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Review

HIV-1 Nef: a master manipulator of the membrane trafficking machinery mediating immune evasion

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ABSTRACT

Background: Many viral genomes encode a limited number of proteins, illustrating their innate efficiency in bypassing host immune surveillance. This concept of genomic efficiency is exemplified by the 9 kb RNA genome of human immunodeficiency virus 1 (HIV-1), encoding 15 proteins sub-divided according to function. The enzymatic group includes proteins such as the drug targets reverse transcriptase and protease. In contrast, the accessory proteins lack any known enzymatic or structural function, yet are essential for viral fitness and HIV-1 pathogenesis. Of these, the HIV-1 accessory protein Nef is a master manipulator of host cellular processes, ensuring efficient counter-attack against the host immune response, as well as long-term evasion of immune surveillance. In particular, the ability of Nef to downmodulate major histocompatibility complex class I (MHC-I) is a key cellular event that enables HIV-1 to bypass the host's defenses by evading the adaptive immune response.

Scope of Review: In this article, we briefly review how various pathogenic viruses control cell-surface MHC-I, and then focus on the mechanisms and implications of HIV-1 Nef-mediated MHC-I downregulation via modulation of the host membrane trafficking machinery.

Conclusion: The extensive interaction network formed between Nef and numerous membrane trafficking regulators suggests that Nef's role in evading the immune surveillance system intersects multiple host membrane trafficking pathways.

Significance: Nef's ability to evade the immune surveillance system is linked to AIDS pathogenesis. Thus, a complete understanding of the molecular pathways that are subverted by Nef in order to downregulate MHC-I will enhance our understanding of HIV-1's progression to AIDS.

The parasitic activity of viruses is counteracted by innate and adaptive immune responses, aimed at halting viral replication to prevent the spread of disease. The adaptive immune response includes the destruction of virally infected cells by a specific type of immune cell, the circulating $CD8^+$ cytotoxic T lymphocytes (CTLs). Specifically, CTLs recognize virally infected cells when they present viral peptides on cell surface major histocompatibility complex class I (MHC-I) receptors [1]. CTLs produce cytotoxic enzymes that induce the death of these virally infected cells. However, many viruses have evolved elaborate mechanisms to counteract or evade the host CTL response, specifically by decreasing the amount of cell surface MHC-I. The following sections will describe our current understanding of MHC-I trafficking and how HIV-1 Nef subverts host membrane trafficking pathways to evade detection by the immune system.

1. The subversion of host cellular membrane trafficking by pathogenic viruses

The transit of proteins within cells is controlled by membrane trafficking regulators, including a vast network of organelles and vesicles, mediated by specific membrane effector proteins [2]. Viruses hijack host membrane trafficking pathways to divert host cellular proteins or deliver viral proteins to a subcellular location supporting viral replication. Furthermore, viruses that mediate sustained infection, such as HIV-1, often subvert host cell membrane trafficking to evade immune surveillance, in large part by modulating cell surface MHC-I.

Multiple membrane trafficking regulator proteins ensure the transit of MHC-I complexes to the cell surface. The transporters associated with antigen presentation-1 and -2 (TAP-1 and TAP-2) form a transmembrane heterodimer in the membrane of the endoplasmic reticulum (ER). The TAP-1 and TAP-2 proteins facilitate the active transport of cytosolic peptides generated by the immunoproteasome. The immunoproteasome cleaves viral peptides and enables their loading onto MHC-I complexes. This peptide loading step is facilitated by numerous ER resident chaperones. The resulting MHC-I complexes are comprised of the MHC-I

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alpha, or heavy chain, β_2-microglobulin and the cytosolic viral peptide. Properly folded MHC-I-peptide complexes exit the ER and are transported through the Golgi apparatus, where they undergo additional post-translational processing. From the trans-Golgi network (TGN), MHC-I-containing vesicles bud off and travel to the plasma membrane, where vesicular membranes fuse (Fig. 1). At the cell surface, trafficking is dynamic and may result in MHC-I secretion or recycling back to a paranuclear compartment in membrane-enclosed vesicles, i.e. exosomes or endosomes, respectively. The aforementioned steps, including formation of MHC-I-peptide complexes and trafficking to the cell surface, are prime targets for pathogenic viruses to interfere with antigen presentation to CTLs, thereby hindering the immune response. Importantly, viral proteins mediate these effects by direct interaction with host cellular proteins, illustrating the importance of protein–protein interactions in viral immune evasion.

Various pathogenic DNA viruses are known to manipulate membrane trafficking steps that control the levels of cell surface MHC-I, including herpes simplex virus (HSV), human cytomegalovirus (HCMV), adenovirus, human papilloma virus (HPV), as well as Kaposi's sarcoma-associated herpes virus (KSHV) (Table 1). The ability of HSV and HCMV to downregulate MHC-I has been well characterized. The HSV and HCMV proteins, US6 and ICP47, respectively, utilize distinct mechanisms to inhibit viral peptide translocation into the ER lumen by targeting the TAP complex. HCMV US6 directly binds the TAP

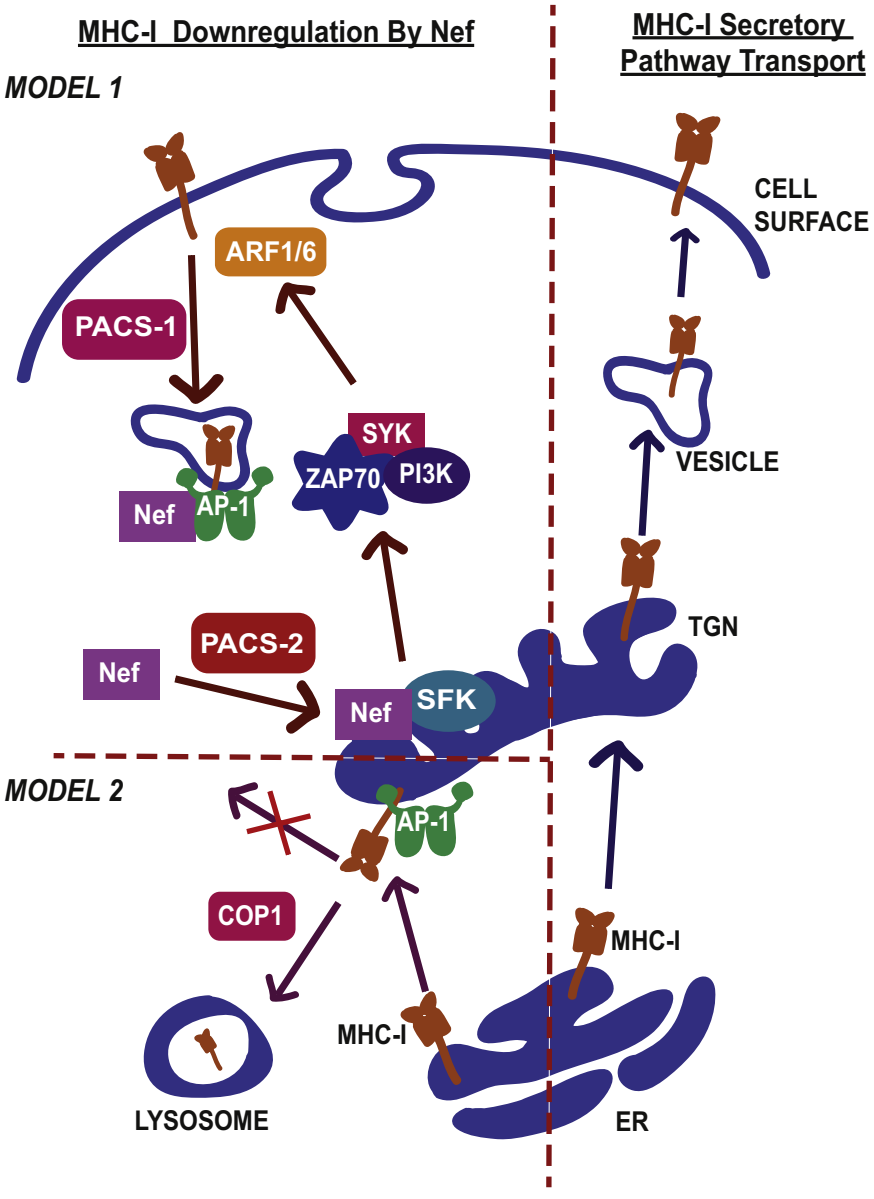

Fig. 1. Trafficking of MHC-I along the secretory pathway to the cell surface is subverted by HIV-1 Nef. Newly synthesized MHC-I molecules are transported along the secretory pathway to the cell surface (right). Properly folded MHC-I:peptide complexes are trafficked from the ER, through the Golgi and to the cell surface within vesicles. The HIV-1 protein Nef disrupts cell surface localization of MHC-I, via two distinct models. The first model (top), constitutes an increase in endocytosis of cell surface MHC-I. This is initiated by the PACS-2 mediated localization of Nef to the TGN, where Nef interacts with and activates an SFK, leading to a signal transduction pathway culminating in the formation of an activated protein complex containing ZAP70 (or Syk in monocytic cells) and PI3K. Activated PI3K causes accumulation of PIP3 on the inner leaflet of the plasma membrane, and cell surface MHC-I is internalized in either ARF1 or ARF6 coated vesicles. Endocytosed MHC-I forms a ternary complex with Nef and AP-1 and via PACS-1 is sequestered in the paranuclear region of the cell. The second model (bottom) comprises a block in transport of newly synthesized MHC-I. It is suggested that Nef binds to MHC-I in the ER, leading to the sequestration of Nef in the paranuclear region of the cell in a process requiring AP-1. Sequestered MHC-I is then transported in COPI coated vesicles to the lysosome for degradation.

Table 1
Viral proteins that modulate cell surface MHC-I expression.

Virus	Viral protein	Host target and effect	Reference
HSV	ICP47	Binds TAP complex, preventing peptide loading	[5,6]
	US6	Binds TAP complex, prevents ATP hydrolysis	[3,4]
HCMV	US2, US11	Promotes retrotranslocation of MHC-I heavy chain from the ER	[8–12]
	US3	Binds tapasin, inhibits peptide loading onto MHC-I complex, directly binds and impedes MHC-I complex formation in ER	[13–15]
Adenovirus	E3	Interacts with MHC-I complexes and promotes retrotranslocation of MHC-I from cis-Golgi back to ER, may inhibit TAP-MHC-I interaction	[16–18]
HPV types 7, 18	E7	Decreases transcription of genes encoding MHC-I heavy chains and other proteins involved in MHC-I complex formation	[19–21]
KSHV	MIR1, MIR2	Act as ubiquitin ligases to induce MHC-I endocytosis and degradation	[22,23]

complex to inhibit ATP binding, thereby preventing ATP hydrolysis and peptide translocation, while peptide binding remains intact [3,4]. In contrast, HSV ICP47 binds to the peptide-binding site of the TAP complex, preventing peptide loading without affecting ATP binding or hydrolysis [5,6], thereby decreasing cell surface MHC-I and preventing CTL-mediated lysis of HSV infected cells [7].

HCMV encodes three viral proteins in addition to US6 that prevent properly folded MHC-I complexes from exiting the ER: US2, US3, and US11. US11 and US2 promote retrotranslocation of MHC-I heavy chains from the ER, thereby targeting them for ubiquitination and subsequent proteasomal degradation by distinct mechanisms [8–12]. In contrast, HCMV US3 binds and inhibits tapasin, a chaperone protein bridging the TAP complex with partially folded MHC-I prior to peptide loading [13], as well as directly binding and impeding MHC-I complexes in the ER[14,15]. However, the contribution of these individual proteins to the prevention of CTL-mediated killing of infected cells is unknown.

The adenovirus protein E3/19 K (E3) is a well-studied viral protein that, similar to the HSV and HCMV proteins described above, promotes ER retention of MHC-I and formation of MHC-I complexes [16]. Specifically, E3 interacts with MHC-I complexes [16–18], and via a carboxyl-ER retention signal promotes retrograde transportation of any MHC-I-E3 complexes reaching the cis-Golgi [17]. E3 may additionally inhibit the TAP-MHC-I interaction [16]. Moreover, the E7 proteins from HPV types 16 and 18, decrease transcription at the locus encoding MHC-I molecules [19] via modulation of histone deacetylases (HDACs) at the MHC-I promoter [20]. HPV types 16 and 18 E7 also decrease transcription of genes encoding proteins important for peptide loading, including the TAP complex subunit TAP-1, by a yet undefined mechanism [19,21].

While many viral proteins target MHC-I complexes in the ER, MHC-I complexes successfully reaching the plasma membrane are also targeted. For example, KSHV genes K3 and K5 encode the ubiquitin ligases modulator of immune recognition -1 and -2 (MIR1 and MIR2), respectively, which decrease the stability of cell surface MHC-I by inducing MHC-I endocytosis and subsequent degradation in an allele-specific manner [22]. Indeed, expression of K3 (encoding MIR1) efficiently downregulated both classical (HLA-A and HLA-B) and non-classical MHC-I alleles (HLA-E), while K5 (encoding MIR2) did not downregulate non-classical MHC-I alleles [23]. However, the relative contributions of these genes to MHC-I downregulation in the context of pathogenesis is only beginning to be elucidated, and appears dependent on the stage of infection [24].

2. Modes of HIV-1 Nef-mediated MHC-I downregulation

The formerly presented viral proteins are produced by DNA viruses, with double-stranded DNA genomes encoding an array of proteins contributing to their ability to evade detection by the host immune system. For example, HCMV has an over 230 kb dsDNA genome, encoding at least seven proteins that inhibit antigen presentation by MHC-I molecules (reviewed in [25]). This is in stark contrast to the approximately 9 kb single-stranded RNA HIV-1 genome, which encodes only ten genes; with a single accessory protein, Nef, primarily responsible for modulating cell surface MHC-I.

In 1996 Schwartz et al. [26] demonstrated that Nef downregulates MHC-I in HIV-1-infected U937 monocytic cells when compared to equivalent cells infected with HIV-1 lacking a functional Nef gene. The authors suggested that Nef causes perturbed MHC-I endocytosis and MHC-I accumulation in vesicles recycling from the plasma membrane, specifically early endosomal compartments, rather than decreased biosynthesis of MHC-I. This was the first demonstration of a viral protein interfering with antigen presentation through MHC-I endocytosis. Since this discovery, Nef-mediated MHC-I downregulation has been extensively studied, and is now known to be critical for the onset of AIDS [27]. Currently, there are two primary models of HIV-1 Nef-mediated MHC-I downregulation, both of which entail Nef forming protein–protein interactions with numerous host membrane trafficking proteins. While the complete mechanisms of these models have yet to be elucidated, the molecular details of many of the known Nef-host protein interactions have been well characterized and are described in the following sections.

2.1. Nef promotes the endocytosis of cell surface MHC-I

The model developed by Thomas and colleagues (Fig. 1) proposes that the membrane trafficking proteins phosphofurin acidic cluster sorting proteins -1 and -2 (PACS-1 and -2) bind Nef directly and promote endocytosis of cell surface MHC-I. Specifically, Nef binding to the cargo sorting protein PACS-2 is required for trafficking of Nef to the TGN, where Nef then directly binds and activates an Src family kinase (SFK) [28]. Interestingly, whereas Nef binds at least nine SFKs *in vitro*, it only leads to activation of the SFKs Hck, Lyn and c-Src [29]. Indeed, simultaneous knockdown of Hck, Lyn and c-Src was necessary to block Nef-mediated MHC-I downregulation in $CD4^+$ T cells, demonstrating that these 3 SFKs have overlapping functions in Nef action [30]. The PACS-2-dependent, Nef-mediated activation of TGN-localized SFKs triggers a phosphorylation cascade leading to formation of a protein complex comprised of the protein tyrosine kinase Zeta-chain-associated protein kinase 70 (ZAP-70) (or spleen tyrosine kinase (Syk) in monocytic cells) and the signaling kinase phosphoinositide 3-kinase (PI3K), resulting in activation of PI3K [31]. Studies utilizing small molecule inhibitors of PI3K demonstrated that activation of PI3K is essential for Nef-mediated MHC-I downregulation [32]. Moreover, Atkins et al. [28] used siRNA knockdown and cells from PACS-2 knockout mice to demonstrate that TGN-localization of Nef, and subsequent PI3K activation and MHC-I endocytosis is PACS-2 dependent. Furthermore, Nef is unable to recruit activated PI3K in splenocytes derived from PACS-2 knockout mice [28].

Activated PI3K produces phosphatidylinositol (3,4,5)-triphosphate (PIP3) on the inner leaflet of the plasma membrane, but how this ultimately results in the internalization of cell surface MHC-I is unclear. Based on studies in HeLa or CEM-SS T cells, it has been reported that MHC-I is internalized into endosomal compartments coated with the small membrane associated GTPases ARF6 or ARF1 [33,34], which regulate membrane trafficking by inducing changes to actin dynamics. More research is needed to identify the specific membrane trafficking regulators mediating this process.

Following Nef-induced endocytosis, internalized MHC-I is inhibited from recycling back to the cell surface and is observed in endocytic vesicles in complex with Nef and adaptor protein 1 (AP-1), a vesicular adaptor protein that functions in transport between endosomes and the TGN. AP-1 is essential, as knockdown of the AP-1 subunits μ1 or γ abolishes Nef-mediated MHC-I downregulation [34]. Noviello et al. [35] proposed a model of cooperative binding of Nef to AP-1 and MHC-I, wherein a ternary complex is formed. More recently, a crystal structure was solved demonstrating the interactions between Nef in complex with the cytoplasmic tail of MHC-I and the μ1 subunit of AP-1 [36], a more detailed description of which is given below (Table 2). These MHC-I/Nef/AP-1 complexes are trafficked through the endocytic pathway and ultimately sequestered within the paranuclear region of the cell in a step requiring binding of Nef to PACS-1 [37].

Interestingly, this model of Nef-mediated MHC-I endocytosis is akin to that utilized by Nef in the downregulation of other cell surface molecules, such as chemokine receptors, via an endocytic pathway [38]. Further research is needed to determine the precise endocytic pathways utilized by these chemokine receptors, but there is evidence suggesting it is analogous to MHC-I downregulation, in that it requires intact SFK and PACS-1 interaction motifs on Nef [39]. Additionally, it has been shown that downregulated chemokine receptors co-localize with downregulated MHC-I and Nef in the paranuclear region [38,39]. Moreover, while the well-characterized downregulation of the HIV-1 receptor CD4 also occurs via increased endocytosis, this pathway is conversely AP-2 and clathrin-dependent, utilizing interfaces on Nef distinct from MHC-I downregulation [40]. Thus, the Nef-CD4 interaction is bridged by AP-2, while the Nef-MHC-I interaction requires specific interactions with AP-1 and PACS-1.

2.2. Nef blocks the transport of newly synthesized MHC-I

A separate model has been proposed in which Nef primarily blocks the transport of newly synthesized MHC-I from the TGN to the cell surface (Fig. 1). In this model, Nef binds to immature, hypo-phosphorylated MHC-I in the TGN, leading to MHC-I sequestration and inhibition of further transport [41]. These studies demonstrated that Nef preferentially binds hypo-phosphorylated MHC-I, which is present early in the secretory pathway. Indeed, an MHC-I mutant that mimics phosphorylated MHC-I is less efficiently downregulated by Nef. Furthermore, Nef co-immunoprecipitates with tapasin, an ER resident chaperone that associates with MHC-I, suggesting that Nef can bind MHC-I in the ER [41].

As in the previously described model of Nef-mediated MHC-I endocytosis, Nef forms a ternary complex with MHC-I and AP-1, and is sequestered in the TGN before eventual transport in coatomer protein complex subunit beta (beta-COPI) coated vesicles to lysosomes for degradation [41]. The role of beta-COPI was validated by siRNA knockdown, as well as expression of a Nef mutant deficient in beta-COPI binding, both of which inhibit MHC-I downregulation [42,43]. Additionally, lysosomal inhibitors prevented the decrease in MHC-I seen in the presence of Nef [44], while beta-COPI knockdown increased accumulation of MHC-I, but decreased co-localization with the lysosomal marker LAMP-1 [42]. It has been argued that similar to MHC-I, Nef facilitates CD4 downregulation by trafficking CD4 in beta-COPI coated vesicles to the lysosome for degradation [42].

The relative distinction and/or overlap between these two modes of MHC-I downregulation have yet to be clearly defined. It has been suggested that these modes occur at different time points of viral infection and may be temporally linked [30]. Specifically, it was proposed that at early time points of infection, during the first 48 hours, the dominant mode of downregulation is endocytosis, while later in infection the dominant mode becomes a block in the secretory pathway to the cell surface. Nonetheless, it appears as though these two modes are not mutually exclusive. It was found that blocking SFK activation in H9 $CD4^+$ T cells with the small molecule inhibitor 2c, which inhibits SFK activation and the downstream signaling pathway that leads to endocytosis of MHC-I, also inhibits the secretory pathway blockade later in infection [30]. Furthermore, both models include modulating the subcellular localization of MHC-I through a ternary MHC-I/Nef/AP-1 complex, which is sequestered intracellularly [37,41], therefore presenting a possible point of intersection between these two models.

Taken together, these models suggest a possible mechanism for Nef-mediated MHC-I downregulation (Fig. 1), in which during the first 48 hours of infection Nef mediates the assembly of a kinase complex which induces a signal transduction pathway to cause endocytosis of cell surface MHC-I, followed by subsequent paranuclear sequestration of MHC-I. After the first 48 hours of infection, cell surface MHC-I decreases and newly synthesized MHC-I is inhibited, via the MHC-I/Nef/AP-1 ternary complex, from being transported to the cell surface, and is degraded in lysosomes. However, as the half-life of an activated $CD4^+$ T cell is only 48 hours, endocytosis of cell surface MHC-I may be more relevant to HIV-1 infected T cells, whereas later secretory pathway inhibition may be applicable in HIV-1 infected monocytes, which have a half life of two weeks [45]. Ultimately, it is still unknown how the temporal regulation or switch between modes is mediated and the relative contribution of cell-type and length of infection.

Table 2
Nef host protein interacting partners implicated in MHC-I downregulation.

Interacting host protein	Motif on Nef implicated in binding	Reference
PACS-1, PACS-2	$EEEE_{65}$, P_{78}, W_{113}, Y_{120}	[37,73]
SFKs (Hck, Lyn, c-Src)	$PxxP_{75}$	[49–51]
AP-1*	$W_{13}/V_{16}/M_{20}$, $EEEE_{65}$	[35,36]
β-COPI	$R_{17/19}$	[42,47]

3. Nef motifs and binding partners implicated in MHC-I downregulation

The downregulation of MHC-I requires numerous Nef-host protein interactions mediated by various interfaces on Nef (Fig. 2). As Nef lacks enzymatic function, it relies on interactions with numerous host cell proteins, including signaling molecules with critical roles in membrane trafficking events [29], itinerant cargo [46], components of vesicular coats [35,47], and membrane trafficking regulators that direct proper subcellular localization [37]. Of these, the major players in Nef-mediated MHC-I downregulation are outlined in the following sections.

3.1. Signaling molecules: SFKs

The induction of MHC-I downregulation by Nef requires the direct activation of a TGN-localized SFK to trigger a PI3K-dependent signal transduction pathway. The interface of the Nef-SFK complex is comprised of a type II polyproline helix on Nef including P_{69}xRPxxPxPR_{78} and the RT loop of the SH3 domain in the SFK. This type of interaction is typical of many SH3 domain mediated interactions, [48], suggesting that viruses have evolved to mimic host protein interactions to exert their functions. The importance of the Nef-SFK interaction has been demonstrated using a Nef $PxxP_{75}$ ➔ $AxxA_{75}$ mutant, which is deficient in SFK activation and MHC-I downregulation [29].

The molecular details of the Nef-SFK interaction interface have been characterized using a combination of X-ray crystal structures, solution NMR studies and mutational analysis. Lee et al. [49] analyzed the kinetics of the Nef-SFK interaction using surface plasmon resonance and isothermal titration calorimetry to demonstrate that additional Nef residues near the $PxxP_{75}$ motif also contribute to Nef's interaction with SFKs [49]. This is consistent with X-ray crystallography structures showing that additional residues beyond the polyproline motif interact with SFKs [50,51], and suggests that Nef may bind to SFKs using an allosteric mechanism, as mutations in Nef outside of the SH3 binding pocket weaken the Nef-SFK interaction [52]. The Nef-SFK interaction also has important implications in MHC-I downregulation by Nef, as illustrated

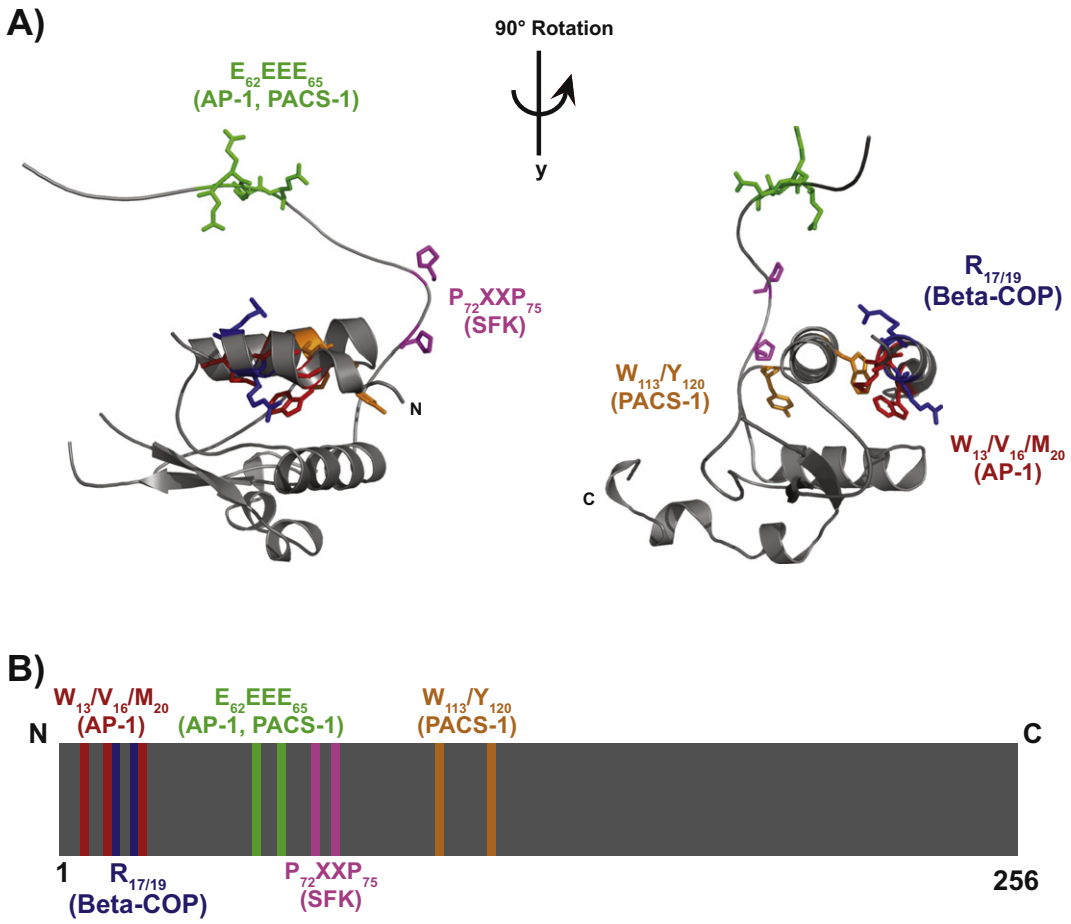

Fig. 2. Crystal structure of Nef and its interaction interfaces utilized in MHC-I downregulation. Shown is (A) a crystal structure of a Nef monomer from a ternary complex with AP-1 and MHC-I (PDB: 4EMZ) and (B) a stick diagram depicting various Nef interaction interfaces. Not shown is the disordered region comprising amino acids 24–54. Shown are residues of Nef implicated in interacting with PACS-1 ($E_{62}EEE_{65}$ and W_{113} and Y_{120}), AP-1 ($E_{62}EEE_{65}$ and W_{13}, V_{16}, M_{20}), Beta-COP ($R_{17/19}$) and SFKs ($PxxP_{75}$). Numbering refers to HIV-1 Nef pNL4.3.

using the small molecule inhibitor 2c, which blocks the Nef-SFK interaction by directly targeting the Nef-SFK interaction interface [30]. 2c was shown to inhibit MHC-I downregulation in infected H9 $CD4^+$ T cells, and this was attributed to a block in endocytosis of cell surface MHC-I [30].

In addition to the requirement of an intact Nef $PxxP_{75}$ motif for activating SFKs to mediate MHC-I downregulation, an intact Nef $PxxP_{75}$ motif is important for Nef to mediate other functions, as conservation of Nef's polyproline helix across numerous Nef variants suggests it is essential for viral fitness. Indeed, yeast-based studies, *in vitro* binding assays, and *in vivo* kinase activation assays have demonstrated that binding and subsequent activation of Hck is a conserved ability across multiple Nef alleles [52–54]. However, the conservation of this motif is not necessarily due to a selective pressure to maintain MHC-I downregulation, as the Nef-SFK interaction is implicated in other Nef functions, such as modulation of calcium stores [55], viral infectivity [56], viral replication [57], and T cell activation and signaling [58–60]. As in MHC-I downregulation, these effects require Nef to interact with host cell proteins and modulate intracellular trafficking. For example, Nef interacts with the SFK Lck to re-organize its subcellular localization from the plasma membrane to intracellular compartments, thereby activating T cell survival signals and promoting HIV-1 replication [61]. Due to use of the same interaction interface on Nef to interact with numerous host cell proteins, it is possible there is competition between various Nef binding partners, which may play a role in regulating these functions, though this has not yet been investigated.

As Nef $P_{72}xxP_{75}$ is well conserved across various Nef alleles and plays an important role in pathogenicity, it has become the target of a number of inhibitors (reviewed in [62]). Betzi et al. [63] were the first to target the Nef-SFK interaction using *in silico* modeling to identify two small molecules that block the Nef-Hck interaction. Subsequent studies screened small molecules for their ability to inhibit Nef-mediated SFK (Hck) activation, leading to identification of diphenylfuropyrimidine (DFP) compounds, which were also found to inhibit Nef-mediated enhancement of HIV-1 replication [64]. However, the interface(s) targeted by these inhibitors remain unknown. Further screening led to the identification of a diphenylpyrazole compound, termed B9, which was shown to target the Nef dimerization interface [65]. A single domain antibody was also designed to target Nef [66], and based on this antibody a small peptide, termed Neffin, was produced containing a portion of the anti-Nef antibody linked to a recombinant form of the Hck SH3 domain [67]. This peptide binds Nef with high affinity and inhibits a number of Nef functions including MHC-I downregulation and Nef-mediated increases in infectivity [67,68]. The development of Nef inhibitors is currently an area of interest, as Nef inhibition represents a novel HIV-1 targeting strategy. Current drug therapies target viral enzymes to which resistance often develops, highlighting the need for novel therapeutics [69]. Blocking Nef-mediated immune evasion would benefit HIV-1 infected patients, as this action would permit the immune system to more efficiently target HIV-1 infected cells for permanent destruction by increasing antigen presentation. Moreover, increased antigen presentation by Nef inhibition may represent a novel paradigm allowing the reactivation of latently

infected cells. Reactivation may permit eradication of the virus by antiretroviral therapies, thus realizing a key goal in the field of HIV cure research.

3.2. Vesicular coat proteins: COPI and AP-1

Nef downregulates MHC-I in a clathrin-independent manner by subverting vesicular coat proteins including COPI and AP-1, which form spherical structures around tubulated membranes to facilitate budding. COPI coated vesicles are homologous to clathrin coated vesicles in that they are made up of an inner "adaptor protein like" layer and an outer "clathrin like" complex. COPI specifically mediates budding from the Golgi complex, and Nef binds the COPI beta-subunit to facilitate budding of MHC-I loaded vesicles from the Golgi [42]. Nef binds beta-COPI using a positively charged $R_{17/19}$ patch, and mutation of these residues decreases MHC-I downregulation [42].

The adaptor protein AP-1 is also required for efficient Nef-mediated MHC-I downregulation, as demonstrated by siRNA knockdown studies [44]. Specifically, AP-1 is thought to be essential for Nef-mediated sequestration of endocytosed MHC-I molecules, as well as MHC-I lysosomal degradation. While Dikeakos et al. [30] demonstrated that knockdown of AP-1 perturbs Nef-mediated paranuclear sequestration of MHC-I, Roeth et al. [44] found that AP-1 knockdown inhibited Nef-mediated transport and subsequent degradation of MHC-I in lysosomes. To mediate this, Nef forms a ternary complex with MHC-I and AP-1 by binding the AP-1 μ-1 subunit and the C-terminal cytoplasmic domain of MHC-I. Specifically, Nef residues $W_{13}/V_{16}/M_{20}$ form a hydrophobic pocket, while an acidic cluster, Nef $_{62}EEEE_{65}$, mediates electrostatic interactions with the AP-1 μ-1 subunit. Additional Nef residues act to stabilize the complex, including the $PxxP_{75}$ motif [36,70].

The crystal structure of this complex further suggests that AP-1 bridges the interaction between Nef and MHC-I by forming weak Van der Waals forces with the MHC-I cytoplasmic tail, but does not form close contacts with MHC-I itself [35,36]. Indeed, Jia et al. [36] suggest neither AP-1 nor Nef form more than a transient interaction with MHC-I based on small buried interaction interfaces. Importantly, mutational studies demonstrate that residues in AP-1, Nef and MHC-I essential for formation of the ternary complex, including the acidic cluster ($E_{62}EEE_{65}$) and $W_{13}/V_{16}/M_{20}$ of Nef, are essential for MHC-I downregulation [35,70,71].

3.3. Vesicular sorting proteins: PACS-1 and PACS-2

Nef subverts the host cellular trafficking machinery in a multitude of ways to mediate MHC-I downregulation, including interacting with the vesicular sorting protein family phosphofurin acidic cluster sorting proteins (PACS-1 and PACS-2). PACS-1 was initially characterized based on its ability to bind to the protease furin and mediate its proper localization [72]. Subsequently, PACS-1 was implicated in Nef-mediated MHC-I downregulation and shown to bind to the Nef acidic cluster ($_{62}EEEE_{62}$) [73]. More recent mapping suggests Nef Y_{120} and W_{113} are additionally required for direct binding with PACS-1/2, and that P_{78} supports the Nef-PACS interaction [37]. Interestingly, similar to Nef, PACS-1 binds AP-1 directly [74], and PACS-1 is known to form a ternary complex with furin and AP-1 [74]. This raises the possibility that Nef may form a similar complex with PACS-1 and AP-1. Consistent with this notion, the Nef-MHC-I fusion protein utilized in producing the ternary complex crystal structure with AP-1 also interacts with PACS-1 [30], suggesting PACS-1 may form a ternary complex with AP-1 and MHC-I that is involved in MHC-I downregulation.

The interactions of PACS-1 with AP-1 and Nef are important in MHC-I downregulation. A PACS-1 mutant unable to bind to AP-1, termed Admut (adaptor mutant), acts as a dominant negative, causing mislocalization of PACS-1 cargo and inhibition of MHC-I downregulation [74]. Furthermore, expression of PACS-1 or PACS-2 mutants unable to bind Nef inhibits MHC-I downregulation [37]. The interaction between PACS-1 and Nef is additionally modulated by post-translational modifications, as PACS-1 is regulated by phosphorylation of an internal acidic cluster ($S_{278}EEEEE$). In its dephosphorylated state, the PACS-1 acidic cluster binds the cargo-binding pocket, blocking cargo binding. This inhibition is relieved via phosphorylation of S_{278}, as a phosphorylation mimic (PACS-$1S_{278}D$) binds more readily to Nef. Conversely, non-phosphorylatable PACS-1 ($S_{278}A$), binds Nef less efficiently, resulting in mislocalization of Nef and inhibition of MHC-I downregulation [75].

While there is evidence that the distinct interactions of PACS-1 with AP-1 or Nef are important for MHC-I downregulation, it is unclear whether a Nef/AP-1/PACS-1 ternary complex is formed. The Nef acidic cluster ($E_{62}EEE_{65}$) is required for direct interaction with both PACS-1 and AP-1 [35,73]. Therefore, a Nef/AP-1/PACS-1 ternary complex would require Nef to interact with either of these proteins via an undefined secondary motif. Alternatively, this may suggest that Nef interacts with PACS-1 and AP-1 in distinct compartments or time points, being tightly regulated by competition for binding to the same site.

3.4. Nef Dimerization in MHC-I downregulation

While Nef oligomers were discovered prior to elucidation of Nef's functions [76], dimerization of Nef has since been determined to be integral to Nef function [77,78]. Through crystal structures, modeling, and mutational analysis, residues critical for dimerization have been identified. Specifically, Nef D_{123} forms ionic interactions with R_{105} on another Nef monomer, flanking an interacting hydrophobic interface constituting I_{109}, L_{112}, Y_{115} and F_{121} [77–79]. Importantly, it has been shown that D_{123} is necessary for dimerization and MHC-I downregulation [77,78]. However, the crystal structure of a Nef dimer in complex with the SH3 and SH2 domains of the SFK Hck revealed four novel dimerization interfaces. This Nef-Hck crystal structure furthermore suggested that upon binding to Hck, Nef homodimerizes leaving D_{123} solvent exposed, despite this residue's previous implication in dimerization [80]. Therefore, Nef may form distinct contacts with other Nef monomers upon binding to host cell proteins, such as SFKs. While it has yet to be directly shown that Nef-mediated MHC-I downregulation requires Nef dimerization, this would not be surprising as Nef dimerization is required for Nef to activate the SFK Hck [81], and SFK activation is an essential step in the signaling pathway of MHC-I endocytosis [30]. Furthermore, Nef dimers co-localize with the TGN marker TGN-46 [78], raising the possibility that Nef dimerization is required for PACS-2 dependent trafficking of Nef to the TGN wherein SFKs are activated.

4. Functional consequences of Nef-mediated MHC-I downregulation

Cell surface MHC-I molecules function to present peptides to external receptors present on other host cells. In healthy cells, these peptides are derived from the cell itself, but during an HIV-1 infection these peptides may be derived from a viral protein. The presentation of a foreign peptide, as in the case of an HIV-1-derived peptide, on MHC-I can then be detected by $CD8^+$ cytotoxic T lymphocytes, which then mediate the killing of these infected cells. Therefore, downregulation of cell surface MHC-I by Nef allows HIV-1 infected cells to evade detection by CTLs by limiting the presentation of viral peptides on the cell surface. Indeed, CTLs are less effective at killing HIV-1-infected cells containing a functional Nef gene, as opposed to HIV-1 lacking Nef expression or expressing a mutant Nef that is unable to downregulate MHC-I [82,83].

Clinically, there appears to be a correlation between rate of progression to AIDS and downregulation of MHC-I, supporting the notion that Nef-mediated MHC-I downregulation allows the virus to evade immune surveillance and spread more rapidly. Recently, a study from Kuang et al. [84] revealed that untreated patients in early stages of HIV-1 infection that maintained a low viral load were more likely to be infected with HIV-1 viruses encoding Nef alleles significantly less efficient at downregulating MHC-I than alleles from patients that did not maintain

low viral loads [84]. An additional cohort of 4244 HIV-1 infected individuals in India demonstrated that the frequency of mutations in the $PxxP_{75}$ motif of Nef, which is essential for MHC-I downregulation, was greater in patients that progress more slowly, if at all, to AIDS [85]. Interestingly, while other studies have not observed a correlation between rate of progression to AIDS and MHC-I downregulation [86–88], these studies were limited by small sample size, with less than a dozen patients per experimental group and likely under-powered to detect significant differences, suggesting further analysis with larger cohorts of HIV-1 infected individuals is warranted.

The Simian immunodeficiency virus (SIV) also encodes a Nef gene that induces MHC-I downregulation. However, the mechanism utilized by SIV Nef to downregulate MHC-I is not as well characterized as that for HIV-1 Nef. Furthermore, certain SIV subtypes, specifically those derived from infection of rhesus macaques (SIVmac 239), may utilize a mechanism distinct from HIV-1 Nef to downregulate MHC-I [89,90]. Despite conservation of the $PxxP_{75}$ motif in SIV Nef [91], the $AxxA_{75}$ mutation in SIVmac 239 Nef does not abolish its ability to downregulate MHC-I, nor was the interaction between SIVmac 239 Nef and AP-1 essential for MHC-I downregulation. Furthermore, the C-terminal 27 amino acids of SIVmac 239 Nef, which are absent in HIV-1 Nef, contains residues essential for MHC-I downregulation [89]. Nonetheless, the ability of SIVmac 239 Nef to downregulate MHC-I correlates with faster progression to disease, weaker $CD8^+$ T cell responses and higher viral load in infected monkeys [92–95]. Additionally, rhesus macaque infection with SIV encoding mutations in the Nef gene that disrupt MHC-I downregulation, while retaining other Nef functions, demonstrate that MHC-I downregulation offers a selective advantage, as Nef reverts to regain MHC-I downregulation [92]. This is supported by elegant *in vivo* studies demonstrating that HIV-1 encoding a Nef allele competent in downregulating MHC-I outcompetes a virus encoding a downregulation-defective allele in the presence of HIV-1 specific CTLs [96].

Along with CTLs, the immune system also utilizes natural killer cells (NK cells) to detect unhealthy cells. NK cells are cytotoxic lymphocytes that induce cell lysis upon binding to cell surface receptors or molecules on neighboring cells. NK cells bind to molecules on the cell surface of neighboring cells via inhibitory or activating receptors, and this balance determines NK-mediated destruction. NK cells bind specific classes of MHC-I molecules and cells lacking sufficient cell surface MHC-I molecules are often targeted for NK-mediated destruction [97]. HIV-1 infected cells avoid this fate by preferential downregulation of specific classes of MHC-I. Nef primarily downregulates MHC-I classes HLA-A, HLA-B, and to a lesser extent HLA-C, while leaving HLA-C, HLA-E and HLA-G relatively unaffected [98–100]. Resistance to Nef-mediated MHC-I downregulation exhibited by certain MHC-I alleles is due to differences in their cytoplasmic tail sequences [100]. A critical residue in the MHC-I cytoplasmic tail implicated in forming a stable ternary complex with Nef and AP-1 is Y_{320}. This conserved residue in HLA-A and HLA-B has been shown to bind a hydrophobic tyrosine binding pocket in AP-1 [36]. Mutation of Y_{320} to A_{320} disrupts co-immunoprecipitation between Nef and AP-1 [44].

5. Conclusion

In the last three decades there have been significant achievements in the understanding of how HIV-1 persists in infected individuals through evasion of the immune system. During this time, it was established that viral proteins, including the HIV-1 protein Nef, allow viruses to hijack host cells to evade detection by the immune system via decreasing cell surface MHC-I. Much progress has been made toward elucidating how Nef mediates MHC-I downregulation, despite lacking enzymatic activity. Our current understanding suggests that Nef forms interactions with host cell proteins to induce both the endocytosis of mature MHC-I from the cell surface, and the lysosomal degradation of MHC-I blocked early in the secretory pathway. However, there is still much we do not know about Nef-mediated MHC-I downregulation, and many questions still remain regarding the connection between the two current models of MHC-I downregulation.

Furthermore, while it is well-established that Nef activates a signaling cascade causing endocytosis of cell surface MHC-I, the remaining membrane trafficking proteins involved in the sequestration of endocytosed MHC-I remain to be elucidated. Evidence suggests that PACS-1 and AP-1 are involved in this process, but these proteins are unlikely to act alone, suggesting that additional endo-lysosomal membrane trafficking factors are likely involved. This could be addressed through experiments with cell free systems to determine the minimal membrane trafficking regulators required to mediate Nef's subversion of the membrane trafficking machinery. Additionally, questions remain regarding the paradox of a potential Nef/AP-1/PACS-1 ternary complex forming while both PACS-1 and AP-1 independently interact with the same interface on Nef.

Future research into Nef's functions will be assisted by recent developments in tools and models that can be translated to study HIV-1 infection. For example, further characterization of MHC-I downregulation could be achieved using live cell imaging of HIV-1-infected cells expressing tagged versions of these proteins. Also, utilization of a mouse model that closely phenotypes HIV-1 infection would prove highly effective for identification and testing of Nef inhibitors. Indeed, it has been suggested that humanized bone marrow-liver-thymus (BLT) mice present a good model for HIV-1 infection, in particular for studying MHC-I downregulation, as the $CD8^+$ T cell response in this model mimics the human response [101].

Importantly, with the high rate of mutation in enzymes targeted by current HIV-1 treatments, there remains a need to develop novel therapies. The ability to inhibit Nef-mediated MHC-I downregulation early in infection could facilitate the host immune system to target HIV-1 infected cells for permanent destruction and thus slow progression of the virus. However, since efficiency of MHC-I downregulation via Nef differs between various Nef alleles in HIV-1 [102], identification of subsets of patients likely to benefit from this approach would need to be determined.

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