Mechanisms of CD8+ T Cell Mediated Virus Inhibition in HIV-1 Virus Controllers

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Molecular Genetics and Microbiology in the Graduate School of Duke University

2014
ABSTRACT

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Abstract

CD8+ T cells are associated with long term control of virus replication to low or undetectable levels in a population of HIV+ therapy-naïve individuals known as virus controllers (VCs; <5000 RNA copies/ml and CD4+ lymphocyte counts >400 cells/µl). These subjects’ ability to control viremia in the absence of therapy makes them a gold standard for the type of CD8+ T-cell response that should be induced with a vaccine. Studying the regulation of CD8+ T cells responses in these VCs provides the opportunity to discover mechanisms of durable control of HIV-1. Previous research has shown that the CD8+ T cell population in VCs is heterogeneous in its ability to inhibit virus replication and distinct T cells are responsible for virus inhibition. Further defining both the functional properties and regulation of the specific features of the select CD8+ T cells responsible for potent control of viremia in VCs would enable better evaluation of T cell-directed vaccine strategies and may inform the design of new therapies.

Here we discuss the progress made in elucidating the features and regulation of CD8+ T cell response in virus controllers. We first discuss the development of assays to quantify CD8+ T cells’ ability to inhibit virus replication. This includes the use of a HIV-1 panel which can subsequently be used as a tool for evaluation of T cell directed vaccines. We used these assays to evaluate the CD8+ response among cohorts of HIV-1 seronegative, HIV-1 acutely infected, and HIV-1 chronically infected (both VC and chronic viremic) patients. Contact and soluble CD8+ T cell virus inhibition assays (VIAs)
are able to distinguish these patient groups based on the presence and magnitude of the responses. When employed in conjunction with peptide stimulation, the soluble assay reveals peptide stimulation induces CD8+ T cell responses with a prevalence of Gag p24 and Nef specificity among the virus controllers tested. Given this prevalence, we aimed to determine the gene expression profile of Gag p24-, Nef-, and unstimulated CD8+ T cells. RNA was isolated from CD8+ T-cells from two virus controllers with strong virus inhibition and one seronegative donor after a 5.5 hour stimulation period then analyzed using the Illumina Human BeadChip platform (Duke Center for Human Genome Variation). Analysis revealed that 565 (242 Nef and 323 Gag) genes were differentially expressed in CD8+ T-cells that were able to inhibit virus replication compared to those that could not. We compared the differentially expressed genes to published data sets from other CD8+ T-cell effector function experiments focusing our analysis on the most recurring genes with immunological, gene regulatory, apoptotic or unknown functions. Some of the most commonly identified genes in these studies were tumor necrosis factor receptor superfamily member 9 TNFRSF9 and the macrophage inflammatory proteins MIP-1α, and MIP-1αP. Using PCR in a larger cohort of virus controllers we confirmed the up-regulation of these genes in Gag p24 and Nef-specific CD8+ T cell mediated virus inhibition. We also observed increase in the mRNA encoding antiviral cytokines macrophage inflammatory protein MIP-1β, interferon gamma (IFN-γ), granulocyte-
macrophage colony-stimulating factor (GM-CSF), and recently identified lymphotactin (XCL1).

Our previous work suggests the CD8+ T-cell response to HIV-1 can be regulated at the level of gene regulation. Because RNA abundance is modulated by transcription of new mRNAs and decay of new and existing RNA we aimed to evaluate the net rate of transcription and mRNA decay for the cytokines we identified as differentially regulated. To estimate rate of mRNA synthesis and decay, we stimulated isolated CD8+ T-cells with Gag p24 and Nef peptides adding 4-thiouridine (4SU) during the final hour of stimulation, allowing for separation of RNA made during the final hour of stimulation. Subsequent PCR of RNA isolated from these cells, allowed us to determine how much mRNA was made for our genes of interest during the final hour which we used to calculate newly transcribed RNA. To assess if stimulation caused a change in RNA stability, we calculated the decay rates of these mRNA over time. In Gag p24 and Nef stimulated T cells, the abundance of the mRNA of many of the cytokines examined was dependent on changes in both transcription and mRNA decay with evidence for potential differences in the regulation of mRNA between Nef and Gag specific CD8+ T cells. The results were highly reproducible in that in one subject that was measured in three independent experiments the results were concordant.

This data suggests that mRNA stability, in addition to transcription, is key in regulating the direct anti-HIV-1 function of antigen-specific memory CD8+ T cells by
enabling rapid recall of anti-HIV-1 effector functions, namely the production and increased stability of antiviral cytokines. We have started to uncover the mechanisms employed by CD8+ T cell subsets with antigen-specific anti-HIV-1 activity, in turn, enhancing our ability to inhibit virus replication by informing both cure strategies and HIV-1 vaccine designs that aim to reduce transmission and can aid in blocking HIV-1 acquisition.
Dedication

To my family, everything I am is a reflection of the love and patience you have poured into me over all of these years, my success is a testament to your abilities and faith.

To my grade-school friend, Sandie, whose untimely death first piqued my interest in the disease that claimed your young life and to my mentor, Mr. Toliver, who dared me to dream bigger and push harder while secretly being in cahoots with my mother to make sure I one day get a Ph.D., I dedicate this document to your memories.
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Acknowledgements

First, I must thank Georgia Tomaras for being a steady voice of encouragement and for so skillfully guiding my scientific growth. To the Tomaras lab, my second family that I spent more time with than my actual family, I am forever appreciative of you all.

I thank my committee members past and present for your unwavering support. I am grateful to the smiling faces that I encountered daily throughout MSRB2, SORF, and the larger Duke community. I am indebted for the assistance of the Duke CFAR, and the patients, physicians, and staff of the Duke Adult Infectious Diseases Clinic, because of whom our work is possible. Thank you my financial supporter, the National Institute of Allergy and Infectious Disease (F31-AI-078715).

To my many personal and professional mentors, thank you for the lessons. A humongous thank you to my friends that I met before and during Duke who were always where I needed you to be whether you were there or not there.

Finally to the smartest, truthiest (yes, “truthiest”) and most humorous group of people that ever walked the face of God’s green earth - Olive, Franklyn, Odetta, Keisha, and Michael (aka my family) - doth not exist the words to adequately express all you mean to me. Thank you for being my cheerleaders, listening board, source of reason, prayer partners, and tiny voice that whispered “get the hell over it” anytime I was discouraged.
Chapter 1 Introduction

1.1 Global Impact of HIV-1/ AIDS

Human Immunodeficiency Virus-1 (HIV-1) is the causal agent of Acquired Immunodeficiency Syndrome (AIDS) (1). Since the identification of AIDS in the 1980s (2, 3), AIDS-related illnesses have caused more than 39 million deaths worldwide (4). The epicenter of the pandemic is in Sub-Saharan Africa where more than 70% of HIV-1 infected persons live and ~1/20 adults are infected with HIV-1 (4). While Sub-Saharan Africa is bearing the brunt of the HIV-1 pandemic, the impact of infection is clear across the globe. It is estimated that more than 35 million are infected with HIV-1 globally, with 2.1 million new cases of infection reported each year (4). The burden of HIV-1 and AIDS does not end with individual health outcomes as there are social, educational, and economic reverberations at the population level (5-7). With such a significant global impact, HIV-1 has been a premier research interest for over 30 years [reviewed in (8)].

1.2 Virology of HIV-1

1.2.1 HIV-1 Structure and Genome

HIV-1 is a lentivirus that is part of the retrovirus family (2). At the core of the HIV-1 virion is viral RNA, RNA-associated proteins, and enzymes enclosed by a cone shaped capsid wrapped in matrix protein scaffold with an outer viral envelope (9). Each HIV-1 virion contains two copies of the HIV-1 genome which is a single strand positive sense RNA measuring 9.7 kilobases. The nine protein products of the HIV-1 genome can
be classified as structural, regulatory, and accessory [reviewed in (10) and (11)]. The structural genes \textit{gag}, \textit{pol}, and \textit{env} encode essential components of the virion. Gag and Pol are made as a precursor myristylated Gag-Pol protein. Gag is later processed to p17 matrix, p24 capsid, and p7 nucleocapsid by the viral protease. The catalytic \textit{pol} gene encodes the enzymes protease, reverse transcriptase (RTase), and integrase. Env glycoproteins are made as precursor gp160, which is processed to gp120 and gp41 by a cellular protease as Env is trafficked to the cell surface (12). The accessory genes vpu, vif, vpr, and nef while not necessary for viral propagation, do serve to influence the viral life cycle and pathogenesis (10, 11). \textit{tat} and \textit{rev} are regulatory genes which modulate the transcriptional and post-transcriptional phases of viral gene expression (11).

\textbf{1.2.2 Life Cycle of HIV-1}

HIV-1 infects by first binding gp120 to the CD4 molecule found on dendritic cells, macrophages, and the virus’ primary target, CD4+ T cells (13) or by using non-classical receptors (14). Once gp120 is bound to CD4, conformational changes in gp120 expose structural elements in the V3 hypervariable region of gp120 [reviewed in (15)]. This exposed region then binds to a co-receptor, most commonly C-C chemokine receptor type 5 (CCR5) (16) or C-X-C chemokine receptor type 4 (CXCR4) (17). CCR5 serves as a co-receptor early in infection and CXCR4 serves as the main co-receptor later in infection (15). Binding of V3 to the co-receptor causes a structural rearrangement in gp41 which leads to interaction of the gp41 fusion peptide with a fusion receptor on the
target cell membrane and subsequent fusion (18). After fusion of the viral envelope and cell membrane, the virus’s RNA and RTase, integrase, and protease enzymes enter the cytoplasm. The RTase transcribes the single-stranded RNA of the virus creating anti-sense cDNA. This cDNA is used as a template to generate a sense strand which, with the anti-sense cDNA, forms a double-stranded DNA (dsDNA) (19). RTase has a very high error rate which leads to great variation in the strands of the dsDNA (20). The newly created dsDNA is transferred into the cell’s nucleus and integrated into the host chromosome as a provirus by the viral enzyme integrase (21, 22).

If cellular conditions allow for productive infection, the provirus is replicated and transcribed into mRNA. The resultant mRNA is spliced, exported to the cytoplasm, and then translated by host enzymes to make the gene products including those of early infection, Tat and Rev (23). Tat and Rev allow for enhanced transcription of the provirus and late viral gene expression which produces the other viral gene products that act to protect the infected host cell from destruction and allow for virion budding. The Gag protein is the primary orchestrator of HIV-1 budding (24). The N-terminus of the gag precursor polypeptides Gag and Gag-Pol are myristoylated which allows association with the inner surface of the cell membrane (25). Two RNA copies and enzymes are recruited by this membrane-associated Gag, initiating virion budding [reviewed in (26)]. In addition, gp41 and gp120 Env molecules are transported to the cell membrane when they become anchored to serve as part of the virions’ outer coating. The emergent virion
is a non-infectious immature particle with a full Gag polyprotein (27). Once the virion budds, the Gag precursor polyprotein is cleaved by protease to give the viral capsid, matrix, and nucleocapsid shells. The mature virion also contains host proteins that aid in infectivity (27, 28).

Tat is a trans-acting protein that enhances viral transcription up to 1000-fold (11) and can activate uninfected CD4+ T cells (29). Rev induces the transition from early to late viral gene expression and helps export un-spliced viral RNA from the nucleus to the cytoplasm (30). Nef downregulates the expression of CD4 and MHC class I molecules, perturbing T cell activation and presentation of HIV-1 to immune cells (31, 32). Nef also maintains high viral loads and CD4+ T-cells loss (33). VPU acts to enhance virion release by countering the action of tetherin (34) and targets CD4 for degradation (35). Vif prevents the activity of APOBEC3G, a major innate immunity factor (36). Vpr causes the cell to freeze at the G2/M stage allowing for an optimal environment for viral replication (37).

1.2.3 HIV-1 Groups and Subtypes

The high error rate of reverse transcriptase and fast replication pace of HIV-1 leads to great genomic diversity which has resulted in the generation of multiple subtypes (38). HIV-1 strains can be phylogenetically divided into 4 groups pandemic M (“major”), O (“outlier”), N (“non-M, non-O”), and P (“pending the identification of
further human cases”) (39, 40). Each of the 4 groups represents an independent transmission event in which SIV was introduced to the human population (38, 41).

More than 90% of HIV-1 strains belong in the M group. The M-group can be further divided into clades loosely correlating to geographical location (42). In addition to the clades, group M also includes circulating recombinant forms that result from the combination of viruses from different clades (43, 44). To date, group O has been limited to central Africa with most cases in Cameroon (45, 46). Discovered in Cameroon in the late 1990s, Group N infection is also very limited (47). The newest group, P, stems from a single case reported in 2009 in which the virus sequence bared greater similarity to SIV isolated from gorillas than to that isolated from chimpanzees, similar to Group O (48-50).

Group M Subtype B is the dominant viral subtype in industrialized countries (42). While understanding subtype B is important for those in the developed world, more than 90% of infected persons in Africa and Asia, regions hit especially hard by the pandemic, have non-subtype B infection (42). Among these infected persons, subtype C viruses are responsible for more than 50% of infection and C is the dominant subtype in sub-Saharan Africa and India (51, 52). Only ~10% of infections globally can be attributed to infection by subtype B virions (51). Given these data, it is important that non-subtype B viruses be considered in the development of HIV-1 therapies (53).
1.3 Immunopathogenesis of HIV-1 Infection

HIV-1 is spread via the transfer of bodily fluids (54). The majority of adult HIV-1 infection occurs through mucosal surfaces (most commonly, sexual contact) while about 20% of infections are via cutaneous routes with most cases resulting from drug use or transfusion of blood or blood products (52). Transmission and subsequent establishment of infection are influenced by host factors as well as characteristics of the virus including the amount of infectious virion that is in the transmitted fluid (55). For this reason, over 50% of new HIV-1 infections can be attributed to transmission from someone who is in the acute stage of infection when the virus load is highest (56). In addition to the high levels of viremia, the properties of viruses present during acute infection have characteristics that are advantageous for transmission (57, 58). While the risk of transmission varies depending on the route of exposure (59) the characteristics and timing of resultant infection are generally uniformed (60, 61).

Primary or acute HIV is the period from the time of infection to the start of clinical latency. During this time, 70% of infected persons experience mononucleosis-like symptoms (fever, rash, swollen lymph glands) (62). Acute HIV-1 infection is marked by the sequential appearance of viral and immune markers in the blood (60, 63). As the immune response to HIV-1 kicks in and begin to inhibit virus spread to a stable set-point, the asymptomatic phase begins (64).
1.3.1 The Transmitted Founder Virus

In addition to host factors, viral factors can influence the ability of HIV-1 to cross mucosal barriers and establish infection (55, 57, 58). Transmission of HIV-1 across the mucosa is an inefficient process [reviewed in (65)]. The inoculum of HIV-1 transferred during transmission exists as complex mixture of viruses with varying genomes referred to as quasispecies (66). Previous research suggests that there is a biological bottleneck at the mucosa that selects for viruses with properties that favor the establishment of new infection once transmitted (67). Studies of HIV-1 quasispecies in patients with early HIV-1 infection have helped to define the characteristics that are advantageous to virion transmission.

Biological characterization of HIV-1 transmitted/ founder (T/F) virus genomes and envelope sequences have revealed fundamental properties of these viruses that favor crossing of mucosal surfaces to establish productive infection (67). These viruses primarily utilize the CCR5 co-receptor, mask co-receptor binding regions, exhibit higher infectivity and more Env particles per virion, are resistant to fusion inhibitors, broadly neutralizing antibodies (see Section 1.3.3.2), and interferons, while the env sequences of these viruses have shorter V1/V2 loops and fewer N-linked glycosylation sites (67-75). Models that allow inference of the genetic sequence of transmitted viruses (73) and subsequent experiments indicate that in many cases transmission can be attributed to one or few T/F virus(es) (76, 77) with multivariant transmission being more common in
intravenous drug user cohorts (78), supporting the hypothesis of the mucosal barrier providing a sieve for T/F selection (79).

1.3.2 Early HIV-1 Infection

The majority of HIV-1 transmission events occur via sexual contact. Therefore, most models of early infection are vaginally and rectally based and much of the current understanding of transmission and early infection come from in vitro and in vivo simian studies. It is unclear whether HIV-1 virions are transferred as free virion or within an infected cell. Once inside a new host, the virion or cell crosses the mucosal epithelium within a few hours (80). Previous research has revealed that T/F viruses infect CD4+ T cells more efficiently than other cell types and that established infection likely occurs via a founder population of infected CD4+ T cells (80, 81).

Once initial localized HIV-1 replication is complete, virions and/or infected cells reach draining lymph nodes, where amplification of infection and systemic spread initiates. In the draining lymph nodes virions encounter activated CD4+ T cells, dendritic cells, and B cells all of which potentially aid the early spread of infection through the lymphoid and circulatory systems (82, 83). While some research suggests gp120’s affinity for integrin α4β7 (84), the homing receptor for the gut mucosa, leads to HIV-1’s specific targeting of the gut-associated lymphoid tissues (GALT) (85), recent evidence indicates this may not be true for infectious virions (86). Regardless of the mechanism, the large CD4+ T cell population in the GALT is depleted by HIV-1 in acute
infection (87, 88) while the T/F virus mutates rapidly producing a new quasispecies swarm that goes through selection for viral strains that escape the initial immune responses to HIV-1 (89).

During acute infection, CD8+ T cells strongly contribute to controlling HIV-1 viremia (see Section 1.3.4) and establishing viral set point (90). At this point CD4+ T cell numbers return to near normal levels in the blood but not in the GALT (88). Replication continues at a limited pace as the chronic phase of infection begins. The chronic phase ends with the onset of AIDS which is characterized by opportunistic infections.

1.3.3 The Immune Response to HIV-1

Activation of the innate and adaptive branches of the immune system is a central feature of acute HIV-1 infection that persists, to varying degrees, into chronic infection. Early immune responses to HIV-1 are important in determining disease progression (57, 91) and analyses of T/F and asymptomatic phase viruses show that the acute immune response drives the selection of virus escape (57, 68, 92). Early activity of innate immunity components combat early viremia while priming the HIV-1 specific adaptive response [reviewed in (93)]. It is believed that the adaptive immune response limits viremia via multiparameter activities that include neutralizing and non-neutralizing antibodies as well as both CD4+ and CD8+ T cell functions.
1.3.3.1 Innate Immune Responses to HIV-1 Infection

The innate immune system is the earliest line of defense against virus. As HIV-1 in plasma increases, there is an onslaught of cytokines, small proteins that act as immune system communicators and effectors (94, 95). These cytokines act to inhibit HIV-1 replication and enhance both the innate and adaptive immune responses (96-98). This bolstering of antiviral immunity is caused by the generated “cytokine storm” inadvertently contributes to viral replication and CD4 loss via recruitment of susceptible T cells to the site of HIV-1 foci (99). In a similar fashion, the presence of innate immunity cells also acts as a double-edged sword working to limit infection while sometimes leading to the destruction of other immune cells (99, 100).

Dendritic cells (DCs) can be infected via CD4 and C-type lectin receptors such as DC-SIGN (82). DCs are found in the squamous (such as vagina, cervix and foreskin) and columnar (such as the cervix and rectum) epithelial surfaces making DCs good candidates for the primary cell type infected by HIV-1 (101). Animal models of infection reveal antigen-loaded DCs can reach draining lymph nodes as early as 18 hours post-exposure (102). DCs can be derived from myeloid or plasmacytoid lineages with myeloid-derived Langerhan Cells (LCs) being found within the squamous epithelia where they interact with HIV-1 virions and Plasmacytoid DCs (pDCs) being found in the lymph nodes and blood. pDCs activated by HIV-1 secrete large amounts of type I interferons, a group of pro-inflammatory cytokines that carry out antiviral functions and
initiate signaling cascades that result in the formation of other cytokines and non-specific CD8+ T cell proliferation. DCs link the innate and adaptive immune systems. DCs located in other peripheral tissues and blood can bind and endocytose foreign antigen which they later present to CD4+ or CD8+ T cells in mucosal or lymphatic sites, beginning the adaptive response to the infection (103-105). In addition, DCs can interact with and enhance the function of Natural Killer and B cells (106, 107).

During acute infection, Natural killer (NK) cells become activated and show an enhanced capacity to kill cells infected with HIV-1 (108). NKs have direct cytolytic functions as well as an immune-regulatory role. NKs are CD3- lymphocytes that can kill infected cells (via lysis or apoptosis) and act as immune mediators, attracting adaptive immune cells to sites of infection through direct interaction and the release of cytokines (103, 109). Multiple receptors for NK cells have been associated with HIV-1 transmission and disease course (110-112). In addition, certain virion mechanisms are known to be evasive of NK responses indicating NKs may exert selective pressure on HIV-1 virions [reviewed in (113)].

1.3.3.2 Humoral Immunity: The Repertoire of the Antibody Response

Most effective anti-viral immune responses include the induction of virus-specific antibodies that can neutralize (cause loss of infectivity via blocking of the biological activities) the antigen (114-116). This loss of infectivity can occur via multiple mechanisms including blocking virion binding to receptors, uptake, and causing
aggregation of virus particles (117-119). In addition to neutralization, antibodies can also carry out non-neutralizing functions (120, 121). Env’s crucial role in virus entry and the requirement of some of Env’s conserved regions for viral functionality make Env a prime target for HIV-1 antibodies.

Antibodies consist of two Fab (antigen-binding) arms and a Fc (crystalizable) domain (122). Each Fab arm has a heavy and a light chain and has two variable and two constant regions which dictate antigen specificity. Within each variable domain, there are complementarity determining regions (CDRs) which provide the ability to recognize antigen (122). The Fab and Fc domains of antibodies are responsible for distinct functions with the Fab recognizing antigen and the Fc interacting with components of the immune system to cause pathogen deletion. In humans, there are 5 isotypes IgA, IgD, IgE, IgG and IgM with IgG being the most abundant subclass in human serum and IgA being the primary antibody in mucosal secretions (saliva, tears, and milk) (122).

Antibodies have several functions (119). In antibody-mediated neutralization of HIV-1, antibodies with Fab regions specific to the HIV-1 envelope prevent infection of target cells by inhibiting fusion of the host cell membrane and viral envelope. In addition to neutralizing functions, antibodies are able to carry out non-neutralizing effector functions. These functions include antibody dependent cellular cytotoxicity (ADCC), antibody-dependent cellular viral inhibition (ADCVI), virion capture, inhibition of transcytosis, aggregation of virus, and phagocytosis. With ADCC, the
engagement of Fc and FcR on innate cells results in the secretion of cytolytic cytokines which causes death of HIV-1 infected cells (123-127). Antibody-dependent cellular virus inhibition (ADCVI) is the ability of virus-specific antibodies and effector cells to inhibit viral replication in a target cell population in vitro due to ADCC and non-cytolytic mechanisms such as β-chemokine release from the effector cells (119, 127). Antibodies can also cause cell lysis through activation of complement dependent cytotoxicity (128).

1.3.3.3 CD4+ T cell Functions in Response to HIV-1

CD4+ T cell functions include the induction and maintenance of CD8+ T cell responses, involvement in B cell proliferation and the maturation of antibodies, cytotoxicity, and maintaining the integrity of the mucosa. CD4 helper T cells are critical for the cytolytic activity of CD8+ T cells including the reversal of exhausted CD8 phenotypes during chronic infection (129, 130) (131) including the reversal of exhausted CD8 phenotypes during chronic infection (132)(129) . CD4+ follicular helper T cells are essential in the maintenance of germinal centers (133, 134), the main sites of B cell affinity maturation and proliferation (134, 135). In germinal centers, CD4 help provides the signals needed for B cell proliferation and differentiation (136). In addition, these CD4 cells are crucial in the process of somatic hypermutation which is key in the development of bnAbs (137). CD4+ T cells recognize HLA class II bound epitopes and can respond with secretion of antiviral and cytolytic cytokines (138, 139) which, when present during acute infection, can work to influence disease course (140). Previous
research has indicated a role for CD4+ T cells in maintaining the integrity of the mucosa (141).

1.3.3.4 The CD8+ T cell Response to HIV-1

CD8+ T cells are one of the body’s major defenses against HIV-1 infection. Initial CD8+ T cell responses appear near the time of peak viremia with viral escape mutants exhibiting signs of CD8+ T cell pressure (68, 89, 142-144). Depleting CD8+ T-cells from the peripheral blood mononuclear cells (PBMCs) of HIV-1+ individuals in vitro and in macaque models in vivo leads to a significant increase in HIV-1 replication compared to non-depleted populations in acute and chronic infection (145). CD8 alleles have been associated with control of viremia in populations of chronically infected patients (see Section 1.5). Studies of chronically infected patients and the results of vaccine trials, have shown that CD8+ T cells exhibit some level of poly-functionality, responding to stimulation by producing multiple effector molecules which enable the carrying out of cytolytic (killing) and/or non-cytolytic (viral suppression in the absence of killing) functions (146, 147) (Figure 1).

Cytotoxic T cells (CTLs) have been defined by their capacity to recognize viral antigen presented in conjunction with the major histocompatibility complex (MHC) molecule on the surface of antigen presenting cells (158). Antigen presentation to CTLs leads to lysis of the infected cell via the release of cytolytic proteins including chemokines (C-C motif) ligand 4 (CCL4) and perforin (159). Traditionally, the ability of CD8+ T cells to lyse cells
was measured a $^{51}$C assay in which target CD4+ cells are labeled with the radioactive nucleotide $^{51}$Cr which is released when target cells are killed by cytolysis (160). Recently, $^{51}$Cr have been replaced with assays such as ELISpot and intracellular flow cytometry, which measure the production of various cytokines that act as surrogate markers for cytolysis (126, 161). Clearance of virus during acute infection correlates with the rise of HIV-1-specific CTLs (155, 156). These responses are typically to HIV-1 proteins Env, Nef, Gag, and Pol and select for escape mutations and subsequent T-cell responses to other epitopes as virions begin to mutate epitopes recognized by CD8+ T cells. These mutations are optimized for viral fitness and can impact HLA allele binding, TCR recognition, and processing of epitope by antigen presenting cells (157). In addition to classical, MHC Class I restricted cytolytic CD8+ T cell function, recent reports from Picker et al have opened the door to the possibility of non-canonical MHC Class II restricted CD8+ T cells with anti-HIV-1 activity. The Picker group reported that a rhesus macaque CMV vector expressing SIV elicited CD8+ T cells able to recognize a variety of epitopes including some that were restricted to class II (162). Whether there is MHC class II restricted CD8+ T cell activity in humans warrants further investigation. The non-cytolytic CD8+ T cell response suppresses HIV-1 replication without causing lysis of the infected CD4+ cell (146). Non-cytolytic activity does not depend on cell-cell contact but is carried out through soluble mediators (146, 148, 149). There is a positive
correlation between the non-cytolytic CD8+ T-cell response and a healthy clinical status (150-154).

In the absence of well-defined correlates of protection from an anti-HIV-1 T cell mediated vaccine study, the full repertoire of molecules derived from CD8+ T cells that mediate potent anti-HIV-1 effects is not known [reviewed in (163) (164)]. Correlational studies indicate that the effector molecules are likely to be co-expressed with commonly measured determinants of cytotoxicity CD107a, perforin, and granzyme along with β-chemokines and other soluble factors that can act to block infection directly (165, 166). Studies with agents known to impact histone modification have shown that differences in chromatin, specifically the acetylation state of histones, influence the CD8+ T cells’ ability to inhibit HIV-1 virus (167, 168) and may provide a mechanism in which to identify effector molecules.

1.3.3.5 Immune Dysfunction in HIV-1 Infection

While no one immune change that occurs with HIV-1 infection can fully account for the scale and diversity of immune dysfunction associated with HIV-1 infection and pathogenesis, the dysfunction of immune cells and loss of CD4+ T cells can account for much of the dysregulation seen in the responses of the adaptive immune system. The earliest immune dysfunction seen with HIV-1 infection is that of CD4+ T cells as depletion of CD4+ T-cells is a hallmark of infection. Because most transmitted/ founder viruses use the co-receptor CCR5 for entry, activated and memory CD4+ T cell subsets
which express high CCR5 (compared to naïve cells which are CCR5-) are the primary targets of HIV-1 (169). Due to the large-scale killing of these cells by cytopathic effects and cytolytic cells, direct functions of CD4+ T cells and immune functions aided by the activity of CD4+ T cells become highly compromised during the course of infection. CD4+ T cells able to withstand deletion are functionally abnormal in the majority of HIV-1+ individuals. These abnormal functions include decreased poly-functionality, marked by a decrease in IL-2 production, limited proliferation, defective up-regulation of activation markers with concurrent up-regulation of the inhibitory molecules and increased expression of the apoptosis markers (170). These changes are accompanied, and likely partially the result of blunted T cell signaling and exhaustion (171).

A crucial emerging role for CD4+ T cells, specifically follicular helper T cells (TFH), is providing help for antibody maturation (137, 172, 173). TFH are antigen-experienced CD4+ T cells that constitutively express CXCR5, the homing receptor of the B cell follicles (174). TFH migrate to B cells where they interact with cognate B cells, subsequently triggering germinal center formation (136, 175). Germinal centers are composed of rapidly dividing and mutating B cells (135). TFH can enter into germinal centers where they are critical in mediating B cell selection and survival which dictates which cells differentiate into plasma cells capable of producing antibodies and subsequently forming memory B cells that monitor for antigen re-encounter. With CD4 loss, there is inadequate TFH help resulting in improper B cell responses (176). The high
viral load and storm of pro-inflammatory cytokines seen during acute infections leads to dysregulation of B cells in various tissues. These changes also contribute to poor HIV-specific B cell and antibody responses and a loss of germinal centers and increased B cell lysis and apoptosis (177).

The reduced efficiency of CD8+ T cell responses against HIV-1 escape mutants, compared with CD8+ T cell mediated responses to transmitted/ founder viruses is likely a consequence of waning CD4 T helper cell function (178). Similar to CD4+ T-cells, CD8+ T-cells become dysfunctional and exhibit an exhausted phenotype during chronic infection, contributing to failure of HIV-1 control (179). High levels of antigenic stimulation and repeated activation during the early phases of acute infection drive the induction of a senescent/ exhausted phenotype. CD8+ T cell exhaustion is characterized by expression of negative regulatory cell surface molecules such as PD-1 (programmed cell death protein 1), Tim-3 (T cell immunoglobulin domain- and mucin domain-containing protein 3), and CD57 (180, 181). These cells experience loss of proliferative capacity and anti-HIV-1 effector functions in part due to decreased responsiveness to TCR stimulation (182). CD4+ regulatory T cells also work to control CD8 activation effector functions (183).

With HIV-1 infection, dendritic cells (DCs) become defective with diminished function (184). This dysfunction can be a direct consequence of interaction with virus or a result of indirect effects such as the production of IL-10, which blocks the maturation
of DCs, by other immune cells. Impairment of DC function likely contributes to insufficient priming of adaptive immune responses and NK cell activation. Contributing to CD4+ T cell loss, activated DCs can transmit virus to CD4+ T cells chemokines produced by pDCs can cause the chemotaxis of CD4+ T cells to foci of infection).

1.4 Preventing and Treating HIV-1 Infection

1.4.1 Interventions for Preventing HIV-1 Infection

The best solution to the HIV-1 pandemic is to stop virus transmission. While there is high hope for therapies that enhance the immune response before and right after transmission, population- and individual-level strategies that have great potential of immediate impact by reducing the chance of HIV-1 acquisition in persons at risk for exposure. There is no mistaking the need for biomedical interventions but it is clear that effective stymieing of the HIV-1 pandemic requires complementing scientific advances with behavioral and structural interventions (185-187).

Structural interventions are public health initiatives that work to promote the health of the population by addressing the higher level determinants associated with disease risk (186, 188). This multi-disciplinary approach incorporates public health, sociology, and politics among other fields (188, 189). In the case of HIV-1 infection, several modalities including screening of blood donors and campaigns addressing poverty, insufficient education, and poor access to healthcare services have the potential to limit transfer of virus (190, 191). With the rate of infection being the highest among
young people, traditional and social media outlets can be used to push for change in social norms concerning high risk behaviors and to advocate community mobilization to work towards reinforcing HIV prevention messages (192, 193). In areas of extremely high incidence, school-based testing for HIV (to allow for immediate post-exposure care) as well as the distribution of condoms and contraception can be effective interventions in slowing virus spread (194, 195). Widespread HIV-1 testing and counseling for infected persons can also contribute to the decline of virus transmission (196, 197).

In addition to structural changes, implementation of biomedical interventions that make the human body less susceptible to HIV acquisition or transmission can significantly reduce the rate of HIV-1 incidence. These can include the use of antiretroviral therapy for prevention of infection of those populations highest at risk for infection (198). While the efficacy of treatment as prevention indicated by these results is astounding, implementation of widespread ART should be cautiously approached due to the therapies’ less than ideal characteristics (see Section 1.4.3). Other biomedical interventions that can prove to be efficacious with less effort include male circumcision and use of vaginal microbicides (68, 199).

Even the best structural and biomedical effort will have little effect on the rate of HIV-1 transmission if not complemented with changes in the behavior of persons at risk, no matter how small, of infection and those already infected. In those currently infected with HIV-1 there is discordance in knowledge of infection and control of viremia with
decline of adherence in every step of treatment. Addressing this gap is crucial to preventing virus spread as low, controlled levels of viremia significantly reduce chance of transmission. A component of this gap is chosen non-adherence due to the discrepancy between the number of patients linked to/ retained in care and those that are controlling their viremia. While access, prohibitive costs, and severe side-effects do factor into this discrepancy, some patients simply choose not take their medication everyday (200). Conscious, safe sexual behaviors such as limiting rates of partner change/ practicing monogamy and regular condom use are personal choices that, when complemented with regular testing for infection, can go a long way to stopping virus spread.

1.4.2 Current Treatments

Current therapy for HIV is a combination of anti-retroviral drugs. These drugs can fall into one of many classes depending on the step of HIV-1 replication blocked. Fusion Inhibitors block virus entry by perturbing the fusion of viral envelope and host cell membranes (201-206). These medicines work by targeting host and virion surface molecules that usually interact to allow fusion. Reverse transcription of HIV-1 can be blocked by 2 classes of medicines Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs) and Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) (207). Both classes work by inhibiting the activity of reverse transcriptase which turns the virus’ single strand RNA in dsDNA which is later integrated into the host
chromosome. NRTIs provide faulty DNA building blocks that stunts replication (208). NNRTIs directly inhibit the function of the reverse transcriptase enzyme (207). Protease inhibitors cause dysfunction in the protease enzyme which usually functions to cleave newly synthesized HIV-1 poly-proteins into functional proteins (209). Integrase Inhibitors prevents integration of viral genetic material into the host genome (210). While each of these medicines are efficacious in blocking replication alone, combatting infection by employing combination of drugs is key to effectively treating infection. These combinations are fixed-dose of 2 or more medications from 1+ class(es) combined into one pill at specific ratios (211).

In 2010, the World Health Organization updated its recommendation on use of ART (185). When ART is initiated, patients should be given a combination of reverse transcriptase inhibitors a NNRTI and two NRTIs, one of which should be zidovudine or tenofovir. If first-line therapy fails, second-line ART should include a protease inhibitor (Ritonavir-boosted atazanavir or lopinavir/ritonavir) and two NRTIs, one of which should be zidovudine or tenofovir. In addition to new recommendations for first- and second-line therapies, WHO recommends continuous laboratory monitoring in order to optimize care pre- and during ART to monitor ART failure and drug toxicity. HIV-1+ patients co-infected with tuberculosis or hepatitis B virus (HBV) should be started on ART when treatments for co-infections are initiated.
In addition to the above recommendations, WHO suggests those infected with HIV-1 with CD4 counts of ≤350 cells/ul should be given antiretroviral therapy. HIV-1 infected subjects designated as having stage 3 (marked by severe weight loss, chronic diarrhea and persistent fever, pulmonary tuberculosis) or 4 (opportunistic infection) clinical disease are started on ART regardless of CD4 cell count. This change increases the CD4 T cell threshold for ART from 200 cells/µl to 350 cells/µl. This change came about as the result of scientific evidence that starting ART at CD4 levels higher than 250 cells/µl reduces mortality rates in HIV-1+ asymptomatic persons and reduces risk of developing opportunistic disease and AIDS (212, 213) and is expected to reduce HIV-related mortality 20% by 2015 (UNAIDS. Cost estimates: AIDS financing and economics. 2009).

Use of ART to treat HIV-1 infection has transformed a prognosis of likely death to one of a near normal life-span. In the early 2000s, there was a > 30-fold increase in the number of infected persons on ART in developing nations (214, 215). Grouped with previously mentioned interventions (Section 1.4.1), ART has led to the reduction in HIV-1 prevalence rates in a growing number of countries as new HIV infections on the decline (216). In addition, ART has contributed to the lowering of the mortality rate which has resulted in an increased the life span in infected persons due to a delay the onset of AIDS (217).
1.4.3 Limitations to Current Treatments

The impact of ART, while significant, has proven to be a less than ideal. There is great disparity in the number of infected persons and those who are currently controlling their viremia with ART even in developed nations (Figure 1.2). While it is believed more than 35 million are infected with HIV-1 globally, only 9.7 million are currently on ART, which means that a significant portion of those eligible for therapy are not being treated (214). When only taking into consideration those who know of their status, there is still a discrepancy in treatment and controlling viremia (Figure 2). Several factors including high cost, possible severe side-effects due to drug toxicity, needed compliance to lifelong therapy, variable pharmacokinetics, and less than optimal efficacy contribute to treatment failure (218).

The 2010 WHO recommended scale-up in ART initiation have considerable costs in price and possible side effects. The number of persons eligible for ART increased by 49% with life-expectancy for HIV-1+ individuals increasing 1-2 year (219). This increase in population needing therapy and time of therapy is of concern as not everyone has access to drugs due to availability and costs (220) and changing the threshold for ART initiation can result in a 57% increase in the cost for providing therapy for infected persons (219). This adds to the already high costs of ART resulting from the need for lifelong treatment given increased life expectancy for those on therapy and the prevalence of risk and infection among young populations. Increase in exposure to ART due to longer
treatment periods will likely lead to greater chance of developing ART-related side-effects and resistance. The greatest contributor to resistance is poor treatment adherence which results in the revival of active infection and emergence of drug-resistant HIV-1 variants.

1.4.4 Ideal Characteristics of an HIV-1 vaccine

To date, there is no therapeutic or functional cure for HIV-1. While there have been publicized special cases of a “cure” (221, 222), the biology of HIV-1, specifically the establishment of latent reservoirs, does not allow for a sterilizing or functional cure (223). In lieu of a cure, therapies are needed to protect from infection and provide control of disease while avoiding the limitations of ART. While the sterilizing immunity offered by modalities that block infection in uninfected persons is the ultimate goal, a valuable complement would be a vaccine that reduces risk of HIV-1 transmission and slows progression in infected persons by inhibiting virus replication and subsequently maintaining low viral load. Ideally, this therapy would be cheap, globally dispensable and provide long-term protection and/ or control with a single or few dose(s).

1.4.4.1 Antibodies with Neutralizing and Non-neutralizing Functions

Antibodies will likely be the primary mediators of a protective immune response to HIV-1. Given the high mutation rate of HIV-1, a successful vaccine will bring about the induction of durable broadly neutralizing antibodies (bnAbs) that can protect
against infection regardless of virus clade and difficulty of neutralization (63, 118, 120). Previous research has shown that only a small percentage of HIV-1+ persons produce antibodies that can neutralize a range of subtypes (224). A great deal of effort is now placed on the design of a vaccine regimen that will elicit these bnAbs. Analysis of isolated bnAbs revealed many shared characteristics including 17-49% somatic hypermutations from germline (225), a high frequency of insertions and deletions (226), long CDRH3s (heavy-chain complementarity-determining regions) (227, 228), high levels of poly-reactivity, and evidence that the virus/antibody interplay drives maturation over a period of months/years (229, 230). Longitudinal studies of bnAb development (230, 231) indicate that induction of bnAbs may require priming the antibody response via binding HIV-1 Env to a naive B with subsequent sequential immunizations with immunogens inferred from studying the lineage of bnAbs in HIV-1–infected individuals (232), a challenging and exceptional task (229, 233).

While the design of bnAb-inducing vaccine is the penultimate goal of anti-HIV-1 humoral vaccine design, credence is still to be given to the induction of conventional Abs (234). Vaccines that induce conventional Abs have been successful against several pathogens including HIV (235). The activity of Abs can be synergistic meaning that a polyclonal response to HIV-1 may have the capacity to drastically reduce risk of HIV-1 infection (236). IgA and IgG can capture free virion inhibiting transcytosis at the mucosa and systemic replication (237, 238). Studies aimed at understanding and editing highly
conserved N-glycosylation site of the Fc regions shows enhancement of Fc receptor-mediated activity of Abs (121). Specifically, certain Fc glycan structures selectively promote Fc mediated effector functions without regards to Fab specificity (121, 239, 240). The greatest support for the role of Abs in protecting from HIV-1 comes from the RV144 “Thai trial”. For RV144, subjects were given fours doses of recombinant HIV–avian pox virus and two doses of gp120 proteins. Efficacies of 60% and 31% were observed 1 and 3 years after immunization, respectively (241). Independent analyses of correlates of risk have shown that levels of plasma IgG Abs to gp70-V1V2 were inversely correlated with infection (242-244). Interestingly, IgA antibodies to gp120 directly correlated with infection Env IgA/IgG ratios directly correlated with HIV-1 infection risk indicating that IgA likely interfered with IgG function (245). This is further supported by the inverse correlation of Ab-dependent cellular cytotoxicity (ADCC), neutralizing Abs (NAbs), and CD4+ T cell responses with infection risk in the presence of low IgA (242). The results from primary and secondary analyses of RV144 indicate that despite lesser potency and breadth compared to bnAbs, conventional Abs can protect from infection and have the potential to impact the HIV-1 epidemic. Identification of immunogen alterations and adjuvants to enhance the RV144 response are underway in follow-up studies.
1.4.4.2 T cell Help and CD4 Effector Functions

In addition to humoral responses, strong T cell responses are needed to aid in preventing protection and controlling virus replication. CD4+ T cell functions needed for protection against and control of HIV-1 are 3 pronged: aid in development (176) and function (178) of other cell types, CD4+ CTL killing of infected cells, and CD4+ T cell mediated mucosal protection (246). Although these CD4 functions are crucial in allowing for potent immune responses, it is important to remember that CD4 function in response to HIV-1 is double-edged, allowing for enhance protection and infection. To this end, the main focus of CD4 directed therapies is the elicitation of balanced, potent CD4+ T cell responses that bias the expression of HIV-1 co-receptors to limit the spread of virus (247).

1.4.4.3 CD8+ T cell Responses to aid in Protection and Limit Viremia

Eliciting a CD8+ T cell response will be an important component of a HIV-1 vaccine. CD8+ T cells can aid in providing protection from acquisition (248) and in controlling HIV-1 viremia by exerting antiviral pressure (63, 249). CD8+ T-cells inhibit HIV-1 replication by cytolysis of infected CD4+ T-cells and non-cytolytic suppression of virus replication [reviewed in (250)]. Studies from multiple groups suggest the molecules responsible for CD8+ T-cell mediated virus inhibition are likely to involve a profile of several cytokines (reviewed in (250)). Earlier efforts aimed at identifying phenotypic markers of CD8+ T-cells that can potently inhibit virus replication have had varied
results and rarely identify viable candidates (251-253). Studies in vaccinated monkeys show that memory CD8+ T-cells are crucial to protection from acquisition (254, 255) and in cases of breakthrough infection where vaccinated animals become infected (256). While these studies collectively represent an important step forward in the field of CD8+ T-cell inhibition of HIV-1, analyses of previous CD8+ T cell vaccine trials highlight the field’s current lack of understanding of the genes/mechanisms needed to control virus which inhibits vaccine design. The STEP study (257) aimed at inducing potent CD8 responses using an Ad5 vector expressing HIV Gag, Pol, and Nef. Results of this trial indicate that the induced T cell responses were weak and limited antigen specificity. Interestingly, when evaluated side-by-side against a Duke University cohort of patients with natural control of viremia (see Section 1.5.2), STEP trial vaccinees were shown to have less broad and weaker CD8+ T cell responses to HIV-1 (Dorrel et al, submitted). Importantly, these results brought into question the use of animal models to predict vaccine efficacy (258) and the predictive capacity of the widely accepted IFNγ ELISpot assay (259). Additionally, this trial highlighted the heterogeneity of the anti-HIV-1 T cell response among CD8+ T cell subsets and the need to find and characterize the “right” responses (260). A CD8+ T cell directed vaccine would work by introducing viral epitopes to CD8+ T cells through MHC class I, and possibly class II (see Section 1.3.3.4), complexes. In response to these epitopes, CD8+ T cells would exhibit cytolytic and non-cytolytic effector functions to inhibit virus replication. Regarding protection from
infection, localization of HIV/SIV-specific CD8+ T cells in sites of viral replication during early acute infection could aid in stopping dissemination of HIV-1 (261). Thus, studies aimed at understanding the cytokine profile and regulation of lytic and non-lytic capabilities of antigen specific CD8+T cells systemically and mucosally are central in gaining adequate understanding of the type of durable CD8+T-cell response that should be elicited via vaccination to control HIV-1 replication.

Assays used to assess cellular and molecular functions, must be carefully evaluated to ensure they measure a true surrogate of relevant in vivo activity. Conventionally, T cell directed therapies were evaluated by their ability to secrete IFNγ measured by enzyme-linked immunosorbent spot or intracellular cytokine staining assays (262). The Merck STEP trial highlighted the inability of these assays to adequately measure the CTL killing or noncytolytic suppression of HIV-1 in autologous CD4+ T cells or in cell lines in evaluation of therapeutic modalities needed to determine which CD8+ T cell responses actually control HIV-1 replication in vitro (260). Additionally, caution must be taken in how identification of “need to elicit” responses is undertaken. To determine what is needed for a potent bnAb response, researchers used a bnAb isolated from a patient and to infer the ontogeny of the response including the identification of the unmutated ancestor and intermediates. These sequences were used as templates for immunogen design in an effort to bring about these responses in vaccines. Similarly, for T cells directed therapies, the identification and characterization of cells with potent
inhibitory potential must first be carried out in patient populations which possess strong anti-HIV-1 CD8+ T cell mediated activity allowing for subsequent immunogen design and recapitulation in T cell directed therapies. Acutely infected individuals and long term non-progressor patients with strong T cell immunity are such populations, therefore, detailed analyses of functional properties of CD8+ T cells that contribute to initial viremic control during acute infection and long-term control in the absence of therapy will inform vaccine design.

1.5 Acutely Infected Subjects and Virus Controllers: Gold Standards of a Potent CD8 Response

1.5.1 CD8+ T cell Effector Function in Acute HIV-1 Infection

The CD8+ T cell response to HIV-1 arise early in acute infection and contributes to the viral resolution seen (63, 263, 264). These responses drive escape mutations in the transmitted/founder viruses (see Section 1.3.1) during acute infection, demonstrating that CD8+ T cells apply selective pressure on virus replication. Freel et al assessed longitudinally autologous and heterologous CD8+ T cell antiviral functions (total function and soluble function only) during acute infections in five persons (92). Results indicate there is rapid elicitation of autologous and heterologous HIV-1-specific CD8+ T cell mediate inhibition of transmitted founder viruses. While the response to autologous virus was durable out to 48 weeks, the ability to inhibit heterologous virus wanes by the time of resolution of viremia. Additionally, these experiments indicate CD8+ T cells that mediate soluble inhibition are specific to the same antigens as CD8+ T cell previously
shown to drive virus escape (265). These results are complemented by studies Ferrari et al which showed the earliest T cell responses are predominantly oligofunctional with the magnitude of T cell responses associating with selection of escape mutants (68). The observed contribution of CD8+ T cells producing MIP1β, a cytokine that can block virus entry but has not cytolytic function, to the CD8+ T cells response in the Ferrari paper agrees with the Freel et al result that noncytolytic mechanism exert immune pressure during acute infection.

1.5.2 Virus Controllers: Chronically Infected Patients Controlling Infection

1.5.2.1 Categorization of Controllers

The course of HIV-1 infection varies between individuals with some experiencing a rapid progression from acute infection to AIDS, while others remain asymptomatic. These non-progressing asymptomatic individuals represent 1-2% of the HIV-1 infected population and share the ability to naturally maintain control of virus load and/or HIV-1-specific immunity have been termed “long term non-progressors” (LTNPs) or “controllers. These individuals are subdivided by the viral load, CD4+ T cell counts, and time since diagnosis of HIV-1 infection (266, 267). LTNPs are therapy-naïve patients that have remained asymptomatic for a period greater than 10 years maintaining CD4 counts > 500 cells/μL (268, 269). Controllers have been diagnosed for less than 10 years and can be further subdivided (154, 166, 266). Virus controllers have been infected for at least 1 year and maintain viral loads below 5,000 copies/mL and CD4
counts above 385 cells/ μL (165-167, 270). Viremic controllers are ART-naïve the year preceding classification and maintain viral loads below 2,000 copies/mL (267). HIV-1 controllers are infected for more than five, years ART-naïve, and maintain viral loads below 500 copies/mL (266). Elite controllers have viral loads that are below the threshold of detection for current technologies and are ART-naïve for the year preceding classification (267, 271-273)

1.5.2.2 Genetic Correlates of Control in Virus Controllers

Genome-wide association studies of HIV controller patients have identified genetic correlates of non-progression (274, 275). Many of these correlates are polymorphisms localized to MHC class I alleles HLA-B57 and -B27. These alleles may recognize highly conserved epitopes in which mutation will cause significant loss of viral fitness (276). CD8+ T cells restricted to these alleles express less TIM3 indicating these cells are less prone to inhibition by regulatory T cells, likely contributing to positive clinical outcomes (277). Another MHC class I region correlated with control of HIV viral load is a single nucleotide polymorphism upstream of the HLA-C gene (reviewed in (278) which presents antigenic peptides to CTLs and can serve as ligands for NK cell function. Correlation of protection was also associated with some MHC class II alleles acting either independently or in combination with protective MHC class I alleles (279, 280). While these host functions influence control, not all controllers possess these alleles leaving some aspect of control unexplained.
1.5.2.3 CD8+ T cell Memory in Controlling HIV-1

Studies from Freel et al. and Killian et al. (166, 281) showed that CD8+ T-cells sorted on differentiation state (naïve, memory, and terminal effector) exhibit heterogeneous effector function. Results from Freel reveal that with the exception of naïve cells, all CD8+ T-cell differentiation stages are able to inhibit virus replication in CD4+ T-cells, as expected (166). When inhibition is measured on a per cell basis, central memory cells seemingly exhibit a higher level of activity although statistical significance was not achieved (166). Similar to Freel, Killian concluded that memory CD8+ T-cells are most important to virus inhibition. From phenotypic analysis of cells from controllers, Kilian found that controllers had an elevated level of transitional (intermediate of central and effector) memory CD8+ T-cells (CD45RA-CD27+PD-1+). When used in a functional assay, these cells had maximum inhibitory activity (281). These studies confirm the crucial role of memory CD8+ T-cells to inhibiting the replication of HIV-1 in CD4+ T-cells seen in controllers (153).

1.6 Understanding the Dynamics of Immune Effector Function Recall

Quick and timely control of pathogen requires a dynamic and temporally regulated immune response. To achieve a proper and efficient immune response, hundreds of genes must be coordinately turned “on” and “off”, which calls for multiple layers of gene regulation. This is especially important for regulating the expression of cytokines, molecules that function as immune effectors and immune system
communicators. Improper regulation of cytokines and their networks can lead to prolonged, inappropriately scaled inflammation and disease development (282, 283). Thus, being able to finely tune cytokine expression is critical.

1.6.1 Mechanisms that Regulate Gene Expression

Cells respond to changes in their environment through dynamic regulation of gene expression which includes a variety of mechanisms that result in an increase or decrease in the production of specific gene products. The central dogma of molecular biology once held that DNA is transcribed into RNA which is later translated to protein. However, more than seven decades of research has revealed that the DNA → RNA → protein path is an oversimplification of the flow of genetic information in a biological system. The pathway from DNA to final gene product(s) (whether RNA or protein) includes multiple steps, each of which can be regulated in response to cellular stimuli to increase an organism’s adaptability.

Eukaryotic chromatin consists of nucleosomes, subunits of DNA wrapped around histone proteins (284), packed to create the higher order structure most commonly associated with chromosomes. This higher order structure limits spatial access to DNA. To allow transcription factors and the transcription machinery access to DNA, nucleosome packing can be regulated by epigenetic mechanisms (285). The presence of epigenetic marks at gene loci can allow for DNA to be in an open confirmation allowing access to transcription activators and repressors, as well as the
transcriptional machinery leading to rapid induction of gene expression. Post transcription, primary RNA transcripts are processed to create mature mRNA. Once, mRNA leaves the nucleus, factors that impact cytoplasmic turnover and stability of mRNA plays a crucial role in mRNA fate. Translation of RNA can be altered by various mechanisms [reviewed in (286)] and the resultant protein can also be modified to carry out different functions in response to changing cellular environments and needs.

1.6.2 Effector Function Recall as a Result of Gene Regulation

The T cell arm of the adaptive immune response is characterized by phases with transcriptionally, phenotypically, and functionally distinct cells (287-289). T cells are derived from hematopoietic progenitor stem cells from the bone marrow that migrate to the thymus (290). In the thymus, these cells expand to a large population of thymocytes (122) which undergoes receptor recombination as well as positive and negative selection leading to deletion of 98% of thymocytes. The remaining 2% of cells leave the thymus as naïve cells. When activated by antigen presenting cells presenting its cognate antigen, naïve cells are brought out of their resting state and proliferate and differentiate as they transition into effector and memory cells (291-296). Effector cells mediate the destruction and clearance of pathogens by direct cytolysis and production of cytokines, small protein molecules that act as the messengers of the immune response, regulating cellular behavior and processes via interactions with plasma membrane receptors (285, 297, 298). Once antigen is cleared, there is a contraction of terminal effector cells leaving behind a
population of resting memory cells which monitors the host and is capable of starting an effector function recall, quickly and robustly producing cytokines upon pathogen re-encounter (299, 300), a phenomena known as effector function recall.

Previous research has shown that effector function recall is, in part, brought about by priming of T cells during the transition from naïve to memory cell. This transition is associated with extensive nuclear remodeling which includes the addition/subtraction of epigenetic marks at the loci of genes important to effector function (301-306). Opening of these loci allow the aforementioned “easy” access to these genes by the transcriptional machinery and for a more rapid and robust expression of gene products, including cytokine genes, in effector and memory cells than naïve (307-311). The role of epigenetic priming in CD8+ T cells in relation to anti-HIV-1 function has been characterized recently with specific focus on the involvement of histone acetylation (167, 168, 312). Because cellular mRNA levels are established by mRNA production and degradation, the involvement of post-transcriptional mechanisms that control mRNA degradation can prove to equal importance in dictating the immune response as the mechanisms that dictate transcription. Studies by other groups showed that while transcription is the primary contributor to RNA level (313), duration of rapid and transient responses (similar to those of cytokines) is determined at the post-transcriptional level (58, 313, 314).
1.6.3 mRNA Stability Influences Cytokine Expression

At the mRNA level, abundance is driven by two regulatory processes: transcription of new mRNAs and RNA decay of new and existing RNA (315). Mature mRNAs within the same cells have distinct stabilities with their half-lives ranging from minutes to days. By having mRNAs with various half-lives, cells can rapidly alter protein synthesis in response to its changing needs without need for transcription. A primary mediator of RNA half-life is the binding of the 3’ untranslated region of mRNAs by a subset of RNA-binding proteins (RBPs) which can act to stabilize or destabilize mRNAs, increasing or decreasing half-life, respectively. The importance of the 3’ UTR in cellular immunity was uncovered by Caput et al (316) who identified a highly conserved adenosine-uracil rich nucleotide sequence in the 3’ UTR of cytokines. These AU-rich elements (AREs) were later shown to be the major and most common regulators of mammalian RNA stability found in 5-10% of mRNA transcripts (317, 318) including many cytokine genes (319, 320). AREs can be bound by RBPs (321) (322), and microRNAs (miRNAs) (323) causing mRNA stabilization or destabilization. ARE binding proteins have been shown to destabilize several cytokines including those previously implicated as potential mediators of the anti-HIV-1 CD8 T cell mediated response (324-330). The 3’ UTR of IFN-γ can be bound and destabilized by the RBP tristetraprolin (TTP) in T cells stimulated with anti-CD3 (331). The RBP Roquin is known to regulate IFN-γ in CD8+ T cells (332), recognizing a specific structural element to drive
constitutive decay of target messages (333). Usually, cytokine mRNAs are degraded rapidly as their half-lives are shorter than 2 hours but (de)stabilization of cytokine mRNAs by AREs significantly impact cytokine mRNA half-life which can result changes in cellular function and the ability to limit disease (334). Although previous research indicates the involvement of post-transcriptional regulation in innate and adaptive immunity, the role of mRNA turnover was largely unexplored in memory CD8+ T cell response, specifically as it pertains to HIV-1. Exploring this phenomenon within the HIV-1 context would enable better understanding of CD8+ T cell effector function in VCs and aid the creation of assays to evaluate vaccine modalities.

1.7 Focus of this Work.

By studying the immune responses of virus controllers we can identify naturally occurring factors can be exploited with a vaccine. Over the years, research has shown that one crucial contribution to the strong immune response seen in virus controllers is the activity of CD8+ T cells (66-68). This dissertation builds significantly upon recently published work using the innovative combined strategies of CD8+ T-cell virus inhibition assays, flow cytometry (with cell sorting), and molecular techniques to define the genes and regulatory mechanisms that play a role in the CD8+ T-cell response of a virus controller cohort.

Several genes expressed in HIV-1 CD8+ T-cells have been identified as correlating or mediating HIV-1 inhibition in controllers. However, the regulation of gene
expression as well as the full repertoire of genes that mediate CD8+ T-cells’ HIV inhibitory activity are unknown. This gap limits the ability to harness the immunological protection provided by CD8+ T-cell mediated antiviral mechanisms. In chapter 2, we detail the development of a suite of assays to determine the presence, breadth, magnitude, MHC class restriction, and antigen specificity of the CD8+ T cell response. The efficacy of these tools was evaluated in 4 patient cohorts: HIV-1 seronegative control, chronic viremic, virus controllers, and elite controllers. We found that our assays stratified these patients accurately reflecting their clinical status and indicated both MHC class I and II restricted CD8+ T cell activity. We further probed the virus controller cohort with the same peptides used to determine antigen specificity of the CD8+ T cell function to interrogate the gene expression profile of cells with potent antiviral activity from 2 VCs. Validation of this profile was then carried out in the larger cohort. Efforts to identify an appropriate marker to sort cells activated with antigen stimulation to provide finer definition of the genes associated with control in our cohort were begun.

We previously found that the antiviral CD8+ T-cell mediated response is modulated by epigenetic mechanisms, providing the paradigm of the induction of effective CD8+ T-cell mediated inhibition of HIV-1 at the level of gene regulation. However, it is likely that a timely and robust CD8 immune response to HIV-1 requires the involvement of additional modes of gene regulation. It is possible, yet not
specifically shown that post-transcriptional regulation fills this gap directly impacting the expression of anti-viral genes and their functions. Implications of correlating both of these regulatory activities with in the CD8 function would include identification of new epigenetic- and RNA stability-bases assays to pinpoint genes involved in the anti-HIV-1 CD8 response. Once we identified the epitope specificity and began to build the gene expression profile of cells with potent CD8 function in VCs we followed up with studies to characterize the regulatory mechanisms at work in these antigen-specific cells. In Chapter 3, we measure the amount of newly transcribed RNA rate and RNA stability of the mRNAs for the genes identified in Chapter 2. Indeed, we revealed the role of both transcription and stability in modulating antigen-specific CD8+ T cell responses.

We summarize this work and its implications for future experimentation in Chapter 4. Discussion includes elucidation the mechanisms of the increased transcription and MHC class II restriction observed in chapter 2 as well as use of cells and RNA-seq technologies to further explore the milieu of antiviral genes regulated at the level of RNA stability.
Figure 1.1: The sum CD8+ T cell response to HIV-1.

The CD8+ T cell effector response to HIV-1 includes cytolytic killing of CD4+ T cells (blue) by CD8+ T cells (pink) via the interaction of T cell receptors and MHC class I on CD4+ T cells. In addition, CD8+ T cells can release soluble mediators that act to inhibit virus replication without causing lysis of the infected cell. Adapted from (250).
Figure 1.2: The HIV-1 treatment cascade in the United States.

Bars illustrate the sequential steps of HIV diagnosis, care with antiretroviral therapy (ART), and control of viremia. About 20% of those infected are unaware of their infection. Less than 40% of infected persons are retained in care with <25% controlling their viremia. A meaningful component of this cascade is due to non-adherence to therapy. Therefore, being able to understand and exploit mechanisms that allow for durable control of viremia is needed. Adapted from (335) with information from (336) and (337).
Chapter 2 Elucidation of the Antigen-Specificity and Cytokine Profile of Functional CD8+ T Cells in the Duke Virus Controller Cohort

2.1 Introduction

CD8+ T cells are heterogeneous in their abilities to mediate antiviral functions. The capacity of CD8+ T cells to limit HIV-1 infection is not limited to cytolytic killing but can also include non-cytolytic suppression of virus replication (146, 147). Cytotoxic T-cells (CTLs) recognize viral antigen presented in conjunction with the major histocompatibility complex class I (MHC Class I) molecule on the surface of antigen presenting cells (158). Antigen presentation to CTLs leads to lysis of the infected cell via the release of cytolytic proteins (159). The non-cytolytic response suppresses HIV-1 replication without causing lysis (146) and without requiring cell-to-cell contact. While β-chemokines have been shown to mediate the non-cytolytic response (338), it is clear that the nature of the CD8+ T cell antiviral activity is multifactorial, involving a profile of several cytokines (165, 338-344) that can act at gene expression as well as entry. Currently employed surrogate markers of T cell function rely heavily on the expression of IFNγ (262). As exemplified by the Merck STEP trial, IFNγ is not an accurate marker, likely due to IFNγ expression even in nonfunctional exhausted cells (294, 345). In order to identify which CD8 subsets are functional and to evaluate vaccine induced T cell responses, there needs to be tools to assess total CD8 function including cytolytic and noncytolytic responses (346).
Towards this end, our lab quantifies CD8+ T cell function with the use of virus inhibition assays. Our contact virus inhibition assay allows assessment of the sum CD8+ T cells function (cytolytic and non-cytolytic). For this assay, we co-cultured a known number of CD8+ and CD4+ T cells with a specified amount of HIV-1. To determine CD8+ T cell function, we measure the virus produced in the wells with CD8+ T cells and compare it to virus production in the absence of CD8+ T cells. Our soluble virus inhibition assay works using the same principle but only measures soluble, non-contact mediated activity as these assays are carried out in a trans-well system which allows for separation of CD8+ and CD4+ T cells. Separation of these cellular populations allows for exchange of soluble factors but not cell-to-cell contact, limiting any observed suppression from being contact-mediated.

The infidelity of HIV-1’s reverse transcriptase enzyme and the high transcription pace of HIV-1 replication has caused the generation of a variety of virus sub-types. While these sub-types are loosely divided by geographical regions, there are an increasing number of different subtypes being found in the same countries and of subtype recombinants being uncovered. This recombination of viral subtypes along with the current virus heterogeneity has dual implications for HIV-1 T cell directed vaccine design. First, the T cell response developed for induction by vaccination should show efficacy across clades. Secondly, candidate vaccines will have to be evaluated against various viral strains representative of different populations.
In this chapter, we develop and test a multi-clade HIV-1 panel to assess the breadth of CD+ T cell function in cohorts of HIV-1 seropositive subjects with emphasis on controllers. Once we determined these responses do indeed show breadth across clades, we began to investigate the specific features of CD8+ T cell antiviral functions in the Duke virus controller cohort. We found Gag p24 and Nef CD8+ T cell specific soluble inhibition was common among the VCs and correlated with substantial increases in the abundance of mRNAs encoding the antiviral cytokines MIP-1α, MIP-1αP (CCL3L1), MIP-1β, GMCSF, XCL1, TNFRSF9 and IFN-γ. Efforts were then initiated to sort cells activated with peptide stimulation to further probe these potent antiviral CD8+ T cells. In assessing the possible presence of MHC class II restricted CD8+ T cells in humans, we developed an antibody blocking virus inhibition assay which we used to assess the reliance of CD8+ anti-HIV function on MHC class II. Results indicate that many of our virus controllers possess some level of MHC class II restricted CD8+ T cell function.

2.2 Materials and Methods

**Patient cohorts.** (Table 2.1) Antiretroviral therapy (ART)-naive HIV-1-infected virus controllers (Tables 2.2.A and 2.2.B) (maintaining plasma HIV-1 loads of <5,000 RNA copies/ml and CD4+ lymphocyte counts of >400 cells/μl), one ART-experienced individual (VC15), were enrolled through the Infectious Diseases Clinic at the Duke University Medical Center. These patients were studied throughout this work. VC15 was previously on ART but naturally controlled his/her viremia (maintaining a VL of
<5,000 copies/ml and a CD4 count of >800 cells/µl) for two years post-therapy before being enrolled in our study. While in the study, VC15 had VLs of 1,590 to 2,950 copies/ml and CD4 counts of 721 to 801 cells/µl. VC27 maintained VLs of <100 to 2,690 copies/ml with the exception of two draw dates on which his/her VLs were 5,190 and 5,360 copies/ml. **Details of other VC patient exceptions.** Seven VCs were HLA typed, two of these patients had alleles known to be associated with CD8⁺ T cell control. **Six patients** (VC28, VC29, and VC30) maintained viral loads below 48 copies/ml (infected for >6 years, with viral loads measured for >8 months) and were categorized as **elite controllers.** **Chronic viremics** were recruited in North Carolina, U.S.A. as part of the CHAVI001 cohort. **Healthy uninfected donors** were recruited through the Duke Virologic Basis for Specific Immune Defects in AIDS program (kindly provided by Kent Weinhold, Department of Surgery, and Duke University). The studies were reviewed and approved by the Duke University Medical Center Institutional Review Board, and all participants provided written informed consent.

**PBMC isolation and cellular subset separation.** Peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation [adapted from (347, 348)]. Isolated PBMCs were then frozen in a 90% fetal bovine serum (FBS) 10% dimethyl sulfoxide freezing media and stored at −180°C until thawed for analysis. For analysis, cells were thawed in a 37°C water bath and washed twice with pre-warmed RPMI 1640 media supplemented with 10% heat-inactivated FBS and penicillin/ streptomycin then
examined for recovery and viability. Cells were then activated by OKT3 and anti-CD28 antibodies for 3 in RPMI 1640 supplemented with 20% FBS, penicillin-streptomycin, and 20 U/ml recombinant human interleukin-2 (IL-2) or treated otherwise according to specific assay protocols (see below). T cells activated on day of thaw were separated into CD8+ and CD8− subsets using a CD8+ T cell isolation kit and CD8+ depletion beads, respectively on day 3 of activation.

**Transmitted/ Founder virus.** Full-length infectious molecular clones (IMC) expressing the Transmitted/Founder (T/F) virus sequences inferred from CHAVI 001 subjects infected with clades B, C, and A/E HIV-1 viruses. The T/F sequence derivation for and construction of the clade C IMC were conducted as previously described (349, 350). Replication-competent virus stocks were generated as described previously (166, 270, 349, 350). Briefly, proviral DNA was transfected into 293T cells using Fugene HD (Roche). Working stocks were amplified by passaging virus in human PBMC from seronegative donor pools (American Red Cross). PBMC-derived virus supernatants were collected every 2 to 3 days for up to 16 days and filtered through a 0.45-μm syringe filter. Viral titers were determined on TZM-bl cells (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from John C. Kappes, Xiaoyun Wu, and Tranzyme, Inc.).
**Virus Inhibition Assays.** The virus inhibition assay (VIA) quantifies CD8+ T-cells’ ability to control viral replication in target T-cells by co-culturing CD8+ T-cells, CD4+ target T-cells, and a known amount of HIV-1 (165, 167, 270).

**Contact-mediated Virus Inhibition Assay.** CD4+-enriched cells were infected with a full length transmitted/ founder virus for 2 h in a 50 mL conical tube at 1200 x g or regular infection. HIV-1 infected CD4+ cells were plated with autologous CD8+ T cells at varying effector to target ratios or infected CD4+ T cells were cultured alone. 3, 7, and 10 days post-infection, the cellular supernatants were collected and applied to TZM-bl cells in the presence of DEAE Dextran. TZM-bl cells are a HIV-susceptible and permissive indicator cell line that offers increased sensitivity due to high expression of CD4 and co-receptors used by HIV-1. These cells are stably transduced with a HIV-1 LTR driven firefly luciferase gene, allowing for luciferase production when cells become infected with HIV (351). After 48 hours, TZM-bl cells were lysed and the firefly luciferase content of the lysate quantified with the Luciferase Assay System (Promega) on a Centro LB luminometer (Berthold). The level of HIV replication in the co-cultures containing CD8+ T cells and infected CD4+ T cells was expressed as the percent of the HIV replication in CD4+ T-lymphocytes alone.

**Antigen-specific soluble virus inhibition assay (sVIA).** HIV-1-specific soluble VIAs with primary CD8+ T cells were performed as previously described (92, 270). Briefly, twelve pools of HIV-1 PTE (Potential T cell Epitopes; NIH AIDS Reagent Program,
Division of AIDS, NIAID, NIH) (352) peptides representing the following HIV-1 regions: Env1 4 to 296 (gp120), Env2 297 to 488 (gp120), Env3 489 to 602 (gp41), Env4 603 to 840 (gp41); Gag1 1 to 128 (p17), Gag2 131 to 361 (p24), Gag3 362 to 486 (p17); Pol1 1 to 152 (protease), Pol2 156 to 447 (p51), Pol3 452 to 709 (p51 + p15), Pol4 711 to 988 (p31); Nef 1 to 193 or HIV clade B consensus peptides were used (0.2 μg/ml) to stimulate isolated activated or unactivated CD8+ T cells (unless otherwise noted) patients for 5.5 hours. Post stimulation, these CD8+ cells were placed in the upper chambers of a 96-well trans-well plate. The bottom chamber contained TZM-bl cells, DEAE-dextran, and a transmitted/ founder virus which was added at the same time as CD8+ T cells. After 48 hours, TZM-bl cells were lysed and luciferase content measured. Virus inhibition was calculated as the log reduction in relative light units (RLU; luciferase) of wells with CD8+ T cells compared to control wells without CD8+ T cell effectors. The cutoff for significant virus inhibition (>0.39 log reduction) was determined using sero-negative control subjects.

**Antibody blocking virus inhibition assay.** CD4+ T cells are infected via spinoculation with T/F virus (1200xg, room temperature, 2 hours) then incubated with monoclonal antibodies targeting MHC Class I or MHC Class II molecules for 30 minutes. These infected cells are then cultured with autologous CD8+ T cells. Virus production in these wells is compared to production in the absence of antibodies and/ or CD8+ T cells and the presence of a negative control antibody. Blocking of CD8+ virus inhibition was
calculated as the increase in virus production compared to control wells without antibodies or compared to the isotype negative control.

**Measurement of gene expression via Illumina.** To determine the differences in RNA expression of CD8 cells stimulated with HIV peptide vs. unstimulated cells, we used the Illumina HumanHT-12 v4.0 Expression BeadChip. The BeadChip supports human genome-wide transcriptional coverage. The BeadChip array targets more than 47,000 probes corresponding to characterized genes, gene candidates, and splice variants from NCBI RefSeq Release 38 (November 7, 2009) and UniGene content. Of the targets 43,270 were coding and 3961 were non-coding. For this experiment, CD8+ T cells were isolated from PBMCs of 2 virus controllers (VC20 and VC28) via negative depletion. After an overnight incubation in RPMI10 culture media (10% FBS + penicillin-streptomycin), cells were stimulated with HIV peptide (Gag or Nef) + co-stimulatory antibodies for 5.5 hours. RNA was then isolated from multiple time-points after stimulation (0, 12, 24, 36, 48 hours) for VCs. Unstimulated and 48 hour Gag stimulated samples from a seronegative patient were used as an additional negative control. Total RNA was quantified by NanoDrop 8000 spectrophotometer (Thermo Scientific). The RNA 260/280 values for samples ranged from 2.13 to 3.38. RNA was used to generate biotin-labeled cDNAs which were loaded onto the same BeadChip to avoid batch effects (Duke University Institute of Genome Sciences & Policy).
**Analysis of Illumina data.** Lower-level analysis of differential gene expression via comparison of probe intensity from microarray data was performed using the lumi package of BioConductor, an open source R based project (353, 354). Before comparison of samples was made, the data was pre-processed. Pre-processing transforms the raw fluorescence signal from the microarray into a normalized signal. Pre-processing starts with variance stabilizing transformation (355) which stabilized the data’s variance, making it independent of the mean which is necessary given the complicated error structure of the data. The data is then quantile normalized via multiple algorithms which make data from all samples identical in statistical properties. Post-normalization, transcript probes with low intensities were non-differentially filtered to remove probes with intensity below detection, leaving 20,661 probes. The nucleotide universal identifier annotation package is then applied to annotate each probe (354) and changes in gene expression between control and experimental samples are quantified.

**Gene Set Enrichment Analysis.** Post-processing, Gene set enrichment analysis (GSEA) of GO biological processes was performed as previously (356). GSEA uses statistics and changes in gene expression compared to a control to identify biological pathways impacted by experimental conditions. The log2 value of the probe intensity of the 20661 genes with detectable expression was calculated and rank order listed from the most up-regulated to the most down-regulated. The list of genes was then used to run GSEA, allowing us to obtain the identity of functionally related genes whose
expression changed in a coordinated manner. These changes were compared across samples to compile a list of differentially expressed pathways based on a GSEA permutation familywise error rate (FWER P) value <0.5. Gene pathways are defined by Gene Ontology (http://geneontology.org/), the Molecular Signatures Database (http://www.broadinstitute.org/gsea/), the Reactome Pathway Database (http://www.reactome.org/), BioCarta (http://www.biocarta.com/), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway data-bases (http://www.genome.jp/kegg/).

**Measurement of mRNA abundance.** CD8+ T cells were stimulated with select PTE peptide pools (Gag p24, Gag p17, or Nef) as described above then harvested in 1 ml of Trizol and RNA extracted following manufacturer’s protocols (Life Technologies). RNA was DNase (Ambion) treated after initial RNA extraction from cells then reverse transcribed into cDNA using iScript kit (Bio-Rad). Real time quantitative PCR was performed using gene-specific primers (Appendix C). The resultant values were normalized for relative amount of RNA input based on the amounts of the housekeeping gene GAPDH.

**Multi-parameter cytokine-staining assay.** Flow cytometric analyses of HIV-1 specific CD8+ T cells were performed as previously described (68, 92, 166). For cytokine expression in peptide stimulated cells, PBMCs were stimulated with the PTE peptide pools as described above for 5.5 hours. Stimulation with 0.2 μg/ml SEB also for 5.5 hours
was used as a positive control. The titer of each antibody was determined to obtain the saturating concentration used for the final staining. Stimulations were conducted in the presence of 0.5 µg/ml anti-CD107a phycoerythrin (PE)-Cy5 (clone H4A3; eBioscience), 5 µg/ml brefeldin A (Sigma), and 4ul/6mL Golgi Stop(BD) for 5.5 h at 37°C in 5% CO2. After washing, the cells were stained with a viability indicator (LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit; Molecular Probes®) in phosphate-buffered saline for 20 min at room temperature. Cells were then washed and stained for 20 min at 4°C with a surface stain cocktail containing anti-CD3–APC-H7 (clone SK7; BD Biosciences), anti-CD4-BV605 (clone RPA-T4; BD Horizon™), anti-CD8-PacBlue (clone RPA-T8; BD Pharmingen™), anti-CD27-Cy7-PE (clone M-T271; BD Pharmingen™), anti-CD45RO-ECD (clone UCHL1; Beckman Coulter), and anti-CCR7- Alexa Fluor® 700 (clone 150503; BD Pharmingen™). PBMC were subsequently washed twice, and then fixed and permeabilized with Fixation/Permeabilization solution (BD Cytofix/Cytoperm™) for 20 min at 4°C. After incubation the cells were washed twice in Perm/Wash buffer (BD Perm/Wash™) diluted 1:10 with distilled water. Cells were then stained with anti-IFN-γ-BV650™ (4S.B3; Biolegend®), anti-IL-2–APC (clone MQ1-17H12; BD Pharmingen™), anti-MIP-1α-Fluorescein (clone 93342; R&D Systems®), anti-MIP-1β-PE (clone D21-1351; BD Pharmingen™), and anti-TNFα-PerCP-Cyanine5.5 (clone MAb11; eBioscience®) for 45 minutes at 4°C. After washing and fixation, all samples were acquired on a custom-made LSRII (BD Bioscience, San Jose, CA) within the next 24 h.
Gates were set to include singlet events, lymphocytes, live CD3+ cells, CD4+, and CD8+ subsets. From the total CD4+ and CD8+ populations, the naïve subset was identified as CD45RO− CD27+. This subset was excluded from the subsequent analysis, including only the memory population. Antigen-specific populations were identified within the memory population as single-function cells shown in the sequential single-/cytokine-/chemokine/-degranulation gates. Responses were considered positive if the percentage of antigen-specific cells was 3-fold above the background and greater than 0.05% after background subtraction. Data analysis was performed using FlowJo 9.6.4 software (TreeStar).

**Statistical analyses.** Statistical analyses were performed using GraphPad Prism (GraphPad Software) and SAS v9.3 (SAS Institute). Correlations between cytokine expression level and virus inhibition were calculated using the Spearman’s rank correlation co-efficient (GraphPad Software). Appropriate SAS PROC tests were used to calculate raw p-values using Wilcoxon Exact Tests and for controlling the FDR [False Discovery Rate] using the Benjamini & Hochberg method (357).

**2.3 Results**

Virus inhibition assay reveals that CD8+ T cells from virus controllers are capable of potent and broad *in vivo* CD8+ T cell inhibitory responses.

We first assessed the potency and breadth of virus inhibition among the VCs in our cohort using contact-mediated virus inhibition assay against a panel of viruses.
Results indicate that while magnitude of activity is varied, patients in the Duke VC cohort possess the ability to potently suppress replication of lab-adapted (NL4-3) and full length (WITO, WEAU3, CH040.c, CH058, and CH077) virus strains in autologous primary CD4+ T cells with an observed average of a 23-fold reduction in virus replication (compared to control wells without CD8+ T cells) (Figure 2.1). An ideal T cell-directed therapy would provide efficacious control of multiple viruses to be globally relevant and address the diversity of virus strains. Unpublished work by Dorrell et al indicates a breadth of CD8+ T cell responses in some of the Duke VCs with indication of clade- matched responses having the greatest magnitude. This breadth among VCs could be due to cross reactivity of CD8+ T cells in VCs or conservation of epitopes CD8+ T cells from VCs recognize. Determining which of these hypotheses is true would require finer definition of epitope specificity of these CD8+ T cells for different clades. We have begun to undertake this task with clade B matched CH040.c virus as detailed in the next section. We are currently completing studies to assess CD8+ T cell mediated antiviral activity using a panel of full length infectious molecular HIV-1 clones representing clades B, C, and A/E (Payne et al, in preparation). This would allow for further comparison of clade-specific and cross-clade inhibitory activity among the virus controllers.

**sVIA can distinguish chronically infected cohorts and reveal a prevalence of Gag p24- and Nef-specific CD8+ T cell anti-HIV-1 activity in virus controllers.**
To begin to evaluate the antigen-specificity of CD8+ T cells that inhibit HIV-1 via soluble mechanisms and assess if we could also distinguish our cohorts by their non-cytolytic function we used an HIV-1 specific transwell soluble virus inhibition assay (sVIA) (92) to determine the presence and magnitude of non-cytolytic function among clade B patients via sVIA. For these experiments, we use Potential T cell Epitopes (PTE) (352) peptides to stimulate CD8+ T cells. PTE peptides are overlapping fifteen-mers derived from circulating viruses and represent 85% of worldwide virus sequences (92). These peptides are pooled based on frequency of recognition and sequence so that all peptides in a pool align to the same region of the HIV genome. Stimulation with pools of HIV-1 PTE peptides can cause the secretion of factors that act to control viral replication, replicating activity seen in vivo (358). Primary CD8+ T cells from patients were stimulated with Env, Nef, Pol, or Gag HIV-1 PTE peptide pools for 5.5 hours, then tested for their ability to inhibit a R5 tropic clade B founder virus, CH040.c (350, 359) (Figure 2.2). In eleven of twelve controller patients, antigen specific stimulation of CD8+ T cells mediated soluble inhibition of HIV-1 replication (Tables 2.C). The most common epitope specificities of cells that mediated soluble virus inhibition were Gag p24 (Gag2) (nine out of twelve controller patients) and Nef (seven out of twelve controller patients). The second most common CD8+ T cell specificities that inhibited virus replication of controllers were to Gag p17 (Gag1) and protease (Pol1) (four out of twelve controller patients each). Almost half of those with ART suppressed viremia also have Gag2
specific responses. A prevalence of Gag p24 and Nef is not seen in the chronic viremic patients. Non-stimulated JR-HVS cells, a virus controller cell line with potent inhibition (360), was used as positive controls for inhibition. CD8+ T-cells from HIV-1 seronegative donors were used as negative controls given that CD8+ T-cell mediated inhibition is a hallmark of those previously exposed to HIV-1 (167).

Identification of differentially expressed genes antiviral genes in Gag p24 and Nef stimulated cells in 2 Virus Controllers

To determine which genes are differentially expressed in the cellular populations with and without inhibitory activity, RNA was isolated from CD8+ T-cells from two virus controllers with strong virus inhibition and one seronegative donor after a 5.5 hour incubation with PTE peptide pools. RNA was analyzed using the Illumina Human BeadChip platform (Duke Center for Human Genome Variation). There were 12 total samples analyzed – 5 from VC20 and VC28 (0 hour Un-stimulated and 48 hours Un-stimulated, Gag, Nef, and Scramble stimulation) and 2 from a seronegative patient (48 hours unstimulated and Gag stimulated. Analysis of probe intensity revealed that samples from the seronegative patient were not properly detected so they were therefore excluded from further analysis. Analysis of data by Duke Center for Human Genome Variation and Vaccine & Gene Therapy Institute of Florida revealed that multiple genes and pathways were differentially expressed in CD8+ T-cells that were able to inhibit virus replication compared to those that cannot. To guide the analysis on genes that
were likely involved in the CD8+ response, I focused further analysis on genes with immunological or unknown function with a special focus on cytokines. Cytokines are small protein molecules that act as the messengers of the immune response, regulating cellular behavior and processes via interactions with plasma membrane receptors (338, 361, 362). These interactions lead to the activation of intracellular signal transduction cascades (285). Given the small sample size and chosen time-points, the changes in expression were small with a maximum change of 60%. To add another level of power to the analysis, I compared the list of most likely candidate genes from our list to published data sets (Table 2.4) focusing on genes that were reoccurring through multiple studies (165, 167, 252, 253, 256, 363), had a greater than 20% change in our studies, and a function that was unknown or could be related to CD8+ activity. Interestingly, when I compared the identified genes of interest from our study to published data sets from other CD8+ T-cell effector function experiments, the most commonly reoccurring gene was TNFRSF9 (Table 2.4). In our studies TNFRSF9 was found to increase in expression 20% in cells with antigen-specific inhibition compared to autologous cells without. The differential expression of TNFRSF9 is of interest not only because of its known role in CD8+ function but also its role in enhancing apoptosis, a cellular function highlighted in recent literature from other labs for its link to the T-cell response to HIV-1 (364). MIP-1α and its isoform MIP-1αP, the second most reoccurring genes (also
increasing about 20%), belong to a family of cytokines known as β-chemokines which are known to block HIV-1 entry (16, 165, 338, 341).

**HIV-1 antigen specific CD8+ T cells have increased expression of select cytokines that correlate with virus inhibition.**

To validate the results of the microarray screen from VC20 and VC28, we assessed expression of a panel of cytokines in the larger Duke VC cohort (Table 2.2.B). This panel included effector molecule targets identified from the screen (TNFRSF9, MIP-1α, and MIP-1αP), genes our lab has previously identified as correlating with CD8 function (MIP-1β, IFN-γ, and GM-CSF), a recently characterized lymphokine that can block HIV-1 entry (XCL1) (365), and a β-chemokine that did not correlate with CD8 function in our cohort previously as a control (RANTES). We and others have shown that IFN-γ and the β-chemokines MIP-1α and MIP-1β were associated with CD8+ T cell inhibition of HIV-1 (166, 251-253, 338, 361, 366). MIP-1α, MIP-1αP, and MIP-1β bind CCR5 and block the entry of R5-tropic viruses (including transmitted/founder viruses) in CD4+ T cells (16, 338, 367). The gene copy number of MIP-1αP varies greatly among the population and was linked to HIV-1 infection and disease progression (368). XCL1 is a recently characterized CD8+ T cell derived anti-HIV chemokine which works to block HIV-1 attachment via direct interaction with gp120 (365). The interaction between TNFRSF9 and its ligand, TNFRSF9, is known to enhance the proliferation and activity of CD8+ T cells (369) while GMCSF can also influence the T cell response (370). Additional
motivation for including RANTES and GM-CSF in the panel came from their known regulation by post-transcriptional mechanisms that would be crucial for later experiments (see section 3.3). To determine whether these cytokines are up-regulated in CD8+ T cells from VCs with antigen-specific antiviral activity, we first measured mRNA cytokine levels, in three independent experiments, in primary CD8+ T cells from a virus controller patient, VC30 who possess mid-range Gag p24 specific inhibition. After a 5.5 hour Gag p24-peptide stimulation, the cytokine expression in Gag p24 stimulated cells was compared to un-stimulated autologous cells. p24 stimulated CD8+ T cells had an increase in mRNA for IFN-γ, MIP-1α, MIP-1αP, MIP-1β, TNFRSF9, XCL1, and GMCSF (Figure 2.3). In contrast, no change was seen in mRNA expression of RANTES, another β-chemokine with HIV-1 suppressive activity (16, 33).

We next measured levels of mRNA expression of the aforementioned cytokines, the CXCR4 interacting cytokines macrophage-derived chemokine (MDC) (371), and thymus and activation regulated chemokine (TARC) (372) in un-stimulated and 5.5 hour stimulated (Gag p24, Nef, or Gag p17) CD8+ T cells from ten additional VC patients. For the Gag p24 experiments, we divided the patients into two groups - those with p24-specific soluble virus inhibition (VC11, -23, -26, -27, -28, and -29; n=6) and those without (3 HIV-1 seronegative patients, VC16, and -24; n=5) (Table 2.3.A). Amounts of mRNA in p24-stimulated CD8+ T cells from VCs without p24-specific inhibition and seronegative patients were largely unchanged (fold-change values close to 1) compared to un-
stimulated cells (Figure 2.4). However, levels of mRNA that encode IFN-γ, MIP-1α, MIP-1αP, MIP-1β, GM-CSF, TNFRSF9 and XCL1 increased in p24-stimulated CD8+ T cells from VCs with p24-specific inhibition compared to un-stimulated autologous cells. When comparing the fold-increases in cells from patients with p24 specific inhibition to patients without p24 specific inhibition, the increases in IFN-γ, MIP-1α, MIP-1αP, MIP-1β, and GM-CSF are statistically significantly (p<0.05, Wilcoxon Exact Test, controlled for false discovery rate using Benjamini & Hochberg (357)) higher in those with antigen specific inhibition. CD8+ T cells from VCs with p24-specific inhibition had increased mRNA levels for at least three of the cytokines, indicating some degree of poly-functionality in their responses. We next compared Nef stimulated CD8+ T cells from patients with Nef-specific inhibition (VC26, -27, -29, and -16; n=3) and those without (3 HIV-1 seronegative patients, VC11, -23, -28, and -24; n=7). Nef-stimulated cells had an up-regulation in mRNA expression for the queried genes, similar to p24-stimulated cells (Figure 2.4). Although these values did not reach statistical significance when correcting for multiple comparisons, however some of the un-adjusted p-values did reach significance (Appendix E). These changes were specific as no change was observed in mRNA levels of RANTES in any of the subject cohorts or stimulation conditions, consistent with our previous report that RANTES did not correlate with virus inhibition (92). Levels of MDC and TARC were also unchanged (data not shown) and serve as additional negative controls. Because Gag p17 (Gag3)-specific inhibition was not
common among the VCs (Table 2.3.A and 2.3.B) we compared abundance of cytokine mRNA in p24- and Nef-stimulated CD8+ T cells to autologous Gag p17-stimulated cells to determine if the observed changes in gene expression were global responses to antigen stimulation or were antigen specific. The fold-changes in mRNA abundance observed when p24- and Nef-stimulated cells were compared to p17-stimulated cells (Figures 2.4 and 2.5, bottom panels) mirrored that of comparison to un-stimulated indicating that the up-regulation of gene expression is antigen-specific. The increased expression of IFN-γ, MIP-1α, MIP-1αP, MIP-1β, and GM-CSF were statistically significant in p24- compared to p17-stimulated CD8+ T cells (p<0.05, Wilcoxon Exact Test, controlled for false discovery rate using Benjamini & Hochberg (357)), however, the observed increased abundance in Nef-stimulated samples did not reach statistical significance.

**CD107 as a phenotypic marker of cell activation.**

Using bulk CD8+ T cell populations, we were able to identify genes differentially expressed in antigen-specific CD8+ T cells with and without anti-HIV-1 function. While our approach of comparative analysis of cells from the same patients allowed more accurate assessment than previously used differential gene expression approaches (251-253), it is possible that our results were diluted by the presence of cells not activated by peptide stimulation. To allow finer definition of differential gene expression, we aimed to identify a marker to use to allow isolation of cells activated by peptide stimulation.
We have previously shown antiviral activity is strongly associated with increased numbers of memory CD8^+ T cells expressing poly-functional markers CD107a, IFN-γ, TNF-α, IL2 and MIP-1β (166). Here, we wanted to determine whether cell surface staining with these markers was associated with CD8^+ T cells that mediate virus inhibition which would allow use of associated marker(s) as a surrogate for cell activation. We measured the expression of the cytokines in memory CD8^+ T cell subset of virus controllers with and without p24-specific inhibition by multi-parameter flow cytometry which is represented by the data from VC23 and VC24 in Figure 2.6A.

The percentage of CD8^+ T cells from patient VC23 (Gag p24 specific inhibition) expressing MIP-1β compared to un-stimulated increased from 2.7% to 4.7% of memory cells (Figure 2.6A) while the expression of CD107 increased from 0.5% to 2.8%. These changes were not seen in CD8^+ T cells without soluble virus inhibition (Figure 2.6A, VC24). This trend continued with 2 other VCs (data not shown), thus, MIP-1β and CD107 associate with memory CD8^+ T cell antigen-specific soluble inhibition of HIV-1 in the Duke VC cohort and could potentially serve as a marker of T cell activation. TNFRSF9 which was identified as being differentially expressed in our microarray (Table 2.4) and screen of VCs (Figures 2.3, 2.4, and 2.5) is commonly used an early marker of T cell activation (373). Because many of our assays quantify the expression of MIP-1β, we wanted to exclude its use as a phenotypic marker for cellular isolation and decided to compare the utility of CD107 and TNFRSF9 as early T cell activation markers
in our virus controllers (Figure 2.6B). Preliminary results indicate that expression of CD107 better identifies CD8+ T cells in our cohort activated by antigen stimulation.

**Major Histocompatibility Complex (MHC) Class II restriction of CD8+ T cells with anti-HIV-1 function.**

T cells can inhibit HIV-1 replication via the engagement of T cell receptors with major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs). It has been long thought that CD8+ T cells recognize antigens presented by MHC class I molecules while CD4+T cells are MHC class II restricted. This school of thought was recently challenged by non-human primate studies that found that vaccine induced CD8+ T cell responses that protected from SIV acquisition included MHC class II restricted CD8+ T cell activity. These results by Picker et al have opened the door to the possibility of non-canonical MHC Class II restricted CD8+ T cells with anti-HIV-1 activity in humans. We sought to determine if this activity is present in our VCs by developing a novel MHC blocking CD8+ virus inhibition assay adapted from our previously described virus inhibition assays. In the MHC blocking VIA, CD4+ T cells are infected then incubated with monoclonal antibodies targeting MHC Class I or II molecules for 30 minute. These infected cells are then cultured with autologous CD8+ T cells for 48 hours then virus production in these wells is compared to production in the absence of antibodies and/or CD8+ T cells. We found that the CD8+ T cells from our virus controllers are able to potently inhibit HIV-1 replication in autologous CD4+ T cells
in a dose-dependent manner (Figure 2.7). When MHC class I antibodies are incubated with infected CD4+ T cells before introduction of autologous CD8+ T cells, there is an increase in HIV-1 replication (measured as relative light units (RLUs)) compared to replication in the absence of Class I antibodies (Figure 2.8). This result indicates that, as expected, the anti-HIV-1 activity of CD8+ T cells is restricted, at least in part, to MHC Class I. When antibody to class I is added and becomes bound to class I molecules on APCs, blocking class I presentation there is a reversion of inhibition (indicated by the increase in virus production). Interestingly, the same is seen to varying extent with the addition of MHC class II antibodies to the co-culture of some of the VCs (Figure 2.8).

2.4 Discussion

The sequence diversity presented by different HIV-1 subtypes is important in developing a vaccine for HIV-1. Any HIV-1 prevention strategy should be effective against most, if not all, virus strains to allow for efficacious control of virus spread. Previous research has shown that an immune response triggered by one strain of HIV may not protect against all other strains (374-376), therefore, the ability to control virus replication across clades should be assessed for newly developed prevention or therapeutic strategies. We report here that the CD8+ T cell function found in the Duke virus controller cohort is effective in stymieing virus replication using a multi-clade panel of viruses in our contact VIA. The breadth of inhibition, indicated by CD8+ T cell inhibition of non-clade B viruses, was greater in VCs than non-VCs. This could be due to
cross reactivity of CD8+ T cells in VCs or conservation of epitopes CD8+ T cells from VCs recognize. If this breadth was in fact due to conservation of epitope, epitope-specific assessment of CD8 function would reveal a conservation of specificity for certain HIV-1 proteins across virus clades assessed. While, efforts to assess specificity in a contact VIA are still being optimized, we were able to modify the sVIA to allow assessment of antigen specificity. Utilizing our sVIA, we uncovered that the Duke cohort of virus controllers has substantial Gag p24- and Nef-specific CD8+ T cell mediated non-cytolytic anti-HIV-1 responses to a clade B virus. Determining the antigen specificity of the CD8 response to other viral clades in contact mediated and soluble inhibition will help inform the causes of viral breadth in the Duke virus controller cohort.

Our findings that Gag p24 and Nef-specific CD8+ T cells are most associated with HIV-1 inhibition agree with previous research (377-381). We previously demonstrated that Gag- and Nef-dominant soluble activity mediated by CD8+ T cells during acute HIV-1 infection corresponded with breadth of virus inhibition as well as immune pressure against transmitted founder viruses, but that this activity was diminished by 6 months post-infection in the patients examined (92). Others also reported early Gag and Nef CD8+ T cell antiviral activity in acute infection (382, 383). Walker et al recently published that ¼ of the epitope-specific responses that most correlate to HIV-1 control are specific to p24 (384). Nef-specific responses were also detected. This epitope specific targeting mediated the link between HLA and control of virus. The observance of the
similarity of soluble responses in acute patients and VCs suggests the soluble antiviral activity of CD8+ T cells that broadly develop during acute infection may be maintained in virus controller patients. This evidence complements an earlier report that long-term non-progressors maintain functional cytotoxic CD8+ T cells that are lost in progressors (273) and indicates that further longitudinal studies to investigate soluble CD8+ T cell response retention are warranted. Notably, we also found that Pol specific CD8+ T cells from some virus controllers mediated antigen specific virus inhibition. Borthwick et al. recently reported that an HIV conserved immunogen vaccine (prime-boost) strategy induced CD8+ T cell virus inhibition that correlated with both Gag and Pol CD8+ T cells (385).

Differential gene expression analysis is a powerful tool for detecting differences in cellular phenotypes. However, recent efforts that used this approach by comparing genes between different people to identify genes that regulate the HIV-1 inhibitory response capacities of CD8+ T-cells have had varied results and rarely identify viable candidates (251-253). This lack of concordance makes it clear that currently employed methods of analyzing the genetic profile of different individuals (with different genetic backgrounds) are inadequate. Given this, we opted to evaluate gene expression in cellular subsets without or without inhibitory function brought about by Gag p24 or Nef peptide stimulation. Using this technique, the “noise” that is associated with comparative gene analysis of people with different genetic backgrounds seen in
previous studies will become negligible allowing confidence that any observed changes are not artifacts of genetic differences. We found that Gag p24 and Nef CD8+ T cell specific soluble inhibition was common among the VCs and was associated with increased mRNA encoding macrophage inflammatory proteins (MIP-1α, MIP-1αP, MIP-1β), interferon gamma (IFN-γ), lymphotactin (XCL1), tumor necrosis factor receptor superfamily, member 9 (TNFRSF9), and granulocyte-macrophage colony-stimulating factor (GM-CSF). Several of these cytokines, such as the β-chemokines (MIP-1α, MIP-1αP, MIP-1β) and XCL1 could play pivotal roles in CD8+ T cells’ ability to inhibit virus at entry.

During our studies of the phenotypic and functional characteristics of CD8+ T cells that mediate inhibition of HIV-1 replication, reports from Picker et al indicated that the presence of non-canonical MHC class II may contribute to very potent CD8+ inhibition of SIV, implicating that class II restricted CD8+ T cells may have reverberations in controlling HIV-1 infection. To study if this phenomena could also be detected in humans we first measured the suppressive capacity from CD8+ T cells from controllers in a VIA (Figure 2.7). As expected, we observed dose-dependent inhibition of HIV-1 replication by CD8+ T cells in autologous CD4+ T cells. We then determined if the CD8 function measured in our assays was MHC class II restricted. To this end, we modified our contact VIA to quantify the contribution of MHC class I and II to CD8 function. These assays revealed that surprisingly, addition of MHC class I or II
antibodies led to a reversion of CD8+ T cell mediated inhibition in many of the VCs tested Figure 2.8. These data suggest that CD8+T function in many VCs in the Duke cohort can be blocked by MHC class I and II antibodies. Linking this restriction to CTL function and uncovering the cause of the observed restriction are the aims of current experiments.
### Table 2.A: Average and Range of Clinical Characteristics of HIV+ Patient Cohorts

<table>
<thead>
<tr>
<th>Patient Cohorts</th>
<th>Virus Load</th>
<th>CD4+ T cell count</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chronic viremic patients</strong></td>
<td>138 851 (42075-150 408)</td>
<td>215 (71-281)</td>
</tr>
<tr>
<td><strong>ART-suppressed HIV+</strong></td>
<td>86 (&lt;48-59)</td>
<td>865 (680-974)</td>
</tr>
<tr>
<td><strong>Virus controllers</strong></td>
<td>1781 (710-2657)</td>
<td>813 (671-750)</td>
</tr>
<tr>
<td><strong>Elite Controllers</strong></td>
<td>&lt;48</td>
<td>1390 (1345-1534)</td>
</tr>
</tbody>
</table>

Table indicates average (bold) and interquartile range (parentheses, Q1-Q3) of indicated values used for experiments in these studies.
Table 2.B: Characteristics of Duke Virus Controllers

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>42</td>
</tr>
<tr>
<td>Sex, female (%)</td>
<td>57.1</td>
</tr>
<tr>
<td>Age, average (IQR)</td>
<td>45.9 (41-44)</td>
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<tr>
<td>Time since prognosis</td>
<td>14.35 (9-20)</td>
</tr>
<tr>
<td>Known protective alleles</td>
<td>7</td>
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</table>

Values in boldface are the median. The interquartile range (Q1-Q3) is indicated in the parentheses. Only 7 of the 42 VCs were HLA typed. HLA typing of other VCs is pending.
<table>
<thead>
<tr>
<th></th>
<th>NL4</th>
<th>WITO</th>
<th>WEAU3</th>
<th>CH040</th>
<th>CH058</th>
<th>CH077</th>
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<td>VC18</td>
<td>3.47</td>
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<td>VC23</td>
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<td>2.03</td>
<td>1.91</td>
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<td>VC33</td>
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<td>VC28</td>
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Figure 2.1: CD8+ T cells from virus controller possess breadth of total CD8+ T cell mediated inhibition of replication against a multi-clade HIV-1 panel log10 reduction in virus production in the presence of CD8+ T cells compared to target cell only control. Colors indicate quartile that value falls is categorized as (light yellow=Q1, bright yellow = Q2, orange=Q3, red=Q4). Blanks indicate value not determined.
Figure 2.2: Antigen-specific soluble virus inhibition assay (sVIA) stratifies chronically infected HIV+ patients reflecting clinical status.

CD8+ T cells isolated from patients were stimulated with pools of HIV-1 PTE peptides then used in antigen-specific soluble virus inhibition assay (sVIA) to determine the cells’ ability to inhibit replication of clade B transmitted/ founder virus CH040.c in TMB-bl target cells in a transwell system. Luciferase production by TZM-bl cells were corrected for background then transformed into log10 values. log10 reduction in virus production was then calculated by subtracting log10 values of each well from that of TZM-bl + virus only control well to quantify the reduction in replication as result of CD8+ T cell addition. Results reflect average of duplicate wells. Generated by Tamika Payne and Alyse Frisbee.
Table 2.C: Antigen-specificity of CD8+ T cell inhibition among HIV+ chronically infected cohorts

<table>
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<tr>
<th>Cohort</th>
<th>Envelope (Env1) gp120</th>
<th>Envelope (Env2) gp120</th>
<th>Envelope (Env3) gp41</th>
<th>Envelope (Env4) gp41</th>
<th>Gag (Gag1 P17) p17</th>
<th>Gag (Gag2 P24) p24</th>
<th>Gag (Gag3 P24) p24</th>
<th>Pol (Pol1 P51) protease</th>
<th>Pol (Pol2 p51) p51</th>
<th>Pol (Pol3 p51+p15) p51+p15</th>
<th>Pol (Pol4 p51) p51</th>
<th>Nef (p31)</th>
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<td>&lt;0.39</td>
<td>&lt;0.39</td>
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<td>ART-suppressed HIV+ (n=5)</td>
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<td>&lt;0.39</td>
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<td>Virus Controllers (n=5)</td>
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**Top:** individual patient responses to HIV-1 PTE peptide pools. A yellow box indicates above threshold (0.39 log reduction in virus production compared to virus only control).

**Bottom:** Summary of results for each patient group.

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Table: 75
### Table 2.D: Genes differentially expressed in CD8+ T cells with and without anti-HIV-1 function

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A “•” indicates the specified gene was found to be differentially expressed in CD8+ T cells with and without the ability to inhibit virus replication in the indicated study.
Figure 2.3: Gag p24 stimulated cells from VC30 exhibit greater expression of select cytokines than unstimulated cells.

Values for fold changes (5.5 h p24 stimulation/unstimulated) in total mRNA abundance (real-time [RT]-PCR) in CD8+ T cells from an HIV-1 virus controller (VC30). The results are from three independent experiments. The lines represent the median values. Generated by Tamika Payne and Jeff Blackinton.
Figure 2.4: Gag p24 stimulated CD8+ T cells from VCs with Gag p24-specific inhibition have increased expression of mRNAs for select cytokines.

Fold changes in total mRNA levels: 5.5-h p24-stimulated CD8+ T cells compared to unstimulated (top) and p17-stimulated (bottom) cells from patients with p24-specific inhibition (triangles, n=6: VC11, -23, -26, -27, -28, and -29) and those without (circles, n=5: 3 HIV-1-seronegative patients and VC16 and -24). The lines indicate the medians. mRNA abundance was determined via primer-specific PCR. P values were calculated using the Wilcoxon exact test, controlling for false discovery using the Benjamini and Hochberg method. Generated by Tamika Payne and Jeff Blackinton.
Figure 2.5: Nef stimulated CD8+ T cells from VCs with Nef-specific inhibition have increased expression of mRNAs for select cytokines.

Fold change in mRNA levels: 5.5-h Nef-stimulated CD8+ T cells compared to unstimulated (top) and p17 stimulated (bottom) cells from patients with Nef-specific inhibition (diamonds, n=4: VC26, -27, -29, and -16) and those without (squares, n=7: 3 HIV-1- seronegative patients and VC11, -23, -28, and -24). The lines indicate the medians. mRNA abundance was determined via primer-specific PCR. P values were calculated using the Wilcoxon exact test, controlling for false discovery using the Benjamini and Hochberg method. Generated by Tamika Payne and Jeff Blackinton.
Figure 2.6: Expression CD107 in memory CD8+ T cells is associated with inhibition of HIV-1 replication and serves as an early activation marker in the bulk CD8+ T cell population.

Expression of MIP-1β and CD107 proteins increases in p24-stimulated memory CD8+ T cells in patient VC23 (p24-specific inhibition) and not VC24 (no p24-specific inhibition) compared to unstimulated. Cells shown are live CD3+CD8+ memory cells. Generated by Tamika Payne and Joy Pickeral.
Figure 2.7: CD8+ T cells from virus controllers are able to inhibit virus replication in autologous primary CD4+ T cells in a dose-dependent manner

Effector CD8+ T cells were co-cultured at various E:T ratios with target autologous CD4+ T cells and 0.1 MOI clade B CH040.c transmitted founder virus for 3 days. Supernatants were collected and virus production quantified using TZM-bl cells. Percent virus inhibition is reduction of virus produced compared to wells with no CD8+ T cells. Percent inhibition increases with E:T ratio for all VCs tested. HIV-1 patient (circle) was used as a negative control and showed no difference in inhibition. Generated by Tamika Payne and Alyse Frisbee.
Figure 2.8: Addition of MHC class I or Class II monoclonal antibodies reverses CD8+ T cell mediated virus inhibition to varying degrees in the Duke virus controller cohort.

CD4+ T cells from each VC were infected via spinoculation (1200xg, 2hrs) with 0.1MOI clade B CHO40.c virus. Cells were then incubated with monoclonal antibody (mAb) for MHC Class-I, -II, or a negative control antibody for 30 minutes at 37 degrees. Autologous CD8+ T cells were then added at an E:T ratio of 0.5:1 and cells were co-cultured for 72 hours. Collected supernatants were analyzed for virus production via TZM-bl cells. Virus production was compared to inhibition observed at a 0.5:1 E:T ratio in the absence of antibody (Figure 2.7) to calculate percent inhibition blocked. Cutoff for positive percent suppression blocked > 28.0% (3 STDEVs + mean) is indicated by dotted line. p-values calculate using Wilcoxon Sign Test (n=7) and indicate that the median of the differences in response between MHC I or MHC II and negative control is significantly different from zero. Generated by Tamika Payne and Alyse Frisbee.
3.1 Introduction

During immune response to pathogen, gene regulation drives the processes of cellular maturation and differentiation, leading to the creation of immune cell subsets that possess different gene expression profiles, which can subsequently produce different proteins, tailoring the response to the invading micro-organism. Assays to assess T-cell function in response to HIV-1 show that CD8+ T-cell mediated inhibition arises within 48 hours of re-stimulation and is principally mediated by memory CD8+ T cells (166, 281, 386). There are two subtypes within the memory cell population, central and effector memory cells, which differ in formation and transcriptional profiles [reviewed in (387)]. Central memory cells are primarily found in secondary lymphoid organs and have a greater proliferative potential; effector memory cells exhibit constitutive effector function and are preferentially found in non-lymphoid tissue (292, 388). While there is still debate on which subset of these cells is the most important for anti-HIV-1 CD8 function, fine control of gene expression in both memory subsets is important for the immune response to HIV-1 as rapid initiation of responses is crucial for timely control of infection and prolonged immune responses can prove detrimental (389). A number of studies have demonstrated the importance of regulating gene
expression during the immune response providing a balanced cellular response (390-395). However, identification of mechanisms used to regulate the expression of effector molecules remains largely unexplored in the CD8+ T cell responses to HIV-1. Characterization of these mechanisms could lead to novel correlates of CD8+ T cell-mediated inhibition of HIV-1, new therapeutics, and more accurate means of evaluating vaccine efficacy.

Given the rapidity of the CD8+ T-cell inhibition we hypothesized that, in addition to controlled transcription, the CD8+ T-cell effector response to HIV-1 is regulated at the level of post-transcriptional regulation. In this chapter we evaluated the regulation of gene expression in the antigen-specific CD8+ T cell response to HIV-1. We first determined the rate of mRNA transcription for each gene then quantified the contribution of transcription to the total change in mRNA abundance observed in chapter 2. We found that while the amount of newly transcribed RNA increased in patients with antigen-specific inhibition this change did not sufficiently account for change in mRNA abundance. Modeling of our data revealed that a change in stability of RNA was needed to fully account for change in abundance. We therefore measured the decay rate of the mRNAs of our genes of interest and used these values to calculate half-lives. Analysis of calculated half-lives revealed that there was an increase in half-life in patients with antigen-specific inhibition. We consequently have begun to define the
roles of transcriptional and post-transcriptional gene regulation in genes that correspond to virus inhibition within a cohort of virus controller patients.

3.2 Materials and methods

**Patient cohort.** Twelve HIV-1 virus controllers (see Section 2.2) with CD8+ T cell mediated virus inhibition were studied in the experiments detailed in this chapter. Seven of these VCs were HLA typed, and only two of the patients used in our assays have alleles known to be associated with CD8+ T cell control. Three patients (VC28, VC29 and VC30) were elite controllers. Healthy uninfected donors were recruited through the Duke Virologic Basis for Specific Immune Defects in AIDS program (Dr. K. Weinhold). Studies were reviewed and approved by Duke University Medical Center’s Institutional Review Board and all participants provided written informed consent.

**Transmitted/ Founder virus.** Replication-competent virus stocks from a full-length infectious molecular clone (IMC) expressing a transmitted founder virus (CH040.c) were generated as described previously [(92, 166, 350, 359), Section 2.2].

**4sU incorporation and PCR analysis.** To assess transcription and mRNA decay independently, 4-thiouridine (4sU; 200 uM) was added one-hour prior to harvest of cells (4.5 hours after the start of peptide stimulation). Cells were harvested in 1 ml of Trizol and RNA extracted following manufacturer’s protocols (Life Technologies). Separation of 4sU labeled RNA from unlabeled RNA was performed using a highly efficient (>90%) biotinylation method as in (396) with minor modifications. Notably, RNA was DNase
(Ambion) treated after initial RNA extraction from cells and streptavidin MyOne C1 Dynabeads (Invitrogen) were used to extract biotinylated 4sU RNA. Subsequently, three populations of RNA for each sample were reverse transcribed into cDNA using iScript kit (Bio-Rad): Total RNA (T), Labeled RNA (L), and Unlabeled RNA (U). Real time PCR was performed on each population of RNA then normalized for relative amount of RNA input. Abundance of mRNA was represented by the measurement of the total RNA sample (T). Newly transcribed RNA was represented by the labeled fraction of mRNA (L). The decay rate (DR) was calculated from measurements of labeled (L) and unlabeled (U) mRNA, as a function of L/U: ln(1-L/U). An apparent RNA half-life was calculated using the decay rate via \(-t*\ln(2)/DR\), where \(t\) = the time of 4sU incorporation (1 hour for the purposes of these experiments). Two assumptions of this method are that transcription and stability are constant over the period of measurement. These assumptions result in more conservative conclusions regarding changes in stability between samples, especially among short-lived mRNAs. The mRNA stabilities calculated using 4sU based measurements correlate very well with other established methods such as actinomycin D (396). To compensate for a bias against labeling of short or U depleted mRNAs, we calculated the stochastic likelihood of labeling based on conservative incorporation rates of 1 4sU for every 100 uridines taken from (396-398) and our own measurements (data not shown) using an equation described in (58). All half-lives were normalized to GAPDH, where GAPDH half-life was defined to be 8
hours based on previous publications and other global analyses (399, 400). This method led to calculated half-lives that were consistent across the patients tested. The average apparent half-life (in hours) of RANTES mRNA was 5.56 ± 0.73, half-life of the IFN-γ mRNA was 0.63 ± 0.039, MIP-1α was 0.82 ± 0.10, MIP-1αP was 1.05 ± 0.19, MIP-1β mRNA was 2.30 ± 0.37 hours, GMCSF was 0.96 ± 0.12 and TNFRSF9 was 1.57 ± 0.20 (Figure 3.5). As expected, the half-life for XCL was more varied among different patients due to XCL’s larger value for half-life (average = 8.39 hours) and that RNA stability calculations are prone to greater error with longer intrinsic mRNA survival.

**Time course prediction modeling.** 4sU labeling was performed in one hour intervals for 6.5 hours after stimulation (Figure 3.10A), resulting in continuous measurement of transcription. Amounts of RNA in each population were measured as above. Predictions for RNA abundance that assumed a constant decay rate were determined based on a previously developed model (314), that uses the un-stimulated RNA abundance, then iteratively adds amounts of transcribed RNA in each hour and subtracts a decay rate dependent on the amount of decayed RNA in that hour. For time $i$,

$$T_i = T_0 + [N_i - T_{i-1}]DR_0.$$

where $T_0 = \text{initial measured total}$, $N_i = \text{the amount of newly transcribed RNA at time} = i$ and $DR_0 = \text{initial calculated decay rate}$. Because several of the mRNAs are short (~1000 bp) and contain relatively few uridines, we corrected for the stochastic likelihood
of failing to include a 4sU in the place of a uridines for an entire mRNA, outlined in (58).

Subsequently, modeling the optimized decay rate solved for the decay rate in the above equation, since the totals are observed.

$$\text{DR}_{\text{optimized}} = (T_{i+1} + N_{i+/T_i}) / T_{i-1}$$

Data from two biological replicates were independently treated through the modeling then averaged using fold-change measurements in predicted and observed abundances.

Statistical analyses. Performed as described in Section 2.3.

3.3 Results

CD8+ T cells with antigen-specific inhibition exhibit more newly transcribed RNA for IFN-γ, MIP-1α, MIP-1αP, MIP-1β, GM-CSF, XCL1, and TNFRSF9.

To investigate the mechanisms behind the up-regulation of IFN-γ, MIP-1α, MIP-1αP, MIP-1β, GM-CSF, TNFRSF9, and XCL1 mRNA in CD8+ T cells with p24- and Nef-specific inhibition of HIV-1, we performed 4-thiouridine (4sU) analysis after antigen stimulation (396). 4sU analysis uses incorporation of 4sU into nascent RNAs to physically isolate mRNA synthesized before 4sU addition from those synthesized after 4sU addition. For a fixed period of 4sU incorporation, comparing 4sU labeled mRNA across samples allows calculation of newly transcribed RNA (total RNA minus the amount of 4sU labeled RNA to decay prior to cell harvest), while using the ratio of labeled to unlabeled mRNA to infer a decay rate (396). This method has been employed
extensively to simultaneously measure mRNA transcription and decay across a variety of systems (314, 396, 401, 402) with greater reproducibility than using actinomycin D (396). We added 4sU to cells 4.5 hours after the start of antigen stimulation then allowed 4sU incorporation for 1 hour before lysing cells and harvesting RNA. In triplicate experiments in CD8+ T cells from patient VC30, we observed highly reproducible increases in newly transcribed RNA of several cytokines in p24-stimulated compared to autologous un-stimulated cells (Figure 3.1). The rate of transcription for mRNAs for most of the genes measured in CD8+ T cells with Gag p24- and Nef-specific inhibition (Figures 3.2 and 3.3) also increased compared to unstimulated and p17-stimulated cells. The exceptions to this observation were XCL1 and TNFRSF9, for which measured changes in newly transcribed RNA were greater than changes in abundance. In patients without antigen specific virus inhibition, we observed no changes in the amount of newly transcribed RNA.

**Change in transcription solely does not account for mRNA change.**

Notably, the change in newly transcribed RNA was consistently less than the change we observed in total mRNA abundance (Figure 3.4). In a majority of the cases in which there was a significant increase in gene expression (compared to patients without antigen specific inhibition) we also observed a less dramatic, albeit significant, increase in the rate of newly transcribed RNA) for IFN-γ, MIP-1α, MIP-1αP, MIP-1β, GM-CSF. For each of these messages, increases in total mRNA for these markers were 1.5- to 3-
fold more than the newly transcribed RNA (Figure 3.4). In comparison, total mRNA levels from an mRNA that is known to be transcriptionally induced, TNFRSF9, increased similarly to newly transcribed RNA. Equivalent changes in total RNA and the amount of newly transcribed mRNA is seen for XCL-1 indicating that abundance of RNA for this gene is likely primarily dictated by transcription. This indicates the potential for the contribution of additional regulatory mechanisms to the control of gene expression of MIP-1α, MIP-1αP, MIP-1β, IFNγ, and GM-CSF indicated. Though there are several possibilities for the other mechanism(s) involved, one logical explanation is the stabilization of the mRNA itself.

mRNA encoding IFN-γ, MIP-1α, MIP-1αP, and MIP-1β are stabilized after stimulation, contributing to the observed increase of RNA expression

The observed expression of mRNA is the balance between synthesis of new mRNA (transcription) and degradation (decay) of mRNA over time (403). To investigate the role of decay, we used the ratio of 4sU labeled mRNA over unlabeled mRNA to calculate mRNA decay rates for the selected panel of antiviral cytokines in CD8+ T cells. Using these decay rates, we calculated an apparent half-life, the instantaneous measure of the predicted half-life given the cellular conditions, of each message with and without antigen stimulation. For messages from un-stimulated cells from VCs and seronegatives, these apparent half-lives were consistent across the patients tested (Figure 3.5). For example, in the 12 VC and 2 HIV-1 seronegative subjects examined, the average
apparent half-life of the IFN-γ mRNA was 0.63 ± 0.039 hours (Figure 3.5), similar to previously published values for the stability of IFN-γ mRNA (331, 404, 405).

We next calculated the half-lives from VC30 CD8+ T cells without stimulation and from autologous cells 4.5 to 5.5 hours after stimulation with Gag p24 (Figure 3.6). We observed a consistent increase in the apparent half-life after stimulation with Gag p24 for IFN-γ, MIP-1α, MIP-1αP, MIP-1β and GM-CSF. We extended this analysis to additional virus controller patients and HIV-1 seronegative individuals. After stimulation, cells from VCs without an antigen-specific antiviral CD8+ T cell response had mRNA half-lives that remained highly consistent with those in un-stimulated cells. Cells with a p24-specific response showed increases in stability with MIP-1αP being the only statistically significant increase (Figure 3.7; P<0.05, Wilcoxon Exact Test, controlling for FDR (357)). While changes in apparent half-life in Nef-stimulated (compared to un-stimulated) cells did not reach significance when comparing patients with Nef-specific inhibition to those without and correcting for multiple comparisons, the values for IFN-γ and MIP-1β did reach significance when not adjusting for comparisons (Figure 3.8, Appendix E). In addition, when compared to Gag p17 stimulated cells, the increase in stability observed in Nef-stimulated CD8+ T cells is significant for IFN-γ, MIP-1α, and MIP-1αP (Appendix E). Taken together, the sum of change in newly transcribed RNA and change in mRNA stability corresponded with the differences in the mRNA abundance after antigen stimulation (Figure 3.10 E-F).
Temporal Dynamics of CD8+ T cell Responses

Measuring mRNA stability at fixed times after stimulation allowed us to observe differences in apparent half-life in the immediately preceding hour. However, the possibility remained that transcription rate dynamics occurring before the addition of 4sU at 4.5 hours could explain the observed differences in apparent half-life. To independently observe the impact of transcription and stability on total mRNA abundance, we performed 4sU analysis on p24-stimulated CD8+ T cells from VC20 every hour for a full 6.5-hour time course to capture all newly transcribed RNA (Figure 3.9.A). From this, we quantified total mRNA abundance and newly transcribed RNA at each hour (starting at 0.5 hours) after stimulation. Using the decay rate from un-stimulated cells (Figure 3.5), the initial abundance and newly transcribed RNA over every hour period, we predicted mRNA abundance at each time-point assuming no change in mRNA stability and compared to the measured total (Figure 3.9). In cases where mRNA abundances are constant or derive primarily from transcriptional variation, this model is highly accurate (314). Two controls confirmed the accuracy of this model for both stable (RANTES; Figure 3.9.B) and a known transcriptionally driven messages (TNFRSF9; Figure 3.9.C). Substantial deviations from the predictions given by the model suggest that RNA stability is the likely variable. If the observed totals are greater than predicted, it suggests the RNA is stabilized, while if they are less than predicted, it suggests the RNA is destabilized. As shown in Figure 3.9 D-F, predicted levels of RNA for MIP-1α,
MIP-1β and IFN-γ were substantially less than the RNA abundances that were observed, thus suggesting that the RNA is stabilized. For example, the predicted levels of IFN-γ and MIP-1β were approximately half of the observed values (Figure 3.9.D, F), while the observed abundance of MIP-1α was ~25% higher than the model predicted (Figure 3.9.E). These results suggest that a substantial stabilization of the target mRNAs took place in these cells. We further incorporated the variance in stability we observed across VCs, predicting abundances using apparent half-lives that represented the extremes of the observed un-stimulated stabilities in the VCs (Figure 3.5). These extreme values could still not mimic the observed results.

We subsequently allowed stability to vary and solved the equation for the optimized mRNA stability for the observed total mRNA abundances. While RANTES and TNFRSF9 minimally change in mRNA stability (Figure 3.9.G, H), IFN-γ, MIP-1α and MIP-1β robustly increased in stability in coordination with the induction of transcription (Figure 3.9.I-K). Most messages followed a peaked response, where stability was highest during the time of greatest transcription, resulting in maximal increases in mRNA abundance (Figures 3.10.G-K).

3.4 Discussion

Induction of CD8+ T cells capable of inhibiting HIV-1 replication is important for both development of cure therapies (406) as well as HIV-1 vaccine strategies. In this chapter, we report on the regulation of antiviral gene expression in CD8+ T cells that
inhibit virus replication in virus controllers. Through 4sU RNA analysis, we report the novel observation that the expression of antiviral genes MIP-1α, MIP-1αP (CCL3L1), MIP-1β, GMCSF, XCL1, TNFRSF9 and IFN-γ is controlled at the level of mRNA abundance and is driven through a coordinated response of both increased transcription and stabilization of mRNA. Together, these changes accounted for increase in mRNA abundance observed in chapter 2. This coordinated response allows for a rapid and robust induction of mRNA messages that can enhance the CD8+ T cells’ ability to inhibit virus upon antigen encounter.

The expression of cytokines has long been associated with antiviral functions. The regulation of these cytokines, however, is poorly understood. We assessed transcription and decay rates for mRNAs in un-stimulated and stimulated (p24, p17, and Nef) CD8+ T cells and found that both mechanisms drive induction of key cytokines with maximum increases in stability and newly transcribed RNA occurring at the same time. This induction of gene expression via the cooperation of signaling pathways to bring about increased transcription and enhanced stability to induce multiple cytokines with antiviral activity is an interesting contrast to the usual gene expression buffering seen in eukaryotes (407) which is the result of a balanced interplay of transcription, post-transcriptional regulation, and post-translational modifications (408).

The finding that RNA stability is involved in the CD8+ T cell response allows possible future identification of other markers of CD8+ T cell effector function using
techniques aimed at globally identifying mRNAs that exhibit increased stabilization and other post-transcriptional changes in CD8+ T cells with anti-HIV-1 effector function. Additionally, we uncovered evidence for potential differences in the regulation of mRNA between Nef and Gag specific CD8+ T cells which may inform the temporal arising of antigen specific responses during infection and warrants further investigation.
Figure 3.1: Gag p24 stimulated cells from VC30 exhibit greater newly transcribed RNA of select cytokines than unstimulated cells.

Fold change (5.5-h p24-stimulated/unstimulated) in newly transcribed RNA in CD8+ T cells from an HIV-1 virus controller (VC30). Results are from three independent experiments. The lines represent the median values. Generated by Tamika Payne and Jeff Blackinton
Figure 3.2: Gag p24 stimulated CD8+ T cells from VCs with Gag p24-specific inhibition have increased newly transcribed RNA of mRNAs for select cytokines. Fold changes in newly transcribed RNA: 5.5-h p24-stimulated CD8+ T cells compared to unstimulated (top) and p17-stimulated (bottom) cells from patients with p24-specific inhibition (triangles, n=6: VC11, -23, -26, -27, -28, and -29) and those without (circles, n=5: 3 HIV-1-seronegative patients and VC16 and -24). The lines indicate the medians. Newly transcribed RNA was determined via primer-specific PCR of 4sU-containing RNA. p values were calculated using the Wilcoxon exact test, controlling for false discovery using the Benjamini and Hochberg method. Generated by Tamika Payne and Jeff Blackinton.
Figure 3.3: Nef stimulated CD8+ T cells from VCs with Nef-specific inhibition have increased newly transcribed RNA of mRNAs for select cytokines.

Fold change in newly transcribed RNA: 5.5-h Nef-stimulated CD8+ T cells compared to unstimulated (top) and p17 stimulated (bottom) cells from patients with Nef-specific inhibition (diamonds, n=4: VC26, -27, -29, and -16) and those without (squares, n=7: 3
HIV-1- seronegative patients and VC11, -23, -28, and -24). The lines indicate the medians. Newly transcribed RNA was determined via primer-specific PCR of 4sU-containing RNA. p values were calculated using the Wilcoxon exact test, controlling for false discovery using the Benjamini and Hochberg method. Generated by Tamika Payne and Jeff Blackinton.
Figure 3.4: Measured changes in newly transcribed RNA do not match observed changes in mRNA abundance for some markers.

The change in total mRNA abundance (green) from Gag p24-stimulated (top) and Nef-stimulated (bottom) CD8+ T cells from VCs with antiviral activity does not match the change in newly transcribed RNA (blue) for MIP-1α, MIP-1αP, MIP-1β, IFNγ, and GM-CSF. Boxes indicate the median as well as the upper and lower quartiles with whiskers being the minimum and maximum values. Line at y=1 indicates no change in value (compared to unstimulated). Generated by Tamika Payne and Jeff Blackinton.
Calculated apparent half-lives are consistent across patients. Apparent half-lives were calculated for the unstimulated CD8+ T cells from 12 VCs (VC9, -11, -15, -16, -20, -23, -24, -26, -27, -28, -29, and -30). The lines represent the mean values, while the error bars represent standard errors of the mean. Generated by Tamika Payne and Jeff Blackinton.
Figure 3.6: Apparent half-lives of select cytokines are greater in Gag p24-stimulated cells from VC30 than unstimulated cells.

Raw GAPDH-normalized apparent half-life values for unstimulated and 5.5-h p24-stimulated CD8+ T cells from VC30. The results are from three independent experiments. The lines represent the median values. Generated by Tamika Payne and Jeff Blackinton.
Figure 3.7: Gag p24 stimulated CD8+ T cells from VCs with Gag p24-specific inhibition have increased apparent half-life of mRNAs for select cytokines.

Fold changes in apparent half-life: 5.5-h p24-stimulated CD8+ T cells compared to unstimulated (top) and p17-stimulated (bottom) cells from patients with p24-specific inhibition (triangles, n=6: VC11, -23, -26, -27, -28, and -29) and those without (circles, n=5: 3 HIV-1-seronegative patients and VC16 and -24). The lines indicate the medians. Apparent half-life was calculated as described in section 3.2. p values were calculated using the Wilcoxon exact test, controlling for false discovery using the Benjamini and Hochberg method. Generated by Tamika Payne and Jeff Blackinton.
Figure 3.8: Nef stimulated CD8+ T cells from VCs with Gag Nef-specific inhibition have increased apparent half-life of mRNAs for select cytokines.

Fold change in apparent half-life: 5.5-h Nef-stimulated CD8+ T cells compared to unstimulated (top) and p17 stimulated (bottom) cells from patients with Nef-specific inhibition (diamonds, n=4: VC26, -27, -29, and -16) and those without (squares, n=7: 3 HIV-1- seronegative patients and VC11, -23, -28, and -24). The lines indicate the medians. Apparent half-life was calculated as described in section 3.2. p values were calculated using the Wilcoxon exact test, controlling for false discovery using the Benjamini and Hochberg method. Generated by Tamika Payne and Jeff Blackinton.
Figure 3.9: Temporal expression of antiviral cytokines is quicker and more robust in CD8+ T cells with higher RNA stability.

Cells were labeled with 4sU for 1-h segments after stimulation for 6.5 h with p24 peptide, and then the RNA was separated to measure the newly transcribed RNA. The levels of total mRNA were predicted using a pre-stimulation total, newly transcribed RNA over each hour-long time period, and the pre-stimulation decay rate. These predictions were then compared to observed total mRNA abundances. (B and C) For unchanged (RANTES) (B) or transcriptionally induced (TNFRSF9) (C) genes, the model (red lines) accurately predicted the observed levels of mRNA abundance (blue lines). For IFNγ (D), MIP1α(E), and MIP-1β (F), however, the levels of total mRNA observed...
were much greater than a constant decay rate predicted. (G to K) We calculated optimized mRNA stabilities based on the observed mRNA totals (see Section 3.2). The optimized stability of mRNA increased in coordination with increases in newly transcribed RNA, so that mRNAs underwent a “peaked” stability response that correlated with the transcriptional induction. Generated by Tamika Payne and Jeff Blackinton.
Figure 3.10: Changes in transcription and stability do account for observed changes in mRNA abundance.

The observed total mRNA abundance across VCs, predicted mRNA abundances based on the observed newly transcribed RNA only, predicted mRNA abundances based on the calculated apparent half-lives only, and predicted mRNA abundances based on calculations that included observed newly transcribed RNA and apparent half-life values are shown. Boxes indicate the median as well as the upper and lower quartiles with whiskers being the minimum and maximum values. Line at y=1 indicates no change in value (compared to unstimulated). All values were normalized to the observed total mRNA abundance of GAPDH of each message. Generated by Tamika Payne and Jeff Blackinton.
Chapter 4 Discussion and Implications

4.1 Apoptosis and CD8+ T cell Mediated Inhibition of HIV-1

In addition to understanding how to bring about humoral and T cell functions that will both inhibit infection and control viremia, it is necessary to elucidate mechanisms important to establishing response durability allowing for long-lasting protection. Therefore, an important aspect to address is the apoptosis of functional cells. Apoptosis, programmed cell death, occurs via an extrinsic or intrinsic pathway [reviewed in (409)]. The intrinsic pathway is initiated intra-cellularly and is primarily regulated by the Bcl-2 family of proteins, which control the release of cytochrome c. The extrinsic pathway of apoptosis is mediated by surface receptors. The two pathways converge on the effector caspase, caspase-3, which, once cleaved, forms an active enzyme which preferentially recognizes the peptide sequence Asp-Glu-Val-Asp-Gly, cleaving at the carboxy side of the second aspartic acid in a variety of host cellular proteins which leads to subsequent cell death (410). Apoptosis of the bulk CD+8 T cell population was shown to correlate with progression from acute infection to AIDS with chronic viremic patients having highest susceptibility (411, 412). In infected persons,
HIV-specific cells are more prone to apoptosis (likely due to reduced expression of anti-apoptotic markers) than autologous cells specific to other viruses (413, 414). Interestingly, analysis of our transcriptome data indicates a striking increase in the expression of pro-survival/ anti-apoptotic genes TNFRSF9, BIRC3, ATF3, YAF2, ROG, ZBTB32 (Appendix D). The up-regulation of these genes in cells with potent inhibition suggests that similar to what is seen with other LTNP populations (271, 415) the cohort of Duke virus controllers may have fewer apoptotic CD8+ T cells. If this hypothesis is validated, it would be additional evidence for promoting the capacity for survival (via resistance of apoptosis) of cells with potent effector function as a complement to studies aimed at blocking expression of PD1, Tim-3, and other cell exhaustion markers to allow for effector functions (416, 417). Further analysis of apoptosis-associated markers with concurrent analysis of inhibitory factors can inform our understanding of why protective responses are lost in chronic viremic patients by providing insight into the regulation of exhaustion and thus identify mechanisms to revert or prevent apoptosis and to enhance vaccine induced immune response to be long lived.
4.2 Differential Regulation of Antigen-specific CD8+ T cell Responses

In our analysis of regulation of epitope-specific responses, we observed a difference in the significance of changes in newly transcribed RNA and RNA decay/half-life in cells with Gag p24- and Nef-induced inhibition with Gag p24 responses showing greater significant change in transcription. These results bring up an interesting concept of possible differences among epitope-driven responses by different CD8+ T cell subpopulations. While changes in total RNA did occur in CD8+ T cells with p24 and Nef stimulated inhibition, statistical analyses revealed a possible difference in the regulation of RNA abundances in p24- and Nef-specific cells. We observed that there was a marked statistically significant increase in the newly transcribed RNA for several mRNAs encoding antiviral cytokines in cells with p24-specific inhibition of virus while changes in Nef-stimulated cells were less dramatic and not significant. Interestingly, when comparing p24 and Nef stimulated cells to autologous p17 stimulated cells, we observed significant changes in the stability of IFNγ, MIP-1α, and MIP-1αP in Nef stimulated cells while only observing significance in the fold-change in stability of MIP-1αP in p24 stimulated cells (Appendix E). These observations fit with previous reports that Nef responses are the first to come up in acute infections (380, 418), which may be a reflection of the rapid expression that can be garnered through regulation of RNA stability allowing for a more immediate display of effector function. However, further studies are needed to examine the potential differences in the p24- and Nef-specific CD8+ T cell responses.
T cell responses to determine if regulation by transcription and RNA stability are dependent on antigen specificity and, if so, whether the stage of memory cell differentiation (419) of these antigen specific CD8+ T cells plays a role.

### 4.3 The Potential of MHC Class II-restricted CD8+ T cells

Classical theories of immunology hold that human CD8+ T cells recognize peptides presented by MHC class I molecule and CD4+ T cells recognize peptides bound to MHC class II. Reports by Picker et al. challenges this long held paradigm with their discovery of MHC class II restricted T cells in NHP in response to CMV/SIV vector. In our studies outlined in chapter 2, we determined that the CD8+ T cell mediated inhibition observed in our VIA is blockable by a MHC class II antibody. This exploratory study opens the door to the possibility of existing MHC class II restricted CD8+ T cells. Next steps should include confirming the existence of MCH-class II restricted CD8+ T cells in humans via the use of MHC class II tetramers. Flow cytometric analysis should include phenotypic markers to identify tetramer+CD3+CD8+ lymphocytes. A cytometry panel should also include CD4 antibody to determine if any identified tetramer+CD8+ cells are also CD4+. Additional markers should include natural killer markers (CD56 and CD16) as well as memory cell markers (CD45, CCR7, and CD28) to generate a full phenotypic profile of any tetramer+CD8+ cells. As it is doubtful that the number of tetramer positive cells will be sufficient to allow use in traditional functional assays, assessment of function would likely be limited to use of staining for surrogate markers.
of function and subsequent flow cytometry or single cell genomics based applications. Once fully optimized, single cell functional assays similar to the technology developed by Love et al (420), could proof to be very valuable to the study of MHC class II CD8+ T cells. Confirmation of this population and its involvement with inhibiting virus replication would expand the defined set of HIV-1 specific CD8+ T cells that should be induced with a vaccine.

4.4 Epigenetic Modification and Genomic Copy Number Variation as a Likely Determinants of Transcription Rate

Once stimulated with cognate antigen, naïve cells differentiate to effector and memory phenotypes that are more functional than their naïve precursor. There are multiple hypotheses for this disparity in functionality including a difference in proliferation [19] and activity in T-cell receptor signaling (421, 422). During the transition from naïve to effector, there is a priming of genes and regulatory elements which allows for display of effector functions upon antigen re-encounter (423). This modification of accessibility can include several mechanisms that can affect DNA, histones, and the spatial organization of genes and regulatory elements in the nucleus such as the existence of transcription factories [(clustered unit of gene transcription bound within a discrete nuclear site; (424)] to yield a continuum of accessibility. This continuum is reflected on the gene’s ability to be transcribed, rate of transcription, and the stability of transcription. Our results indicate there is a difference in the rate of newly
transcribed RNA of mRNAs for select genes in CD8+ T cells capable of HIV-inhibition compared to those that are not.

One plausible explanation for the observed increase in transcription could be the presence of epigenetic marks at the loci of these genes in memory cells that contribute to a primed state. Epigenetics is the collection of heritable changes in gene expression not encoded in DNA sequence (425). Eukaryotic chromatin consists of nucleosomes, subunits of DNA wrapped around histone proteins (284). The higher order structure created by nucleosome packing allows 2m of DNA to fit into a 6μm space but limits spatial access to DNA. To allow transcription factors and the transcription machinery access to DNA, nucleosome packing can be regulated by epigenetic mechanisms which include methylation of DNA and modification of histones (285). DNA methylation is the covalent addition of a methyl (-CH3) group to the carbon at position 5 of cytosine rings (426). Methylation usually occurs when the cytosine is adjacent to guanine, a conformation known as a “CpG dinucleotide” (427-429). In addition to methylation, gene expression can be epigenetically regulated by modification of histones, the proteins which form the core of the nucleosome (reviewed in (430)). Histones-2A, -2B, -3, and -4 are core histones which dimerize and assemble to form the octameric nucleosome protein. The histones’ amino acids can be modified by the addition or removal of –R groups. Examples of modifications include are lysine acetylation (-COCH3) (431), lysine and arginine methylation (-CH3). Histone modification changes the electrostatic charge
of histones, affecting how the histone associates with its DNA and other nucleosomes. Histone modification also creates context-influenced “histone codes” which act as binding sites for proteins that cause chromatin remodeling (430).

The chromatin changes brought about by epigenetic mechanisms play a crucial role in gene expression during the immune response [reviewed in (432, 433)]. There is evidence that rapid recall abilities of memory cells are associated with the acquisition of pro-transcription and loss of anti-transcription epigenetic marks at effector cytokine loci (308, 434) with Araki et al. showing that acetylation controls expression of the transcription factor eosmoderin and the genes it regulates including perforin and granzyme B (308). These and similar results give credence to the theory that upon primary stimulation and subsequent differentiation of naïve cells, effector cytokine loci in naïve cells undergo changes that result in memory cells with higher levels of pro-transcription epigenetic markers at these loci. These changes can cause a poised “ready-to-respond” state which results in almost immediate gene transcription upon secondary antigenic stimulation of memory cells (308, 310). We have previously shown that treatment of CD8+ T cells with valproic acid, a chemical that alters histone acetylation, impacts the CD8+ T cells’ ability to inhibit HIV-1 (167). This finding was supported by a recent study by Walker et al. which demonstrated that CD8+ T cell anti-HIV function is impacted by stimulation with HDAC inhibitors to varying degrees (435).
To determine if there is epigenetic priming of memory cells at candidate gene loci in response to primary HIV-1-infection, measurements of the amount of pro-transcription epigenetic marks at promoter, enhancer, and coding regions of candidate gene loci in resting naive and memory cells can be taken. Assessment would include promoter proximal and distal as well as intragenic sites as it has previous research indicates that cell culturing leads to artificial epigenetic remodeling at promoter distal sites (309). Candidate loci can be interrogated for pro-transcription histone markers H4 acetylation (H4ac) and tri-methylation of H3 lysine 4 (H3K4me3). For this analysis, a live cell sorting scheme to isolate naive-like (CD45RO− CCR7+ CD28+) and memory (CD45RO+) cells should be considered to isolate cells of interest. No significant difference in the levels of mRNA production and gene product secretion in resting and naïve memory cells, would be indicative of epigenetic priming at these loci is in preparation for antigen encounter, in a ready-to-respond state prepared to rapidly express these genes. To confirm the hypothesis of a ready-to-respond state of candidate genes epigenetically primed but not differentially expressed in resting memory versus naïve cells, protein production in memory and naïve cells post-re-exposure to antigen could then be measured.

While it is likely that epigenetics, specifically, differential histone modification plays a role in the difference in transcription observed among patients with and without antigen specific CD8 function, there are several mechanisms known to enhance
transcription and could be acting in our system (408, 436). An alternative, or complementary, explanation for difference in transcription rates is a disparity in the presence of transcription factor(s) that regulate the expression of genes relevant to CD8+ T effector function.

In addition, genomic variation, specifically, copy number variation could be attributing to differences in response. Copy number variation (CNV) is a significant source of genomic diversity and disease [reviewed in (437, 438)]. Because gene regions can range from 0 to 48 copies per diploid genome in the general population (438)] and population studies have revealed important roles for CNV in disease association [reviewed in (439)], CNV in immune related genes may prove to be important in HIV-1 susceptibility and immune response. CCR5 ligands include the macrophage inflammatory proteins (MIPs) MIP1α (CCL3) and MIP1β (CCL4) as well as their isoforms MIP1α-AP (CCL3L1) and MIP1β2 (CCL4L1). The ligands can act to block HIV-1 entry into CD4+ T cells and can influence cell mediated immunity (338, 440-444)]. These ligands are encoded in a region of chromosome 17q (445) that is a hotspot for segmental duplications which leads to great variation in the copy number of these genes found in the population (446). Low copy number of these chemokine genes has been previously associated with reduced chemokine expression (368), increased risk of HIV-1 infection through mucosal, cutaneous, and vertical routes (368, 447-451). Determining if
copy number variation of the CCR5 ligands plays a role in the differential rate of transcription observed in our experiments would be an informative follow-up study.

4.5 Molecular Mechanisms that Impact RNA Stability

Analysis of RNA decay rates and subsequent calculation of apparent half-lives revealed that for many of the genes we assessed in our panel, there was increased mRNA stability. These results place us in a position to not only further evaluate the posttranscriptional mechanisms at work in CD8+ T cells that inhibit virus but also comprehensively identify other genes important to anti-HIV-1 function. Follow-up studies should aim to show that mRNA stabilization translates to faster, more robust expression/secretion of cytokine at the protein level and subsequent enhancement of the CD8+ T cell response to HIV-1. To go about this, western blot, Luminex, or flow cytometry can be used to determine the kinetics of protein production in these cells post antigen stimulation. Cells and cellular supernatants can be analyzed at 2 hour time increments starting at 0 hours post exposure to 12 hours post exposure to determine the presence and quantity of protein production. Correlation of faster and higher magnitude protein expression of genes with stabilized mRNA in antigen-stimulated cells would confirm that increases in mRNA half-life primes these cells in a ready-to respond state allowing for rapid and robust effector molecule expression upon antigen encounter.

In chapter 3, we observe increases in the stability of the CCR5 ligand family as well as IFNγ and GMCSF, all genes known to have AREs in their 3′ UTR. Coupled with
previous research that showed mirrored changes to some of these genes in response to stimulation with antigen or other cytokines such as IL10 (452, 453), these data suggest a concomitant signaling pathway may regulate the stability of mRNAs encoding HIV-1 antiviral cytokines by predictable transacting factors. RNA stability is primarily controlled by RNA binding proteins (RBPs) and miRNAs. The most common RBP regulators of cytokine stability are HuR (454, 455), Roquin (333), and TTP (324, 331, 456, 457). We measured the abundance of the mRNA for these RBPs simultaneously with our genes of interest (Figure 4.1) but did not observe a change in abundance as we did with the other genes. While the RNA abundances of these RBPs did not change in these studies, it is known that post-translational modifications (including phosphorylation and glycosylation) can affect their mRNA targeting (458, 459). Therefore, measuring the expression of the post-translationally modified versions of the protein products of these RBP genes may uncover differential expression of functional RBPs in our cellular populations of interest. Post detection of differential expression of RBPs, confirmation of RBP association with the mRNAs for our cytokines of interest will be needed. To go about this biotinylated mRNA can be used to probe cellular lysates. Streptavidin can then be used to isolate the exogenous mRNA and any proteins that associated with the RNA during incubation with the lysate. Associated proteins can then be dissociated, identified via mass spectrometry, and confirmed as associating by running streptavidin isolated fractions via western blot and probing the membrane with antibody for
identified proteins. Alternatively, methods such as RIP-ChIP (460) can be used to isolate RBP's and interrogate associated mRNAs.

Changes in miRNA regulation may also underlie changes in mRNA stability. In previous reports, elimination of Dicer, a protein required for miRNA processing, resulted in CD8⁺ T cells that responded faster after anti-CD3 and anti-CD28 stimulation but were not able to resolve activating responses (461). After stimulation, miR-130/301 (461) and miR-155 (462) were strongly up-regulated in CD8⁺ T cells. Additionally, large-scale differences in miRNA profiles were observed in naïve, effector and memory CD8⁺ T cells (295). Therefore, interrogation of the miRNA profile of these cells, beginning with miRNAs, shown to be regulators of cytokine expression can inform our understanding of the regulation seen in our results. The importance of identified regulatory mechanisms to the CD8⁺ T cell response should be assessed via perturbation of any identified mechanism in CD8⁺ T cells and subsequent measurement of impact on the ability of said cells to inhibit HIV-1 replication.

4.6 Concluding Remarks

Throughout these studies we addressed aspects of CD8⁺ T cells that potently inhibit HIV-1 replication in our virus controller cohort. We showed that our virus inhibition assays (VIAs) allow us to stratify HIV-1+ patients according to clinical groupings and can be used as an effective tool to evaluate vaccine modalities. In addressing the potency of response among patient cohorts, we showed that CD8⁺ T cells
from virus controllers are able to inhibit a multi-clade panel of viruses demonstrating breadth of response which may include MHC class II restricted CD8+ T cells. Determining if this response is due to the presence of a highly conserved epitopes across clades could inform vaccine design strategies with subsequent identification of said epitope for vaccine targeting. With a clade B matched transmitted/ founder virus, our virus controller cohort had a prevalence of Gag p24 and Nef specific CD8+ T cell response. Moreover, evaluation of antigen-induced gene expression in p24- and Nef – stimulated cells from 2 virus controllers allowed us to begin to build a profile of the gene signature of cells with potent inhibitory capacity. We evaluated this profile for accuracy and measured the expression of previously identified effector molecules in the larger VC cohort and found a strong association of antigen specific CD8 function as measured by our VIA and the mRNA abundance of select genes. Lastly, Gag p24 and Nef stimulated cells were shown to control the expression of these genes, to varying extents, via the regulation of transcription and post-transcriptional mechanisms to enhance mRNA stability. Thus, we have advanced the field of HIV immunology by starting to define the molecular signature of anti-HIV CD8+ T-cells and demonstrating the regulatory mechanisms responsible for virus inhibition. The results of this approach can be applied toward the design of vaccine immunogens in order to target well-defined HIV-1 specific CD8+ T cells that mediate the stabilization and rapid release of β-chemokines and other antiviral cytokines upon antigen encounter to block HIV-1 acquisition. Of importance to
HIV-1 acquisition, our results can be applied to the study CD8+ T-cells in the mucosa, most often the site of initial exposure to HIV-1. Previous research has shown that weak or late CD8+ responses at these sites allows for viral dissemination \((463)\). This indicates induction and maintenance CD8+ protective immune response at mucosal sites is an important goal in efforts to produce an efficacious preventative vaccine.

Finally, the overall results and conclusions from these studies can serve as a platform to 1. evaluate potency and breadth of CD8+ T cell function in HIV-1+ persons and vaccinees 2. identify CD8+ immune response-relevant genes by interrogating the gene expression profile of cells activated via antigen stimulation and by comprehensive identification of post-transcriptionally regulated mRNAs in antigen stimulated CD8+ T cells from virus controllers using labeled RNA-seq. Follow-up work to identify the important regulatory RBPs and miRNAs driving the post-transcriptional responses in HIV-1 antigen stimulated CD8+ T cells we would not only uncover exploitable mechanisms to bring about a durable, long-lived CD8 response but also allow for global interrogation of these factors to uncover new potential effector targets for therapy. Of global significance to human health, the capacity to harness the immunological protection provided by this CD8+ T-cell antiviral mechanism through vaccination or novel therapeutics could significantly decrease viremia during acute infection, impacting HIV-1 transmission rates and/or provide component of a HIV-1 cure.
Figure 4.1: mRNA abundance of RNA-binding proteins HuR and TTP does not change with Gag p24 stimulation of cells with p24-specific inhibition.

CD8+ T cells from VC20 (patient with Gag p24 specific inhibition) were stimulated with Gag p24 for 5.5 hours. A fraction of cells were collected every hour starting at hour 0.5, lysed, and lysate analyzed for the presence of mRNAs for HuR and TTP via primer specific PCR. Abundance of housekeeping gene GAPDH and RANTES, cytokine without Gag p24-induced up-regulation of mRNA was also measured. MIP1-α was used as a positive control. Generated by Tamika Payne and Jeff Blackinton.
Figure A.1: Geographical distribution of HIV-1 viral subtypes. Genetic forms predominant in different world regions are shown. Adapted from (42).
## Appendix B

### Table A.1: HLA type of Virus Controllers

<table>
<thead>
<tr>
<th></th>
<th>HLA-A</th>
<th>HLA-B</th>
<th>HLA-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC2</td>
<td>0203</td>
<td>2301</td>
<td>1503g</td>
</tr>
<tr>
<td>VC3</td>
<td>3002</td>
<td>7401g</td>
<td>1503g</td>
</tr>
<tr>
<td>VC11</td>
<td>0101g</td>
<td>0201g</td>
<td>1503g</td>
</tr>
<tr>
<td>VC12</td>
<td>0201g</td>
<td>6801</td>
<td>3902/04/12</td>
</tr>
<tr>
<td>VC13</td>
<td>3303</td>
<td>3601</td>
<td>1402</td>
</tr>
<tr>
<td>VC14</td>
<td>2402g</td>
<td>7401g</td>
<td>5703</td>
</tr>
<tr>
<td>VC15</td>
<td>0301</td>
<td>2501/02</td>
<td>0702/07</td>
</tr>
<tr>
<td>VC16</td>
<td>2902/10</td>
<td>3301/05</td>
<td>4501g</td>
</tr>
<tr>
<td>VC20</td>
<td>0301</td>
<td>-</td>
<td>4403</td>
</tr>
<tr>
<td>VC21</td>
<td>2301</td>
<td>3201</td>
<td>5802</td>
</tr>
<tr>
<td>VC22</td>
<td>4402g</td>
<td>4403/07</td>
<td>4403/07</td>
</tr>
<tr>
<td>VC23</td>
<td>0201g</td>
<td>0301</td>
<td>-</td>
</tr>
<tr>
<td>VC24</td>
<td>0201g</td>
<td>2301</td>
<td>4101</td>
</tr>
<tr>
<td>VC25</td>
<td>0301</td>
<td>-</td>
<td>0702g</td>
</tr>
</tbody>
</table>

The listed alleles are either the only allele detected or the common allele of a group that includes 1 common and 1 or more rare alleles (464). A “/” indicates loci could not be fully resolved and one of the indicated alleles is present. “g” indicates that alternate alleles are possible but they differ outside exons 2 and 3. Thus, they have identical antigen recognition sites compared to the listed alleles.
## Appendix C

Table A.2: Sequences of primers used for PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CATGTTCGTCATGGGTGTGAACCA</td>
</tr>
<tr>
<td></td>
<td>AGTGATGGCATGGACTGTGGCAT</td>
</tr>
<tr>
<td>RAN7TES</td>
<td>TACATTGGCCCAGGCCCCACTGCC</td>
</tr>
<tr>
<td></td>
<td>GGGTGGCCACACACTTGGCG</td>
</tr>
<tr>
<td>IFNγ</td>
<td>ACGAGATGACTTGGAAAAGCTGACT</td>
</tr>
<tr>
<td></td>
<td>CCTCGAAAACAGCATCTGACTCCT</td>
</tr>
<tr>
<td>MIP1-α</td>
<td>AGAAGGACACGGGCAGCAGACACA</td>
</tr>
<tr>
<td></td>
<td>ACAGCAAGGGGACAGCGAGGAG</td>
</tr>
<tr>
<td>MIP1-αP</td>
<td>GAAGGACACGGCCAGCGCAAG</td>
</tr>
<tr>
<td></td>
<td>GCAGTGAGACGGACCATGAT</td>
</tr>
<tr>
<td>MIP1-β</td>
<td>TAGCTGCTTCTCTGCTCCAGCG</td>
</tr>
<tr>
<td></td>
<td>GCAGAGGGCTGCTGCTCAGGATGA</td>
</tr>
<tr>
<td>TNFRSF9</td>
<td>CCGGGAGGACTGGACAGTGAC</td>
</tr>
<tr>
<td></td>
<td>CGCCAGCTCTACTGTGGAAA</td>
</tr>
<tr>
<td>XCL1</td>
<td>ATCCGACCTGCTCTGCTGCTGCTGCTCAAG</td>
</tr>
<tr>
<td></td>
<td>GCCGCAACACGAGGTCTCTTATCG</td>
</tr>
<tr>
<td>TARC</td>
<td>AGGGAGCCATTCCTCTAGA</td>
</tr>
<tr>
<td></td>
<td>CTCTTTTGGTTGGGTCCGGA</td>
</tr>
<tr>
<td>MDC</td>
<td>GCGTGGTGTTGCTAACCCTTC</td>
</tr>
<tr>
<td></td>
<td>CCACGGGTCATCAGAGTAGGCC</td>
</tr>
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</table>
### Appendix D

Table A.3: Functions of select genes differentially expressed in transcriptome analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFRSF9</td>
<td>T-cell co-stimulatory molecule. Blocks apoptosis.</td>
</tr>
<tr>
<td>MIP1A and MIP1AP</td>
<td>HIV-1 suppressive factor</td>
</tr>
<tr>
<td>BIRC3</td>
<td>Caspase inhibition</td>
</tr>
<tr>
<td>RBM28</td>
<td>snRNP component</td>
</tr>
<tr>
<td>LSM14A</td>
<td>Viral nucleic acid sensor; promotes p-bodies</td>
</tr>
<tr>
<td>ATF3</td>
<td>Transcription factor; histone acetyltransferase</td>
</tr>
<tr>
<td>YAF2</td>
<td>Enhances MYCN-dependent transcriptional activation; chromatin remodeling; inhibits CASP8 apoptosis in zebrafish</td>
</tr>
<tr>
<td>ZBTB32 (ROG)</td>
<td>Transcriptional repressor helper T-cell differentiation; apoptosis</td>
</tr>
<tr>
<td>IFITM1</td>
<td>IFN-induced antiviral protein that mediate cellular innate immunity (required for interferon responses); inhibit HIV-1 infection</td>
</tr>
<tr>
<td>STAT1</td>
<td>Signal transduction pathway of cytokines</td>
</tr>
<tr>
<td>GNLY</td>
<td>T-cell cytotoxic granule component; tf is ATF2</td>
</tr>
<tr>
<td>TMEM140</td>
<td>Transmembrane protein with unknown function</td>
</tr>
<tr>
<td>EGR2</td>
<td>Bound by Tat to induce FasL apoptosis</td>
</tr>
<tr>
<td>FLJ43681</td>
<td>Ribosomal protein pseudogene</td>
</tr>
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</table>
Appendix E

Table A.4.A: Calculated p-values for observed fold-change differences in Gag p24 stimulated cells.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Gene</th>
<th>Raw p-value</th>
<th>FDR corrected p-value</th>
<th>Raw p-value</th>
<th>FDR corrected p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>IFNγ</td>
<td>0.004</td>
<td>0.030</td>
<td>0.008</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>MIP-1α</td>
<td>0.004</td>
<td>0.030</td>
<td>0.008</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>MIP-1αP</td>
<td>0.004</td>
<td>0.030</td>
<td>0.016</td>
<td>0.054</td>
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<tr>
<td></td>
<td>MIP-1β</td>
<td>0.004</td>
<td>0.030</td>
<td>0.008</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>GMCSF</td>
<td>0.004</td>
<td>0.030</td>
<td>0.008</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>TNFRSF9</td>
<td>0.017</td>
<td>0.054</td>
<td>0.032</td>
<td>0.080</td>
</tr>
<tr>
<td></td>
<td>XCL1</td>
<td>0.017</td>
<td>0.054</td>
<td>0.008</td>
<td>0.030</td>
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<tr>
<td>Transcription</td>
<td>IFNγ</td>
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<td>0.030</td>
<td>0.008</td>
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<tr>
<td></td>
<td>MIP-1α</td>
<td>0.052</td>
<td>0.104</td>
<td>0.008</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>MIP-1αP</td>
<td>0.004</td>
<td>0.030</td>
<td>0.008</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>MIP-1β</td>
<td>0.004</td>
<td>0.030</td>
<td>0.008</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>GMCSF</td>
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<td>0.104</td>
<td>0.008</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>TNFRSF9</td>
<td>0.017</td>
<td>0.054</td>
<td>0.008</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>XCL1</td>
<td>0.004</td>
<td>0.030</td>
<td>0.008</td>
<td>0.030</td>
</tr>
<tr>
<td>Half-life</td>
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<td>0.017</td>
<td>0.054</td>
<td>0.095</td>
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<tr>
<td></td>
<td>MIP-1α</td>
<td>0.082</td>
<td>0.147</td>
<td>0.151</td>
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</tr>
<tr>
<td></td>
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<td>0.004</td>
<td>0.030</td>
<td>0.032</td>
<td>0.080</td>
</tr>
<tr>
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<td></td>
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<td>1.000</td>
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<tr>
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<td>XCL1</td>
<td>0.429</td>
<td>0.500</td>
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</tr>
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</table>

p values were calculated using the Wilcoxon exact test. Raw p-values as well as those calculated while controlling for false discovery using the Benjamini and Hochberg method are reported. Cells highlighted in yellow indicate a statistically significant value.
Table B.4.B: Calculated p-values for observed fold-change differences in Nef stimulated cells.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Gene</th>
<th>Raw p-value</th>
<th>FDR corrected p-value</th>
<th>Raw p-value</th>
<th>FDR corrected p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>IFNγ</td>
<td>0.042</td>
<td>0.091</td>
<td>0.117</td>
<td>0.185</td>
</tr>
<tr>
<td></td>
<td>MIP-1α</td>
<td>0.042</td>
<td>0.091</td>
<td>0.183</td>
<td>0.257</td>
</tr>
<tr>
<td></td>
<td>MIP-1αP</td>
<td>0.042</td>
<td>0.091</td>
<td>0.262</td>
<td>0.349</td>
</tr>
<tr>
<td></td>
<td>MIP-1β</td>
<td>0.042</td>
<td>0.091</td>
<td>0.067</td>
<td>0.127</td>
</tr>
<tr>
<td></td>
<td>GMCSF</td>
<td>0.164</td>
<td>0.245</td>
<td>0.833</td>
<td>0.875</td>
</tr>
<tr>
<td></td>
<td>TNFRSF9</td>
<td>0.024</td>
<td>0.068</td>
<td>0.833</td>
<td>0.875</td>
</tr>
<tr>
<td></td>
<td>XCL1</td>
<td>0.073</td>
<td>0.133</td>
<td>0.383</td>
<td>0.454</td>
</tr>
<tr>
<td></td>
<td>IFNγ</td>
<td>0.315</td>
<td>0.389</td>
<td>0.383</td>
<td>0.454</td>
</tr>
<tr>
<td></td>
<td>MIP-1α</td>
<td>0.109</td>
<td>0.180</td>
<td>0.667</td>
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</tr>
<tr>
<td></td>
<td>MIP-1αP</td>
<td>0.230</td>
<td>0.312</td>
<td>0.383</td>
<td>0.454</td>
</tr>
<tr>
<td></td>
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<td>0.133</td>
<td>0.117</td>
<td>0.185</td>
</tr>
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<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
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<td>XCL1</td>
<td>0.315</td>
<td>0.389</td>
<td>0.183</td>
<td>0.257</td>
</tr>
<tr>
<td></td>
<td>IFNγ</td>
<td>0.024</td>
<td>0.068</td>
<td>0.033</td>
<td>0.080</td>
</tr>
<tr>
<td></td>
<td>MIP-1α</td>
<td>0.109</td>
<td>0.180</td>
<td>0.033</td>
<td>0.080</td>
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<tr>
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<td>MIP-1αP</td>
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<td>0.389</td>
<td>0.033</td>
<td>0.080</td>
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<tr>
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<td>0.067</td>
<td>0.127</td>
</tr>
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<td>0.183</td>
<td>0.257</td>
</tr>
<tr>
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<td>0.950</td>
<td>0.667</td>
<td>0.727</td>
</tr>
<tr>
<td></td>
<td>XCL1</td>
<td>0.164</td>
<td>0.245</td>
<td>0.667</td>
<td>0.727</td>
</tr>
</tbody>
</table>

P values were calculated using the Wilcoxon exact test. Raw p-values as well as those calculated while controlling for false discovery using the Benjamini and Hochberg method are reported. Cells highlighted in yellow indicate a statistically significant value.
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Biography

Tamika Payne was born Tamika John in Georgetown Guyana on October 17, 1987. Tamika received her Bachelors of Science in Biological Sciences from the University of Maryland Baltimore County (UMBC) in May 2009. Later that year, Tamika joined the Duke community as a graduate student in Molecular Genetics and Microbiology. After completing her rotations, Tamika chose Dr. Georgia Tomaras as her research mentor and began researching the CD8+ T-cell response to HIV-1 infection in virus controllers. This project resulted in one published first author paper, “Transcriptional and Post-transcriptional Regulation of Cytokine Gene Expression in HIV-1 Antigen-specific CD8+ T Cells that Mediate Virus Inhibition” (J Virology, 88 (17):9514-9528.), and three other papers (one first and one second author) in preparation at the time of this dissertation. Tamika’s graduate work was supported by the Viral Oncology Training Program and NIH Ruth L. Kirschstein National Research Service Awards for Individual Pre-doctoral Fellowship (F31). While research was her priority as a graduate student, Tamika was consistently engaged in the Duke community serving on the Executive Boards of the Duke University Bouchet Society and Black Graduate and Professional Student Association. In 2010-12 Tamika was as a mentor for the Building Opportunities and Overtures in Science and Technology (B.O.O.S.T), a mentoring program which expose underprivileged middle-school children to science. In 2012, she was hired as an assistant coordinator. In recognition of her work in and out of the lab,
Tamika was selected as the Graduate Life of the Mind Award 2013 recipient in which is given to a student that demonstrates personal and academic excellence in both the Duke and Durham communities. In 2014, Tamika was awarded the American Society for Microbiology-Centers for Disease Control Resident Postdoctoral Research Fellowship.