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The Selective Downregulation of Class I Major Histocompatibility Complex Proteins by HIV-1 Protects HIV-Infected Cells from NK Cells

George B. Cohen,*†‖ Rajesh T. Gandhi,*#
Daniel M. Davis,† Ofer Mandelboim,†
Benjamin K. Chen,* Jack L. Strominger,†
and David Baltimore*‡§
*Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139
†Department of Molecular and Cellular Biology
Harvard University
Cambridge, Massachusetts 02138

Summary

To avoid detection by CTL, HIV encodes mechanisms for removal of class I MHC proteins from the surface of infected cells. However, class I downregulation potentially exposes the virus-infected cell to attack by NK cells. Human lymphoid cells are protected from NK cell cytotoxicity primarily by HLA-C and HLA-E. We present evidence that HIV-1 selectively downregulates HLA-A and HLA-B but does not significantly affect HLA-C or HLA-E. We then identify the residues in HLA-C and HLA-E that protect them from HIV downregulation. This selective downregulation allows HIV-infected cells to avoid NK cell–mediated lysis and may represent for HIV a balance between escape from CTL and maintenance of protection from NK cells. These results suggest that subpopulations of CTL and NK cells may be uniquely suited for combating HIV.

Introduction

Viruses that infect humans have evolved mechanisms of avoiding the immune response. A component of cellular immunity, cytotoxic T lymphocytes (CTL) control viral infections by recognizing virus-encoded peptides presented on the surface of infected cells by class I MHC molecules. As is true for several herpes viruses (Ploegh, 1998), human immunodeficiency virus-1 (HIV-1) regulates cell surface expression of class I proteins (Schwartz et al., 1996), and this process permits infected cells to resist CTL killing (Collins et al., 1998). However, removal of cell surface class I proteins may expose the infected cell to attack by natural killer (NK) cells. The role of NK cells in fighting viral infections is well established (Tay et al., 1998). NK cells preferentially lyse target cells that lack cell surface class I expression. NK cells are prevented from killing lymphoid cells primarily by NK inhibitory receptors that are specific for HLA-C and HLA-E (reviewed in Leibson, 1998; Yokoyama, 1998; Lanier, 1998a). When engaged by the proper class I protein, these receptors send inhibitory signals to the NK cell that prevent lysis of the target cell. In contrast, the majority of CTL recognize peptides presented by HLA-A and -B proteins (Littaua et al., 1991). Therefore, we were interested in determining which class I MHC proteins are downregulated by HIV and whether downregulation of class I proteins exposed HIV-infected cells to NK cell lysis.

Two previous studies had looked at the issue of class I downregulation by HIV and both found that the HIV protein Nef can downregulate HLA-A and -B (Collins et al., 1998; Le Gall et al., 1998). However, they came to different conclusions as to whether HLA-C is downregulated by HIV. Le Gall et al. (1998) used cells transfected with HIV *Nef* alone and found that there was no downregulation of HLA-C in cells stained with a monoclonal antibody (mAb) specific for denatured HLA-C protein. In contrast, Collins et al. (1998) detected apparent downregulation of HLA-C in cells infected with full-length HIV through staining with a HLA-Cw3-specific antibody. Neither of these studies looked at the downregulation of HLA-E. Interpreting the results of these prior studies is complicated by the differences in experimental techniques, the paucity of antibodies specific for HLA-C, the cross-reactivity of these antibodies with other HLA proteins (Zemmour et al., 1992a), and the low cell surface expression level of HLA-C compared to HLA-A and -B (Lawlor et al., 1990; Zemmour et al., 1992b). We avoid these complications by using the well characterized lymphoblastoid cell line 721.221 (221 cells) as a system to investigate HIV downregulation of class I molecules. 221 cells do not express HLA-A, -B, or -C proteins. However, upon transfection with the genes encoding these alleles, 221 cells readily express class I proteins on their surface (Shimizu and DeMars, 1989). Thus, the complicated processes involved in class I protein presentation are still intact in this cell line.

To study MHC protein downregulation by HIV, we created a series of 221 cell lines stably expressing both CD4 and defined class I proteins. These cells were then infected with HIV. The whole virus was used in this study since viruses that downregulate class I proteins often have multiple genes devoted to disrupting class I expression. For example, human cytomegalovirus has at least four proteins that disrupt class I presentation (Ploegh, 1998). In HIV, three viral proteins, Nef, Vpu, and Tat, have had ascribed to them the ability to decrease class I cell surface levels (Howcroft et al., 1993; Schwartz et al., 1996; Kerkau et al., 1997).

Using this system, we find that HIV-1 removes HLA-A and -B proteins from the surface of infected cells by a mechanism that depends on Nef expression. In contrast, HIV-1 does not significantly downregulate surface HLA-C or -E. This specificity is determined by two residues that are encoded in the cytoplasmic tail of all HLA-A and

‡To whom correspondence should be addressed (e-mail: baltimo@its.caltech.edu).
§Present Address: Office of the President, California Institute of Technology, Pasadena, California 91125.
‖Present Address: AIDS Research Center, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts 02129.
#Present Address: Division of Infectious Diseases, Johns Hopkins Hospital, Baltimore, Maryland 21205.

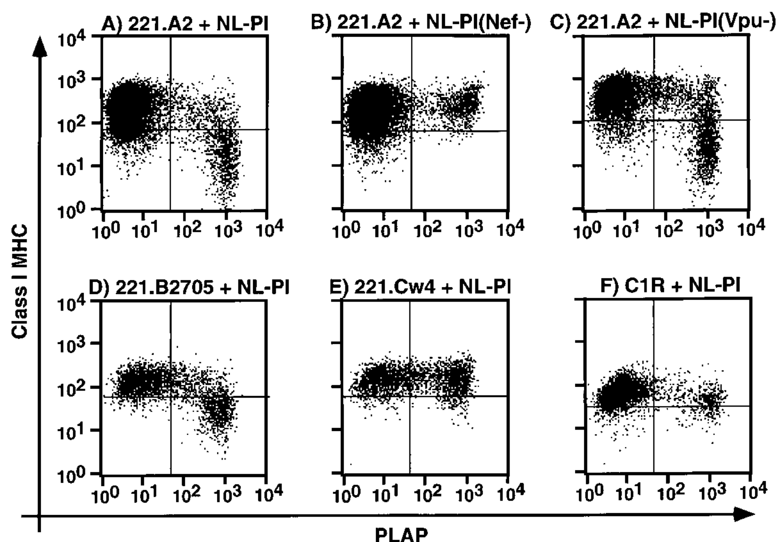

Figure 1. HIV-1 Selectively Downregulates Class I MHC Proteins

221 cells stably expressing defined class I proteins were infected with the NL-PI reporter virus. At 40–48 hr post infection, these cells were stained with a PLAP mAb (x axis) and a pan-class I MHC mAb (y axis). The NL-PI reporter virus used to infect these cells carries the full complement of HIV-1 genes except for (B) and (C), which used, respectively, a *Nef*-minus and *Vpu*-minus NL-PI virus. The infected 221 cells expressed (A) HLA-A2, (B) HLA-A2, (C) HLA-A2, (D) HLA-B2705, and (E) HLA-Cw4. In (F), we infect C1R cells.

-B alleles but that are not found in any HLA-C allele. Similarly, a single residue substitution in the cytoplasmic tail of HLA-E accounts for the resistance of this protein to HIV downregulation. We then show that the HLA-C and -E left on the surface of HIV-infected cells protects the cells from NK cell lysis.

Results

HIV-1 Selectively Downregulates Class I MHC Proteins

221 cells stably expressing CD4 and defined class I allotypes were made using retrovirus-mediated gene transfer. These stable cell lines were then infected with HIV strain NL43 (NL-PI) carrying the placental alkaline phosphatase (*PLAP*) reporter gene (Chen et al., 1996). Infection with NL-PI marks infected cells with the PLAP cell surface protein. The effect of HIV infection on cell surface levels of class I MHC proteins was assessed 40–48 hr post infection by staining for class I protein and PLAP with fluorescently conjugated antibodies followed by flow cytometry. Figure 1A presents results for HLA-A2-positive 221 cells (221.A2) infected with HIV NL-PI. Three populations of cells are distinguished by the applied gates. In the upper left quadrant are the uninfected cells. In the upper right quadrant are the cells infected with HIV that have not downregulated class I MHC. In the lower right quadrant are the cells infected with HIV that have downregulated HLA-A2. To facilitate comparisons of the extent of class I downregulation among different cell lines, the horizontal line that demarcates class I high from low cells is drawn throughout this paper such that 90%–95% of the uninfected cells lie above this line (except where noted otherwise). The downregulation of HLA-A2 by HIV in 221 cells is comparable to that previously reported in HIV-infected-A2-positive peripheral blood mononuclear cells stained with an HLA-A2-specific monoclonal antibody (Collins et al., 1998). This downregulation was Nef dependent because infection with the same reporter virus having a frame shift in the *Nef* coding region resulted in no MHC downregulation (Figure 1B). Mutations in other HIV gene products such as *Vpu* (Figure 1C) or *Vpr* (data not shown) had little effect on HLA-A2 downregulation. HIV also downregulated HLA-B2705 (Figure 1D) and -B702 (data not shown) in a Nef-dependent manner. The downregulation of the HLA-B allotypes was consistently less dramatic than that of HLA-A2.

In contrast, HIV infection of 221 cells that express HLA-Cw4 (Figure 1E), -Cw3, or -Cw7 (data not shown) did not result in significant class I downregulation. Similarly, no downregulation was seen in HIV-infected 221 cells expressing the nonclassical class I MHC molecule, CD1d. These results held over a wide range of viral multiplicity of infections and expression levels of the class I allotypes. Similar results were obtained using HIV strain HXB-2 repaired in the *Nef* and *Vpu* reading frames and carrying the *PLAP* reporter gene. Equivalent downregulation of class I protein was seen whether the cells were stained with a pan-class I MHC mAb or with fluorescently labeled β2-microglobulin (Davis et al., 1997) (data not shown).

Because HIV may affect class I protein expression at the transcriptional level (Howcroft et al., 1993), and the class I alleles in 221 cells are expressed from a heterologous promoter, the effect of HIV on endogenous HLA-C in the cell line C1R was also examined. C1R is a well characterized cell line that expresses endogenous HLA-Cw4, low amounts of HLA-B35 (approximately 15% of that of -Cw4), and little or no HLA-A (Zemmour et al., 1992b). Therefore, the level of cell surface HLA-C in HIV-infected C1R cells can be assessed with a pan-class I mAb. Following HIV infection of C1R cells, only a very small amount of class I downregulation was seen, probably due to the residual HLA-B35 (Figure 1F). We conclude that HIV-1 does not downregulate HLA-C.

Region on Class I MHC Protein Required for Downregulation by HIV-1

To identify the region on HLA-A2 that accounted for its Nef-dependent downregulation, a chimeric molecule was made between human CD8α and HLA-A2. This chimera contained the extracellular region of CD8α joined to the transmembrane (Tm) and cytoplasmic tail region of HLA-A2 (CD8α/A2-Tm&tail). Upon HIV infection, wild-type CD8α did not undergo appreciable Nef-induced

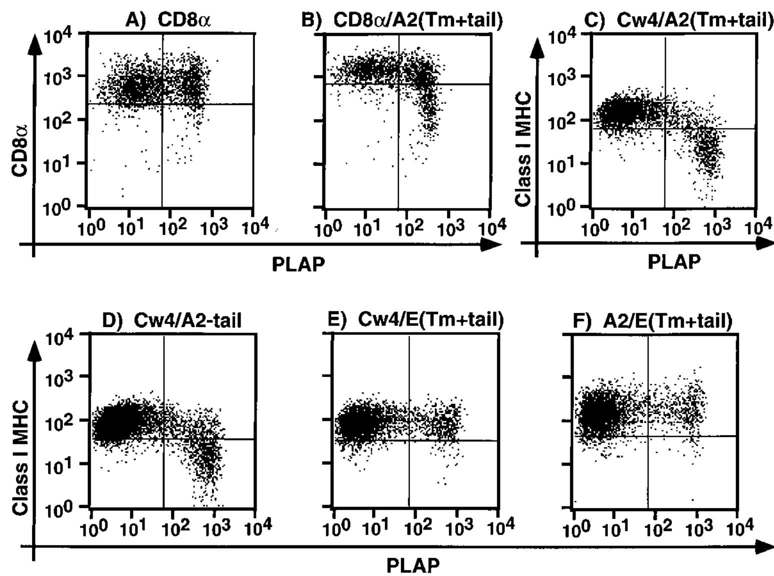

Figure 2. Region on Class I MHC Molecules Required for Downregulation by HIV-1

221 cells expressing class I chimeric molecules were infected with the NL-PI virus. The cells were stained 40–48 hr post infection with a PLAP mAb (x axis) and either a CD8 mAb (2A-2B; y axis) or a pan-class I MHC mAb (2C-2F; y axis). The infected 221 cells expressed (A) CD8α, (B) CD8α/A2(Tm&tail), (C) HLA-Cw4/A2(Tm&tail), (D) HLA-Cw4/A2-tail, (E) HLA-Cw4/E(Tm&tail), and (F) HLA-A2/E (Tm&tail).

downregulation (Figure 2A), while CD8α/A2-Tm&tail was downregulated (Figure 2B) in a Nef-dependent manner.

As with CD8α, transfer of the Tm and cytoplasmic tail region of HLA-A2 to HLA-Cw4 (Cw4/A2-Tm&tail) conferred Nef-dependent downregulation on Cw4 (Figure 2C). Moreover, a second HLA-Cw4 and -A2 chimeric molecule, containing only the cytoplasmic tail region of HLA-A2 fused to the extracellular and Tm region of HLA-Cw4 (Cw4/A2-tail), was downregulated by HIV (Figure 2D) to a similar extent as HLA-Cw4/A2-Tm&tail. Chimeras containing either the Tm and tail of HLA-B27 joined to HLA-Cw4 (Cw4/B27-Tm&tail) or containing the HLA-B27 tail alone (Cw4/B27-tail) were also downregulated (data not shown). Therefore, the cytoplasmic tail region of HLA-C is responsible for its resistance to downregulation by HIV.

In contrast to the results with the HLA-Cw4/A2 and -Cw4/B27 chimeras, transfer of the HLA-E transmembrane and cytoplasmic tail region to HLA-Cw4 (Cw4/E-Tm&tail; Figure 2E) or to -A2 (A2/E-Tm&tail; Figure 2F) did not permit significant Nef-dependent downregulation. HLA-A2 and -Cw4 chimeras containing only the cytoplasmic tails of HLA-E (A2/E-tail and Cw4/E-tail) also did not undergo Nef-dependent downregulation (data not shown). Attempts to assess the effect of HIV on endogenous HLA-E using an HLA-E-specific mAb (Braud et al., 1998a) were inconclusive because the staining with this reagent was not sufficiently strong to give interpretable results.

Identification of Residues in the Cytoplasmic Tails of Class I MHC Molecules that Are Critical for Nef-Dependent Downregulation

Since the selective downregulation of class I proteins is due to differences in the cytoplasmic tail region, we examined this region of the molecule in more detail. Differences within the cytoplasmic tail regions of HLA-A, -B, and -C are shown in Table 1 along with the sequences of HLA-E and CD1d. The site where the cytoplasmic tails were swapped to create the chimeras is shown by the arrow above the class I consensus sequence. Five residues of HLA-C are different from the consensus sequence beyond this point (indicated by dark circles, Table 1).

To define the residues that were critical for allowing HLA-A and -B downregulation, point mutations were made in the -Cw4/B27-tail chimeric molecule. Since we were interested in the residues in HLA-C that account

Table 1. Class I MHC Cytoplasmic Tail Sequences

	310		320	330	340
	|	↓	|● ○ ○○ ●	| ● ●	| ●
Consensus	RRKSSGGKGG		SYSQAASSDS	AQGSDVSLTA	CKV
HLA-A	-----DR---		----------	----------	---
HLA-B	----------		----------	----------	...
HLA-C	----------		-C------N-	-----E--I-	--A
HLA-E	-K--------		---K-EW---	----ESHSL.	...
CD1d	TSRFKRQT..		--QGVL....	....	...

The top row shows the class I MHC consensus sequence and is followed by the consensus sequences for HLA-A, -B, -C, and the nonclassical class I molecules, HLA-E and CD1d. The vertical arrow above the class I consensus sequence marks the site where cytoplasmic tails were swapped to create chimeric molecules. Dashes represent residues that match the consensus sequence. Dots are residues that are deleted in the sequence shown. Dark circles indicate residues that differ between the consensus sequence and HLA-C. Open circles indicate residues that differ between the consensus sequence and HLA-E that are discussed in the text. The numbering above the consensus sequence corresponds to the residue number in HLA-Cw4.

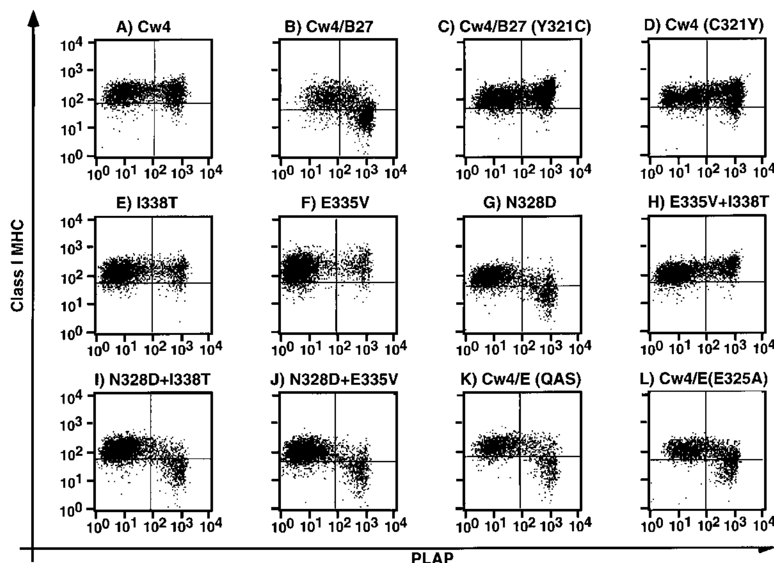

Figure 3. Identification of Residues in the Cytoplasmic Tail Region of Class I MHC Proteins that Are Critical for Nef-Dependent Downregulation

221 cells expressing the indicated class I allotypes were infected with the NL-PI virus and stained for PLAP (x axis) and class I MHC (y axis) 40–48 hr post infection. The infected 221 cells expressed (A) HLA-Cw4, (B) -Cw4/B27-tail, (C) -Cw4/B27(Y321C), and (D) -Cw4 (C321Y). In (E)–(J), HLA-Cw4(C321Y ΔC3) was further mutated to make -Cw4 progressively more like HLA-B with the following mutations: (E) I338T; (F) E335V; (G) N328D; (H) E335V and I338T; (I) N328D and I338T; and (J) N328D and E335V. In (K) and (L), the HLA-Cw4/E-tail chimera was made more like the class I consensus cytoplasmic tail with the following mutations: (K) HLA-Cw4/E (QAS) contains K323Q, E325A, and W326S; and (L) HLA-Cw4/E (E325A).

for its resistance to Nef-induced downregulation, the mutations were made such that the HLA-B27-tail became progressively more like that of HLA-C. Compared to the -Cw4/B27-tail chimera (Figure 3B), the -Cw4/B27-tail chimera containing the single point mutation Y321C was not downregulated by HIV (Figure 3C), consistent with previous reports that mutations at Tyr-321 abrogate Nef-dependent downregulation (Le Gall et al., 1998) and colocalization of Nef with HLA-B (Greenberg et al., 1998). However, inserting Tyr-321 into -Cw4 (Cw4 C321Y, Figure 3D) or -Cw6 (data not shown) did not make these molecules susceptible to HIV-induced downregulation.

To identify other residues necessary for conferring Nef-dependent downregulation on HLA-C, additional mutations were made in the -Cw4(C321Y) molecule. Neither deleting the last three carboxy-terminal amino acids to make the HLA-C-tail more like HLA-B, -Cw4 (C321Y ΔC3) nor changing the last amino acid to Val as it is in HLA-A, -Cw4 (C321Y A342V) had any appreciable effect (data not shown). There were only three remaining amino acids that differed between the consensus sequence and HLA-C in the cytoplasmic tail region. All six possible combinations of these three amino acids were made in the context of the -Cw4 (C321Y ΔC3) mutant. All of the mutants that contained both C321Y and N328D were downregulated. Mutants that did not contain both the tyrosine and aspartic acid were not downregulated (Figures 3E–3J). Therefore, both Tyr-321 and Asp-328 are critical for Nef-dependent class I downregulation.

HLA-E contains both Tyr-321 and Asp-328, yet chimeras containing the cytoplasmic tail region of HLA-E were not downregulated by HIV (Figures 2E and 2F). However, HLA-E differs from the cytoplasmic tail consensus sequence at a number of other residues, (Table 1) including three residues between Tyr-321 and Asp-328. A chimeric molecule containing the HLA-E cytoplasmic tail but mutated to match the class I consensus sequence at these three residues, HLA-Cw4/E-tail (K323Q, E325A, W326S), was downregulated by HIV (Figure 3K). Moreover, this downregulation was retained in the mutant, -Cw4/E-tail(E325A) (Figure 3L), which contains only a single point mutation in the HLA-E cytoplasmic tail. Thus, in addition to Tyr-321 and Asp-328, Ala-325 is also critical for Nef-dependent downregulation of class I proteins.

HLA-C Protects HIV-Infected Cells from NK Lysis

To determine whether the HLA-C left on the surface of HIV-infected cells protected infected cells from NK cells, flow cytometry (Slezak and Horan, 1989; Collins et al., 1998) was used to follow NK lysis of HIV-infected target cells. NK-killing assays are generally done using either Cr^{51} or S^{35}-methionine release as an indicator of target cell lysis (Mandelboim et al., 1996). However, neither of the radioisotope release assays is as sensitive as flow cytometry in discerning the killing efficiency of NK cells on a heterogeneous target cell population in which only a fraction of the cells are HIV infected and have downregulated class I MHC proteins.

Two different sources of NK cells were used as the effector cells in these assays. First, primary NK cells carrying the NK inhibitory receptor, NKIR1 (which is inhibited by a subset of HLA-C allotypes: HLA-Cw2, -Cw4, -Cw5, and -Cw6), were cloned from peripheral blood (Mandelboim et al., 1996). An S^{35} release assay established that these NK cells (called NK.NKIR1) had the predicted specificity for class I proteins. Second, we used an immortalized cell line that we genetically altered to have a defined killing specificity. Prior work (Wagtmann et al., 1995) had shown that the killing specificity of immortalized NK cells could be changed by transferring in genes coding for NK inhibitory receptors. However, because vaccinia virus was used as the viral vector, the infected NK cells lived only transiently. We therefore used a retrovirus vector to stably introduce the NKIR1 gene into the YTS cell line. The resulting immortalized cell line, called YTS.NKIR1, should be selectively inhibited from killing target cells that express HLA-Cw2, -Cw4, -Cw5, or -Cw6 relative to the parental cell line (YTS), which does not express any NKIRs and therefore kills cells indiscriminately. The surface level expression of NKIR1 in YTS.NKIR1 and NK.NKIR1 cells

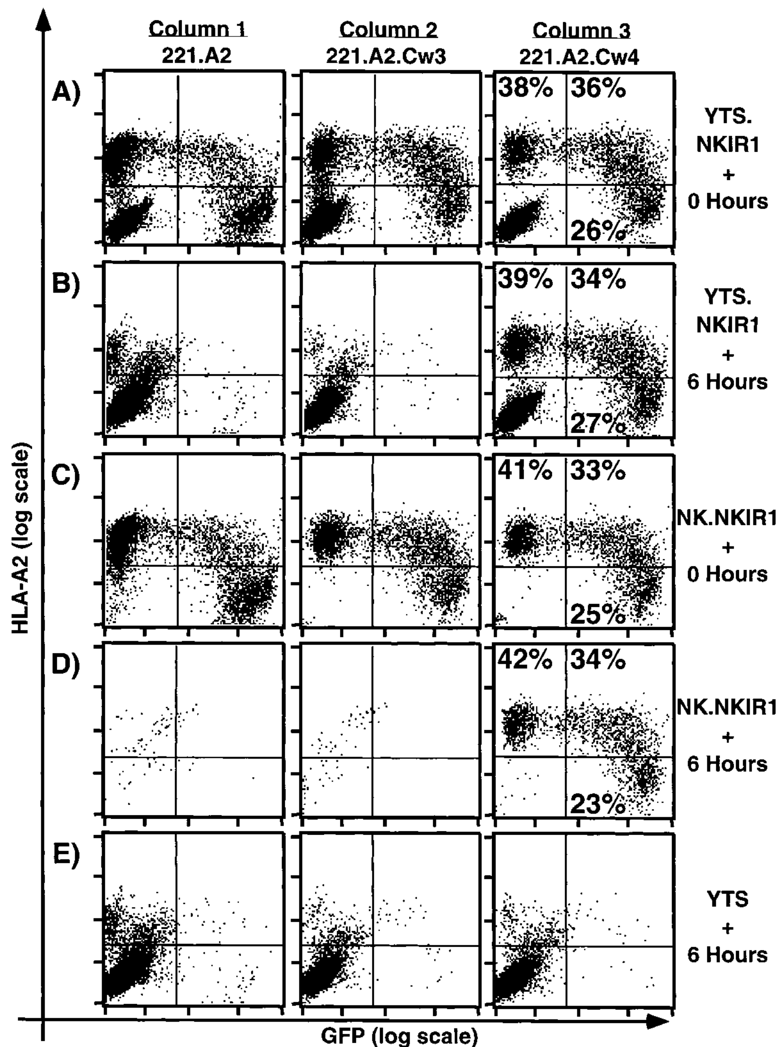

Figure 4. HLA-Cw4 Protects HIV-Infected Cells from NK Cell Lysis

221 cells expressing HLA-A2 alone (column 1), -A2 and -Cw3 (column 2), and -A2 and -Cw4 (column 3) were infected with HIV carrying a GFP reporter, NL-GI. At 35–45 hr post infection, the infected cells were mixed with either YTS.NKIR1 cells (A and B), NK.KIR1 cells (C and D), or YTS cells (E) at a 4:1 effector:target cell ratio. Cells were either stained immediately (time = 0 hr) for GFP (x axis) and HLA-A2 (y axis) (A) and (C) or incubated for 6 hr at 37°C (time = 6 hr) and then stained (B, D, and E). Not shown are YTS cells mixed with target cells at time 0 hr as these looked identical to the YTS.NKIR1 cells at 0 hr (A). YTS and YTS.NKIR1 cells are uninfected and HLA-A2-negative and therefore appear near the origin (lower left quadrant) in (A), (B), and (E). The cells shown in Figures 4–6 have been electronically gated based on light scattering to exclude dead cells. Primary NK.NKIR1 cells are much smaller than 221 cells and have mostly been excluded from the analysis presented in (C) and (D) based on light-scattering properties. To facilitate comparisons of the ability of NK cells to selectively kill HIV-infected cells, the proportion of 221.A2.Cw4 cells (column 3) that are uninfected (upper left quadrant), infected but have not yet downregulated HLA-A2 (upper right quadrant), or infected and have downregulated HLA-A2 (lower right quadrant) are reported in (A)–(D). No such numbers are reported for 221.A2.Cw4 cells mixed with YTS cells (E), as too few cells survive to make the numbers meaningful.

was comparable as judged by flow cytometry (data not shown). That the YTS.NKIR1 cell line had the desired killing specificity was confirmed in NK killing assays done using S^{35}-release or FACS analysis (data not shown). In these assays, YTS.NKIR1 cells efficiently lysed 221 cells that expressed either no class I protein or expressed HLA-A2, -B2705, or -Cw3, but they did not lyse 221 cells that expressed HLA-Cw4. In contrast, the parental YTS cell line, lacking NKIR1 expression, lysed 221.Cw4 cells as effectively as the other 221 cells (data not shown). NK lysis of 221 target cells in the FACS assay was followed based on the decrease in light-scattering signal of cells as they die or by the uptake of the fluorescent dye 7-aminoactinomycin D, 7AAD, which is specific for dead cells (Schmid et al., 1994). The killing of 221 cells by NK cells in the flow cytometry assay occurred at effector to target ratios (1:1–10:1) and within times (3–6 hr) comparable to those of S^{35}-release assays (data not shown).

The flow cytometry NK lysis assay was then used to test whether the HLA-C left on the surface of HIV-infected cells was sufficient to protect 221 cells from NK cells. 221 cells were made that expressed either HLA-A2 alone, -A2 and -Cw3, or -A2 and -Cw4. HLA-A2 was added to the 221.Cw3 and 221.Cw4 cells so that class I downregulation could be followed when these cells were infected with HIV. These cells were infected with HIV carrying the gene for green fluorescent protein (NL-GI). We used *GFP* as the reporter construct in HIV for these experiments because of concerns that the cell surface protein PLAP might influence the ability of NK cells to recognize target cells. At 35–45 hr post infection, we mixed the target cells with YTS.NKIR1 cells (defined as time t = 0) and stained with an mAb specific for HLA-A2 (Figure 4A). At this time point, the NK cells have not yet begun to lyse the target cells. YTS cells are HLA-A2-negative and are not infected with HIV and therefore appear in the lower left quadrant of Figure 4A. All three populations of 221 target cells (columns 1–3, Figure 4A) show signs of infection and HLA-A2 downregulation.

The mixed effector/target cell populations were then incubated together for 6 hr (Figure 4B). Strikingly, almost all of the 221 cells that express -A2 alone or -Cw3 with -A2 have been lysed and no longer appear in the live cell gate. In contrast, HIV-infected cells that express -Cw4 with -A2 were protected from the YTS.NKIR1 cells. A quantitative analysis showed that -Cw4 protected 221 cells from the YTS.NKIR1 effectors regardless of the

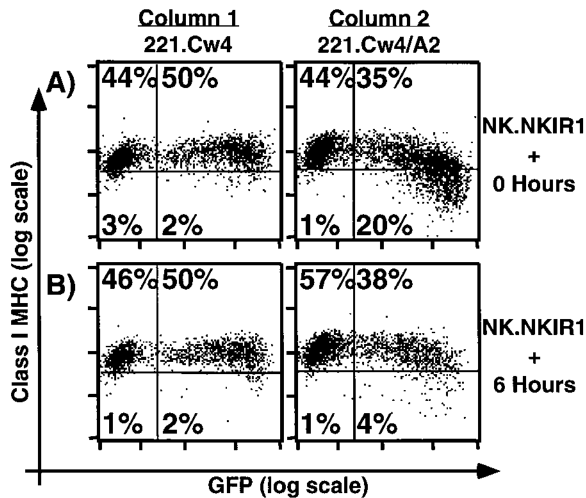

Figure 5. NK.NKIR1 Cells Can Selectively Kill 221 Cells that Downregulate a HLA-Cw4/A2-Tail Chimera

221 cells expressing either -Cw4 (column 1) or the -Cw4/A2-tail chimera (column 2) were infected with the NL-GI virus and mixed with the primary NK.NKIR1 cells. Cells were either stained immediately (time = 0 hr) for GFP (x axis) and class I MHC protein (y axis) (A) or incubated for 6 hr at 37°C (time = 6 hr) and then stained (B). Primary NK.NKIR1 cells are much smaller than 221 cells and have been excluded from the analysis presented based on light-scattering properties. To facilitate comparisons of the ability of NK cells to kill HIV-infected cells, the proportion of 221.Cw4 and 221.A2.Cw4 cells are reported in the respective quadrants.

extent of HIV infection or HLA-A2 downregulation (compare the percentages in the respective quadrants of Figures 4A and 4B in column 3). Similar results were seen when primary NK.NKIR1 cells were used as the effector cells (Figure 4C and 4D; primary NK.NKIR1 cells are much smaller than 221 cells and have mostly been excluded from the analysis presented in Figures 4C and 4D based on light-scattering properties). In contrast, YTS cells lacking NKIR1 lysed all target cell populations indiscriminately (Figure 4E shows time = 6 hr; the cells at time = 0 hr were indistinguishable from those shown in Figure 4A and are not shown). Therefore, the NKIR1 receptor is responsible for the inability of YTS.NKIR1 cells to lyse HIV-infected 221.A2.Cw4 cells. These findings indicate that the -Cw4 left on the surface of HIV-infected cells is functional and able to protect the infected cells from NK cells.

Last, we asked whether removal of -Cw4 from the surface of an HIV-infected cell exposes that cell to killing by NK cells. 221 cells expressing either the chimeric molecule, HLA-Cw4/A2-tail, or a control population of 221 cells that express Cw4 alone were infected with HIV. The infected cells were then mixed with NK.NKIR1 cells and either analyzed immediately (Figure 5A) or after a 6 hr incubation (Figure 5B). As expected, in the HIV-infected 221.Cw4 cells class I protein was not downregulated (Figure 5A, column 1). In contrast, when 221.Cw4/A2-tail cells were infected with HIV, the chimeric molecule was downregulated (Figure 5A, column 2). Following incubation with the NK.NKIR1 cells, the 221.Cw4 cells showed little change in the relative ratios of uninfected to infected cells (50% were infected in both Figures 5A and 5B, column 1). In contrast, those 221.Cw4/A2-tail cells that had downregulated surface -Cw4/A2 were selectively killed, as seen by the selective loss of the lower right-hand quadrant cells (20% in Figure 5A and 4% in Figure 5B, column 2). Similar results were also seen using YTS.NKIR1 cells as the effectors. In contrast, YTS cells killed both cell populations equally as well (data not shown). The results with the HLA-Cw4/A2-tail chimeras show that the NK lysis flow cytometry assay is sensitive to small changes in surface HLA-C expression levels. We conclude that HLA-C is not downregulated by HIV and that the selective downregulation of class I proteins by HIV protects HIV-infected cells from natural killer cells.

HLA-E Protects HIV-Infected Cells from NK Cell Lysis

Surface expression of HLA-E requires binding of a nonamer peptide derived from the leader signal sequence of an HLA-A, -B, or -C protein to HLA-E (Braud et al., 1998a; see also Lanier, 1998a, and references within). In the absence of this nonamer peptide, HLA-E does not efficiently make its way to the cell surface. Therefore, although 221 cells express intracellular endogenous HLA-E, unless the cells are transfected with another class I MHC protein, they do not express HLA-E on the cell surface.

The NK inhibitory receptor that is specific for HLA-E is a heterodimer of CD94 and NKG2A (Borrego et al., 1998; Braud et al., 1998b; Lee et al., 1998). One of the NK cell lines that we generated from peripheral blood was found to be largely devoid of the NK receptors, NKIR1 and NKIR2, specific for HLA-C proteins. This cell line, called NK.CD94, was therefore a good candidate for an NK cell line specific for HLA-E. The ability of this cell line to lyse 221 cells expressing various MHC class I proteins infected with HIV NL-GI was tested. In addition to infecting a number of 221 cell lines previously described in this paper, a 221 cell line that expressed a construct encoding the leader peptide of HLA-A2 fused to HLA-E (HLA-A2 leader/E) was also infected. Adding the HLA-A2 leader peptide to HLA-E allows surface expression of the full-length mature form of HLA-E in the absence of other class I proteins. Consistent with our mutagenesis analysis, cells expressing the HLA-A2 leader/E protein did not show significant class I downregulation upon HIV infection (Figure 6A, column 3).

HIV-infected 221 cells that expressed either no class I protein or expressed HLA-B2705, -Cw3, or -A2 leader/E were then mixed with the NK.CD94 cells for either 0 or 6 hr. HLA-B2705 contains a leader peptide that does not bring HLA-E to the cell surface, HLA-Cw3 has a leader peptide that will bring the endogenous HLA-E to the surface, and HLA-A2 leader/E has a leader peptide that will bring both the endogenous HLA-E as well as the transfected HLA-E to the cell surface (Braud et al., 1998a). 221 cells expressing no class I protein (data not shown) or HLA-B2705 were efficiently lysed by NK.CD94 regardless of HIV infection (compare Figures 6B, time = 0 hr, and Figure 6C, time = 6 hr, column 1). In contrast, 221 cells expressing HLA-Cw3 or the -A2 leader/E construct were inefficiently lysed by NK.CD94 (compare Figures 6B, time = 0 hr, and Figure 6C, time = 6 hr, column 2, -Cw3 cells; column 3, -A2 leader/E cells). Preincubation of the NK.CD94 cell line with an antibody to CD94,

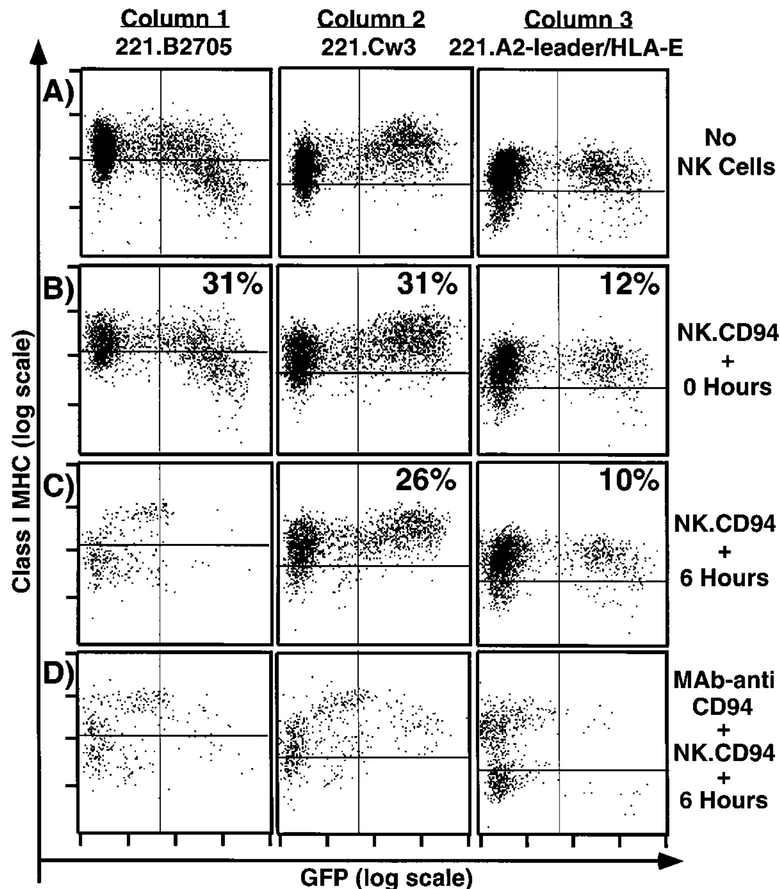

Figure 6. HLA-E Protects HIV-Infected Cells from NK Cell Lysis

221 cells expressing HLA-B2705 (column 1), HLA-Cw3 (column 2), and HLA-A2 leader/E (column 3) were infected with HIV carrying a GFP reporter, NL-GI. At 35–45 hr post infection, the infected cells were analyzed for class I downregulation (A) or mixed with NK.CD94 cells (B–D) at effector to target cells ratios of 5:1–10:1. Cells were either stained immediately (time = 0 hr) for GFP (x axis) and MHC class I (y axis) (B) or incubated for 6 hr at 37°C (time = 6 hr) and then stained (C and D). NK cells in (D) were preincubated with an anti-CD94 mAb to block the CD94/NKG2A interaction with HLA-E. Primary NK.CD94 cells are much smaller than 221 cells and have mostly been excluded from the analysis presented in (B)–(D) based on light-scattering properties. To facilitate comparisons of the ability of NK cells to selectively kill HIV-infected cells, the proportion of HIV-infected cells are reported in (C) and (D). No such numbers are reported when too few cells survive to make the numbers meaningful.

which blocks the CD94/HLA-E interaction, reversed this inhibition and promoted killing of these cells (Figure 6D). Control antibodies, which block the interaction of NKIR1 or NKIR2 with their respective ligands, had little or no effect on the killing potential of NK.CD94 cells (data not shown). These results suggest that HIV-infected cells expressing either HLA-Cw3 or HLA-A2 leader/E are protected from lysis by NK.CD94 cells through an inhibitory signal transduced by the CD94/NKG2A complex upon HLA-E recognition.

Discussion

Viruses that infect humans and last for the lifetime of the host have evolved mechanisms of avoiding the host's CTL response. Among these mechanisms is downregulation of surface expression of class I MHC protein (Ploegh, 1998). However, downregulation of class I MHC proteins comes with a potential cost because this exposes the infected cell to NK cells. NK killing is inhibited primarily by the presence of either HLA-C or HLA-E and to a lesser extent by some HLA-B alleles (Valiante et al., 1997; Lanier, 1998a; Yokoyama, 1998). Given the specificity of inhibition of NK cells by specific MHC allotypes, it was of particular interest to determine which class I alleles are downregulated by HIV.

Using the 721.221 cell line stably infected with defined class I alleles, we found that HIV downregulates HLA-A and -B alleles from the cell surface. In contrast to its effect on HLA-A and HLA-B, we find that HIV does not downregulate HLA-C in either 221 cells or the endogenous HLA-C found in C1R cells. Our results support the conclusion of Schwartz and colleagues that Nef alone does not downregulate HLA-C (Le Gall et al., 1998), and we further demonstrate that full-length HIV also does not downregulate HLA-C. We suspect that a previous report from this laboratory (Collins et al., 1998), that in HIV-infected cells there is a Nef-dependent downregulation of HLA-C, was due to cross-reactivity of the anti-HLA-Cw3 mAb with other class I allotypes (Zemmour et al., 1992a; Collins and D.B., unpublished data).

Our studies suggest that in addition to HLA-C, HLA-E is also not downregulated by HIV. Surface expression of HLA-E requires that the peptide presentation site on HLA-E is bound to a nonamer peptide derived from the leader signal sequence of either HLA-A, -B, or -C (see Leibson, 1998, and references within). Since HLA-C is not downregulated by HIV, its leader-derived nonamer peptide should be available to bring HLA-E to the surface.

In direct killing assays, we find that the HLA-C and HLA-E left on the surface of HIV-infected cells protects those cells from NK lysis. This implies that HIV escapes from the immune response by downregulating those class I proteins recognized by the majority of known CTL (HLA-A and -B) while retaining on the cell surface those HLA molecules that inhibit the majority of NK cells (HLA-C and -E). This selective downregulation may represent for HIV an optimal balance of escape from CTL and maintenance of protection from NK cells.

This strategy of selective MHC downregulation is reminiscent of that used by the fetus to avoid the maternal immune system. The MHC that the fetus carries is half derived from the father and therefore could be recognized by maternal CTL as foreign. Placental trophoblast cells therefore do not express HLA-A or -B but do express class I proteins that inhibit NK cell lysis (Pazmany et al., 1996; Lanier, 1998b, and references within). Therefore, HIV may have adopted a strategy similar to that of the fetus: keep those class I MHC proteins that protect from NK cells on the surface of the cell and remove those that present antigens to the most effective CTL.

Mechanism of Class I MHC Downregulation by Nef

We found that the ability of Nef to downregulate HLA-A2 could be transferred to a heterologous cell surface protein, human CD8α, by replacing the Tm and cytoplasmic-tail sequence of CD8α with that of HLA-A2. Thus, at least part of the Nef-induced downregulation is independent of peptide presentation by the class I protein, consistent with reports that Nef accelerates the surface endocytosis rate of class I molecules (Schwartz et al., 1996; Greenberg et al., 1998).

Similar chimeras made between HLA-Cw4 and -A2 or between HLA-Cw4 and -B27 further narrowed Nef's ability to selectively downregulate class I to the cytoplasmic tail region of the class I protein. There is very little sequence variability within the cytoplasmic tail region of class I molecules. Therefore, we have tested either directly or indirectly (by using chimeric molecules) the susceptibility of the vast majority of class I molecules to Nef-dependent downregulation. All HLA-A and -B alleles tested were downregulated whereas all HLA-C alleles were not.

Upon further mutagenesis, we found that mutation of two residues in the cytoplasmic tail of HLA-C, C321Y and N328D, were required to make HLA-C susceptible to Nef-induced downregulation. All HLA-A and -B allotypes have Tyr and Asp at these positions; all HLA-C allotypes have Cys and Asn. Our results on Tyr-321 are in agreement with recent reports on the role of Tyr-321 in the Nef-dependent downregulation of HLA-B (Greenberg et al., 1998; Le Gall et al., 1998). However, in our system, Tyr-321 was necessary but not sufficient to induce downregulation. Neither a -Cw4 nor a -Cw6 allotype carrying the single substitution, C321Y, was downregulated. Similarly, -Cw4 with the C321Y mutation and truncated after Ala-324 was not downregulated by HIV (unpublished data) nor was the class I MHC molecule, CD1d, which contains a tyrosine in its cytoplasmic tail that is required for the spontaneous endocytosis of CD1 proteins from the cell surface (Jackman et al., 1998).

We also found that mutation of a single amino acid in the cytoplasmic tail of HLA-E, E325A, is required to make the -E cytoplasmic tail responsive to Nef-induced downregulation. Glu-325 is unique to HLA-E as all HLA-A, -B, and -C alleles contain an alanine at this position. Tyr-321, Ala-325, and Asp-328 lie within seven amino acids of each other, perhaps suggesting that this region of the class I molecule needs to adopt an α-helical conformation for HIV-dependent downregulation. Ala-325 would lie on the same face of this proposed helix as Tyr-321 and Asp-328.

Implications

These results suggest that in vivo the majority of CTL (Collins et al., 1998) and NK cells will not recognize HIV-infected cells that have selectively downregulated cell surface class I proteins. However, small populations of NK cells and CTL may be uniquely suited for eliminating HIV-infected cells. For instance, NK cells that express an inhibitory receptor, NKB1, specific for a subset of HLA-B molecules, in theory, should be capable of lysing HIV-infected cells that downregulate HLA-B if the NK cells do not also express inhibitory receptors for HLA-C and HLA-E. However, the in vivo importance of the NKB1 inhibitory receptor in NK biology is unclear because it is rarely expressed in the NK cells of individuals who carry the appropriate HLA-B ligands (Valiante et al., 1997). Interestingly, those HLA-B alleles that have been associated with a slower progression to AIDS predominantly belong to the subset of HLA-B alleles that are recognized by NKB1, while HLA-B alleles associated with a fast progression tend to belong to the subset of HLA-B alleles that are not recognized by NKB1 (Kaslow et al., 1996). There has also been a report that low NK activity is associated with rapid progression to AIDS (Bruunsgaard et al., 1997). Therefore, the role of NK cells during HIV infection warrants further study.

There is also an unusual HLA-B allele, HLA-B4601, that apparently arose from recombination between HLA-Cw1 and -B15. HLA-B4601 inhibits killing by NK cells expressing NKIR2 (like NKIR1, NKIR2 is specific for a subset of HLA-C allotypes) (Barber et al., 1996). However, the cytoplasmic tail of HLA-B4601 matches the HLA-B consensus sequence and should be downregulated by HIV. Therefore, in HLA-B4601-positive individuals infected with HIV, the NKIR2 NK cells may be able to preferentially kill HIV-infected cells that have downregulated -B4601 if these individuals do not also contain HLA-C alleles recognized by NKIR2. Thus, it will be interesting to determine if the HLA-B4601 allele is associated with a slower progression to AIDS.

Similarly, just as NK cells inhibited by HLA-B might selectively be used to fight HIV, the fact that HIV leaves HLA-C on the cell surface suggests that CTL restricted to HLA-C-presented antigens might be particularly effective at killing HIV-infected cells. However, the role of HLA-C in CTL responses in vivo is enigmatic (Lawlor et al., 1990). Many known CTL are restricted by HLA-A and -B allotypes but few HLA-C-restricted CTLs have been described. This could reflect a true immunological bias or a technical one. Few HLA-C-specific antibodies exist and, therefore, typing HLA-C-restricted CTL is difficult. Interestingly, of the few known CTL restricted to HLA-C, many are directed against HIV antigens (Littaua et al., 1991; Johnson et al., 1993). There has also been a report of HIV-positive long-term nonprogressors with an HLA-C-restricted immunodominant CTL response (Nehete et al., 1998) and that HLA-C-restricted CTL occur frequently during the acute phase of HIV-infection (D. Nixon, personal communication). That allotype-specific populations of NK cells or CTL may be particularly effective in the clinical setting has been suggested by a recent report that patients undergoing bone marrow transplantation have a lower risk of a leukemia relapse if the donor marrow is mismatched at the HLA-C locus

(Sasazuki et al., 1998). This result may suggest a unique role for HLA-C within the human immune response.

The failure of HIV to downregulate HLA-C and the existence of HLA-C-restricted CTL raises questions about the utility to the virus of class I downregulation as a mechanism of immune evasion. However, HLA-C is expressed on the cell surface at lower levels than HLA-A and -B (Lawlor et al., 1990; Zemmour et al., 1992b) and CTL restricted to HLA-C-presented antigens may therefore be less effective at recognizing viral antigens due to the lower cell surface density of HLA-C. Immune evasion by class I downregulation would then serve as a mechanism of weakening the immune response but would not give the virus absolute protection from the immune system. In addition, HLA-A and -B downregulation by HIV is not absolute and in vivo occurs concurrent with CTL recognition of class I–presented viral antigens. Therefore, class I downregulation will afford the virus only partial protection from even HLA-A- and HLA-B-restricted CTL. However, given the enormous numbers of replication cycles that HIV goes through within a single infected individual, the selective advantage that downregulating class I proteins confers on the virus would not have to be large to account for its existence. Class I downregulation may then allow HIV to walk the thin line between too little immune evasion, which would result in decreased viral loads and transmission, and too much immune evasion, which would make the virus more pathogenic and also does not favor transmission.

Experimental Procedures

Cell Lines

C1R cells (Zemmour et al., 1992b), 721.221 cells (Shimizu and DeMars, 1989), YT cells (Yoneda et al., 1992), and the retrovirus packaging cell lines Bing and Bosc (Pear et al., 1993) are as previously described. YTS cells (provided by Z. Eshhar, Rehovoth, Israel) are a subline of the YT NK tumor cell line.

Constructs

The retroviral vectors: pM5-neo containing the mouse ecotropic receptor (Baker et al., 1992), MSCV-neo, and pBABE-puro were used as described (Ausubel et al., 1996) except that the MSCV vector was modified so that it contained no BamHI site. The mouse ecotropic receptor was digested out of the pM5-neo vector and ligated into the pBABE-puro vector to create the vector pBABE-ecotropic receptor-puro. The puromycin resistance gene was then deleted from this vector to create the vector pBABE-ecotropic receptor without a drug selection marker.

The genes for human CD8α and the class I alleles HLA-A201, -B2705, -Cw304, and -Cw401 were cloned into the MSCV-neo vector. Human CD4 was cloned into the pBABE-puro retrovirus vector. The *NKIR1* gene was cloned into the vector pBABE-puro that had been modified so that expression of the *Sa pac* gene that confers puromycin resistance was driven off of the retroviral LTR. A CMV promoter driving expression of the *NKIR1* gene was then inserted immediately downstream of the *Sa pac* gene. This vector was also used to drive expression of the HLA-A2 leader/E construct that contained the HLA-A2 leader (residues Met-(−24) to Ala-(−1)) joined to the mature HLA-E molecule (Gly-1 to Leu-337).

Class I MHC chimeric molecules containing the extracellular and transmembrane (Tm) portion of Cw4 and the cytoplasmic tail of either HLA-A1, -A2, -B15, -B27, or HLA-E were made as follows. PCR was used to introduce a BamHI site in the cytoplasmic tail of HLA-Cw4 at residues 319 and 320. The BamHI restriction sequence, GGA TCC, introduces a silent mutation into Cw4 as it encodes for the amino acids, Gly-319 and Ser-320, found in virtually all class I proteins. This modified -Cw4 was cloned into the EcoRI–XhoI site of the MSCV-neo vector. The MSCV.Cw4 plasmid was then digested with BamHI and XhoI to excise the cytoplasmic tail of Cw4. The cytoplasmic tail of the other class I molecules, which had been generated by PCR to contain a similar BamHI–XhoI site, were then cloned in to create the chimeric molecules. A similar sequence was used to create the HLA-A2/E-tail chimera and to create point mutations in the cytoplasmic tail of -Cw4.

The chimeras that contain the extracellular domain of HLA-Cw4 and the Tm and cytoplasmic tail of either HLA-A2, -B27, or -E were generated directly by PCR with overlapping primers. HLA-Cw4/A2(Tm-tail) contains residues Met-(−24)-Lys-275 from Cw4 and Pro-276-Val-342 from HLA-A2. HLA-Cw4/B27(Tm-tail) contains residues Met-(−24)-Trp-274 from Cw4 and Glu-275-Ala-339 from HLA-B27. HLA-Cw4/E (Tm-tail) contains residues Met-(−24)-Ser-277 from -Cw4 and Ser-278-Leu-338 from HLA-E. The CD8α/HLA-A2 chimeric molecule contains the extracellular region of CD8α, Met(1)-Gly-176, and the Tm and cytoplasmic tail of HLA-A2, Pro-276-Val-342 (CD8α/A2-Tm&tail). All of these constructs were cloned into the MSCV-neo vector.

HIV strain HXB2 (HXB-PI) or strain NL43 (NL-PI) carrying the *PLAP* reporter gene have been described (Chen et al., 1996; Collins et al., 1998; Gandhi et al., 1998). HIV strain NL43 carrying an enhanced *GFP* gene, NL-GI (*GFP* from Clonetech), is identical to NL-PI except that *GFP* replaces *PLAP* as the reporter. NL-PI defective in *Vpu* (NL-PI Vpu-) was generated by PCR such that the Vpu initiating ATG codon was changed to CTG and the second codon of *Vpu*, CAA, was changed to TAA so that it encodes a premature stop codon. All gene products generated by PCR were fully sequenced.

Use of Retroviruses to Create Stable Cell Lines

Retrovirus gene–mediated transfer was used to create 221 cell lines stably expressing defined class I alleles. Retroviruses were generated by transfecting cultures of the packaging cell lines, Bosc (for ecotropic viruses) or Bing (for amphotropic viruses) with retroviral vectors. The resulting viral supernatant was then harvested and used to infect cells as described (Pear et al., 1993; Ausubel et al., 1996). In brief, 4 ml of viral supernatant supplemented with 4 μg/ml hexadimethrine bromide (Sigma) was used to infect $\sim 3.0 \times 10^5$ 221 cells. The 221 cell/virus mixture was transferred to a tissue culture plate and centrifuged for 1.5 hr at $\sim 1{,}000 \times g$ and 32°C. Air-tight centrifuge rotor buckets were used when the spin-infection was done with amphotropic virus. After centrifugation, the cells and virus were incubated for 3–12 hr at 37°C before the media was changed.

Stable 221 cell lines expressing CD4 and defined class I proteins were created by the following scheme. First, 221 cells expressing the murine ecotropic receptor were made by infecting 221 cells with an amphotropic retrovirus carrying the mouse ecotropic receptor. Subsequently, genes could be introduced into these 221 cells with high efficiency using the less hazardous murine retrovirus. Approximately 1 week after infecting 221 cells with the ecotropic receptor, these cells were infected with a murine virus carrying the pBABE-CD4-puro construct. CD4-positive 221 cells were selected in puromycin and sorted by FACS for CD4 expression. These cells were then infected with retroviruses carrying defined class I genes. Class I MHC–positive 221 cells were selected in neomycin and bulk sorted by FACS for class I expression. This scheme for making CD4 and class I–positive 221 cells was followed for all cell lines generated during the course of this work with the following exceptions: HLA-Cw702, -B702, -Cw6(C321Y) and CD1d. The genes for these class I alleles were introduced into 221 cells by electroporation as previously described (Mandelboim et al., 1996).

C1R and YTS cells carrying the *CD4* and *NKIR1* genes, respectively, were generated using retroviral vectors in a procedure similar to the one outlined above for 221 cells.

HIV Infection, NK Assays, and Flow Cytometry

HIV was generated from transfected 293 cells and HIV infection of target cells was done as described previously (Chen et al., 1996). NK assays were performed 35–45 hr post HIV infection. 221 target cells (1×10^4 to 4×10^4) were mixed with NK effector cells (at the effector to target cell ratios indicated in the text) and incubated together in a U-bottomed 96-well microtiter plate at 37°C for 0–6 hr.

Staining of HIV-infected cells is as described (Chen et al., 1996). HIV-infected cells were stained 40–48 hr post infection with the following reagents: a pan-class I MHC mAb, W6/32 (Harlan Sera-lab), rabbit anti-human PLAP (Biomeda), Cy-chrome-conjugated anti-CD8α and 7-aminoactinomycin D (PharMingen), anti-HLA-A2 (One Lambda), and anti-NKIR1 (Immunotech). Secondary antibodies were goat anti-mouse IgG FITC and goat anti-rabbit IgG PE (Caltag). Stained HIV-infected cells were fixed overnight with 2% formaldehyde in phosphate-buffered saline at 4°C. NK receptor blocking antibodies were: HP3E4, anti-NKIR1 (Perez-Villar et al., 1997); GL183, anti-NKIR2 (Immunotech); and HP-3D9, anti-CD94 (PharMingen). Blocking of NK inhibitory receptors was done using 2–5 μg of mAb mixed with NK cells on ice for 30 min prior to incubation with target cells at 37°C. Results shown are typical of at least duplicate experiments.

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