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New Insights into the Functionality of a Virion-Anchorered Host Cell Membrane Protein: CD28 Versus HIV Type 1

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It is now well established that the HIV type 1 (HIV-1) incorporates a vast array of host-encoded molecules in its envelope during the budding process. Interestingly, it was demonstrated that the attachment process is accentuated by supplementary interactions between virion-anchored host molecules and their cognate ligands. Such an enhancement of the viral attachment process was found to result in an increase of infectivity for both T and macrophage-tropic strains of HIV-1. Given that previous work indicates that HIV-1 is budding at the site of cell-to-cell contact, a location rich in the costimulatory CD28 glycoprotein, we investigated whether CD28 could be efficiently acquired by HIV-1. We have been able to generate progeny viruses bearing or not bearing on their surfaces host-derived CD28 using our previously described transient transfection and expression system. The physical presence of CD28 was found to markedly increase virus infectivity in a CD28/B7-dependent manner following infection of two human lymphoid cell lines expressing high levels of surface B7-1/B7-2, two natural ligands of CD28. The physiological significance of CD28 incorporation was provided by the observation that an anti-CD28 Ab decreased replication in primary human mononuclear cells of clinical isolates of HIV-1 propagated in such cells. A virus precipitation assay revealed that M-, T-, and dual-tropic clinical strains of HIV-1 produced in primary human mononuclear cells do indeed incorporate CD28. These results show for the first time that HIV-1 can incorporate CD28 and the acquisition of this specific host surface glycoprotein modulates the virus life cycle. *The Journal of Immunology*, 2002, 169: 2762–2771.

Enveloped retrovirus particles are formed by extrusion through the host cell membrane. During this process, the newly formed viral entities become coated with a lipid bilayer derived from the cell membrane. The specificity of this process is questioned by the observation of viral pseudotyping. Pseudotyping with heterologous viral glycoproteins has been observed on many occasions, e.g., murine leukemia virus envelope proteins in HIV type 1 (HIV-1) particles (1), envelope proteins from human T cell leukemia virus types 1 and 2 in vesicular stomatitis virus (2), influenza virus hemagglutinin in Rous sarcoma virus particles (3), human T cell leukemia virus-1 envelope glycoprotein in HIV-1 (4), and vesicular stomatitis virus G protein in both murine retroviral and lentivirus vector particles (5). The low specificity of the process governing glycoprotein incorporation into retroviral particles is most likely responsible for the observation that retroviruses have been found to incorporate certain cell-derived proteins in their envelope. For example, HIV-1 has been reported to acquire a considerable number of cell-surface proteins including CD3, CD11a (LFA-1), CD11b (Mac-1), CD18, CD25, CD43, CD44, CD54 (ICAM-1), CD55, CD59, CD63, CD71 (transferrin receptor), HLA-DR, HLA-DP, and HLA-DQ (6).

Several studies have scrutinized the functionality of some virion-anchored host proteins with respect to the biology of HIV-1. The physical presence of host-encoded CD55 and CD59 was found to protect the virus from complement-mediated virolysis (7). Castilletti and coworkers (8) have reported that virus infectivity is increased following treatment of U937 monocytes with IFN-γ due to an enhancement of virion-bound host ICAM-1 and HLA-DR proteins. MHC class-II (MHC-II) proteins on purified HIV-1 particles can present super Ag to human T cells (9). In addition, a synergy in virus neutralization was demonstrated to occur between an anti-LFA-1 mAb and polyclonal anti-HIV-1 Abs (10). The incorporation of ICAM-1 rendered HIV-1 virions less susceptible to Ab-mediated neutralization (11, 12). Data from time-course and infectivity experiments revealed that the kinetics of infection was more rapid for virosions bearing host-derived MHC-II glycoproteins than for HIV-1 particles devoid of host MHC-II (13). Additional experiments revealed that the presence of host-derived HLA-DR1 and ICAM-1 on HIV-1 led to an enhancement of virus infectivity (1.6- to 2.3-fold increase for HLA-DR1; 4.6- to 9.8-fold increase for ICAM-1) by accelerating the kinetics of virus entry and/or increasing the efficiency of the early steps in the viral life cycle (14, 15). Other studies revealed that surface expression of LFA-1 in its high-affinity state for ICAM-1 markedly enhanced susceptibility of human cells to infection by ICAM-1-bearing HIV-1 particles (11, 16). Recently, infectivity of primary and laboratory Tropic isolates of HIV-1 was increased upon incorporation of foreign MHC-I molecule (17).
The homodimeric CD28 glycoprotein is not present on the surface of cells of the monocyte/macrophage lineage, but is constitutively expressed on virtually all human CD4+ T lymphocytes and ~50% of CD8-expressing T cells (18, 19). The CD28-mediated signal transduction pathway is considered as one of the dominant costimulatory pathways to achieve the complete activation of the T cell (20). The CD28 cosignal is triggered by the ligation of CD28 with its physiological B7-1 (CD80) and B7-2 (CD86) counterligands, which are normally expressed on the surface of the APC. CD28 must be physically located near the cell-to-cell contact site between the T cell and the APC to be able to bind to its natural counterreceptors. Given that a unidirectional budding of HIV-1 has been shown to occur at the site of cell-to-cell contact (21), it can be proposed that CD28 is also present within the virus envelope. The possible acquisition of CD28 by HIV-1 might reveal functional implications considering that B7-1 and B7-2 molecules are expressed on activated monocytes/macrophages as well as on activated T lymphocytes (18, 22–25), two cell types recognized as reservoirs of HIV-1 in infected individuals. Thus, the primary objective of the current work was to define whether host-derived CD28 is incorporated into the HIV-1 envelope, and if so, to study the functional effect(s) on the HIV-1 biology of such a virus-anchored host constituent.

Materials and Methods

Cells

RAJI is an EBV-carrying B cell line that has been reported to express very high levels of cell surface MHC-II molecule (26). Moreover, RAJI cells have been shown to express similar high levels of B7-1 (CD80) and B7-2 (CD86) (27). The RAJI cell line was rendered susceptible to HIV-1 infection by stable transfection with a cDNA encoding for human CD4 (i.e., RAJI-HIV). Flow cytometric analysis indicated that RAJI and RAJI-CD4 cell lines express comparable levels of CCR5 and are both negative for CCR2 (data not shown). The LuSIV cell line was derived from the CEM × 174 cells and was stably transfected with the HIV-Vpr239 long terminal repeat (~225 → +149) cloned upstream of the firefly luciferase reporter gene (kindly supplied by Dr. J. E. Clements, Johns Hopkins University School of Medicine, Baltimore, MD). LuSIV cells are highly sensitive to HIV and SIV, resulting in a rapid and strong expression of the reporter gene, which correlates with viral infectivity (28). FACS analysis revealed that LuSIV cells express high levels of both B7.1 and B7.2 molecules (data not shown). RAJI, RAJI-CD4, and LuSIV cell lines were maintained in complete culture medium made of RPMI 1640 supplemented with 10% FBS (Invitrogen, San Diego, CA), glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (100 μg/ml). 293T cells are human embryonic kidney cells that express the simian virus 40 large T Ag and were kindly supplied by Dr. W. C. Greene (J. Gladstone Institutes, San Francisco, CA). These cells were cultured in DMEM supplemented with 10% FBS, 1-glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (100 μg/ml). Flow cytometric analysis revealed that 293T cells are negative for CD28 expression (data not shown). Primary human PBMCs from healthy donors were isolated by Ficoll-Hypaque density gradient centrifugation. To obtain monocyte-derived macrophages (MDM), PBMCs were incubated for 3 h at 37°C in 48-well flat-bottom tissue culture plates (Microtest III, Falcon; BD Biosciences, Lincoln Park, NJ) (3 × 10^6 cells/ml, 500 μl/well). Cells were next washed twice with PBS to remove unadhered cells and kept in culture for 3 days in RPMI 1640 supplemented with 20% FBS in the presence of IFN-γ (500 U/ml). Human tonsillar tissues were filtered through a 0.45-μm cellulose acetate membrane to remove cellular debris. The standardization on p24 contents is based using an in-house double Ab sandwich ELISA specific for the major viral p24 protein (29). The immobilization of p24 contents is based on our previous observation indicating that virus preparations harvested from transfected 293T cells contain minimal amounts of p24 that are not associated with infectious virions (15). In some experiments, clinical strains of HIV-1 were produced in acutely infected PBMCs from healthy donors. At the maximal virus production and before extensive cytopathic effects were seen, cells were centrifuged at 300 × g for 5 min and the virus-containing supernatants were clarified at 2000 × g for 30 min and filtered through a 0.45-μm cellulose acetate membrane to remove cellular debris. Therefore, the virus-containing supernatants were stored at ~80°C in aliquots. Two X4 Tropic (i.e., 92HT599 and 93UG070), one R5 macropage tropic (i.e., 92HT026), and one R5X4 dual tropic (i.e., 92R9009) primary isolates of HIV-1 were used in our studies (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD).

Abs and purified fusion/recombinant proteins

The mAb 9.3 is specific for human CD28 and inhibits interaction between CD28 and B7-1/B7-2 (32, 33), while BB-1 is a neutralizing mAb directed against B7-1 (CD80) (34). Both Abs were kindly provided by Dr. J. A. Ledbetter (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). The two mAbs, BU-63 and FUN-1, were shown to block the B7-1 (CD80)/CD28 interaction by 10% (35). BU-63 has been supplied by Dr. D. L. Hardie (University of Birmingham, Birmingham, U.K.) and FUN-1 is a kind gift from Dr. Y. Nozawa (Fukushima Medical University, Fukushima, Japan). CTLA-4lg is constituted of the extracellular domain of CTLA-4 fused to the Fc fragment of IgG1. Previous experiments have indicated that this fusion protein demonstrates a strong affinity for B7-1 and B7-2 molecules and blocks the interaction between CD28 and B7.2 (36). FITC-conjugated goat anti-mouse Ab was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Sim.2 is a mAb specific for human CD4 that binds to a different epitope than Leu 3a, whereas Sim.4 is also an anti-CD4 Ab, but recognizes the same epitope as Leu 3a (37). Both Abs block the process of HIV-1 infection, although Sim.2 shows a stronger anti-HIV-1 capacity (38).

Cytometric analyses

Expression of CD28 on the surface of transiently transfected 293T cells was monitored with the anti-CD28 9.3 Abs. Briefly, an aliquot of transfected 293T cells (1 × 10^6) was washed with PBS (pH 7.4). Pelleted cells were incubated for 30 min on ice with a saturating concentration of the primary 9.3 Ab (1 μg/10^6 cells) in a final volume of 100 μl of PBS. The cells were washed twice with 500 μl of PBS and incubated for 30 min with a saturating concentration of a FITC-conjugated goat anti-mouse Ab (1 μg/10^6 cells) in a final volume of 100 μl of PBS. Finally, cells were washed twice with PBS and resuspended in 300 μl of PBS containing 1% (w/v) paraformaldehyde before cytometric analysis (EPICS Elite ESP; Coulter Electronics, Miami, FL). The same technical strategy was used for monitoring expression of B7-1 and B7-2 on the surface of target cells used for HIV-1 infection. In this case, monoclonal anti-B7-1 (clone BB-1) and anti-B7-2 (clone FUN-1) Abs were used. Surface expression of CD28 on CD4-positive T lymphocytes from PBMCs originating from a single healthy donor was monitored by two-color flow cytometry. PBMCs (1 × 10^6) were first incubated for 30 min on ice with a saturating concentration of the 9.3 Ab (1 μg/10^6 cells) in a final volume of 100 μl of PBS. The cells were washed twice with 500 μl of PBS and incubated for 30 min with a saturating concentration of PE-conjugated goat anti-mouse Ab (1 μg/10^6 cells) in a final volume of 100 μl of PBS. Cells were then washed twice and incubated for 30 min with an FITC-conjugated anti-CD4 Ab (i.e., SIM.4). Cells were then washed twice and resuspended in 300 μl of PBS containing 1% (w/v) paraformaldehyde before cytometric analysis.

Virus capture assay

The physical presence of host-encoded CD28 glycoproteins on the surface of HIV-1 particles was investigated using streptavidin-coated magnetic beads in combination with a biotinylated monoclonal anti-CD28 Ab as described previously (39). In brief, after incubation of streptavidin-coated magnetic beads with biotinylated Ab (1 h at room temperature), beads were washed twice and resuspended in PBS + 0.1% BSA before used for capture assay. Immunomagnetic beads were incubated overnight at 4°C on a rotating plate with studied virus preparations (2.5–10 ng of p24). Next, beads were extensively washed and resuspended in PBS + 0.1% BSA + viral p24 contents were quantified by the p24 assay. A complex made of streptavidin-coated magnetic beads and biotinylated monoclonal anti-CD45 Ab (clone UCHL-1) was used as a negative control based on the
observation that CD45 is excluded from the virus envelope (40). A more physiological assay was used to assess the presence of foreign CD28 proteins on the exterior of viral entities. This biological test is based on incubation of virus preparations with RAJI, a CD4-negative cell line expressing similar high surface levels of B7-1 and B7-2 (27). Briefly, virus stocks (20 ng of p24) were exposed to 2.5 × 10^3 RAJI 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 PBS and the pellet was resuspended in 150 μl PBS before monitoring for p24 content. Appropriate controls consisted of the RAJI/virus mixture incubated in the presence of either CTLA-4Ig, an anti-B7-1 (clone BB-1), or an anti-B7-2 (clone FUN-1) Ab.

Virus infection and luciferase assay

Similar amounts of each recombinant luciferase-encoding virus stocks (10 ng of p24 for CD28^- and CD28^+) were used to inoculate RAJI-CD4 cells (1 × 10^5) in a 96-well flat-bottom tissue culture plate (Microtest III, Falcon; BD Biosciences). In some experiments, cells were either left untreated or were treated with CTLA-4, the anti-B7-1 Ab BB1, or the anti-B7-2 Ab BU-63 (10 μg/ml) for 30 min at 4°C before inoculation with HIV-1. Cells were also treated when appropriate for 5 min at 37°C with increasing concentrations of the anti-CD4 SIM.2 Ab (0, 0.2, 1, 2.5, 5, and 10 μg/ml) before virus infection (5 ng of p24 for CD28^- or CD28^+ virions). Virus preparations used for the infection of LuSIV cells (strain 89.6) were pretreated or not for 30 min at 37°C with the anti-CD28 mAb (clone 9.3) or an isotype-matched irrelevant control Ab (1 μg/ml final concentration) before infection with HIV-1 (10 ng of p24 and 1 × 10^5 cells). Cells were kept incubated at 37°C for 48 h (RAJI-CD4 and LuSIV). Finally, cells were lysed and luciferase activity was monitored using a microplate luminometer (MLX; Dynex Technologies, Chantilly, VA). Luciferase activity is expressed as relative light units.

HIV-1 infection of MDM and tonsils

MDM were infected with the 89.6 strain of HIV-1 as follow. Culture medium was first removed from the 48-well plates and replaced by an equal amount of each virus preparation (CD28^- and CD28^+) for 1 h at 37°C (20 ng of p24 in a final volume of 200 μl). The cells were next washed twice with PBS and 500 μl of fresh medium was added. After 3 days of infection, the supernatant was harvested and p24 was measured by ELISA. Tonsil preparations used for the infection of LuSIV cells (strain 89.6) were pretreated or not for 30 min at 37°C with the anti-CD28 mAb (clone 9.3) or an isotype-matched irrelevant control Ab (1 μg/ml final concentration). Cells were kept incubated at 37°C for 48 h (RAJI-CD4 and LuSIV). Finally, cells were lysed and luciferase activity was monitored using a microplate luminometer (MLX; Dynex Technologies, Chantilly, VA). Luciferase activity is expressed as relative light units.

Viral entry assay

RAJI-CD4 cells (5 × 10^6 cells/ml, 250 μl/well) were exposed to similar amounts of CD28^- and CD28^+ virus preparations (50 ng of p24) in complete culture medium for 2 h at 37°C. Cells were washed twice with 250 μl of ice-cold PBS and were next incubated for 5 min at 4°C with 250 μl of cold RPMI 1640 (without FBS) supplemented with pronase (Boehringer Mannheim, Laval, Quebec, Canada) at 0.1 mg/ml. Cells were washed immediately with 2 ml of ice-cold RPMI-10 containing 10% FBS and three times with ice-cold PBS to eliminate pronase. Cells were resuspended in 1 ml of complete culture medium to which was added 200 μl of disruption buffer (0.5% Triton X-100 in PBS). Finally, cells were agitated for 10 min at room temperature and then stored at −20°C until assayed for p24 content.

Results

The host-derived costimulatory molecule CD28 is efficiently incorporated within the HIV-1 envelope

We initially used our previously described transient transfection and expression system (15, 16) to define whether host cell surface CD28 glycoprotein is efficiently acquired by mature HIV-1 particles. To this end, we transiently cotransfected 293T cells with an infectious molecular clone of HIV-1 (pHXB-Luc) and a mammalian expression vector coding for the human CD28 glycoprotein (pHB-Apr-1-neo). Flow cytometric studies indicated that cotransfection with both vectors resulted in expression of a high level of CD28 in 293T cells that do not constitutively express this cell surface constituent (Fig. 1). Next, the presence of the costimulatory molecule CD28 on the exterior of HIV-1 particles was assessed using a functional binding assay. Given that RAJI cells are CD4-negative and are expressing similar elevated levels of the cognate ligands of CD28, i.e., B7-1 and B7-2 (data not shown), we hypothesized that such cells could capture virions in a CD28-B7-1/B7-2-dependent mode. To directly assess the role played by CD28-B7-1/B7-2 interaction in the possible attachment of CD28-bearing progeny viruses on the surface of RAJI, experiments were also conducted in the presence of the CTLA-4Ig fusion protein because it binds to both B7-1 and B7-2, but with a 20- to 100-fold higher affinity than CD28 (41). As shown in Fig. 2, CD28^- viruses were found to bind more significantly to RAJI cells as compared with CD28^+ virions (4.5-fold increase, 416 vs 93 pg of captured viral particles per 10^5 RAJI cells). Similar amounts of each recombinant luciferase-encoding virus stocks (10 ng of p24 for CD28^- and CD28^+) were used to inoculate RAJI-CD4 cells (1 × 10^5) in a 96-well flat-bottom tissue culture plate (Microtest III, Falcon; BD Biosciences). In some experiments, cells were either left untreated or were treated with CTLA-4, the anti-B7-1 Ab BB1, or the anti-B7-2 Ab BU-63 (10 μg/ml) for 30 min at 4°C before inoculation with HIV-1. Cells were also treated when appropriate for 5 min at 37°C with increasing concentrations of the anti-CD4 SIM.2 Ab (0, 0.2, 1, 2.5, 5, and 10 μg/ml) before virus infection (5 ng of p24 for CD28^- or CD28^+ virions). Virus preparations used for the infection of LuSIV cells (strain 89.6) were pretreated or not for 30 min at 37°C with the anti-CD28 mAb (clone 9.3) or an isotype-matched irrelevant control Ab (1 μg/ml final concentration). Cells were kept incubated at 37°C for 48 h (RAJI-CD4 and LuSIV). Finally, cells were lysed and luciferase activity was monitored using a microplate luminometer (MLX; Dynex Technologies, Chantilly, VA). Luciferase activity is expressed as relative light units.

FIGURE 1. Flow cytometric analysis of 293T cells transfected with pHXB-Luc and a vector coding for human CD28. 293T cells were transiently transfected with pHXB-Luc alone (A) or were cotransfected with pHXB-Luc and pHB-Apr-1-neo (B). Surface expression of CD28 was monitored by labeling cells with a monoclonal anti-CD28 Ab (clone 9.3, dotted lines) or an isotype-matched irrelevant commercial control Ab (straight lines) followed by an FITC-conjugated goat anti-mouse IgG Ab.

FIGURE 2. Capture of isogenic CD28^- and CD28^+ HIV-1 particles with RAJI cells. Similar amounts of CD28^- and CD28^+ virions, standardized in terms of p24 content, were incubated with RAJI cells for 1 h at 37°C in the absence or the presence of the CTLA-4Ig fusion protein. Cells were next washed several times with PBS and the levels of RAJI-associated viral p24 were estimated by performing an in-house p24 enzymatic assay. Data shown represent the means ± SD from triplicate samples and these results are representative of three different experiments.
viral p24). The observed enhancement of attachment of CD28+ viral particles to RAJI cells was abrogated by CTLA-4Ig, thus indicating that it is conferred by the interaction between virion-anchored host CD28 and cell surface B7-1/B7-2.

*Infectivity of CD28-bearing virions is markedly enhanced for target cells expressing high levels of B7-1/B7-2*

We next assessed whether the acquisition of host-derived CD28 by HIV-1 could modulate virus infectivity by up-regulating the virus attachment/entry process. This possibility was tested using RAJI-CD4, a RAJI derivative stably expressing CD4 that has been shown to be highly susceptible to HIV-1 infection (13). It should be stressed that flow cytometric analyses revealed that RAJI-CD4 cells, as it is the case for the parental RAJI cell line, express considerable amounts of surface B7-1/B7-2. Virus infection experiments were performed with three distinct virus stocks originating from independent transfections. As illustrated in Fig. 3A, CD28 increases virus infectivity for RAJI-CD4 cells by up to 12.3- to 17.2-fold. Virus infection studies were next conducted in the presence of CTLA-4Ig to demonstrate without any doubt the crucial role played by virus-anchored host CD28 in the noticed increase in virus infectivity. Treatment of the RAJI-CD4 and CD28+ HIV-1 particles mixture with CTLA-4Ig was found to totally abrogate the increase in virus-encoded reporter gene activity conferred by virally embedded host CD28 (Fig. 3B). Similar results were obtained following infection of another B7-1/B7-2-expressing cell line, i.e., LuSIV, with the dual-tropic strain B9.6. In this experimental setting, an 8-fold increase of HIV-1 infectivity was conferred by the presence of host CD28 in the viral envelope (Fig. 3C). Again, the enhancement of virus infectivity was abrogated by a pretreatment with a blocker of the interaction between CD28 and B7-1/B7-2. Interestingly, an isotype-matched irrelevant Ab (control Ab) had no effect on infectivity of CD28-bearing virions.

It has been shown that B7-1 and B7-2 bind to overlapping but not identical sites on CD28 (42–45). Thus, we investigated the involvement of either CD28/B7-1 or CD28/B7-2 interaction in the observed enhancement of virus infectivity. As expected, infection of RAJI-CD4 cells with virions devoid of host-encoded CD28 (CD28−) was unaffected by treatment with either BB-1 (anti-B7-1) or BU-63 (anti-B7-2) (Fig. 4A). Infectivity of progeny viruses bearing host CD28 in their envelope was more markedly affected by the addition of the monoclonal anti-B7-1 Ab than by treatment with the monoclonal anti-B7-2 Ab. The combined action of the two Abs resulted in an almost complete inhibition of the increase in virus infectivity conferred by the virion-anchored host CD28 glycoprotein. We next studied in further detail the ability of BB-1 and BU-63 to abrogate the increase in virus infectivity of CD28+ HIV-1 particles for RAJI-CD4 cells. As expected, treatment of the mixture composed of CD28+ virions and RAJI cells (CD4-negative) with either BB-1 or BU-63 had no noticeable effect on viral binding to RAJI cells (Fig. 4B). However, the overall attachment process of CD28+ viruses to RAJI cells was more markedly diminished by BB-1 as compared with BU-63.

We also investigated whether the increase in the virus attachment of CD28-bearing virions to susceptible target cells could lead to a concomitant augmentation of virus internalization. For this purpose, we performed a virus entry assay based on the use of a potent mixture of proteolytic enzymes (pronase) that eliminate noninternalized virus particles attached to the cell surface (46, 47). Results from Table I indicate that attachment of CD28+ HIV-1 particles to RAJI-CD4 cells is quantitatively more important as compared with the binding efficiency of isogenic progeny viruses devoid of host-encoded CD28 (3166 vs 536 pg/ml, i.e., a 6-fold increase). The removal of noninternalized virus particles attached to the cell surface by pronase demonstrate that intracellular viral p24 represented 12.3 and 33.7% of the total input for CD28+ and
CD28 + virions, respectively. In these experiments, treatment of the mixture composed of CD28 - progeny viruses and RAJI-CD4 cells with CTLA-4 Ig decreased the percentage of virus entry to 14.9%, a value comparable to the one seen when RAJI-CD4 cells are inoculated with CD28 - HIV-1 particles (i.e., 12.3%).

To explore the relative importance of the alternative infection pathway (i.e., CD28 and B7-1/B7-2) by comparison with the major pathway (i.e., gp120 and CD4), RAJI-CD4 cells were treated with a known blocker of the gp120-CD4 interaction. As shown in Fig. 5A, the anti-CD4 SIM.2 Ab was found to mediate a dose-dependent inhibition of infection by both CD28 - and CD28 + viruses. However, CD28 + progeny viruses were found to be more resistant to neutralization by the anti-CD4 Ab than were CD28 - viruses (Fig. 5B). Such results demonstrate the importance of the additional secondary CD28-B7-1/B7-2 interactions when the primary CD4-gp120 association is compromised.

The degree of virion-anchored host CD28 and HIV-1 infectivity are both influenced by the level of CD28 expression on the surface of virus producer cells

The biological significance of our findings was investigated by first defining whether the incorporation rate of CD28 and virus infectivity were affected by the amounts of surface CD28 on 293T cells and second by comparing the levels of expression of CD28 between 293T cells and natural cellular reservoirs of HIV-1, i.e., CD4-expressing T lymphocytes. To test whether differences in surface expression levels of CD28 can quantitatively affect the amounts of virion-bound foreign CD28, different virus stocks were made by transfecting 293T cells with increasing amounts of the CD28-encoding vector. Results from flow cytometric analyses indicate that there is a correlation between the introduced CD28 expression plasmid and the levels of CD28 expressed on the surface of 293T cells (Fig. 6A). The next step was to evaluate the degree of virally embedded host CD28 in progeny virions produced by 293T cells that express varying levels of surface CD28. The presence of these proteins of host origin on the exterior of the virion makes them accessible to reagents such as Abs specific for the protein of interest. This method has been used with success to immunoprecipitate the viral entity from a variety of preparations.

We previously developed a virus capture assay based on magnetic beads coated with an Ab directed against the studied cellular protein(s) (48). A more sensitive version of this virus capture test was used in the present work that relies on the use of streptavidin-coated magnetic beads in combination with a biotinylated monoclonal anti-CD28 Ab (39). An isotype-matched biotinylated anti-CD45 Ab (clone UCHL-1) was used as a control to estimate the nonspecific binding of viruses to magnetic beads. As shown in Fig. 6B, the virus recovery rates were directly influenced by the levels of CD28 expressed on the surface of 293T cells. Interestingly, the observed quantitative changes in the amounts of host-encoded CD28 acquired by HIV-1 seem to correlate in an almost linear way with virus infectivity (Fig. 6C). Because CD4-expressing T lymphocyte represents the major CD28-positive cell type that is infected by HIV-1 in vivo, we next examined surface levels of CD28 on such primary human cells. We noticed that human CD4 + T cells express a significant amount of surface CD28 that is comparable to the amount of CD28 found on the surface of 293T cells transiently transfected with 2–10 μg of the CD28-encoding plasmid (compare Fig. 6, A and D). This suggests that viral entities will emerge from target cells in vivo expressing levels of surface CD28 protein sufficient to modulate HIV-1 infectivity.

The phenomenon of CD28 incorporation has physiological significance for the HIV-1 life cycle

To directly demonstrate the relevance of CD28 incorporation in HIV-1 biology, isogenic CD28 - and CD28 + dual-tropic virus stocks were used to infect more natural B7-1/B7-2-expressing target cells. For this purpose, MDM were derived from fresh PBMCs and grown for 3 days in the presence of IFN-γ to induce expression of the costimulatory B7-1 and B7-2 molecules. FACS analyses revealed that this treatment led to a noticeable up-regulation of B7-1 and B7-2 on the surface of MDM (data not shown). Such IFN-γ-treated MDM were next inoculated with the dual-tropic HIV-1 strain 89.6 bearing or not on its surface the CD28 glycoprotein. As depicted in Fig. 7A, CD28-bearing virions were found to be more infectious for MDM than isogenic viruses devoid of foreign CD28 (compare 7300 and 3400 pg/ml of p24). Pretreatment of our viral stocks with the anti-CD28 mAb 9.3 diminished the process of infection with CD28 + virions, while it had no effect on the replicative capacity of CD28 - HIV-1 particles.
A tissue culture system has been previously developed as a model for studying HIV-1 pathogenesis (49). This experimental system consists of histocultures of human lymphoid tissues (i.e., tonsils) that preserve their general cytoarchitecture. Human lymphoid tissues cultured ex vivo harbor different cell subsets which may express B7-1 and/or B7-2 molecules (e.g., activated T lymphocytes, macrophages, and dendritic cells). Histocultures of human tonsils were thus infected with isogenic CD28+/− and CD28++ 89.6 virus stocks in the absence or the presence of the blocking anti-CD28 Ab. CD28-bearing HIV-1 particles were again more infectious than their CD28− counterparts (compare 66,000 and 32,000 pg/ml of p24) and pretreatment with 9.3 reduced infectivity of CD28++ only (Fig. 7B). To more clearly establish the physiological significance of the presence of host-derived CD28 when inserted within the virus envelope, we assessed whether a blocking anti-CD28 Ab could affect replication of a clinical strain of HIV-1 produced in PBMCs. Treatment of the R5X4 clinical isolate of HIV-1 92RW009 with 9.3 resulted in a diminution of virus production in IFN-γ-treated MDM (Fig. 8). Replication of this clinical strain of HIV-1 was unaffected by an isotype-matched irrelevant Ab (data not shown).

Host-derived CD28 is detected on the surface of clinical isolates of HIV-1 produced in primary human mononuclear cells

The virus capture assay was used to assess the physical presence of foreign CD28 on the surface of clinical strains of HIV-1 produced during normal infection of human PBMCs. Two different T cell tropic (X4) clinical isolates of HIV-1 expanded on human PBMCs were more efficiently captured by an anti-CD28 Ab than by an Ab recognizing CD45 (Fig. 9A). The use of RAJI cells to capture such progeny viruses showed that the binding of 92HT599 and 93UG070 to these cells is partly mediated through an interaction between CD28 and B7-1/B7-2 because treatment with CTLA-4Ig diminishes the amounts of viruses attached to the cell surface (Fig. 9B). Virus capture studies were also performed with macrophage (R5) and dual-tropic (R5X4) primary isolates of HIV-1 because CD28-expressing CD4+ T lymphocytes are susceptible to infection by such viruses under in vivo situations. These viruses were expanded in PBMCs from two healthy donors. Precipitated viruses are presented as the ratio between virions captured with anti-CD28 and viruses captured with anti-CD45 because there were quantitative differences in the virus-associated p24 content for the virus preparations tested (data not shown). As depicted in Fig. 9C, magnetic beads coated with anti-CD28 efficiently captured T-, M-, and dual-tropic field isolates of HIV-1 produced by PBMCs from two healthy donors.

Discussion

The binding event represents the first step in the virus replicative cycle. The attachment of viruses to the surface of the target cell was previously considered to involve simple recognition to a single-cell surface molecule by virus attachment proteins. Results from studies aimed at understanding virus-host interactions revealed that virus binding is a more complex phenomenon. For example, some viruses recognized more than one receptor and sometimes a secondary binding step has been shown to follow the

Table I. Internalization of isogenic CD28− and CD28+ virions in RAJI-CD4 cells

<table>
<thead>
<tr>
<th>Virus Stocks (treatment)</th>
<th>Total p24 Levels in the Absence of Pronase (pg/ml)</th>
<th>Intracellular p24 Levels in the Presence of Pronase (pg/ml)</th>
<th>Percentage of Virus Entry (intracellular/total p24 levels)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD28− (without CTLA-4Ig)</td>
<td>536 ± 96</td>
<td>66 ± 12</td>
<td>12.3</td>
</tr>
<tr>
<td>CD28+ (without CTLA-4Ig)</td>
<td>3166 ± 58</td>
<td>1066 ± 57</td>
<td>33.7</td>
</tr>
<tr>
<td>CD28− (with CTLA-4Ig)</td>
<td>623 ± 130</td>
<td>93 ± 12</td>
<td>14.9</td>
</tr>
</tbody>
</table>

a Results shown are the mean number ± SD for triplicate samples.

b NT, Not tested; NA, not applicable.

FIGURE 5. Sensitivity of CD28− and CD28+ virions to neutralization by an agent blocking the gp120-CD4 interaction. A, RAJI-CD4 cells were incubated for 5 min at 37°C with the indicated amounts of the anti-CD4 SIM.2 Ab. Such treated cells were inoculated with CD28− and CD28+ luciferase-encoding virus stocks (5 ng of p24). Cells were next incubated at 37°C for 48 h before evaluation of the luciferase activity. B, Inhibition of virus infectivity was calculated with the following formula: percentage of inhibition = [1 − (virus treated with SIM.2/untreated virus)] × 100%. Results shown are the mean ± SD for triplicate samples and are representative of two independent experiments.
initial binding event (50). This is exemplified by the observation that HIV-1 recognizes both CD4 and galactosyl ceramide as primary cellular receptors and some members of the seven-transmembrane chemokine receptor family served as coreceptors for virus entry (51). More evidence suggests that other surface molecules might also be playing an active potentiating role in the whole process of HIV-1 binding. Given that the interaction between gp120 from primary viruses and cellular CD4 is often weak, these secondary interactions would act by stabilizing or strengthening the initial virus-cell contact and would allow ligation of gp120 to the appropriate chemokine coreceptor. This type of secondary interaction can be biologically important for a pathogen such as HIV-1 found in fluids with flow where a rapid and firm docking of the virus to its host cell is essential. Previous work has indicated that incorporation of foreign HLA-DR and ICAM-1 proteins in HIV-1 contributes to virus infectivity by increasing the binding/entry process (12, 14, 15). In the present study, we provide evidence indicating that the costimulatory molecule CD28, which is constitutively expressed on virtually all human CD4$^+$ T lymphocytes, is incorporated within the HIV-1 envelope and is enhancing the process of infection through a potentiating effect on the early events of virus replication. The noticed enhancement of virus infectivity conferred by the acquisition of host CD28 is primarily due to the interaction between CD28 and B7-1. Our observation is consistent with a previous work that has reported that although B7-1 and B7-2 bind CD28 with similar low affinities and CTLA-4 with similar high affinities (52), B7-2 has faster dissociation kinetic than B7-1 (53). Given that the increase in virus infectivity is resulting from an augmentation of the overall attachment process, it is thus logical to observe that infectivity of CD28-bearing HIV-1 particles is more affected when CD28/B7-1 interaction is blocked than when CD28/B7-2 interaction is abrogated. However, it is important to note that two lines of evidence suggest that the sole CD28-B7 interaction is not sufficient per se to allow HIV-1 infection. First, infection with CD28-bearing virions is completely abolished when using an anti-CD4 Ab that blocks the gp120-CD4 interaction (Fig. 5, A and B). Second, the CD4-negative parental RAJI cell line is
not susceptible to infection with CD28-bearing HIV-1 particles despite surface expression of CXCR4 and high levels of B7-1/B7-2 (data not shown). The interaction between the external viral envelope and CD4 remains thus essential for infection of B7-expressing cells with HIV-1 carrying foreign CD28 in their envelope.

Results from infectivity studies conducted in MDM are particularly informative since induction of the two costimulatory B7-1 and B7-2 molecules after activation of this cell type is a well-established phenomenon. For example, microbial components such as bacterial LPS (54) and some cytokines have the capacity to up-regulate expression of these two costimulatory molecules on MDM. Among the various cytokines involved during the immune response, IFN-γ is recognized as the major macrophage-activated molecule able to increase surface expression of B7-1/B7-2 on cells of the monocyte/macrophage lineage (55–57). The presence of the additional CD28/B7 interaction between the virus and its target could represent an important step in the establishment of the infection as indicated by the observation that IFN-γ-treated MDM are more susceptible to infection with CD28-bearing virions. Members of the B7 family are also expressed on a variety of APCs and CD4-positive T lymphocytes, particularly in human lymphoid tissue where a high activation state is prevailing (58). Virus infectivity was also positively affected by the acquisition of CD28 when human lymphoid organs cultured ex vivo were used as targets. Such data reveal some clinical significance since these tissues are considered major reservoirs of HIV-1 and sites of intense virus replication. Interestingly, T lymphocytes have been demonstrated to express both B7-1 and B7-2 following stimulation (22–25), and their expression levels are augmented in HIV-1-infected individuals (59–61).

As stated above, one obvious mechanism to explain the noticed increase of HIV-1 infectivity conferred by the presence of host-encoded CD28 is by a direct contribution to the early events of the viral life cycle, namely binding and entry. The fact that CD28 increases virus entry into RAJI-CD4 cells by >3-fold entirely supports this idea. It should be stated that a similar conclusion has been drawn to explain the previously described augmentation of infectivity for progeny viruses bearing host cell membrane CD44, MHC-I, MHC-II, and ICAM-1 proteins (12–15, 62). Maréchal and colleagues (46) have previously shown that HIV-1 entry occurs both through plasma membrane fusion, leading to productive infection, and through endocytosis, usually leading to nonproduction infection. Their work has also revealed that most of the virus p24 taken up by cells is not due to infectious virus, but to particles that

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**FIGURE 8.** Replication of a clinical isolate of HIV-1 in human MDM following treatment with a blocking anti-CD28 Ab. The macrophage-tropic clinical strain of HIV-1 92RW099 that was produced in PBMCs was either left untreated or was treated with the anti-CD28 9.3 before inoculation of IFN-γ-treated MDM. Virus-infected target cells were analyzed for p24 concentration in the culture medium at the indicated times. Data shown represent the means ± SD of three determinations and these results are representative of three different experiments.

**FIGURE 9.** Capture of virions with immunomagnetic beads and RAJI cells. A, Virus stocks from transiently transfected 293T cells (CD28+ and CD28−) and clinical isolates of HIV-1 expanded in primary human cells were incubated with a combination made of streptavidin-coated magnetic beads and a monoclonal anti-CD28 or anti-CD45 Ab. B, Such virus preparations were also incubated with RAJI cells as described in Fig. 2. C, T-, M-, and dual-tropic clinical isolates of HIV-1 were produced by infecting PBMCs from two healthy donors. Such virus stocks were subjected to the virus capture assay. Results shown are the mean ± SD of triplicate samples and are representative of three different experiments.
undergo endocytosis and end up in lysosomes. Although the experimental approach used in the present study to assess virus entry does not permit discrimination between cytosolic p24 and nonspecific vesicular uptake, our observation that the process of HIV-1 infection is modulated by incorporation of host CD28 and blockers of CD28-B7-1/B7-2 interactions is an indication that cytosolic p24 is also augmented by the presence of virion-anchored host CD28. Our findings indicate also that the presence of the additional CD28 interaction in fulfills a certain role in the infection process when the major CD4/gp120 interaction is compromised by a blocking agent. Our observations might reveal great physiological relevance considering that the stabilization of the viral particle on the host-cell membrane by this additional interaction will favor the ensuing gp120-CD4 association. The process of HIV-1 attachment to host cells is considered to be essentially mediated by the association between cellular CD4 and virion-associated gp120. This postulate originates from early studies suggesting that virus attachment to T cells correlates with CD4 expression (63, 64) and is gp120-dependent (64, 65). However, the binding of gp120 to cellular CD4 might not be sufficient per se for an efficient docking of the virus to cell types such as macrophages that express little CD4 (66, 67). Given that macrophages express both B7-1 and B7-2 molecules, it is possible that the additional interaction mediated by CD28 and B7-1/B7-2 facilitates the binding and entry process of HIV-1. Moreover, follicular dendritic cells that are CD4-negative have the capacity to trap large quantities of HIV-1 on their surfaces via dendritic cell-specific ICAM-3 grabbing nonintegrin (68, 69). It is believed that this process could be involved in the migration of the virus from the mucosa to the lymphoid organs. It is conceivable that other virus-cell interactions mediated by adhesion molecules could participate in this process. The interaction between CD28 and B7 family members represents a good candidate because follicular dendritic cells express large amounts of B7 molecules (70).

The current work has not explored the possible effect of CD28-B7-1/B7-2 signal transduction pathway in HIV-1 replication. This scenario should not be ignored considering the observation that B7-2-bearing HIV-1 particles mediate signaling events in human T lymphocytes resulting in activation of some specific transcription factors such as NF-kB and NF-AT (71). It should also be noted that cross-linking of B7-1 resulted in tyrosine phosphorylation of several proteins in RAJI cells (72). Proliferation of B cells was found to be arrested upon engagement of B7-1 on the surface of B cells while occupancy of B7-2 promoted growth and differentiation of these cells (73). Studies are underway to define whether signal transduction events resulting from the interaction between virion-bound host CD28 and cell surface B7-1/B7-2 can affect HIV-1 transcriptional activity and/or target cell function(s).

The physiological relevance of the insertion of host-derived CD28 in the HIV-1 envelope is high considering that CD28 is constitutively expressed on virtually all CD4+ T lymphocytes, a cellular subpopulation considered as a major reservoir of HIV-1, and that the level of CD28 found on freshly isolated CD4+ T cells is comparable to the amount of CD28 expressed on transfected 293T cells used in the present study. Moreover, the observation that treatment of clinical virus isolates with a blocking anti-CD28 Ab leads to a decrease of HIV-1 production in MDM is an indication that the level of foreign CD28 acquired by clinical strains of HIV-1 is sufficient to play a role in the virus life cycle.

In conclusion, these experiments further reinforce the idea that the biology of HIV-1 is influenced by the nature of host cell membrane constituents found embedded within virions. Our data confirm the high degree of complexity of interactions occurring between a pathogen such as HIV-1 and its cellular target. A better understanding of the exact contribution of virion-anchored host molecules to the life cycle of HIV-1 is crucial to provide insights into the pathogenesis of this retroviral disease.

Acknowledgments

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References


