

**INTRACELLULAR TRAFFICKING OF MHC-I IN NORMAL AND HIV-1 NEF
EXPRESSING CELLS**

by

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**A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Immunology)
in The University of Michigan
2008**

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ACKNOWLEDGEMENTS

I would like to start by thanking my parents, Ruth and John Schaefer. They have always given me unconditional love and support. I would also like to thank my sisters; Dawn, Wendy, Anna, Beth and Rachael. They have always made time to talk with me and offer support and a listening ear.

I would like to express my appreciation to my mentor, Dr. Kathleen Collins. She has been an excellent mentor and is an outstanding scientist. I feel very fortunate to have been trained in her laboratory. She has always exhibited the highest scientific integrity and I have learned many skills that will be extremely valuable throughout my career.

I would like to thank the past and present members of the Collins Laboratory, Chris, Jason, Elizabeth, Jolie, Anna, Adewunmi, Deanna, Maya, Matt, Jeremiah, Tracey, and Penney. They have always freely offered scientific suggestions and advice and have made the lab an enjoyable place to work. Specifically, I would like to thank the individuals that contributed excellent work to the individual chapters. Jeremiah Roeth, Elizabeth Wonderlich, and Jolie Leonard contributed to Chapter II. Deanna Kulpa, Maya Williams and Penelope Blakely contributed to Chapter III.

I would also like to thank my thesis committee members, Keith Bishop, Colin Duckett, Nick Lukacs and Malini Raghavan, for their guidance and support throughout my graduate career. I also thank the Graduate Program in Immunology, Ellen Elkin and Zarinah Aquil for wonderful support during my graduate career.

Additionally, I thank the Microscopy and Image analysis Laboratory and the Vector Core for excellent technical support. I also thank Patrizio Giacomini for the generous gift of the L31 antibody used in Chapter III.

TABLE OF CONTENTS

Acknowledgements	ii
List of Figures.....	vi
Abstract.....	viii
CHAPTER I.....	1
INTRODUCTION	
Introduction.....	1
HIV Biology.....	2
HIV Disease Progression.....	3
The Role of HIV-1 Nef in Disease Progression.....	5
Cellular Trafficking Pathways Affected by Nef.....	7
Nef Mediated CD4 Downmodulation.....	10
Nef Mediated Disruption of the Immune Response.....	13
MHC-I Biology.....	15
Nef Mediated MHC-I Downmodulation.....	18
Selective Downmodulation by Nef.....	26
HLA-C Biology.....	27
HLA-C and NK Cell KIRs.....	28
HLA-C Questions.....	32
Overview of Thesis.....	32
CHAPTER II.....	52
HIV-1 NEF TARGETS MHC-I AND CD4 FOR DEGRADATION VIA A FINAL COMMON β -COP-DEPENDENT PATHWAY IN T CELLS	
Abstract.....	52
Introduction.....	53
Results.....	57
Discussion.....	67
Materials and Methods.....	74
References.....	99

CHAPTER III.....	105
------------------	-----

A NOVEL TRAFFICKING SIGNAL WITHIN THE HLA-C CYTOPLASMIC
TAIL ALLOWS REGULATED EXPRESSION UPON DIFFERENTIATION OF
MACROPHAGES

Abstract.....	105
Introduction.....	106
Results.....	109
Discussion.....	123
Materials and Methods.....	128
References.....	152

CHAPTER IV.....	155
-----------------	-----

DISCUSSION

Significant Conclusions.....	155
HIV-1 Targets MHC-I and CD4 for Degradation via a β -COP dependent Pathway.....	156
Future Directions for Nef Mediated Disruption of Intracellular Trafficking Pathways.....	164
A Novel Trafficking Signal in the HLA-C Cytoplasmic Tail Regulates Its Cell Surface Expression Upon the Differentiation of Macrophages.....	167
Future Directions for HLA-C Biology and Trafficking Pathways.....	172
Concluding Remarks.....	176
References.....	178

LIST OF FIGURES

Figure

1-1.	Genomic Map of HIV-1.....	34
1-2.	Disease Progression in an untreated HIV infection.....	35
1-3.	Domains in HIV-1 Nef required for MHC-I and CD4 downmodulation and degradation.....	36
2-1.	The cytoplasmic tail domain of MHC-I and CD4 determines the mechanism by which Nef affects trafficking.....	82
2-2.	MHC-I and CD4 co-localize in a subset of vesicles in Nef-expressing T cells....	84
2-3.	Bafilomycin treatment increases MHC-I and CD4 co-localization in Nef expressing cells.....	86
2-4.	Knockdown of β -COP does not affect HLA-A2 transport to the cell surface or the overall structure of the Golgi apparatus.....	88
2-5.	A second siRNA directed at β -COP disrupts Nef-dependent MHC-I trafficking.	89
2-6.	Nef requires β -COP to reduce HLA-A2 cell surface expression and target it for degradation.....	91
2-7.	Nef requires β -COP to target HLA-A2 and CD4 for degradation.....	93
2-8.	β -COP does not disrupt co-localization of CD4 and HLA-A2, but does increase the amount of stainable protein within the cell.....	94
2-9.	Binding of HLA-A2 and CD4 to HIV-1 Nef and cellular co-factors in T cells...	96
2-10.	V ₁₀ E Δ 17-26 Nef is defective at MHC-I, but not CD4, degradation.....	98
3-1.	The extracellular and cytoplasmic domains of HLA-C influence surface expression.....	137
3-2.	Expression of HA-tagged chimeric MHC-I molecules.....	138

3-3.	The HLA-C tail does not affect transport from the ER into the Golgi apparatus.....	139
3-4.	The HLA-C tail disrupts protein transport and accelerates internalization from the cell surface.....	141
3-5.	The cytoplasmic tail of HLA-C does not inhibit recycling.....	142
3-6.	The HLA-C cytoplasmic tail promotes lysosomal targeting.....	144
3-7.	Expression of A2/C cytoplasmic tail point mutants.....	145
3-8.	Isoleucine at position 337 (I ₃₃₇) is required for accelerated internalization and lysosomal targeting.....	146
3-9.	Aspartic acid at position 333 (D ₃₃₃) in the HLA-C cytoplasmic tail attenuates the internalization and lysosomal targeting signal.....	147
3-10.	Macrophage differentiation upregulates HLA-C and HA-A2/C.....	148
3-11.	Serine 335 (S ₃₃₅) regulates HLA-Cw*0401 expression in macrophage cell lines.....	151
4-1.	Model for the mechanism by which Nef affects CD4 and MHC-I trafficking...	177

ABSTRACT

HIV-1 establishes a chronic infection. To facilitate viral infection and spread, HIV-1 Nef disrupts the surface expression of the viral receptor (CD4) and antigen presenting molecules (MHC-I). Nef binds the cytoplasmic tails of both molecules and disrupts their trafficking using separate mechanisms that are incompletely understood. We demonstrate here that these distinct mechanisms are dictated by differences in the cytoplasmic tails of CD4 and MHC-I. Despite these differences, we demonstrate that MHC-I and CD4 are ultimately targeted to the same degradative compartments via interaction of Nef with the cellular protein β -COP. Moreover, we demonstrate that the N-terminal α -helical and dimerization domains of Nef are required for the Nef/ β -COP interaction. These findings have revealed the convergence of separate degradative pathways for CD4 and MHC-I, providing insight into the mechanisms used by HIV to evade the immune response.

The removal of MHC-I from the cell surface protects HIV-infected cells from cytotoxic T lymphocytes. However, complete downmodulation of MHC-I by HIV Nef would render the infected cell susceptible to NK cell-mediated destruction. This scenario may be avoided by Nef's inability to downmodulate certain MHC-I alleles, HLA-C and HLA-E, which inhibit NK cells. In the present work, we sought to better understand the biology of HLA-C in order to discern its role in HIV infection.

Compared to other classical MHC-I allotypes, HLA-C has low cell surface expression and altered intracellular trafficking. Our studies have revealed that aspects of HLA-C trafficking are controlled by both the extracellular and cytosolic domains, in both CD4+ T cells and macrophages. Interestingly, we also found that HLA-C expression is upregulated upon macrophage differentiation, revealing the possibility of previously uncharacterized roles for HLA-C in the immune system. These studies bring us closer to understanding the role of HLA-C in the immune system and in HIV infection.

The studies described in this thesis further our understanding of the interplay between HIV and the host immune system and provide insight into the biology of HLA-C in T cells and macrophages. Collectively, these data contribute significantly to our knowledge in areas crucial for the development of better antiretroviral therapies and vaccine strategies.

CHAPTER I

INTRODUCTION

In 1983, when a novel human retrovirus was determined to be the causative agent of Acquired Immunodeficiency Syndrome (AIDS) (14, 56, 107) it seemed certain that a cure would be available soon and a vaccine shortly thereafter. Later, the virus was named Human Immunodeficiency Virus (HIV) (31). Sadly, more than 25 years later we have no cure or vaccine, only treatments that can improve the quality of life and prolong an infected individual's life expectancy. As a scientific community, we have answered many of the important questions concerning the origins, life cycle and pathogenesis of HIV, but there are just as many questions that remain unanswered and these questions are proving to be more challenging than the previous ones. However, it is essential that we continue to ask and answer these questions. Worldwide, 40 million people are infected with HIV, 22 million have died (197), and 14 million have been orphaned. In addition to loss of life, HIV has also wreaked economic havoc worldwide, but especially in developing countries. Thus, it is essential that we continue to study HIV so that we can

continue to improve the quality of life for those who are HIV positive and living with AIDS.

HIV Biology

HIV-1 belongs to the *Retroviridae* family, indicating that the RNA genome is transcribed into DNA within the cell using a viral enzyme called reverse transcriptase. Within the retrovirus family, HIV-1 is in the genus *Lentivirus*. The HIV-1 RNA genome is 9 kilobases in length and encodes 15 different proteins (53, 131). The main proteins have been classified into three groups; structural, regulatory, and accessory proteins. The structural proteins are Gag, Pol and Env; the regulatory proteins are Tat and Rev; and the accessory proteins are Vpu, Vpr, Vif, and Nef (Figure 1-1).

To date, three major classes of HIV-1 have emerged: M (main), N (new/neither M or O), and O (outlier). Group M accounts for more than 90% of worldwide HIV infections and has been further divided into 9 subtypes, or clades, that are designated by the letters A-D, F-H, J and K (72, 168). Among Western European and American infections, Clade B is the most common and most of the current drug development has targeted this clade.

During HIV infection, the viral tropism is determined by the envelope protein (Env). HIV uses a primary receptor, CD4, and a coreceptor, most often CXCR4 or CCR5 (3, 45, 51, 128). CXCR4 is mainly expressed on CD4+ T cells, and HIV virions that use this coreceptor are referred to as X4 viruses or T-cell tropic. Alternatively, CCR5 is mainly

expressed on macrophages, and HIV virions that use this coreceptor are referred to as R5 viruses or macrophage-tropic. Interestingly, after seroconversion, R5 viruses are the first strains isolated and are thus considered to be responsible for HIV transmission (6). Indeed, some individuals tend to exhibit a strong resistance to HIV because they are homozygous for a mutant allele of CCR5 that contains a 32-base pair deletion (CCR5-delta-32) and is not expressed on the cell surface (39, 110, 160).

HIV Disease Progression

CD4+ T cell depletion through peripheral destruction and decreased production is the hallmark of HIV disease progression (69, 71, 126, 158, 190). As plasma viral titers rise, untreated individuals experience continual decline in CD4+ T cells levels to the point that the immune system is disabled and they are overcome by opportunistic infections. HIV disease progression can be divided into three phases: primary HIV infection, chronic HIV infection and clinical AIDS (Figure 1-2). Markers present in each phase have been characterized and correlate with the rate at which an infected individual will progress through HIV infection to AIDS.

Primary HIV infection is the time from initial infection to the development of an antibody response detectable by standard tests. Infected individuals will often experience mononucleosis-type symptoms lasting for a few days to a few weeks (34, 86). A correlation between more severe clinical symptoms in primary HIV infection with a more rapid clinical course of HIV disease progression has been reported (143). During primary

HIV infection, CD4+ T cell counts decline as viral titer levels increase, sometimes low enough to allow opportunistic infections to develop (67, 142, 181). As CD4+ T cell levels rebound and viral titer levels decrease, a steady state viral load, termed the viral set-point, is established. Generally, the time to reach the viral set point ranges from 3 to 5 months (90). In addition to the severity of clinical symptoms in primary HIV infection, a high viral set-point has also been correlated with increased HIV disease progression (73, 121, 122).

In contrast to the dramatic fluctuation in CD4+ T cell levels and viral load experienced during primary HIV infection, chronic HIV infection is characterized by equilibrium between viral replication and the host immune response. But, despite equilibrium being reached, most infected individuals will have progressive loss of CD4+ T cells (12, 94, 104, 130). Additionally, a higher viral load is correlated with the rate of CD4+ T cell decline. Together the rate of CD4+ T cell loss and viral load can predict how quickly an individual will progress to clinical AIDS (121, 137).

Clinical AIDS can be diagnosed by either laboratory methods (CD4+ T cell levels fall below 200 cells/ μ L) or by clinical criteria (development of an opportunistic illness). Prior to antiretroviral treatment, survival time in the United States after diagnosis of AIDS was 10-12 months (52).

Despite the grim prognosis of HIV infection, some individuals progress to AIDS more slowly than others. A number of host factors have been identified to contribute to HIV

disease progression. Individuals that become infected with HIV at an older age tend to have more rapid disease progression and shorter survival times (11, 130). As mentioned previously, individuals homozygous for CCR5-delta-32 exhibit a strong resistance to HIV infection and, while heterozygous individuals have normal infections rates, they do display delayed progression to AIDS (109). In addition, various HLA alleles have been associated with the rate of disease progression. Individuals that have general HLA homozygosity or express HLA-B35 and HLA-Cw4 alleles tend to have accelerated disease progression (75, 83, 176, 178), while HLA-B27 and B57 alleles are associated with long-term nonprogression of HIV (87).

The Role of HIV-1 Nef in Disease Progression

In addition to host factors, viral factors have been shown to influence disease progression. The HIV accessory protein Nef was once thought to be a **negative factor** that inhibited viral replication (28) but has since been shown to be necessary for optimal viral infection, inhibiting infected cell death and increasing viral spread. Individuals infected by an HIV with a defective Nef protein do not display the typical progression to clinical AIDS (42), however, after an extended time some of these individuals have begun to show declining CD4 counts (18, 102). In SCID mice infected with a *nef* deleted HIV-1 mutant, the characteristic decline of CD4⁺ T cells was not observed; additionally, the viral *nef*-mutants had attenuated growth rates (76). Rhesus macaques infected with a simian immunodeficiency virus (SIV) lacking a functional Nef protein also did not show the typical disease progression (92). These examples illustrate the importance of Nef in

disease progression, and the inability of HIV to fully evade the host's immune system without a functional Nef protein. Also, because this viral protein can influence disease outcome so dramatically, these examples highlight the need to more fully understand Nef's functions.

Nef is expressed early in the viral life cycle and is one of the HIV accessory proteins. Although no enzymatic activities have been reported, it is myristoylated and is known to interact with many host cellular proteins. Consequently, Nef is thought to alter cellular pathways by acting as a multifaceted molecular adaptor protein. Structurally, Nef exhibits an overall flexibility that has been speculated to allow multiple conformations (8, 55). Indeed, Nef has been reported to affect a wide array of cellular functions, such as protein trafficking, signal transduction cascades and apoptotic pathways.

To enhance viral infection, Nef activates CD4⁺ T cells, making them more susceptible to HIV infection. Nef alters signal transduction pathways downstream of the TCR by modulating Vav (48), p21-activated kinase 2 (47, 136, 151), Rac (184), CDC42 (111), and the DOCK2/ELMO1 complex (77). In addition to enhancing viral infection, Nef also increases infectivity by removing CD4 from the cell surface, which can interfere with viral budding and release (49, 58).

In addition to enhancing viral infectivity, Nef also promotes viral spread. Infected macrophages are induced to produce macrophage inflammatory protein 1 α and 1 β and other soluble factors to recruit T cells (174, 175) and dendritic cells (DC) that become

infected upregulate DC-SIGN, which can capture virions and transmit them to target cells (167).

When a cell is infected, it is in the best interest of the host to eliminate that cell and several mechanisms have evolved to detect and eliminate virally infected cells. HIV has subsequently evolved to counter these mechanisms through Nef. By altering intracellular trafficking and signaling pathways Nef can inhibit apoptosis of an infected cell and avoid cytotoxic T lymphocyte (CTL) recognition of infected cells. Nef inhibits apoptosis by modulating p53 mediated apoptosis (63) and ASK1 mediated apoptosis (54). To avoid CTLs, which recognize infected cells via peptides presented on major histocompatibility complex class 1 molecules (MHC-I), Nef removes MHC-I from the cell surface (33).

Cellular Trafficking Pathways Affected by Nef

To accomplish several of the goals stated above, Nef modulates intracellular trafficking pathways involving adaptor protein complexes, the V1H subunit of the vacuolar ATPase, PACS-1, and COP1 vesicles.

Adaptor Protein Complexes

Proteins are trafficked through the cell by recruitment into coated vesicles. Recruitment can be mediated via the heterotetrameric adaptor protein (AP) complexes, of which there are four, AP-1, AP-2, AP-3 and AP-4 (for a review, see reference (152)). Proteins are

recruited when the AP complexes bind to motifs present in the cytoplasmic tail domain. All four AP complexes are able to recognize two signals: tyrosine based (YXX Φ) or dileucine based ([D/E]XXXL[L/I]) (where X is any amino acid and Φ is a large hydrophobic amino acid). Each AP complex has a unique intracellular distribution which affords it a certain amount of protein recruitment specificity. AP-1 is localized to the TGN and cycles between the TGN and endosomal network. AP-2 is located at the plasma membrane (PM) and also traffics proteins to the endosomal network. AP-3 is localized to the endosomal network and traffics proteins to lysosomes. AP-4 is less well characterized but it is known that it is localized to the TGN, similarly to AP-1. Both AP-1 and AP-2 recruit proteins into clathrin-coated vesicles, while AP-3 and AP-4 have been reported to function independently of clathrin.

Nef contains a dileucine (LL₁₆₇, Figure 1-3) binding motif and has been shown to interact with AP subunits and AP complexes through yeast two-hybrid (37, 46, 82, 100, 144) and GST-pull down assays (22, 80, 101, 144). Furthermore, the dileucine residues are needed for CD4 cell surface removal (60, 118).

VIH

VIH is the H subunit of the vacuolar ATPase, a multisubunit enzyme that facilitates the acidification of intracellular compartments and plays a role in receptor mediated endocytosis, intracellular trafficking and protein degradation (120, 169). VIH is able to bind AP-2 and is thought to be a connector between Nef and the endocytic machinery, as

Nef interacts with VIH through its dileucine (LL₁₆₅) and diaspartic acid (DD₁₇₅, Figure 1-3) motifs in the C-terminal flexible loop (56, 112).

PACS-1

When phosphofurin acid cluster sorting protein 1 (PACS-1) was first reported, its initial function was to control the transport of furin and manose-6-phosphate receptor trafficking from endosomes to TGN (188). It does this by linking the acidic-cluster in the cytoplasmic tail domain with AP-1 (188). When Nef is present in a cell, MHC-I accumulates in the region of the Golgi/TGN (155, 172). Since Nef contains an N-terminal acidic-cluster (EEEE₆₅, Figure 1-3), it has been suggested that the Nef-mediated Golgi/TGN accumulation of MHC-I is due to a Nef/PACS-1 interaction (19, 147).

COP-I

Traditionally, COP-I vesicles are made of coatomer consisting of seven subunits (α , β , β' , γ , δ , ϵ , and ζ) and mediate retrograde transport between the Golgi and endoplasmic reticulum (ER) by binding to KKXX motifs (for review see reference (13)). Recently, however, COP-I vesicles have been found in the endosomal network, mediating transport between early and late endosomes (5, 66, 191). These COP-I vesicles are distinct from the COP-I vesicles involved in Golgi-ER transport, in that not all seven subunits are required for coatomer formation; γ -COP and δ -COP are not found on endosomes (5, 66, 191). Also, the function of the endosomal COP subunits can be dissected; β , β' , ζ -COPs

are able to mediate membrane association, while α and ε -COP are needed for coat activity (66). Equally interesting, the membrane association of β -COP and the formation of transport vesicles is dependent of luminal pH (5).

Nef also interacts with β -COP and has been implicated the in CD4 degradation pathway (50, 145). The Nef/ β -COP interaction was first reported from a yeast two-hybrid screen (14). It has been suggested that a diglutamic acid (EE₁₅₅, Figure 1-3) in Nef mediates this interaction (145) but this result has been controversial (81). Subsequently, it was reported that this diglutamic acid motif interacts with ARF1, a small GTPase important for endosomal trafficking, and, in turn, this recruits β -COP (50). The significance of the Nef/ β -COP interaction is not well understood, and hopefully, future work will be able to clarify the existing questions.

Nef Mediated CD4 Downmodulation

Nef modulates the intracellular trafficking of a wide variety of host cellular proteins, including: CD4, MHC-I, MHC-II, CD28, transferrin receptor, mannose receptor, CD80, CD86, CD8, CD1d, CCR5, TNF, LIGHT, DC-SIGN, and the invariant chain (4, 26, 27, 96, 116, 163, 164, 167, 171, 173, 182). The intracellular trafficking pathways of CD4 and MHC-I and how Nef influences them have been well studied and will be reviewed here.

CD4 is the main receptor used by HIV for cell entry. However, once viral entry has occurred the presence to CD4 on cell surface can be detrimental to HIV pathogenesis. CD4 on the surface of an HIV infected cell can promote viral superinfection and inhibit the release of new virions (15, 21, 35, 192). Prior studies of Nef mediated CD4 downmodulation have demonstrated that Nef promotes increased internalization of cell surface CD4 and degradation through transport to acidic compartments. This model is generally agreed upon as it is based on work from several labs (for review see references (10, 40, 146)).

Nef Binds CD4

The interaction between Nef and the CD4 cytoplasmic tail has been well studied. Indeed, Nef has been shown to bind CD4 through yeast two-hybrid assay systems (157), purified protein systems (65, 68, 148), in cell lysates (85) and in living cells (16, 30). NMR structural analysis has indicated the Nef core domain interacts with the CD4 cytoplasmic tail (65). Originally, the interaction was thought to occur between the core domain of Nef and a 13 amino acid stretch (QIKRLLSEKKT) of CD4. Even though this interaction was highly specific, it was quite weak. Further experiments demonstrated a more stable interaction when full length Nef was used, indicating other Nef domains may contribute to the interaction (148).

Nef Increases CD4 Internalization

Two domains in Nef are required for increased CD4 internalization: a dileucine motif (LL₁₆₅) and a diaspatic acid motif (DD₁₇₅). The dileucine and diaspatic acid domains are needed for V1H binding (55, 56, 117) and the dileucine domain is needed for AP-2 binding (37). Interestingly, both V1H and AP-2 are involved in internalization at the PM. A popular hypothesis is that Nef recruits both of these molecules and uses them to facilitate CD4 internalization; however, this hypothesis is based on correlative findings that mutations in the domains required for increased internalization also bind these cellular factors.

To strengthen this model, several groups have used RNA interference to dissect the molecular interactions required for Nef mediated CD4 internalization. Unfortunately, this approach has not provided the desired clarity, as different conclusions have been drawn from different groups. One group reported that knockdown of AP-2 had no effect on HIV-1 Nef CD4 downmodulation, whereas SIV Nef CD4 downmodulation was inhibited and transferrin receptor internalization was blocked (156). Other studies have concluded that AP-2 is necessary (84, 171), although one study found the dependency on AP-2 was only if Eps-15 was knocked down also (84). Discrepancies between the studies could be due to the cell type used and that SIV Nef contains N-terminal tyrosine amino acids that bind AP-2 (117, 144). This model would be greatly strengthened if a three way interaction between Nef/CD4/AP-2 could be demonstrated.

Nef Targets CD4 to Acidic Compartments for Degradation

The mechanism by which Nef targets CD4 for degradation in acidic compartments is also not well understood. It has been reported the Nef diglutamic acid domain (EE₁₅₅) recruits β -COP via Arf1 (50, 145) and this complex targets CD4 for degradation. However, subsequent studies were unable to demonstrate a role for the diglutamic acid domain in CD4 downmodulation (81). Thus, these data remain controversial and additional studies will be needed to determine the role of the diglutamic acid domain in β -COP recruitment and CD4 downmodulation and degradation.

Nef Mediated Disruption of the Immune Response

The immune system is constantly surveying the intracellular content of nucleated cells looking for the presence of intracellular pathogens. This immune surveillance occurs through a process known as antigen presentation. Antigen presentation takes place when intracellular proteins are displayed on the cell surface as peptides in complex with MHC-I. In a sense, these peptides represent a snapshot of the intracellular protein content. How these peptides are derived and how they are associated with MHC-I is discussed below.

The specialized immune cell capable of recognizing the MHC-I/peptide complex is the CD8⁺ T cell. Recognition of the MHC-I/peptide complex requires direct cell-cell contact with a CD8⁺ T cell. If the CD8⁺ T cell recognizes the peptide, meaning the cell is virally infected, the CD8⁺ cell is activated, and becomes a cytotoxic T lymphocyte (CTL). CTLs target infected cells for destruction through perforin, granzymes, and *fas*-mediated

apoptosis (17). Additionally, CTLs can secrete cytokines and chemokines to suppress viral infections. In HIV infection, the β -chemokines, macrophage inflammatory protein 1 α and RANTES, suppress HIV infection through competition for CCR5 (3, 29) and IFN- γ suppresses HIV replication by inducing cellular antiviral proteins (123). CTLs also secrete a soluble factor called CD8⁺ cell antiviral factor (CAF) (23, 187). Although CAF has not been formally identified, studies have demonstrated CAF blocks viral transcription (115) and can be detected by adding supernatants from CTLs directly onto infected CD4⁺ T cells (115, 189). Clearly, this is an intriguing factor and formal identification will aid in the development of new antiviral therapies.

Similar to other viruses, HIV has evolved mechanisms to avoid immune detection. To evade the CTL response HIV had developed several mechanisms, including: mutation of immunodominant epitopes (20, 59) and induction of CTL apoptosis by Fas and tumor necrosis factor (199). An additional mechanism to evade CTLs is disruption of antigen presentation by the removal of MHC-I from the cell surface through Nef. Removing MHC-I from the cell surface effectively makes the infected cell invisible to CTLs, as evidenced by the demonstration that HIV-1 specific CTLs were able to recognize CD4⁺ T cells infected with a *nef*-defective HIV-1, but were unable to recognize cells infected with an HIV-1 with an intact Nef protein (33).

Disruption of antigen presentation via MHC-I cell surface removal allows several CTL effects to be abrogated, such as: specific cytotoxic activity, cytokine production and suppression of HIV replication. CD4⁺ T cells infected with either a wild type HIV or an

HIV expressing a Nef protein defective in MHC-I downmodulation only were cultured with HIV specific CTLs. The CTLs cultured in the presence of the nef-defective HIV showed greater levels of cytokine production, specifically IFN- γ , and stronger suppression of HIV replication compared to CTLs cultured in the presence of wild type HIV (177). Collectively, these data indicate that disruption of antigen presentation by Nef mediated removal of MHC-I from the cell surface is vital to disrupting the CTL immune response.

MHC-I Biology

Antigen presentation allows the immune system to monitor the intracellular protein content through peptides that are derived from intracellular proteins. Under normal conditions the peptides are derived from host cellular proteins, but when a cell becomes infected, the pathogen's proteins are also degraded into peptides. Peptides are displayed, or presented, on the cell surface by MHC-I molecules and this is how the immune system is able to detect an infected cell.

A functional MHC-I molecule consists of a transmembrane heavy chain, a light chain called β 2 microglobulin, and a peptide 8 to 10 amino acids in length. MHC-I protein folding and peptide generation have been subject of numerous studies (for review see reference (38, 44, 64, 165)) and is reviewed below.

Peptide Processing and Transport into the ER Lumen

Peptides are generated from ubiquitinated intracellular proteins by the proteasome. The proteasome is multisubunit threonine protease complex that is abundant throughout the cell. The complex consists of a central cylindrical barrel (the 20S part) and one or two caps (the 19S part) (64). Studies have suggested the proteasome generates peptides 15 amino acids in length, which are then further trimmed by cytosolic peptidases (25, 149, 150).

Once peptides have been generated in the cytosol, they are translocated into the ER lumen by the transporter associated with antigen processing (TAP). TAP, a member of the ATP-binding cassette transporter family, is composed of two subunits, TAP1 and TAP2 (64). TAP acts as a transmembrane pore and ATP hydrolysis by TAP1 and TAP2 is needed for peptide transport activity (9). Similar to MHC-I, TAP prefers peptides 8-12 amino acids in length (95, 153).

MHC-I Assembly and Peptide Loading

As the MHC-I heavy chain is synthesized into the ER lumen it binds the membrane associated ER chaperone calnexin (38). Calnexin facilitates proper folding and intrachain disulfide bond formation (38, 204). β 2 microglobulin binds the heavy chain upon dissociation from calnexin and both are incorporated into the peptide loading complex. The peptide loading complex consists of TAP, the transmembrane glycoprotein tapasin, the soluble ER chaperone, calreticulin, and the soluble thiol oxidoreductase, ERp57 (38,

204). At this point, peptides are loaded into the peptide binding groove and bind to specific amino acids termed anchor residues (38, 64). The anchor residues confer the MHC-I peptide specificity. Once fully assembled, MHC-I molecule leaves the ER and travels to the plasma membrane.

MHC-I alleles

There are several MHC-I gene products and they can be subdivided into classical and nonclassical groups. Human leukocyte antigen (HLA)-A, -B, and -C are considered classical MHC-I molecules. They fulfill the “classical” role by presenting peptides to CTLs, although HLA-B and -C have been shown to inhibit natural killer (NK) cells as well (97, 179, 202). Each of these genes is very polymorphic, HLA-A has 303 alleles, HLA-B has 559 alleles and HLA-C has 150 alleles (78). Polymorphisms among the MHC-I alleles is an important strength, in that different MHC-I allotypes have different affinities for peptide sequences. Since certain allotypes of MHC-I have greater affinity for some peptides, it has been hypothesized that this could exert a protective effect for one disease, but could exert negative effect for others. It is thought that this heterogeneity arose to ensure that a particular pathogen would not exterminate an entire population. An example of this is the overrepresentation of HLA-B*5701 in long-term nonprogressor HIV patients (124); these individuals appear to be especially equipped to control HIV.

The nonclassical MHC-I are HLA-E, -F, -G, -H, and -J. These MHC-I are nonpolymorphic and serve specialized roles in the immune system. HLA-E binds peptides derived from the leader sequence of other MHC-I (79) and can interact with the receptor NKG2A. NKG2A is an inhibitory ligand present on NK cells and stimulation inhibits the cytotoxic activity of the NK cell. HLA-G interacts with the inhibitory receptor, ILT-2, and also inhibits the cytotoxic activity of NK cells. HLA-G is expressed only on fetus-derived placental cells. As these cells do not express other types of MHC-I and it is hypothesized that HLA-G expression protects these cells from NK cell mediated killing (79). Not much is known about the other nonclassical MHC-I molecules, HLA-F, -H, and -J.

Nef Mediated MHC-I Downmodulation

The first reports that HIV reduces the cell surface expression of MHC-I came in 1989 (91, 162), but it was not until 1996 that this reduction was attributed to Nef and a mechanism based on increased internalization was proposed (164). Subsequently, it was demonstrated the reduction was enough to enable HIV infected cells to evade CTL recognition and killing (33).

Nef directly binds the MHC-I cytoplasmic tail. Mutagenesis studies have shown the amino acid sequence YSQAASS in the MHC-I cytoplasmic tail is needed for Nef binding (194). Interestingly, this is the same amino acid sequence needed for downmodulation and MHC-I molecules that lack this sequence (HLA-E and HLA-C) are not

downmodulated and do not bind Nef (32, 194, 196). There are several domains in Nef needed for MHC-I downmodulation including: the N-terminal alpha helix, polyproline, acidic-cluster and oligomerization domains (Figure 1-3) (2, 32, 60, 118, 193, 200). The N-terminal alpha helix has been reported to interact with the tyrosine kinase, Lck, and is required for optimal infection of resting PBMC (14, 62). The polyproline domain constitutes an SH3 binding domain and interacts with Hck and Lyn (159) and has been reported to be required for viral infectivity (161, 170). The acidic-cluster domain has been reported to interact with PACS-1 and is thought to contribute to the intracellular accumulation of MHC-I in Nef expressing cells (19, 147). Recently, the polyproline and acidic-cluster domains have also been reported to stabilize the Nef/MHC-I/AP-1 interaction (196). The oligomerization domain is required for Nef to form dimmers, interact with human thioesterase, downmodulate CD4 and enhance viral infectivity (108). In addition, these domains are also required for Nef to bind the MHC-I cytoplasmic tail (193). Interestingly, with exception of the oligomerization domain, these domains are not required for CD4 downmodulation, demonstrating that Nef downmodulates CD4 and MHC-I through separate mechanisms.

Nef Binds to MHC-I Early in the Secretory Pathway

Early models proposed that Nef downmodulates MHC-I by increasing the internalization rate and targeting MHC-I to the TGN through retrograde transport (61, 100, 101, 164). Since Nef is affecting MHC-I at the cell surface, one would expect Nef to bind to MHC-I at the plasma membrane or, at least, later in the secretory pathway, if this model is

correct. In contrast, Nef was shown to interact with MHC-I in an early compartment of the secretory pathway, the ER (89). Immunoprecipitation experiments showed Nef in a complex with MHC-I and a resident ER protein, tapasin (89). This finding is further strengthened by the knowledge that Nef prefers to bind a hypophosphorylated MHC-I cytoplasmic tail (89) and the MHC-I cytoplasmic tail is not phosphorylated until the TGN (24, 43, 107). Thus, Nef binds the MHC-I cytoplasmic tail in the ER, not at the plasma membrane or as MHC-I is trafficking to the cell surface.

Nef Blocks transport of MHC-I in T Cells

Even though early models indicated that Nef mediated downmodulation of MHC-I was via increased internalization (100, 164), the increase in internalization was modest. Since Nef downmodulates MHC-I to the extent that CTLs are no longer able to detect HIV infected cells (33), it was questioned if the modest increase in internalization caused by Nef was enough to account for these dramatic effects. Many of the experiments that contributed to this model were performed in easily manipulated cell types, such as HeLa cells, 293 cells and astrocytic cells, because T cell lines and primary T cell can be difficult to manipulate. However, the degree of MHC-I downmodulation in these cell types compared to T cells was quite different, ranging from 2- to 4-fold in HeLa cells to up to 200-fold in T cells (33, 100, 101, 164). This discrepancy suggested MHC-I was downmodulated through a different mechanism in T cells, a physiologically relevant cell type. Several studies promoted the model of increased internalization and retrograde

transport to intracellular organelles (61, 101, 147, 164), whereas other studies demonstrated that Nef can inhibit transport to the cell surface (172).

To address these discrepancies, HeLa cells were directly compared to a CD4⁺ T cell line, CEM (88). When expressing equal amounts of Nef, both cell types showed only a small effect of internalization. Nef expression increased HLA-A2 internalization by only 25% in HeLa cells and 15% in CEM cells (88). However, in the CEM cells Nef downmodulated MHC-I more efficiently than in the HeLa cells (88). These data indicated an additional mechanism may be involved. When the transport rate of newly synthesized MHC-I was examined in CEM and HeLa cells expressing Nef, a more dramatic effect was seen. In CEM cells expressing Nef an 8-fold reduction of newly synthesized MHC-I arriving at the cell surface was observed, whereas in HeLa cells expressing Nef only a 1.3-fold reduction in MHC-I arriving at the cell surface was observed (88). These results demonstrated that the primary mechanism Nef utilizes to downmodulate MHC-I in T cells is by blocking the transport of MHC-I to the cell surface. Moreover, these results showed that Nef behaves differently in different cell types.

Nef Recruits AP-1 to Redirect MHC-I

Once it was established that Nef blocks the transport of newly synthesized MHC-I to the cell surface in T cells, attention was turned to deciphering the mechanism. Nef has been shown to interact with several adaptor proteins, including AP-1 (see above), which is

known to facilitate trafficking between the TGN and endosomes (41, 186). This adaptor protein seemed like a promising candidate and, in fact, it was demonstrated through RNA interference that AP-1 is required for Nef mediated MHC-I downmodulation (155). AP-1 was also found associated with the Nef/MHC-I complex in T cells (155). Previous studies had indicated the dileucine motif of Nef was needed to recruit AP-1 but this domain had not been shown to be required for MHC-I downmodulation. Mutagenesis studies revealed that the alpha helical domain was instead needed for AP-1 recruitment, and specifically the methionine at position 20 was essential (155). Additionally, the MHC-I cytoplasmic tail contains a tyrosine at position 320 that was also found to be vital for AP-1 recruitment (155). Thus, sequences in both Nef and MHC-I cytoplasmic tail were required for AP-1 recruitment and redirection into the endosomal network.

The dileucine motif was not required for AP-1 recruitment (155, 196), which is surprising as this motif was originally reported to interact with AP-1. The studies reporting the Nef/AP-1 interaction via the dileucine motif were *in vitro* studies and lacked the additional cellular proteins that would be present in an *in vivo* setting, such as MHC-I, and did not characterize the interaction in the context of the MHC-I cytoplasmic tail. The Nef/MHC-I cytoplasmic tail complex appears to render the Nef dileucine motif inaccessible to AP-1, as the dileucine motif is not required in a more physiological setting.

The data indicating the dileucine motif was not needed to recruit AP-1 to the MHC-I cytoplasmic tail led to several new questions about how Nef was recruiting adaptor

proteins and which Nef domains were involved in the recruitment of adaptor proteins. It became apparent that while in vitro studies such as yeast two hybrid and GST-pull downs provided necessary information, they were unable to tell the complete story. Further experiments in vivo went on to identify additional amino acids in the MHC-I cytoplasmic tail, A₃₂₄ and D₃₂₇, that helped to stabilize Nef/AP-1 binding (196). Two additional Nef domains, the acidic-cluster and polyproline domains, were also found to stabilize the Nef/MHC-I/AP-1 complex (196). Intriguingly, it was also demonstrated that Nef was stabilizing the natural interaction between MHC-I cytoplasmic tail and the tyrosine binding pocket in AP-1 (196).

Nef delivers MHC-I to Acidic Compartments for Degradation

Ultimately, when Nef is expressed in cells MHC-I is targeted for degradation. This degradation can be stabilized by ammonium chloride or bafilomycin 1A (a specific inhibitor of the vacuolar ATPase) but not lactacystin (a proteasome inhibitor) (155), indicating the degradation occurs in acidic compartments, such as lysosomes. Unfortunately, the cellular factors and the mechanism Nef utilizes are unknown at this time. Since Nef commandeers β -COP to target CD4 for degradation (145), it is possible that β -COP is part of the MHC-I degradation pathway also. AP-3 is known to traffic proteins between endosomes and lysosomes and interaction studies have indicated Nef binds to AP-3 (37, 80, 82); so it is also possible that AP-3 is the link to the lysosomal compartments.

Additional Cellular Factors and Other Models for MHC-I Downmodulation

Several studies have also demonstrated that MHC-I downmodulation by Nef is mediated through a phosphoinositide 3-kinase (PI 3-kinase) dependent pathway (19, 74, 99, 172). The involvement of PI-3 kinase is specific to the MHC-I pathway, as the PI 3-kinase inhibitor, LY294,002, had no effect on CD4 downmodulation (172). The part of the downmodulation pathway effected by PI 3-kinase has yet to be fully characterized. In T cells, LY294,002 did not reverse the Nef mediated MHC-I block in transport (88) and there is evidence that Nef recruits PI 3-kinase in the TGN through interactions with Src family tyrosine kinases and ZAP-70 via the polyproline domain (74). Although these data are intriguing, an inhibitor of tyrosine kinases, herbimycin A, did not block MHC-I downmodulation (118), thus questioning the role of tyrosine kinases in the Nef mediated mechanism for MCH-I downmodulation. Clearly, PI 3-kinase is involved in MHC-I downmodulation, but the specific step in the pathway is not known and future studies will be needed to fully characterize its requirement.

The Nef polyproline domain is also required for MHC-I downmodulation (61, 118, 193) and Nef binding to the MHC-I cytoplasmic tail (193). Recently, this domain has been reported to be stabilize the Nef/MHC-I/AP-1 interaction (196). The polyproline domain has also been shown to bind SH3 domains and to be important for binding and activating Src family kinase members (7, 14, 103, 159). However, as mentioned above, the inhibitor of tyrosine kinases, herbimycin A, was not able to block MHC-I downmodulation (118), thus questioning the role of Src family kinases in the MHC-I

downmodulation pathway. Further studies will be needed to fully characterize the role of the polyproline domain in Nef induced MHC-I downmodulation.

An alternative model and additional cellular factors have also been proposed for the Nef mediate MHC-I downmodulation pathway. The Nef acidic-cluster domain (EEEE₆₅) has been reported to bind to PACS-1, a cellular factor that mediates transport between the endosomal network and the TGN (19, 74, 147). This model proposes that the PACS-1/Nef interaction directs Nef to the TGN and allows the Nef polyproline domain to bind to the SH3 domain of Src family kinases (SFK) (74). The Nef associated SFK promote recruitment and activation of ZAP-70. The Nef/SFK/ZAP-70 complex then recruits PI 3-kinase, which stimulates the Arf 6 mediated internalization of MHC-I. Nef then prevents recycling of newly internalized MHC-I, which is directed to the TGN through retrograde transport.

When the alternative model is contrasted against the first model described in detail above which suggests Nef mediated MHC-I downmodulation is due to a block in MHC-I transport to the cell surface (referred to from here as the “block in transport model”), it is readily apparent that the two are very different. Each model suggests different host cellular factors are involved and the mechanism used to downmodulate MHC-I is different. The block in transport model indicates it is a block in transport mediated through AP-1, while the alternative model indicates Nef induces accelerated internalization of MHC-I through a SFK/ZAP-70/PI 3-kinase mechanism. However, the alternative model involving PACS-1 and SFK is controversial. Recent studies using

RNA interference have been unable to confirm the role of PACS-1 in Nef induced downmodulation of MHC-I (113). Moreover, these studies were unable to confirm the role of PACS-1 on the localization of other acidic-cluster containing cellular proteins (113), suggesting that PACS-1 may not interact with acidic-cluster domains as reported (147, 188). These data weaken the alternative model demonstrating the requirement of PACS-1 for Nef mediated MHC-I downmodulation. Additionally, the alternative model presented above indicates SFK are needed to for Nef to downmodulation MHC-I. However, studies with the inhibitor of tyrosine kinases, herbimycin A, were unable to block MHC-I downmodulation (118), indicating SFK are not required for Nef mediated MHC-I downmodulation, further weakening this model. Thus, based on these data, the block in transport model is the preferred model.

Selective MHC-I downmodulation by Nef

It is well established that removing the MHC-I from the cell surface protects HIV infected cells from CTLs (33). However, the immune system has evolved to recognize cells with low MHC-I surface levels as infected or abnormal and targets these cells for destruction via NK cells. Thus if Nef downmodulates all the MHC-I from an infected cell it could render it susceptible to attack from NK cells. Certain MHC-I molecules, HLA-C and HLA-E, are known to inhibit NK cells and, interestingly, these are not downmodulated by Nef (32, 101). HLA-C and -E are resistant to Nef mediated downmodulation because they lack the required amino acid sequence needed for Nef binding. HLA-C is missing both the critical tyrosine and aspartic acid, and HLA-E is

missing a critical alanine (32). Importantly, using an MHC-I deficient B cell line, 721.221, HLA-C and -E were found to protect HIV infected cells from NK cell lysis (32). Thus, it appears HIV has developed a mechanism to evade both the CTL and NK cell responses.

Surprisingly, HIV may also leave HLA-C on the cell surface to increase viral infectivity (36). HIV virions bud from infected cells and as a result host surface proteins are incorporated into the viral envelope. Virions produced from cells expressing HLA-C have faster growth kinetics and are more infectious than virions produced from non-HLA-C expressing cells (36). Additionally, virions produced from HLA-C expressing cells have lower susceptibility to antibody neutralization (36). Interestingly, HLA-C has been demonstrated to co-precipitate with *Env* proteins, gp120/gp160 (36), suggesting it may be preferentially recruited into the virion envelope.

HLA-C Biology

Based on amino acid sequence at position 80, HLA-C alleles can be divided into two groups. C1 allotypes are HLA-Cw1, -Cw7, and -Cw8 and have an asparagine at position 80, whereas C2 allotypes are HLA-Cw2, -Cw4, Cw5, and Cw6 and have a lysine at position 80 (129, 140). Interesting, HLA-C is most related to HLA-B and it has been suggested that HLA-C differentiated from an HLA-B-like ancestor after the separation of hominoid and monkey ancestors (1).

Even though HLA-C is considered a classical MHC-I molecule, there are several significant differences between HLA-C and the other classical molecules, HLA-A and HLA-B. HLA-C has much lower cell surface expression (166), even though HLA-A, -B and -C are synthesized at similar rates (132, 203). This has been attributed to poor association with β 2-microglobulin (132, 203), the ability to only bind a restricted peptide repertoire, and a more stable interaction with TAP (133) resulting in the majority of HLA-C remaining in the ER. Some investigators have attributed the low cell surface expression of HLA-C to increase turnover of heavy chain mRNA (119), but the majority of data suggests the low cell surface expression is due to events in the ER. In addition to low cell surface expression, HLA-C differs from HLA-A and -B in that it is thought the main function of HLA-C is to provide inhibitory/activating signals to NK cells by acting as the ligand for killer-cell immunoglobulin-like receptors (KIRs).

HLA-C and NK Cell KIRs

NK cells are part of the innate immune response and are important for recognizing abnormal and virally infected cells. Once NK cells have identified an abnormal or virally infected cell, they eradicate the cell through lytic mechanisms and cytokine production, most notably IFN- γ . Their activity is controlled by the balance of signals from inhibitory and activating cell surface receptors, KIRs (98, 185). The natural ligands for KIRs are MHC-I molecules, mainly, HLA-C, -E, -G and some HLA-Bw4 alleles. C1 group alleles are the ligands for two inhibitory KIRs, KIR2DL2 and KIR2DL3, while C2 group alleles are the ligands for the inhibitory KIR, KIR2DL1. HLA-C alleles are also thought to

interact with the activating KIRs (KIR2DS), but these interactions are incomplete in their characterization (140). Interestingly, certain HLA-C and KIR combinations have been noted to promote either greater inhibition or greater activation and the balance between activation and inhibition signals has been implicated in a number of disease outcomes (140).

Infectious Disease

Hepatitis C virus (HCV) is a worldwide viral infection that can lead to hepatocellular cancer and liver failure through cirrhosis. It is estimated that 170 million people are chronically infected worldwide and 3 to 4 million new people are infected each year (198). Only about 20% of newly infected individuals are able to resolve acute infection. Recently, an association between expression of the inhibitory KIR, KIR2DL3 and C1 alleles and resolution of acute HCV infection has been reported (93). Individuals homozygous for C1 alleles and KIR2DL3 alleles were significantly more likely to resolve HCV infection than individuals with a combination of C1 and C2 alleles or with other combinations of the KIR2DL alleles (93). Even though C1 alleles can bind both KIR2DL2 and KIR2DL3, the interaction between C1 alleles and KIR2DL2 is stronger than with KIR2DL3. The stronger interaction leads to a greater inhibitory signal in the NK cell (195). A model was proposed that a weaker inhibitory receptor-ligand (KIR2DL3-C1) interaction would be protective because it could be more easily overridden by activating signals, allowing the NK cells to be more active and clear more virally infected cells (93).

Autoimmune disease

HLA-C and NK cell KIRs have also been implicated in psoriasis. It has been known for some time that the frequency of HLA-Cw6 is increased among individuals with psoriasis (57), and HLA-Cw*0602 is associated with an earlier age of onset of psoriasis (57). Studies have also found an association of the activating KIR, KIR2DS1, and HLA-Cw6 with a predisposition for psoriasis (114). The current model for the influence of KIRs and HLA-C on the disease course of psoriasis indicates that certain combinations of KIR2DL (inhibitory), HLA-C and KIR2DS (activating) will be protective towards the development of disease. Combination that promote more inhibition of NK cells will confer protection while combinations that promote activation of NK cells will support the development of autoimmunity (134). HLA-C and KIRs have also been implicated in the development of type 1 diabetes (180), scleroderma (127), and rheumatoid vasculitis (201).

Reproduction

NK cells make up 50-90% of the leukocytes in the decidua, the uterine lining during pregnancy. Based on surface expression of CD56, NK cells fall into two categories: CD56 low cells, which specialize in cytolysis, and CD56 high cells, which specialize cytokine production (183). Uterine NK (uNK) cells express very high levels of CD56 and are known to produce a combination of cytokines associated with angiogenesis and

vascular stability – such as vascular endothelial growth factor C, placental growth factor and angiopoietin 2 (106). Additionally, uNK cells produce GM-CSF, IFN- γ , colony-stimulating factor 1, and TNF (125).

During pregnancy, development of the placenta is critical for proper health of both the mother and fetus. Placental development is partially mediated by interactions between maternal uNK cells and fetal extravillous trophoblast (EVT) cells. It is thought the uNK cells influence the EVT to invade the arterial wall of the spiral arteries and modify them to allow enough blood supply to nourish the fetus (70). If adequate blood flow does not reach the placenta, pre-eclampsia can develop. Pre-eclampsia is a condition that affects 2-10% of pregnancies and is the main cause of pregnancy-related mortality worldwide (140). It is characterized by hypertension and proteinuria and can damage the maternal endothelium, kidneys and liver.

EVT express a combination of HLA-C, HLA-E and HLA-G. HLA-C is the only polymorphic HLA expressed by EVT. Interestingly, an association between the combination of the HLA-C group (C1 versus C2) expressed by the EVT and the KIR2DLs expressed by the maternal uNK cells with the development of pre-eclampsia has been reported (70, 125, 140, 141). Studies have indicated that combinations of HLA-C and KIR2DLs that send a more inhibitory signal to uNK cells are more likely to develop pre-eclampsia, whereas combinations that send activating signals are less likely to develop pre-eclampsia (70, 125, 139, 140). However, if there is too much uNK cell activation spontaneous abortion can occur. This has been reported in mothers with fewer

inhibitory KIRs (135). Clearly, a balance needs to be reached in which the uNK cell receives enough activation to allow adequate cytokine production and EVT stimulation but not to much that spontaneous abortion occurs. The balance is dictated by the combination of HLA-C alleles on the fetal EVT and the KIR2DLs on the maternal uNK cells.

HLA-C Questions

Even though many studies have been conducted to further understand HLA-C biology and its role in antigen presentation, many questions remain regarding its role in the immune system. Why does HLA-C have such low cell surface expression compared to the other classical MHC-I molecules? Why did HLA-C evolve to be retained in the ER? Why would a cell make a protein only to sequester it? Why did this MHC-I molecule evolve to bind a restricted peptide repertoire? As discussed above, the HLA-C cytoplasmic tail sequence varies from that of HLA-A and -B, does this affect its function or intracellular trafficking? Does HLA-C contribute to immune function through a mechanism that is yet to be discovered? Did HLA-C evolve to confer protection against a specific pathogen? Clearly, questions remain and future work will hopefully provide insights.

Overview of Thesis

The goal of this thesis was to examine MHC-I in the context of HIV-1 infection and in normal cells. It was hypothesized that HIV-1 Nef recruits β -COP to target MHC-I for degradation in lysosomal compartments and chapter two describes how HIV-1 Nef targets MHC-I and CD4 for degradation via a β -COP dependent pathway. It was also hypothesized that since HLA-C is not downmodulated by HIV-1 Nef, it may serve a specialized role in an HIV relevant cell type and chapter three describes the regulation of HLA-C cell surface expression in T cells and macrophages. Chapter three is relevant to HIV research because HLA-C has been shown to be important for HIV immune evasion and viral infectivity, and, since, T cells and macrophages are both targets of HIV it is important to understand HLA-C biology in these two cell types. Chapter four discusses the implications of the findings presented in chapters two and three and future directions for these projects.

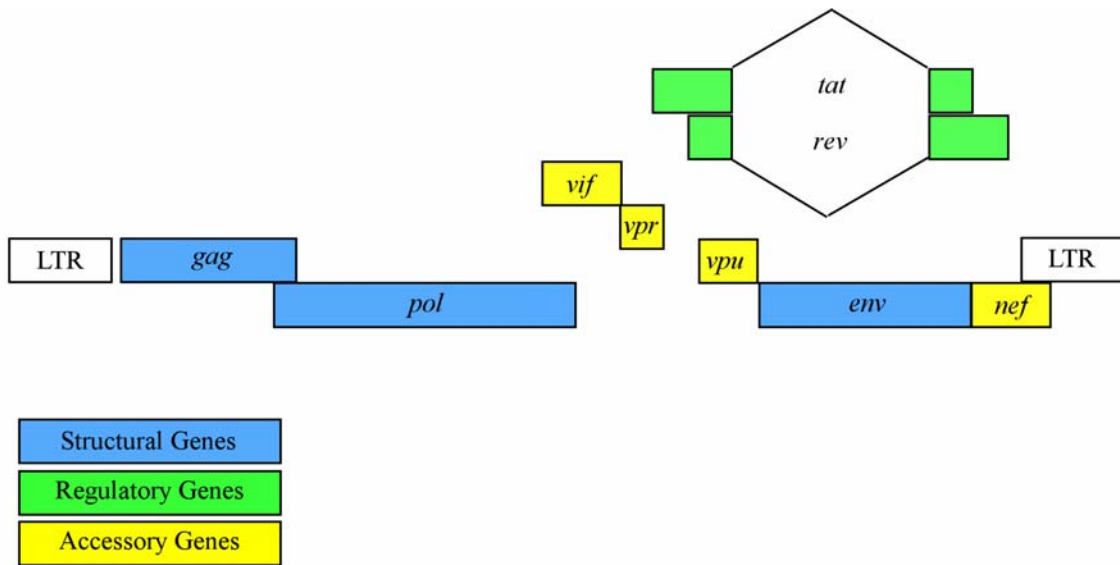


Figure 1-1. Genomic Map of HIV-1. HIV-1 encodes nine genes. The structural genes are highlighted in blue, the regulatory genes are highlighted in green and the accessory genes are highlighted in yellow. [Adapted from reference (105)]

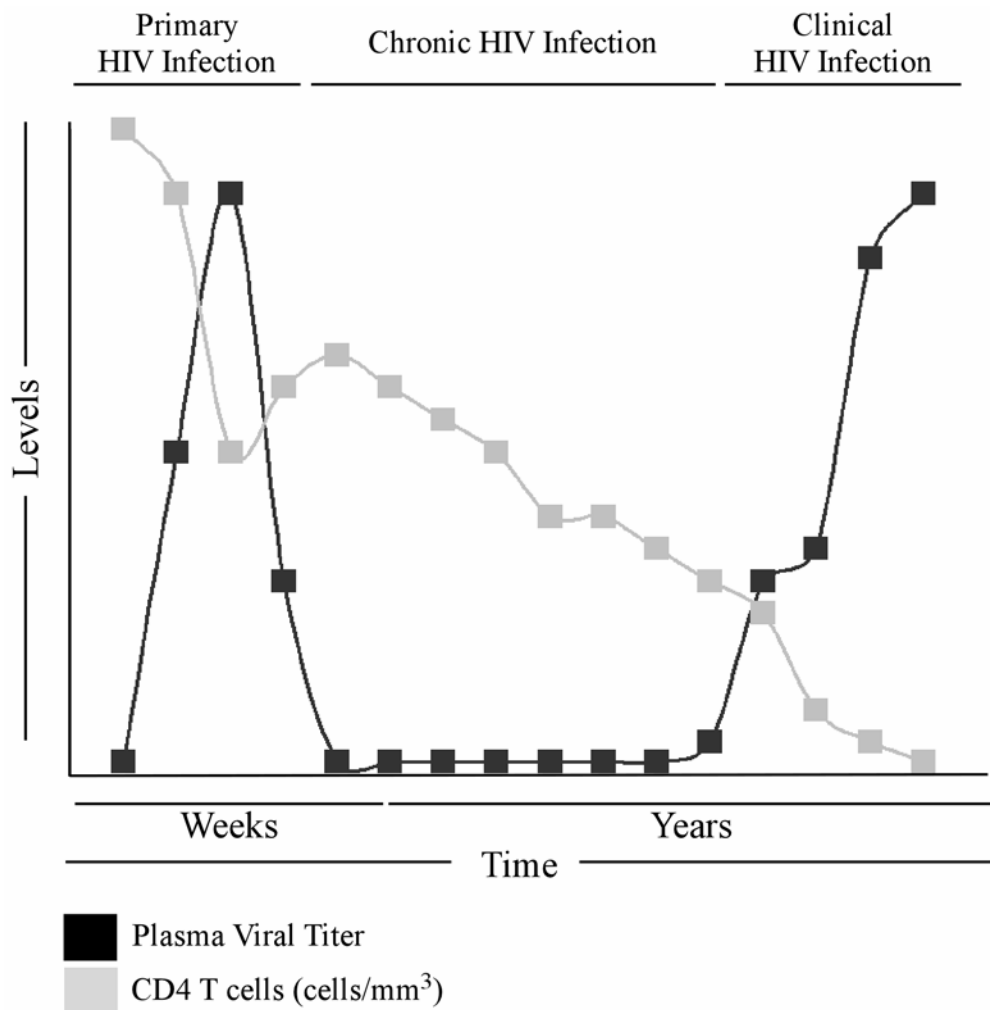


Figure 1-2. Disease Progression in an Untreated HIV Infection. During primary infection plasma viral titers rise and CD4+ T cell levels decline. Chronic HIV infection is marked by further continual decline of CD4+ T cell levels and the viral set point is established. During clinical HIV infection viral titers rise dramatically and CD4+ T cell levels decline to very low levels. The HIV infected individual develops AIDS and succumbs to opportunistic infections. [Adapted from reference (138)]

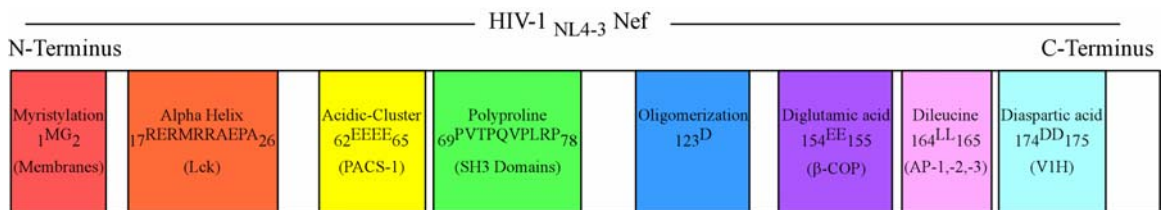


Figure 1-3. Domains in HIV-1 Nef Required for MHC-I and CD4 Downmodulation and Degradation. The domains in HIV-1_{NL4-3} Nef necessary for MHC-I and CD4 downmodulation and degradation are highlighted. The amino acid sequence and location of the domains are presented. The adaptor protein reported to interact with the each domain is in parentheses. [Adapted from reference (154)]

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CHAPTER II

HIV-1 NEF TARGETS MHC-I AND CD4 FOR DEGRADATION VIA A FINAL COMMON β -COP-DEPENDENT PATHWAY IN T CELLS

Abstract

To facilitate viral infection and spread, HIV-1 Nef disrupts the surface expression of the viral receptor (CD4) and molecules capable of presenting HIV antigens to the immune system (MHC-I). To accomplish this, Nef binds to the cytoplasmic tails of both molecules and then, by mechanisms that are not well understood, disrupts the trafficking of each molecule in different ways. Specifically, Nef promotes CD4 internalization after it has been transported to the cell surface whereas Nef utilizes the clathrin adaptor, AP-1, to disrupt transport of MHC-I from the TGN to the cell surface. It is not understood how the same initial event (Nef binding) can lead to targeting of different molecules to distinct intracellular pathways. We demonstrate here that these distinct mechanisms are dictated by amino acid differences in the cytoplasmic tails of CD4 and MHC-I. Despite these differences in initial intracellular trafficking, we demonstrate that MHC-I and CD4 are ultimately found in the same Rab7⁺ vesicles and are both targeted for degradation via the

activity of the Nef-interacting protein, β -COP. Moreover, we demonstrate that mutations in the N-terminal α helical and dimerization domains of Nef dramatically reduced co-precipitation of Nef and β -COP. We furthermore show that the amino acid requirements for AP-1 binding are distinct from those necessary for the interaction of Nef with β -COP. These data provide crucial new information, which improves our understanding of how Nef functions.

Introduction

The HIV-1 accessory protein, Nef, affects the biology of the infected cell in several ways to achieve conditions optimal for viral replication and spread. Nef alters the intracellular trafficking of important immune molecules, such as class I and II major histocompatibility complex proteins (MHC-I and MHC-II), CD4, CD28, and DC-SIGN (23, 58, 59, 61, 64). Nef-dependent reduction of surface MHC-I protects HIV-infected PBMCs from recognition and killing by HIV-specific cytotoxic T lymphocytes (CTLs) *in vitro* (12). Moreover, disruption of MHC-I expression by HIV-1 and SIV Nef provides a selective advantage under immune pressure *in vivo* (8, 9, 48, 63). CD4 downregulation by Nef is also essential for efficient viral spread. The rapid removal of CD4 prevents viral superinfection (6), and enables optimal viral particle production by eliminating detrimental CD4/HIV envelope interactions in the infected cell (41, 57).

Mutagenesis of protein-protein interaction domains has revealed that Nef uses genetically separable mechanisms to affect MHC-I and CD4 transport. Specifically, disruption of MHC-I surface expression requires an N-terminal α helix, a polyproline repeat, and an

acidic domain in Nef (27, 47), while CD4 downregulation requires an intact dileucine motif, two diacidic motifs, and a hydrophobic pocket in Nef (7, 13, 47, 52). Amino acids necessary for the myristoylation (1, 50) and oligomerization (45) of Nef are required for the disruption of both MHC-I and CD4 surface expression.

Nef has the capacity to affect MHC-I transport at multiple subcellular locations; Nef blocks the export of newly-synthesized MHC-I from the secretory pathway and Nef expression results in a small increase in the rate of MHC-I internalization (39). To accomplish this, Nef directly binds to the cytoplasmic tail of MHC-I early in the secretory pathway (40, 56, 69, 70). The Nef-MHC-I complex then actively recruits the clathrin adaptor protein complex AP-1, which targets MHC-I from the TGN to the endolysosomal network where it is ultimately degraded (56). Recruitment of AP-1 requires a methionine at position 20 in the N-terminal α helical domain of Nef and a tyrosine residue in the cytoplasmic tail of MHC-I. The normal function of AP-1 is to target proteins into the endolysosomal pathway and then recycle them back to the TGN. Thus, the AP-1 interaction with the Nef/MHC-I complex explains the targeting of MHC-I containing vesicles to the endosomal pathway and to the TGN. However, it does not explain accelerated degradation of MHC-I, hence other cellular factors may be involved (56).

The mechanism of Nef-induced CD4 internalization and degradation has been derived, in part, from correlating Nef function with the requirement for domains in the C-terminal flexible loop region of Nef that bind to cellular factors. The Nef dileucine motif

(ExxxLL₁₆₅) is needed for CD4 internalization and it binds to adaptor protein complexes AP-1, AP-2, and AP-3 (2, 7, 14, 20, 26, 34, 36, 42, 51). In addition, a diacidic motif, which is also required, binds to the H subunit of the vacuolar ATPase (V1H) (46), and may promote AP-2 recruitment (24). Because the normal role of AP-2 is to link cargo to clathrin and promote internalization, it makes sense that this molecule would be necessary and indeed, the involvement of AP-2 has now been confirmed using RNAi knockdown in a number of cell systems (11, 37, 60).

After CD4 is internalized, it is targeted to lysosomes for degradation. There is evidence that this step requires β -COP (52), a component of COP-1 coats implicated in endosomal trafficking as well as trafficking through the early secretory pathway (4, 15, 28). Specifically, there are defects in the Nef-dependent transport of CD4 into acidified vesicles at the non-permissive temperature in cells harboring a temperature sensitive ϵ -COP mutant (52). Nef has been reported to directly interact with β -COP (5), but it is unclear as to which domain of Nef is involved in this interaction (21, 35).

To more clearly understand the mechanism of altered MHC-I and CD4 trafficking observed in Nef-expressing cells, we directly compared these two processes in T cells that expressed Nef. We confirmed that Nef primarily affected MHC-I and CD4 at different subcellular locations and we demonstrated that the cytoplasmic tails of the respective molecules dictated these differences. Despite the differences in initial trafficking, we found that HLA-A2 and CD4 co-localized in a discrete subset of vesicular structures. Upon further inspection, we determined that these structures also contained

markers of late endosomes (Rab7) and to a lesser extent, the lysosomal marker (LAMP-1). Electron microscopy (EM) revealed that CD4 and HLA-A2 were found within MVBs of Nef-expressing T cells. HLA-A2 (but not CD4) was also found in tubulovesicular structures adjacent to the Golgi. In Nef expressing cells, reduction of β -COP expression reduced the targeting of HLA-A2 from the TGN to LAMP-1⁺ compartments and stabilized CD4 expression within endosomal compartments. Finally, we identified two separate domains within Nef that were necessary for Nef activity and for β -COP binding. These data support a model in which both MHC-I and CD4 are ultimately targeted to the lysosomes in Nef expressing cells by a final common pathway.

Results

The cytoplasmic tail dictates the pathway utilized by Nef to eliminate MHC-I and CD4 surface expression. It is known that Nef binds to the cytoplasmic tails of both CD4 and MHC-I, but that it affects them differently. To better understand the similarities and differences governing these two pathways, we examined the trafficking of CD4, HLA-A2 and a chimeric molecule in which the wild type HLA-A2 cytoplasmic tail was substituted with the CD4 cytoplasmic tail (HA-A2/CD4). A flow cytometric analysis of steady state surface expression revealed that Nef dramatically reduced steady state surface expression of all three molecules (Figure 2-1A). Consistent with prior studies, we found that CD4 was rapidly internalized from the cell surface in Nef expressing T cells, whereas wild type HLA-A2 was not (Figure 2-1B). Substitution of the CD4 tail for the HLA-A2 cytoplasmic tail was sufficient to confer this phenotype (Figure 2-1C). Conversely, prior studies have shown that Nef disrupts cell surface expression of MHC-I by blocking the transport of newly synthesized MHC-I. As shown in Figure 2-1D, Nef inhibited HLA-A2 forward transport by approximately 75%, whereas CD4 was unaffected at Nef levels that had a clear effect on HLA-A2 transport. Slight effects on CD4 could be observed at higher Nef levels (Figure 2-1D, lane 8). The substitution of the HLA-A2 cytoplasmic tail with the CD4 tail reduced the ability of Nef to disrupt forward trafficking (Figure 2-1E). Thus, sequences in the cytoplasmic tails of CD4 and HLA-A2 determine how Nef disrupts their trafficking.

CD4 and a subset of HLA-A2 proteins are found in late endosomes and lysosomes of Nef-expressing T cells. To better understand the similarities and differences between

MHC-I and CD4 trafficking in Nef-expressing cells, we compared the steady-state distribution of these molecules in T cells using confocal microscopy (Figure 2-2A). We found that Nef expression caused the bulk of MHC-I to cluster in the perinuclear region where, in agreement with many other studies (27, 42, 62). Interestingly, we also identified a substantial subset of HLA-A2 that co-localized with CD4 in vesicular structures (Figure 2-2A, arrows). To further identify these structures, we simultaneously stained for HLA-A2, CD4, and organelle markers using 3-color confocal microscopy (summarized in Table 2-1). Our results indicated that CD4 was mainly found in discrete vesicular structures, which also contained HLA-A2 (91.9% CD4⁺ vesicles co-localized, Table 2-1) and markers of late endosomes and lysosomes. Overall, the best marker for structures containing both HLA-A2 and CD4 was Rab7 (94%, of CD4⁺ vesicles co-localized, Table 2-1 and Figure 2-2A, arrowheads mark example vesicles). CD4 and HLA-A2 were also found to co-localize with markers of lysosomes, such as LAMP-1. However, the vesicles with the most intense LAMP-1 staining did not contain either HLA-A2 or CD4, possibly because of degradation. Consistent with this, the co-localization of HLA-A2 and CD4 was dramatically increased when the cells were treated with bafilomycin, which inhibits degradation in acidic compartments (Figure 2-3). Thus, the normal steady-state co-localization of HLA-A2 and CD4 in Nef expressing cells was limited because degradation prevented accumulation in this compartment.

Colocalization of HLA-A2 and CD4 in MVBs. To further discern these structures, we also examined them using electron microscopy (EM). In agreement with the confocal data, our EM analysis revealed that compared with control cells in which both HLA-A2

and CD4 were found on the cell surface (Figure 2-2B, panel 1), in Nef-expressing T cells, the majority of CD4 was found in MVBs, co-localizing with HLA-A2 (Figure 2-2B, panel 2). In addition, we also noted substantial HLA-A2, but not CD4, accumulating in tubulovesicular structures adjacent to Golgi stacks (Figure 2-2B, panel 3). In separate experiments these structures were also found to contain AP-1 (Figure 2-2C). Based on these studies, it appears that the majority of HLA-A2 resides in tubulovesicular structures in the region of the TGN with AP-1, whereas at any given time, a small subset can be found in the endosomal compartment with CD4.

Required cellular co-factors. To further elucidate the similarities and differences between these pathways, we examined the role of known Nef-interacting proteins implicated in intracellular trafficking. AP-1 is a heterotetrameric adaptor protein involved in protein sorting from the TGN and it has been previously demonstrated to interact with MHC-I molecules in Nef expressing cells and to direct MHC-I into the endolysosomal pathway (56). Nef is also known to interact with β -COP (5), a component of COP-1 vesicles also involved in endosomal trafficking (4, 15, 28). Indeed, expression of wild type β -COP is needed for targeting CD4 into acidic vesicles in Nef-expressing cells (52).

To compare and contrast the requirement for these factors in Nef-dependent CD4 and HLA-A2 trafficking, we knocked down their expression using lentiviral vectors expressing short hairpin RNAs (shRNAs) (54). All of these studies were performed in T cells and new cell lines were generated for each experiment to eliminate the possibility

that long term growth in culture would select for cells that had compensated for the defect. Using this system, we obtained good knock down of the $\mu 1$ subunit of AP-1 and β -COP (Figure 2-4A-C). [A small apparent effect of sh β -COP on $\mu 1$ levels observable in Figure 2-4A was not significant when adjusted for protein loading in the experiment shown here or in replicate experiments (Figure 2-4B). We also did not observe any effect of another siRNA directed against a different target site in β -COP on $\mu 1$ expression (Figure 2-5).]

The effect of knocking down β -COP expression on the structural integrity of the Golgi.

Because β -COP is known to be important for intra-Golgi and ER-to-Golgi trafficking, we asked whether the Golgi structure or MHC-I trafficking were drastically affected by reduced β -COP expression. We found that there was only a small reduction in the normal transport of MHC-I to the cell surface (35% reduction, Figure 2-4D). In addition, cells lacking β -COP generally maintained overall Golgi structure as assessed by the intracellular localization of giantin, a transmembrane protein normally residing in the cis and medial Golgi (43) (Figure 2-4E). In contrast, brefeldin A, an inhibitor of an ARF1 GEF necessary for β -COP activity obliterated the normal Golgi staining (Figure 2-4E, panel 9). The relatively mild phenotype of this knock-down compared to the drastic effects of brefeldin A, suggests that brefeldin A has effects other than just disrupting COP 1 coats by blocking ARF1 activity.

Having established that knocking down β -COP allowed relatively normal forward trafficking of HLA-A2, we proceeded to assess the effect of knocking down β -COP or

AP-1 in Nef-expressing cells. Consistent with previous publications (56), we found that knocking down the ubiquitously expressed form of AP-1 (AP-1A (22)) largely reversed the effect of Nef on HLA-A2, but had no effect on downmodulation of CD4 or the HA-A2/CD4 chimera (Figure 2-6A and B). Surprisingly, we also observed that knocking down β -COP expression inhibited MHC-I downmodulation by Nef and also had a smaller effect on CD4 and A2/CD4 downmodulation (Figure 2-6A and B).

A role for β -COP in promoting degradation of Nef cellular targets. Prior studies had determined that expression of β -COP was necessary for acidification of CD4-containing vesicles and thus it was hypothesized that β -COP was needed to target vesicles containing internalized CD4 for lysosomal degradation. Therefore, we asked whether the role of β -COP in MHC-I trafficking was also to promote MHC-I degradation. To examine this, we utilized an assay we had developed, which measures the loss of mature, endo H resistant HA-tagged HLA-A2 in Nef expressing cells by western blot analysis. This assay system is based on previous data demonstrating specific degradation of the mature form of MHC-I in a manner that is reversible by inhibitors of lysosomal degradation (56). As shown in Figure 2-6C, under normal, steady state conditions, most of the HLA-A2 is resistant to endo H digestion, indicating that it has matured through the Golgi apparatus (Figure 2-6C, lane 2). However, when Nef was expressed, we observed a dramatic reduction in total MHC-I and a decrease in the ratio of endo H resistant to sensitive protein (Figure 2-6C compare lanes 2 and 18). Consistent with a role for AP-1, we observed that AP-1A shRNA largely reversed this effect of Nef (Figure 2-6C, compare lanes 18 and 20. See also Figure 2-6D for quantification). To detect degradation

of molecules containing a CD4 tail, we used an HA-A2/CD4 chimera, which was identical to HA-tagged-HLA-A2, except that it had the CD4 cytoplasmic tail. We found that Nef expression accelerated the degradation of endo H resistant protein (Figure 2-6C, compare lanes 6 and 22). However, we found that there was no effect of reduced AP-1A expression on Nef-dependent degradation of molecules containing the CD4 tail (Figure 2-6C, compare lanes 22 and 24. See also Figure 2-6D for quantification).

When β -COP expression was reduced, we observed a small increase in the amount of immature, endo H sensitive protein (Figure 2-6C, compare lanes 10 and 12), consistent with the 35% reduction in export of MHC-I to the cell surface shown in Figure 3D. However, we also noted that reduction in β -COP expression reduced the Nef-dependent degradation of the mature, endo H resistant form of these molecules (Figure 2-6C, compare lanes 26 and 28. See also Figure 2-6D for quantification) implicating β -COP in this pathway. We were also able to confirm the model that β -COP is involved in Nef-dependent CD4 degradation as reducing β -COP with shRNA reversed the degradation of the A2/CD4 chimeric molecule (Figure 2-6C, compare lanes 30 and 32. See also Figure 2-6D for quantification).

β -COP is required for targeting internalized CD4 for degradation in Nef-expressing T cells. We next directly examined the effect of reducing β -COP expression on Nef-dependent trafficking by confocal microscopy. For these experiments, cells were infected with HIV or were transduced with Nef-expressing adenoviral vectors and then the fate of internalized CD4 was assessed by confocal microscopy. Using this assay system, we

observed fairly rapid internalization of CD4 in Nef-expressing cells, followed by loss of CD4 staining by 30 minutes (Figure 2-7A, compare control cells in row 1 to Nef-expressing cells in row 3). However, in T cells expressing β -COP shRNA, there was a three-to-four fold increase in the number of CD4-containing vesicles, consistent with a role for β -COP in promoting maturation of these vesicles into degradative compartments (Figure 2-7A, compare control treated Nef-expressing cells in row 3 to sh β -COP-expressing cells in row 4). Reduction of β -COP expression yielded similar results whether Nef was introduced using HIV infection or via adenoviral vectors (Figure 2-7B and C).

β -COP is required for targeting MHC-I to LAMP-1⁺ compartments in Nef-expressing T cells. Confocal analysis of MHC-I intracellular localization revealed that expression of β -COP shRNA in control cells increased the intracellular accumulation of MHC-I, consistent with the slowing of export we observed in cells deficient in β -COP (Figure 2-7D, compare rows 1 and 2). Infection with Nef-expressing HIV resulted in the loss of cell surface MHC-I and an increase in intracellular MHC-I, some of which co-localized with LAMP-1 under normal conditions (Figure 2-7D, compare rows 1 and 3). Under these conditions, reduction of β -COP expression slightly reduced the degree of colocalization with LAMP 1 (Figure 2-7D, compare rows 3 and 4).

To enhance our ability to observe trafficking of MHC-I into LAMP-1⁺ compartments, we treated the cells with bafilomycin, which inhibits the vacuolar ATPase and thus acidification and degradation within lysosomal compartments. As previously reported

(56), bafilomycin treatment allowed us to detect MHC-I in LAMP-1⁺ compartments in Nef-expressing cells (Figure 2-7D, compare rows 3 and 7). The expression of β -COP shRNA decreased LAMP-1 colocalization with MHC-I, consistent with a role for β -COP in targeting MHC-I for degradation in lysosomal compartments in Nef expressing T cells (Figure 2-7D, compare rows 7 and 8). Similar results were observed whether Nef was introduced using HIV or adenoviral vectors (Figure 2-7E and F).

We also examined co-localization of HLA-A2 and CD4 in cells that expressed β -COP shRNA. We observed that reduction of β -COP expression resulted in increased staining of both proteins, and did not disrupt their co-localization (Figure 2-8). Thus, β -COP is not necessary for targeting these proteins into a common endosomal pathway, but rather is needed for their subsequent targeting into a degradative pathway.

The cytoplasmic tail of MHC-I is necessary for AP-1 binding in Nef-expressing T cells.

To further explore the molecular mechanism for the similarities and differences in MHC-I and CD4 trafficking in Nef-expressing T cells, we asked whether these molecules differed as to how well they bound Nef or cellular factors. As expected, we found that HIV Nef bound to both the HLA-A2 and the CD4 tail (Figure 2-9A, right panel). However, AP-1 only co-precipitated with molecules containing the HLA-A2 cytoplasmic tail (Figure 2-9A, right panel). The chimeric molecule with the CD4 cytoplasmic tail did not bind AP-1 in Nef-expressing T cells (Figure 2-9A, right panel). In these experiments, we noted that the expression level of A2/CD4 was lower than for wild type HLA-A2, which could explain this difference. Therefore, we confirmed these data using a fusion

protein containing either HLA-A2 or A2/CD4 directly fused to full length HIV-Nef protein. In previously published experiments it was shown that the HLA-A2/Nef fusion protein co-precipitated AP-1 in a manner that depended on sequences both in Nef and in the HLA-A2 cytoplasmic tail (56). Here we show again that the HLA-A2 cytoplasmic tail was necessary for this interaction and, moreover, that the CD4 tail could not substitute for it (Figure 2-9B, right panel).

In contrast, despite multiple attempts, we were unable to demonstrate Nef-dependent co-precipitation of β -COP with HLA-A2, A2/CD4 or wild type CD4. Difficulty detecting a three way complex containing HLA-A2, Nef and β -COP, may result from the fact that most of the HLA-A2 in Nef-expressing cells is complexed with AP-1 and is engaged in a recycling pathway, whereas at any given time, a much smaller fraction is contained within Nef- β -COP complexes and is rapidly targeted for degradation.

Despite the difficulties with detecting a three-way complex, we were nevertheless able to confirm that Nef co-precipitated with endogenous β -COP in CEM T cells (Figure 2-9C, right panel). The Nef- β -COP interaction is well-described in the literature (5), however the domain of Nef that interacts with β -COP has been controversial. An initial report that β -COP interacts with a diacidic motif (EE₁₅₅QQ) within the Nef C-terminal loop (52) has been challenged in a more recent publication (35) and appears to be only partially needed for β -COP binding through a primary interaction with ARF1 (21). To determine whether there was a domain in Nef that affected both MHC-I degradation and β -COP binding, we screened a panel of mutations that are specifically defective at disrupting

MHC-I trafficking (27, 47, 65). As shown in Figure 2-9C, we found that the V₁₀E Δ17-26-Nef mutant did not efficiently bind β-COP. Deletion of this region (RERMRRAEPA) removed a number of arginine residues and it is notable that β-COP is known to interact with arginine rich domains (67). Interestingly, this mutant is also defective at interacting with AP-1 (56). However, the β-COP binding site was separable from the AP-1 interaction site because M₂₀, which is needed for AP-1 interaction (56), was not necessary for β-COP binding to Nef (Figure 2-9C). Mutation of the Nef dimerization motif [D₁₂₃G, (45)], which disrupts a number of Nef functions, including MHC-I and CD4 downmodulation, also reduced binding to β-COP (Figure 2-9C). Finally, mutation of the Nef acidic domain (E₆₂₋₆₅Q), which disrupts binding to MHC-I (69) and PACS-1 (53), did not affect binding to β-COP.

As expected, we found that V₁₀EΔ17-26 Nef was defective at inducing the degradation of the endo H resistant form of HLA-A2 (Figure 2-10A). In contrast, V₁₀EΔ17-26 Nef was not defective at A2/CD4 degradation based on western blot analysis (Figure 2-10A). These data suggested that there may be another interaction domain that recruits β-COP to the Nef-CD4 complex to promote CD4 degradation. This would be consistent with the faint band observable in the V₁₀EΔ17-26-Nef mutant immunoprecipitation (Figure 2-9C, longer exposure) and prior publications demonstrating that mutation of EE₁₅₅ reduced, but did not eliminate, β-COP binding (21). Thus, there may be two independent binding sites for β-COP within Nef, each of which governs the degradation of a different cellular factor.

Discussion

Expression of HIV Nef in infected cells protects them from lysis by CTLs and this activity of Nef is due to downmodulation of MHC-I surface expression. The Nef protein also prevents superinfection and promotes viral spread by removing the viral receptor, CD4 from the cell surface (for review see (55)). We provide evidence that sequences in the cytoplasmic tail of these molecules are important for determining whether Nef disrupts their trafficking from the cell surface or at the TGN. Despite these differences, we report here the novel finding that the final steps leading up to the degradation of these proteins are remarkably similar. These data have allowed us to provide a model that unifies two functions of the HIV Nef protein (Figure 2-10B).

As shown in Figure 2-10B, Nef binds to hypo-phosphorylated MHC-I cytoplasmic tail early in the secretory compartment (40), but allows normal transit through the Golgi apparatus and into the TGN (56). The Nef-MHC-I complex then recruits the AP-1 heterotetrameric clathrin adaptor protein using a binding site that is created when Nef binds the MHC-I cytoplasmic tail. This binding site requires a methionine from the N-terminal α helix of Nef and a tyrosine residue in the MHC-I cytoplasmic tail (56). Formation of this complex results in the re-direction of MHC-I trafficking in such a way that it is targeted to lysosomes for degradation (56). However, cellular proteins that normally bind AP-1 are not degraded, but rather recycled to the TGN. Thus, we hypothesized that another cellular factor may be required for the final step.

Our intriguing observation that CD4 co-localizes with a subset of MHC-I in late endosomal compartments allowed us to postulate that cellular factors implicated in targeting CD4 to acidic vesicles may be crucial for both pathways. In contrast to MHC-I, CD4 traffics to the cell surface normally in Nef-expressing cells, but is then internalized rapidly and targeted to lysosomes for degradation. It has been proposed that the rapid internalization results from Nef recruiting AP-2, a heterotetrameric clathrin adaptor protein that normally promotes endocytosis (11, 37, 51, 60). Once internalized, CD4 traffics to progressively more acidic compartments in a process that can be blocked in cells harboring a temperature sensitive allele of the ϵ subunit of COP 1 coats (5, 52). Data indicating that Nef directly interacts with the β subunit of this complex [β -COP (5, 52)] led to the model that Nef recruits β -COP to promote trafficking to degradative compartments. We present data here that knocking down the expression of β -COP with shRNA stabilized newly internalized CD4 in Nef expressing cells, confirming the role of β -COP in Nef-dependent CD4 degradation. Also, we present the more surprising result that reduced levels of β -COP abrogated the degradation of MHC-I and it did so by blocking the transport of MHC-I from intracellular vesicles to LAMP-1⁺ compartments. These results indicate that the final step in the destruction of these disparate molecules is shared in common.

Although we found that knocking down β -COP resulted in stabilization of internalized CD4, we observed almost no effect of β -COP knock-down on CD4 surface expression. In contrast, there was a substantial effect of β -COP knock-down on HLA-A2 surface expression (Figure 2-6A). This might suggest that the role of beta-COP in the modulation

of these targets was different, rather than the same. However, this apparent paradox can be explained by our model shown in Figure 2-10B. As indicated, differences in response to β -COP knockdown can be explained by differences in the intracellular pathways of these proteins before they interact with β -COP. MHC-I is engaged in an AP-1-dependent endosome-to-TGN loop, and MHC-I could “leak” out to the cell surface from the TGN in the absence of β -COP, whereas CD4 may be unable to return to the cell surface from its endosomal compartment. Alternatively, there may be a slow default degradation pathway for internalized CD4 in T cells that prevents its return to the cell surface.

We were also able to confirm that endogenous β -COP and Nef interacted in T cells (5). Moreover, we were able to identify two domains that are needed for this interaction to occur efficiently. One of these domains (D_{123}), is required for dimerization of Nef and is needed to affect a variety of Nef functions (45). The other is a region within the N-terminal α helical domain of Nef that is specifically required for disruption of MHC-I trafficking and for interactions with AP-1 (56). The binding site for β -COP is distinct from that used by AP-1, because recruitment of β -COP does not require M_{20} , whereas AP-1 does (56). The fact that Nef $M_{20}A$ binds β -COP, but does not promote MHC-downmodulation (65) makes sense, because this mutant is unable to bind the MHC-I cytoplasmic tail (69).

The identification of a β -COP binding domain within a region of Nef that is also required for Nef to accelerate MHC-I degradation confirms the requirement for β -COP in this pathway. The residual binding of β -COP to this mutant provides suggestive data that

another binding site for β -COP exists. Indeed, there is evidence that a diacidic motif found in the C-terminal loop of Nef interacts with ARF 1, and this in turn promotes an interaction with β -COP (21). Mutation of this di-acidic motif only partially affected β -COP binding (21), supporting the notion that there are two regions within Nef that recruit β -COP; one located in the N-terminal α helix that targets MHC-I for degradation and the other in the C-terminal flexible loop that targets CD4 for degradation. There is precedent for such redundancy. For example, there are two AP-1 binding sites within Nef; a dileucine motif within the C-terminal flexible loop (7, 14, 26, 36) as well as a second site that forms upon binding of Nef to the MHC-I cytoplasmic tail. Despite the presence of two AP-1 signals, only one is active in the context of the Nef-MHC-I complex (56, 71).

We also present evidence that knock down of β -COP yielded a distinct phenotype from BFA treatment. As described above, BFA is a chemical inhibitor of ARF1, that is known to trigger the reversible collapse of the cis-medial Golgi compartments to the ER (18, 25, 44) by inhibiting an ARF-specific guanine nucleotide-exchange protein (ARF-GEF) (17, 30). Because ARF1 activity is necessary for recruitment of β -COP to membranes (16), it was possible that the dramatic effects of BFA resulted from the inability for β -COP to function normally. However, our results demonstrating that knock-down of β -COP had no effect on overall Golgi structure indicate that the dramatic effects of BFA are not due to disruption of β -COP function.

The data presented here, that CD4 and MHC-I are degraded at an accelerated rate in Nef-expressing cells via a common final pathway point to a number of new and exciting areas

of investigation that should yield important insights into the mechanism of Nef. If Nef binds the cytoplasmic tail of both MHC-I and CD4, how does Nef use different initial mechanisms to target MHC-I and CD4 for destruction? Our previously published data indicate that Nef preferentially binds to immature forms of MHC-I found in the ER, which have hypo-phosphorylated cytoplasmic tail domains (40). As the Nef-MHC-I complex migrates through the secretory pathway it recruits AP-1, and in some cell types that traffic MHC-I rapidly through the secretory pathway, we have found that reducing the trafficking rate accelerates AP-1 recruitment. Intriguingly, we have previously shown that the AP-1 binding site needed for this process is created when Nef binds to MHC-I. This binding site requires a methionine residue in the N-terminal α helical domain of Nef and, a tyrosine residue in the MHC-I cytoplasmic tail plus two additional domains within Nef that stabilize the complex (56, 71). Hence, in this case, the cytoplasmic tail of the affected cellular factor dictates which trafficking factor binds. Accordingly, Nef may fail to affect CD4 early in the secretory pathway because there are post-translational modifications that prevent binding until CD4 has reached the cell surface or because the CD4 cytoplasmic tail lacks necessary signals for recruitment of trafficking factors found early in the secretory pathway. Of note, we have also found that late forms of MHC-I that are phosphorylated on their cytoplasmic tail domain bind Nef poorly (40). In sum, differential effects of Nef on these two molecules are likely to result from natural differences in their primary sequence, post-translational processing and natural trafficking pathways.

The data we present here, that the cytoplasmic domains are responsible for the differing initial pathways taken by HLA-A2 and CD4 in the presence of Nef, may seem somewhat obvious; since Nef is always the same, one might conclude that this information has to be contained in the modulated protein. However, it was also possible that the ectodomain affected Nef responsiveness by binding to other transmembrane proteins or by altering intracellular trafficking. This was certainly a possibility for MHC-I in which it is clear that the efficiency of peptide loading can affect trafficking and we have found that trafficking rates affect responsiveness to Nef and AP-1 binding (40).

A recent report indicates that the effect of Nef on internalization of MHC-I occurs via a PI3-kinase dependent pathway (33). This publication reported that CEM cells, which were used in our study, have less PTEN (a phosphatase that inhibits PI3-kinase). This deficiency might make it more difficult for us to detect an effect of chemical PI3-kinase inhibitors, but would not affect our ability to detect a PI3-kinase-dependent pathway. In fact, one would expect the opposite, that the PI3-kinase-dependent pathway would be more active in our system. However, we have found that Nef has a relatively small effect on internalization of MHC-I, and mainly affects MHC-I protein export and degradation. These data have been corroborated in HIV-infected primary T cells (39, 69), which were also found to have relatively low levels of PTEN (33).

From a teleological perspective, it makes sense that Nef would have evolved to target early forms of MHC-I, which harbor antigens derived from the newly synthesized viral proteins. Older forms of MHC-I already on the cell surface would be bound to normal

cellular antigens and would in fact be protective as they would inhibit killing by natural killer cells that are stimulated to lyse cells with abnormally low MHC-I expression. On the other hand, it makes sense that Nef, an early viral protein, would have evolved to target surface CD4 to rapidly and efficiently remove CD4 in order to prepare the cell for rapid release of viral particles and to render the cell resistant to re-infection. Meanwhile, a late protein, Vpu, is expressed in infected cells and specifically targets the newly synthesized CD4 for degradation, preventing any additional CD4 from reaching the cell surface (68).

How do MHC-I and CD4 end up in the same vesicles? Our model is that both Nef-dependent pathways target proteins into endosomal vesicles, albeit from different starting places (in one case from the TGN and in the other from the cell surface). The fact that β -COP is responsible for targeting both types of vesicles for degradation suggests the intriguing possibility that Golgi-derived and cell-surface derived vesicles can merge into the same degradative pathway.

In sum, we have found that the HIV Nef protein commandeers the cellular trafficking machinery efficiently by using and abusing their natural activities. The fact that these pathways may end in a final common step raises the important possibility that inhibitors might be developed that could block multiple Nef functions.

Materials and Methods

Cell lines

CEM T cells stably expressing HA-tagged HLA-A2 (CEM HA-HLA-A2) have already been described (56). Cell lines stably expressing YFP-tagged Rab7 or HA-HLA-A2/CD4 were made by transducing cells with murine retroviral constructs (MSCV YFP-Rab7 or MSCV HA-A2/CD4) as previously described (39), followed by culture in selective media.

DNA constructs

MSCV YFP-Rab7 was constructed by cloning a filled-in a *Kpn* I-*Xho* I fragment from pEYFP-Rab7 (31) into MSCV puro (29). MSCV HA-A2/CD4 was constructed using PCR mutagenesis of the CD4 open reading frame. The first round PCR produced two products: the first utilized 5' primer (primer 1) 5'-CGGGATCCACCATGCGGGTCACGGCG-3' and 3' primer (primer 2) 5'-CTCTGCTTGGCGCCTTCGGTGCCACATCACAGCAGCGACCAC-3' with MSCV HA-HLA-A2 as the template (56). The second utilized 5' primer (primer 3) 5'-GTGGTCGCTGCTGTGATGTGGCACC GAAGGCGCCAAGCAGAG-3' and 3' primer (primer 4) 5'-CCTCGAGTCAAATGGGGCTACATGTCTTTGAAATCGGTGAGGGCACTGG-3'. The second round utilized primers 1 and 4 from the previous PCR reactions plus 1 µl of each purified first round PCR reactions as template. The resulting product was digested with *Bam* HI and *Xho* I and ligated into MSCV 2.2 (29) digested with *Bgl* II and *Xho* I.

MSCV A2/Nef has been described (69). MSCV HA-A2/CD4/Nef was constructed using a PCR mutagenesis approach. The first round PCR produced two products: the first utilized 5' primer (primer 1) 5'-CGGGATCCACCATGCGGGTCACGGCG-3' and 3' primer (primer 2)

5'-CCACTTGCCACCCATACTAGTAATGGGGCTACATGT-3' with MSCV HA-A2/CD4 as the template. The second utilized 5' primer (primer 3) 5'-ACATGTAGCCCCATTACTATGATGGGTGGCAAGTGG-3' and 3' primer (primer 4) 5'-GCGAATTCTCAGCAGTTCTTGAAGTACTC-3' with an NL43 Nef open reading frame as template. The second round utilized primers 1 and 4 from the previous PCR reactions plus 1 µl of each purified first round PCR reactions as template. The resulting product was digested with *Bam* HI and *Eco* RI and ligated into MSCV IRES GFP (69) digested with *Bgl* II and *Eco* RI.

The FG12 shRNA lentiviral vectors were constructed as previously described (54). Briefly, complementary primers were annealed together and ligated into vector pRNAi (38) digested with *Bgl* II and *Hind* III. The sequences of the primers are as follows (the target sequence is underlined): shNC is an siRNA directed at GFP, with several base

changes (56) sense 5'-
 GATCCCCGCTCACACTGAAGTTAATCTTCAAGAGAGATTAAGTTCAGTGTGAGCTTTTGGAAA-3', antisense 5'-
 AGCTTTTCCAAAAGCTCACACTGAAGTTAATCTCTTGAAGATTAAGTTCAGTGTGAGCGGG-3', shβ-COP
 sense 5'-GATCCCCTGAGAAGGATGCAAGTTGCTTCAAGAGAGCAACTGCATCCTTCTCATTTTTGGAAA-3',
 antisense 5'-AGCTTTTCCAAAATGAGAAGGATGCAAGTTGCTCTCTTGAAGCAACTGCATCCTTCTCAGGG-3';
 shµ1A (a mixture of two lentiviruses was used) sense
 5'-GATCCCCTGAGGTGTTCTTGGACGCTTCAAGAGAGACGTCCAAGAACACCTCATTTTTGGAAA-3',
 antisense 5'-AGCTTTTCCAAAATGAGGTGTTCTTGGACGCTCTCTTGAAGACGTCCAAGAACACCTCAGGG-3',
 sense 5'-GATCCCCGACAAGGTCCTCTTTGACTTCAAGAGAGTCAAAGAGGACCTGTGCGTTTTTGGAAA-3', and
 antisense 5'-AGCTTTTCCAAAACGACAAGGTCCTCTTTGACTCTCTTGAAGTCAAAGAGGACCTGTGCGGGG-3'.

The pRNAi constructs were digested with *Xba* I and *Xho* I to remove the promoter and

shRNA sequence. The resulting fragment was ligated into FG12 (54). digested with *Xba I* and *Xho I*.

Viral Transductions

Adenoviral and HIV (HXB-EP (12)) transductions of T cells (56) or 373 mg astrocytoma cells (62) have been described previously.

Flow cytometry and internalization assays

Intact cells were stained for flow cytometry analysis as previously described (70). Briefly, the HLA-A2 extracellular domain was stained with BB7.2 (49) that had been purified as previously described (39). Endogenous CD4 was stained with RPA-T4 from Serotec. The secondary antibody was goat α -mouse-phycoerythrin (BioSource, 1:250). Endocytosis assays were performed as previously described with minor modification (39). Briefly, cells were washed once with Endocytosis Buffer [D-PBS, 10 mM HEPES, 10 μ g/ml BSA (NEB)], then stained with primary antibody (described above) for 20 minutes on ice. After washing, the cells were resuspended in RPMI supplemented with 10% fetal bovine serum, 10 mM HEPES buffer, 2 mM L-glutamine, penicillin and streptomycin (R10) (pre-warmed to 37°C) and replicate aliquots were removed and placed on ice for each time point. Cells were then washed and stained with goat α -mouse-phycoerythrin (BioSource, 1:250) and the samples were analyzed using a FACScan flow cytometer (Becton Dickinson). Flow cytometry data was processed using FlowJo v4.4.3 software (Treestar Corp.). The mean fluorescence at time zero was set to

100%, and this value was used to calculate the relative surface staining at each subsequent time point.

Cell Surface Transport Assay

CEM cells transduced with adenoviral vectors as previously described (39) were first incubated in pre-label media [RPMI –Cys –Met (Specialty Media, Inc.) + 10% dialyzed FBS (Invitrogen)] for 15 minutes at 37°C. Pulse labeling was performed in pre-label media with 150-200 µCi/ml Pro-mix-L [³⁵S] (>1000 Ci/mmol; Amersham Pharmacia) for 30 minutes at 37°C. The cells were then chased in R10 media for 15 minutes at 37°C, followed by two washes with D-PBS. To label the protein that reached the cell surface, the cells were resuspended in D-PBS containing 0.5 mg/ml EZ-Link sulfo-NHS-LC-Biotin (Pierce), and incubated at 37°C for 1 hour. Surface biotinylation was quenched by washing the cells in D-PBS + 25 mM Lysine (Fisher).

For Figure 2-1D, immunoprecipitation of proteins from cell lysates was performed as previously described (56), except that one-third of the total lysate was used for the HLA-A2 immunoprecipitation while two-thirds of the material was used to recover CD4. For immunoprecipitations of ³⁵S labeled proteins, 5 µg of BB7.2 and 2.5 µg RPTA4 (BD Pharmingen) were used for HLA-A2 and CD4 respectively. In Figure 2-1E and 3D, the total cell lysate was immunoprecipitated with anti-HA ascites (HA.11, Covance).

For Figures 2-1D and E, recovered proteins were released from the beads by boiling in 100 µl of 10% SDS. One third was analyzed directly by SDS-PAGE (Total). The

remaining two thirds was brought to a total volume of 1 ml in RIPA Buffer (56), and 40 μ l of avidin-agarose (Calbiochem) was added to recover biotinylated proteins. After 2 hours at 4°C, the beads were washed three times with 1 ml RIPA buffer and proteins were separated by SDS-PAGE (Surface).

Immunofluorescence Microscopy

Adeno-transduced CEM cells were adhered to glass slides, fixed, permeabilized, and stained for indirect immunofluorescence as previously described (56). Bafilomycin treatment was performed as described previously (56). The following antibodies were utilized to localize proteins via microscopy: Figures 2-1, 2-2, and 2-4; anti-CD4 (S3.5, Caltag Laboratories) and anti-HLA-A2 (BB7.2); Figure 2-4; anti-giantin (Covance); Figure 2-7; anti-CD4 antibody (S3.5, Caltag Laboratories), anti-LAMP-1 (H4A3, BD Pharmingen) and anti-HLA-A2 (BB7.2). Secondary antibodies were obtained from Molecular Probes and were used at a dilution of 1:250: Giantin, Alexa Fluor 546 goat anti-rabbit; CD4, Alexa Fluor 546 goat anti-mouse IgG2a; LAMP, Alexa Fluor 546 goat anti-mouse IgG1; BB7.2 (Figures 2-2, 5D and Figure 2-8), Alexa Fluor 647 goat anti-mouse IgG2b; BB7.2 (Figure 2-3), Alexa Fluor 488 goat anti-mouse IgG2b

For the microscopy based internalization assay in Figure 2-7A, CEM cells were allowed to adhere to glass slides, and placed on ice. The cells were washed once with wash buffer (D-PBS, 10 μ g/ml BSA (NEB) and 2% goat serum), incubated with anti-CD4 antibody (S3.5, Caltag Laboratories, IF, 1:25) for 20 minutes, washed once with wash buffer, incubated with Alexa fluor 546 goat anti mouse IgG2a (Molecular Probes, 1:250) for 20

minutes and washed once with wash buffer. The zero time point was fixed with 2% paraformaldehyde, while the remaining time points incubated at 37°C for the indicated time. The cells were then fixed with 2% paraformaldehyde. Images were collected using a Zeiss LSM 510 confocal microscope and processed using Adobe Photoshop software. Three-dimensional projections of cells were generated from Z-stacks using Zeiss LSM Image Examiner software. Otherwise, single Z sections through the center of the cell were displayed.

Electron Microscopy

Electron microscopy with CEM cells transduced with adenovirus was performed by the Harvard Medical School (HMS) Electron Microscopy Facility. Frozen samples were sectioned at -120°C, the sections were transferred to formvar-carbon coated copper grids and floated on PBS until the immunogold labeling was carried out. The gold labeling was carried out at room temperature on a piece of parafilm. All antibodies and protein A gold were diluted in 1% BSA. The diluted antibody solution was centrifuged 1 minute at 14,000 rpm prior to labeling to avoid possible aggregates. Grids were floated on drops of 1% BSA for 10 minutes to block for unspecific labeling, transferred to 5µl drops of primary antibody and incubated for 30 minutes. The grids were then washed in 4 drops of PBS for a total of 15 minutes, transferred to 5µl drops of Protein-A gold for 20 minutes, washed in 4 drops of PBS for 15 minutes and 6 drops of double distilled water. Contrasting/embedding of the labeled grids was carried out on ice in 0.3% uranyl acetate in 2% methyl cellulose for 10 minutes. Grids were picked up with metal loops (diameter slightly larger than the grid) and the excess liquid was removed by streaking on a

filterpaper (Whatman #1), leaving a thin coat of methyl cellulose (bluish interference color when dry). The grids were examined in a Tecnai G² Spirit BioTWIN transmission electron microscope and images were recorded with an AMT 2k CCD camera.

Lentiviral shRNA preparation and transduction

Lentiviruses expressing the shRNA were generated using an approach similar to that already described (54). Briefly, 293 cells were transfected with the FG12 constructs described above plus pRRE (19), pRSV-Rev (19) and pHCMV-G (32) using Lipofectamine 2000 (Invitrogen). Supernatants from the transfected cells were collected and used to transduce CEM T cells using a spinnoculation protocol.

Western Blot analyses and Immunoprecipitations

For the western blot analysis in Figures 2-4A, 2-5, 2-6C, and 2-10C cells were lysed in PBS 0.3% CHAPS, 0.1% SDS pH 8, 1mM PMSF, normalized for total protein and separated by SDS-PAGE. Endo H (NEB) digestion was performed according to the manufacturer's protocol. Staining of the western blot was performed using anti-Nef [AG11, (10)] and anti- β -COP [M3A5 (3)], which were purified as previously described (39). Additional antibodies used were HA (Covance) and μ 1 (RY/1 (66)). The secondary antibody for anti-Nef, β -COP, and HA was HRP-rat anti-mouse IgG₁ (Zymed) and for anti- μ 1 was HRP-goat anti-rabbit (Zymed).

For Figure 2-9B, the IP-western experiment was performed as previously published (69). Briefly, parental CEM T cells were spin-transduced with murine retroviral supernatant expressing either empty vector, A2/Nef or A2/CD4/Nef. Seventy two hours post

transduction, the cells were incubated in 20 mM NH₄Cl for 4 hours. The cells were then treated with DTBP (Pierce) for 40 minutes, quenched per the manufacturer's protocol, and lysed in PBS with 0.3% Chaps and 0.1% SDS. The lysate was pre-cleared and immunoprecipitated with HLA-A2 with BB7.2 chemically crosslinked protein A/G beads (Calbiochem) (56). The immunoprecipitates were washed in TBS with 0.3% Chaps and 0.1% SDS. A more stringent IP protocol was used in Figure 2-9A and C. For these experiments, CEM cells were transduced with either control, wild type Nef, or mutant Nef expressing adenovirus. Forty eight hours post-transduction, the cells were incubated in 20mM NH₄Cl for 16 hours. The cells were not crosslinked and were lysed in digitonin lysis buffer (1% digitonin (Wako), 100 mM NaCl, 50 mM Tris pH 7.0, 1 mM CaCl₂, and 1 mM MgCl₂). After pre-clear, the lysates were immunoprecipitated with either BB7.2 (Figure 2-9A) or M3A5 (Figure 2-9C) crosslinked to beads. The immunoprecipitates were eluted and analyzed by western blot as described previously (69).

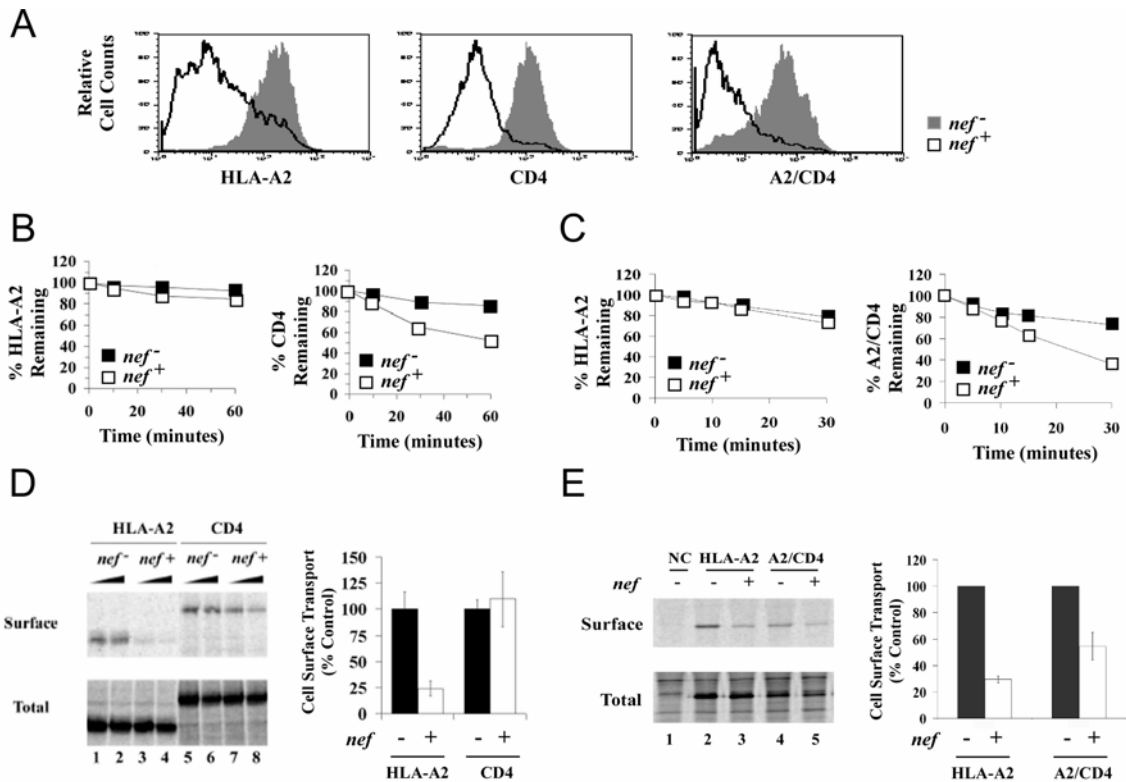


Figure 2-1. The cytoplasmic tail domain of MHC-I and CD4 determines the mechanism by which Nef affects trafficking (A) Reduction of surface expression of HLA-A2, CD4 and A2/CD4 as measured by flow cytometry. CEM HA-HLA-A2 and CEM HA-A2/CD4 cells were transduced with a control adenovirus (*nef*⁻) or adeno-Nef (*nef*⁺) and stained for surface HLA-A2 and CD4. The histograms shaded gray represent cells treated with control adenovirus, and the solid black line indicates cells treated with adeno-Nef. (B-C) Measurements of surface stability. CEM HA-HLA-A2 and HA-A2/CD4 cells were treated with adenovirus as in part (A) and the internalization of endogenous CD4 (B) or A2/CD4 (C) was compared to the internalization of HLA-A2. The filled squares represent control (*nef*⁻) cells, and the open squares represent adeno-Nef (*nef*⁺) cells. Quantitation of part B was compiled from four independent experiments performed in duplicate. Part C is representative of two experiments performed in triplicate. (D-E) HLA-A2 is inefficiently transported to the cell surface in T cells expressing Nef. CEM HA-HLA-A2 and CEM HA-A2/CD4 cells were transduced as in part A. Metabolic labeling with continuous surface biotinylation was performed in the presence of a cell-impermeable biotinylation reagent [NHS-biotin, (Pierce)] to label cell surface proteins. The cells were lysed and immunoprecipitated first with an antibody against HLA-A2 or CD4 (part D) or anti-HA (part E), then 2/3 was re-precipitated with avidin beads to selectively precipitate the HLA-A2 on the cell surface. Normalized surface MHC-I was calculated as follows: ((surface MHC-I / total MHC-I X 2) X 100). Quantitation for parts D and E represents the mean +/- standard deviation for four and three independent experiments respectively. For part D, quantitation is derived from data for the lower of the two Nef levels shown.

Figure 2-2. MHC-I and CD4 co-localize in a subset of vesicles in Nef-expressing T cells. (A) Three way co-localization of HLA-A2, CD4 and Rab7. CEM HLA-A2 cells stably expressing YFP-Rab7 were transduced with adeno-Nef. HLA-A2 (red), CD4 (green), and YFP-Rab7 (blue) were simultaneously detected using three-color confocal microscopy. The top row shows the x-y projection of the cell, while the bottom row displays the x-z projection. Ten sequential optical sections were compiled to generate a projection of each cell about the x-z plane *Scale bar = 5 microns* (B) Immunogold labeling of HLA-A2 and CD4. Representative electron micrographs of CEM HA-HLA-A2 cells treated with control adeno (column 1) or adeno-Nef (columns 2 and 3). Thawed cryosections of cells were labeled with anti-HA (HLA-A2) and anti-CD4 antibodies followed by 15 and 10nm protein A-gold respectively. (C) Immunogold labeling of HA-HLA-A2 and γ -adaptin in Nef-expressing CEM T cells.

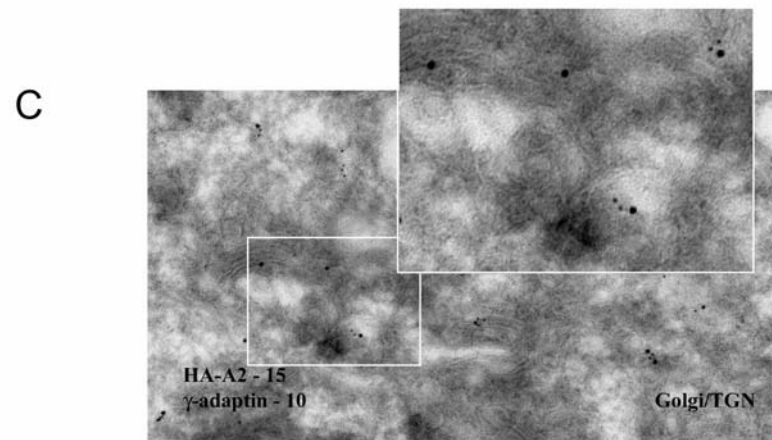
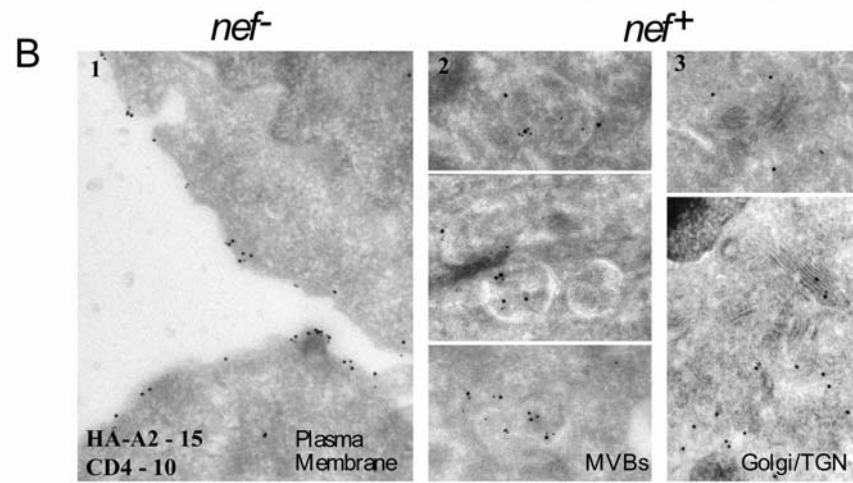
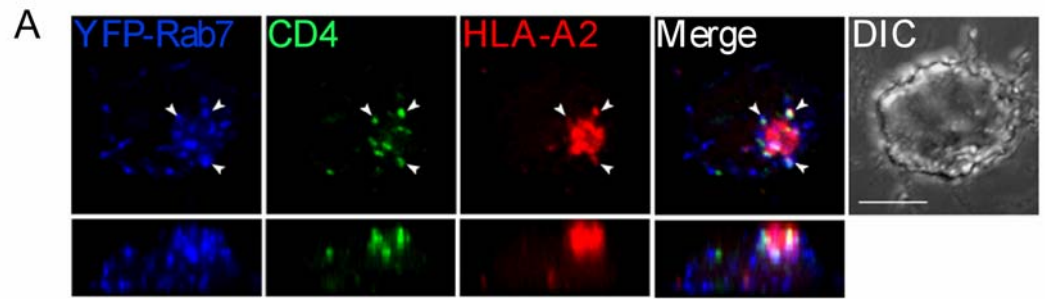


Table 2-1. Co-localization of HLA-A2 and CD4 with Organelle Markers in HIV-1 Nef expressing CEM cells.

Marker	Organelle	Number of co-localizing vesicles	Total Number of CD4 positive vesicles	% CD4 Co-localization
HLA-A2	N/A	306	333	91.9
γ-adaptin	TGN/Endosomes	29	78	37.2
EEA1	Early Endosomes	48	125	38.4
YFP-Rab7	Late Endosomes	49	52	94.2
Lamp1	Lysosomes	92	130	70.8¹

¹The majority of CD4 co-localized with weakly Lamp1-positive structures.

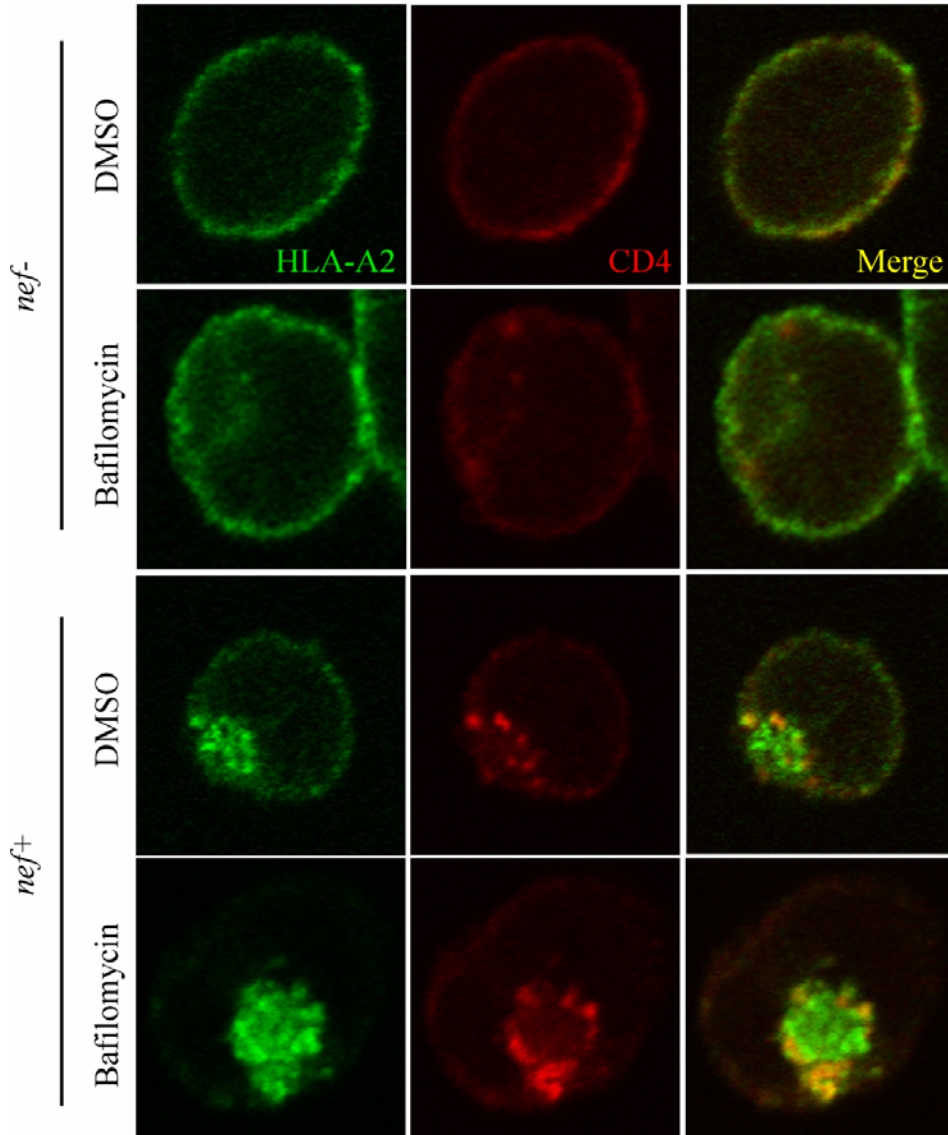
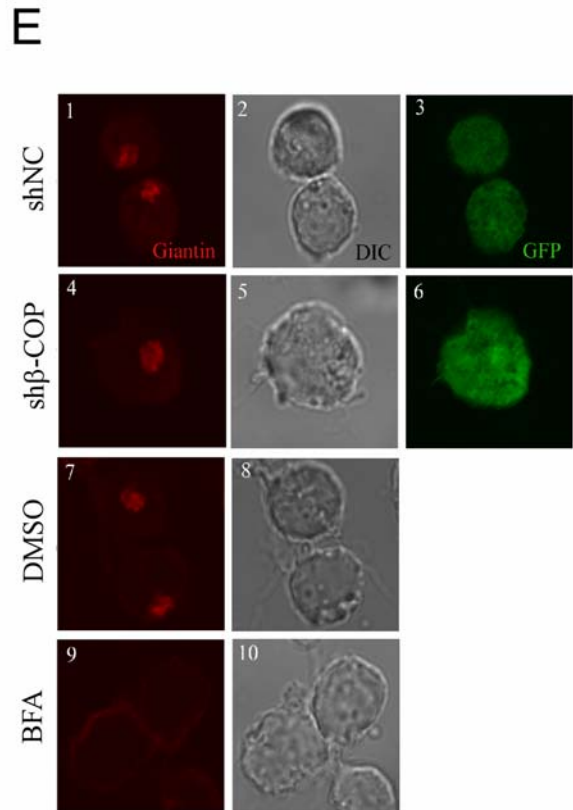
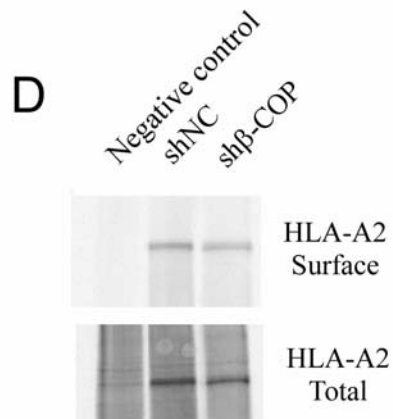
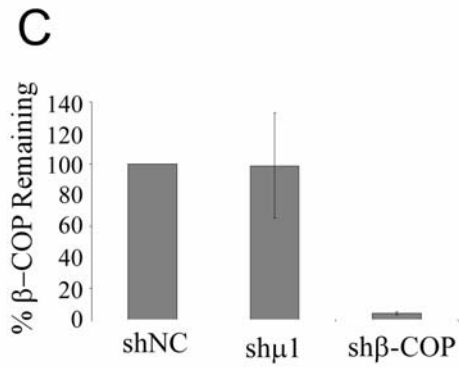
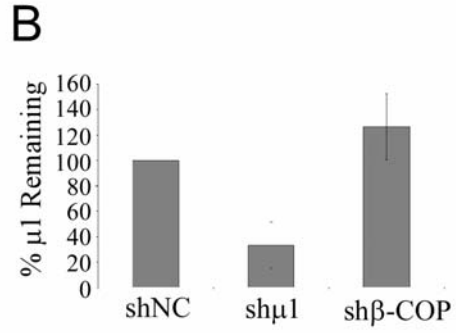
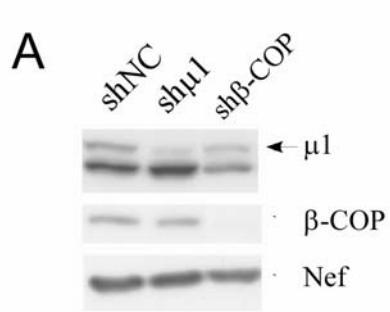


Figure 2-3. Bafilomycin treatment increases MHC-I and CD4 co-localization in Nef expressing cells. CEM HA-HLA-A2 cells were transduced with a control adenovirus (*nef*⁻) or adeno-Nef (*nef*⁺) as described in Materials and Methods. 72 hours later were treated with bafilomycin or solvent control (DMSO) and stained with antibodies directed against HLA-A2 and CD4 as described in Materials and Methods. Images were taken with a Zeiss confocal microscope and processed with LSM Image Browser and Adobe Photoshop software. Single Z-sections are shown.

Figure 2-4. Knockdown of β -COP does not affect HLA-A2 transport to the cell surface or disrupt the Golgi apparatus. (A) Analysis of protein expression in β -COP and μ 1 knockdown cells. CEM HA-HLA-A2 cells were transduced with a lentivirus expressing control shRNA (shNC) or an shRNA targeting either β -COP (sh β -COP) or μ 1 (sh μ 1). 72 hours later, they were transduced with adeno-Nef or control adenovirus. Three days later they were harvested and western blot analysis was used to assess protein levels of β -COP, μ 1 and Nef. Data is representative of seven independent experiments. (B and C) Quantification of μ 1 and β -COP expression in shRNA treated cells. The amount of either μ 1 (B) or β -COP (C) was quantified using Adobe Photoshop software. The average percent remaining \pm standard deviation for four experiments (B) and three experiments (C) is shown. To adjust for protein loading in part B, the nonspecific background band directly below μ 1 (shown in part A) was used to normalize protein loading. (D) Knockdown of β -COP does not affect HLA-A2 transport to the cell surface. CEM HA-HLA-A2 cells were transduced with lentivirus expressing either shNC or sh β -COP as in part A. Cell surface transport was assessed using a metabolic labeling assay with biotinylation as described in Figure 2-1D. (E) Knockdown of β -COP does not disrupt the Golgi apparatus. CEM HA-HLA-A2 cells were transduced with lentivirus expressing the indicated shRNA and GFP as in part A and treated with brefeldin A (BFA) at 50 μ M or DMSO for 30 minutes. The integrity of the Golgi apparatus was assessed by immunofluorescence staining for giantin and analyzed by confocal microscopy. Images were taken using a Zeiss confocal microscope and analyzed with LSM Image Browser and Adobe Photoshop software. Single Z-sections are shown. The results shown for parts D and E are representative of three independent experiments.



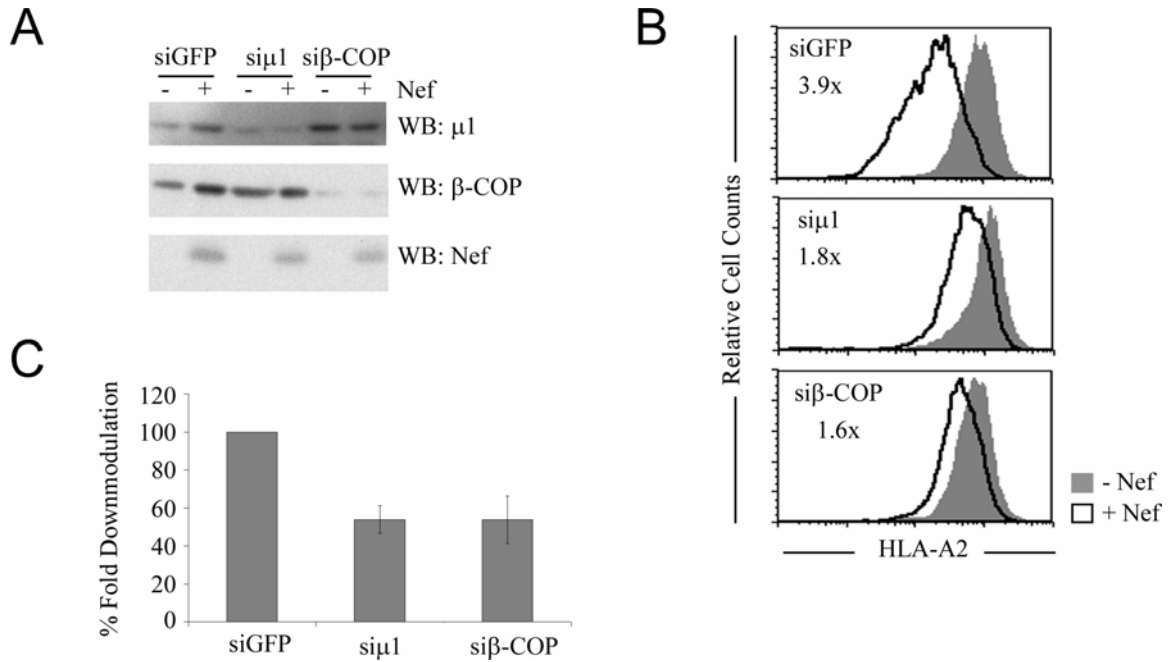


Figure 2-5. A second siRNA directed at β -COP disrupts Nef-dependent MHC-I trafficking. (A) Western blot analysis of protein expression in 373 mg astrocytoma cells transfected with the indicated siRNA. Previously published protocols (56) were used to transfect 373mg astrocytoma cells with control siRNA [(siGFP (56)] an siRNA targeting β -COP (si β -COP, sense 5'-GGAGAUGUAAAGUCAAGA-3', antisense 5'-UCUUUGACUUUACAUCUCC-3', Ambion) or an siRNA targeting the AP-1 μ subunit [si μ 1 (56)]. The data is representative of three experiments. (B) β -COP and μ 1 are required for Nef to efficiently reduce cell surface expression of HLA-A2. HLA-A2 cell surface expression on astrocytoma cells from (A) was assessed by flow cytometry as described in Materials and Methods. The fold downmodulation of HLA-A2 (mean fluorescence intensity of control/ mean fluorescence intensity of Nef expressing cells) for each condition is shown in the upper left corner. (C) Quantitation of HLA-A2 fold downmodulation in Nef expressing cells treated with siRNA. The mean fold downmodulation +/- standard deviation from three experiments is shown.

Figure 2-6. Nef requires β -COP to reduce HLA-A2 cell surface expression and target it for degradation. (A) β -COP and μ 1 are required for Nef to reduce cell surface expression of HLA-A2. CEM HA-HLA-A2 and CEM HA-A2/CD4 cells were transduced with a lentivirus expressing control shRNA (shNC), shRNA targeting β -COP (sh β -COP) or μ 1 (sh μ 1) and transduced with control adenovirus (*nef*⁻) or adeno-Nef (*nef*⁺). Cell surface expression was assessed by flow cytometry. The gray shaded histogram represents control adenovirus (*nef*⁻) treated cells and the solid black line represents adeno-Nef (*nef*⁺) treated cells. (B) Quantitation of HA-HLA-A2, CD4 and HA-A2/CD4 downmodulation in Nef expressing cells transduced with shRNA. The mean fold downmodulation (mean fluorescence of control/mean fluorescence of Nef-expressing cells) +/- standard deviation derived from seven (HLA-A2), six (CD4) and two (A2/CD4) independent experiments. (C) Knockdown of β -COP stabilizes intracellular levels of HLA-A2 and A2/CD4 in Nef expressing cells. CEM HA-HLA-A2 and CEM HA-A2/CD4 were treated as in part A. Lysates from these cells were generated and treated with endoglycosidase H (endo H). Protein levels of HLA-A2 and A2/CD4 were assessed by western blot using an anti-HA antibody. Endo H resistant bands are marked with an R and endo H sensitive bands are marked with an S. The results shown are representative of three independent experiments for HLA-A2 and two independent experiments for A2/CD4. (D) Quantification of endo H resistant protein. Adobe Photoshop software was used to quantify each band. The percentage of endo H resistant protein in each condition was calculated as follows: [resistant band/(resistant band + sensitive band)] X 100. The fold stabilization was then calculated as: (% endo H resistant in experimental sample)/[% endo H resistant in control (shNC)]. The data shown is the mean of two experiments +/- standard deviation.

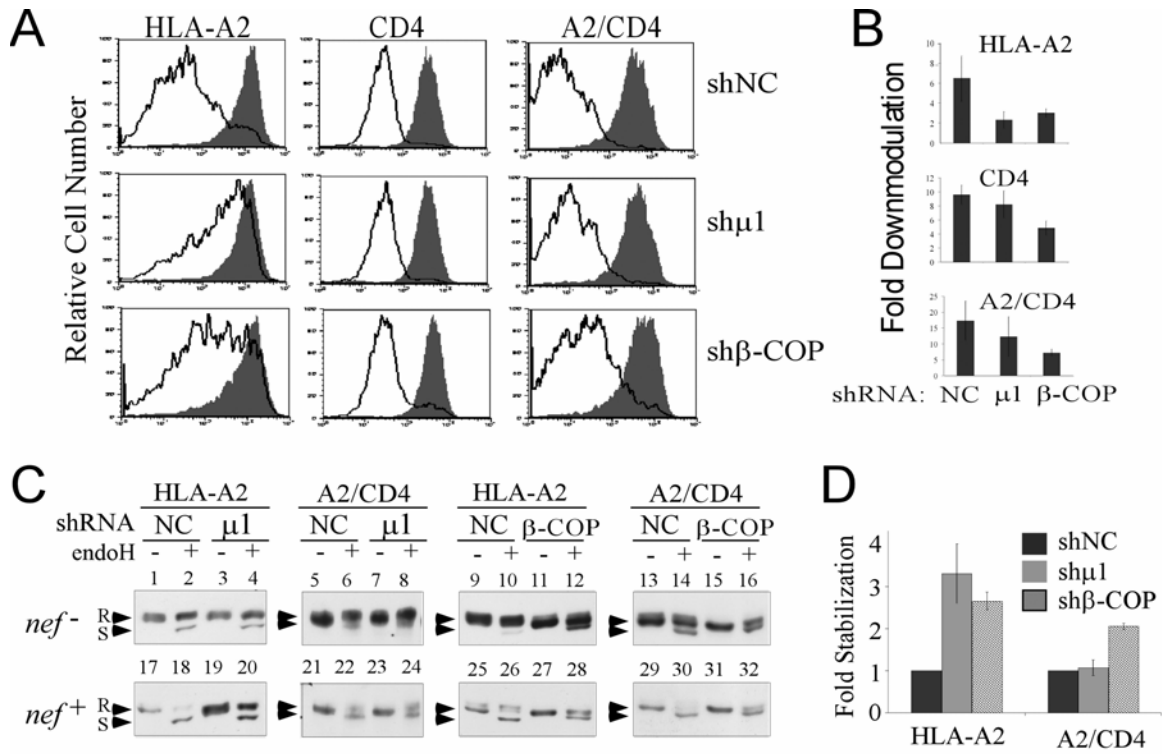
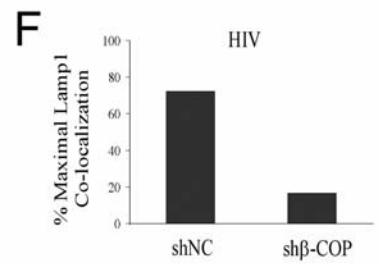
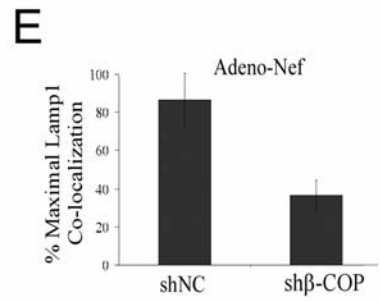
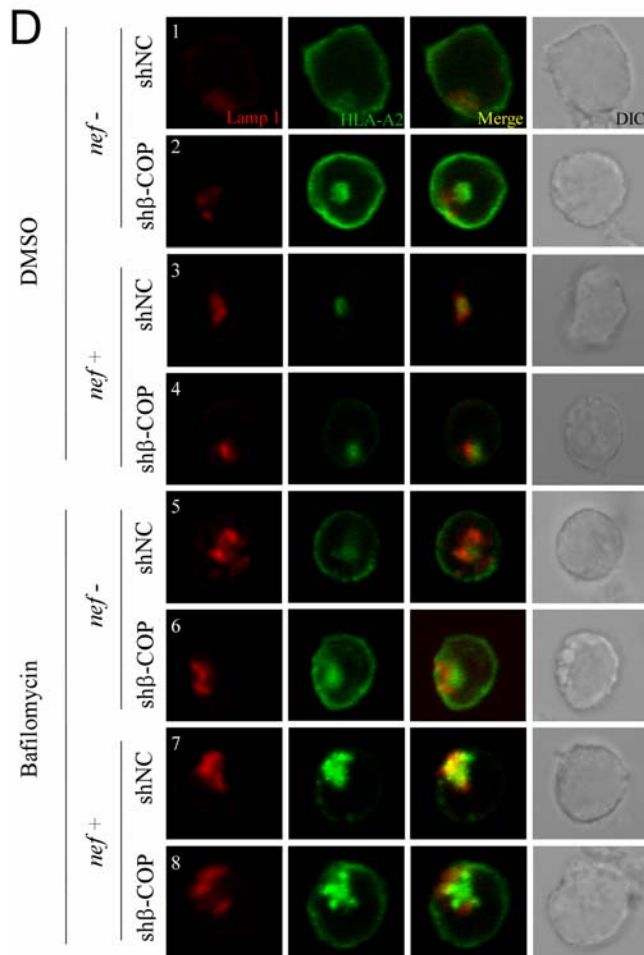
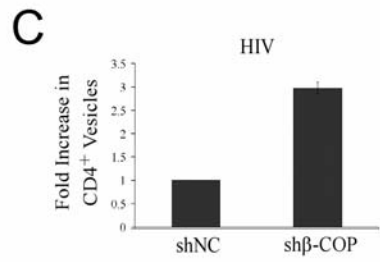
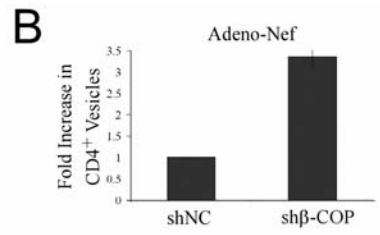
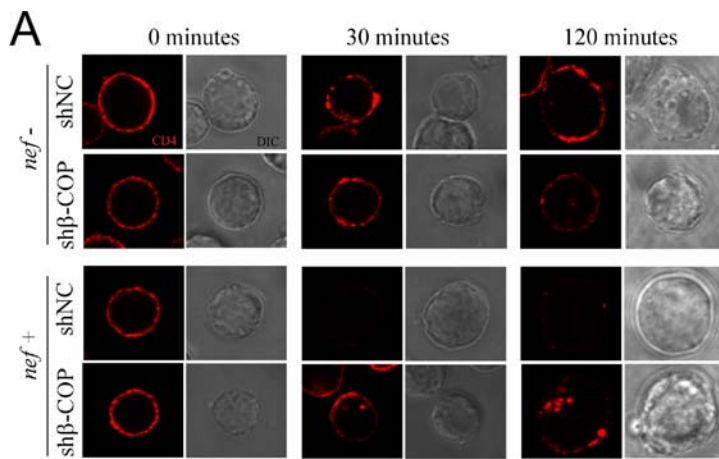


Figure 2-7. Nef requires β -COP to target HLA-A2 and CD4 for degradation. (A) Knockdown of β -COP stabilizes CD4⁺ vesicles in Nef expressing cells. CEM HA-HLA-A2 cells transduced with a lentivirus expressing either control shRNA (shNC) or shRNA targeting β -COP (sh β -COP) were transduced with control adenovirus (*nef*⁻) or adeno-Nef (*nef*⁺). The cells were incubated with CD4 antibody on ice and then shifted to 37°C for internalization for the indicated times. Images were taken with a Zeiss confocal microscope and processed using LSM Image Browser and Adobe Photoshop software. Single Z-sections are shown. (B) Quantitation of CD4⁺ vesicles is shown for 15 cells treated with shNC and 17 cells treated with sh β -COP. The mean +/- standard deviation is shown. (C) Quantitation is shown for 5 cells treated with shNC and 5 cells treated with sh β -COP. The mean +/- standard deviation is shown. (D) CEM HA-HLA-A2 cells were transduced with a lentivirus expressing either control shRNA (shNC) or shRNA targeting β -COP (sh β -COP), infected with HIV, treated with Bafilomycin or DMSO and stained for HLA-A2 and LAMP-1 as previously described (56). Images were taken with a Zeiss confocal microscope and processed as in part A. Single Z-sections are shown. (E) Relative co-localization of HLA-A2 with LAMP-1 in 10 adeno-Nef-expressing T cells treated with shNC and 15 adeno-Nef-expressing T cells treated with sh β -COP. (F) Relative co-localization of HLA-A2 with LAMP-1 in 6 HIV-infected T cells treated with shNC and 6 HIV-infected T cells treated with sh β -COP. Quantitation of microscopy data was performed independently by two, blinded investigators who scored maximal observable co-localization amongst all cells at an arbitrary value of 5. Each cell was then scored relative to that. The mean +/- stand deviation is shown.



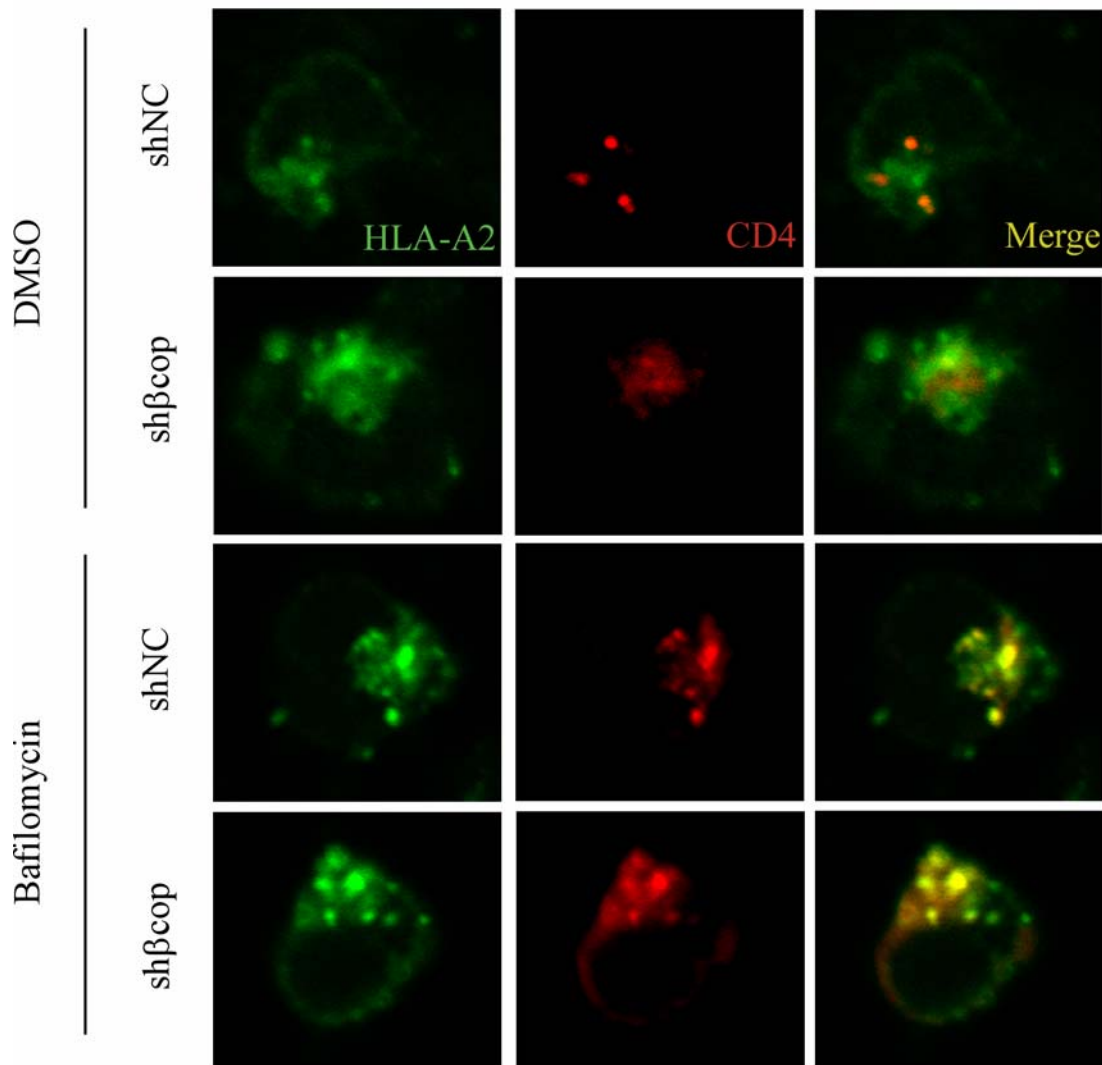


Figure 2-8. β -COP does not disrupt co-localization of CD4 and HLA-A2, but does increase amount of stainable protein within the cell. HLA-A2 CEM cells were transduced with a lentivirus expressing control shRNA (shNC) or an shRNA targeting β -COP (sh β -COP). After three days, the cells were transduced with adeno-Nef. After three additional days, the cells were stained with antibodies directed against HLA-A2 and CD4 as in Figure 2-3. Images were taken with an Olympus FV-500 confocal microscope and processed with Adobe Photoshop software. Single Z-sections are shown.

Figure 2-9. Binding of HLA-A2 and CD4 to HIV-1 Nef and cellular co-factors in T cells. (A) The HLA-A2 cytoplasmic tail is necessary for co-precipitation of AP-1. Parental CEM T cells (CEM) or CEM T cell lines expressing HA-HLA-A2 or HA-A2/CD4 were transduced with adeno-Nef or a control adenovirus. Lysates were immunoprecipitated with an antibody directed against HLA-A2 (BB7.2) and the presence of Nef or AP-1 were detected by western blot analysis. Results are representative of three independent experiments. (B) The cytoplasmic tail is necessary for the HLA-A2/Nef fusion protein to co-precipitate AP-1 in Nef expressing T cells. CEM T cells were transduced with a murine retroviral vector expressing no protein (vector), A2/Nef or A2/CD4/Nef fusion proteins. These cells were immunoprecipitated with an anti-HLA-A2 antibody (BB7.2) and western blot analysis was performed to detect co-precipitation of AP-1. (Spaces between lanes indicate where intervening lanes were cropped out to remove irrelevant data.) Results are representative of two independent experiments. (C) Nef D₁₂₃G and V₁₀EΔ17-26 mutants are defective at β-COP binding. CEM T cells were treated with control adenovirus (*nef*⁻), adeno-Nef (*nef*⁺) or the indicated mutant and immunoprecipitated with a control antibody (BB7.2) or an antibody directed against β-COP (M3A5). The presence of Nef was detected by western blot analysis. Arrows indicate the positions of wild type Nef and Nef V₁₀EΔ17-26. Results are representative of at least two independent experiments.

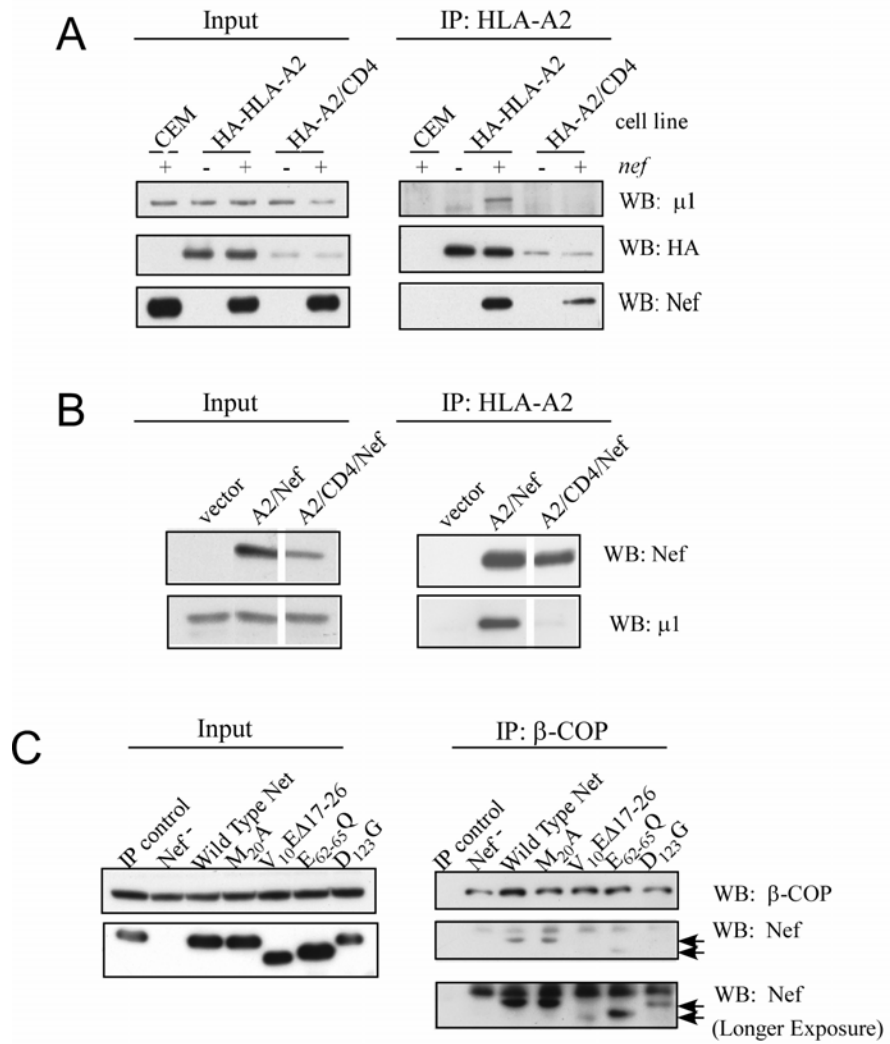
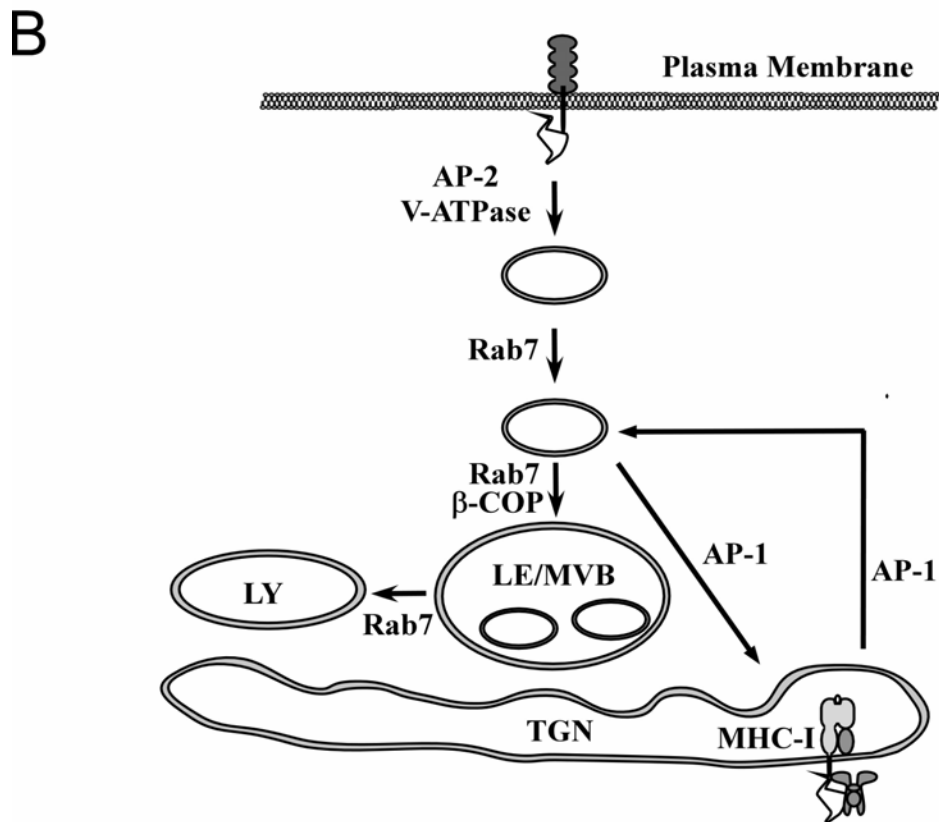
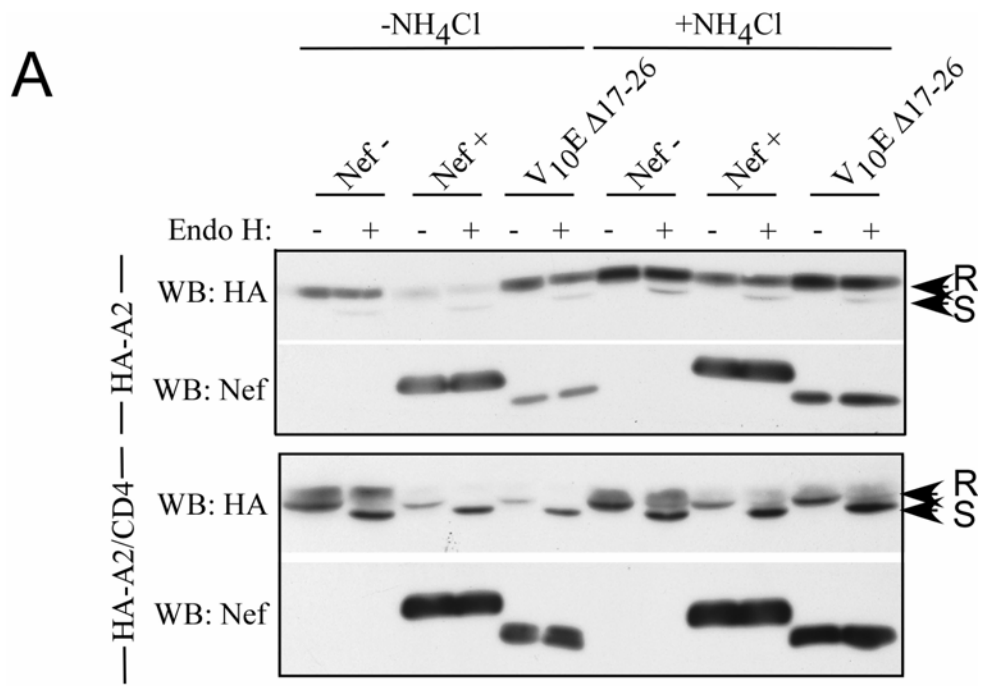


Figure 2-10. (A) V₁₀ED17-26 Nef is defective at MHC-I, but not CD4, degradation. CEM cells expressing HA-HLA-A2 and HA-A2/CD4 were transduced with adeno-viral vectors encoding wild type Nef (Nef⁺), or V₁₀EΔ17-26 Nef or a control adenoviral vector (Nef⁻). Two days later, the media on half of the cells was replaced with media containing 20mM ammonium chloride. The next day, the cells were harvested, lysed, and normalized. Each sample was split equally and one set was treated with endo H. Protein levels of HA-HLA-A2 and HA-A2/CD4 were assessed by western blot analysis using an anti-HA antibody. Endo H resistant bands are marked with an R and endo H sensitive bands are marked with an S. **(B) Model for the mechanism by which Nef affects CD4 and MHC-I trafficking.** HIV Nef binds the CD4 cytoplasmic tail at the cell surface, and recruits AP-2 and/or the vacuolar-ATPase to facilitate internalization. CD4 is internalized and is transported to an endosomal compartment associated with Rab7 and β-COP. Conversely, Nef binds the MHC-I cytoplasmic tail early in the secretory pathway, AP-1 is recruited and facilitates transport to an intermediate endosomal compartment marked with Rab7 and β-COP. If AP-1 falls off the Nef-MHC-I complex after arrival in the endosome, Nef binds β-COP and targets MHC-I (and CD4) to lysosomes for degradation. If AP-1 remains bound, it promotes recycling of the Nef-MHC-I complex to the TGN. LY=lysosome, LE/MVB =late endosome/multi-vesicular body.



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CHAPTER III

A NOVEL TRAFFICKING SIGNAL WITHIN THE HLA-C CYTOPLASMIC TAIL ALLOWS REGULATED EXPRESSION UPON DIFFERENTIATION OF MACROPHAGES

Abstract

Major histocompatibility complex class I molecules present peptides to cytotoxic T lymphocytes (CTLs). In addition, HLA-C allotypes are also recognized by killer cell Ig-like receptors (KIR) found on natural killer cells and effector CTLs. Compared to other classical MHC-I allotypes, HLA-C has low cell surface expression and an altered intracellular trafficking pattern. We present evidence that this results from effects of both the extracellular and the cytoplasmic tail. Notably, we demonstrate that the cytoplasmic tail contains a dihydrophobic (LI) internalization and lysosomal targeting signal that is partially attenuated by an aspartic acid residue (DXSLI). In addition, we provide evidence that this signal is specifically inhibited by hypophosphorylation of the adjacent serine residue upon macrophage differentiation and that this allows high HLA-C expression in this cell type. We propose that tightly regulated HLA-C surface expression

facilitates immune surveillance and allows HLA-C to serve a specialized role in macrophages.

Introduction

Major histocompatibility complex class I molecules (MHC-I) are necessary for presentation of antigens to naïve CD8⁺ cytotoxic T lymphocytes (CTLs) through engagement with the T cell receptor and CD8. There is evidence that professional antigen presenting cells (APCs) are required for this step, as they have unique co-stimulatory molecules needed for this process (32). APCs are thought to activate naïve CD8⁺ T cells by internalizing exogenous antigens and presenting them in association with MHC-I in a process known as cross-presentation (39). Activated CTLs then mature into effector cells, which have the capacity to kill cells bearing foreign epitopes through the release of perforin, granzymes and via the activation of apoptotic pathways (2).

MHC-I can also be recognized by MHC class I-specific inhibitory receptors, such as killer cell Ig-like receptors (KIR) (23). These receptors are classically thought to be expressed on natural killer (NK) cells to facilitate the eradication of virally infected or tumorigenic cells that have downmodulated MHC-I. More recently evidence has been accumulating that these receptors are also up-regulated with the acquisition of cytotoxic function in CD8⁺ T cells (1). In this case they may serve as an important negative feedback mechanism that aids in the prevention of autologous damage by raising the threshold for cell lysis (1).

There are three major sub-types of classical MHC-I molecules that serve these roles. HLA-A, B and C all have the capacity to present viral antigens to CTLs. Additionally, almost all HLA-C molecules are recognized by KIRs. Perhaps because of the crucial role of HLA-C as an inhibitory molecule capable of sending a dominant negative signal (9), HLA-C is normally expressed at low levels at the cell surface. HLA-C heavy chains are expressed at similar levels compared to HLA-A and B (21, 35). However, the HLA-C heavy chain is not stably expressed at the cell surface, and does not associate efficiently with the MHC-I light chain (β 2-microglobulin) (21, 30, 31). Additionally, HLA-C presents a more restricted repertoire of peptides causing it to be retained in the endoplasmic reticulum (ER) in complex with the transporter associated with antigen processing (TAP), which is responsible for transporting peptides into the ER for MHC-I loading. The retained HLA-C is then eventually degraded in the ER (21). The addition of HLA-C specific peptides has been shown to release HLA-C from TAP *in vitro* (21) and to increase the cell surface expression of HLA-C (16).

To better understand elements governing HLA-C surface expression, we examined the intracellular trafficking of chimeric molecules that contained the HLA-A*0201 extracellular domain and the HLA-C cytoplasmic tail (A2/C) or the HLA-Cw*0401 extracellular domain and the HLA-A cytoplasmic tail (Cw4/A). We found that HLA-Cw*0401 was poorly expressed on the cell surface relative to HLA-A*0201 in a variety of cell types, including T cell lines, astrocytoma cells, primary T cells and monocytic cell lines. Not surprisingly, the extracellular domain of HLA-C was responsible for

promoting retention in the ER. Remarkably, however, the cytoplasmic tail also had an effect on cell surface expression by increasing internalization at the cell surface and targeting the molecules for degradation in acidic organelles. Mutagenesis studies revealed that aspartic acid at position 333, serine at position 335 and isoleucine at position 337 were key amino acids that affected the activity of this motif. Finally, we found the complex regulation of HLA-C surface expression allowed the specific upregulation of HLA-C upon differentiation of primary monocytes and monocytic cell lines into macrophage-like cells. The specific induction of HLA-C expression with differentiation strongly suggests that there is a unique role for HLA-C in antigen presenting cells. We propose that HLA-C plays a role in downmodulating the normal CD8⁺ cellular immune response and/or that it functions to specifically limit the lysis of APCs that are cross-presenting antigen.

Results

HLA-C is a classical MHC-I molecule that plays a dual role, as it is able to present antigens to the immune system and to send inhibitory signals to natural killer cells. However, for unclear reasons, HLA-C molecules are known to be expressed at much lower levels on the cell surface than other classical MHC-I molecules. The extracellular domains of MHC-I proteins are known to be highly polymorphic, whereas the cytoplasmic tail domains are generally highly conserved within allotypes (Figure 3-1A). A comparison amongst the three types of classical MHC-I molecules revealed that there were four amino acids unique to HLA-C (Figure 3-1A).

To determine whether these amino acid differences played a role in reducing HLA-C surface expression, we attached an HA tag onto the N-terminus of HLA-A*0201 and HLA-Cw4*0401 (HA-A2 and HA-Cw4, Figure 3-1B). The HA tag, which was inserted just after the leader cleavage site, allowed us to compare the expression of heterologous proteins using the same antibody so that differences in antibody affinity did not confound our results. In prior publications, we have demonstrated that the presence of this tag did not affect the maturation and expression of HLA-A2 (27). Additionally, we have demonstrated that this tag did not affect recognition by the conformationally sensitive anti-HLA-A2 antibody, BB7.2 (24, 27).

We also made chimeric molecules in which the cytoplasmic tail domains of HLA-A or HLA-C were fused just after the transmembrane domain of HA-Cw4 and HA-A2 to create HA-Cw4/A and HA-A2/C respectively (Figure 3-1B). To accomplish this cDNAs

encoding these proteins were cloned into murine retroviral vectors and viral supernatants were used to transduce CEM T cells at a low multiplicity of infection to limit the number of transductants with multiple integrated copies. Bulk cell lines were then grown in selective media to obtain a uniform population. To ensure that our results were not influenced by arbitrary variations introduced by individual transfections or transductions, the cell lines used in our investigations were re-made with new transfections and transductions on three separate occasions and in each case yielded similar data.

Because, the translation initiation and leader sequences were the same for each molecule, we were able to measure initial protein synthesis as an estimate of the amount of translatable RNA in the cell. As shown in Figure 3-2A (and quantified in Figure 3-2B), the expression level of HA-A2 and HA-A2/C was not significantly different ($p=0.12$). However, expression of constructs containing the HLA-C extracellular domain were expressed at significantly lower levels than HA-A2 [$p < 0.01$ (HA-Cw4) and 0.02 (HA-Cw4/A)], consistent with the reported instability of HLA-C mRNA (19) (Figures 3-2A and B).

Despite the fact that there were similar amounts of HA-A2 and HA-A2/C protein initially synthesized, we found that there was approximately three-fold less HA-A2/C on the cell surface [mean fluorescence intensity (MFI) = 50 ± 4 for HA-A2/C, compared with 149 ± 18 for HA-A2] (Figure 3-1C). These data were corroborated by confocal microscopy, confirming that HA-A2 was largely expressed on the cell surface, where it co-localized significantly with concanavalin A (ConA, Figure 3-1D). In comparison, HA-A2/C had a

staining pattern that was distinctly different relative to that of ConA (compare panels 3 and 7 in Figure 3-1D). Consistent with prior reports, we also found that molecules with an HLA-C extracellular domain were expressed poorly on the cell surface relative to those containing HLA-A extracellular domains [(MFI)= 12 +/- 3 for HA-Cw4 and 20 +/- 2 for HA-Cw4/A2 (Figure 3-1C)].

To verify these results in a different system, we cloned HA-A2 and HA-Cw4 into a bicistronic vector, which also expressed GFP. This vector system allowed us to monitor the number of gene copies present in the cell by measuring GFP expression by FACS. These constructs were directly transfected into activated primary T cells and the surface expression levels of HA-A2 and HA-Cw4 in primary T cells expressing equivalent amounts of GFP was measured and compared to what was observed in CEM T cells. As shown in Figures 3-1E, at similar GFP expression levels the cell surface expression of HA-A2 and HA-Cw4 was comparable to that achieved in stable CEM T cell lines (Figures 3-1C and 3-1E). Moreover, the pattern we observed was not changed by adding twice as much DNA to the transfection, indicating that the surface expression of HA-Cw4 and HA-A2 was not substantially affected by differences in transfection conditions.

The HLA-C cytoplasmic tail contains an internalization and lysosomal trafficking signal. To determine how the cytoplasmic tail affected cell surface expression, we utilized pulse-chase labeling with endo H digestion to measure the rate at which molecules harboring this domain were transported into the Golgi apparatus where they become modified such that they are resistant to endo H digestion. We observed that the cytoplasmic tail did not influence the rate at which molecules acquired resistance to

digestion by endo H (Figure 3-3A and B). In contrast, we confirmed prior indications that the extracellular domain of HLA-C promoted ER retention and degradation (Figure 3-3A)

We then used pulse-labeling followed by a chase period in the presence of a cell impermeable biotinylation reagent, which labels molecules that reach the cell surface during this time. The lysates were first immunoprecipitated with anti-HA. Then, one-third of the cell lysates was analyzed directly (total), and the remaining two-thirds was eluted from the beads and re-precipitated with avidin-agarose to isolate the subset of MHC-I at the cell surface (Figure 3-4A and B). As determined by phosphorimager analysis, we found that HA-A2/C was transported to the cell surface approximately two-fold more slowly than HA-A2 ($p < 0.01$, $n = 2$).

We then compared the internalization rate of HA-A2 and HA-A2/C using a FACS based assay in which cells were incubated on ice with a primary antibody directed against an HLA-A2 specific epitope (BB7.2). The cells were then shifted to 37°C for the indicated time period after which they were labeled with a secondary antibody. Flow cytometry was then used to quantify the amount of antigen remaining on the cell surface (15). As shown, we observed that, HA-A2/C was internalized twice as fast as HA-A2 ($p < 0.01$, $n = 3$, Figure 3-4C). In contrast, we did not observe any decrease in recycling of HA-A2/C that would explain its lower expression levels. In fact, a slightly higher percentage of HA-A2/C was recycled to the cell surface from internal compartments ($p = 0.036$, $n = 3$, Figure 3-5).

We then examined the ultimate fate of HA-A2/C with longer pulse-chase experiments to determine whether it was targeted to lysosomal compartments after internalization. As shown in Figure 3-6A and quantified in Figure 3-6B, we found that mature, endo H resistant HA-A2/C was degraded approximately twice as fast as HA-A2. (HA-A2 had a half-life of 10.5 hours, compared to 6 hours for HA-A2/C). These data were confirmed by confocal microscopy in which we noted that HA-A2/C displayed several areas of co-localization with LysoTracker, a marker of acidic organelles, whereas this was not observed with HA-A2 (Figure 3-6C).

Finally, to provide further evidence that the degradation of HA-A2/C occurred in acidic compartments, such as lysosomes, we treated CEM T cells with bafilomycin, an inhibitor of the vacuolar ATPase that is required for efficient acidification and degradation in lysosomal compartments. As shown in Figures 3-6D and quantified in Figure 3-6E, bafilomycin treatment resulted in a six-fold increase of HA-A2/C compared with a 2.2-fold increase for HA-A2 ($p=0.015$, $n=3$). In sum, these data indicate that the HLA-C cytoplasmic tail contains an internalization and lysosomal targeting signal.

Identification of a trafficking signal in the HLA-C cytoplasmic tail that promotes intracellular localization and lysosomal targeting. To determine which amino acids were responsible for the effects of the HLA-C tail, we focused on four amino acid differences between HLA-C and HLA-A/B molecules (Figure 3-1A). Each of these amino acids was mutated in HA-A2/C, and stable CEM T cell lines were made as

described above for HA-A2/C. Initial protein synthesis measurements indicated that each of these molecules was expressed similarly to HA-A2/C, except for HA-A2/C D₃₃₃A, which was expressed slightly less ($p=0.04$, Figure 3-7).

We then performed flow cytometric analysis on cells stained with an anti-HA antibody to measure surface expression. As shown in Figure 3-8A and B, only one of the amino acids substitutions reversed the effect of the HLA-C tail. Specifically, changing isoleucine at position 337 in the HLA-C tail to the threonine found in HLA-A and B tails (I₃₃₇T) increased surface expression by three-fold compared with HA-A2/C ($p<0.001$, $n=3$). Conversely, we found that the reciprocal mutation in the HLA-A cytoplasmic tail (T₃₃₇I), reduced surface expression of HA-A2 by 3-fold ($p<0.0001$, $n=3$, Figure 3-8A and B).

An analysis of intracellular transport, using the assay described above, revealed that HA-A2/C I₃₃₇T transport was reduced compared with wild type HA-A2 ($p<0.001$, $n=3$), but was not significantly different than A2/C (Figure 3-8C). Whereas, the flow cytometric internalization assay (described above) revealed that substitution of I₃₃₇ reduced the internalization rate 15-fold (from 3.73% per minute to 0.25% per minute $p<0.01$, Figure 3-8D).

Finally, we used pulse-chase analysis plus or minus bafilomycin to measure the degree to which wild type and mutant molecules were degraded in acidic compartments. As shown in Figure 3-8E and quantified in Figure 3-8F, substitution of I₃₃₇ in HA-A2/C increased

the amount of recovered protein four-fold in the control, DMSO treated, sample (compare lanes 5 and 8 in Figure 3-8E, $p=0.0013$), resulting in expression that was similar to that of HA-A2 (compare lanes 3 and 8 in Figure 3-8E). Thus, I₃₃₇ is a determinant required for accelerated internalization and degradation of molecules containing an HLA-C cytoplasmic tail domain.

To further define the internalization and lysosomal targeting motif in HLA-C, the highly conserved aspartic acid (D) at position 333 was changed to an alanine. Based on initial protein synthesis, this mutant was expressed slightly less than HA-A2/C ($p=0.04$), but was expressed similarly to HLA-A2 and most A2/C mutants (C₃₂₀Y, N₃₂₇D, I₃₃₇T, T₃₃₇I, Figure 3-7). Substitution of D₃₃₃ resulted in a substantial loss of cell surface expression compared to HA-A2/C, HA-A2 and the other HA-2/C mutants (Figure 3-8A and B, p values ranged from $< 1 \times 10^{-4}$ - 10^{-6}). The intracellular transport rate was not the explanation for the reduction in surface expression as this was similar to HA-A2/C (Figure 3-9A).

However, we found that substitution of D₃₃₃ resulted in a two-fold increase in internalization rate compared with HA-A2/C ($p < 1 \times 10^{-4}$) and a five-fold increase relative to wild type HA-A2 ($p < 1 \times 10^{-4}$, Figure 3-9B). Additionally, pulse-chase analysis revealed that mutation of D₃₃₃ also caused an increase in turnover of mature, endo H resistant molecules (Figure 3-9C and D). In sum, these data indicate that D₃₃₃ functions to attenuate the downstream dihydrophobic internalization and lysosomal targeting signal.

Evidence for regulated cell surface expression of HLA-C in antigen presenting cells.

The data we have acquired indicates that there are multiple mechanisms by which cells precisely regulate HLA-C expression. The extracellular domain promotes retention and degradation in the ER and the cytoplasmic domain promotes internalization and degradation. Thus, it seems that complex mechanisms exist to maintain significant intracellular levels of HLA-C while limiting (but not eliminating) surface expression. It makes sense that a molecule capable of sending a dominant inhibitory signal to killer T cells should be tightly regulated, as high expression could result in higher activation thresholds for the detection of virally infected or tumorigenic cells. However, the fact that intracellular pools of HLA-C are maintained suggests that there may be conditions in which it is advantageous to rapidly upregulate HLA-C cell surface expression. We were unable to specifically induce HLA-C surface expression in T cells with a variety of stimuli, such as interferons alpha and gamma or with compounds that stimulate T cells, such as IL-2 and PHA. Additionally, we were unable to upregulate HLA-C expression by treatment of T cells with specific pathogens, such as HIV and adenovirus.

It remained possible, however, that HLA-C had evolved to present peptides from certain types of pathogens, or that it functions to inhibit killing under some conditions. For example, when antigen presenting cells (APCs) activate naïve CTLs by cross-presenting exogenous antigen, it would not be advantageous to lyse the uninfected, cross-presenting APC. Thus it would make sense for these cells to upregulate molecules that would send inhibitory signals to effector T cells that might otherwise mistake the APC for an infected

target cell and lyse it. Indeed, it has recently been reported that co-incident with acquisition of killer T cell effector functions, CTLs up-regulate the KIR family of inhibitory receptors (1).

To examine this possibility further, we isolated primary human CD14⁺ mononuclear cells from a normal, healthy donor's peripheral blood. Some of the cells were stained immediately for HLA-C and HLA-B allotypes and the remainder were incubated in GMCSF for seven days to induce macrophage differentiation. To measure HLA-C surface expression, we obtained an antibody that specifically recognizes most HLA-C allotypes, albeit like most allotype-specific antibodies, it is not completely specific and does cross-react with some HLA-B allotypes. As shown in Figure 3-10A, HLA-C staining was low in freshly isolated, undifferentiated CD14⁺ cells (about 2.8-fold above background and more than forty-fold less than Bw6). After seven days of culture in GMCSF, HLA-C surface staining increased more than twenty-fold relative to that of Bw6.

The data described above confirms that HLA-C expression is normally much lower than that of HLA-A and HLA-B allotypes in undifferentiated primary monocytes using antibodies that recognize natural epitopes. However, it is difficult to compare staining by different antibodies as it can be confounded by differences in antibody affinity. Moreover, this approach does not allow the determination of which domains of HLA-C are responsible for regulated expression in differentiated macrophages. To further investigate this observation in a more well-defined system, we expressed HA-A2, HA-

Cw4 and HA-A2/C in cell lines (THP1 and U937) that were capable of differentiating into macrophage-like cells by the addition of LPS and PMA. For comparison, we also expressed HA-tagged B*4405 and B*4402 in these cells. These two molecules differ by only a single amino acid, but are known to vary substantially in terms of peptide loading and rates of ER egress (34).

In undifferentiated non-adherent solvent (DMSO)-treated cells, HA-A2, HA-A2/C and HA-Cw4 were expressed in a manner that was very similar to what we observed in primary cells and stable CEM T cell lines (Figure 3-10B, top row). HA-A2 was expressed at comparably high levels (Figure 3-10B, top row) and was largely endo H resistant (Figure 3-10C lanes 2 and 6), whereas HA-Cw4 was expressed at very low levels at the cell surface (Figure 3-10B, top row) and was largely endo H sensitive (Figure 3-10C lanes 18 and 22). HA-A2/C was expressed at intermediate levels (Figure 3-10B, top row) and had reduced amounts of endo H resistant material (Figure 3-10C lane 10 and 14), presumably due to increased internalization and degradation in acidic compartments as was observed in other cell types (Figures 3-4 and 3-6). HA-B*4405 was expressed well on the cell surface (Figure 3-10B, lower plots) and was largely endo H resistant (Lanes 34 and 38 in Figure 3-10C), whereas HA-B*4402 was expressed comparatively less well on the cell surface (Figure 3-10B, lower plots) and was largely endo H sensitive due to ER-retention [lanes 26 and 30 in Figure 3-10C, see also (34)].

When U937 and THP1 cell lines were treated with LPS and PMA to induce differentiation into macrophage-like cells, we observed no change in the surface

expression of HA-A2 or HA-B*4402 and we noted a decrease in the surface expression of HA-B*4405 (Figure 3-10B). Whereas, we observed that A2/C cell surface expression was increased to achieve levels that were similar to wild type HA-A2 (Figure 3-10B, top row). Additionally, we also observed an increase in full length HA-Cw4 cell surface expression (Figure 3-10B, top row).

The increase in surface expression of A2/C was reflected by an increase in the amount of endo H resistant protein detected by western blot analysis (Figure 3-10C, compare lanes 10 and 12 for U937 cells or lanes 14 and 16 for THP1 cells). We also noted an increase in the ratio of endo H resistant:sensitive forms of full length HLA-Cw*0401 (Figure 3-10C compare lanes 18 and 20 for U937 or lanes 22 and 24 for THP1). Albeit, most of the full-length HLA-C molecules remained endo H sensitive.

The relative amount of endo H resistant material for HA-A2 and HA-B*4405 remained unchanged. However, we did note an increase in the fraction of HA-B*4402 that became resistant to endo H (lanes 25-32). Thus, macrophage differentiation resulted in a complex set of effects that enhanced ER exit of some MHC-I molecules, like HA-B*4402, that are normally retained in the ER because of problems with protein loading (34). In addition, macrophage differentiation increased the amount of endo H-resistant HA-A2/C, which is normally low because of lysosomal targeting of mature molecules.

To better understand the striking upregulation of surface HA-A2/C and the stabilization of mature, endo H resistant forms of A2/C in differentiated macrophage-like cells, we

first examined internalization rate. As shown in Figure 3-10D, we found that HA-A2/C was internalized 3.6 times more rapidly than HA-A2 in DMSO treated monocytic cells. However, after treatment with LPS and PMA, HA-A2 and HA-A2/C were internalized at very similar rates, suggesting that the activity of the cytoplasmic tail signal was modified with differentiation.

Evidence that HLA-C Cell Surface expression in Macrophages is Regulated by

Phosphorylation. The HLA-C internalization and lysosomal targeting signal is surrounded by serine residues (SDXSLI) and thus could be regulated by phosphorylation (20, 38). To examine this, we mutated these residues to alanine residues to prevent phosphorylation (A2/C SSAA). In undifferentiated U937 and CEM cells, we found that mutation of both serines rescued HA-A2/C expression to that of wild type HA-A2 (Figure 3-11A). Mutation of the serine residue at position 335 to glutamic acid (S₃₃₅E), to mimic phosphorylation, did not affect expression of HA-A2/C in undifferentiated cells. Remarkably however, when macrophage differentiation was induced in U937 cells with LPS and PMA, we found that mimicking phosphorylation prevented upregulation, whereas unmodified HA-A2/C was upregulated 4.7-fold ($p < 1 \times 10^{-6}$, Figure 3-11A and B).

To examine the mechanism by which S₃₃₅ affected HA-A2/C expression, we examined degradation by pulse-chase analysis. As shown in Figure 3-11C, HA-A2/C expression was reduced by 97% by 12 hours (compare lanes 15 and 16 with lanes 17 and 18 in Figure 3-11C). Inhibition of phosphorylation by alanine substitutions (SSAA) resulted in stabilization of the molecule nearly four-fold (compare lanes 17 and 18 with lanes 29 and

30 in Figure 3-11C). In contrast, mimicking phosphorylation of S₃₃₅ by glutamic acid substitution resulted in degradation similar to HA-A2/C (compare lane 17 and 18 with lanes 41 and 42 in Figure 3-11C). In all cases, the observed degradation was rescued with bafilomycin.

Following treatment with LPS and PMA to induce macrophage differentiation, we observed a four-fold increase in HA-A2/C protein at the 12 hour chase point compared to DMSO-treated cells (compare lanes 17 and 18 with lanes 23 and 24 in Figure 8C). Mimicking phosphorylation at position 335 (S₃₃₅E) prevented differentiation-induced stabilization of HA-A2/C (compare lanes 17 and 18 with lanes 47 and 48 Figure 3-11C). Conversely, inhibiting phosphorylation by substituting alanine at the same position maintained protein stability (compare lanes 17 and 18 with lanes 35 and 36). In sum, these data strongly indicate that phosphorylation of S₃₃₅ in the HLA-C tail is necessary for lysosomal targeting and that macrophage differentiation inhibits phosphorylation of this residue.

Finally, we examined the effect of mimicking or inhibiting phosphorylation on internalization rate using the FACS-based internalization assay described above. As shown in Figure 3-11E (left graph), mimicking phosphorylation accelerated internalization 1.7-fold ($p < 0.01$), whereas preventing phosphorylation at this position inhibited it 2.0 fold ($p < 0.01$). Following treatment with LPS and DMSO, we again observed a decrease in the internalization rate of HA-A2/C (Figure 3-11E, right graph). However, the phosphorylation mimic (S₃₃₅E) did not respond, and

continued to be internalized at a rapid rate. These data suggest that phosphorylation is necessary for the internalization and degradation of HLA-C, and that macrophage differentiation resulted in hypophosphorylation, and increased expression of HLA-C.

Discussion

In this study, we demonstrated that HLA-C is a highly regulated MHC-I molecule and in most cell types its expression is limited at almost every step in the biosynthetic pathway. This low level may allow a balance between the signals needed for antigen presentation and appropriate inhibition of NK cells, without sending strong dominant signals that might overly increase activation thresholds. The extracellular domain of HLA-C reduces expression by promoting ER retention whereas the cytoplasmic tail contains an internalization and lysosomal targeting signal.

The influence of the extracellular domain of MHC-I on its surface expression is not surprising. This region of the molecule dictates the peptides MHC-I will bind and ultimately regulates its release from the ER and transport to the cell surface. Indeed, the addition of HLA-C specific peptides has been reported to promote the release of HLA-C from TAP *in vitro* (22), and thus would be expected to decrease ER retention. Based on these data, it is tempting to speculate that HLA-C expression would increase with a broadening of intracellular peptides such as would occur with infection by viruses or intracellular bacteria. To this end we did examine several viruses (HIV and adenovirus) without detecting any significant change in HLA-C expression. Obviously, however, we cannot rule out the possibility that specific peptides found in other kinds of pathogens might stimulate the release of HLA-C from the ER.

The bigger surprise was the discovery of an internalization and lysosomal targeting signal within the HLA-C cytoplasmic tail. This motif was identified by the demonstration that

mutating isoleucine at position 337 to a threonine reversed the phenotype conferred by swapping the HLA-C cytoplasmic tail for the HLA-A2 cytoplasmic tail. Interestingly, this motif more closely resembles a Golgi localized, gamma-ear containing, ARF-binding (GGA) consensus binding motif (DXXLL) than the consensus signal recognized by the heterotetrameric clathrin adaptor protein AP-2 (DXXXLL; reviewed in (6)). GGAs are localized to the TGN and endosomal compartments, and are thought to play a role in trafficking between the TGN and endosomes. Thus, it was possible that GGAs played a role in targeting HLA-C into the endolysosomal pathway from the cell surface or the TGN. However, while we observed some reduction in surface expression and some alteration in intracellular localization with knockdown of GGA-2 and -3, we observed no significant change in the surface transport rate, internalization, recycling or degradation rates. Also, arguing against a role for the GGAs, we found that mutation of the required aspartic acid residue at position 333 (DXSLI) to an alanine, actually increased the activity of the signal. Based on these data, the role of this amino acid was not to provide a GGA binding site, but rather to attenuate the dihydrophobic signal so as to allow some HLA-C to remain on the cell surface. Thus, we have defined a set of amino acids in the HLA-C cytoplasmic tail, which comprise a novel signal that serves to maintain a precise, low level of HLA-C surface expression. Further work will be needed to identify the corresponding trafficking protein that binds it.

Interestingly, the activity of the HLA-C internalization and lysosomal targeting signal also depended on an adjacent serine (DXSLI). Changes in this position increased or decreased internalization and degradation, depending on the substitution that was made.

When the serine was changed to a glutamic acid residue, which mimics the negative charge provided by phosphorylation, internalization and degradation occurred rapidly. When phosphorylation was prevented by changing the serine to an alanine, internalization and degradation were inhibited and cell surface expression was increased.

The complex regulation of HLA-C trafficking was puzzling in that HLA-C cell surface expression was kept low while HLA-C intracellular levels remained fairly high. These observations suggested that under most conditions it is beneficial to keep HLA-C expression low to reduce inhibitory signals that might limit immune surveillance, but that there might be some circumstances in which HLA-C might be rapidly up-regulated, either to increase the capacity of the cell to present certain types of antigens, or to turn down an immune response by increasing signaling to KIRs.

To examine whether HLA-C might be specifically upregulated under some conditions of immune activation, we treated CEM cells with interferons (alpha and gamma) or with chemicals known to activate T cells (PHA and IL-2), without success. Additionally, we infected the cells with viral pathogens such as adenovirus and HIV, again without significant affect. We then turned to APCs, because these cells have unique roles in antigen presentation (e.g. the capacity to present exogenous antigens in association with MHC-I).

We found that undifferentiated primary monocytes and monocytic cell lines expressed low levels of HLA-C, similar to the other cell types we examined. Upon differentiation,

however, we observed a reduction in internalization and degradation and a corresponding upregulation of HLA-C and molecules bearing the HLA-C cytoplasmic tail, whereas the surface expression of HLA-A and HLA-B molecules remained essentially unchanged or was even reduced somewhat. The dependence of this effect on the cytoplasmic tail, which we demonstrated governs post-Golgi trafficking, ruled out the possibility that this was due to a change in the peptide loading capacity of the APCs in the ER. Upregulation of expression with differentiation depended on the serine adjacent to the dihydrophobic motif (DXSLI). When this serine was modified to a glutamic acid, mimicking phosphorylation, low expression and rapid internalization was maintained upon induction of differentiation. When phosphorylation was inhibited by changing the serine to an alanine residue, high surface expression and reduced internalization resulted and was maintained upon induction of differentiation.

These observations, together with the strong evidence that HLA-C plays a crucial role as an inhibitor of NK cell lysis by virtue of its specific binding of KIRs, suggests that HLA-C is upregulated on macrophages to downregulate and/or specifically inhibit lysis of cells bearing these receptors. Interestingly, it has recently been demonstrated that CTLs acquire KIRs coincident with acquisition of effector functions (1). Thus, HLA-C may be upregulated to provide feedback inhibition of CTLs, once they have fully matured. Alternatively, another, perhaps more intriguing possibility is that HLA-C is specifically upregulated on APCs to protect them from lysis by mature CTLs while they are cross-presenting exogenous antigens to naïve CTLs. The capacity to specifically prevent the lysis of cross-presenting APCs would be advantageous in the setting of a chronic

infection in which it was necessary to continuously present antigens over an extended period of time. In preserving these cells by such a mechanism, the resulting increased threshold to lysis may inadvertently create a protected reservoir that aids in the persistence of certain organisms. Indeed, there is a long list of persistent pathogens that can be found in macrophages, including HIV, leishmania, brucella, salmonella, herpes viruses, tuberculosis, legionella, plus others (3, 4, 8, 11, 13, 17, 26, 29). Thus, HLA-C may be precisely regulated in order to balance the need for continued immune activation by APCs presenting antigens against the cost of allowing some pathogens to persist.

Materials and Methods

DNA constructs.

MSCV 2.1 HA-HLA-A*0201 was constructed as previously described (28). For MSCV 2.1 HLA-Cw*0401, the HLA-Cw*0401 open reading frame (Peter Parham, Stanford University) was amplified with the following primers, 5'-CAATCTCCCCAGACGCCGAGATGCG-3' and 5'-CCGCTCGAGTCAGGCTTTACAAGCGATGAGAGA-3'. The PCR product was digested with *Nae* I and *Xho* I and the 3' fragment was gel purified. This fragment was then ligated to the 5' leader sequence plus the HA tag from HA-HLA-A*0201 (isolated by digesting MSCV 2.1 HA-HLA-A*0201 with *Eco* RI and *Nae* I) and MSCV 2.1 digested with *Eco* RI and *Xho* I.

MSCV 2.1 HA-Cw4/A2 was constructed by digesting MSCV 2.1 HA-HLA-Cw*0401 with *Eco* RI and *Xho* I, sub-cloning this fragment into the same sites in Litmus 29 to generate Litmus 29 HLA-Cw*0401. A three-way ligation was then performed with a *Eco*RI to *Sap* I fragment from Litmus 29 HLA-Cw*0401 that encodes the extracellular domain of HLA-Cw*0401, a DNA fragment encoding the HLA-A*0201 cytoplasmic tail digested with *Sap* I and *Xho* I (generated by PCR amplification of MSCV 2.1 HA-HLA-A*0201 with the following primers 5'-GTGATCACTGGAGCTGTGGTCGCTGCT-3' and 5'-CCGCTCGAGTCACACTTTACAAGCTGT GAGAGACAC-3'), and MSCV 2.1 digested with *Xho* I and *Eco* RI.

MSCV A2/C was constructed by first PCR amplifying the Cw*0401 cytoplasmic tail was using the following primers 5'-CAGGTGACCGGTGCTGTGGTCGCTGCTGTGATGTGGA

GGAGGAAGAGCTCAGGTGGA-3' and 5'-CACCTGCAGCTGTCAGGCTTTACAAGCGATGAG-3' and then digesting with *Age* I. This fragment was then ligated into pcDNA3.1 HLA-A*0201 *Age* I (a plasmid containing an HLA-A*0201 open reading frame with a silent sequence change to introduce an *Age* I site (10)) digested with *Age* I and *Eco* RV to generate pcDNA3.1 A2/Cw4. A DNA fragment encoding part of the HLA-A*0201 extracellular domain and the HLA-Cw*0401 cytoplasmic tail was isolated by digesting pcDNA3.1 A2/Cw4 with *Pml* I and *Eco* RV. This fragment was then ligated into MSCV2.1 HA-HLA-A*0201 digested with *Pml* I and *Hpa* I.

pcDNA3.1 (+) IRES GFP was generated by isolating the IRES GFP cassette from MSCV IRES GFP (36) digested with *Xho* I and *Sal* I. This cassette was then ligated into pcDNA3.1(+) digested with *Xho* I. pcDNA3.1(+) HA-HLA-A*0201 IRES GFP and pcDNA3.1(+) HA-HLA-Cw*0401 were generated by isolating HA-HLA-A*0201 or HA-HLA-Cw*0401 from MSCV 2.1 HA-HLA-A*0201 or MSCV2.1 HA-HLA-Cw*0401 as described below. MSCV 2.1 HA-HLA-A*0201 or MSCV 2.1 HA-HLA-Cw*0401 were digested with *Eco*RI, filled in using Klenow, and then digested with *Xho* I. These fragments were then ligated into pcDNA3.1(+) IRES GFP digested with *Eco*RV and *Xho* I.

The MSCV 2.1 HA-A2/Cw4 point mutations N₃₂₇D, E₃₃₄V, I₃₃₇T, D₃₃₃A, SSAA, S₃₃₅E and MSCV 2.1 HA-A*0201 T₃₃₇I were introduced using PCR. The 5' primer for all the constructs was 5'-CGACCGCCTCGATCCTCC-3'. The 3' primers used are as follows:
N₃₂₇D 5'-CCGCTCGAGTCAGGCTTACAAGCGATGAGAGACTCA

TCAGAGCCCTGGGCACTGTCGCTGGACGC-3', E₃₃₄V 5'-CCGCTCGAGTCAGGCTTTACA
AGCGATGAGAGATACATCAGAGCCCTG-3', I₃₃₇T 5'-CGGCTCGAGCTGTCAGGCTTTACAA
GCTGTGAGAGACTC-3', D₃₃₃A 5'-GCCCTCGAGTCAGGCTTTACAAGCGATGAGAGACTC
TGCAGAGCCCTGGGCACTGTTGCTGGA-3', SSAA 5'-CCGCTCGAGCGGTCAGGCTT
TACAAGCGATGAGTGCCTCATCTGCGCC-3' and S₃₃₅E 5'-CCGCTCGAGCG
GTCAGGCTTTACAAGCGATGAGCTCCT CATCAGA-3'. The template for all the PCR
reactions was MSCV 2.1 HA-A2/Cw4. The resulting PCR product was digested with
EcoR I and *Xho* I and ligated into MSCV 2.1 digested with the same enzymes. The
C320Y point mutation was generated using a two round PCR mutagenesis approach. The
first round PCR consisted of two reactions; reaction 1 contained the primers 5'-
CGACCGCCTCGATCCTCC-3' and 5'-AGCCTGAGAGTAGCTCCCTCC-3', and reaction 2
contained the primers 5'-GGAGGGAGCTACTCTCAGGCT-3' and 5'-
CCGCTCGAGTCAGGGTTTACAAGCGATGAGAGA-3'. The template for both reactions was
MSCV 2.1 HA-A2/Cw4. The second round PCR reaction contained primers 5'-
CGACCGCCTCGATCCTCC-3' and 5'- CCGCTCGAGTCAGGGTTTACAAGCGATGAGAGA-3'.
The template for this reaction was one microliter from each of the round one PCR
reactions. The resulting PCR product was digested with *EcoR* I and *Xho* I and ligated
into MSCV 2.1 digested with the same enzymes.

Cell lines

THP-1 and U937 macrophage cell lines were obtained from the ATCC. THP-1 cells
were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 10 mM HEPES, 1

mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10% fetal bovine serum, 0.05 mM beta-mercaptoethanol, and 2mM penicillin, streptomycin and glutamine. U937 and CEM cells were cultured with RPMI 1640 supplemented with 10% fetal bovine serum, 10 mM HEPES, and 2 mM penicillin, streptomycin and glutamine (R10). Cell lines expressing various MHC-I molecules were generated using murine retroviral vectors as previously described (25, 37) except that they were pseudotyped with pCMV VSV-G (Dr. Nancy Hopkins, MIT). 1×10^6 cells were spin infected with the retroviral supernatants by centrifuging at 2500 RPM in a table top centrifuge for two hours with $8 \mu\text{g/ml}$ polybrene. The cells were then selected with neomycin (1mg/ml).

PBMC isolation and electroporation

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque centrifugation. Following isolation they were stimulated with $10 \mu\text{g/ml}$ PHA (Sigma); 24 hours later interleukin (IL)-2 was added at 50 units/ml, and fresh IL-2 was added after 3 days. Five days after isolation, 5×10^6 stimulated PBMCs were electroporated using the Amaxa Nucleofector system. Electroporations were performed according to the manufacturer's protocol except that following electroporation the cells were placed in $500 \mu\text{l}$ of media in 1.5ml eppendorf tubes and incubated for 10 minutes at 37°C before being placed in a 12-well dish.

Macrophage Differentiation Buffy coats provided by the New York Blood Center were purified over a ficoll gradient. CD14^+ mononuclear cells were prepared from the ficoll-purified PBMCs. To assess MHC-I expression levels, freshly purified, undifferentiated

cells were pre-incubated with 10% Fc Block (Accurate) in FACS buffer (10mM Hepes, 2% fetal bovine serum, 1% human serum, 0.02% azide) for 20 min on ice and then stained with antibodies directed against HLA-A2 (BB7.2), HLA-C (L31, gift of Patrizio Giacomini, Regina Elena Cancer Institute, Italy), Bw4 (One Lambda) and Bw6 (One Lambda), depending on the donors MHC-I phenotype. The cells were also stained with matched, isotype control antibodies (protein A purified IgG2b for BB7.2, IgG1 ascities for L31 and IgM for anti-Bw4 and anti-Bw6.). For staining with L31, a citrate-phosphate buffer (pH 3.0) was used to release β_2 microglobulin and expose the epitope as described previously (5, 12). To induce maturation, the CD14⁺ cells were plated at 1×10^6 /ml in R10 plus GM-CSF for five days. The cells were then harvested and stained again with anti-MHC-I antibodies as described above.

For differentiation of monocytic cell lines, one million THP-1 or U937 cells were treated with lipopolysaccharide (LPS, 100 ng/mL for THP1 and 10ng/ml for U937) solubilized in DMSO in 1 mL of media in a 24-well plate. Twenty four hours later, another 1 mL of media was added containing phorbol myristal acetate (PMA, 200 ng/mL for THP1 and 10ng/ml for U937) and LPS. After 72 hours at 37°C, cells were harvested by treatment with cell dissociation solution (Sigma) for 20 min at 37°C.

Western blot analysis

Cells were lysed in PBS 0.3% CHAPS, 0.1% SDS pH 8, 1mM PMSF. They were then normalized for total protein and separated by SDS-PAGE. Western blot analysis was

performed with the following antibodies: HA (HA.11, Covance, 1:5,000), and rat anti-mouse-HRP (Zymed, 1:25,000).

Immunofluorescence microscopy

CEM T cells were prepared for immunofluorescence microscopy as previously described (28) except that they were permeabilized with 0.1% digitonin (Wako Chemicals USA) diluted in D-PBS with calcium and magnesium and cells were blocked with equal parts wash buffer and F_c receptor blocker (Accurate Chemical and Scientific Corp). To identify acidic compartments, LysoTracker (Molecular Probes) was diluted 1:150 in culture media and incubated with cells for 5 minutes at 37°C. To identify cell surface staining, concanavalin A conjugated to Alexa Fluor 488 (Molecular Probes) was diluted to 40µg/ml and incubated with cells for 5 minutes on ice. HA-tagged molecules and KDEL were stained using mouse monoclonal antibodies (HA.11, 1:50, Covance, and anti-KDEL, 1:200, Stressgen, respectively) followed by staining with appropriate secondary antibodies (goat anti-mouse IgG₁ Alexa Fluor 488, and goat anti-mouse IgG_{2a} Alexa Fluor 546, 1:250, Molecular Probes). Images were collected using a Zeiss LSM 510 confocal microscope and processed with Adobe Photoshop software.

Flow cytometry.

Stains were performed as previously described (33) using an anti-HA antibody (HA.11, 1:50, Covance) or an anti-HLA-A2 antibody (BB7.2 (24)) and goat-anti-mouse-phycoerythrin (1:250, Biosource). FACS analysis of the THP-1 and U937 cells was the

same except the cells were incubated with Fc receptor blocker (Accurate Chemical and Scientific Corp.) for 20 min at 4°C prior to the anti-HA antibody incubation.

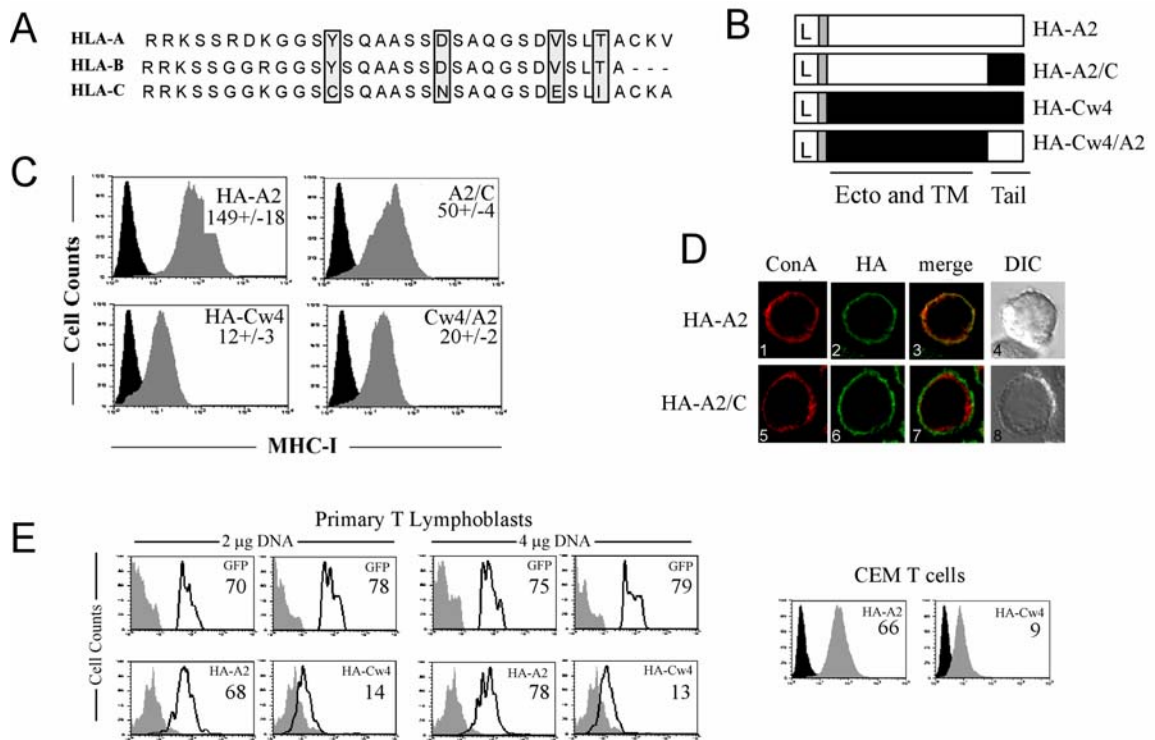
Transport, internalization and metabolic labeling assays.

The transport and endocytosis assays were performed essentially as previously described (15) except that an antibody directed against the HA tag (HA.11, Covance) was used. For metabolic labeling, fifteen million CEM T cells were pulse labeled for 15 minutes with [³⁵S]-methionine and cysteine. For inhibitor studies a third of the sample was harvested after the pulse while the remaining cells were then chased for 12 hours in either RPMI with DMSO or 100nM bafilomycin A (Sigma). Lysates were generated in PBS 0.3% CHAPS, 0.1% SDS pH 8, 1mM PMSF and precleared over night. They were immunoprecipitated for two hours with an antibody against HA (HA.11, Covance) and washed three times in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS). The immunoprecipitates were then eluted by boiling in 10% SDS, reprecipitated with an antibody against HA, and washed three times in RIPA buffer. The final immunoprecipitates were then separated by SDS-PAGE.

Recycling assays. A measurement of the rate of recycling of internalized molecules was performed as described previously (18), except that washes were performed at room temperature to avoid inhibiting recycling with cold temperature (7). Briefly, cells were incubated with 150µg/ml cycloheximide (Sigma) for 2-3 hours in RPMI plus 10% serum. Then cells were harvested and an aliquot was removed and placed on ice. The remainder of the samples were stripped of stainable HLA-A2 by washing in 50 mM glycine, 100 mM NaCl, (pH 3.4), twice, at room temperature for one minute.. The stripped cells were then washed in PBS and incubated at 37°C, 5% CO₂ in media without serum for the indicated period of time, in the presence of 150 µg/ml cycloheximide. (Samples were

incubated without serum to avoid substitution of bovine β_2 -microglobulin present in serum for the human β_2 -microglobulin removed with the stripping protocol.) Cells were then placed on ice and stained for HLA-A2 with BB7.2, using wash buffers that included BSA instead of serum.

Figure 3-1. The extracellular and cytoplasmic tail domains of HLA-C influence surface expression. (A). The cytoplasmic tail sequences of HLA-A, HLA-B and HLA-C molecules. Amino acid differences unique to HLA-C are boxed. (B) Schematic diagram of HA-tagged MHC-I molecules. Open boxes represent HLA-A*0201 sequences, black boxes represent HLA-Cw*0401 sequences, the gray box represents the HA-tag and L indicates the position of the leader sequence. (C) Cell surface expression of HA-A2, HA-Cw4, HA-A2/C and HA-Cw4/A. CEM T cell lines were generated expressing the indicated MHC-I molecules. The cells were stained with an antibody directed against the HA-tag and analyzed by flow cytometry. The filled gray curve represents the expression of the indicated molecules while the filled black curve represents control cells stained with the HA antibody. The mean fluorescence intensity (MFI) +/- standard deviation (SD) from 6 experiments is shown in the upper right hand corner. (D) Subcellular distribution of MHC-I molecules. The indicated CEM T cell lines were incubated with concanavilin A (ConA) to label the cell surface (green). They were then fixed, permeabilized, and stained with HA to examine MHC-I localization (red). Merge panels represents the product of these two overlapping images in which regions of overlap are highlighted in yellow. All images were collected on a Zeiss LSM 510 confocal microscope and single z-sections are displayed. (E) Cell surface expression of HA-A2 and HA-Cw4 in primary T cells. PBMCs were electroporated with a bi-cistronic GFP-expressing construct as described in Materials and Methods. Twenty-four hours post electroporation PBMCs were stained using an antibody directed against HA and analyzed by flow cytometry. Cells with equal GFP expression were selected and their GFP expression is shown in the upper panels. The corresponding HA stain is shown in the panels below. In the lower panels, the filled curve is the background staining on mock-transfected cells and the black line represents HA staining for cells transfected with the indicated construct. As a control, stable CEM T cells expressing the same molecules were also stained and are shown in the panels on the right.



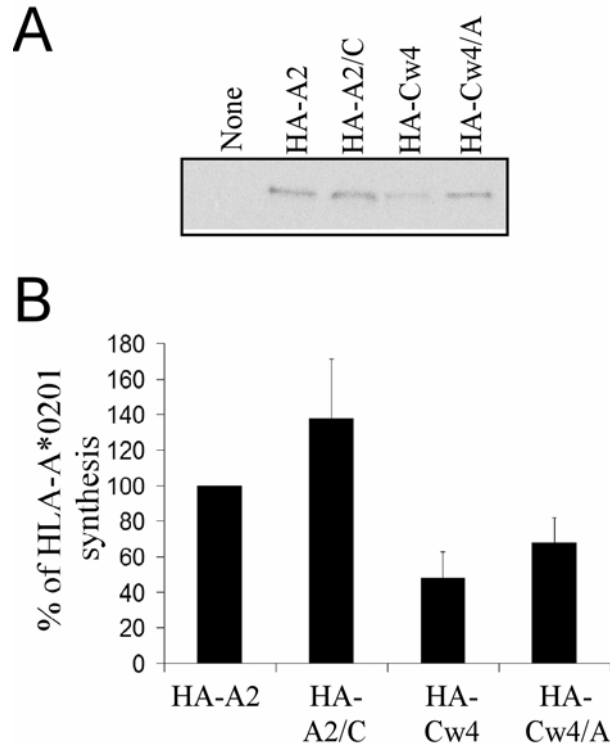


Figure 3-2. (A) Expression of HA-tagged chimeric MHC-I molecules. CEM T cells (negative control) or CEM T cells expressing the indicated MHC-I were pulse labeled with [³⁵S]-methionine and cysteine for 15 minutes. Lysates from these samples were immunoprecipitated with an antibody against HA, and separated by SDS-PAGE. (B) Quantitation of pulse labeling. A phosphorimager and ImageQuant software were used to quantify the samples. Values were corrected for differences in methionine and cysteine content. The counts from the HLA-A*0201 sample were set to 100% and the values from other samples were expressed relative to it. The results are displayed as the means +/- SD from 3 experiments.

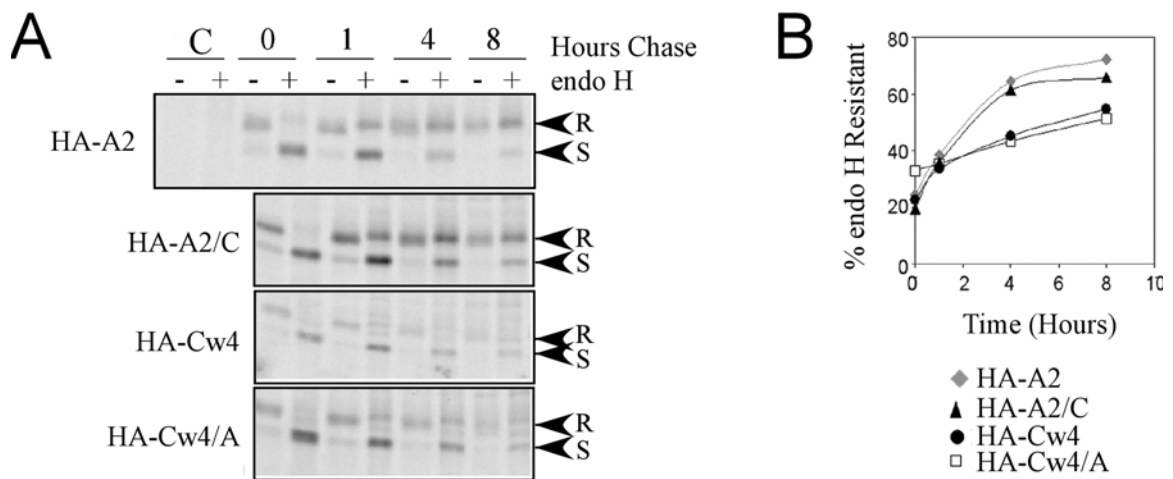
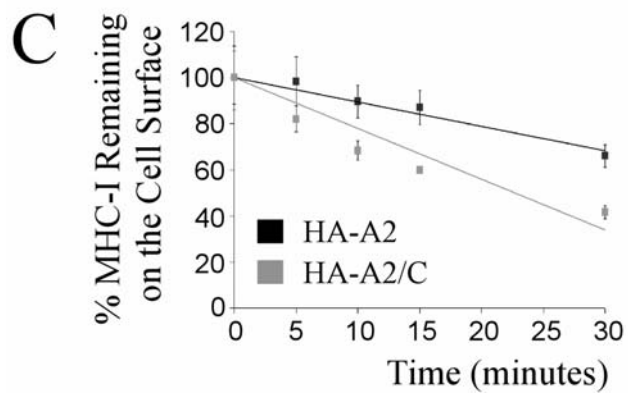
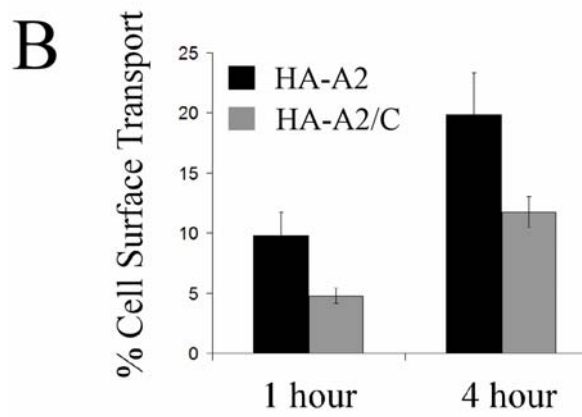
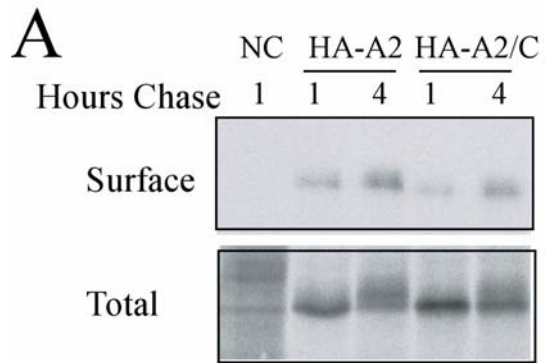


Figure 3-3. The HLA-C tail does not affect transport from the ER into the Golgi apparatus. (A and B) CEM T cells (negative control) or CEM T cells expressing the indicated MHC-I were pulse labeled with [³⁵S]-methionine and cysteine for 15 minutes, chased for the indicated time period and harvested. Lysates were immunoprecipitated with an antibody against HA, treated with endo H, and separated by SDS-PAGE. “C” indicates control cells that do not express the HA tag. (B) A phosphorimager and ImageQuant software were used to quantify the gel shown in part A. The percentage of molecules that were resistant to endo H digestion over time were calculated as follows: $[\frac{\text{endo H resistant}}{\text{endo H resistant} + \text{endo H sensitive}}] \times 100$.

Figure 3-4. The HLA-C tail disrupts protein transport and accelerates internalization from the cell surface. (A and B) CEM T cells (negative control) or CEM T cells expressing the indicated MHC-I were pulse labeled with [³⁵S]-methionine and cysteine. They were then chased for one or four hours in biotin. Lysates from these samples were immunoprecipitated with an antibody against HA and one-third of the immunoprecipitate was analyzed by SDS-PAGE (total). The remaining two-thirds was reprecipitated with avidin-agarose and then analyzed by SDS-PAGE (surface). (B) Quantitation of cell surface transport. A phosphorimager and ImageQuant software were used to quantify recovered protein. The percentage of MHC-I molecules that had reached the cell surface at the indicated time point was calculated as follows: $[(\text{surface MHC-I})/(\text{Total MHC-I} \times 2)] \times 100$. The mean from two experiments +/- standard deviation is shown. (C) A2/C is internalized at an accelerated rate. CEM T cells stably expressing the indicated protein were incubated on ice with the anti-HLA-A2 antibody, BB7.2. The cells were shifted to 37°C for the indicated time and then stained with a secondary antibody to determine the percentage of HA-A2 (black square) or HA-A2/C (gray square) remaining on the cell surface over time. The mean +/- standard deviation for an experiment performed in quadruplicate is shown.



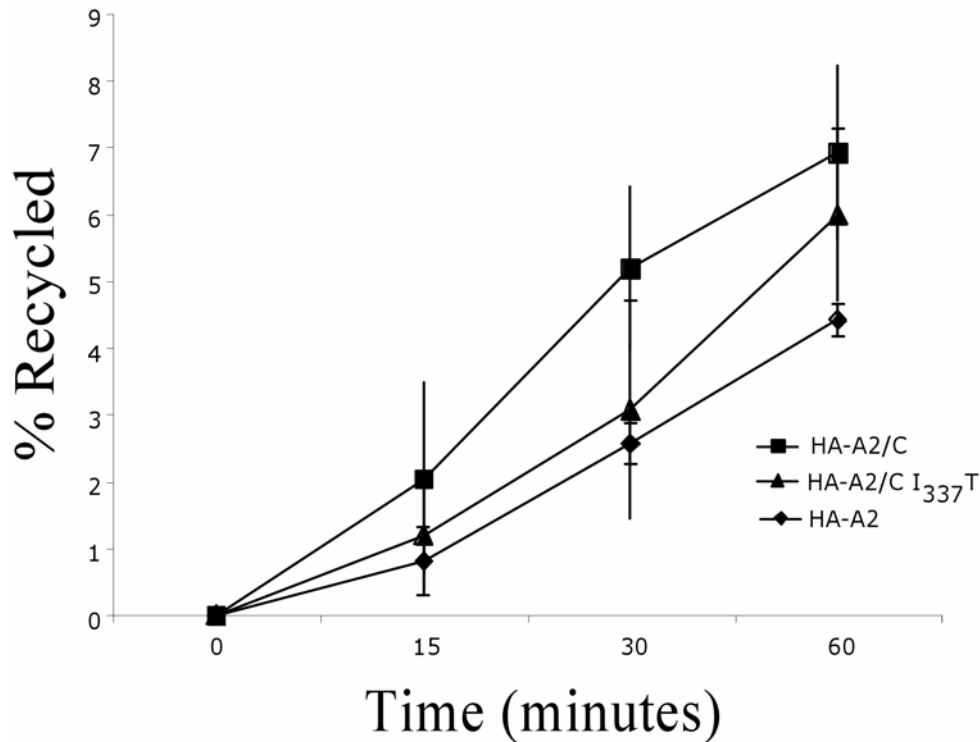
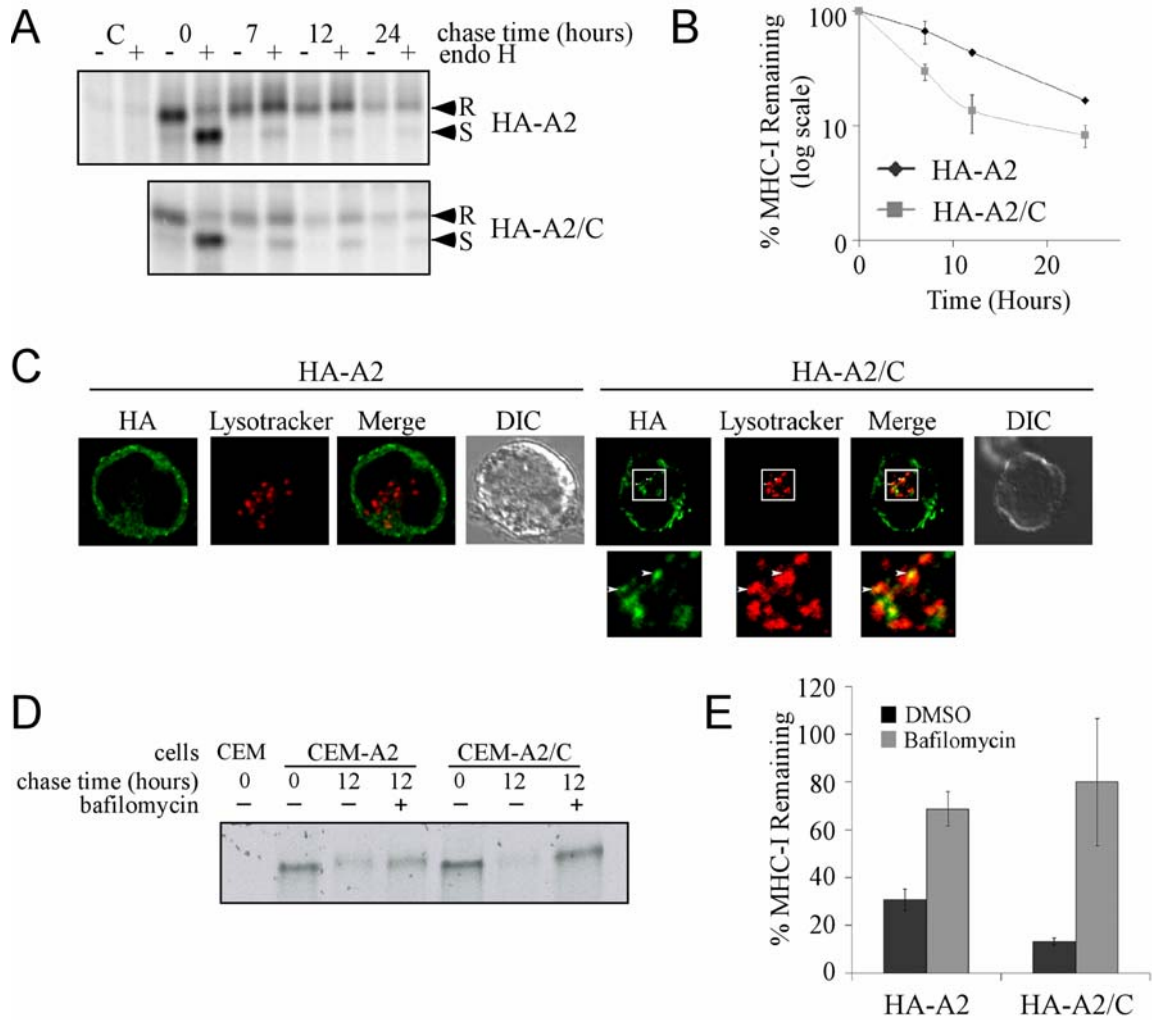


Figure 3-5. The cytoplasmic tail of HLA-C does not inhibit recycling. The rates of HA-A2/C, HA-A2/C I337T and HA-A2 recycling in CEM T cells were measured using a FACS based assay. The indicated CEM cell lines were incubated in 150 μ g/mL cyclohexamide for 2-3 hours. An aliquot of cells was set aside on ice to represent steady state cell surface expression of the indicated MHC-I. The stainable MHC-I was then stripped off the surface with a 50mM glycine 100mM NaCl (pH 3.4) buffer. The stripped cells were washed and incubated at 37°C with media without serum in the presence of cyclohexamide for the indicated period of time. The cells were then placed on ice and stained for MHC-I using the BB7.2 antibody. The percent recycled was calculated by ((steady state mean)/(time point mean))*100. The mean +/- standard deviation for an experiment performed in quadruplicate is shown.

Figure 3-6. The HLA-C cytoplasmic tail promotes lysosomal targeting. (A and B) CEM T cells (control) or CEM T cells expressing the indicated MHC-I molecule were pulse labeled with [³⁵S]-methionine and cysteine for 15 minutes, and chased for the indicated time. Lysates from these samples were immunoprecipitated with an anti-HA antibody as in figure 3-3A. (B) Quantitation of pulse-labeling. A phosphorimager and ImageQuant software was used to determine the amount of MHC-I at each time point. The amount at time 0 was set to 100% and the percentage remaining was then calculated for each time point. The mean +/- standard deviation from two experiments is shown. (C) HA-A2/C is directed to acidic compartments. The localization of the indicated MHC-I was compared to Lysotracker, which marks acidic compartments. The merge panel represents the overlap of MHC-I with the respective counterstain. A portion of the AHA-A2/C images with Lysotracker were enlarged (boxed area) and shown below the original images. Areas of co-localization are indicated by arrows. The images were collected using a Zeiss 510 LSM and single z-sections are displayed. (D and E) Bafilomycin stabilizes the degradation of HA-A2/C. Pulse-chase analysis was performed as in (A) except that the cells were chased in either 100nM bafilomycin A or solvent (DMSO). (E) A phosphorimager and ImageQuant software was used to determine the percentage of MHC-I remaining [(value following chase/initial value) x100]. The mean +/- standard deviation is shown for three experiments.



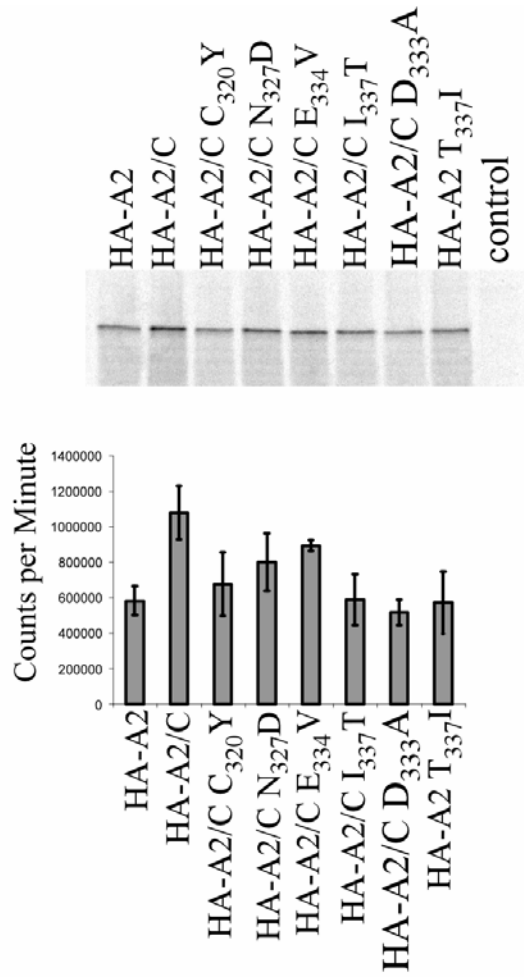
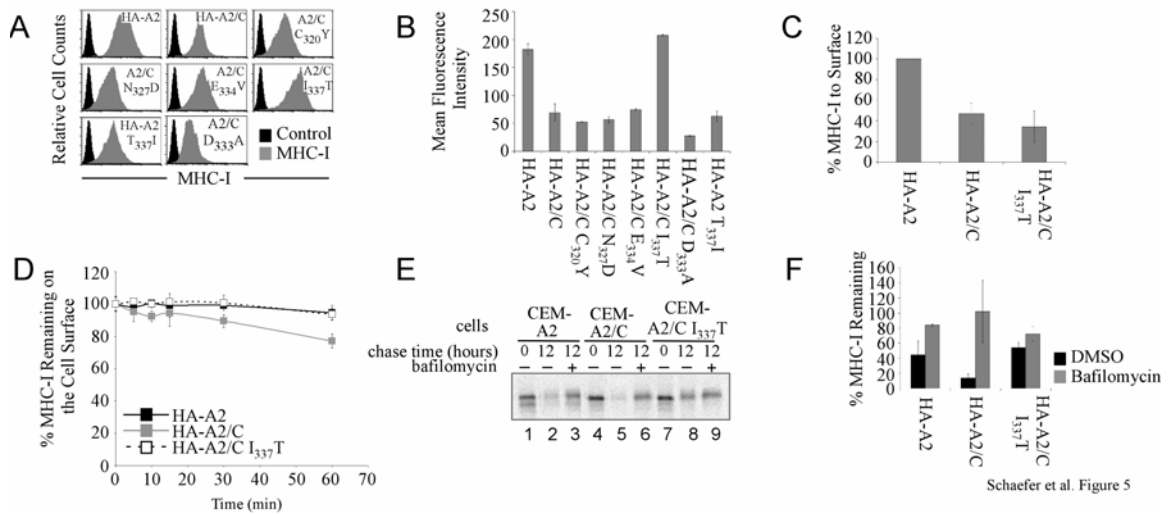


Figure 3-7. Expression of A2/C cytoplasmic tail point mutations. CEM T cells (negative control) or CEM T cells expressing the indicated MHC-I were pulse-labeled as described in Figure 3-1, and analyzed by SDS PAGE. Bands were quantified using a phosphorimager and ImageQuant software. The results are displayed as the means +/- SD from three experiments.



Schaefer et al. Figure 5

Figure 3-8. Isoleucine at position 337 (I₃₃₇) is required for accelerated internalization and lysosomal targeting. (A and B) I₃₃₇ is necessary for reduced steady state cell surface expression of HA-A2/C. CEM T cell lines were generated expressing the indicated MHC-I. (A) The cells were stained with an antibody directed against the HA-tag and analyzed by flow cytometry. The filled gray curve represents the expression of the indicated molecule while the filled black curve represents untagged cells stained with the HA antibody. (B) Quantitation of the MFI of the HA-A2/C cytoplasmic tail mutants. The results are depicted as MFI +/- SD from three experiments. (C) I₃₃₇ is not required for slow export of HA-A2/C. A transport assay was performed as in Figure 3-4A, except cells were chased in biotin for only 1hr. The transport assay was quantified as in Figure 3-4B, except that percent of HA-A2 transported to the cell surface was set to 100%. The mean +/- standard deviation is shown for two experiments. (D) I₃₃₇ is necessary for accelerated internalization of HA-A2/C. A FACS-based assay was used as in Figure 3-4C to determine the percentage of each molecule remaining on the cell surface over time. The mean +/- SD for an experiment performed in triplicate is shown. (E and F) I₃₃₇ is necessary for lysosomal targeting of HA-A2/C. A pulse chase was performed as in Figure 3-6D and was quantified as in Figure 3-6E. The mean +/- SD is shown for two experiments.

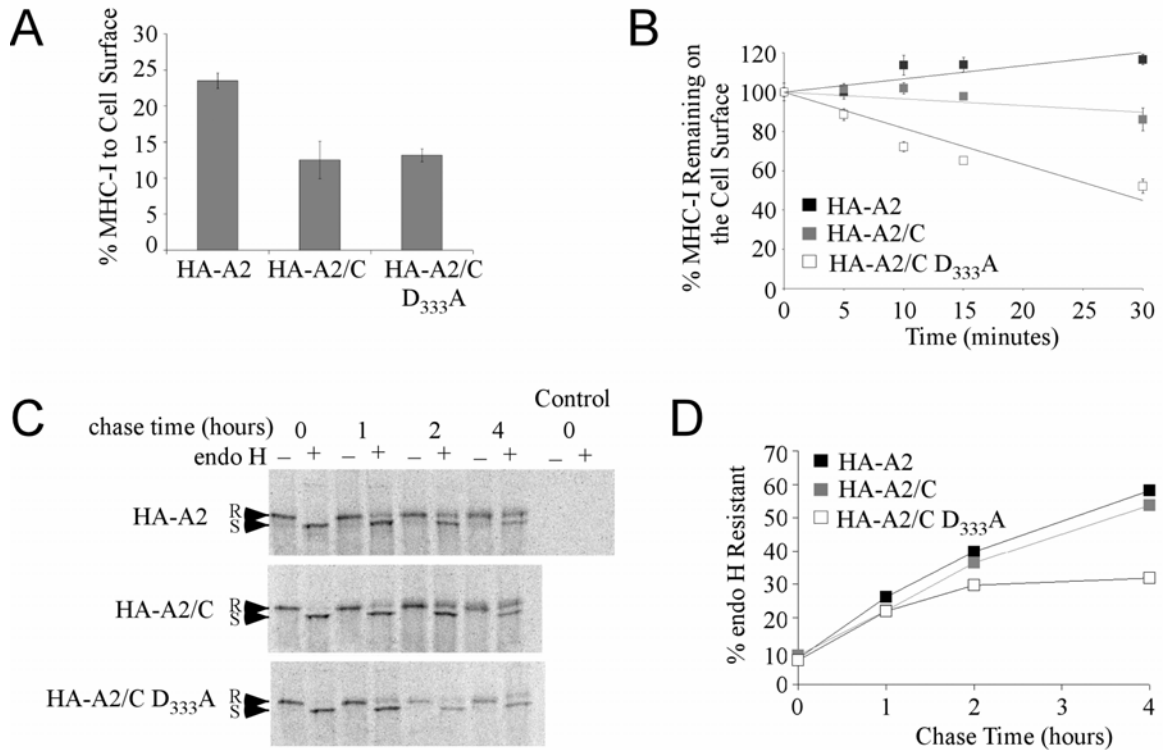


Figure 3-9. Aspartic acid at position 333 (D₃₃₃) in the HLA-C cytoplasmic tail attenuates the internalization and lysosomal targeting signal. (A) D₃₃₃ is not needed for reduced HA-A2/C cell surface transport rate. CEM T cells expressing the indicated protein were utilized for a transport assay performed as described in Figure 3-4A and quantified as in Figure 3-4B. (B) Substitution of D₃₃₃ accelerates internalization. The rate of internalization was determined using CEM T cells stably expressing the indicated mutant. A FACS based assay was used as in Figure 3-4C to determine the percentage of MHC-I remaining of the cell surface over time. The mean +/- SD for an experiment performed in triplicate is shown. (C and D) Substitution of D₃₃₃ enhances lysosomal targeting. To assess the degradation rate of each construct A pulse chase was performed using CEM T cells expressing the indicated mutant as described in Figure 3-3A and quantified as in Figure 3-3B.

Figure 3-10. Macrophage differentiation upregulates HLA-C and HA-A2/C. (A) Upregulation of HLA-C upon differentiation of primary CD14⁺ cells into macrophages. CD14⁺ mononuclear cells were isolated from the blood of a normal donor. A subset of the cells were stained immediately (untreated) with anti-HLA-C (L31), an antibody against a subset of HLA-B allotypes (HLA-Bw6) expressed by this donor or isotype control antibodies. The remainder of the cells were cultured for seven days in GM-CSF, harvested and stained with the same antibodies. (B) Upregulation of HA-Cw4 and HA-A2/C with differentiation of monocytic cell lines into macrophage-like cells. U937 (left panels) and THP1 (right panels) cell lines were derived as described in Material and Methods. Cell surface expression was determined using an antibody to HA for undifferentiated (DMSO) and differentiated (LPS/PMA) by FACS. (C) Macrophage differentiation stabilizes the endo H resistant form of HA-A2/C and HA-Cw4. U937 and THP1 cell lines were treated with LPS and PMA as in (B). Lysates were generated, digested with endo H, and analyzed by western blot with an antibody directed at HA. The endoH resistant band is indicated by “R” and the endoH sensitive band is indicated by “S”. (D) Macrophage differentiation inhibits internalization of HA-A2/C. The FACS-based internalization assay described in Figure 3-4C was used to determine the percentage of HA-A2 or HA-A2/C remaining on the cell surface over time in undifferentiated (DMSO) or differentiated (LPS/PMA) U937 cell lines. For these experiments an HLA-A2-specific monoclonal antibody (BB7.2) was used. The mean +/- standard deviation for an experiment performed in quadruplicate is shown.

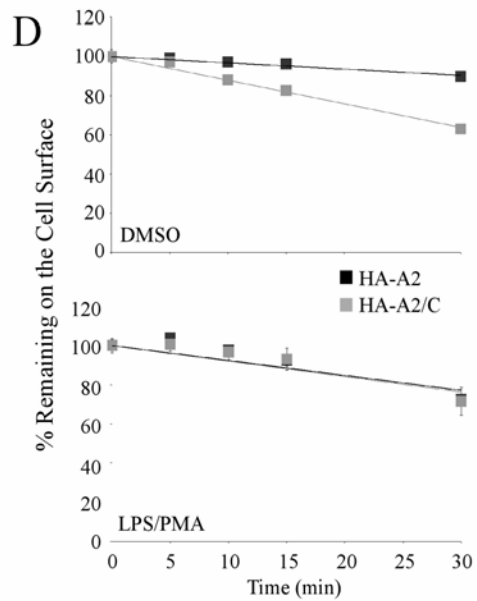
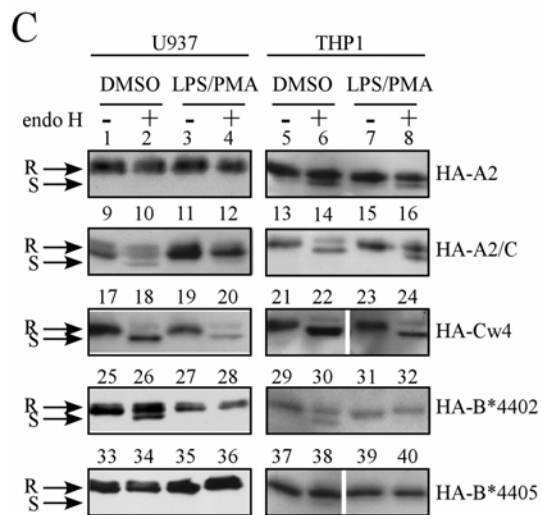
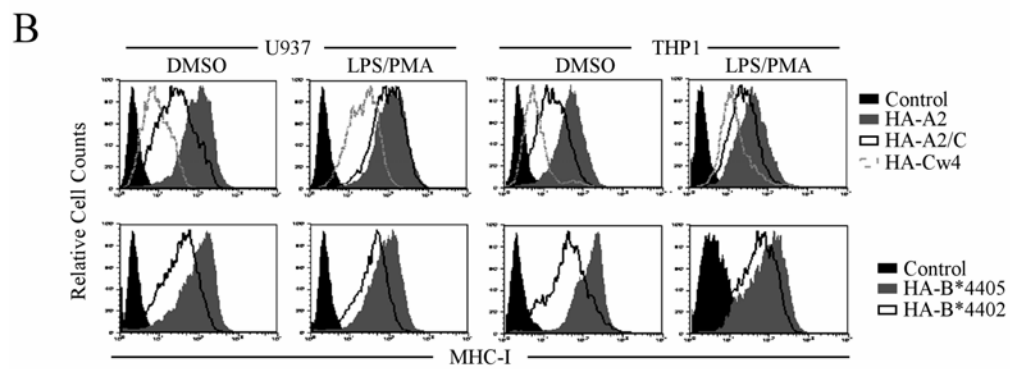
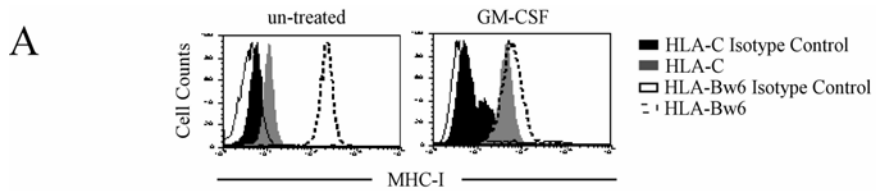
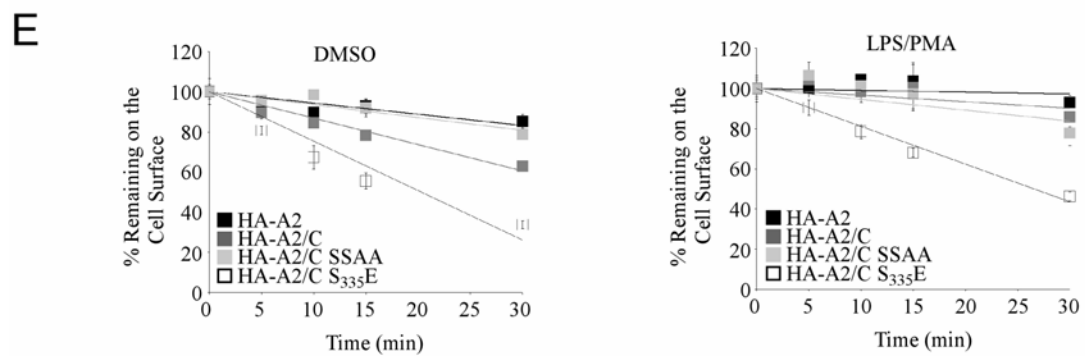
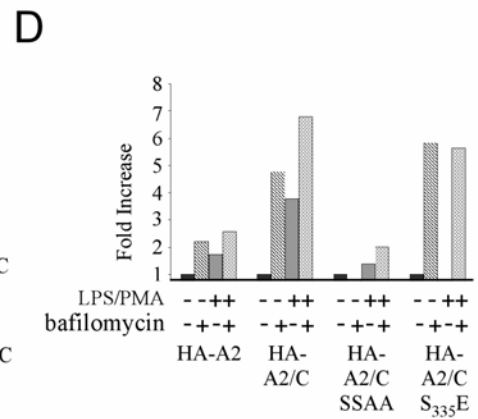
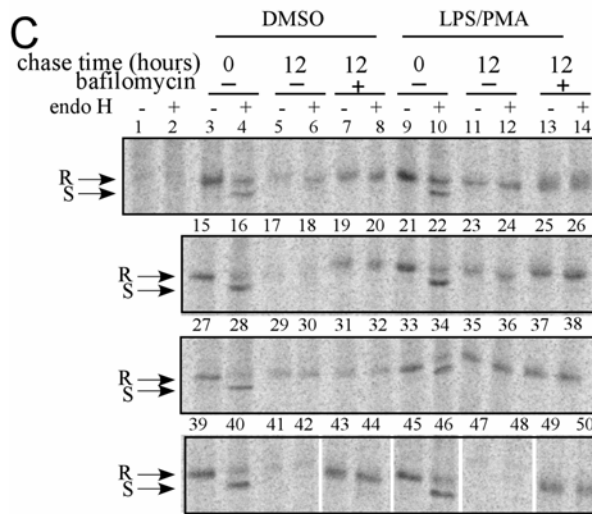
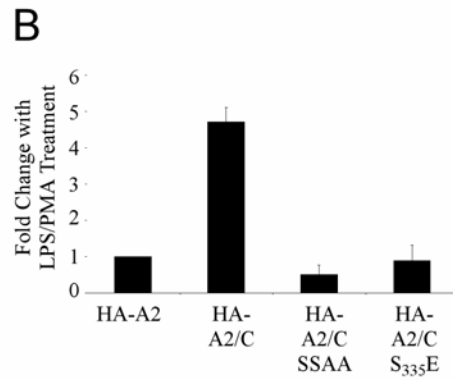
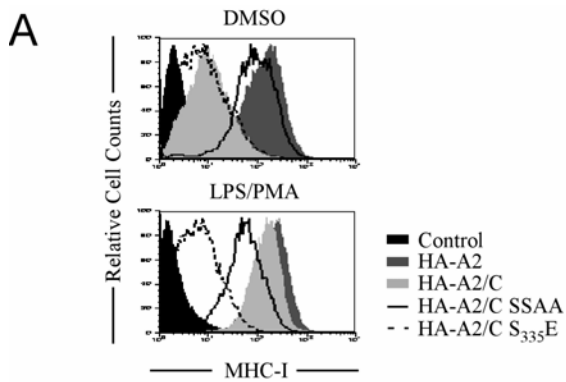


Figure 3-11. Serine 335 (S₃₃₅) regulates HLA-Cw*0401 expression in macrophage cell lines. (A and B) The serine residues in the HLA-C cytoplasmic tail at positions 332 and 335 were substituted with alanine (HA-A2/C SSAA) and the serine at position 335 was changed with a glutamic acid residue (HA-A2/C S₃₃₅E). U937 cell lines were generated as previously described and cell surface expression was determined using the anti-HLA-A2 antibody, BB7.2, for undifferentiated (DMSO) and differentiated (LPS/PMA) cells by FACS. (B) The fold increase in MFI relative to HA-A2 is shown. The mean +/- standard deviation for three experiments is shown. (C and D) Macrophage differentiation inhibits lysosomal targeting of HA-A2/C through hypophosphorylation of S₃₃₅. The indicated U937 cell lines were pulse-labeled with ³⁵S and chased for 12 hours plus or minus bafilomycin as in Figure 3-6D. The indicated protein was immunoprecipitated with BB7.2 and separated by SDS PAGE. White lines indicate places where the gel image was cropped and rearranged to place samples in a consistent order relative to one another. (D) A phosphorimager and ImageQuant software were used to quantify each band. Fold stabilization was calculated as follows: [% remaining for each condition/ % remaining for the control]. (% Remaining was calculated as in Figure 3-3E). (E) Macrophage differentiation inhibits internalization of HA-A2/C through hypophosphorylation of S₃₃₅. An internalization assay performed as described in Figure 3-3C was used to determine the percentage of the indicated MHC-I molecules remaining on the cell surface over time in DMSO versus LPS/PMA-treated U937 cells. The anti-HLA-A2 monoclonal antibody BB7.2 was used to detect surface expression in these experiments. The mean +/- standard deviation for an experiment performed in triplicate is shown.



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Chapter IV

Discussion

Significant Conclusions

The findings presented here provide a more complete model of the mechanism used by HIV-1 Nef to target MHC-I and CD4 for degradation. If new and useful treatments to inhibit HIV are to be designed, a complete understanding of the pathways and mechanisms used by HIV proteins is essential. In addition, these studies shed light on the role of HLA-C in CD4⁺ T cells and macrophages. These findings are significant in that HLA-C cell surface expression has been shown to be important for HIV immune evasion and little is known about HLA-C biology in these cell types. The most noteworthy contributions are summarized below and will be discussed in detail in this chapter.

- i) The cytoplasmic tail domain of MHC-I and CD4 determines the pathway by which HIV-1 Nef alters intracellular trafficking.
- ii) HIV-1 Nef requires β -COP to disrupt MHC-I cell surface expression, and to target MHC-I and CD4 for degradation.

iii) HIV-1 Nef requires the N-terminal alpha helical domain and oligomerization domain to recruit β -COP.

iv) Low cell surface expression of HLA-C is due to effects of both the extracellular domain and the cytoplasmic tail domain; the extracellular domain retains HLA-C in the ER, while the cytoplasmic tail domain promotes accelerated internalization and degradation in lysosomal compartments.

v) HLA-C cell surface expression increases upon macrophage differentiation.

vi) HLA-C internalization and degradation are regulated by serine 335.

HIV-1 Nef targets MHC-I and CD4 for degradation via a β -COP dependent pathway

Nef reduces the cell surface levels of both MHC-I and CD4 and targets them for degradation but does so by seemingly different mechanisms. Evidence is presented here that indicates the mechanism utilized by Nef is determined by the cytoplasmic tail amino acid sequence of the affected molecule. Interestingly, the data presented here demonstrate that the different pathways utilized by Nef converge in the endosomal network and β -COP is used to target MHC-I and CD4 for degradation. Additionally, the β -COP/Nef interaction was shown to require the N-terminal alpha helical domain and the

oligomerization domain (D₁₂₃). These data have allowed us to provide a model that unifies two functions of the HIV Nef protein.

The MHC-I and CD4 degradation pathways converge

The finding that the seemingly disparate MHC-I and CD4 downmodulation and degradation pathways converge is noteworthy because it demonstrates that Nef utilizes the same pathway to target MHC-I and CD4 for degradation even though the mechanisms to reduce cell surface expression are different, MHC-I is redirected from the TGN to the endosomal network via AP-1 (33), whereas CD4 is subjected to accelerated internalization and directed to the endosomal network (reviewed in (32)). Confocal microscopy indicated HLA-A2 and CD4 co-localized in Rab7+ vesicles and immunogold labeling showed HLA-A2 and CD4 together in the same multivesicular body. Since these two mechanisms start very differently, the demonstration that they converge in the same vesicles is a significant finding because it demonstrates these two different mechanisms of Nef are combined at this intracellular location. This finding also suggests that Nef could target other host cellular factors for degradation through this mechanism. In addition to CD4 and MHC-I, Nef has been reported to affect other host cellular molecules. For example, Nef has been reported to promote increased internalization of CD1d, CD28, CD80, CD86, and CD8 $\alpha\beta$ (8, 11, 35, 36). Interestingly, these investigations have suggested Nef promotes the internalization of these molecules through different mechanisms. CD28, CD8 $\alpha\beta$ and CD1d are thought to be internalized through an AP-2 dependent mechanism (10, 35, 36), while CD80 and CD86 are thought

to be internalized via Rac-based actin polymerization (9). Perhaps these Nef mediated pathways also converge in Rab7+ vesicles.

β-COP is Required for MHC-I and CD4 degradation

Recent reports have demonstrated that after Nef redirects MHC-I from the TGN to the endosomal network, it then targets MHC-I for degradation in lysosomal compartments (33). However, it was unknown which cellular factors Nef recruited and the pathway utilized by Nef to target MHC-I and Nef to the lysosomes. Since MHC-I and CD4 were shown to co-localize in Rab7+ vesicles and since β-COP had already been postulated to be involved in the CD4 degradation pathway, it was hypothesized that β-COP was part of the MHC-I degradation pathway. Using RNA interference, it was demonstrated that β-COP was required for disruption of MHC-I cell surface expression as assessed by FACS analysis, degradation as assessed by western blot and targeting of MHC-I to Lamp1+ vesicles as assessed by confocal microscopy. Identification of β-COP as a host cellular factor required for Nef mediated MHC-I degradation is significant in that it furthers our understanding of how Nef disrupts MHC-I trafficking and antigen presentation. Also, since β-COP has been identified as part of the CD4 degradation pathway and since Nef affects several other host cellular molecules, these data hint that β-COP could also be involved their degradation pathways. If this hypothesis is true, it would be noteworthy because it would indicate that several known Nef mediated degradation pathways traffic into the endosomal network and require β-COP to target for degradation. If these molecules all share a common degradation pathway, this pathway is a potential target for

novel antiviral therapies. A complete understanding of how Nef commandeers intracellular trafficking pathways to disrupt antigen presentation and the immune response is crucial if we are to develop new antiviral therapies and vaccine strategies.

Additionally, using RNA interference the role of β -COP in the CD4 degradation pathway was confirmed. The first reports to suggest β -COP played a role in the CD4 degradation used a Nef diglutamic acid mutant that was deficient in CD4 downmodulation (31). However, subsequent studies were unable to confirm the role of the Nef diglutamic acid motif in CD4 downmodulation (23) and subsequently, the role of β -COP in CD4 degradation was questioned. These results provide the necessary confirmation for the role of β -COP in CD4 degradation.

Nef requires the oligomerization and alpha helical domains to interact with β -COP

Nef was first reported to interact with β -COP through yeast two-hybrid experiments (4) and data presented here were able to confirm that endogenous β -COP and Nef interact in T cells. This result is noteworthy in that it was generated using endogenous β -COP, not an overexpressed protein as is often used for interaction studies. Additionally, it was performed in T cells, the natural target of HIV infection, as opposed to more easily manipulated cell types where interaction experiments are often performed, such as HeLa cells or 293 cells.

Further characterization of the β -COP/Nef interaction was performed and the results indicated the oligomerization (D_{123}) and alpha helical domains were necessary for the interaction. The requirement of the dimerization domain suggests two hypotheses, either β -COP directly binds to D_{123} , or this mutation allosterically disrupts the structure of the actual binding site, which is located elsewhere. The involvement of the alpha helical domain is also interesting. This domain includes M_{20} , which has been shown to be important for AP-1 recruitment (33). But this amino acid is not required for β -COP recruitment, as a Nef mutant containing a mutation at M_{20} only interacts with β -COP. The alpha helical domain is an arginine-rich domain of Nef and arginine residues have recently been reported to serve as a β -COP binding motif (37), thus, these arginine residues could serve as the β -COP binding motif in Nef. Alternatively, the alpha helical domain is a large part of the Nef protein and undoubtedly contributes to its overall structure. Generating a mutant without a functional alpha helical domain could have deleterious effects on the Nef protein structure and if the β -COP binding site requires a specific confirmation, it could be disrupted and unable to interact with β -COP.

The involvement of the alpha helical domain was surprising, in that it has not been associated with CD4 degradation. The participation of a Nef domain in the β -COP interaction that is required for MHC-I degradation but not for CD4 degradation suggests Nef has developed two mechanisms to recruit β -COP. The hypothesis that Nef recruits β -COP differently for MHC-I and CD4 is supported by the co-immunoprecipitation experiments presented here in that some residual β -COP binding is seen when the oligomerization and alpha helical domains are mutated individually.

The identification of the Nef domains required for β -COP recruitment is important since the requirements for the β -COP interaction have been controversial. Previous studies have reported Nef recruits β -COP via the diglutamic acid domain (31) but further investigations were unable to confirm the role of this domain in the β -COP interaction (23). This report now demonstrates two domains, the alpha helix and oligomerization domain, are required for the β -COP interaction.

The cytoplasmic tail domain determines how Nef will disrupt intracellular trafficking

Nef commandeers several different trafficking pathways to disrupt intracellular trafficking. Evidence has been provided here to indicate the cytoplasmic tail determines which pathway Nef will utilize to disrupt trafficking. This is demonstrated by the observation that when the HLA-A2 cytoplasmic tail is replaced with the CD4 cytoplasmic tail, HLA-A2 now has accelerated internalization, and does not interact with AP-1. The demonstration that the cytoplasmic tail of the affected molecule dictates which mechanism will be used may seem unimportant because since Nef is always the same the information must be encoded in the affected molecule. But this is not the case. It is possible the extracellular domain could affect Nef responsiveness by binding to other transmembrane proteins or by altering intracellular trafficking. This is especially true for MHC-I, in that the efficiency of peptide loading can affect trafficking and it has been shown that trafficking rate can affect the magnitude to which Nef is able to downmodulate MHC-I.

Implications of this Work

Implications for the HIV field

These findings has allowed us to develop a model that unifies two mechanisms used by Nef to disrupt protein trafficking (Figure 4-1). In this model, Nef binds the MHC-I cytoplasmic tail early in the secretory pathway and redirects MHC-I from the TGN to vesicles containing Rab7 and β -COP via AP-1. Conversely, Nef binds the CD4 cytoplasmic tail at the cell surface and promotes accelerated internalization by recruitment of AP-2 and the vacuolar-ATPase. CD4 is also directed to vesicles containing Rab7 and β -COP. After arrival at the Rab7+ vesicle, Nef recruits β -COP and targets MHC-I and CD4 for degradation.

Previously, β -COP had not been implicated in the MHC-I degradation pathway, therefore, this model represents a more complete understanding of how Nef targets MHC-I for degradation and, ultimately, disrupts antigen presentation. Since disruption of antigen presentation is one of the strategies HIV utilizes to evade the immune response, advancing our knowledge as to how HIV evades the immune system will allow for better design of antiviral drugs and development of vaccine strategies.

Additionally, this study confirmed the role of β -COP in CD4 degradation. Previously, the data implicating β -COP in CD4 degradation was controversial. Now, we can more confidently place β -COP in the pathway Nef exploits to degrade CD4.

Implications for General Biology

This work also further characterizes the role of β -COP in intracellular trafficking, specifically in the endosomal network. These data confirm a role in intracellular trafficking within the endosomal network for β -COP. Additionally, to date, this is the first report of a viral protein commandeering β -COP to target specific proteins for degradation.

Furthermore, these data show that β -COP is not necessary to maintain the structure of the Golgi. Golgi structure was assessed by examining the localization of Giantin, a resident Golgi protein, through confocal microscopy. Treatment with brefeldin A (BFA), a chemical inhibitor of Arf1 known to cause loss of Golgi structure (18), caused mislocalization of Giantin, whereas knockdown of β -COP did not affect the localization of Giantin. Since Arf1 is needed for recruitment of β -COP to membranes (17) it would seem in the BFA treatment that the loss of Giantin localization was not due to the inability of β -COP to associate to membranes, because cells in which β -COP had been knocked down showed normal Giantin localization, suggesting other undescribed mechanisms exist to ensure Golgi structure.

Future Directions for Nef Mediated Disruption of Intracellular Trafficking Pathways

Even though much is known about HIV-1 Nef, there are still many unanswered questions. This section will discuss future directions related to this body of work.

β-COP Recruitment to Nef

The data presented here indicates that Nef recruits β-COP in order to direct MHC-I to lysosomal compartments for degradation. However, it is unknown how Nef recruits β-COP to the MHC-I cytoplasmic tail. In normal trafficking within the Golgi stacks, β-COP is recruited to membranes by Arf1, a small GTPase (17). β-COP recruitment in the endosomal network is quite different. There, β-COP is recruited to membranes via a pH dependent mechanism; as the pH drops β-COP is recruited (2). In the CD4 degradation pathway, it has been reported the Nef recruits β-COP through Arf1 (19). As for MHC-I, there are several possible recruitment mechanisms for β-COP: Nef could directly bind β-COP without the need of an additional cellular factor such as Arf1, Nef could utilize the pH dependent mechanism, or, as with CD4, Nef could recruit β-COP via Arf1. Future experiments will be needed to elucidate the required mechanism.

The alpha helical domain has been implicated in Nef/β-COP binding. This domain is arginine-rich and arginine residues have recently been shown to be a binding motif for β-COP (37). Mutational analysis of this region needs to be performed to determine if the

arginine residues are important for β -COP binding. This analysis would also determine if the alpha helical domain is directly binding β -COP or if destroying the alpha helical domain disrupted the structure of Nef so as to not allow β -COP binding.

When co-immunoprecipitation experiments were performed there was some residual β -COP binding when the alpha helical domain was mutated. It remains to be determined if there are additional β -COP binding domains within Nef. This question is especially relevant since the β -COP is needed for CD4 degradation but the alpha helical domain is not required for CD4 degradation.

It also remains to be determined if there are any amino acids in the MHC-I cytoplasmic tail that are required for β -COP binding. It has been shown that Nef binding to the MHC-I cytoplasmic tail creates a unique AP-1 binding site that requires residues from both Nef and the cytoplasmic tail (33). Perhaps a similar binding site is created for β -COP.

It has been observed that some Nef alleles are less efficient at disrupting MHC-I cell surface expression than others (20). Amino acid sequence alignments between these Nef alleles and Nef alleles that are efficient at MHC-I downmodulation have shown differences in some conserved regions (20). Are these Nef alleles deficient at MHC-I downmodulation because they fail to interact with necessary adaptor proteins, such as AP-1 and β -COP? Or are they deficient at binding MHC-I itself? Answers to these questions would allow us to better understand Nef biology.

Nef mediated Degradation

Nef also disrupts the intracellular trafficking of several other molecules, including CD1d, CD28, CD80, CD86, and CD8 $\alpha\beta$ (8, 11, 35, 36). Since the findings presented here have demonstrated that the MHC-I and CD4 Nef degradation pathways converge, and it would be informative to determine if other Nef mediated degradation pathways also converge and use β -COP to target for degradation. If this is so, perhaps new therapies could specifically target this section of the mechanism and inhibit degradation of several molecules with one treatment.

Nef has also been reported to interact with AP-3, which traffics between endosomes and lysosomes, via the dileucine motif (15) and stabilize it on membranes (24). Yet AP-3 has not been associated with downmodulation or degradation of any affected host proteins. It is possible this protein is also needed to target proteins for degradation in lysosomes. Studies have suggested that β -COP traffics between early and late endosomes (2), while AP-3 traffics between the late endosomes and lysosomes (reviewed in (7, 32)), perhaps β -COP is needed for transport in the endosomal network and AP-3 is needed for transport to the lysosomes.

Involvement of GGAs

AP-1 is known to traffic between the TGN and endosomal network, and is necessary for Nef mediated redirection of MHC-I from the TGN to the endosomal network (33). The recently discovered Golgi-localized, gamma-ear containing, Arf-binding proteins (GGAs) have also been reported to transport cargo along this pathway and have been associated with AP-1 (reviewed in (6)). Even though these proteins have not been associated with Nef, they associate with AP-1 (3), and since AP-1 is necessary for Nef to disrupt intracellular trafficking pathways, these proteins may also be involved.

CD4 Internalization

Much work has been done to determine the mechanism by which Nef accelerates CD4 internalization (see chapter 1). AP-2 mediates internalization at the plasma membrane and it has been hypothesized that Nef utilizes AP-2 to internalize CD4. Several groups have used RNA interference to determine if AP-2 is required, but this approach has not provided clarity, as different groups have drawn different conclusions (8, 25, 34, 35). The involvement of AP-2 would be greatly strengthened if a Nef/CD4/AP-2 complex could be demonstrated.

A Novel Trafficking Signal in the HLA-C Cytoplasmic Tail Regulates Its Cell Surface Expression Upon the Differentiation of Macrophages

HLA-C has very low cell surface expression. Evidence is presented here indicating the low surface expression is due to effects from both the extracellular and cytoplasmic tail

domains. The extracellular domain retains HLA-C in the ER, while the cytoplasmic tail domain promotes internalization and degradation in lysosomal compartments. Despite the low surface expression, HLA-C was found to be upregulated upon macrophage differentiation. Furthermore, the upregulation was regulated by serine 335.

The extracellular and cytoplasmic domains contribute to HLA-C low surface expression

Previous investigations had concluded the extracellular domain was primarily the cause of HLA-C low cell surface expression because it associated poorly with β -2 microglobulin, bound a restricted peptide repertoire, and is more stably associated with TAP (reviewed in chapter 1). Evidence is presented here that demonstrates the cytoplasmic tail also contributes to low cell surface expression. The effect of the cytoplasmic tail was characterized by constructing chimeric molecules in which the cytoplasmic tail domains of HLA-A2 and HLA-Cw4 were switched. When the HLA-Cw4 cytoplasmic tail was placed on the HLA-A2 extracellular domain (A2/C), HLA-A2 had decreased transport to the cell surface, a greater internalization rate and increased degradation in lysosomal compartments. This report is the first to implicate the cytoplasmic tail in the determination of HLA-C surface expression.

Also, many previous reports examined HLA-C in the context of B cell lines, specifically the MHC-I deficient B cell line, 721.221 (12, 16, 21, 26, 27, 30, 38). The study presented here is the first to examine HLA-C and directly compare it to HLA-A in T cells and macrophages, both of which are targets of HIV.

The HLA-C cytoplasmic tail contains a novel internalization signal

One of the surprising results of the study presented here was the identification of a novel internalization signal in the HLA-C cytoplasmic tail. Mutagenesis experiments revealed that isoleucine at position 337 in the HLA-C cytoplasmic tail was required for the increased internalization. When this residue was changed to the corresponding threonine in the HLA-A cytoplasmic tail, the increased internalization was ablated. Isoleucine residues can serve as a binding motif for AP-2, but the sequence surrounding the isoleucine at position 337 is DESLI and does not match the AP-2 binding motif, DxxxLL. Interestingly, the sequence does mimic the GGA binding motif, DxxLL. However, GGAs are not localized to the plasma membrane and have not been implicated in internalization. RNA interference was used to determine that GGAs are not involved in HLA-C internalization and degradation. Further experiments went on to determine the role of the aspartic acid residue at position 333. When this residue was mutated to an alanine, the rate of internalization was increased, indicating this residue serves to attenuate the isoleucine residue at position 337. These experiments reveal a novel trafficking motif (DESLI), and characterization of this novel motif furthers our understanding of cell biology and intracellular trafficking pathways. Future experiments will be needed to determine the cellular factor facilitating the internalization.

HLA-C is upregulated upon Macrophage differentiation

Another surprise generated by this study was the demonstration that HLA-C is upregulated upon macrophage differentiation. In undifferentiated macrophages, HLA-C has low cell surface expression and is internalized rapidly, as in T cells. However, upon differentiation, HLA-C cell surface expression increased to levels comparable to other HLA-A and -B alleles and the rate of internalization was similar to that of HLA-A2. This upregulation was seen in two macrophage cell lines, U937 cells and THP1 cells, and in primary CD14⁺ cells. Also, the upregulation was specific for HLA-C, because HLA-A and -B alleles did not exhibit an upregulation. These results indicate HLA-C expression in differentiated macrophages may contribute to immune function through a mechanism that is yet to be characterized. Additionally, this result is significant as it is the first report of HLA-C being upregulated and having cell surface expression similar to other classical MHC-I molecules.

The HLA-C upregulation is controlled by serine 335

Mutagenesis experiments were performed to determine the role of the serine residues surrounding the DESLI motif. Serine 335 (A2/C S335E) was mutated to a glutamic acid residue to mimic the negative charge provided through phosphorylation, and serines 332 and 335 (A2/C SSAA) were mutated to alanine residues to mimic a neutral charge and represent an unphosphorylated state. In undifferentiated macrophages, A2/C SSAA was expressed on the cell surface and internalized similarly to HLA-A2, whereas, A2/C S335E was expressed on the cell surface and internalized similarly to A2/C. Upon differentiation, however, A2/C S335E was not upregulated like A2/C and continued to be

rapidly internalized, indicating the serine at this position is controlling the internalization and cell surface expression of HLA-C through phosphorylation.

Implications of this Work

These findings have allowed us to further characterize the mechanism of HLA-C retention and low cell surface expression. The data indicating the cytoplasmic tail domain plays a significant role in the intracellular trafficking of HLA-C will contribute to both the immunology and the protein trafficking fields. Prior research efforts had attributed the low cell surface expression to the HLA-C extracellular domain, but here the cytoplasmic tail is shown to also contribute. This study also reports a novel trafficking signal in the HLA-C cytoplasmic tail domain that is controlled by phosphorylation. Greater understanding of protein trafficking signals is vital to developing a more complete understanding of how cells function and is necessary for development of new therapies, as many protein trafficking pathways are hijacked by viral and bacterial proteins.

The finding that HLA-C was upregulated on differentiated macrophages was quite surprising as there are no other reports of conditions that will increase HLA-C cell surface expression. This result indicates that HLA-C may serve a specialized role in macrophages and, possibly, antigen presenting cells in general. HLA-C is a ligand for KIR receptors (reviewed in chapter 1) and CTLs have recently been shown to express KIR (1). HLA-C may have evolved to protect cross-presenting antigen presenting cells

from CTLs that could mistake them as infected and target them for destruction. Further experiments will be needed to determine the functional significance of HLA-C upregulation in macrophages and, ultimately, its role in the immune response.

Future Directions for HLA-C Biology and Trafficking Pathways

HLA-C internalization

This study reports a novel internalization signal in the HLA-C cytoplasmic tail. However, it is unknown which adaptor protein mediates the accelerated internalization. Additionally, the internalization signal is regulated through phosphorylation. To fully understand the trafficking pathway of HLA-C, further experiments will need to be done to identify the required adaptor protein. A possible candidate are the β -arrestins. These trafficking proteins have been reported to facilitate internalization, and furthermore, they are recruited to phosphorylated sequences in cytoplasmic tail domains (reviewed in (28)).

HLA-C upregulation

The experiments presented here only examined HLA-C upregulation in differentiated macrophages. Since macrophages are antigen presenting cells is it a natural progression to examine the trafficking of HLA-C in other antigen presenting cells, such as dendritic cells (DC), to determine if this upregulation is specific to macrophages or is a general occurrence in differentiated antigen presenting cells.

Additionally, the study presented here only examined the internalization rate of HLA-C in differentiated macrophages. Perhaps additional changes in intracellular trafficking contribute to the upregulation of HLA-C. For example, the block in transport observed in T cells could also be reversed upon differentiation. Also, since the extracellular domain significantly contributes to the intracellular retention of HLA-C, it should be determined if HLA-C is released from the ER upon macrophage differentiation.

Macrophages can be differentiated to develop into classically activated macrophages or, the more recently described, alternatively activated macrophages (reviewed in (22, 29)). Macrophages exposed to IFN- γ and LPS develop into the classically activated macrophage that is associated with a Th1 response. Alternatively activated macrophages are exposed to IL-4 or IL-13 and are associated with a Th2 response. It should be determined if the upregulation of HLA-C is primarily associated with a particular immune response, such as Th1 versus Th2, or occurs irrespective of the source of activation.

It has been hypothesized in this thesis that upregulation of HLA-C protects cross-presenting macrophages from CTL mediated lysis. Experiments need to be performed to test this hypothesis and determine if the degree of HLA-C upregulation on differentiated macrophages is enough to inhibit CTL mediated lysis. Also, since HLA-C can present peptides in addition to inhibiting KIR expressing cells, can HLA-C perform the dual role of presenting peptides to CD8⁺ T cells and inhibit CTL mediated lysis simultaneously?

HLA-C and HIV Infection

The role of HLA-C in HIV infection also requires further investigation. Experiments have shown the HIV protein Nef is selective as to which MHC-I it downmodulates, HLA-C and -E are left on the cell surface, while HLA-A and -B are downmodulated (13). That investigation also reported the HLA-C on the surface was sufficient to protect these cells from NK cell mediated lysis (13). However, these experiments were performed in the B cell line, 721.221, which is MHC-I deficient and is not a natural target of HIV. Studies performed with T cells and undifferentiated macrophages have shown HLA-C surface expression is very low and it is questionable if this very low surface expression is indeed enough to protect HIV infected cells from NK cell mediated lysis. The hypothesis that selective MHC-I downmodulation functions to protect HIV infected cells from NK cell mediated lysis is well known and makes sense given the role of HLA-C in the inhibition of NK cells. Experiments performed to address this hypothesis blocked the KIR/HLA-C interaction with antibodies specific for the KIR (5). These experiments showed an increase in the specific lysis of HIV infected cells when the KIR/HLA-C interaction was blocked, indicating that blocking the interaction between KIR and HLA-C was protective for the HIV infected cell (5). However, the increase in specific lysis was only modest. Also, since the blocking antibody was directed against the KIR receptor, one could question if the blocking antibody could signal through the KIR receptor to inhibit the NK cell, masking the magnitude of specific lysis that would have occurred had the blocking antibody been directed against HLA-C. Further

experiments need to be performed to fully address the hypothesis that Nef does not downmodulate HLA-C and –E to protect HIV infected cells from NK cell mediated lysis.

There is an alternative hypothesis as to why HIV leaves HLA-C on the cell surface. It has been reported that virions that incorporate HLA-C have increased infectivity (14). HLA-C incorporation was also associated with lower susceptibility to antibody neutralization (14). Perhaps HLA-C left of the cell surface promotes increased infectivity of newly budded virions and shields them from neutralizing antibodies. The mechanism by which HLA-C promotes increased virion infectivity and lowers susceptibility to antibody neutralization has not been fully characterized, but the observation that HLA-C and Env interact (14) may provide a useful clue. Perhaps the HLA-C/Env interaction alters Env to allow for more efficient receptor binding, rendering the virion more infectious. Alternatively, HLA-C could change the epitope on Env recognized by the neutralizing antibodies, making it less susceptible to antibody neutralization. It is unknown if the aforementioned observations are specific to HLA-C or if HLA-E also increases viral infectivity and lowers susceptibility to antibody neutralization. Since HLA-E is also not downmodulated by Nef, experiments should be performed to determine if HLA-E can increase viral infectivity and lower susceptibility to neutralizing antibodies.

The upregulation of HLA-C upon macrophage differentiation in the context of HIV infection also needs to be investigated. HLA-C surface expression is very low on T cells and undifferentiated macrophages and it also is questionable if this amount of HLA-C

would be able to confer increased virion infectivity and lowered susceptibility to antibody neutralization. However, the increase of HLA-C surface expression upon macrophage differentiation could provide HLA-C levels sufficient to confer these beneficial effects. Additionally, it is unknown if HIV activates macrophages upon infection. Nef encodes mechanisms to activate T cells, perhaps similar mechanisms are utilized to activate macrophages, and thus upregulate HLA-C to increase viral infectivity and shield new virions from neutralizing antibodies.

Concluding Remarks

HIV infection is a growing problem with over 40 million people infected worldwide. The studies described in this thesis add to our knowledge of HIV biology, specifically the strategies employed by HIV to disrupt antigen presentation and evade the immune response. Additionally, these investigations provide greater insight into HLA-C and macrophage biology, both of which are involved in HIV infection. Collectively, these data may someday allow better therapies and vaccines strategies to be designed and implemented.

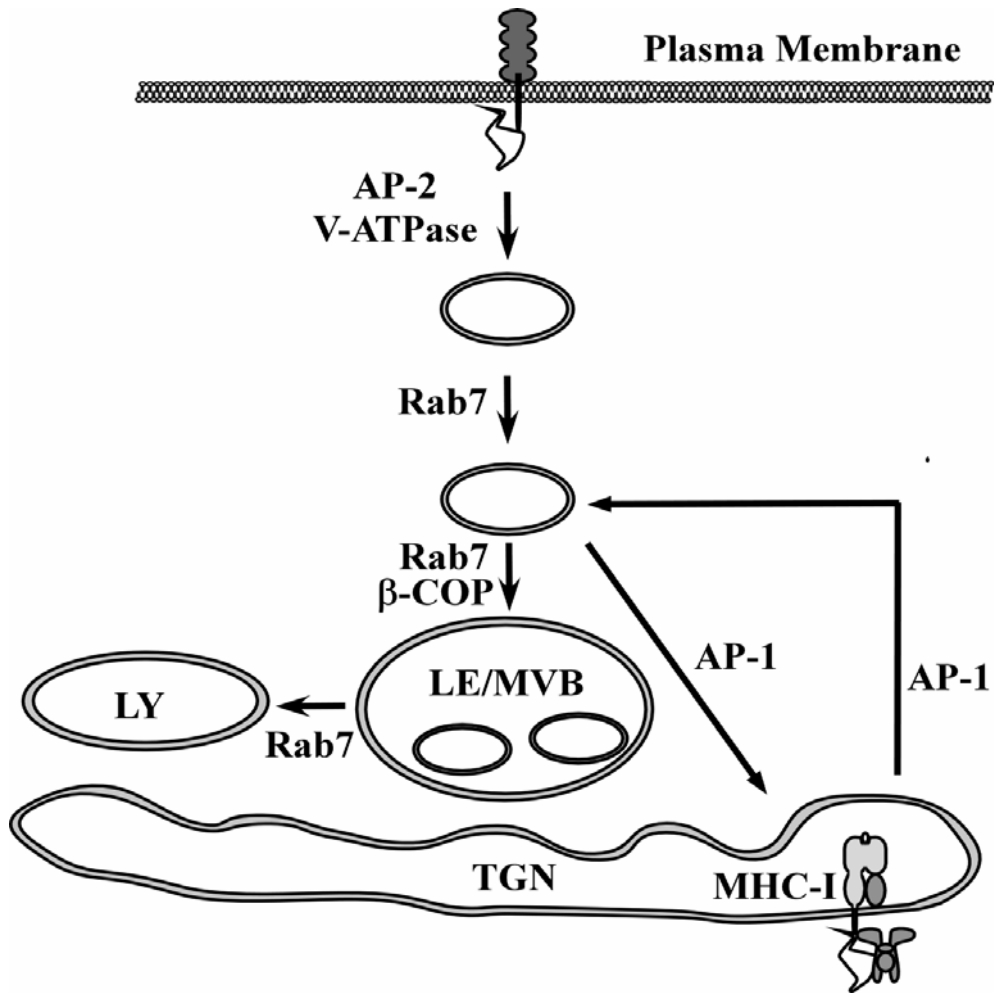


Figure 4-1. **Model for the mechanism by which Nef affects CD4 and MHC-I trafficking.** HIV Nef binds the CD4 cytoplasmic tail at the cell surface, and recruits AP-2 and/or the vacuolar-ATPase to facilitate internalization. CD4 is internalized and is transported to an endosomal compartment associated with Rab7 and β -COP. Conversely, Nef binds the MHC-I cytoplasmic tail early in the secretory pathway, AP-1 is recruited and facilitates transport to an intermediate endosomal compartment marked with Rab7 and β -COP. If AP-1 falls off the Nef-MHC-I complex after arrival in the endosome, Nef binds β -COP and targets MHC-I (and CD4) to lysosomes for degradation. If AP-1 remains bound, it promotes recycling of the Nef-MHC-I complex to the TGN. LY=lysosome, LE/MVB =late endosome/multi-vesicular body

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