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Primary Human Mammary Epithelial Cells Endocytose HIV-1 and Facilitate Viral Infection of CD4$^+$ T Lymphocytes$^\nabla$

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The contribution of mammary epithelial cells (MEC) to human immunodeficiency virus type 1 (HIV-1) in breast milk remains largely unknown. While breast milk contains CD4$^+$ cells throughout the breast-feeding period, it is not known whether MEC directly support HIV-1 infection or facilitate infection of CD4$^+$ cells in the breast compartment. This study evaluated primary human MEC for direct infection with HIV-1 and for indirect transfer of infection to CD4$^+$ target cells. Primary human MEC were isolated and assessed for expression of HIV-1 receptors. MEC were exposed to CCR5-, CXCR4- and dual-tropic strains of HIV-1 and evaluated for viral reverse transcription and integration and productive viral infection. MEC were also tested for the ability to transfer HIV to CD4$^+$ target cells and to activate resting CD4$^+$ T cells. Our results demonstrate that MEC express HIV-1 receptor proteins CD4, CCR5, CXCR4, and galactosyl ceramide (GalCer). While no evidence for direct infection of MEC was found, HIV-1 virions were observed in MEC endosomal compartments. Coculture of HIV-exposed MEC resulted in productive infection of activated CD4$^+$ T cells. In addition, MEC secretions increased HIV-1 replication and proliferation of infected target cells. Overall, our results indicate that MEC are capable of endosomal uptake of HIV-1 and can facilitate virus infection and replication in CD4$^+$ target cells. These findings suggest that MEC may serve as a viral reservoir for HIV-1 and may enhance infection of CD4$^+$ T lymphocytes *in vivo*.

In the more than 2 decades since the discovery of human immunodeficiency virus (HIV) transmission via breast milk, little progress has been made in understanding the contribution made by the mammary gland to HIV replication in mammary tissue and breast milk. Mammary epithelial cells (MEC), the milk-secreting cells of the mammary gland, play a unique functional role within breast tissue. While most epithelial layers in the body serve a protective function, MEC selectively take up components of the plasma and manufacture constituents of breast milk to secrete a nutrient- and immune factor-rich fluid (22).

In a nonpregnant or nonlactating woman, the mammary gland contains a slowly dividing population of mammary epithelial cell progenitors, known as mammary stem cells, which are present in the breast tissue from birth and persist throughout most of a woman's premenopausal life (14). Hormonal changes associated with pregnancy and lactation induce rapid proliferation and terminal differentiation of these cells into mammary epithelial secretory alveolar cells, epithelial ductal cells, or myoepithelial cells (14, 25, 34, 39, 43). Stem cell division is primarily asymmetric, such that one daughter cell differentiates while the other retains the stem cell phenotype (11, 13, 43). Due to the steep rise in prolactin in the first half of pregnancy, hormone-induced proliferation and differentiation of mammary stem cells are largely complete by the second trimester (25). Whether mammary stem cells or their differentiated progeny are susceptible to human immunodeficiency virus type 1 (HIV-1) infection or sequestration of virions in endosomal compartments is largely unknown.

Milk is a very cellular fluid throughout the lactation period, with more than 10^6 leukocytes/ml in the colostrum (5, 26), followed by a decline over time to approximately 10^5 to 10^4 leukocytes/ml in mature milk (9, 15, 16, 26). To reach the milk, lymphocytes and monocyte/macrophage populations must traffic paracellularly or transcellularly through a normally impermeable MEC monolayer on their way into the milk duct. Epidemiologic studies show that HIV-1 RNA and proviral DNA in breast milk are significantly associated with virus transmission (19, 28–30); however, little is known about the interaction between MEC and leukocytes in the mammary glands of HIV-infected women. Limited attention has been paid to the concept that MEC may contribute to the HIV-1 viral load in breast milk. This may be due, in part, to an early study of primary human MEC using a laboratory-adapted strain of HIV-1, which demonstrated low levels of viral replication in the MEC (40). However, even low-level HIV-1 replication or transcytosis of virions across MEC without direct infection could significantly impact the persistence and spread of virus within the mammary gland. MEC and leukocytes entering breast milk share close physical contact. Therefore, cell-to-cell contact between MEC, which either harbor HIV-1 or bind and transmit the virus to susceptible target cells in transit to the milk, and leukocytes could provide a potential mechanism for seeding newly infected cells into the breast milk.

There is growing evidence that the mammary gland contributes to breast milk transmission of HIV-1 beyond simply allowing the passive flow of HIV-1 virions and infected cells into the milk. Treatment of HIV-1-infected pregnant and lactating women with highly active antiretroviral therapy (HAART) has been shown to decrease breast milk cell-free HIV-1 RNA but

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not cell-associated HIV-1 DNA (23, 36). In addition, phylogenetic analyses of HIV-1 DNA sequences derived from paired plasma and breast milk of HIV-positive African women show genetic differences between HIV-1 from blood and that from breast milk (4, 36). A recent study of lactating rhesus monkeys chronically infected with simian immunodeficiency virus (SIV) demonstrated the presence of phylogenetically similar or identical groups of SIV variants in the milk, suggesting local replication of virus within the mammary gland (27).

The mechanism(s) of HIV-1 infection, replication, and dissemination in human mammary tissue is unknown. We undertook the present study to examine basic mechanistic questions, including characterization of primary human MEC for expression of HIV-1 receptors, coreceptors, and alternate receptors; evaluation of the susceptibility of MEC to direct infection with HIV-1; and investigation of indirect modes whereby MEC might facilitate HIV-1 infection of target cells. Our results indicate that, while MEC do not support integration of HIV-1, they do sequester and release infectious virions, facilitating infection of $CD4^+$ cells. In addition, secretions from the apical (luminal) surface of MEC increase HIV-1 replication and proliferation of HIV-infected cells.

MATERIALS AND METHODS

Subjects. Breast tissue was obtained from patients undergoing breast reduction surgery, following informed consent. Patients who were eligible to donate tissue to this study included premenopausal women of <40 years of age scheduled for bilateral mammoplasty, with no personal history of breast cancer and no prior abnormal mammogram or breast biopsy.

As the standard of care, the pathology department at the Dartmouth-Hitchcock Medical Center (DHMC) requires that all breast tissue samples removed at surgery be tested for occult neoplasia. This process requires up to 24 h, necessitating the immediate placement of study tissue into fresh medium (Dulbecco's modified Eagle's medium [DMEM]–F-12 medium with 10% fetal bovine serum [FBS], antibiotics, and amphotericin B) and storage at 4°C until release by the pathologist. In addition, to maintain the DHMC standard of care for mammoplasty patients, only women who had ≥500 g of tissue removed per breast were eligible to donate tissue to the study.

The protocol for obtaining tissue for this study was approved by the Dartmouth College Committee for the Protection of Human Subjects.

Dissociation of human mammary tissue and enrichment for $BerEp4^+$ cells. Methods for dissociation of tissue and enrichment for $BerEp4^+$ cells were adapted from published protocols for isolation and enrichment of MEC from human breast tissue (10, 13, 38). In brief, human mammary tissue was minced and placed into dissociation beakers (Bellco Glass, Vineland, NJ) with digestive medium containing DMEM–F-12 medium, bovine serum albumin (BSA), collagenase, hyaluronidase, insulin, hydrocortisone, and ampicillin, streptomycin, and amphotericin B and shaken at 70 rpm in a 37°C incubator at 5% CO_2 overnight. The following day, the mixture was filtered through a 250-μm wire mesh, and the undigested tissue was placed into fresh digestion medium for an additional 6 to 8 h. The filtrate was centrifuged at 80 × *g* for 4 min until the supernatant was clear. To aid in removal of fibroblasts in the remaining supernatant, Accumax (Innovative Cell Technologies, Inc., San Diego, CA), an enzymatic reagent used to disaggregate cell clusters, was added in a 1:1 volume ratio with the remaining cell pellet and medium after the last wash, and the solution was mixed by pipetting, incubated at 37°C for 7 min, and pipetted multiple times to separate the remaining fibroblasts from the MEC. Cells were washed with phosphate-buffered saline (PBS) containing 0.1% BSA-0.6% sodium nitrate at 80 × *g* for 4 min until the supernatant was clear, followed by a last wash at 600 × *g* for 10 min. The cell pellet was resuspended in PBS-BSA-sodium nitrate solution, and enrichment for $BerEp4^+$ cells was performed using immunomagnetic beads coated with anti-BerEP4 (EpCAM) monoclonal antibody (MAb) (CELLection epithelial enrich dynabeads; Invitrogen, Carlsbad, CA). Enriched MEC were either immediately placed into culture with mammary epithelial growth medium (MEGM) (Lonza, Rockland, ME) or suspended in 90% FBS-10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen for future use.

The purity of $BerEp4^+$ cells was assessed by staining enriched cell cultures for BerEp4 and the panleukocyte marker CD45. Enriched MEC were cultured on glass coverslips (Fisherbrand, Pittsburgh, PA) in MEGM until reaching a minimum of 70 to 80% confluence. Cells were washed with PBS, fixed with 4% methanol-free paraformaldehyde (PF) for 10 min at room temperature (RT), and washed once with PBS, followed by three PBS rinses of 5 min each. Cells were then blocked with 1% BSA-1% FBS in PBS for 30 min at RT. After aspiration of the blocking solution, purified mouse anti-human BerEp4 IgG1 (Dako, Carpinteria, CA) and mouse anti-human CD45 IgG1 conjugated to Alexa Fluor 405 (Invitrogen, Carlsbad, CA) were added to designated coverslips at a concentration of 10 μg/ml in blocking solution and incubated for 1 h at RT. Cells were washed five times with 0.1% BSA in PBS. Goat $F(ab')_2$ anti-mouse IgG–fluorescein isothiocyanate (FITC) secondary antibody (R&D Systems, Minneapolis, MN) was added to cells stained for BerEp4, and cells were incubated for 1 h at RT in the dark. Cells were washed five times with 0.1% BSA in PBS, followed by three washes in PBS for 15 min each, and incubated for 5 min at RT with 4% methanol-free paraformaldehyde, followed by three washes with PBS. After being air dried, the coverslips were mounted onto glass microscope slides with Prolong Gold antifade reagent (Invitrogen). The mammary epithelial cell line MCF-10A was used as a positive control for BerEp4 staining, and peripheral blood mononuclear cell (PBMC)-derived macrophages were utilized as a positive control for CD45 staining. Slides were imaged using a Bio-Rad MR1000 confocal scanning laser microscope system equipped with a krypton/argon laser and a 40× oil immersion objective (Hercules, CA). Images were captured using Zeiss LSM Image software. Simultaneous imaging took place using differential interference contrast (DIC) for visualizing cell morphology to assess whether fibroblasts were present.

Detection of HIV-1 receptors CD4, CXCR4, CCR5, and GalCer. Enriched MEC were cultured on glass coverslips (Fisherbrand) in MEGM until reaching a minimum of 50% confluence. Cells were washed with PBS, fixed with 4% methanol-free paraformaldehyde for 10 min at RT, and washed once with PBS, followed by three PBS rinses of 5 min each. Cells were then blocked with 1% BSA-1% FBS in PBS for 30 min at RT. After aspiration of the blocking solution, purified primary antibodies mouse anti-human CD4 IgG1 (BD Pharmingen, San Jose, CA), mouse anti-human CD184 (CXCR4) IgG2a(κ) (BD Pharmingen), mouse anti-human CD195 (CCR5) IgG2a(κ) (BD Pharmingen), and mouse anti-human galactocerebroside MAb IgG3 (Millipore, Billerica, MA) were added to designated coverslips at a concentration of 10 μg/ml in blocking solution, and the mixtures were incubated for 1 h at RT. Cells were washed five times with 0.1% BSA in PBS, goat $F(ab')_2$ anti-mouse IgG–FITC (R&D Systems) secondary antibody was added, and cells were incubated for 1 h at RT in the dark. Cells were washed five times with 0.1% BSA in PBS, followed by three washes in PBS for 15 min each, and incubated for 5 min at RT with 4% methanol-free paraformaldehyde, followed by three washes with PBS. After being air dried, the coverslips were mounted onto glass microscope slides with Prolong Gold antifade reagent with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen). TZM-bl cells were used as a positive control for expression of CD4, CXCR4, and CCR5. HT-29 cells served as a positive control for galactosyl ceramide (GalCer) expression. FITC-conjugated mouse isotype controls (Invitrogen) were also used in all staining experiments. Slides were imaged using a Bio-Rad MR1000 confocal scanning laser microscope system equipped with a krypton/argon laser and a 40× oil immersion objective (Hercules, CA). Images were captured using Zeiss LSM Image software.

Detection of CD4 mRNA. Detection of CD4 mRNA was quantified by real-time PCR with a commercially available kit (TaqMan Gene Expression Cells-to-CT kit; Applied Biosystems, Austin, TX) using the CD4 expression assay (Applied Biosystems), and the human beta-actin gene expression assay (Applied Biosystems) was used as a positive control. All key reagents and protocols for PCR cycling were followed per the manufacturer's instructions. Samples were amplified on an ABI Prism 7000 sequence detection system and data collected on Applied Biosystems 7000 sequence detection software.

PCR and p24 ELISA. Enriched MEC were cultured in 48-well plates until reaching at least 80% confluence. CCR5-tropic HIV-1_{BaL}, CXCR4-tropic HIV-1_{HC4}, and dual-tropic HIV-$1_{C7/86}$ strains were grown and DNase treated, titers in PBMCs were determined, and strains were added at 300 50% tissue culture infective doses ($TCID_{50}$) to wells in 200 μl of medium and incubated for 2 h at 37°C. Wells were washed three times, and the MEC were incubated for 14 days at 37°C. Phytohemagglutinin (PHA)-activated HIV-infected and uninfected PBMCs were used as controls. Wells were replenished with fresh medium on days 4, 7, 10, and 14. Supernatants were collected on days 0, 7, and 14 and measured for HIV p24 antigen by use of a commercially available enzyme-linked immunosorbent assay (ELISA) (Perkin Elmer, Waltham, MA) that has a limit of sensitivity of 4.3 pg/ml. Total genomic cellular DNA was extracted from MEC on day 14 by use of a Qiagen blood minikit (Qiagen, Valencia, CA). Reverse transcription and

integration of HIV-1 proviral DNA were assessed by PCR. PCR for reverse transcription was performed using the long terminal repeat (LTR) sense primer M667 (5′-GGCTAACTAGGGAACCCACTG-3′) (Integrated DNA Technologies [IDT], Coralville, IA) and the antisense primer AA55 (5′-CTGCTAGAG ATTTTCCACACTGAC-3′) (IDT). The first round of the HIV-1 integration PCR was performed by using the Alu sense primer (5′-GCCTCCCAAAGTGC TGGGATTA-3′) (IDT), which binds to short DNA sequences occurring in multiple locations throughout the human genome, and the HIV-1 Gag antisense primer (5′-CCTGCGTCGAGAGAGCTCCTCTGG-3′) (IDT) (3). Five microliters of first-round product was then used in nested PCR, with the LTR sense primer M667 (IDT) and the antisense primer AA55 (IDT) (3). Products from the second-round PCR were run on 1% agarose gels with ethidium bromide to detect an expected 140-bp fragment. HIV-exposed and washed PBMCs were used as positive controls, and HIV-unexposed MEC and PBMCs were used as negative controls. PCR for actin was performed using a human beta-actin gene sense primer (5′-CACTCTTCCAGCCTTCCTTCC-3′) and antisense primer (5′-CTG TGTTGGCGTACAGGTCT-3′). Experiments were performed in triplicate under each condition.

GFP reporter virus. MEC were cultured on glass coverslips in 24-well plates in MEGM until reaching a minimum of 50% confluence. Fifty microliters of NLENG1i_BaL.ecto, a green fluorescent protein (GFP) reporter virus designed to fluoresce within a cell after HIV-1 integration into the genome (provided courtesy of John Kappes and Christina Ochsenbauer-Jambor, University of Alabama, Birmingham), was added to MEC in 200 μl of medium, and the mixture was incubated for 2 h, followed by addition of 2 ml of medium. HIV-exposed and -unexposed TZM-bl cells were used as controls, as were HIV-unexposed MEC. Cultures were examined after 48 h and again after 5 days under bright-field light microscopy and GFP-filtered microscopy, utilizing an inverted Olympus IX50 microscope system with a mercury bulb and a GFP filter, attached to an Olympus digital camera, and QCapturePro imaging software.

Electron microscopy. MEC were cultured on 6-well culture plates until reaching at least 80% confluence. Test wells were exposed to either a minimum of 200 μl cell-free HIV-1_{BaL} or HIV-1_{HC4} or a minimum of 8×10^6 PHA-activated PBMCs preinfected for 48 h with the same viral strains. Unexposed MEC and PBMCs were used as controls. MEC exposure to cell-free HIV-1 ranged from 0.5 h to 7 days, and MEC exposure to infected PBMCs ranged from 2 to 7 days. Cells were washed 3 times with PBS and fixed in 2% glutaraldehyde (GTA)-1% paraformaldehyde (PF) in 0.1 M Na-cacodylate buffer at RT. After 15 min, solutions were gently replaced with fresh fixative. After 1 h, a cell scraper was used to lift the cell monolayer, which was transferred to 15-ml tubes. The cells were gently centrifuged, and fresh fixative was added. After an additional hour of fixation, the samples were stored overnight at 4°C. Samples were washed three times for 15 min each in 0.1 M Na-cacodylate–0.15 M sucrose buffer, postfixed with 1% OsO_4 in 0.1 M Na-cacodylate–0.15 M sucrose buffer for 2 h at RT, and en bloc stained in 2% aqueous uranyl acetate. Cells were dehydrated through a graded series of ethanols and propylene oxide and embedded in Epon (LX112 kit; Ladd, Inc., Williston, VT). Thin sections were mounted on 400HH Cu grids (Electron Microscopy Sciences, Hatfield, PA), stained with methanolic uranyl acetate for 15 min and Reynold's lead citrate for 3 min. All images were taken at 100 kV on a JEOL 1010 transmission electron microscope equipped with an XR-41B AMT digital camera.

Coculture of $CD4^+$ T lymphocytes with HIV-1-exposed MEC. MEC were seeded into 48-well plates and cultured until reaching at least 80% confluence. HIV-1_{BaL} (300 $TCID_{50}$) was added to designated wells in 200 μl medium and incubated for 2 h at 37°C. Wells were washed three times, and supernatant was collected after the last wash (day 0) and stored at −70°C. Two million PHA-activated or nonactivated $CD4^+$ T lymphocytes were negatively selected from PBMCs by use of EasySep CD4 T Cell Enrichment immunomagnetic beads (Stem Cell Technologies, Vancouver, BC, Canada). The enriched $CD4^+$ T cells were added to each test well immediately after day 0 supernatant collection. Supernatants were collected and wells replenished with fresh medium at days 4, 7, 10, and 14. HIV-exposed PBMCs and -unexposed MEC and PBMCs were used as positive and negative controls, respectively. Samples were evaluated for HIV-1 p24 antigen by use of a commercially available ELISA (Perkin Elmer). Cell viability of PHA-activated and nonactivated $CD4^+$ T lymphocytes in culture was measured at the end of the experiment (day 30) by using CellTiter Aqueous One solution (Promega, Madison, WI). Experiments were performed in triplicate under each condition.

Activation of GFP-labeled Jurkat cells latently infected with HIV-1. MEC were seeded at 80,000 cells/0.4-μm transwell insert (BD, Franklin Lakes, NJ) and cultured until a tightly polarized monolayer was achieved, as determined by a transwell epithelial resistance (TER) of >1,000 Ω measured using an epithelial voltohmmeter (EVOM; World Precision Instruments, Sarasota, FL). After 48 h, apical- and basolateral-surface conditioned media were removed and placed in separate wells. J-Lat Tat-GFP clone 82 Jurkat cells (kindly contributed to the NIH AIDS Research and Reference Reagent Program by Eric Verdin), a Jurkat cell GFP reporter model for latent HIV infection that increases in fluorescence intensity if activated, were added to the cell-free MEC conditioned supernatants (1×10^6/well) and allowed to incubate overnight at 37°C. In parallel experiments, MEC were seeded in 48-well plates and cultured until reaching at least 80% confluence. J-Lat Tat-GFP clone 82 Jurkat cells (1×10^6/well) were added to evaluate the effect of direct contact with MEC on fluorescence intensity after overnight incubation at 37°C. J-Lat Tat-GFP cells cultured overnight in fresh medium alone served as negative controls, while those cultured in medium with 10 ng/ml human recombinant tumor necrosis factor alpha (TNF-α) (Sigma, St. Louis, MO) served as positive controls. Wild-type (WT) Jurkat cells were used to measure cellular autofluorescence. Cells were washed in PBS and fixed in 1% paraformaldehyde. GFP fluorescence was measured using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). A gate (R1) was drawn to include live cells; from the live-cell population, gate R2 was drawn to exclude autofluorescence exhibited by WT Jurkat cells (18). Data comparing mean fluorescence intensities among the test and control conditions were obtained utilizing information from gate R2.

Effects of MEC conditioned medium on PBMC proliferation and HIV-1 replication in infected PBMCs. MEC were cultured in MEGM on 0.4-μm transwell inserts (BD) placed in 24-well plates until a minimum TER of 1,000 Ω was reached. Conditioned medium from the apical compartment was collected and replaced with fresh medium every 48 to 72 h and stored at −20°C. PHA-activated PBMCs were infected with HIV-1_{BaL} (300 $TCID_{50}$/well) and cultured with MEC apical-surface conditioned medium for 7 days. HIV-infected PBMCs cultured in normal medium were used as controls. The cultures were tested for HIV-1 p24 antigen by ELISA (Perkin Elmer) and for cell viability/proliferation by use of CellTiter Aqueous One reagent (Promega) at day 7.

Measurement of cytokines and chemokines from apical surfaces of MEC. MEC were cultured in MEGM on 0.4-μm transwell inserts placed in 24-well plates until a minimum TER of 1,000 Ω was reached. Conditioned medium from the apical compartment was collected every 48 to 72 h, and results were assessed against a panel of 27 human cytokines, chemokines, and growth factors by utilizing a multiplex human Luminex assay (Bio-Rad). Because interleukin-8 (IL-8) concentrations were beyond the upper level of detection of the Luminex assay, conditioned medium was measured again using an IL-8 ELISA kit (Bio-Legend, San Diego, CA).

Statistical analysis. SPSS version 11.5 (Chicago, IL) was used to calculate statistical significance (two-tailed *P* value), with Student's *t* test for comparison.

RESULTS

Study subjects. MEC from adult subjects of <40 years of age consenting to breast reduction were utilized for this study. Eligible subjects had no history of mammary neoplasia, and clinical and microscopic examination of the tissue indicated no pathology associated with the donated tissue. No association between subject age and number of MEC enriched from mammary tissue was observed.

MEC isolation yields a highly pure population. In the nonlactating mammary gland, MEC are the minority cell type amid adipocytes and connective tissue (21). By use of methods adapted from established protocols (10, 13, 38), 2×10^6 to 10×10^6 MEC were reliably obtained from approximately 100 g of tissue, depending on the patient sample. In order to preserve live cells for use in subsequent experiments, we chose to use confocal microscopy rather than flow cytometry for confirmation of MEC purity. After optimization of the isolation protocol, enriched MEC were cultured on glass coverslips. After 7 to 10 days in culture (70 to 80% cell confluence), MEC were stained for expression of the epithelial cell marker BerEp4, as well as the leukocyte marker CD45. Differential interference contrast (DIC) was carried out using Zeiss LSM software to simultaneously view cells in grayscale in order to confirm the morphology of MEC. Visualization of the entire coverslip using the

10× objective, followed by that of a minimum of 20 fields per coverslip using the 40× objective, resulted in observation of only BerEp4$^+$ cells. Moreover, DIC visualization confirmed the morphology of enriched cells as epithelial in nature.

MEC express HIV-1 receptors and coreceptors in the cytoplasm and nucleus. To identify whether MEC might be susceptible to direct infection with HIV, we first characterized MEC for expression of HIV-1 receptors and coreceptors. Enriched MEC were grown on glass coverslips, and the cells were stained with antibodies specific for the HIV-1 receptors and coreceptors CD4, CCR5, and CXCR4 and the alternate receptor galactosyl ceramide (GalCer). Our results demonstrate that MEC consistently express CCR5, CXCR4, and GalCer (Fig. 1). *Z*-stack imaging using confocal microscopy confirmed expression of these receptor proteins throughout the cytoplasm, compared to staining only on the outer membrane of the control epithelial cell lines TZM-bl and HT-29. MEC from a minimum of three subjects stained negative for CD4; however, cells from one subject stained positive for CD4 expression, with *Z*-stack imaging confirming the presence of CD4 protein in the cell nucleus (Fig. 1G). Real-time PCR confirmed the presence of mRNA for CD4 in MEC from three subjects tested, suggesting that MEC are transcriptionally active for CD4 mRNA expression (data not shown) but that protein expression is restricted or absent. Taken together, these results indicate that primary human MEC express HIV-1 coreceptors, including CCR5, CXCR4, and GalCer, while expression of CD4 on the cell surface is not detected.

MEC are not directly susceptible to infection with CCR5- and CXCR4-tropic strains of HIV-1. An early study of primary MEC and immortalized mammary epithelial cell lines, using HIV-$1_{HTLV\text{-}IIIB}$ cultured in chronically infected H9 cells and HIV-1_{P1}, a highly cytopathic patient strain, cultured in C8166 cells, reported low-level productive infection of MEC (<100 pg/ml of HIV p24 antigen) (40). However, the use of laboratory-adapted and highly pathogenic strains of HIV-1 may not be representative of physiologically relevant conditions. We utilized CCR5-tropic HIV-1_{BaL}, a strain known to readily infect susceptible CD4$^+$ target cells, as well as two primary patient isolates, CXCR4-tropic HIV-1_{HC4} and dual-tropic HIV-$1_{C7/86}$. In addition to the assessment of HIV-1 p24 antigen, HIV-1 infection of MEC was evaluated by PCR and by using a green fluorescent protein (GFP)-labeled reporter virus.

Figure 2 shows a representative PCR result for HIV-1 reverse transcription and integration in MEC and PBMCs. While PBMCs support HIV-1 reverse transcription and viral integration with CCR5-, CXCR4-, and dual-tropic strains, there was no evidence of the presence of HIV-1 reverse transcription or integration in MEC with any of the HIV-1 isolates tested. In addition, while PBMC controls demonstrated significant productive HIV-1 infection with all three HIV-1 strains (>200 ng/ml p24 by day 14), MEC cultures yielded no detectable HIV-1 p24 antigen in culture supernatants under the same conditions (data not shown).

To further substantiate our findings, we tested a recently developed GFP reporter virus, NLENG1i_BaL.ecto (courtesy of John Kappes and Christina Ochsenbauer-Jambor, University of Alabama, Birmingham). While TZM-bl cells exposed to GFP–HIV-1_{BaL} were positive for GFP fluorescence as observed by microscopy at 48 h and 5 days after virus inoculation, no evidence of GFP fluorescence was observed in MEC from three subjects over the same time course (data not shown).

Taken together, our results provide evidence to indicate that MEC are not directly susceptible to infection with CCR5-, CXCR4-, and dual-tropic HIV-1 strains. Our findings do not preclude the possibility of MEC infection as a rare event but suggest that these cells are relatively refractory to HIV-1 entry, reverse transcription, and integration through normal fusion/entry pathways. Detection of CD4, CCR5, CXCR4, and GalCer expression by MEC raises the possibility that HIV-1 may bind surface receptors on MEC and may be internalized through alternate mechanisms.

Endocytosis of HIV-1 by MEC. Using electron microscopy (EM), we investigated the possibility that HIV-1 entry into MEC may occur through endocytosis. After MEC were cultured with HIV-infected PBMCs in 6-well plates, cell layers were fixed, removed, and prepared for EM. HIV-1 virions were observed in membrane-coated vesicles inside MEC, suggesting endocytic uptake of virions (Fig. 3). Interestingly, close contact of MEC with lymphocytes was also observed, both by formation of junctions and clefts (Fig. 4A and B) and by total engulfment of lymphocytes by MEC (Fig. 4C and D). In the latter case, both cell types were healthy and intact, indicating that lymphocytes were not being degraded by MEC. The formation of cell-to-cell junctions and total engulfment suggest that lymphocytes and MEC communicate with one another via specific cellular interactions.

MEC facilitate CD4$^+$ T-cell infection with HIV-1. The finding that MEC can endocytose HIV-1 virions led to further investigation of the ability of MEC to transfer virions to CD4$^+$ target cells. This process has previously been demonstrated in nonepithelial cells, such as dendritic cells (DC) (45). As milk is a cellular fluid, lymphocytes presumably come into close contact with MEC as they travel from the underlying basolateral tissue and circulate into the milk lumen. It is reasonable to assume that endocytic uptake of HIV-1 by MEC might lead to subsequent release of virions into the milk lumen or directly to uninfected CD4$^+$ cells in close proximity.

To evaluate this possibility, MEC were exposed to HIV, washed extensively to remove unbound virus, and cocultured with uninfected, PHA-activated CD4$^+$ T lymphocytes. CD4$^+$ T cells consistently became HIV infected after coculture with HIV-exposed and washed MEC (Fig. 5). Target cell infection occurred even after trypsin treatment of HIV-exposed and washed MEC (data not shown). These results indicate that HIV capture by MEC and transfer of virions to nearby CD4$^+$ target cells constitute a possible mechanism by which uninfected CD4$^+$ T lymphocytes traversing the mammary epithelium into breast milk may become HIV-1 infected.

MEC sustain but do not activate resting CD4$^+$ T cells. The persistence of HIV-1 proviral DNA in breast milk of women on HAART has led to speculation that a quiescent reservoir of HIV-infected cells may home to the breast milk (23, 36). It is not known whether MEC play a role in maintaining this reservoir of HIV-infected resting cells within breast tissue. In experiments carried out to evaluate this *in vitro*, HIV-exposed and washed MEC were cocultured with resting CD4$^+$ T lymphocytes. After 10 days, the cocultures did not yield detectable productive infection as measured by HIV p24 antigen in the

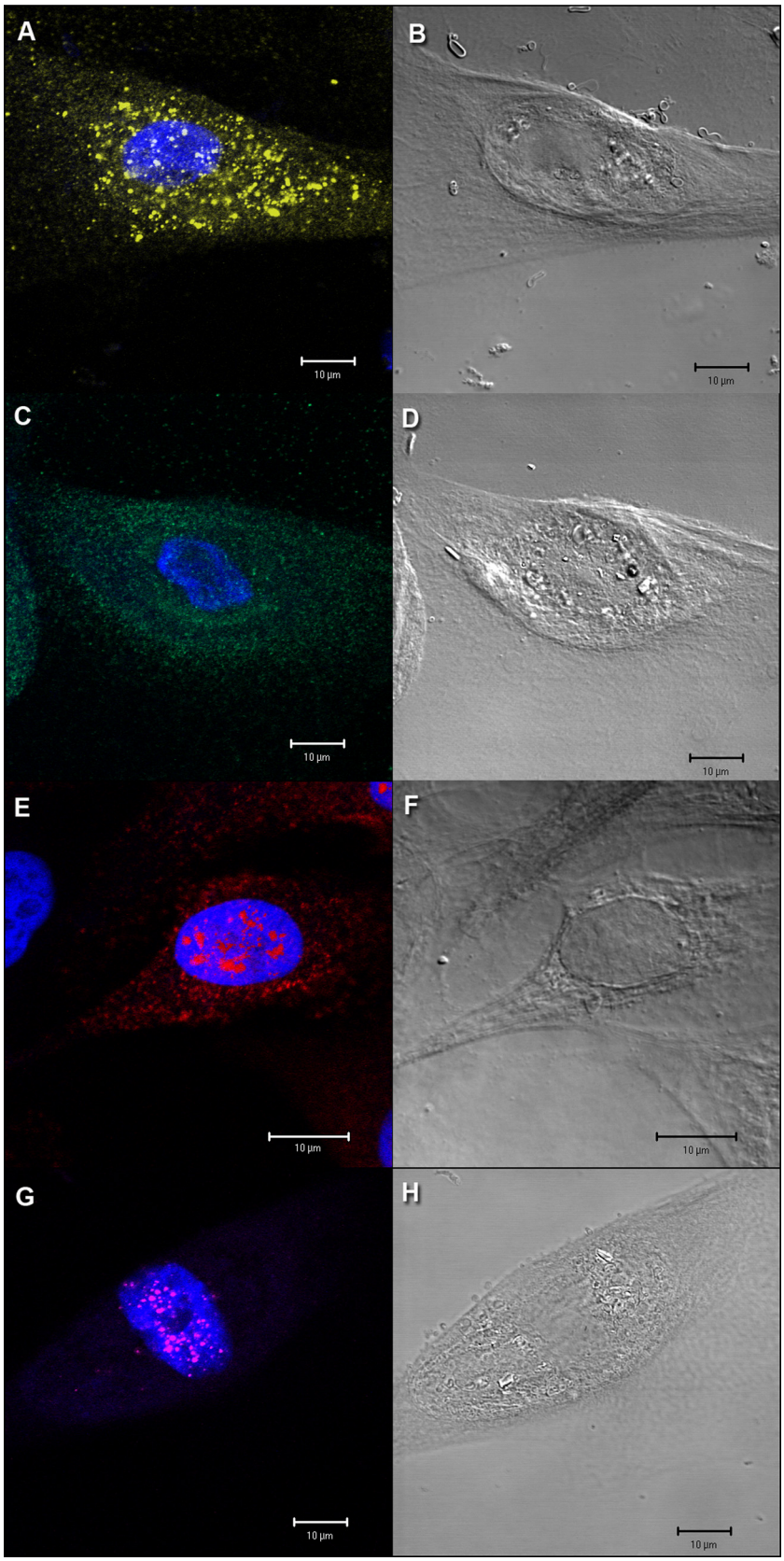

FIG. 1. Primary human MEC express HIV-1 receptors, coreceptors, and an alternate receptor. Confocal microscopy images of MEC stained for HIV receptors (left) and the same cells imaged by DIC (right). Nuclei are stained blue with DAPI. An immunomagnetic bead can be observed in the DIC image shown in panel D. (A) GalCer expression (yellow). (C) CCR5 expression (green). (E) CXCR4 expression (red). (G) CD4 expression (pink). *Z*-stack imaging confirmed the presence of receptors within the cytoplasm and/or nucleus as shown. Similar results were obtained in two additional subjects, with the exception of CD4 expression. CD4 mRNA was confirmed in three subjects by use of real-time PCR.

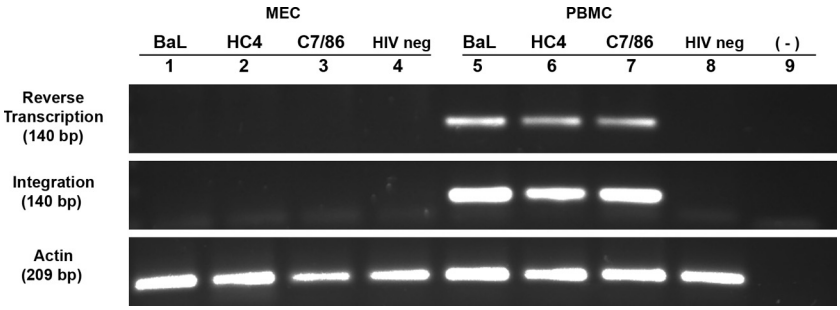

FIG. 2. Primary human MEC do not support HIV reverse transcription or integration of the CCR5-, CXCR4-, or dual-tropic strains tested. PCR results for HIV-1 reverse transcription and integration of CCR5-tropic HIV-1_{BaL}, CXCR4-tropic HIV-1_{HC4}, and dual-tropic HIV-$1_{C7/86}$ in both primary human MEC and PBMC controls. The bottom row shows PCR results for human beta-actin genomic DNA. Lanes 1 to 4, MEC; lanes 5 to 8, PBMCs; lane 9, negative control (no DNA). Experiments were performed in triplicate under each condition. Similar results were obtained with MEC from three additional subjects.

culture supernatants (Fig. 5), nor was productive infection detectable by p24 antigen ELISA after 4 weeks. After 30 days of coculture with MEC, the resting cells were removed, and their viability was assessed relative to that of resting $CD4^+$ T cells cultured alone under the same conditions. Interestingly, the viability of resting $CD4^+$ T cells cocultured with MEC (optical density at 490 nm [OD_{490}] of 0.446, 0.514, and 1.022 in independent experiments) was on average 1.65-fold (range, 1.51- to 1.86-fold) greater than that of resting $CD4^+$ T cells cultured alone for the same time period (OD_{490} of 0.292, 0.276, and 0.644). The medium control OD_{490} in each experiment was less than 0.175, suggesting that MEC may play a role in sustaining the resting T-cell population.

In further experiments, we tested whether MEC could activate T cells latently infected with HIV-1. J-Lat Tat-GFP clone 82 Jurkat cells (kindly contributed to the NIH AIDS Research and Reference Reagent Program by Eric Verdin). These cells were cocultured overnight directly with MEC and, in separate experiments, were exposed to the apical or basolateral secretions obtained from culturing MEC on transwell inserts. Our flow cytometry results demonstrate no effect on cell activation or GFP expression when cells latently infected with HIV were exposed directly to MEC or to MEC conditioned medium, while treatment of Jurkat-GFP cells with TNF-α resulted in high levels of GFP expression (data not shown).

Secretions from polarized MEC increase HIV replication and proliferation of HIV-infected target cells. After observing increased survival of resting $CD4^+$ T cells cocultured with MEC, we further examined whether MEC might also affect HIV replication in activated target cells. Culturing MEC on transwell inserts allows epithelial cells to form a tightly polarized monolayer, more closely mimicking MEC growth *in vivo*.

The results of these experiments demonstrate a statistically significant increase in viral replication in HIV-exposed PBMCs when cells were cultured in the presence of conditioned medium from the apical surface of polarized MEC compared to that for cells cultured in medium alone (Fig. 6A). Proliferation of PBMCs was also significantly greater when cells were cultured in the MEC conditioned medium (Fig. 6B). One interpretation of these findings is that increased viral replication is directly associated with increased target cell proliferation. These results indicate that MEC secretions from the apical side of the cells facilitate target cell viability and proliferation and HIV-1 replication.

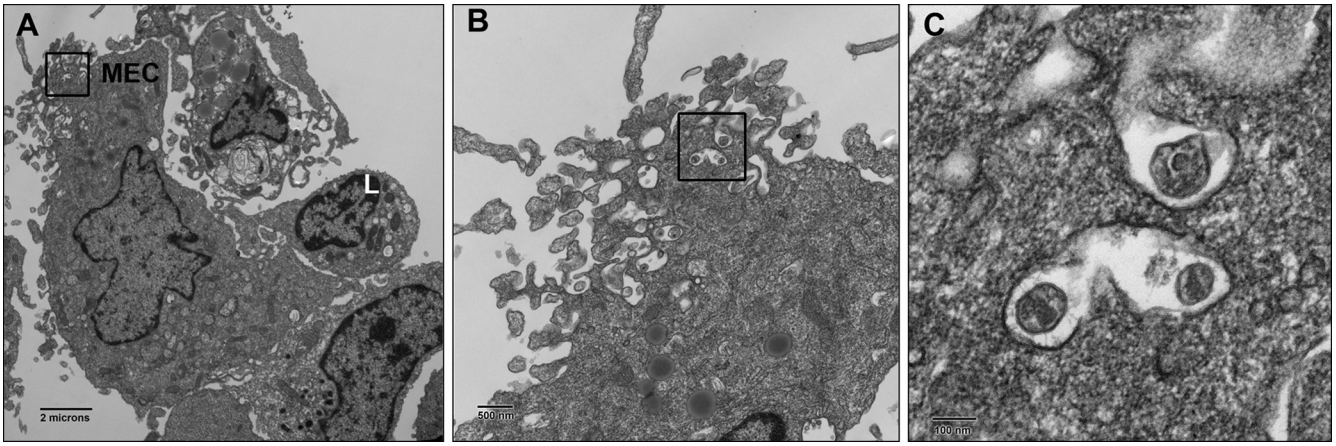

FIG. 3. MEC endocytose HIV-1 virions from infected PBMCs. Transmission electron microscopy images showing HIV-1 virions inside MEC endocytic compartments, with panels from left to right exhibiting progressive enlargement of the boxed area. The image shows HIV-1_{HC4} virions. Experiments were performed in duplicate with both CCR5-tropic HIV-1_{BaL} and CXCR4-tropic HIV-1_{HC4}. Similar experiments were repeated with MEC from two additional subjects. L, lymphocyte.

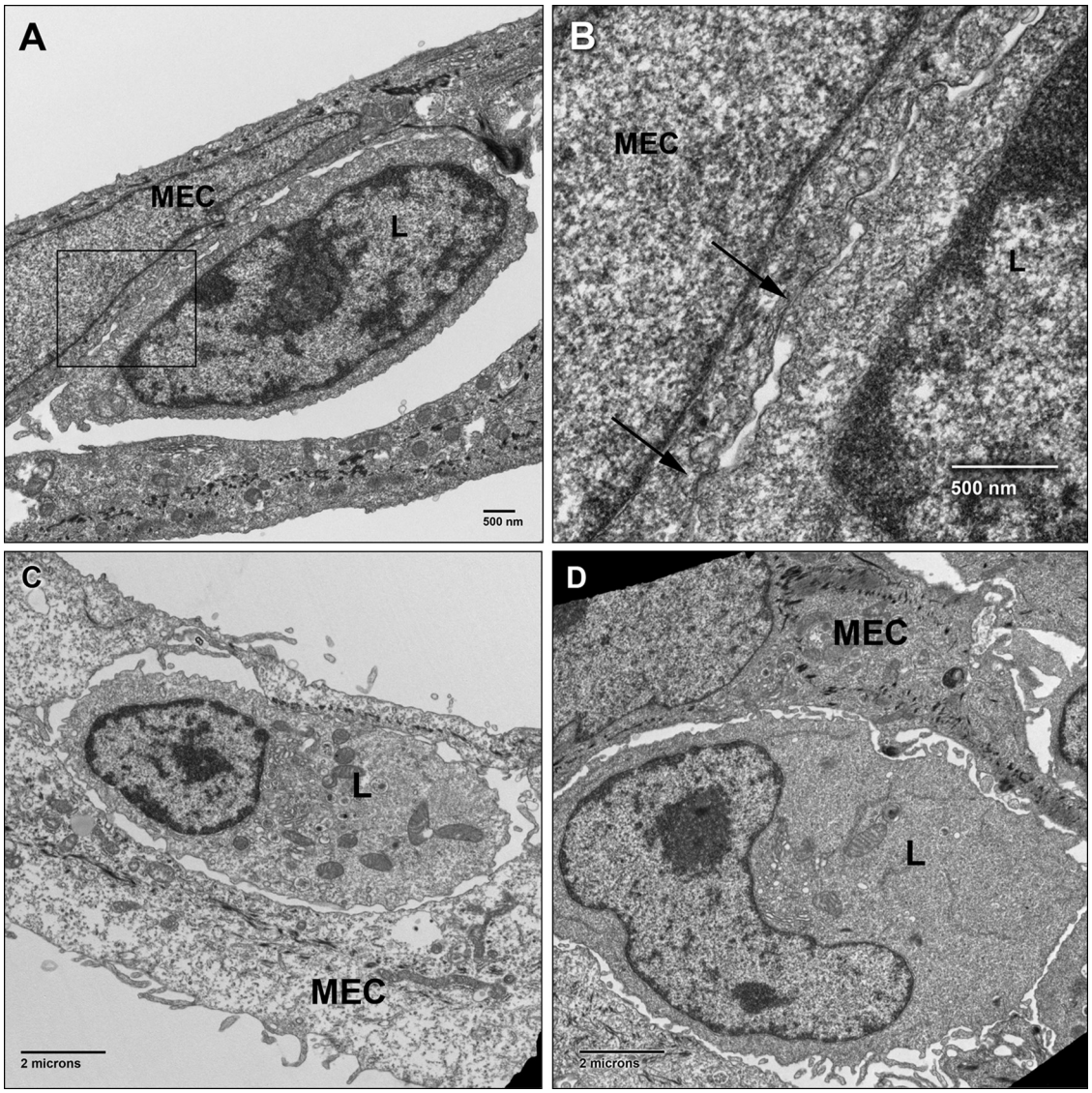

FIG. 4. MEC maintain close physical contact with $CD4^+$ T lymphocytes. (A) Transmission electron microscopy image showing junctions and clefts between MEC and lymphocytes (L). (B) Enlarged image of boxed area, showing junctions between cells (black arrows). Junctions between MEC and lymphocytes were observed in all samples from two individual subjects, with conditions tested in duplicate, irrespective of HIV-1 infection of lymphocytes. (C and D) Images of separate observations of lymphocyte engulfment by MEC from two individual subjects. Lymphocyte endocytosis was not dependent on HIV-1 infection of lymphocytes.

Analysis of Luminex and ELISA data from MEC secretions (Fig. 6C) (mean concentrations of >10 pg/ml) revealed that MEC are highly secretory cells that release a variety of immune mediators with widely ranging concentrations. Several of these factors, including IL-8 and IL-6, have been shown to upregulate HIV-1 replication *in vitro* (7, 12, 20) and may contribute to the observed increase in virus replication in PBMCs exposed to MEC secretions.

DISCUSSION

This is the first study to demonstrate endocytosis of HIV-1 virions by primary human MEC. In addition, HIV-exposed MEC facilitate infection of activated target cells, presumably by release of previously endocytosed HIV virions. Moreover, MEC appear to form intimate junctions with lymphocytes and in some cases completely engulf intact lymphocytes, suggesting close cell-to-cell communication between MEC and lymphocytes. Furthermore, MEC secretions from the apical surface significantly increase cell proliferation and HIV-1 replication in infected target cells.

In contrast to findings from an early study by Toniolo et al. (40), we did not find evidence to suggest that MEC are susceptible to direct infection by HIV-1, based on PCR evaluation of HIV-1 reverse transcription or integration in MEC, HIV p24 release, and the use of a sensitive GFP reporter virus. This discrepancy may be due in part to the techniques used to isolate pure populations of MEC and to the use of different isolates of HIV-1. In the present study, we adapted protocols

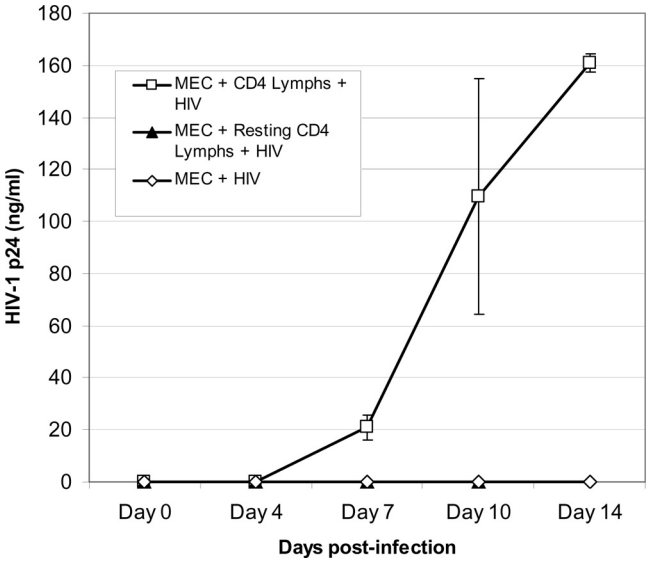

FIG. 5. Activated $CD4^+$ T lymphocytes are productively infected with HIV-1 after coculture with HIV-exposed MEC. Mean p24 antigen concentrations in culture supernatants up to 14 days after coculture of activated versus resting $CD4^+$ T lymphocytes with HIV-exposed and washed MEC. Each experiment was conducted in triplicate wells. Data represent the means ± standard deviations (SD) from three individual subjects.

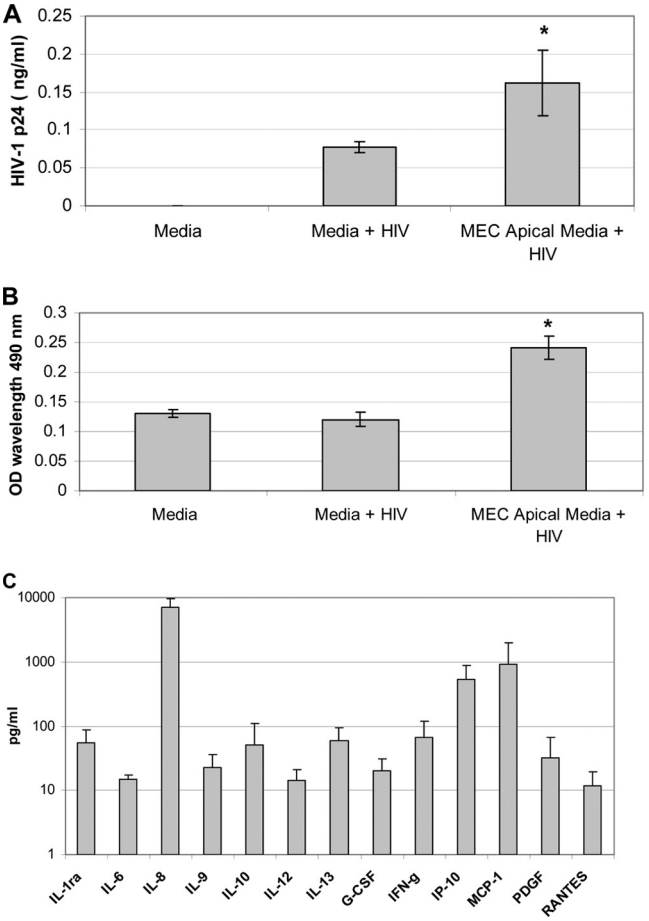

FIG. 6. MEC secretions from the apical surface increase HIV-1 replication and cellular proliferation in infected PBMCs. (A) HIV-1-infected PBMCs were cultured in MEC conditioned medium and compared to controls cultured in medium alone. Mean HIV p24 antigen concentrations on day 7 of the culture (*, $P < 0.02$). Each experiment was conducted in triplicate wells. Data represent mean values ± SD from two subjects. (B) Mean proliferation of HIV-1-infected PBMCs, measured by a 3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)-based assay (*, $P < 0.0001$). Data represent mean values ± SD from the same two subjects represented in panel A. (C) Cytokines, chemokines, and growth factors (>10 pg/ml) measured in MEC apical-surface conditioned medium. Data represent the mean values ± SD from four subjects. IL-1ra, IL-1 receptor antagonist; G-CSF, granulocyte colony-stimulating factor; IFN-g, gamma interferon; MCP-1, monocyte chemoattractant protein 1; PDGF, platelet-derived growth factor.

for MEC isolation to yield a highly purified population of cells that was free of contaminating fibroblasts and $CD45^+$ leukocytes. Direct infection of MEC was evaluated using primary HIV-1 isolates, including two patient isolates with tropism for either CCR5 or CXCR4. By comparison, the previous study of MEC relied on the use of a T-cell-adapted strain of HIV-1 ($HIV\text{-}1_{HTLV\text{-}IIIB}$) and a highly cytopathogenic isolate ($HIV\text{-}1_{P1}$).

However, our findings of HIV-1 receptors and coreceptors CD4, CXCR4, CCR5, and GalCer on MEC indicate that direct infection of MEC cannot be ruled out. A previous HIV-1 infectivity study using primary human MEC also reported CD4 mRNA transcripts by use of PCR, as well as "faint" expression of CD4 and GalCer on MEC membranes by use of a radiobinding assay (40). While expression of CCR5 and CXCR4 was not evaluated in the report by Toniolo et al., our findings are consistent with studies of murine mammary cells, which have been found to express CCR5 and CXCR4 (2). In addition, breast cancer studies have demonstrated the presence of CXCR4 in human mammary tissue by use of immunohistochemistry (31). Our findings also confirmed the widespread expression of the glycosphingolipid GalCer, suggesting that HIV-1 transcytosis across MEC via this receptor may be possible. Previous studies have demonstrated GalCer-dependent transcytosis of HIV-1 across human intestinal cell lines, initiated by GalCer binding to the ELDKWA epitope of the HIV-1 envelope glycoprotein gp41 (1).

Direct infection of MEC with HIV appears unnecessary for these cells to actively contribute to the transmission of HIV to target cells trafficking through the mammary epithelial layer on their way into the breast milk. Our EM results demonstrate the presence of intact HIV-1 virions within endosomal compartments in primary human MEC, suggesting that these cells are capable of active uptake and intracellular sequestration of virions. Presumably, virus harbored within MEC may be targeted for degradation; however, our coculture results suggest that infectious virus may also be released extracellularly as free virus or across cell-cell junctions toward proximal target cells. Vesicular transcytosis of HIV-1 across tightly polarized intestinal and endometrial epithelial cell layers has previously been demonstrated during investigations of HIV-1 passage through a mucosal layer (6, 17). In a nonepithelial cell model, dendritic cells (DC) have been shown to capture infectious HIV-1 virions and subsequently transmit them to T cells via exocytosis,

further supporting the concept of viral transfer to target cells without *de novo* infection (45).

Our observations by EM also suggest that transcytosis of lymphocytes across MEC may occur by direct migration of lymphocytes through the MEC. In the rat mammary model, lymphocytes have been observed to traverse through intraepithelial gaps between MEC into the ductal lumen (33). However, this phenomenon has not been observed directly in human tissue, despite observations of T lymphocytes in close proximity to the mammary epithelial layer (32).

To determine whether MEC can effectively capture and transfer HIV-1, we performed coculture experiments using MEC previously exposed to the virus. After extensive washes (and in some cases trypsinization) to remove unbound virus, MEC were cocultured with both activated and resting $CD4^+$ target cells. These conditions resulted in robust infection of the virus in activated $CD4^+$ cells, confirming that HIV-1 captured by MEC remains infectious and can initiate a productive infection in susceptible target cells. In the absence of evidence supporting direct infection of MEC by HIV-1, these data suggest that MEC may internalize the virus and subsequently release infectious virions.

Our study findings also suggest that while MEC do not activate or lead to productive infection of quiescent T cells, they may enhance their survival. One interpretation of these findings is that T cells latently infected with HIV homing to the mammary gland are not activated but rather are viably sustained by MEC. Unlike in the rodent mammary gland, leukocytes begin to accumulate in the lumen of human mammary ducts prior to parturition (at least by week 34 of gestation) (32). Since low levels of cell-to-cell transmission of HIV-1 occur among latently infected T cells in order to maintain an infected-cell reservoir (37), it is possible that cell-to-cell HIV-1 transmission among both activated and resting T cells could occur in the mammary gland during the formation of the cell-rich colostrum. Therefore, physiologically relevant opportunities for HIV infection of activated target cells and for accumulation of latently infected cells in the mammary gland may exist *in vivo*.

Results from this study indicate an important effect of MEC secretions on HIV-1 infection and replication in susceptible target cells. As mentioned previously, MEC secretions may also contribute to sustaining the viability of $CD4^+$ resting cells. In an effort to better understand the environment created by MEC in culture, we sought to characterize the soluble factors released by MEC. Analysis of the apical secretions from the polarized cells by multiplex assays revealed the presence of a complex array of cytokines, chemokines, and growth factors. Breast milk is known to contain many chemokines and cytokines (24, 41), some of which may influence HIV-1 infection and transmission (7, 12, 20). Epidemiologic studies have sought to find correlations between individual breast milk components and transmission of HIV-1 through breast-feeding or between HIV-infected and noninfected women (8, 35, 42, 44). However, a clear relationship has not been established, making it likely that multiple components interact to affect cell activation and virus replication within the breast milk milieu.

In summary, findings from this study demonstrate that MEC play an active role in uptake of HIV-1 and can facilitate $CD4^+$ target cell infection, HIV replication, cell proliferation, and survival of resting $CD4^+$ T cells. These results highlight the complexity and importance of MEC and their potential contribution to sustaining cell-free and cell-associated HIV-1 in breast milk. Epidemiologic studies of breast milk from HIV-infected, lactating women on HAART have demonstrated little or no impact on cell-associated HIV proviral DNA, compared to a rapid reduction in cell-free HIV RNA (23, 36). Our findings support the concept proposed by these and other studies which suggests that mammary tissue may represent a distinct site of virus replication that is refractory to antiretroviral (ARV) intervention. Further studies are needed to determine whether mammary tissue harbors HIV-1 *in vivo* and whether this site forms a reservoir for virus replication and persistence within the host.

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