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Relative Resistance of HLA-B to Downregulation by Naturally Occurring HIV-1 Nef Sequences

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ABSTRACT HIV-1 Nef binds to the cytoplasmic region of HLA-A and HLA-B and downregulates these molecules from the surface of virus-infected cells, thus evading immune detection by CD8+ T cells. Polymorphic residues within the HLA cytoplasmic region may affect Nef’s downregulation activity. However, the effects of HLA polymorphisms on recognition by primary Nef isolates remain elusive, as do the specific Nef regions responsible for downregulation of HLA-A versus HLA-B. Here, we examined 46 Nef clones isolated from chronically HIV-1 subtype B-infected subjects for their ability to downregulate various HLA-A, HLA-B, and HLA-C molecules on the surface of virus-infected cells. Overall, HLA-B exhibited greater resistance to Nef-mediated downregulation than HLA-A, regardless of the cell type examined. As expected, no Nef clone downregulated HLA-C. Importantly, the differential abilities of patient-derived Nef clones to downregulate HLA-A and HLA-B correlated inversely with the sensitivities of HIV-infected target cells to recognition by effector cells expressing an HIV-1 Gag-specific T cell receptor. Nef codon function analysis implicated amino acid variation at position 202 (Nef-202) in differentially affecting the ability to downregulate HLA-A and HLA-B, an observation that was subsequently confirmed by experiments using Nef mutants constructed by site-directed mutagenesis. The in silico and mutagenesis analyses further suggested that Nef-202 may interact with the C-terminal Cys-Lys-Val residues of HLA-A, which are absent in HLA-B. Taken together, the results show that natural polymorphisms within Nef modulate its interaction with natural polymorphisms in the HLA cytoplasmic tails, thereby affecting the efficiency of HLA downregulation and consequent recognition by HIV-specific T cells. These results thus extend our understanding of this complex pathway of retroviral immune evasion.

IMPORTANCE Recognition of genetically diverse pathogens by the adaptive immune system represents a primary strategy for host defense; however, pathogens such as HIV-1 can evade these responses to achieve persistent infection. The HIV-1 nef gene and the HLA class I locus rank among the most diverse genes of virus and host, respectively. The HIV-1 Nef protein interacts with the cytoplasmic region of HLA-A and HLA-B and downregulates these molecules to evade cellular immunity. By combining molecular, genetic, and in silico analyses, we demonstrate that patient-derived Nef clones downregulate HLA-A more effectively than HLA-B molecules. This in turn modulates the ability of HIV-specific T cells to recognize HIV-infected cells. We also identify a naturally polymorphic site at Nef codon 202 and HLA cytoplasmic motifs (GGG_314,315 and CKV_339–341) that contribute to differential HLA downregulation by Nef. Our results highlight new interactions between HIV-1 and the human immune system that may contribute to pathogenesis.

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The HLA class I (HLA-I) gene region, comprising the HLA-A, HLA-B, and HLA-C loci, ranks among the most polymorphic regions in the human genome, with 2,735 HLA-A, 3,455 HLA-B, and 2,259 HLA-C alleles identified to date (International ImMunoGeneTics project [IMGT] HLA database; http://www.ebi.ac.uk/ipd/imgt/hla/) (see reviews in references 1 and 2). HLA-I polymorphism is mainly concentrated within exons 2 and 3 (1), which primarily form the antigenic peptide-binding groove of the HLA-I complex (3) and play an important role in restricting CD8+ T lymphocyte specificity. Other exons also exhibit polymorphism, albeit to a lesser extent. For example, HLA-A, HLA-B, and HLA-C alleles can be classified into 5, 2, and 7 polymorphic types, respectively, based on sequence variations within their cytoplasmic domains (encoded by exons 5 to 7 for HLA-B or 5 through 8 for HLA-A and HLA-C). Polymorphism in the cytoplasmic domain also influences receptor expression: for example,
a unique amino acid conserved in all HLA-C allotypes (Ile at codon 337 [Ile-337], rather than Thr-337 as in HLA-A and HLA-B) yields lower cell surface expression of HLA-C than of HLA-A and HLA-B (4). However, the implications of HLA cytoplasmic polymorphisms for modulation of antiviral immunity remain incompletely understood.

HLA-I-restricted CD8+ cytotoxic T lymphocyte (CTL) responses are important for controlling a wide range of viral infections (5, 6), including HIV-1 (7, 8), human T-cell leukemia virus type 1 (HTLV-1) (9), cytomegalovirus (10), and herpes simplex virus (11) infections. In turn, viruses have evolved various mechanisms to evade HLA-I-restricted antiviral immunity, such as inhibiting intracellular antigen-processing pathways and down-regulating HLA-I molecules from the infected cell surface (see reviews in references 12 to 14). In HIV-1, the 27- to 35-kDa accessory protein Nef downregulates HLA-A and HLA-B molecules from the surface of HIV-1-infected cells (15, 16). Nef does not downregulate HLA-C molecules due to the presence of unique residues at codons 320 and 327 in their cytoplasmic regions (17). As such, the antiviral activities of HLA-A and HLA-B-restricted CTLs are substantially reduced by Nef expression (18–20), whereas the antiviral activities of HLA-C-restricted CTLs are unaffected by Nef (21). Maintenance of HLA-C expression allows virus-infected cells to escape recognition by the innate immune system, as downregulation of all HLA-I molecules would render HIV-infected cells susceptible to recognition by natural killer cells (22). Importantly, it was recently demonstrated that chimeric HLA-A02 molecules expressing various HLA-A and HLA-B cytoplasmic tails are differentially susceptible to Nef-mediated downregulation and that this in turn has implications for infected-cell recognition by HLA-A02-restricted CTLs (23). However, all prior studies focused on a limited number of prototypic laboratory-adapted HIV-1 strains (22, 23). It is thus unclear whether highly diverse naturally occurring (patient-derived) Nef sequences also display differential abilities to downregulate HLA-A and HLA-B, and if so, which Nef residue(s) modulate these interactions.

Nef ranks among the most diverse HIV-1 proteins (24, 25). Primary Nef clones isolated from patients at various infection stages and/or with different disease phenotypes exhibit substantial functional heterogeneity (26–31), including wide-ranging HLA-I downregulation capacities (26, 28–30, 32–35). However, previous studies investigated a variety of HLA-I allotypes using different target cells and antibodies; as such, the possibility that these differences were influenced in part by the experimental conditions cannot be conclusively ruled out. In this study, we assessed 46 subtype B Nef clones isolated from the same number of chronically HIV-1-infected patients for their ability to downregulate various HLA-A, HLA-B, and HLA-C allotypes. Individual primary Nef clones exhibited differential abilities to downregulate HLA-I allotypes, with HLA-B molecules exhibiting decreased susceptibility to Nef-mediated downregulation compared to the susceptibility of HLA-A (whereas HLA-C was resistant to Nef’s effects). Differential Nef-mediated downregulation of HLA-A versus HLA-B molecules in turn modulated the ability of HIV-specific effector T cells to recognize HIV-infected target cells. By combining statistical analysis, site-specific mutagenesis, and structural interpretation, we identified natural polymorphisms within Nef and HLA cytoplasmic sequences that contribute to Nef’s differential abilities to downregulate HLA-A and HLA-B molecules.

**RESULTS**

**Differential downregulation of HLA-A, HLA-B, and HLA-C by HIV-1 laboratory strains.** To investigate differential downregulation of various HLA-A, HLA-B, and HLA-C allotypes by HIV-1 Nef, we stably transduced HLA class I-deficient 721.221 cells with HLA-A*02:01 (A02), HLA-A*24:02 (A24), HLA-A*33:01 (A33), HLA-B*35:01 (B35), HLA-B*57:01 (B57), or HLA-C*04:01 (C04). The cytoplasmic tails of HLA-B molecules are 3 amino acids shorter than those of HLA-A and HLA-C molecules (Fig. 1A). Moreover, HLA-A allotypes harbor aspartic acid and arginine at codons 314 and 315, respectively (DR1,13,15), whereas HLA-B and HLA-C allotypes harbor dual glycines (GG14,15). There are additional amino acid differences between individual allotypes within the cytoplasmic regions (Fig. 1A). The cell surface expression of HLA-I molecules on 721.221 cells was stable and substantial (Fig. 1B, top row). Specifically, the levels of surface expression of the three HLA-A (A02, A24, and A33) and two HLA-B (B35 and B57) allotypes were comparable when cells were stained with the pan-HLA-specific monoclonal antibody (Mab) w6/32, whereas the surface expression of the HLA-C allotype (C04) was lower, as expected. No changes in cell surface HLA-I expression were observed in cells infected with the HIV-1 reference strain NL43 engineered to lack Nef (NL43-A nef) (Fig. 1B, middle row). In contrast, when cells were infected with vesicular stomatitis virus envelope glycoprotein (VSV-g)-pseudotyped HIV-1 NL43 expressing the Nef gene from the prototypic laboratory strain SF2 (NL43-NefSF2), all HLA-A and HLA-B allotypes were downregulated from the cell surface, whereas the HLA-C allotype was unaffected (Fig. 1B, bottom row). As described in Materials and Methods, HLA-I downregulation activity was quantified using a scale from 0% (denoting no HLA-I downregulation activity in virus-infected cells) to 100% (denoting complete downregulation activity). The greater the downregulation activity value, the lower the residual cell surface expression of HLA-I. NefSF2’s ability to downregulate individual HLA-A and HLA-B molecules varied to some extent; the downregulation values (mean ± standard deviation [SD]) were 71.4% ± 3.1% for A02, 61.2% ± 3.7% for A24, 57.4% ± 2.4% for A33, 50.1% ± 2.4% for B35, and 52.4% ± 3.1% for B57 (Fig. 1C). Importantly, NefSF2’s ability to downregulate HLA-A alleles consistently exceeded that of HLA-B (Mann-Whitney test, P < 0.001). Two other laboratory HIV-1 Nef strains, NefNL43 and Nefref, were also evaluated for their ability to downregulate A24 and B35 (see Fig. S1 in the supplemental material). In both cases, their ability to downregulate HLA-A exceeded their ability to downregulate HLA-B (Mann-Whitney, P < 0.001), and neither downregulated C04.

**Differential downregulation of HLA-A, HLA-B, and HLA-C by natural Nef sequences.** We next assessed whether patient-derived Nef sequences differed in their ability to downregulate HLA-A, HLA-B, and HLA-C allotypes. For these experiments, we used 721.221 cells stably expressing A24, B35, and C04 as allotype representatives. A24 and B35 were chosen because they exhibited levels of susceptibility to Nef-mediated downregulation similar to the median values of the HLA-A and HLA-B allotypes tested (Fig. 1C), while C04 was chosen because the 721.221 cells expressing this allele exhibited the most stable cell surface HLA-C expression among our panel of 721.221 cells engineered to express various HLA-C allotypes. Representative HLA-I downregulation...
data, derived from cells infected with HIV-1 NL43-derived strains encoding Nef clonal sequences from three chronic progressors (subjects CP66, CP84, and CP90) are shown in Fig. 2A. The three patient-derived Nef clones varied in their ability to downregulate A24 and B35, but none downregulated C04. For example, CP66-Nef’s B35 downregulation activity was 23%, which was approximately half of that of NefSF2 (49%, Fig. 1B). In contrast, CP84-Nef’s A24 and B35 downregulation activities, 66% and 56%, respectively, were comparable to those of NefSF2. CP90-Nef downregulated A24 and B35 by equivalent, relatively lower levels (38% and 41%, respectively).

To quantify the ability of naturally occurring Nef sequences to downregulate A24 and B35, we expanded this analysis to 46 patient-derived Nef clones. All 46 Nef clones displayed greater ability to downregulate A24 (median activity of 60.8% [interquartile range {IQR}, 54.2 to 65.5]) than B35 (median activity, 49.3% [IQR, 41.0 to 55.5]) (Fig. 2B), a difference that was highly statistically significant (Mann-Whitney, *P* < 0.001). No clone downregulated C04. To quantify each virus’ ability to differentially downregulate A24 versus B35, we expressed the downregulation values as pairwise ratios. Overall, the median ratio of A24/B35 downregulation activities of patient-derived Nef clones was 1.25.
TABLE 1  Nef amino acid residues associated with differential HLA-A/HLA-B downregulation ratios in 721.221 cells

| Nef codon | Amino acid | HLA-A/HLA-B downregulation ratio for Nef clone | No. of subjects whose Nef clone |
|----------|-------------|-------------------------------------------------|--------------------------------|
|          |             | With residue | Without residue | Had residue | Did not have residue | P value | q value |
| 158      | Glu         | 1.30         | 1.07            | 33          | 12                     | 0.00064 | 0.13    |
| 202      | Tyr         | 1.23         | 1.65            | 40          | 6                      | 0.0017  | 0.17    |

\(^a\) Nef codon numbers are based on the sequence of NefSF2.

\(^b\) Median HLA-A*24/HLA-B*35 downregulation activity ratio.

\(^c\) The row total varies due to the gap in the aligned sequence.
tions at codon 202 (wild-type Tyr). These substitutions included naturally occurring residues (Ala, Lys, and Met at 158 and His, Leu, and Phe at 202), as well as residues not observed in natural Nef sequences (His at 158 and Ala at 202), as determined in our cohort and in 1,470 publicly available subtype B Nef sequences in the Los Alamos HIV sequence database. The steady-state levels of the wild-type Nef protein and the variants were comparable in virus-producing cells (data not shown). We then tested the Nef variants for A02 and B51 downregulation activity in T1 cells. No mutation at codon 158 substantially influenced A02 or B51 downregulation activity or A02/B51 downregulation ratios (Fig. 3A). In contrast, all mutations at codon 202, except Phe, significantly decreased Nef-mediated downregulation of both A02 and B51 in T1 cells compared to the downregulation activity of wild-type Tyr-202 (Fig. 3B). Moreover, these mutations impaired B51 downregulation activity to a greater extent than they impaired A02 downregulation, resulting in corresponding A02/B51 downregulation ratios that were also significantly higher (Fig. 3B). Specifically, the A02/B51 downregulation ratios for the Ala-, His-, and Leu-202 mutations were 2.47/0.18, 1.72/0.21, and 2.55/0.42, respectively, values that were 2.1-, 1.5- and 2.2-fold higher, respectively, than that of wild-type NefSF2 (Tyr-202). Consistent results were also obtained in 721.221 cells expressing A24 and B35 (see Fig. S3 in the supplemental material).

To further confirm the effect of Nef-202 on differential HLA-A and HLA-B downregulation, we introduced a His-to-Tyr mutation into patient CP66’s Nef clone at codon 202, thereby reverting this clone to the wild-type sequence at this position. This particular patient clone was chosen because it exhibited substantially impaired HLA-B downregulation activity in T1 cells (A02/B51 downregulation ratio, 2.30/0.28), 721.221 cells (A24/B35 downregulation ratio, 1.86/0.04), and primary T cells (A02/B35 downregulation ratio, 1.66/0.04).
downregulation ratio, 2.16 ± 0.25 (Fig. 3C). The introduction of the His-to-Tyr reversion substantially increased A02 and B51 downregulation activity in T1 cells, A24 and B35 downregulation activity in 721.221 cells, and A02 and B35 downregulation activity in primary T cells (Fig. 3C). As the extent of functional rescue by this reversion was more pronounced for HLA-B than for HLA-A allotypes, the ratio of HLA-A/HLA-B downregulation activities of CP66-Nef decreased to 1.29 ± 0.06 in T1 cells, 1.23 ± 0.01 in 721.221 cells, and 1.43 ± 0.07 in primary T cells, values that are comparable to the median for patient-derived Nef clones (Fig. 2C). These data indicate that amino acid polymorphism at Nef-202 alone can modulate Nef’s ability to downregulate HLA-B and, to a lesser extent, HLA-A allotypes.

**HLA-I cytoplasmic motifs that modulate sensitivity to Nef-mediated downregulation.** Nef-mediated downregulation of cell surface HLA-I molecules occurs through interaction between the HLA cytoplasmic domain and Nef in conjunction with the clathrin adaptor protein complex 1 (AP1) (17, 36, 37). We sought to identify HLA-I cytoplasmic motifs that determine sensitivity to Nef-mediated downregulation. As shown by the sequences in Fig. 1A, HLA-A and HLA-B alleles differ in their amino acid residues at codons 314 and 315, as well as in the presence (HLA-A) or absence (HLA-B) of the C-terminal Cys-Lys-Val (CKV339–341) motif. We therefore created a chimeric A02 molecule possessing the cytoplasmic tail of B35 by introducing a GG314,315 mutation into A02 and additionally deleting its C-terminal CKV339–341 motif; we designated this sequence A02GG. We then established Jurkat T cells stably expressing A02 and A02GG, which exhibited similar cell surface expression of these molecules upon staining with anti-HLA-A2 serotype antibody (mean fluorescence intensity; MFI, 5,664 ± 226 and 4,989 ± 263, respectively) (Fig. 4A). Infection of these cells with an HIV-1 NL43 strain encoding NefGG resulted in downregulation of A02 and A02GG to similar levels (Fig. 4A and B). As expected, the introduction of the His-202 mutation into NefSF2 impaired its ability to downregulate both A02 and A02GG to a greater extent for the latter. Specifically, the introduction of His-202 increased the A02/ A02GG downregulation ratio from 1.03 to 1.82, a 1.8-fold increase (Fig. 4C). Complementary results were obtained with the parental (His-202) and wild-type revertant (Tyr-202) CP66-Nef sequences. Specifically, the CP66 Tyr-202 revertant mutant exhibited A02 downregulation activity comparable to that of NefSF2 and A02GG downregulation activity moderately lower than that of NefSF2 (Fig. 4A and B). In contrast, the parental CP66-Nef (His-202) exhibited substantially impaired A02GG downregulation activity. Overall, the Tyr-to-His substitution increased the A02/A02GG downregulation ratio of CP66-Nef from 1.15 to 2.86, a 2.5-fold increase (Fig. 4C).

We further investigated potential interactions between Nef codon 202 and the cytoplasmic tails of A02 and A02GG by examining the existing crystal structure of the A02 cytoplasmic tail and Nef in complex with the μ1 subunit of API (37). This crystal structure exhibits an involvement of Tyr-202 of Nef in contacting a portion of the μ1 subunit of API and forming a part of the groove for HLA-I binding (Fig. 5A and B) (37). Although the crystal structure did not include A02’s cytoplasmic tail in its entirety (the last residue observed in the structure is Gly-331, which lies seven residues upstream from the C-terminal CKV339–341 that is present in A02 but absent in HLA-B alleles, since the remaining region is disordered in three-dimensional space), the proximity of Gly-331 to the main chain atoms of Nef Tyr-202 suggested that CKV339–341 could also be located nearby. Indeed, modeling of a 7-amino-acid spacer following Gly-331 indicated that CKV339–341 could readily be positioned next to the side chain of Nef Tyr-202 for potential interaction (Fig. 5B). In contrast, the DR314,315 residues of HLA-A, present in the crystal structure, do not directly contact any Nef residues (Fig. 5A). These data suggest that Nef codon 202 and the C-terminal CKV339–341 motif that is present in HLA-B but not HLA-A alleles, in conjunction with the μ1 subunit of clathrin API, may form an interaction that enhances the downregulation of HLA-A over that of HLA-B.

**Effects on T cell recognition.** We postulated that Nef’s differential abilities to downregulate HLA-A and HLA-B molecules would have consequences for T cell recognition of viral antigens presented on the surface of HIV-infected cells (18–20, 35). To test this hypothesis, we used a published reporter cell coculture assay that features HLA-A*02:01-expressing target cells and HIV-1-specific effector cells that transiently express a T-cell receptor (TCR) specific for an HLA-A*02:01-restricted HIV-1 epitope in Gag (FK10; Gag433–442 [FLGK1WPSYK]), human CD8α chain, and an nuclear factor of activated T cells (NFAT)-driven luciferase construct (see Materials and Methods). When target cells are infected with HIV, endogenously derived viral peptide antigens are

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**FIG 4** Effects of HLA-I cytoplasmic tail polymorphisms on Nef-mediated HLA-A and HLA-B downregulation activity. (A) The amino acid sequence of the cytoplasmic region of A02 was mutated to encode GG314,315 and a C-terminal deletion mutant of the CKV339–341 motif, resulting in a cytoplasmic sequence identical to that of B35 (A02GG; see Fig. 1A). Jurkat transfectants stably expressing A02 and A02GG were infected with recombinant NL43 viruses encoding various Nef clones or remained uninfected. The Nef clones tested were NefSF2 with Tyr-202 or His-202 and CP66-Nef with Tyr-202 or His-202. Representative flow cytometry plots are shown, and the levels of HLA downregulation activity are indicated in the plots. (B and C) Quantitative assessment of the downregulation activities (B) and the activity ratios (C) of A02 and A02GG are shown. The data shown are the mean results ± SD from 3 to 5 independent assays. Statistical analysis was performed by paired t test. n.s., not significant.
processed and presented in complex with A02 on their surface, though the HIV Nef protein counteracts this by downregulating HLA-I. When HIV-infected target cells are cocultured with A02-FK10-specific reporter cells, TCR-dependent signaling can be quantified based on luminescence. The ability of a given Nef sequence to downregulate HLA-I on the target cell thus correlates inversely with the TCR-mediated luminescence signal in effector cells.

We first undertook the following control experiments. Target cells expressing either A02 or A02GG,\textsubscript{H9004}CKV were pulsed with increasing concentrations of FK10 peptide and cocultured with A02-FK10-specific reporter cells, and the TCR-mediated signal was quantified based on luminescence. The ability of a given Nef sequence to downregulate HLA-I on the target cell thus correlates inversely with the TCR-mediated luminescence signal in effector cells.

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HIV-1-CP66-Nef (Tyr-202) revertant virus were comparable in magnitude and similar to those induced by NefSF2, we observed significantly greater T cell responsiveness toward A02GG,ΔCKV-expressing target cells infected with HIV-1 encoding the parental CP66-Nef (i.e., carrying the natural polymorphism His-202) than toward A02 target cells infected with the same virus (t test, \( P < 0.0001 \)) (Fig. 6C). Moreover, the magnitude of responses against the parental HIV-1–CP66-Nef virus was higher in both cells than in cells infected with HIV-1 strains encoding NefSF2 or the CP66-Nef (Tyr-202) revertant. Finally, the T cell responses as measured in this assay and Nef’s HLA-I downregulation activity as measured by flow cytometry showed a significant inverse relationship (Pearson’s correlation coefficient, \( r = -0.926, P = 0.0008 \)) (Fig. 6D). Together, these results are consistent with our observation that the naturally occurring His-202 mutation attenuates Nef’s ability to downregulate HLA-I, with a more pronounced impairment seen for HLA-B (modeled herein by the A02GG,ΔCKV construct) than for HLA-A. Our results further suggest that inefficient removal of HLA-B from the infected cell surface preferentially renders these cells more detectable by HIV-specific, HLA-B-restricted effector T cells.

**DISCUSSION**

In this study, we assessed the interplay between naturally occurring host (HLA-I) and virus (HIV-1 Nef) polymorphisms. We observed that HLA-B cytoplasmic tails displayed significantly decreased susceptibility to downregulation by primary HIV-1 Nef clones compared to the susceptibility of HLA-A cytoplasmic tails. On the host side, the relative resistance of HLA-B to downregulation by patient-derived Nef clones appeared to be modulated by the GG314,315 motif and/or the lack of the C-terminal CKV339–341 motif in the HLA-B cytoplasmic tail. On the viral side, HIV-1 Nef codon 202 was responsible, at least in part, for differential downregulation of HLA-A and HLA-B allotypes by this protein. Impor-
nantly, the results from our TCR recognition assays indicated that the differential susceptibilities of HLA-I molecules to downregulation by patient-derived Nef clones are likely to modulate T cell recognition of HIV-infected target cells presenting viral antigens in complex with HLA-I on their surface.

While it has long been known that HLA-B-restricted T cell responses exert a dominant influence on HIV-1 immune control (38, 39), it was only recently that the relative resistance of HLA-B to Nef-mediated downregulation was put forward as a possible underlying mechanism (23). The present study extends the previous work, which was performed using laboratory-adapted HIV-1 reference strains, by demonstrating that the majority of naturally occurring Nef sequences also downregulate HLA-A to a greater degree than HLA-B. Our results thus identify differential levels of susceptibility to HLA-A and HLA-B as a fundamental property of HIV-1 subtype B Nef sequences.

The introduction of various amino acid substitutions at Nef codon 202 (wild-type Tyr) substantially affected the protein’s ability to downregulate HLA-I, with greater impairment of HLA-B downregulation than of HLA-A downregulation. In particular, the uncommon natural variants Leu and His and the unnatural variant Ala displayed this phenotype, while the common natural variant Phe conferred only modest effects compared to the activity of Nef with the wild-type Tyr. This provides a possible explanation for the relative frequency of these residues in natural subtype B HIV-1 Nef sequences. Of interest, the Nef Phe-202 polymorphism has been identified as being associated with host expression of HLA-A*0301 and HLA-B*1501 (40), suggesting that it may arise under immune selection pressure by these alleles in vivo; however, we were unable to confirm this experimentally due to limited availability of peripheral blood mononuclear cells (PBMC) from this cohort. Also of interest, while Tyr and Phe are frequently observed at Nef codon 202 in all HIV-1 group M subtypes, the consensus at this residue in HIV-1 group O is Leu (Los Alamos HIV sequence database). Given our observation that the Leu-202 substitution in NefSF2 substantially increased the HLA-A/HLA-B downregulation ratio (Fig. 3B), it would be intriguing to investigate downregulation of HLA-I molecules by naturally occurring HIV-1 group O Nef sequences.

Some limitations of this study merit mentioning. Although we investigated 46 patient-derived HIV-1 subtype B Nef clones, this panel of sequences did not capture the entirety of HIV-1 subtype B Nef genetic diversity. Nevertheless, this relatively small subset of patient isolates exhibited a substantial dynamic range of HLA-A and HLA-B downregulation function. Importantly, the majority of Nef clones were less able to downregulate HLA-B than HLA-A—an observation that remained true for all cell lines and primary T lymphocytes, as well as all individual HLA alleles and cyttoplasmic variants tested. As our goal was to investigate Nef-mediated HLA-A and HLA-B downregulation on the surface of HIV-infected cells, we employed recombinant virus approaches (as opposed to transient-transfection systems, which are limited by Nef overexpression and potential cytotoxicity during plasmid delivery). Nevertheless, recombinant approaches are inherently limited by potential incompatibilities between the insert and the backbone (though all of the recombinant viruses used were replication competent and demonstrated Nef polyfunctional activity in various cell lines and primary T cells, as demonstrated in a previous published study of these clones [41]). The crystal structure of a ternary complex formed by Nef, the HLA-A02 cyttoplasmic tail, and the cargo-bonding μ1 subunit of AP1 was recently solved (37), yielding new insight into the molecular basis of Nef-mediated HLA-I downregulation function. However, due to the disordered nature of the C-terminal end of the HLA-A02 cytoplasmic tail, this region was not included in the structure (37), rendering it impossible to directly investigate interactions between this region (notably, the C-terminal CKV<sub>339-341</sub> motif) and Nef-202. Nevertheless, modeling of a 7-amino-acid spacer following Gly-331, the final residue of the HLA-A02 cyttoplasmic tail observed in the structure, indicated that the CKV<sub>339-341</sub> motif could readily be positioned next to the side chain of Nef Tyr-202, supporting a possible interaction.

Despite these limitations, our study extends the current understanding of Nef-mediated HLA-I downregulation function by demonstrating that, on average, subtype B Nef sequences from untreated, chronically HIV-infected patients downregulate HLA-A ~1.25-fold more efficiently than HLA-B. Moreover, these effects appear to be modulated, at least in part, by polymorphisms at Nef residue 202, as well as those located within the HLA-I cytoplasmic domain. Importantly, the differential levels of Nef-mediated HLA-A and HLA-B downregulation on HIV-infected cells modulate their subsequent recognition by HIV-specific T cells. Together, these results identify a new motif in HIV-1 Nef that differentially alters its ability to counteract HLA-A- and HLA-B-restricted CTL responses. Further studies will be necessary to determine whether Nef polymorphisms at residue 202 are associated with variations in immune-mediated control of infection or viral pathogenesis.

**MATERIALS AND METHODS**

**Patient-derived Nef clones and recombinant virus preparation.** The recombinant viruses expressing patient-derived Nef clones in an HIV-1 NL4.3 proviral backbone were generated as part of previously published studies (28, 30, 41). Briefly, Nef sequences were isolated from plasma viral RNA of 46 individuals chronically infected with HIV-1 subtype B, recruited in the Boston area, who were untreated at the time of sample collection and had a median plasma viral load of 80,500 (IQR, 25,121 to 221,250) RNA copies/ml and a median CD4 count of 292.5 (IQR, 72.5 to 440) cells/mm<sup>3</sup>, as described previously (30, 42, 43). This study was approved by the Institutional Review Boards at all relevant institutions, and all participants provided written informed consent. The GenBank accession numbers for clonal nef sequences used in this study are JX440926 to JX440971 (41). To facilitate a consistent codon numbering scheme (based on the sequence of the Nef<sub>JKR</sub> reference strain), all clonal Nef sequences were pairwise aligned to that of Nef<sub>JKR</sub> and insertions stripped out. Patient-derived nef clones isolated as described above were transferred into plasmid pNL43 and confirmed by sequencing as described previously (41). In addition, specific mutations (i.e., at Nef codons at 158 and 202) were introduced into the nef clone of strain SF2 using conventional overlapping PCR (33, 35), and the entire nef sequence was reconfirmed after subcloning into pNL43. The resultant DNA (5 μg), along with a plasmid encoding vesicular stomatitis virus envelope glycoprotein (VSV-g) (1 μg), was transfected into 10<sup>6</sup> 293T cells, and the virus-containing culture supernatants were harvested 48 h later. Recombinant viruses harboring nef from HIV-1 subtype B reference strain SF2 (Nef<sub>JKR</sub>) or lacking nef (∆Nef) were used as positive and negative controls, respectively, as previously described (30, 35).

**Cells and antibodies for HLA analysis.** Nef-mediated downregulation of cell surface expression of HLA-I molecules was assessed using the following four different cell systems: (i) 721.221, an HLA-A,-B,-C null human lymphoblastoid cell line, which was transfected to express a single HLA-I allele; (ii) T1, a human CD4<sup>+</sup> monocyte cell line that expresses 6 different autologous HLA-I alleles (2 alleles each of HLA-A,-B,
and -C); (iii) human primary T lymphocytes isolated from HIV-negative donors expressing autologous HLA-A*02 and HLA-Bw6 serotype alleles; and (iv) Jurkat, a human CD4+ T cell line that was stably transfected with genes encoding HLA-A*02:01 or its cyclospastic tail mutant. For the first system, we used the HLA-A-A, and -C null 721.221 cell line (44) that had been engineered to stably express a single HLA class I allele encoding HLA-A*02:01, HLA-A*2402, HLA-A*3303, HLA-B*3501, HLA-B*57:01, or HLA-C*04:01. These transfectants were kindly provided by M. Takiguchi, Kumamoto University, Japan, or T. Yamamoto, Vaccine Research Center, NIH, United States. HLA-I expression for each of the 721.221 lines was validated by HLA genotyping methods as described previously (45), and HLA-I cell surface expression was confirmed by staining with a pan-HLA-I-specific antibody (clone w6/32; BioLegend Co.), followed by flow cytometry. For the second system, the human CD4+ monocyte cell line T1 was used. The HLA-I genotyping results for T1 cells were A*02:01, A*31:01, B*40:01, B*51:01, C*01:02, and C*03:04. In the case of T1 cells, the cell surface expression of the specific HLA-I alleles HLA-A*02:01 and HLA-B*51:01 was evaluated using an HLA-A2 serotype-specific MAb (clone BB7.2; BioLegend) and an HLA-Bw4 serotype-specific MAb (clone TU109; kindly provided by M. Takiguchi), respectively. For the third system, primary T lymphocytes were isolated from PBMC of HIV-negative donors, followed by activation with phytohemagglutinin for 5 days. For the fourth system, the human CD4+ Jurkat T cell line was used. Human cDNA encoding HLA-A*02:01 was cloned from PBMC of a healthy HLA-A*02:01-positive volunteer into plasmid pcDNA3.1. Mutations (D83R, G85R, and A133V, deletion of CKV339–341) were introduced into the cytoplasmic tail region of HLA-A*02:01, giving rise to HLA-A02/GGDAKKV, in which the cytoplasmic tail region is equivalent to that of HLA-B*3501. In all cases, the levels of cell surface expression of HLA-A02 and its cyclospastic tail mutant were evaluated by an HLA-A2 serotype-specific MAb as described above.

HLA-I downregulation assay. The 721.221, Jurkat, T1, and primary T cells were infected with the VSV-g-pseudotyped recombinant HIV-1 and harvested 48 h later for staining with a pan-HLA-I specific antibody (clone w6/32; BioLegend Co.) or the serotype-specific MAb (clone BB7.2; BioLegend) and an HLA-Bw4 serotype-specific MAb (clone TU109; kindly provided by M. Takiguchi), respectively. For the third system, primary T lymphocytes were isolated from PBMC of HIV-negative donors, followed by activation with phytohemagglutinin for 5 days. For the fourth system, the human CD4+ Jurkat T cell line was used. Human cDNA encoding HLA-A*02:01 was cloned from PBMC of a healthy HLA-A*02:01-positive volunteer into plasmid pcDNA3.1. Mutations (D83R, G85R, and A133V, deletion of CKV339–341) were introduced into the cytoplasmic tail region of HLA-A*02:01, giving rise to HLA-A02/GGDAKKV, in which the cytoplasmic tail region is equivalent to that of HLA-B*3501. In all cases, the levels of cell surface expression of HLA-A02 and its cyclospastic tail mutant were evaluated by an HLA-A2 serotype-specific MAb as described above.

T cell recognition assay. The effects of Nef-mediated HLA-I downregulation on T cell recognition were analyzed using a T cell receptor (TCR)-based reporter cell assay as previously described (46, 47). Briefly, the effector cells were prepared by electrotransfection of Jurkat cells with expression plasmids encoding TCR-α and -β chains specific for the HLA-A*02:01-restricted HIV-1 Gag FK10 epitope (Gag333–444) (FLGK1WP2SKYK), human CD8-α chain (InivigoGen), and NFAI-luciferase reporter (Affymetrix). The resultant cells were incubated for 24 h, followed by separation of the CD8-expressing fraction by magnetic bead sorting (Miltenyi). Target cells were prepared by infection of parental Jurkat cells (lacking A02) or Jurkat cells transfected with A02 or A02/GGDAKKV with recombinant HIV-1 encoding nef alleles of interest. For control experiments, uninfected target cells were pulsed with the synthetic FK10 peptide. Effector cells (5 × 10^5 cells) were cocultured with target cells (5 × 10^4 cells, unless otherwise specifically indicated) for 6 h and then harvested for the luciferase assay (steady-Glo luciferin kit; Promega). Luminescence was measured by using a plate reader (CentroXS3; Berthold Technologis) with the following conditions: 3,000 ms integration and 100 ms settle time.

Western blotting. HEK-293T cells were transfected with proviral DNAs encoding NefSF2 or mutants for preparation of cell lysates as described previously (30, 31). Briefly, lysates were prepared in duplicate, subjected to SDS-PAGE, transferred to nitrocellulose membranes, and stained with rabbit anti-Nef polyclonal antiserum (NIH AIDS Research and Reference Reagent Program), followed by secondary enhanced chemiluminescence rabbit IgG (horseradish peroxidase-linked whole antobody; GE Healthcare Life Sciences), as reported previously (30). Band intensities were quantified using the Amersham Imager 600 (GE Healthcare Life Sciences).

Statistical analysis. Statistical analyses were performed using Graph Pad Prism 6.0. For Nez codon function analyses, the Mann-Whitney U test was used to identify amino acids associated with differential abilities to downregulate HLA-A and HLA-B (expressed as HLA-A/HLA-B downregulation ratios). Multiple comparisons were addressed using q values, the P value analogue of the false discovery rate (FDR), which denotes the expected proportion of false positives among results deemed significant at a given P value threshold (48). For example, at a q value of ≤0.2, we expect 20% of identified associations to represent false positives. In the present study, statistical significance was defined as a P value of <0.05 for (univariate analyses) or a P value of <0.05 and a q value of <0.2 (for analyses correcting for multiple hypothesis testing).

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01516-15/-/DCSupplemental.

Figure S1, EPS file, 0.5 MB.
Figure S2, EPS file, 0.8 MB.
Figure S3, EPS file, 0.6 MB.

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