Human immunodeficiency virus 1 Nef protein downmodulates the ligands of the activating receptor NKG2D and inhibits natural killer cell-mediated cytotoxicity

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Natural killer (NK) cells are a major component of the host innate immune defence against various pathogens. Several viruses, including Human immunodeficiency virus 1 (HIV-1), have developed strategies to evade the NK-cell response. This study was designed to evaluate whether HIV-1 could interfere with the expression of NK cell-activating ligands, specifically the human leukocyte antigen (HLA)-I-like MICA and ULBP molecules that bind NKG2D, an activating receptor expressed by all NK cells. Results show that the HIV-1 Nef protein downmodulates cell-surface expression of MICA, ULBP1 and ULBP2, with a stronger effect on the latter molecule. The activity on MICA and ULBP2 is well conserved in Nef protein variants derived from HIV-1-infected patients. In HIV-1-infected cells, cell-surface expression of NKG2D ligands increased to a higher extent with a Nef-deficient virus compared with wild-type virus. Mutational analysis of Nef showed that NKG2D ligand downmodulation has structural requirements that differ from those of other reported Nef activities, including HLA-I downmodulation. Finally, data demonstrate that Nef expression has functional consequences on NK-cell recognition, causing a decreased susceptibility to NK cell-mediated lysis. These findings provide a novel insight into the mechanisms evolved by HIV-1 to escape from the NK-cell response.

INTRODUCTION

Human immunodeficiency virus 1 (HIV-1) has evolved several mechanisms to evade the immune defence of the host and to establish a chronic infection (Peterlin & Trono, 2003). The escape from HIV-specific cytotoxic T lymphocytes (CTLs) is achieved by viral epitope mutation, as well as by non-mutational mechanisms such as downregulation of human leukocyte antigen (HLA)-I expression on infected cells (McMichael, 1998). The Nef protein of HIV-1 has been clearly implicated in this phenomenon, causing an accumulation of HLA-I molecules in clathrin-coated vesicles in the Golgi area (Le Gall et al., 1998) and thus protecting infected cells from CTL recognition and killing (Collins et al., 1998). However, HLA-I downregulation can alert NK cells that preferentially lyse target cells with reduced HLA-I expression. NK cells are regulated by a delicate balance of inhibitory and activating signals and they are kept in an ‘off’ state by inhibitory receptors recognizing HLA-I (Lanier, 2005). Upon HLA-I downregulation, as occurs during HIV-1 infection, triggering signals may prevail, thus leading to activation of NK cells. However, Nef selectively decreases HLA-A and HLA-B, whilst leaving the levels of HLA-C and HLA-E unchanged (Cohen et al., 1999). This selective HLA-I downregulation has been shown to protect HIV-infected cells from lysis mediated by NK cells expressing inhibitory receptors that are specific for HLA-C or HLA-E (Cohen et al., 1999). Nevertheless, an effective virus evasion strategy would also require interference with the expression of NK cell-activating ligands, as has been shown for some herpesviruses (Lodoen & Lanier, 2005). Here, the ligands of NKG2D, an activating receptor expressed on all NK cells,
CD8⁺ T cells and γδ T cells (Raulet, 2003), were studied. NKG2D ligands (NKG2DLs) are HLA class I-like molecules: the highly polymorphic major histocompatibility complex (MHC)-I-related chains A and B (MICA and MIBC) and the UL16-binding complex 1, 2, 3 and 4 (ULBP1, ULBP2, ULBP3 and ULBP4) (Raulet, 2003). MICA and MIBC contain the z₁, z₂ and z₃ MHC-like domains, but they do not associate with β₂-microglobulin or peptides. Cell-surface expression of MIC proteins, which is normally restricted to the gastrointestinal epithelium, can be induced by tumour transformation, heat shock, DNA damage, and infection by Mycobacterium tuberculosis, Escherichia coli and cytomegalovirus (CMV) (Gasser et al., 2005; Lodoen & Lanier, 2005). The ULBP molecules do not associate with β₂-microglobulin or peptides, they lack the z₃ domain and, with the exception of ULBP4, are attached to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor (Chalupny et al., 2003). The ULBPs are expressed in a variety of human tumours and transformed cell lines and they can be induced in fibroblasts upon CMV infection (Rölle et al., 2003; Welte et al., 2003). Apparently, NKG2DLs behave as danger signals that alert the immune system of a distressing event occurring within a cell. Through NKG2D binding, MIC and ULBP molecules trigger the effector function of NK cells in a very efficient manner that, in some cases, overrides inhibitory signals delivered by the HLA-specific inhibitory receptors (Bauer et al., 1999; Cosman et al., 2001; Pende et al., 2001). Thus, to efficiently escape from NK-cell recognition, a virus should prevent cell-surface expression of NKG2DLs, as shown for the UL16 protein of CMV (Rölle et al., 2003; Welte et al., 2003). In this study, the possible development of a similar mechanism by HIV-1 to escape from NK cells was investigated. Modulation of NKG2DLs during HIV-1 infection was analysed and, in particular, the role of the HIV-1 Nef protein in this phenomenon, given its capacity to downregulate classical HLA-1 and other cell-surface molecules, including CD4 (Baur, 2004; Doms & Trono, 2000; Peterlin & Trono, 2003), was studied.

**METHODS**

**Cells and antibodies.** 293T and Phoenix-ampho cells (kindly provided by G. Nolan, Stanford, CA, USA) were maintained in Dulbecco’s modified Eagle’s medium. Jurkat E6-1 and CEM-GFP cells (Gervaix et al., 1997) were maintained in RPMI 1640 medium. Both media were supplemented with 10% fetal bovine serum, 2 mM l-glutamine and 100 units penicillin/streptomycin ml⁻¹. The medium for CEM-GFP was also supplemented with 100 μg G-418 ml⁻¹. NKL [kindly provided by M. Robertson, Indiana University, Indianapolis, IN, USA (Robertson et al., 1996)] and Nishi (Cerboni et al., 2001) cells were maintained as described previously. All tissue-culture reagents were from Gibco-BRL.

For flow-cytometric analysis of NKG2DLs, the following IgG1 mAbs were used (Cosman et al., 2001): anti-ULBP1 (M295), -ULBP2 (M311), -ULBP3 (M550) and -MICA (M673). Anti-NKG2DL antibodies from R&D Systems were also used (data not shown). Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-HLA-1 and PE-conjugated anti-CD4 mAbs were from BD Biosciences. The IgG1 mAbs anti-NKG2D (149810; R&D Systems) and anti-CD56 (C218; ATCC) were used in cytotoxicity assays.

**DNA constructs.** Construction of a Pinco-Nef retroviral clone expressing the nef gene of the HIV-1 NL4-3 virus has been described previously (Casartelli et al., 2003b). To create the mutant Pinco-Nef clones, specific mutations were introduced into the nef gene by standard site-directed mutagenesis based on recombinant overlapping PCR. All mutants were sequenced on both strands.

**Patient-derived nef alleles.** The nef genes were derived from HIV-1-infected patients as described previously (Casartelli et al., 2003a). Briefly, the patients were perinatally infected Italian children classified as rapid progressors (RP1, RP2, RP3 and RP4), slow progressors (SP1 and SP2) and non-progressors (NP1, NP2, NP3, NP4, NP5 and NP6). Isolation, subcloning in the Pinco retrovirus and functional characterization of patients’ nef genes were also described previously (Casartelli et al., 2003a, b).

**Infection of cells with recombinant retroviruses and HIV-1.** Production of Pinco-based retroviral particles and infection of cells have been described elsewhere (Casartelli et al., 2003b). In brief, Phoenix cells were transfected with the Pinco-Nef clones by the calcium phosphate/chloroquine method. After 48 h post-transfection, cell-culture supernatants were collected and clarified by low-speed centrifugation and aliquots were stored at −80 °C. The infectious units concentration (IU ml⁻¹) was determined by infecting CEM-GFP indicator cells with serial dilutions of virus preparation and scoring the number of green fluorescent protein-positive (GFP⁺) cells after 48 h by flow cytometry. Virus stocks were also titrated by anti-p24 ELISA (Immugenetics) according to the manufacturer’s instructions. As PDS is less infectious than NL4-3 due to the absence of Nef expression (Chowers et al., 1994), 1 IU PDS corresponded to p24 amounts that were three to five times higher than those in 1 IU NL4-3. For both virus strains, at least three different stocks were used.

For HIV-1 infection, Jurkat cells were resuspended at 10⁶ cells ml⁻¹ in medium with 8 μg polybrene ml⁻¹, either alone or together with NL4-3 or PDS virus at an m.o.i. of 0.003 (corresponding to 0.5 or 1.5–2.5 μg p24 ml⁻¹, respectively) and centrifuged at 2500 r.p.m. for 90 min at 30 °C. Between each infection cycle, cells were cultivated for at least 4 h. Cells were harvested 72 h after the first infection cycle and analysed.

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For HIV-1 infection of primary human T lymphocytes, peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by Ficoll-Hypaque (Amersham Biosciences) density-gradient sedimentation of buffy coats and CD4⁺ cells were purified with anti-CD4 mAb-coated magnetic beads (MACS Miltenyi Biotec) according to the manufacturer’s instructions. The purity of CD4⁺ T cells was >95% as determined by flow cytometry. CD4⁺ T cells were infected by incubation with NL4-3 or PDS virus at an m.o.i. of 0.003 (50 or 150–250 ng p24 per 10⁶ cells, respectively) for 4 h at 37 °C. Cells were then washed twice, resuspended at 1.5 × 10⁶ ml⁻¹ in the same medium used for Jurkat cells supplemented with 100 IU human recombinant interleukin-2 (IL-2) ml⁻¹, and stimulated by the addition of...
staphylococcal enterotoxin B superantigen (Sigma) at a final concentration of 100 ng ml\(^{-1}\) and irradiated allogeneic PBMCs at a 1:1 ratio with infected CD4\(^+\) T cells.

**Flow cytometry.** The following procedures were performed at 4 °C in PBS containing 0.5 % BSA and 0.1 % sodium azide unless otherwise specified. For simultaneous detection of surface NKG2DLs and intracellular p24, 5 \times 10\(^7\) HIV-1-infected or uninfected cells (Jurkat or CD4\(^+\) T cells) were incubated with anti-NKG2DL mAb or mouse IgG\(_1\) (BD Biosciences). After three washes, cells were incubated with Cy5-conjugated goat anti-mouse IgG (GAM) (Southern Biotechnology Associates). Alternatively, cells were incubated with FITC-conjugated anti-HLA-I mAb. Cells were then washed, fixed and permeabilized with reagents from BD Biosciences and incubated with the PE-conjugated anti-HIV p24 mAb (KC57-RD1; Coulter Immunology). Cells were washed, resuspended in 1 % paraformaldehyde and analysed (FACSCalibur; BD Biosciences).

Uninfected Jurkat cells or Jurkat cells infected with retroviruses were stained with PE-conjugated anti-HLA-I, anti-CD4 or anti-NKG2DL mAbs as described above, but with PE-conjugated GAM (Jackson Immunoresearch Laboratories) and analysed by two-colour fluorescence-activated cell-sorting analysis. Cell-surface expression of HLA-I, CD4 or NKG2DLs was determined as the geometric mean fluorescence intensity (MFI) of cells gated for medium GFP (10\(^{-1}\)–10\(^{-3}\) MFI). The level observed with Pinco-infected cells was taken as 100 % cell-surface expression.

**Cytotoxicity assay.** NK cell-mediated cytotoxicity was assessed by standard 4 h \(^{51}\)Cr-release assays (Rolle et al., 2003). Where indicated, NK cells were incubated with saturating amounts of anti-NKG2D or anti-CD56 mAbs as described above, but with PE-conjugated GAM (Jackson Immunoresearch Laboratories) and analysed by two-colour fluorescence-activated cell-sorting analysis. Cell-surface expression of HLA-I, CD4 or NKG2DLs was determined as the geometric mean fluorescence intensity (MFI) of cells gated for medium GFP (10\(^{-1}\)–10\(^{-3}\) MFI). The level observed with Pinco-infected cells was taken as 100 % cell-surface expression.

RESULTS

**Nef protein of HIV-1 downregulates cell-surface expression of MICA, ULBP1 and ULBP2**

To investigate whether the HIV-1 Nef protein has the capacity to affect cell-surface expression of NKG2DLs, Nef of the NL4-3 virus strain was expressed in the T-lymphoblastoma Jurkat cell line that constitutively expresses MICA, ULBP1 and ULBP2, but not ULBP3. A transduction system based on a retroviral vector expressing GFP alone (Pinco) or together with NL4-3-derived Nef (Pinco-Nef) and two-colour flow cytometry were employed. The intracellular concentrations of Nef resulting from retroviral transduction are similar to those observed upon HIV-1 infection (Liu et al., 2001; our unpublished data). The HLA-I and CD4 downregulation activities of Nef could be measured readily in this system (Fig. 1a), whereas cell-surface expression of CD3, which is not modulated (Schrager & Marsh, 1999), was the same in Pinco- and Pinco-Nef-infected cells (data not shown). Fig. 1(a) shows that the fluorescence intensities of MICA, ULBP1 and ULBP2 on GFP\(^+\) Pinco-Nef-infected cells were 65, 50 and 40 %, respectively, of the values measured on control GFP\(^+\) Pinco-infected cells. The same results were obtained by using different mAbs (data not shown), suggesting that the effect on NKG2DL expression did not result from the masking of antigenic epitopes. These data demonstrate that the HIV-1 Nef protein reduces cell-surface expression of MICA, ULBP1 and ULBP2, with the highest efficiency on the latter molecule.

**NKG2DL downmodulation by Nef mutants**

In order to gain insights into the mechanism of Nef-mediated downregulation of NKG2DLs, a panel of NL4-3 Nef proteins with mutations at various amino acid residues that mediate specific Nef functions was tested for this activity. It is well documented that, to downregulate HLA-I and CD4, Nef uses different residues/domains and interacts with distinct components of the endocytic and sorting pathways (Arold & Baur, 2001; Geyer et al., 2001). However, an N-terminal myristoylation signal, required for localization at the plasma membrane, has been shown to be critical for all Nef activities, including enhancement of HIV-1 infectivity/replication and alteration of signalling pathways. Nef proteins with mutations that abolish myristoylation (G\(_A\)), the capacity to downmodulate HLA-I (M\(_{G2A}\), EEEE\(_{GQQQ}\), P\(_{78L}\)) or CD4 (LL\(_{165AA}\), DD\(_{175AA}\), and, in part, P\(_{78L}\) and RR\(_{106AA}\)), or the ability to associate with SH3 domains (AxxA\(_{75}\)) or p21-activated PAK kinase (RR\(_{106AA}\)) (Fig. 1b) were tested. When compared with wild-type Nef, some mutants had a slightly reduced activity on NKG2DLs (i.e. G\(_2A\) and RR\(_{106AA}\) on ULBP2, AxxA\(_{75}\) on MICA and ULBP2, and LL\(_{165AA}\) on ULBP1 and ULBP2), although differences were not statistically significant. In general, all tested mutants retained the capacity to downmodulate MICA, ULBP1 and ULBP2, indicating that, to interfere with NKG2DL expression, Nef uses residues and/or domains that differ from those required for CD4 and HLA-I downmodulation and for other known Nef activities.

**Ability to downmodulate NKG2DLs is variably conserved in nef genes isolated from HIV-1-infected patients**

Conservation of the capacity to downmodulate NKG2DLs among primary nef genes was investigated. Twelve nef allelic variants were analysed, each one derived from a perinatally infected child and found previously to be as efficient as wild-type NL4-3-derived nef at downregulating both CD4 and HLA-I molecules (Casartelli et al., 2003a, b). To avoid possible bias due to the patients’ stage of disease, the nef genes were derived from four rapid-progressor (RP1–RP4), two slow-progressor (SP1 and SP2) and six non-progressor (NP1–NP6) patients. As shown in Fig. 2, strong downmodulating activity on ULBP2 was observed with all Nef variants, the majority of which, if compared with the NL4-3-derived Nef protein, reduced ULBP2 expression with a higher efficiency (up to 88 % of reduction with the SP2-1 variant, compared with control cells). Also, MICA was
downmodulated by all patient-derived Nef proteins, although to levels that were similar to those measured with NL4-3 Nef (data not shown). Thus, the ability to reduce the surface expression of ULBP2 and MICA is conserved in nef genes derived from patients. Conversely, the down-

**Fig. 1.** Downregulation of cell-surface NKG2DLs in cells expressing the HIV-1 Nef protein. (a) Jurkat cells were infected with empty (Pinco) or Pinco-Nef (NEF) retrovirus. After 72 h, expression of cell-surface CD4, HLA-I, MICA, ULBP1 and ULBP2 was analysed together with GFP expression by two-colour flow cytometry. Compared with uninfected cells (not shown), 100% of the cells were GFP⁺. For each surface molecule, the percentage of negative cells and the geometric mean intensity of fluorescence (MFI) in cells expressing medium GFP levels (10⁻²–10⁵ MFI, R1 gate) are indicated. IgG₁ control staining is also shown. The expression of each surface molecule in Pinco-infected cells was identical to that in uninfected cells (data not shown). Differences for all surface molecules between Pinco- and Pinco-Nef-infected cells were statistically significant (P<0.002 by Student’s paired t-test). This experiment is one representative out of six. (b) Jurkat cells were infected with Pinco, Pinco-Nef or retroviruses expressing mutant Nef proteins in which the indicated substitutions were introduced. Cells were analysed as described in (a). The specific fluorescence values for cell-surface molecules were determined in cells expressing wild-type (filled bars) or mutated Nef proteins (shaded bars) by considering their values relative to Pinco-infected cells (taken as 100%; empty bars). Reported values are the means±SD of at least three independent experiments.

**Fig. 2.** ULBP2 downmodulation activity of primary nef genes. Jurkat cells were infected with Pinco, Pinco-Nef or retroviruses expressing the indicated nef genes isolated from RP, SP and NP HIV-1-infected patients. Cells were analysed as described in Fig. 1(a). The geometric MFI specific for cell-surface ULBP2 in GFP⁺ cells is reported. Values are the means±SD of three independent experiments.

In conclusion, these data suggest that the ability to reduce cell-surface expression of ULBP2 and MICA is conserved in nef genes derived from patients and is not restricted to a laboratory-grown strain of HIV-1.
Infection with HIV-1 modulates the expression of NKG2DLs in a Nef-dependent manner

Next, we asked whether infection with HIV-1 resulted in the modulation of cell-surface NKG2DLs and whether Nef could affect the expression of these molecules on HIV-1-infected cells. Jurkat cells were infected with NL4-3 and monitored for intracellular expression of the viral p24 capsid antigen and for cell-surface expression of NKG2DLs. Productively infected p24+ Jurkat cells were not detected until 4 days after HIV-1 infection (data not shown). As for NKG2DLs, no significant changes in their cell-surface expression were detected until 4 days post-infection, when an almost twofold increase in MICA was observed on p24+ cells (Fig. 3). The relative amount of ULBPs remained unchanged, with the exception of a small decrease in ULBP2 (of about 20%). Cells were also infected with a mutated NL4-3 virus, PDS, that contains two stop mutations in the nef coding region, thus differing from wild-type NL4-3 only in its inability to express the Nef protein (Chowers et al., 1994). As expected, the reduction of cell-surface HLA-I was observed upon infection with wild-type virus, but not with PDS (Fig. 3). Compared with NL4-3-infected cells, MICA expression on PDS-infected cells was increased by an additional 30%, to a level corresponding to a 150% increase compared with the level on uninfected cells. Moreover, in PDS-infected cells, 30 and 77% increases were observed in ULBP1 and ULBP2 expression, respectively, compared with NL4-3-infected cells. Conversely, ULBP3 expression was not affected by HIV-1 infection even in the absence of Nef. In cells that were exposed to either NL4-3 or PDS virus, but that were not productively infected (p24<sub>seg/low</sub> cells gated in the R1 region; Fig. 3a), cell-surface expression of the NKG2DLs did not vary significantly compared with uninfected cells, with the exception of a small increase in MICA expression. Moreover, infection with heat-inactivated NL4-3 and PDS viruses did not result in detectable p24 expression or NKG2DL modulation (data not shown), indicating that the effect on NKG2DLs cannot be ascribed to cell-surface perturbations by input virus sticking nonspecifically to cells, but was instead due to virus infection. Results in Fig. 3 were also obtained by using a different set of mAbs (data not shown). These findings demonstrate that, in HIV-1-infected Jurkat cells, Nef completely inhibits the virus-induced increase in cell-surface expression of ULBP1, ULBP2 and, at least in part, MICA.

Analysis was also extended to primary cells by infecting purified CD4+ T lymphocytes with NL4-3 or PDS virus and analysing cell-surface expression of NKG2DLs and intracellular accumulation of p24 at various time points. To obtain a high percentage of infected p24+ T cells, infection was performed with high virus doses followed by antigen stimulation (Maier et al., 2000). Under these conditions, p24+ cells were detected 4 days post-infection (1–5%), reached a maximum after 5 days (20–30%) and then started to decline due to massive cell death. Although primary CD4+ T cells do not normally express NKG2DLs, ULBP1 and ULBP2 were expressed in p24+ T cells infected productively with the NL4-3 virus (Fig. 4). Moreover, in PDS-infected cells, the expression of ULBP1 and ULBP2 was increased by an additional 30% compared...

Fig. 3. NKG2DL surface expression upon HIV-1 infection. (a) Jurkat cells were not infected (n.i.) or infected with wild-type NL4-3 or a Nef-defective NL4-3 strain (PDS) of HIV-1. After 4 days, expression of cell-surface MICA, ULBP1, ULBP2, ULBP3 and HLA-I was analysed together with intracellular p24 expression by two-colour flow cytometry. Isotype-control IgG1 staining is also shown. Values corresponding to geometric MFI specific for IgG1 and for each NKG2DL of total n.i. cells and of NL4-3- and PDS-exposed cells, either infected productively (p24+, gate R2) or not (p24<sub>seg/low</sub>, gate R1), are indicated. Data representative of one out of five independent experiments are shown. (b) The mean ± SD intensity of fluorescence relative to the cell-surface expression of MICA, ULBP1 and ULBP2 in n.i. cells and in p24+ cells (gate R2) was determined as shown in (a) in five independent experiments. Data were calculated by subtracting from each sample the corresponding isotype-control staining and normalized by considering as 100% NKG2DL cell-surface expression on n.i. cells. Significant differences, as calculated by the paired t-test, are indicated: *P<0.05; **P<0.01; ***P<0.001.

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with NL4-3-infected cells. Cell-surface MICA was undetectable in cells infected with either NL4-3 or PDS virus. In contrast to Jurkat cells, which constitutively express these NKG2DLs on their surface, in acutely infected CD4\(^+\) T-cell blasts, the expression of ULBP1 and ULBP2 was also affected by wild-type HIV-1, although at lower levels than compared with a Nef-deficient virus, and MICA was not modulated by HIV-1 with or without expression of Nef, at least under these experimental conditions. It is likely that the mechanisms through which HIV-1 regulates MICA expression differ from those that control the expression of ULBP1 and ULBP2 and these aspects need further investigation.

**Nef expression protects cells from NK cell-mediated cytotoxicity**

The susceptibility of Pinco-infected Jurkat cells to NK-cell lysis was compared with that of Pinco-Nef-infected cells that had reduced levels of cell-surface MICA, ULBP1 and ULBP2. \(^{51}\)Cr-release assays were performed using two NK leukaemia cell lines, NKL and Nishi, characterized previously for their NKG2D expression and NKG2D-mediated effector functions (Bauer et al., 1999; our unpublished data), as effector cells. The percentage of NK cell-mediated lysis, converted into lytic units (LU), was 13 LU (Pinco) compared with 4 LU (Pinco-Nef) with Nishi and 28 LU (Pinco) compared with 12 LU (Pinco-Nef) with NKL. Thus, upon Nef expression, NK cell-mediated lysis was inhibited by \(~70\) and \(~57\%) with Nishi and NKL, respectively (Fig. 5a, b). The increased resistance to lysis of Pinco-Nef cells cannot be explained on the basis of HLA-I expression, which is reduced by Nef and should probably result in increased lysis (Lanier, 2005). Instead, it is likely that, due to NKG2D downregulation by Nef, triggering signals delivered via NKG2D are decreased, which results in a weaker activation of NK cells and in reduced lysis. The contribution of NKG2DLs to cell lysis was investigated by preincubating effector cells either with an anti-NKG2D mAb or with an isotype-control mAb (anti-CD56). As expected, treatment of NK cells with the anti-NKG2D mAb reduced their capacity to kill Jurkat cells. Conservation of the capacity to inhibit NK-cell lysis in Nef variants derived from primary isolates, which have been shown to be able to downmodulate NKG2DLs (Fig. 2; data not shown), was then investigated. Jurkat cells were infected with recombinant retroviruses expressing RP1-12 or NP3-4 nef genes derived from a rapid-progressor and a non-progressor patient, respectively. As shown in Fig. 5(c), primary nef genes were able to inhibit NK lysis at levels comparable to the NL4-3-derived nef gene. Treatment with the anti-NKG2D mAb resulted in inhibition of lysis (data not shown). Taken together, these results show that Nef expression protects cells from NK cell-mediated cytolysis and suggest that the NKG2D–NKG2DL interaction contributes to this evasion.

**DISCUSSION**

In this study, it has been shown for the first time that the HIV-1 Nef protein downmodulates MICA, ULBP1 and ULBP2. Such findings were obtained both in cells expressing only the Nef protein – thus indicating that HIV-1 replication and expression of other virus proteins are not required for this activity of Nef – and in cells infected with HIV-1. In particular, it was observed that, during HIV-1 infection, T cells (both freshly isolated CD4\(^+\) T cells and Jurkat cells) responded to the infection by increasing cell-surface expression of some NKG2DLs. This cellular response was particularly evident if the virus did not express Nef, indicating that the viral protein limits the expression of NKG2DLs. The activity of Nef on ULBP1 and ULBP2 was observed in both CD4\(^+\) T- and Jurkat-infected cells and the expression of these molecules was always higher if Nef was not expressed. The constitutive expression of MICA in Jurkat cells was increased upon HIV-1 infection, with highest levels observed with the Nef-deficient virus. However, in CD4\(^+\) T cells, cell-surface MICA was not...
observed in uninfected cells or in cells infected with either wild-type or Nef-deficient virus. It is possible that the role of HIV-1 and, in particular, of the Nef protein, on MICA expression may become apparent in CD4+ T cells under other conditions, such as co-infection with opportunistic micro-organisms that induce NKG2DLs.

Apparently, conserved residues and motifs of Nef that have been shown previously to mediate downmodulation of either HLA-I or CD4 molecules are dispensable for its activity on NKG2DLs. In addition, the tyrosine-based and the di-leucine sorting motifs present in HLA-A/-B and CD4 molecules, respectively, that are required for Nef-mediated downregulation (Cohen et al., 1999; Grzesiek et al., 1996), are absent in the GPI-anchored ULBPs, as well as in the cytoplasmic domain of MICA (Raulet, 2003). It is thus possible that Nef activity on NKG2DLs occurs through mechanisms unrelated to those used to downmodulate other cell-surface molecules. Interestingly, NKG2DL downregulation capacity differs from any other previously described biological activity of Nef because it does not require Nef localization at the plasma membrane. As Nef also binds several signalling molecules and interferes with cellular activation pathways (Baur, 2004), the expression of NKG2DLs could be regulated by Nef not only during their trafficking within the cell, but also at various steps of their biosynthesis. Moreover, Nef might use different mechanism(s) to downregulate each NKG2DL, as all Nef proteins analysed in this study had a stronger activity on ULBP2 and patient-derived Nef variants were active on MICA and ULBP2, but not on ULBP1. All of these aspects should be investigated in future studies.

Our results on NKG2DLs enlarge the variety of molecules downmodulated by the HIV-1 Nef protein (Baur, 2004; Doms & Trono, 2000) and suggest that Nef stimulates endocytosis in a non-specific manner. However, the fact that Nef does not affect the expression of several cell-surface molecules (e.g. IL-2 receptor α chain, CD45RO, the transferrin receptor, CD3) (Schrager & Marsh, 1999; Schwartz et al., 1993; data not shown), rather indicates that Nef interacts specifically with cellular pathways to affect the expression of selected molecules.

Downregulation of NKG2DLs by Nef is not as efficient as CD4 downmodulation and it requires higher expression levels of Nef (i.e. GFP intensity of fluorescence above 102 in Fig. 1a). Nevertheless, HLA-I downmodulation by Nef, which also requires higher protein levels compared with the activity on CD4 (Liu et al., 2001; Fig. 1a), was shown to be sufficient to strongly reduce recognition and killing of HIV-1-infected cells by CTLs (Collins et al., 1998). Remarkably, by interfering with NKG2DL expression, Nef might help HIV-1 to evade the NK cell-mediated immune responses of the host and establish a state of chronic infection. This issue has been investigated here for the first time and it was found that Nef-expressing cells with reduced levels of surface MICA, ULBP1 and ULBP2 molecules were protected from NK cell-mediated lysis. The possibility that Nef may affect the expression of other NK cell-activating ligands cannot be excluded. However, downmodulation of NKG2DLs by Nef might have important functional consequences on NKG2D+ cells during HIV-1 infection. Despite a dramatic decrease in HLA-I cell-surface molecules, HIV-1-infected T cells are not killed by autologous NK cells (Bonaparte &
Barker, 2003), suggesting that the virus has evolved efficient mechanisms to evade NK-cell recognition. The selective retention of cell-surface expression of HLA-C and -E molecules, which are not modulated by Nef (Cohen et al., 1999), may, at best, prevent killing of HIV-1-infected cells by those NK cells expressing HLA-C and -E inhibitory receptors. Conversely, given that all human NK cells express NKG2D, downmodulation of NKG2DLs provides a general mechanism that could explain the resistance of HIV-infected cells to NK cell-mediated lysis, independently from the repertoire of HLA-I-specific inhibitory receptors.

As Nef is a crucial determinant for disease progression in humans and animal models (Deacon et al., 1995; Hanna et al., 1998; Kirchhoff et al., 1995), it will be relevant to examine the contribution of its NKG2DL downmodulation activity to virus pathogenicity in vivo. The conserved ability of patient-derived Nef proteins to downregulate MICA and ULBP2 suggests that Nef activity on NKG2DLs may influence the pathogenesis of the virus in its host. In addition, results show that nef genes isolated from patients are also able to inhibit lysis by NK cells, indicating that this activity of Nef is conserved in vivo and is not restricted to the laboratory-grown NL4-3 strain. Thus, the in vivo selective pressure exerted by NK cells, considering their important role in controlling virus infections, has induced HIV-1 to evolve mechanisms for modulating NK-cell activity. The selection of Nef proteins that efficiently interfere with the expression of NKG2DLs could allow the virus to evade recognition by NKG2D+ cells and favour in vivo virus spread. A larger screening is needed to test whether Nef activity on each NKG2DL is modulated during the course of HIV-1 infection, as shown previously for other Nef functions (Carl et al., 2001; Casartelli et al., 2003b). The role of Nef activity on NKG2DLs in the perturbation of NK-cell functions observed during HIV-1 infection should also be investigated. Indeed, in viraemic patients, NK cells are functionally defective and display phenotypic alterations, including slightly decreased expression of the NKG2D receptor (Eger & Unutmaz, 2004). Interestingly, downregulation of NKG2D also occurs in some tumour patients as a consequence of a soluble form of MICA released by tumour cells upon proteolytic cleavage (Groh et al., 2002; Salih et al., 2003). By analogy, it should be analysed whether, as a result of Nef activity, HIV-1-infected cells release soluble MICA (or other NKG2DLs) to desensitize the NKG2D receptor and evade NKG2D-mediated immunity.

NK cells and CTLs are the main effector cells in the immune defence against persistent virus infections and, consequently, many viruses have evolved molecular mechanisms to evade their recognition. HIV-1 shares with CMV the capacity to downmodulate HLA-I and NKG2DLs to avoid recognition by CTLs and NK cells, respectively. Although the Nef protein is not able to fully protect cells from killing by NK cells (this study) or CTLs (Collins et al., 1998), in accordance with its inability to completely downmodulate NKG2DLs or HLA-I, respectively, it is likely that a short delay in the destruction of virus-producing cells by the immune system may increase in vivo virus loads significantly and influence the pathogenesis of the virus in its host. Our findings strengthen the importance of NK-cell functions in the host anti-HIV defence and highlight the need for therapeutic strategies to reinforce the innate immune surveillance against HIV infection.

ACKNOWLEDGEMENTS

This work was supported by V National Program of AIDS Research (Istituto Superiore di Sanità, grant 4OF.35), Ricerche Correnti of Children’s Hospital Bambino Gesù, Associazione Italiana per la Ricerca sul Cancro, Istituto Pasteur-Fondazione Cenci Bolognetti and Ministero dell’Istruzione Università e Ricerca. We thank Marina Potestà for excellent technical assistance and Egidio Bottini for help with statistics.

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