Elucidating the consequences of HIV-1 immune escape from host CTL selection pressure

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Abstract

This thesis focuses on HIV-1 adaptation to CTL immune response and presents original data on the consequences of 10 published HLA-B*13-associated CTL escape mutations in Gag, Pol and Nef on HIV-1 replication capacity (RC) and Nef-mediated CD4 and HLA class I downregulation. B*13-driven immune escape at Gag-I147L and -I437L incurred replicative costs of 5% and 17% on *in vitro* viral RC, which was rescued to wild-type (HIV-$1_{NL4.3}$) levels by Gag-A146S/K436R and Gag-K436R, respectively. One major observation was that B*13-driven double mutation, $Nef_{E24Q/Q107R}$ impaired this protein's HLA class I downregulation ability by 40%, with no evidence of replicative or expression defects. Moreover, cells infected with double mutant virus were "visible" to HIV-specific T cells. Our results thus suggest that B*13-mediated protective effects on HIV-1 disease progression may be attributable, in part to a novel mechanism – namely, the selection of escape mutations in Nef that dampen one of HIV-1's key immune evasion strategies.

Keywords: human leukocyte antigen class I (HLA class I); cytotoxic T lymphocyte (CTL); escape mutation; replication capacity; HLA class I downregulation

Dedication

This thesis is dedicated in the loving memory of my late father, Shahid Hussain. May his soul continue to rest in peace, Ameen!

A deep and sincere feeling of gratitude to my mother, Masroor Jehan, my siblings, Aafia Shahid and Zia Ahmed; their kind words have motivated and encouraged me throughout these years.

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List of Acronyms

AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
CCR5	C-C chemokine receptor type 5
CTL	Cytotoxic T lymphocyte
CXCR4	C-X-C chemokine receptor type 4
ELISpot	Enzyme-linked immunosorbent spot
GALT	Gut-associated lymphoid tissue
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
MHC	Major histocompatibility complex
PBMC	Peripheral blood mononuclear cell
PR	Protease
PrEP	Pre-exposure prophylaxis
RC	Replication capacity
RT	Reverse transcriptase
SIV	Simian immunodeficiency virus
START	Strategic timing of antiretroviral treatment
TasP	Treatment as prevention
TCR	T cell receptor
WHO	World Health Organization

Thesis overview

This thesis is a compilation of original work published, or submitted for publication on the topic of HIV-1 evolution under host immune selection pressures, particularly $CD8^+$ cytotoxic T lymphocytes (CTLs). The thesis is structured in a manuscript-based format and comprises four chapters:

Chapter 1 is a brief overview of HIV-1 epidemiology, genome organization, life cycle, pathogenesis, natural history, and host cellular immune responses.

Author contributions: I wrote this chapter as an introductory section. My senior supervisor read the text and edited it for coherence and clarity.

Chapter 2 is a literature review on HIV-1 immune escape and viral adaptation to host immune responses mediated by $CD8^+$ cytotoxic T lymphocytes (CTL), humoral (antibody), innate and vaccine-induced immune responses.

Author contributions: This chapter was a combined effort of my supervisor and I, which has been recently published as a book chapter in "*Global Virology - Identifying and Investigating Viral Diseases*" (Springer publications).

Chapter 3 is an original research chapter that addresses the thesis' key question, which is to identify the impact of HLA-B*13-associated immune escape mutations on HIV-1 replication capacity and Nef-mediated CD4 and HLA class I downregulation function.

Author contributions: My supervisor and I designed the study. I was directly involved in data collection and analysis. I performed the following experiments: PCR amplification and DNA sequencing, replication capacity, Nef-mediated CD4 and HLA class I downregulation assay, Nef TCR co-culture assay and western blots. Our collaborator, Alex Olvera (IrsiCaixa AIDS Research Institute - HIVACAT, UAB, Barcelona, Spain) performed the interferon-gamma (IFN-γ) assay for epitope mapping. I originally wrote the chapter in form of a manuscript, which was extensively edited by my supervisor before submission to an international peer-reviewed journal.

Chapter 4 provides a summary of the thesis and proposes future directions on studies related to HLA-associated immune escape mutations.

Author contributions: I wrote this chapter as a summary section. My senior supervisor read the text and edited it for coherence and clarity.

Chapter 1.

Introduction to HIV/AIDS

1.1. The HIV/AIDS pandemic

If left untreated, human immunodeficiency virus (HIV)-1 infection is characterized by the progressive depletion of $CD4^+$ T cells, rendering the host susceptible to a variety of opportunistic infections that define the acquired immunodeficiency syndrome (AIDS) [1-3]. Although both HIV-1 and HIV-2 can cause AIDS, HIV-1 is the leading cause of AIDS pandemic that has claimed approximately 39 million lives, with nearly 37 million people currently living with HIV-1 worldwide [4,5]. Despite concerted efforts, an HIV vaccine remains a distant goal and faces enormous challenges [6]. One of them is HIV's extremely high mutational plasticity that yields extensive viral genetic diversity both within and among human hosts globally [7]. Furthermore, although antiretroviral drugs [8] given in combination as Highly active antiretroviral therapy (HAART) [9] have resulted in significant declines in HIV-related morbidity and mortality [9-12], global disparities in HAART implementation [13,14] and drug resistance remain ongoing challenges for controlling HIV-1 spread. Finally, although HAART effectively suppresses active viral replication, viral latency remains another major hurdle for HIV-1 eradication strategies. HIV-1 latently infected cells contain a stably integrated HIV-1 provirus that remains quiescent in the post-integration state [15]. However these viruses can emerge upon cessation of antiretroviral therapy (ART) and contribute to increased viremia [16]. As such, HIV-1 eradication remains a challenge [17].

1.2. Organization of the HIV-1 genome

HIV-1 is an enveloped RNA virus containing a ≈9.8Kb single stranded genome packaged in a ≈100-300 nm viral particle [18]. The RNA genome encodes nine genes,

which translate into 15 proteins; these include structural (*gag*), non-structural (*pol*), envelope (*env*) [19], regulatory (*tat* and *rev)* and *accessory* genes (*vif, vpr, vpu* and *nef)* (Figure 1.1) [20,21]. HIV-1 Gag is transcribed as a single precursor polyprotein, $pr55^{Gag}$ [22,23], which is subsequently cleaved by the HIV-1 protease enzyme [24] into matrix (MA, p17), capsid (CA, p24), spacer peptide 1 (SP1; p2), nucleocapsid (NC, p7), spacer peptide 2 (SP2, p1), and p6 proteins. Pol is expressed as a Gag-Pol precursor polyprotein ($pr160^{Gag\text{-}Pol}$) [25,26], which is generated by a ribosomal frameshift during the translation of viral mRNA [27]. It is further cleaved by viral protease into enzymes, including protease (PR, p16), reverse transcriptase (RT, p51) plus the ribonuclease H domain (RNase H), and integrase (INT, p31) [25]. The *env* gene encodes glycoprotein precursor gp160, which is subsequently cleaved by the host protease, Furin to yield envelope surface glycoprotein (gp120) and the transmembrane glycoprotein (gp41) [28] [29]. The expression of regulatory (*tat* and *rev*) and accessory genes (*vif, vpr, vpu* and *nef*) are associated with various functions, summarized in Table 1 [30,31].

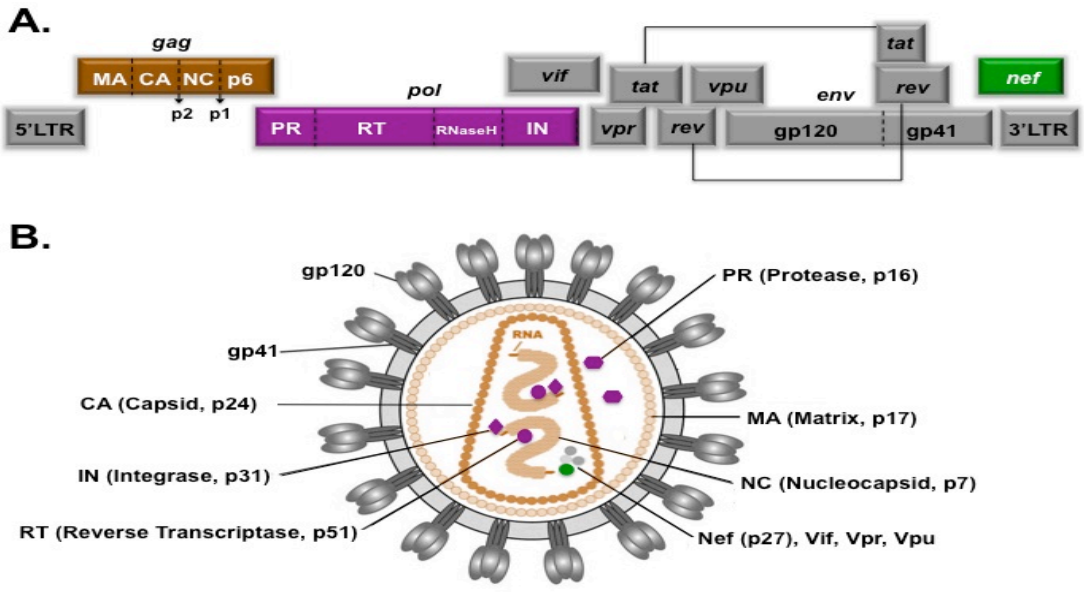

Figure 1.1. Organization of the HIV-1 genome.

A: Genomic organization of HIV-1, with each rectangle representing a major gene encoding structural (Gag), non-structural (Pol), envelope (Env), regulatory (Rev and Tat), accessory (Vif, Vpr, Vpu and Nef) proteins, and long terminal repeats (LTRs). **B:** HIV-1 virion structure. Note that Gag, Pol and Nef are colored to match the color scheme used in chapter 3. (Virion structure modified from: http://web.archive.org/web/20050531012945/http://www.niaid.nih.gov/factsheets/howhiv.htm).

Table 1.1. List of HIV-1 proteins and their functions

Gene/Protein	Function [19,30]
LTR: (Long terminal repeat)	Region required for viral genome transcription
Gag (Group specific antigen)	**Matrix (MA):** provides icosahedral shape to the virus **Capsid (CA):** forms conical structure; encapsulates viral RNA **Spacer peptide 1 (SP1, p2):** no known function **Nucleocapsid (NC, p7):** RNA/DNA binding protein, critical for chaperoning of viral genomic RNA during virion assembly **p6:** regulates budding of nascent virions from cell membrane **Spacer peptide 2 (SP2, p1):** no known function
Pol (Polymerase)	**Protease (PR):** performs proteolytic processing of polyproteins **Reverse Transcriptase (RT):** transcribes single-stranded RNA into double-stranded DNA **RNaseH:** specifically degrades RNA in RNA:DNA hybrids after reverse transcription **Integrase (IN):** integrates viral DNA into host chromosomal DNA
Env (Envelope)	**gp120:** binds to host CD4 and co-receptors CCR5 and/or CXCR4 to initiate viral entry **gp41:** promotes fusion of virion with plasma membrane
Vif (Virulence infectivity factor)	Restricts inhibitory function of host factor (APOBEC3G)
Vpr (Virulence protein R)	Promotes nuclear import of pre-integration complex
Vpu (Viral protein U)	Promotes intracellular degradation of CD4 and virion release
Tat (Transactivator protein)	Promotes viral RNA transcription
Rev (Regulator of viral expression)	Regulates viral RNA nuclear export
Nef (Negative infectivity factor)	Promotes CD4 lymphocyte activation, cell cycle arrest, enhances infectivity by downregulating CD4, downregulates HLA-A and -B to escape host CTL recognition

1.3. HIV-1 life cycle

HIV-1 preferentially infects $CD4^+$ T cells, however macrophages and dendritic cells are also susceptible to infection [32-34].

The HIV-1 life cycle begins with the attachment of envelope glycoprotein to the CD4 cell surface receptor, followed by attachment to co-receptors CCR5 and/or CXCR4 on the host cell surface [35-37]. This allows the viral envelope to fuse with the host cell membrane, facilitating viral entry [38] and release of the viral capsid containing the RNA genome and viral enzymes inside the host cell [38]. Following entry, reverse transcriptase initiates the formation of one double-stranded molecule of viral DNA by copying the sequence of the RNA genome [39]. This viral DNA enters the nucleus of the host cell and viral integrase facilitates the integration of viral DNA into the host's DNA. The integrated form of the HIV-1 genome is known as the provirus. The proviral DNA serves as a template for transcription of viral messenger RNAs (mRNAs) [40]. The production of viral mRNA and its translation in the cytoplasm is regulated by host and viral mechanisms. The newly formed viral RNA is transported to the cytoplasm [41], where it is translated into polypeptide chains, which mature to form viral proteins and enzymes. The newly formed viral RNA genome along with proteins move toward the cell membrane where they accumulate and virion assembly is initiated [42,43]. The newly assembled viral particles bud out of the plasma membrane, maturing into infectious virions, which subsequently infect other cells [30,41].

1.4. Natural history of HIV-1 infection in the era of HAART

The natural course of HIV-1 infection has radically changed over the past two decades [3]. The advent of HAART has dramatically reduced HIV-1-related morbidity and mortality [44] such that HIV-1 infection has now become a manageable chronic condition in patients who achieve durable virologic suppression [45]. As per World Health Organization (WHO) guidelines, antiretroviral treatment should be initiated in HIV-infected adults who have a CD4 count below 500 cells/mm^3 [46]. HAART effectively decreases plasma viral loads to undetectable levels in the blood and other body fluids in the majority of treated patients [47,48], thereby slowing host immune deterioration and

preventing the emergence of opportunistic infections.

Suppression of HIV-1 RNA in the blood and sexual fluids to undetectable levels by HAART reduces an individual's risk of HIV transmission to other individuals to near zero [49,50]. This observation is the key to “treatment as prevention” (TasP) [51] strategies that use antiretroviral therapy to reduce HIV-1 incidence [52]. Antiretroviral therapy can also be used for pre-exposure prophylaxis (PrEP), which involves the use of antiretroviral drugs orally or topically (either vaginally [53] or rectally[1]) to prevent sexual or parenteral HIV-1 infection. The first clinical trial that demonstrated prophylactic effects of antiretroviral drugs was the CAPRISA 004 trial that evaluated a microbicide gel containing tenofovir (a drug that blocks viral reverse transcription). A total of 889 rural and urban South African women were enrolled in the study, with the recommendation to use the gel before and after sex. HIV-1 acquisition was reduced up to 39% in these women [53]. Subsequent studies have since confirmed the benefits of PrEP [54-56]. Based on these findings, PrEP has now become a part of the comprehensive HIV prevention plan, where the U.S. Food and Drug Administration (FDA)-approved drug Truvada®, is recommended for daily use in high-risk populations, such as homosexual men who engage in unprotected sex, uninfected heterosexual individuals who engage in sex with intravenous drug users, or with HIV-positive individuals, and/or individuals who share needles or inject drugs. Truvada® is a commercial formulation of two HIV ARV drugs, tenofovir and emtricitabine, which block HIV-1 reverse transcription [57,58].

With no effective vaccine currently available against HIV [8, 9], HAART remains the mainstay of HIV treatment [10]. In fact, a major international randomized clinical trial, Strategic timing of antiretroviral treatment (START), recently reported that early initiation of HAART, irrespective of the CD4 count, improves clinical outcomes for HIV-1-infected individuals [59]. Specifically, HIV-1-infected individuals who initiate HAART early, have a 53% lower risk of developing AIDS or other associated illnesses compared to those who

[1] The phase II clinical trial of the rectally-applied microbicide gel, known as MTN-017 is currently underway, that aims to enroll a total of 186 HIV-negative men who have sex with men (MSM) and transgender women in Peru, South Africa, Thailand and the United States. http://fenwayfocus.org/2013/10/fenway-one-of-8-sites-worldwide-enrolling-participants-in-first-ever-phase-ii-microbicide-trial/.

initiate HAART when CD4 counts fall below 350 cells/mm^3. Therefore, early HAART offers a dual benefit; not only does it improve clinical outcomes in HIV-1-infected persons but it also reduces the risk of HIV-1 transmission to others [59].

1.5. HIV-1 and host cellular immune responses

The natural history of HIV-1 infection is influenced by host cellular immune responses. Most HIV-1-infected individuals mount a robust immune response to the virus during the first few months of infection, however these responses become less effective over time [60]. Therefore, it is imperative to understand the immunological outcomes that may influence HIV-1 disease progression.

There are four major types of host immune T cells among others: $CD4^+$ T-helper (Th1), $CD8^+$ (cytotoxic T-lymphocyte, CTL), suppressor (Treg) and effector (Th17) T cells [61]. However, antigen-specific cellular immune responses include the role of $CD4^+$ and $CD8^+$ T cells. Both cell types express a unique T cell receptor (TCR) on their cell surface, which determines antigen specificity.

T cell recognition of virally-infected cells is dependent upon the interaction of two cells; *Antigen-presenting cells* (APCs) which process and present viral antigens, loaded onto major histocompatibility complex (MHC) molecules, also known as human leukocyte antigen (HLA) in humans. However, *Antigen recognizing cells (ARCs)* ($CD4^+$ and $CD8^+$ T cells) recognize the peptide antigen bound to an HLA molecule presented on the APCs. [62-64].

1.5.1. HIV-1 antigen processing and presentation of HLA-restricted peptides to T cells

HLA class I and II molecules are crucial for T cell mediated immune responses. T cells recognize foreign antigenic peptides that are generated by intracellular protein degradation, bound to HLA class I or II, and presented at the surface of infected cells.

$CD8^+$ T cell responses are triggered upon the recognition of HLA class I-bound peptide *via* the specific TCR. These peptides are mainly derived from the digestion of

proteins in the cytosol of an infected cell, known as *endogenous* or *intracellular* proteins. For HLA class I antigen processing, proteasomal degradation disrupts cytosolic viral (antigen) proteins into small fragments called “peptides”, that are generally 8-11 amino acids in length. Transporter associated proteins (TAPs) translocates the peptides into the lumen of the endoplasmic reticulum (ER). Followed by gene expression, HLA class I molecules are concurrently synthesized, translocated and assembled into the lumen of ER, where the peptides bind to these molecules and after final processing in Golgi apparatus, the peptide-HLA class I complex is presented on the infected cell surface to be recognized by antigen-specific TCR on $CD8^+$ T cell (CTL) [61,65-67].

On the other hand, $CD4^+$ T cell responses are triggered upon the recognition of HLA class II-bound peptide complex *via* the antigen-specific $CD4^+$ TCR. For HLA class II antigen processing, extracellular proteins are endocytosed by antigen-presenting cells (APCs) and degraded into small antigenic peptides that are generally 12-18 amino acids long. Endocytic vesicles fuse to the peptides, where they interact with HLA class II molecules. HLA class II α and β chains, class II-invariant peptide (CLIP) and invariant chain (Ii) are simultaneously assembled in the ER lumen, however they cannot bind peptides, as the CLIP complex occupies the peptide-binding site. The heterodimer complexes leave the ER, pass *via* Golgi apparatus to fuse with endocytic vesicles. The Ii component is degraded, and with the assistance of HLA-DM and HLA-DO (intracellular proteins involved in class II antigen presentation), the peptide binds to HLA class II molecules. The peptide/HLA class II complex is finally translocated to the plasma membrane, where the complex is recognized by $CD4^+$ T cells [67].

1.5.2. $CD4^+$ T helper (Th1) immune responses

$CD4^+$ T helper cells are immunomodulatory cells. As mentioned above, $CD4^+$ T cell immune responses are triggered upon the recognition of HLA class II-bound peptide on the surface of a professional antigen presenting cell by the specific TCR on $CD4^+$ T cell. For a particular phenotype to be differentiated, cytokine-mediated $CD4^+$ T cell signaling pathways are required. Activated T helper type 1 (Th1) cells secrete cytokines such as interleukin-2 (IL-2) and interferon-gamma (IFN-γ) which help sustain CTL responses, whereas T helper type 2 (Th2) cells secrete IL-4, IL-5, IL-6 and IL-10,

which help in promoting B-cell responses against viral infection [68].

HIV-1 mainly targets $CD4^+$ T cells. If left untreated, the resulting loss of these cells alters host immune function and leads to the progression to AIDS [69,70]. Upon HIV-1 infection, 30-60% of $CD4^+$ T cells are destroyed in the gut-associated lymphoid tissue (GALT) within four days [71] (though HAART can partially rescue these losses [72-74]).

Despite the fact that $CD4^+$ T cells are depleted upon infection, strong HIV-1-specific $CD4^+$ T cell responses are attributed to control of viral replication. For instance, a vigorous $CD4^+$ T cell response in acute HIV-1 infection leads to the subsequent control of viral replication [75], and viral escape from $CD4^+$ T cell-targeted epitopes have been observed, where mutations arise in recognized $CD4^+$ epitopes found in Gag, Nef and Integrase [76,77], suggesting that $CD4^+$ T cells can exert selection pressure on the virus. Whether or not CD4 T cell responses are required for CTL responses [78], remains inconclusive, because CTL response can be generated in the absence of $CD4^+$ T cells as well [79].

1.5.3. $CD8^+$ T lymphocyte (CTL) immune responses

$CD8^+$ T cells recognize and kill HIV-infected cells, thereby playing an essential role in controlling acute viremia and long-term disease suppression [80]. The early CTL response is directed against a few conserved epitopes and follows a hierarchical immunodominance pattern [81]. Within weeks to months post infection, plasma HIV-1 levels typically decline and stabilize at a host-specific 'set point'. Several lines of evidence suggest that CTLs are crucial in controlling HIV-1 viremia in infected individuals [82]. HIV-1-specific CTL responses have been temporally associated with the establishment of viral load set point [83-86]. CTLs can inhibit viral replication *ex vivo* [87]. Moreover, a strong correlation is observed between CTL response and early viral set point, suggesting that the immunodominant CTL responses in acute infection play an important role in viral set point and subsequent disease progression [88]. Third, based on animal studies of the simian immunodeficiency virus (SIV)-infected rhesus macaques strong $CD8^+$ T cell immune responses have been observed. These studies showed that experimental depletion of $CD8^+$ T cells in rhesus macaques resulted in an inability to

control SIV infection in early and chronic infection [86,89]. Finally, escape mutations in CTL-targeted epitopes develop *in vivo* during infection [90].

1.5.3.1 Structure and diversity of HLA class I alleles

HLA class I molecules are highly polymorphic and polygenic in nature [91], allowing the presentation a broad range of peptide repertoires at both the individual and population levels [92,93]. The peptide-specificity of HLA class I proteins is determined by the HLA heavy chain, encoded by HLA-A, -B, and -C whereas the β2-microglobulin polypeptidic light chain interacts with the heavy chain, maintaining the structural stability of the HLA class I molecule [94].

HLA class I molecules are encoded by eight exons. However, the α1 and α2 domains encoded by exons two and three, respectively. These exons mainly form the groove that interacts with antigenic peptides; their variability is responsible for extensive polymorphism of HLA class I molecules. The HLA peptide-binding groove has six binding pockets: A, B, C, D, E and F. Typically, amino acid side chains of the residues at position 2 and the carboxyl (C) - terminus of the peptides binds to the B and F-pockets of the HLA molecule, respectively. Apart from these anchor sites, the residues that lie in between are termed 'auxiliary anchor positions', and can substantially affect the HLA-peptide interaction [95]. Therefore, each HLA class I molecule binds a large, yet finite, number of correctly processed peptides that contain a specific peptide binding motif and lead to the presentation of a diverse set of peptides to CTLs.

1.5.3.2 Influence of HLA class I genotype on HIV-1

Expression of certain HLA class I molecules has been consistently linked to HIV-1 control [96-100] and the rate of progression to AIDS [101,102], with HLA-B playing a particularly critical role. First, the majority of the detectable HIV-specific $CD8^+$ T cell responses are restricted by HLA-B alleles [103]. Second, certain HLA-B alleles are associated with either slow disease progression (most notably HLA-B*57 and B*27) whereas some are associated with relatively ineffective control of viral replication and rapid disease progression, including B*35 and B*5802 [104]. Third, HLA-B-restricted $CD8^+$ T cells exert the strongest selection pressure on HIV-1 as measured by Matthews *et al.* [105].

It is thus imperative to explore why HLA-B dominantly influences HIV-1 disease progression. HLA-B is the most polymorphic of all HLA loci (3887 allelic variants) compared to HLA-A and HLA-C alleles (3107 and 2623 alleles, respectively) (http://www.ebi.ac.uk/ipd/imgt/hla/stats.html), suggesting that the HLA-B locus is diversifying more rapidly than HLA-A and -C [98]; the reasons for which remain unknown.

From a biochemical perspective, amino acid differences in the peptide-binding grooves of the HLA class I molecules may explain the relatively greater diversity of antigenic peptides presented by HLA-B. The amino acids that bind the B pocket of HLA-A alleles are broadly hydrophobic residues, excluding proline. In contrast, the B pocket of HLA-B alleles can accommodate hydrophobic residues; but also proline, histidine, glutamine, and other positively and negatively charged residues. HLA-B diversity, however, arises from intra-locus recombination events within exon 3, mainly affecting the F, C and D pockets [95,106]. For example, three amino acid differences between HLA-B*5801 and B*5802, are predicted only to affect the C pocket [98]. In this scenario, the rapidly evolving pathogens, such as HIV-1 may adapt more readily to the relative homogeneity offered by HLA-A alleles, whereas less so to the functional diversity offered by HLA-B. Therefore, this functional diversity of HLA-B suggests that, as the epidemic evolves, dominant immune-mediated selection pressures will remain connected to HLA-B. Lastly, HLA-B may be more protective against HIV-1 infection than other alleles as it is least affected by Nef-mediated cell-surface downregulation of HLA class I molecules [107].

1.6. HLA-mediated HIV-1 immune escape

A major mechanism whereby HIV-1 evades host CTL immune responses is *via* the selection of HLA class-restricted CTL escape mutations that allow the virus to evade recognition by these cells [108-111]. CTL escape mutations may interfere with intracellular viral peptide processing [90,112], abrogate peptide/HLA binding [113,114], and/or disrupt recognition of the HLA/peptide complex by the peptide-specific TCRs on CTLs [115,116].

Achieving a deeper understanding of the pathways, mechanisms, and implications of HIV-1 mutational immune escape is of paramount importance. Therefore, we provide an extensive literature review on this topic and explore it further in the next chapter. Taken together, this information will help us design novel immune intervention strategies that will bring us closer to our ultimate goal of ending the HIV-1 pandemic.

1.7. References

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Chapter 2.

Mutational immune escape in HIV-1 infection

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2.1. Abstract

Adaptive, and possibly innate, host immune responses represent major selective forces driving the evolution of the human immunodeficiency virus 1 (HIV-1) group M "pandemic" strain. This immune-driven viral evolution occurs *via* the selection of escape mutations in the viral genome that allow HIV-1-infected cells to evade detection by cellular immune responses and/or that allow individual virions to escape neutralization by host antibodies. The first part of this chapter provides an overview of past and recent advances in our understanding of HIV-1 mutational immune escape (and the associated process of reversion) as highly specific and reproducible processes both within and between hosts. HIV-1 escape from adaptive cellular immune responses are covered in detail, but escape from neutralizing antibodies and natural killer (NK) cells are also discussed. In particular, we highlight recent advances in our understanding of early escape dynamics, molecular mechanisms, and the consequences of escape for viral fitness and diversity. We also describe insights recently gained from statistical association studies of immune-driven polymorphisms in HIV-1. The second part of the chapter outlines the potential biological and clinical implications of immune escape for the pandemic's future, including the evidence supporting gradual HIV-1 evolution towards increasing "resistance" to host immunity *via* the spread of immune escape mutations in circulating HIV-1 sequences. Finally, we discuss the evidence supporting vaccine-induced immune responses as potential drivers of rapid within-host viral adaptation, and its potential implications for the transmission, selection and evolution of HIV-1 as the epidemic progresses.

2.2. Introduction and overview

HIV-1 group M "pandemic" strain originated from a single zoonotic chimpanzee-to-human transmission event approximately 100 years ago [1,2]. Since then, HIV-1 group M has diversified into 9 subtypes and more than 60 circulating recombinant forms that differ by up to 30% in their envelope amino acid sequence (Figure 2.1) [3,4,5,6]. This extraordinary global diversity has arisen as a result of evolutionary selection pressures imposed on HIV-1 by the estimated 75 million individuals infected since the epidemic's genesis [7]. Among the strongest of these evolutionary pressures is the human immune response itself.

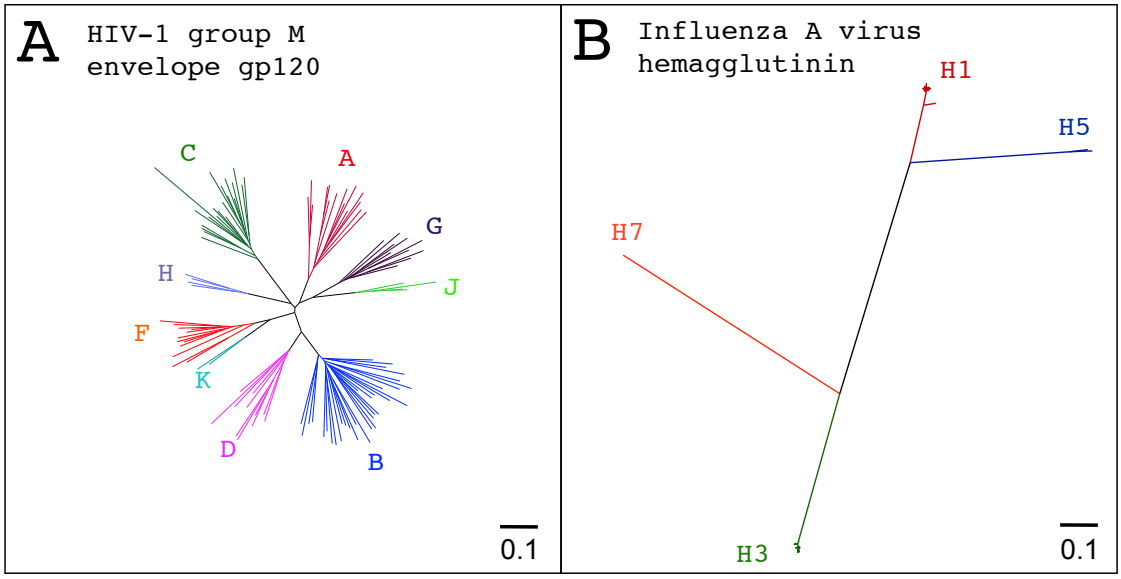

Figure 2.1. Global genetic diversity of HIV-1 group M envelope and influenza A virus hemagglutinin, 2012.

Unrooted maximum likelihood phylogenetic trees depicting global genetic diversity of N=112 HIV-1 group M envelope gp120 (panel A) and N=138 influenza A virus hemagglutinin (panel B) sequences sampled in the year 2012, drawn on the same genetic distance scale. HIV-1 group M sequences were obtained from the Los Alamos HIV Database 2012 compendium [273] and http://www.hiv.lanl.gov/); influenza A virus sequences were obtained from the NCBI Influenza virus resource and flu database (http://www.ncbi.nlm.nih.gov/genomes/FLU/aboutdatabase.html). Colored letters denote HIV-1 group M subtypes A-K and influenza A virus hemagglutinin subtypes H1, H3, H5, H7. Intra-subtype diversity of HIV-1 group M vastly exceeds that of influenza A virus sequences circulating within a given year (though influenza A virus exceeds HIV-1 group M in terms of total inter-subtype diversity).

It is now understood that a severe genetic bottleneck occurs at the time of HIV-1 infection [8,9,10] such that, depending on the transmission route, a single [8,9] or a limited number [11,12] of closely related founder viruses establish productive infection in the recipient. As a result of HIV-1's high replication rate [13], the high error rate of the virally-encoded reverse transcriptase enzyme [14,15], frequent recombination [16] and the mutation-inducing effects of host RNA editing enzymes such as APOBEC3G [17], this transmitted/founder virus rapidly gives rise to a swarm of related viral variants within the infected individual [18]. This genetic pool becomes the evolutionary substrate upon which antibodies [19], T lymphocytes [20] and possibly innate responses [21] exert immune pressures, driving the selection of escape mutations in the viral genome [22,23,24,25,26]. Mutational immune escape is a dynamic process that continues over the life of the infected person, shaping HIV-1 diversity within individuals [27,28,29,30] and host populations globally [31,32]. Understanding the pathways, mechanisms and biological implications of immune-mediated HIV-1 evolution is therefore of great importance to HIV-1 biomedical and clinical research, in particular to HIV-1 vaccine design.

Towards this end, this chapter provides an overview of past and recent advances in our understanding of mutational immune escape in HIV-1. Reflecting the authors' expertise, HIV-1 escape from cellular immune responses mediated by CTLs comprise a major focus, but mutational escape from humoral (antibody), innate, and vaccine-induced immune responses are also covered. The relevance of immune escape to HIV-1 vaccine research, and its potential implications on the pandemic's future are also discussed.

2.3. HIV-1 mutational escape from immune recognition

2.3.1. Recognition and HIV-1 immune control by CTLs

CTL eliminate HIV-1-infected cells *via* the recognition of short, virus-derived peptide epitopes that are produced and processed within the infected cell and loaded onto human leukocyte antigen (HLA) class I molecules for presentation at the cell

surface (Figure 2.2A). Located within the major histocompatibility complex (MHC) region on the short arm of chromosome 6, the HLA class I genes (comprising the HLA-A, B, and C loci) are among the most polymorphic in the human genome [33]. Peptide-HLA binding is defined by HLA allele-specific amino acid motifs within the peptide, most commonly involving positions 2 and the C-terminus [34], allowing CTL to recognize a broad range of pathogen-derived epitopes in an HLA-restricted manner.

HLA-restricted CTLs play a major role in immune control of HIV-1 *in vivo*. It was long observed that HIV-1-specific CTLs first appear around the time of the dramatic acute phase viremia decline [35,36] that occurs approximately 3-4 weeks following infection [37]; it is now known that the earliest CTLs emerge *prior* to peak viremia and play an active role in its control to setpoint levels [24]. Similarly, experimental depletion of $CD8^+$ T cells in rhesus macaques resulted in an inability to control simian immunodeficiency virus (SIV) infection in early and chronic infection [38,39,40]. Strong epidemiological links between host carriage of specific HLA class I alleles and HIV-1 disease progression have also been demonstrated in natural history [41,42,43,44,45] and genome-wide association [46,47,48,49] studies. In particular, HLA-B*57 and B*27 are associated with lower viral loads and slower progression [44,46,50,51,52], whereas certain HLA-B*35 subtypes are associated with faster progression [41]. Independent effects of HLA-C expression level on HIV-1 control have also been demonstrated [53]. HLA class I alleles and their associated CTLs responses may also protect against HIV-1 acquisition [54,55] (though this remains controversial [56]). Similarly, evidence also suggests that vaccine-induced CTL could protect against SIV and HIV-1 acquisition and/or disease progression [57,58,59] (though readers new to the field should be aware that incomplete vaccine protection observed in the only “successful” HIV-1 vaccine trial to date was not likely attributable to CTL [60], and that three other trials evaluating a cellular HIV-1 vaccine delivered *via* a human adenovirus 5 vector ended in failure [61,62,63]). It is also worth noting that the efficacy of CTL-mediated control of HIV-1 differs based on the viral protein (and/or epitopes) targeted. In particular, recognition of key conserved epitopes in $p24^{Gag}$ [64,65,66] and to a lesser extent Pol may be most beneficial, whereas targeting of envelope may have negative clinical consequences [65]. However, the observation that HLA-restricted CTL exert potent immune pressures on HIV-1 *in vivo* is perhaps most clearly demonstrated by the virus’s ability to escape this

pressure *via* mutation. We now turn to the history of discovery in this area.

2.3.2. CTL escape in HIV-1: early evidence and mechanisms

Of the host immune responses targeting HIV-1 *in vivo*, those exerted by CTLs are the best understood with respect to the specific mutational strategies employed by HIV-1 to evade them. Though CTL directly mediate the elimination of HIV-1-infected cells *in vivo*, HIV-1 mutational escape from CTL pressures is commonly conceptualized as "HLA-driven" or "HLA-associated" due to the requirement that the viral epitope be bound and presented by a specific HLA molecule for CTL recognition.

Selection of HIV-1 mutants capable of evading CTL recognition *in vivo* was first described in the early 1990s [20,67,68] when it was observed that "*accumulation of such mutations in T cell antigenic targets…provides a mechanism for immune escape*" [20]. In the original 1991 study, researchers noted temporal shifts in the dominant HLA-B*08-restricted HIV-1 Gag epitopes targeted by patient-derived CTL, with some epitopes exhibiting permanent loss of recognition over time. These shifts in CTL epitope recognition coincided with the appearance of viral mutations within them. Whereas some mutants abolished *in vitro* CTL recognition, others retained some ability to be recognized by certain autologous CTL populations. The former observation led researchers to conclude that a major mechanism of *in vivo* CTL escape was the selection of a mutant epitope no longer capable of forming complexes with the relevant HLA, whereas the latter observation revealed that some escape mutants retained the ability to bind HLA, but stimulated a smaller and/or different pool of CTLs following their selection [20].

CTL escape mutations can be broadly classified into three mechanistic categories (two of which were inferred in the original 1991 study [20]). The most intuitive is escape *via* mutation(s) that reduce or abrogate viral epitope binding to HLA, thereby impairing CTL recognition of infected cells (Figure 2.2B). These mutations usually occur at HLA-specific epitope "anchor" residues - typically peptide positions two and/or the C-terminus - and are commonly referred to as "anchor-residue escape" mutations.

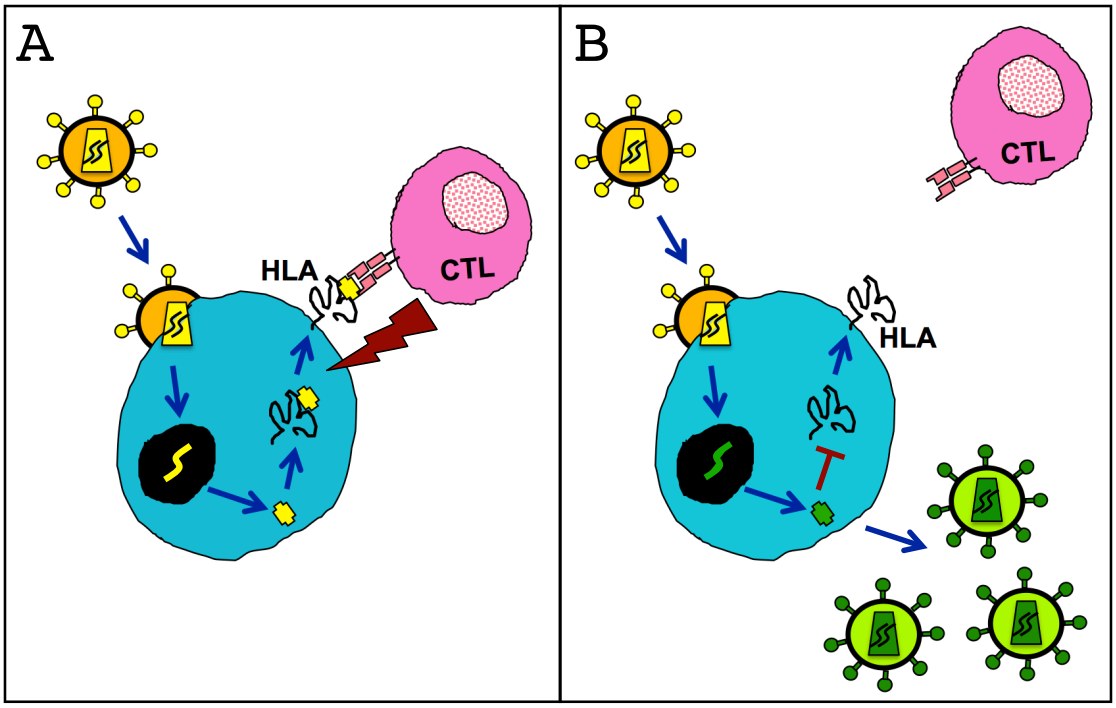

Figure 2.2. Mutational escape in HIV-1 allows infected cells to evade detection and elimination by HLA class I-restricted CTLs.

A: A simplified diagram depicting HIV-1 infection of a target cell, and the subsequent intracellular production of short virus-derived peptide epitopes that are processed and loaded onto HLA class I molecules for presentation at the cell surface. Recognition of the viral epitope-HLA complex by the T cell receptor (TCR) complex of an HLA-restricted CTL results in elimination of the infected cell. For more details, see sections 2.3.1.

B: Under immune pressure, mutations are selected in HIV-1 that allow infected cells to evade detection by CTL. In this simplified diagram, a mutation occurs during the reverse transcription of RNA to proviral DNA, that, when translated into protein, abrogates the ability of the original encoded virus-derived epitope to bind HLA. As such, the viral epitope is not presented at the cell surface, allowing the infected cell to evade detection by the original HLA-restricted CTLs. This results in the release of escape mutant HIV-1. In addition to the mechanism depicted here, CTLs escape mutations may also interfere with proper processing of HIV-derived peptide epitopes prior to loading onto HLA ("antigen processing escape") and/or may abrogate CTL recognition of the mutant peptide-HLA complex ("TCR escape"), as described in section 2.3.2.

A well-known example is the B*27-associated R264K mutation selected at position 2 of the B*27-restricted KK10 epitope in Gag (that spans codons 263-272) [69]. CTL escape can also act upon processes that occur prior to, or following, peptide-HLA binding. For example, some CTL escape mutant epitopes retain the capability to bind HLA, but reduce or abrogate recognition of the peptide-HLA complex by the TCR

expressed by some or all members of the original selecting CTL pool. Such "TCR escape mutations" usually occur at central epitope positions. The B*27-associated L268M mutation selected at position 6 of the KK10 epitope provides an example [20]. L268M-containing KK10 retains the ability to bind HLA-B*27, but abrogates its recognition by key B*27-restricted CTL clonotypes in the repertoire [70]. Other examples of TCR escape abound [70,71,72,73,74,75]. The original 1991 study was also the first to document *de novo* recognition of variant peptides by novel CTL populations following TCR escape [20], a now well-described phenomenon [76,77] that underscores the dynamic and adaptable nature of the host cellular response to a rapidly evolving pathogen. The third category of CTL escape mutations inhibit epitope formation by interfering with their proper processing within the host cell. The first such "antigen processing escape mutation" to be mechanistically characterized was B*57:03-restricted Gag-A146P, occurring at the residue immediately upstream of the IW9 epitope (Gag codons 147-155) that acts *via* prevention of N-terminal aminopeptidase-mediated trimming of this epitope [78]. Though antigen processing mutations often occur at positions flanking the N and/or C-terminal epitope boundaries, they can also occur within the epitope [79]. For example, a mutation occurring at position 5 of a B*07-restricted epitope in a cryptic Gag reading frame acted *via* introduction of a proteasomal cleavage site at this position, yielding a profound reduction in epitope formation [80]. Antigen processing CTL escape mutations may also occur at positions distal to the epitope [81]. Arguably the most foundational observation made in the original 1991 study was the HLA-restricted nature of CTL escape. Since "*different HLA class I molecules select distinct HIV-derived epitopes to stimulate CTL responses*" wrote the researchers, then "*HLA type could have an effect on virus escape*" [20]. This realization was key to the next major development in the field - namely, that the kinetics and nature of *in vivo* CTL escape was specific to, and thus broadly reproducible based on, the HLA class I alleles expressed by the host.

2.3.3. The timing and mutational pathways of CTL escape are reproducible based on host HLA

Despite HIV-1's genetic plasticity, the timing and mutational pathways of CTL escape are broadly predictable based on host HLA – a phenomenon most strikingly

illustrated by the near-identical patterns of CTL epitope targeting and escape in identical adult twins infected on the same date with the same virus *via* injection drug use [82]. Importantly though, CTL escape is also reproducible across unrelated hosts sharing the same HLA. For example, three-quarters of HIV-1 subtype B infected persons expressing the protective HLA-B*57 allele select a T-to-N mutation at Gag codon 242 (position three of the $p24^{Gag}$ TW10 epitope at Gag codons 240-249), usually within the first weeks to months following infection [83,84]. Fifty percent of B*57-expressing persons will additionally select G248A at position 9 of this epitope [84,85,86]. Together, these two mutations confer complete escape from B*57-restricted, TW10-specific CTL [83]. In contrast, in B*27-expressing individuals, targeting of the immunodominant $p24^{Gag}$ KK10 epitope begins in early infection and is often sustained for years thereafter [42]. KK10 escape begins *via* selection of the L268M mutation at position 6 of the epitope a few months after infection [20], that abrogates its recognition by certain autologous B*27-restricted CTL [70]. Complete escape from KK10-expressing CTL does not generally occur until years later, *via* selection of R264K at epitope position 2 [69] that abrogates epitope binding to B*27 [87]. The lengthy timeline of selection of R264K is now known to be due to its substantial fitness cost, which necessitates the development of a distal compensatory mutation prior to its selection *in vivo* [88]. Though epitopes besides KK10 are targeted in B*27-expressing persons [89], KK10 escape remains one of the most clear-cut examples where *in vivo* HLA-mediated control of HIV-1 replication is largely mediated by sustained targeting of a single key epitope, and where escape leads directly to loss of HIV-1 control [27,90].

That the first CTL escape mutations emerge rapidly following infection has long been known [22,91,92]. Recently however, major advances in our understanding of the dynamics of HIV-1 infection and subsequent escape have been achieved *via* detailed studies of intra-host HIV-1 evolution using single-genome amplification (e.g., [9]) or next-generation sequencing (e.g., [93]). We now appreciate that HIV-1 transmission is characterized by a severe genetic bottleneck, where an estimated 80% of heterosexual transmissions are productively initiated by a single transmitted/founder virus [8,9,10], whereas infection in men who have sex with men or persons who inject drugs is generally established by a limited number of closely related donor/founder viruses [11,12]. We also now appreciate that CTL-mediated killing of infected cells begins *prior*

to acute-phase peak viremia, and that selection of the first CTL escape mutations occurs during this time [24,93]. Indeed, the selection (and in some cases the fixation) of CTL escape variants has been observed as early as 21 days post-infection in humans [18,24] and 17 days in macaque models of SIV infection [94].

The evolutionary pathways along which these early mutations arise have also recently been elucidated in detail. In the earliest days following infection, HIV-1 undergoes rapid population growth and exhibits star-like diversification, but immune selection (notably by CTLs) dominates thereafter, leading to the survival of viral lineages harboring escape mutations [18]. As it turns out, the conceptually straightforward pathway, whereby the first selected escape mutation gradually outcompetes the original transmitted form, is likely to be true for only a minority of cases [24]. More commonly, the first escape variant tends to be rapidly followed by the emergence of numerous others, from which the "final" escape form is ultimately selected [24]. This is likely because the initially-appearing pool of low frequency mutants often retain some ability to be targeted by existing (or *de novo*) CTL [95]. This drives the selection of more effective escape variants, often at HLA-anchor residues that ultimately outcompete both transmitted founder and initial variants [10,93]. For example, in a B*57:03-expressing individual, initial escape within the $p24^{Gag}$ TW10 epitope occurred approximately 5 months post-infection *via* a transient, minority G-to-E mutation at position 9 (G248E) that retained the ability to bind B*57:03 and reduced CTL recognition only modestly [95]. By approximately 1.5 years following infection, this mutation was outcompeted by variants expressing the "canonical" B*57-restricted G248A mutation at this position (along with T242N and V247I at epitope positions 3 and 8).

It is also now understood that CTL escape accounts for a major proportion of within-host HIV-1 evolution in the first year of infection [24,84,93]. To provide context, a detailed study of seven newly-infected individuals revealed that, approximately 6 months following infection, between 9 to 18 positively-selected substitutions were observed throughout the HIV-1 proteome [18], whereas another estimated that a minimum of 30% of observed substitutions in Gag/Pol and 60% in Nef were attributable to HLA-driven selection [84]. HLA-driven CTL escape continues to occur (albeit at a slower rate [96,97]) over the infection course, with some escape mutations occurring on a

timecourse of years [27,98,99].

2.3.4. Reversion, compensation, and fitness costs of CTL escape

When CTL escape mutations selected in a previous host are transmitted to an individual lacking the restricting HLA allele(s), many will revert to the original (usually subtype consensus) amino acid [83,100,101,102,103]. Like escape, reversion is also HLA-restricted, though in the opposite context (as it occurs in the *absence* of selection pressure by the original restricting HLA). The timing of reversion is also predictable to some extent. Whereas some escape mutations, for example the B*57-associated Gag T242N, revert consistently and rapidly following transmission [83,93,102], most revert more slowly [98,104]. Yet others are so stable that they revert rarely or not at all [32,105,106,107].

Certain CTL escape mutations occur at a cost to viral fitness [108]. Like the reversion of certain drug resistance mutations upon transmission to a therapy-naïve host [109], fitness costs of CTL escape mutations can be inferred by their tendency to revert following transmission to an HLA-mismatched host [83,100,108,110]. Fitness costs of escape vary widely, depending on their location in the viral proteome. Broadly speaking, escape mutations within conserved viral regions tend to exhibit more pronounced fitness costs, whereas escape in more variable regions tends to be fitness-neutral [111]. An example of a highly fitness-costly mutation is the B*27-associated R264K substitution in the $p24^{Gag}$ KK10 epitope, which essentially abolishes *in vitro* viral replication when engineered alone into the reference strain NL4-3 ($HIV\text{-}1_{NL4\text{-}3}$), likely because this variant is unable to replicate efficiently in the presence of normal cellular levels of cyclophilin A [88]. Generally however, *in vitro* fitness costs of escape mutations observed *in vivo* tend to be subtler, often requiring multiple substitutions to reduce function. Alone, the B*57-driven Gag-T242N mutation reduces viral replicative capacity only modestly [112,113], but dose-dependent replicative reductions are observed when it is present alongside other common B*57-driven mutations in $p24^{Gag}$ [114,115,116]. Fitness-costly escape mutations are numerous, widespread throughout the HIV-1 genome, and are restricted by a broad range of HLA alleles. Examples include A*74:01 [117] and Cw*03 [118]–driven mutations in $p24^{Gag}$, B*13-driven mutations in $p1^{Gag}$ [119], B*35-driven mutations

in Nef [120], and Cw*05-driven mutations in integrase [121]. Furthermore, the consistent reversion of fitness-costly escape mutations explains why certain HIV-1 residues that are highly conserved at the population level remain so despite being under strong selection by one or more HLA alleles. For example, the Gag-T242N mutation is reproducibly selected in the vast majority of individuals expressing HLA-B*57 and/or B*58, but its consistent reversion [83] ensures that its prevalence remains ≈1% among individuals lacking these alleles [83,122].

Fitness costs associated with the primary escape event can be offset by the selection of compensatory mutations at secondary sites [123]. This was first demonstrated in the simian/human immunodeficiency virus (SHIV) model, where the fitness costs of a rare *in vivo* escape mutation in capsid were rescued by the selection of compensatory mutations 21 codons upstream and/or 24 codons downstream of the primary escape site [123]. Due to its routine late emergence following other clustered mutations within the $p24^{Gag}$ KK10 epitope, the B*27-restricted R264K mutation was long suspected to require compensation [69], however, it was not until 2007 that its compensatory mutation was identified to be S173A, nearly 100 residues upstream [88]. Indeed, the requirement that S173A be present prior to R264K selection *in vivo* provided an explanation for the latter's lengthy timeline of selection and also resolved the seemingly paradoxical initial observation that R264K abolished HIV-1 replication when engineered alone *in vitro* (S173A rescues R264K replication to near wild-type levels [88]). Examples of compensatory mutations now abound. Whereas most occur in relatively close proximity to the primary escape site (e.g., S165N with A163G in B*5703-KF11 [98]; E260D with R264K in B*27-KK10 [124]; H219Q, I223V and M228I with T242N in B*57-TW10 [112,113], all in $p24^{Gag}$), others, such as S173A with R264K in B*27-KK10 [88], occur a substantial linear distance away, but may reside nearby in the folded protein structure. Compensatory mutations are also highly reproducible in context of their associated primary escape site. Indeed, the reproducibility of HLA-driven escape, reversion and compensation is most clearly revealed by population-level studies [125,126,127], the subject of the following section.

2.3.5. Identification of CTL escape mutations "at the population-level" by statistical association: overview and methods

The predictable nature of HIV-1 adaptation to HLA has facilitated the systematic identification of HLA-associated viral polymorphisms "at the population level" – a term loosely used to describe the identification, *via* statistical association, of viral polymorphisms significantly over- (or under)-represented among persons expressing a given HLA allele, in cross-sectional datasets [31,86,126,128]. An advantage of these approaches is that they are comprehensive and largely unbiased, allowing the identification of HLA-associated viral polymorphisms regardless of their proximity to known CTL epitopes. A disadvantage is their correlative nature, thus necessitating experimental validation to confirm HLA-associated polymorphisms as mutations directly conferring CTL escape, and to elucidate their mechanisms.

Population-level analyses identify two types of associations: adapted and nonadapted (Figure 2.3). Adapted associations are viral polymorphisms that are significantly *over-represented* in individuals harboring a particular HLA allele; these are likely to represent CTL escape mutations. Conversely, nonadapted forms are viral polymorphisms that are significantly *under-represented* in individuals harboring a particular HLA allele; these represent the immunologically susceptible form for the HLA allele in question. In most cases, both nonadapted and adapted forms are identified at a given HIV-1 codon for a particular HLA allele. For example, at Gag codon 242, T and N represent nonadapted and adapted forms associated with HLA-B*57. Sometimes an HLA allele can select multiple escape pathways at a given viral site, yielding multiple adapted associations. For example Nef codon 94, position 5 of the B*08-restricted FL8 epitope, harbors four B*08-associated adapted forms: "E", "M", "N" and "Q" (whereas the subtype B consensus "K" represents the B*08-associated nonadapted form at this position) [126,129]. In the majority of cases, nonadapted forms represent the HIV-1 subtype consensus whereas adapted forms represent variants, but exceptions occur. For example at Gag codon 147, "L" and "I" represent the nonadapted and adapted forms associated with B*14:02 and B*15:01 (but the subtype B consensus is "I") [86]. HIV-1 codons harboring diametrically opposed HLA associations also exist, where a given viral polymorphism represents the nonadapted form for one HLA allele but the adapted form

for another [129]. Gag codon 147 again provides an example: in opposition to the B*14:02 associations described above, “L” and “I” represent the adapted and nonadapted forms associated with A*25:01, B*13:02 and B*57:01, among others [86].

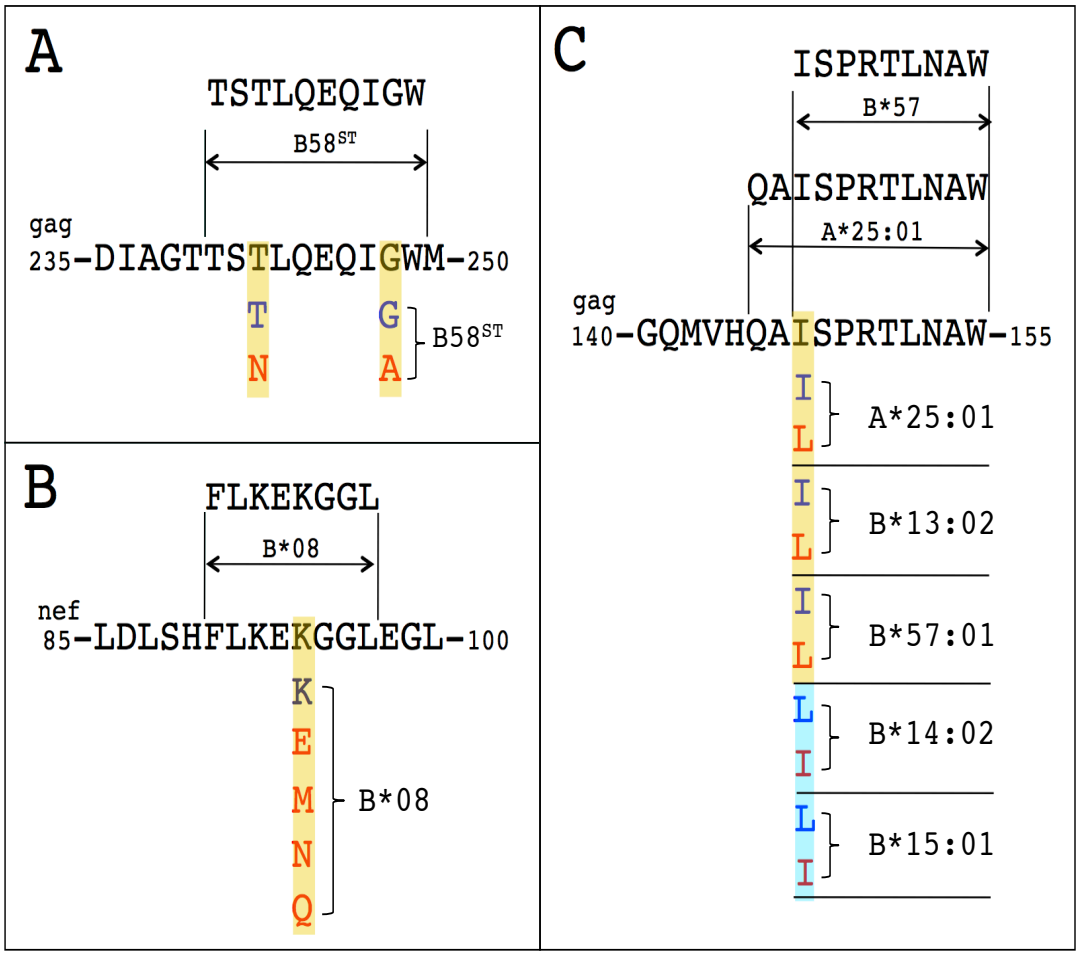

Figure 2.3. Examples of HLA-associated polymorphisms in HIV-1 identified “at the population level”.

The predictable nature of HIV-1 adaptation to HLA has facilitated the systematic identification of HLA-associated viral polymorphisms “at the population level”, *via* statistical association approaches. These associations can be depicted in “HIV-1 immune escape maps” which indicate their locations, specific amino acid residues and HLA restrictions. A selection of HLA-associated HIV-1 sites, and their polymorphisms are shown here. The HIV-1 subtype B consensus amino acid is used as a reference. Known $CD8^+$ epitopes in HIV-1 (available at http://www.hiv.lanl.gov/content/immunology), and their HLA-restrictions, are indicated above the consensus sequence. HLA-associated polymorphisms are listed below the consensus sequence: Nonadapted associations (HIV-1 residues under-represented among persons expressing the HLA) are in blue, whereas adapted associations (HIV-1 residues enriched among persons expressing the HLA) are in red (continued on next page).

A: At HIV-1 Gag codon 242, residue 3 of the TW10 epitope recognized by HLA alleles belonging to the B58 supertype, T and N represent nonadapted and adapted forms associated with HLA B58 supertype alleles, respectively. At Gag codon 248, position 9 of this epitope, G and A represent nonadapted and adapted forms associated with the HLA B58 supertype alleles, respectively.

B: Sometimes HLA-driven escape can occur along multiple pathways at a given viral site, yielding multiple adapted associations. For example, at Nef codon 94, position 5 of the B*08-restricted FL8 epitope, the subtype B consensus "K" represents the B*08-associated nonadapted form at this position, whereas four B*08-associated adapted forms are observed: "E", "M", "N" and "Q".

C: Some HIV-1 codons harbor a large number of associations with different HLA alleles, some of which occur in opposing directions. Gag codon 147, which lies within numerous CTL epitopes, provides an example. Here, "L" and "I" represent the adapted and nonadapted forms associated with A*25:01, B*13:02 and B*57:01. In diametric opposition, "I" and "L" represent the adapted and nonadapted forms associated with B*14:02 and B*15:01. In the majority of cases, nonadapted forms tend to represent the HIV-1 subtype consensus whereas adapted forms represent variants, but exceptions occur. The associations with B*14:02 and B*15:01 at this position also illustrate "exceptional" cases where the adapted form represents the subtype consensus whereas the nonadapted form represents a polymorphic variant (in most cases - including all other examples depicted in this Figure - nonadapted forms represent the HIV-1 subtype consensus whereas adapted forms represent variants). For more detail, see section 2.3.5.

Published in 2002, the first study to identify HLA-associated polymorphisms "at the population level" identified nearly 100 polymorphisms in HIV-1 RT in a cohort of ≈400 patients, illustrating the extensive impact of CTL pressures on HIV-1 [31]. Since then, analytical methods have been refined. In particular it has been recognized that, since HIV-1 sequences are related to one another through descent from a common ancestor (some more closely than others, for example a transmission pair), they should not be analyzed using standard tests of association that assume independence of observations. The problem can be illustrated by the extreme example of a heterogeneous dataset comprising different host populations infected with different HIV-1 subtypes (e.g., Europeans infected with subtype B, and Africans infected with subtype C). Here, HIV-1 sequences belonging to each subtype will share lineage-specific polymorphisms reflecting their descent from the most recent common ancestor at the root of that lineage. Similarly, HLA allele frequencies will differ between the two host groups due to their descent from different ancestral populations.

As such, standard tests of association would identify strong relationships between HIV-1 subtype C lineage-specific polymorphisms and HLA alleles enriched among Africans (and likewise HIV-1 subtype B lineage-specific polymorphisms and HLA alleles enriched among Europeans). However, such associations would be attributable

to confounding due to viral lineage ("founder") effects, rather than escape mutations directly selected by the associated HLA [130]. Though this is an extreme example, analyses comprising only a single HIV-1 subtype could similarly be confounded by subtle lineage effects.

To address this, "phylogenetically-informed" methods that correct for the underlying evolutionary relationships (that is, the inferred phylogeny) linking HIV-1 sequences in the dataset have been developed, with the goal of distinguishing HIV-1 sites under HLA-driven selection in the present host from those likely to be explained by founder effects (i.e., neutral evolution in the tree) [125,130,131]. More recent strategies also correct for the confounding effects of linkage disequilibrium between HLA class I alleles [125] (thus identifying the HLA allele directly responsible for selecting the viral variant, rather than alleles in linkage disequilibrium with it) and HIV-1 amino acid co-variation [125,132,133] (thus discriminating the specific viral variants directly selected by HLA, from those that indirectly arise as secondary or compensatory mutations). Strategies to address lineage effects and population stratification in both virus and host have also been implemented [134].

2.3.6. Insights from population-level studies of CTL escape

2.3.6.1 CTL escape pathways are highly HLA-specific and a major driver of viral diversity

Population-level studies have yielded comprehensive maps of the locations, specific amino acids, relative frequencies, and statistical strengths of selection of HLA-associated polymorphisms in HIV-1 [125,126,129,131,135,136,137,138]. These "immune escape maps" are most detailed for HIV-1 subtype B [86,126,129,138], followed by C [125,128,131,139] and CRF01 (AE) [140], though other subtypes remain understudied in this context. Population-level studies have also confirmed escape (and reversion) as highly reproducible processes in the context of host HLA. For example, the strongest HLA association in subtype B is the HLA-A*24:02-restricted Y135F escape mutation in Nef. In chronic infection, 81% of A*24:02-expressing persons harbor this mutation, compared to only 12% of persons who do not express an allele belonging to the A24 supertype, yielding a statistical association of very high magnitude (in this case,

an odds ratio of approximately 30 and a *p*-value of $8x10^{-118}$) [86]. By definition, such a strong statistical association can only be achieved if the mutation is near-universally selected in persons harboring the HLA, and reverts consistently in individuals lacking it [141]. Escape is also highly HLA-specific. When population-level analyses are undertaken at various HLA resolution levels (e.g., supertype, type, subtype), the majority (>60%) of HLA-associated polymorphisms are identified as specific to a particular HLA subtype, whereas <10% are identified as shared across HLA supertypes [86]. This high HLA-specificity remains true even for closely related HLA subtypes that present the same viral epitopes [128,138]. For example, HLA-B*57:02, B*57:03 and B*58:01 all bind Gag-TW10, but they drive significantly different escape pathways within this epitope. In particular, escape at position 3 (*via* selection of T242N) is significantly stronger for B*57:02 compared to the others, escape at position 7 occurs *via* I247M in B*57:02 versus I247V in B*57:03, whereas escape at position 8 is essentially B*58:01-specific [128]. The specificity of HIV-1 adaptation to HLA may also shed mechanistic light on the longstanding observation that HLA alleles, sometimes differing by as little as one amino acid between them, can mediate differential rates of disease progression [142,143,144]. This observation also underscores the importance of identifying HLA-associated polymorphisms at the HLA subtype level.

Population-level studies also confirm immune escape as widespread throughout HIV-1. Over 2100 HLA-associated polymorphisms, occurring at ≈35% of the virus' nonconserved codons have been identified across the HIV-1 subtype B proteome [86], though their distribution is somewhat non-uniform. Whereas HLA pressures greatly influence diversity of certain viral genes (e.g., the highly diverse *nef* gene and the relatively mutationally-constrained $p24^{Gag}$ harbor HLA-associated polymorphisms at ≈70% and ≈40% of their nonconserved residues, respectively), the highly variable *vpu* gene exhibits evidence for HLA-mediated selection at only one-quarter of its nonconserved sites [86,145]. Despite this, HLA is likely to represent the single most important host genetic factor influencing global HIV-1 diversity – an observation recently confirmed *via* genome-wide association. In a "genome-to-genome" analysis of >1000 individuals for whom human genetic variation (assessed in terms of single nucleotide polymorphisms [SNPs]) and HIV-1 sequences were available, 48 HIV-1 amino acids associated with human SNPs were identified, all of which mapped to the HLA class I

region [134]. Similarly, a study investigating immune-driven evolution of HIV-1 Gag and Nef during the North American subtype B epidemic observed that HIV-1 sites under HLA selection have diversified to the greatest extent over time, supporting a significant role of HLA in driving global HIV-1 diversification [146].

2.3.6.2 Population-level studies illuminate escape mechanisms, aid novel epitope discovery, and reveal correlates of protective immunity

Though the mechanisms of immune escape cannot be determined *via* association studies alone, analysis of the distribution of HLA-associated polymorphisms within (or flanking) known or inferred epitopes can shed light on which mechanisms predominate. In particular, epitope-HLA anchor residues are significantly enriched for HLA-associated polymorphisms, identifying abrogation of peptide-HLA binding as a predominant *in vivo* escape mechanism [86,147]. Moreover, bioinformatic predictions estimate that the "average" HLA-restricted anchor residue polymorphism confers a ten-fold reduction in peptide binding affinity to HLA [86]. Inferred escape *via* TCR and/or antigen processing mechanisms also occur, but less frequently than anchor residue escape. Population-level studies have also aided CTL epitope discovery, as the presence of HLA-associated viral polymorphisms generally indicates the presence of a CTL epitope nearby. Bioinformatic approaches can then be applied to predict its location and sequence for experimental validation – a type of "rational", polymorphism-guided approach to epitope discovery. Numerous CTL epitopes have been identified this way [121,130,148,149], including HLA-restricted epitopes in nonstandard ("cryptic") [150] and/or antisense [151] HIV-1 reading frames.

Association studies of HLA-driven escape can also shed light on why certain HLA alleles are more effective at controlling HIV-1 than others [86]. This is because HLA-associated polymorphisms mark viral sites under strong and reproducible *in vivo* selection by a particular HLA allele. As such, analysis of the properties of these sites (i.e., their location, frequency, distribution, sequence conservation and their strength of association with the restricting HLA) can be used to identify features that discriminate protective from nonprotective HLA alleles. Analyses of this type have identified CTL response breadth as the most consistent correlate of immune protection: in general, protective HLA-A and -B alleles exert immune pressure on a larger overall number of

HIV-1 sites compared to nonprotective alleles [86]. The strength and location of selection pressure is also important: protective HLA alleles also tended to strongly target highly mutationally constrained sites, notably in Gag and to a lesser extent Pol. Protective HLA alleles also exhibited a higher average number of escaping sites per epitope in Gag, supporting diversity of selection pressure (e.g., in terms of the clonal composition and/or diversity of epitope-specific CTL repertoire [70,152]) on key viral areas as an additional correlate of protection. It is important to emphasize that the unit of analysis in these investigations is the HLA allele, not the individual, and that conceptualizing HIV-1 codons as sites under HLA-mediated selection does not imply that CTL escape is protective at the individual level (on the contrary, escape is generally linked to negative clinical outcomes [27,90,128,153]). Rather, these sites represent the total potential of individual HLA alleles to effectively target HIV-1. In support of the potential *in vivo* relevance of this novel analytical perspective, a recent population-level analysis in HIV-1 subtype C identified HLA-restricted viral polymorphisms as stronger predictors of HLA-plasma viral load correlations than CTL responses measured by traditional *in vitro* assays [128].

2.3.7. HLA class II-driven immune escape

Effective antiviral immunity generally requires $CD4^+$ T-lymphocyte help. HLA class II-restricted HIV-1-specific $CD4^+$ T cell responses emerge rapidly following infection [154] (e.g., Gag-specific $CD4^+$ T cell responses peak at a median of 28 days [155]), but the HIV-1-specific $CD4^+$ response rapidly becomes dysfunctional, in part because of the specific elimination of virus-specific $CD4^+$ cells [156,157]. As such, the extent, durability and contribution of $CD4^+$ T cells to HIV-1 control *in vivo* remains incompletely understood. It remains similarly unclear whether mutational escape from $CD4^+$ T cell responses occurs to any great extent *in vivo*: whereas some early studies supported this possibility [158], others did not [159,160]. Furthermore, attempts to identify HLA class II-restricted viral polymorphisms by statistical association have yielded no strong evidence of their existence [161], suggesting that mutational escape from HLA class II-restricted $CD4^+$ T cells is far weaker, less specific and/or less reproducible compared to HLA class I-restricted escape from CTL pressures.

2.3.8. Escape from neutralizing antibodies

The HIV-1 *env* gene evolves rapidly within a host after infection and has diversified to an extraordinary extent at the population level [4]. Although CTL escape contributes to this process, the most significant factor driving HIV-1 envelope evolution is the autologous neutralizing antibody response. Beginning at approximately three months post-infection [162,163] (though earlier in some [164]), HIV-1-infected individuals begin to develop antibodies capable of neutralizing their own virus (termed "autologous" or "strain-specific" neutralizing antibodies; NAbs) [19,165]. (Non-neutralizing antibodies, directed against envelope and non-envelope targets, emerge earlier [166]). In contrast to acute-phase HIV-1-specific CTL responses, autologous NAbs do not contribute to virus containment to any appreciable extent, likely because they drive the rapid selection and outgrowth of neutralization-resistant escape mutants [19,162]. Initial NAb escape exposes novel envelope epitopes against which subsequent waves of autologous NAbs arise, driving further envelope evolution. That antibodies and virus co-evolve in cycles of response and escape was first inferred *via* the ability of autologous sera to neutralize viral variants present in the infected individual 6 (or 12) months prior, but not those present at the time of serum sampling [23].

It is now understood that in approximately 80% of infected individuals, this process results in the continued production of autologous NAbs that remain largely specific to the individual's evolving virus. However in approximately 20% of individuals, this process [30,167,168] leads to the emergence of antibodies that are capable of neutralizing a broad range of HIV-1 isolates across subtypes [169,170,171]. Though individuals producing such "broadly neutralizing antibodies" do not likely derive clinical benefit from them (presumably because their own virus has already escaped) [169,170], the evolutionary mechanisms driving their development are of paramount interest as an effective preventive HIV-1 vaccine will likely require their elicitation (along with effective cellular responses) [172]. For this reason, HIV-1 antibody escape is being elucidated in the context of co-evolution of HIV-1 founder viruses and their autologous NAbs, towards the goal of exploiting this natural process in HIV-1 vaccine design.

Initial studies of HIV-1 neutralization escape, the earliest of which date back to

the late 1980s [173,174], hinted at a variety of escape pathways including the accumulation of amino acid changes in envelope [23] (suggestive of escape through the selection of specific point mutations), changes in *N*-linked glycosylation patterns [162] and lengthening of certain hypervariable domains in gp120, notably V1/V2 [175,176]. However, the identification of specific genetic events conferring escape from individual NAb responses began only recently (e.g., the first specific identification of an envelope escape mutation conferring neutralization escape at the single antibody level was not achieved until 2009 [177]). Unlike CTL epitopes whose (linear) sequences can be predicted from HLA anchor residue motifs without knowledge of the T cell receptor sequence or structure, antibodies directly recognize three-dimensional epitopes whose sequences can span discontinuous sites on one or more members of the envelope trimer, rendering their locations difficult to predict based on HIV-1 sequence alone.

Recent studies have therefore taken the approach of longitudinally characterizing envelope evolution while simultaneously attempting to isolate individual neutralizing antibodies (and/or the B-cell clonal lineages producing them) in individual patients [30,167,168,177,178]. From these studies, a central role of immune-driven envelope evolution in driving autologous neutralization breadth is emerging. In one individual, initial autologous NAbs were directed against epitopes in the first and second hypervariable loops of gp120 (V1/V2), and escape was achieved *via* point mutations in this region including one in V2 that created a putative *N*-linked glycosylation site conferring escape from two distinct monoclonal antibodies isolated from this patient [177]. In a second individual, escape from the initial NAb pool occurred *via* convergent evolutionary pathways (one involving changes in the V3-V5 gp120 outer domain and the other involving co-dependent changes in V1/V2 and gp41), whose lineage members subsequently oscillated in frequency over time [177]. NAb escape *via* distinct evolutionary pathways within a single host was confirmed in an individual in whom escape in a V3-proximal epitope occurred along three divergent viral lineages, each featuring a unique amino acid change [167]. A subsequent study of three acutely-infected individuals whose initial response was directed against different conformational epitopes in envelope, where each escaped along distinct pathways [164], also supports the strain- and host-specific nature of initial epitope targeting and autologous neutralization escape. That escape occurs *via* distinct mechanisms (e.g., point

mutations, glycan shifts, and co-operative conformational changes between two domains) both within and among hosts indicates that HIV-1 employs multiple mutational strategies to escape early autologous neutralizing antibodies [177].

Though autologous NAbs appear after HIV-1-specific CTL, NAb escape shares some similarities with CTL escape. Analogous to other HIV-1 regions, within-host envelope diversification is initially starlike - but, after the appearance of the first NAbs, multiple amino acids often transiently appear in regions under pressure, from which the final neutralization mutant(s) ultimately emerge [164,168]. Of interest, the timecourse of selection (and subsequent fixation) of NAb escape mutations is on average slower than the corresponding process of CTL escape in early infection [164]. Moreover, fitness costs ranging from 0% to 24% were observed for early envelope escape mutants, indicating that NAb escape can also be fitness-costly [164]. The extent to which neutralizing antibody epitopes - and their escape pathways - are shared across patients also remains a key question. The observation that, compared to transmitted/founder viruses, chronic subtype C viruses are significantly enriched for a glycan at envelope codon 332 (whose presence can help trigger the evolution of broadly neutralizing antibodies against this key conserved epitope region [30]), supports the idea of shared evolutionary pathways of neutralization escape. Finally and importantly, the discovery that broadly-neutralizing antibodies evolve *via* complex and dynamic interplay between virus and host immune response has led to the hypothesis that this process could be recapitulated *via* vaccination with specific transmitted/founder envelopes and their sequential escape variants [168]. Though some experimental support already exists for such a strategy [179], further research will be required to move exciting new idea forward.

2.3.9. Innate immune responses: KIR-driven HIV-1 polymorphisms?

Host-driven polymorphisms that do not map to known CTL or NAb escape sites are often identified in individual and population-level studies. Though many are likely attributable to incomplete epitope mapping, some could represent polymorphisms selected by immune responses other than CTL or NAb. In particular, evidence supports innate immune responses, notably natural killer (NK) cells, as mediators of HIV-1

immune control and potential drivers of immune escape.

NK cells express cell-surface receptors belonging to the highly polymorphic killer cell immunoglobulin-like receptor (KIR) gene family, which comprise a variety of inhibitory and activating receptors that interact with HLA class I ligands on target cells [180]. Engagement of activating KIR (which generally exhibit short cytoplasmic tails, denoted by "S" in the gene name) delivers a stimulatory signal, whereas engagement of inhibitory KIR (which generally exhibit long cytoplasmic tails, denoted by "L" in the gene name) delivers a tolerance signal; when the former overcome the latter, NK effector functions are initiated [180]. Indeed, a major trigger for enhanced NK cell-mediated recognition of HIV-1-infected cells is the selective downregulation of their HLA-A and -B (though not C) ligands by the viral Nef protein [181], leading to a reduction in signaling through inhibitory KIR. Inhibitory KIR bind their HLA class I ligands in an allotype-specific manner. For example, KIR3DL1 receptors interact with HLA-B molecules belonging to the Bw4 allotype (determined by amino acids 77-83 of the HLA coding region), notably those harboring isoleucine at position 80 (Bw4-80I), and to a lesser extent those harboring threonine at this position (Bw4-80T) [182,183,184]. Some activating KIR also recognize HLA class I in an allotype-specific manner, though generally at lower avidity than their inhibitory counterparts [185]. An example is KIR2DS1, which binds HLA-C molecules belonging to the C2 allotype (determined by amino acids 77-80 of the HLA coding region) [186,187]. Of note, despite high similarity between the extracellular domains of activating KIR to those of their inhibitory counterparts, many ligands for activating KIR remain unknown.

KIRs, alone and in combination with their allotype-specific HLA ligands, may modulate HIV-1 susceptibility and pathogenesis [185]. HIV-1-infected individuals expressing the activating KIR3DS1 allele in combination with HLA-Bw4-80I exhibit lower viral loads [188], delayed clinical progression [189] and protection from opportunistic infections [188], though not in all studies [190,191]. Higher frequencies of KIR3DS1 homozygosity [192,193] and higher KIR3DS1/3DL1 transcript ratios [194] have been observed in HIV-1 exposed seronegative individuals, suggesting that activating KIR may also confer some level of protection against HIV-1 acquisition. Though protection *via* engagement of an activating receptor seems intuitive, the underlying mechanism

remains unknown (KIR3DS1-expressing NK cells can inhibit Bw4-80I–expressing cells *in vitro* [195], but there remains no evidence that KIR3DS1 directly binds HLA-Bw4-80I [196]). Intriguingly, KIR3DL1 alleles possessing a high-expression, high-inhibitory phenotype (termed KIR3DL1*h/*y) may also be protective [197]. When present in combination with HLA-Bw4-80I alleles, notably HLA-B*57, KIR3DL1*h/*y alleles were associated with lower viral loads and conferred protection against HIV-1 disease progression [198]. KIR3DL1*h/*y-HLA-B*57 co-expression may also protect against HIV-1 acquisition [199] (though another study that did not discriminate KIR3DL1 alleles based on expression reported the opposite [193]). That highly inhibitory KIR receptor-ligand interactions can be protective seems somewhat counterintuitive, especially given that the opposing signals of activating KIR may also be protective. Nevertheless, the data support a role, albeit complex and incompletely elucidated, of KIR in HIV-1 control.

KIR-associated immune pressures may also drive the selection of viral polymorphisms that allow infected cells to evade NK-mediated killing. To shed light on how such mutations could arise in a reproducible manner, we must first briefly re-visit KIR-ligand binding. Though not antigen-specific in the classical sense, KIR receptor-ligand interactions are nevertheless modulated in part by HLA polymorphism (within members of the relevant allotype [200]) and the sequence of the HLA-bound peptide [201,202,203,204,205]. In particular, C-terminal proximal epitope residues may play a role in KIR-HLA interaction [186,203,206]. The idea that naturally-arising HIV-1 variants could affect KIR-HLA binding was supported by reduced *in vitro* binding of KIR3DL1 to its HLA B*57:03 ligand in the presence of the TW10 epitope harboring a G-to-E substitution at position 9 (though this was not claimed to be an *in vivo* NK-driven escape mutation, as failure to engage KIR3DL1 would render infected cells more, not less, susceptible to NK-mediated killing [95]). Rather, NK cell escape could theoretically be achieved *via* viral polymorphisms that reduce recognition by activating KIR, or enhance recognition by inhibitory KIR. Towards the identification of such mutations, statistical association approaches were applied to N=91 linked KIR/HIV-1 sequences, yielding 22 KIR-associated viral polymorphisms. The researchers identified two linked polymorphisms in Vpu (71M/71H) that were particularly overrepresented among KIR2DL2-expressing persons [21]. Consistent with the greater affinity of KIR2DL2 for HLA-C group 1 compared to group 2 ligands [207], these polymorphisms were even

more enriched among KIR2DL2^{+} individuals homozygous for HLA-C group 1 alleles [21]. Researchers further showed *in vitro* that the presence of these polymorphisms enhanced the ability of the inhibitory KIR2DL2 to bind HIV-1-infected cells, that KIR2DL2^{+} NK cells failed to become activated in the presence of polymorphism-containing HIV-1, and that cells infected with polymorphism-containing HIV-1 were not inhibited by KIR2DL2^{+} NK cells [21]. However, researchers were unable to elucidate the mechanism whereby these polymorphisms reduced the ability of KIR2DL2^{+} NK cells to recognize variant virus-infected cells, nor were they able to identify whether specific peptide(s) played a role in this interaction.

Despite remaining questions, these findings suggest that immune pressure by an inhibitory KIR could select *in vivo* escape mutations conferring enhanced binding of the inhibitory receptor to HIV-1-infected cells, thereby allowing them to escape NK cell-mediated elimination. The recent identification of an HLA-C*01:02-restricted p24^{Gag} peptide variant that bound KIR2DL2, that conferred functional inhibition of KIR2DL2-expressing NK cells *in vitro* [208], provides theoretical support for this model. The idea that NK cells recognize antigen in a manner that is to some extent specific, leading to the reproducible selection of escape mutations *in vivo,* is intriguing. Further research is required to confirm and to elucidate the extent to which innate immune responses drive HIV-1 evolution.

2.4. Immune-driven HIV-1 evolution: consequences and implications

2.4.1. Fitness consequences of escape for infection and transmission

The protective effects of certain HLA class I alleles are attributable, at least in part, to their ability to mount strong CTL responses against mutationally constrained HIV-1 regions where escape can only occur at a functional and/or replicative cost to the virus (e.g., [209]). In these cases, the viral advantage gained *via* immune escape is offset in part by its associated replicative costs, thus conferring some residual biological "benefit" to the host in terms of lower viral loads. For example, HLA-B*81 is a protective

allele in context of the South African HIV-1 subtype C epidemic. The B*81-driven Gag T186S escape mutation (selected at position 7 of the immunodominant B*81-restricted TL9 epitope spanning Gag codons 180-188) is fitness-costly [210] and difficult to compensate [211]. Thus, although Gag-T186S confers escape from the B*81 TL9-mediated recognition of infected cells, the sustained replication defects conferred by this substitution may contribute to the long-term clinical benefits associated with HLA-B*81 expression. It is important to note however, that initial immune-driven viral fitness reductions are often of limited duration. For example, recombinant HIV-1_{NL4-3} encoding acute/early Gag-Protease sequences derived from individuals expressing protective HLA display relative replicative reductions in acute/early infection, but these defects are largely undetectable by chronic infection due to the selection of compensatory mutations [212].

The clinical "benefits" of immune-driven viral replicative costs can also be detected when viruses containing such mutations are transmitted to persons lacking the restricting HLA. Indeed, lower viral loads in individuals acquiring HIV-1 with key fitness-costly escape mutations in Gag [213,214] (though not Nef [214]) have been demonstrated. That immune-driven mutations selected by past hosts inherently influence the pathogenicity of a given HIV-1 sequence is supported by the observation that a substantial fraction of set-point plasma viral load is "heritable" from one infection to the next [215]. That HIV-1 sequences are inherent determinants of pathogenesis is also supported by the observation that viral replication capacity correlates positively with viral load (and negatively with $CD4^+$ T cell count) at various infection stages [139,210,212,216,217]. Extending these observations, one could hypothesize that the acquisition of attenuated HIV-1, followed by further within-host selection of fitness-costly escape mutations, would provide maximal clinical "benefit" to the host. Indeed, elite controllers, rare individuals who are able to spontaneously suppress plasma HIV-1 RNA to below limits of clinical detection without the need for antiretroviral therapy [218], provide a model for this phenomenon [219]. Elite controller-derived HIV-1 sequences generally exhibit reduced function compared to HIV-1 from noncontrollers at both early [220,221] and chronic [116,222,223,224] infection stages, supporting the acquisition of attenuated HIV-1 in at least some of these individuals. Two lines of evidence suggest that these relative defects are also attributable to the within-host selection of fitness-

costly mutations. First, elite controllers expressing protective HLA alleles exhibit even greater HIV-1 attenuation than those who lack them [222,223], in a manner that is “dose dependent” on the number of mutations selected [223,224]. Second, elite controllers harbor non-canonical escape mutations that confer greater fitness costs than conventional ones [225,226], possibly as a result of enhanced immune recognition of common CTL escape variants in these persons [227].

The study of fitness consequences of escape in general - and in elite controllers in particular - is relevant to HIV-1 vaccine research. Specifically, immune-mediated containment of HIV-1 replication to levels that slow disease progression and possibly reduce transmission might be achievable through the design of vaccines that stimulate CTL responses focused against critically conserved viral regions where escape can only occur at substantial fitness costs [228,229]. A related strategy would be to design immunogens featuring both “nonadapted” (susceptible) and “adapted” (escape variant) forms - provided the latter retain the ability to bind the relevant HLA molecules - with the goal of generating broad, potent, variant-reactive CTL responses that, upon infection, will drive HIV-1 evolution down unconventional pathways not unlike those selected in elite controllers [225,226]. Strategies to comprehensively identify HLA-driven immune escape mutations, compensatory pathways and “vulnerable” sites across HIV-1 are thus paramount to achieving such goals.

2.4.2. Differential HIV-1 adaptation to global populations

It is commonly said that HIV-1 adapts to its human hosts “at the population level” [31,32]. This refers to the observation that, since HIV-1 genomes residing in an individual will exhibit adaptations to its host’s immunogenetic profile, then HIV-1 sequences circulating in a given population will, by extension, exhibit adaptations that reflect the distinct immunogenetic profile of that host population [32,136]. The existence of CTL escape mutations “unique” to particular host populations, because they are restricted by HLA alleles specific to these populations, provides one illustration of this phenomenon. For example, >50% of HLA-associated polymorphisms identified in HIV-1 subtype B sequences in Mexico [136] and nearly two-thirds of those identified in Japan [230] are distinct from those observed in subtype B-infected cohorts from

Canada/USA/Australia, because the former populations exhibit “unique” HLA alleles (e.g., B*39 in Mexico and B*67:01 in Japan) that are not found in the latter populations [136,230]. That HIV-1 polymorphisms correlate with host ethnicity (a surrogate of HLA) also demonstrates population-specific viral adaptation [231].

Population-specific HIV-1 adaptation also manifests itself in terms of differential HIV-1 polymorphism frequencies among host groups. In particular, of HLA-associated polymorphism frequencies among viral sequences circulating in a given population will generally reflect the frequencies of their restricting HLAs in that population [32]. This remains true even among individuals lacking the restricting HLA. This is because higher numbers of persons expressing the HLA will generally translate to higher numbers of polymorphisms selected and thus transmitted (though many factors, including the wide-ranging probabilities of polymorphism selection in context of their viral location and restricting HLA, the fact that multiple HLA alleles select the same - or opposing – mutations at a given viral location, the existence of “consensus” HLA-associations, and the timing of escape/reversion, will render this correlation less than perfect). The B*51-associated I135X mutation in Reverse Transcriptase (at the C-terminus of the B*51-TI8 epitope, RT codons 128-135) provides an example. In an analysis of nine cohorts spanning five continents, HLA-B*51 and RT-I135X prevalence exhibited a strong positive correlation [32], indicating that the more frequent an HLA allele is in a population, the more frequent its associated adaptations will be observed in circulating HIV-1 sequences.

Although a major portion of population-specific HIV-1 adaptation to host cellular immune responses is attributable to population-specific differences in HLA alleles and their frequencies, other host factors (e.g., variability in T cell receptor genetics) also likely plays a role. A recent study comparing HLA-associated polymorphisms in HIV-1 subtype B cohorts in Japan versus Canada/USA/Australia identified numerous cases where the same HLA allele selected significantly different escape pathways across cohorts [230], implying factors beyond HLA in driving these differences. HLA-driven escape pathways also differ across HIV-1 subtypes, presumably as a result of genetic differences in the viral backbone. For example, Gag-T242N is commonly selected by B*57 in HIV-1 subtypes B [83,126,137,138], C [125] and D [232] but rarely in subtype A1

[232]. Similarly, the fitness costs of escape can differ across subtypes. For example, Gag-M250I confers profound fitness costs in subtype B (where it represents a rare escape mutation selected by HLA B58 supertype alleles) but not subtype C (where it appears to be a minor non-HLA-associated polymorphism) [226]. Together, these observations highlight the relevance of HLA, along with other host and viral genetic determinants of HIV-1-specific CTL responses, in driving HIV-1 evolution. As such, cellular vaccine designs featuring immunogens that incorporate immune-relevant HIV-1 diversity may require us to distinguish escape pathways that are "universal" across host populations and/or HIV-1 subtypes, from those that are population and/or HIV-1 subtype-specific.

2.4.3. Is HIV-1 becoming increasingly "resistant" to host immunity as the epidemic progresses?

As described in previous sections, many - though not all - immune escape mutations selected in the previous host will revert to consensus upon transmission to a host lacking the restricting HLA allele. As such, the persistence of certain escape mutations following transmission has led to concerns that these could gradually spread throughout the population (Figure 2.4) [32,105,233,234,235,236,237]. Analogous to the negative impact of transmitted drug resistance mutations on treatment efficacy [238], acquisition of "escape mutant" HIV-1 by persons expressing the relevant HLA could undermine the ability of their CTL to control infection. As such, the spread of HIV-1 strains harboring escape mutations throughout the population could gradually undermine host antiviral immune potential, and potentially diminish the protective effects of certain HLA alleles as the epidemic progresses [31,32,106]. Indeed, the S173A compensatory mutation has been shown to stabilize the B*27-associated R264K mutation in $p24^{Gag}$ upon transmission [106,107] and the S165N compensatory mutation has been shown to stabilize B*57-associated mutations within the $p24^{Gag}$ KF11 epitope [98], supporting this concern. That certain (though not all) escape mutations are capable of spreading in HIV-1-infected populations has also been demonstrated *via* mathematical modeling [104].

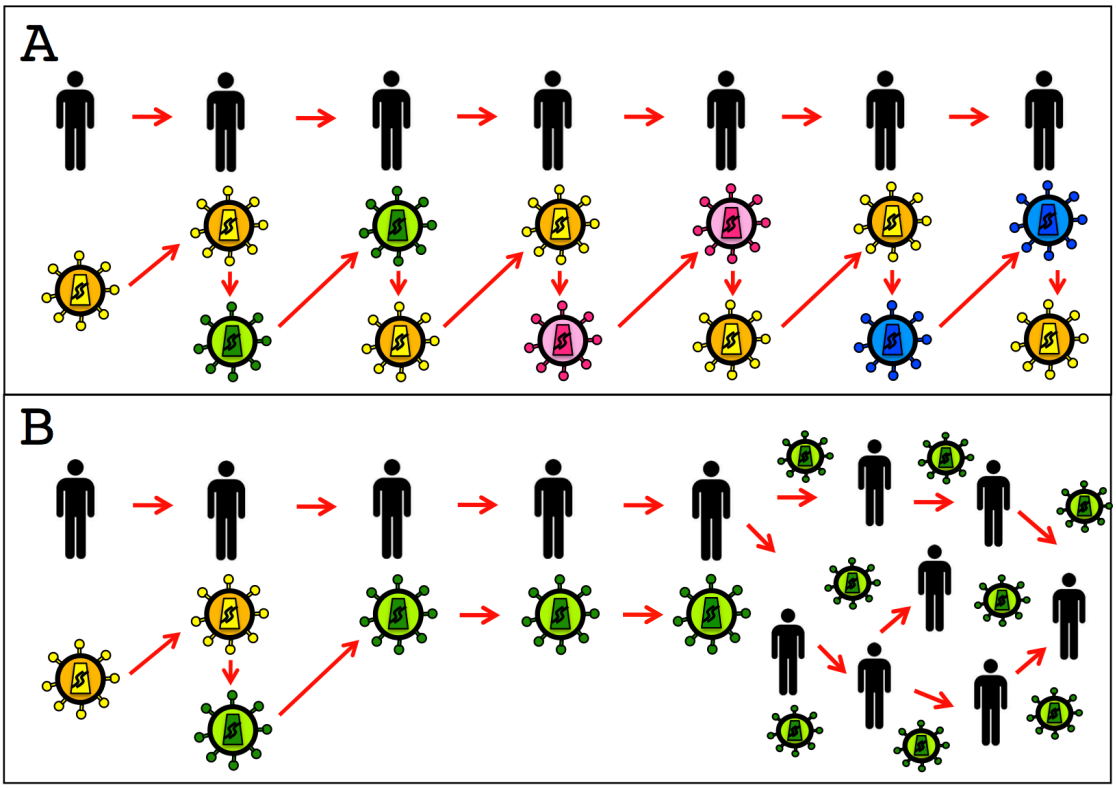

Figure 2.4. The persistence of HIV-1 immune escape mutations upon transmission could lead to their gradual spread in the population over time.

In this simplified diagram of HIV-1 transmission, viruses harboring the consensus amino acid at a particular codon are colored yellow, whereas other colors denote viruses harboring various HLA-associated escape mutations. Arrows depict HIV-1 transmission from host to host, and the subsequent selection and/or reversion of escape mutations within these hosts.

A: In this scenario, HIV-1 escape mutations selected in previous host(s) revert consistently upon transmission. When sampled at a given point in time, sites of viral escape would exhibit diversity at the population level (as the relevant polymorphism would be present in some hosts but not others), however the population consensus sequence would remain stable (unchanged) over time.

B: In this scenario, an HIV-1 escape mutation stably persists upon transmission to hosts lacking the relevant HLA allele. Such mutations could gradually spread throughout the population, causing the subtype consensus to shift over time. For more details, see section 2.4.3.

The extent to which HLA-associated polymorphisms are spreading in HIV-1-infected populations remains incompletely known, in part due to the scarcity of historic data. Nevertheless, it has been suggested that CTL epitopes in European HIV-1

sequences are being “lost” through mutational escape from HLA-B mediated selective pressures [235]; similarly, higher viral polymorphism frequencies have been reported in modern compared to historic HIV-1 subtype B and F sequences in South America [236]. The high frequency of the B*51-associated HIV-1 Reverse Transcriptase (RT) I135X mutation in Japan, a population where B*51 prevalence approaches 20%, is also suggestive of escape mutation accumulation [32] (though the possibility that the Japanese epidemic was founded by an HIV-1 sequence containing RT-I135X cannot be ruled out). A recent comparative study of historic (1979-1989) versus modern (2000+) HIV-1 subtype B cohorts in North America revealed modest, though statistically significant increases in the average background frequencies of HLA-associated polymorphisms, notably in Gag, over the study period which paralleled an approximate twofold increase in HIV-1 diversity during this time [146]. Although the extent of polymorphism spread appears relatively modest for the North American HIV-1 epidemic, corresponding rates of immune-driven polymorphism spread in regions with high HIV-1 prevalence, older epidemics, differential transmission dynamics and/or where host HLA diversity is relatively limited may be higher, and thus possess more immediate implications for host immunity in these populations.

The accumulation of CTL escape mutations in circulating HIV-1 sequences is paralleled by a similar phenomenon driven by humoral immunity. Two recent studies evaluating antibody neutralization resistance of historic versus modern HIV-1 envelope sequences suggest that HIV-1 is drifting towards a more neutralization-resistant phenotype over time [239,240]. Furthermore, contemporary sera exhibited lower heterologous neutralizing activity than historic sera, consistent with a gradual undermining of humoral immunity as HIV-1 becomes increasingly neutralization resistant [240]. Taken together, evidence suggests that HIV-1 is becoming - albeit gradually - more “pre-adapted” to host immunity as escape mutations spread in circulation. Further studies are therefore warranted to explore the extent of HIV-1 adaptation to cellular and humoral immune pressures in different host populations as their respective epidemics increase in age and diversity, and the potential implications of this adaptation for natural and vaccine-induced immunity over time.

2.4.4. Implications of immune escape for antiretroviral therapy

Immune-driven HIV-1 polymorphisms can also influence HIV-1 susceptibility to antiretroviral drugs [241,242]. Bevirimat, an HIV-1 $p24^{Gag}$ (capsid) maturation inhibitor whose development was halted in 2010 following poor efficacy in individuals harboring common Gag polymorphisms, provides an example. Bevirimat prevents capsid formation by inhibiting cleavage at the CA/SP1 site in Gag [243], but its activity is reduced in HIV-1 harboring naturally-occurring substitutions within the QVT motif of SP1 (Gag codons 369-371) and/or Gag substitutions V362I, S373P and I376V [243,244,245,246]. Perhaps unsurprisingly, these "naturally-occurring" substitutions are largely HLA-driven. Gag V362I, conferring high-level bevirimat resistance [245], and S373P, potentially associated with low-level resistance [244,245] are selected by HLA-B*35 [126], whereas a variety of HLA alleles including C*03 select polymorphisms within the QVT motif [126]. Indeed, up to 50% of subtype B sequences (and >90% in other subtypes) harbor polymorphisms within the QVT motif [247], underscoring the relevance of immune-driven polymorphisms to drug development.

By definition, licensed antiretrovirals will have demonstrated potent activity against a range of HIV strains, so any impact of immune-driven polymorphisms on their activities will be subtler (or the relevant polymorphisms more rare) than the above example. Nevertheless, such effects have been documented for non-nucleoside reverse transcriptase inhibitors (NNRTIs). The first evidence arose *via* primary HIV-1 drug resistance surveys that identified a minority of HIV-1 RT sequences exhibiting intermediate-level reduced *in vitro* susceptibility to NNRTIs in the absence of major resistance mutations [248,249]. These observations were not explained by shared viral ancestry, but rather by the presence of polymorphisms at sites not previously associated with resistance [249]. In particular, I135T/V/L and 283I/L in RT, present alone or in combination, conferred up to 3-fold reduced *in vitro* susceptibility to NNRTIs [250]. Notably, these polymorphisms are immune-driven: those at RT codon 135 are selected by a variety of HLA alleles including B*51 [32,126,149,242] and B*52 [86,149], whereas 283L is selected by B*15 [126]. Polymorphisms at RT codon 138 (E138G/A/K), selected by B*18 [86,241] and B*46 [86], may also mediate up to 7-fold decreased susceptibility to the second-generation NNRTI rilpivirine [241]. Naturally-occurring polymorphisms

modulating *in vitro* NNRTI susceptibility have also been identified in non-subtype B contexts [251].

Other immune-driven polymorphisms do not directly influence drug susceptibility, but rather facilitate the selection of major resistance mutations *in vivo*. The presence of I135T has been associated with the subsequent selection of the K103N resistance mutation during NNRTI therapy [252,253], possibly because it enhances stability of the mutant enzyme's active site [252]. Furthermore, *in vitro* passage of HIV-1 containing the I135V/T/R polymorphisms in the presence of NNRTI led to the selection of E138K, which together with I135V/T/R conferred significantly reduced *in vitro* susceptibility to both first and second-generation NNRTIs [242]. Similarly, the A*11-associated V106I polymorphism [86] confers significant *in vitro* resistance to some NNRTIs when present in combination with natural polymorphism V179D [254].

Despite the observations described above, it is important to note that no studies have conclusively demonstrated that specific HLA alleles (and/or the presence of specific immune-driven viral polymorphisms) enhance risk of treatment failure [253,255], an observation attributable in part to the widespread use of HIV-1 drug resistance testing to guide treatment choices [256,257]. Such risks nevertheless remain a concern, and argue for enhanced collaboration across the fields of viral immune adaptation and drug resistance. A comprehensive understanding of HLA-associated polymorphisms across HIV-1 subtypes and host populations could facilitate the identification of immune escape mutations capable of modulating the efficacy of current and future antiretroviral agents.

2.4.5. Escape from vaccine-induced antiviral immunity

Our discussion of mutational HIV-1 escape has thus far focused on natural immune responses. However, vaccine-induced immune responses could also exert sufficient pressures to drive viral evolution [258,259,260]. Analysis of "breakthrough" HIV-1 sequences infecting participants of recent vaccine trials supports this idea. A recognized challenge in designing vaccines against genetically heterogeneous pathogens such as HIV-1 is the possibility that vaccine-induced immunity may protect against infection by strains most similar to the vaccine immunogen(s), but not against

genetically divergent strains. That vaccine-induced immunity could induce a partial barrier through which antigenically divergent HIV-1 strains could penetrate has been termed the “acquisition sieve effect” [261,262,263]. A related - yet mechanistically distinct - possibility is that vaccine-induced immunity would fail to block HIV-1 infection regardless of strain, but would instead drive the outgrowth of escape variants at rates exceeding those observed in natural infection [259,262], a phenomenon termed “postinfection sieve effect”. The latter is particularly relevant to vaccines designed to stimulate cellular responses, as these are unlikely to block HIV-1 transmission. Vaccine sieve effects can be identified by retrospectively comparing the HIV-1 sequences of vaccine versus placebo trial participants who subsequently became infected, to determine differences between them (e.g., in terms of specific HIV-1 polymorphisms and/or differences in their average genetic distance from the vaccine strain) [261,262,263]. Notably, acquisition and post-infection sieve effects are difficult to distinguish from one another, as both may occur before HIV-1 RNA can be reliably detected in the bloodstream [37], and/or may manifest themselves *via* the presence of identical immune-associated polymorphisms.

HIV-1 vaccine sieve effects were first suggested by the presence of atypical V3 amino acid motifs in HIV-1 *env* sequences from individuals vaccinated with recombinant HIV-1_{MN} gp120 [258]. Recent comparisons of founder HIV-1 strains from vaccine- and placebo recipients of the RV144 “Thai” vaccine trial [60] identified differential amino acid frequencies at *env* V2 codons 169 and 181 between the two groups [260], suggesting that the vaccine preferentially blocked viruses harboring specific substitutions at these positions [260], possibly *via* vaccine-induced V2-specific antibodies possessing antibody-dependent cellular cytotoxicity activity [264]. Rapid selection of CTL escape mutations by vaccine-induced cellular immune responses may also have occurred in the failed STEP vaccine trial [61,265]. Inferred T cell epitope sequences within Gag/Pol/Nef (the regions contained within the vaccine) from infected vaccine recipients exhibited greater genetic distances to the immunogen sequence compared to those of infected placebo recipients, presumably as a result of extensive and rapid immune escape [259]. The lack of such differences for epitopes within other HIV-1 proteins also supported this conclusion [259]. HIV-1 sequences from vaccine recipients also exhibited substitutions at Gag codon 84 more frequently than placebo recipients, identifying this as a putative

signature site of HIV-1 evolution in response to vaccine-induced CTL responses [259].

The implications of vaccine-induced immune responses on the transmission, selection and evolution of HIV-1 are potentially profound. Rapid vaccine-driven immune escape could yield clinical consequences for the infected individual [266], whereas the use of partially-effective vaccines capable of blocking infection by certain HIV-1 strains raises concerns regarding potential shifts in HIV-1 strain and lineage distributions (and their clinical and pathogenic consequences) at the population level. That vaccine-induced immune responses (notably CTL) may target slightly different epitopes than those generally targeted in natural infection [267] may further complicate this issue, and highlights it as an area worthy of future investigation.

2.5. Concluding remarks

Since its identification as a novel human retrovirus just over 30 years ago [268,269], HIV-1 has claimed the lives of an estimated 40 million individuals, with approximately 35 million additional persons currently infected [7]. Although expansion of HIV-1 treatment can help to stem the pandemic's tide [270,271,272], HIV-1's substantial capacity for host adaptation and ever-increasing global diversification, driven in large part by selection pressures imposed by the host immune response, remain major challenges for the design of interventions, notably a vaccine [3,4]. Achieving a deeper understanding of how immune selection pressures drive the evolution and diversification of HIV-1 both within and among hosts, and how these viral changes in turn affect our immune responses to the virus, will bring us closer to our ultimate goal of ending the HIV-1pandemic.

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Chapter 3.

Consequences of HLA-B*13-associated escape mutations on HIV-1 replication and Nef function

3.1. Abstract

HLA-B*13 is associated with superior *in vivo* HIV-1 viremia control. Protection is thought to be mediated by sustained targeting of key CTL epitopes and viral fitness costs of CTL escape in Gag, although additional factors may contribute. We assessed the impact of 10 published B*13-associated polymorphisms in Gag, Pol and Nef, in 23 biologically relevant combinations, on HIV-1 replication capacity and Nef-mediated reduction of cell surface CD4 and HLA class I expression. Mutations were engineered into HIV-$1_{NL4.3}$ and replication capacity (RC) was measured using a GFP-reporter T cell line. Nef-mediated CD4 and HLA-A*02 downregulation was assessed by flow cytometry, and T cell recognition of infected target cells measured via co-culture with an HIV-specific luciferase-reporter cell line. When tested individually, only Gag-I147L and Gag-I437L incurred replicative costs (5% and 17% respectively), consistent with prior reports. The Gag-I437L-mediated replication defect was rescued to wild-type levels by the adjacent K436R mutation. A novel B*13 epitope, terminating at Gag-147, was identified in $p24^{Gag}$ ($GQMVHQAI_{Gag140-147}$). No other single or combination Gag, Pol or Nef mutants impaired viral replication. Single Nef mutations did not affect CD4 or HLA downregulation; however, the double mutant E24Q-Q107R showed 40% impairment in HLA downregulation with no evidence of Nef stability defects. Moreover, target cells infected with HIV-1-$Nef_{E24Q-Q107R}$ were recognized nearly two-fold better by HIV-specific T cells compared to those infected with HIV-$1_{NL4.3}$ or single Nef mutants. Our results indicate that CTL escape in Gag and Nef can be functionally costly and suggest that these effects may contribute to long-term HIV-1 control by HLA-B*13.

3.2. Introduction

The natural history of HIV-1 is influenced by human leukocyte antigen (HLA) class I polymorphisms [1-4]. Notably, HLA-B*57 and B*27 are associated with viral load control and slower disease progression [5,6], but other "protective" HLA class I alleles have also been identified [4,7,8]. HLA-B*13 is one such allele, but it remains understudied in part because it is relatively rare in many populations (expressed in less than 4% of Caucasians [9] and Africans [10,11], although its frequency exceeds 10% in some Asian populations [12-14]). HLA-B*13, of which the most common subtype is B*13:02 [15], is associated with lower viral loads in chronic infection [10,11,14,16,17], lower hazard ratios of progression to AIDS in historic natural history studies [18] and is also enriched among HIV controllers [6,19,20].

B*13-mediated protective effects are attributed, at least partially, to sustained targeting of conserved B*13-restricted cytotoxic T-lymphocyte (CTL) epitopes, particularly in HIV-1 Gag [11] and Nef [21]. Optimally-described B*13 epitopes have also been identified in Pol [22], although these responses appear to contribute less to control [11]. Similar to B*57 [23-25] and B*27 [26], the protective effects of B*13 may also be mediated to some extent by costs to viral replicative fitness incurred as a result of immune escape within certain CTL epitopes, notably in Gag [27]. These replicative costs may limit the net viral "benefit" gained via immune evasion, leading to relative viral control or reduced pathogenesis. In particular, selection of Gag-I437L or I437M escape mutations at the C-terminal anchor residue of the B*13-restricted $p1^{Gag}$ RI9 epitope ($RQANFLGKI_{429-437}$) substantially impaired *in vitro* HIV-1 replicative capacity [27,28]. Likewise, the B*13-driven Gag-I147L polymorphism, located four codons downstream of the $p24^{Gag}$ VV9 epitope ($VQNLQGQMV_{135-143}$) confers minor replication defects [28].

Though fitness-reducing CTL escape mutations in HIV-1 commonly occur in Gag [28,29], escape in Pol can also incur replicative costs [30,31]. In addition, naturally-occurring sequence variation in Nef [32-34], including HLA-driven escape mutations [35,36] can modulate this protein's major functions, including cell-surface CD4 [37] and HLA class I [38] downregulation. However, the functional costs of B*13-driven immune escape outside of Gag and their possible contributions to B*13-mediated protective effects have not been investigated. Moreover, the extent to which secondary

(compensatory) polymorphisms can restore fitness costs of primary B*13-driven escape mutations also remains incompletely known. Such effects however are supported by observations of reduced replication capacity of patient-derived B*13 Gag sequences in early, but not chronic infection [25].

To further elucidate the replicative and functional consequences of B*13-driven HIV-1 evolution, we investigated the 10 polymorphisms in Gag, Pol and Nef that are most commonly selected in B*13-expressing individuals [39]. Specifically, we tested these polymorphisms alone and in biologically observed combinations for effects on *in vitro* HIV-1 replication capacity and Nef-mediated CD4 and HLA class I downregulation activity. Our results extend current knowledge of B*13-driven Gag replicative costs [27,28] by identifying Gag-K436R as a compensatory mutation for Gag-I437L. We also provide evidence for a novel B*13-restricted CTL epitope terminating at Gag codon 147, B*13's most frequent escape site in HIV-1 [39]. Notably, although B*13-associated substitutions in Pol and Nef did not impair viral replication, the B*13-associated Nef polymorphisms E24Q-Q107R, when expressed together, reduced Nef's ability to downregulate HLA class I from the infected cell surface. This in turn enhanced the recognition of infected cells by HIV-1-specific T cells *in vitro.* Though this mutation combination is observed in less than 5% of B*13-expressing persons, it nevertheless represents a novel consequence of CTL escape in HIV-1. Specifically, our results suggest that B*13-mediated immune control may be attributable, at least in part, to CTL escape-driven attenuation of Nef's immune evasion function.

3.3. Materials and methods

3.3.1. Definition of HLA-B*13-associated polymorphisms

Ten published HLA-B*13 and/or B*13:02-associated polymorphisms in HIV-1 Gag (n=4), Pol (n=4) and Nef (n=2), originally identified via statistical association with phylogenetic correction in the International HIV Adaptation Collaborative (IHAC) cohort comprising >1800 antiretroviral-naïve chronically HIV-1 subtype B infected individuals [39], were selected for analysis (Fig. 3.1 and 3.2). These polymorphisms represent all B*13/B*13:02-associated sites in Gag, Pol and Nef for which a specific 'adapted' (CTL

escaped form) amino acid was identified at $p<0.0001$ and $q<0.05$ in the original study [39]. At two of these codons (protease 63 and RT 369) the original study identified two possible adapted forms; the one with the lower p-value was selected for the present analysis. The extent to which these polymorphisms are enriched in B*13:02+ versus B*13:02- individuals in the original study [39], along with their locations respective to published or bioinformatically-predicted B*13-restricted CTL epitopes. (For more details, see Figure 3.2 in results section).

3.3.2. Characterization of escape rates and frequencies in B*13+ individuals

The frequencies and kinetics of selection of B*13-associated polymorphisms in acute/early HIV-1 subtype B infection were defined using longitudinal plasma HIV-1 RNA sequences from nine B*13:02+ seroconverters from cohorts in Berlin (Jessen-Praxis Medical Clinic, Germany), New York (Aaron Diamond AIDS Research Center, USA), Boston (Massachusetts General Hospital, USA) and Vancouver (British Columbia Centre for Excellence in HIV/AIDS; Vanguard and Vancouver Injection Drug Users Study [VIDUS] Canada) [40,41]. All patients provided written informed consent and the cohort studies were approved by their respective institutional review boards. For four patients, HIV-1 sequences were already published [41]. For others, HIV-1 RNA was extracted from plasma and Gag, Pol and Nef were amplified in independent nested RT-PCR reactions using HIV-1-specific primers. Amplicons were bidirectionally sequenced on a 3130xl automated DNA sequencer (Applied Biosystems, Inc.) and chromatograms were analyzed using Sequencher v5.0 (Genecodes). Sequences were aligned to the HIV-1 subtype B reference strain HXB2 (GenBank accession # K03455) using an algorithm based on the HyPhy platform [42]. Time from estimated infection date to the first detection of the specific B*13-associated polymorphism was determined using Kaplan-Meier methods; these data were also used to calculate escape prevalence after one year of infection. The prevalence of these mutations in chronic infection was calculated using published plasma HIV-1 RNA sequences from 69 B*13:02+ individuals in the IHAC cohort [39].

3.3.3. Generation of mutant HIV-1

B*13-associated polymorphisms were engineered into the HIV-1 subtype B reference strain NL4.3 (NIH AIDS Research and Reference Reagent Program, contributed by Dr. Malcolm Martin, Catalog # 114 [43]). First, pNL4.3 *gag*, *pol* (covering *protease* and the first 400 codons of *reverse transcriptase*) and *nef* were subcloned into pCR2.1-TOPO (Life Technologies). Individual mutations were introduced into these plasmids using the QuikChange II XL site-directed mutagenesis kit (Stratagene) using custom designed primers (Table 3.1). Combination mutants were generated by successive rounds of mutagenesis. All mutants were confirmed by DNA sequencing. Mutant subcloned genes were then used to generate recombinant NL4.3 viruses by homologous recombination [25]. Briefly, mutant *gag, pol and nef* were re-amplified from subclones using 100-mer primers complementary to NL4.3. After verification on an agarose gel, each amplicon was co-transfected, along with the relevant linearized pNL4.3 vector (Δ*gag*, Δ*pol* or Δ*nef*), into a Tat-inducible CEM-GFP-reporter T cell line (GXR25) [44] via electroporation. Cells were incubated for 10 days at 37°C in R20+ media [RPMI with phenol red, supplemented with 20% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin] to allow the generation and propagation of infectious virions. GFP expression (indicating HIV-1 infection) of these cultures was monitored daily by flow cytometry (Guava easyCyte 8HT; Millipore); once this reached >15%, supernatants containing infectious mutant virions were harvested, aliquoted and frozen at -80°C. The median time to harvest was 13 (range: 11-14) days. HIV-1 RNA from each virus stock was re-extracted (Invitrogen PureLink™ Genomic DNA/RNA kit; Life Technologies), re-amplified and the relevant gene was re-sequenced to confirm the presence of the desired substitution(s) and the absence of any others.

Table 3.1. List of forward primers used in site-directed mutagenesis

Gene	Mutation	Forward Primer
gag	A146S	CCAGGGGCAAATGGTACATCAG**TCC**ATATCACCTAGAACTTTAAATG
	I147L	CAGGGGCAAATGGTACATCAGGCC**TTA**TCACCTAGAACTTTAAATGCATGG
	K436R	GAGAGACAGGCTAATTTTTTAGGG**AGA**ATCTGGCCTTCCCACAAGGGAAGG
	I437L	GAGACAGGCTAATTTTTTAGGGAAG**TTA**TGGCCTTCCCACAAGGGAAGGCCAG
pol	PR-L63S	CAAAGTAAGACAGTATGATCAGATA**AGC**ATAGAAATCTGCGGACATAAAGCTATAGG
	RT-Q334N	AATAGCAGAAATACAGAAGCAGGGG**AAT**GGCCAATGGACATATCAAATTTATC
	RT-T368A	CCCACACTAATGATGTGAAACAATTA**GCA**GAGGCAGTACAAAAAATAGCCACAG
	RT-K374R	GAAACAATTAACAGAGGCAGTACAA**AGA**ATAGCCACAGAAAGCATAGTAATATGGGG
nef	E24Q	GAAAGAATGAGACGAGCT**CAA**CCAGCAGCAGATGGGGTGGGAGCA
	Q107R	CTAATTCACTCCCAAAGAAGA**AGA**GATATCCTTGATCTGTGGATCTACC

3.3.4. *In vitro* HIV-1 replication capacity assays

Viral titers were assessed as described previously by infecting GXR25 reporter cells in order to determine the volume necessary to achieve an MOI=0.003 (0.3% HIV-infected cells) at 2 days after infection [25,31,45,46]. For subsequent viral replication assays, GXR25 cells were infected with control (wtNL4.3) and mutant viruses at MOI=0.003, after which the percentage of GFP^+ (HIV-infected) cells was monitored daily by flow cytometry until day 6. For each virus, the natural log of the slope of viral spread (measured as % infected cells) was calculated during the exponential phase. This value was then normalized by the mean rate of spread of wtNL4.3 such that RC values <1.0 or >1.0 indicate rates of viral spread slower than or faster than NL4.3, respectively. Each virus was assayed in a minimum of two independent experiments each containing three replicates.

3.3.5. Characterization of a novel putative HLA-B*13:02-restricted epitope in p24 Gag

The presence of a novel B*13:02-restricted epitope overlapping Gag_{146} and Gag_{147} was investigated bioinformatically and experimentally. First, the HIV-1 consensus B amino acid sequence spanning $Gag_{135-155}$ was scanned for the presence of B*13:02-restricted 8-11mer epitopes using NetMHCpan 2.8 [47,48] (http://www.cbs.dtu.dk/services/NetMHCpan/). This algorithm identifies candidate epitopes based on their predicted half-maximal inhibitory concentrations (IC50), where values <500 nM, 500-5,000 nM and >5,000 nM are predicted as strong, weak and non-binders, respectively [47,48]. After candidate epitopes were experimentally verified, NetMHCpan 2.8 binding predictions were repeated for variants containing B*13-associated polymorphisms at Gag_{146} and/or Gag_{147}. PBMC from seven B*13:02+ chronically HIV-1 subtype B-infected individuals recruited at Hospital Clinic Barcelona (n=4) and at IrsiCaixa AIDS Research Institute, Badalona (n=3), Spain collected with written informed consent were screened for responses against C- and N-terminally extended versions of the predicted optimal epitope using a gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assay (as described, [31]). The threshold for positive responses was defined as the highest of the following three criteria: a

minimum of five spots per well, the mean number of SFC of the negative-control wells plus three times its standard deviation, or three times the mean of negative-control wells [49]. Plasma samples were also available from three of the patients; these were subjected to HIV-1 RNA Gag sequencing.

3.3.6. Assessment of Nef-mediated CD4 and HLA class I downregulation in infected cells

Nef-mediated CD4 and HLA class I downregulation was assessed in a GXR25 cell line stably transduced to express HLA-A*02:01 (GXR-A*02). Briefly, one million GXR-A*02 cells were infected with Nef control and mutant viruses at an MOI=0.01. Control viruses included wtNL4.3, ΔNef and Nef_{M20A} (the latter two kindly provided by Takamasa Ueno, Kumamoto University) as well as an HIV-uninfected culture. Nef_{M20A} was used as a control, since it is impaired for HLA class I downregulation [50]. The NL4.3-ΔNef virus was pseudotyped with vesicular stomatitis virus G protein (VSV-G) to enhance infection in GXR-A*02 cells [51]. Once HIV-1 infection reached ≈10-15%, 500,000 cells were stained with APC-labeled anti-CD4 and PE-labeled anti-HLA-A*02 antibodies (BD Biosciences) and cell surface expression of these molecules was measured by flow cytometry. The level of Nef-mediated CD4 downregulation of each virus was expressed as the ratio of the median fluorescence intensity (MFI) of CD4 expression in GFP^+ (HIV-infected) cells to the MFI of CD4 in the uninfected cultures (the latter was used because CD4 downregulation precedes GFP expression in GXR25 cells, leading to lower MFIs in the GFP^- gate in infected cultures). These values were then normalized to that of the wtNL4.3 control. The calculation was as follows: $[(1-(\text{MFI } GFP^+_{\text{mutant}}/\text{MFI } GFP^-_{\text{uninfected}}))/(1-(\text{MFI } GFP^+_{\text{wtNL4.3}}/\text{MFI } GFP^-_{\text{uninfected}}))]*100$. As such, normalized values <100% and >100% indicate CD4 downregulation functions lower than or higher than wtNL4.3, respectively. The same calculation was used to quantify Nef-mediated HLA-A*02 downregulation. Viruses were assessed in a minimum of three independent experiments.

3.3.7. Impact of Nef-associated HLA class I downregulation on infected cell recognition by HIV-1 specific effector T cells

To determine the impact of Nef-mediated HLA-A*02 downregulation on the recognition of infected target cells by HIV-1 specific effector T cells, we employed a reporter T cell co-culture assay. Briefly, one million GXR-A*02 target cells were infected with control (wtNL4.3-Nef, ΔNef, Nef_{M20A}) or Nef mutant viruses at an MOI=0.01 in R20+ media without phenol red. Cultures were monitored daily by flow cytometry and used as "targets" when the proportion of GFP^+ (HIV-infected) cells reached ≈10-15%. HLA-A*02-restricted reporter "effector" T cells were generated using a modification of previously described methods [52] (Anmole G et al, submitted for publication). Briefly, Jurkat T cells were co-transfected with four expression plasmids: 3 µg each of $pSELECTGFP_{Zeo}$ (Invivogen) containing TCRα and TCRβ genes isolated from a CTL clone specific for the A*02-restricted $p24^{Gag}$ $FK10^{Gag}$ epitope (FLGKIWPSYK, Gag 433-442) (kindly provided by Mario Ostrowski and Brad Jones, University of Toronto); 5 µg of a CD8α expression plasmid (Invivogen); and 10 µg of an NFAT-driven luciferase reporter plasmid (Panomics/Affymetrix). Reporter "effector" T cells were electroporated using the Gene Pulser Xcell™ Electroporation System (Bio-Rad Laboratories) (square-wave protocol; 500 V, 2000 µF, 3 msec and 1 pulse), recovered at room temperature for 10 minutes and incubated for 18 hours in R10+ to allow the expression of the TCR. A total of 50,000 HIV-1-infected target cells were co-cultured with 50,000 effector cells (1:1 E:T ratio) for 6 hours and TCR-mediated signaling was detected as NFAT-driven luciferase expression using the Steady-Glo luciferase system (Promega) and quantified using a Tecan Infinite M200 plate reader. Prior studies have indicated that this signal is dependent on endogenously processed HIV-1 Gag $FK10^{Gag}$ peptide antigen presented on HLA-A*02.

3.3.8. Western blots

Western blots were performed as described previously [36]. GXR-A*02 cells were infected with control or mutant viruses. When cultures reached ≈10-15% infection, 800,000 cells were pelleted and lysed. Due to the similar molecular weights (≈27kDa) of Nef and GFP (HIV-1 infection control), lysates were split in half and two blots processed in parallel (with Nef and housekeeping gene control β-actin on one and GFP on the

other). Cell lysates were subjected to SDS-PAGE using Mini-Protean TGX 4% to 20% gels (Bio-Rad Laboratories), and proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (GE healthcare). Nef was detected using a polyclonal rabbit antibody (NIH AIDS Research and Reference Reagent Program, contributed by Dr. Ronald Swanstrom, Catalog # 2949) (1:4,000) [53] followed by staining with secondary donkey anti-rabbit antibody (GE Healthcare) (1:30,000). Expression of β-actin and GFP were assessed using primary mouse anti-actin (Sigma) (1:20,000) and primary mouse anti-GFP (1:10,000) respectively, followed by secondary goat anti-mouse (Jackson ImmunoResearch) (1:20,000). Western blots were developed using Clarity™ Western ECL Substrate (Bio-Rad Laboratories), based on the chemiluminescent detection method. Blots were developed with ImageQuant LAS 400 (GE Healthcare).

3.3.9. Statistical analyses

The one sample t-test was used to assess whether replication capacities and CD4/HLA-A*02 downregulation functions of mutant NL4.3 viruses differed significantly from that of the wtNL4.3 reference strain (whose function was set to 1.0 [for RC] or 100% [for CD4/HLA downregulation]). The Bonferroni correction was used to adjust for multiple tests. Spearman's correlation was used to assess the relationship between Nef-mediated HLA-A*02 downregulation in HIV-infected target cells and NFAT-mediated TCR signaling in HIV-specific effector T cells. Statistical analyses were performed in Prism 5.0 (Graphpad Software). All tests of significance were two-tailed.

3.4. Results

3.4.1. Rates and frequencies of B*13-mediated escape in Gag, Pol and Nef

Ten published HLA-B*13 and/or B*13:02-associated polymorphisms in HIV-1 were investigated: A146S, I147L, K436R and I437L in Gag; L63S in protease; Q334N, T369A, and K374R in reverse transcriptase; and E24Q and Q107R in Nef (Fig. 3.1) [39]. These polymorphisms are significantly enriched in B*13:02+ individuals with chronic HIV-1 subtype B infection, and nine of them lie within or near to published or predicted B*13-restricted CTL epitopes (Fig. 3.2). In addition, Gag-I437L (and to a lesser extent Gag-K436R) are experimentally verified B*13-driven escape mutations within the RI9^{Gag} epitope (RQANFLGKI$_{429-437}$) [11] while Nef-Q107R confers escape from CTL responses against the RI9^{Nef} epitope (RQDILDLWI$_{106-114}$) [21]. Together, these observations support these 10 polymorphisms as the most commonly selected B*13 escape mutations *in vivo*.

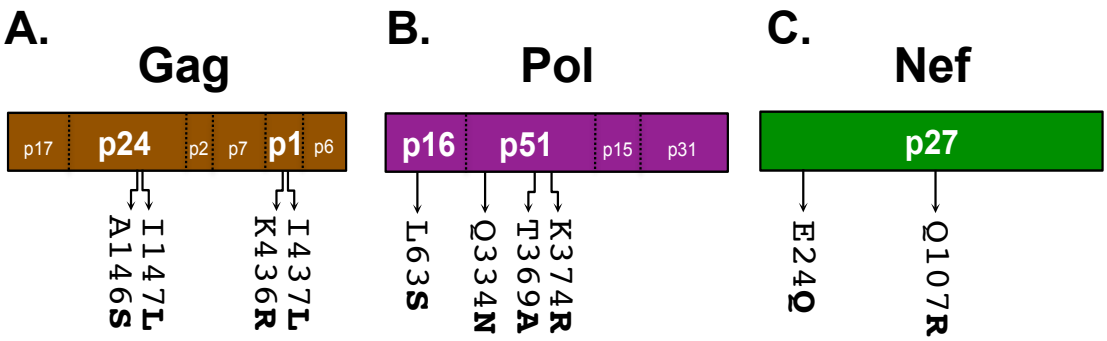

Figure 3.1. HLA-B*13-associated polymorphisms in HIV-1 A. Gag, B. Pol and C. Nef.

Locations of 10 published HLA-B*13-associated polymorphisms in HIV-1 subtype B Gag, Pol and Nef (HXB2 codon numbering).

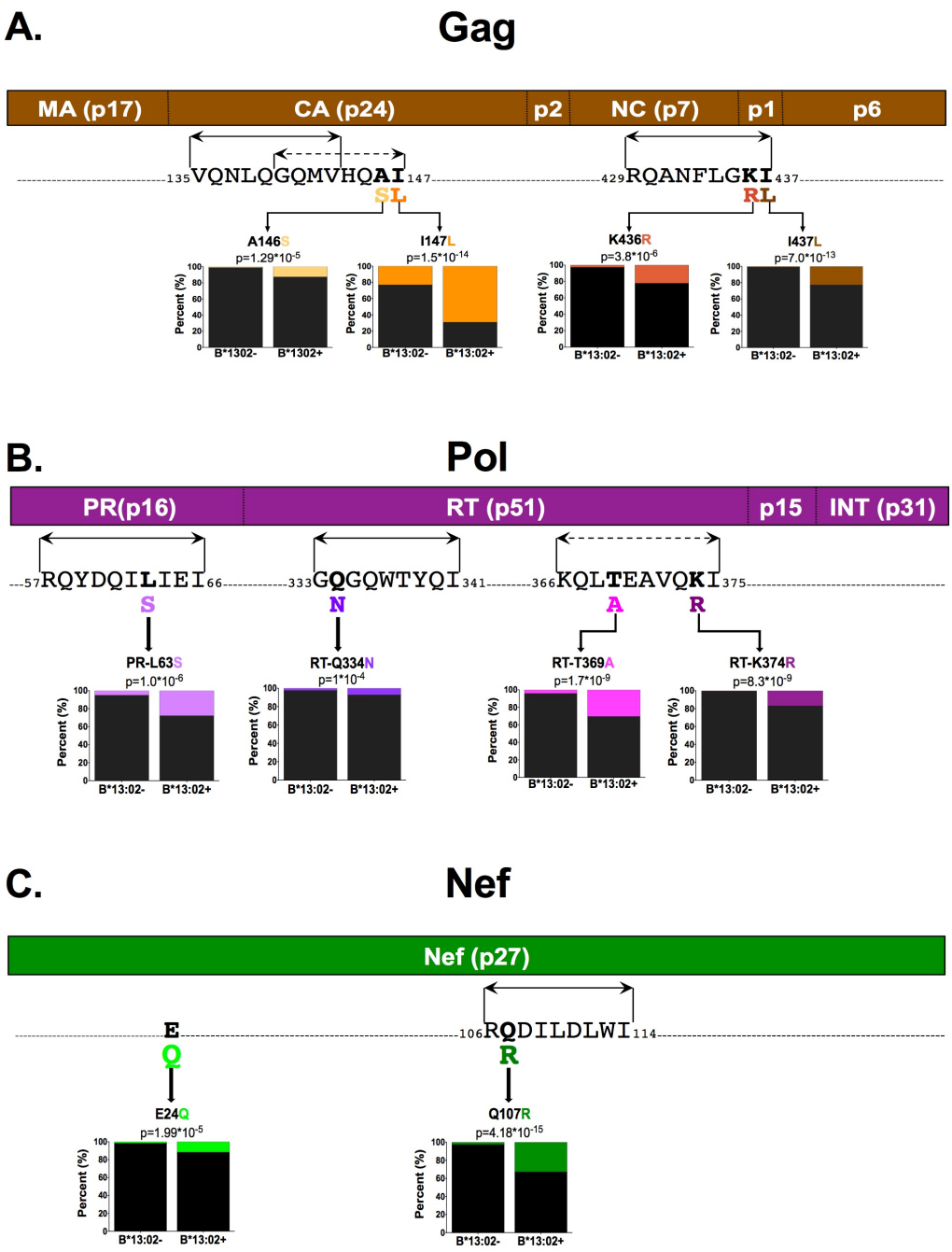

Figure 3.2. HLA-B*13-associated polymorphisms and their locations with respect to published or predicted CTL epitopes

Relative enrichment of the 10 studied polymorphisms in **A.** Gag, **B.** Pol and **C.** Nef in B*13:02+ versus B*13:02- individuals is depicted as colored bar graphs (data, including significance values, are derived from (39)). Polymorphisms are shown with respect to the locations and sequences of published (*solid lines*) and predicted (*dotted lines*) B*13-restricted epitopes.

To infer the extent and timing of HLA-B*13-driven escape during HIV-1 infection, we analyzed longitudinal plasma HIV-1 RNA sequences from nine B*13:02+ seroconverters [40,41] and cross-sectional sequences from 69 B*13:02+ individuals with chronic infection [39]. In HIV-1 Gag, 36% and 13% of B*13:02+ individuals harbored Gag-I147L and -I437L respectively at one year post-infection; by the chronic stage, 69% and 26% of B*13:02+ individuals harbored these substitutions (Fig. 3.3A top and middle). In contrast, Gag-A146S and -K436R emerged later in infection (reaching 17% and 26%, respectively, by the chronic stage). For HIV-1 Pol, protease-L63S, RT-T369A and RT-K374R were observed in ≈12% of B*13:02+ individuals in early infection, and 25-37% in chronic infection (Fig. 3.3B, top and middle). RT-Q334N frequency was higher in early (25%) compared to chronic (9%) sequences, suggesting it as a possible transient escape form in some individuals [40]; however, our ability to conclude this is limited since the data are derived from independent cross-sectional early and chronic cohorts, rather than longitudinal samples from the same individuals. For HIV-1 Nef, E24Q was observed in 0% and 12% of early and chronic sequences whereas Q107R was observed in 25% and 33% of sequences at these stages, respectively (Fig. 3.3C top and middle).

Chronic HIV-1 sequences were additionally analyzed for the presence of naturally occurring mutant combinations (Fig. 3.3A,B,C bottom). We began by engineering all combinations observed at >5% frequency (plus the Nef double E24Q/Q107R mutation, observed at 4% frequency) into an HIV-$1_{NL4.3}$ backbone. Later, we engineered two additional Gag combinations for our analyses of compensatory mutation pathways. Together a total of 23 mutant viruses (10 single, 13 combinations) were constructed.

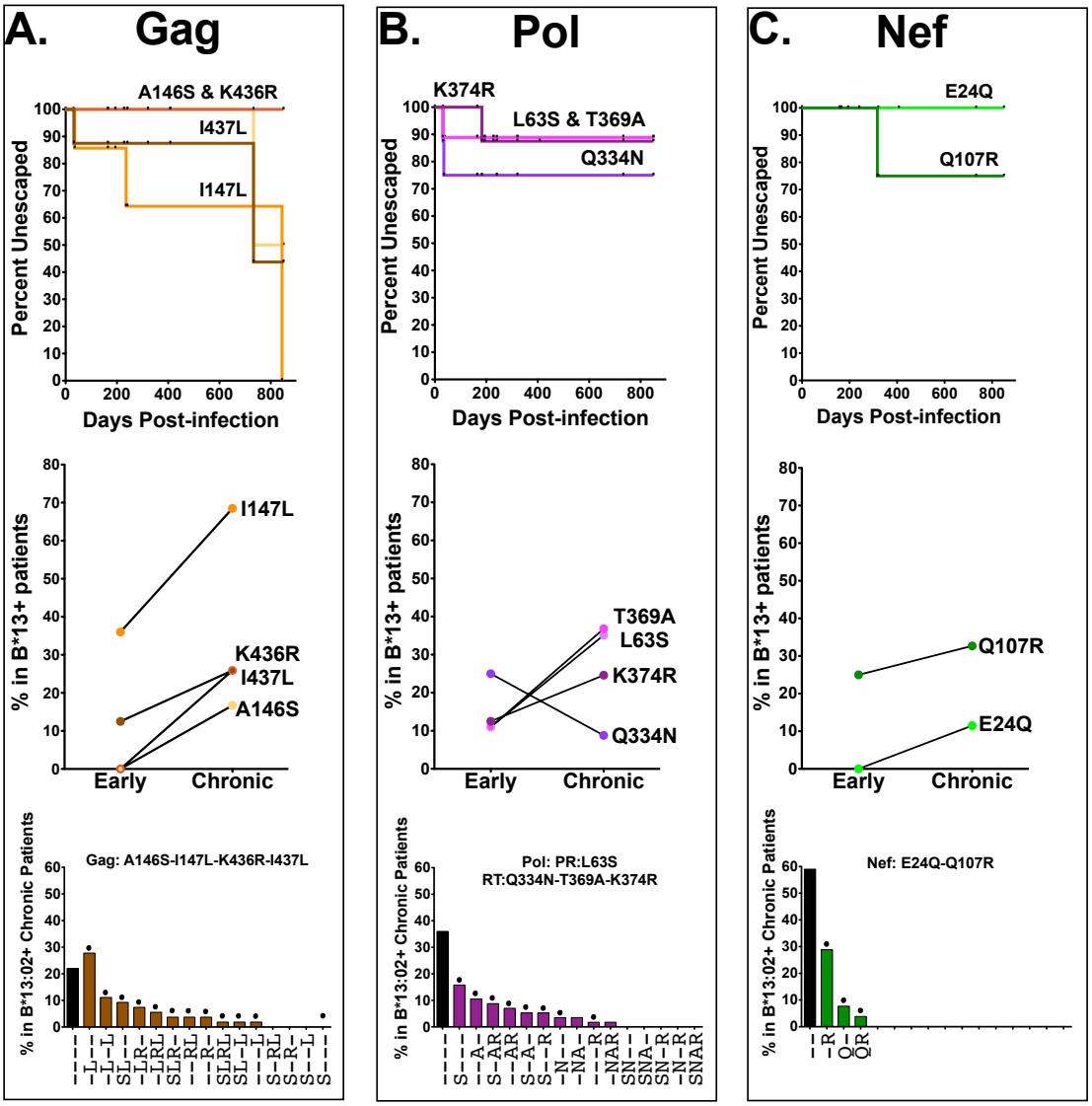

Figure 3.3. Rates of escape and prevalence of HLA-B*13-associated polymorphisms in Gag, Pol and Nef.

A-C (top). Rates of escape of 10 B*13-associated polymorphisms calculated using longitudinal plasma HIV-1 RNA sequences from nine B*13:02 seroconverters. Note that curves for Gag-A146S/K436R and Pol-L63S/T39A are superimposed. **A-C (middle)**. Prevalence of these polymorphisms in B*13:02+ individuals in early (one year post-infection; N=9) and chronic (N=69) infection. **A-C (bottom)**. Frequencies of these polymorphisms, alone and in combination, in 69 B*13:02+ individuals with chronic infection. Polymorphisms are listed in order, with hyphens denoting the consensus (non-escaped) form. Single and combination mutants marked with a dot were engineered into NL4.3 and tested for *in vitro* viral RC.

3.4.2. Impact of HLA-B*13-associated polymorphisms in Gag on viral RC

Mutant HIV-$1_{NL4.3}$ viruses were evaluated using a multi-cycle *in vitro* replication capacity (RC) assay using the GXR25 GFP reporter cell line [25,44]. Representative data are shown in Fig 3.4, including flow cytometry results, viral growth curves and wtNL4.3-normalized replication capacity measurements for uninfected cultures and those infected with wtNL4.3 and Gag-I437L (which is known to impair *in vitro* RC [27,28]). When engineered alone into HIV-$1_{NL4.3}$, two of the four B*13-associated Gag polymorphisms - Gag-I147L and Gag-I437L - reduced RC by 5% and 17% respectively ($p<0.001$), while replication of Gag-A146S and Gag-K436R were comparable to wtNL4.3 (Fig. 3.5A,B). The RC measurements for Gag-I147L, Gag-K436R and Gag-I437L are consistent with previous studies conducted in Jurkat T cells and $CD8^+$-depleted peripheral blood mononuclear cells (PBMCs) [27,28]. To our knowledge, the lack of RC impact of Gag-A146S has not been reported, although A146P, which is selected by HLA-B*57 and other alleles [39,54], does not carry a replicative cost in PBMCs [28,54,55]. B*13-associated polymorphisms have not been evaluated previously in combination for their effects on RC; in particular it is unclear whether they all represent primary escape mutations or may also include secondary (compensatory) changes. Eight B*13-associated polymorphism combinations in Gag were therefore assessed (Fig. 3.5C,D). The two combinations that contained Gag-I437L in the absence of the adjacent K436R (i.e I147L-I437L and A146S-I147L-I437L) exhibited 9% and 17% reductions in RC, respectively. These values were significantly lower than wtNL4.3 ($p<0.001$) but not significantly different than Gag-I437L alone, indicating that neither A146S nor I147L compensates for Gag-I437L-mediated replicative costs. In contrast, mutant combinations that included Gag-I437L in the presence of K436R (K436R-I437L, I147L-K436R-I437L and A146S-I147L-K436R-I437L) exhibited RCs comparable to wtNL4.3, indicating that the replicative cost of Gag-I437L was compensated by the adjacent K436R. Similarly, the three combined mutants with Gag-I147L in the presence of the adjacent A146S and/or the downstream K436R also exhibited RCs that were not significantly different than wtNL4.3. This suggests that the modest replicative cost of Gag-I147L in the absence of I437L might be compensated by A146S or K436R, although further study will be required to elucidate these mechanisms - particularly in the case of K436R, which lies in a different Gag protein subunit (p1).

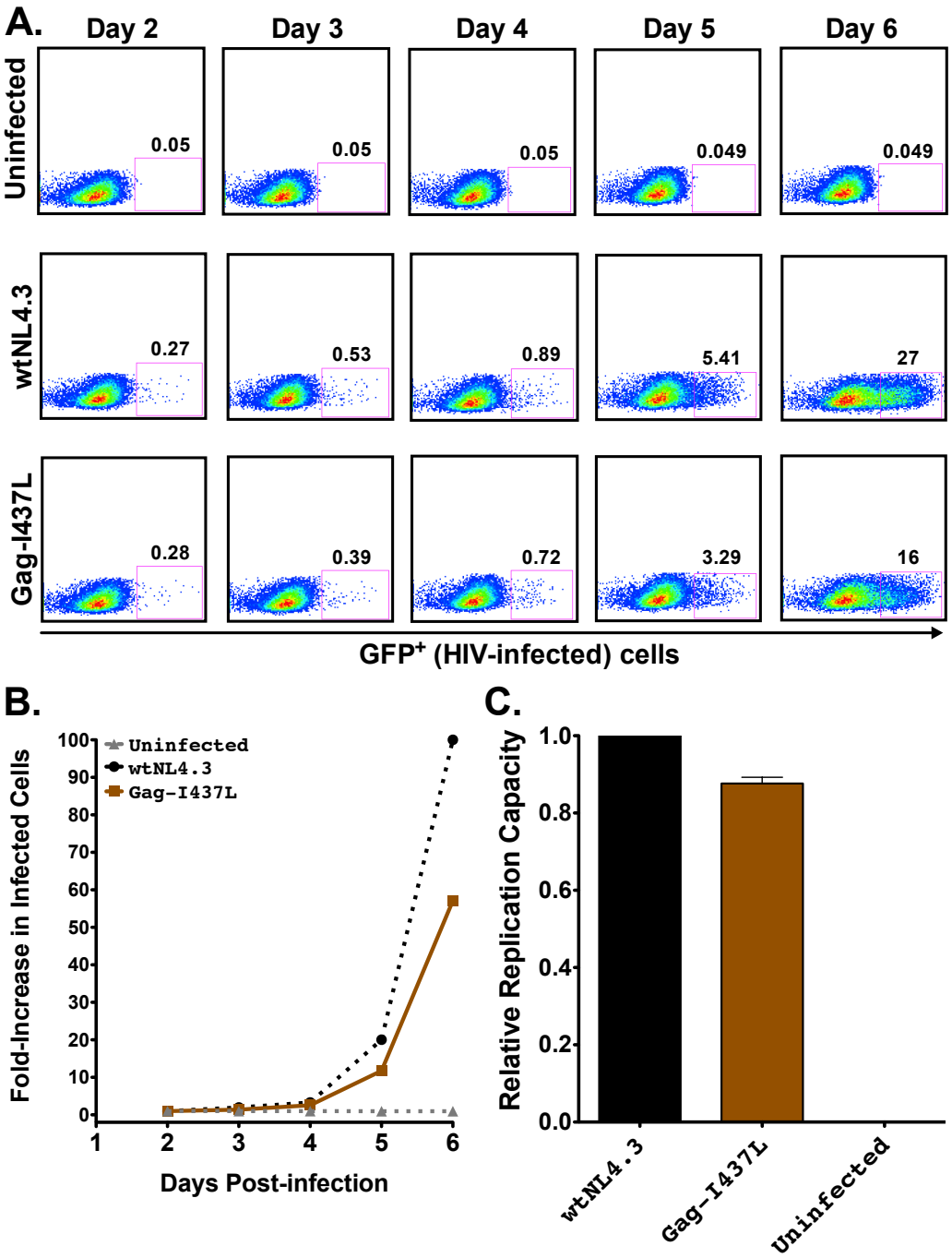

Figure 3.4. HIV-1 replication capacity: control data.

A. Representative flow plots of viral spread in culture (measured as % GFP+ [HIV-infected] cells), for uninfected control, and cells infected with wtNL4.3 and mutant Gag-I437L viruses. **B.** Growth curves for data shown in panel A, expressed as the fold-increase in infected cells from the day 2 value. **C.** Mean RC value for Gag-I437L normalized to the wtNL4.3 control, from one representative experiment containing three replicates. Error bars denote standard error of mean.

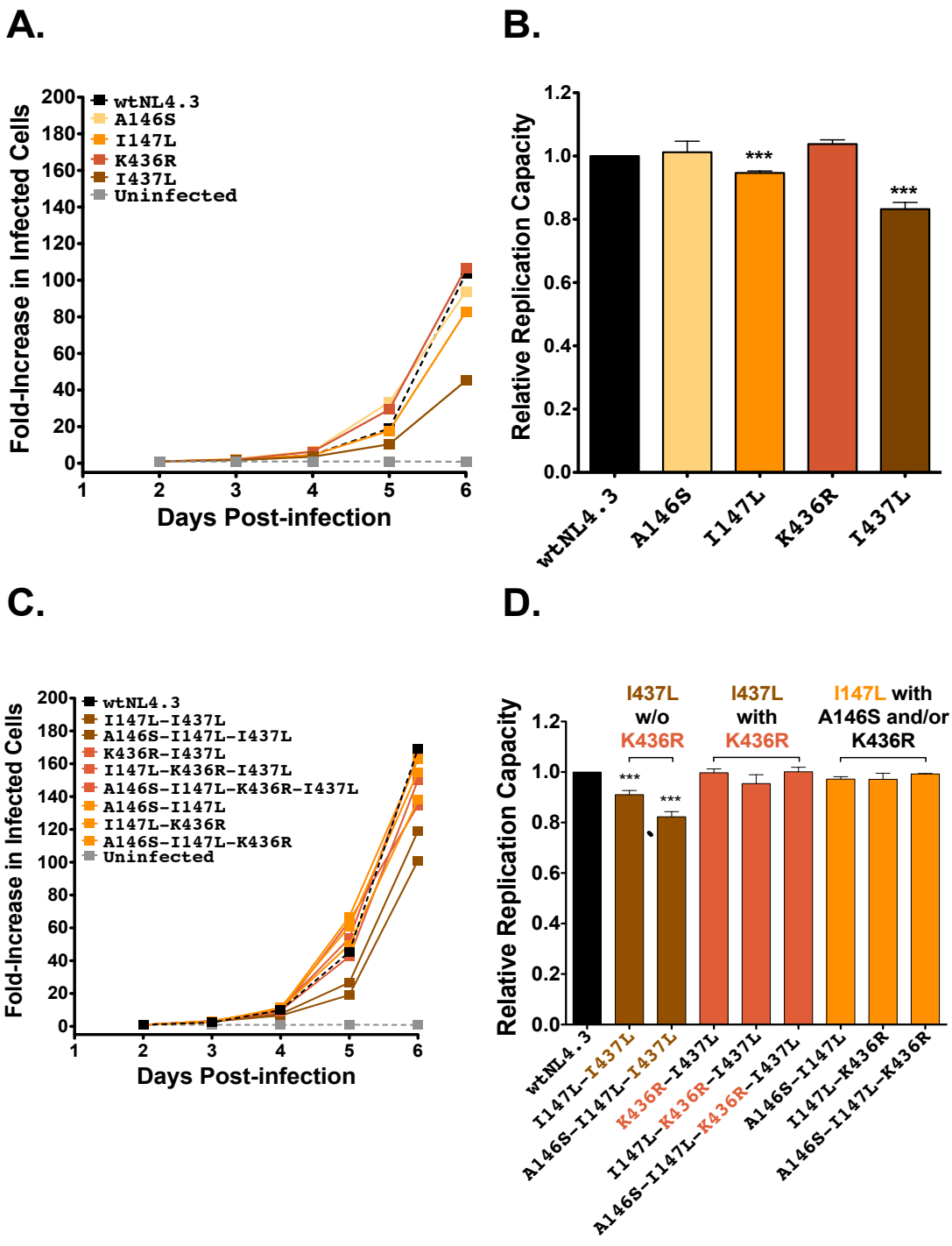

Figure 3.5. Replication capacities of NL4.3 viruses encoding B*13-associated polymorphisms in Gag.

A. Representative growth curves of single Gag mutants over a 7-day assay. **B**. NL4.3-normalized RC values of each single Gag mutant. (Triple asterisks *** denote significance at p<0.001). **C and D**. Same as panels A and B, but for combination Gag mutants.

3.4.3. A novel putative B*13-restricted epitope in p24 Gag

The region spanning $Gag_{135-155}$ is dense in CTL epitopes [22]. It has previously been hypothesized that Gag-I147L confers escape from CTL targeting the upstream B*13:02-restricted VV9 epitope ($VQNLQGQMV_{135-143}$) (Fig. 3.6) via an antigen processing mechanism [11]. Another possibility is that Gag-A146S and/or I147L confer escape from an undiscovered B*13:02-restricted epitope at this site, a hypothesis that is consistent with the higher prevalence of escape within (versus outside) CTL epitopes [39]. To investigate this, we utilized NetMHCpan2.8 to predict B*13:02-restricted epitopes in the HIV-1 subtype B consensus sequence spanning $Gag_{135-155}$. We thus identified two overlapping candidate epitopes, LI10 ($Gag_{138-147}$) and GI8 ($Gag_{140-147}$), where the latter is fully embedded in the former. Predicted half-maximal (IC_{50}) HLA binding affinities were 3324 nM (LI10) and 3181 nM (GI8), classifying them as weak binders of potentially low immunogenicity [56] (Fig. 3.6). However, for context, the published adjacent VV9 epitope has a predicted IC_{50} of 9174 nM, suggesting that responses to both LI10 and GI8 are feasible. Of note, Gag-A146S and -I147L lie at the penultimate and C-terminal residues of both LI10 and GI8.

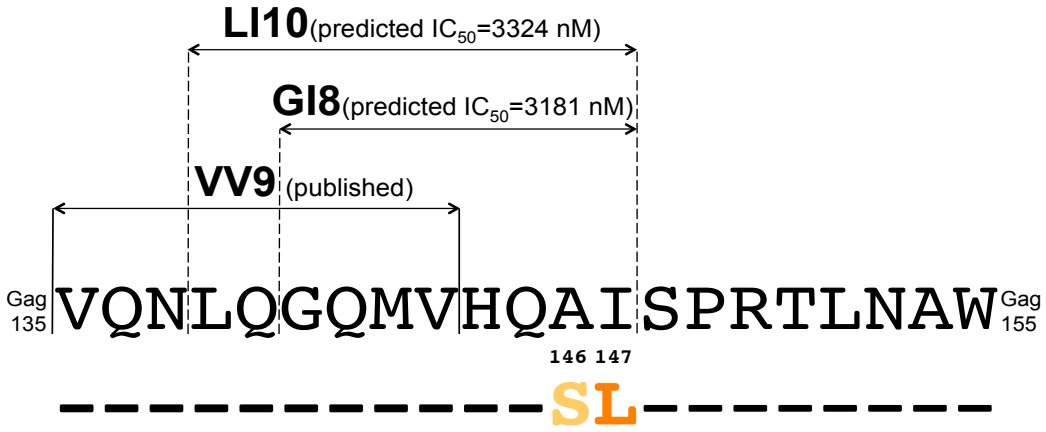

Figure 3.6. Prediction of a novel putative B*13-restricted CTL epitope in p24 Gag.

Locations and sequences of published and bioinformatically predicted B*13:02-restricted epitopes are shown above the HIV-1 consensus B amino acid sequence spanning $Gag_{135-155}$.

IFNγ-ELISpot assays were performed using PBMC from two chronically infected B*13:02+ individuals with responses to this region. In both cases, GI8 elicited the

strongest response to a series of N-terminally extended peptide sequences that included LI10 (Fig. 3.7A). Removal of the C-terminal Isoleucine abrogated responses, suggesting that this position served as the F-pocket anchor residue for HLA-B*13 (data not shown). Limited cell numbers precluded HLA restriction confirmation and experimental validation of A146S and/or I147L as *bona fide* escape mutations. Still, the predicted IC_{50} values of GI8 variants harboring Gag-I147L were nearly threefold higher than wild-type, supporting I147L as a potential escape mutation (Fig. 3.7B). This conclusion is reinforced by the observation that responding patient 1 harbored wild-type I147 whereas patient 2 harbored I147L in plasma (not shown): the latter's lower GI8 response is thus consistent with waning of CTL responses to the wild-type epitope following *in vivo* escape.

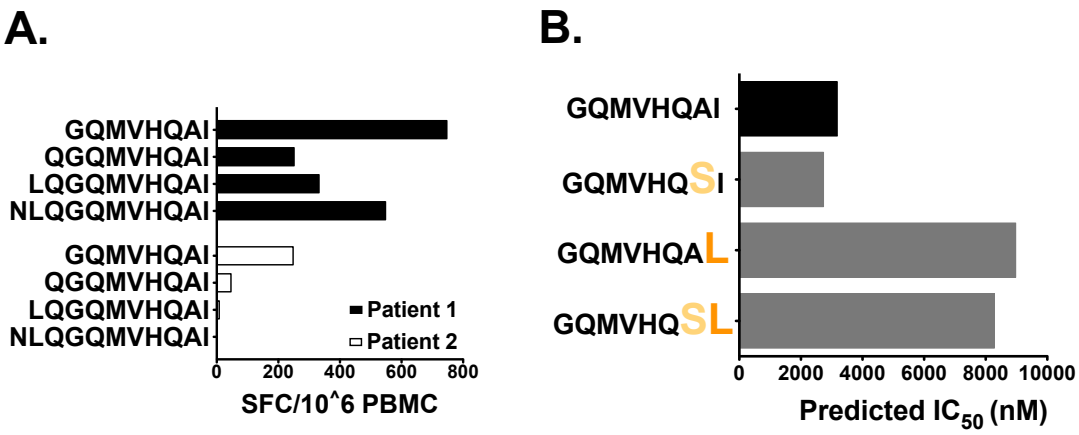

Figure 3.7. IFN-γ responses and bioinformatic predictions of GI8 variants.

A. IFN-γ ELISpot responses, measured in spot-forming cells (SFC) per million PBMC, to predicted epitopes and their N-terminal extensions in two B*13:02+ chronically HIV subtype B-infected patients who responded to this region (This data was contributed by Alex Olvera). **B.** NetMHCpan predicted IC_{50} values for GI8 and variants harboring B*13:02-associated polymorphisms Gag-A146S and/or I147L.

3.4.4. HLA-B*13-associated polymorphisms in Pol and Nef do not impair viral RC

We next examined the effects of B*13-associated polymorphisms in Pol (L63S in protease, and Q334N, T369A and K374R in RT) and in Nef (E24Q and Q107R) on viral replication. None of the Pol substitutions significantly altered *in vitro* RC when engineered alone into NL4.3 (Fig. 3.8A,B), and the four most common Pol mutant combinations observed in natural sequences also did not significantly affect RC (Fig. 3.8C,D). Similarly, the two B*13-associated Nef polymorphisms did not impair RC, either

alone or in combination (Fig. 3.9A,B). Note that the Nef observations are not simply attributable to the lack of requirement of this protein for viral replication *in vitro,* as NL4.3-ΔNef replicated poorly in GXR25 cells (not shown).

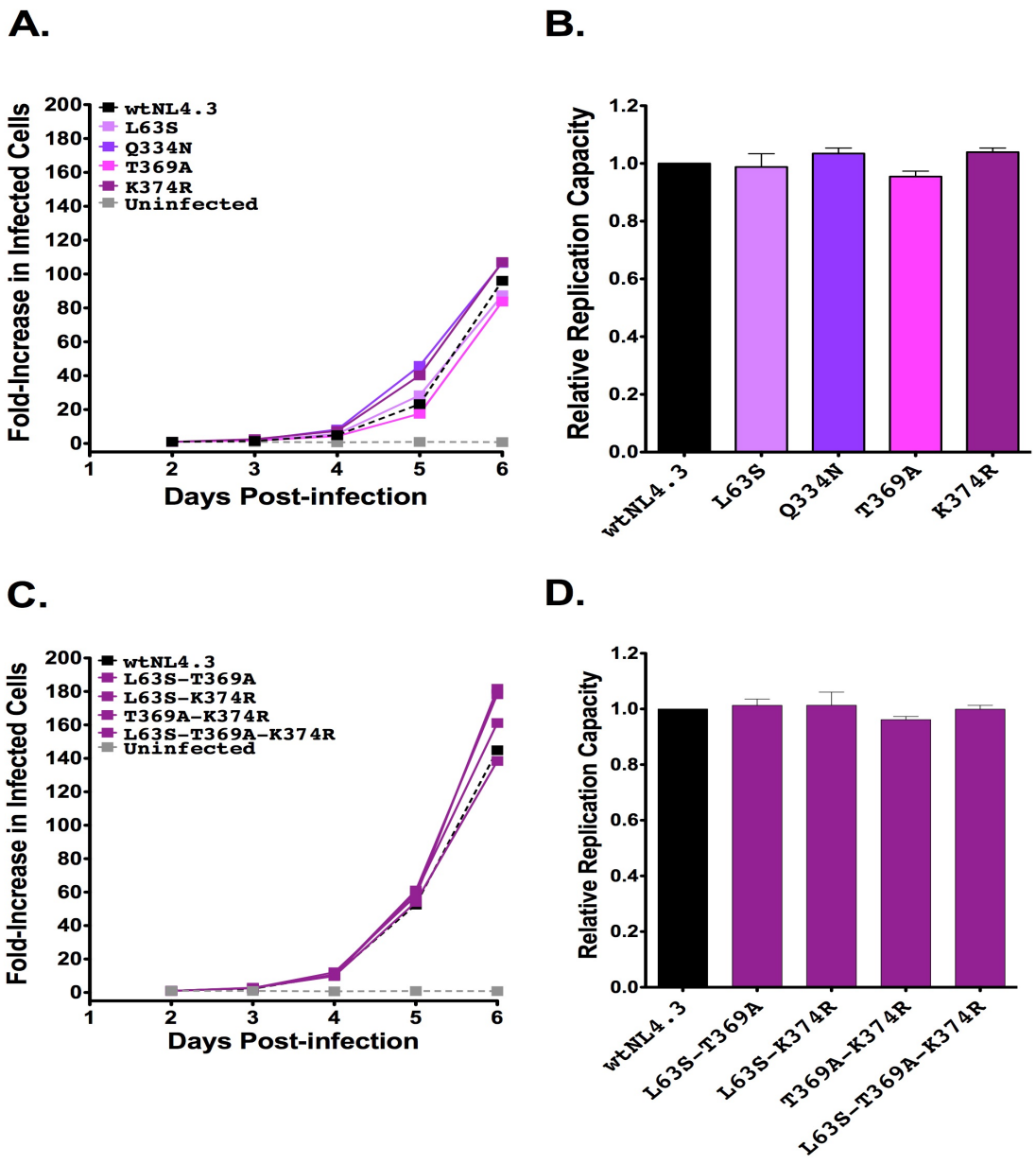

Figure 3.8. Replication capacities of NL4.3 viruses encoding B*13-associated polymorphisms in Pol.

A. Representative growth curves of single Pol mutants over a 7-day assay. **B**. Mean NL4.3-normalized RC values of each single Pol mutant. **C and D**. Same as panels A and B, but for combination Pol mutants.

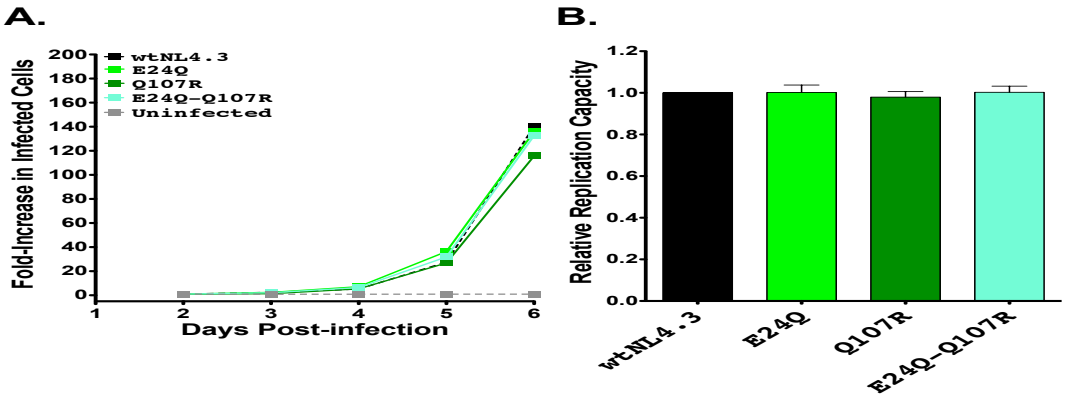

Figure 3.9. Replication capacities of NL4.3 viruses encoding B*13-associated polymorphisms in Nef.

A. Representative growth curves of single and double Nef mutants over a 7-day assay. **B**. Mean NL4.3-normalized RC values of each Nef mutant.

3.4.5. Impact of HLA-B*13:02-associated Nef polymorphisms on CD4 and HLA class I downregulation

Naturally occurring sequence variation in Nef can modulate its CD4 and HLA class I downregulation activities [33-36,57]. We therefore investigated the impact of B*13-associated polymorphisms on Nef function. To this end, the GXR25 cell line was stably transduced to express HLA-A*02:01 (GXR-A*02) as a representative HLA class I molecule. GXR-A*02 cells were infected with control viruses (wtNL4.3, ΔNef, $Nef_{M20A;}$ the latter is impaired for HLA class I downregulation [50] but replicates comparably to wtNL4.3; not shown) or mutant Nef viruses (E24Q, Q107R, and E24Q-Q107R) and stained for surface CD4 and A*02 expression once infection reached ≈10-15%. Representative data for uninfected and control-infected cultures are shown in Fig. 3.10 A,B. Compared to wtNL4.3, ΔNef virus displayed a modest (5%) reduction in CD4 downregulation that was nevertheless statistically significant and a substantial (66%) defect in HLA class I downregulation activity ($p \leq 0.01$; Fig. 3.10C,D). Efficient downregulation of CD4 by this virus is likely due to the actions of Vpu and Env in our culture system [58]). CD4 downregulation ability of Nef_{M20A} was comparable to wtNL4.3, but its HLA downregulation function was impaired by 60% ($p<0.01$). Neither Nef-E24Q nor Q107R significantly affected CD4 or HLA class I downregulation alone; moreover, CD4 downregulation function of the E24Q-Q107R double mutant was comparable to wtNL4.3. However, the ability of the double mutant to downregulate HLA class I was

42% reduced compared to wtNL4.3 (p=0.007).

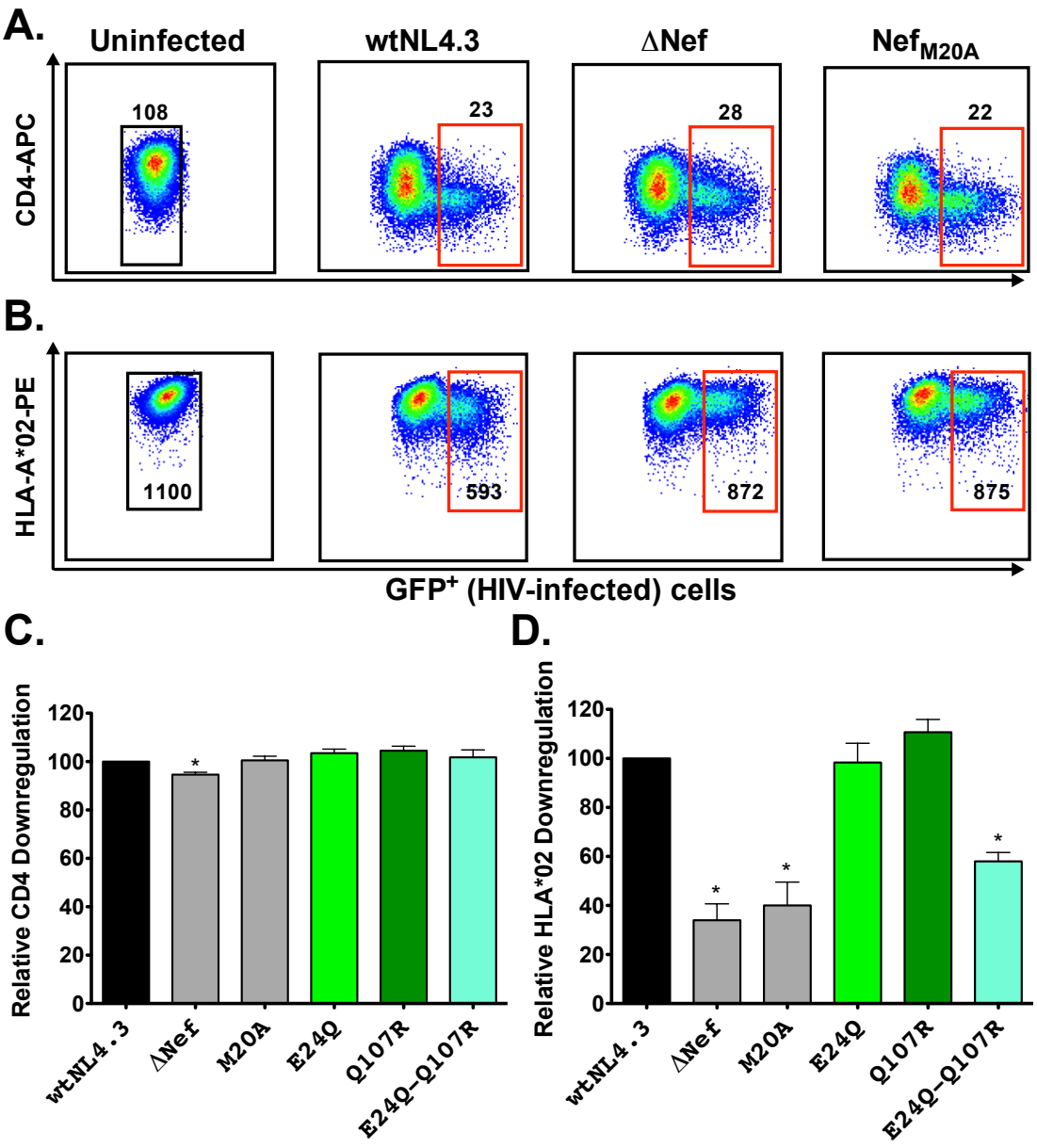

Figure 3.10. Impact of B*13-associated polymorphisms on Nef-mediated CD4 and HLA class I downregulation.

A and B. Representative flow plots of CD4 (A) and HLA class I (B) downregulation for uninfected cells, and cells infected with wtNL4.3, ΔNef and Nef$_{M20A}$ control viruses. Median fluorescence intensities (MFI) of CD4 and HLA class I expression (y-axis) are shown for GFP$^+$ (HIV-infected, ***red*** gates) versus GFP$^-$ (HIV-1 uninfected, ***black*** gates). **C and D.** Impact of control viruses and those harboring B*13-associated Nef polymorphisms on CD4 and HLA-A*02 downregulation, normalized to wtNL4.3. Single asterisk (*) denotes significance at p≤0.01.

Intracellular Nef expression was readily detectable in cultures infected with control and mutant HIV-1, suggesting that the attenuated HLA downregulation activity of the Nef double mutant was not simply due to poor expression or a stability defect (Fig. 3.11).

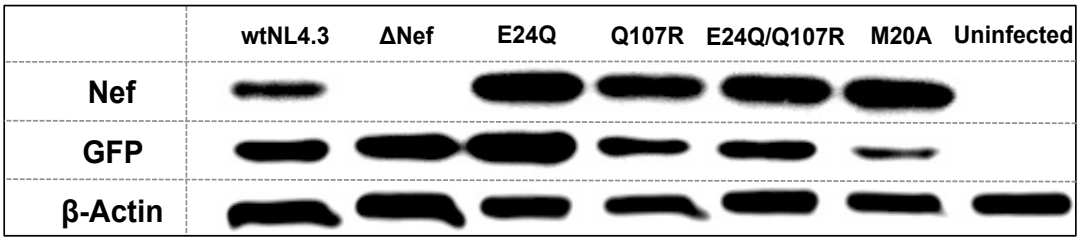

Figure 3.11. Detection of Nef, GFP and β-actin protein expression.

Detection of Nef, GFP and β-actin protein expression by Western Blot in GXR-A*02 reporter T cells infected with NL4.3 viruses encoding control or mutant Nef alleles.

3.4.6. Impaired HLA class I downregulation of Nef double mutant renders infected cells more susceptible to recognition by HIV-1-specific T cells

An impaired ability of the Nef E24Q-Q107R double mutant to downregulate viral peptide/HLA complexes implies that cells infected with this virus should become more visible to $CD8^+$ T cells, including those restricted by B*13 and non-B*13 epitopes. To test this hypothesis, we used a novel co-culture assay that features "target" cells (GXR-A*02 cells infected with control or mutant HIV-1) and HIV-1-specific "effector" cells transiently expressing an A*02-restricted $FK10^{Gag}$-specific TCR-α/β, CD8α and an NFAT-driven luciferase reporter construct (Fig. 3.12). In this assay, TCR-dependent signaling is quantified based on luminescence following co-culture with A*02/FK10 expressing target cells. Downregulation of HLA-A*02 by wild type Nef from the infected target cell surface is expected to lead to reduced TCR signaling in effector cells, while target cells infected with HIV-1 containing defective Nef sequences that fail to downregulate HLA-A*02 should lead to increased TCR signaling.

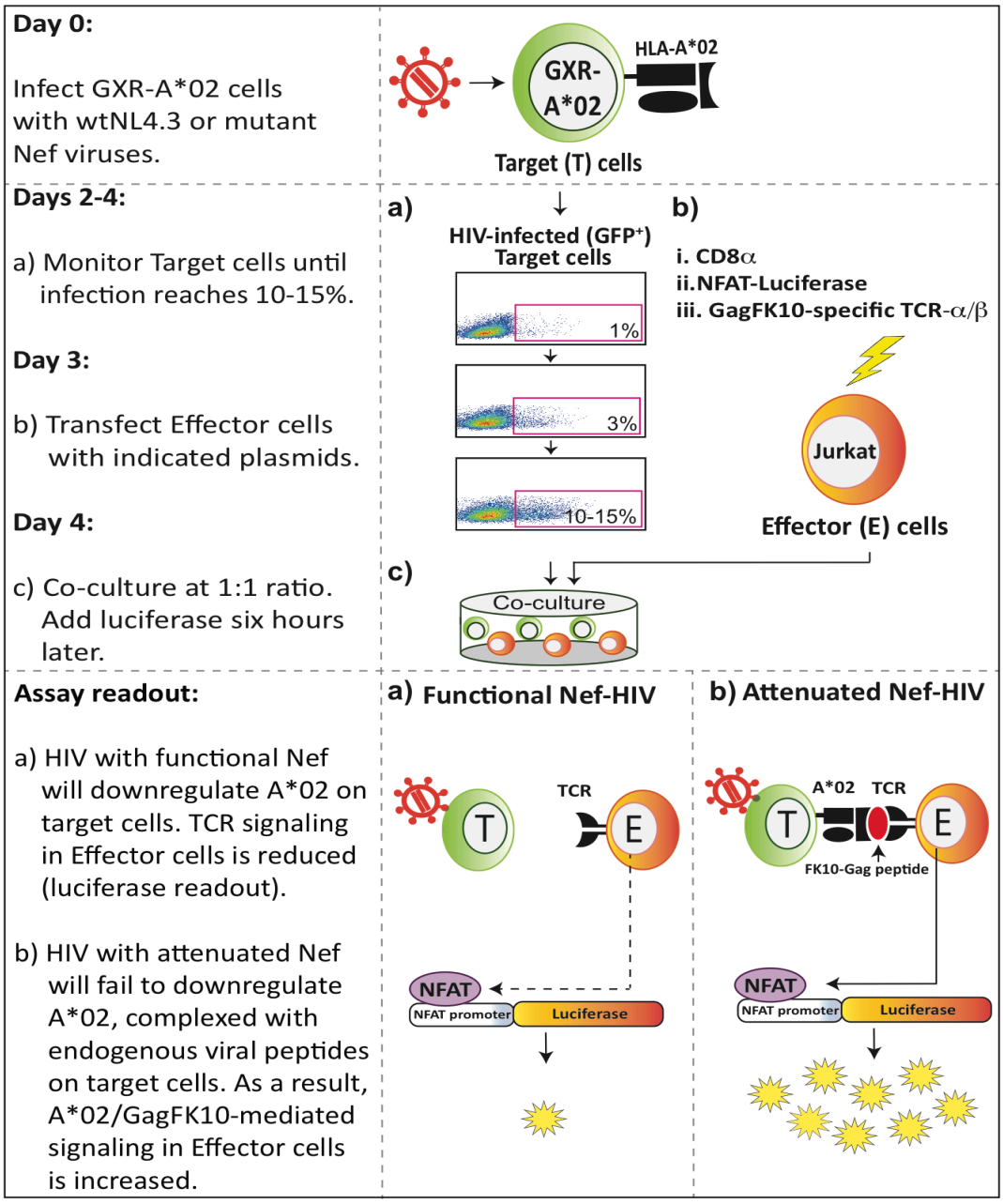

Figure 3.12. ***in vitro*** **co-culture assay to assess the impact of Nef-mediated HLA downregulation on infected cell recognition by HIV-1 specific effector T cells.**

Depiction of the TCR luciferase reporter cell assay used to assess the functional significance of Nef-mediated HLA class I downregulation on CTL evasion. "Target" GXR-A*02 cells were infected with wtNL4.3 or Nef variant viruses at an MOI=0.01 and used once infection (% GFP^+ cells) reached 10-15%. "Effector" Jurkat luciferase reporter cells transiently expressing TCR specific for the A*02-restricted $FK10^{Gag}$ epitope were generated as described in Methods. Luciferase expression (indicating TCR-dependent recognition of viral antigen on target cells) was detected after 6 hours of co-culture by luminescence.

Consistent with this, we observed a significant negative correlation between the extent of Nef-mediated HLA-A*02 downregulation on target cells and TCR-driven luciferase signal in HIV-1-specific effector cells (Spearman's R=-0.88, p=0.03) (Fig. 3.13). This was confirmed in three independent experiments (Spearman's R= -0.82 to-0.89, p=0.03 to 0.06). Importantly, control experiments using uninfected GXR-A*02 target cells and parental GXR25 target cells (lacking A*02) did not induce luciferase upon co-culture with effector cells (not shown), indicating that target cell recognition was HIV-1 epitope and HLA-specific. Taken together, our results suggest that the impaired HLA class I downregulation activity of the B*13:02-associated Nef E24Q-Q107R double mutant could render HIV-1-infected cells more vulnerable to $CD8^+$ T cell responses *in vivo*.

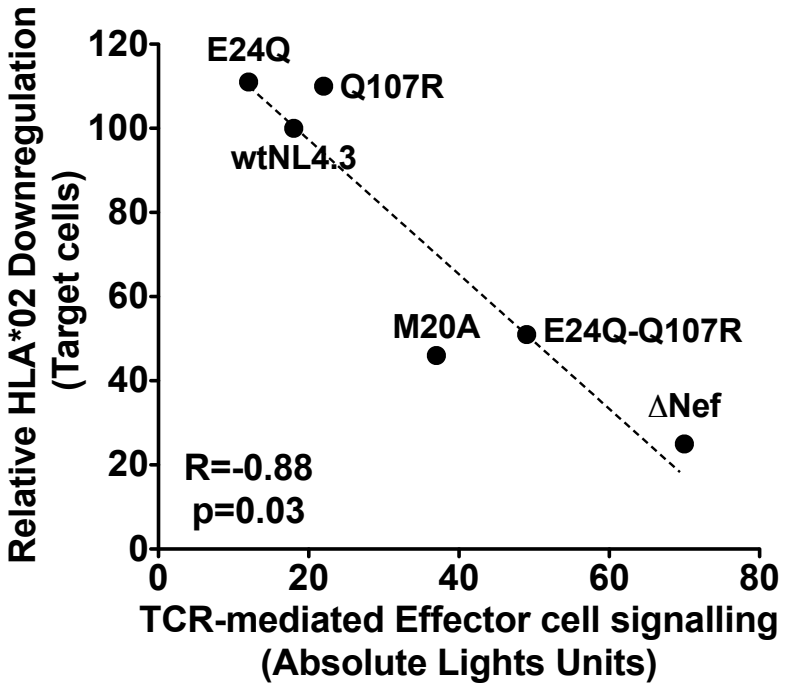

Figure 3.13. Impact of Nef double mutant E24Q-Q107R on recognition of HIV-infected target cells by HIV-specific effector T cells.

Representative data depicting a negative correlation between Nef-mediated ability to downregulate HLA-A*02 on GFP-reporter "target" cells infected with control and mutant Nef viruses, and TCR-driven luciferase signal in A*02-$FK10^{Gag}$-specific Jurkat "effector" cells following co-culture (Spearman's R=-0.88, p=0.03).

3.5. Discussion

We engineered a total of 23 mutant HIV-$1_{NL4.3}$ recombinant viruses harboring B*13-associated polymorphisms either alone (n=10) or in naturally occurring combinations (n=13) and investigated their effects on HIV-1 replication and Nef protein function. Our results extend current knowledge in multiple ways. First, we confirmed that escape via Gag-I437L incurs a substantial (17%) fitness cost [27,28], and we extend this observation by demonstrating that the adjacent K436R mutation rescues *in vitro* RC to wild-type levels. This compensatory relationship is corroborated by the time course of selection of these polymorphisms *in vivo* (I437L is ultimately selected in 26% of B*13:02+ individuals; of these, 33% also developed K436R), the highly significant negative association reported between I437L and wild-type K436 in HIV-1 subtype B sequences [39], and evidence that despite its strong association with B*13:02, K436R alone does not confer escape in the majority of HIV-infected individuals [27,59]. Similarly, we confirmed that Gag-I147L, the most rapidly and frequently-selected B*13:02 escape mutation in HIV-1 [39], incurs a minor (5%) replicative cost, which appears to be rescued by the adjacent A146S and/or distal K436R mutations. Again, these putative compensatory relationships are corroborated by their time course of selection (I147L is ultimately selected in 70% of B*13:02 individuals; of these, 13% also develop A146S or K436R). Taken together, we hypothesize that replicative costs of Gag-I147L and I437L escape mutations, which usually occur within the first year of infection and may only be compensated later in fewer than half of cases, contribute to lower viral loads that are observed long-term in B*13:02-expressing persons.

While B*13-mediated escape in Pol and Nef do not substantially impair viral RC, the B*13-driven Nef E24Q-Q107R double mutant conferred a >40% reduction in Nef's ability to downregulate cell surface HLA class I. This defect was nearly as profound as that conferred by ΔNef or the Nef-M20A mutation (which does not occur naturally) in our assay system. Moreover, the inability of Nef E24Q-Q107R to downregulate HLA class I enhanced the recognition of infected cells by HIV-1-specific T cells *in vitro.* In other words, this naturally occurring mutant combination, selected *in vivo* under B*13-mediated CTL pressure, conferred a functional cost to Nef's immune evasion activity. Although attenuation of Nef's HLA class I downregulation function as a consequence of

CTL escape has been described previously [35,36], the mutations required to confer these effects are rare *in vivo*. The HLA-B*35-driven Nef R75T and Y85F escape mutations, when present together, reduced Nef's HLA class I downregulation activity nearly twofold, but this combination occurs in <0.2% of natural HIV-1 sequences [35]. Similarly, a Nef clone exhibiting ≈15% lower HLA downregulation activity was isolated from an acutely HIV-infected individual who subsequently controlled HIV-1 viremia spontaneously to <2000 copies/ml, however this defect required the selection of four Nef mutations in the context of two independent HLA alleles [36]. Although the E24Q-Q107R double mutant is observed in only 4% of B*13-expressing individuals and this combination tends to arise late in infection, it nevertheless represents the most frequent *in vivo* CTL escape pathway identified to date that significantly compromises this Nef function. Importantly, once this double mutant arises *in vivo*, its HLA downregulation defect would serve to boost the levels of viral epitopes complexed with all HLA-A and HLA-B alleles expressed by the individual (not just those restricted by HLA-B*13), thus rendering infected cells more susceptible to recognition by numerous HIV-1-specific T cells (or at least those specific for viral epitopes that had not yet developed CTL escape mutations).

Several limitations of the study merit comment. First, we focused on escape in Gag, Pol and Nef because all known B*13-restricted epitopes lie in these proteins. However, undiscovered B*13 epitopes are likely to exist in other regions, as indicated by the existence of B*13-associated polymorphisms in gp41, Tat, Vif and Vpr [39], and their impact on HIV-1 replication and protein function remain unknown. Second, due to the relatively large number of mutants assessed here, all replication capacity assays were performed in an immortalized GFP reporter T cell line. However, the magnitude of replication defects observed for Gag-I437L and Gag-I147L corroborated previous results in PBMCs [27,28], indicating that RC measurements in our assay system are generally representative of those in other cell types. Third, due to insufficient PBMCs from B*13+ individuals, we were unable to fully characterize the novel GI8 epitope in $p24^{Gag}$, although its bioinformatically-guided discovery (combined with reduced predicted binding affinities of its escape variants) supports it as such. Fourth, we assessed the impact of Nef-E24Q and/or Q107R on only three of Nef's *in vitro* functions (viral replication and CD4/HLA class I downregulation), but their impact on other Nef functions, including

upregulation of HLA class-II invariant chain (CD74) [60], enhancement of virion infectivity [61], and alteration of T cell receptor signaling [62] remain to be determined. Moreover, to evaluate the effect of Nef mutations on HLA downregulation and its consequences for T cell-mediated recognition of infected cells, we used HLA-A*02 and an $FK10^{Gag}$-specific TCR as representative indicators of this process. Since HLA-B molecules may generally be more resistant to Nef-mediated downregulation compared to HLA-A [63] and the impact of Nef-mediated HLA downregulation may be to some extent HIV gene and/or epitope-specific [64-66], recognition of other CTL epitopes might differ from that observed here for A*02-$FK10^{Gag}$. However, using a transient transfection system, we previously demonstrated a strong correlation between the ability of natural Nef sequences to downregulate HLA-A*02 and HLA-B*07 [34], and Gag-specific CTL in general may be less susceptible to Nef-mediated HLA downregulation than CTL targeting other HIV-1 proteins [66], possibly due to presentation of Gag epitopes before Nef-mediated HLA downregulation is complete [67]. Together, these observations suggest that our luciferase reporter T cell approach should be broadly representative of CTL recognition and may be a conservative measure of Nef function. Finally, while we have demonstrated enhanced recognition of target cells infected with Nef E24Q-Q107R mutant virus using a reporter T cell assay, we have not performed inhibition studies or assessed other effector functions directly with primary CTL clones.

The mechanism whereby Nef-E24Q and Q107R together confer such a profound HLA downregulation defect requires further study. While these codons have not been explicitly identified as being critical for Nef function, both lie within or adjacent to important motifs (Fig. 3.14). Nef-E24 is located in the N-terminal alpha helix within a region (amino acids 17-26) that, when deleted and engineered along with a V10E substitution, renders Nef defective for HLA downregulation [68]; it also lies 4 codons downstream of M20, which, when artificially mutated to alanine, produces a severe HLA downregulation defect [50]. Nef-Q107 lies adjacent to arginine residues R105/R106 that are essential for several Nef functions, including dimerization [69-71]; it is therefore possible that the creation of a triple arginine ($RRR_{105-107}$) via Q107R may alter this protein-protein interface. Finally, mutations at the adjacent residue 106 (*e.g.*, R106K, R106L) confer modest to severe HLA class I downregulation defects [72]. Further

mechanistic studies will be required to examine this double mutant and to identify compensatory pathways in natural sequences.

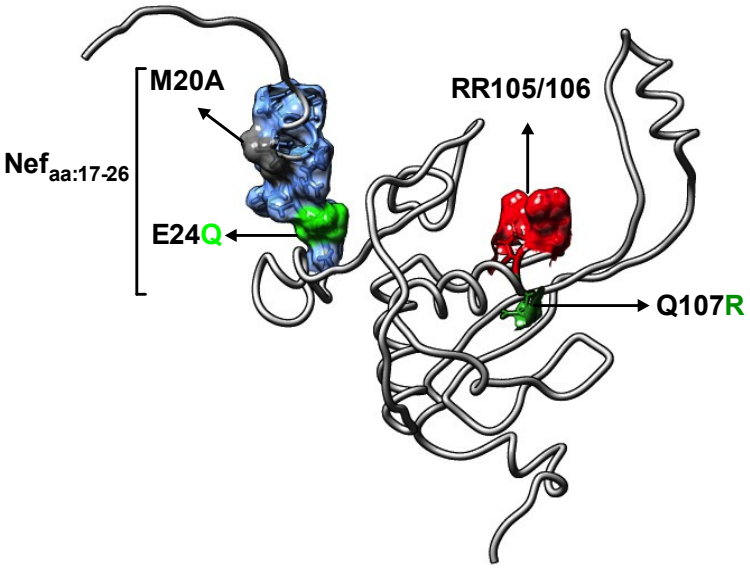

Figure 3.14. Location of B*13-associated Nef mutations on three dimensional protein structure.

Nef Ribbon structure showing the location of E24Q and Q107R mutations (green) along with residues previously associated with HLA class I downregulation function and/or Nef dimerization: Δ17-26 (blue), M20A (grey) and RR105/106 (red). Nef composite structure was obtained from Dr. Art F. Poon [4].

Taken together with the previous literature, our results suggest that B*13-mediated viremia control is achieved *via* multiple mechanisms. The first, demonstrated by other studies, is sustained targeting of CTL epitopes in multiple HIV-1 proteins [11,21]. The second is *via* fitness costs of the resulting escape mutations that presumably limit the net viral "benefit" gained from evading the CTL response. Specifically, B*13 escape mutations may impair HIV-1 by two distinct mechanisms: by reducing Gag fitness and dampening Nef's immune evasion function by compromising its HLA downregulation activity. Functional costs of CTL escape in Nef have been reported by others [35,36,73]; our results extend these observations by suggesting that such costs may be biologically relevant. More broadly, our study highlights the potential

utility of HIV-1 T cell vaccines designed to target vulnerable epitopes in Nef where escape mutations impair its viral immune evasion activity.

3.6. References

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Chapter 4.

Thesis Summary

This thesis focuses on HIV-1 adaptation to host selection pressure by HLA class I-restricted CTL immune responses. In particular, it provides evidence for viral replication defects and functional implications of published HLA-B*13:02-associated CTL escape mutations selected in HIV-1.

HIV-1 escape from CTL is a well-reported mechanism of host immune evasion [1], allowing ongoing viral replication and progressive loss of $CD4^+$ T cells in HIV-1-infected individuals. Antiviral CTL immune pressure on HIV-1 favors the outgrowth of escape variants, with the 'most optimal' biological properties for replication in that particular host. However, certain escape mutations occur at the cost of reduced replication capacity or protein function in HIV-1. Escape mutations that arise in CTL epitopes restricted by HLA-B*57/58:01 and B*27 have been attributed to viral fitness defects. For instance, Gag-A163G mutation in the B*57:03-restricted $KF11^{Gag}$ epitope ($KAFSPEVIPMF_{162\ to\ 172}$) [2] and Gag-R264K mutation in the B*27-restricted $KK10^{Gag}$ epitope ($KRWIILGLNK_{263\ to\ 272}$) [3] have been reported to impair viral replication. The reduction in viral replicative fitness is believed to contribute, at least in part, to the protective effects of these HLA-B alleles in conferring long-term control of HIV-1.

B*13:02, a relatively rare allele in Caucasian and African populations, is also associated with slower HIV-1 disease progression. Antiviral CTL responses in B*13:02+ individuals mainly target Gag [4], though Nef-specific responses have also been described [5]. Out of the 10 B*13:02-associated escape mutations included in our study that lie in Gag (A146S, I147L, K436R, I437L), Pol (PR-L63S, RT-Q334N, T369A, K374R) and Nef (E24Q, Q107R) [6], two of them, i.e., Gag-I147L and -I437L showed modest and severe impacts on HIV-1 replication, respectively. However, the replication-

impaired phenotype was rescued by the development of compensatory mutations.

The Gag-I147L and -I437L escape mutations lie at the C-terminal anchor residues of putative CTL epitope-GI8 and immunodominant published CTL epitope-RI9 [4,7], respectively. These mutations arise early in B*13:02+ HIV-infected individuals. The viral replication capacity defects associated with these mutations prevail for a considerable time before the selection of compensatory mutations Gag-A146S and/or -K436R (for I147L) or Gag-K436R (for I437L). These mutations offer complete compensation of the viral fitness. Therefore, these findings suggest that since a fair number of HIV-1infected B*13:02+ individuals develop fitness-costly GI8 and RI9-associated escape mutations early in infection that are not compensated until later and the viral fitness costs associated with escape contribute, at least in part, to B*13:02-mediated immune control.

We have also demonstrated that B*13:02-associated escape mutations E24Q and Q107R in Nef, when found in combination, have drastic consequences on this protein's HLA class I downregulation function. These results indicate that HIV-1 becomes more susceptible to host CTL immune surveillance and killing. Although the combination rarely exists *in vivo*, it depicts a novel CTL immune evasion strategy whereby HIV-1's evolutionary struggle confers a negative impact on Nef's key function [8]. It is potentially due to this severe impairment that the combination mutation (E24Q-Q107R) is infrequently selected in natural HIV-1 sequences.

Therefore, the dual impairment of viral replication and Nef-mediated HLA class I downregulation as a consequence of B*13:02-associated immune escape provides new horizons to further explore the functional consequences of other HLA-driven escape mutations in HIV-1. It is also important to identify other amino acid residues in Nef that lie in close proximity to published residues, involved in CD4 and/or HLA class I downregulation function, as the characterization of immune-driven Nef mutations affecting this protein's functions could reveal regions of greater potential as a component for HIV-1 attenuation-vaccine design [9]. Essentially, Nef mutations that abrogate both CD4 and HLA class I downregulation function but retain CTL, T-helper cell and antibody responses may be beneficial for a vaccine against HIV-1 [10].

In conclusion, HIV-1's mutational plasticity and capacity for diversification represents a major challenge for vaccine development. Since the immune response is an important driver of HIV-1 evolution, it is imperative to enhance our current understanding of the mechanisms of immune evasion strategies adapted by HIV-1.

4.1. References

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