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Enhanced Susceptibility to Human Immunodeficiency Virus Infection in CD4+ T Lymphocytes Genetically Deficient in CD43

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ABSTRACT

CD43 is a cell surface sialoglycoprotein expressed by most cells of hematopoietic origin, including all T lymphocytes. Elimination of CD43 expression by gene targeting in the CEM T cell line results in its increased homotypic adhesion and binding to HIV-1 gp120. Here we report that the CD43-negative CEM cells show increased susceptibility to HIV-1 infection and increased viral replication compared with the parental CD43+ CEM cell line. Increased HIV-1 replication also was observed in CEM cells with diminished CD43 expression secondary to functional inactivation of a single CD43 allele. The CD43− CEM cells were more susceptible to HIV-1-induced cytopathicity than their CD43+ counterparts. HIV-1 replication also was increased in the CD43− CEM cells after transfection with the infectious HIV molecular clone pNL4-3. These data suggest that factors that diminish CD43 expression on T lymphocytes may enhance their susceptibility to HIV-1 infection.

INTRODUCTION

CD43 (leukosialin or sialophorin) is an integral membrane sialoglycoprotein expressed by virtually all cells of hematopoietic origin.[1,2] CD43 is heavily glycosylated by O-linked oligosaccharides, the structures of which differ in complexity, depending upon cell lineage, degree of differentiation, and state of activation.[3] In T lymphocytes, CD43 constitutes a major portion of the cell membrane glycocalyx.[4–7]

The physiologic functions of CD43 are not completely understood, although it is thought to have an important role in leukocyte adhesion. Depending upon the experimental conditions, CD43 has been observed to either promote or inhibit cellular adhesion. Treatment of lymphocytes or monocytes with anti-CD43 monoclonal antibodies can induce homotypic adhesion[8,9] and CD43 has been shown to be a ligand for ICAM-1.[10] By contrast, we have observed that expression of CD43 in HeLa cell transfectants decreases their heterotypic adhesion to T lymphocytes.[11] Elimination of CD43 expression in the human T cell line CEM by gene targeting enhances its homotypic adhesion and binding to purified HIV-1 gp120.[12] These latter data suggested that diminished CD43 expression might enhance susceptibility of T lymphocytes to HIV-1 infection. In the current study, we tested whether alterations in the level of CD43 expression by the CEM T cell line would influence its susceptibility to HIV-1 infection. Loss of CD43 expression results in enhanced susceptibility to HIV-1 infection and increased virus production in CEM cells. The results also suggest that CD43 interference with HIV-1 infection may involve intracellular events that occur after virus binding effect.

MATERIALS AND METHODS

Cells and viruses

The A3.01 subline of the CD4+ T lymphocyte line CEM,[13] CEM-SS,[14] H9, and H9/HTLV-IIIB cells (H9 chronically infected with HIV-1_{IIIB}),[15] was obtained from the NIH/NIAID AIDS Research and Reference Reagent Repository (Ogden BioServices, Rockville, MD). Knock-out mutants of the A3.01 CEM cell line that express reduced levels of CD43 (SKO) or are CD43-negative (DKO) were derived as described previously.[12] All cell lines were maintained in RPMI medium supplemented with 20% fetal bovine serum. An infectious molecular clone of HIV-1, pNL4-3,[16] was obtained from the AIDS

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Research and Reference Reagent Repository. Plasmid preparation and cesium chloride density gradient centrifugation were performed according to standard procedures.

Virus infection

Cell free HIV-1_{IIIB} was used for all the infections. Unless otherwise specified, cell-free virus was propagated on H9 cells; H9 and H9/HTLV-IIIB cells were mixed at a ratio of 4:1 and the culture supernatants were harvested after 1 week incubation. The virus stocks were stored at −70°C until use. The infectivity of the virus stocks was titered by syncytium formation in CEM-SS cells. CEM and the various CD43 mutants were infected with cell-free HIV-1_{IIIB} at different multiplicities of infection and 1 week later, the culture supernates were harvested, clarified by centrifugation, and filtered through 0.45-μm Millipore filters. Where HIV infection of CEM and DKO cells was initiated by transfection with pNL4-3 plasmid DNA, 15 μg of plasmid DNA was electroporated into exponentially growing cultures using a Cell-porator (Bethesda Research Laboratories, Gaithesburg, MD).

Assays for HIV infection

A reverse transcriptase (RT) assay or HIV p24 antigen capture assays were performed on culture supernatants of HIV-infected cells to measure virus yield. RT assays were performed according to previously described procedures[17] with minor modifications. Briefly, the reactions were carried out in a total volume of 50 μl, using 5 μg/ml poly(A), 1.6 μg/ml oligo(dT_{12-18}) and 1 μCi [^{3}H]TTP in 50 mM Tris (pH 7.8), 75 mM potassium chloride, 5 mM magnesium chloride, 2 mM dithiothreitol, and 0.05% NP-40. The reaction was initiated by addition of 10 μl of virus-containing tissue culture supernatant. After 1 hr incubation at RT, 10 μl of the reaction mixture was spotted onto a Whatman DE-81 filter paper, air dried, and washed four times in 2× SSC. The filter papers were dried and transferred to a plastic bag containing scintillation cocktail, sealed, and counted in an LKB Betaplate reader. p24 antigen capture assays were performed using a commercially available kit (DuPont or Coulter) according to manufacturer's instructions.

XTT assay

The susceptibility of cells to HIV-induced killing was monitored by XTT assay, a chromogenic dye-conversion assay to monitor cell viability, and was performed as previously described.[18] Briefly, six replicates of serial 4-fold dilutions of an HIV stock were prepared in 96-well microtiter plates, and CEM or CD43-negative mutant CEM cells (5000 cells/well) were added to the virus dilutions. After incubation at 37°C for 7 days, 50 μl of XTT solution (0.1% in 25 mM phenoxymethosulfate) was added to each well, and the extent of dye reduction was monitored spectrophotometrically (A_{450}) as a measure of cell viability.

Flow cytometry analysis of virus-infected cells

Cells (2–10 × 10^5) were harvested and resuspended in 50 μl cold FACS buffer (PBS with 1% BSA and 0.02% sodium azide) containing either no antibody, a 1:25 dilution of human HIV immune globulins (HIVIG, AIDS Research Reagent Program), or 5 μl of CD4-FITC (Becton-Dickinson, Mountain View, CA). After incubation on ice for 20 min, the cells were washed twice with FACS buffer. Cells were pelleted, washed, and incubated on ice for 20 min with a 1:20 dilution of FITC-conjugated goat antihuman immunoglobulins (TAGO Inc., Burlington, CA). The cells were again pelleted, washed twice with FACS buffer, and resuspended in 500 μl of FACS buffer containing 1% formaldehyde before analysis on a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA with LYSYS II software).

RESULTS

HIV Infection of CEM and CD43-deficient mutant cell lines

CEM and DKO (CD43 double knock-out) cells were infected with cell-free HIV at a multiplicity of infection of 0.5, 5, or 7.5, and then analyzed by flow cytometry after immunofluorescent staining with HIVIG and FITC-conjugated goat antihuman immunoglobulins. At all multiplicities of infection, the mean fluorescence intensity of infected $CD43^-$ cells (DKO) was approximately twice that of the $CD43^+$ cells (CEM) at the time of peak virus infection (Fig. 1). The peak virus infection was attained on day 7 postinfection (at a multiplicity of 0.5 or 5) or day 5 (at a multiplicity of 7.5) in both the $CD43^+$ and $CD43^-$ cell lines. At the highest moi (7.5), a virus dose that would be expected to infect the majority of cells, the percentage of DKO cells displaying viral glycoproteins was 15-fold greater than the CEM cells at day 3 (Fig. 2). At this moi, few viable cells were present at days 7 and 10 (not shown). Taken together, these data suggest that CD43 interfered with HIV infection of the CEM cells.

We also measured virus production from CEM variants infected with HIV-1 at different multiplicities. CEM, SKO (CD43 single knock-out), or DKO cells were infected with serial dilutions of a high-titered virus stock, and the amount of reverse transcriptase in the culture supernatants was determined after 7 days. Compared to the parental CEM cells, consistently greater amounts of virus were produced from both SKO ($p < 0.01$) and DKO ($p < 0.001$) cells at all multiplicities (Fig. 3). DKO cells do not express CD43, whereas SKO cells express CD43 at 50–80% of wild-type levels.[12] The virus yield from DKO was significantly greater than the virus yields from SKO supernatants ($p < 0.001$).

Effect of CD43 expression on cell survival after HIV infection in culture

CD43 expression by CEM cells has been shown to interfere with their homotypic adhesion.[12] Since cell fusion contributes to HIV-induced cytopathicity in culture, and the extent of cell fusion is influenced by intercellular adhesive interactions,[19–25] we hypothesized that HIV-induced syncytium formation and cytopathicity would be enhanced in $CD43^-$ DKO cells. We therefore monitored cell fusion and virus-induced cytopathicity in HIV-infected CEM and DKO cells for 7 days. Cell fusion or syncytium formation was not very pronounced either in HIV-infected $CD43^+$ (CEM) or $CD43^-$ (DKO) cells (not

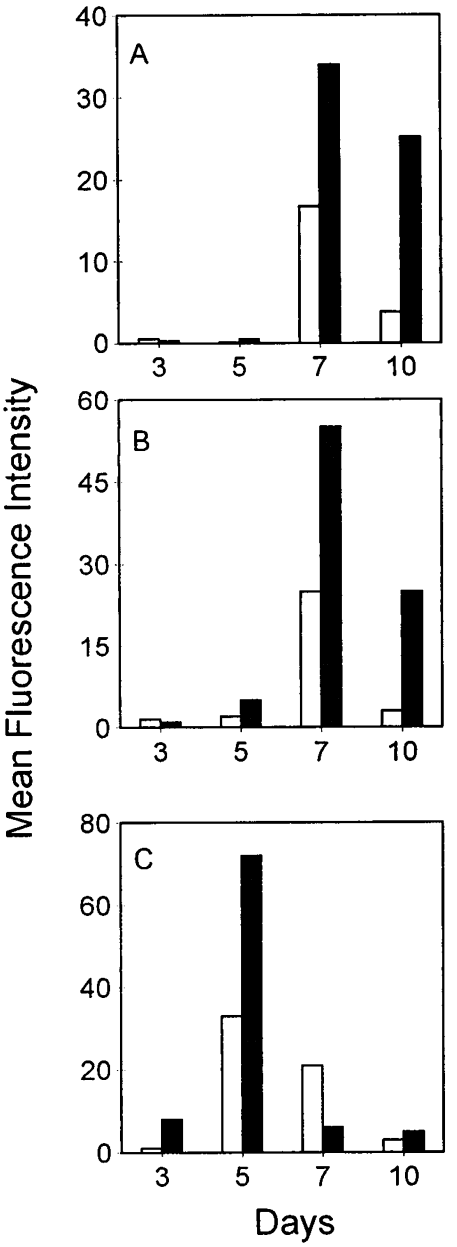

FIG. 1. HIV-infection of CEM and DKO cells. CEM (open bars) or DKO (solid bars) cells were infected with HIV-1_{IIIB} at a multiplicity of 0.5 (A), 5 (B), or 7.5 (C) and analyzed by flow cytometry after immunofluorescent staining with HIVIG and FITC-conjugated goat antihuman immunoglobulins. The mean fluorescence intensity of the cultures at different times after infection are shown as a measure of HIV infection.

shown), although the virus stocks used in this study are highly fusogenic and cause extensive syncytium formation in other lymphocytic cell lines including CEM-SS or MT-2. The poor syncytium-forming ability of the A3.01 CEM and DKO cells may be related to their low levels of LFA-1 and ICAM-1 expression,[12] adhesion counterreceptors that contribute to syncytium formation in CEM cells.[26]

Cell viability was assayed by a colorimetric dye-conversion assay (XTT-assay, Fig. 4) and by viable cell counting after try-

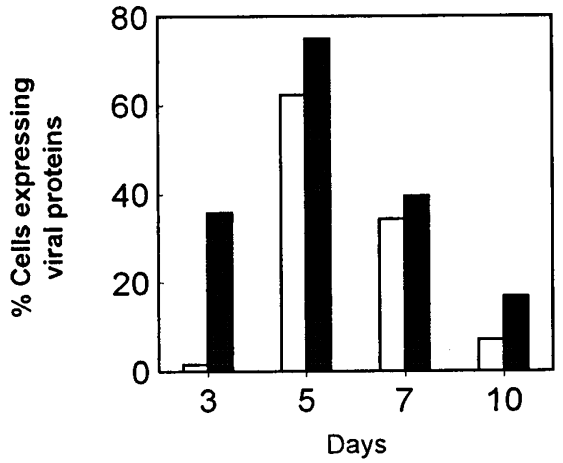

FIG. 2. Number of virus-infected cells at different days postinfection. CEM (open bars) or DKO (solid bars) cells were infected with HIV at a multiplicity of 7.5, and the percent of HIV-infected cells in culture at different days postinfection was determined by flow cytometry after immunofluorescent staining with HIVIG and and FITC-conjugated goat antihuman immunglobulins.

pan-blue dye exclusion (Fig. 5). Despite the infrequency of syncytia in HIV-infected CEM and DKO cell cultures, the viability of the infected DKO cells was reduced compared with that of the CEM cells. The reduced viability of the DKO cells may

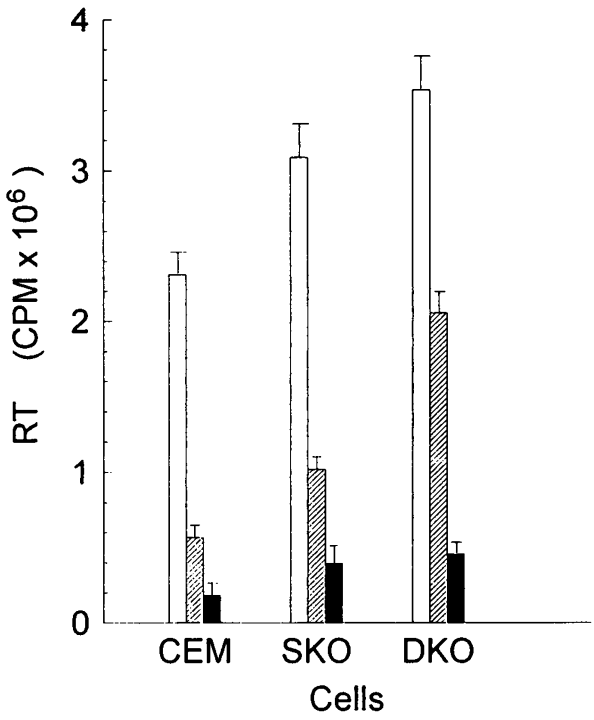

FIG. 3. Virus production from HIV-infected wild-type or mutant CEM cells. CEM, SKO, or DKO cells were infected with a 1:64 (open bars), 1:256 (hatched bars), or 1:1024 (solid bars) dilution of a HIV-1_{IIIB} virus stock, and the extent of virus production was monitored by RT assay after 7 days.

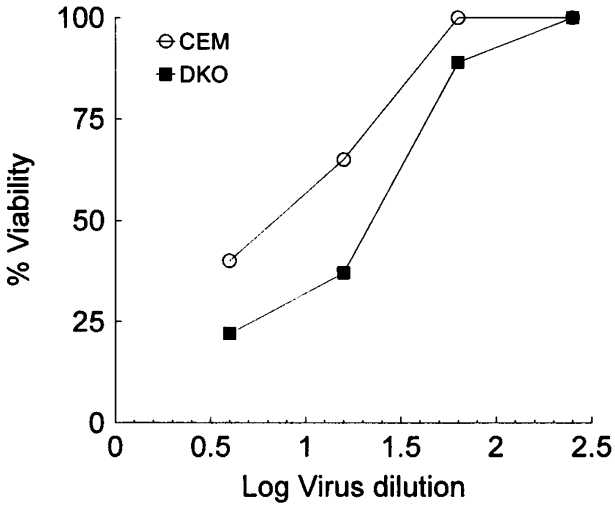

FIG. 4. XTT assay showing the susceptibility of CEM and DKO to HIV-induced killing. CEM (circles) or DKO (squares) cells were infected with various dilutions of a HIV-1_{IIIB} virus stock, and the viability of the cultures was monitored by colorimetric XTT dye-conversion assay after 7 days.

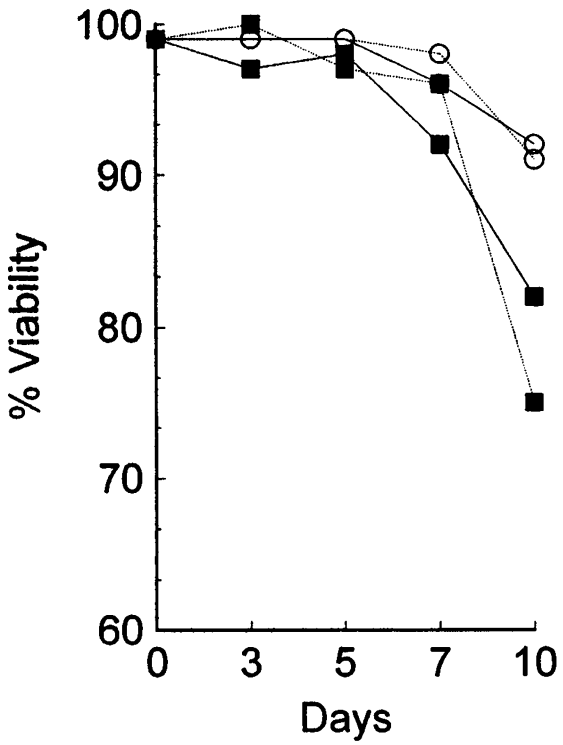

FIG. 5. Viability of HIV-infected CEM and DKO. CEM (circles) or DKO (squares) cells were infected with HIV-1_{IIIB} at a multiplicity of 5 (dotted lines) or 0.5 (solid lines), and the viability of the cultures was monitored on different days after infection by trypan blue dye exclusion.

reflect either their greater susceptibility to infection (Fig. 2) or increased virulence of HIV-1 in CD43-deficient cells.

Kinetics of HIV replication in CD43$^+$ and CD43$^-$ cells

CEM or DKO cells were infected with HIV-1_{IIIB} at a low multiplicity (0.01 $TCID_{50}$ per cell) and virus production was monitored by RT assays over a 10-day period. The peak RT activity in DKO culture supernatants was ~2-fold greater than the peak RT activity in supernatants from CEM cultures (Fig. 6). Both CEM and DKO showed a mean doubling time of ~20 hr (not shown). The differences in virus yield from these cell lines could not be attributed to differences in cell viability or cell number between the two cultures at the time that peak virus release was detected (Fig. 6).

Effect of CD43 expression on HIV replication in pNL4-transfected cells

We have shown previously that loss of CD43 expression by CEM cells increases their binding to purified HIV gp120.[12] To investigate whether the increased susceptibility of DKO cells to HIV infection is related to enhanced HIV binding or to a postbinding event, we investigated HIV replication in cells transfected with an infectious molecular clone of HIV. As shown in Table 1, the p24 antigen levels in culture supernatants of pNL4-3 transfected DKO cells, measured at days 3, 5, and 7, was consistently ~2-fold greater than that seen with CEM cells, comparable to the results seen after infection with cell-free virus (Fig. 3). The observed differences were not related to differences in transfection efficiency since comparable lev-

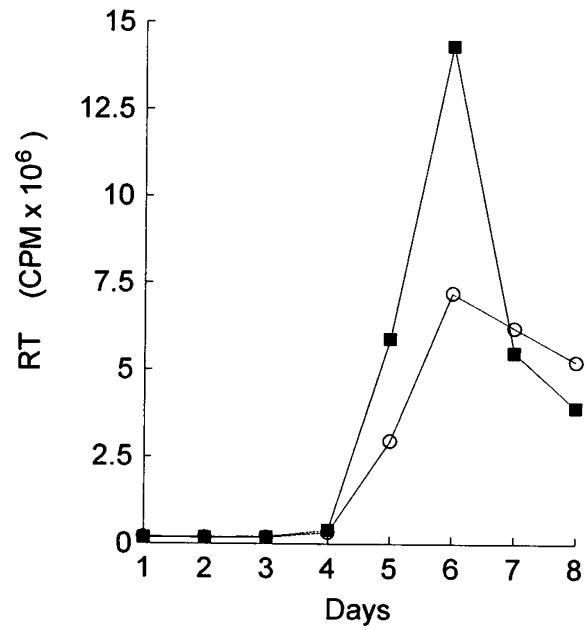

FIG. 6. Kinetics of HIV replication in CEM and DKO cells. Virus production from HIV-infected (moi = 1) CEM (circles) or DKO (squares) was monitored by reverse transcriptase assay at different days postinfection.

TABLE 1. VIRUS PRODUCTION FROM pNL4-TRANSFECTED CEM OR DKO CELLS

Cell type	*Days*	*p24 ng/ml*[a]
CEM ($CD43^+$)	3	0.29 ∓ 0.08
	5	2.20 ∓ 0.19
	7	12.50 ∓ 1.80
DKO ($CD43^-$)	3	0.62 ∓ 0.07
	5	4.56 ∓ 0.29
	7	28.30 ∓ 3.21

[a]Exponential cultures of CEM or DKO cells were adjusted to a density of 1×10^6 cells/ml and transfected with 10 μg of the plasmids pNL4-3 (containing an entire infectious HIV-1 proviral DNA) by electroporation. Culture supernatants were collected from the transfected cells on indicated days and assayed for HIV p24 antigen levels using commercially available (Coulter) kits. Each figure represents a mean of two experiments.

els of expression were observed with an indicator β-gal expression vector (data not shown).

DISCUSSION

We have previously generated CD43-mutant CEM T lymphocyte lines that are either deficient for CD43 expression or express diminished levels of CD43 using gene targeting and negative immunophenotypic selection.[12] Using these genetically defined lines, we show in this study that loss of CD43 expression is associated with enhanced susceptibility to HIV-1 infection and increased virus production by infected lines. The increased susceptibility of the CD43-mutant CEM cells to HIV-1 infection appears specifically related to diminished or lack of CD43 expression. All of these cell lines express similar levels of the HIV-1 receptor CD4,[12] and the doubling time of the CEM and CD43-mutant sublines in culture was similar, precluding the possibility that the enhanced virus production of the CD43-mutant cells resulted from more rapid growth kinetics.

The difference between the $CD43^+$ and $CD43^-$ cells in their susceptibility to infection appeared to be substantial. Three days after HIV-1 infection using an moi of 7.5, the $CD43^-$ cell (DKO) culture contained several-fold more cells expressing HIV-1 glycoproteins compared to a similarly infected $CD43^+$ cell (CEM) culture (Fig. 2). The magnitude of differences in viral production between the $CD43^+$ and $CD43^-$ cells were less marked (approx. 2-fold). However, HIV-1-infected DKO cells consistently yielded greater levels of p24 antigen and RT activity in culture supernatants than did the parental CEM line, and were more susceptible to HIV-1-induced cytopathicity. Moreover, culture supernatants from HIV-1-infected SKO cells, a mutant CEM cell line that expresses intermediate CD43 levels, contained intermediate levels of RT activity compared to the DKO and parental CEM cell lines. These latter data suggest that the enhanced HIV-1 production by the CEM cells and mutant sublines was inversely proportional to their level of CD43 expression.

Several reports have shown that adhesion receptors normally expressed by T lymphocytes including LFA-1, ICAM-1, and LFA-3 may play an important role in the cell-to-cell spread of HIV infection in cell culture.[19–25] The LFA-1/ICAM-1 counterreceptors, depending upon their levels of expression, may also contribute to HIV-1-induced syncytium formation.[26] It is unlikely that the LFA-1/ICAM-1 counterreceptors are involved in the enhanced HIV infection of the $CD43^-$ cells observed in this study: (1) the various CD43 mutants and the parent CEM used in this study all express very low levels of LFA-1 and ICAM-1; and (2) the enhanced homotypic adhesion of DKO cells is not inhibited by the anti-β_2-integrin antibody TS1/18.[12] In contrast to adhesion molecules that enhance the transmission and effects of HIV-1 in culture, CD43 expression appears to interfere with susceptibility to HIV infection and subsequent virus production by infected cells. To our knowledge, this is the first example of a naturally expressed lymphocyte surface protein that interferes with HIV-1 infection.

Several mechanisms may be involved in the enhanced HIV replication in $CD43^-$ cells. Earlier, we demonstrated that CD43 inhibits CEM cell binding to various protein ligands including gp120,[12] and thus an increased virus binding to $CD43^-$ cells may be responsible for enhanced HIV replication. The differences in viral replication in $CD43^+$ and $CD43^-$ cells after transfection with pNL4-3, however, suggest that the anti-HIV effects of CD43 may go beyond its potential to interfere with HIV-1 binding to the CEM cell surface or subsequent HIV-1 internalization. Rather, a postinternalization effect is suggested, one that might affect viral replication and possibly cell–cell transmission. These results should be interpreted with caution since multiple rounds of HIV infection may have ensued during the long time course of this experiment. In preliminary studies, we were unable to demonstrate appreciable differences in virus binding to $CD43^+$ and $CD43^-$ cells by a modified virus capture assay.[27] It is possible that the enhanced HIV infection of $CD43^-$ cells is mediated entirely by their increased homotypic cell adhesion. The adhesive interactions facilitate syncytium formation and enhance cell-to-cell spread of HIV. Finally, CD43 deficiency *in vivo* is associated with increased T cell activation (unpublished results), and it is possible that cellular factors associated with this activation may directly enhance HIV LTR expression. Whether HIV LTR expression is enhanced in $CD43^-$ cells remains to be investigated.

We have shown earlier that a decrease in the extent of CD43 sialylation can diminish its antiadhesive effect.[11] It is likely that the antiadhesive and antiviral functions of CD43 are functionally related. Since CD43 is a major component of the cell surface glycocalyx and contributes a large proportion of the negatively charged sialic acid on T lymphocyte surfaces,[4–7] diminished sialylation of CD43 might result in enhanced HIV-1 binding and cell–cell transmission. Indeed, immature thymocytes naturally express incompletely sialylated CD43 forms[1,28] and these cells are particularly susceptible to HIV-1 infection.[29–31] Whether T lymphocytes from patients with Wiscott–Aldrich syndrome show an enhanced susceptibility to HIV infection has not been investigated. These investigations have been hampered by the fact that the defects in CD43 expression (decreased amount, reduced molecular weight) seen in circulating lymphocytes from Wiscott–Aldrich syndrome are not maintained in cell lines established from these patients.[32]

Although our study did not address the specific influence of CD43 sialylation on viral transmission, it may have important

consequences for HIV-1 infection *in vivo*. The short-term enhancement in both virus infection and production that we noted in CD43-deficient cells suggests that factors that diminish or alter CD43 expression *in vivo* may have an impact upon viral burden in HIV-1-infected individuals. Defects in CD43 sialylation are prominent in CEM cells infected by HIV-1.[33] Taken together, these observations raise the possibility that HIV-1 infection of cells may diminish CD43 interference with cell-to-cell viral transmission.

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