Examining The Role of Nef in the Resistance of SIV–Infected Macrophages to CD8+ T Cell Suppression

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EXAMINING THE ROLE OF NEF IN THE RESISTANCE OF SIV-INFECTED MACROPHAGES TO CD8+ T CELL SUPPRESSION

By

Jennifer Nina Rainho

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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EXAMINING THE ROLE OF NEF IN THE RESISTANCE OF SIV–INFECTED
MACROPHAGES TO CD8⁺ T CELL SUPPRESSION

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SIV–specific CD8⁺ T cells kill SIV–infected CD4⁺ T cells in an MHC Class I (MHC–I) dependent manner. However, they are reportedly less efficient at killing SIV–infected macrophages. Since the viral accessory protein, Nef, has been shown to down-regulate MHC–I molecules and enhance CTL evasion in HIV–1 infected CD4⁺ T cells, we examined whether Nef played a role in protecting SIV–infected macrophages from killing by SIV–specific CD8⁺ T cells.

To explore the role of Nef in CD8⁺ T cell evasion, we compared the ability of freshly sorted SIV–specific CD8⁺ T cells to readily suppress viral replication or eliminate CD4⁺ T cells or monocyte–derived macrophages infected with SIV variants containing wild–type (WT) or mutated nef genes. In Chapter 3, we show that SIV–specific CD8⁺ T cells suppressed viral replication and eliminated the majority of SIV–infected CD4⁺ T cells. Additionally, suppression of viral replication was enhanced in CD4⁺ T cells infected with a SIV harboring a nef variant containing a point mutation (Y223F) that has been shown to impair MHC–I down–regulation. However, infection with the Y223F Nef variant did not promote killing of macrophages by freshly sorted SIV–specific CD8⁺ T
cells. Furthermore, we show elimination of WT–infected macrophages by CD8⁺ T cells lines in a MHC–I dependent manner. These results suggest that mechanisms other than Nef–mediated MHC–I down–regulation govern the resistance of SIV–infected macrophages to killing by freshly sorted CD8⁺ T cells.

Chapter 4 evaluates the ability of freshly sorted SIV–specific CD8⁺ T cells to kill macrophages infected with a nef deletion mutant (Δnef). Despite the fact that Δnef restored the MHC–I expression, it did not sensitize infected macrophages to CD8⁺ T cell elimination or suppression of viral replication after 24 h of co–culture. Thus, we show that although macrophages infected with SIV nef mutants that increase MHC–I expression, and entirely disrupt Nef function, this is not sufficient to impact their sensitivity to CD8⁺ T cell killing, as observed in infected CD4⁺ T cells. Therefore, these results suggest that Nef appears neither necessary nor sufficient for the resistance of infected macrophages to elimination or suppression of viral replication by “unstimulated” freshly sorted SIV–specific CD8⁺ T cells. This study has implications for viral persistence and suggests that macrophages may afford primate lentiviruses some degree of protection from immune surveillance.
To my parents, Joseph and Gloria Rainho
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Chapter 1

Introduction

1.1 HIV and SIV

1.1.1 Discovery of HIV and Therapeutics

In 1981, a “mysterious immunodeficiency disease” among gay men was reported and later known as Acquired Immune Deficiency Syndrome (AIDS). Two years later, Luc Montagnier’s group at the Pasteur Institute isolated a novel retrovirus named lymphadenopathy-associated virus (LAV) (1). LAV was very similar to a newly identified virus named human T–cell leukemia virus III (HTLV–III) isolated from Robert Gallo’s group at NIH (2). These viruses were determined to be the same and renamed Human Immunodeficiency Virus–1 (HIV–1), the causative agent of AIDS. In 1985, Ronald Desrosiers group at Harvard University isolated the simian T–cell leukemia virus III (STLV–III), or simian immunodeficiency virus (SIV), from rhesus macaques (3). SIV had similar morphology, pattern of growth and antigenic properties as HIV–1, and would prove to be an exceptional animal model for HIV (3). It is believed that SIV has been transmitted to humans from African primates on several occasions resulting in different HIV strains, HIV–1 from infected chimpanzees and HIV–2 from sooty mangabeys (4-7).

HIV and SIV are related members of the genus lentivirus of the family Retroviridae. Retroviruses replicate in a host cell through the process of reverse transcription using its own reverse transcriptase enzyme. Retroviruses are enveloped viruses with a genome comprised of two copies of single stranded RNA molecules and
share the same basic structure encoded by the *gag*, *pol*, *env*. Lentiviral–infections classically show a chronic phase of disease, followed by an extended clinical latency period, and persistent viral replication.

Since its discovery, HIV infection has become a global epidemic affecting millions of individuals. In 1987, the first anti–HIV therapeutic, a nucleoside analog called AZT, appeared and provided scientists with optimism. Unfortunately, hopefulness soon faded as doctors realized the emergence of AZT–resistant strains in infected patients soon after AZT monotherapy (8). However, with the development of new classes of drugs and combination therapy, a regimen referred to as Highly Active Antiretroviral Therapy (HAART), this approach proved to be effective (9). In 1997, the significance of HAART was evident as the CDC reported that incidence of HIV and annual AIDS death declined in the US for the first time since its discovery, sadly the global pandemic was still on the rise with the most affected region in sub–Saharan Africa (10).

Despite the significant advancement in drug therapies a revolution in preventing HIV infection or the AIDS pandemic is in the development of an anti–HIV vaccine. Efforts to develop an effective vaccine are underway and have proven to be difficult. Unfortunately, in 2003, the first large-scale AIDS vaccine clinical trial, AIDSVAX failed. Evaluations of the trial revealed that in more than 2,000 intravenous drug users the vaccine did not reduce the incidence of HIV infection (11, 12). In 2007, the STEP trial was abruptly terminated when the data showed that the administration of the vaccine presented no benefit and the adenovirus (Ad5) vector used to deliver the HIV antigens might have increased the risk of HIV infection in some of the trial participants (13). It
was reasoned that the Ad5 cold virus could have interfered with its efficacy as a vaccine vector and possibly increased the risk of HIV infection. More recently, the HVTN-505 vaccine trial that included 2,500 individuals with no prior exposure to the Ad5 cold virus did not prevent HIV infection (14). Scientists hoped the antibodies directed at the cold virus could have interfered with its efficacy as a vaccine vector, but this proved to be false. Fortunately, the RV144 trial involving 16,000 volunteers from Thailand showed a modest efficacy using a canarypox prime–recombinant envelope (Env) protein boost design vector. In this study, the rate of HIV infection was lowered by only 31 percent in vaccinated individuals compared to placebo groups, providing scientists with encouragement that a protective vaccine is possible (13). Additionally, the use of bone marrow or stem cell transplantation to alter the immune system of HIV–infected patients may offer a promising alternative to the development of novel drugs and vaccines in finding a cure (15).

1.1.2 HIV Genome and Viral Proteins

The HIV–1 genome is approximately 9.7 kilobases (kb) in length and is flanked with two identical “Long Terminal Repeats” (LTR) at each end of the genome, consisting of RNA/DNA sequences serving regulatory and structural functions. Between these LTRs are 9 open reading frames (ORFs), which encode 15 distinct proteins, including the fundamental genes common to all retroviruses gag, pol, env that encode three major polyproteins precursors Gag, Gag–Pol and Env. The viral proteins of HIV–1 can be characterized by their individual function into four groups: structural (MA, CA, NC and p6), enzymatic (PR, RT, IN), regulatory (tat and rev), and accessory proteins (Vif, Vpr, Vpu and Nef) are described in this section.
Figure 1.1 Genomic map of HIV-1. The HIV-1 and SIV genome is compromised of nine genes and is flanked with identical long terminal repeats (LTRs) at each end of the proviral genome. The nine genes encode fifteen proteins that are required for HIV-1/SIV pathogenesis. The structural genes are comprised of \textit{gag}, \textit{env} and \textit{pol}. The regulatory proteins made up of are \textit{tat} and \textit{rev}. The HIV–1 accessory genes are \textit{vif}, \textit{vpr}, \textit{vpu} and \textit{nef}. SIV encodes the same structural and regulatory proteins but differ in the accessory genes, which are \textit{vif}, \textit{vpx}, \textit{vpr} and \textit{nef}. Each of these genes are described in the text. Figure adapted from (16).
HIV-1 Gag is necessary for the assembly of virions, maturation of virions after particle budding, and stimulating virus replication post entry. The gag gene encodes the Pr55\textsuperscript{gag} (Gag) polyprotein precursor and is cleaved by the viral protease upon particle maturation yielding separate viral proteins: the matrix protein (MA/p17), capsid (CA/p24), nucleocapsid (NC/p7) proteins, p6, and two additional spacer peptides, p1 and p2. The MA domain directs Gag to the cellular membrane and facilitates the incorporation of Env glycoproteins into nascent virions (17, 18). The CA domain is downstream of MA and is important in packaging Gag–Pol precursors and cyclophilin A (CypA) into nascent particles to form the viral core (19). CA promotes viral replication by its interaction with CypA facilitating HIV–1 genome delivery into target cells (20). NC tightly associates with unspliced viral RNA at the packaging signal guiding its dimerization and encapsidation (21). In the virus life cycle, NC plays a role in virus assembly with tight packaging of Gag and efficient Gag membrane trafficking and binding. Additionally, NC contributes to efficient reverse transcription and preintegration complex (PIC) stability (22). P6 incorporates Vpr into virions and is required for efficient virus release by interacting with the Tsg101 host protein (23, 24).

The HIV envelope protein is critical for viral entry. The env gene encodes a single gp160 protein, which undergoes intracellular processing, and gives rise to the glycoprotein complex: the surface unit (SU) gp120 and transmembrane unit (TM) gp41. The gp120 protein binds to the CD4 receptor and subsequently associates with the chemokine receptors CXCR4 or CCR5. The gp41 binds gp120 and this complex
undergoes conformational changes to promote fusion of the viral particle and target host cell lipid membranes allowing for viral entry (25).

A negative ribosomal frameshift error during Gag translation events leads to the synthesis of the pol gene encoding three enzymes: protease (PR), reverse transcriptase (RT) and integrase (IN). PR requires dimerization to be active and it is possibly its own first substrate so that it can be released and continue with the processing of the Gag and GagPol precursors that follows (26). Polyprotein processing is the key step in virion maturation and produces changes to the morphology of the viral particle that is required for virus infectivity.

Following the PR protein domain, RT encodes an RNA–dependent DNA polymerase responsible for producing new double stranded DNA from the viral single–stranded RNA in infected cells (Reviewed in ref. (27)). RT biochemical activities include RNA– and DNA–dependent polymerase activity and RNaseH ribonuclease activity. During DNA synthesis a RNA:DNA hybrid is formed and RNaseH specifically degrades the genomic RNA template required for the completion of the process. Throughout viral replication, RT causes a high rate of mutations, as it is an error prone polymerase (28).

After viral DNA is synthesized in the cytoplasm by reverse transcription, IN binds the double–stranded viral DNA and is imported into the nucleus for subsequent integration into the host genome. IN forms an active intermediate of the viral DNA by removing two nucleotides from the 3’ end of the blunt ended linear viral DNA. The 3’ ends of the viral DNA are covalently attached to the host chromosome, in a process referred to as DNA–strand transfer. The host machinery repairs the gaps at the 5’ ends of the viral DNA completing the provirus integration process.
Tat and Rev are two regulatory proteins that enhance the expression of viral genes and are necessary in HIV–1 replication. As one of the earliest expressed viral proteins, Tat, functions to stimulate the LTR–directed transcriptional elongation with binding to the transactivation response (TAR) in the 5’–LTR of nascent HIV–1 mRNAs and recruiting a cyclin T1/CDK9 complex to hyper–phosphorylate the C–terminal domain of RNA polymerase II (Pol II) (29, 30). Rev mediates nucleocytoplasmic export of unspliced and partially spliced viral transcripts containing the Rev responsive element (RRE) (31, 32). Tat and Rev are essential for the synthesis of full–length HIV–1 transcripts and required for a complete viral replication cycle.

The HIV–1 genome encodes four additional “accessory” proteins, viral infectivity factor (Vif), viral protein u (Vpu), viral protein r (Vpr), and negative factor (Nef). The term “accessory” proteins were given because their absence has little bearing on viral replication in cell cultures. However, recent studies have revealed that they support viral replication in vivo. Vif counteracts antiviral effect of apolipoprotein B mRNA–editing enzyme catalytic polypeptide–like 3G (APOBEC3G) to enhance virus infectivity (33). Vpu overcomes host protein B cell stromal factor 2 (BST-2) or tetherin’s ability to prevent virus release and also is involved in the down–regulation of intracellular CD4 expression (34, 35). In macrophages, Vpr enhances viral replication probably by facilitating nuclear import of the HIV–1 PIC and arresting cells in the G2 phase of the cell cycle (36–40). Nef is important in multiple functions linked to pathogenicity and disease progression in vivo. Nef is the main focus of my thesis research and will be reviewed in detail in Chapter 1, 3 and 4. The accessory proteins encoded by the SIV genome are Vif, Vpx, Vpr, and Nef. Vpr and Vpx have been reported to play a role in
enhancing viral replication in myeloid cells (41, 42). Vpx specifically targets SAM domain HD domain-containing protein 1 (SAMHD1) for degradation and depletion of the deoxynucleotide triphosphates (dNTPs) pool needed for transcription (43).

1.1.3 Replication Cycle of HIV–1

The HIV–1 replication cycle is a multi–step process compromised of events divided into two distinct phases: the early phase and the late phase. The early phase includes the critical steps of viral attachment, viral membrane fusion and penetration, viral uncoating, reverse transcription, PIC nuclear import and viral DNA integration. The late phase includes viral RNA production, nuclear export, viral protein translation, virion assembly, budding and maturation. The HIV replication cycle is depicted in Figure 1.2.

HIV can enter target cells (macrophages and CD4+ T cells) through the Env glycoproteins that are embedded in the membrane of the virus and able to bind specific receptors on host target cells. The viral attachment step begins when the viral gp120 protein binds to the CD4 receptor on the host cell. This interaction induces a conformational change to expose the chemokine binding domains of gp120 and subsequent binding to the host CCR5 or CXCR4 co–receptor. Co–receptor binding initiates gp41 enabling fusion of the viral and the target cell plasma membrane (44).

The viral core is delivered into the cytoplasm of the target cells and is uncoated to expose the reverse transcription complex (RTC) composed of the viral genomic RNA and the viral proteins (NC, RT, IN, Vpr, and MA) that remain to be associated with it (19, 45). The genomic RNA is reverse transcribed by RT into a double–stranded proviral cDNA copy, referred to as PIC. The cellular nuclear transport machinery transports PIC
across the nuclear envelope and the viral cDNA is integrated by IN into the host chromosome, resulting in the permanent integration of HIV–1 in the infected host cell (46, 47).

The late phase of the replication cycle begins with the transcription of integrated proviral DNA and is associated with the activation state of the cell. The host RNA polymerase II synthesizes HIV mRNA transcripts from the 5’ LTR promoter in the proviral template, which is greatly enhanced by Tat (48). Partially spliced and unspliced viral mRNAs containing the RRE sequence are bound and transported by Rev into the cytoplasm via the cellular nuclear export machinery (48). Translation of viral proteins occurs yielding the expression of Env, Gag and Gag–Pol polyprotein precursors that gather at the plasma membrane for assembly and budding. In macrophages and dendritic cells, membranes of intracellular compartments are thought to be the site of viral assembly. The viral genomic RNA is recognized by NC at the Psi site and is subsequently packaged into virions that are newly assembled. As the virus is released, the virion maturation is finalized as Gag and Gag-Pol undergo proteolytic processing by PR to form a distinctive condensed, conical core of the mature HIV–1 infectious virions (49).
Figure 1.2 HIV–1 replication cycle. The HIV–1 replication cycle is compromised of the following main steps: virus entry, uncoating of the capsid core and release into the cytoplasm, reverse transcription of the genomic RNA, dsDNA is imported into the nucelus, integration of the proviral DNA into the host chromosome, exportation of the transcribed mRNA, translation of viral proteins followed by assembly, budding of new virions from the cell and maturation. Figure adapted from (50).
1.1.4 Immunopathogenesis of HIV–1 Infection and Elite Control

The CD4 receptor is the main receptor required for HIV–1 infection. This receptor is highly expressed on T helper lymphocytes and at low levels in dendritic cells, monocytes, and macrophages. The second interaction required for infection is the interaction of gp120 with the chemokine co-receptor CCR5 or CXCR4 on a target cell, and is dependent on the amino acid sequence of the viral envelope (51). The chemokine receptor usage by a virus can determine its cellular tropism. Macrophage tropic, termed M–tropic or R5–tropic, strains use the CCR5 receptor and T–tropic, termed R4–tropic, strains use the CXCR4 co–receptor for entry. There are dual tropic (X4R5) strains that can infect both cell types. The majority of infected individuals at the primary stage are infected with R5–strains, where X4–strains appear in the late stages of infection as CD4+ T cell population sharply decreases.

In a typical progressor infected with HIV, infection can be separated into three stages: acute infection, chronic infection and AIDS. The earliest stage of HIV infection is the acute infection and generally develops within 2 to 4 weeks after an individual is infected. Upon infection, there is a drastic loss of CD4+ T cells in the peripheral blood and an initial peak of viremia. Virus replication decreases with the emergence of anti–HIV antibodies and cytotoxic T lymphocytes (CTL) resulting in a viral set–point. This robust early immune response eventually fails to abolish the virus since most individual’s transition to the chronic phase of infection.

During the chronic phase there is partial control of viral replication and systemic CD4+ T cell depletion in the peripheral blood. HIV is actively replicating in the lymphoid organs resulting in its destruction and deterioration of the immune system. There is a
steady decline in the naïve and memory T-cell population and persistent immune activation occurs. Approximately 9–10 years after the primary infection the immune system can no longer control the virus, the virus rebounds and the decline in CD4+ T cells is accelerated. Opportunistic diseases develop resulting in AIDS and the patient dies. Similar disease progression is seen with SIV infection in a rhesus macaque, but in a shorter time span of 2 years.

There are a small subset of individuals (less than 5%) that are HIV–infected and control viral replication in the absence of retroviral therapy, termed long–term non–progressors (LTNP) or elite controllers (EC) (52). Long–term non–progressors experience low level of viremia and normal CD4+ T cell counts without antiretroviral treatment for more than 5 years. However, these individuals eventually develop AIDS. Elite controllers are able to completely control viral replication, remain undetectable with clinical assays, and do not display any disease progression (52). Both of these individuals have a preserved immune system or lymphoid tissues. It is not completely understood why these individuals, in the absence of therapy, can control disease progression. However, there is an association with expression of certain protective HLA class I alleles, in humans and macaque models (52, 53). Peptides are presented to CD8+ T cells by class I molecules and therefore it is possible that protective CTL responses can be elicited by over–representing certain class I molecules, which may be the situation in these patients. Unfortunately, not all EC expressing protective HLA class I alleles control disease. Furthermore, EC tend to have “polyfunctional” CTL in mucosal tissue and peripheral blood, and tend to have CTL that are more efficient at lytic granule loading and granzyme B delivery to kill infected CD4+ T cells (54-56). This may explain the
ability of these over-represented CTLs to control disease in these individuals. Understanding the mechanisms of elite control may provide valuable insight into eliminating HIV.

1.2 Nef

1.2.1. Nef and HIV–1/SIV Pathogenesis

NEgative Factor (Nef), is a non-structural viral accessory protein encoded by HIV and SIV and contributes to viral pathogenesis in the infected host. It is essential for optimal viral replication, accelerated disease progression in vivo, and required for eventual collapse of the immune system (57, 58). In fact, juvenile rhesus monkeys infected with SIV containing mutations disrupting Nef function control viral loads to a low level and severely attenuate the development of AIDS (59, 60). Furthermore, infecting rhesus macaques with SIV strains containing a HIV-1 nef were capable of efficient viral replication and exhibited a significantly higher progression to AIDS (61, 62). Comparably, inactivating deletions or point mutations in nef can be detected in HIV–1 infected human non-progressors (63-67). Thus, despite the difference in sequence, HIV–1 and SIV Nef proteins seem to be functionally similar.

1.2.2. Nef Gene and Protein Structure

The nef gene is located at the 3’ end of the env gene and overlaps with the 3’–LTR. It encodes a 27–35 kDa myristylated protein and is one of the first viral proteins synthesized (68). Nef is predominantly located in the cytosol but a third of the Nef proteins are membrane–associated (69, 70). Additionally, Nef is located in virus particles and specifically processed by the viral protease (71, 72).
Nef is an accessory protein with a multitude of molecular functions. The variety of motifs in its sequence allow for interactions with a variety of binding partners that contribute to the post–translational modification of Nef, altering of signaling events and trafficking. The Nef protein can be comprised of two domains that can be cleaved by PR and separated into a variable N–terminal membrane anchor region and a highly conserved core domain. Additionally, there is a C–terminal flexible loop important for interactions with diverse host cellular proteins including adapter proteins, coatomer proteins and a vacuolar ATPase (reviewed in (73)).

The N–myristoylation of Nef is required for cell membrane attachment and many of its biological functions. The first amino acid sequence (MG$_{xxx}$(S/T) in the N–terminal arm of HIV–1 Nef is a substrate for N-myristoyl transferase proteins, as all myristoylated proteins begin with the sequence Met-Gly (74). The initiating methionine is removed and the glycine at residue 2 is the site where a myristate is covalently linked resulting in the stabilization of a second alpha helix. Alternatively, if Nef is not myristoylated the N–terminal domain is left unstructured with one single short alpha helix (75). An additional modification of Nef results from cleavage by the viral protease between residues W57 and L58 within the virion separating Nef into the anchor and core domain (76). The HIV–1 Nef domain structures have been described in crystal form and in NMR solution and crystal form (75, 77-79).

The core domain of Nef forms well–defined structures that are highly conserved and relatively stable permitting for interactions with a variety of cellular proteins. The N–terminal region in the Nef core domain contains a proline rich motif (P$_{xx}$P) that interacts with tyrosine kinases containing a SH3 domain, such as Hck, Lck, Fyn and Vav (77, 78,
The ability of Nef to stimulate cell activation and promoting viral replication in resting cells, is dependent on these protein interactions (83, 84). When a SH3 domain binds to this motif in Nef, a left–handed polyproline helix II shape is formed. Downstream from the polyproline helix, two–pairs of anti–parallel α–helices strands containing a flexible loop twist a central beta sheet structure producing two short α–helices, resulting in an α–β structure (85). The core domain also contains another long flexible loop between β3 and β4, and is likely open to bind the β– or μ–subunit of adaptor proteins (86).

1.2.3. Nef’s Cellular Functions

The HIV Nef protein is a multifunctional protein that does not have enzymatic or structural function. Instead, it is key in manipulating host cell signaling and trafficking pathways in an infected cell. Nef contains several effector domains and can physically interact with certain cellular proteins in various pathways to alter normal biological activity. Thereby, contributing to viral spread by enhancing viral replication, infectivity, and down–regulation of cell surface markers to evade the anti–viral immune response. This section will discuss the individual cellular functions of Nef.

1.2.3.1. Infectivity Enhancement

The importance of Nef in viral replication stems from studies suggesting a Nef function in increasing the infectivity of progeny viral particles that is dependent on its association with the cellular membrane. Specifically, abrogating the myristoylation of Nef or disrupting its membrane targeting can result in dramatic decrease in infectivity (51, 69, 72). More so, the attachment of Nef to lipid rafts may also enhance infectivity of virus particles (69). However, this theory is still debated since a Nef mutant that highly
increases its incorporation into lipid rafts did not further increase virion infectivity (87, 88). The presence of Nef may be required during the production of virions to enhance infectivity. Expressing Nef after virion production does not rescue infectivity, indicating Nef may alter virions and change their behavior throughout target cell infection (89). Moreover, enhancement of infectivity is completely inhibited when the incorporation of Nef into virions is prevented by fusing the N–terminus of Nef to Cyclophylin A, a protein that interacts with Gag and is important in virion incorporation (90). Additionally, delivery of Nef into virions after cleavage by viral protease in a Nef–Cs–Vpr fusion construct did not result in the increase of infectivity (91).

Infectivity of HIV–1 viruses with different cellular tropism, both X4– and R5–tropic envelopes, are enhanced by Nef and is dependent on the virus entry pathway (92). The ability of Nef to enhance HIV–1 infectivity can be complemented by disrupting the actin cytoskeleton, but pseudotyping virus particles to enable entry into cells via an endocytosis–dependent pathway at a low pH naturally penetrated the cortical actin barrier (93). Moreover, Nef is no longer required for optimal infectivity when HIV–1 virus particles are VSV–G– and Ebola–GP–pseudotyped, resulting in the entry of virions via endocytosis (94).

The interaction of Nef with cellular proteins can also contribute to enhancing viral infectivity. For instance, the infectivity of Nef is impaired when disrupting the dileucine motif in Nef (L\textsubscript{164}/165L) that is responsible for AP–mediated CD4 down–regulation (95). Furthermore, the interaction of Nef with Dynamin 2 results in clathrin–dependent endocytosis and is responsible in optimizing virus infection possibly by altering the
amount of lipid of the viral envelope (96). However, disrupting the association of Nef with p21–associated kinase 2, PAK2, can still enhance infectivity of viral particles (92).

### 1.2.3.2. Cell signaling

#### 1.2.3.2a. PAK2 Activation

PAK2 is a serine kinase complex that is a well defined Nef associating kinase (NAK) (97). A variety of cellular stresses induce PAK2 activation resulting in the regulation of multiple cellular activities, including: reorganization of the cytoskeleton, cellular mobility, gene transcription, program cell death, and changes in the morphology of a cell (98-101). Nef specifically mediates the activation of PAK2 and induces the formation of a Nef/activated PAK2 complex that is poorly understood and whose components have been difficult to identify. (102, 103). This interaction is conserved amongst diverse primate lentiviral groups (104, 105).

In HIV–1 infected T cells, the Nef–PAK2 complex may induce activation and possibly enhance viral replication (106-110). This complex has been proven to induce the expression of Fas–Fas ligand (111). Multiple studies have identified important residues involved in the interaction of the Nef–PAK2 complex. PAK2 binding is highly dependent on residues R104 and R105, and an accurate structure of the Nef core domain. Unfortunately, altering these residues do not specifically prevent PAK2 binding, but can lead to disruption of multiple Nef functions (112, 113). A more recent study has identified residues 85, 89, 187, 199 and 191 in a hydrophobic binding surface of Nef that is selectively important for PAK2 association, dispensable for down–regulation of CD4 or MHC class I molecules (114). Moreover, residue F191R is important in disrupting the
association of Nef with PAK2 and specifically impairing the enhancement of viral replication in P4–CCR5 cells (101). This residue did not alter the ability to activate T cells

1.2.3.2b. HCK

Multiple Src family tyrosine kinases (SFK) have been reported to interact with Nef. The Nef P72xxP motif has affinity for the Src homology (SH3) domain and has been shown to interact with Hck, Lyn, Lck, Fyn and Src (103). The interaction with Hck is best characterized and shows the highest affinity. A triple amino acid substitution in the “hydrophobic pocket” of Nef can result in HIV–1 and SIV Nef to target different Src family kinases (115). HIV–1 targets mostly Lyn and Hck, and SIV favors Src and Fyn (115).

Nef binds to Hck, a Src family member, mainly expressed in macrophages/monocytes (116). The interaction of Nef with Hck leads to an increase in Hck catalytic activity and constitutive activation, possibly contributing to macrophage survival and possibly HIV–1 replication (117, 118). In the presence of Hck, Nef significantly inhibited the activity of macrophage colony–stimulating factor (M–CSF) because of the down–regulation of the M–CSF receptor (119). The Nef–Hck interaction may contribute to the development of AIDS, as seen in a study completed in HIV–1 transgenic mice expressing an intact Nef gene. These mice were crossed with an hck−/− background and showed a drastic delay in AIDS progression (120). It is not currently known how Hck activation may contribute to HIV–1 pathogenesis.
1.2.3.3. Dysregulation of Immune Cell Receptors

As a multivalent adapter protein, Nef can alter the signaling and trafficking of multiple immune cell surface receptors including the following: CD3, CD4, CD8, MHC–I, CD1a, CD1d, CD74, MHC–II, DC–SIGN, mannose receptor, tumor necrosis factor, CD80, CD86, transferrin receptor, CTLA–4, hemochromatosis protein HFE, CCR5 and CXCR4 (reviewed in (121)). In this section, I will focus on the mechanisms of Nef–induced CD4 and MHC–I down–regulation.

1.2.3.3a. CD4

The down–regulation of CD4 molecules on the cell surface is the first most extensively studied effect of Nef (122, 123). CD4 is expressed on a subset of T lymphocytes and cells of the macrophage/monocyte lineage. CD4 receptors bind peptide bound MHC complexes (pMHCs) on the surface of antigen presenting cells (APCs). This interaction is critical in the development and function of the immune system, specifically in T cell antigen recognition and activation. However, if CD4$^+$ T cells are depleted, the body would no longer be able to clear standard infections and become vulnerable to a wide range of opportunistic infection due to immune dysfunction, as seen in AIDS.

CD4 is the primary co–receptor for HIV–1, HIV–2 and SIV infection. The binding of virions to CD4 initiates the viral entry process into the target cell of interest. HIV may benefit from down–regulating its own CD4 receptor to prevent super–infection and stimulate viral particle release, resulting in an infection that is productive but controlled (124-127).
The mechanism in which Nef down-regulates CD4 has been extensively studied, but not fully elucidated. One model implicates Nef assisting in the removal of CD4 molecules from the surface of the plasma membrane through endocytosis (128-130). The other model suggests Nef plays a role in altering normal sorting of newly synthesized CD4 from the trans golgi network (TGN) to the plasma membrane (131, 132). Instead CD4 molecules are redirected to lysosomes and degraded. The exact mechanism of Nef-mediated CD4 down-regulation is uncertain.

It is widely accepted that the N–terminal myristoylation signal of Nef localizes it to membranes (75). The Nef amino acids centered at W_{57,58}L and L_{164,165}L dipeptide on Nef are necessary for CD4 down-regulation in both of these models in host cells (95, 129, 133-137). Specifically, in the folded region of Nef the W_{57,58}L residue is important in Nef’s physical interaction with the cytosolic tail of CD4 (138). CD4 down-regulation is also dependent on clathrin and involves the direct interaction of Nef with adapter protein (AP) complexes, AP–1 or AP–2 (95, 129, 133, 134, 138). The recruitment of adaptor complexes are dependent on a dileucine motif (L_{164,165}L) found in a Nef flexible loop (129). Each adapter protein mediates CD4 down-regulation differently. The AP–2 protein is localized at the plasma membrane and mediates the capture of CD4 molecules expressed on the cell surface into clathrin–coated vesicles, resulting in increased internalization of CD4 into endosomes (139). Alternatively, AP–1 interacts with clathrin–coated vesicles and transports proteins between the TGN and endosomes preventing the expression of newly synthesized CD4 molecules on the surface of the cell by sorting it directly to endosomes (129). In the endosome, the E_{155}E diacidic sequence in Nef mediates the binding of beta subunit of COPI coatomers (beta-COP) routing Nef–bound
CD4 molecules to the lysosome for degradation (140). Most recently, the ubiquitination of HIV–1 Nef at residue K_{144} is required for CD4 down–regulation (141).

1.2.3.3b. MHC–I

The HIV–1 Nef protein interferes with the normal trafficking of MHC–I molecules to the cell surface leading to the disruption of antigen presentation, and in turn, reduced recognition by CTL and elimination of infected cells (142). CTLs are a very important part of the adaptive immune system and in the elimination of intracellular pathogens. The T cell receptor (TCR) of a CTL can recognize “non–self” viral peptides presented by MHC–I molecules on the surface of an infected cell. Upon recognition, the CTL is activated and releases perforin and granzymes to kill the infected target cell and control viral infection by preventing viral spread (reviewed in (143)). All nucleated cells express three classical MHC–I genes (HLA–A, HLA–B, and HLA–C) that are very polymorphic encoding many alleles. A less polymorphic non–classical MHC–I gene, HLA–E is also expressed by the same cells. HLA–A and HLA–B are important in presenting antigen to CTLs, and HLA–C and HLA–E are important in inhibiting natural killer cell (NK) function (reviewed in (144)). Nef can selectively bind to a sequence present in the cytoplasmic tail of HLA–A and HLA–B leading to its down–regulation and evasion from CTL recognition (145). Variations in the cytoplasmic tail of HLA–C and HLA–E prevent Nef interaction and protects from NK cell lysis (145, 146).

The molecular mechanism of Nef–mediated MHC–I down–regulation has become more defined but is not completely understood. In HIV–1, Nef–mediated MHC–I down–regulation requires Nef to bind directly to a conserved tyrosine residue Y320 in the B7 heavy chain of the cytoplasmic tail of MHC–I (147). The three essential domains in Nef
include the following: polyproline (P$_{72/75/78}$P), a N–terminal α–helix (R$_{17}$ERM$_{20}$RRAEPA$_{26}$ and precisely M$_{20}$A), and an acid cluster (E$_{62-65}$E) (147). Presently, two models of MHC–I down–regulation exists involving disrupting the trafficking of newly synthesized MHC–I molecules by different pathways. The first model of MHC–I down–regulation involves the interaction of Nef with phosphofurin acidic cluster sorting proteins (PACS–2) (148, 149). There is evidence that the Nef acidic cluster (E$_{62-65}$E) interacts with PACS–2 targeting Nef to the paranuclear region where a multi–kinase cascade is assembled. The Nef polyproline (P$_{72/75/78}$P) region can now bind and activate the TGN–localized Src family kinase (SFK), to phosphorylate zeta chain associated protein 70kDa (ZAP–70) and subsequent recruitment of phosphoinositide 3–kinase (PI3K) resulting in the loading of GTP to ARF6 (148, 150). Therefore, PACS–2 is essential for Nef sorting of MHC–I molecules to the TGN/endosomal system and preventing its expression on the plasma membrane.

The alternate model of MHC–I down–regulation is based on the interactions of HIV–1 Nef, AP–1, MHC–I complex. Nef strongly interacts with the μ subunit of AP–1, which is necessary for down–regulation of MHC–I (151-153). However, AP–1 does not directly bind to the cytoplasmic tail of MHC–I. Therefore, the complex may be important in MHC–I down–modulation by targeting newly synthesized MHC–I protein from the TGN to lysosomes leading to its degradation (152, 153). Interestingly, the complex requires the same E$_{62-65}$E and P$_{72/75/78}$P motifs as the PACS model.

SIV Nef, like HIV–1 Nef, have the ability to down–regulate the cell surface expression of MHC–I molecules (135, 142, 154, 155). Both Nef proteins have the capability to interact with the same adapter protein complexes (AP–1 and AP–2) and
protein serine kinases to alter protein sorting and signal transduction mechanisms (149, 150, 153, 155, 156). Furthermore, the conserved Y320 residue in MHC–I molecules is required for both Nef proteins to increase the rate of endocytosis of the MHC–I complex (157).

The important residues in Nef required for MHC–I down–regulation differ in SIV. The C–terminal sequence in SIV–Nef is required for the down–regulation of MHC–I, unlike the P_xP helix in HIV–1 (135, 154). Disrupting the P_xP motif in SIV–Nef is not essential for this function. In the SIVmac239 clone (239–Nef), residue Y_{223} in the C–terminus of Nef is specifically responsible for disrupting MHC–I surface expression (158). In addition, mutating residue Y_{223} from a tyrosine to a phenylalanine reveals that this residue is dispensable for other 239–Nef functions, and does not affect the Nef to interaction with AP–1 or AP–2 (157). Moreover, mutations in other regions, such as D_{155}L, G_{238}*, and Y_{226}A, of the C–terminus can disrupt interactions of Nef with cellular factors and direct binding to MHC–I heavy chains. The binding of AP–1 and AP–2, and the down–regulation of MHC–I, are separate functions of 239–Nef since disrupting the interaction with AP–2 does not interrupt MHC–I down–regulation (157). Interestingly, SIV strains isolated from other simian species, for examples, SIVsyk, SIVagm, and SIVlhoest, encode Nef proteins that are similar to HIV–1 Nef with a shorter sequence lacking a C–terminal region or an N–terminal residues important in AP–2 interaction, as seen in 239–Nef (135, 154).

1.3. Macrophage and HIV–1 / SIV Infection

The primarily targets of HIV–1 infection are CD4^+ T cells and macrophages. During HIV–1 infection, a decline in CD4^+ T cell numbers is observed and strongly
correlated with the risk of immune dysfunction and the development of opportunistic infections. Monocytes and macrophages also play important roles as HIV reservoirs (159). Monocytes can circulate throughout the body and enter into anatomical compartments and tissues, while only 0.01–1% of monocytes contain proviral DNA (160), they may persist in sites where penetration of antiretrovirals is limited and establish viral reservoirs (161, 162). An infected macrophage can survive for long periods (weeks to months) and store infectious virions in cytoplasmic compartments that are capable of transmitting virus *in trans* (163, 164).

Macrophages play an important role in the pathogenesis of HIV–1/SIV. With the ability of monocytes to cross the blood–brain barrier, differentiation into macrophages can establish infection in the central nervous system (CNS) (165). Infection of microglia and perivascular macrophages actively mediate entry of HIV–1 into the CNS, resulting in HIV–associated neurocognitive disorders (HAND) including, HIV–associated dementia, encephalitis, cognitive disorder, peripheral neuropathy (166). Rhesus macaques infected with a highly pathogenic hybrid simian–human immunodeficiency virus (SHIV) resulted in the rapid depletion of CD4+ T cells such that macrophages were the principal reservoir that sustained high viral loads in the SHIV–infected animals (167). In another study, rhesus macaques depleted of CD4+ T cells exhibited higher viral loads than non–depleted animals, *in situ* staining performed on gut tissue revealed that macrophages were the primary source of viral replication (168). Additionally, artificial depletion of CD4+ T cells in SIV–macaques revealed extensive infection of macrophages in the lymph nodes
and mucosal tissues, and microglial cells in the brain (169). It has also been suggested that the level of monocyte turnover is responsible for disease progression in the macaque model of AIDS (170).

The viral accessory protein, Nef, has been shown to be important in the infiltration of myeloid cells into the brain which is associated with HIV–associated dementia (HAD) in infected individuals (171). Interestingly, in animal models injected with Nef into the brain revealed an increase in the migration of monocytes and macrophages to that site (172). A study by Spoerer et al. found that Nef uses a matrix metalloproteinase–9 mechanism to disrupt the blood brain barrier (BBB) (173). Other groups, revealed an increase in monocyte migration across the BBB by Nef–expressing astrocytes with up–regulated CCL2/MCP–1 chemokine secretion (174-176). Additionally, neuropathogenesis of HIV–1 is linked to Nef–induced apoptosis in primary microvascular endothelial cells in the brain (177). Furthermore, evaluating CNS– and lymphoid–derived HIV–1 isolates have revealed conserved functions in Nef alleles that include CD4 and MHC–I down–regulation, which may be important in inhibiting recognition by CD8+ T cells and viral dissemination (178).

Although, viremia present in the peripheral blood can be suppressed with HAART treatment, HAND is a complication many HIV–infected individuals continually suffer with (179). Despite HAART, ongoing HIV replication and immune activation can be detected in the CNS (180-182). This phenomenon may be facilitated by the low level of antiretroviral penetration observed in the cerebrospinal fluid (183, 184). The dissemination of the virus in the CNS reservoir may be mediated by cell–to–cell transfer of the virus, but this has not been confirmed. As such, macrophages may be relevant to
strategies aimed at elimination of HIV–1 from infected individuals. In this regard, researchers have begun to explore approaches to eradicate persistent viral reservoirs. One method is to stimulate viral activity in the reservoir with the hope that the HIV–infected cells are eliminated by viral cytopathic effects and/or cell–mediated immune clearance (185).

HIV cell–to–cell transfer is several thousand fold more efficient in spreading virus between cells than cell–free virion infection (186, 187). Specifically, virological synapses are normally formed among cells that express HIV–1 co–receptors via, such as T–cells and T–cells or macrophages (188-190). Infected macrophages can persist for several weeks and possibly infect more than one T–cell every 6 h, proving that they may contribute greatly to dissemination of HIV viral particles (164, 191). Moreover, infected macrophage expressing Nef can permit infection in resting lymphocytes via the CD40L signaling pathway (192). An alternate cell–to–cell infection method of macrophage involves the phagocytosis of HIV–1 infected CD4+ T cells (193). Interestingly, macrophages have the ability to selectively capture and engulf HIV–1 infected CD4+ T cells through an Env–receptor independent mechanism resulting in a productive infection (194). HIV–1 can also be transmitted via bodily fluids in which macrophages are prevalent, including vaginal secretions and semen (195).

Macrophages present unique obstacles to infection by primate lentiviruses. Some studies suggest that macrophages are resistant to the cytopathic effects of viral replication in comparison to activated CD4+ T cells (167, 195-197), and that HIV–1 has evolved mechanisms to prolong the life span of infected macrophages (197, 198). The expression of cell surface CD4 is much lower in macrophages than T–cells, this may be important in
not only limiting viral entry but also superinfection. The lower cytopathic effect may be associated with the limited dNTP pool in macrophages resulting in slow reverse transcription kinetics and virus life cycle, following postentry viral replication events (199). This has to be accommodated in order for primate lentiviruses to establish a productive infection (200, 201). In infected macrophages, the assembly of virions have been observed in intracellular vesicle–like compartments enabling virus particles to hide and possibly evade the host immune responses (202, 203). Therefore, as HIV–1 infection progresses to the late stage and most CD4\(^+\) T cells have been depleted, macrophages may still be present.

HIV–1 infection induces anti–apoptosis signaling in macrophages and, in turn, prompts apoptosis of T cells. Upon infection, macrophages release pro–survival cytokines in an Env–mediated mechanism responsible in maintaining cell survival in the face of apoptotic stimuli (197). Apoptotic sensitivity was restored when those pro–survival effectors were inhibited and HIV–1 infected macrophages succumb to apoptosis (197). Moreover, NF–κB levels are higher in persistently infected macrophages that are resistant to TNFα–induced apoptosis (204). In turn, macrophages can determine the fate of T cells. Macrophages have the ability to attract CD4\(^+\) and CD8\(^+\) T cells with the release of CC–chemokines (204). Once activated, the HIV–1 infected macrophages can up–regulate the cell surface expression, and secreted form, of TNFα involved in inducing apoptosis in susceptible CD8\(^+\) T cells (205, 206). During HIV–1 infection, apoptosis of CD8\(^+\) T cells can result from the interaction of TNFR2 on the surface of CD8\(^+\) T cells and TNF–α on the cell surface of activated macrophages, this may be a result on the decreased expression of anti–apoptotic proteins in CD8\(^+\) T cells (207). Macrophages may
up–regulate FasL upon infection and induce apoptosis in CD4+ T cells (206, 208). Thus, macrophages are long–term survivors capable of establishing and maintaining a continuous hidden virus reservoir in HIV–1 infection.

1.4. CD8+ T Cells in HIV/SIV Infection

CD8+ T cells have been demonstrated in reducing HIV–1 viral load shortly following transmission and delaying the onset of disease. Particularly, antigen–specific CD8+ T cell responses were associated in the rapid control of acute phase HIV–1 viremia (209). In 1986, the first in vitro study revealed HIV–1 viral replication could be suppressed with the addition of CD8+ T cells (209). Subsequent reports using an ex vivo model in infected individuals, revealed improved clinical prognosis correlated with the reduction of HIV replication and higher HIV–specific CD8’ T cells responses (210, 211).

The discovery of the similar disease progression in the macaque model of SIV–infection and that of HIV–1, enabled scientists to study the biology of HIV–1 infection and the contribution of the host cellular immune responses (3, 212, 213). Studies, in which CD8+ T cells were depleted from non–human primates infected with SIV showed a dramatic increase in virus replication and accelerated disease progression (214, 215). Unfortunately, CD8’ T cells exert selective pressure on the virus and “virus escape” mutations can develop, preventing the binding of the epitope to MHC–I molecule and elimination of viral control (216, 217). Studies completed in long–term non–progressors and elite controllers revealed that certain HLA or MHC class I alleles are enriched and is associated with HIV–1 control. HLA–B*57, and to a lesser extent, HLA–B*27 alleles in humans and Mamu–B*08, Mamu–B*17, and Mamu–A*01 in rhesus macaques are
overrepresented in individuals that naturally control HIV or SIV replication (218-222). Not every individual expressing these alleles control virus replication, but have a higher probability of mounting the effective CD8+ T cell responses. Furthermore, a dramatic rise plasma viremia was observed in rhesus macaque elite controllers after the in vivo depletion of CD8+ T cells, which is rapidly reversed by the re-emergence of SIV-specific CD8+ T cells (214, 215, 223, 224). Moreover, the TCR repertoire of the responding CD8+ T cell population may also strongly influence an individual’s ability to control infection and disease progression (225).

In addition to the antigen specificity of the CD8+ T-cell response, the ‘quality’ of the virus–specific CD8+ T cells is equally important in controlling HIV replication (226, 227). In addition to having a greater quantity of virus–specific CD8+ T cells, elite controllers also have a portion of CD8+ T cells that are polyfunctional and may contribute significantly in the ability to kill HIV–1 infected CD4+ T cells compared to HIV–specific CD8+ T cells from progressors (55). These factors include the capability of rapid proliferation, up-regulation of perforin and granzyme B, lytic granucale loading, low PD-1 expression, and secretion of multiple chemokines or cytokines including secrete IFN gamma, TNF alpha, IL–2 CCL3 and CD107a (reviewed in (228)). Aside from this, little is known about the mechanisms by which this control of viral replication by CD8+ T cells occurs.

HIV–1 infected macrophages seem to have developed mechanisms to evade CD8+ T cell mediated control of viral replication in infected macrophages. Understanding how CD8+ T cells control viral replication in CD4+ T cells has been the focus of many studies, but little attention has been paid to understanding CD8+ T cell mediated control of viral
replication in infected macrophages. Previously, CD4+ T and CD8+ T cell lines or clones have been shown to rapidly suppress viral replication in HIV/SIV–infected macrophages (229, 230). However, less is known about the ability of unstimulated primary CD8+ T cells to control viral replication. A recent report demonstrated that freshly sorted SIV–specific CD8+ T cells have the ability to suppress viral replication and eliminate SIV–infected CD4+ T cells after 2 days of infection, but are not able to eliminate SIV–infected macrophages (231). Another study demonstrated that viral replication in HIV–1 infected macrophages could be suppressed 5–7 days after infection by ex vivo freshly sorted bulk CD8+ T cells (232). In order to generate CD8+ T cell lines, B–lymphoblastoid cell lines (BLCLs) are repeatedly pulsed with peptide and CD8+ T cell lines are repeatedly stimulated in vitro and maintained in interleukin–2 (IL–2) containing tissue culture media. These cell lines may become addicted to IL–2 and have less physiologic relevance in the study of CD8+ T cell killing than freshly sorted CD8+ T cells and may not accurately reflect what happens in vivo.

1.5. Study Aims

The studies conducted in the thesis attempt to determine the mechanism responsible for protecting SIV–infected macrophages from killing by freshly sorted CD8+ T cell mediated, specifically focusing on the ability of Nef to alter MHC–I surface expression. Using an ex vivo viral suppression assay, we confirm that SIV–infected macrophages are not eliminated, or viral replication is not suppressed, by freshly sorted SIV–specific CD8+ T cells isolated from elite controllers, as seen in co–cultures completed with CD8+ T cell lines as effectors. I determined that disrupting Nef’s ability to down–regulate MHC–I in SIV–infected macrophages does not sensitize them to rapid
killing, as seen in autologous SIV–infected CD4+ T cells. Furthermore, macrophages infected with a Nef–deficient virus did not lead to an increase in elimination or suppression of viral replication, as seen in autologous infected CD4+ T cells. Collectively, the data presented here indicate that Nef appears to neither be necessary nor sufficient for the resistance of infected macrophages to elimination or suppression of viral replication by unstimulated freshly sorted SIV–specific CD8+ T cells. These findings may provide some insight into macrophages as persistent viral reservoirs and suggests that macrophages may afford primate lentiviruses some degree of protection from immune surveillance.
CHAPTER 2

Materials and Methods

2.1. Animals

Indian rhesus macaques (Macaca mulatta) used in this study were housed at the Wisconsin National Primate Research Center. The animals were typed for various MHC–I alleles, including Mamu–B*08, Mamu–A*01, Mamu–A*02, Mamu–B*17 and Mamu–B*03 (Table 1). The animals were cared for according to the regulations and guidelines of the University of Wisconsin Institutional Animal Care and Use Committee.

2.2. Target Cell Isolation and Culture

To generate target cells, peripheral blood mononuclear cells (PBMC) were freshly isolated from SIV-naïve Indian rhesus macaque whole blood by Ficoll–Plaque Plus density centrifugation (GE Healthcare Life Sciences). CD14+ target cells were isolated by positive selection using CD14 microbeads (Miltenyi Biotec) and LS columns (Miltenyi Biotec) and used according to the manufacturer’s instructions. CD14+ cells were re-suspended in macrophage media: comprised of DMEM (Gibco) containing 10% heat inactivated human serum (Sera Care Life Sciences), 2mM L–glutamine (Gibco), 10µg/ml gentamicin (Sigma–Aldrich) and 10 ng/ml rhMCSF (R&D System); seeded at 1 million cells per well in a 24 well plate (Corning) and cultured for 6 days at 37°C with 5% CO₂. CD4+ T target cells were isolated from the CD14 negative PBMC fraction using positive selection with CD4 microbeads (Miltenyi Biotec) and LS columns. Target CD4+ T cells were activated for three days with 5 mg/ml phytohemagglutinin (PHA) (Roche Diagnostics) and 10 U of interleukin–2 (IL–2)ml and further cultured in R10–100 (RPMI 1640 (Gibco) containing 10% FBS (Hyclone), supplemented with 2mM L–glutamine.
Gibco), 100 I.U./ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and 100 U of IL–2/ml. IL–2 was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

2.3. Isolating Ex Vivo SIV-specific CD8\(^+\) T Cells

SIV–specific CD8\(^+\) T cells were purified from PBMC isolated from elite controller, infected or vaccinated Indian rhesus macaques blood (Table 1) using Ficoll–Plaque Plus density centrifugation. T cells were isolated by negative selection using a Pan T cell isolation kit (Miltenyi Biotec) and LS columns and used according to the manufacturer’s instructions. The T cell fraction was re–suspended at 40 million cells/ml in R10-100 and incubated with one of the following tetramers for 1 h at 37°C: Gag\(_{181-189}\) CM9–APC (MBL), Nef\(_{137-146}\) RL10–APC, Vif\(_{97-104}\) WY8–PE, Vif\(_{66-73}\) HW8–APC or Vif\(_{100-109}\) VL10–APC from the NIH Tetramer Core Facility (Table 2). Tetramer bound CD8\(^+\) T cells were enriched using APC or PE microbeads (Miltenyi Biotec) depending on the fluorophore used and incubated overnight in R15–100 (RPMI 1640 containing 15% FBS and 100U IL–2/ml) at 2 million cells/ml in a 24 well plate. Surface staining was performed post sort to ensure that epitope–specific CD8\(^+\) T cells were at least 50% of the purified cells.
Table 2.1. Rhesus macaques used in this study.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Vaccine Regimen</th>
<th>SIV inserts</th>
<th>Strain used for infection</th>
<th>Viral load at the time of sampling (vRNA copies/ml)</th>
<th>Relevant MHC-I genotype</th>
<th>Sex</th>
</tr>
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<tbody>
<tr>
<td>r04106</td>
<td>EP rDNA + pIL-12/rAd5/HSV1/RRV</td>
<td>Env, Vif, Nef, and Tat</td>
<td>SIV Negative</td>
<td>N/A</td>
<td>Mamu-A*01</td>
<td>Female</td>
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<tr>
<td>r08061</td>
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<td>Gag, Vif, Nef, and Tat</td>
<td>SIV Negative</td>
<td>N/A</td>
<td>Mamu-A*01</td>
<td>Male</td>
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<tr>
<td>r04134</td>
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</tr>
<tr>
<td>r00068</td>
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<td>N/A</td>
<td>Mamu-B*08</td>
<td>Female</td>
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<tr>
<td>r02019</td>
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<td>&lt;50</td>
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<td>Female</td>
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<td>r90067</td>
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<td>SIVmac239</td>
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<td>Mamu-A<em>01, -B</em>08</td>
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<td>rh2361</td>
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<td>Male</td>
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<td>rRRV/rAd5/HSV1VSV</td>
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<td>Mamu-A<em>01, -A</em>02</td>
<td>Male</td>
</tr>
<tr>
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<td>Mamu-B*17</td>
<td>Male</td>
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*NA, Not applicable

*ND, Not determined
### Table 2.2: Freshly sorted SIV—specific CD8⁺ T cells used in this study

<table>
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<tr>
<th>Protein</th>
<th>Amino acid position</th>
<th>Core epitope</th>
<th>MHC-I restriction</th>
<th>Epitope sequence</th>
</tr>
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<td>181-189</td>
<td>CM9</td>
<td>Mamu-A*01</td>
<td>CTPYDINQM</td>
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<tr>
<td>Nef</td>
<td>137-146</td>
<td>RL10</td>
<td>Mamu-B*08</td>
<td>RRHRILDIYL</td>
</tr>
<tr>
<td>Vif</td>
<td>100-109</td>
<td>VL10</td>
<td>Mamu-A*01</td>
<td>VTPNYADILL</td>
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<tr>
<td>Vif</td>
<td>97-104</td>
<td>WY8</td>
<td>Mamu-A*02</td>
<td>WTDVTPNY</td>
</tr>
<tr>
<td>Vif</td>
<td>66-73</td>
<td>HW8</td>
<td>Mamu-B*17</td>
<td>HLEVQGYW</td>
</tr>
</tbody>
</table>
2.4. Generation of SIV Viruses and Spinoculation Infection

SIVmac239wt (WT), SIVmac239Δnef (Δnef) and SIVmac239–Y223F (Y223F) pseudotyped with vesicular stomatitis virus glycoprotein (VSV–G) were generated by co-transfection of 293T cells using Lipofectamine 2000 (Invitrogen) containing 12 µg of SIV proviral plasmids and 1 µg of the pCMV–VSV–G plasmid. The SIVmac239wt and SIVmac239Δnef plasmids were provided by Dr. Ronald Desrosiers, and the SIVmac239–Y223F plasmid were obtained from Dr. Frank Kirchhoff. Virus containing supernatants were harvested at 48 h and 72 h post-transfection. Supernatants were pooled, centrifuged for 5 min at 530 rcf, filtered through a 0.1µM filter, purified through a 20% sucrose cushion and frozen at -80ºC.

2.5. Infection Protocols

Monocyte–derived macrophages (macrophages) were infected with 50 ng of p27\textsuperscript{gag} of WT, Y223F or Δnef (VSV–G–pseudotyped) virus per million cells for 6 h, 48 h prior to the co-cultures. Subsequent experiments were completed with macrophages infected with 2 ng of p27\textsuperscript{gag} to reduce infection levels to approximately 35%, and will be referred to as “low multiplicity of infection” (low MOI). Viral input was washed and macrophages were cultured in 1 ml of macrophage media. Since, the Nef variants (Y223F or Δnef) were available in the SIVmac239 backbone, a lymphocyte tropic virus, we chose to use pseudotyped virus to facilitate viral infectivity in macrophages. To confirm the viability of our obtained plasmids, replication curves were completed with each virus in both CD4\textsuperscript{+} T cells and macrophages over 13 days (Fig. 1). Verifying SIVmac239 replicated in CD4\textsuperscript{+} T cells, but peaked in macrophages at day 3. As a control,
macrophages were infected in the presence of Tenofovir at 400µM for 72 h to ensure that productive infection was being quantified by flow cytometry in Gag p27+ target cells (Fig. 2). Tenofovir was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Furthermore, infecting target cells with pseudotyped virus did not effect the Nef–mediated modulation of MHC–I (Chapter 3).

CD4+ T cells were infected with 400 ng of WT, Y223F or Δnef (VSV–G–pseudotyped) virus per 1 million cells in a 12 well plate (Corning) via spinoculation at 1200 × g for 2 h, 24 h prior to co–culture (233). Cells were allowed to sit for an additional 2 h at 37°C, washed twice and cultured in R15–100.
Figure 2.1: Replication curve of target cells infected with SIV. Line graph showing the replication kinetics using an RT-Assay in a) macrophages and b) CD4⁺ T cells infected with pseudotyped WT, Y223F or Δ nef at 50 ng of p27 gag /million target cells over 13 days. Supernatant was harvest every 48 h and frozen at -20°C. Each time point was complete in triplicate in a single experiment.
Figure 2.2: Testing productive virus infection in macrophage with tenofovir. Contour plots representing intracellular p27 staining of macrophages infected with WT and Nef variants. The plot of the right shows WT–infected macrophages in the presence of tenofovir. Contour plots were generated gating on live CD14+ macrophages from one representative experiment, 72 h after infection.
2.6. *Ex vivo* Viral Suppression Assay

The viral suppression assay (VSA) involves the co–culture of primary WT–, Y223F– or Δnef–infected CD4+ T lymphocytes or monocyte–derived macrophages (target cells), with enriched, primary unstimulated SIV–specific CD8+ T cells (effector cells) from several elite controller, infected or vaccinated animals (Table 2) for 24 h (Fig. 3). In the majority of the co–cultures containing WT– and Y223F–infected target cells freshly sorted Gag\textsubscript{181-189}CM9– and Nef\textsubscript{137-146}RL10–specific CD8+ T cells (Table 2) were used from elite controller animals (Table 1). The co–cultures with Δnef–infected target cells, or target cells infected at a low MOI, were performed with freshly sorted effector cells from infected or vaccinated animals (Table 1) that were elicited from SIV–specific alleles that mounted alternate target peptides (A*01 Gag\textsubscript{181-189}CM9, A*02 Vif\textsubscript{97-104}WY8, A*02 Vif\textsubscript{100-109}VL10, B*17 Vif\textsubscript{66-73}HW8) (Table 2). Suppression of viral replication was determined by an antigen capture assay for Gag p27 (Zeptometrix) in culture supernatants. Elimination of infected target cells was assessed by flow cytometric quantification of Gag p27+ target cells in the presence and absence of effectors. SIV–infected target cells cultured in the absence of effectors (target cells only) and SIV–infected target cells isolated from animals with mismatched MHC–I alleles were included as biological controls. Target and effector cells were not completely autologous but matched in one MHC–I allele. Therefore, MHC–I specific elimination will be determined by subtracting the non–specific killing observed in mismatched target cell co–cultures.

Infected CD4+ T lymphocytes (2.5 × 10^4) or infected macrophages (2.5 × 10^4) were co–cultured at 37°C with SIV–specific CD8+ T cells at an effector / target (E/T) ratio of 1:1 in 200 µl of R15-100 or macrophage media, respectively. The following day,
co–cultures were centrifuged and supernatants were collected and frozen for p27 ELISA analysis. The cells of the co–cultures were harvested and stained with antibodies against CD14 (BV421, Clone M5E2, Biolegend) or CD4 (BV421, Clone L200, BD), CD3 (PerCPCy5.5, Clone SP43-2, BD), CD8a (BV605, Clone, RPA-T8) and HLA–A,B,C (APC, Clone W6/32, Biolegend), followed by intracellular Gag p27 staining using BD’s Cytofix/Cytoperm with a primary 55–2F12 Gag p27 antibody at 20 µg/ml and 5 ng/ml Goat α-Mouse IgG2b R–PE conjugated secondary antibody (Invitrogen) for 20 minutes at room temperature. Live/Dead fixable near–IR (Invitrogen) was used to exclude dead cells from the analysis. Data were collected using an LSR II flow cytometer (BD biosciences) and analyzed using FlowJo software (version 10.0.6.3). MHC–I specific elimination of target cells were calculated as the percent of p27 positive cells eliminated after 24 h co–culture with effector cells, using the following equation: 

\[ \frac{((\% \text{ of p27 positive cells in target cells only} - \% \text{ of p27 positive cells in target cells with fresh CD8}^+ \text{ T cells}) / \% \text{ of p27 positive cells in target cells only}) \times 100}{\% \text{ elimination of mismatch controls.}} \]

Percent maximum suppression of viral replication in target cells was calculated from the concentration of p27^{gag} present in the supernatant collected at 24 h of co–culture with effector cells, using the following equation: 

\[ \frac{((\text{p27 ng/ml in target cells only} - \text{p27 ng/ml in target cells with fresh CD8}^+ \text{ T cells}) / \text{p27 ng/ml in target cells only}) \times 100}{\% \text{ elimination of mismatch controls.}} \]

Using tetramer-bound CD8^+ T cells in the co–culture assays did not prevent their effector function, as they were able to kill infected CD4^+ T cells. The tetramer–bound CD8^+ T cells were not readily phagocytized by infected macrophages as their presence and viability was confirmed using a live/dead gating by flow cytometry and they were 80% viable.
**Figure 2.3: Ex vivo viral suppression assay scheme.** a) Target cells (macrophages and CD4\(^+\) T cells) were isolated from uninfected MHC–I–matched and mismatched rhesus macaque PBMCs and infected *in vitro*. b) Effector SIV–specific CD8\(^+\) T cells were enriched from a SIV–infected elite controller, infected or vaccinated animals using MHC–I tetramers specific for the epitope of interest (Gag\(^{181-189}\) CM9–APC, Nef\(^{137-146}\) RL10–APC, Vif\(^{97-104}\) WY8–PE or Vif\(^{100-109}\) VL10–APC). c) Freshly sorted SIV–specific CD8\(^+\) T cells were co–cultured with SIV–infected macrophages or activated SIV–infected CD4\(^+\) T cells at a 1:1 ratio for 24 h. d) Supernatant was collected from each co–culture condition and p27 content was determined using a Gag p27 ELISA to calculate the percent of viral suppression. e) Flow cytometry was used to analyze intracellular staining of Gag p27 and used to calculate the percent elimination of target cells (234).
2.7. Harvesting Supernatant for ELISA

In order to determine the ability of CD8$^+$ T cells to suppress viral replication in target cells, supernatant was harvested from each of the co–cultures. At the end of each experiment, the 96 well plates containing the co–cultures are centrifuged for 5 min at 530 rcf, 70 ul of supernatant was harvested from each culture condition and frozen at -80ºC. Each sample is diluted 1:10 according to the p27 Zeptometrix manual. The remaining supernatant was refrozen at -80ºC. For macrophage cultures completed at a low MOI, supernatants are diluted 1:5.

2.8. Generation and Maintenance of SIV-specific CD8$^+$ T Cell Lines

In order to generate peptide–specific CD8$^+$ T cell lines, we used previously described methods (235, 236). CD8$^+$ T cell lines were started using freshly isolated PBMC, and autologous B–lymphoblastoid cell lines (BLCL) were used as antigen presenting cells. BLCL were irradiated (9,000 rads) then pulsed with 0.05 mM Nef$^{137-146}$ RL10 peptide (Genescript) in $1*10^6$ cells/500ul for 1 to 2 hours at 37°C. After one wash, BLCL were mixed with whole PBMC at a ratio of 1:1 R15 with 10 ng/ml of recombinant human interleukin–7 (Sigma-Aldrich) and incubated for 48 hours. Every 3 to 5 days after, cells were cultured with R15–100. Every 7 days, the CD8$^+$ T cell lines were re–stimulated using peptide–pulsed, irradiated BLCL. After 14 days in culture, CD8$^+$ T cell lines were tested for epitope specificity after by either MHC–I tetramer assays or either intracellular cytokine staining (ICS) assays as previously described (235-237).
2.9. Reverse Transcription (RT) Activity Assay

The RT activity of SIV was measured using a previously described protocol (238). To lyse each sample, 10 µL Solution A (300 mM KCl, 100mM Tris–HCl pH 7.9, 0.1% NP-40, 10 mM DTT) is added to 10 µL of culture supernatant in a 96 well plate, mixed and incubated at 37° C for 15 minutes. Followed by the addition of 25 ul of Solution B (150 mM KCl, 50 mM Tris-HCl pH 7.9, 15 mM MgCl₂, 5 mM DTT, 0.05% NP-40, 0.250 U/ml oligo dTTP, 10µCi/ml ³H-TTP, 10µg/ml poly A) and incubated overnight at 37°C. RT activity determined as previously described (239).
Chapter 3

Determining the Role of MHC–I Down–regulation in the Resistance of SIV–infected Macrophages to CD8\(^+\) T Cell Killing

3.1. Introductory Remarks

Macrophages are long–lived reservoirs that are resistant to the cytotoxic effects of HIV–1 and possibly CD8\(^+\) T cell mediated killing, compared to CD4\(^+\) T cells. Previous reports have shown that CD8\(^+\) T cell lines or clones can rapidly suppress viral replication in HIV/SIV infected macrophages (229, 230). Recent reports, completed ex–vivo have revealed that HIV/SIV–infected macrophages are not readily eliminated and viral replication is not suppressed by freshly sorted CD8\(^+\) T cells obtained from elite controllers (231, 232). Using highly stimulated CD8\(^+\) T cell lines or clones to evaluate the killing of macrophages may have less physiologic relevance than freshly sorted CD8\(^+\) T cells, which may not accurately reflect what happens in vivo.

To determine the mechanism responsible for the reported protection of infected macrophages from rapid killing by freshly sorted unstimulated CD8\(^+\) T cells, we chose to focus on the viral accessory Nef protein. Nef can down–regulate MHC–I molecules thereby facilitating evasion from CD8\(^+\) T cell recognition and increased viral replication in infected CD4\(^+\)T cells (142, 155, 240). We examined if Nef–mediated MHC–I down–regulation underscored the ability of infected macrophages to resist CD8\(^+\) T cell surveillance. An ex vivo viral suppression assay was used in a previous study to evaluate the ability of SIV–specific CD8\(^+\) T cells to eliminate and suppress viral replication in SIV–infected CD4\(^+\) T cells and macrophages (231). This viral suppression assay was used to examine the capacity of freshly isolated SIV–specific CD8\(^+\) T cells to control
viral replication in primary target cells (CD4+ T cells and macrophages), infected with a nef variant containing a point mutation (Y223F) that has been shown to impair MHC–I down–regulation. As effectors, we used freshly sorted SIV–specific CD8+ T cells from elite controller animals that have high levels of CD8+ T cells with defined target specificities (A*01 Gag181–189 CM9– and B*08 Nef137–146 RL10) (Table 2). Co-cultures completed with macrophages infected at a low MOI, used freshly sorted effector cells from infected or vaccinated animals (Table 1) that were elicited from SIV–specific alleles that mounted alternate target peptides (A*01 Gag181–189 CM9, A*02 Vif97–104 WY8, A*02 Vif100–109 VL10, B*17 Vif66–73 HW8) (Table 2).

In this chapter, we first confirmed the ability of SIV–specific CD8+ T cells to eliminate and suppress viral replication in SIV–infected CD4+ T cells, but not the majority of SIV–infected macrophages infected at different MOIs. We next tested the ability of SIV–specific CD8+ T cells to eliminate and suppress viral replication in target cells (CD4+ T cells and macrophages) infected with a point mutant (Y223F) that impairs the down–regulation of cell surface MHC–I molecules by nef. Revealing that disrupting Nef–mediated MHC–I down–regulation in macrophages is not sufficient to impact their sensitivity to CD8+ T cell killing, as seen in Y223F–infected CD4+ T cells. Moreover, we evaluated the ability of SIV–specific CD8+ T cell lines to readily eliminate WT–infected macrophages, compared to the same SIV–specific primary CD8+ T cells. Confirming that SIV–infected macrophages are rapidly eliminated by CD8+ T cell lines, but not freshly sorted SIV–specific CD8+ T cell lines.
3.2. RESULTS

3.2.1. Confirming the Ability of SIV–specific CD8+ T Cells to Eliminate and Suppress Viral Replication in WT–infected CD4+ T Cells, but not WT–infected Macrophages

HIV/SIV–specific CD8+ T cells control viral replication in HIV/SIV-infected CD4+ T cells in an MHC Class I (MHC–I) dependent manner (142, 231, 241, 242). However, they are less efficient at controlling viral replication in SIV–infected macrophages (231). To confirm and possibly extend these results, monocyte–derived macrophages and CD4+ T cells were infected in vitro with pseudotyped SIVmac239 and their elimination and the suppression of the viral replication were evaluated by freshly sorted SIV–specific CD8+ T cells ex vivo (Fig. 1). Single cycle infection of macrophages is very inefficient, even with macrophage–tropic viruses. Therefore, the viruses were pseudotyped with VSV–G, that has the ability to transduce a wide range of mammalian cells, to ensure synchronous and efficient infection of macrophages. Tetramer–sorted SIV–specific CD8+ T cells were effective at eliminating SIV–infected MHC–I matched CD4+ T cells (Fig. 3.1a, 3.1e), but not at killing SIV–infected MHC–I matched macrophages at a high MOI (Fig. 3.1c, 3.1e). When the percent of elimination was calculated by subtracting the MHC–I mismatched non–specific killing (Figure 3.1e), it was shown that SIV–specific CD8+ T cells were able to eliminate a mean of 36% of WT–infected CD4+ T cells (Fig. 3.1e), but only a mean of 3% of WT–infected macrophages (Fig. 3.1e) and this difference was statistically significant (P–value: <0.0001). Furthermore, suppression of viral replication was significantly higher (P–value: 0.0002) in WT–infected CD4+ T cells than in WT–infected macrophages at a high MOI (Fig. 3.2a). A 32% mean of maximum suppression was observed in WT-infected CD4+ T cells,
compared to 9% in WT–infected macrophages (Fig. 3.2a). When the percent of suppression of viral replication was calculated by subtracting the MHC–I mismatched non-specific suppression of viral replication (Figure 3.2b, 3.2c), it was shown that SIV–specific CD8$^+$ T cells were able to eliminate a mean of 28% of WT–infected CD4$^+$ T cells (Fig. 3.2c), but only a mean of 5% of WT–infected macrophages (Fig. 3.2c) and this difference was statistically significant ($P$–value: <0.0028). Indeed, the majority of WT–infected CD4$^+$ T cells exhibited evidence of suppression in viral replication by CD8$^+$ T cells (Fig. 3.2a, 3.2c).

To ensure that the higher levels of infection in macrophages were not saturating the capacity of freshly sorted SIV–specific CD8$^+$ T cells to kill them, co-cultures with macrophages infected at a lower MOI were also completed. At the time of these experiments our availability for SIV–specific CD8$^+$ T cells from elite controllers were limited. Therefore, co–cultures were completed with macrophages infected at a low MOI using SIV–specific CD8$^+$ T cells isolated from an infected elite controller, infected or vaccinated animals as effectors. Lowering the MOI did significantly increase in the elimination of macrophages (from 3% mean to 8% mean, Fig. 3.3g). However, significant differences ($P$–value: <0.0001) were still observed in the ability of SIV–specific CD8$^+$ T cells to eliminate CD4$^+$ T cells compared to macrophages infected at a low MOI (Fig. 3.3g). Likewise, suppression of viral replication was significantly higher ($P$–value: 0.0092) in WT–infected CD4$^+$ T cells than in macrophages infected at a low MOI (Fig. 3.3h).
Figure 3.1: Freshly sorted SIV–specific CD8\(^+\) T cells are more effective at eliminating WT–infected CD4\(^+\) T cells, than in WT–infected macrophages. Contour plots representing intracellular p27 staining of infected target cells: a) MHC–I matched or b) MHC–I mismatched WT–infected CD4\(^+\) T cells and c) MHC–I matched or d) MHC–I mismatched WT–infected macrophages. Contour plots of the target cells were generated by gating on live CD8\(^-\) T cells or live CD14\(^+\) macrophages from one representative experiment. Comparison of the e) elimination in target cells by tetramer–sorted SIV–specific CD8\(^+\) T cells after subtracting MHC–I mismatched non-specific killing. Gag CM9 and Nef\(_{137-146}\), RL10–specific CD8\(^+\) T cells isolated from multiple elite controller animals were used as effectors in this data set. The black bars represent the e) mean percent elimination as black lines and each data point represents co–cultures from different animals.
Figure 3.2: Freshly sorted SIV–specific CD8+ T cells are more effective at suppressing viral replication in CD4+ T cells than macrophages infected with WT virus at a high MOI. a) Comparison of the percent maximum suppression viral replication in a) MHC–I matched or b) MHC–I mismatched WT-infected target cells c) MHC–I specific suppression of viral replication in WT–infected target cells after subtracting MHC–I mismatched non-specific killing. Macrophages are infected at a high MOI. The mean percent suppression of viral replication is represented as black lines and each data point represents an independent experiment. Gag181-189 CM9 and Nef137-146 RL10–specific CD8+ T cells isolated from elite controller animals were used as effector cells.
Figure 3.3: Freshly sorted SIV–specific CD8\(^+\) T cells are more effective at eliminating WT–infected CD4\(^+\) T cells and suppressing their viral replication, than
in WT–infected macrophages at different MOIs. Contour plots representing intracellular p27 staining of infected target cells: a) MHC–I matched or b) MHC–I mismatched WT–infected CD4⁺ T cells and c) MHC–I matched high MOI or d) MHC–I mismatched high MOI WT–infected macrophages and e) MHC–I matched low MOI f) MHC–I mismatched low MOI WT–infected macrophages. Contour plots of the target cells were generated by gating on live CD8⁻ T cells or live CD14⁺ macrophages from one representative experiment. Comparison of the g) elimination and h) suppression of viral replication in target cells by tetramer–sorted SIV–specific CD8⁺ T cells after subtracting MHC–I mismatched non-specific killing. The SIV–specific CD8⁺ T cells listed in Table 2 were used in this dataset. The black lines represent the mean percent g) elimination or h) suppression of viral and each data point represents co–cultures from different animals.
3.2.2. Confirming the Ability of SIV–specific CD8⁺ T Cells Lines to be more Effective at Eliminating WT–infected Macrophages than Freshly Isolated SIV–specific CD8⁺ T Cells

Previous studies have demonstrated that HIV/SIV–specific CD8⁺ T cell lines rapidly eliminate the majority of HIV/SIV–infected macrophages (229, 230). Other studies have shown freshly sorted SIV–specific CD8⁺ T cells do not suppress viral replication or elimination of SIV–infected macrophages (231, 232). Therefore, to confirm this we decided to generate SIV–specific CD8⁺ T cell lines from the same SIV–specific primary CD8⁺ T cells isolated ex vivo. The primary Nef₁₃₇₋₁₄₆RL₁₀–specific CD8⁺ T cells isolated ex vivo were repeatedly stimulated with peptide and expanded in vitro every week for one month. Nef₁₃₇₋₁₄₆RL₁₀–specific CD8⁺ T cell lines were confirmed to be responding to peptide with an ICS assay (Fig. 3.4a), and co–culture experiments were completed in parallel with freshly isolated Nef₁₃₇₋₁₄₆RL₁₀–specific CD8⁺ T cell lines ex vivo (Fig. 3.4b, 3.4c). As a positive control for the ICS, we incubated CD8⁺ T cell lines with leukocyte activation cocktail (LAC). Moreover, co–cultures were also blocked with an MHC–I (W6/32) monoclonal antibody to determine if killing was MHC–I dependent (Fig. 3.4c).

In agreement with published studies, Nef₁₃₇₋₁₄₆RL₁₀–specific CD8⁺ T cell lines generated from the same Nef₁₃₇₋₁₄₆RL₁₀–specific primary CD8⁺ T cells, eliminated 78% of WT–infected macrophages at a high MOI (Fig. 3.4b) (229, 230). However, freshly isolated, Nef₁₃₇₋₁₄₆RL₁₀–specific CD8⁺ T cells eliminated approximately 18% of the same WT–infected macrophages (Fig. 3.4b). Furthermore, elimination of macrophages by Nef₁₃₇₋₁₄₆RL₁₀–specific CD8⁺ T cell lines was MHC–I dependent. Blocking MHC–I on the surface of SIV–infected macrophages greatly reduced elimination of macrophages by
Nef<sub>137-146</sub>–specific CD8<sup>+</sup> T cell lines to only 5% (Fig. 3.4c). This confirms the ability of CD8<sup>+</sup> T cell lines to eliminate macrophages, and to a lesser extent, freshly sorted SIV–specific CD8<sup>+</sup> T cells in a MHC–I dependent manner.

### 3.2.3. Evaluating the Role of Nef–mediated MHC–I Down–regulation in Sensitizing Infected Macrophages to Killing by SIV–specific CD8<sup>+</sup> T Cells

HIV–1 infected CD4<sup>+</sup> T cells have been shown to be eliminated by HIV–specific CD8<sup>+</sup> T cells lines primarily in an MHC–I–dependent manner (142, 231, 241, 242). Therefore, we investigated whether the ability of Nef to disrupt the down–regulation of MHC–I molecules would sensitize the infected macrophages to elimination and suppression of viral replication by freshly sorted SIV–specific CD8<sup>+</sup> T cells early in co–culture (142). To validate the ability of Nef variants to disrupt MHC–I down–regulation, CD4<sup>+</sup> T cells and macrophages were infected with VSV–G–pseudotyped SIVmac239wt (WT) and SIVmac239–Y<sub>223</sub>F (Y223F). Y223F contains a substitution for a tyrosine to a phenylalanine at position Y223 that impairs the down–regulation of cell surface MHC–I molecules by nef (158). Since, Y223F was readily available in the SIVmac239 backbone, a lymphocyte tropic virus that efficiently replicates in lymphocytes, but poorly in macrophages (243). Infection with VSV–G–pseudotyped viruses facilitates synchronous presentation of peptides on MHC–I molecules. Because of the efficiency of pseudotyped infections, virus spread was not an issue. Flow cytometry analysis of the MHC–I surface expression on target cells infected with the point mutant showed that infecting with pseudotyped virus did not affect the Nef–mediated modulation of MHC–I (Figure 3.5). When compared to wild–type SIV, the point mutant was impaired in its ability to down–regulate MHC–I (Fig. 3.5a, 3.5b). MHC–I expression in uninfected cells was used as a comparative, but may differ from infected cells due to the activation state of the cell.
Figure 3.4: SIV-specific CD8\(^+\) T cells lines are more effective at eliminating WT-infected macrophages than freshly isolated SIV-specific CD8\(^+\) T cells, in a MHC-I dependent manner. A) Intracellular cytokine staining of a Nef\(_{137-146}\)RL10-specific CD8\(^+\) T cells line stimulated with Nef\(_{137-146}\)RL10 peptide, leukocyte activation cocktail (LAC), or no stimulus. Bar graph comparing b) the percent elimination of WT-infected macrophages by freshly isolated Nef\(_{137-146}\)RL10–specific CD8\(^+\) T cells and Nef\(_{137-146}\)RL10–specific CD8\(^+\) T cells lines c) and elimination of WT-infected target cells is MHC-I dependent. Bar graph represents percent elimination data calculated from the absolute counts of contour plots generated from intracellular p27 staining of live CD14\(^+\)p27\(^+\) macrophages after subtracting MHC-I mismatch non–specific killing. Each bar represents the mean and standard deviation of two independent experiments.
Figure 3.5. Disrupted MHC–I expression profile of Y223F–infected target cells. The MHC–I histograms were generated from gating on live infected a) CD4⁺ T cells and b) macrophages infected with WT and Y223F virus for 72 h from a representative experiment. Uninfected cells were used as a comparative. The geometric means of the MHC–I profile are included below the histograms.
To validate the ability of freshly isolated tetramer sorted SIV–specific CD8$^+$ T cells from elite controllers to kill target cells infected with a Nef variant capable of disrupting MHC–I down–regulation, \textit{ex vivo} viral suppression assays were completed with infected CD4$^+$ T cells and macrophages (infected at a high MOI) isolated from the same animal in parallel. Co–cultures of infected CD4$^+$ T cells with effector cells showed a non–significant modest increase in the MHC–I specific elimination of Y223F–infected cells (44% elimination) compared to WT–infected CD4$^+$ T cells (40% elimination) (Fig 3.6e). However, in the co–cultures with macrophages infected at high MOI, a mean of 2% of the WT–infected cells were eliminated and no increase in the MHC–I specific elimination of Y223F–infected macrophages was observed (Fig. 3.6e). A modest increase was observed in the mean of MHC–I specific suppression of viral replication in Y223F–infected CD4$^+$ T cells (27%) compared to WT–infected CD4$^+$ T cells (15%), no statistical significant difference was observed (Fig. 3.7e). \textit{Ex vivo} sorted SIV–specific CD8$^+$ T cells that suppressed viral replication in WT– and Y223F–infected CD4$^+$ T cells, suppressed viral replication to a lesser extent in WT– and Y223F–infected macrophages at a high MOI (Fig.3.7e). The difference in the suppression of Y223F–infected targets was statistically significant ($P$–value: 0.0016).
Figure 3.6: freshly sorted SIV-specific CD8+ T cells are modestly more effective at eliminating CD4+ T cells than macrophages when infected with Y223F at a high
**MOI.** Contour plots representing intracellular p27 staining of infected target cells: a) MHC–I matched or b) MHC–I mismatched WT– and Y223F–infected CD4+ T cells and c) MHC–I matched or d) MHC–I mismatched WT– and Y223F–infected macrophages at a high MOI. Contour plots were generated gating on live CD8+ T cells or live CD14+ macrophages from one representative experiment. e) MHC–I specific elimination in WT– and Y223F–infected target cells after subtracting MHC–I mismatched non-specific killing. The mean percent elimination of target cells is represented as black lines and each data point represents an independent experiment. Gag_{181-189} CM9 and Nef_{137-146} RL10–specific CD8+ T cells isolated from multiple elite controller animals were used as effectors.
Figure 3.7: Freshly sorted SIV–specific CD8+ T cells are modestly more effective at suppressing viral replication in CD4+ T cells than macrophages infected with Y223F at a high MOI. a) Comparison of the percent maximum suppression viral replication in a) MHC–I matched or b) MHC–I mismatched CD4+ T cells infected with WT or Y223F and c) MHC–I matched or d) MHC–I mismatched macrophages infected at a high MOI with WT or Y223F by tetramer-sorted SIV–specific CD8+ T cells. e) MHC–I specific suppression of viral replication in WT– and Y223F–infected target cells after subtracting MHC–I mismatched non-specific killing. The mean percent suppression of viral replication is represented as black lines and each data point represents an independent experiment. Gag_{181-189} CM9 and Nef_{137-146} RL10–specific CD8+ T cells isolated from elite controller animals were used as effectors.
Figure 3.8: Freshly sorted SIV–specific CD8⁺ T cells are more effective at eliminating CD4⁺ T cells than macrophages when infected with Y223F at a low MOI.
Contour plots representing intracellular p27 staining of infected target cells: a) MHC–I matched or b) MHC–I mismatched WT– and Y223F–infected CD4⁺ T cells and c) MHC–I matched or d) MHC–I mismatched WT– and Y223F–infected macrophages. Contour plots were generated gating on live CD8⁻ T cells or live CD14⁺ macrophages from one representative experiment. e) MHC–I specific elimination in WT– and Y223F–infected target cells after subtracting MHC–I mismatched non-specific killing. The mean percent elimination of target cells is represented as black lines and each data point represents an independent experiment. The majority of the co-cultures were completed with Gag₁₈₁₋₁₈₉ CM9– and Vif₆₆₋₇₃ HW8–specific CD8⁺ T cells isolated from infected or vaccinated animals were used in this data set. One experiment included Nef₁₃₇₋₁₄₆ RL10–specific CD8⁺ T cells from an elite controller as effectors.
To confirm that the higher levels of infection in macrophages were not overwhelming the ability of freshly sorted SIV–specific CD8⁺ T cells to kill infected macrophages with disrupted MHC–I down–regulation, co–cultures with Y223F–infected macrophages at a lower MOI were also completed. As stated previously, the majority of the co–cultures were completed with Gag₁₈₁–₁₈₉ CM9– and Vif₆₆–₇₃ HW8–specific CD8⁺ T cells isolated from infected or vaccinated animals as effectors. One experiment included Nef₁₃₇–₁₄₆ RL10–specific CD8⁺ T cells from an elite controller as effectors. Interestingly, reducing the infection levels of macrophages did not significantly increase the MHC–I specific elimination of Y223F–infected macrophages, as observed in Y223F–infected CD4⁺ T cells (P–value: 0.0445). SIV–specific CD8⁺ T cells were able to eliminate a mean of 25% of Y223F–infected CD4⁺ T cells, but only a mean of 8% of WT–infected macrophages with reduced infection levels (Fig. 3.8e) and this difference was statistically significant (P–value: <0.0322). Furthermore, we did not see an increase in the MHC–I specific elimination Y223F–infected macrophages (mean of 6%), compared to WT–infected macrophages (mean of 8%) (Fig. 3.8e).

In order to compare the ability of SIV–specific CD8⁺ T cells to eliminate and suppress viral replication in macrophages infected at different MOIs. We compiled the MHC–I specific elimination data from the co–cultures completed with macrophages infected at a high MOI (Fig 3.6e) and low MOI (Fig 3.8e). Though this data was completed on different days with different effector cells, it would still allow us to observe the trend in killing amongst target cells infected with wild–type and Nef mutant. Co–cultures of infected CD4⁺ T cells with effector cells showed a non–significant modest increase in the elimination of Y223F–infected cells (36% elimination) compared to WT–
infected cells (29% elimination) (Fig 3.9a). However, in the co–cultures with macrophages infected at a high MOI, only a mean of 2% of the WT–infected cells were eliminated and no increase in the MHC–I specific elimination of Y223F–infected macrophages was observed (1%). Similar results were seen in WT–infected macrophages (mean of 8%) compared to Y223F–infected macrophages (mean of 6%) with reduced infection. When infected with the point mutant an increase in the elimination is observed in CD4+ T cells compared to WT–infected cells, which was not seen in macrophages (Fig. 3.9b). Ex vivo sorted SIV–specific CD8+ T cells that eliminated WT–infected CD4+ T cells (Fig. 3.9a), eliminated less WT–infected macrophages at a high MOI (P–value: 0.0025) and low MOI (P–value: 0.0093). The same was observed for Y223F infections comparing CD4+ T cells and macrophages at a high MOI (P–value: 0.0001) and low MOI (P–value: 0.0001). No differences were detected in the ability of the different SIV–specific CD8+ T cells to kill target cells (Fig. 3.9a). Furthermore, we did not see an increase in the MHC–I specific elimination Y223F–infected macrophages at a high (mean of 1%) or low MOI (mean of 6%), compared to WT–infected macrophages. In fact, an increase was observed in MHC–I specific elimination in WT–infected macrophages with reduced infection (mean of 8%), compared to macrophages with a higher infection (mean of 2%) and this difference was statistically significant (P–value: 0.0415). The same was shown in Y223F–infected macrophages (P–value: 0.0276).

To evaluate the ability of SIV–specific CD8+ T cells to suppress viral replication in Y223F–infected macrophages at different MOIs, we compiled the data into one graph depicted in Figure 3.9. An increase was observed in the mean of MHC–I specific suppression of viral replication in Y223F–infected CD4+ T cells (mean of 25%)
compared to WT–infected CD4⁺ T cells (mean of 9%) \( (P\text{-value } 0.0113, \text{Fig. 3.10a}) \). However, we did not see a significant increase in the ability of SIV–specific CD8⁺ T cells to eliminate Y223F–infected macrophages, compared to WT–infected macrophages irrespective of the MOI used in macrophages. Freshly sorted SIV–specific CD8⁺ T cells that suppressed viral replication in Y223F–infected CD4⁺ T cells, suppressed viral replication to a lesser extent in Y223F–infected macrophages at a high MOI \( (P\text{-value: } 0.0003) \) or low MOI \( (P\text{-value: } 0.0023) \). Additionally, when infected with the point mutant an increase in the suppression of viral replication was observed in CD4⁺ T cells compared to WT–infected cells, which was not seen in the majority of macrophages (Fig. 3.10b). Due to limited availability of elite controllers for our studies, the majority of co–culture experiments completed with macrophages infected with a low MOI were completed with effectors from infected or vaccinated experiments which could explain the reduced elimination and suppression of viral replication observed in WT–infected CD4⁺ T cells controls (Fig. 3.8, Fig. 3.9, Fig. 3.10). No differences were detected in the ability of the different SIV–specific CD8⁺ T cells to kill target cells. (Fig. 3.10b) Together, it appears that disrupting Nef’s ability to down–regulate MHC–I does not further sensitize infected macrophages to killing.
Figure 3.9: Elimination is not increased in macrophages infected with Y223F at different MOIs. a) MHC–I specific elimination in WT– and Y223F–infected target cells by freshly sorted SIV–specific CD8⁺ T cells, including macrophages infected with a low MOI, after subtracting MHC–I mismatched non-specific killing. The mean percent elimination of target cells is represented as black lines and each data point represents an independent experiment. b) Represents the same data depicted as a before and after graph to compare the trend in killing. Co–cultures of macrophages infected with high MOI were performed with Gag₁₈₁–₁₈₉ CM9 and Nef₁₃₇–₁₄₆ RL10–specific CD8⁺ T cells isolated from multiple elite controller animals.

Co–cultures with low MOI–infected macrophages
included Gag\textsubscript{181-189} CM9– and Vif\textsubscript{66-73}HW8– specific CD8$^{+}$ T cells isolated from infected or vaccinated animals. Two experiments included Nef\textsubscript{137-146}RL10–specific CD8$^{+}$ T cells from an elite controller as effector cells.
Figure 3.10: Suppression of viral replication is not increased in macrophages infected with Y223F at different MOIs. a) MHC–I specific suppression of viral replication in WT– and Y223F–infected target cells by freshly sorted SIV–specific CD8+ T cells, including macrophages infected with a low MOI, after subtracting MHC–I mismatched non-specific killing. The mean percent suppression of viral replication of target cells is represented as black lines and each data point represents an independent experiment. b) Represents the same data depicted as a before and after graph to compare the trend in killing. Macrophage high MOI co-cultures included Gag_{181-189} CM9 and Nef_{137-146} RL10–specific CD8+ T cells isolated from multiple elite controller animals were used in this data set. Macrophage low MOI co-cultures included Gag_{181-189} CM9– and
Vif$_{66-73}$HV8–specific CD8$^+$ T cells isolated from infected or vaccinated animals were used in this data set. Two experiments included Nef$_{137-146}$RL10–specific CD8$^+$ T cells from an elite controller as effectors.
3.3. Discussion

Indeed, it has been shown that SIV–infected macrophages are resistance to killing by freshly sorted SIV–specific CD8⁺ T cells (231, 232). One study revealed that it may take up to 5 days to eliminate HIV–1 infected macrophages by bulk freshly sorted CD8⁺ T cells isolated from elite controllers, and still have the ability to spread infection to CD4⁺ T cells (232). Therefore, determining a mechanism to rapidly eliminate macrophages is of importance. A known function of the viral accessory protein, Nef, is to down–regulate MHC–I expression in infected targets, and has been reported to protect HIV–infected primary CD4⁺ T cells from lysis by CD8⁺ T cell lines (142, 157, 231, 241, 242). We, therefore, sought to determine if SIV–infected macrophages are protected from recognition by freshly sorted SIV–specific CD8⁺ T cells due to the Nef–mediated down–regulation of MHC–I molecules.

In this chapter, I have confirmed that the majority of SIV–infected macrophages infected at different MOIs are not significantly eliminated or viral replication is not suppressed by SIV–specific CD8⁺ T cells, as seen in SIV–infected CD4⁺ T cells. Furthermore, SIV–infected macrophages at a high MOI can be rapidly eliminated in a MHC–I dependent manner by SIV–specific CD8⁺ T cell lines, but not freshly sorted cells. Based on our results, freshly sorted effector cells eliminated and suppressed viral replication in WT–infected CD4⁺ T cells and most of the killing was MHC–I dependent. We reasoned that the ability of Nef to down–regulate MHC–I was protecting macrophages from SIV–specific CD8⁺ T cell recognition. However, infecting macrophages with a point mutant (Y223F) that alters Nef’s ability to down–regulate MHC–I did not further sensitize macrophages to killing by primary effector cells. We
observed mixed results in SIV–infected CD4+ T cells with increased MHC–I expression. We show consistent significant differences in the increased suppression of viral replication in Y223F–infected CD4+ T cells compared to the same cells infected with wild–type. However, in co–cultures completed with SIV–specific CD8+ T cells from elite controllers did not reveal a significant increase in elimination when infected with the point mutant, as observed in co–cultures completed with effectors from infected or vaccinated animals. It could be that freshly sorted CD8+ T cells from elite controllers can effectively eliminate WT–infected CD4+ T cells and increasing MHC–I expression in Y223F–infected CD4+ T cells does not lead to significantly more elimination. Therefore, disrupting MHC–I down–regulation does not play a significant role in the resistance of infected macrophages to elimination or suppression of viral replication by “unstimulated” freshly sorted SIV–specific CD8+ T cells.

The experimental design explained in my study does have limitations, as described here. First, non–specific killing controls for the co–cultures were completed with target cells mismatched at one MHC–I allele. However, co–cultures completed with MHC–I mismatched CD4+ T cells, we observed a considerable amount of non–specific killing, which could be the result of an allogeneic reaction. In the future, as a control, the assay could be improved by completing co–cultures with target cells pre–incubated with a MHC–I antibody to block MHC–I specific killing, instead of co–cultures with mismatched target cells. A recent study, evaluated the ability of bulk HIV–specific CD8+ T cells from elite controllers to eliminate autologous infected macrophages.
isolated from the same individual (232). I believe this may be a better approach to look at the killing of infected target cells, but can be difficult in a rhesus macaque system with the limitations in blood draws.

We report here that disrupting MHC–I down–regulation does not play a significant role in the resistance of infected macrophages to killing by “unstimulated” freshly sorted SIV–specific CD8+ T cells. We did see significant killing of infected macrophages by SIV–specific CD8+ T cell lines. HIV/SIV–infected macrophages may be more resilient to the effector molecules secreted by unstimulated freshly sorted SIV–specific CD8+ T cells. The elimination we observed in our infected macrophages by the CD8+ T cell lines that were repeatedly stimulated with the Nef_{137-146}RL10 peptide may elicit abnormal antiviral responses upon recognition of their specific epitope that may overcome defense mechanisms by macrophages. A study completed by Shan et. al., revealed the up–regulation of granzyme B (GrB), IFN–γ, CD107α and perforin production in primary CD8+ T cells stimulated with Gag–specific peptides, compared to non–stimulated cells (244). It is possible that infected macrophages are targeted more efficiently by the excessively primed Nef_{137-146}RL10–specific CD8+ T cell lines and are able to deliver effector molecules more robustly that may overcome defense mechanisms by infected macrophages. Whereas, freshly–sorted SIV–specific CD8+ T cells may be a more physiological representation.

The mechanism by which HIV/SIV–infected macrophages are resistant to CD8+ T cell mediated killing is unknown. Are the CD8+ T cells effectively delivering effector molecules to infected macrophages, or are delivered effector molecules inhibited by a factor expressed in infected macrophages? An interesting approach could be to evaluate
and compare the ability of freshly sorted SIV–specific CD8\(^+\) T cells to deliver effector molecules to infected macrophages and CD4\(^+\) T cells. One study assessed the ability of different HIV–specific CD8\(^+\) T cells from progressor or elite controller individuals to load granules and deliver granzyme B to HIV–infected CD4\(^+\) T cell targets, a similar experiment has not been completed in macrophages (55). Therefore, it would be exciting to look at the ability of CD8\(^+\) T cells to deliver effector molecules to SIV–infected macrophages. Understanding the mechanism of macrophage resistance to CD8\(^+\) T cell recognition or killing can help in developing targeted drug therapies that be used in conjunction with vaccines that boost antigen–specific CD8\(^+\) T cells.
Chapter 4

Determining the Role of Nef in the Resistance of SIV–infected Macrophages to CD8\(^+\) T Cell Killing

4.1. Background

The HIV–1/SIV Nef protein contributes to viral spread by enhancing viral replication, infectivity, and down–regulation of cell surface markers to evade the anti–viral immune response (121, 143, 240). Nef interferes with the normal trafficking of MHC–I molecules to the cell surface leading to the disruption of antigen presentation, and in turn, reduced recognition by CD8\(^+\) T cells and elimination of infected target cells (135, 142, 242). Specifically, Nef protects HIV–1 infected primary CD4\(^+\) T cells against killing by CD8\(^+\) T cells, and this is correlated with nef–mediated MHC–I down–regulation (231). The majority of assays investigating the role of nef or MHC–I down–regulation in restricting CD8\(^+\) T cell–mediated killing have been completed with primary CD4\(^+\) T cells or macrophage cell lines as infected target cells (142, 155, 231, 240, 241). Infecting macrophages with a point mutant that alters the ability of Nef to down–regulate MHC–I molecules, did not result in increased killing by SIV–specific CD8\(^+\) T cells. Therefore, we investigated whether entirely disrupting Nef function in our infected macrophage would render them sensitive to elimination and suppression of viral replication by freshly sorted SIV–specific CD8\(^+\) T cells.
4.2. Results

4.2.1. MHC-I Expression of Target Cells Infected with Nef Variants

Figure 4.1. MHC–I expression profile of target cells infected with SIV Nef variants.

The MHC–I histograms were generated from gating on live infected a) CD4+ T cells and b) macrophages infected with WT and Nef variants for 72 h from a representative experiment. Uninfected cells were used as a comparative. The geometric means of the MHC–I profile are included below the histograms.
Accordingly, it was reasoned that the interference of MHC–I down–regulation may not be fully recapitulated in the point mutant (Fig. 3a,b). The ex vivo viral suppression assay was used to evaluate the ability of SIV–specific CD8⁺ T cells to eliminate and suppress viral replication in CD4⁺ T cells and macrophage infected with pseudotyped SIVmac239wt (WT) and SIVmac239Δnef (Δnef), a 181–bp deletion at nucleotide 175 in the beginning of the nef coding sequence that would be expected to inactivate all activities of nef (157, 245).

To validate and compare the ability of Nef variants to disrupt MHC–I down–regulation, CD4⁺ T cells and macrophages were infected at a high MOI with VSV–G–pseudotyped SIVmac239wt (WT), SIVmac239Δnef (Δnef) and SIVmac239–Y₂₂₃F (Y223F). Flow cytometry analysis revealed impaired MHC–I down–regulation in target cells infected with the point mutant and deletion mutant, and this disruption was more pronounced for the deletion mutant when compared to wild–type SIV (Fig. 4a, 4b). MHC–I expression in uninfected cells was used as a comparative, but may differ from infected cells due to the activation state of the cell.

4.2.2. Evaluating the Role of Nef in Sensitizing Infected Macrophages to Killing by SIV–specific CD8⁺ T Cells.

Accordingly, it was reasoned that the interference of MHC–I down–regulation may not be fully recapitulated in the point mutant (Fig. 3a,b). Therefore, co–cultures were completed in target cells infected with the deletion mutant that best restored the MHC–I expression. Since the deletion used to construct the Δnef variant resulted in a frameshift that disrupts the Nef₁₃₇–₁₄₆RL10 epitope, co–cultures with Δnef–infected target cells were performed with freshly sorted effector cells from infected or vaccinated animals, which may explain the lower values in viral suppression in CD4⁺ T cell co–
cultures. In addition to MHC–I down–regulation, Nef is able to down–regulate CD4 surface expression (246). Therefore to accurately analyze the elimination of ∆ nef–infected CD4+ T cells, the % elimination of CD4+p27+ and CD4p27+ T cells was reported. Co–cultures of infected CD4+ T cells with effector cells showed a modest increase in the mean of MHC–I specific elimination of ∆ nef–infected CD4+ T cells (27%) compared to WT–infected CD4+ T cells (19%), no increase in the mean of MHC-I specific elimination of WT–infected macrophages (2%) compared to ∆ nef–infected macrophages (3%) was observed when infected at a high MOI (P–value: 0.0239, Fig. 4.2e).

As expected, there was significant MHC–I specific suppression of viral replication in ∆ nef–infected CD4+ T cells (P–value: 0.0418), but not in the majority of WT–infected CD4+ T cells (Fig. 4.3a, Fig 4.3e). Most ex vivo sorted SIV–specific CD8+ T cells that suppressed viral replication in WT– and ∆ nef–infected CD4+ T cells, did not suppress viral replication WT– and ∆ nef–infected macrophages (Fig. 4.3c, 4.3e), and a statistical significant difference was observed in the mean of maximum suppression in ∆ nef–infected CD4+ T cells (41%) than in ∆ nef–infected macrophages (0%) (P–value: 0.0393, Fig. 4.3e). Despite the fact that ∆ nef restored the MHC–I expression, it did not sensitize macrophages infected at a high MOI to CD8+ T cell elimination or suppression of viral replication after 24 h of co–culture (Fig. 4.2c, 4.3c, 4.3e).

To confirm that the higher levels of infection in macrophages were not overwhelming the ability of freshly sorted SIV–specific CD8+ T cells to kill infected macrophages with disrupted Nef function, co–cultures with ∆ nef–infected macrophages at a lower MOI were also completed. Interestingly, reducing the infection levels of
macrophages did not significantly increase the MHC–I specific elimination of \( \Delta \text{nef} \)-infected macrophages, as observed in \( \Delta \text{nef} \)-infected CD4\(^+\) T cells (\( P \)-value: 0.0153). SIV–specific CD8\(^+\) T cells were able to eliminate a mean of 32% of \( \Delta \text{nef} \)-infected CD4\(^+\) T cells, but only a mean of 3% of \( \Delta \text{nef} \)-infected macrophages with reduced infection levels (Fig. 4.4e) and this difference was statistically significant (\( P \)-value: \(<0.0270\)). Furthermore, we did not see an increase in the MHC–I specific elimination \( \Delta \text{nef} \)-infected macrophages (mean of 3%), compared to WT–infected macrophages (mean of 2%) (Fig. 4.4e).

In order to compare the ability of SIV–specific CD8\(^+\) T cells to eliminate and suppress viral replication in macrophages infected at different MOIs when infected with a Nef deficient virus, we compiled the MHC–I specific elimination data from the co–cultures completed with macrophages infected at a high MOI (Fig. 4.2e) and low MOI (Fig. 4.4e). Co–cultures of infected CD4\(^+\) T cells with effector cells showed a significant increase in the mean of MHC–I specific elimination of \( \Delta \text{nef} \)-infected CD4\(^+\) T cells (29%) compared to WT–infected CD4\(^+\) T cells (16%) (\( P \)-value: 0.0064, Fig. 4.5a). However, we did not observe in \( \Delta \text{nef} \)-infected macrophages (3%) at a high MOI when compared to WT–infected macrophages (2%). Similar results were also seen in macrophages infected at a low MOI when comparing \( \Delta \text{nef} \) and WT infections (5% and 8%, respectively). No differences were detected in the ability of the different SIV–specific CD8\(^+\)T cells to kill target cells (Fig. 4.5a). Additionally, when infected with the Nef–deficient virus an increase in the elimination is observed in CD4\(^+\) T cells compared to WT–infected cells, which was not seen in macrophages (Fig. 4.5b). As stated previously, reducing the infection levels slightly increased the MHC–I specific elimination in WT–infected
macrophages (mean difference of 6%), but the difference was not significant. The same was true for Δnef–infected macrophages (mean difference of 2%). As observed in previous experiments (Fig. 3.3 and 4.4e), effector cells were more efficient in eliminating WT–infected CD4$^+$ T cells than macrophages infected at high ($P$–value: 0.0064; Fig. 4.5a) or low MOI. Similar results were obtained when the percent of elimination of Δnef–infected CD4$^+$ T cells was compared to macrophages infected at a high ($P$–value: 0.0005) or low ($P$–value: 0.0025) MOI. Co–cultures were completed with SIV–specific CD8$^+$ T cells from infected or vaccinated animals due to limitation on the availability of elite controller animals with the desired SIV–specific CD8$^+$ T cell responses, which may explain the lower values in viral suppression in CD4$^+$ T cell co–cultures.

To compare the ability of SIV–specific CD8$^+$ T cells to suppress viral replication in Δnef–infected macrophages at different MOIs, we compiled the data into one graph depicted in Figure 4.6a,b. Consistently, there was a significant increase in the suppression of viral replication in Δnef–infected CD4$^+$ T cells (mean of 32%) ($P$–value: 0.0075) when compared to the WT–infected (mean of 6%) (Fig. 4.6a). Interestingly, ex vivo sorted SIV–specific CD8$^+$ T cells, which suppressed viral replication in Δnef–infected CD4$^+$ T cells, were not able to suppress viral replication in Δnef–infected macrophages at a high (mean of 0%) or low (mean of 6%) MOI, and these differences were statistically significant ($P$–values: 0.0062 and 0.0098, respectively). No differences were detected in the ability of the different SIV–specific CD8$^+$ T cells to kill target cells (Fig. 4.6a). Additionally when infected with the point mutant an increase in the suppression of viral replication was observed in CD4$^+$ T cells compared to WT–infected cells, which was not seen in macrophages (Fig. 4.6b). Furthermore, reducing the infection levels of
macrophages did not reveal significant differences in elimination by SIV–specific CD8\(^+\) T cells used in this dataset. Therefore, despite the fact that \(\Delta\text{nef}\) restored the MHC–I expression, it did not sensitize infected macrophages to CD8\(^+\) T cell elimination or suppression of viral replication after 24 h of co–culture (Fig. 4.5, Fig. 4.6a,b). Thus, Nef is not required, nor sufficient, to control viral replication in infected macrophages by freshly isolated SIV–specific CD8\(^+\) T cells.
Figure 4.2: Freshly sorted SIV–specific CD8+ T cells are more effective at eliminating Δnef–infected CD4+ T cells than Δnef–infected macrophages at a high
MOI. Contour plots representing intracellular p27 staining of infected target cells: a) MHC–I matched or b) MHC–I mismatched WT– and Δnef–infected CD4⁺ T cells and c) MHC–I matched or d) MHC–I mismatched WT– and Δnef–infected macrophages infected at a high MOI. Contour plots were generated gating on live CD8⁻ T cells or live CD14⁺ macrophages from one representative experiment. e) MHC–I specific elimination in WT– and Δnef–infected target cells after subtracting MHC–I mismatched non-specific killing. The mean percent suppression of viral replication is represented as black lines and each data point represents an independent experiment. Macrophage co-cultures were performed with Gag₁₈₁-₁₈₉ CM9– or Vif₉₇-₁₀₄ WY8–specific CD8⁺ T cells isolated from infected or vaccinated animals and used as effector cells.
Figure 4.3: Freshly sorted SIV–specific CD8⁺ T cells are more effective at suppressing viral replication in ∆nef–infected CD4⁺ T cells, than in ∆nef–infected macrophages at a high MOI. Comparison of the percent maximum suppression viral replication in a) MHC–I matched or b) MHC–I mismatched CD4⁺ T cells infected with WT or ∆nef and c) MHC–I matched or d) MHC–I mismatched macrophages infected with WT or ∆nef at a high MOI by tetramer–sorted SIV-specific CD8⁺ T cells. e) MHC–I specific suppression of viral replication in WT– and ∆nef–infected target cells after subtracting MHC–I mismatched non-specific killing. The mean percent suppression of viral replication is represented as black lines and each data point represents an independent experiment. Macrophage co-cultures were performed with Gag_{181-189} CM9– or Vif_{97-104} WY8–specific CD8⁺ T cells isolated from infected or vaccinated animals and used as effector cells.
Figure 4.4: Freshly sorted SIV–specific CD8⁺ T cells are more effective at eliminating Δnef–infected CD4⁺ T cells than Δnef–infected macrophages at a low
MOI. Contour plots representing intracellular p27 staining of infected target cells: a) MHC–I matched or b) MHC–I mismatched WT– and Δnef–infected CD4+ T cells and c) MHC–I matched or d) MHC–I mismatched WT– and Δnef–infected macrophages infected at a low MOI. Contour plots were generated gating on live CD8+ T cells or live CD14+ macrophages from one representative experiment. e) MHC–I specific elimination in WT– and Δnef–infected target cells after subtracting MHC–I mismatched non-specific killing. The mean percent suppression of viral replication is represented as black bars and each data point represents an independent experiment. The mean percent elimination of target cells is represented as black lines and each data point represents an independent experiment. Macrophage co-cultures included Gag_{181-189} CM9–, Vif_{97-104} WY8–, Vif_{66-73} HW8– or Vif_{100-109} VL10– specific CD8+ T cells from infected or vaccinated animals and used as effectors in this dataset. Co-cultures (in Fig. a and b) or (Fig. c and d) were completed with target cells from the same animal using the same effector cells.
Figure 4.5: Elimination if not increased in macrophages infected with Δnef at different MOIs. a) MHC–I specific elimination in WT– and Δnef–infected target cells by freshly sorted SIV–specific CD8+ T cells, including macrophages infected with a low MOI, after subtracting MHC–I mismatched non-specific killing. The mean percent elimination of target cells is represented as black lines and each data point represents an independent experiment. b) Represents the same data depicted as a before and after graph to compare the trend in killing. Macrophage co-cultures included Gag_{181-189}CM9–, Vif_{97-104}WY8–, or Vif_{100-109}VL10– specific CD8+ T cells from infected or vaccinated animals and used as effectors in this dataset.
Figure 4.6: Suppression of viral replication is not increased in macrophages infected with Δ nef at different MOIs. a) Comparison of the MHC–I specific suppression of viral replication in WT– and Δ nef–infected target cells by freshly sorted SIV–specific CD8 T cells after subtracting MHC–I mismatched non–specific killing. The mean percent suppression of viral replication is represented as black bars and each data point represents an independent experiment. b) Represents the same data depicted as a before and after graph to compare the trend in killing. All co-cultures included Gag_{181-189} CM9–, Vif_{97-104} WY8–, or Vif_{100-109} VL10–specific CD8^+ T cells from infected or vaccinated animals and used as effectors in this dataset.
4.3. Discussion

Previously, we reasoned that the ability of Nef to down-regulate MHC–I was protecting macrophages from SIV–specific CD8⁺ T cell recognition. However, altering the ability of Nef to down-regulate MHC–I in infected macrophages with a point mutant, did not further sensitize macrophages to killing by primary effector cells. Along with its ability to down-regulate MHC–I, HIV–1 Nef protects macrophages from HIV–1 induced apoptosis (247). Therefore, it is possible that infected macrophages are recognized by CD8⁺ T cells, but may be protected by anti-apoptotic factors induced by Nef. Therefore, I propose that abrogating Nef’s functions will sensitize macrophages to killing by SIV–specific CD8⁺ T cells.

In this chapter, we decided to entirely disrupt Nef function and evaluate the killing of macrophages by freshly sorted “unstimulated” SIV–specific CD8⁺ T cells using the same *ex vivo* viral suppression assay mentioned in Chapter 3. We noticed that infecting target cells with a Nef–deficient virus was more effective at disrupting MHC–I down-regulation, compared to Y223F– and WT–infected target cells. Therefore, it could be possible that we did not see elimination of target cells because the phenotype may not be fully recapitulated in the point mutant. Moreover, we infected CD4⁺ T cells and macrophages with the Nef–deficient virus and hoped that abolishing Nef functions would promote rapid killing in our infected macrophages. However, entirely eliminating Nef did not sensitize infected macrophages to killing, as seen in SIV–infected CD4⁺ T cells. Therefore, Nef appears to be neither necessary nor sufficient for the resistance of infected macrophages to elimination or suppression of viral replication by “unstimulated” freshly sorted SIV–specific CD8⁺ T cells.
Completing *ex vivo* viral suppression assays with a non–human primate model can have many limiting factors. Initially, co–cultures were to be completed with effector cells from rhesus macaque elite controllers, but we were limited in the availability of animals with the desired SIV–specific CD8$^+$ T cell responses. The SIVmac239Δnef variant contained a 181 bp deletion upstream from the Nef$^{137-146}$ RL10 epitope, resulting in a frame–shift that abrogated this downstream epitope. At the time of the co–cultures, the elite controllers available at the Wisconsin National Primate Center only had Nef$^{137-146}$RL10–CD8$^+$ T cell responses. Therefore, our experiments with this mutant were completed with SIV–specific CD8$^+$ T cells from infected or vaccinated animals. Since, we completed our experiments in parallel with infected CD4$^+$ T cells, it provided a good positive control in tracking the ability of the SIV–specific CD8$^+$ T cells to kill infected target cells. The effector cells were less efficient at killing WT–infected CD4$^+$ T cells, but significant killing was observed when infected with the Nef–deleted variant. Thereby, implying if Nef did, in fact, play a significant role in preventing killing by CD8$^+$ T cells we should have detected an increase in Δnef–infected macrophages.

We had hoped to complete additional experiments in parallel using alternative approaches to evaluate the ability of freshly sorted SIV–specific CD8$^+$ T cell to kill/respond to macrophages, compared to the infected CD4$^+$ T cells. Unfortunately, we were very limited in the availability of macrophages and SIV–specific CD8$^+$ T cell from rhesus macaques. Therefore, we decided to keep the experimental approach simple with repeated measurements, in order to observe a trend in the killing of target cells infected with Nef variants. As stated previously, it would have been beneficial to look at the ability of CD8$^+$ T cells to deliver effector molecules or respond to antigen presented on
target cells. Unfortunately, it would require additional co–cultures completed in parallel and analyzed differently, which was not possible with the limited cell number. Acquiring, blood samples from rhesus macaques is dependent on the weight of the animals limiting blood draws to once every 2 to 3 weeks. Thereby, restricting the number of experiments that could be completed.

We report here that entirely disrupting Nef function did not sensitize infected macrophages to rapid killing, as seen in SIV–infected CD4+ T cells. Therefore, it is not known if macrophages are intrinsically resistant to CD8+ T cell mediated killing or does infection of macrophages by HIV–1/SIV induce a protective mechanism. Our results imply that macrophages are resistant to CD8+ T cell killing by a Nef–independent mechanism. CD8+ T cells are able to lyse peritoneal macrophages infected with Lymphocytic choriomeningitis virus (LCMV), suggesting macrophages can be killed by virus–specific CD8+ T cells (248, 249). If LCMV–infected macrophages are susceptible to killing by primary CD8+ T cells, this would suggest HIV–1 may employ a mechanism to circumvent recognition or killing by CD8+ T cells. We have previously demonstrated that HIV–1 infected macrophages release pro–survival cytokines induced by the viral envelope protein that maintains cell survival in the face of apoptotic stimuli (197). Apoptotic sensitivity was restored when those pro–survival effectors were inhibited and macrophages succumbed to apoptosis (197). This same process may be involved in protecting infected macrophages from CD8+ T cell suppression.
Chapter 5

Final Conclusion and Perspective

5.1. Motivation

Monocytes and macrophages play important roles as HIV reservoirs. Macrophages can persist in sites where penetration of antiretrovirals is limited, such as the CNS, and establish viral reservoirs (165). Additionally, studies completed in rhesus macaques have revealed that macrophages are the principal reservoir after CD4⁺ T cell depletion (167). As a reservoir, macrophages present unique obstacles to infection. The infected macrophage tends to be resistant to the cytopathic effects of viral replication in comparison to activated CD4⁺ T cells (167, 195-197), and that HIV–1 has evolved mechanisms to prolong the life span of infected macrophages (197, 198). Interestingly, an infected macrophage can survive for long periods and store infectious virions that can be transmitted in trans (164). Other studies completed using ex vivo assays, have demonstrated that freshly sorted HIV/SIV–specific CD8⁺ T cells are less effective at suppressing viral replication in macrophages early in co–culture, and can spread infection to CD4⁺ T cell effector cells (231, 232). Collectively, it seems that infected macrophages have the ability to establish a persistent viral reservoir and have developed mechanisms to evade CD8⁺ T cell recognition and control of viral replication. In this regard, researchers have begun to explore approaches to eradicate persistent macrophage viral reservoirs. The studies conducted in the thesis attempt to determine the mechanism responsible for protecting SIV–infected macrophages from killing by freshly sorted CD8⁺ T cells, specifically focusing on the ability of Nef to alter MHC–I surface expression.
5.2. Summary

The work presented in this thesis evaluated the role of Nef–mediated MHC–I down–regulation, and general Nef function, in protecting infected macrophages from rapid killing by freshly sorted unstimulated CD8+ T cells. We examined the capacity of freshly isolated SIV–specific CD8+ T cells to control viral replication in primary target cells (CD4+ T cells and CD14+ monocyte–derived macrophages) infected with SIV’s harboring a nef variant containing a point mutation (Y223F) that has been shown to impair MHC–I down–regulation and a nef deletion mutant (Δnef) that would be expected to abrogate all activities of Nef, including MHC–I down–regulation. We show that although macrophages infected with SIV nef mutants that increase MHC–I expression, and entirely disrupt Nef function, this is not sufficient to impact their sensitivity to CD8+ T cell killing.

5.3. Involvement of Disrupting MHC–I Down–regulation in Infected Macrophages and Evasion from CD8+ T cell Recognition

HIV–1 infected CD4+ T cells have been shown to be eliminated by HIV–specific CD8+ T cells that were freshly sorted (231) or generated into cell lines (142, 231, 241) primarily in an MHC–I–dependent manner. Therefore, we investigated whether the disruption of the ability of Nef to down–regulate MHC–I molecules would sensitize the infected macrophages to elimination and suppression of viral replication by freshly sorted SIV–specific CD8+ T cells early in co–culture (142). However, altering MHC–I down–regulation by infecting macrophages with a Nef point mutant, did not sensitize macrophages to killing by freshly sorted SIV–specific CD8+ T cells. Furthermore, we did see significant elimination of WT–infected macrophages by CD8+ T cells lines in a
MHC–I dependent manner. These studies suggest that the interference of antigen presentation via MHC–I regulation does not affect CD8+ T cell killing in SIV–infected macrophage. Future experiments, could include evaluating the ability of freshly sorted SIV–specific CD8+ T cells to deliver effector molecules to SIV-infected macrophages, and determine if macrophages are expressing an inhibitor of effector molecules to protect them from CD8+ T cell mediated killing. It is possible that infected macrophages degrade effector molecules elicited by CD8+ T cells, for example by serine protease inhibitor 9 (PI–9). Interestingly, alveolar macrophages infected with *M. tuberculosis* (MTB) induce the expression of PI–9, an inhibitor of granzyme B (GrB), protecting infected macrophages from GrB–mediated apoptosis and promoting their survival (250, 251). Furthermore, monocytes from HIV–infected patients revealed high expression of PI–9, contributing to successful MTB infection and supported higher MTB growth (252). Thus, it may be worthwhile to screen PI–9 levels in our SIV–infected macrophages, and determine if silencing its expression would sensitize SIV–infected macrophages to killing by freshly sorted SIV–specific CD8+ T cell killing.

### 5.4. Nef is Dispensable in Sensitizing Macrophages to CD8+ T Cell Killing

HIV/SIV have developed protective mechanisms to prolong the life span of infected macrophages. Specifically, Nef has been suggested in protecting monocyte–derived–macrophages from HIV–1 induced apoptosis, as well as, down–regulate MHC–I expression (135, 242, 247). Accordingly, we reasoned that SIV–infected macrophages have developed resistance to CD8+ T cell–mediated killing via a Nef–dependent mechanism. It is possible that abrogating Nef function would promote killing in our resistant infected macrophages. However, disrupting the Nef function, did not further
sensitize macrophages to killing by primary effector cells, as observed in infected CD4⁺ T cells. Therefore, my results suggest that Nef appears neither necessary nor sufficient for the resistance of infected macrophages to elimination or suppression of viral replication by “unstimulated” freshly sorted SIV–specific CD8⁺ T cells. HIV may have acquired Nef to protect infected CD4⁺ T cells from CD8–mediated killing, but macrophages seem to have developed alternate mechanisms for preserving viability in the face of the antiviral CD8 T cell responses.

It is important to state that the results presented in this thesis do not suggest that Nef is not significant in establishing the macrophage as a HIV/SIV reservoir in the CNS. As mentioned in the introduction, Nef contributes to the pathogenesis observed in the brain in infected individuals (171). Nef has been proven to attract monocytes and macrophages into the brain where their infection can result in a cytotoxic environment (174-176). The expression of Nef can lead to a dysfunctional blood brain barrier (BBB) (177). Specifically, Nef can induce apoptosis in human brain microvascular endothelial cells leading to blood brain barrier (BBB) dysfunction (177). Furthermore, the disruption of tight junctions by an impaired matrix-metalloproteinase–9 mechanism can result in a dysfunctional BBB that is Nef–mediated (174, 176). Nef may also contribute to escape from immune surveillance and viral replication in the brain. Interestingly, CNS– and lymphoid derived HIV–1 isolates have revealed the highly conserved functions of CD4 and MHC–I down–modulation among Nef alleles (178). Therefore, Nef plays a significant role in HIV/SIV pathogenesis but possibly not macrophage resistance to CD8⁺ T cell killing.
Are macrophages intrinsically resistant to CD8–mediated T cell killing or does infection of macrophages by HIV–1/SIV induce a protective mechanism? CD8+ T cells are able to lyse peritoneal macrophages infected with Lymphocytic choriomeningitis virus (LCMV), suggesting that macrophages can be killed by virus–specific CD8+ T cells (248, 249). If LCMV–infected macrophages are susceptible to killing by primary CD8+ T cells, this would suggest HIV–1 may employ a mechanism to circumvent recognition or killing by CD8+ T cells. Many viruses have adopted various strategies to preserve host cell viability in the face of cytopathicity and cell–mediated clearance forces. In particular, Kaposi sarcoma-associated human herpes virus–8, and related gammaherpesviruses, prevents the triggering of TRAIL–induced apoptosis by encoding viral FLICE–inhibitory proteins (vFLIPs) that interact with the Fas–associated death domain (FADD) to inhibit active caspase 8 generation (253). Additionally, T cells infected with Human T cell leukemia virus type 1–infected are resistant to TRAIL–mediated apoptosis by the viral transactivator Tax (254). Furthermore, three proteins (E3 RID) encoded by the human adenovirus type 5 induce the internalization of TRAIL receptors from the cell surface targeting lysosomal degradation (255). Moreover, the human herpesvirus 7 is resistant to TRAIL–mediated cytotoxicity and this is associated with down–regulation of the TRAIL–R1 receptor from the surface of cells (256). Also in B cells, the BHRF1 protein is encoded by the Epstein–Barr virus and is responsible for the inhibition of apoptosis by TRAIL (257, 258).

We have previously demonstrated that HIV–1 induces pro-survival factors (MCSF) that preserve host cell viability in the face of cytopathicity. In HIV–infected macrophages, the envelope glycoprotein induces the pro-survival cytokine, Monocyte
Colony Stimulating Factor (MCSF). This circumvents TRAIL–mediated apoptosis to maintain cell survival in the face of apoptotic stimuli, thereby affording macrophages protection from the cytopathic effects of the virus (197). Apoptotic sensitivity could be restored when those pro–survival effectors were inhibited by treating infected macrophages with Imatinib, an anti–cancer drug, that has the ability to block signaling by the MCSF receptor (197). Induction of pro–survival cytokines, such as MCSF, appears to be envelope–dependent and independent of Nef, and indicates that Nef is dispensable for the resistance of infected macrophages to CD8\(^+\) T cell killing. The fact that inactivating mutations in Nef do not restore susceptibility to CD8\(^+\) T cell surveillance implies that the induction of pro–survival pathways in infected macrophages was Nef–independent. This further suggests that the induction of pro–survival pathways creates a phenotype that overrides the impact of cytotoxic CD8\(^+\) T cells on infected macrophages. Therefore, the results in the current study, that Nef does not underscore resistance to CD8\(^+\) T cell killing, can be reconciled by our previous observations that the viral envelope may harness activities that maintain the viability of the infected macrophage in the face of immune (CD8\(^+\) T cell killing) or viral (cytopathicity) assault. Studies are underway to determine whether induction of pro–survival cytokines in infected macrophages underscores the resistance of infected macrophages to CD8\(^+\) T cell killing, and we are now exploring whether pharmacologic inhibition of these pro–survival pathways can restore viral cytopathicity and the killing of infected macrophages by CD8\(^+\) T cells. Determining the intrinsic factors in HIV/SIV–infected macrophages that protect from elimination by CD8\(^+\) T cells, could help in developing drug treatments to eradicate the macrophage reservoir.
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