the index peptide. CTL pre-exposed to the index and variant peptides exhibited no notable decrease in subsequent killing of index peptide-loaded target cells compared to control CTL that had not been pre-exposed to any peptide (Figure 6.5). The level of killing remained stable despite pre-exposure to



**Figure 6.5. Suboptimal pre-stimulation of HIV-1-specific CTL does not reduce subsequent cytolytic function.** CTL clones S00001-SL9-3.23T (A), S42758-RL10-3.22 (B), and S00076-KK10-10.37 (C) were pre-exposed to the indicated concentrations of index (●), variant 1 (▲), or variant 2 (■) peptide presented by irradiated APC matched by the restricting HLA type. After 48 hours, killing of <sup>51</sup>Cr-labeled target cells pulsed with the index epitope was assayed. Target cells for clones S00001-SL9-3.23T, S42758-RL10-3.22, and S00076-KK10-10.37 were T1, Jurkat, and autologous EBV-transformed B cells respectively. Dotted horizontal lines represent the levels of killing by control CTL pre-exposed to APC without peptide. Graphs are representative examples from three independent experiments for each CTL clone.

the variant peptides across a wide spectrum of concentrations yielding CTL stimulation ranging from minimally to fully activating. These results demonstrated maintenance of CTL cytolytic function after weak stimulation by APL, further supporting a lack of anergy following suboptimal stimulation by APL.

### 6.3 Discussion

HIV-1-specific CTL dysfunction is thought to play a pivotal role in the pathogenesis of infection, yet the precise factors determining this phenomenon remain incompletely understood. This dysfunction has been observed in assays such as “polyfunctionality” in cytokine production and cytolytic function marker expression [83] and capacity to suppress viral replication [84, 85], both of which correlate to immune containment of infection. A leading candidate mechanism is CTL exhaustion driven by viral persistence causing continuous antigenic stimulation, which is supported by data such as studies showing shortened telomeres in CD8<sup>+</sup> T cells [86] and upregulation of cell surface exhaustion markers [87, 88] in HIV-1 infection.

Another mechanism considered here is anergy due to suboptimal signaling by viral epitope variants that serve as weak agonist APL, which has been well documented in other experimental systems primarily involving CD4<sup>+</sup> T cells [56, 57, 89]. While APL-induced anergy has been shown for an HIV-1 gp120-specific CD4<sup>+</sup> T cell clone [90], it has not been clearly defined for HIV-1-specific CD8<sup>+</sup> T cells. Early studies have raised the question of CD8<sup>+</sup> T cell anergy in HIV-1 infection, but not via APL stimulation, and they defined “anergy” solely based on the criterion of reduced proliferative capacity [91-93], which is nonspecific and insufficient to differentiate anergy from other dysfunctional processes such as exhaustion or senescence. Thus, our study is the first to directly address APL-induced anergy in HIV-1-specific CTL.

Contrary to the strong evidence for APL inducing T cell anergy through suboptimal signaling, we did not observe evidence for anergy when index and weak agonist epitope variants were tested over a range of concentrations that span a spectrum of CTL triggering ranging from none

to full agonism. This range of activity was confirmed by measuring target cell killing and cytokine release. However, anergy-associated genes were not selectively induced by suboptimal stimulation. Indeed, differential transcription compared to unstimulated CTL (whether up or down with increasing stimulation) demonstrated simple dose-dependence for index and APL epitopes, rather than any pattern of an anergic profile specific to weak stimulation. Furthermore, there was no direct evidence for dysfunction in terms of target cell killing after pre-exposure to APL. These results argue against weak agonist APL-induced anergy being a key mechanism for CTL dysfunction in HIV-1 pathogenesis.

Lack of epitope variation causing anergy is also consistent with findings of Streeck *et al* [94], who examined the functional outcomes of HIV-1-specific CTL responses in a cohort of persons with acute/early infection. They noted that when epitopes remained fixed the CTL targeting those epitopes became dysfunctional, whereas CTL targeting epitopes that exhibited sequence evolution maintained function. These results suggested that exhaustion plays a major role in loss of CTL function even relatively early in infection, because epitope mutation and presumably diminished recognition was associated with preserved CTL function. Consistent with our results, epitope mutation did not drive CTL dysfunction, as would be expected if altered peptide ligand induced anergy.

The current study is indirectly relevant to another proposed avenue of HIV-1-specific CTL dysfunction termed “antagonism,” in which reduced killing of target cells with the index epitope has been observed in the presence of poorly recognized epitope variants [37, 38, 95]. The precise mechanism has not been defined, but these data suggest that it is not mediated by anergy.

Observations of antagonism have required excess APL concurrently presented with index epitope, which is more supportive of direct competition for TCR engagement. Moreover, prior reports of antagonism have not tested pre-exposure of HIV-1-specific CTL to APL alone, or explored changes in CTL phenotype specifically induced by APL. Thus the potential role for anergy has not been directly addressed in past demonstrations of antagonism, and these complementary data are not consistent with anergy mediating antagonism.

There are technical caveats that should be considered in interpreting these results. The anergy markers selected for this study were based on prior characterizations of anergy mostly using murine CD4<sup>+</sup> T cells, and to a lesser extent in murine CD8<sup>+</sup> T cells. While anergy has been evaluated in human T cells [49, 56, 57, 62, 63, 90, 96], the mechanisms and molecular signatures are less defined than for their murine counterparts [48], particularly for CD8<sup>+</sup> T cells, and thus it is possible that different factors are involved in human CTL anergy. Indeed, most of the T cell function markers have functional overlap outside the processes they were chosen to represent. Further, the 6-hour timepoint chosen for analysis of gene expression was based on three factors: pilot data that preferentially favored detection of the activation- and exhaustion-associated markers (data not shown); concern over the possibility that longer duration would lead to increased CTL-on-CTL killing, or “CTL fratricide” (data not shown); and experimental logistics. It is possible that the kinetics of differential transcription may vary between genes involved in activation and genes associated with anergy; most if not all demonstrations of anergy have been shown at a time point of 16 hours post-stimulation or longer. Also, while defective proliferation and IL-2 production are considered defining hallmarks of anergy in CD4<sup>+</sup> T cells [51, 97], the impact of anergy on CTL cytolytic function is less clear [61], although it might be expected to



diminish since the putative biologic role of anergy is avoidance of autoimmunity [49]. As for any *in vitro* studies of anergy, experimental conditions differed from those *in vivo*, including the use of synthetic peptide epitopes, non-physiologic antigen-presenting cells and feeder PBMC from healthy donors that could either artificially provide or lack co-stimulatory ligands (see Section 7.4.1 for further detail), and the use of CTL clones that have been selected to proliferate well over repeated rounds of stimulation.

In summary, our study examined the consequences of weak agonist APL stimulation of HIV-1-specific CTL clones. Despite the firm rationale that this could trigger anergy, supported by studies of suboptimal T cell signaling in other systems, these results demonstrated no evidence for a transcriptional profile or loss of cytolytic function that would indicate anergy. With the caveats that the anergy-mediating factors and/or conditions could differ between our experimental system (using human CD8<sup>+</sup> T cell clones) and those of other anergy studies (predominately using murine CD4<sup>+</sup> T cells) and conditions *in vivo* (discussed further in Section 7.4.3), these findings suggest that anergy is not a major mechanism of HIV-1-specific CTL dysfunction.

## **Chapter 7: Conflicting Unpublished Preliminary Data and Extended Discussion**

## 7.1 Abstract

Prior to the experiments described in Chapters 5 and 6, we investigated the hypothesis of suboptimal stimulation-induced anergy using the previously described peptide pre-treatment killing assays, but under different experimental conditions. The preliminary data presented here, while incomplete, support the trend that pretreatment of an SL9-specific CTL clone with suboptimal doses of either index SL9 or two SL9 variants resulted in dysfunction to index epitope, as evidenced by a loss of cytolytic activity against index SL9-bearing target cells. One major difference in these experiments was the absence of PBMC, which threw into question the biologic relevance of such a trend (as there would be PBMC present *in vivo*); for this reason, those experiments were abandoned for several years, which is why the result of dysfunction was not reproduced. At a later date, the investigation into these experiments was reopened, and led to a result that was suggestive that CD28-independent costimulatory ligands on both feeder PBMC and the APC can block the induction of CTL dysfunction. Incorporating these data with additional literature on the role of costimulation for effector CTL, I propose a speculative model of how HIV-1-specific CTL may be rendered anergic by HIV-1-infected cells presenting weak agonist epitope variants within the context of inadequate costimulation through CD28-independent pathways *in vivo*.

## 7.2 Materials and Methods

HIV-1-specific CTL clone. CTL clone S00001-SL9-3.23T recognizing the epitope SLYNTVATL (SL9, Gag 77-85, A\*02-restricted) was described in Chapter 5.

Target cell line. T1 (HLA-A\*02<sup>+</sup>) cells were maintained as described previously in Chapter 5 and were used as HLA-matched antigen-presenting cells (APC) and target cells for the CTL clone S00001-SL9-3.23T in all <sup>51</sup>Cr-release killing assays.

Peptides. Peptides were purchased from Sigma and were >70% pure by standard desalting. Lyophilized peptides were reconstituted at 2 mg/mL in 10% DMSO and stored in aliquots at -80°C. These included: the SL9 index epitope, SLYNTVATL (Gag 77-85) and its corresponding variants SLYNTIATL (“V82I”) and SLYNTIAVL (“NL4-3”).

Peptide pre-treatment killing assays. T1 cells were irradiated with 10,000 rads to serve as antigen-presenting cells (APC). CTL were pretreated with APC pulsed with or without different concentrations of either index SL9, V82I or NL4-3 variant at a 1:2 ratio (2 x 10<sup>4</sup> CTL and 4 x 10<sup>4</sup> APC per well) in 200 µL R10 per well in a 96-well flat-bottom plate for 24-72 hours at 37°C. Each pretreatment condition was then split into duplicate wells of a 96-well U-bottom plate (100 µL pretreatment culture per well), and the CTL then were assayed for killing activity by addition of 1 x 10<sup>4</sup> target cells (100 µL/well) that had previously been <sup>51</sup>Cr-labeled and pulsed with either 6 ng/mL or 10 ng/mL of index SL9 peptide. After 4 hours coculture at 37°C, lytic capacity

against the index epitope was determined by measurement of released  $^{51}\text{Cr}$  as described in Materials and Methods in Chapter 5.

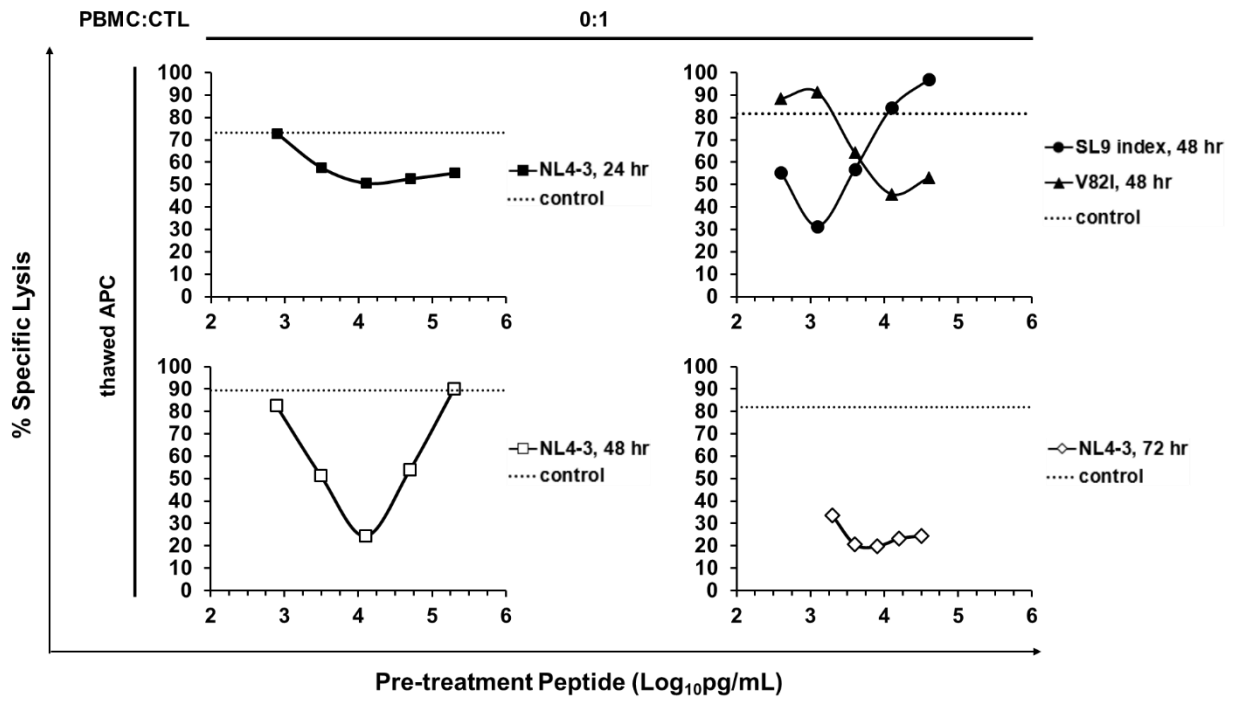
### 7.3 Results

*The degree of CTL dysfunction following pretreatment with suboptimal stimulation appears to be dependent on the pretreatment duration, as well as the peptide sequence and concentration.*

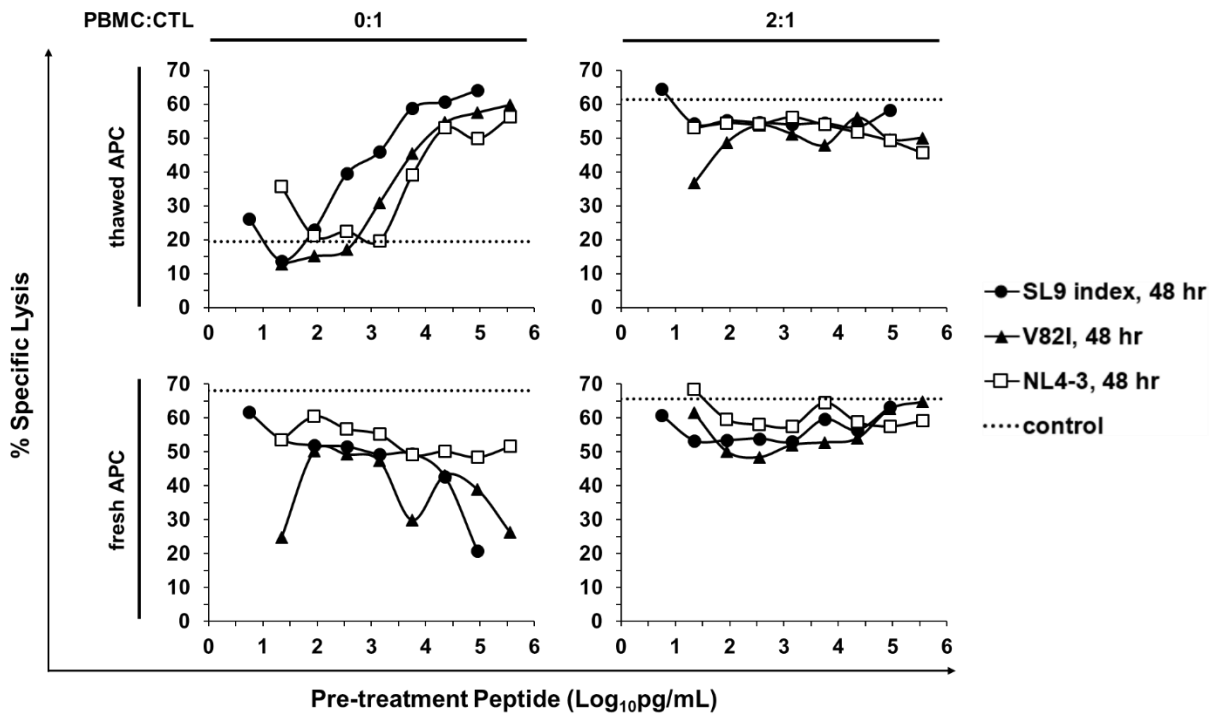
Because there is no positive control experiment for inducing anergy in human effector CTL, it was not immediately clear how we should proceed. In the beginning of my project we did pretreatment assays similar to what was described in Chapter 6, including the use of irradiated allogeneic feeder PBMC (“feeders”). When we did the early pilot experiments on the SL9-specific clone 3.23T this way, we never observed any noticeable loss in killing of target cells (data not shown), consistent with the later findings that are shown in Chapter 6. The results of these early pilots did not look promising regarding our hypothesis.

A change in the result came when we decided to try setting up the assays without feeder PBMC. We made this change to the protocol because we suspected that perhaps the feeders were providing some form of costimulation (based on the fact that CTL die if stimulated with anti-CD3 without feeders but survive if feeders are included at a 10:1 ratio or more), and since classic anergy in naïve T cells is caused by a lack of costimulation through CD28, we speculated that maybe there was some kind of rescue occurring through a costimulatory pathway independent of CD28. When we performed the pretreatment assays on the SL9 specific clone 3.23T using peptide-loaded T1 cells but omitting the feeders, the following results followed (Figure 7.1, panel A). Varying degrees of dysfunction seemed to occur under different conditions of pretreatment duration (24, 48, or 72 hours), different peptides (SL9 subtype B consensus “index,” “V82I” or “V82I/T84V” variants) and different concentrations, with the strongest inhibition of killing occurring after 48- or 72-hour pretreatment with the “V82I/T84V” variant

**A**



**B**



**Figure 7.1. Dysfunction following suboptimal stimulation appears to be influenced by peptide affinity and dosage, the condition of the antigen-presenting cells, and the**

**presence or absence of feeders. A.** The specific lysis of  $^{51}\text{Cr}$ -labeled T1 target cells pulsed with 6 ng/mL of index SL9 epitope done by the CTL clone S00001-SL9-3.23T is plotted in relation to the concentration of peptide used for pretreatment. Pretreatment peptide conditions were: index SL9 for 48 hours (filled circle), “V82I” for 48 hours (filled triangle), “V82I/T84V” (*displayed in figure as “NL4-3”*) for 24 hours (filled square), “V82I/T84V” for 48 hours (open square), or “V82I/T84V” for 72 hours (open diamond). As a control, the killing done by CTL pretreated with APC without peptide is shown as horizontal dotted lines. In each of the 4 graphs, the pretreatment APC were frozen/thawed irradiated T1 cells, and no allogeneic feeder PBMC (PBMC:CTL= 0:1) were present. Each of the 4 graphs was from a different experiment but was only done once.

**B.** The specific lysis of  $^{51}\text{Cr}$ -labeled T1 target cells pulsed with 10 ng/mL of index SL9 epitope done by the CTL clone S00001-SL9-3.23T is plotted in relation to the concentration of either index SL9 (filled circle), “V82I” (filled triangle), or “V82I/T84V” (*displayed in figure as “NL4-3”*; open square) epitope that the clone was pretreated with for 48 hours. As a control, the killing done by CTL pretreated with APC without peptide is shown as horizontal dotted lines. In this single (n=1) experiment, four variables were tested in parallel: the pretreatment epitope sequence, its concentration, the condition of the APC (frozen/thawed cells, top panel; vs. fresh cells, bottom panel), and the presence (PBMC:CTL= 2:1) or absence (PBMC:CTL= 0:1) of feeders.



(SLYNT**I**A**V**L) of the SL9 epitope (Figure 7.1, panel A). The duration of pretreatment appeared to be important, as the inhibition of killing was much less at the 24-hour time point compared to the 48- or 72-hour timepoints. Most protocols for anergy induction by weak peptide stimulation in CD4<sup>+</sup> T cells use pretreatment periods between 18 and 48 hours, so our data hinting at an effect at ~48 hours in our CTL clones is fairly consistent with that. Interestingly, pretreatment with the index sequence of SL9 (SLYNT**V**A**T**L) also inhibited target cell killing (Figure 7.1, panel A), consistent with a published report that low densities of cognate peptide can trigger anergy in a human CD4<sup>+</sup> Th1 clone [16]. Dysfunction was also observed after pretreatment with the variant “V82I”, albeit with less efficiency than the index (Figure 7.1, panel A). The peptide concentrations that appeared to most efficiently cause dysfunction were close to what was required to trigger 50% of maximal lysis. As these experiments were not repeated multiple times using exactly the same conditions, they do not meet the criteria to be considered reproducible. Also, these data only come from one CTL clone, so it’s difficult to make the case that this is a general phenomenon. However, given that the setup and results were similar between experiments, it suggests a positive trend that weak stimulation with peptide may be capable of causing anergy in virus-specific CTL under certain conditions.

*The induction of CTL dysfunction seems to be dramatically affected by the presence of allogeneic feeder PBMC during pretreatment or the condition of the pretreatment APC.*

Several years after the experiments in Figure 7.1 panel A were performed, I re-evaluated those data and decided to test whether my suspicion about the feeders blocking anergy might be correct. In addition to rescue by feeders, I also suspected that the APC themselves (which are T1

cells and therefore not physiologic) might be expressing something unnatural on their surface that was sending the CTL abnormal signals. Cell signaling pathways are interconnected and demonstrate a lot of cross-talk. Considering this, I designed and performed an experiment that tested four different variables simultaneously. As before, I tested titrations of index epitope and select variants, but I added two new variables: presence or absence of feeders, and the condition of the APC (frozen/thawed cells, or cells fresh from culture). The hypothesis was that if feeders did block anergy, there would be reduction in target cell killing in the plate that did not receive feeders, but there would be little to no change in the killing in the plate that received feeders; and if the T1 cells were expressing something on their surface that was sending rescuing signals, the process of freezing and thawing might disrupt that from happening. The results of that experiment are shown in Figure 7.1, panel B. Under conditions similar to those used in the pretreatment assays described in Chapter 6, which featured a 2:1 ratio of feeders to CTL and freshly-irradiated T1 cells as the pretreatment APC, a lack of dysfunction was once again observed across all tested peptides and concentrations (Figure 7.1, panel B, lower right graph). In sharp contrast, in the plate where the pretreatment APC were instead irradiated then subsequently frozen and later thawed, and the feeders were omitted (Figure 7.1, panel B, upper left graph), considerable dysfunction manifested. Note that the pretreatment concentration of index SL9 that had the greatest effect (~1.3 logs pg/mL) is far too low (i.e. the TCR signaling is far too weak) to trigger cytotoxicity [5]; a similar pattern was observed for the two variants. This supports the notion that the dysfunction is truly mediated by suboptimal stimulation, and *not* overstimulation.

Now here is where things get even more interesting. In the plate that served as an experimental replicate of the one shown in the upper left graph, with the exception that feeders *were* added

(Figure 7.1, panel B, upper right graph), *the dysfunction is almost completely abolished.*

Similarly, the induction of dysfunction seems moderately to severely blocked by the use of freshly-irradiated APC even in the absence of feeders (Figure 7.1, panel B, lower left graph).

This experiment shown in Figure 7.1 panel B was only performed once, as it was very large in scale. Nevertheless, the result that simply including feeders (or using freshly-irradiated T1 cells as the pretreatment APC) during a suboptimal stimulation can prevent the subsequent loss of cytolytic activity against index epitope is consistent with our early pilot data that showed a lack of dysfunction in every experiment where feeders were included (data not shown), as well as the pretreatment experiments from Chapter 6 that also utilized feeders and freshly-irradiated APC.

Collectively, the experiments shown in Figure 7.1 (panels A and B), while not necessarily reproducible, suggest a positive trend that suboptimal stimulation of HIV-1-specific CTL may be capable of rendering them dysfunctional, and if so, this mechanism is consistent with anergy.

## 7.4 Discussion

### 7.4.1 Making Sense of a Contradiction

After comparing the contradictory results of pretreatment killing assays described in Chapter 6 with those described in this chapter, several key questions linger: why would pretreatment assays result in dysfunction when feeders are absent, but show no discernable dysfunction when feeders are included? Secondly, what is the mechanism explaining why dysfunction occurred when the APC underwent a freeze/thaw cycle post-irradiation, yet functionality was maintained when the APC were freshly irradiated?

To address the first question, feeder PBMC provide costimulation for CTL. They are crucial for maintaining CTL viability during *in vitro* stimulation with anti-CD3 antibody, as rapid death occurs when HIV-1-specific CTL are stimulated in the absence of feeders (our unpublished observations). While necessary for CTL survival during activation, feeders appear to be dispensable for resting CTL, as evidenced by maintenance of CTL viability during rest periods in the absence of feeders. A publication by Giuntoli et al. [98] gave further evidence that PBMC provide costimulation for CTL: CTL clones stimulated with anti-CD3 in the presence of either PBMC depleted of activated CD4<sup>+</sup> T cells, or whole PBMC combined with blocking antibodies to either CD137L or CD70, displayed markedly reduced expansion. This suggested that the reason why feeder PBMC are required for the *in vitro* expansion of CTL clones is that activated CD4<sup>+</sup> T cells present the costimulatory ligands 4-1BBL and CD27L to their respective receptors (4-1BB and CD27) expressed on the surface of CTL. Furthermore, Wilcox et al. [99] showed that activation of the 4-1BB signaling pathway via agonistic antibody administration blocked or reversed CTL anergy. Due to its potent costimulatory capacity, the 4-1BB pathway is also

currently being utilized to augment the functionality of chimeric antigen receptor (CAR)-modified T cells; inclusion of the cytoplasmic domain from 4-1BB into the CAR construct increases the cytotoxicity, cytokine release, and persistence of the modified T cells. Thus, in the pretreatment killing assays discussed in Chapter 6—which featured PBMC from healthy donors—there would presumably have been normal frequencies of healthy CD4<sup>+</sup> T cells (in contrast to the severe depletion of CD4<sup>+</sup> T cells in HIV-1 infection *in vivo*) as well as professional APC that are capable of presenting costimulatory ligands that may prevent anergy. Additionally, the PBMC our lab works with are often pooled together from multiple donors, which would result in a mixed lymphocyte reaction (MLR). This MLR would increase the activation of the CD4<sup>+</sup> T cells, and likely lead to increased costimulatory ligand expression. Taken together, this provides a plausible explanation for how feeder PBMC might costimulate CTL through the 4-1BB and CD27 pathways and block the induction of anergy.

In answer to the second question I posed, I will refer to the study by Marc Jenkins and Ron Schwartz which made the discovery of T cell anergy [50], previously mentioned in Chapter 4. The authors found that when peptide-pulsed APC were fixed, they induced functional hyporesponsiveness in antigen-specific Th1 cell clones. In contrast, T cells incubated with unfixed peptide-pulsed APC retained functionality on subsequent challenge. It is important to understand WHY this is: fixation disrupts the native folding of cell surface proteins, which includes T cell costimulatory molecules. Similarly, DMSO can disrupt the structure of surface proteins by removing water and acting as a mild oxidant. Our observation that fresh feeders generally appear to work better than frozen feeders at costimulating expansion of our HIV-1-specific CTL clones *in vitro* is consistent with the idea that DMSO disrupts, to some extent, the feeders' costimulatory capacity. Recall from Chapter 3 the very high level of complexity of

antigen stimulation, which is comprised of TCR-pHLA ligations, adhesion molecule linkages, costimulatory molecule binding, cytokine signaling, etc. By disrupting surface protein structures, the freezing and thawing process can heavily influence the quality of the stimulation that T cells receive. A common misconception is that T cell activation is only dependent on antigen density and antigen affinity for the TCR. In truth, the decision to activate the cell or render it tolerant in response to antigen is heavily dependent on the signals that are delivered by a plethora of other proteins on the surface of the target cell, as well as by the precise balance of cytokines in the local environment. In light of these facts, it is now easy to understand why freezing and thawing of APC can help create conditions that provide for a suboptimal stimulation.

However, the story does not end there. There is another piece to the puzzle of why freezing/thawing of the APC seemed to allow the induction of dysfunction whereas fresh APC did not. The APC used (T1 cells) are an immortalized T/B cell hybrid line; it has been shown that several T and B cell lymphoma lines have high expression levels of 4-1BBL [100].

Additionally, the use of lethal-dose radiation has been demonstrated to cause upregulation of surface levels of this molecule, which increases the immunogenicity of tumor cells [101, 102].

Taken together, this suggests that the use of irradiated T1 cells fresh from culture may be more immunogenic, and that the accumulation of surface damage due to freezing/thawing may counteract this greater immunogenicity. This may explain why suboptimal doses of peptide presented in the context of freeze/thawed APC were able to cause dysfunction, whereas those same peptide conditions presented in the context of fresh APC did not cause dysfunction (Figure 7.1, panel B).

#### 7.4.2 Traversing the Murky Field of T Cell Anergy

As a field of study, T cell anergy is still not well understood. There are several major challenges to the clear understanding of T cell anergy. The first is that the field is plagued by confusion brought on by widespread misuse of the terms “exhaustion”, “anergy”, “tolerance” and “ignorance”. These terms are often mistakenly used interchangeably in publications, which in turn spreads the confusion to the readers. Second, there are cases where the authors’ methodology is inadequately controlled and/or misinterpretation of the data leads them to a conclusion that is not well supported; this can influence future work by other groups and help perpetuate incorrect or outdated notions of how anergy works. The third challenge to a clear understanding of anergy is perhaps the most important one: the use of many different model systems for anergy has led to the usage of a variety of terms to describe it, such that even a very knowledgeable reader would have difficulty avoiding confusion. Models for anergy have included: bacterial superantigens, such as the staphylococcal enterotoxins A and B [103-109]; polyclonal T cell stimuli, such as ionomycin without phorbol 12-myristate 13-acetate (PMA) [52-54] or immobilized anti-CD3 without anti-CD28 [66, 68, 69, 73, 110-112]; peptides, either with low doses of index peptide [55, 56], or higher doses of weak agonist altered peptide ligands (APL) [57, 90]; oxidized phospholipids [113]; and Galectin-1 [114]. These diverse models of anergy have produced a wide variety of terminology for anergy, which include: “split anergy”; “division arrest anergy”; “clonal (in vitro) anergy”; “in vivo anergy/adaptive tolerance”; “self-tolerance”; “allograft tolerance”; “tolerization”; “activation-induced nonresponsiveness (AINR)”; “antigen-induced (hypo/un/non)-responsiveness”; “TCR antagonism”; “T cell antagonism”; and “T cell interference”. Making matters worse, a Pubmed search for “T cell anergy” will bring up publications about B cell anergy, NK cell anergy, skin test anergy, and

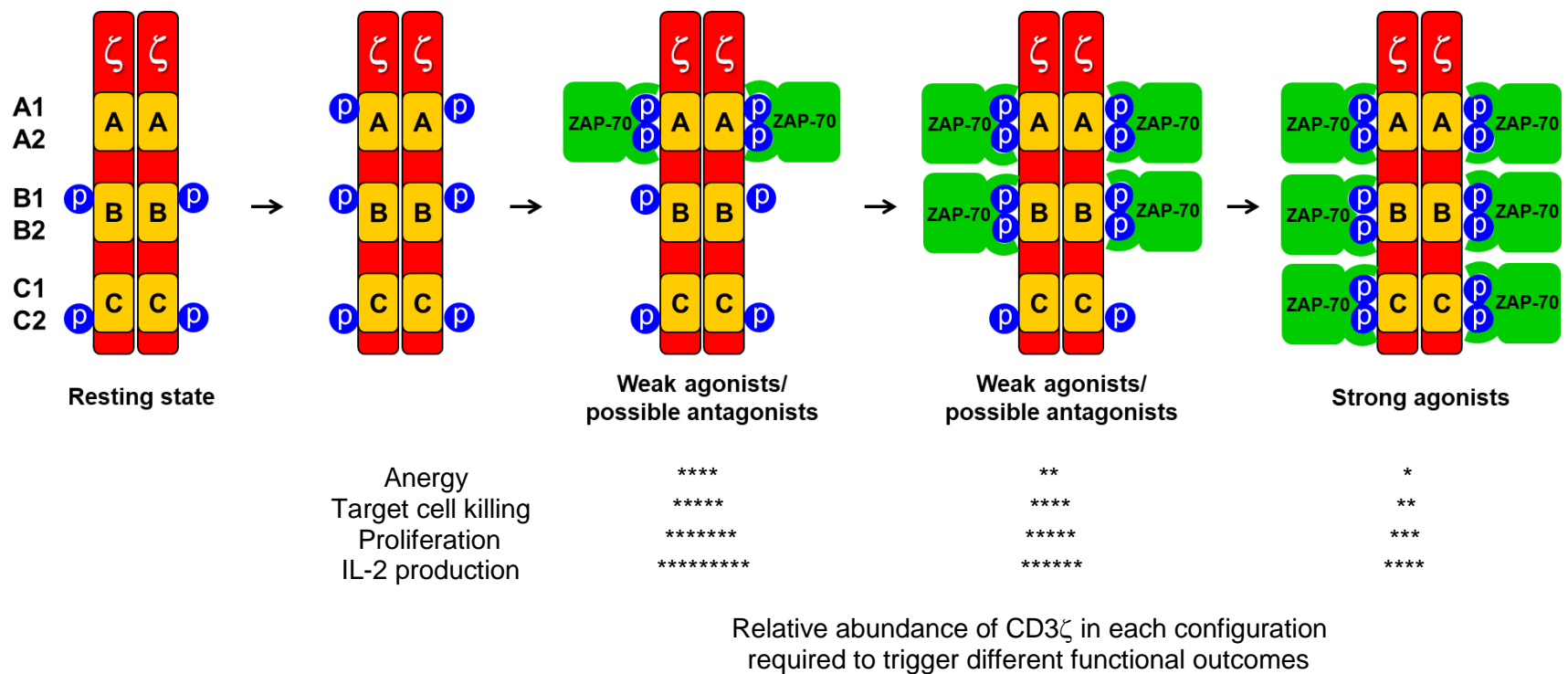
other topics completely unrelated to T cell anergy. The Pubmed search algorithm also contributes to this problem; if the form of the search term is even slightly different (e.g. “tolerized” instead of “tolerization”, or “anergy” instead of “anergic”), the search results will include certain publications while excluding others that may be relevant. Locating appropriate literature for T cell anergy without being misdirected by these non-related topics can be quite a challenge.

It should also be pointed out that there is, unfortunately, a prevalence of circular logic that impedes the progress toward a better understanding of anergy. The problem is that often how one *defines* anergy determines how one *looks* for anergy, which has a substantial influence on what one *concludes* about anergy, which in turn forms the basis of the definition. To put this in perspective, imagine that a researcher defines anergy as Schwartz did [51]—a defect in proliferation and IL-2 production. Under this mindset, it would be a very natural response for the researcher to then design experiments to test for anergy by measuring proliferation and IL-2 production in T cells activated under different conditions. If XYZ stimulation condition causes the proliferation or IL-2 production to decrease, the researcher would naturally feel excited to publish that ‘XYZ condition causes anergy’. There needs to be greater awareness that just because a treatment decreases T cell proliferation or IL-2 production, that is *not* enough to say that it causes anergy. If a treatment condition showed less IL-2 by ELISA or less proliferation by tritiated thymidine incorporation (both of which are bulk methods), and the cells were not counted at the time of harvest, then the cause could be cell death rather than anergy. Conversely, a treatment that increases proliferation (such as blocking PD-1 pathway) does not necessarily indicate prevention or reversal of anergy. There is often too much focus on the *effect* of anergy rather than the *cause*; this has resulted in widespread misbelief that anergy and exhaustion are



highly similar or even identical, because they can both lead to reduced proliferation and IL-2 production under certain conditions. It is important that the topic of anergy be approached with an open mind and an awareness that it is a highly complex process; oversimplification can sometimes do more harm than good.

While still being far from clear—more studies are needed—evidence suggests anergy may be caused by suboptimal avidity engagement resulting in suboptimal ZAP-70 activation. Different conditions of peptide stimulation may be capable of producing a similar low-avidity engagement [57, 115] and triggering a comparable low-level activation of ZAP-70 (Figure 7.2). Since peptide recognition is a spectrum [75], varying levels of peptide affinity and density can produce comparable recognition and therefore also potentially anergy. Thus far a connection has not been formally established between suboptimal ZAP-70 activation and the selective activation of NFAT in the absence of AP-1 (Figures 3.1 and 4.1); such a connection would be highly interesting. In principle, a connection is possible if low-level activity of ZAP-70 results in low-level PLC- $\gamma$ 1 activity leading to an amount of IP<sub>3</sub> sufficient for activating NFAT but an inadequate amount of DAG for activating AP-1.

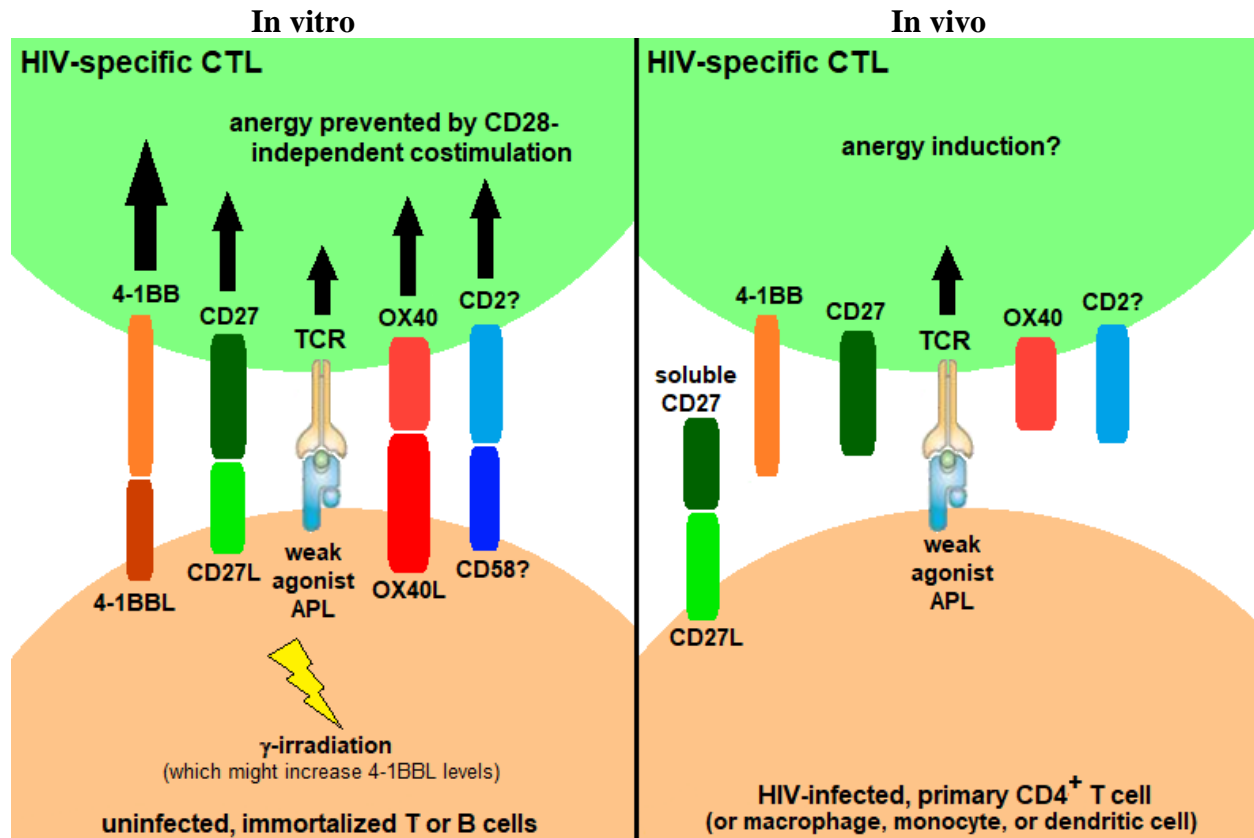


**Figure 7.2. Steps of TCR-stimulated CD3ζ ITAM phosphorylation and the contribution of each phosphorylated species to effector function and possible energy.** This *oversimplified* schematic of the steps of TCR stimulation-induced phosphorylation of CD3ζ highlights the progressively increased recruitment of the kinase ZAP-70 as phosphorylation approaches completion. In accordance with studies of peptide-induced differential signaling and/or anergy [55-60, 89, 115-117], I propose that different numbers of TCR-CD3 complexes engaged by pMHC ligands of varying affinity may be capable of triggering anergy through suboptimal recruitment and activation of ZAP-70. In this speculative model, a low copy number of CD3ζ chains containing three doubly-phosphorylated ITAMs and a high copy number of CD3ζ chains containing only one doubly-phosphorylated ITAM may both result in a comparable, suboptimal level of ZAP-70 activation. Supported by the data presented in Figure 7.1, the degree of TCR engagement required for anergy is likely to be less than that which is required for target cell killing.

#### 7.4.3 HIV-1 Epitope Variant-Induced CTL Anergy In Vivo: A Proposed Speculative Model

In spite of the confusion that still surrounds T cell anergy and the conclusion of the data presented in Chapter 6, it nevertheless remains possible that HIV-1-specific CTLs may be rendered anergic by epitope variants of HIV-1 that serve as weak agonist APL *in vivo*. Many viruses induce differential expression of T cell costimulatory and co-inhibitory molecules to aid in their persistence [118]. Because TCR signaling and CTL responses are modulated by costimulatory pathways independent of the classic CD28 pathway, it is potentially feasible that an HIV-1-infected cell *in vivo* may have reduced expression of one or more of these CD28-independent costimulatory ligands, which when presenting a weak agonist epitope variant, may send a weak TCR signal that activates NFAT but fails to activate AP-1, thereby triggering anergy rather than activation. Alternatively, it could be that HIV-1 infection may not necessarily decrease the surface expression of the costimulatory ligands but may instead increase the levels of the soluble forms of the respective receptors, as exemplified by the elevation of soluble CD27 in HIV-1-infected persons [119, 120].

Combining these ideas with those discussed in Section 7.4.1, a plausible model unfolds where irradiated pooled PBMC from healthy donors and irradiated uninfected T and B leukemia cells *in vitro* have higher expression of costimulatory ligands (of which 4-1BBL and CD27L seem the most likely) and send rescuing signals to block the induction of anergy; in contrast, a primary CD4<sup>+</sup> T cell, macrophage, monocyte, or dendritic cell that is infected with an HIV-1 epitope variant *in vivo* may have reduced costimulatory potency (either through virus-driven ligand downregulation or soluble receptor upregulation, or possibly both) and may therefore present a weak TCR ligand in the absence of rescuing signals, resulting in NFAT but not AP-1 activation leading to anergy induction.



**Figure 7.3** A proposed speculative model of how APL-induced CTL anergy may occur in HIV-1 infection *in vivo* despite the evidence to the contrary *in vitro*. HIV-1-specific CTL might be rendered anergic *in vivo* if they encounter an HIV-1-infected primary CD4<sup>+</sup> T cell (or macrophage, monocyte, or dendritic cell) presenting a weak agonist epitope variant within the context of either reduced surface expression of T cell costimulatory ligands (such as 4-1BBL, CD27L, OX-40L, CD58, or others), or elevated levels of soluble forms of the respective receptors, as is the case with soluble CD27 in HIV-1 infection [119, 120]. In this scenario, anergy in effector memory CTLs might possibly be caused by a lack of costimulation, similar to the mechanism described for naïve CD4<sup>+</sup> T cells but with the difference that the blockade is of CD28-independent costimulatory receptors. However, blockade of CD28-independent costimulatory pathways alone would be insufficient to drive anergy, which would require a suboptimal antigenic stimulation that sends weak TCR signals capable of activating NFAT but incapable of activating AP-1 and NFκB. Selective activation of NFAT in the absence of AP-1 and NFκB (whose activation can be rescued by CD28-independent costimulatory pathways) may drive anergy in human effector/memory CTLs in a similar mechanism as was described for murine Th1 cells [12].

## **Chapter 8: Concluding Remarks**

## **8.1 How to Completely Exclude (or Confirm) Anergy As A Mechanism of HIV-1-Specific CTL Dysfunction—the Final Nail in the Coffin**

*What WOULD be the ideal way to truly exclude (or confirm) anergy in vitro?*

This should be achievable using a modified version of the methods we used, where “feeders” from healthy donors are omitted, and uninfected peptide-loaded immortalized cells would be replaced by HLA-matched primary cells that are infected with HIV-1 containing either index epitope or a variant (and it would be interesting to compare CD4<sup>+</sup> T cells against macrophages and monocytes). The caveat is that primary cells would have to be activated first in order to make them permissive to HIV-1 infection, but this is feasible. Using VSV-G pseudotyped virus should not be a problem, and if an MOI of 2-3 is used, this should achieve ~86-95% infection.

*How would one identify markers of anergy in human CTL?*

The absence of a well-established control protocol for generating anergic antigen-specific effector/memory CTL remains an obstacle. However, what one could do is use established protocols of inducing anergy with polyclonal stimuli (e.g. plate-bound anti-CD3 without anti-CD28, or ionomycin without PMA) on *ex vivo*-isolated naïve human CD8<sup>+</sup> T cells to elucidate markers of anergy in that subset. Although the markers of anergy in cells that were previously naïve might be different than markers of anergy in cells that had already differentiated into effector or memory phenotype, it can nevertheless be useful to confirm similarity or differences between markers of anergy in humans compared to mice. Cells stimulated with both anti-CD3 and anti-CD28 would serve as a positive control for productive activation and effector cell differentiation. Full-genome mRNA expression profiling by microarray would likely be the

fastest and most cost-effective way to scan the entire transcriptome for protein biomarkers that are differentially expressed during anergy.

*Can anergy be excluded (or confirmed) with an in vivo model?*

Testing for anergy induced by replication-competent HIV-1 *in vivo* would require the humanized bone marrow/liver/thymus (BLT) mouse model [121], since HIV-1 cannot infect normal mice. The BLT mice could be modified with, for example, the SL9-specific 1.9 TCR transduced into CD34<sup>+</sup> stem cells which are then transplanted into the mice. The CD8<sup>+</sup> T cells that come out of the thymus should be naïve, SL9-specific CD8<sup>+</sup> T cells. It should be noted that in this model immune reconstitution is far from normal, and it would be difficult to assess for anergy in these transgenic T cells at different stages of differentiation simultaneously (naïve, effector memory, central memory, lytic effectors) post-infection. This is because naïve cells have different signaling requirements for activation compared to antigen-experienced cells. Anergy may occur in naïve and antigen-experienced cells to different extent, and may depend on the duration of infection, viral sequence, host restriction factors, amount of inflammation, and professional APC maturation and cross-talk.

For simplicity, prior to virus infection one could immunize the mice with SL9 index epitope to generate effector SL9-specific CTL, and these cells would be still relatively fresh (functionally speaking). HLA-A\*0201<sup>+</sup> primary CD4<sup>+</sup> T cells infected with single-round HIV-1 containing an SL9 variant (pick as many different variants as you like to test) could be injected into the mice as the “pretreatment” step to try to induce anergy in the effector 1.9 TCR-transgenic cells via the presentation of the weak agonist APL. To confirm whether the 1.9 TCR-transgenic effector CTL

are anergic (or if they are still functional), they can be isolated *ex vivo* and tested in assays to measure killing, cytokines, and proliferation in response to challenge with the SL9 index epitope presented on the surface of a primary HIV-1-infected cell. Important controls would be a group of mice that were pretreated with the index SL9 peptide, which would serve as a control for potential exhaustion that might occur after two rounds of ‘strong’ stimulation by the well-recognized peptide, and a group of mice pretreated with no antigen, whose CTL should remain highly functional during challenge.



## 8.2 Personal Statement

Working on this project has been the most challenging thing I have ever done. Anergy is a most stubborn, elusive, grueling puzzle, with so many different pieces and a level of complexity that is nothing short of mind-boggling, even for someone such as myself who loves signaling and gene regulation. The most difficult part about this project was the fact that there is no established positive control for inducing anergy in human effector CD8<sup>+</sup> T cell clones, particularly those derived from HIV-1-infected subjects that are already dysregulated because of the broad immune activation they were exposed to *in vivo*. It is my profound hope that one day, hopefully soon, someone more intelligent and talented than I will come along and resolve these confounding issues regarding anergy; if this dissertation assists with that, I will be grateful. Once we know how anergy works in effector T cells, it will give us control over the switch that determines immunity vs. tolerance. With that knowledge, I believe we will be able to selectively induce antigen-specific tolerance to treat T-cell mediated autoimmune diseases and break antigen-specific tolerance to boost the T cell response to cancer and viral infections.

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