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Novel Pathway for Induction of Latent Virus from Resting CD4$^+$ T Cells in the Simian Immunodeficiency Virus/Macaque Model of Human Immunodeficiency Virus Type 1 Latency$^\nabla$

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Although combination therapy allows the suppression of human immunodeficiency virus type 1 (HIV-1) viremia to undetectable levels, eradication has not been achieved because the virus persists in cellular reservoirs, particularly the latent reservoir in resting CD4$^+$ T lymphocytes. We previously established a simian immunodeficiency virus (SIV)/macaque model to study latency. We describe here a novel mechanism for the induction of SIV from latently infected resting CD4$^+$ T cells. Several human cell lines including CEMx174 and Epstein-Barr virus-transformed human B-lymphoblastoid cell lines mediated contact-dependent activation of resting macaque T cells and induction of latent SIV. Antibody-blocking assays showed that interactions between the costimulatory molecule CD2 and its ligand CD58 were involved, whereas soluble factors and interactions between T-cell receptors and major histocompatibility complex class II were not. Combinations of specific antibodies to CD2 also induced T-cell activation and virus induction in human resting CD4$^+$ T cells carrying latent HIV-1. This is the first demonstration that costimulatory signals can induce latent virus without the coengagement of the T-cell receptor, and this study might provide insights into potential pathways to target latent HIV-1.

Advances in the antiretroviral therapy, particularly the introduction of highly active antiretroviral therapy (HAART), now allow the control of viral replication in patients with human immunodeficiency virus type 1 (HIV-1) infection. HAART reduces plasma HIV-1 RNA levels to below the limit of detection of current assays in many patients (20, 21, 37). However, HIV-1 persists even in successfully treated patients whose plasma virus levels have fallen to undetectable levels (10, 14, 16, 17, 23, 49, 54). A major viral reservoir in which long-term persistence has been extensively documented consists of latently infected resting CD4$^+$ T lymphocytes (10, 16, 17, 37, 38, 54). These latently infected cells carry stably integrated proviruses (9, 10, 22) but do not produce virus unless they are activated through encounters of antigen and/or cytokines (9, 44). Such induction events are one likely source of viral rebound after the interruption of therapy (12). In addition to the stable form of HIV-1 latency that involves integrated proviruses, a more labile preintegration form of latency was observed in resting CD4$^+$ T cells from viremic patients (2, 15, 22, 39, 51, 55, 56).

HIV-1 and HIV-2 appear to have originated from simian immunodeficiency viruses (SIVs) that naturally infect many species of African primates (46). HIV-1 is closely related to SIVcpz, which is found in chimpanzees (19), and HIV-2 resembles SIVsm, which is found in sooty mangabeys (6). SIV infections are apparently nonpathogenic in their natural hosts (4, 42), and immunodeficiency is extremely rare (35). However, cross-species transmission can result in AIDS-like syndromes, with high levels of viremia, a loss of CD4$^+$ T cells, and opportunistic infections. An AIDS-like disease was first noted in rhesus macaques infected with SIV from sooty mangabeys (29). Although SIV infection of macaques is an excellent model for HIV-1 pathogenesis, it has only recently been used to model the treatment of HIV-1 infection (11, 34, 47, 57), including the SIV/macaque model that we recently developed to study HIV latency under suppressive therapy (47). We showed that SIV established latent infection in resting macaque CD4$^+$ T cells and that these latently infected cells persisted in the peripheral lymphoid organs despite suppressive antiretroviral therapy of the infected animals.

In the process of developing the SIV/macaque model, we discovered a novel approach to reactivating latent virus from resting CD4$^+$ T cells in either the pre- or postintegration states of latency. We have reported results from infected aviremic animals in a previous report (47). All of the experiments reported here were done with viremic animals. Coculturing of the human lymphoid cell line CEMx174 with resting CD4$^+$ T cells from infected macaques on HAART resulted in T-cell activation and induction of latent SIV. In previous studies, induction of latent virus was accomplished through mitogen stimulation (9), engagement of T-cell receptor (TCR) and

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major histocompatibility complex (MHC) (33), antibodies to CD3 and CD28 (3), cytokines (8, 44), or pharmacologic stimuli that activate downstream signaling molecules in the T-cell activation pathways (25). The pathway for the activation of latent SIV described here is not principally dependent on TCR-MHC interactions or cytokines. Rather, the activation is dependent upon the interaction between the costimulatory molecule CD2 on T cells and its ligand, CD58. Pioneering work by Meuer and colleagues showed that resting $CD4^+$ T cells can be activated through the CD2 pathway alone without the coengagement of the TCR (31). We now show that latent SIV or HIV-1 can be induced through the CD2 pathway. These results provide the first evidence that the engagement of costimulatory molecules can induce latent virus in T cells without the coengagement of the TCR with the MHC. Therefore, these studies suggest new strategies for targeting the latent reservoir in HIV-1 infection.

MATERIALS AND METHODS

The protocols involving human patients and macaques were approved by an institutional review board of the Johns Hopkins University School of Medicine.

Isolation of resting $CD4^+$ T cells. Resting $CD4^+$ T cells were isolated as described previously (47). Briefly, macaque or human blood was centrifuged through discontinuous density gradients to obtain peripheral blood mononuclear cells (PBMC). In some of the experiments, a positive selection step with antibody-conjugated magnetic beads (Miltenyi Biotech) was used prior to the sorting for resting $CD4^+$ T cells. The positively selected $CD4^+$ T cells were then stained with phycoerythrin-conjugated antibody to CD4 (Pharmingen) and fluorescein isothiocyanate (FITC)-conjugated antibodies to CD25 and HLA-DR (Becton Dickinson). The $CD4^+$ $CD25^-$ DR^- fraction was collected.

Activation of resting $CD4^+$ T cells by PHA and irradiated PBMC. Resting macaque $CD4^+$ T cells were cultured with phytohemagglutinin (PHA) (0.5 μg/ml) and irradiated human or macaque PBMC (10×10^6 irradiated PBMC per 10^6 resting $CD4^+$ T cells). Resting human $CD4^+$ T cells were cultured with PHA and irradiated allogeneic human PBMC. PHA was removed by changing the culture medium 1 day later. Cells were cultured in interleukin-2 (IL-2)-containing medium for 2 weeks or as indicated. SIV released by the activation of latently infected cells was amplified by the addition of CEMx174 cells to the culture, and HIV-1 was amplified by the addition of $CD4^+$ T lymphoblasts prepared by the stimulation of PBMC from healthy human donors with PHA and IL-2 for 3 days, followed by the isolation of $CD4^+$ cells.

Cell lines. CEMx174, a gift from James Hoxie of the University of Pennsylvania, is a hybrid cell line generated by fusing 721.174, a mutagenized, MHC class II-negative, Epstein-Barr virus (EBV)-transformed, human B-lymphoblastoid cell line (LCL), with CEM, a $CD4^+$ human T-cell tumor line (43). B-LCL is an EBV-transformed B-cell LCL generated from healthy human B lymphocytes. Jurkat is a human $CD4^+$-T-cell line (ATCC TIB-152). 293T is a human embryonic kidney epithelial cell line (36). CA46, a gift from Richard Ambinder (Johns Hopkins University), is an EBV-negative Burkitt's lymphoma cell line. Rael, also a gift from Richard Ambinder, is an EBV-positive Burkitt's lymphoma cell line.

Resting T-cell activation and latent virus induction by various cell lines. Resting macaque $CD4^+$ T cells were cocultured with the indicated cell lines in RPMI 1640 with glutamine and 10% fetal bovine serum. The ratio of primary cells to the cell lines was usually 4:1 on the day of coculturing. The primary cell concentration was kept between 100,000 and 250,000 cells/ml of medium. The cell lines continued to multiply during the culture period, and the cultures were split on the fifth day and every other day thereafter or as needed. The cultures were kept 1 to 2 weeks, and growth of virus was detected by measuring p27 antigen in culture supernatants by enzyme-linked immunosorbent assay (ELISA) (Beckman Coulter). Quantification of the frequency of latently infected $CD4^+$ T cells was carried out as previously described (47). Results were expressed as infectious units per million (IUPM) resting $CD4^+$ T cells.

In the experiments involving human resting $CD4^+$ T cells, human PBMC were sorted and cocultured with CEMx174 as described above for macaque cells. Human $CD4^+$-T-cell lymphoblasts were added to the culture on the third day to amplify HIV-1 released from latently infected cells. At the end of culture period, the release of HIV-1 was detected by ELISA for HIV-1 p24 antigen (Beckman Coulter).

In the blocking assays, antibodies were added 5 h before combining primary T cells with the cell lines. Antibodies were all obtained from Pharmingen, and each one was used at a saturating concentration of 4 μg/ml.

T-cell activation by CD25 and Ki67. On day 5 or 6 after the initiation of the coculture, cells were stained with FITC-conjugated antibodies to CD25 (CD25-FITC) or Ki67 (Ki67-FITC) to detect cellular activation and proliferation, respectively. For Ki67 staining, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences). To distinguish between the primary T cells and the cell lines, CD6-phycoerythrin (Pharmingen) was used to mark primary T cells, and CD45-PerCP-Cy5.5 (BD Bioscience) or CD22-Cychrome (Pharmingen) was used to mark human cell lines (the anti-CD45 antibody used, clone 2D1, does not cross-react with macaque, and CD22 is expressed only in B-cell lines and not in primary T cells). From forward- and side-scatter profiles, the smaller primary lymphocyte population was gated (see Fig. 3A), and on the fluorescence channels, CD6-phycoerythrin-positive and CD45-PerCP-Cy5.5- or CD22-Cychrome-negative cells were then gated (see Fig. 3B) and analyzed for CD25 or Ki67 expression using a flow cytometer (FACScan from BD Biosciences).

In vitro infection of T cells. Resting macaque $CD4^+$ T cells were obtained as described above and infected with SIVmac239, SIVmac251, or SIV/17E-Fr (18, 30) with a multiplicity of infection (MOI) of 0.02 or 0.03. After spin inoculation at $1,200 \times g$ for 2 h, the cells were washed three to four times before being cocultured with the indicated cell lines. Supernatants were taken at various days postinfection for analysis of SIV p27 antigen. For the limiting-dilution experiments, T cells were diluted in fivefold serial dilutions in duplicate, as previously described (47).

Induction of HIV with anti-CD2 antibodies. Resting human $CD4^+$ T cells were purified, as described above, from patients on HAART with prolonged suppression of viremia to <50 copies/ml. Purified resting $CD4^+$ T cells (10^6) were resuspended in 1 ml RPMI 1640 supplemented with 10% fetal calf serum and penicillin-streptomycin and placed in one well of a 48-well plate. For reactivation with anti-CD2 antibodies, two different anti-CD2 antibodies (T112 and T113) (31) were used to stimulate the cells. As a positive control, cells were also costimulated with 1 μg of anti-CD3 monoclonal antibody per ml (BD Biosciences) immobilized on tissue culture plates (Fisher) and 1 μg/ml of soluble anti-CD28 antibody (BD Biosciences). Beginning on day 4, 0.5 ml of medium was taken from each well and replaced with fresh medium every other day. Culture supernatants were harvested on day 8, and cell-free HIV-1 virions were quantitated using the Roche Amplicor 1.5 ultrasensitive assay for HIV-1 RNA (detection limit of 50 copies/ml).

Generation of 293T-58H cell lines. 293T cells expressing CD58 were generated by transfection with CD58 expression vectors created using pORF-hLFA-3 (Invitrogen), which contains the intronless open reading frame of the human CD58 gene, and pSELECT-hygro-mcs (Invitrogen), which contains a cytomegalovirus promoter driving a hygromycin resistance gene. After a fragment flanked by NotI/NheI (containing the human elongation factor 1α [hEF1]/human T-cell leukemia [HTLV] promoter) was removed from pSELECT-hygro-mcs, a fragment from pORF-hLFA-3, flanked by NotI/NheI (containing the hEF1-eIF4g promoter and the CD58 gene), was cloned into pSELECT-hygro-mcs to generate pORF58H. Similarly, fragments from pORF-hLFA-3, flanked by NcoI/NheI (containing only the CD58 gene), were cloned into pSELECT-hygro-mcs immediately downstream from the hEF1/HTLV promoter, resulting in pSEL58H. These two vectors were transfected into 293T cells using Lipofectamine 2000 (Invitrogen), and transfectants were selected with hygromycin B (Invitrogen) with increasing doses from 200 μg/ml to 400 μg/ml. Surviving cells were tested for CD58 expression. The cell line stably transfected with pSEL58H was named 293T-58H(SEL), and the cell line stably transfected with pORF58H was named 293T-58H(ORF).

Infection of human resting T cells with HIV-GFP pseudotype virus. The generation of HIV pseudovirions carrying the green fluorescent protein (GFP) gene has been described previously (39). Briefly, a reporter provirus that encodes enhanced GFP in place of HIV-1 *env* (pNL4-3-GFP) was cotransfected with an expression vector carrying HIV *env*. The resulting pseudovirus was used for infection. Human resting $CD4^+$ T cells were purified as described above and precultured with various cell lines before being infected at an MOI of 0.2. The supernatant was collected 4, 6, and 10 days postinfection.

RESULTS

Coculture with CEMx174 cells is more efficient in the induction of latent SIV from resting $CD4^+$ T cells of infected macaques than standard mitogen stimulation. The original method for activating latent HIV-1 from resting $CD4^+$ T cells

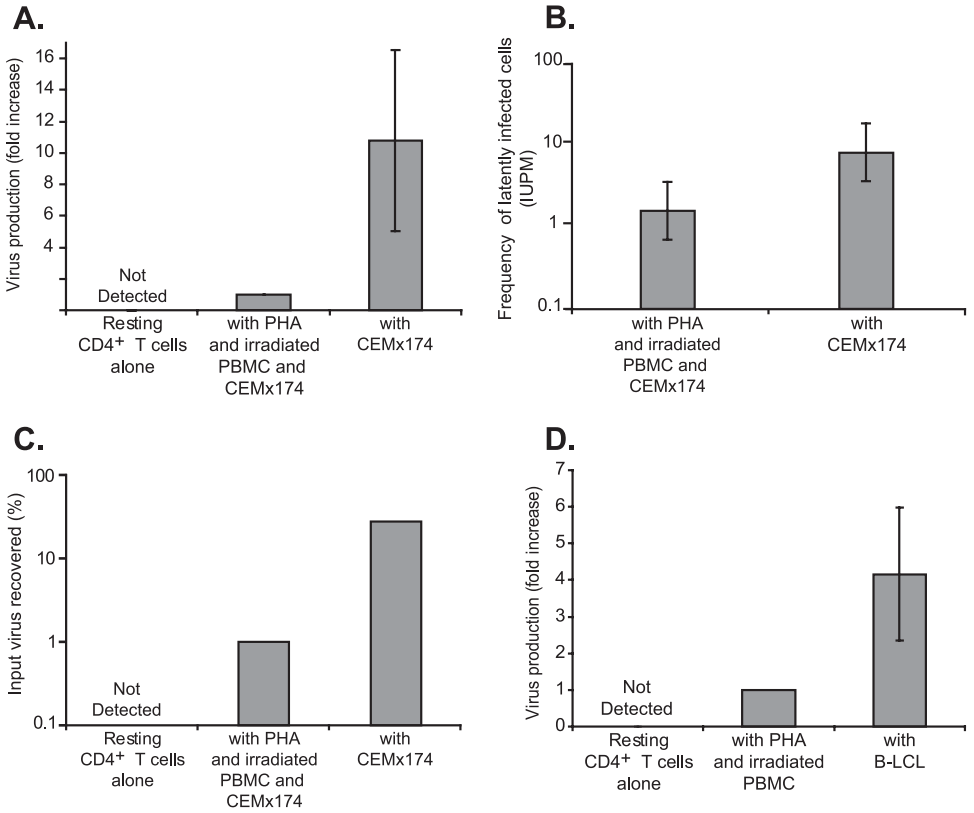

FIG. 1. Coculture with CEMx174 cells is more efficient in induction of latent SIV from resting $CD4^+$ T cells than is standard mitogen stimulation. (A) Resting $CD4^+$ T lymphocytes from SIV-infected pig-tailed macaques were cultured alone without stimulation, were activated with PHA and irradiated human PBMC and cocultured with CEMx174 cells, or were cocultured with CEM cells without PHA activation. Release of virus from latently infected cells was measured by ELISA for SIV p27 antigen in the culture supernatant after 2 weeks of coculture. No p27 antigen was detected when resting $CD4^+$ T cells were cultured alone. p27 antigen production was readily detected when resting $CD4^+$ T cells were activated with PHA and irradiated PBMC and cocultured with CEMx174. Levels of p27 production under these conditions were set to 1 so that multiple independent experiments could be compared. Virus production was invariably higher when resting $CD4^+$ T cells were cocultured with CEMx174 cells without PHA activation. Virus production under these conditions was expressed as the increase (*n*-fold) relative to PHA activation plus CEMx174 coculture. (B) Limiting-dilution analysis of the frequency of latently infected resting $CD4^+$ T cells capable of releasing replication-competent SIV following PHA activation and CEMx174 coculture or CEMx174 coculture alone. Results are expressed as IUPM resting $CD4^+$ T cells. T cells were obtained from SIV-infected pig-tailed macaques. (C) Coculture with CEMx174 cells alone is more efficient than PHA activation followed by CEMx174 coculture in rescuing virus from preintegration latency. Uninfected resting macaque $CD4^+$ T cells were first infected with SIVmac239 at an MOI of 0.02 and then activated in limiting dilutions by the addition of PHA and irradiated human PBMC followed by the addition of CEMx174 cells or were cocultured with CEMx174 cells alone. The level of SIV p27 (ng/ml) was measured at 1 week of coculture in a limiting dilution. IUPM were calculated based on the number of positive wells as described previously (17). The percentage of virus recovery was calculated by dividing the observed IUPM by the theoretical input virus based on the MOI. A representative experiment is shown here. (D) Another human B-LCL also induces SIV from resting macaque $CD4^+$ T cells. Viral release was measured on day 6 of culture by ELISA for p27 levels in culture supernatant.

of infected individuals involved stimulating the cells with the mitogen PHA and irradiated allogeneic PBMC (7, 9, 17, 50). This approach, referred to hereafter as PHA activation, was used because it induced the uniform activation of human $CD4^+$ T cells (24). The released virus can be amplified by coculture with uninfected $CD4^+$ lymphoblasts. This approach allows the rescue of replication-competent virus from recently infected resting $CD4^+$ T cells that contain unintegrated HIV-1 DNA (3, 39, 45) or from stably infected resting $CD4^+$ T cells that carry integrated HIV-1 provirus (32). To activate latent SIV from resting macaque $CD4^+$ T cells, we used PHA and irradiated human PBMC to induce T-cell activation. The SIV released from latently infected cells was subsequently amplified by coculturing with the human B-T-hybrid cell line CEMx174. CEMx174 was selected because previous studies showed that SIV replicated well in this cell line (1). During the course of these studies, we found that compared with the PHA activation/CEMx174 coculture method, coculture with CEMx174 cells alone (in the absence of PHA activation) was more efficient in recovering replication-competent SIV from the latently infected resting $CD4^+$ T cells of infected pig-tailed macaques (Fig. 1A). Presumably, some released virus bound to irradiated PMBC during PHA activation, resulting in lower levels of virus detected. Purified resting $CD4^+$ T cells from infected animals did not produce detectable levels of SIV antigen when cultured alone, consistent with latent infection (Fig. 1A). Following PHA activation, resting $CD4^+$ T cells from infected macaques released virus that could be amplified by coculture with CEMx174 cells and detected as SIV p27 antigen in the supernatant by ELISA. Surprisingly, levels of virus pro-

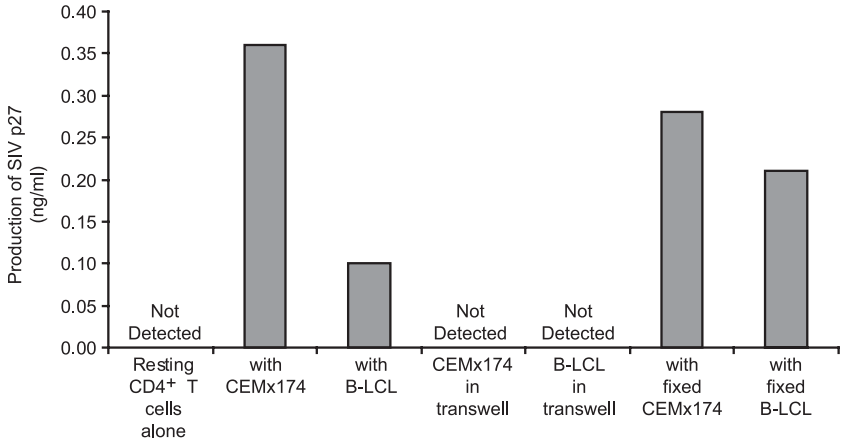

FIG. 2. Viral induction requires cell-cell contact but not viable stimulating cells. Latently infected resting $CD4^+$ T cells from infected macaques were cultured either alone or under the indicated conditions. Where indicated, stimulating cell lines were fixed with 2% paraformaldehyde for 1 h on ice. Virus production was measured by the p27 level in the culture supernatant on day 6 of culture. The experiment shown is representative of more than three experiments.

duction were on average 10 times higher if resting $CD4^+$ T cells were cocultured with CEMx174 cells without PHA activation. This effect could be seen by monitoring the amount of viral antigen in the culture supernatant (Fig. 1A) or by measuring the frequency of $CD4^+$ T cells capable of releasing replication-competent virus (Fig. 1B). The frequency of latently infected cells was measured by culturing cells in limiting dilutions of resting $CD4^+$ T cells from infected macaques under different conditions and measuring virus production. In the representative experiment shown in Fig. 1B, the number of latently infected cells detected by the CEMx174 coculture method was comparable to the number detected with PHA activation and CEMx174 coculture (Fig. 1B). It should be noted that this culture assay measures the frequency of cells carrying latent SIV, including both the preintegration and postintegration forms of latent infection. We previously showed that virus could be induced from resting $CD4^+$ T cells of macaques on suppressive antiretroviral therapy (47). Because of the short functional half-life of unintegrated viral DNA (2, 55, 56), the majority of the latent viruses rescued were likely to have come from cells harboring integrated viral DNA. In this report, we used viremic animals. Presumably, latent virus in both the preintegration and postintegration forms was induced.

Coculture with CEMx174 cells is more efficient in rescuing SIV from preintegration latency. In vitro infection of resting $CD4^+$ T cells with HIV-1 leads to a transient state of preintegration latency in which no virus is produced until the cells are rendered permissive for virus production by cellular activation (5, 17, 54, 55). To determine whether coculture with CEMx174 cells allowed the rescue of SIV from the state of preintegration latency, we carried out in vitro infections of resting macaque $CD4^+$ T cells with a known multiplicity of SIV (MOI of 0.02). After extensive washes, infected T cells were activated with PHA and irradiated human PBMC and then cocultured with CEMx174 or were cocultured with CEMx174 only. Cultures were carried out in limiting dilutions in duplicate, and the percentage of virus recovery was calculated by dividing the IUPM of recovered virus by the input virus. In the representative experiment shown, after 1 week in culture, 28% of the input virus was recovered in CEMx174-only cultures, whereas only 1% was recovered using the PHA activation method and CEMx174 coculture (Fig. 1C). In vitro infection of resting T cells alone was not productive. Thus, coculturing with CEMx174 cells rendered resting macaque T cells permissive for productive SIV infection in vitro with an extremely high efficiency.

CEMx174 is a hybrid between a $CD4^+$ human T-cell line, CEM, and an EBV-transformed human B-lymphoblastoid cell line, 721.174 (43). We found that other EBV-transformed human B-LCL could also induce SIV from resting macaque $CD4^+$ T cells (Fig. 1D). In these experiments, virus amplification was likely occurring in macaque $CD4^+$ T cells that became activated. The amount of viral production seen with PHA activation was set to 1, and viral release from B-LCL cocultures was expressed as an increase (*n*-fold) compared with that with PHA activation. In multiple experiments, B-LCL induced, on average, four-times-higher levels of virus production than that seen with PHA simulation. Other cell lines, such as Jurkat (a human T-cell line) and 293T (a human epithelial cell line) did not exhibit such activity (see Fig. 5A).

Induction of latent SIV virus requires cell-cell contact but not viable stimulating cells. To examine the mechanism of latent virus induction through coculture with particular human cell lines, we first tested whether induction required cell contact or was mediated by soluble factors. Contact dependence was assessed in transwell experiments in which resting $CD4^+$ T cells from aviremic infected macaques were cultured in the top chambers of wells separated from stimulating cell lines in the bottom chambers by a membrane with a 0.4-μm pore size. While both the CEMx174 cell line and B-LCL could induce latent SIV in standard cocultures, the same cell lines could not induce latent virus in the transwell system (Fig. 2). Other experiments also showed that cell-free supernatants from either CEMx174 or B-LCL cells could not induce latent virus (data not shown). However, CEMx174 and B-LCL cells that were fixed with 1% paraformaldehyde were able to induce latent virus from resting T cells (Fig. 2). In control experi-

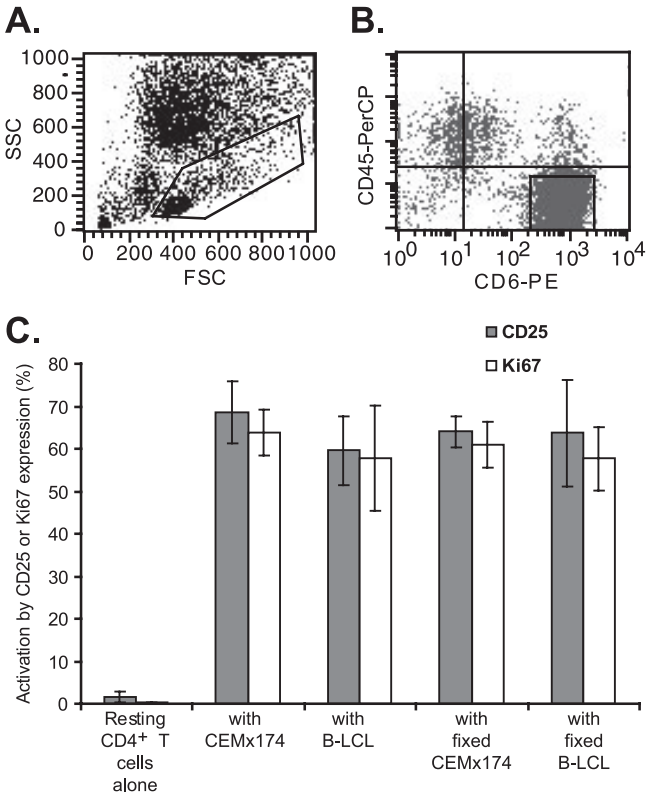

FIG. 3. Induction correlates with T-cell activation and proliferation. Resting macaque $CD4^+$ T cells were cultured alone or with the indicated stimulating cells. On day 6 of coculture, flow cytometry was used to measure T-cell activation and proliferation. Macaque T cells were distinguished from stimulating cell lines by forward-scatter (FSC) and side-scatter (SSC) profiles (A) and surface marker staining using human-specific anti-CD45 antibody and antibody to CD6, which is expressed on macaque T cells but not the stimulating cell lines (B). Gated small cells stained by CD6 but not CD45 were analyzed for activation by the expression of CD25 and for proliferation by the expression of Ki67 (C). PE, phycoerythrin.

ments, we showed that fixed Jurkat or 293T cell lines (which did not induce virus in standard coculture) could not induce latent virus (data not shown). Taken together, these results suggest that latent virus induction is mediated through direct contact with certain human cells rather than through soluble factors and that induction does not require viable stimulating cell lines.

Induction of latent SIV correlates with T-cell activation and proliferation. To determine whether this novel method of inducing latent SIV was correlated with T-cell activation and proliferation, we measured CD25 and Ki67 expression in macaque $CD4^+$ T cells cocultured with CEMx174 or B-LCL. CD25, the α chain of the IL-2 receptor, is expressed transiently after T-cell activation (23). Ki67 is a human nuclear antigen associated with cell proliferation. To distinguish between macaque T cells and the stimulating human cell lines, we used size (as assessed by forward- and side-scatters plots) (Fig. 3A) as well as antibodies to CD6 (expressed in macaque T cells but not in CEMx174 or B-LCL) and to human CD45 (which does not cross-react with macaque CD45 cells) (Fig. 3B). $CD6^+$ and $CD45^-$ cells were gated for analysis of CD25 or Ki67 expression. As shown in Fig. 3C, both CEMx174 and B-LCL induced cell activation and proliferation in macaque $CD4^+$ T cells. Activation was induced whether the stimulating cells were viable or fixed. These results suggest that in this novel method, the induction of latent SIV may be due to the activation of macaque T cells.

Involvement of MHC class II and costimulatory molecules in T-cell activation and induction of latent virus. To determine whether xenogeneic TCR-MHC interactions were involved in the T-cell activation and latent virus induction observed in this system, we used the broadly reactive anti-human HLA-DR antibody L243, which is known to block mixed-lymphocyte reactions. Saturating concentrations of L243 did not block macaque T-cell activation or latent virus induction in coculture. A representative experiment is shown in Fig. 4A. T-cell activation was measured by CD25 expression (Fig. 4A, bars, left *y* axis), and virus production was measured by p27 levels in the culture supernatant (triangles, right *y* axis). Irrelevant antibodies were included in some experiments and showed no effect (data not shown). In control experiments, we showed that L243 completely inhibited the activation of macaque $CD4^+$ T cells by irradiated human PBMC, an activation event that depends on xenogeneic TCR-MHC interactions (not shown). Thus, the activation and induction of latent virus by CEMx174 and B-LCL do not depend on the recognition of MHC class II in these human cell lines. We also cocultured macaque $CD4^+$ T cells with two other human B-cell lines, CA46, an EBV-negative Burkitt's lymphoma cell line, and RAEL, an EBV-positive Burkitt's lymphoma cell line. Both cells lines express high levels of HLA-DR (as high as those of B-LCL) (data not shown). These two MHC class II-positive human B-cell lines did not induce the activation of macaque $CD4^+$ T cells or the production of virus from latently infected cells (Fig. 4A). These results suggest that xenogeneic recognition of human MHC class II by TCRs on macaque $CD4^+$ T cells is not responsible for the T-cell activation and induction of latent SIV in this system.

In light of the observation that human B cells, through costimulatory molecules along with other soluble factors, are able to render human resting $CD4^+$ T cells permissive for HIV infection (52), we tested whether costimulatory molecules were involved in the induction of latent SIV (Fig. 4B). Stimulating cell lines were pretreated with antibodies to CD80 (B7-1), CD86 (B7-2), CD54 (ICAM-1), and CD58 (LFA-3) at a concentration of 4 μg/ml to block these costimulatory molecules on the surface of the stimulating cell lines before they were cocultured with macaque $CD4^+$ T cells. Virus production from a coculture with B-LCL was set to 100% and served as the positive control, and levels of virus production from cultures treated with antibodies were expressed as percentages of the positive control. As shown in Fig. 4B, partial inhibition with antibodies to the costimulatory molecules CD80 and CD86 and with antibodies to the adhesion molecule CD54 was observed. Most notably, antibody to CD58 had a dramatic inhibitory effect on the induction of latent virus. It completely inhibited viral induction. As a negative control, antibodies to CD14 had no effect. Antibody against CD58 had the same inhibitory effect on both B-LCL-mediated stimulation (Fig. 4B) and CEMx174-mediated stimulation (Fig. 4C). CD58 is a ligand for the T-cell surface protein CD2, which is involved in

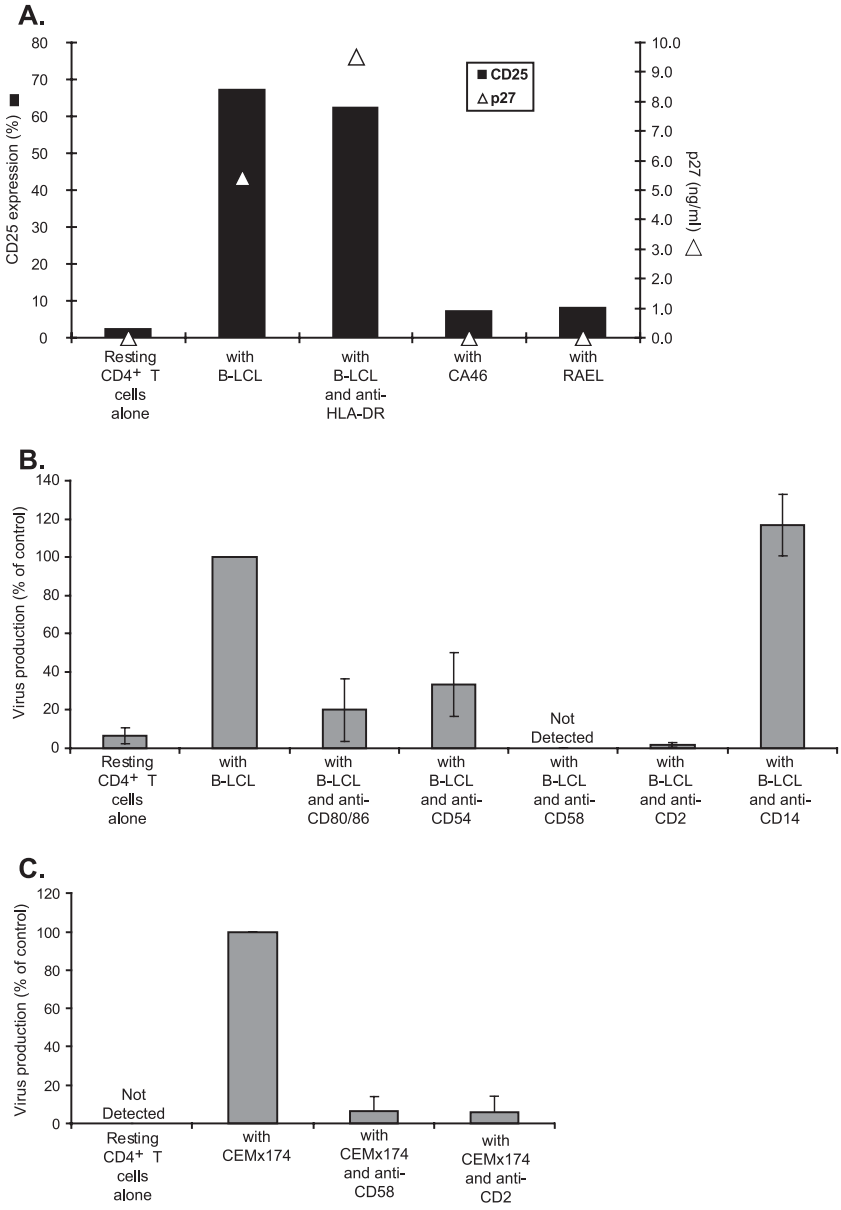

FIG. 4. Involvement of MHC class II and costimulatory molecules in T-cell activation and induction of latent SIV virus. (A) MHC class II molecules do not play a major role in activation. Resting $CD4^+$ T cells from infected macaques were cocultured in the presence of the indicated cell lines with or without anti-HLA-DR antibody (L243). Activation was measured by CD25 expression (bars, left *y* axis), and virus production was measured by the p27 level in the supernatant (triangles, right *y* axis). The experiment shown is representative of more than four similar experiments. (B and C) CD58-CD2 interactions are essential for induction of latent SIV. Resting $CD4^+$ T cells from infected macaques were cocultured with B-LCL or CEMx174 in the absence or presence of different blocking antibodies to the indicated costimulatory molecules. Antibodies to CD80 and CD86 were used in combination. Antibodies were used at saturating concentrations (4 μg/ml).

cell-cell adhesion and signaling for T-cell activation. Therefore, we examined whether anti-CD2 antibody had a similar inhibitory effect. As shown in Fig. 4B and C, both anti-CD2 antibody and anti-CD58 antibody inhibited the B-LCL- or CEMx174-stimulated release of SIV from resting $CD4^+$ T cells, suggesting that the CD58-CD2 interaction is essential for the induction of latent SIV in this system.

CD58-CD2 interactions in T-cell activation and induction of latent SIV. To explore the relationship between CD58 expression and the induction of latent SIV further, we examined various human cell lines for their levels of CD58 expression and ability to induce latent SIV. As shown in Fig. 4A, the Burkitt's lymphoma cell lines CA46 and RAEL did not induce SIV. In addition, neither the T-cell line Jurkat nor the embryonic kidney cell line 293T was able to induce latent virus (Fig. 5A). CD58 expression on these cell lines was compared with that on B-LCL (Fig. 5B). All four cell lines expressed CD58 but at a much lower level than B-LCL. Thus, high levels of CD58 may be required for virus induction, or these cell lines may lack additional components needed for induction.

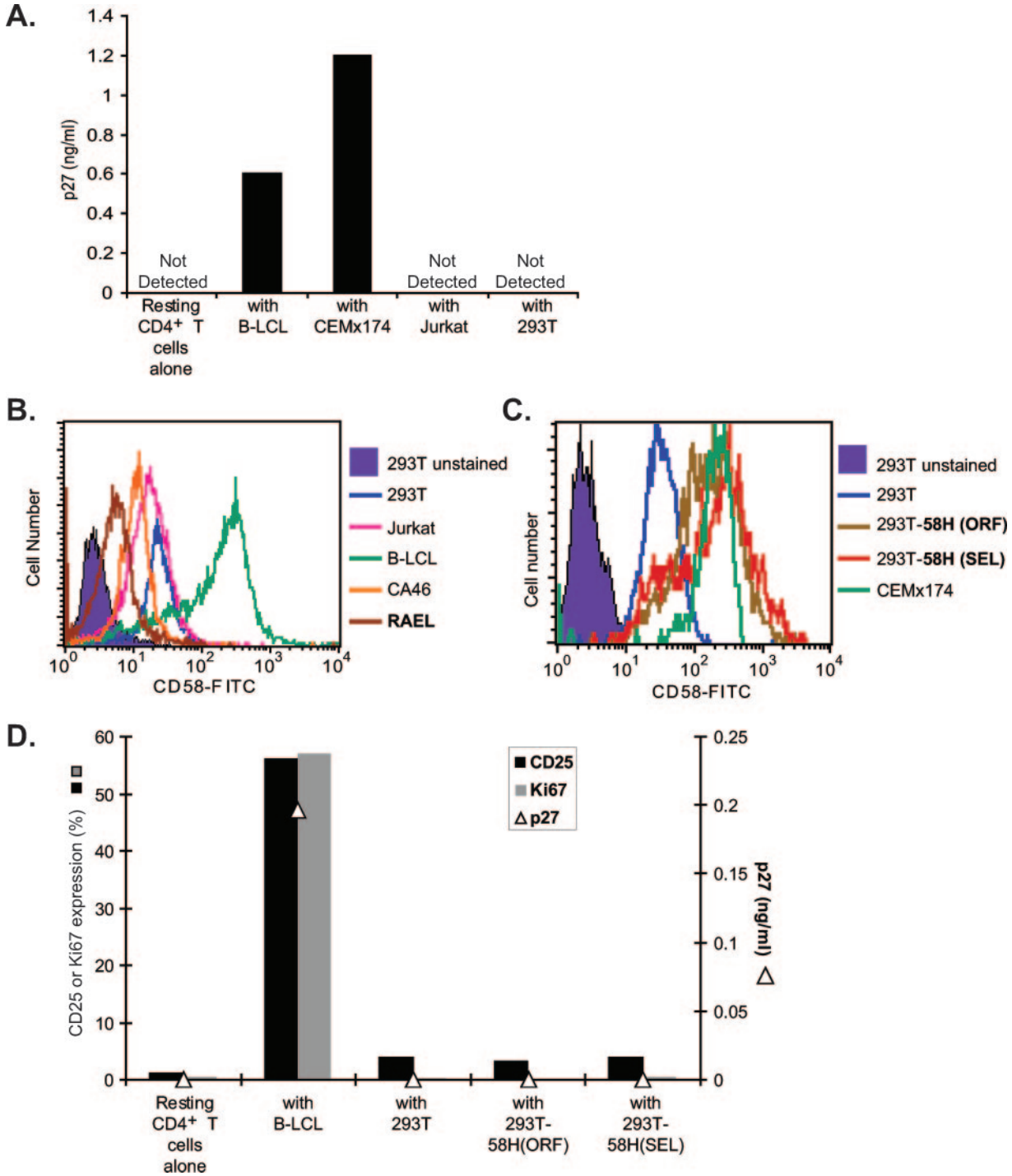

FIG. 5. CD2-CD58 interactions in T-cell activation and induction of latent SIV. (A) Viral induction in cocultures of resting $CD4^+$ T cells from infected macaques with different stimulating cell lines. The experiment shown is representative of more than two experiments with similar results. (B) CD58 expression levels on different cell lines measured by antibody staining and flow cytometry. (C) CD58 expression levels in 293T cell lines overexpressing CD58 compared with that on CEMx174. (D) Macaque T-cell activation and latent SIV induction in cocultures with various cell lines. Cells were cultured for 6 days, and T-cell activation parameters (CD25 and Ki67 expression levels) (bars on the left *y* axis) were measured by flow cytometry. Virus production was measured with ELISA for p27 in the supernatant (triangles on right *y* axis). The experiment shown is representative of two experiments with similar results.

In order to test whether the CD58-CD2 interaction was sufficient to elicit virus induction, we transfected a nonhematopoietic cell line (293T) with CD58. We generated two 293T cell lines using different plasmids: 293T-58H(ORF) and 293T-58H(SEL). The CD58 gene was driven by different promoters, but both of them expressed high levels of CD58 (Fig. 5C). About 80% of the cells in each cell line expressed CD58 to a level comparable to that seen with CEMx174. However, nei-

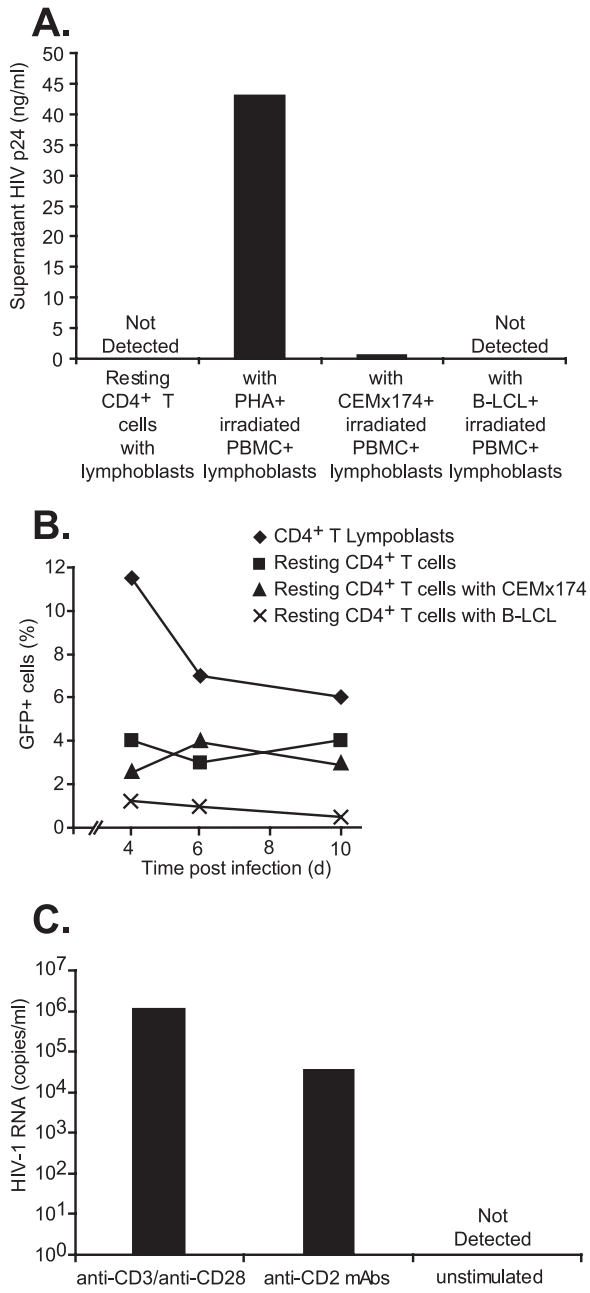

FIG. 6. Activation of human CD4$^+$ T cells and reactivation of latent HIV-1. (A) Failure of CEMx174 and B-LCL to activate latent HIV-1. Purified resting CD4$^+$ T cells from aviremic patients on HAART were cocultured with CEMx174 or B-LCL or were activated with PHA and irradiated allogeneic PBMC. Subsequently, CD4$^+$ lymphoblasts from healthy donors were added to all wells to amplify any HIV-1 released from latently infected cells. As a negative control, resting CD4$^+$ T cells were cultured with lymphoblasts alone. Virus production was measured by p24 levels in the culture supernatant at 2 weeks. (B) Infection of resting human CD4$^+$ T cells by HIV-1 pseudovirions. Resting CD4$^+$ T cells from a healthy donor were subjected to PHA activation to generate lymphoblasts or were cultured alone or with B-LCL or CEMx174 for 2 days. Cells were then infected with a pseudotyped recombinant HIV-1 carrying the GFP gene at an MOI of 0.2. The level of infection was measured by GFP expression on days 4, 6, and 10 postinfection. (C) Induction of latent HIV-1 in human T cells with anti-CD2 antibodies. Resting CD4$^+$ T cells from aviremic patients on HAART were activated by anti-CD3 and anti-CD28 antibodies or by a combination of two anti-CD2 antibodies, T112 and T113. Induction of latent HIV-1 was measured by quantitative PCR in the culture supernatant on day 8 of culture. Unstimulated cells served as a control.

ther of the stably transfected 293T cell lines was able to activate the resting macaque CD4$^+$ T cells or to induce latent virus from resting macaque CD4$^+$ T cells (Fig. 5D). Therefore, CD58 expression alone is not sufficient for the induction of latent SIV. Taken together, these results suggest that CD2-CD58 interactions are necessary but not sufficient for the induction of latent SIV virus from resting macaque CD4$^+$ T cells in this system.

Latent HIV induction in human T cells with the cell lines. The induction of T-cell activation and latent SIV production were observed for three different macaque species, *Macaca nemestrina* (pig-tailed macaques), *Macaca mulatta* (rhesus macaques), and *Macaca fascicularis* (cynomolgus macaques), when macaque resting CD4$^+$ T cells were cocultured with either CEMx174 or B-LCL (see Fig. 1 for pig-tailed macaques and Fig. 2 to 5 for rhesus macaques; cynomolgus macaque data are not shown here). In order to examine whether this approach for the induction of latent virus was applicable to HIV-1 and human T cells as well, resting CD4$^+$ T cells from HIV-1-infected patients on HAART were stimulated either by the standard PHA method or with CEMx174 or B-LCL, and human CD4$^+$ T lymphoblasts were added to all the cultures to amplify HIV-1 released from latently infected cells. In several experiments with different human donors, neither CEMx174 nor B-LCL was able to induce latent HIV-1, while virus induction was readily achieved with the standard PHA activation method. A representative experiment is shown in Fig. 6A. In addition, despite likely HLA differences, resting human CD4$^+$ T cells were not significantly activated when cocultured with CEMx174 or B-LCL, as measured by the expression of CD25 and Ki67 (data not shown).

Susceptibility to HIV-1 infection is a sensitive indicator of the degree of T-cell activation (56). To examine whether coculture with CEMx174 or B-LCL would render human resting CD4$^+$ T cells permissive for HIV-1 infection, a previously described recombinant HIV-1 reporter virus system was used (39). An Env-defective provirus carrying a form of GFP was cotransfected into 293T cells along with an expression vector for a standard X4 HIV-1 Env. The resulting pseudovirions were used to infect human resting CD4$^+$ T cells preactivated (cocultured for 5 days) with the cell lines. On various days postinfection, the fraction of GFP-positive cells was determined. Readily infectible PHA-activated human T lymphoblasts were included as a positive control. No increase in viral infection was induced by coculture with either of the cell lines compared to resting T cells alone (Fig. 6B). Thus, coculture with CEMx174 or EBV LCL does not render resting human T cells permissive to productive HIV-1 infection.

The above-described results suggest that interactions between CD2 on resting macaque CD4$^+$ T cells and CD58 on CEMx174 cells can both activate macaque T cells and induce latent SIV infection, while the same stimulating cells have little effect on HIV-1 infection of resting human CD4$^+$ T cells. These results suggest that some aspect of the xenogeneic CD2-

CD58 interaction is particularly suited to stimulating latent virus. To determine whether latent HIV-1 in resting human $CD4^+$ T cells could be induced by other forms of stimulation through CD2, we tested combinations of anti-CD2 antibodies for their ability to induce HIV-1 in latently infected human resting $CD4^+$ T cells. Previous studies have shown that human T cells can be activated by ligating CD2 with particular monoclonal antibodies (31). Cross-linking with CD2 antibodies in the absence of TCR engagement induces T-cell activation, proliferation, and effector functions. To determine whether this form of stimulation could induce latent HIV-1, resting $CD4^+$ T cells from patients on HAART who had suppression of viremia to below the limit of detection were purified. In this situation, latent HIV-1 is found mostly in the postintegration form. Resting $CD4^+$ T cells were cultured in the presence of the combination of two anti-CD2 antibodies, T112 and T113. This combination of CD2 antibodies led to the release of virus from latently infected human T cells, which was comparable to that seen following traditional activation through the TCR and CD28 (Fig. 6C). This demonstrates that latent HIV-1 can be induced through the costimulatory molecule CD2.

DISCUSSION

In the process of developing an SIV/macaque model for HIV-1 latency, we discovered a novel way to activate macaque resting $CD4^+$ T cells and induce latent SIV from these cells. This method involves coculturing resting macaque $CD4^+$ T cells with the human B-T-hybrid cell line CEMx174 or with EBV-transformed human B-LCL. By days 5 to 6 of coculture, resting T cells became largely activated and produced detectable levels of virus. T-cell activation and virus induction required cell-cell contact. Soluble factors from the cell lines did not have any effect (Fig. 2A). Xenogeneic TCR-MHC interactions were not responsible for the activation, as HLA-DR antibody did not block T-cell activation, and other human B-cell lines with similar levels of MHC class II expression did not elicit such activation (Fig. 4A). Antibody-blocking studies showed that CD2-CD58 interactions were involved in the activation process (Fig. 4B and C). However, CD2-CD58 interactions alone were not sufficient to activate resting T cells or induce latent virus (Fig. 5). The activation may require additional surface molecules. Future studies will investigate what other factors are involved in the activation process. Induction of latent SIV and T-cell activation were observed following interactions between human B-LCL and resting $CD4^+$ T cells from at least three different macaque species (rhesus, pigtailed, and cynomolgus macaques). However, such interactions neither activated human resting $CD4^+$ T cells nor induced latent HIV-1, suggesting that the xenogeneic CD2-CD58 interaction may be unusually strong, explaining its essential role in this process. Interestingly, other forms of signaling through human CD2 were sufficient to activate latent HIV-1.

A major motivation for developing an animal model for HIV-1 latency is to provide a means for testing strategies for the elimination of the latent reservoir. Since the discovery of the latent reservoir, several approaches for eliminating the reservoir have been proposed. It has been suggested that the intensification of the HAART regimen with more drugs will lead to a more rapid decay of the reservoir by stopping replenishment from ongoing viral replication that may continue despite HAART (41). However, even in patients on HAART who have had no detectable viremia for as long as 7 years, there is no substantial decay of this reservoir (48). Other approaches involve the activation of latently infected cells in the presence of HAART. Methods of activation include the engagement of the TCR or CD3 (27, 33, 40); use of pharmacological agents that activate downstream in the TCR pathway, such as the phorbol ester prostratin (25, 26); and use of cytokines, such as IL-2 (8) and IL-7 (3, 44, 53), and combinations of cytokines (8, 28). Early in vivo approaches with IL-2 proved to be ineffective and extremely toxic (13), but recently, in vitro studies with prostratin and IL-7 showed promise, as such agents induced latent virus without causing T-cell replication. The method of activation in resting T cells reported in this study did not resemble any of the approaches mentioned above. It relies on neither the TCR-MHC interaction nor cytokines or soluble factors but involves costimulatory interactions between CD2 on the T cell and CD58 on the stimulating cell. The level of T-cell activation and latent viral induction is similar to that with mitogen/PHA stimulation (Fig. 1A and D and 2C). Interestingly CD2-CD58 interactions were necessary but not sufficient for this activation effect. Further studies will be conducted to investigate additional factors involved in this process.

In summary, we have discovered a novel way to activate resting T cells and induce latent SIV that is dependent upon the interaction between CD2 and CD58. The identification of a novel method may contribute to the search for new ways of reactivating/purging the latent viral reservoir without causing vast damage to the immune system. This approach also highlights the potential for SIV models to provide new insights and approaches for HIV-1 treatment research.

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