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HIV's evasion of the cellular immune response

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Summary: Despite a strong cytotoxic T-lymphocyte (CTL) response directed against viral antigens, untreated individuals infected with the human immunodeficiency virus (HIV-1) develop AIDS. We have found that primary T cells infected with HIV-1 downregulate surface MHC class I antigens and are resistant to lysis by HLA-A2-restricted CTL clones. In contrast, cells infected with an HIV-1 in which the *nef* gene is disrupted are sensitive to CTLs in an MHC and peptide-specific manner. In primary T cells HLA-A2 antigens are downmodulated more dramatically than total MHC class I antigens, suggesting that *nef* selectively downmodulates certain MHC class I antigens. In support of this, studies on cells expressing individual MHC class I alleles have revealed that *nef* does not downmodulate HLA-C and HLA-E antigens. This selective downmodulation allows infected cells to maintain resistance to certain natural killer cells that lyse infected cells expressing low levels of MHC class I antigens. Downmodulation of MHC class I HLA-A2 antigens occurs not only in primary T cells, but also in B and astrocytoma cell lines. No effect of other HIV-1 accessory proteins such as vpu and vpr was observed. Thus Nef is a protein that may promote escape of HIV-1 from immune surveillance.

Introduction

A dramatic increase in our knowledge of the natural history of human immunodeficiency virus (HIV) infection has yielded insights into mechanisms of HIV disease pathogenesis. After exposure to HIV an infected individual becomes acutely viremic. This phase subsequently resolves coincident with the appearance of anti-HIV cytotoxic T lymphocytes (CTLs) (1, 2). Thereafter infected individuals experience a period of clinical latency that is variable in length and proportional to the amount of circulating virus (3). It is not until much later, when HIV has destroyed most of the host's $CD4^+$ T cells, that the infected individual becomes symptomatic with opportunistic infections that the weakened immune system can no longer contain. Coincident with the drop in $CD4^+$ T cells and the appearance of opportunistic infections, there is also a rise in HIV loads presumably because the immune system completely fails to contain HIV (4–6).

A shortened half life of infected $CD4^+$ T cells has been demonstrated both *in vivo* and *in vitro* and is due at least in part

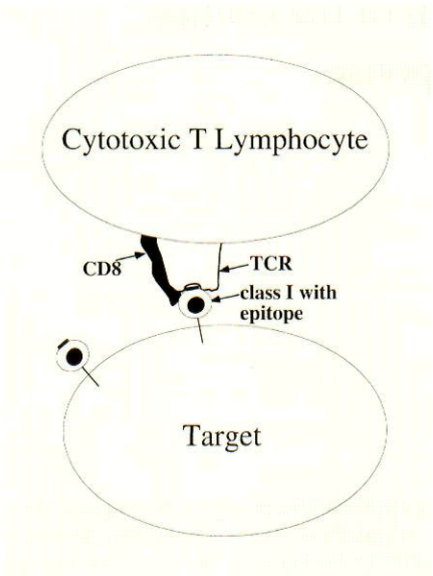

Fig. 1. Antigen processing and presentation in association with MHC class I antigens. Schematic diagram of cytotoxic T cells recognizing a target cell by contact of the T-cell receptor and CD8 molecules with MHC class I antigens and peptide antigen.

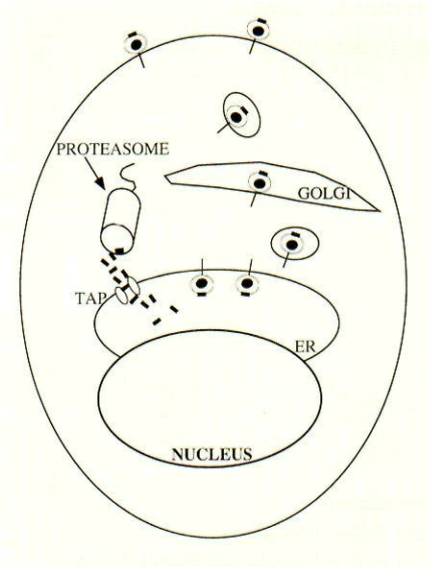

Fig. 2. Schematic diagram of antigen processing and presentation in association with MHC class I antigen heavy and light chains. TAP: transporter associated with antigen processing; ER: endoplasmic reticulum.

to the direct toxic effects of HIV (7–14). Additional destruction may be mediated by the host's immune response against infected cells. Thus a disease model has evolved in which viral loads are maintained by successive rounds of newly infected CD4+ T cells that live just long enough to produce the next round of viral progeny. The precise contribution of sequestered reservoirs and longer lived cells such as infected macrophages has not yet been well worked out (for review see (15)).

In an infected individual multiple factors determine viral levels including the effectiveness of the host's immune response and the genetics of the particular infecting viral strain. In support of the importance of host factors, studies using MHC class I HLA-tetrameric complexes to identify anti-HIV CTLs have indicated an inverse correlation between the quantity of anti-HIV CTLs and viral load (16). In addition, a role for CTLs in controlling HIV-1 infection *in vivo* is supported by individual patient studies in which variant HIV epitopes appear to have been selected by a strong immune response (17–19). These studies suggest an important role for CTLs in the control of virus load.

CTLs detect infected cells through the T-cell receptor and CD8 molecules which recognize foreign peptides assembled in the groove of MHC class I proteins and expressed on the surface of almost all mammalian cells (*Fig.* 1). The peptides are derived from cytosolic proteins which are digested by the proteasome and transported into the endoplasmic reticulum where they assemble with the MHC class I heavy and light chains (*Fig.* 2). In addition to this classical mode of CTL activation, CTLs can also become activated by other routes: for instance, phagocytes and dendritic cells can ingest antigens and present them in association with MHC class I antigens (20–22).

CTLs are well known for their ability to lyse cells expressing foreign antigens by the release of granzymes and perforins and by the activation of Fas-mediated killing (23). However they may have more than one defense mechanism against HIV. In addition to the traditional cytolytic responses, activated anti-HIV CTLs secrete soluble substances including chemokines that potently inhibit infection by HIV (24–26). It is possible that secretion of soluble inhibitory factors by CTLs plays an important role in the control of virus levels. Consistent with this, McMichael and colleagues reported a strong inverse correlation between CTLs and viral load, but were unable to detect an inverse correlation between CTLs and life span of infected cells (16). Perhaps activated CTLs reduce viral loads by inhibiting new infection with soluble factors, but fail to kill already infected cells efficiently. The surviving cells would continue to generate virus with variant antigens that could potentially escape immune recognition. Such a model would help explain why the potent anti-HIV CTL response fails to prevent progression to the acquired immunodeficiency syndrome (AIDS) in almost every untreated individual.

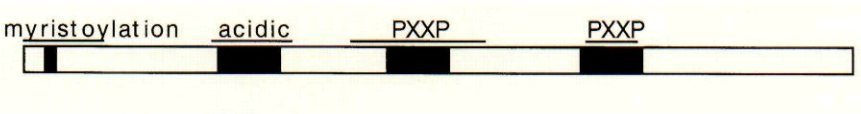

Fig. 3. Conserved domains within the *nef* open reading frame.
Schematic diagram of the conserved domains of Nef (65, 66).

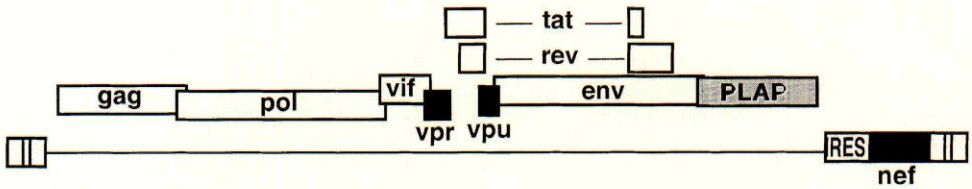

Fig. 4. Schematic of HIV-PLAP genome. Genomic organization of HIV-1 vectors containing PLAP. The HIV-PLAP vectors HXB-PI and HXB-EP were derived from the molecular clone HXB2D. Like their parent, they do not express functional vpu and vpr proteins. In addition, HXB-EP lacks a functional envelope gene. The NL-PI vectors have a similar construction, but were derived from the molecular clone NL4-3. NL-PI has open reading frames for all accessory proteins. For detail on the construction of these vectors, please refer to Chen et al. (32) and Collins et al. (33).

The critical role of viral determinants in the establishment of high viral loads is best illustrated by the requirement of an intact *nef* gene for the development of AIDS in humans and monkeys (27–29). Nef is a 27–34 kDa myristoylated protein that has several conserved domains which appear to be important for its function (*Fig.* 3). Notably, there is a conserved acidic domain of unclear function and several proline-rich domains. The first proline-rich domain has been shown to be important for the interaction of Nef with SH3 domain containing kinases such as Hck (30). Cell culture studies have delineated multiple activities of Nef including increased viral infectivity, T-cell activation, CD4 downmodulation and MHC class I antigen downmodulation (for review see (31)). It is still unclear whether all the reported cell culture activities of *nef* are required for HIV disease pathogenesis in infected people.

Role of Nef in protection of HIV-infected cells from CTLs

We recently reported that expression of *nef* in an infected cell protects it from anti-HIV CTL recognition. To infect primary $CD4^+$ T cells, we used an HIV that placed the cell surface marker placental alkaline phosphatase (PLAP) on the infected cell (32), (*Fig.* 4). Thus in a mixture of infected and uninfected cells we were able to directly examine what happened to the infected cells after adding CTLs. Specifically, we used CTL clones restricted to MHC class I HLA-A2 antigens and that recognized the HXB Gag epitope (SLYNTIAVL (SL9)) (33). We found that the ability of these CTLs to recognize and kill infected target cells expressing *nef* was reduced up to 24-fold in some experiments. Because of the importance of MHC class I antigens for CTL recognition, we stained infected cells with anti-MHC class I antibodies and found that infected cells expressing *nef* had reduced levels of MHC class I antigens on their cell surface. The loss of MHC class I antigens was dramatic, especially when we used monoclonal antibodies specific to antigens encoded by the MHC class I HLA-A2 allele (*Fig.* 5*A*). A nef^- HIV did not cause downmodulation of HLA-A2 and an $A2^-$ donor showed no reactivity to the antibody (*Fig.* 5*A*).

We then demonstrated that the ability of CTLs to recognize infected cells could be restored by preincubating infected cells with synthetic peptide epitope (33). We were thus able to demonstrate that HIV-infected cells escape CTL recognition by reducing the density of antigenic peptide complexed to MHC class I antigen on the cell surface. A partial reduction in surface MHC class I antigen with HIV-1 infection has been reported previously (25, 34, 35), and has been attributed variably to *nef*, *vpu* and *tat* (36–38). Interestingly, the downregulation of MHC class I HLA-A2 antigens we observed on primary T cells was more dramatic than what had previously been reported. We found that primary T cells infected with a nef^+ virus had up to 300-fold less surface HLA-A2 by flow cytometry. It is worth noting that Tsomides et al. (39) demonstrated that there are

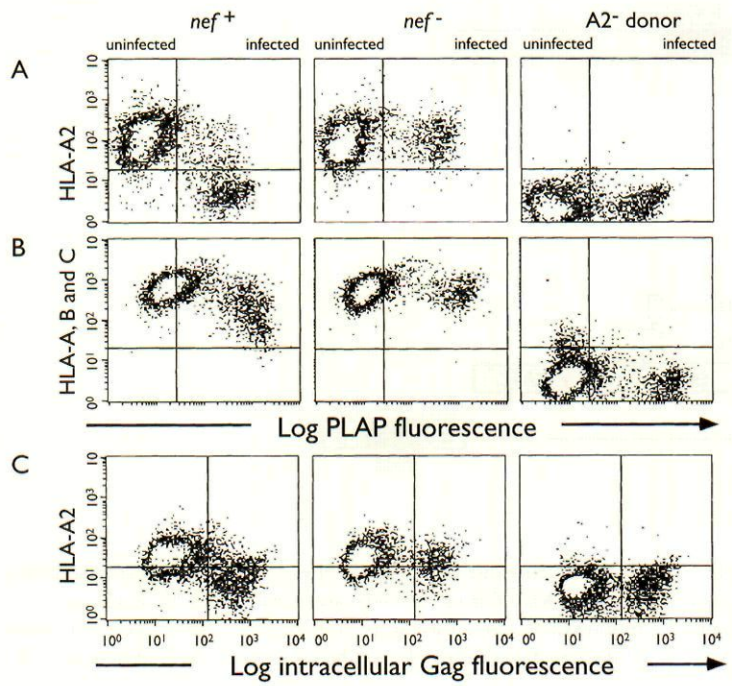

Fig. 5. Downmodulation of MHC class I antigens by HIV-1. Primary CD4+ T cells were infected with HIV-PLAP and were stained with the indicated antibodies. **A.** Cells were stained with monoclonal antibodies specific for HLA-A2 (obtained from One Lambda) and a polyclonal antibody against PLAP (obtained from Dako, Zymed or Biomeda). **B.** Cells were stained with monoclonal antibody W6/32 (Hartlan) which recognizes HLA-A, B and C antigens and a polyclonal antibody against PLAP. **C.** Cells were stained with a monoclonal antibody specific for HLA-A2 antigens and a flourescein isothiocyanate (FITC)-conjugated monoclonal antibody that recognizes HIV-1 Gag. The FITC-conjugated anti-Gag antibody was KC57 (Coulter). Intracellular staining was as described previously (25).

approximately 400 Gag SL9 epitopes presented on the surface of Jurkat cells chronically infected with a virus lacking *nef*. Thus, expression of *nef* may eliminate almost all Gag SL9 epitopes from the surface of some infected cells.

When monoclonal antibodies were used that recognized epitopes common to MHC class I HLA-A, B and C antigens, downmodulation was apparent, but was less impressive (compare *Figs 5A* and 5B). There are at least three possible explanations for this phenomenon. One is that not all types of MHC class I antigens are downmodulated. Another is that loss of monoclonal antibody binding does not reflect loss of protein from the cell surface, but rather loss of the monoclonal antibody epitope (as could occur from a conformational change). A third is that this monoclonal antibody is not completely specific and recognizes antigens other than those related to MHC class I HLA-A, B and C. In support of the first explanation, Schwartz and colleagues have reported that when *nef* is expressed alone it is able to downmodulate exogenously expressed MHC class I HLA-A and HLA-B antigens, but not HLA-C antigens (40). This is because HLA-C antigens lack a tyrosine residue in their cytoplasmic tails that is required for *nef*-mediated downmodulation (40, 41). Consistent with the findings of Schwartz and colleagues, we have not observed *nef*-mediated protection of infected cells by HLA-Cw8-restricted CTLs (K. L. Collins, B. D. Walker, D. Baltimore, unpublished results). In addition, selective *nef*-dependent downmodulation of HLA-A and -B antigens but not HLA-C or HLA-E antigens was observed in HIV-infected 721.221 cells expressing individual MHC class I genes (G. B. Cohen, R. T. Gandhi, D. M. Davis, O. Mandelboim, B. K. Chen, J. L. Strominger, D. Baltimore, unpublished results).

We have found it difficult to examine directly whether endogenous HLA-C antigens on primary T cells are downmodulated with HIV infection, due to the low level of MHC class I HLA-C antigens normally expressed on the surface of cells (42–45) and due to the relative lack of commercially available HLA-C-specific monoclonal antibodies. Although initial studies led us to believe that HLA-Cw3 was downmodulated in a *nef*-dependent manner (33), this observation may have been due to antibody cross-reactivity. HIV-mediated downmodulation of HLA-Cw3 is certainly less apparent when it is overexpressed in 721.221 cells (G. B. Cohen, R. T. Gandhi, D. M. Davis, O. Mandelboim, B. K. Chen, J. L. Strominger, D. Baltimore, unpublished results).

A rationale for the specificity of MHC class I downmodulation is that HLA-A and -B are the major MHC class I encoded proteins known to present antigens to CTLs while HLA-C and -E can interact with various inhibitory receptors on natural killer (NK) cells and can protect cells against these killers. Thus HIV may direct its attention against the major subset of MHC class I antigens that allow for CTL killing while sparing others that might make the cells sensitive to NK-cell killing. In support of this theory, infected 721.221 cells were resistant to certain CTL and NK cells (G.B. Cohen, R. T. Gandhi, D. M. Davis, O. Mandelboim, B. K. Chen, J. L. Strominger, D. Baltimore, unpublished

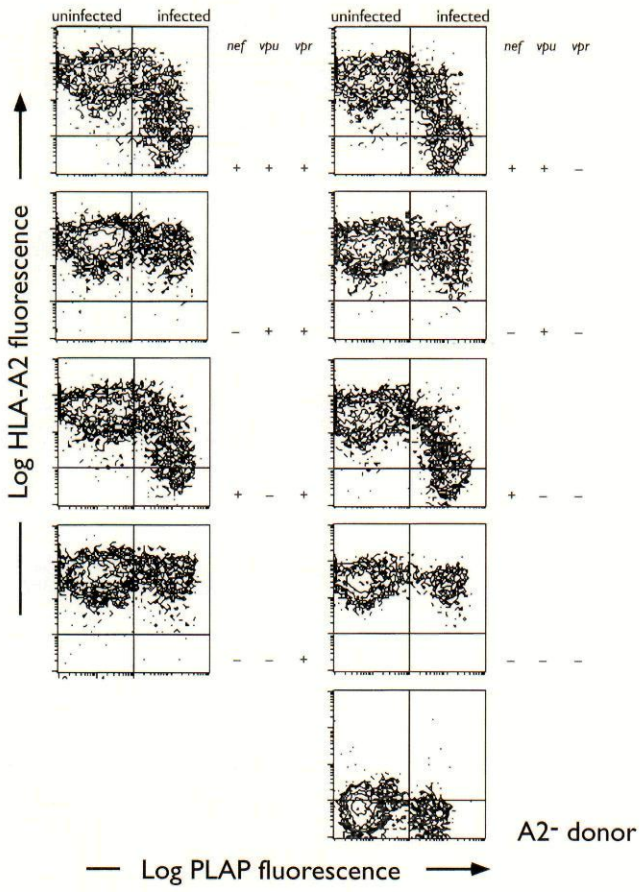

Fig. 6. *Nef* is required for MHC class I downmodulation whereas *vpu* and *vpr* are not. Primary CD4+ T cells were infected with NL-PI containing mutations in *nef*, *vpu* or *vpr* genes. Infected cells were then stained with antibodies against HLA-A2 antigens and against PLAP. A null mutation was introduced in *vpu* by using a PCR-based approach to change the ATG to ACG. A frameshift mutation was introduced into *vpr* by filling in the Afl II site in the *vpr* open reading frame. The mutant containing both *vpu* and *vpr* mutations was constructed by substituting the Sal I-Spe I fragment of NLPI*gag*^{hxb}*vpu*$^{-}$ with the Sal I-Spe I fragment of NLPI*gag*^{hxb}*vpr*$^{-}$. All *nef* mutations were made by ablating the N-terminal unique Xho I site (32, 33). The absence of the mutant protein was confirmed for each by western blot.

results). It is important to point out, however, that some NK cells may require MHC class I HLA-B antigens for inhibition (46), that there are anti-HIV-HLA-C-restricted CTL (47–49) and that HLA-E can on some occasions activate NK cells (50).

All of our experiments were performed with cells infected with a lab-adapted virus that expresses PLAP (Fig. 4). It will thus be important to demonstrate that our results are generalizable to cells infected with naturally occurring viruses or so-called "primary isolates". As a first step towards this goal we asked whether we could detect MHC class I antigen downmodulation by staining cells for intracellular Gag rather than with PLAP. Surprisingly we found that it was much more difficult to detect the effects of *nef* using an intracellular stain. It is probably the fixation process required for intracellular staining that makes the experiment so unconvincing (Fig. 5C). The diminished ability to detect the effect of *nef* on MHC class I antigens with this staining technique is consistent with what others have reported (25). Although not useful for examining MHC class I antigen downmodulation, staining with intracellular Gag was helpful in confirming the ability of PLAP expression to identify infected cells. The percentage of PLAP positivity correlated nicely with the percentage of cells positive for intracellular Gag.

The role of other HIV accessory proteins

To explore the role of *vpu* and *vpr* in MHC class I downmodulation and protection of infected cells from CTLs, we inserted PLAP into a HIV-1 molecular clone, NL-PI, which expresses these genes (and contains many other variations in sequence (33)). In NL-PI there are two amino acid changes within the relevant *gag* epitope which result in non-recognition by the anti-*gag* CTL clones described above (S. Kalams, manuscript in preparation). Therefore, to create a version that could be recognized by the CTL clones, the N-terminus of HXB-PI *gag* was inserted into NL-PI (NL-PI*gag*^{HXB} (33)).

The results we obtained with NL-PI*gag*^{HXB} were very similar to what we observed with HXB-PI. Cells infected with NL-PI depended on *nef* for MHC class I downregulation; there was no more than twofold residual downregulation in the absence of a functional *nef* gene. A CTL killing assay suggested that there might be another protective factor in NL-PI because *nef*$^{-}$ NL-PI constructs survived better than *nef*$^{-}$ HXB constructs (40% versus 10% (33)).

To further explore the role of other HIV genes we constructed mutants in NL-PI*gag*HXB that did not express *vpu*

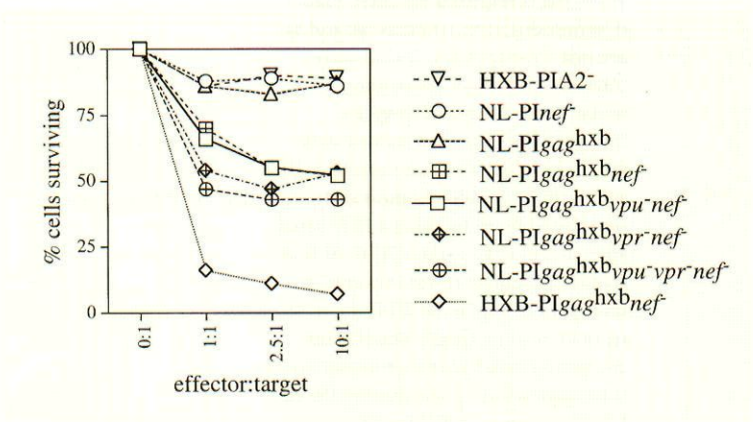

Fig. 7. HIV-1 Nef protects infected cells from CTLs, but Vpu and Vpr do not. Primary CD4+ T cells were infected with the indicated virus and were then treated with CTL clone 161JXA14 as described (33). All cells were then stained with antibodies against HLA-A2 antigens, PLAP and CD8. Surviving CD8⁻ cells were identified as described (1) and the percentage of surviving cells was quantitated: [(percentage PLAP+ in CTL-treated sample)/ (the average percentage PLAP+ of duplicates to which no CTLs were added)] × 100. HXBnef⁻A2⁻ refers to target cells isolated from an HLA-A2-negative donor and infected with HXB-PInef⁻. All other cells were isolated from an HLA-A2-positive donor. NL-PInef⁻ refers to a virus that lacks the cognate epitope recognized by the CTL clones (33). The correct antigen was inserted to create NL-PIgag^hxb (33).

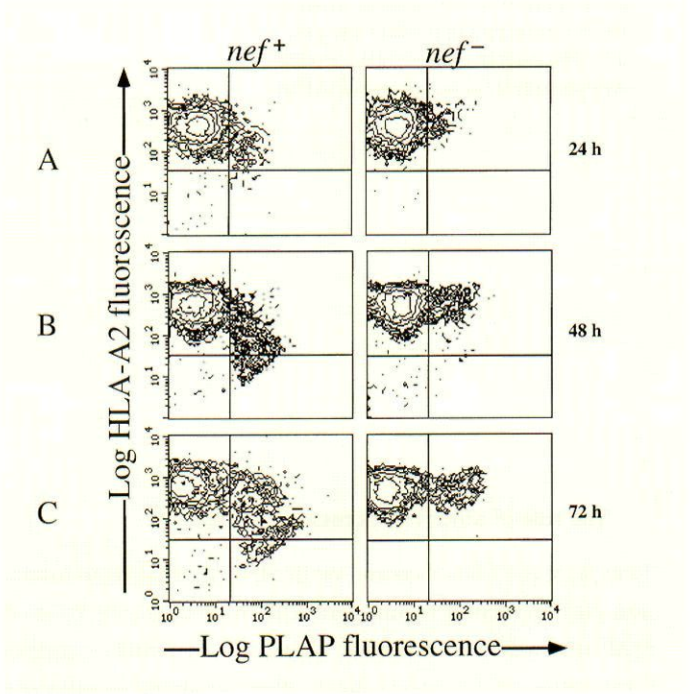

Fig. 8. Time course of MHC class I downmodulation. Primary CD4+ T lymphocytes were infected with HIV PLAP, harvested at (A) 24, (B) 48 and (C) 72 h post-infection and stained with antibodies directed against HLA-A2 antigens and PLAP.

and/or *vpr*. In contrast with the dramatic effect of *nef*, we observed no significant effect of *vpu* and *vpr* expression on MHC class I downmodulation (Fig. 6). Additionally, we were unable to detect any effect of *vpu* and/or *vpr* expression on the ability of CTLs to recognize and lyse infected primary T cells that did not express *nef* (Fig. 7). Most of our work is based on HLA-A2-restricted CTLs recognizing a Gag epitope and we cannot rule out an effect of these genes on other MHC class I antigens and on the ability of CTLs to recognize other viral antigens.

Timing of MHC class I antigen downmodulation

All of our CTL assays were performed by mixing CTLs with target cells that had already been infected for at least 48 h. This system differs from the *in vivo* situation where CTLs are continuously exposed to virally infected cells at all stages of infection. To determine whether there might be a window of time before MHC class I downmodulation occurs when CTLs could recognize infected cells, we performed a time course in which cells were sampled and stained at varying times after infection. We found that at 24 h, we could just begin to see PLAP staining and downmodulation of MHC class I antigens (Fig. 8A). By 48 h we could detect brightly staining PLAP-positive cells and complete downmodulation of MHC class I antigens (Fig. 8B). We have observed on multiple occasions that Nef is fully active in protecting infected cells as early as 48 h post-infection (data not shown). However, due to the lack of PLAP staining, we have not been able to test the efficacy of CTLs earlier than 48 h post-infection.

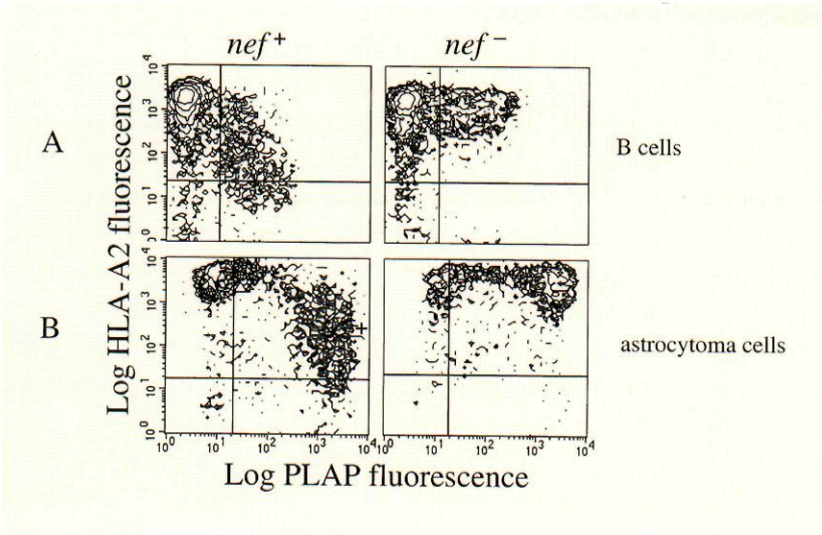

Fig. 9. MHC class I downmodulation occurs in infected astrocytoma cells and B cells. The JY EBV-transformed B-cell line (39) and 373MG astrocytoma cells (67) were infected with an HIV-1 molecular clone (HXB-EP (33)) pseudotyped with VSV-G (68). Forty-eight hours post-infection cells were harvested and stained with antibodies directed against HLA-A2 antigens and PLAP.

Effect of *nef* on MHC class I antigens expressed by other cell types

To examine whether the effect of *nef* on MHC class I antigens extends to cell types that lacked the required HIV receptors, we used an envelope-defective form of HIV pseudotyped with vesicular stomatitis virus G protein (VSV-G). An Epstein–Barr virus (EBV)-transformed B-cell line (JY cells) and an astrocytoma cell line (373 MG) were infectable with the VSV-G pseudotyped virus (*Figs 9A & 9B*). We found that HIV expressing *nef* dramatically downmodulated MHC class I HLA-A2 antigens on both cell lines in a *nef*-dependent manner. Interestingly, both cell lines express higher levels of HLA-A2 antigens than primary T cells, and *nef* leaves a correspondingly higher residual amount of MHC class I antigens on these cells. It will be important to determine whether related cell types such as primary macrophages important for infection *in vivo* similarly have high residual MHC class I antigens. If true, these cells may be more susceptible to CTLs than the primary $CD4^+$ T cells we have tested thus far. Interestingly, macrophages have recently been shown to be important for high viral loads in end stage individuals with low CD4 counts (and presumably poor CTL function) (51).

Closing remarks

The development of the HIV PLAP system has provided us with a unique probe to better understand HIV-induced disease pathogenesis. Using this approach various groups in the Baltimore laboratory have demonstrated the independent contributions of *nef*, *vpu* and *env* genes in the downmodulation of CD4 (32), the role of *nef* in protecting primary T cells from CTLs (33), the direct toxicity of HIV on T cells (14) and the ability of NK cells to recognize infected cells (G. B. Cohen, R. T. Gandhi, D. M. Davis, O. Mandelboim, B. K. Chen, J. L. Strominger, D. Baltimore, unpublished results). The benefit of this marker over staining methods that rely on fixation and permeabilization is evident in the work presented here.

The mechanism of Nef-mediated MHC class I downmodulation is currently being explored by a number of groups and is gradually coming to light. Interestingly, it appears that different domains of *nef* are required for MHC class I downmodulation versus *nef*-mediated CD4 downmodulation. Recent data has implicated the acidic and proline-rich regions of *nef* as being important for MHC class I downmodulation (41), whereas we have not found the proline-rich region to be important for CD4 downmodulation (30). Additional evidence supporting a separate mechanism for MHC class I antigen downmodulation is that expression of *nef* leads to localization of CD4 molecules to membrane-associated clathrin-coated pits (52), whereas Nef directs MHC class I antigens to the *trans*-Golgi (40, 41). How a single 27 kDa protein mediates the downmodulation of two very different cell surface molecules by two different mechanisms will be very interesting to learn. Nef has been reported to interact with a host of cellular factors and it is possible that *nef*'s differential association with these factors is important for its multiple activities. Three different cellular factors have now been implicated in CD4 downmodulation based on mutagenesis studies: a thioesterase (53) (G. B. Cohen, D. Baltimore, unpublished results), the υ subunit of a clathrin adaptor (40, 54) and a vacuolar ATPase (55). However, none have yet been directly implicated in MHC class I downmodulation. A greater understanding of the relevant cellular partners will help speed the development of effective anti-*nef* pharmaceuticals. Hopefully these would help control virus infection by allowing the host's immune system to be more effective.

Our studies demonstrate that *nef*-mediated MHC class I downmodulation can be profound and that *nef*-expressing cells with low levels of MHC class I antigens have a survival advantage in the presence of CTLs. Based on these findings we expect that Nef-expressing cells will survive longer *in vivo*, produce more infectious viral particles, and expose the immune system to a greater diversity of antigenic variants. Thus the activity of *nef* is likely to be synergistic with other reported mechanisms of HIV escape from CTLs that rely on antigenic variation.

A growing number of studies suggest that antigenic variation is an important element of HIV immune evasion. Individual patients with immunodominant CTL responses can escape the CTL response by generating antigenic variants not recognized by CTLs (17–19). In addition, some individuals who mount a CTL response to the immunodominant strain appear to lack the ability to respond to immunogenic variants that arise later (56–58). Furthermore, it has been reported that certain types of peptides that are sequence variants of the cognate peptide antigens recognized by antigen-specific T-cell receptors can act as antagonists (59, 60).

The effects of *nef* are likely to be aided by other mechanisms that diminish the anti-HIV CTL response. Macrophages (and possibly T cells) can directly kill some anti-HIV CTLs (61, 62). In addition, preferential depletion of anti-HIV $CD4^+$ T cells probably leads to anti-HIV CTL anergy because of a lack of T-cell help (63, 64). It is also probable that reservoirs of HIV-infected cells exist that are sequestered in sites where access to CTL is restricted, such as the brain. Finally, truly latent cells with integrated provirus that does not express viral proteins are likely to be resistant to CTL (for review see (15)). It is not surprising that HIV employs multiple mechanisms to evade the host's immune system given the almost universal lethality of virus infection in untreated individuals. Hopefully a greater understanding of precisely how the immune system is weakened will provide ways of designing more effective therapeutics and better vaccination strategies.

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