DC-SIGN on B Lymphocytes Is Required For Transmission of HIV-1 to T Lymphocytes

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Infection of T cells by HIV-1 can occur through binding of virus to dendritic cell (DC)-specific ICAM-3 grabbing nonintegrin (DC-SIGN) on dendritic cells and transfer of virus to CD4+ T cells. Here we show that a subset of B cells in the blood and tonsils of normal donors expressed DC-SIGN, and that this increased after stimulation in vitro with interleukin 4 and CD40 ligand, with enhanced expression of activation and co-stimulatory molecules CD23, CD58, CD80, and CD86, and CD22. The activated B cells captured and internalized X4 and R5 tropic strains of HIV-1, and mediated trans infection of T cells. Pretreatment of the B cells with anti–DC-SIGN monoclonal antibody blocked trans infection of T cells by both strains of HIV-1. These results indicate that DC-SIGN serves as a portal on B cells for HIV-1 infection of T cells in trans. Transmission of HIV-1 from B cells to T cells through this DC-SIGN pathway could be important in the pathogenesis of HIV-1 infection.

Introduction

HIV-1 can bind to the type II C-type lectin receptor, dendritic cell (DC)-specific ICAM-3 grabbing nonintegrin (DC-SIGN; CD209), on myeloid DC and be transferred to CD4+ T cells [1,2]. An important feature of this trans pathway is that the virus does not establish an efficient, productive infection in these DC. Rather, it is captured by the DC and internalized in distinct intracellular compartments, and then transmitted to CD4+ T cells wherein it undergoes productive replication [3]. This is considered to be an alternative pathway to HIV-1 cis infection of T cells, macrophages, and DC that occurs through binding to the primary CD4 receptor and either of the chemokine coreceptors CXCR4 or CCR5.

B lymphocytes have also been implicated in trans infection of T cells with HIV-1 [4]. Given the intimate association of B cells and T cells in lymphoid tissue, B cell-mediated trans infection pathways could be important in the spread of virus to T cells. B cells derived either from lymphoid tissue or from the peripheral blood of HIV-1–infected persons carry replication-competent virus of either the CXCR4 (X4) or CCR5 (R5) tropic strain [5]. The mechanism by which B cells have been shown to transmit the virus involves binding of HIV-1 immune complexes to CR2 or CD21 on the surface of B cells and subsequent passage to the T cells [6–10]. Other reports have proposed a role for B cells in HIV-1 infection involving B cell activation processes induced by nef-expressing macrophages [11].

B cells express some C-type lectin receptors [12–15], with conflicting reports on expression of DC-SIGN [16,17]. Transfer of HIV-1 by B cells via a C-type lectin receptor pathway described for DC could be of significance in HIV-1 pathogenesis. We therefore investigated B cells for a DC-SIGN–mediated, trans pathway for HIV-1 infection of CD4+ T cells. We found that a subset of B cells in the peripheral blood and tonsils of healthy, HIV-1 seronegative donors expressed DC-SIGN, and that DC-SIGN expression increased after stimulation with interleukin 4 (IL-4) and CD40 ligand (CD40L). The stimulated, DC-SIGN+ B cells mediated trans infection of T cells.

Results

DC-SIGN Expression in B cells and Enhancement by Stimulation with IL-4 and CD40L

Our initial, three-color flow cytometric analysis of peripheral blood mononuclear cells (PBMC) showed that DC-SIGN was expressed on a small but distinct subset of CD19+ B cells within the CD45+CD19+ gated population of PBMC of normal, HIV-1–negative persons (representative example, Figure 1A). To extend this finding, we analyzed DC-SIGN expression on B cells that were purified from PBMC of 33 normal donors by sorting with anti-CD19 monoclonal antibody (mAb)-coated magnetic beads. Flow cytometric results showed that DC-SIGN was expressed on 7.9 ± 1.8% (mean ± standard error [SE]) of purified, peripheral blood CD20+ B cells.

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Abbreviations: ABC, antibody binding capacity; ART, antiretroviral therapy; CD40L, CD40 ligand; DC, dendritic cell; DC-SIGN, DC-specific ICAM-3 grabbing nonintegrin; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; mAb, monoclonal antibody; MESF, molecules of equivalent soluble fluorochrome; MFI, mean fluorescence intensity; MOI, multiplicity of infection; PBMC, peripheral blood mononuclear cells; SE, standard error

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A cell surface molecule, DC-SIGN, is known to bind the AIDS virus, human immunodeficiency virus 1 (HIV-1), on dendritic cells. HIV-1 can then be transferred from these dendritic cells to CD4+ T cells, in which the virus replicates and kills the T cells. Here, Rappocciolo and colleagues present their findings that DC-SIGN serves a similar function on a subset of B cells of the peripheral blood and tonsils. Although B cells that express DC-SIGN do not replicate HIV-1, they serve as portals for transfer and enhanced HIV-1 infection of CD4+ T cells, the major site of virus replication in the host. This newly described pathway for HIV-1 infection of T cells via B cells could be important in the pathogenesis of the virus infection.

We next addressed whether expression of DC-SIGN on this subset of B cells was related to a state of cellular activation. For this, we determined the proportion of B cells that expressed CD23, another type II C-type lectin receptor that is a low-affinity Fc receptor for IgE (Fc epsilon RI) and is associated with B cell activation and differentiation [15]. We found that DC-SIGN and CD23 were coexpressed on 8.1 ± 1.7% of B cells in normal persons (Figure S1A, T0). Indeed, 80% of DC-SIGN+ B cells also expressed CD23 (unpublished data). To further delineate the relationship of B cell activation to DC-SIGN, we examined whether stimulation of B cells with the T helper type 2 (Th2) cytokine interleukin 4 (IL-4) alone or in combination with the CD4+ T cell activation factor CD40L could alter expression of DC-SIGN and CD23, a procedure that mimics activation of B cells by CD4+ T helper cells during antigen processing [18,19]. Also, IL-4 has been shown to up-regulate expression of DC-SIGN on monocyte-derived DC [20], breast-milk macrophages [21], and the THP-1 cell line [22]. Our data demonstrate that treatment of purified B cells with a range of concentrations of either IL-4 or CD40L alone had little effect on the expression of DC-SIGN or CD23 by 24 h (Figure 1D, top; Figure S1B, top) or 48 h (unpublished data). In contrast, treatment with a combination of different concentrations of IL-4 and CD40L had a synergistic effect on expression of DC-SIGN and CD23 on B cells, with the greatest increase in the number of B cells expressing DC-SIGN being induced by stimulation with 1,000 U/ml of IL-4 and 1 μg/ml of CD40L for 24 h (Figure 1D, top and bottom, respectively; Figure S1B, bottom). This treatment was therefore used for stimulation of B cells in subsequent experiments.

Cumulative data from 33 normal donors showed that expression of DC-SIGN by purified, peripheral blood CD20+ B cells significantly increased after stimulation of the cells for 24 h with IL-4 and CD40L (representative example, Figure 1B) from a mean level of 7.9 ± 1.8% to 28.2 ± 3.3% (p = 0.0001) (Figure 1C). Moreover, the mean fluorescence intensity (MFI) of DC-SIGN on the B cells increased 3-fold after stimulation, from 10.8 ± 2.4 to 30.2 ± 6.6 (p < 0.005). The DC-SIGN+ CD23+ B cell population also increased from 8.1 ± 1.7% at 0 h to 25.7 ± 3.2% at 24 h (n = 33; p < 0.0002; Figure S1A, T24), with 86% of DC-SIGN+ cells expressing CD23 after 24 h of stimulation (unpublished data).

To confirm the increase in DC-SIGN expression in B lymphocytes stimulated by IL-4 and CD40L, we measured the level of DC-SIGN mRNA by real-time reverse transcriptase PCR [23,24] in B cells stimulated with IL-4 and CD40L compared to unstimulated B cells. B cells from five of seven normal donors (donors 1, 2, 4, 5, and 7) had large, 6.1- to 122.4-fold increases in levels of DC-SIGN mRNA, accompanied by an increase in surface expression of DC-SIGN (Table 1). B lymphocytes from the other two donors (donors 3 and 6) had low-to-moderate, 1.1- to 2.1-fold increases in DC-SIGN mRNA levels together with increases in surface expression of DC-SIGN after stimulation.

We next examined whether HIV-1 infection altered the number of B cells expressing DC-SIGN in the peripheral blood of HIV-1-infected persons, and the capacity of their B cells to respond to stimulation with IL-4 and CD40L. We found that the percentage of DC-SIGN+ B cells in the blood of HIV-1-infected persons with chronic HIV-1 infection who were antiretroviral therapy (ART) naive or those who had suppressed viral infection on ART was similar to uninfected persons (Figure 1C). However, expression of DC-SIGN was not enhanced on B cells from the HIV-1–infected subjects on ART in response to stimulation with IL-4 and CD40L, whereas DC-SIGN expression was enhanced in B cells from HIV-1–uninfected persons and ART-naive, HIV-1–infected subjects.

Next we compared the level of expression of DC-SIGN to other surface molecules that are known to increase during B cell activation and that play an important role in the interaction between B and T lymphocytes. Flow cytometry analysis of purified, IL-4– and CD40L-stimulated B cells for expression of DC-SIGN, CD23, the B cell signal transduction molecule CD22, and T cell co-stimulatory molecules CD58, CD80, and CD86 showed that there was an increase in coexpression of all of these markers with DC-SIGN at 24 h compared to 0 h (p < 0.005; T0 and T24, Figure 2A) or mock-stimulated B cells at 24 h (p < 0.05; unpublished data).

Finally, we determined the expression of CD4 and the chemokine receptors CXCR4 and CCR5 on B cells, which are the primary receptor and coreceptors involved in HIV-1 cis infection. Although a small subset of B cells from the blood expressed low levels of CD4, this marker was not detectable after 24 h (Figure 2B) or 48 h (unpublished data) of stimulation of the B cells with IL-4 and CD40L. High levels of expression of the CXCR4 coreceptor for HIV-1 were evident on most B cells before and after stimulation with IL-4 and CD40L, whereas the CCR5 coreceptor was not expressed.

Taken together, these results indicate that a distinct population of activated B lymphocytes constitutively expressed DC-SIGN in the blood of normal donors. Moreover, there was a significant increase in the frequency and intensity of DC-SIGN–expressing B cells derived from HIV-1–negative subjects and HIV-1–infected persons not on ART, which was not observed in HIV-1–infected persons on ART, after 24 h of stimulation with IL-4 and CD40L. Finally, the activated B cells expressed the CXCR4 coreceptor for HIV-1, but not the primary CD4 receptor or the CCR5 coreceptor for the virus.
to T cells [25]. We therefore determined the level of expression of DC-SIGN on blood-derived B cells as compared to DC and Raji–DC-SIGN cells by antibody binding capacity (ABC) and the number of molecules of equivalent soluble fluorochrome (MESF). The results show that IL-4– and CD40L-stimulated B cells expressed 137,870 ± 22,432 ABC (n = 6) for DC-SIGN compared to mock-stimulated B cells (36,946 ± 1,125, n = 6), DC (213,350 ± 43,370 ABC; n = 6), and Raji–DC-SIGN cells (225,750 ± 19,880 ABC; n = 4) (Figure 3A). A similar quantitative expression of DC-SIGN was observed on activated B cells by MESF (Figure 3B). The MESF and ABC histogram profiles for DC-SIGN on activated B cells compared to DC and Raji–DC-SIGN cells are shown in Figure 3C. These data support that IL-4– and CD40L-stimulated B cells expressed a sufficient number of DC-SIGN molecules for transfer of HIV-1 to T cells.

Activated B Cells Transmit HIV-1 X4 and R5 Strains to T Lymphocytes

Our finding that B lymphocytes expressed DC-SIGN led us to investigate whether these cells could be exploited by HIV-1 as a means for enhanced infection of T cells, as has been demonstrated for DC [1,26]. We first examined whether HIV-1 was able to bind to B cells and be transmitted to T lymphocytes through DC-SIGN using a low concentration of HIV-1 (multiplicity of infection [MOI] = 10⁻⁴, corresponding to 10 pg of p24 per 10⁶ cells), similar to that used in studies of DC-SIGN–related transmission of HIV-1 from DC to T cells [1,27]. This low amount of virus usually does not result in efficient cis infection of T cells. As displayed in Figure 4A, when the purified IL-4– and CD40L-stimulated B cells were incubated with X4 tropic HIV-1 (strain IIIb) and then co-cultured with autologous CD4⁺ T cells, virus replicated in the cultures as shown by an increase from undetectable, <1 × 10⁴

![Figure 1. Expression of DC-SIGN on B Cells](image_url)
transduced with IL-4 and CD40L, and loaded with HIV-1 X4 and cultured with T cells for 24 h, were not capable of enhancing HIV-1 infection in the co-cultures. Levels of HIV-1 X4 remained below detection in IL-4- and CD40L-stimulated B cells, mock-stimulated B cells, and T cells alone (Figure 2B). This indicates that virus replication in the B–T cell co-cultures was not a result of direct, cis infection of the T cells or B cells by HIV-1. For these HIV-1 transmission experiments, we used B cells obtained from magnetic bead–purified fractions with >96% CD20+ cells and <1% T cells and monocytes. Moreover, to ensure that the observed results were not due to contamination with other cell types, we also found that fractions of >96% pure B cells obtained by flow cytometry sorting were able to transmit HIV-1 to T cells with the same efficiency as the magnetic bead–purified B cells (unpublished data).

Further experiments demonstrated that IL-4– and CD40L-activated B cells loaded with three different concentrations of HIV-1 X4 resulted in a dose response–related level of infection in the B–T cell co-cultures (Figure 4B). Levels of HIV-1 p24 increased from undetectable, <1 × 10^3 pg per ml on day 4, to ≥5 × 10^5 pg of p24 per ml on day 16 at the highest input MOI (i.e., 10^4), and >1.7 × 10^4 and ≥8 × 10^5 pg per ml at the lower, 10^3 and 10^2 MOI, respectively. Virus did not replicate at any of these input concentrations in B cells or T cells alone (Figure 4B).

We next investigated whether expression of CD4, CXCR4, or CCR5 by B cells was related to trans infection of T cells. As shown in Figure 2B and in the literature [28], low numbers of unstimulated B cells in blood express very low levels of CD4. Moreover, expression of CD4 was down-regulated after stimulation of the B cells with IL-4 and CD40L for 24 h (Figure 2B). We next demonstrated that activated B cells enhanced trans infection of CD4+ T cells with a low MOI of the HIV-1 R5 tropic, Ba-L strain (Figure 4C), comparable to HIV-1 X4, even though B cells do not express CCR5 (Figure 2B) [28]. Moreover, cis infection of either unstimulated B cells (unpublished data), stimulated B cells (Figure 4C), or T cells (Figure 4C) with HIV-1 R5 at this low MOI did not result in virus replication.

Taken together, our data indicate that IL-4– and CD40L-activated B cells from HIV-1–negative persons mediated efficient trans infection of autologous CD4+ T cells with either X4 or R5 tropic HIV-1. Furthermore, this trans infection was not related to CD4, CXCR4, or CCR5 expression on the B cells. The fact that these were purified B cells from HIV-1–negative persons indicates that HIV-1 immune complexes and other types of cells were not involved in this process. It should also be noted that we did not include the lectin polybrene, a commonly used, receptor-independent enhancer of HIV-1 infection [29], in any of the cultures.

Table 1. Real-Time RT-PCR Measurement of DC-SIGN mRNA Levels in B cells of Seven Healthy, HIV-1 Seronegative Donors

| Donor | Time | CT | Fold Change (2^(-ΔΔCT)) | MFIa | % CD209+/CD20−c |
|-------|------|----|------------------------|------|------------------|
| 1     | T0   | >40.0 | 1                       | 16.1 | 12               |
| 2     | T0   | >40.0 | 1                       | 10.0 | 1.1              |
| 3     | T0   | >40.0 | 1                       | 20.7 | 5.5              |
| 4     | T0   | >40.0 | 1                       | 33.7 | 11.5             |
| 5     | T0   | >40.0 | 1                       | 15.2 | 8.2              |
| 6     | T0   | >40.0 | 1                       | 58.4 | 55.0             |
| 7     | T0   | >40.0 | 1                       | 4.3  | 0.3              |
| DC    |      | >40.0 | 1                       | 1.3  | 4.1              |
| K562  |      | >40.0 | 1                       | 1.1  | 13.0             |
| K562–DC-SIGN cellsd | 26.4 | >17,560 | 3,377 | 88.8% CD209+     |

aA maximal measure of DC-SIGN mRNA expression of CT = 40 was used for all calculations using K562 cells and CD19+ (CD19+K562) B cells (time 0 h = T0) as the appropriate calibrators. Therefore, the fold-increase measurements at time 24 h (T24) are minimal estimates.
bMFI of DC-SIGN on B cells analyzed by flow cytometry.
cPercent of DC-SIGN+/CD20− B cells as calculated above the background levels given by the isotype controls.
dK562–DC-SIGN cells are K562 cells stably transfeccted with a plasmid encoding DC-SIGN.

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findings that human herpesvirus 8 infects DC and macrophages via DC-SIGN and results in loss of surface expression of DC-SIGN [30]. Thus, after exposure of IL-4– and CD40L-activated B cells to HIV-1, the virus is associated with DC-SIGN⁺ but not DC-SIGN⁻ B cells, and there is no alteration in DC-SIGN expression in these cells as detected by flow cytometry.

We next determined the role of DC-SIGN on B cells in trans infection of T cells by treating IL-4– and CD40L-activated B cells with anti–DC-SIGN mAb or two nonspecific IgG as controls, one with the same isotype as the anti–DC-SIGN mAb (IgG2b) and the other of a different isotype (IgG1), prior to incubation with HIV-1 X4 or HIV-1 R5 and co-culture with T cells. The results show that pretreatment of B cells with anti-DC-SIGN mAb inhibited HIV-1 X4 and R5 replication in the B–T cell co-cultures, whereas pretreatment with the control IgG had no effect (Figures 6A and 6B). Also, inhibition of HIV-1 transmission by anti-DC-SIGN mAb was dose-dependent (Figure 6C). Because T cells do not express DC-SIGN [17], this virus-inhibitory effect was related to blocking of DC-SIGN on the activated B cells. These results indicate that HIV-1 X4 and R5 strains can be associated with B cells via DC-SIGN and that this leads to trans infection of T cells.

Although activated B cells did not express the primary CD4 receptor or the CCR5 coreceptor necessary for conventional infection by HIV-1 R5 (Figure 2B), they did express high levels of the CXCR4 coreceptor for HIV-1 (Figure 2B) [28]. Even though this is an inefficient receptor for HIV-1 infection in the absence of CD4 [31], we examined whether HIV-1 X4 trans infection of T cells was related to CXCR4 expression on the B cells. We treated activated B cells with anti-CXCR4 mAb or an IgG control, and showed that this did not inhibit HIV-1 X4 trans infection of T cells, whereas trans infection was blocked by pretreatment of the B cells with anti-DC-SIGN mAb (Figure 6D).

Taken together, these results show that DC-SIGN expression on purified, IL-4– and CD40L-activated, peripheral
blood B cells is directly related to trans infection of T cells. We hypothesized that this process could occur during B–T cell interactions in lymphatic tissues. In support of this, we found that DC-SIGN was expressed by 26.4 ± 6% (n = 5) of single-cell suspensions of purified tonsil B cells (representative example, T0, Figure 7A). In contrast to blood B cells, there was little coexpression of CD23 by freshly purified, tonsil B cells, but comparable up-regulation of CD23 and coexpression with DC-SIGN after 24-h stimulation with IL-4 and CD40L (T24, Figure 7A). Importantly, trans infection of T cells was mediated by the stimulated tonsil B cells, as demonstrated by an increase in p24 levels from <1 × 10^3 pg per ml at day 4 to >3 × 10^3 pg per ml at days 8 to 12 in the B–T cell co-cultures (Figure 7B). Over 90% of this virus replication was blocked by pretreatment of the B cells with anti–DC-SIGN mAb. Thus, both DC-SIGN expressing blood and lymphatic tissue B cells can mediate efficient trans infection of T cells.

**HIV-1 Is Internalized in Activated B Cells**

HIV-1 is predominantly found within cytoplasmic vacuoles in DC after binding to DC-SIGN [3]. This could relate to different pathways of HIV-1 that are required for subsequent trans infection of T cells. We therefore conducted a series of

![Figure 3](image-url)
B cells incubated with HIV-1 for 1 h at 4°C had viral particles bound only to their outer surface (Figure 8B). However, activated B cells exposed to HIV-1 for 2 h at 37°C had viral particles internalized in vacuoles of the cell cytoplasm (Figure 8C), with no intracellular virus apparent outside of vacuoles. Both intact viral particles and possible degraded virions were present in the vacuoles, similar to those observed after internalization of HIV-1 in DC (Figure 8D; [3]).

To prove further that HIV-1 particles were localized inside B lymphocytes and not merely bound to their surface, we incubated activated B cells with AT-2 inactivated HIV-1 X4 particles for 2 h at 37°C to allow internalization, and then treated the cells with trypsin to cleave virions still bound on the cell surface. The culture medium was removed, and the cells were washed and lysed. HIV-1 p24 contained in the wash supernatants and cell lysates was measured by enzyme-linked immunosorbent assay (ELISA). Under these conditions, essentially all of the p24 was found in the whole cell lysate, as very little was detected in the cell culture supernatants collected after the trypsin treatment (Figure 8E). To prove that internalized virus and not surface-bound virus was transferred from B cells to the T cells, we next performed a protease protection transmission assay. Cells were loaded with HIV-1 R5 at 4°C (wet ice) for 1 h, washed, and then shifted to 37°C for the times indicated prior to treatment with trypsin or medium (mock treatment). As shown in Figure 8F, very brief proteolysis of the B cells that had been exposed to HIV-1 at 4°C to inhibit virus entry and then shifted to 37°C (i.e., 0 min incubation) prevented transmission of HIV-1 to the T cells. In contrast, proteolysis of the HIV-1-loaded B cells after shifting to 37°C for 30 min did not prevent trans infection of the T cells. These data support that HIV-1 was internalized by the activated B cells prior to transmission to T cells.

Finally, we examined the ability of HIV-1 to remain infectious in B cells over time, as has been shown for HIV-1 infection of DC [32]. For this, we exposed activated B cells to infectious X4 or R5 strains of HIV-1 for 2 h at 37°C, and then either mixed them immediately with T cells or kept them at 37°C for 2 d before mixing with T cells. As shown in Figure 9A and 9B, B cells that were loaded with HIV-1 and cultured alone for 2 d could still transmit virus to T cells, although at a lower efficiency than when T cells were added immediately to the HIV-1-exposed B cells. Taken together, these results indicate that HIV-1 was internalized by the activated B cells prior to transmission to T cells.

Discussion

Our results demonstrate that activated B lymphocytes derived from peripheral blood and lymphatic tissue express DC-SIGN, and that these cells mediate HIV-1 trans infection of T lymphocytes. Evidence for trans infection was an increase of HIV-1 p24 from undetectable levels (<1 × 10^3 pg/ml) to >10^3–10^4 pg/ml after 12–16 d of co-culture of the activated B cells with autologous CD4^+ T cells. The trans infection was not due to direct cis infection of the B cells, as virus replication was not detectable in B cells alone after exposure to HIV-1. Likewise, there was little or no detectable replication of HIV-1 after direct infection of T cells alone with the low input of HIV-1 used in our experiments. Expression of DC-SIGN by
the B cells was associated with this enhanced HIV-1 infection of CD4\(^+\) T cells in \textit{trans}. That is, treatment with IL-4 and CD40L, which are mediators of B cell activation by CD4\(^+\) T cells [19], enhanced DC-SIGN expression on the B cells. A total of 80\% of the HIV-1–infected B cells were DC-SIGN\(^+\) by flow cytometry. This was supported by immunofluorescence microscopy analysis showing that DC-SIGN and HIV-1 were coexpressed in the B cell cytoplasm. Association of virus with a small portion of non-DC-SIGN–expressing B cells could be related to expression of other C-type lectin receptors by B cells that bind gp120 [16]. Most importantly, DC-SIGN expression by the B cells was required for \textit{trans} infection of CD4\(^+\) T cells with both X4 and R5 tropic strains of HIV-1, as we could block essentially all of the \textit{trans} infection by pretreating the B cells with mAb specific for DC-SIGN.

In further support of a central role for DC-SIGN on B cells in HIV-1 \textit{trans} infection, we found that the number of DC-SIGN molecules expressed on activated B cells was similar to that known to be sufficient to sustain capture of HIV-1 and \textit{trans} infection of T cells by DC [25]. Notably, the mechanism of \textit{trans} infection of T cells by B cells did not involve HIV-1 infection of B cells by the conventional, CD4–CXCR4/CCR5 pathway. This was supported by the fact that B cells expressed little or no CD4 and did not express CCR5. Moreover, although B cells expressed high levels of CXCR4, treatment with anti-CXCR4 mAb prior to virus binding to the B cells did not inhibit \textit{trans} infection of the T cells. Thus, although other C-type lectin receptors are expressed on B cells and can bind gp120 [16], our results support a requirement for DC-SIGN in B cell–mediated, \textit{trans} infection of T cells.

HIV-1 was internalized by B cells as determined by resistance of B cell–associated virus to treatment with trypsin, and predominance of virus particles in cytoplasmic vacuoles of B cells after binding to DC-SIGN. Both intact and apparently degraded particles were present within the vacuoles, similar to HIV-1 internalization in DC via DC-SIGN [3]. Internalized and not extracellularly bound virus resulted in \textit{trans} infection of T cells, which was demonstrated by lack of effect of trypsin treatment of HIV-1–loaded B cells on their ability to mediate \textit{trans} infection. These results suggest a role for internalization of HIV-1 by B cells that is similar to that of DC \textit{in trans} infection of T cells [33]. We further observed that virus was maintained for at least 2 d in an infectious form in B cells, comparable to its association with DC [32]. It is not clear how this virus infectivity persists. Recent studies indicate that most of the captured virions are destroyed in DC-SIGN–expressing DC and B cell lines engineered to express DC-SIGN [3,34]. However, a portion

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**Figure 5. Expression of DC-SIGN and HIV-1 in B Cells**

(A) Immunofluorescent microscopy showing coexpression of DC-SIGN and HIV-1 in IL-4 and CD40L purified, activated B cells after 2 h of exposure to HIV-1 X4 (MN) at 37\°C. B cells not exposed to HIV-1 were used as controls; these included a subset that expressed DC-SIGN and were negative for HIV-1 (unpublished data). (600\times magnification) Green indicates DC-SIGN, red indicates p24, and blue indicates DAPI.

(B) Flow cytometry histograms showing coexpression of DC-SIGN and HIV-1 in IL-4 and CD40L purified, activated B cells after 2 h of exposure to HIV-1 X4 (MN) at 37\°C.

(C) HIV-1 R5 (Ba-L) had no effect on expression of DC-SIGN by purified, activated B cells (left) or DC (middle), and minimally inhibited DC-SIGN expression in Raji–DC-SIGN cells (right) after 24 h incubation with the virus. Full histogram represents uninfected B cells; purple overlay histogram represents HIV-1–infected cells; and black overlay histogram represents isotype controls. Data are from single experiments representative of five independent experiments.

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of the input virus can rapidly trans infect T cells [3,35]. In contrast, the longer term, persistent infectivity of DC-SIGN–expressing DC and B cell lines has been related to low levels of de novo HIV-1 replication [3,35]. It is possible that very low, subdetectable levels of HIV-1 replication occurred in our IL-4– and CD40L-stimulated B cells, and resulted in persistence of infectious virus involved in trans infection of T cells. Further work is in progress to delineate the mechanisms by which DC-SIGN-expressing B cells lead to HIV-1 trans infection of T cells.

The DC-SIGN–mediated, B-to-T cell trans infection pathway appears to be distinct from previously described B–T cell infectious processes. That is, it has been reported that transmission of HIV-1 from B cells to T cells involves virus trapped in immune complexes on the surface of the B cells [6–10]. This is not involved in our system, since in our studies, HIV-1 trans infection of T cells was mediated by B cells from normal, HIV-1 antibody–negative persons. Finally, in contrast to Swingler et al. [11], where nef-induced soluble factors released by infected macrophages work together with B cells to lead to trans infection of T cells, we observed trans infection of T cells by purified, DC-SIGN+ B cells in the absence of macrophages.

Notably, we found that approximately 8% of B cells in the blood of normal donors expressed DC-SIGN. Stimulation for 24 h with IL-4 and CD40L resulted in an increase in the number of B cells expressing DC-SIGN (i.e., 28%) and the level of DC-SIGN expression on the B cells. Approximately 80% of the DC-SIGN+ B cells in blood also expressed the type II C-type lectin receptor and B cell activation marker, CD23, which increased to 86% after stimulation in vitro with IL-4 and CD40L. Activation of these stimulated, DC-SIGN+ B cells was confirmed by elevated levels of T cell coreceptors CD58, CD80, and CD86, as well as increases in coexpression of the B

**Figure 6.** B Cell–Mediated Transmission of HIV X4 and R5 to T Cells Is Blocked by Anti–DC-SIGN mAb  
(A) and (B) HIV-1 p24 levels in cultures of activated B cells that were treated with anti–DC-SIGN mAb, incubated with either HIV-1 X4 (IIIb) (Figure 6A) or R5 (Ba-L) (Figure 6B) for 2 h at 37 °C, washed and cocultured with autologous T cells. B and T cells cultured alone with each virus were used as controls.  
(C) HIV-1 p24 levels in cultures of activated B cells that were incubated with decreasing amounts of anti–DC-SIGN mAb prior to exposure to HIV-1 R5 (Ba-L) and culture with autologous T cells for 8 d. Treatment with mouse IgG or an unrelated mAb (anti-CD11a) had no effect on HIV-1 R5 (Ba-L) trans infection.  
(D) HIV-1 p24 levels in cultures of activated B cells incubated with anti–DC-SIGN mAb or anti-CXCR4 mAb, washed, loaded with HIV-1 X4 (IIIb), and cocultured with autologous T cells for 12 d. Data are represented as mean of triplicates ± SE. Data are from single experiments representative of eight independent experiments.

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**A**

- B + X4 + T
- B + anti-DC-SIGN + X4 + T
- B + mouse IgG + X4 + T

**B**

- B + R5
- T + R5
- B + R5 + T
- B + R5 + anti-DC-SIGN + T
- B + R5 + mouse IgG + T

**C**

- untreated
- anti-DC-SIGN
- mouse IgG
- anti-CD11a

**D**

- untreated
- anti-CXCR4 treated
- anti-DC-SIGN treated
cell signal transduction molecule CD22. Expression of DC-SIGN was not restricted to blood B cells, as we found that approximately 26% of tonsil B cells constitutively expressed DC-SIGN, and that this increased to 39% after stimulation of the cells with IL-4 and CD40L. Interestingly, our study showed that few B cells directly isolated from the tonsils expressed CD23, but that this number increased after stimulation with IL-4 and CD40L. Expression of CD23 on some but not all DC-SIGN⁺ B cells could be related to differential expression of CD23 by B cells in distinct areas of tonsils [36], and a pronounced cleavage and shedding of soluble CD23 by activated B cells [37].

Of interest is that there was a comparable number of DC-SIGN-expressing B cells in the blood of uninfected persons as in those with chronic HIV-1 infection who were treatment naive or receiving ART. In support of these findings, although B cells can harbor HIV-1 in blood [6], we found that HIV-1 did not kill B cells or inhibit DC-SIGN expression within 24–

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**Figure 7.** DC-SIGN-Expressing Tonsil B Cells Mediate trans Infection of T Cells

(A) Expression of CD20 and DC-SIGN or CD23 and DC-SIGN in fresh (time 0 h; T0) and IL-4- and CD40L-activated (time 24 h; T24) B cells derived from tonsils. Data are from one experiment representative of five independent experiments.

(B) Levels of HIV-1 p24 in co-cultures of B and T cells derived from tonsils. B cells were stimulated with IL-4 and CD40L for 24 h, treated with anti-DC-SIGN mAb or mouse Ig (20 µg/ml) or left untreated, and loaded with 10⁻⁷ MOI of HIV-1 R5 (Ba-L) for 2 h at 37 °C, then extensively washed in cold medium and incubated with purified, autologous CD⁴⁺ T cells (T). B cells and T cells directly loaded with HIV-1 served as controls. Amount of HIV-1 p24 in the cell culture supernatants was determined by ELISA. Data are from one experiment representative of two independent experiments.

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48 h of exposure to virus in vitro. Our observation that IL-4 and CD40L stimulation of B cells from persons receiving ART failed to enhance expression of DC-SIGN suggests a negative effect of ART on this process. This could be related to the reported inhibitory effect of protease inhibitors, which are common components of ART, on expression of DC-SIGN [38], and requires further study.

Expression of DC-SIGN on B cells suggests that it is operative in their normal function. This type II C-type lectin receptor could be involved in B cell trafficking and antigen presentation to T cells, similar to its function in DC [39]. Indeed, high levels of expression of DC-SIGN were induced in B cells by a combination of T cell factors IL-4 and CD40L and not by either alone. These results support the concept that DC-SIGN is involved in the interaction of activated B and T cells during antigen processing and presentation.

Our data indicate that DC-SIGN–expressing B cells could become vehicles for HIV-1 infection of T cells during their cognate interactions in the lymphatics. High concentrations of HIV-1 have been found associated with B cells and CD4+ T cells in lymph nodes [40], where B cells are activated and proliferate through cytokine and CD40L–CD40 interactions [41]. This has been related to virus-containing immune complexes on the surface of B cells that could lead to infection of CD4+ T cells during “cross talk” in the micro-environment of lymphoid tissues. The presence of a large amount of unspliced simian immunodeficiency virus RNA in B cell–rich areas of lymphoid tissues [42], is also consistent with virions being associated with B cells and follicular DC in germinal centers. B cells have therefore been proposed as an important, lymphatic reservoir in the pathogenesis of HIV-1 [28]. Our results suggest that B cells play a previously unrecognized role in replication of HIV-1 in T cells and viral pathogenesis through a DC-SIGN–dependent mechanism.

Materials and Methods

Donors. PBMC were obtained from healthy, HIV-1–seronegative (n = 33) and HIV-1–seropositive (n = 20) adult volunteers and subjects enrolled in the Multicenter AIDS Cohort Study. Informed consent was obtained following institutional guidelines. ART-naive, HIV-1–seropositive subjects (n = 10) had mean (± SE) of 658 ± 124 CD4+ T cells per ml of blood, and HIV-1 RNA loads of 6,410 ± 3,701 copies per ml of plasma; HIV-1–seropositive subjects on ART (n = 10) had
**Figure 9. HIV-1 Is Maintained in an Infectious Form by B Cells**

Activated B cells were loaded with HIV-1 X4 (IIIb) or HIV-1 R5 (Ba-L) for 2 h at 37°C, extensively washed and then either cultured immediately with autologous T cells (panel A) or incubated for 2 d prior to adding autologous T cells (panel B). B cells and T cells directly loaded with HIV-1 served as controls.

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CD4+ T cell counts of 939 ± 82 and viral RNA loads of 66 ± 20. Human tonsils were obtained from patients (n = 5) undergoing therapeutic surgery, in accordance with institutional guidelines.

**Preparation of B and T cells.** PBMC were isolated by Ficoll-Hypaque density gradient separation and used immediately for surface phenotype staining or further purification. For B cell purification, monocytes were depleted with two rounds of anti-CD14 mAb-coated immunomagnetic microbeads (Miltenyi) according to the manufacturer's instructions. B cells (CD19+ cells) were then isolated from the CD14+ cell fraction by incubation with anti-CD19 mAb-coated, magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the fractionated B cells was 96.4% ± 0.4 (mean ± SE; n = 33) as determined by staining with anti-CD20 mAb, with <1% CD14+ and CD3+ cells. The immunomagnetic bead purification procedure did not alter expression of DC-SIGN or CD23 (unpublished data). Autologous CD4+ T cells were obtained from the remaining CD14+ CD19+ cell fraction by CD4+ cell purification using anti-CD4 mAb-coated microbeads (Miltenyi). Activated B cells were generated by culture of CD19+ cells in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 1,000 U/ml of rhIL-4 (R & D Systems, Minneapolis, Minnesota, United States), and 1 ug/ml of soluble trimeric CD40 L (Amgen, Thousand Oaks, California, United States). CD4+ T lymphocytes were cultured in RPMI1640 medium supplemented with 20% FCS and phytohemagglutinin (PHA) and IL-2, as described [43].

Tonsils were immediately transferred to the laboratory in cold PBS supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), gentamicin (5 µg/ml), amphotericin B (0.5 µg/ml), and 5% FCS. The tissues were cut into fragments and pushed through a stainless steel sieve with a 250-µm mesh, using the flat end of a plastic syringe plunger, to remove the connective capsula. Lymphocytes from the collected cell suspension were isolated by Ficoll-Hypaque density gradient centrifugation. The cells collected at the interface were stained with anti-CD20, anti-CD3, and anti-CD14 mAb to determine the relative percentage of B and T lymphocytes, and were shown to contain 60%–70% B lymphocytes, 30%–40% T lymphocytes, and 1% or less monocytes. B and T cells were purified by immunomagnetic bead separation and activated as described above.

**Preparation of DC.** DC were generated from CD14+ cells as described [44]. Briefly, CD14+ cells were enriched from PBMC with anti-CD14 mAb magnetic beads (>95% lineage negative, HLA DR+ cells) and cultured for 5 d in the presence of 1,000 U/ml of rhIL4 and rGM-CSF.

**Antibodies.** The following mAbs were used in this study: anti-DC-SIGN (CD209) (clone 120507; R & D Systems); anti-CD4, anti-CD14, anti-CD19, anti-CD20, anti-CD22, anti-CD45, anti-CD58, anti-CD80, anti-CD86, anti-CXCR4, and CCR5 (BD Pharmingen, San Diego, California, United States); anti-CD23 (Caltag Laboratories, Burlingame, California, United States); anti-HIV-1 p24 (KC57; Beckman-Coulter, Fullerton, California, United States). These mAbs were used either unlabeled or conjugated with FITC, PE, PE-Cy5, or PE-Cy7 as indicated below. Appropriate isotype-matched controls were used for background staining evaluation. For the virus receptor blocking experiments, we used sodium azide–free, low-endotoxin, purified, anti–DC-SIGN clone 120507 mAb conjugated with IgG1 of the relevant isotype (Becton Dickinson, Palo Alto, California, United States) reconstituted in sterile PBS.

**Flow cytometry and quantification of DC-SIGN expression.** Expression of cell surface molecules was examined by flow cytometry with a Beckman Coulter XL flow cytometer. Cells were incubated with the desired antibodies or isotype controls for 30 min at 4°C in buffer consisting of PBS supplemented with 0.1% FCS and 0.1% NaN3. After extensive washing with the buffer, the cells were resuspended in 1% paraformaldehyde in PBS for flow cytometric analysis. HIV-1 intracellular p24 was identified using PE-labeled KC57 anti–HIV-1 Gag mAb, following cell permeabilization using Perm-flow (Invirion Diagnostics, Oak Brook, Illinois, United States) according to manufacturer's instructions. Results were expressed either as percent positive cells above the isotype control threshold or as MFI above the isotype controls.

For quantification of DC-SIGN expression on DC, Raji–DC-SIGN cells, and activated B cells, we used the Quantum Simply Cellular kit (Bangs Laboratories, Fishers, Indiana, United States) according to the manufacturer's instructions. Quantification was performed by converting the geometric mean channel fluorescence (GMCF) into ABC. The kit contains five microbeads of uniform size coated with different amounts of goat anti-mouse IgG (Fc-specific) on their surface that have different abilities to bind mouse antibodies (ranging from 0 to about 250,000 molecules). Both beads and cells were labeled with saturating amounts of FITC-conjugated, anti–DC-SIGN mAb, processed, and analyzed by flow cytometry under identical conditions. A calibrating curve was derived from the bead samples using QuickCal (Bangs Laboratories). The GMCF of the samples was converted to ABC per cell by comparison with the regression curve generated with the beads. Samples were also evaluated for fluorescence intensity as expressed by MESF units, using the Quantum MESF kit (Bangs Laboratories). The kit consists of five bead populations having different levels of FITC fluorescence intensity. As described above, a regression curve was generated by plotting the GMCF of the beads against their known MESF using QuickCal. The MESF of the cell samples was then determined as described above for ABC.

**Real-time RT-PCR measurement of DC-SIGN mRNA.** Total RNA was extracted from cells using Trizol (Invitrogen Life Sciences, Carlsbad, California, United States) according to the manufacturer's instructions, DNase-treated (Ambion, Austin, Texas, United States) and affinity column purified (RNAasy, Qiagen, Valencia, California, United States).
The sequences of the primers and probe for Taqman amplification and detection of DC-SIGN mRNA were kindly provided by B. Lee (University of California, Los Angeles) and were DC-SIGN.F1, 5’-GCCTGAAGACCTGGATGCC-3’; DC-SIGN.R1, 5’-GAAGCAGTGGAAAGAGAATGTTG-3’; and DC-SIGN.probe, 5’-6FAM-ACAGTCGTGAAGACACCGTGCC-ATCT-MGB-3’. The comparative
Ct method [23,24] was used to determine relative mRNA expression levels. The primer and probe set for
β-glucoronidase (β-GUS) (Applied Biosystems, Foster City, California,
United States) was used as the endogenous control. Across all samples, mean β-GUS Ct values were 29 ± 2 (unpublished data).
cDNA synthesis with 400-ng input RNA was performed in duplicate with
Superscript II reverse transcriptase (Invitrogen) and random hexamers as described [24], in parallel with control reactions lacking
RT. Amplification and detection for 40 cycles was performed on a
Prism 7000 sequence detection system (Applied Biosystems).

amplicons were then incubated with 200 units of Taqman enzyme
in a 50-μl reaction volume for 2 h. The MOI was based on tissue culture infectious dose 50%
(TCID50) assay for each virus.

Virus. HIV-1 IIIB (X4 tropic virus) and HIV-Ba-L (R5 tropic virus) were propagated in
PHA-activated normal donor PBMC and purified as described [43]. Virus titers were determined as pg/ml
by p24 ELISA (DuPont, Wilmington, Delaware, United States), with a
lower limit of 1 pg/ml and upper limit of ≥ 5 × 1010 pg/ml. AT-2–
infected HIV-1 ML (CL.4SUF1; X4, tropic), and ADA (MSU1T1-
CCR5; R5, tropic) were a gift from J. D. Lifson, National Cancer
Institute, Frederick, Maryland.

HV-1 infection and transmission assay. Purified, IL-4– and CD40L-
stimulated B lymphocytes (1 × 106) were incubated with different amounts of HIV-1 IIIB or HIV-1 Ba-L, i.e., 10−2, 10−3, or 10−4 MOI at 37
°C for 2 h. The MOI was based on tissue culture infectious dose 50%
determination on C8166 cells, PHA-stimulated human lymphocytes,
and confirmed by spectrophotometric analysis of 10-fold serial dilutions on TZM-bl indicator cell line. Unless otherwise specified, cell-
free supernatants were taken at various time intervals for titration of virus by p24 ELISA. No difference was observed in viability of mock-
treated and HIV-1–treated B cells as measured by trypan blue dye
exclusion. In some experiments, stimulated B cells were incubated with
anti–DC-SIGN mAb (clone 120507) or CD11a/LFA-1 (clone HI111, BD
Pharmingen) or mouse IgG (R & D Systems) for 30 min at 4°C prior to incuba-
cion with virus. The specificity of anti–DC-SIGN mAb clone
120507 was confirmed by binding to DC-SIGN–transfected K-562 cells
and lack of binding to K562 cells transfected with the DC-SIGN–
related, type II C-type lectin, DC-SIGN.R1 [30].

Loading of DC and activated B cells with HIV-1 for electron microscopy. A total of 1 × 106 DC or purified activated
B cells were incubated in a 1.5-ml Eppendorf tube with AT-2–infected
HIV-1 (5 ng of p24 Ag/106 cells) in a total volume of 100 μl at 37°C for up to 2 h. After
the incubation, cells were extensively washed in cold medium using a
refrigerated microfuge, and the cell pellets were fixed in PBS with
2.5% glutaraldehyde for 1 h. Pellets were washed three times in PBS
and then post-fixed in 1% aqueous osmium tetroxide supplemented
with 1% K3Fe(CN)6 for 1 h. Pellets were then washed three times in
PBS and then dehydrated through a series of 30%–100% ethanol, 100%
propylene oxide, and then infiltrated with 1:1 mixture of propylene
oxide–polishable 812 epoxy resin (Polysciences, Warrington,
Pennsylvania, United States) for 1 h. After several changes of 100%
resin over 24 h, the pellet was embedded in a final change of resin,
fixed with 4% paraformaldehyde for 20 min, and then permeabilized
with 0.5% triton X-100, 0.1% saponin, 0.1% NaN3 for 20 min at room
temperature. Cells were stained with FITC-conjugated, anti-DC-
SIGN mAb and PE-labeled anti-HIV-1 p24 mAb. To avoid non-
specific binding of IgG, all incubations and dilutions of reagents were
done in Super-Block blocking buffer (Pierce Biotechnology, Rock-
ford, Illinois, United States). Controls included activated B cells not
exposed to HIV-1.

Supporting Information

Figure S1. CD40L and IL-4 Act Synergistically in Inducing Enhanced DC-SIGN and CD23 Expression on B Cells
(A) Coexpression of DC-SIGN and CD23 on B cells from healthy, HIV-1–seronegative donors (n = 20). Hour 0 [T0] = 0 h; hour 24 [T24] = 24 h.
(B) Time-dependent expression of DC-SIGN and CD23 in response to IL-4 and CD40L. Purified B cells from a normal donor were cultured in the presence of IL-4 or CD40L or a combination of the two. Untreated cells were used as controls (NT). DC-SIGN and CD23 coexpression on activated B cells was greatest using a combination of 1,000 U/ml of IL-4 and 1 μg/ml of CD40L for 24 h. Single concentrations of 100, 500, and 10,000 U/ml of IL-4 and 0.1, 0.5, and 10 μg/ml of CD40L induced similar, low levels of DC-SIGN expression (unpublished data). Also, various combinations of these concentrations of IL-4 and CD40L induced less DC-SIGN expression than this combination (unpublished data).

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Accession Numbers
The GenBank accession number (http://www.ncbi.nlm.nih.gov/Genbank) for the gene mentioned in this paper is CD209 (NM_021155).

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and CRR analyzed the data and wrote the paper.

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