HLA-C is necessary for optimal human immunodeficiency virus type 1 infection of human peripheral blood CD4 lymphocytes

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The hypothesis that open conformers of HLA-C on target cells might directly exert an effect on their infectability by human immunodeficiency virus (HIV) has been suggested previously. This was tested by exploiting the peculiar specificity of monoclonal antibody (mAb) L31 for HLA-C open conformers to show that normal levels of Env-driven fusion were restored in HLA-C transfectants of a major histocompatibility complex-deleted (fusion-incompetent) cell line. The physiological relevance of this finding is now confirmed in this report, where small interfering RNA (siRNA) technology was used to silence HLA-C expression in peripheral blood lymphocytes (PBLs) from 11 healthy donors. Infectability by HIV (strains IIIB and Bal and primary isolates) was significantly reduced (P = 0.016) in silenced cells compared with cells that maintained HLA-C expression in 10 of the 11 PBL donors. Normal infectability was resumed, together with HLA-C expression, when the effect of siRNA interference waned after several days in culture. Additional confirmation of the HLA-C effect was obtained in several assays employing HLA-C-positive and -negative cell lines, a number of HIV strains and also pseudoviruses. In particular, viruses pseudotyped with env genes from HIV strains AC10 and QH0692.42 were assayed on siRNA-silenced lymphocytes from three healthy donors: the differences in infection with pseudoviruses were even higher than those observed in infections with normal viruses.

INTRODUCTION

Three recent whole-genome association studies have demonstrated and confirmed that minor allele variants of single-nucleotide polymorphisms in the major histocompatibility complex (MHC; in particular HLA-C) gene regions are associated with a lower viral load at the set point and additionally with delayed human immunodeficiency virus type 1 (HIV-1) disease progression (Fellay et al., 2007; Limou et al., 2009; van Manen et al., 2009). These findings, which echo a previous report on the association between HLA-C alleles and rapid progression to AIDS (Carrington et al., 1999), have prompted wide speculations on the role of HLA (and HLA-C in particular) in HIV biology and immunology. HLA-C differs from HLA-A and -B because HIV-1 selectively downregulates HLA-A and -B, but does not significantly affect HLA-C (or HLA-E). Therefore, infected cells display reduced detection by both cytotoxic lymphocytes and natural killer (NK) cells (Cohen et al., 1999). The hypothesis that HLA-C might exert an effect directly on HIV infectivity was originally suggested by De Santis et al. (1996), exploiting the peculiar specificity of monoclonal antibody (mAb) L31 to show that normal levels of Env-driven fusion were restored in HLA-C transfectants (Grassi et al., 1991; Setini et al., 1996) of an MHC-deleted (fusion-incompetent) cell line. The physiological relevance of this finding is confirmed in this report, where small interfering RNA (siRNA) technology was used to silence HLA-C expression in peripheral blood lymphocytes (PBLs), the natural target of HIV-1. Two different kinds of single-cycle infection assay and a fusion assay demonstrated that the presence of HLA-C on recipient PBLs is necessary for optimal HIV-1 entry.

RESULTS

HLA-C affects HIV-1 infectability of cell lines

It was shown previously that HLA-C-deprived virions are severely restricted in their infectivity (Cosma et al., 1999). To evaluate the relevance of cellular HLA-C on the infectability of PBLs by HIV-1, it was therefore necessary
to use reliable single-cycle infection methods to distinguish between entry and secondary virus replication. We showed that one such quantitative assay (Mascola et al., 2002) is suitable for this purpose by demonstrating (i) that it could be used to effectively measure the action of known entry inhibitors such as IZN17 (Eckert & Kim, 2001) and T20 (Rusconi et al., 2007) on the infection of PBLs by HIV-1 strain IIIB, giving a linear dose response for both inhibitors (Fig. 1a), and (ii) that the lack of HLA-C had a detrimental effect on the infectability of two cell lines that have the same CD4 expression (not shown) and differ only for HLA-C expression. These cell lines were 221/4+/C+/ [a 721.221 cell line (Shimizu et al., 1988) with a homozygous deletion of the whole MHC region, transfected with two plasmids carrying CD4 or HLA-Cw4] and 221/4+/C− [an HLA-C-negative segregant of the same cell line sorted by fluorescence-activated cell sorting (FACS)] (Fig. 1b). HIV-1 entry was significantly enhanced (P=0.0006) by the presence of HLA-C molecules on the recipient cell line (Fig. 1c). The HLA-C effect became even more evident when infecting a mixture of the two cell lines and visualizing their differing infectabilities by FACS analysis (Fig. 1b, Mix): HLA-C-positive cells (stained with mAb L31) were 2.23-fold more susceptible to infection with HIV than HLA-C-negative cells.

HLA-C siRNA silencing in cell lines

A custom-made mixture of two HLA-C-specific siRNAs (Matucci et al., 2008) has been shown previously to be effective in silencing the expression of HLA-C in the TZM-bl cell line (Kirchherr et al., 2007). RNA analysis (Fig. 2a) demonstrated the effectiveness and selectivity of siRNA silencing, showing that expression of HLA-A and -B and various other cellular genes, including HIV-1 receptors, was unaltered by HLA-C siRNA treatment. FACS analysis (Fig. 2b) showed an almost complete downregulation of HLA-C [measured using mAb L31 (Grassi et al., 1991; Setini et al., 1996), which is specific for HLA-C open conformers]. The bulk of MHC class I, measured by mAb W6/32 (Natali et al., 1989), specific for all trimeric-conformation class I molecules, showed a reduction compatible with the silencing of HLA-C and the conservation of HLA-A and -B, as already seen by RNA analysis. Among the HIV-1 receptors, CD4 and CXCR4 expression was unaltered by the treatment, whilst CCR5 shows a minor reduction in expression (not evidenced by RNA analysis).

HLA-C-silenced TZM-bl cells were used in a fusion assay with a panel of HeLa derivatives bearing HIV-1 env from different viral strains (Joliot et al., 2001; Schwartz et al., 1994), such as ADA (R5), LAI (X4) and NDK (X4). HLA-C silencing in TZM-bl cells significantly reduced the fusion index for the three envelope types tested (ADA, P=0.0298; LAI, P=0.0192; NDK, P=0.0007; Fig. 2c). Furthermore, the infection efficiency of engineered pseudoviruses [expressing the Env protein of the HIV-1 strains NDK, QH0692.42 and pRHPA4259.7 or of vesicular stomatitis

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**Fig. 1.** Analysis of infection using a single-cycle intracellular p24 assay (Mascola et al., 2002). (a) Effect of entry inhibitor peptides T20 and IZN17 (1.1 μM each) on human PBLs infected with HIV IIIB and dose–effect regression. (b) Infectability by HIV IIIB of cell lines 221/4+/C+ and 221/4+/C− assayed in separate cultures and after mixing at a 1 : 1 ratio in a single culture. The insert in the middle dot plot shows the intracellular fluorescence of a p24-positive cell among p24-negative cells. (c) Infection with HIV IIIB of 721.221 cells with or without HLA-C expression. Means±sd and P values (Student’s t-test, one-tailed) are shown for six replicates. Each stock of virus was titrated initially on the different cell types to define the concentration giving up to 5 % infection of PBLs or 10 % of 721.221 cells.
virus (VSV-G) on HLA-C-silenced target cells was measured using TZM-bl cells in a single-cycle assay. The efficiency of HIV pseudovirus infection (measured by Tat-driven luciferase production) was significantly reduced in HLA-C-silenced cells (NDK, \( P = 0.0028 \); QH0692.42, \( P = 0.002 \); pRHPA4259.7, \( P = 0.0001 \); Fig. 2d). Conversely, the control VSV pseudovirus showed only a marginal (non-significant) difference in the infection of HLA-C-silenced and non-silenced TZM-bl target cells.

In conclusion, X4 and R5 isolates were affected to the same extent, indicating that the observed minor reduction in CCR5 expression does not play a role in the reduction in infectability.

**HLA-C siRNA silencing in human PBLs**

As a prelude to addressing the HLA-C effect on the physiological target of HIV-1, the specificity of the siRNAs in human PBLs was evaluated by FACS analysis (Fig. 3a). HLA-C was indeed also strongly silenced in PBLs, and the expression of MHC class I molecules, CD4, CXC4 and CCR5 behaved exactly as in the TZM-bl cell line. HLA-C silencing was optimal at 24–48 h after treatment and its expression returned to pre-treatment levels after 7 days, as shown by Western blot analysis (Fig. 3b). The single-cycle infection of PBLs showed that HLA-C siRNA silencing reduced infection of all HIV-1 strains tested (Fig. 3c, IIIB (X4), Bal (R5) and J45CPp (R5X4)). Again, R5 and X4 viruses were equally affected by the treatment. At 7 days after silencing, siRNA-treated PBLs regained full infectability together with full expression of surface HLA-C (not shown).

A marked reduction (60–90%) in infectability of HLA-C-silenced PBLs from three healthy donors with the luciferase-expressing pseudoviruses (Connor et al., 1995; He et al., 1995) QH0692.42 (R5) and AC10 (R5) confirmed the HLA-C effect (Fig. 4a). The reduction in infectability following HLA-C silencing was evaluated further in the single-cycle assay using PBLs from 11 blood donors and three viruses, i.e. IIIB (X4), Bal (R5) and J45CPp (R5X4) (in two cases), two viruses (in one case) or one virus only (in eight cases). The HLA-C effect was observed in 10 of 11 patients (Fig. 4b). The fraction of p24-positive cells was significantly reduced (28–58%) in HLA-C-silenced PBLs.
**Fig. 3.** HLA-C siRNA silencing in human PBLs. (a) siRNA selectivity in human PBLs studied by FACS analysis (negative control, black line; mock-treated, blue line; siRNA-silenced, red line). HLA-C was silenced and MHC class I molecules were only marginally affected, whilst CD4, CXCR4 and CCR5 were unaffected. (b) Western blot analysis showing that the effect of HLA-C silencing is completely lost by 7 days after treatment. (c) HLA-C siRNA silencing reduces infection of human PBLs with viruses IIIB (X4), Bal (R5) and J45CPp (X4) (virus input 1050, 30 and 20 ng ml\(^{-1}\), respectively).

**Fig. 4.** HLA-C siRNA silencing in human PBLs. (a) Infection with luciferase-labelled pseudoviruses with the R5 phenotype (QH0692.42 and AC10) of PBLs from three healthy donors. Infection of non-silenced PBLs (open bars) was higher than infection of HLA-C-silenced PBLs (filled bars) for all donor cells tested. Data are means of three replicates. c.p.s., Counts per second. (b) Infection with HIV strains. The statistical significance of the reduced infectability of HLA-C-silenced PBLs was evaluated for 11 blood donors (whose PBLs were effectively HLA-C silenced for at least 3 days, retaining >50% viability) employing various combinations of viruses (a, IIIB; b, Bal; c, J45CPp). The HLA-C effect was observed in 10 of 11 patients. Data are means of three replicates. The fraction of p24-positive cells (pooled data) was significantly reduced (\(P=0.016;\) Student’s t-test, one-tailed) in HLA-C-silenced PBLs (filled bars) compared with mock-treated control samples (open bars).
when compared with mock-treated control samples ($P=0.016$ for pooled data).

**DISCUSSION**

The data presented in this paper strongly support the view that HLA-C open conformers on the surface of PBLs (and other recipient cells) are necessary for optimal HIV-1 entry (i.e. for cell infectability) by both X4 and R5 HIV viruses, by analogy with the findings obtained with MHC-deleted cell lines. These results suggest an interaction in *trans* between HLA-C molecules on the lymphocyte membrane and Env structures on the virions.

As already mentioned, the role of HLA-C antigens in virus infectivity has been demonstrated previously (Cosma et al., 1999) by comparing the infectivity of HIV-1 virions grown in the MHC-deleted mutant cell line 721.221 (CD4 +) with that of virions grown in HLA-C-positive transfectants of the same cell line. This showed that the infectivity of HLA-C-negative viruses was largely reduced on indicator cell lines and PBLs. Using the same siRNAs described in that report, we also recently showed that the infectivity of HLA-C-deprived viruses is reduced (Matucci et al., 2008) in PBLs and other recipient cells. This result strongly indicates a role for HLA-C molecules interacting in *cis* with Env molecules within the virion membrane and confirms our previous report (Cosma et al., 1999) using a new and different experimental strategy. Therefore, HLA-C open conformers have been shown conclusively by highly stringent methods to be necessary for optimal HIV-1 infection on ‘both sides’ of viral entry, i.e. virion as well as cellular components. The relationship between the two HLA-C effects (on virus infectivity and target cell infectability) remains unclear.

The specificity of mAb L31 (Grassi et al., 1991) has been documented extensively (Setini et al., 1996): L31 binds the KYK z1 motif, characteristic of all HLA-C alleles and accessible only on open conformers (i.e. molecules devoid of peptide with or without β2-microglobulin). As recently reviewed elsewhere (Arosa et al., 2007), open conformers of MHC molecules are involved in homotypic and heterotypic *cis* and *trans* interactions with other surface proteins. This notion is probably the key to understanding how HLA-C could affect HIV-1 entry from both sides of the interaction (virion and cellular). The particular interaction(s) necessary for optimal HIV-1 infectability/infectivity is still unknown, although a direct interaction with Env proteins has been suggested by the demonstration that HLA-Cw4 molecules co-precipitate with Env proteins (Cosma et al., 1999) in lysates from HIV-1-infected cells. Furthermore, the accessibility of some gp120 epitopes (on the surface of virions or infected cells) is regulated by the presence/absence of HLA-C (Cosma et al., 1999).

Due to the flexibility of membrane proteins, the mechanisms of the two types of interaction might well be similar. However, some differences between the two interactions may also exist: the fusion capacity of NDK Env is not influenced by the presence of HLA-C in the virions or when HLA-C is co-expressed with Env proteins in the donor cell line (Cosma et al., 1999; Matucci et al., 2008), but is enhanced when HLA-C is co-expressed with the receptors, as reported here (Fig. 2c, d).

This view is consistent with recently reviewed experimental data (Arosa et al., 2007) showing that ‘...HLA open conformers adopt a supine orientation with the long heavy chain axis parallel to the plasma membrane (like HFE and M10) (Mitra et al., 2004), in a position that favours *cis* interactions between the z1 and z2 domains and adjacent receptors’. In addition, some NK receptors recognize peptide-empty MHC molecules (HLA-G and H-2Kb; Benoit et al., 2005; Gonen-Gross et al., 2005), thus supporting the view that open MHC-I conformers can serve as *cis–trans* recognition structures. Although our results agree with the genomic/epidemiological data to support an important role for HLA-C in HIV-1 infection, the molecular mechanisms of this role remain obscure. However, given their potential basis for novel intervention strategies, they clearly deserve further attention.

**METHODS**

**Vectors and cell lines.** HeLa cells expressing the HIV-1 Env protein of different isolates were used as effector cells. The cell line 221/4+/C- [a B-lymphoblastoid 721.221 cell line (Shimizu et al., 1988) with a homozygous deletion of the whole MHC region] transfected with two plasmids carrying CD4 or HLA-Cw4 was provided by Dr M. Malnati (HSR, Scientific Institute, Milan, Italy). In order to compare two cell lines differing only in Cw4, the cell line 221/4+/C- (a HLA-C-negative segregant) was obtained by FACSs from 221/4+/C+ cells cultured without the selection antibiotic for the plasmid carrying HLA-C. The two cell lines expressed the same level of CD4 (data not shown). The TZM-bl indicator cell line (Wei et al., 2002) was obtained from EVA/MRC, Centre for AIDS Reagents (CFAR), National Institute for Biological Standards and Control (NIBSC). TZM-bl cells express CD4, CCR5 and CXCR4 and the HIV-1 LTR-driven *Escherichia coli* β-galactosidase and firefly luciferase reporter cassettes, both activated by HIV Tat expression.

**Viruses and antibodies.** J45CPp (NSX4R3 phenotype) is a primary isolate from a patient cohort followed at the Infectious Disease Department of San Raffaele Hospital, Milan, Italy. HIV strains IIIB and Bal are X4 and R5 viruses, respectively, grown in PBL cultures. ADA, LA1 and NDK (Joliot et al., 2001; Rousseau et al., 2006; Schwartz et al., 1994) HeLa cell lines were kindly provided by Dr M. Alizon and Dr U. Hazan (Institut Cochin, Paris, France). VSV-G enveloped and the HIV-1 env and rev sequences of the HIV-1 subtype B strains QH0692.42, AC10, 6535.5 and pHPA4259.7 and subtype D strain NDK were obtained through the MRC CFAR NIBSC programme.

mAbs L31 (Grassi et al., 1991; Setini et al., 1996) and W6/32 (Natali et al., 1989) were provided by Dr P. Giacomini (Laboratory of Immunology, Regina Elena Cancer Institute CRS, Rome, Italy). mAb M01 16/41 (anti-p24) was donated from Polymun, Germany. mAb mouse anti-CXCR4 antibodies were provided by the MRC CFAR NIBSC programme. Other antibodies used were: mouse mAb anti-CD4 (Becton Dickinson), rat mAb anti-CCR5 (Leukosite), fluorescein isothiocyanate (FITC)-labelled goat polyclonal anti-mouse...
Single-round infection protocol. HIV-1 infection of phytohaemagglutinin (PHA)- and interleukin (IL)-2-stimulated PBLs was performed in 96-well round-bottomed culture plates by combining 40 μl virus stock with 30 μl PBLs (1.5 × 10^6 cells per well). The PBLs were resuspended in IL-2 culture medium (RPMI 1640 with 10% fetal calf serum, 1% penicillin–streptomycin and 200 U IL-2 ml^-1) containing 1 μM indinavir (Merck) and this concentration was maintained throughout. After overnight incubation at 37 °C, 150 μl IL-2/indinavir culture medium was added, and the cell cultures were maintained at the same temperature (Mascola et al., 2002). The PBLs were harvested for intracellular p24 antigen staining at day 2 of infection and transferred into FACS tubes. For intracellular p24 staining, cells were washed twice in PBS, fixed with 4% paraformaldehyde in PBS and permeabilized with saponin buffer PBS containing 1% BSA, 0.5% saponin (Sigma). Cells were resuspended for 30 min at 4 °C with 50 μl mAb M01 16/4/1 (20 μg ml^-1). After two washes with saponin buffer, cells were resuspended for 30 min at 4 °C with 50 μl of a 1:250 dilution of FITC-labelled goat polyclonal anti-mouse antibody. After four additional washes, HIV-1- or mock-infected PBLs were analysed with a CyAn flow cytometer (Dako) and data analysis was performed using FlowJo software. The single-round infection protocol was the same for 221 cell lines, but used 10^5 cells per well cultured without IL-2. The fraction of p24-positive cells increased linearly with increasing virus dose up to 5% for PBLs and 10% for 221 cells. Each virus stock was titrated on the different cell types to define the concentration giving approximately 5% infection of PBLs or 10% of 221 cells.

Entry peptidyl inhibitors IZN17 (41 residues, 4810 Da; Eckert et al., 2007) were a kind gift of Professor R. Cortese (IRBM, Pomezia, Rome).

siRNA design and transfection protocols. The HLA-C-specific siRNAs used were J-017513-06 and J-017513-08 (Dharmacon; Matucci et al., 2008) for the experiments with TZM-bl cells; only the latter was used in the experiments with PBLs. These two HLA-C-specific siRNAs were chosen as the best inhibitors from a series of siRNAs that were tested initially. Several siRNAs had no effect, ruling out the possibility of non-specific activation of the interferon system.

TZM-bl cells were added to six-well plates at a concentration of 2 × 10^5 cells per well; 24 h later, the cells were transfected with 100 nmol siRNA per well using DharmaFECT 1 reagent (Dharmacon) as recommended for HeLa and HeLa-derived cells (such as TZM-bl cells), following the manufacturer’s instructions. The specificity of RNA silencing of HLA-C expression was confirmed by Western blotting of cytoplasmic protein lysates, FACS analysis and RT-PCR after 72 h of culture, as described previously (Matucci et al., 2008).

PHA- and IL-2-stimulated PBLs (5 × 10^6 cells) were transfected with 4 μM siRNA (or without siRNA as a mock control), using a Human T Cell Nucleofector kit for Stimulated Human T Cells (Amaxa) following the manufacturer’s instructions. The RNA silencing effect on HLA-C expression was tested after 24 and 48 h of culture by FACS and Western blot analysis. Only PBLs that were effectively HLA-C silenced whilst retaining >50% viability were subjected to infection.

Western blot analysis. PBLs (9 × 10^6 cells) were lysed in 100 μl TNN buffer [50 mM Tris/HCl (pH 8.0), 250 mM NaCl, 0.5% NP-40] with protease inhibitors [200 μg PMSF ml^-1 (Sigma–Aldrich) and Complete Protease Inhibitor Cocktail (Roche)] and 20 mM of N-ethylmaleimide (Sigma–Aldrich). Six micrograms of protein was separated on an 11% polyacrylamide gel and transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell). HLA-C protein was identified using mAb L31 and HRP-labelled goat anti-mouse polyclonal antibody by a chemiluminescence reaction (ECL; Amersham Biosciences) and autoradiography.

TZX-bl luciferase and β-galactosidase reporter gene assays. The fusion process between gp120/gp41 effector cells (HeLa, HeLa-ADA, HeLa-LAI and HeLa-NDK) and TZM-bl cells was assessed by measuring luciferase gene activity and X-Gal cell staining (KPL). TZM-bl cells (5 × 10^4 cells per well) were co-cultured in microtitre plate wells (Corning) with an equivalent number of effector cells for 3–7 h at 37 °C. The luciferase activity resulting from fusion and transactivation was analysed using brillet reagent (Perkin Elmer Life Sciences) according to the manufacturer’s instructions and read using a Victor3 luminometer (Perkin Elmer Life Sciences). All assays were performed in triplicate.

In situ staining of fusion cells for β-galactosidase gene activation was performed in a 24-well plate format (Corning) after co-cultivation of a total of 5 × 10^4 cells. Fusing cells were fixed in 0.25% glutaraldehyde for 15 min at room temperature, washed three times in PBS and stained with a solution containing 0.2% X-Gal. The staining solution was removed and cells were overlaid with PBS for microscopic analysis. Blue-stained syncytia were photographed using a Nikon Eclipse 80i microscope; means ± SE were calculated by counting blue syncytia in five different fields, and fusion efficiency was determined by calculating the fusion index (White et al., 1981).

Pseudovirus infection assay. Pseudoviruses expressing VSV-G env or the HIV-1 env and rev sequences of the HIV-1 subtype B strains QH0692.42, pRHPA4259.7 and subtype D NDK were prepared as described previously (Li et al., 2005). A luciferase reporter gene expression assay was performed using TZM-bl cells pre-treated for 48 h with HLA-C siRNA and compared with un-treated TZM-bl cells. Serial fivefold dilutions of pseudovirus were made in quadruplicate wells in 96-well culture plates in a total volume of 100 μl growth medium for a total of five dilution steps. Freshly trypsinized cells (10^4 cells) were added to each well. The pseudovirus was removed after 10 h of infection and, at 48 h post-infection, luciferase activity was determined using brillet reagent in a Victor3 luminometer as described above.

Pseudoviruses expressing HIV-1 env and rev sequences of the HIV-1 subtype B strains QH0692.42 and AC10 and carrying the luciferase gene were prepared as described previously (Connor et al., 1995; He et al., 1995) and used to infect human PBLs: 1.5 × 10^6 PBLs in 30 μl were infected with 40 μl of twofold dilutions of pseudovirus (in quadruplicate). After 72 h, the luciferase activity, which is proportional to the infectivity, was determined using a Luciferase Assay System (Promega) and measured with a Mitras luminometer (Berthold Technologies) according to the manufacturer’s instructions.

Statistical analysis. A one-tailed Student’s t-test with Welch’s correction was used to analyse the results.

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