JOBNAME: joa 38#1 2005 PAGE: 1 OUTPUT: Thu December 2 16:31:39 2004
lww/joa/91064/V8756R1

Replication of M-tropic HIV-1 in Activated Human Intestinal Lamina Propria Lymphocytes Is the Main Reason for Increased Virus Load in the Intestinal Mucosa

Sheriff Aziz, PhD, Oliver T. Fackler, PhD,‡ Andreas Meyerhans, PhD,†*
Nikolaus Müller-Lantzsch, PhD,† Martin Zeitz, MD,§ and Thomas Schneider, MD, PhD§

Summary: The gastrointestinal tract is the site of early abundant HIV replication and associated marked $CD4^+$ T-cell depletion. The aim of this study was to characterize the basis for the increased HIV replication in this compartment. Isolated mononuclear cells of the peripheral blood (PBMCs), the intestinal lamina propria (LPMCs), and purified gut lamina propria $CD4^+$ T-cell subpopulations (LP T cells) were isolated, phenotypically characterized, and infected in vitro with 2 different HIV-1 strains. T-cell subpopulations were analyzed by fluorescence-activated cell sorter. HIV-1 core protein p24 was determined in supernatants after in vitro infection. Furthermore the effect of T-cell stimulation on the replication of M- and T-tropic HIV strains was studied. In vitro replication of HIV-1 was significantly increased in $CD69^{high}$ compared with $CD69^{low}$ $CD4^+$ LP T cells, while there was no difference between $CD103^-$ and $CD103^+$ $CD4^+$ LP T cells. Experimental stimulation of LPMCs, which mimics activation by intestinal pathogens frequently present in the bowel of HIV-infected patients, further dramatically enhances HIV replication (24.5-fold) compared with nonstimulated LPMCs. M-tropic HIV-1 showed a preferential replication in LPMCs, while T-tropic HIV-1 strain showed a preferential replication in PBMCs. Thus, the elevated activation state of target cells in the intestine and not the expression of the homing marker CD103 is directly linked to massive HIV production.

Key Words: HIV replication, intestinal lymphocytes, CD103, CD69, T-cell activation, M-tropic HIV-1 strain

(*J Acquir Immune Defic Syndr* 2005;38:23–30)

Received for publication April 22, 2004; accepted July 12, 2004.
From *Internal Medicine II and †Institute of Virology, University Saarland, Homburg/Saar, and ‡Institute of Hygiene, Department of Virology, University of Heidelberg; and §Internal Medicine I, Department of Gastroenterology and Infectious Diseases, Charite-Campus Benjamin Franklin, Free University, Berlin, Germany.
Supported by DFG Grant Schn616.
Reprints: Thomas Schneider, Internal Medicine I, Charite-Campus Benjamin Franklin, Department of Gastroenterology and Infectious Diseases, Hindenburgdamm 30, 12200 Berlin, Germany (e-mail: thomas.Schneider@charite.de).

Early immunologic and virologic events in lymph nodes and other lymphoid organs may influence the clinical outcome of HIV infection.[1,2] Studies in the simian immunodeficiency virus (SIV) model identified a crucial role for the gastrointestinal tract during this latent phase of infection.[3–6] This important role of the intestine may reflect that, in contrast to peripheral blood mononuclear cells (PBMCs), gastrointestinal lamina propria mononuclear cells (LPMCs) are permissive for HIV infection without prior stimulation,[7] leading to fast $CD4^+$ T-cell depletion accompanied by increased virus replication in the gastrointestinal mucosa.[4,5] Reflecting the differences in permissivity of target cells in these compartments, no correlation was found in HIV-infected humans between changes in T-cell subpopulations in the peripheral blood and the intestinal mucosa.[8] Furthermore, CD4 T-cell depletion is more pronounced in the intestinal tract compared with the peripheral blood.[8–10] In rectal biopsies of HIV-infected patients, a 200–1000 times higher p24 concentration was measured in comparison to autologous blood.[11] In a further study, we found that the increased viral replication in the gastrointestinal tract is due to increased transcription or translation and not to a higher number of infected cells.[12] Recent studies show that lymphocytes rather than macrophages are the primary target of HIV in the gastrointestinal tract.[13]

Thus, the gastrointestinal tract has been recognized as a major site of HIV replication and T-cell depletion.[14] However, the reasons for these phenomena remain mostly unclear but likely reflect intrinsic properties of the respective target cells in that compartment.[15] Indeed, LPMCs can be distinguished from PBMCs, as almost all LPMCs express the memory marker CD45RO[16] and show an increased expression of activation markers like CD25[17] or CD69.[18] Additionally approximately 15% of $CD4^+$ lymphocytes of the intestinal mucosa express the human mucosal lymphocyte receptor (HML-1, CD103), which is usually absent on lymphocytes of the peripheral blood (PBLs).[19] CD103 is a $\alpha_E\beta_7$ integrin that is expressed on mucosal lymphocytes in the process of homing into the intestinal lamina propria recirculating from lymph nodes after being primed in the intestinal mucosa.[20]

HIV replication is tightly coupled to the activation state of its target cell and preferentially multiplies in memory CD4 T cells.[21,22] It has recently become clear that changes in the activation state can significantly alter the ability of T cells to

propagate HIV.[23] Thus HIV/SIV may better replicate in LPMCs due to their increased activation status, thereby increasing virus burden and causing accelerated $CD4^+$ T-cell depletion in the intestinal mucosa. Recently it was reported that M-tropic in contrast to T-tropic HIV strains are effectively transported through the epithelial layer of the mucosa.[24] The higher cytopathogenicity of CCR5 using HIV strains[25,26] in the setting of immune activation and the observed higher replication rates of M-tropic primary isolates from AIDS progressors in comparison to isolates from long-term-survivors[27] results from the early and complete loss of intestinal CD4 T cells in the intestinal mucosa observed in HIV-infected humans.

The major aim of this study was to identify mucosal T-cell subpopulations that are the main producers of HIV in the intestinal tract. Furthermore, we analyzed the effect of stimulation on HIV replication in $CD4^+$ LPMCs, as the altered mucosal barrier and frequent intestinal infections with a diverse number of pathogens that are observed in HIV-infected patients may further activate mucosal T cells.[11,28,29] We also studied whether there is a difference between M- and T-tropic HIV-1 replication in LPMCs compared with autologous PMBCs.

METHODS

Isolation of LPMCs

Human colonic specimens were provided by the Department of Surgery (University of Saarland, Germany) in agreement with the local ethics commission. Mucosa specimens were taken from 29 patients admitted for hemicolectomy. The mucosa used for cell isolation was macroscopically normal and was dissected with a distance of at least 5 cm from malignant tissue or otherwise suspect areas. All patients tested negative for HIV-1. LPMCs were isolated by the previously described d,1-Dithiothreitol-EDTA-collagenase method.[30] Viability of the cells was determined by trypan blue dye exclusion and was >90%.

Isolation of PBMCs

Autologous PBMCs were isolated from venous heparinized blood obtained from patients 1 day after surgery. Mononuclear cells were separated from other cellular elements by layering on a Ficoll density gradient. PBMCs were counted by trypan blue dye exclusion. Viability was >97%.

Immunomagnetic Depletion of $CD8^+$ LPMCs and PBMCs

$CD8^+$ cells were depleted shortly before in vitro infection, before cell sorting or after stimulation using Dynabeads M-450 CD8 (Dynal A.S., Oslo, Norway) according to the manufacturers' protocol. The efficiency of $CD8^+$ mononuclear cell depletion was >98% as assessed by flow cytometric analysis.

Phenotypic Characterization by Flow Cytometry

Triple color staining of cells was performed using fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, and phycoerythrin-cy5 (PECy5)-conjugated, mouse antihuman monoclonal antibodies to the surface antigens CD4 (clone MT310, Coulter Immunotech, Marseille, France), CD8 (clone DK25, Dako, Hamburg, Germany), CD25 (clone ACT-1, Dako), CD45RO (clone UCHL1, Dako), CD3 (UCHT 1, Coulter Immunotech), CD69 (clone TP 1.55.3, Coulter Immunotech), CD103 (clone 2G5, Coulter Immunotech), CXCR4 (clone 12G5, R&D Systems, Wiesbaden, Germany), and CCR5 (clone 45531.1, R&D Systems). Staining was performed as previously described.[5]

Stimulation of PBMCs and LPMCs

An unusual characteristic of LPMCs is their low proliferative response to mitogens and to stimuli of the CD3 pathway, both of which represent potent stimuli for PBMCs.[31] However, stimulation of LPMCs via the accessory signaling pathway by ligation of the CD2 receptor with anti-CD2 produces proliferative responses that are similar or slightly reduced compared with similar stimulation of autologous PBMCs.[32,33] Therefore, for PBMC anti-CD3 plus anti-CD28 and for LPMCs anti-CD2 plus anti-CD28 stimulation, protocols were chosen to achieve a comparable state of proliferation and cytokine responsiveness.[34]

PBMCs were stimulated with anti-CD3 (Immunotech, Marseille, France) and anti-CD28 antibodies (Ancell, Bayport, MN). LPMCs were stimulated as previously described with 2 anti-CD2 antibodies and anti-CD28,[34] respectively. The anti-CD3 and anti-CD28 antibodies were used at a concentration of 1μg/mL. The anti-CD2 antibodies M1 and M2, a kind gift by Prof. Dr. Meuer, were used at a concentration of 0.1 μg/mL. The anti-CD3 antibody was immobilized on the plate at least 3 hours before incubation with the cell suspension. Anti-CD28 was given into 24-well plates (Greiner Labortechnik, Frickenhausen, Germany) shortly before the cell suspension was added. The antibody-cell suspension was mixed thoroughly before incubation. Cells were cultured for 48 hours in a humidifier at 37°C and 5% CO_2.

Isolation of $CD69^{high}$ and $CD69^{low}$ $CD4^+$ LP T Cells, Respectively

$CD69^{high}$ and $CD69^{low}$ LP T cells were isolated using a fluorescence-activated cell sorter (FACS-Vantage; Becton Dickinson, Mountain View, CA). After $CD8^+$ T-cell depletion, cells were stained with anti-CD69 PE, anti-CD8 FITC, and anti-CD3 PECy5 antibodies. Mononuclear cells were electronically gated by forwards/sidewards scatter (FSC/SSC), and T cells were identified by the expression of CD3. Only $CD3^+$ T lymphocytes negative for CD8 were considered $CD4^+$ T lymphocytes. $CD69^{high}$ and $CD69^{low}$ LP T cells were separated by setting sort gates around the desired cell populations. Approximately 1 log phase of CD69 PE fluorescence intensity was chosen between $CD69^{high}$ and $CD69^{low}$ LP T cells. Separated cells were taken into culture overnight to allow detachment of the anti-CD3 and anti-CD69 antibodies. A small proportion of isolated cells were again checked for CD69 and CD4 expression. Cells were held in complete medium until in vitro infection or FACS analysis.

Isolation of $CD103^+$ and $CD103^-$ $CD4^+$ LP T Cells, Respectively

After $CD8^+$ T-cell depletion, $CD4^+$ $CD103^+$ LPLs were isolated using the Dynal CELLection Pan Mouse IgG Kit

(Dynal Biotech Inc, North Deerbrook, WI) with a mouse antihuman anti-CD103 FITC conjugated antibody according to the manufacturer's protocol. $CD4^+$ $CD103^-$ LP T cells, which remained in the supernatant after selection of $CD4^+$ $CD103^+$ LP T cells, were also collected for in vitro infection studies. LP T cells were separated at a purity of at least 96%, $CD4^+$ $CD103^-$ at a purity of at least 93% as determined by FACS analysis.

In Vitro Infection

Cells were cultivated in 1640 RPMI medium supplemented with 10% fetal calf serum (FCS) and antibiotics at a concentration of 1×10^6 cells/mL for variable time lengths in 96-well round-bottom plates (Cellstar, Greiner bio-one, Frickenhausen, Germany) in a humidified incubator at 37°C and 5% CO_2. Cells were infected with T-cell tropic HIV Lai-2, kindly provided by Dr. K. Peden (National Institutes of Health, Bethesda, MD)[35] or the monocytotropic primary virus isolate HIV BaL.[36] For infection, cells were incubated with virus inoculum of 0.001 MOI (multiplicity of infection, as determined by X4/R5 positive Hela-CD4 indicator cells) per 1×10^6 cells/mL overnight, washed 3 times, and recultured for various time lengths. Viral replication was evaluated using a commercially available enzyme-linked immunosorbent assay (ELISA) against the HIV-1 core protein p24 (Abbott Murex, Wiesbaden, Germany). Amounts were determined by the principles of a capture ELISA according to the manufacturers' protocol. Uninfected cells cultured under the same conditions served as negative controls. To exclude the possibility of false-positive p24 ELISA results due to passively released virus particles into the culture supernatants, we infected autologous cells supplemented with 0.1 μg/mL zidovudine at time of infection and after the washing procedure. Supernatants were taken at the described time points for HIV p24 antigen determination and stored at −80°C until quantification.

Quantification of Infectious Particles in HIV-1 Stocks

To determine and normalize the amount of infectious particles in the virus stocks used for in vitro infection studies, we used a CD4, CXCR4, and CCR5 expressing HeLa cells (Magi-CXCR4, CCR5 entry assay). Determination was performed in triplicate in 3 experiments.

Nested PCR for Detection of Proviral DNA

Infection was determined by nested polymerase chain reaction (PCR) as previously described[12] using HIV-1gp120 V1V2-specific primers LV 13: (CTT TAG AAT CGC AAA ACC AGC CG), LV 15: (GCC ACA CAT GCC TGT GTA CCC ACA) and 2 internal primers SK 123: (TAA TGT ATG GGA ATT GGC TCA A) and SK 122: (CAA GCC TAA AGC CAT GTG TA).[24,37] To normalize the amount of cells examined for proviral DNA PCR analysis, we determined GAPDH as a housekeeping gene using following MM primers TGG TAT CGT GGA AGG ACT CAT GAC, AGT CCA GTG AGC TTC CCG TTC AGC with the PCR program previously described.[12]

Statistical Analysis

Quantitative data were presented as medians and quartiles if not otherwise indicated. Significance was determined with GraphPad PRISM software (San Diego, CA) using a 2-tailed Mann-Whitney test with a 95% CI, and P values < 0.05 were considered significant.

RESULTS

Identification of 2 Distinct CD69-Expressing $CD4^+$ LP T Cells

Virtually all $CD4^+$ LP T cells expressed the early activation marker CD69. However, 2 CD69 subpopulations could be clearly distinguished. The majority showed high expression of the CD69 marker (median 95.9%, range 93.9–97%). A small proportion of $CD4^+$ LP T cells shows low CD69 expression (median 3.6%, range 2.1–6.1%) (Fig. 1A). In the following, these subpopulations will be designated $CD69^{high}$ and $CD69^{low}$ LP T cells, respectively. $CD69^{high}$ LP T cells were sorted to a purity of at least 97.7% (Fig. 1C). $CD69^{low}$ LP T cells were >92.8% free of $CD69^{high}$ LP T cells (Fig. 1B).

$CD69^{high}$ and $CD69^{low}$ LP T Cells Differ Also in the Expression of Other Activation Markers, the Expression of the Memory Marker, and the Expression of HIV Coreceptors

To further characterize the phenotype of the separated $CD69^{high}$ and $CD69^{low}$ LP T cells, we determined the expression levels of CD25, CD45RO, CD4, CXCR4, and CCR5 on these subpopulations as fluorescence intensity. The fluorescence intensity of CD4 was similar in both subpopulations (Table 1). However, the fluorescence intensity of CD25, CD45RO, CXCR4, and CCR5 was higher in the $CD4^+CD69^{high}$ population (Table 1).

HIV-1 Replication Is Higher in $CD69^{high}$ Compared With $CD69^{low}$ $CD4^+$ LP T Cells

The presence of 2 intestinal T-cell subpopulations differing in their activation state allowed us to test whether increased HIV replication in the intestine correlates with the activation level of the resident target cells. Thus both subpopulations were separately infected with the M-tropic HIV-1 strain BaL. Significantly higher amounts of p24 were found in the supernatants of $CD69^{high}$ as compared with $CD69^{low}$ $CD4^+$ LP T cells infected with the same HIV-1 strain beginning at postinfection day 5 ($CD69^{high}$: 3710, 3370–3980 p24 pg/mL vs. $CD69^{low}$: 590, 113–880 p24 pg/mL, $P < 0.0023$). The difference in virus production from the 2 subpopulations of LP T cells was also seen at postinfection days 7 and 9 ($CD69^{high}$ at postinfection day 7: 3235 pg/mL, 3060–3980 pg/mL vs. $CD69^{low}$ 780, 0–1300 pg p24/mL, p 0.0023; $CD69^{high}$ at postinfection day 9: 3520 pg/mL, 3150–3710 pg/mL vs. $CD69^{low}$ 790, 139–1487 pg p24/mL, $P < 0.0023$) (Fig. 2A). PCR analysis of proviral DNA suggests that $CD69^{high}$ and $CD69^{low}$ LPLs are both permissive to HIV-1 BaL infection

JOBNAME: joa 38#1 2005 PAGE: 4 OUTPUT: Thu December 2 16:31:42 2004
lww/joa/91064/V8756R1

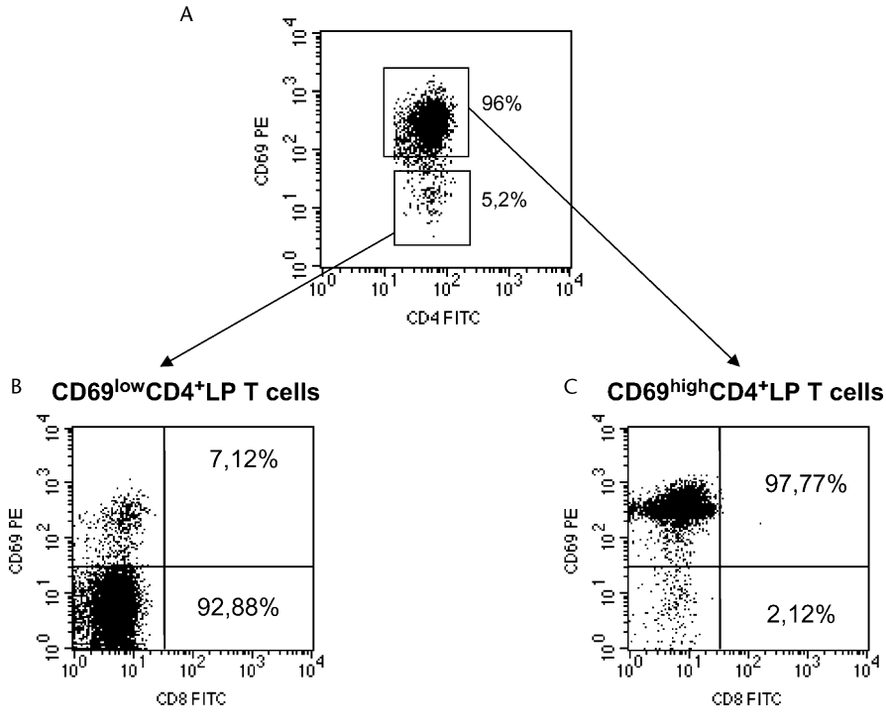

FIGURE 1. Flow cytometric analysis of CD69 expression on $CD4^+$ LP T cells. Cells were stained with anti-CD4 FITC, anti-CD3 PECy5, and anti-CD69 PE. A, Two populations can be discerned by CD69 expression levels in the following, termed as $CD69^{high}$ and $CD69^{low}$ (3.5%) $CD4^+$ LP T cells. For separation cells were stained with anti-CD8 FITC, anti-CD-3 PECy5, and CD69 PE. The 2 subpopulations were separated by FACS sorting. All cells were gated by FSC/SSC lymphocyte gate as well as CD3 positivity. *Dot blots* shown are representative for 6 experiments. B, Shows the separated $CD69^{low}$ $CD4^+$ LP T cells (purity: 92.88%). C, Shows the separated $CD69^{high}$ $CD4^+$ LP T cells (purity: 97.77%).

despite the observed differences in coreceptor surface expression levels (Fig. 2B).

HIV Replication in $CD103^+$ and $CD103^-$ LP T Cells Is Not Different

The amounts of viral p24 determined in the supernatants of in vitro infected $CD103^+$ LP T cells and $CD103^-$ LP T cells in 7 independent experiments showed no significant differences at any time point after infection (Table 2).

Stimulation Results in an Increase of Activation Markers in Both LPMCs and PBMCs, But There Is a Distinct Effect of Stimulation on the Expression of HIV Coreceptor CCR5 in LPMCs and PBMCs

The effect of T-cell stimulation on the phenotype of LPMCs and PBMCs was analyzed by determining the mean fluorescence intensity of the markers CD4, CD25, CD69, CXCR4, and CCR5 before and after stimulation by FACS analysis (Table 3).

The CD4 receptor fluorescence intensity increased after stimulation in PBMCs. A similar phenomenon was observed in LPMCs. As expected, stimulation of PBMCs led to an increase in fluorescence intensity of activation markers CD25 and CD69. Stimulation of LPMCs also resulted in a further increase of CD25. A similar effect was seen for CD69 expression, where the distinct subpopulation of $CD69^{low}$ LPMCs disappeared after stimulation, resulting in a uniformly $CD69^{high}$ LPMC population. In PBMCs, the coreceptor for T-tropic HIV-1 strains increased after stimulation. However, the expression of the coreceptor for M-tropic HIV-strains CCR5 remained low even after stimulation.

In contrast to PBMCs, LPMCs show no changes of the T-tropic coreceptor CXCR4 after stimulation. Instead LPMCs increased the expression of the M-tropic coreceptor CCR5 ($P < 0.008$; Table 3).

TABLE 1. Phenotypic Characterization of $CD69^{high}$ and $CD69^{low}$ $CD4^+$ LP T Cells

Marker	$CD69^{high}$ $CD4^+$ LP T Cells [Fluorescence Intensity] Median (Range)	$CD69^{low}$ $CD4^+$ LP T Cells [Fluorescence Intensity] Median (Range)	*P*
CD4	59 (56–61)	60 (57–63)	NS
CD25	231 (220–239)	25 (23–28)	<0.008
CD45RO	1023 (803–1202)	18 (16–20)	<0.008
CXCR4	888 (789–1083)	18 (16–20)	<0.008
CCR5	170 (154–189)	20 (17–22)	<0.008

Five individual measurements.

Inverse Replication of M-tropic HIV-1 Strain BaL and T-Tropic HIV-1 Strain Lai in Stimulated LPMCs and PBMCs

To compare the effect of stimulation on viral replication in LPMCs and PBMCs, we infected both cell types after stimulation for 48 hours. Maximal p24 values were reached 7 days postinfection in PBMCs infected with the T-tropic strain Lai (145.3 ng/mL, 142.8–177.6 ng/mL) and in LPMCs infected with the M-tropic strain BaL (152.9 ng/mL, 74–158.8 ng/mL) (Fig. 3A and B).

T-tropic HIV-1 strain Lai produced higher amounts of p24 in the supernatants of stimulated PBMCs 7 days postinfection (145.3 ng/mL, 142.8–177.6 ng/mL) compared with

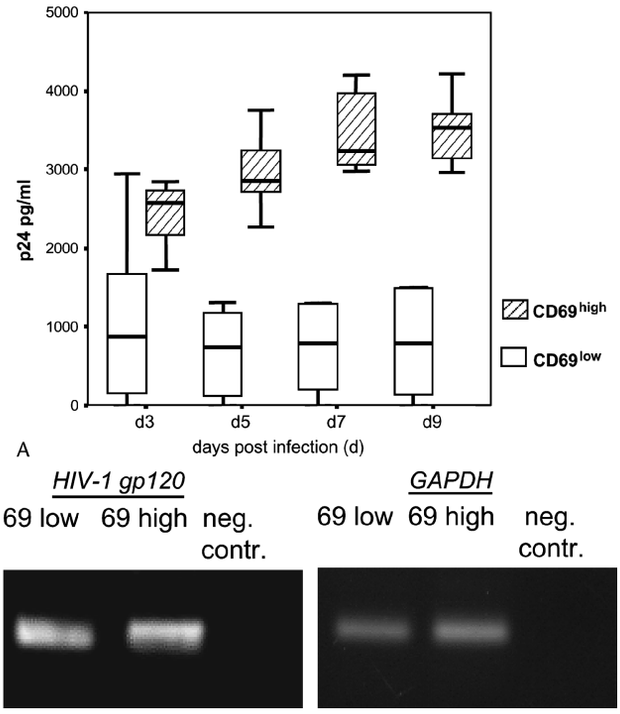

FIGURE 2. A, In vitro HIV-1 BaL replication of CD69^{high} and CD69^{low} CD4^{+} LP T cells. HIV replication was measured by HIV-1 core protein p24 antigen ELISA in the supernatants of cultured cells. There is a higher replication of HIV-1 BaL in CD4^{+} CD69^{high} (*hatched bars*) compared with CD69^{low} CD4^{+} LP T cells (*open bars*) as soon as 5 days postinfection, further increasing 7 days postinfection. Data shown represent 6 experiments. Results are presented as 25th and 75th percentiles (*upper and lower mark*), quartiles (*boxes*), and median (*bold line*). B, PCR analysis of HIV-1 proviral DNA in CD4^{+} CD69^{high} and CD4^{+} CD69^{low} LPLs. As a negative control (neg. contr.) only buffer solution was used. To normalize the number of cells in both LPL subpopulations, GAPDH PCR analysis was performed. The results show a signal in both subpopulations indicating that both CD69^{high} and CD69^{low} CD4^{+} LP T cells are permissive to HIV-1 Infection. The results shown are representative for 3 experiments.

PBMCs infected with M-tropic HIV-1 strain BaL (32.6 ng/mL, 15.2–40.14 ng/mL; $P < 0.0016$) (Fig. 3A).

In contrast, M-tropic HIV-1 strain BaL showed significantly higher replication in stimulated LPMCs as compared with T-tropic strain Lai 7 days postinfection (152.9 ng/mL, 74–158.8 ng/mL vs. 7.5 ng/mL, 6–13 ng/mL; $P < 0.008$; Fig. 3B). Thus PBMCs and LPMCs show comparable virus turnover when stimulated, showing preference for the T-tropic strain BaL in PBMCs and the M-tropic strain Lai in LPMCs.

Nonstimulated LPMCs and autologous PBMCs infected under similar conditions served as controls. As expected, no viral replication was detected in nonstimulated PBMCs after infection with either of the HIV-1 strains. Analogous to stimulated LPMCs, infection of primary, nonstimulated LPMCs showed higher replication of M-tropic HIV-1 BaL in comparison to T-cell tropic HIV-1 Lai 7 days postinfection (6.2 ng/mL p24, 5.5–7.1 ng/mL p24 HIV-1 BaL vs. 1.4 ng/mL p24, 1–1.77 ng/mL p24 HIV-1 Lai; data not shown). Stimulation of LPMCs resulted in a 24.5 higher p24 production of HIV-1 BaL as compared with nonstimulated LPMCs at 7 days postinfection (152.9 ng/mL, 74–158.8 ng/mL, vs. median: 6.2 ng/mL, 5.5–7.1 ng/mL). In contrast, stimulation of LPMCs led only to a 3.75-fold increase in p24 production after infection with Lai as compared with nonstimulated LPMCs (p24 values in stimulated LPMCs 7.5 ng/mL, 6–13 ng/mL, vs. nonstimulated LPMCs 1.4 ng/mL, 1–1.77 ng/mL p24).

DISCUSSION

The gastrointestinal tract is a major site of lentiviral replication and destruction of the immune system.[5,10] Previous studies have shown that in contrast to PBMCs, LPMCs are permissive for HIV infection without prior stimulation.[7] However, the reasons for these differences between peripheral and intestinal mucosal T cells concerning productive HIV infection are not known. In the search for determinants leading to the particular HIV replication in this compartment, this study suggests the elevated activation state of intestinal T cells as the major prerequisite for preferential HIV replication in these cells. We demonstrate the presence of 2 distinct populations of CD69^{+} CD4^{+} T cells in the intestine. While the majority of CD4^{+} LP T cells express CD69 at high intensity, a minority of CD4^{+} LPL T cells shows low levels of CD69 expression. These 2 subpopulations also clearly differ in the fluorescence intensity of CXCR4, CCR5, CD45RO, and CD25 as an indication for expression of these markers per cell. Our subsequent in vitro infection studies show that both subpopulations are permissive of HIV-1 infection. However, HIV replication was much higher in the nonstimulated CD4^{+} CD69^{high} LP T-cell subpopulation compared with the nonstimulated CD4^{+} CD69^{low} LP T-cell subpopulation. These results are in marked contrast to infection of nonstimulated PBLs that do not

TABLE 2. In vitro Infection of CD103^{+} and CD103^{-} CD4^{+} LP T Cells, Respectively

Time Point After Infection	p24 in Supernatant of CD103^{+} CD4^{+} LP T Cells [pg/mL] Median (Range)	p24 in Supernatant of CD103^{-} CD4^{+} LP T Cells [pg/mL] Median (Range)	Significance
3 days PI	4260 (0–6459)	2884 (0–4316)	NS
5 days PI	5350 (3782–8190)	5001 (2884–5329)	NS
7 days PI	7216 (4294–8100)	5965 (3369–6990)	NS
9 days PI	7301 (4295–8640)	7273 (4081–8190)	NS

Seven individual measurements, PI, post infection.

JOBNAME: joa 38#1 2005 PAGE: 6 OUTPUT: Thu December 2 16:31:56 2004
lww/joa/91064/V8756R1

TABLE 3. Phenotypic Characterization of LPMCs and PBMCs Before and After Stimulation

Marker	LPMCs Nonstimulated [Fluorescence Intensity] Median (Range)	LPMCs Stimulated [Fluorescence Intensity] Median (Range)	*P*	PBMCs Nonstimulated [Fluorescence Intensity] Median (Range)	PBMCs Stimulated [Fluorescence Intensity] Median (Range)	*P*
CD4	68 (65–69)	159 (135–174)	<0.016	159 (144–163)	385 (370–398)	<0.008
CD25	135 (130–136)	662 (650–663)	<0.008	18 (13–19)	600 (589–627)	<0.008
CD69	341 (328–353)	1521 (1420–1548)	<0.008	12 (11–14)	371 (362–380)	<0.008
CXCR4	298 (278–310)	241 (204–268)	NS	123 (118–132)	237 (228–240)	<0.008
CCR5	40 (39–41)	180 (158–200)	<0.008	4 (3–8)	19 (16–22)	<0.008

Five individual measurements.
LPMCs were stimulated with anti-CD28 and anti-CD2 antibodies; PBLs were stimulated with anti-CD3 and anti-CD28 antibodies.

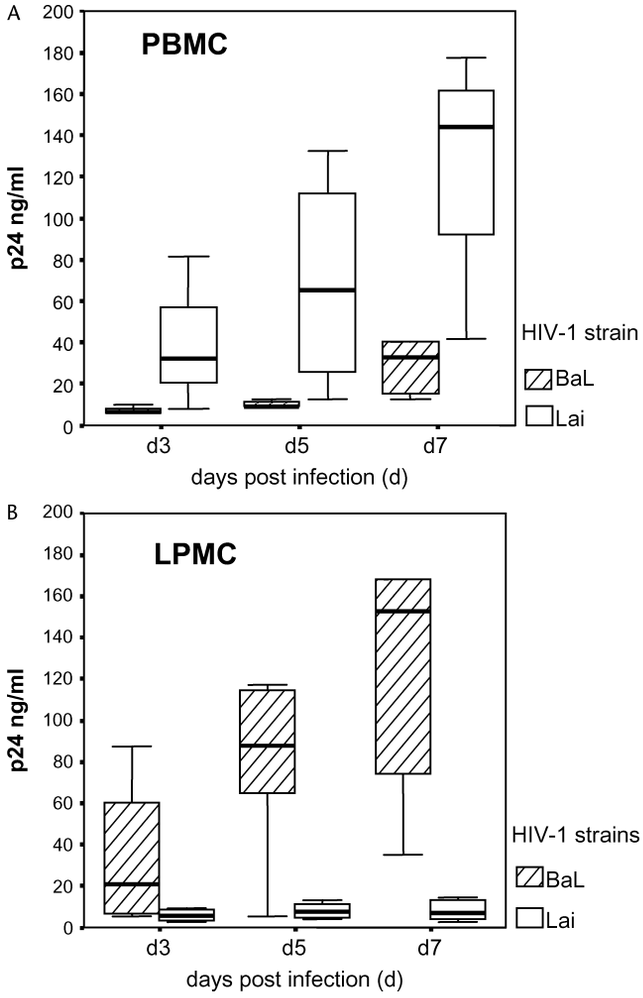

FIGURE 3. CD4+ PBMCs (A) and autologous CD4+ LPMCs (B) were stimulated with anti-CD3-and anti-CD28 antibody (PBMCs) or anti-CD2 antibodies and anti-CD28 (LPMCs). CD8+ cells were depleted prior to infection. Cells were infected with either M-tropic HIV-1 strain BaL (*hatched bars*) or T-tropic HIV-1 strain Lai (*open bars*). Higher replication of T-tropic HIV-1 strain Lai in PBMCs is observed in comparison to infection with the M-tropic strain (A). LPMCs show better replication of M-tropic HIV-1 strain BaL in comparison to T-tropic strain Lai 7 days postinfection (B). Results are presented as 25th and 75th percentiles (*upper and lower mark*), quartiles (*boxes*), and median (*bold line*).

support HIV replication. In agreement with these observations, a T-cell subpopulation with a comparable phenotype was described in PBMCs that is permissive for HIV-1 infection and shows high virus production.[21,22] These results demonstrate that the high levels of HIV-1 replication in intestinal LP lymphocytes are caused by intrinsic features of HIV target cells in the gastrointestinal tract.

Several mechanisms may account for the increased replication of HIV-1 in the CD69^{high} LP T cells. Based on the results presented here, we conclude that the intrinsically high activation state, mirrored by the elevated levels of CD69 and CD25, is central for the replication of HIV in LP T cells. LP T cells with a lower state of activation did not efficiently support viral spread. As shown in our experimental setting, exogenous stimulation further increased dramatically HIV replication in LP T cells.

Since both CD4$^+$ LP T-cell subpopulations, CD69^{high} and CD69^{low}, seem to be permissive to HIV infection, the increased HIV replication seen in CD69^{high} LP T cells seems to be due to a scenario of regulation at the transcriptional/translational level rather than a difference at the virus entry to the cell. However, we cannot completely rule out that some contaminating CD69^{high} LP T cells in the sorted CD69^{low} LP T-cell subpopulation may be responsible for the positive signal. As both signals show similar intensities, this seems to be unlikely.

Given the strict dependence of HIV transcription on the cellular activation state,[37,38] it seems plausible that HIV production is enhanced in CD69^{high} LP T cells. This model would also be consistent with our previous observation that p24 production, but not proviral load, is increased in the intestine as compared with the periphery in HIV patients.[12]

The mucosa homing marker CD103 seems not to be crucial for HIV-1 replication in LP T cells, as no statistically significant difference in p24 content in supernatants of CD103$^+$ and CD103$^-$ LP T cells was seen in our experiments. Although the expression of this marker is associated with T-cell activation on PBLs,[19] the difference of the activation status between CD103$^+$CD4$^+$ and CD103$^-$CD4$^+$ LP T cells seems not to be associated with a difference in HIV replication. One explanation may be that the experiments in which expression of CD103 was linked to activation markers were performed on PBMCs and not in LPMCs. Furthermore, even in PBMCs, CD103 was not correlated with the expression of CD25.[19] In vitro stimulation of PBMCs seems to enhance activation marker more on CD8$^+$ T cells than on CD4$^+$ T cells.[39]

Importantly, robust viral spread in LP T cells was detected only with the M-tropic and not with the T-tropic HIV-1 strain. This preferential replication of M-tropic HIV-1 strains in intestinal $CD4^+$ lymphocytes does not seem to be due to increased viral entry mediated by the elevated levels of CCR5 expression, because p24 intracellular staining revealed similar amounts of p24 in CCR5 high and low subpopulations (data not shown). In contrast, the T-tropic strain Lai replicated more efficiently in PBLs than M-tropic BaL. Thus, the 2 HIV-1 strains behave differently in intestinal and peripheral $CD4^+$ T cells. It is tempting to speculate that the differential coreceptor usage of these HIV variants determines these characteristics. This would predict that among the $CD69^{high}$ LP T cells, a sub-population expressing CCR5 levels sufficient for viral entry is the source of massive virus production. Preferential replication of M-tropic HIV in LP T cells in vitro is in line with the observation that only M-tropic HIV strains are transferred through the epithelial layer of the mucosa.[24] Given the higher cytopathogenicity of CCR5 using HIV strains,[25,26] the early and complete loss of intestinal CD4 T cells in the intestinal mucosa observed in HIV-infected humans[8–10] and in SIV-infected animals[4,5] may be explained in part by the selection of M-tropic virus through transepithelial transport and the preferential replication of these virus strains in intestinal $CD4^+$ T cells. Future experiments with more viral isolates with distinct coreceptor usage, preferentially including Env recombinant viruses in isogenic backbones, and infection experiments analyzing coreceptor-specific subsets of LP T cells will be required to address these questions.

Taken together our results suggest that the elevated activation status of LPMCs is the major reason for high and early virus replication in the intestinal tract. The mucosal HIV replication may be further enhanced in vivo by the tremendous amount of bacteria present in the intestinal tract, while this is not the case in the peripheral blood, which is usually sterile. Therefore as simulated in our in vitro infection studies, viral replication may be increased in the intestine by T-cell stimulation through frequently observed intestinal infection in HIV-infected patients. Moreover HIV-infection itself as well as opportunistic infections induce mucosal inflammation[11] and barrier dysfunction,[28,29] which may further enhance T-cell stimulation and viral replication.[40]

ACKNOWLEDGMENTS

The authors thank Marco Schäfer, Andreas Jung, Tanja Tänzer, and Sabine Ring for expert technical assistance.

REFERENCES

1. Mellors JW, Rinaldo CR Jr, Gupta P, et al. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science*. 1996;272:1167–1170.
2. Lefrere JJ, Roudot-Thoraval F, Mariotti M, et al. The risk of disease progression is determined during the first year of human immunodeficiency virus type 1 infection. *J Infect Dis*. 1998;177:1541–1548.
3. Heise C, Miller CJ, Lackner A, et al. Primary acute simian immunodeficiency virus infection of intestinal lymphoid tissue is associated with gastrointestinal dysfunction. *J Infect Dis*. 1994;169:1116–1120.
4. Veazey RS, DeMaria M, Chalifoux LV, et al. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science*. 1998;280:427–431.
5. Kewenig S, Schneider T, Hohloch K, et al. Rapid mucosal CD4(+) T-cell depletion and enteropathy in simian immunodeficiency virus-infected rhesus macaques. *Gastroenterology*. 1999;116:1115–1123.
6. Harouse JM, Gettie A, Tan RC, et al. Distinct pathogenic sequela in rhesus macaques infected with CCR5 or CXCR4 utilizing SHIVs. *Science*. 1999;284:816–819.
7. Lapenta C, Boirivant M, Marini M, et al. Human intestinal lamina propria lymphocytes are naturally permissive to HIV-1 infection. *Eur J Immunol*. 1999;29:1202–1208.
8. Schneider T, Ullrich R, Bergs C, et al. Abnormalities in subset distribution, activation, and differentiation of T cells isolated from large intestine biopsies in HIV infection. The Berlin Diarrhoea/Wasting Syndrome Study Group. *Clin Exp Immunol*. 1994;95:430–435.
9. Lim SG, Condez A, Lee CA, et al. Loss of mucosal CD4 lymphocytes is an early feature of HIV infection. *Clin Exp Immunol*. 1993;92:448–454.
10. Schneider T, Jahn HU, Schmidt W, et al. Loss of CD4 T lymphocytes in patients infected with human immunodeficiency virus type 1 is more pronounced in the duodenal mucosa than in the peripheral blood. Berlin Diarrhea/Wasting Syndrome Study Group. *Gut*. 1995;37:524–529.
11. Kotler DP, Reka S, Borcich A, et al. Detection, localization, and quantitation of HIV-associated antigens in intestinal biopsies from patients with HIV. *Am J Pathol*. 1991;139:823–830.
12. Fackler OT, Schafer M, Schmidt W, et al. HIV-1 p24 but not proviral load is increased in the intestinal mucosa compared with the peripheral blood in HIV-infected patients. *AIDS*. 1998;12:139–146.
13. Meng G, Sellers MT, Mosteller-Barnum M, et al. Lamina propria lymphocytes, not macrophages, express CCR5 and CXCR4 and are the likely target cell for human immunodeficiency virus type 1 in the intestinal mucosa. *J Infect Dis*. 2000;182:785–791.
14. Veazey R, Lackner A. The mucosal immune system and HIV-1 infection. *AIDS Rev*. 2003;5:245–252.
15. Poles MA, Elliott J, Taing P, et al. A preponderance of CCR5(+) CXCR4(+) mononuclear cells enhances gastrointestinal mucosal susceptibility to human immunodeficiency virus type 1 infection. *J Virol*. 2001;75:8390–8399.
16. Schieferdecker HL, Ullrich R, Hirseland H, et al. T cell differentiation antigens on lymphocytes in the human intestinal lamina propria. *J Immunol*. 1992;149:2816–2822.
17. Zeitz M, Greene WC, Peffer NJ, et al. Lymphocytes isolated from the intestinal lamina propria of normal nonhuman primates have increased expression of genes associated with T-cell activation. *Gastroenterology*. 1988;94:647–655.
18. De Maria R, Fais S, Silvestri M, et al. Continuous in vivo activation and transient hyporesponsiveness to TcR/CD3 triggering of human gut lamina propria lymphocytes. *Eur J Immunol*. 1993;23:3104–3108.
19. Schieferdecker HL, Ullrich R, Weiss-Breckwoldt AN, et al. The HML-1 antigen of intestinal lymphocytes is an activation antigen. *J Immunol*. 1990;144:2541–2549.
20. Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. *Science*. 1996;272:60–66.
21. Schnittman SM, Lane HC, Greenhouse J, et al. Preferential infection of CD4+ memory T cells by human immunodeficiency virus type 1: evidence for a role in the selective T-cell functional defects observed in infected individuals. *Proc Natl Acad Sci USA*. 1990;87:6058–6062.
22. Spina CA, Prince HE, Richman DD. Preferential replication of HIV-1 in the CD45RO memory cell subset of primary CD4 lymphocytes in vitro. *J Clin Invest*. 1997;99:1774–1785.
23. Fackler OT, Baur AS. Live and let die: Nef functions beyond HIV replication. *Immunity*. 2002;16:493–497.
24. Meng G, Wei X, Wu X, et al. Primary intestinal epithelial cells selectively transfer R5 HIV-1 to CCR5+ cells. *Nat Med*. 2002;8:150–156.
25. Fais S, Lapenta C, Santini SM, et al. Human immunodeficiency virus type 1 strains R5 and X4 induce different pathogenic effects in hu-PBL-SCID mice, depending on the state of activation/differentiation of human target cells at the time of primary infection. *J Virol*. 1999;73:6453–6459.
26. Picchio GR, Gulizia RJ, Wehrly K, et al. The cell tropism of human immunodeficiency virus type 1 determines the kinetics of plasma viremia

in SCID mice reconstituted with human peripheral blood leukocytes. *J Virol*. 1998;72:2002–2009.
27. Ofori H, Jagodzinski PP. Increased in vitro replication of CC chemokine receptor 5-restricted human immunodeficiency virus type 1 primary isolates in Th2 lymphocytes may correlate with AIDS progression. *Scand J Infect Dis*. 2004;36:46–51.
28. Stockmann M, Fromm M, Schmitz H, et al. Duodenal biopsies of HIV-infected patients with diarrhoea exhibit epithelial barrier defects but no active secretion. *AIDS*. 1998;12:43–51.
29. Stockmann M, Schmitz H, Fromm M, et al. The mechanism of diarrhea in HIV is based on an impaired epithelial barrier function that could be induced by a specific cytokine pattern. *Ann N Y Acad Sci*. 1998;859:267–270.
30. Bull DM, Bookman MA. Isolation and functional characterization of human intestinal mucosal lymphoid cells. *J Clin Invest*. 1977;59:966–974.
31. Zeitz M, Quinn TC, Graeff AS, et al. Mucosal T cells provide helper function but do not proliferate when stimulated by specific antigen in lymphogranuloma venereum proctitis in nonhuman primates. *Gastroenterology.* 1988;94:353–366.
32. Targan SR, Deem RL, Liu M, et al. Definition of a lamina propria T cell responsive state: enhanced cytokine responsiveness of T cells stimulated through the CD2 pathway. *J Immunol*. 1995;154:664–675.
33. Qiao L, Schurmann G, Betzler M, et al. Functional properties of human lamina propria T lymphocytes assessed with mitogenic monoclonal antibodies. *Immunol Res*. 1991;10:218–225.
34. Fuss IJ, Neurath M, Boirivant M, et al. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease: Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol*. 1996;157:1261–1270.
35. Peden K, Emerman M, Montagnier L. Changes in growth properties on passage in tissue culture of viruses derived from infectious molecular clones of HIV-1LAI, HIV-1MAL, and HIV-1ELI. *Virology.* 1991;185: 661–672.
36. Gartner S, Markovits P, Markovitz DM, et al. Virus isolation from and identification of HTLV-III/LAV-producing cells in brain tissue from a patient with AIDS. *JAMA*. 1986;256:2365–2371.
37. Lawn SD, Butera ST, Folks TM. Contribution of immune activation to the pathogenesis and transmission of human immunodeficiency virus type 1 infection. *Clin Microbiol Rev.* 2001;14:753–777.
38. Folks TM, Clouse KA, Justement J, et al. Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. *Proc Natl Acad Sci USA*. 1989;86:2365–2368.
39. Brew R, West DC, Burthem J, et al. Expression of the human mucosal lymphocyte antigen, HML-1, by T cells activated with mitogen or specific antigen in vitro. *Scand J Immunol*. 1995;41:553–562.
40. Toossi Z, Johnson JL, Kanost RA, et al. Increased replication of HIV-1 at sites of Mycobacterium tuberculosis infection: potential mechanisms of viral activation. *J Acquir Immune Defic Syndr.* 2001;28:1–8.

