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## The Role of the Nef Protein in MHC-I Downregulation and Viral Immune Evasion by HIV-1

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### Abstract

The Nef protein is a major determinant of pathogenicity caused by the human immunodeficiency virus (HIV) and is encoded by the *nef* gene within the genomes of primate lentiviruses HIV-1, HIV-2 and simian immunodeficiency virus (SIV). The HIV Nef protein subverts the intracellular membrane traffic to mediate endocytosis of a number of cell surface receptors to accelerate their degradation. In this review we will examine how the multifunctional Nef can mediate down regulation of the Major histocompatibility Complex (MHC) I complex proteins from the surface of infected cells as a means of immune evasion by HIV. By selectively downregulating MHC-I HLA-A and HLA-B haplotypes, while maintaining the expression of HLA-C, HLA-E and HLA-G the HIV virus is able to avoid recognition by both the NK and cytotoxic CD8<sup>+</sup> T cell effector responses. This protects the virus from cell lysis and enables it to hide from the cell-mediated immune system.

**Keywords:** HIV-1; HLA; MHC; Nef; NK cells

### Introduction

The human immunodeficiency virus (HIV) is a major global health issue that has claimed 39 million lives since its discovery in 1983 [1,2]. In 2013 HIV had a worldwide incidence of 2.1 million cases and a prevalence of approximately 35 million individuals [2]. The virus arose from cross-species transmission with HIV-1 originating from the chimpanzee simian immunodeficiency virus (SIVcpz) and HIV-2 arising from the sooty mangabey virus (SIVsmm) [1]. As a result of the different origins of HIV-1 and HIV-2 their nucleic acid sequences are only 40% homologous and HIV-2 is less virulent with 98% of acquired immunodeficiency syndrome (AIDS) cases arising from HIV-1 infection [1]. The HIV virus can be further broken down into subtypes, with HIV-1 consisting of three subtypes: M, N and O [3]. Group M is the most virulent and is responsible for 90% of the AIDS epidemic worldwide with Group O and N remaining confined within Africa [3,4].

HIV is an obligate intracellular pathogen it must gain entry into the host and hijack its cellular machinery in order to replicate [5]. HIV is a small RNA virus consisting of nine genes [1]. Five of these genes are involved in viral replication and consist of *gag*, *pol*, *env*, *tat* and *rev* and the other four genes *nef*, *vpr*, *vif* and *vpr* are termed accessory proteins [1]. These accessory genes are primarily responsible for HIV's ability to evade the immune response and are dispensable for viral replication [6]. Nef (negative effective factor), a 27-30 kDa myristoylated protein, has been the most extensively researched and is required to help the virus evade the innate and adaptive immune defense and supports AIDS progression [5,7,8]. Nef's crucial role in pathogenesis is due to its ability to markedly increase virus titres while allowing the virus to evade the host immune response [9]. It is expressed early in the virus life cycle and is postrationally modified

by the irreversible attachment of myristic acid to its N-terminus creating an important N-terminus myristoylation site that is required to target Nef to the cellular membrane and can become phosphorylated [10]. Nef has no demonstrable enzymatic function [7,11]. Instead it acts as an adaptor protein by linking molecules to specific cellular factors to propagate its downstream effects [11,12]. These effects include downregulation of CD4, retardation of the immunological synapse and regulation of apoptosis [9,13-16]. Another important function of Nef is its ability to downregulate major histocompatibility complex (MHC) class I molecules and thus protects the virus from cell-mediated and humoral immunity [17,18]. By targeting MHC-I proteins the virus will abrogate immune recognition and activation of by HIV-specific CD8<sup>+</sup> T cells. Therefore, the aim of this review was to highlight the mechanisms by which Nef downregulates class I MHC molecules and the cellular and clinical effects of this function.

### MHC class I downregulation by Nef occurs by two different pathways

MHC-I molecules on virally infected cells present viral antigens to the corresponding antigen-specific CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) [19,20]. The processed peptide/MHC-I molecules are assembled in the endoplasmic reticulum (ER) of the target cell and once loaded with the viral peptide are transported to the cell surface [21-23]. The antigen specific TCR on the CD8<sup>+</sup>CTL recognizes the peptide/MHC-I molecule and it becomes activated and rapidly differentiates into an effector cell that secretes perforin proteins which assemble to form a pore in the cell membrane of the target cell and secreted granzymes enter the cytoplasm of the target cells *via* the perforin pore that results in cell lysis [17,24].

The HIV accessory proteins Nef, Tat and Vpu (viral unique protein) have all been linked to viral modulation of MHC-I expression [25,26]. Tat has been associated with the alteration of MHC-I transcription ... Whereas Vpu's ability to affect MHC-I is similar to Nef in that it downregulates surface expression albeit to a far lesser extent. Although, Tat and Vpu have been shown to modulate MHC-I expression Nef is the key player in HIV-1's abrogation of this important pathway. This is demonstrated by the fact that Nef deficient mutants, which still express Vpu and Tat, have markedly decreased MHC-I expression achieving only 7% of MHC-I downregulation compared to wild type strains of HIV-1 virus. Furthermore, *vpu*- and *tat*- deleted HIV viruses that still expressed Nef failed to significantly downregulate MHC-I molecules [27-29].

There have been conflicting results on the mechanisms by which MHC-I is downregulated. For example, some studies reported that phosphatidylinositol 3-phosphate (PIP3) kinase was required for MHC-I downregulation [30] whilst other studies reported that it was dispensable [31]. This conflict was resolved by the understanding of the importance of D-3 lipid phosphatase (PTEN) which dephosphorylates phosphatidylinositol 3-phosphate (PIP3) [4]. High levels of PIP3 present in cells that lack PTEN, such as Jurkat, CEM and U373 T cells, prevent PACS-1 and phosphatidylinositol 3-kinase (PI3K) inhibitors from affecting MHC-I downregulation [4,31]. This resulted in studies proclaiming that Nef acts independently of PI3K as the PI3K inhibitors LY294002 and wortmannin failed to inhibit Nef-mediated MHC-I downregulation in cell lines that did not express PTEN [31]. The rescue of PTEN expression in these cells restored the sensitivity of MHC-I downregulation to PI3K inhibitors [4].

In addition to the requirements of PI3K, the mechanisms by which MHC-I molecules were downregulated by nef was difficult to define. Some debate arose on whether or not MHC-I molecules were endocytosed or whether nef downregulated MHC-I molecules by targeting their transport to the cell surface [32,33]. Nef can subvert intracellular membrane traffic as part of the virus replication life cycle. Clathrin coated vesicles mediate endocytosis and sorting from the trans-Golgi network (TGN) and endosomes to lysosomes to mediate protein destruction [34]. Vesicular coats are required for intracellular membrane traffic that helps to coordinate cellular organization of vesicles. They are composed of scaffold proteins and adaptor elements that link the scaffold to membrane bound proteins and lipids [35]. Clathrin forms the scaffold on the vesicular coat on to which the heterotetrameric adaptor protein (AP) complexes assemble (e.g., AP-1, AP-2). The vesicular coat is responsible for vesicular traffic originating from the cell membrane and TGN [34]. AP complexes help to connect membrane proteins (i.e., cargo) to clathrin [36]. AP1 needs to be modified by the GTPase Arf1 activator that promotes a conformational change in the AP1 protein that allows to bind to cargo and clathrin at the TGN [37].

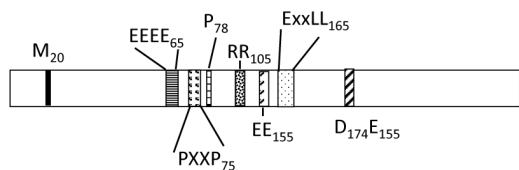
The Nef and Vpu proteins of HIV help to hijack the clathrin pathway [5,6] and are responsible for downregulating the cell surface expression of a range of key target proteins such as CD4, MHC-I and tetherin [38-41]. The AP1 protein plays a critical role in enabling Nef to re-route MHC-I and tetherin from the cell surface and directs them to lysosomes [11,42]. The cargoes carried by AP1 contain either tyrosine based sorting signals which bind to the C terminal domain of clathrin  $\mu$ 1, or dileucine signals, that bind to a site that spans the  $\gamma$  and  $\delta$ 1 subunits of clathrin [43]. As a result Nef can hijack AP-1 to downregulate MHC-I expression by interacting with the  $\mu$ 1 subunit of

clathrin via tyrosine motif binding site on its c- terminal domain [44]. In addition Nef can hijack AP-2 to downregulate CD4 via the dileucine binding site on the clathrin  $\alpha$ - $\sigma$ 2 complex [13,45]. Both these binding sites are normally obstructed when AP1 is in its "locked" conformation. Therefore Nef's ability to subvert membrane traffic relies on the AP1 protein being in the "unlocked" conformation which is linked to the GTPase activity of Arf1. Shen and colleagues have recently shown that Nef can bind to Arf1 and this induces the trimerization of AP-1. These trimers assembled into hexamers to help stabilize the AP-1-Arf1 complexes and in doing so can redirect MHC-I and tetherin to lysosomes [46].

It has now become apparent that there were two distinct pathways of Nef-mediated MHC I downregulation [8]. They are termed the "signalling mode" and the "stoichiometric mode" and act at distinct temporal stages of viral infection [8]. Both pathways are dependent on Nef's N-terminal myristoylation site to assemble within lipid rafts in the plasma membrane. It appears that the "signalling mode" for MHC modulation is utilized in response to acute infection over the first 48 hours that specifically targets the 'mature' MHC-I proteins that are present at the cell surface [8], while the stoichiometric mode is a distinct mechanism utilized by the virus after day 3 of infection and blocks the transport of newly synthesized MHC-I proteins from the ER to the cell surface [8,47-49]. In this way the HIV-1 virus can effectively block immune recognition of virus infected cells by antigen-specific CD8<sup>+</sup> CTLs. It has been suggested that the HIV-1 virus may utilize these different pathways to modulate MHC-I to enable it to adapt to different cellular reservoirs. Infection of CD4<sup>+</sup> T cells become acutely infected with HIV-1 with a half-life of 1.5 days and during this time the virus focuses on the signalling mode to downregulate mature MHC-I proteins from the cell surface [8]. Once the virus has established latency leading to chronic infection of a reservoir (e.g. CD4<sup>+</sup> T cells or monocytes), during this period the virus relies on the stoichiometric mode to block transport of newly synthesized MHC-I proteins to the cell surface.

### Signalling mode

MHC-I proteins have a range of fates within the cell. Newly synthesized MHC I proteins are loaded with peptides within the ER and transported to the cell surface. Protein turnover directs internalized MHC complexes to endosomes which can be targeted to the TGN where they can be ubiquitinated and targeted for degradation. Alternatively the MHC-I proteins may enter a recycling pathway where they can be removed from the cell surface and be recycled back through vesicles to cell surface. These two recycling pathways are ubiquitous in cells and require distinct adaptor proteins known as phosphofurin acidic cluster protein (PACS)-1 and PACS-2 [50]. PACS-1 is involved in recycling of internalized cargo proteins from early endosomes to enable proteins to return to the cell surface [51,52]. PACS-2 on the other hand mediates the trafficking of protein to the ER and can also regulate the trafficking of endocytosed protein cargoes from early endosomes to the TGN [53,54]. Both PACS proteins are multipurpose proteins as they contain furin binding regions that promote binding to cargo proteins, sorting adaptors and kinase enzymes [50]. Evidence suggests that the PACS proteins mediate distinct steps within the signalling pathway. PACS-2 is required for the initial triggering phase and PACS-1 is for the sequestering phase of MHC modulation [8].



**Figure 1:** Functional domains on the Nef protein of HIV-1. The key functional domains of the Nef proteins are illustrated and some of these are described in detail within the text. The M<sub>20</sub>, EEEE<sub>65</sub> acidic cluster and the PXXP<sub>75</sub> SH3 binding domain are the major functional domains on Nef that are required for modulation of MHC-I surface expression.

Nef has the capacity to activate a range of Src family kinases (SFKs) including Hck, Lyn and c-Src and this property appears to be important for HIV disease pathogenesis in animal models [55,56]. In addition Nef can activate PI3K and this is required to block apoptosis and to direct downregulation of MHC-I proteins from the cell surface [30,57]. There are three motifs on the Nef protein that are crucial for its effector activity (Figure 1). The EEEE<sub>65</sub> acidic cluster is required for its interaction with cytosolic sorting protein PACS-1 [58], an SH3 binding domain motif PXXP<sub>75</sub> enables Nef to interact with SFKs [55]. The methionine M<sub>20</sub> residue located at the N-terminus is critical to enable MHC-I proteins to associate with the heterotetrameric sorting adaptor protein AP-1 [11]. Furthermore, the D123 residue is required for Nef to dimerize as the mutant D123G is unable to dimerize and downregulate MHC-I [59]. These motifs are highly conserved in the M group HIV-1 and are responsible for disease pathogenesis [60].

The “signalling mode” is the initial pathway used by the virus to downregulate mature MHC-I molecules that are already expressed at the cell surface of a target cell. The pathway involves two domains on Nef, the EEEE<sub>65</sub> acidic cluster and the PXXP<sub>75</sub> SH3 domain binding site, which are both located at the N-terminus of the protein [30,58,61]. Following viral infection Nef can downregulate MHC-I by activating an endocytic program that requires the Nef’s acidic cluster (EEEE<sub>62-65</sub>) to bind to the endosomal sorting protein PACS-2 and this targets the Nef to a paranuclear region [30,61]. This change to its cellular localization facilitates the interaction of the Nef PXXP<sub>75</sub> SH3 binding domain to form a multiprotein complex involving Src Family kinases (SFK)/zeta associated protein kinase 70 (ZAP70)/phosphatidylinositol kinase 3 (PI3K) i.e. SFK/ZAP70/PI3K [4,8,62]. ZAP-70 is a SFK found in T cells that normally associates with the T cell receptor (TCR) complex. Monocytes can be a source of HIV-1 infection due to expression of CD4 and chemokine receptors. In monocytes the SFK Syk replaces ZAP-70 to form the protein complex with Nef and PI3K. The SFK/ZAP-70/PI3K complex can induce tyrosine kinase signalling and this can increase the rate of endocytosis of cell surface MHC-I proteins from the cell surface [4,30,58]. Once the MHC-I proteins are internalized they can be sequestered within the paranuclear region which requires the M<sub>20</sub> and EEEE<sub>65</sub> domains of Nef interacting with PACS-1 and the AP-1 protein [11,30]. The activation of PI3K triggers the clathrin-independent, GTPase ADP ribosylation factor 6 (ARF6)-dependent endocytosis of MHC-I molecules [8,30]. ARF6 is involved with connecting actin cytoskeletal rearrangements with vesicle trafficking [30]. Once the MHC-I molecules are within the ARF6 endosomes the (M<sub>20</sub>) methionine residue on Nef is required for

sequestering internalized MHC-I molecules to the TGN to prevent further MHC-I recycling [4,30].

### Stoichiometric mode

The stoichiometric mode is utilized after day 3 of infection and affects the transport of newly synthesized MHC-I complexes to the cell surface [8]. By 72 h post infection the stoichiometric mode is upregulated while the signalling mode is extinguished suggesting a temporally determined means of MHC-I downregulation [8]. It is hypothesized that this initial latency in the stoichiometric mode is due to the fact that sustained PI3K signalling driven by the SFK/ZAP-70/PI3K complex is required for its initiation [8]. In contrast to the signalling pathway, the stoichiometric mode is independent of PI3K signalling and involves the diversion of newly synthesized MHC-I molecules in the TGN to lysosomal compartments that is mediated by the M20 residue on Nef /AP-1 and the process is clathrin dependent [8,11,63]. The stoichiometric pathway is initiated when Nef binds to the tyrosine residue at position 320 on the cytoplasmic tail of the MHC-I molecule *via* its acidic cluster (EEEE<sub>65</sub>) and proline rich repeat (PXXP<sub>72-75</sub>) [11,33,64]. As highlighted above the Nef-MHC-I complex creates a hydrophobic region which enables the  $\mu$ 1 subunit of clathrin to bind [11,65]. Once it is bound, AP-1 links clathrin to the cytoplasmic tails of MHC molecules at the TGN and transports these molecules to endosomes where they are tagged for lysosomal degradation by the recruitment of  $\beta$ -COP [11]. A small molecular inhibitor that prevents the SFK-Nef interaction can prevent assembly of the SFK/Zap70/Syk-PI3K cascade and in turn can repress HIV-1-mediated MHC-I downregulation in primary CD4<sup>+</sup> T cells [8]. These studies highlight the crucial role for formation of the multikinase complex for MHC modulation and that signalling by one or more of the kinases influences the membrane trafficking machinery that mediates the switch between the two functional modes [8].

### The importance of the MHC cytoplasmic tail in immune evasion from NK cells and CTLs

The Nef protein has been shown to specifically target the classical MHC-I molecules of the Human Leukocyte Antigen (HLA) complex in particular HLA-A and HLA-B but does not affect the HLA-C, HLA-E or HLA-G molecules [17,66,67]. The ability of Nef to bind to MHC-I proteins is conserved across different HIV-1 M, N and O subtypes as well as primate lentiviruses including Simian immunodeficiency viruses (SIV) SIVcpz and SIVrcm [67]. Nef’s ability to downregulate the MHC-I alleles is determined by the amino acid residues within the cytoplasmic tail of the heavy chain (Table 1). The key amino acid residues on the cytoplasmic domain of MHC-I molecules that facilitates Nef binding to mediate downregulation of MHC-I molecules occur between positions 321 and 328 [17,33,44]. In HLA-A and HLA-B molecules there is a tyrosine residue at 321 and an aspartic acid residue at 328 whereas in HLA-C molecules there is a cysteine occupies position 321 and asparagine at 328 [17,44] (Table 1). These amino acid substitutions in HLA-C are sufficient to prevent the Nef molecule from binding to the HLA-C molecule and thereby prevents its downregulation [17]. The HLA-E molecule also inhibits Nef binding with a substitution of glutamic acid at position 325 on its cytoplasmic tail whereas HLA-A, -B and -C molecules contain an alanine at the same site [17] (Table 1). Mutating the glutamic acid residue at position 325 to alanine enables the HLA-E molecule to be responsive to Nef downregulation, demonstrating the importance of this amino acid in inhibiting this process [17]. In contrast to the other HLA molecules,

the HLA-G molecule evades HIV downregulation by having a premature stop codon which results in a shorter cytoplasmic tail of just six amino acid residues instead of the normal length of 29-33 amino acids [66].

HLA molecule	Amino acid resides at the designated positions on the MHC proteins		
HLA-A	Y321	A325	D328
HLA-B	Y321	A325	D328
HLA-C	C321	A325	N328
HLA-E	Y321	E325	D328

**Table 1: The different cytoplasmic amino acid residues amongst the four HLA haplotypes -A, -B, -C and -E.** The Table shows the amino acid residues on the different Class I HLA molecules based on the primary amino acid sequence. The amino acid residues that Nef requires for downregulation of classical MHC proteins (HLA A & B) to prevent immune recognition by CD8<sup>+</sup> T cells are represented in normal text. The amino acid residues highlighted in bold text are those that prevent Nef mediated MHC downregulation and therefore prevent NK cell activation.

This differentiation between the different HLA haplotypes is important as natural killer (NK) cells target cells can be activated in response to limited MHC-I expression [17]. HLA-C and HLA-E inhibit NK lysis by ligating with the inhibitory receptors on the surface of NK cells (iNKR) [17,68]. These iNKRs are designed to protect normal host cells from NK lysis [68]. Therefore, the maintenance of these specific HLA proteins by Nef promotes survival of the infected cell allowing viral replication to occur as it will avoid NK cell activation [17]. Although the majority of NK cells are inhibited by this method there is a certain subset of NK cells that have an inhibitory receptor (NKB1) that is specific for certain HLA-B molecules [17]. Further research is needed to determine the role these NKB1 expressing NK cells play on HIV disease progression. Nevertheless NK inhibition is further compounded by the Vpu protein of HIV-1 which downregulate NTB-A surface expression to inhibit NK degranulation [68].

In addition to NK cells, downregulation of MHC-I molecules by Nef helps the HIV virus to evade recognition by CTLs [69,70]. Most CTLs are restricted to the HLA-A and HLA-B haplotypes which are targeted by the Nef protein [17]. This is important as CTLs are recognized as an important effector response against HIV infection and without downregulation of MHC-I molecules the infected cells would become susceptible to lysis by HIV-specific CTLs [69]. This selective downregulation of MHC-I of HLA haplotypes is similar to that used by the foetus to maintain tolerance from the maternal immune system [71]. Interestingly, a small subset of CTLs are specific for the HLA-C or HLA-E haplotypes [72]. Further research is needed to determine the role that this CTL subset might have on HIV disease progression.

### Clinical relevance of MHC-I downregulation

The ability of HIV-1 Nef to downregulate MHC-I is dependent on disease progression. MHC-I downregulation is enhanced and maintained in immunocompetent (especially asymptomatic) individuals and lost in those that have progressed to AIDS [73]. This is believed to be due to the high selective pressure exerted by active CTLs in immunocompetent individuals [74].

The importance of Nef's ability to downregulate MHC-I molecules *in vivo* and its impact on disease progression is highlighted by studies on rhesus macaque monkeys that were infected with SIV viral strains that carried specific mutations in the *nef* gene that selectively affects its ability to downregulate MHC-I [75,76]. By mutating the Y223F residue in the *nef* gene the SIVmac239 variant virus blocks MHC-I downregulation without affecting Nef's other functions such as CD4, CD28 and CD3 down-regulation and viral infectivity or viral set point load [75]. Infection with the Y223F-Nef variant virus resulted in a chronic viral infection but the animals remained clinically healthy at 56 weeks post infection [75]. This is in direct contrast with rhesus monkeys that were infected with the wild type SIVmac239 virus which had a 60 to 70% fatality rate within the first year [75]. Interestingly, the Y223F-Nef viral variant reverted to regain its capacity to downregulate MHC-I downregulation within 2-4 weeks post infection [75]. This coincides with the peak in CTL response in SIV-infected macaques which occurs at week 2 [75]. Therefore, this reversion of MHC-I downregulation is likely due to CTL selective pressure [75]. Despite the fact that 4 weeks post infection the virus-mediated reversion of its Y223-Nef to wild type Nef the macaques demonstrated non-progressor or slow-progressor phenotypes [75]. This might indicate a need for MHC-I downregulation in the acute phase of the infection [75].

In both studies the mutated Nef's ability to downregulate MHC-I molecules was regained *via* increased mutation rates facilitated by CTL selective pressure. This increased rate of mutation to recover the MHC-I downregulation function of Nef was so important that its recovery at times occurred at the expense of its other functions such as the downregulation of CD28 [75,76]. This demonstrates that the increase in MHC-I modulation enables the virus to escape CTL detection and is maintained in the presence of these cells [77].

Interestingly, the ability of HIV-1 Nef to downregulate MHC-I changes during disease progression. MHC-I downregulation is 10- to 20-fold higher in immunocompetent (especially asymptomatic) individuals compared to those that have progressed to AIDS [73]. In AIDS patients the Nef protein's functions are centred towards increasing viral replication and infectivity, whilst increasing CD4 downregulation [73]. This is believed to be due to the high selective pressure exerted by active CTLs in immunocompetent individuals, which is lost when the immune system is destroyed and AIDS progression has occurred [74].

### Conclusion

In conclusion, Nef is a multifunctional pathogenicity factor expressed by primate lentiviruses. Disruption of *nef* is associated with defective viral replication. However the Nef protein has a wide range of functions unrelated to infectivity of virions, some of those which are directly related to immune evasion such as down modulation of CD4 [38,39], MHC-I [30,78] and to modulate the threshold of T cell activation between T cells and macrophages [79,80].

The ability of Nef to interfere with the MHC-I complex is an important virulence factor as it enables the HIV virus to evade the host immune response. By selectively downregulating MHC-I HLA-A and HLA-B, while maintaining the expression of HLA-C, HLA-E and HLA-G HIV is able to evade both the innate response mediated by NK cells and the adaptive immune response elicited by CTL effectors. This protects the virus from immune recognition by both arms of the immune response. Further research is needed to determine if any of these pathways used in the downregulation of MHC-I can be safely

inhibited for use as a therapeutic. Furthermore, determining the percentage of long term non-progressors that exhibit CTLs that are specific for HLA-C and HLA-E or NK cells that are specific for HLA-A and HLA-B would also assist in determining the importance of the selectivity in MHC-I downregulation and the possibility of these cells being used as a means of prevention against HIV.

Although we have focused in this review on the function of Nef in modulation of MHC-I proteins. It must not be forgotten that the Nef protein can target a range of other immune signalling proteins e.g., TCR and CD3 as well as the costimulatory receptor CD28 that would effectively block naïve T cell activation. So the HIV virus has a range of weapons in its arsenal to intervene with cellular function and more widely to target specific aspects of cell biology that are going to make it conducive for viral replication and to avoid immune recognition.

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