

Endothelial Cells Promote Human Immunodeficiency Virus Replication in Nondividing Memory T Cells via Nef-, Vpr-, and T-Cell Receptor-Dependent Activation of NFAT

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Human endothelial cells (ECs) enhance human immunodeficiency virus (HIV) replication within CD4⁺ memory T cells by 50,000-fold in a Nef-dependent manner. Here, we report that EC-mediated HIV type 1 replication is also dependent on an intact *vpr* gene. Moreover, we demonstrate that despite a requirement for engaging major histocompatibility complex (MHC) class II molecules and costimulators, EC-stimulated virus-producing cells (p24^{high} T cells) do not proliferate, nor are they arrested in the cell cycle. Rather, they are minimally activated, sometimes expressing CD69 but not CD25, HLA-DR, VLA-1, or effector cytokines. Blocking antibodies to interleukin 2 (IL-2), IL-6, IL-7, or tumor necrosis factor do not inhibit viral replication. Cyclosporine effectively inhibits viral replication, as does disruption of the NFAT binding site in the viral long terminal repeat. Furthermore, in the presence of ECs, suboptimal T-cell receptor (TCR) stimulation with phytohemagglutinin L supports efficient viral replication, and suboptimal stimulation with toxic shock syndrome toxin 1 leads to viral replication selectively in the TCR-stimulated, Vβ2-expressing T cells. Collectively, these data indicate that ECs provide signals that promote Nef- and Vpr-dependent HIV replication in memory T cells that have been minimally activated through their TCRs. Our studies suggest a mechanism for HIV replication in vivo within the reservoir of circulating memory CD4⁺ T cells that persist despite antiretroviral therapy and further suggest that maintenance of immunological memory by MHC class II-expressing ECs via TCR signaling may contribute to HIV rebound following cessation of antiretroviral therapy.

We have recently reported that human endothelial cells (ECs) markedly enhance human immunodeficiency virus (HIV) replication up to 50,000-fold within previously nonactivated memory CD4⁺ T cells that are allogeneic to the ECs (i.e., from a different donor) both in vitro and in vivo in a manner that requires an intact *nef* gene (9). In contrast, Nef does not greatly influence viral replication in T cells cocultured with allogeneic B cells, macrophages, fibroblasts, or dendritic cells. Fluorescence-activated cell sorter (FACS) analysis performed on CD4⁺ T cells cocultured with ECs for 6 or 9 days demonstrated that a discrete subset of T cells (between 5 and 20% of the population) expressed very high levels of intracellular viral proteins and was therefore likely to be responsible for the production of cell-free virus. In EC–T-cell cultures, viral replication depended upon endothelial expression of major histocompatibility complex (MHC) class II molecules (principally HLA-DR), the target molecules recognized by the T-cell receptor (TCR) for antigen of CD4⁺ T cells, as well as on CD2, the target of the EC costimulatory molecule LFA-3 (CD58). Furthermore, only CD45RO⁺ memory T cells supported viral replication, consistent with the capacity of ECs to selectively present antigen to memory cells (reference 14 and unpublished data). Moreover, since ECs lacking MHC class II

molecules could exert this Nef-dependent effect in three cell cultures (involving B cells and ECs allogeneic to the T cells), our data suggested that ECs also provide a unique signal that augments Nef-dependent viral replication. In other words, Nef-dependent enhanced HIV replication required signals for T-cell activation, as well as additional (and as yet undefined) EC-specific signals.

Since activated CD4⁺ T cells are a primary source of HIV production in vitro (4, 44) and in vivo (21, 46, 65), it is reasonable to hypothesize that ECs support viral replication in TCR-activated T cells. The productively infected cells observed on days 6 and 9 of culture, i.e., the p24^{high} T cells, could represent the progeny of the cells that had been fully activated and proliferated in response to high-affinity TCR binding to nonself (allogeneic) MHC molecules. The response of mature CD4⁺ T cells to allogeneic cells is predominantly a cross-reaction of TCRs that were selected during thymic development to recognize peptides derived from foreign proteins bound to self allelic forms of MHC class II molecules with complexes consisting of peptides bound to nonself allelic forms of MHC class II molecules displayed on the surface of the allogeneic cell (MHC alleles are the most diverse genes in the human population). The response to nonself MHC molecules is otherwise identical to activation by foreign proteins (presented as peptides bound to self MHC molecules), depending upon the same intracellular signaling pathways and showing the same requirements for costimulation (41). By limiting dilution analysis, it has been observed that such direct recogni-

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tion of nonself MHC class II molecules on ECs can lead to the proliferation of between 0.1 and 1% of the input peripheral blood T cells, the precise frequency depending on the MHC alleles of the EC and T-cell donors (43). However, these activated cells expand to 10% or more of the total by 6 days, as measured by carboxy-fluorescein diacetate succinimidyl ester (CFSE) dilution assays (which measure cellular proliferation). It is possible that it is these cells that represent the high virus producers ($p24^{\text{high}}$), which is underscored by the ability of HIV proteins to augment T-cell activation (3, 16). Alternatively, the $p24^{\text{high}}$ T cells may represent a population of input cells, larger than the predicted fully activated population (0.1 to 1%), whose TCRs bind to EC MHC class II molecules with moderate to low affinity. While insufficient to support T-cell proliferation, these lower-affinity signals may still be sufficient to activate some specific T-cell functions, including the expression of T-cell activation markers, the elaboration of effector cytokines, and perhaps HIV replication.

Although these are appealing explanations, viral replication may instead, or in addition, be taking place in unactivated or minimally activated T cells. There is growing evidence for circumstances in which T-cell activation and productive viral infection may be uncoupled. In the early and late stages of HIV infection in vivo, most of the cells that express viral RNA do not express markers of cellular activation, and these cells persist despite antiretroviral therapy (ART) (70). Furthermore, resting memory T cells in the gut-associated lymphoid tissue (GALT) are specifically depleted in the initial weeks of simian immunodeficiency virus (SIV) infection, which is thought to establish conditions that eventually lead to progression to AIDS (31, 40). In vitro, infection of thymic stromal histocultures by HIV also leads to replication in cells lacking activation markers (13). High levels of cytokines from highly or moderately activated T cells or ECs could also cause productive infection with minimal activation, thereby enhancing HIV replication in cells that are not receiving a TCR signal (54, 61). Alternatively, low-affinity binding of the TCR with the EC MHC class II molecules may approximate homeostatic signals provided by engagement of the TCR with self MHC class II-peptide complexes. Such signals are not strong enough to induce effector T-cell functions, but they are thought to be necessary for maintenance of $CD4^+$ T-cell memory function (39, 46).

Here, we report that HIV production in this in vitro model requires Vpr, as well as Nef, viral gene products that modulate T-cell signaling pathways (28). Furthermore, despite the requirement for endothelial MHC class II molecules and costimulation, viral replication is largely confined to T-cell populations that do not proliferate and that show minimal evidence of activation. Finally, we present evidence to indicate that viral replication within these cells depends upon suboptimal TCR stimulation and TCR-dependent activation of the transcription factor NFAT, but not cytokine signals.

MATERIALS AND METHODS

Isolation and culture of human cells. All human cells and tissues were obtained under protocols approved by the Yale Human Investigations Committee. Peripheral blood mononuclear cells (PBMCs) were isolated from HIV-seronegative donors by density gradient centrifugation of leukapheresis products by using lymphocyte separation medium (Gibco BRL, Grand Island, N.Y.). $CD4^+$

T cells were isolated from PBMCs by positive selection using Dynabeads (DynaL, Lake Success, N.Y.). The selected population obtained by this procedure was routinely $>97\%$ $CD4^+ CD3^+$ by flow cytometry (data not shown). The cells were subsequently purified by using further negative selection as described previously (12). Activated $CD4^+$ T cells were removed by incubating them with an anti-HLA-DR antibody at a concentration of 5 $\mu\text{g}/\text{ml}$ (LB3.1; a gift of J. Strominger, Harvard University, Cambridge, MA) for 20 min, washing them twice, and depleting them by using magnetic beads conjugated to goat anti-mouse antibody (DynaL).

Human umbilical vein endothelial cells were isolated from umbilical cords by collagenase digestion and maintained in culture by using conditions and reagents as previously described (48). Such cultures are uniformly $CD31^+$ and von Willebrand factor $^+$ and lack $CD45$. When indicated, the cells were treated with 50 ng/ml of gamma interferon ($\text{IFN-}\gamma$) (Biosource, Camarillo, CA) per ml for 3 days prior to coculture with allogeneic $CD4^+$ T cells. After pretreatment with $\text{IFN-}\gamma$, the ECs were uniformly HLA-DR positive (data not shown).

Disruption of the NFAT binding sites in the HIV long terminal repeats (LTRs). The NFAT $^-$ clones were created by site-directed mutagenesis using the Quick Change Site Directed Mutagenesis kit (Stratagene, La Jolla, CA) with p83-2 and p83-10 plasmid DNAs (containing 5' and 3' NL4-3 sequences, respectively) as templates. Individual clones were subjected to fluorescence sequencing (<http://info.med.yale.edu/wmkeck>) to ensure that the exact desired sequence was used in our experiments.

Generation of virus stocks. To generate infectious pNL4-3 (wild type) or derivative HIV (Nef $^-$ or NFAT $^-$), wild-type or mutated p83-2 and p83-10 plasmid DNAs were digested with EcoRI (New England Biolabs, Beverly, MA), column purified (QIAGEN, Valencia, CA), and combined with an equal amount of similarly prepared EcoRI-digested complementary plasmid DNA. The two plasmids were fused by using T4 DNA ligase (New England Biolabs), and the DNA was introduced into the permissive cell line CEMx174 by DEAE-dextran transfection. The transfected cells were cultured in complete medium consisting of RPMI 1640 (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL), penicillin-streptomycin, and L-glutamine (Gibco BRL). HIV lacking Vpr (Vpr $^-$) was described previously (22) and was obtained from the AIDS Research and Reference Reagent Program (Rockville, MD). For the generation of HIV stocks, culture supernatants containing virus were harvested daily beginning 48 h after the first observation of cytopathic effect and stored at -70°C until experimental use.

Viral infection. Approximately 1.0×10^6 primary quiescent $CD4^+$ T cells were cocultured with 1.5×10^5 ECs in C24 well microculture plates (BD Falcon, Bedford, MA). Alternatively, $CD4^+$ T cells were pretreated with PHA-L (5 $\mu\text{g}/\text{ml}$; Fisher Chemical, Fairlawn, NJ) and interleukin 2 (IL-2) (25 IU; Biological Resources Branch, National Cancer Institute, Frederick, MD) for 3 days prior to infection. The cultures were infected with HIV at a multiplicity of infection of 0.05, and cultures containing ECs and T cells were infected within 1 h of coculture. In each case, the infections were carried out in triplicate. After overnight incubation with HIV, nonadherent cells and the culture supernatant were subjected to centrifugation. The supernatant was then removed, the cells were washed with phosphate-buffered saline (PBS) to remove residual virus, and the cells were resuspended with ECs in fresh complete medium. At 3-day intervals, the media were changed; supernatants were aliquoted and stored at -70°C . Where indicated, the cultures were treated with cyclosporine or ethanol vehicle or with rapamycin or dimethyl sulfoxide vehicle (Sigma-Aldrich, St. Louis, MO). These agents were added at the onset of coculture and replaced with every medium exchange.

The growth kinetics of HIV under various experimental conditions were assayed by the concentration of HIV p24 in the culture supernatants as assessed by enzyme-linked immunosorbent assay (ELISA; Coulter, Hialeah, FL).

Immunostaining and flow cytometric analyses. T cells were harvested from the EC-T-cell cocultures as described previously (9). The production of intracellular p24 was assessed by using the fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated anti-p24 monoclonal antibody KC-57 (Coulter). The expression of T-cell surface markers was assessed by using PE-conjugated anti- $CD4$, anti- $CD25$, anti- $CD69$, anti- $CD49a$ (VLA-1), anti-HLA-DR, or immunoglobulin G (IgG) isotype control (Beckman Coulter, Fullerton, CA). For FACS analysis of the $V\beta2^+$ population, the cells were stained first with a monoclonal antibody recognizing $V\beta2$ (MBP2D5) (Beckman Coulter) for 1 h. The cells were then washed and stained with a PE-conjugated goat anti-mouse IgG antibody (Beckman Coulter). For intracellular cytokine staining of T cells, monensin (2 μM) was added for the last 8 h of coculture. For pharmacologically stimulated T cells, 10 ng/ml phorbol myristate acetate (PMA) plus 1 μM ionomycin in the presence of 2 μM monensin were added to replicate cultures for the last 6 h. The cells were then washed, fixed with 4% paraformaldehyde, perme-

abilized with 0.1% saponin, and labeled with anti-cytokine antibody as described previously (34). Intracellular cytokines were assessed by using PE-conjugated anti-IFN- γ , FITC-conjugated anti-IL-4, and FITC-conjugated anti-IL-2 (BD Pharmingen). Extensive fixation was necessary to abolish infectivity of the cells prior to FACS analyses. The absolute intensity of fluorescent staining of fixed cells for various antigens varied among experiments, but the relative intensities among different subpopulations were preserved. We attribute this to effects of the fixative on masking antigenic epitopes.

For proliferation assays, the cells were labeled with 250 nM CFSE (Molecular Probes, Eugene, OR) for 15 min prior to coculture with ECs as described previously (9). The data were acquired with a FACSCalibur flow cytometer and analyzed with CellQuest software (BD Biosciences).

For analysis of DNA content, the cells were fixed with 70% ethanol overnight. The cells were incubated with FITC-conjugated anti-p24^{gag} monoclonal antibody KC-57 for 30 min in the dark. After several washes in PBS, the cells were incubated in PBS containing RNase A (100 μ g/ml; Sigma-Aldrich) for a minimum of 1 h. Propidium iodide (PI) (50 μ g/ml) was added 20 min before FACS analyses.

Superarray RNA analyses of T-cell mRNA. CD4^{low} cells were isolated by magnetic-bead depletion 9 days postinfection in the Nef⁺ virus-infected cultures. In brief, the cells were incubated with 5 to 15 μ g/ml of α -CD4 antibody (R&D Systems, Minneapolis, MN) for 20 min with continuous agitation at 4°C. The cells were then washed two times to remove unconjugated antibody, and the CD4⁺ cells were depleted using magnetic beads conjugated to goat anti-mouse antibody (Dyna). T cells from the same donor were labeled with CFSE prior to EC coculture in the uninfected cultures. After 9 days of coculture, CD4⁺ T cells were harvested, and the CFSE^{high} cells were isolated by FACS using the FACS-Vantage SE (BD Biosciences).

RNAs from these samples were isolated using QiaShredder and an RNeasy MiniPrep kit (QIAGEN). RNA was reverse transcribed using the Amphi-Labelling kit (Superarray, Frederick, MD), and the cDNA was labeled with biotin-16-dUTP (Roche). Cytokine expression was assessed using a cDNA GEArray Human Common Cytokine Microarray (Superarray, Frederick, MD).

Blocking antibodies. Mouse monoclonal antibodies reactive with human cytokines included anti-bone morphogenic protein 6 (BMP6), anti-IL-2, anti-IL-6, anti-tumor necrosis factor (TNF), IgG1 isotype control, and IgG2b isotype control (R&D Systems). The antibodies against IL-7 and its isotype control were rat monoclonal antibodies (BD Pharmingen). The antibodies were used at 10 times the 50% neutralizing dose; the final concentration of the antibodies ranged from 5 to 10 μ g/ml. The antibodies were added at the time of infection, and these antibodies were included in every medium change (every 3 days) and were therefore present throughout the duration of the cocultures.

Polyclonal stimulation of EC-T-cell cocultures. For polyclonal stimulation, phytohemagglutinin L (PHA-L; Sigma), toxic shock syndrome toxin 1 (TSST-1; Toxin Technologies, Sarasota, FL), or vehicle was added to EC-T-cell cocultures prior to infection. After 24 h, the supernatant was harvested so that IL-2 expression could be assayed by ELISA (eBioscience, San Diego, CA). At the time of medium exchange (24 h postinfection), PHA-L and TSST-1 were again added so that they were present throughout the duration of the coculture.

RESULTS

ECs enhance Nef⁺ virus replication in minimally activated CD4⁺ T cells. To determine whether the p24^{high} T cells generated in EC-T-cell cocultures represented the population that had proliferated, we labeled CD4⁺ T cells with CFSE prior to placing them in coculture with allogeneic MHC class II⁺ ECs and infecting them with HIV. In EC-T-cell cocultures infected with a *nef*-deficient strain of virus (Nef⁻) (Fig. 1A), a CFSE^{low} population accumulates, comprising the cells that have proliferated repetitively. This response to IFN- γ -pretreated allogeneic ECs is indistinguishable from that of non-CD4⁺-infected cells (data not shown). However, in EC-T-cell cocultures infected with wild-type virus, we observed a novel population of p24^{high} cells that remained CFSE^{high}, indicating that they had not proliferated (Fig. 1A). The frequency of the p24^{high} CFSE^{high} population ranged between 5 and 20% of the starting population, which is substantially higher than the number of input T cells that can proliferate in response to allogeneic ECs

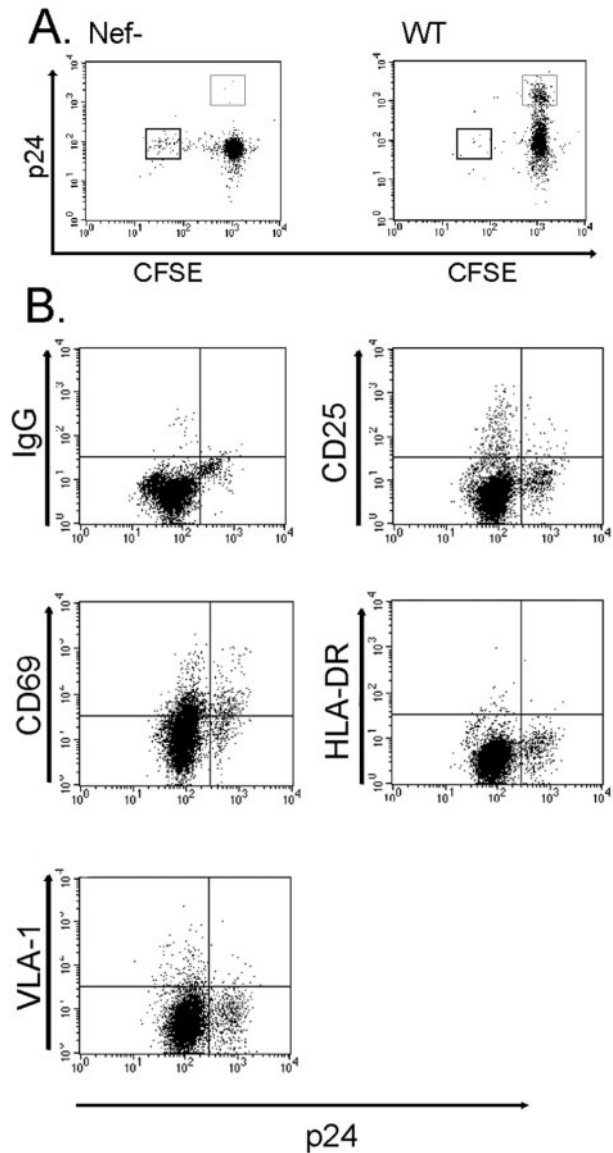


FIG. 1. Characterization of HIV type 1-infected CD4⁺ T cells. (A) FACS analyses of CFSE-labeled CD4⁺ T cells cocultured with ECs, infected with either Nef⁻ or wild-type (WT) HIV, and harvested on day 9 postinfection. The thin-walled box identifies the CFSE^{high} p24^{high} cells, and the thick-walled box identifies the CFSE^{low} p24^{low} cells. Note the expansion of CFSE^{high} p24^{high} cells in the wild-type-infected cultures. (B) FACS analyses of wild-type HIV-infected T cells after 9 days of coculture with ECs stained with antibodies against IgG isotype control, CD25, CD69, HLA-DR, and VLA-1. Note that p24^{high} cells express CD69 but not other markers of activation. The data in panels A and B are from one of three experiments with similar results.

(0.1 to 1.0%) prior to expansion. Therefore, even if this entire population became p24^{high}, without expansion via proliferation, they could not account for the majority of the total p24^{high} population. An unexpected finding was that in cultures infected by wild-type virus, but not by Nef⁻ virus, there was a conspicuous absence of CFSE^{low} cells. We suspect that wild-type virus either inhibited T-cell proliferation or, more likely, mediated the death of infected T cells. We did not investigate this issue further in the present study. In the remainder of

TABLE 1. The p24^{high} cells in EC-T-cell cocultures do not spontaneously produce cytokines

Cytokine	% Positive ^a	
	Monensin alone	Monensin, PMA, ionomycin
IL-2	1.7 ± 1.4	73.5 ± 0.4
IL-4	1.5 ± 0.4	2.6 ± 0.3
IFN-γ	4.4 ± 2.8	32.0 ± 15.6

^a Percentage of the p24^{high} cells that were positive for each intracellular cytokine. These data are from one of three experiments with similar results.

this study, we focused on the characterization of the p24^{high} CFSE^{high} virus producers that emerge when wild-type infected T cells are cocultured with ECs.

We initially examined the activation status of the high-virus-producing T cells. We analyzed the p24^{high} cells for surface expression of early, intermediate, and late activation markers. A few hours after TCR engagement, T cells express the early activation markers CD25, a component of the high-affinity IL-2 receptor, and CD69, a molecule with unknown function. After 24 h of activation, human T cells will stably upregulate MHC class II molecules (e.g., HLA-DR) (15), and after a week will stably upregulate the β₁ integrin, VLA-1 (20). FACS analysis revealed that on day 9 postinfection, the p24^{high} cells did not express CD25, HLA-DR, or VLA-1, although some did express the activation marker CD69 (Fig. 1B). These data are consistent with minimal (and perhaps a very early stage of) activation.

Cytokine synthesis, which can be measured by intracellular staining, is another hallmark of CD4⁺ T-cell activation (29). We therefore proceeded to examine the cytokines expressed by the high virus producers. We harvested the cells 9 days after infection and performed intracellular cytokine staining for effector cytokines, IL-2, IL-4, and IFN-γ, using monensin to enhance the signal by blocking secretion. FACS analysis revealed that the virally infected cells did not appear to be producing cytokines in the presence of MHC class II-expressing ECs (Table 1). Intracellular cytokine staining of replicate cultures showed that these same cells are capable of producing IL-2 and IFN-γ in response to pharmacological stimulation by PMA plus ionomycin (Table 1). EC-stimulated cells after 9 days of culture do not produce IL-4 with or without PMA and ionomycin. The absence of intracellular cytokine staining is thus also suggestive of minimal T-cell activation.

We also characterized the profile of cytokines produced by these cells at the mRNA level. To do so, we took advantage of the well-described abilities of Nef, Vpu, and Env to downregulate surface CD4 in order to isolate a population enriched in virus producers (2, 7, 62, 66). Uninfected T cells are uniformly CD4^{high} p24^{low} (Fig. 2A). In contrast, in the HIV-infected cocultures, we found three populations, a CD4^{high} p24^{low} population, a CD4^{low} p24^{low} population, and a CD4^{low} p24^{high} cell population (Fig. 2B). In other words, the p24^{high} cells are almost exclusively CD4^{low}, presumably due to high levels of expression of HIV protein. By selecting the cells based on CD4 expression, we enriched for a population of the high virus producers (Fig. 2C). In parallel, we isolated the nonproliferating CFSE^{high} cells from an uninfected replicate culture by cell

sorting (Fig. 2D). We thus collected RNAs from CD4^{low} infected cells and CFSE^{high} uninfected cells from replicate EC-T-cell cocultures and analyzed them by using Human Common Cytokine Superarrays. In general, the virally infected cells closely resembled the unactivated T-cell population (data not shown). After normalization to GAPDH (glyceraldehyde-3-phosphate dehydrogenase), these cells actually expressed lower levels of IFN-γ RNA than unactivated T cells isolated from the same donor (Fig. 2E). However, consistent with being HIV infected, the p24^{high} T cells produced more IFN-α7 and IL-8 RNA, the latter presumably due to the effects of Tat (45). The p24^{high} cells also consistently expressed more BMP6 RNA, the function of which is unknown. Despite these few differences, the results from the Superarray analyses generally confirm the lack of T-cell activation.

High virus producers are not arrested in G₂ by Vpr. Since virus-producing cells were not dividing, we tested whether an antiproliferative HIV protein could be responsible for causing cell cycle arrest of the host cell. Specifically, it has been reported that CD4⁺ T cells that have been productively infected with wild-type virus are nonproliferating despite strong stimulation (4), whereas CD4⁺ T cells infected with a *vpr*-deficient strain (Vpr⁻) that were similarly stimulated underwent multiple cell divisions (44). Vpr can arrest infected cells in G₂ (19, 23, 49), which is advantageous to the virus because G₂ is the phase of the cell cycle that is optimal for viral replication (18). Strikingly, Vpr contributes strongly to viral replication in EC-stimulated T cells, since its absence results in a 10- to 20-fold reduction in cell-free virus (Fig. 3A). The number of p24^{high} CFSE^{high} T cells is also drastically reduced in EC-T-cell cocultures infected with Vpr⁻ virus (Fig. 3B), consistent with reduced cell-free virus levels. Because the p24^{high} population is essentially absent in the Vpr⁻-infected cultures, it was not possible to determine if Vpr actually caused growth arrest. To determine whether the cells infected with wild-type virus were, in fact, arrested in a particular stage of the cell cycle, we stained the T cells with propidium iodide. FACS analysis revealed that the p24^{high} cells are not arrested in G₂ or in any other specific stage of the cell cycle but instead represent a heterogeneous population with most of the p24^{high} cells in G₀/G₁ (Fig. 3C). Rapamycin inhibits mTOR, which is required for the progression of IL-2-stimulated cells from G₁ to S (1). To further test if progression beyond G₁ is required for enhanced HIV production, we added rapamycin at concentrations that were sufficient to inhibit or completely block proliferation of polyclonally activated T cells (Fig. 3D). We observed that addition of rapamycin reduced viral replication (though not nearly to the extent of deletion of *vpr*) (Fig. 3E) in our in vitro model using previously resting T cells. Our cumulative observations suggest that the necessity for Vpr for high levels of viral replication in p24^{high} CFSE^{high} cells cannot be explained by its ability to induce cell cycle arrest.

TCRs and not cytokine signals provide minimal activation for viral replication. In our previous study, we showed that the ability of ECs to enhance viral replication in cocultured CD4⁺ T cells requires endothelial expression of MHC class II and costimulators, yet our new data revealed that the p24^{high} T cells showed little evidence of T-cell activation (9). Two models can explain viral replication in minimally activated T cells. In the bystander model, the T cells that are p24^{high} do not

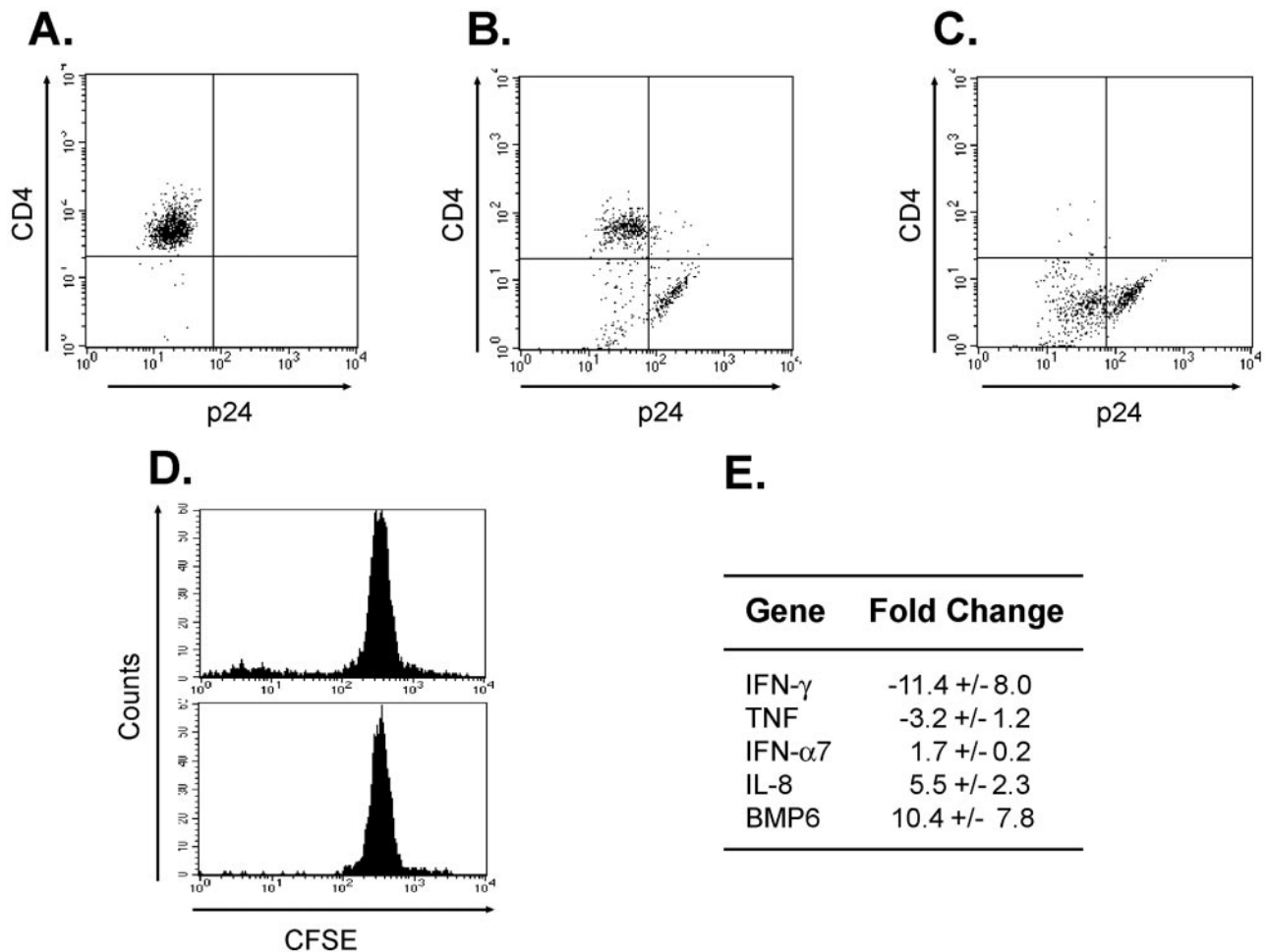


FIG. 2. Changes in gene expression induced by HIV infection. FACS analyses after 9 days of EC-T-cell coculture of (A) uninfected CD4⁺ cells, (B) wild-type HIV-infected T cells without selection, and (C) wild-type HIV-infected T cells following depletion of CD4^{high} cells. (D) FACS histogram of replicate uninfected cultures without sorting (top) and after sorting (bottom) for the CFSE^{high} population. (E) Human Common Cytokine Superarray Analyses of RNAs isolated from CD4^{low} cells from wild-type virus-infected EC-T-cell cocultures compared with unactivated CD4⁺ T cells from the same donor. Cytokine expression levels were normalized to GAPDH. Depicted are genes that displayed a >1.5-fold change in expression in CD4^{low} cells (presumably infected) versus CFSE^{high} cells (from uninfected cultures). The data in panels A to E are from one of three experiments with similar results.

directly recognize MHC class II on the EC surface. Instead, the signals that promote viral replication in these nonproliferating cells, i.e., cytokines, are provided in *trans* by other T cells which are highly or moderately activated. In the suboptimal-signaling model, the T cells that are p24^{high} directly recognize MHC class II on the EC surface but receive signals that are insufficient to fully activate the cells to proliferate and/or elaborate cytokines. Such signals may be provided by EC MHC class II molecules to cells bearing TCRs that interact with low affinity.

Although activated T cells appeared to have died off by day 9 postinfection, we did detect cytokines, such as IL-2, in the medium on day 1 postinfection at levels that were similar to those found in uninfected EC-T-cell cocultures (9). Therefore, to test whether the bystander model was operative in our in vitro system, we added blocking antibodies to IL-2, IL-6, IL-7, TNF, or BMP-6, which have been identified as cytokines that can support TCR-independent viral replication (54, 61, 63), to EC-T-cell cocultures. These treatments did not inhibit viral

replication (Fig. 4A), results that are inconsistent with the bystander model.

To test whether the suboptimal-signaling model was operative, we cocultured CD4⁺ T cells with untreated ECs, added suboptimal doses of PHA-L, and infected them with wild-type virus. Under such conditions, ECs continue to provide costimulation, but the TCR-mediated signals are mediated primarily through PHA-L rather than MHC recognition. PHA-L provides a dose-dependent increase in the IL-2 produced (Fig. 4B) and dose-dependent proliferation (data not shown). Strikingly, suboptimal doses of PHA-L that are insufficient to induce detectable cytokine production still support efficient viral replication (Fig. 4C). We next assessed the ability of suboptimal stimulation with PHA-L to induce a CFSE^{high} p24^{high} population. To do so, we labeled the T cells with CFSE prior to adding them to EC cultures and infecting them. On day 7 postinfection, the T cells were harvested and counterstained for p24. We found that cultures activated with suboptimal

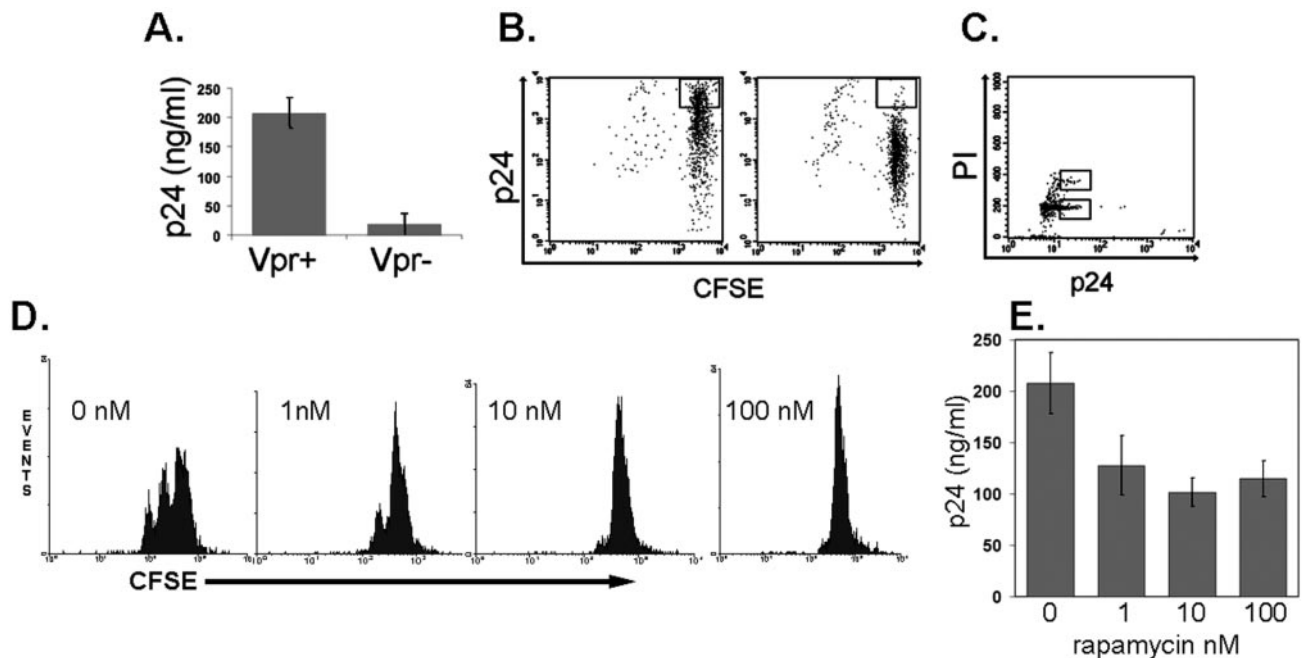


FIG. 3. Characterization of the role of Vpr in enhanced HIV production. (A) Replication of wild-type versus Vpr⁻ HIV in EC-T-cell cocultures 9 days postinfection as assessed by p24 measured in the supernatant by ELISA. (B) CFSE staining of infected cells harvested on day 9 postinfection. The box identifies the CFSE^{high} p24^{high} cells, which are fewer in cultures infected by Vpr⁻ virus (right). (C) PI staining of infected cells harvested on day 9 postinfection. The lower box identifies cells in the G₀/G₁ state, and the upper box identifies the cells in the G₂ state of the cell cycle. Note that the majority of cells are in G₀/G₁. (D) FACS analyses of CFSE-labeled CD4⁺ T cells treated with PHA-L and rapamycin. Note that the cells remain CFSE^{high}. (E) Replication of wild-type HIV in EC-T-cell cocultures treated with rapamycin or vehicle (dimethyl sulfoxide, 0 nM rapamycin) 9 days postinfection as assessed by p24 measured in the supernatant by ELISA. The data in panels A to E are from one of three experiments with similar results. The error bars indicate standard deviations.

stimulation, i.e., 0.0625 μ g/ml of PHA-L, displayed negligible T-cell proliferation, but compared to no PHA-L, they show a significant increase in the number of p24^{high} CFSE^{high} cells (Fig. 4D and E). These data support the idea that a suboptimal TCR signal promotes HIV replication in T cells cocultured with ECs.

To determine more directly whether the p24^{high} cells were receiving MHC class II signals from ECs, we used a different type of polyclonal stimulator, namely, the superantigen TSST-1. TSST-1 binds to a conserved site on MHC class II molecules and to a site on the V β 2 chain of the TCR (17). Therefore, like PHA-L, TSST-1 can activate T cells primarily by simulating TCR-MHC interactions. However, unlike PHA-L, TSST-1 requires expression of endothelial MHC class II, and more importantly, TSST-1-mediated activation, unlike PHA-L-mediated activation, is restricted to those T cells that express TCRs containing the V β 2 receptor chain. We found that TSST-1, like PHA-L, provided a dose-dependent increase in cytokine production (Fig. 5A). If the suboptimal-signaling model were true, TSST-1 should selectively increase viral replication among the cells that express V β 2. To test this, we cocultured MHC class II-expressing ECs with unselected, resting CD4⁺ T cells in the presence of increasing doses of TSST-1. On day 7 postinfection, we harvested the T cells and stained them for extracellular V β 2 and intracellular p24. In the absence of TSST-1 (Fig. 5B), the p24^{high} cells, like the input cells, were predominantly V β 2⁻. In the presence of suboptimal TSST-1, we found that the p24^{high} cells were enriched selec-

tively in the V β 2⁺ population (Fig. 5C). TSST-1 did not change the proportion of V β 2⁻ cells that were p24^{high}. In contrast, suboptimal TSST-1 increased the proportion of V β 2⁺ cells that were p24^{high} by sevenfold (Fig. 5C). These observations directly confirm a role for suboptimal TCR signaling in promoting HIV replication.

Finally, we investigated signals downstream of the TCR that could underlie the EC effect. Cyclosporine inhibits the TCR-dependent activation of calcineurin. Therefore, to assess a role for calcineurin in our in vitro model, we added cyclosporine to EC-T-cell cocultures. Since previous studies had shown that ECs confer cyclosporine resistance on CD4⁺ T cells (24), we chose relatively high doses of cyclosporine, namely, 0.2 μ g/ml and 1.0 μ g/ml. Such concentrations are consistent with doses previously used to examine the impact of calcineurin on HIV replication with minimal effect on cyclophilin A incorporation into virions (11, 26). The addition of cyclosporine significantly inhibited viral replication (Fig. 6A). The major target of cyclosporine is thought to be calcineurin-dependent activation of NFAT. To directly test whether an NFAT-dependent mechanism underlies the EC effect, we disrupted the NFAT binding site in the HIV LTR (26, 27) (Fig. 6B) and tested if this affected the capacity of ECs to enhance HIV production. The recombinant HIV (NFAT⁻) replicated to only very low levels in comparison to the wild-type derivative strain in previously resting CD4⁺ T cells cocultured with IFN- γ -treated ECs (Fig. 6C). To ensure that this phenotype was not due to inherently poor NFAT⁻ replicative capacity, it was also grown in CD4⁺ T

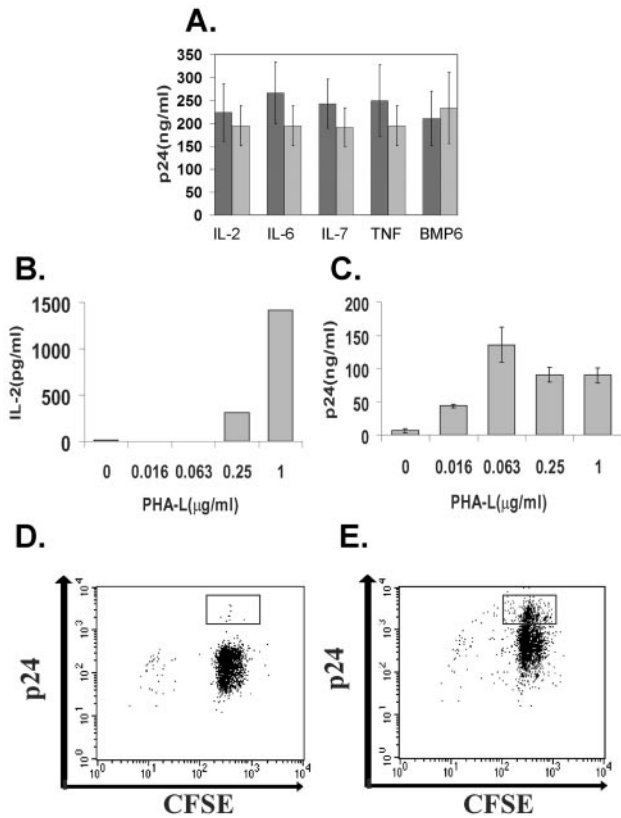


FIG. 4. TCRs and not cytokine signals provide minimal activation necessary for HIV replication. (A) Replication of wild-type HIV in EC-T-cell cocultures 9 days postinfection treated with the indicated blocking antibodies (dark bars) or isotype controls (light bars) as assessed by p24 measured in the supernatant by ELISA. (B) IL-2 measured in the supernatant of infected T cells after 1 day of coculture with ECs in the presence of various doses of PHA-L or vehicle alone. (C) Cell-free virus measured in the supernatant of infected EC-T-cell cocultures on day 7 postinfection as assessed by p24 measured in the supernatant by ELISA. (D and E) FACS analyses of CD4⁺ T cells cocultured with untreated ECs in the presence of (D) 0 μg/ml and (E) 0.063 μg/ml of PHA-L. The boxes identify the CFSE^{high} p24^{high} cells. Note the expansion of CFSE^{high} p24^{high} cells in the wild-type-infected cultures in the presence of 0.063 μg/ml of PHA-L. The data in panels A to E are from one of three experiments with similar results. The error bars indicate standard deviations.

cells pretreated with PHA and IL-2. Under these conditions, NFAT⁻ virus replicated similarly to wild-type virus (Fig. 6D).

DISCUSSION

In the present study, we made the unexpected finding that HIV replication in EC-T-cell cocultures actually occurs exclusively in memory CD4⁺ T cells that are only minimally activated. We cannot rule out the possibility that highly or moderately activated cells contributed to viral replication but died before they could be detected. However, on day 9 postinfection, i.e., the peak of viral replication, the cells that are producing the virus (the p24^{high} T cells) are neither proliferating nor arrested at a specific stage of the cell cycle. Furthermore, other than CD69 expression, the p24^{high} T cells do not express activation markers or effector cytokines. In our system, the

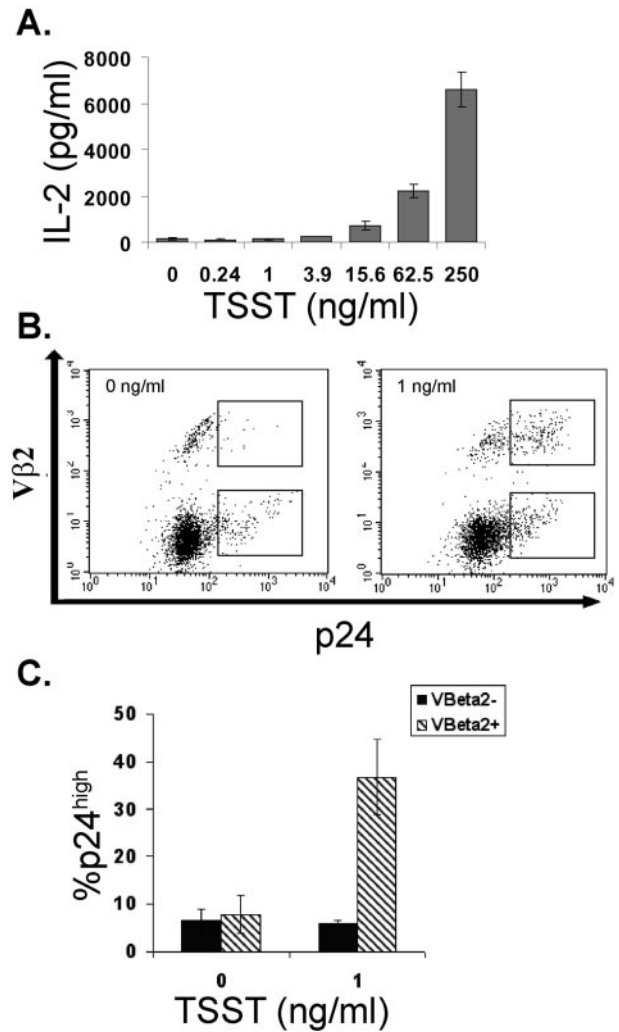


FIG. 5. TSST-1 selectively leads to viral replication in Vβ2⁺ T cells. (A) IL-2 measured in the supernatant of infected T cells after 1 day of coculture with ECs in the presence of increasing doses of TSST-1 or vehicle alone. (B) FACS analyses of CD4⁺ T cells cocultured with IFN-γ-pretreated ECs in the presence of 0 ng/ml or 1 ng/ml of TSST-1. The upper boxes identify the Vβ2⁺ p24^{high} cells, and the lower boxes identify the Vβ2⁻ p24^{high} cells. Note the expansion on the Vβ2⁺ p24^{high} cells in the presence of 1 ng/ml TSST-1. The data in panels A and B are from one of three experiments with similar results. (C) Proportions of p24^{high} cells among the Vβ2⁺ and the Vβ2⁻ populations in infected EC-T cell cocultures stimulated with 0 or 1 ng/ml of TSST-1. Shown are the means from three independent experiments ± standard error.

virally infected T cells are identified by FACS analysis, which can occur only after they express sufficient intracellular viral proteins. Therefore, we cannot rule out the possibility that the cells had previously demonstrated markers of activation, i.e., surface markers and effector cytokines, but had subsequently downregulated them. This seems unlikely, since we examined the cells for early, intermediate, and late activation markers, and we found that the p24^{high} T cells did not express CD25, HLA-DR, or VLA-1 and only small amounts of CD69. Our results using T cells activated by ECs stand in striking contrast to previous studies, which used similar FACS-based techniques

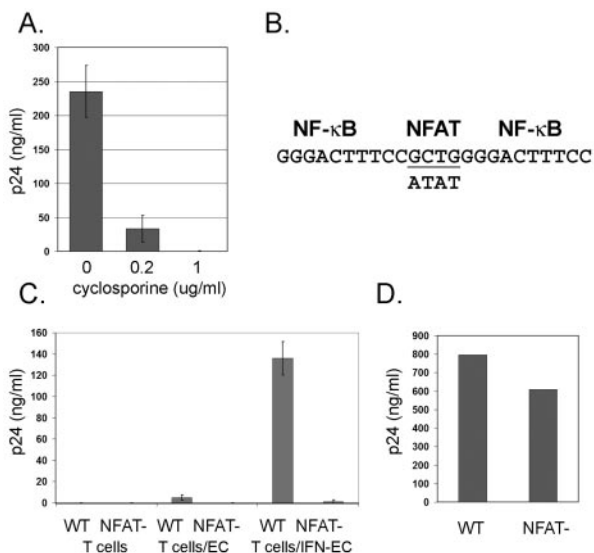


FIG. 6. Characterization of the role of NFAT in HIV production. (A) HIV release on day 9 postinfection (representing the peak) from EC-T-cell cocultures treated with cyclosporine or vehicle (ethanol). The data are from one of three experiments with similar results. (B) Schematic diagram of the region of the HIV LTR that contains the NF-κB and NFAT binding sites. The underlined nucleotides are required for NFAT-LTR interactions. The nucleotides on the lower line indicate the loci that differ between the NFAT⁻ strain and the wild-type derivative strain (upper line). (C) HIV released from T cells on day 9 postinfection (representing the peak). T cells represents T cells alone, T cells/EC represents T cells cocultured with untreated ECs, and T cells/IFN-EC represents T cells cocultured with ECs pretreated with IFN-γ. The data are from one of two experiments with similar results. (D) HIV released from CD4⁺ T cells isolated from PBMCs and pretreated with PHA and IL-2 on day 8 postinfection (representing the peak). Similar results were obtained using the cell line CEMx174 (not shown). HIV release was assessed by p24 measured in the supernatant by ELISA. The error bars indicate standard deviations.

to show that viral replication under other conditions is restricted to highly activated T cells (4, 44).

In a fully quiescent cell, HIV exists as incomplete or complete cDNA, neither of which is stable long term, and both are essentially transcriptionally silent (58, 68, 71). Therefore, even in cells that demonstrate limited markers of activation, viral replication appears to require the activation of specific signaling pathways. In the current study, we tested two possible means by which MHC molecule- and costimulation-dependent signals could support viral replication in EC-stimulated T cells but not proliferation or cytokine production from these same T cells. In the bystander model, the high virus producers receive selective activation via a factor, i.e., a cytokine produced by other CD4⁺ T cells that are highly or moderately activated. This possibility is supported by a previous study, which showed that in autologous dendritic-cell-T-cell cocultures and superantigen, productive HIV replication occurs not only in the superantigen-activated T cells but also in the bystander cells, which did not receive direct TCR activation and were therefore stimulated via cytokines (51). However, in contrast to the previous report on dendritic-cell-T-cell cocultures, we found no direct evidence of highly activated T cells in the EC-T-cell cocultures at the time of peak virus production. In fact, there

was a striking absence of the expected CFSE^{low} population, i.e., the population that proliferated in wild-type-infected cultures. In addition, rapamycin, an inhibitor of cytokine-dependent mTOR activation, only minimally inhibits viral replication, and the addition of blocking antibodies to IL-2, IL-6, IL-7, and TNF, which have been identified as cytokines that can support TCR-independent viral replication (54, 61, 63), had no effect on viral replication in EC-T-cell cocultures. Our data suggest that the bystander model does not explain why the p24^{high} T cells in cocultures with ECs appear minimally activated.

An alternative to the bystander model is the suboptimal-signaling model. There is a hierarchical activation of T-cell functions, and different functions are activated sequentially with increasing TCR stimulus. For example, T-cell proliferation requires higher stimulation than does cytokine production (5). In the suboptimal-signaling model, the high virus producers directly recognize endothelial MHC class II molecules but do not receive a signal sufficiently strong to drive the cell to proliferation or to cytokine production. Our results have led us to favor this model. The experiments with PHA-L demonstrate that suboptimal TCR-mediated stimulation is sufficient for efficient virus replication; furthermore, the experiments with TSST-1 demonstrate that viral replication is restricted to T cells whose TCRs are engaged by endothelial MHC class II molecules. We have also examined the effects of cyclosporine, an inhibitor of the TCR-activated enzyme calcineurin. Although this drug has several nonspecific effects (52, 60), its major action is blocking the calcineurin-dependent activation of the transcription factor NFAT. We have demonstrated that cyclosporine, in contrast to rapamycin, effectively inhibits viral replication. More directly, we also showed that the alteration of the NFAT binding site in the HIV LTR blocks the EC effect on HIV replication.

Studies with altered peptide ligands (APLs) have shown that suboptimal T-cell activation preserves some aspects of full T-cell activation but not others (56). Suboptimal T-cell activation with APLs induces only partial TCR-ζ phosphorylation, which in turn leads to the inefficient recruitment of Src family kinases. The biochemical consequence of this is the selective activation of downstream targets. For example, APL-induced calcium signaling, which is reduced and shorter in duration than agonist peptide-induced calcium signaling, can contribute to the selective activation of NF-κB or of NFAT (30, 57). In contrast to the IL-2 promoter, where NFAT binds as a heterodimer with AP-1, NFAT binds in the LTR enhancer as a homodimer (47). Therefore, while IL-2 expression requires both NFAT activation and AP-1 activation (35), HIV replication can occur with the selective activation of NFAT (26). Independent studies have shown that selective NFAT activation produces a phenotype similar to that of EC stimulation in HIV-infected T cells. NFAT-driven HIV production leads to preferential infection of memory T cells (50), and viral replication occurs without T-cell proliferation, activation marker expression, or cytokine expression (26). Lastly, selective NFAT activation, like HIV replication in our system, can be augmented by EC-specific factors. Wnt, produced by ECs, can inhibit glycogen synthase kinase-3β (GSK-3β), which in turn inhibits the nuclear export of NFAT (42).

A primary role for NFAT in EC-stimulated memory T cells

may explain the important roles of Nef and Vpr in our model system. Nef is expressed even before the virus is integrated (67). In addition, Nef can interact directly with T-cell signaling molecules, including Lck, Vav, Pak2, and PKC- θ (37), and in response to TCR-derived signals, Nef has been shown to potentiate specific TCR-dependent signaling pathways, including those involving ERK (53), MAPK (37), PI-3-kinase (32), and Ca^{2+} (38). The rise in intracellular Ca^{2+} is the mechanism that leads to calcineurin and NFAT activation. The dependence of the EC effect on an intact *nef* gene is reminiscent of the effect that HIV-infected macrophages have on the infectivity of resting $CD4^+$ T cells (59). However, the effect of Nef is operative in the macrophages and not in the resting T cells, whereas in the EC system, the effect of Nef is operative in the resting T cells and not in the ECs. We have shown that ECs, but not other antigen-presenting cells, greatly enhance Nef-dependent viral replication in resting T cells (9). Combined with the findings from this study, we suggest that the primary role of Nef is to serve as a signaling molecule that specifically modulates EC-specific, TCR-dependent signals, notably NFAT. Thus, Nef may play multiple roles in the establishment of an HIV-favorable microenvironment in peripheral tissue.

Vpr, a viral protein that is necessary for viral pathogenesis in vivo (33), contributes to viral replication in EC-T-cell cocultures in a manner that is independent of its ability to induce G_2 arrest. Although we have not determined if the requirement for Vpr occurs prior to or subsequent to integration, we hypothesize that Vpr's ability to promote HIV transcription may underlie its contribution to EC-stimulated viral replication. In support of this, Vpr, a nuclear protein, has been shown to alter signal transduction pathways via direct modulation of transcription factor activity in the nucleus (25, 28), and Vpr-mediated enhancement of HIV transcription is most pronounced under conditions of suboptimal activation. Vpr's ability to activate cyclic AMP responsive element binding factor (CREB) is evident only under conditions that lead to the serine phosphorylation of CREB but not to CREB-dependent gene expression (28). In addition, Vpr's ability to potentiate LTR transcription driven by Sp1, a transcription factor that is active in resting $CD4^+$ T cells (64), is attenuated both by NF- κ B activation and by progression of the cell into G_2 . Moreover, Vpr has been shown to synergize with Nef in augmenting calcineurin-dependent NFAT activation (36–38), which we postulate contributes to the EC effect.

The role of minimal TCR signaling by ECs fits into a general pattern in which HIV subverts normal immune function. $CD4^+$ T cells require TCR-dependent signals for maintenance of immunological memory (39, 55). In humans, MHC class II molecules are displayed on vascular ECs (8). Therefore, as human memory cells circulate through the blood, ECs are well situated to provide the suboptimal TCR signals required for maintenance of immunological memory. We propose that HIV exploits these signals for virus production. Specifically, we hypothesize that EC-expressed MHC class II molecules, perhaps containing self peptides, provide nonredundant signals that enhance virus replication in circulating, latently infected memory T cells.

Our findings have implications for the course of HIV infection in patients. During HIV and SIV infection, cells with limited markers of T-cell activation are productively infected

(70). Furthermore, in the initial weeks of SIV infection, memory T cells are greatly depleted in multiple tissues (which suggests that high-affinity MHC class II-TCR interactions are not required for efficient HIV production in human tissue) and resting memory T cells are specifically depleted in the GALT (31, 40). The loss of these cells (which results directly from viral infection and not via a bystander mechanism) is thought to establish conditions that eventually result in progression to AIDS. Ex vivo analysis of infected patients has shown that resting memory cells represent the T-cell compartment that harbors the highest copy number of viral DNA (6). Furthermore, latently infected memory cells are thought to contribute to the viremic "blips" in patients on ART, as well as to breakthrough virus in patients whose ART regimens are changed and rebound virus in patients who discontinue ART (10, 69). All of these phenomena may involve EC effects on infected memory T cells of the kind described in the present report.

In summary, we have described a novel manner in which vascular ECs can boost HIV replication in minimally activated $CD4^+$ T cells. This response depends upon recognition of molecules on the ECs and involves the viral genes *nef* and *vpr* and TCR-dependent activation of NFAT. These findings illustrate yet another way in which HIV exploits the human immune system and may contribute to rebound following discontinuance of ART in HIV-infected patients and the depletion of resting memory T cells in the GALT in the initial weeks of infection.

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