Human immune response to HIV-1 Nef. II. Induction of HIV-1/HIV-2 Nef cross-reactive cytotoxic T lymphocytes in peripheral blood lymphocytes of non-infected healthy individuals

Maria Lucchiari-Hartz1,3, Monika Bauer2, Gabriele Niedermann1, Bernhard Maier1, Andreas Meyerhans2 and Klaus Eichmann1

1Max-Planck-Institut für Immunbiologie, 79108 Freiburg, Germany
2Institut für Mikrobiologie und Hygiene, Abt. für Virologie, Universität Freiburg, 79104 Freiburg, Germany
3Present address Laboratory of Virus Immunology, Instituto Butantan, 05503-900, São Paulo, Brazil

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Abstract

HIV-specific CD8+ cytotoxic T lymphocytes (CTL) are thought to have a beneficial role in HIV infection. In a previous report we have shown that HIV-1 Nef-specific CTL can be readily induced in peripheral blood lymphocytes of seronegative healthy young adults by in vitro stimulation with autologous Epstein-Barr virus-transformed B lymphoblastoid cell lines transfected with the HIV-1 nef gene. Here we demonstrate that these Nef-specific CTL can efficiently lyse HIV-infected primary CD4+ T lymphocytes. CTL of the blood donor tested were Nef-specific and restricted by the autologous MHC class I molecules HLA-A2 and HLA-B7. They recognized HIV-1 Nef in association with both restriction elements but HIV-2 Nef only in association with HLA-B7. The cross-reactivity of the induced effector cells together with the potent immunogenicity of Nef in healthy seronegatives further support the inclusion of Nef as a constituent of HIV vaccines.

Introduction

A number of observations have suggested that cytotoxic T lymphocytes (CTL) are an important factor in the immune response to HIV. They efficiently inhibit virus replication in vitro (1–4), their appearance early in the infection correlates with the decline of the primary viremia (5–8) and their reduction is associated with disease progression (8–10). Furthermore, a high CTL activity is observed in long-term non-progressors (11–13) and in HIV-exposed but uninfected individuals (14,15).

The HIV Nef protein is of particular interest for the induction of CTL in vaccine strategies. Nef is expressed at a high level early in the HIV replication cycle (16,17) and is very immunogenic (18–21). It is essential for pathogenesis and may determine in part the rapidity of disease progression (22–26). CTL specific for Nef have been demonstrated in infected individuals and the epitopes recognized by these CTL have begun to be defined (27–29). Chenciner et al. (30) generated Nef-specific CTL from peripheral blood lymphocytes (PBL) of HIV-seronegative donors by in vitro stimulation with HIV-infected lymphoblasts. Langlade-Demoyen et al. (15) have found that frequencies of CTL precursors for HIV-1 Nef in uninfected sexual partners of HIV-1-seropositive individuals were higher than in uninfected control individuals, indicating that a CTL response can perhaps protect against low level infections with HIV.

Recently, we have shown that a vigorous CTL response to HIV-1 Nef is induced in PBL of seronegative healthy young adults by stimulation with autologous Epstein–Barr virus (EBV)-transformed lymphoblastoid B cell lines (LCL) expressing a
transfected nef gene (31). The precursor frequencies for Nef-specific CTL in PBL of healthy young adults were as high as that usually seen for memory T cells (31–33). If such CTL would recognize and lyse autologous HIV-infected cells, it is conceivable that they could play a protective role in vivo if activated by appropriate vaccination. Therefore, in the present study, we analysed whether CTL induced in PBL of healthy young adults by Nef-transfected LCL would recognize and lyse autologous HIV infected cells. The observed Nef-specific and HIV-1/HIV-2 cross-reactive CTL activity gives additional support for Nef-based vaccination trials.

Methods

Donors

HIV-infected cells were established from PBL of four healthy HIV-1-seronegative non-exposed individuals PR2 (A2-32, B27-35, Cw2-w4), PR3 (A2-28, B7-40w60, Cw3-w7), PR7 (A9[24]-29, B7-37) and ML (A2-9[23], B12-12). Fresh PBL for induction of Nef-specific CTL were obtained from one of these subjects (subject PR3). The class I HLA type of each donor was determined using standard serological typing methods by Dr A. Urlacher (Centre Regional de Transfusion Sanguine, Strasbourg, France). The donors were tested periodically for HIV antibodies by ELISA (Dr med. Clotten Laboratories, Freiburg, Germany). All studies were performed according to the ethical guidelines of the Max Planck Institute.

HIV production

The viruses used were derived from the molecular clones HIV-1 pLai-2, HIV-2 pRod and HIV-1 pLai Δnef (pLaiga). The infectious recombinant pLaiga was a kind gift of J.-P. Vartanian (Unite de Retrovirologie Moleculaire, Institut Pasteur, Paris). It contains part of the env gene region (6243–6283 of pLai-2) and an in-phase stop codon inserted into the unique XhoI cloning site of nef after amino acid 34. This recombinant therefore expresses only 34 N-terminal amino acids of Nef. Virus containing supernatants were generated by transfecting HeLa cells as previously described (34). The virus was quantified by the activity of RT.

RT assay

HIV viral particles were pelleted from 1 ml supernatant of either transiently transfected HeLa cells or infected CD4+ PBL by centrifugation at 21,000 g and 4°C for 90 min. Pellets were dissolved in virus disruption buffer containing 100 mM Tris–HCl, pH 7.6, 100 mM KCl, 4 mM DTT, 0.2 mM EDTA and 1.25% Triton-X 100. The final polymerase reaction mixture contained 100 mM Tris–HCl, pH 7.6, 100 mM KCl, 4 mM DTT, 0.1 mM EDTA, 0.625% Triton-X 100, 5 mM MgCl₂, 0.05 mM [α-32P]dCTP (50 Ci/mmol), 0.125 mg/ml BSA and 0.05 A₄₉₀nm poly(rA) p(dT)₁₂₋₁₈ (Pharmacia Biotech, Freiburg, Germany). After 1 h incubation at 37°C, the incorporated radioactivity was bound to DE 81 membranes (Whatman, Maidstone, UK), which were then washed with 2.5% sodium pyrophosphate, air dried and counted with a Berthold-Inotech Trace-96 (Berthold, Wildbad, Germany). The values obtained are -50-fold lower than those from conventional scintillation counting.

Preparation of the effector cells

HIV-1 Nef-specific CTL lines were generated from freshly isolated PBL of a healthy seronegative donor (PR3) after stimulation in vitro with Nef-expressing-LCL (Nef+ LCL), as previously described (31,35). In brief, heparinized blood was separated on Ficoll-Hypaque (Sigma, Deisenhofen, Germany) density gradients and the PBL were stimulated with 8000 rad irradiated autologous Nef+ LCL and cultured in RPMI 1640 (Gibco/BRL, Eggenstein, Germany) containing 5% heat-inactivated AB male human serum (Sigma). Human rIL-2 (100 U/ml, Pharma Biotechnology, Hanover, Germany) was added on day 5. Re-stimulation was done on day 14 with irradiated autologous Nef+ LCL and PBL as feeder cells. Cells were cultured 8–10 days later for HIV-1 Nef-specific lytic activity against autologous Nef+ LCL in a standard 4 h 51Cr-release assay, using LCL transfected with the empty vector (NEF- LCL) as control (31). Nef-specific CTL lines were subsequently characterized with regard to surface phenotype, HLA restriction and CTL activity.

Preparation of target cells

Target cells used in the cytotoxicity assays were autologous or HLA class I-matched and -mismatched CD4+ T cells from healthy HIV-1-seronegative non-exposed donors infected in vitro with HIV-1 Lai, HIV-2 Rod and HIV-1 Lai Δnef. PBL were positively selected for CD4+ T cells by Dynabeads M450 CD4 (Dynal, Hamburg, Germany), stimulated with 1 µg/ml phytohaemagglutinin (PHA-P, Sigma), and cultured for 5 days before infection in RPMI 1640 medium containing L-glutamine, antibiotics and 10% FCS (Gibco) Mitogen-stimulated CD4+ T cells (3×10⁶) were infected with cell-free virus-containing supernatants corresponding to 10,000 c.p.m of RT activity. The infected cells were maintained in the presence of 20 U/ml rIL-2 (Amersham, Braunschweig, Germany). Virus production was monitored by testing culture supernatants for RT activity.

Cytotoxicity assay

All CTL assays were conventional 4 h 51Cr-release assays as previously described (34). Briefly, target cells were labelled with 51Cr for 1 h at 37°C and plated out at 5×10⁵ cells/well in 96-well round bottom microtitre plates (Nunc, Wiesbaden, Germany) in a final volume of 200 µl. Effector cells were added at an effector:target (E:T) ratio ranging from 100:1 to 3:1 to experimental wells in triplicate. Controls for background 51Cr release (target cells with medium alone) and maximal 51Cr release (target cells with 1N HCl) were plated in quadruplicate. Plates were incubated for 4 h at 37°C and the percent specific 51Cr release in the supernatant due to cytotoxic lysis was determined in a γ-counter (Canberra-Packard, Frankfurt, Germany). Spontaneous release was <30% of total incorporated radioactivity.

Results

Replication of different HIV isolates in CD4+ PBL of uninfected donors

PHA-activated CD4+ PBL were used as target cells in all CTL assays in order to be able to make comparisons between
targets of different individuals and between targets infected with different HIV isolates, it was necessary to assess the replication efficiencies of the HIV isolates in PBL of all donors under study. PHA-stimulated CD4+ PBL were infected with HIV-1 Lai, HIV-1 Lai Δ nef or HIV-2 Rod and were cultured for 7 days in the presence of IL-2. RT activities were determined daily in the culture supernatants and the kinetics are shown in Fig. 1. All virus isolates replicated efficiently in CD4+ PBL of each of the four donors. When infected with the same virus isolate, RT activities among CD4+ PBL of the four donors varied 2-fold or less. Peak RT activity was observed 4 days after infection in each case.

Lysis of HIV-1 Lai-infected target cells by HIV-1 Nef-induced CTL

HIV-1 Nef-specific CTL were generated by stimulation of PBL of PR3 with autologous Nef-transfected LCL (31) and served as effector cells throughout this study. 51Cr-release assays using HIV-1 Lai-infected CD4+ PBL of the four donors as targets for PR3 CTL are shown in Fig. 2. Uninfected CD4+ PBL of each donor were included as controls and showed up to 25% background lysis. In contrast, autologous HIV-1 infected CD4+ PBL on day 4 of infection reached 80-100% of specific lysis (Fig. 2B). HLA-mismatched HIV-1-infected CD4+ PBL showed only background lysis at all timepoints tested (Fig. 2A). In contrast, HLA-B7-matched 4 day infected CD4+ PBL from PR7 were lysed almost as efficiently as autologous targets (Fig. 2C). HLA-A2-matched 4 day infected CD4+ PBL of donor ML were also lysed, but with lower efficiency (Fig. 2D). The sensitivity to lysis correlated with the kinetics of HIV-1 replication in the CD4+ PBL, which peaked at day 4 in each case (see Fig. 1). However, HLA-matched target cells before the peak of virus replication were often not efficiently lysed. While we do not know the reason for this, it is possible that other, non-matched restriction elements contribute to CTL recognition, so that lysis of heterologous targets is less sensitive.

Lysis of HIV-2 infected cells by HIV-1 Nef-induced CTL

The nef genes of HIV-1 Lai and of HIV-2 Rod share ~37% sequence homology (36). We tested the ability of HIV-1 Nef-specific CTL of donor PR3 to lyse CD4+ PBL of the four
different donors infected with the HIV-2 Rod strain. As shown in Fig. 3(B), PR3 CTL recognized and lysed autologous target cells infected for 4 days with HIV-2. HIV-2-infected, HLA-mismatched targets were not lysed above background (Fig. 3A). Heterologous targets infected with HIV-2 were lysed only when matched for HLA-A2 but not when matched for HLA-A2 (Fig. 3C and D). In general, lysis of HIV-2-infected targets was lower than of HIV-1-infected targets. HIV-2 replicates at lower levels as HIV-1 (see Fig. 1). The less efficient lysis of HIV-2-infected cells may therefore in part be due to a lower level of expression of viral proteins. Thus, the cross-reaction of HIV-1-induced CTL for HIV-2-infected target cells may be more significant than is apparent from the results in Fig. 3. Indeed, data suggest that the Nef proteins of HIV-1 and HIV-2 share (a) common HLA-B7 epitope(s). The failure to detect HLA-A2 restricted cross-reactive lysis may be due to the absence of a shared HLA-A2 epitope.

HIV-1 Nef-induced CTL recognize Nef epitopes on HIV-infected target cells

What are the antigens involved in the recognition of HIV-infected target cells by CTL induced in uninfected individuals by autologous Nef-transfected LCL? We are dealing with heterogeneous CTL populations induced by stimulator cells expressing, in addition to Nef, a number of EBV encoded antigens. We have previously shown that, under the conditions used, Nef is the main antigen recognized by these CTL on Nef-transfected LCL as targets (31). However, a major contribution of Nef to the recognition of HIV-infected CD4⁺ PBL can a priori not be assumed. To test this, we used a HIV-1 Lai construct (HIV-1 Lai ΔNef) in which the nef gene has been disrupted at amino acid 34. CD4⁺ PBL from the four donors were infected with HIV-1 Lai ΔNef, HIV-1 Lai or HIV-2 Rod. After 4 days of infection, these cells were used as targets in ⁵¹Cr-release assays using HIV-1 Nef-induced CTL.
Human HIV-1 Nef-specific CTL recognize HIV-1 and HIV-2

Fig. 3. Cross-reactive lysis of PBL infected with HIV-2 Rod by HIV-1 Nef-specific CTL. Nef-specific CTL from PBL of an uninfected, seronegative, non-exposed donor (PR3) were generated as described in Fig. 2 and were tested in a 4 h standard 51Cr-release assay. Targets tested were CD4+ PBL from uninfected seronegative donors PR2 (A), PR3 (B), PR7 (C) and ML (D) infected with HIV-2 Rod for 1 (closed diamonds), 2 (closed circles), 3 (closed triangles) and 4 (closed squares) days, in the same manner as described in Fig. 1. Uninfected CD4+ PBL (open squares) of each donor were used as negative controls.

of PR3. As shown in Fig. 4, HIV-1 Lai Δnef-infected CD4+ PBL of neither of the four donors were lysed to a level significantly above that of the uninfected control targets. In contrast, HIV-1 Lai- and HIV-2 Rod-infected target cells were again recognized and lysed in a similar way as described above (see Figs 2 and 3). Lack of lysis of HIV-1 Lai Δnef-infected cells cannot be explained by insufficient infection, as HIV-1 Lai Δnef replicated efficiently in all CD4+ PBL, with peak RT activity at day 4 (see Figure 1). These data strongly suggest that HIV-1 Nef-induced CTL of PR3 recognize Nef epitopes on HIV-1 Lai- and HIV-2 Rod-infected CD4+ PBL.

Discussion

In this report we demonstrate that autologous HIV-infected target cells can be recognized and lysed by CTL generated in PBL of healthy seronegative individuals by stimulation with HIV-1 Nef-transfected LCL in vitro. In previous work (31) we have studied Nef-specific CTL responses of four such individuals with very similar results in all parameters tested: Nef-specific CTL reside entirely in the TCRαβ+CD8+ T cell population and are HLA class I restricted. Although the precursor frequencies of these Nef-responsive CTL in all four individuals were typical of that of memory T cells, CTL precursors were predominantly found in the CD45RO+ population. These CTL precursors may therefore represent cross-reactive memory cells having returned to a resting/naive phenotype. Alternatively, the high frequency of Nef-specific CTL precursors in healthy adults may result from a unusually high number of epitopes associated with the Nef protein. Nef carries two epitope clusters, each containing many overlapping epitopes presented by a number of different HLA class I molecules (21).

It is not known whether CTL specific for HIV-1 Nef epitopes could have a protective role in the development of AIDS. HIV Nef-specific CTL are detected in about half of infected individuals with no apparent differences in clinical course between the positive and negative groups (37). While these data argue against a protective role of Nef-specific CTL, the increased precursor frequencies of CTL with Nef specificity in uninfected sexual partners of HIV patients (15) may indicate that low level infections could be controlled by immune
responses that include induction of Nef-specific CTL. We have previously speculated that Nef-specific CTL precursors, although present at high frequency in healthy young adults, may be slow in their activation kinetics due to their naive phenotype (31). Slow activation kinetics are consistent with protection against a low virus dose and the failure to protect against a high dose. Appropriate vaccination could perhaps convert naive Nef-specific CTL precursors into more rapidly responding CD45RO+ memory cells. We therefore think that the data presented in this paper further strengthen the case for Nef as a candidate to be considered in strategies for vaccination against AIDS.

We have previously shown that CTL induced by Nef+ LCL, when harvested from the cultures at appropriate timepoints, primarily recognize Nef epitopes on Nef-transfected target cells (31). In the present work, Nef-specific CTL lysis of HIV-infected cells was ascertained in experiments using a recombinant HIV-1 Lai construct specifically deleted of most of the Nef sequence. Target cells infected with such a recombinant virus were not lysed, strongly suggesting that Nef is the main antigen recognized by our CTL on HIV-infected target cells.

Recognition and lysis of HIV-infected target cells by CTL induced with recombinant Nef is not entirely unexpected but also not self-evident. While most HIV-infected cells are expected to express Nef, it was not known whether the level of expression would be sufficient for recognition by CTL from non-infected donors. In particular, it was conceivable that Nef+ LCL, due to a high expression of Nef, would mostly induce low-affinity CTL unable to recognize the presumably low amounts of Nef expressed by HIV-infected cells. Whereas the Nef sequence remains constant in LCL transfected with the nef gene, HIV-infected cells rapidly generate mutations of viral genes including nef (38-40), possibly further decreasing epitope density by diluting epitopes encoded by the wild-type nef gene. In addition, antigen processing may differ between LCL and HIV-infected CD4+ PBL. This may result in different Nef epitopes generated in the two types of cells, possibly due to competition for MHC class I or other mechanisms influencing epitope hierarchy (41). While none of these possibilities have been ruled out by our experiments, the data show that these putative mechanisms do not preclude efficient recognition of Nef epitopes on HIV-infected target cells.

HIV-1 Nef-expressing LCL were able to stimulate cross-
reactive CTL that efficiently lysed HIV-2 Rod-infected CD4+ PBL, although the sequence identity of both Nef proteins is merely 37% (36). The lysis of HIV-1-infected target cells was restricted by both major class I molecules of the donor (HLA-A2 and HLA-B7). In ongoing and so far incomplete studies on the epitope specificity in this system we identified one epitope for A2 (Nef 180–189) and one for B7 (Nef 68–77) which are recognized by donor PR3, albeit by only a fraction of the Nef-specific CTL (B. Maier et al., unpublished data). We therefore feel that a large proportion of the CTL used in the present study recognize so far unidentified Nef epitopes. Recognition of HIV-2-infected targets was solely restricted by HLA-B7. This might have been predicted from the known HIV-1 Nef CTL epitopes and the homologies between the two Nef sequences (21,28,29,42): the major HIV-1/HIV-2 Nef homology region overlaps to a limited extent with one of the two immunogenic regions of HIV-1 Nef; this region of overlap contains the HLA-B7 epitope Nef 142–149 (PGPVRYPL for HIV-1 Lai; PGPVRYPM for HIV-2 Rod), but no known HLA-A2 epitope (21,29,42). Recognition of Nef 142–149 as the basis of cross-reactivity is presently under study.

Cross-recognition between HIV-1 and HIV-2 proteins by the cellular immune system of infected individuals has already been suggested on the basis of peptide pulsed or recombinant vaccinia virus-infected EBV-immortalized target cells (14,43). HLA-B27 restricted CTL recognized a cross-reactive epitope in the capsid region, HLA-B35-restricted effectors recognized individual epitopes in the matrix, capsid, polymerase and nef proteins. Thus, despite significant sequence divergence between both HIV types, cross-reactivity is clearly possible. The strong degeneracy of effector CTL is certainly one factor facilitating promiscuous recognition (44). Since cross-reactions may be exploited in vaccination strategies, it is of importance to evaluate additional factors by which sequence divergence in epitope regions may influence immunogenicity, such as proteolytic epitope generation, peptide transport to the endoplasmic reticulum and HLA assembly. In conclusion, our demonstration of the induction of a potent and cross-reactive CTL response in seronegatives and the ability of the effector cells to efficiently lyse infected CD4+ PBL, the main targets of HIV in vivo, seems encouraging for vaccination purposes. Together with the high immunogenicity, the early expression in the viral life cycle and the high CTL precursor frequency in seronegatives makes Nef an interesting constituent of HIV vaccines.

References
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Abbreviations

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<tr>
<td>CTL</td>
<td>cytotoxic T lymphocytes</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<td>LCL</td>
<td>EBV-transformed lymphoblastoid B cell lines</td>
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<tr>
<td>PBL</td>
<td>peripheral blood lymphocytes</td>
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<td>PHA</td>
<td>phytohaemaggulutinin</td>
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