Molecules—Peptide-Loaded HLA-DR and CD86 Molecules

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HIV Type 1 Can Act as an APC upon Acquisition from the Host Cell of Peptide-Loaded HLA-DR and CD86 Molecules

Jocelyn Roy, Geneviève Martin, Jean-François Giguère, Dave Bélanger, Myriam Pétrin, and Michel J. Tremblay

It is well documented that a wide range of host-derived cell surface constituents is inserted within HIV type 1 (HIV-1) and located on the exterior of the virion. Although no virus-associated protein of host origin has been shown to be absolutely required for virus replication, studies have revealed that many of these proteins are functional and can affect several steps of the virus life cycle. In this study, we found that HIV-1 acquires peptide-loaded class II MHC (MHC-II) and the costimulatory CD86 molecules from the host cell. Moreover, we present evidence that virions bearing such peptide-loaded MHC-II and CD86 proteins can lead to activation of the transcription factors NF-κB and NF-AT in an Ag-specific human T cell line. A linear correlation was found between activation of NF-κB and the amount of peptide-loaded MHC-II molecules inserted within HIV-1. Finally, transcription of unIntegrated and integrated HIV-1 DNA was promoted upon exposure of peptide-specific human T cells to viruses bearing both peptide-loaded MHC-II and CD86 proteins. These data suggest that HIV-1 can operate as an APC depending on the nature of virus-anchored host cell membrane components. It can be proposed that HIV-1 can manipulate one of its primary targets through the process of incorporation of host-derived proteins.

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H uman immunodeficiency virus type 1 (HIV-1), the etiological agent of AIDS, is released from infected cells by budding through cell membranes. During this process, many host-derived cell surface constituents, such as CD11a, CD18, CD54, CD55, CD59, and class II MHC (MHC-II) molecules, are inserted within the viral envelope (1). Several studies have scrutinized the functionality of these molecules once located onto the exterior of mature HIV-1 particles. For instance, CD55 and CD59 molecules have been shown to protect HIV-1 against destruction by complement proteins (2). The acquisition of the costimulatory molecule CD28 has been reported to result in an increase of virus infectivity (3), whereas insertion of host-encoded CD86 enables whole HIV-1 virions to mediate signal transduction events leading to activation of HIV-1 long terminal repeat (LTR)–driven gene expression in human CD4+ T cells via the mammalian ubiquitous transcriptional factor NF-κB (4). Incorporation of CD54 (ICAM-1) has been reported to promote infection of CD4-negative cells, enhance virus infectivity, and diminish sensitivity to Ab-mediated neutralization (5–8).

Two distinct signals are required for induction of cell proliferation and cytokine production in resting T cells (9–11). The first signal is mediated through an interaction between peptide-loaded MHC-II molecules located on APCs and the TCR/CD3 complex located on CD4+ T lymphocytes. Engagement of the TCR will primarily induce an early wave of protein tyrosine kinase phosphorylation, leading to the activation of downstream signaling pathways that activate transcriptional factors such as AP-1, NF-AT, and NF-κB, which ultimately induce the expression of genes controlling cellular proliferation, differentiation, anergy, or apoptosis (12). The second signal is initiated through an interaction between either CD80 or CD86 on the APC and CD28 on CD4+ T cells. The combination of the first and second signal will lead to a complete activation of CD4+ T lymphocyte, an event that is characterized by cell proliferation and IL-2 production. In the context of HIV-1 infection, the proliferative state of CD4+ T lymphocytes influences the replication pattern of HIV-1. Virus will replicate continuously in actively proliferating T cells until these ultimately die, whereas latency will be observed in quiescent T cells, such as memory cells (13).

Interestingly, abundant levels of host MHC-II proteins have been demonstrated to be acquired by newly formed HIV-1 particles. The physiological significance of this phenomenon is provided by the demonstration that MHC-II molecules are found in clinical strains of HIV-1 bearing different coreceptor usages that were grown in both PBMC and human lymphoid tissues cultured ex vivo (14). There is some evidence suggesting that the HLA-DR isoform is acquired preferentially over the DP and DQ isoforms (15–18). The possible functional role played by MHC-II molecules once found inserted within HIV-1 is illustrated by studies showing that this host cell surface constituent increases virus infectivity and can enable virions to present superantigens (19, 20). Given that HIV-1 incorporate the major constituents necessary for Ag presentation, i.e., MHC-II and CD86, the primary objective of the
present study was to assess whether HIV-1 is able to present a nominal Ag, thereby acting as a fully competent APC. In this study, we provide data indicating that peptide-loaded host MHC-II and CD86 molecules are functional once embedded within the viral envelope because this process enables HIV-1 to present a nominal Ag to an Ag-specific human CD4+ T cell line. We demonstrate that HIV-1-mediated Ag presentation to CD4+ T cells leads to nuclear translocation of NF-AT, an event that is further amplified following the presence of host-encoded CD86 on viral entities. We also show that the extent of NF-AT amplification following the presence of host-encoded CD86 on viral entities is proportional to the amount of peptide-loaded MHC-II molecules that is anchored onto HIV-1 envelope. Finally, the presentation of a nominal Ag by HIV-1 was found to up-regulate transcription of both unintegrated and integrated proviral DNA in Ag-specific CD4-expressing T cells.

Materials and Methods

Reagents

PMA, azidothymidine (3’-azido-3’-dideoxthymidine (AZT)), and ioneomycin (Iono) were purchased from Sigma-Aldrich. Hygromycin B and puromycin A were obtained from Cedarlane Laboratories, whereas G418 was purchased from Invitrogen Life Technologies.

Cells and culture conditions

CH7C17 is a human lymphoid T cell line that stably expresses the HA1.7 TCR, which is specific for influenza hemagglutinin (HA) peptide 307–319 in the context of HLA-DR1. These cells were developed from JRST3.5 cells, a VRC8/CD4+ and MHC-II-negative subline of Jurkat cells (21), in which vectors containing puromycin and hygromycin B resistance genes (i.e., pSad puro and pSad hygro) have been added (22). The CH7C17 cells were kindly provided by R. Sekaly (University of Montreal, Montreal, Canada). The cells were maintained in complete culture medium made of RPMI 1640 supplemented with 20% FBS (HyClone), Glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (100 μg/ml) in the presence of hygromycin B (400 μg/ml) and puromycin A (4 μg/ml). CD28 wild-type (CD28WT; clone A14) and CD28Del.30 (clone F5) cell lines were derived from CH7C17 cells. CD28WT and CD28Del.30 were stably transfected with full-length CD28 or a truncated version of CD28 that lacks the cytoplasmic tail, respectively (23). These cells were kindly provided by O. Acuto (Institut Pasteur, Paris, France) and were maintained in complete culture medium composed of RPMI 1640 supplemented with 20% FBS, Glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (100 μg/ml) in the presence of hygromycin B (400 μg/ml), puromycin A (4 μg/ml), and G418 (2–4 mg/ml). The human embryonic kidney cell line 293T, which expresses the SV40 large T Ag (24), was maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS, Glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (100 mg/ml). These cells were kindly provided by J. Gladis (Clayton Research Laboratories, San Francisco, CA). It should be noted that 293T cells expressing HLA-DR-CD4-Molecules that were loaded either with CLIP, heat shock protein (HSP), or HA were first fixed with 2% paraformaldehyde before their use as controls.

Plasmids

pNL4-3 is a full-length infectious molecular clone of HIV-1 (X4-tropic) (25) that was provided by the AIDS Repository Reagent Program. The commercial pNF-AT1 binding site was generously provided by G. Dardé (University of Paris, France). The commercial pNF-AT1 binding site was generously provided by G. Dardé (University of Paris, France). The plasmid contains a 722-bp fragment (pHSP) or HA from Influenza virus. In this molecular construct, the HA peptide is covalently linked in the peptide-binding groove of the MHC-II molecule. We have also used vectors encoding for the invariant chain, in which the core CLIP sequence is either left unchanged (pCLIP) or exchanged with another sequence, i.e., HSP from Mycobacterium tuberculosis (pHSP) or HA from influenza virus (pHA) (kindly supplied by J. Van Bergen, Leiden University Medical Center, Leiden, The Netherlands) (31). The CD86-encoding vector pCN-B7-2 was obtained from A. Truneh (Smith-Kline Beecham Pharmaceuticals, King of Prussia, PA) (32).

Production of virus stocks

Fully infectious NL4-3-based viruses were generated by calcium phosphate cotransfection in 293T cells using a protocol established in the laboratory. Pseudotyped reporter HIV-1 particles were produced by cotransfection of 293T cells with pNL4-3-Luc-E’ R and an expression vector encoding for the VSV-G full-length envelope protein. Virus-containing supernatants were harvested 40 h posttransfection and then centrifuged at 2000 rpm for 30 min at 4°C. The supernatant was then filtered through a 0.22-μm filter. To eliminate free p24, each unfrozen virus preparation was ultracentrifuged in Centriflic Plus-20 Biomax-100 filter devices (Millipore) at 4000 × g, 4°C, until the supernatant had been filtered. Then virus preparations located on the membrane were recovered by an additional centrifugation at 10000 × g, 4°C, for 1 min. Virions were then frozen at −85°C until assayed. Virus preparations were quantified using an in-house sensitive Alu-B and sandwich enzymatic specific for the major core viral p24-antibody (33).

Antibodies

The hybridoma cell line L243 (IgG2a) secretes a mAb directed against a monomorphic epitope of the HLA-DR determinant of MHC-II. The mAb 2B6 is specific for HLA-DR (34). Both L243 and 2B6 hybridomas were obtained from the American Type Culture Collection. The mAb 9.3 specific for human CD28 (35, 36) was kindly provided by J. Ledbetter (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). The anti-CD45RO UCHL-1 mAb (IgG2a) was a generous gift from R. Sekaly. The mAb BU-63 (IgG2a) is specific for human CD86 (37) and was supplied by D. Hardie (University of Birmingham, Birmingham, U.K.). Abs were purified with a HitTrap protein G affinity column (Amersham Biosciences) following the manufacturer’s instructions. For the purposes of the virus capture assay, biotinylation of the Abs was performed using EZ-Link Sulfo-NHS-LC-Biotin (Pierce).

Flow cytometry

Cell surface expression of the various HLA-DR constructs was evaluated by flow cytometry, as follows. The 293T cells (1 × 10^6) were washed once in PBS containing 2% FBS (PBSF). Cells were then resuspended in 100 μl of PBSF, to which was added 1 μg of anti-HLA-DR mAb clone (clone L243), vortexed gently, and incubated for 30 min on ice. Cells were subsequently washed with PBSF, resuspended in 100 μl of PBS supplemented with (R)-PE-conjugated goat anti-mouse IgG (0.5 μg total), and further incubated for 30 min on ice. Cells were finally centrifuged and resuspended in 1% paraformaldehyde in PBS before analysis by flow cytometry (EPICS XL; Corixa).

Optiprep gradients

Virus-containing supernatants from 293T cells were first ultrafiltered using Centricon Plus-20 Biomax-100 filter devices. Next, samples were centrifuged in an Optiprep (60% (w/v) iodixanol) gradient, as previously described (38). In brief, iodixanol gradients were prepared in distilled/deionized water as 11 steps in 1.2% increments ranging from 6 to 18%. Samples were layered on the top of the gradient in an Optiscale tube (Beckman Coulter) and centrifuged using an Optima L-90K apparatus for 75 min at 52,000 rpm in a NV765 rotor. Gradient fractions were collected and were frozen at −85°C until subjected to HLA-DR and p24 ELISA tests.

HLA-DR ELISA test

Measurements of HLA-DR-containing exosomes/micovesicles were achieved through the use of an in-house enzymatic assay. Briefly, flat-bottom 96-well plates (BD Falcon microplate) were initially coated with the anti-HLA-DR mAb. After the wells were washed and blocked with 1% BSA (Sigma-Aldrich), gradient fractions were added to the wells at various dilutions, along with samples of known HLA-DR concentration (G418-purified HLA-DR proteins from RAJI, a B lymphoid cell line expressing high levels of MHC-II molecules), to establish a standard curve. After 60-min incubation at 37°C, the plates were washed, and a second biotinylated anti-HLA-DR mAb (i.e., L243) was then added. After 1 h

Influenza virus. In this molecular construct, the HA peptide is covalently linked in the peptide-binding groove of the MHC-II molecule. We have also used vectors encoding for the invariant chain, in which the core CLIP sequence is either left unchanged (pCLIP) or exchanged with another sequence, i.e., HSP from Mycobacterium tuberculosis (pHSP) or HA from influenza virus (pHA) (kindly supplied by J. Van Bergen, Leiden University Medical Center, Leiden, The Netherlands) (31). The CD86-encoding vector pCN-B7-2 was obtained from A. Truneh (Smith-Kline Beecham Pharmaceuticals, King of Prussia, PA) (32).
incubation at 37°C, the plates were washed, and a streptavidin-peroxidase conjugate (streptavidin-HRP-40; Research Diagnostics) was added; this was followed by the addition of the 3,3',5,5'-tetramethylbenzidine substrate (Research Diagnostics). After 30 min at room temperature, the reaction was terminated by adding 1 M H₃PO₄, and the absorbance was measured at 450 nm. Unknown HLA-DR values were calculated on the basis of a HLA-DR standard curve ranging from 15.6 to 125 ng/ml.

**Virus capture assay**

We used a modified version of our previously described virus precipitation assay, which is based on the capture of HIV-1 particles using immunomagnetic beads (39). Briefly, commercially available streptavidin-coated magnetic beads (8.4 × 10⁴ beads) (Dynal Biotech) were mixed with 2 μg of biotinylated mAbs in a final volume of 1 ml of PBS plus 10% BSA (PBSA) for 1 h at room temperature on a rocking plate. Immunomagnetic beads were next washed three times in PBSA with a magnet support (Dynal Biotech) and resuspended in 50 μl of PBSA. HIV-1 (1 ng of p24) was added to the Abs/beads mixture (50 μl), and the mixture was incubated overnight at 4°C on a rocking plate. Thereafter, immunomagnetic beads were washed four times in 200 μl of PBSA and finally resuspended in 200 μl of PBSA. Viruses captured on magnetic beads were disrupted by incubation for 30 min at room temperature in 50 μl of lysis buffer (PBS containing 2.5% Triton-X-100). Magnetic beads were pelleted with a magnetic plate (Dynal Biotech), and 125 μl of the cleared supernatants was loaded on an ELISA plate for p24 measurement, as mentioned above. Because CD45 has been shown to be excluded from HIV-1 (40, 41), magnetic beads on an ELISA plate for p24 measurement, as mentioned above. Because CD45 has been shown to be excluded from HIV-1 (40, 41), magnetic beads on an ELISA plate for p24 measurement, as mentioned above. Because CD45 has been shown to be excluded from HIV-1 (40, 41), magnetic beads on an ELISA plate for p24 measurement, as mentioned above. Because CD45 has been shown to be excluded from HIV-1 (40, 41), magnetic beads on an ELISA plate for p24 measurement, as mentioned above. Because CD45 has been shown to be excluded from HIV-1 (40, 41), magnetic beads on an ELISA plate for p24 measurement, as mentioned above. Because CD45 has been shown to be excluded from HIV-1 (40, 41), magnetic beads on an ELISA plate for p24 measurement, as mentioned above. Because CD45 has been shown to be excluded from HIV-1 (40, 41), magnetic beads on an ELISA plate for p24 measurement, as mentioned above. Because CD45 has been shown to be excluded from HIV-1 (40, 41), magnetic beads

**Virus attachment**

This biological test is based on incubation of fully competent HIV-1 particles with CH7C17 cells. Briefly, viruses (20 ng of p24) were incubated with 2.5 × 10⁵ CH7C17 cells for 1 h at 37°C in a total volume of 250 μl of complete culture medium. Next, cells were gently washed twice with 500 μl of PBS, and the pellet was resuspended in 150 μl of PBS prior measurements of the p24 content.

**Transient transfection and cell stimulation**

Cells (400-μl aliquots) were electroporated at room temperature using a gene pulser I apparatus (Bio-Rad, 960 μl, 250 V) with 5 μg of a reporter construct (i.e., pNF-κB-Luc, pNF-AT-Luc, or pLTRX-Luc). Cells were concentrated at 37.5 × 10⁶/ml in RPMI 1640 medium. To minimize variations in plasmid transfection efficiencies, cells were transfected in bulk and were separated into various treatment groups at a density of 10⁶ cells/ well (100 μl) in 96-well flat-bottom plates at 36 h posttransfection. A positive control consisted of cells treated with both PMA and Iono (20 ng of p24). In some instances, transfected cells were also incubated with paraformaldehyde-fixed 293T cells at a 4:1 ratio (i.e., 10⁵ T cells for 25 × 10⁵ 293T cells). Transfected cells were also exposed to various virus preparations, as indicated in the figure legends. On some occasions, cells were treated with the antiviral agent zidovudine (AZT) (10 μM) for 1 h and then washed before their use. In all experiments, cells were incubated at 37°C for 8 h in a final volume of 200 μl before monitoring luciferase activity, as described previously (42).

**Results**

**Insertion of peptide-loaded host HLA-DR proteins into HIV-1 is directly influenced by expression levels of such complexes on virus-producing cells**

Two different experimental strategies were used to produce HIV-1 particles bearing host HLA-DR molecules loaded with the HA peptide from influenza virus. The first approach relies on transient cotransfection of 293T cells with an infectious molecular clone of HIV-1 (i.e., pNL4-3) along with plasmids encoding for the α-chain of HLA-DR and β-chain that is covalently linked with the HA peptide (called HLA-DR(HA)). The second strategy is based also on transient cotransfection of 293T cells with pNL4-3, but this time with vectors encoding HLA-DR α- and β-chains in combination with an expression plasmid encoding for the invariant chain in which the core HLA-DR-associated invariant chain peptide (CLIP) sequence was either left unchanged (called HLA-DR(CLIP)) or substituted with the M. tuberculosis HSP 5–13 (called HLA-DR(HSP)) or HA sequence (called HLA-DR(HA)).

Progeny virus from 293T cells expressing the various HLA-DR proteins was captured with a comparable efficiency when using magnetic beads coated with anti-HLA-DR Abs (Fig. 1A), thus indicating that the tested HLA-DR molecules (i.e., empty or peptide loaded) are incorporated to a comparable degree in budding HIV-1 particles. Flow cytometry analyses indicate that, as expected, parental 293T cells are negative for HLA-DR (Fig. 1B). Importantly, comparable levels of HLA-DR molecules are expressed upon cotransfection of 293T cells with pNL4-3 and vectors encoding for the peptide-loaded HLA-DR molecules independently of the peptide nested in the peptide-binding groove (i.e., CLIP, HSP, or HA). Next, we assessed whether the surface level of HA-loaded HLA-DR proteins on 293T cells can modulate the process of incorporation of such complexes in HIV-1. To this end, parental 293T cells (used as a negative control) and 293T cells transfected with increasing amounts of vectors encoding for HA-loaded HLA-DR were used to produce virions. Virus capture assays (Fig. 1C) and flow cytometry analyses (Fig. 1D) revealed that there is a positive correlation between the levels of HA-loaded HLA-DR on the surface of transfected 293T cells and the virus recovery rates, thus suggesting that the degree of virus-anchored HA-loaded HLA-DR is linearly affected by the amounts of such complexes on the surface of virus-producer cells.

**Cells and HIV-1 particles bearing HA-loaded HLA-DR complexes mediate activation of NF-κB in HA-restricted CD4+ T cells**

The functionality of the studied peptide-loaded HLA-DR complexes was measured by using CH7C17, a human CD4+ T lymphoid cell line carrying a structurally and functionally active TCR, which specifically recognizes HA peptide 307–319 in the context of HLA-DR1. We first investigated whether 293T cells expressing HA-loaded HLA-DR molecules can lead to NF-κB induction in CH7C17 cells. Incubation of 293T cells expressing HLA-DR/HA complexes with CH7C17 that were transiently transfected with an NF-κB-driven vector resulted in activation of NF-κB (Fig. 2A). In this series of investigations, the PMA and Iono combination was used as a positive control, while parental 293T cells as well as 293T cells expressing empty HLA-DR molecules were used as negative controls. Similar observations were made when using the other experimental cell system, which relies on 293T cells that bear HLA-DR(CLIP), HLA-DR(HSP), or HLA-DR(HA) (Fig. 2B). For example, NF-κB was not induced by the two nonspecific Ags (i.e., CLIP and HSP), while this transcription factor was readily induced by cells carrying HLA-DR(HA) complexes. The following experiments were all performed with 293T cells expressing either HLA-DR(CLIP), HLA-DR(HSP), or HLA-DR(HA) complexes because it permits the production of HIV-1 particles differing only by the peptide located in the peptide-binding groove. The capacity of HIV-1 particles to present a nominal Ag was next monitored by producing virions in 293T cells either expressing HLA-DR(CLIP) or HLA-DR(HA) complexes. Incubation of such virus preparations with CH7C17 cells transfected with a NF-κB-dependent reporter construct demonstrated that virus-associated HLA-DR(HA) molecules are functional because it resulted in activation of NF-κB (Fig. 3). It should be noted that CH7C17 cells do not express the costimulatory molecule CD28 and, consequently, a high amount of HLA-DR(HA)-bearing viruses is necessary to initiate TCR-mediated signaling events (i.e., 1 μg of p24).

To ensure that the difference between virions bearing HLA-DR(CLIP) and HLA-DR(HA) with regard to NF-κB induction was not linked with a more important attachment of HLA-DR(HA)- vs HLA-DR(CLIP)-bearing HIV-1 particles, a virus attachment test
was performed. The virus stocks were found to bind with a similar efficiency to CH7C17 cells (i.e., $10^{3.0}/H_{11006}^2.2$, $137.0/H_{11006}^2.2$, $106.3/H_{11006}^{13.8}$, $123.3/H_{11006}^{10.5}$ pg/ml p24 for Null, HLA-DR(CLIP), HLA-DR(HSP), and HLA-DR(HA) virions, respectively), therefore indicating that the nature of the peptide loaded within HLA-DR molecules has little influence on the strength of virus binding to human T cells expressing a functional TCR.

**Insertion of the costimulatory CD86 molecule in HLA-DR(HA)-bearing viruses further augments NF-κB activation**

The previous report showing that CD86-bearing HIV-1 can mediate signal transduction (4) prompted us to assess signal transduction events initiated by viruses bearing both peptide-loaded MHC-II and CD86 molecules. To this end, we used CH7C17

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**FIGURE 1.** Peptide-loaded HLA-DR molecules are acquired by HIV-1, and the process of incorporation is under the influence of surface expression levels on virus-producing cells. A, 293T cells were transfected with pNL4-3 (called Null) or cotransfected with pNL4-3 and the listed HLA-DR-encoding vectors. B, Transfected cells were analyzed by flow cytometry. C, 293T cells were cotransfected with pNL4-3 and increasing amounts of a vector encoding for HA-loaded HLA-DR (ranging from 0.31 to 5 μg). D, Transfected cells were analyzed by flow cytometry. A and C, Similar amounts of each virus stock (1 ng of p24) were incubated with magnetic beads coated with anti-HLA-DR Abs (clone L243). Magnetic beads coated with an isotype-matched anti-CD45 Ab served as controls to estimate the background levels of captured viruses. The data shown are the mean (±SD) of quadruplicate samples and are representative of three independent experiments. B and D, Cells were incubated with an Ab specific for HLA-DR (clone L243) in combination with a (R)-PE-conjugated goat anti-mouse IgG (dotted lines). Controls consisted of cells incubated with an isotype-matched irrelevant mAb (solid lines).
derivatives expressing CD28WT or a CD28 signal-defective mutant (called CD28Del.30). A marked activation of NF-κB was seen only in CD28WT cells upon incubation with either 293T cells expressing HLA-DR(HA) or viruses bearing host HLA-DR(HA) complexes (19.2- and 10.6-fold increase, respectively) (Fig. 4A). No such induction of NF-κB could be seen when using CD28Del.30 cells. It is important to emphasize that the CD86-CD28 cosstimulatory pathway allows a significant activation of NF-κB with a much lower concentration of virus (i.e., 25 ng of p24 as opposed to 1 μg of p24 in Fig. 3). Experiments performed with an NF-AT-driven reporter gene indicated that NF-AT was also activated in CD28WT cells when incubated with 293T cells or viruses bearing HLA-DR(HA) (22.9- and 6.23-fold increase, respectively) (Fig. 4B). Again, no such induction was achieved using CD28Del.30 cells. FACS analyses revealed that both CD28WT and CD28Del.30 cell lines express similar levels of CD28 (Fig. 4C). To eliminate the possibility that virus gene expression can be responsible to a certain extent to the observed activation of NF-κB, experiments were also conducted in CD28WT cells that were pretreated with AZT. Addition of this antiviral agent had no effect on NF-κB induction that is seen upon incubation of CD28WT cells with virions bearing host HLA-DR(HA) and CD86 proteins on their surface (data not shown). This confirms that virus-anchored HLA-DR(HA) and CD86 can still interact with their respective ligands on CD4+ T cells (i.e., TCR and CD28), a process culminating in activation of both NF-κB and NF-AT. We tested also whether there is a putative association between the amount of peptide-loaded HLA-DR incorporated in HIV-1 and the virus-dependent NF-κB signaling pathway. Progeny virus were produced in 293T cells cotransfected with a fixed amount of CD86-encoding plasmid and increasing levels of the HLA-DR(HA) expression vector. Thereafter, CD28WT cells were transiently transfected with the NF-κB-directed luciferase vector prior to exposure to the virus stocks. Activation of the studied transcription factor was in a linear relationship with the sum of HA-loaded HLA-DR complexes that are found embedded within HIV-1 particles (Fig. 5).

Exosomes and/or microvesicles are not implicated in HIV-1-mediated activation of NF-κB

Exosomes and microvesicles (E/M) secreted from professional APCs have been shown to carry HLA-DR molecules, and exosomes can present Ags to human T cells. Thus, we tested the possible involvement of HLA-DR-bearing E/M in the present findings and also whether virus gene expression could lead to an increased production of E/M in the tested virus producer cells (i.e., 293T). We could not detect any NF-κB induction when using supernatants from 293T cells that were cotransfected with vectors encoding for HLA-DR(HA) and CD86 (data not shown). Cell-free

![FIGURE 2](image-url)  
**FIGURE 2.** Cells expressing peptide-loaded HLA-DR complexes mediate NF-κB induction in HA-restricted CH7C17 cells. A, CH7C17 cells were first transfected with pNF-κB-Luc. Next, cells (1 × 10⁶) were either left untreated, treated with PMA/Iono (20 ng/ml and 1 μM, respectively), incubated with 293T cells (control), or incubated with 293T cells bearing either HLA-DR or HA-loaded HLA-DR (at a 4:1 ratio). B, CH7C17 cells were first transfected with pNF-κB-Luc. Cells (1 × 10⁶) were next either left untreated, treated with PMA/Iono (20 ng/ml and 1 μM, respectively), incubated with 293T cells, or incubated with 293T cells bearing either HLA-DR or HLA-DR loaded with CLIP, HSP, or HA (at a 4:1 ratio). Cells were incubated at 37°C for 8 h. Finally, the cells were lysed, and luciferase activities were monitored, as described in Materials and Methods. The results shown are from quadruplicate samples and are expressed as the fold induction relative to the basal luciferase activity in the untreated control (i.e., CH7C17 cells transfected with pNF-κB-Luc). These data are representative of three separate experiments.

![FIGURE 3](image-url)  
**FIGURE 3.** NF-κB is induced in CH7C17 cells upon incubation with HIV-1 particles bearing HA-loaded host HLA-DR. CH7C17 cells were first transfected with pNF-κB-Luc. Cells (1 × 10⁶ cells) were next either left untreated, treated with PMA/Iono (20 ng/ml and 1 μM, respectively), incubated with HIV-1-bearing HLA-DR(CLIP), or incubated with HIV-1-bearing HLA-DR(HA) (1 μg of p24). Cells were incubated at 37°C for 8 h. Finally, the cells were lysed, and luciferase activities were monitored, as described in Materials and Methods. The results shown are the means (±SD) from quadruplicate samples and are expressed as luciferase activity (relative light unit (RLU)). These data are representative of three independent experiments.
supernatants from 293T cells transfected either with the vector encoding for HLA-DR(HA) or the infectious molecular clone of HIV-1 and the vector encoding for HLA-DR(HA) were subjected to a velocity-gradient-based method (i.e., OptiPrep gradients) to separate viruses and E/M. From the OptiPrep gradient, 11 fractions were collected and subjected to ELISA tests specific for HLA-DR and p24. Viral p24 Gag proteins were found to peak in fractions 8–10 (data not shown). The highest concentrations of HLA-DR were found in fractions 5–8 when testing supernatants from 293T cells that were transfected only with the vector encoding for HLA-DR(HA) (Fig. 6). Interestingly, a marked reduction in HLA-DR contents in fractions 5–8 was observed in samples from 293T cells that were cotransfected with HLA-DR(HA) and NL4-3 plasmids, therefore suggesting that production of E/M is diminished in the presence of HIV-1.

Transcription of unintegrated and integrated HIV-1 genome in HA-restricted CD4+ T cells is promoted by viruses bearing host HLA-DR(HA) and CD86

Because early phase HIV-1 transcription is regulated by cellular transcription factors, we monitored the impact of the studied virus preparations on HIV-1 LTR transcriptional activity. This goal was reached using CD28WT cells that were transiently transfected with pNF-κB-Luc or pNF-AT-Luc. These data are representative of three independent experiments.

FIGURE 5. Virus-mediated activation of NF-κB correlates positively with the amount of HA-loaded HLA-DR inserted within CD86-bearing HIV-1. CD28WT cells were first transfected with pNF-κB-Luc. Cells (1 × 10⁶ cells) were next either left untreated, treated with PMA/Iono (20 ng/ml and 1 μM, respectively), or incubated with HIV-1 particles produced by 293T cells expressing fixed levels of CD86 and HLA-DR(CLIP) or expressing a fixed level of CD86 and increasing amounts of HLA-DR(HA) complexes (25 ng of p24). Cells were incubated at 37°C for 8 h. Finally, the cells were lysed and luciferase activities were monitored, as described in Materials and Methods. The results shown are from quadruplicate samples and are expressed as the fold induction relative to the basal luciferase activity in the untreated control (i.e., CD28WT cells transfected with pNF-κB-Luc). These data are representative of three independent experiments.

Transcription of unintegrated and integrated HIV-1 genome in HA-restricted CD4+ T cells is promoted by viruses bearing host HLA-DR(HA) and CD86

Because early phase HIV-1 transcription is regulated by cellular transcription factors, we monitored the impact of the studied virus preparations on HIV-1 LTR transcriptional activity. This goal was reached using CD28WT cells that were transiently transfected with pLTRX-Luc, a vector containing the luciferase gene controlled by the complete HIV-1 LTR region. The results shown are from quadruplicate samples and are expressed as the fold induction relative to the basal luciferase activity in the untreated control (i.e., CD28WT cells transfected with pNF-κB-Luc or pNF-AT-Luc). These data are representative of three separate experiments. C, CD28WT and CD28Del.30 cells were incubated with an Ab specific for CD28 (clone 9.3) in combination with a (R)-PE-conjugated goat anti-mouse IgG. Controls consisted of cells incubated with an isotype-matched irrelevant mAb (i.e., IgG2A).
E/M and to confirm our previous findings with respect to NF-κB induction, we used cell-free supernatants from 293T cells transfected with different sets of encoding vectors in the absence of the infectious molecular clone of HIV-1. Activation of HIV-1 LTR-directed reporter gene expression was observed only when cells were incubated with virions bearing host-derived HLA-DR(HA) and CD86 (Fig. 7A). To more closely approximate natural conditions, experiments were performed also with reporter HIV-1 particles pseudotyped with the VSV-G envelope protein. Transcription of integrated HIV-1 in CD28WT cells was increased by 293T cells expressing HLA-DR(HA) and CD86 (Fig. 7B). More importantly, viruses bearing host-encoded HLA-DR(HA) and CD86 were also found to up-regulate transcription of integrated viral genetic material.

Discussion

The initial contact between HIV-1 and its target cell has been demonstrated to result in transduction of biochemical signals. For example, binding of Env glycoproteins to chemokine receptors activates calcium flux and chemokinin CD4+ T cells (43), and gp120 induces apoptosis in neurons (44), CD4+ T cells (45), and CD8+ T lymphocytes (46). Cross-linking of CD4 with heat-inactivated virus induces signal transduction, resulting in activation of the cellular transcription machinery (47). It has been demonstrated that binding of HIV-1 to its target leads to phosphorylation of PI3K (48). Moreover, the HIV-1 Env can interact not only with CD4 and chemokine receptors, but also with polyansonic sulfated chains or lectin-like domains on some specific cell surface receptors that may initiate signal transduction upon their engagement. For example, gp120 have been shown to associate with the dendritic cell-specific ICAM-3-grabbing nonintegrin (also called CD209) (reviewed in Ref. 49), glycolipid galactocerebroside and its sulfated derivative (50, 51), glycosaminoglycan heparan sulfate (52, 53), placental membrane-binding protein (now called dendritic cell-specific ICAM-3-grabbing nonintegrin) (54), and macrophage mannose receptor (55). Interaction between virus-acquired host cell membrane constituents with their respective cellular counterparts may also generate signaling that might result in a change in the activation status of the cell. This is supported by a study showing that interaction between virus-bound costimulatory molecule CD86 and its cellular cognate ligand mediates nuclear translocation of NF-κB and NF-AT upon a concomitant engagement of the TCR (4). This last observation when coupled with the previous demonstration that HLA-DR molecules enable HIV-1 to present superantigens (20) led us to investigate whether HIV-1 particles bearing the appropriate combination of host cell membrane proteins, i.e., HLA-DR loaded with a nominal Ag and CD86, can trigger intracellular biochemical changes similar to those induced upon Ag presentation.
Our experiments showed that not only can HIV-1 particles bearing HA-loaded MHC-II molecules activate NF-κB in an Ag-specific human T lymphocyte cell line, but the induction of this transcription factor is amplified when host CD86 is also inserted within the viral entity. We also provide evidence that the virus adhesion strength is not modified upon insertion of peptide-loaded HLA-DR molecules onto the surface of virions, thus suggesting that the observed signal transduction events are not due to a change in HIV-1-binding capacity, but rather to interactions between virus-anchored host proteins and their natural counterreceptors. Based on the present results, it can be proposed that host cell membrane proteins once found embedded within the newly formed viruses enable the virus to establish in CD4+ T lymphocytes a microenvironment more appropriate for productive HIV-1 infection. This postulate is based on the idea that replication of HIV-1 in T cells is influenced by the proliferative state of the cells. Indeed, productive infection by HIV-1 requires the activation of target cells. Infection of quiescent peripheral CD4+ T cells is influenced by the proliferative state of the cells. Indeed, productive infection by HIV-1 requires the activation of target cells. Infection of quiescent peripheral CD4+ T lymphocytes by HIV-1 results in incomplete, labile, reverse transcripts. More precisely, it means that virions bearing the appropriate combination of host-encoded molecules could potentially activate CD4+ T cells restricted to a specific Ag and then create an intracellular environment that is adequate for a complete virus life cycle. It is of interest to note that a previous work has indicated that HIV-1-specific CD4+ T cells are preferentially infected by HIV-1 in vivo because they contain more virus-specific DNA than other memory CD4+ T cells, at all stages of the disease (56). In contrast, virions bearing an improper set of host-derived molecules could initiate inadequate signaling events in circulating T cells that could be responsible for functional defects observed in CD4+ T cells. In patients infected with HIV-1, the immunosuppressive state is resulting from functional defects in CD4+ T lymphocytes and a progressive loss of this cell type (reviewed in Refs. 57 and 58). Our results may thus in part explain the functional defects observed in patients experiencing HIV-1 infection. Additional experiments will be required to shed light on this matter. A recent study showed that the HIV-1 regulatory Nef protein impairs MHC-II presentation and surface expression (59). These results raise the possibility that HIV-1 can incorporate peptide-loaded MHC-II molecules that are nonfunctional. However, this study was performed with an established cell line (i.e., HeLa) rather than primary human cells. Additional studies are needed to assess whether both Ag presentation and surface expression of MHC-II are affected by Nef in primary human cells, and to evaluate the functionality of MHC-II molecules incorporated in HIV-1 under natural conditions.

All experiments described in the current work were performed with a fully competent X-tropic isolate of HIV-1 (i.e., NLA-4). Unpublished observations from our laboratory indicate that Ag presentation can also be seen when using Env-deficient HIV-1 particles. Given that the viral tropism is conferred by Env, it can thus be proposed that R5 (macrophage-tropic) and R5X4 (dual tropic) HIV-1 variants can also present a nominal Ag, assuming that such virions carry the appropriate host-derived cell surface constituents. Interestingly, R5- and R5X4-tropic primary isolates of HIV-1 do incorporate substantial amounts of host-derived HLA-DR molecules (39). Although the relevance of our findings to in vivo infection of primary cells is difficult to establish, it is important to emphasize that comparative flow cytometry analyses indicated that primary human monocyte-derived macrophages express higher levels of cell surface HLA-DR compared with transiently transfected 293T cells (data not shown). This suggests that the amount of HLA-DR molecules that can be incorporated into HIV-1 upon more natural conditions will be at least equivalent to the level of HLA-DR that is acquired by virions produced through transfection. Moreover, it was previously shown that virally embedded host HLA-DR outnumbered HIV-1-encoded gp120 by a factor of 1.7 to 2.8 (15). This represents ~367–604 HLA-DR molecules per single virion. It has been previously estimated that ~8000 TCRRs must be engaged to lead to T cell activation (60). A study conducted by Lanzavecchia and colleagues (61) proposed that a single MHC-peptide complex can trigger up to 100 TCRRs, thus explaining how a small number of peptide-MHC complexes can achieve T cell activation. Altogether these findings provide some credence and physiological relevance to our observations because a single viral entity bears a number of MHC-II molecules that might be sufficient to achieve the minimal threshold of TCRRs necessary to achieve T cell activation.

Although we present evidence that exosomes are not involved in the observed phenomenon, it can also be proposed that the physical presence of some specific host cell surface membrane proteins onto HIV-1 might affect the progression of the disease through the production of exosomes. Exosomes are small MHC-II-bearing vesicles (50–90 nm) that originate from late endosomes and are secreted by a broad array of hemopoietic and epithelial cells (62–65). The molecular characterization of dendritic cell-derived exosomes highlighted the presence, among others, of HSP hsc73 and CD86. Exosomes have also been shown to carry ICAM-1 on their exterior (66), and more importantly, it was recently shown that TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/ζ complex (67). The selective enrichment on exosomes of proteins involved in cell-to-cell contact (CD11b and tetraspans) and targeting (lactadherin) strongly suggested that such multivesicular bodies might initiate or amplify T cell-based immune response in vivo (68–70). The demonstration that exosomes can stimulate immune responses in vivo and the fact that these small membrane vesicles of endocytic origin have roughly a diameter comparable to HIV-1 reinforce our hypothesis that HIV-1 can present a nominal Ag to T cells. A more complete understanding of the role played by virus-associated proteins of host origin in HIV-1 infection is mandatory because it might help developing better strategies to fight this retrovirus.

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Disclosures

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