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Optimization of Ex Vivo Activation and Expansion of Macaque Primary CD4-Enriched Peripheral Blood Mononuclear Cells for Use in Anti-HIV Immunotherapy and Gene Therapy Strategies

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Summary: The rhesus macaque model is a useful experimental system to evaluate effects of T-cell autotransfusion and gene therapies for HIV-1 infection and AIDS prior to a clinical trial. To obtain sufficient numbers of primary macaque CD4 T lymphocytes for this purpose, we examined the culture conditions that were needed to optimize ex vivo activation and expansion of macaque primary CD4-enriched peripheral blood mononuclear cells (PBMCs). In this report, we compared the effects of various stimulants on cell expansion, surface expression of CCR5 and CXCR4, and levels of transduction with a Moloney leukemia virus (MoLV) vector encoding the phenotypic selection marker truncated human nerve growth factor receptor (ΔNGFR) alone or with the human anti-HIV-1 tat intrabody sFvhutat2. The use of feeder cells strikingly increased the proliferation rate of macaque CD4-enriched PBMCs in vitro. In the presence of an irradiated rhesus macaque B-lymphoblastoid cell line (BLCL), the highest cell expansion over 21 days was achieved with cells activated by Con A (9648-fold), in turn, from high to low, phytohemagglutinin (PHA) (4855-fold), and anti-CD3/CD28-coated beads (2367-fold). Further studies showed that BLCL feeder cells were more effective than human PBMCs (hPBMCs) in promoting proliferation of macaque CD4-enriched PBMCs activated with Con A and anti-CD3/CD28, respectively. The combined use of both BLCL and hPBMC feeder cells did not further increase cell expansion when compared with the use of BLCL cells alone. In addition, the addition of BLCL-conditioned medium (CM) and hPBMC-CM induced cell growth at a rate higher than did the culture medium alone but not as high as with feeder cells. Con A–activated macaque CD4-enriched PBMCs retained 88% of CXCR4 and 39% of CCR5 expression over 17 days compared with PHA-activated cells (50% for CXCR4, 16% for CCR5) and anti-CD3/CD28-activated cells (34% for CXCR4, 37% for CCR5). Finally, PHA, Con A, and CD3/CD28-coated beads supported comparable levels of MoLV transduction. The results should improve the utility of the rhesus macaque model for the testing of T-cell autotransfusion and gene therapies for HIV-1 infection/AIDS. **Key Words:** Macaque CD4 T cells—Proliferation—Coreceptors—Transduction.

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Infection of rhesus macaques with certain simian immunodeficiency viruses (SIVs) induces an immunodeficiency disease that is clinically similar to HIV infection of human beings and serves as a useful model for studying HIV-1 infection and AIDS. Peripheral blood lymphocytes have become a primary target for anti-HIV immunotherapy using ex vivo expanded autologous T cells with or without a transferred gene of interest. A current

development in the field of HIV-1 gene therapy is to use intracellular antibodies, termed *intrabodies*, synthesized in the transduced $CD4^+$ T lymphocytes and targeted to specific cellular components, where they bind to their target HIV-1 protein and inhibit its function (1,2). We have previously shown that a murine single-chain (sFv) intrabody directed against the N-terminal activation domain of HIV-1 tat is a potent inhibitor of *tat*-mediated LTR transactivation and HIV-1 infection (3). Recently, we have characterized the humanized derivative of this anti-HIV-1 tat intrabody and demonstrated that it has potent anti-HIV-1 activity in vitro (4). Evaluation of the effectiveness of this gene therapy strategy in an animal model prior to a clinical trial will help to address basic questions regarding the safety and efficacy of this approach (5).

Transduction with retroviral vectors is currently the most effective approach for the stable introduction of genes into $CD4^+$ T lymphocytes. One of the limiting factors in the use of $CD4^+$ T lymphocytes for gene therapy for AIDS has been the relatively low transduction efficiencies with Moloney leukemia virus (MoLV)–based vectors. In addition, for a T-cell–based gene therapy, there are basic requirements to activate and expand the transduced cells ex vivo to a high therapeutic number (generally 10^8 cells per kilogram of body mass) for autotransfusion. Different types of stimulants activate $CD4^+$ T cells through distinct mechanisms, which may influence their proliferation, surface molecule expression, and capacity to be transduced with retroviral vectors in vitro. Therefore, in the current study, we established conditions that permit large-scale expansion of primary macaque CD4-enriched peripheral blood mononuclear cells (PBMCs) ex vivo. The effects of activation by various stimulants on cell proliferation, expression of CCR5 and CXCR4, and levels of transduction with retroviral vector expressing sFvhutat2 (pLNCΔNGFRhutat2) and a mock vector (pLNCΔNGFR) were examined.

MATERIALS AND METHODS

Preparation of CD4-Enriched Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells were isolated by Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) density gradient centrifugation of fresh rhesus macaque blood obtained from healthy animals provided by the New England Regional Primate Center. Contaminating erythrocytes were lysed with ACK lysing buffer (Biosource International, Camarillo, CA). $CD8^+$ T cells were depleted using Dynabeads M-450 CD8 (Dynal, Lake Success, NY) or the MACS system (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions. Briefly, PBMCs were incubated with M-450 CD8 beads (bead/CD8 cell >4:1) at 4°C for 30 minutes, followed by magnetic bead separation. In the MACS method, up to 10^7 cells were suspended in 100 μL of buffer (0.5% BSA/2 mM EDTA/PBS) containing 20 μL of anti-CD8 mouse IgG1 (Leu-2A; Becton Dickinson, San Jose, CA). After 20 minutes of incubation at 4°C, cells were washed and resuspended in 80 μL of buffer, and 20 μL of anti-mouse IgG(H + L) $F(ab')_2$ microbeads (Miltenyi Biotech) was added. After 20 minutes of incubation at 4°C, the suspension was washed twice and depleted of $CD8^+$ cells over the appropriately sized MACS column for the cell number. The purity of the $CD4^+$ T cells and depletion of the $CD8^+$ T cells were routinely monitored by FACS using anti-CD4-PE (clone SK3) and anti-CD8-fluorescein isothiocyanate (FITC) (clone SK1; Becton Dickinson) and were >85% $CD4^+$ and <10% $CD8^+$.

Cell Culture and Activation

Unless otherwise indicated, CD4-enriched PBMCs were cultured at 1×10^6 cells/mL in six-well plates in 1 mL per well of complete AIM-V medium (Life Technologies, Rockville, MD, U.S.A.) containing 10% heat-inactivated FBS (Sigma, Saint Louis, MO, U.S.A.) and 2mM of L-glutamine (Sigma). Recombinant human IL-2 (Proleukin; Chiron Corporation, Emeryville, CA, U.S.A.) was added at 100 to 200 U/mL. Cells were stimulated with 2 μg/mL phytohemagglutinin (PHA; Murex Biotech Limited, Dartford, U.K.), 5 μg/mL Con A (Sigma), and 10 ng/mL PMA plus 200 ng/mL ionomycin (Sigma) as well as with anti-monkey CD3 (clone FN18; Biosource International) plus anti-CD28 (clone L293; Becton Dickinson) antibodies, respectively. The latter were coimmobilized on M-450 tosyl-activated magnetic beads (Dynal) with an equal amount of each according to the manufacturer's instructions and were used at a rate of three beads per cell. Human PBMCs (hPBMCs) were isolated by Ficoll-Paque gradient centrifugation from leukopacks obtained by apheresis of healthy donors. A rhesus macaque herpesvirus papio transformed B-lymphoblastoid cell line (BLCL) was cultured in complete AIM-V medium and used as feeder cells or as a course of conditioned medium (CM) (6). Where noted, irradiated (50 gy) hPBMCs and/or BLCL cells were used as feeders to support cell expansions at a rate of 10 feeder cells per macaque cell. AIM-V–CM freshly made from cultures of hPBMCs in the presence of 5 μg/mL Con A and 100 U/mL IL-2 or BLCL cells was filtered at 0.4 μm and used in a mixture with fresh complete AIM-V medium at a ratio of 1:1. As required, the cultures were fed by either adding one volume of fresh complete medium or replacing half of the media with fresh media and were maintained at a concentration of 1 to 2×10^6 cells/mL. Stimulants were not removed from culture but were diluted progressively until restimulation. Unless otherwise indicated, cells were counted and monitored for mean cell volume (MCV) on a Coulter Z2 particle analyzer (Coulter, Hialeah, FL). A gate was set to exclude particles <100 fL. For the cells activated with anti-CD3/CD28 immunobeads, beads in the culture were removed by magnetic separation prior to size measurement. The cell cultures were restimulated with the same stimulants when the volume of the T-cell blasts decreased to 350 to 400 fL. Cell counts were determined from the total particles above these gates.

Preparation of Retroviral Vector Supernatant

Two retroviral vectors (Fig. 1), pLNCΔNGFRsFvhutat2 and pLNCΔNGFR, were constructed as previously described (4) by replacing the *neo* coding sequence with the truncated human nerve growth factor receptor (ΔNGFR) in MoLV vectors LN-sFvhutat2 and LNCX

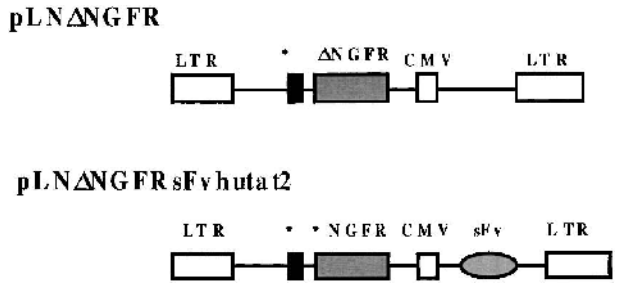

FIG. 1. Schematic diagram of retroviral vectors. The vectors are derived from LNCX (8). The neomycin selection marker was replaced with truncated human nerve growth factor receptor (ΔNGFR) to produce LNCΔNGFR, and sFvhutat2 expression is driven from an internal CMV promoter in LNCΔNGFRsFvhutat2.

(5). The ΔNGFR is used as a phenotypic selection marker as previously described (7). To prepare retroviral vector supernatants, pLNCΔNGFR-sFvhutat2 and pLNCΔNGFR plasmids were transfected into gp + envE86 cells using calcium phosphate (Promega, Madison, WI) according to the manufacturer's recommendations. Transient supernatant was harvested on days 3, 4, and 5. Gibbon ape leukemia virus (GALV) envelope-pseudotyped PG13 packaging cells (ATCC CRL-10686) (8) were infected in the presence of 8 μg/mL polybrene daily for 4 consecutive days. The infected cells were stained with 20 μg of FITC-conjugated (by Molecular Probes Eugene, OR) mouse anti-human NGFR (Chemichon, Temecula, CA) for fluorescent sorting with a FACSTAR PLUS (Becton Dickinson).

The 10% brightest population was selected, and the cells were expanded and stained for two additional sorts. After the third sort, cells were cloned into a 96-well plate at the rate of one cell per well and were split into 6-well plates (Corning, Cambridge, MA). PG13-pLNCΔNGFRsFvhutat2 packaging cell DNA was analyzed by real-time quantitative PCR for sFvhutat2 insert as previously described (5). To detect expression of sFvhutat2 proteins, the packaging cells were intracellularly stained with goat anti-human IgG FITC conjugate (Sigma) using the method described previously, followed by FACS analysis (9) (data not shown). Once producer cell lines were established, cells were cultivated to confluence in 10-cm dishes. After an additional 24 hours of culturing in fresh media, the supernatant was harvested and filtered at 0.45 μm.

Transduction

The retroviral vectors pLNCΔNGFRsFvhutat2 and pLNCΔNGFR were produced in the PG13 GALV packaging cell lines and used for transduction. Transductions were performed at a multiplicity of infection (MOI) of 0.25 measured on CEM × 174 cells, unless otherwise indicated, using an equal volume of vector supernatant. CD4-enriched PBMCs were cultured at 1×10^6 cells/mL in 50:50 complete AIM-V medium/BCLC CM containing 10% FBS and 200 U/mL IL-2. Transduction at 24 hours after stimulation was performed, because in pilot experiments, extended stimulation (e.g., 48 hours) with PHA, Con A, or anti-CD3/CD28 did not improve transduction efficiency of the retroviral vectors (data not shown). These observations are similar to the results obtained by Bunnell et al. (10). Thus, 24 hours after stimulation with PHA, Con A, or anti-CD3/CD28 immunobeads, cells were directly exposed to retroviral supernatants in the presence of 8 μg/mL protamine sulfate (Sigma) plus 200 U/ml IL-2 with centrifugation for 1 hour at 2500 rpm at 32°C, followed by overnight incubation at 37°C. Transduction was performed on 2 consecutive days. Seven days after the second transduction, all cells were analyzed by flow cytometry for the presence of the NGFR on the cell surface. Briefly, after washing twice with 2% FBS in PBS, 1×10^6 cells were incubated with 100 μL of 2 μg/mL of mouse anti-human NGFR (Chemicon). Isotope-matched mouse IgG1 (Pharmingen, San Diego, CA) was used as a control. After washings, cells were suspended in 100 μL of a 1:200 dilution of anti-mouse IgG $F(ab')_2$ FITC (Sigma) and incubated for 30 minutes at 4°C. Cells were then washed, fixed with 2% paraformaldehyde, and analyzed by FACS within 1 week.

Analysis of Expression of CCR5 and CXCR4

CD4-enriched PBMCs were cultured at 1×10^6 cells/mL in a mixture of BLCL-CM and AIM-V medium with 10% heat FBS and 200 U/mL IL-2. After stimulation with PHA, Con A, and anti-CD3/CD28 beads, respectively, cells were harvested on indicated days and analyzed for expression of CCR5 and CXCR4 by flow cytometry, using reagents of anti-CCR5-PE (clone 45531.111; R&D Systems, Minneapolis, MN) and anti-CXCR4-PE (clone 12G5; Pharmingen).

RESULTS

Feeder Cells Augment CD4-Enriched Peripheral Blood Mononuclear Cell Proliferation In Vitro

In a pilot study, we tested whether the use of exogenous feeder cells can improve conditions for ex vivo proliferation of macaque primary CD4-enriched PBMCs. Irradiated hPBMCs and a BLCL (11) were initially chosen as feeder cells. CD4-enriched PBMCs were incubated with Con A in the presence or absence of irradiated BLCL cells and hPBMCs at the rate of 10 feeder cells per macaque cell. The culture was started in six-well plates and later transferred to plastic tissue culture flasks as required. IL-2 was added to all cultures at 100 U/mL. As shown in Figure 2, Con A activation resulted in cell expansion of 8-fold, 65-fold, and 117-fold on days 7, 14, and 21, respectively, in the absence of feeders. The addition of feeder cells significantly increased Con A–activated cell expansion by 29-fold (day 7), 427-fold (day 14), and 4693-fold (day 21), however, indicating that the proliferation of macaque primary CD4-enriched PBMCs can be promoted by coculture with these feeder cells.

Cyclic Changes in Cell Volume Are Dependent in Part on the Type of Stimulant

Several studies have demonstrated that repeated addition of stimulant is necessary to sustain long-term proliferation of CD4 T cells. Experiments undertaken to optimize human $CD4^+$ T-lymphocyte expansion have shown that an optimal interval between stimulations is essential for cell expansion and that MCV is a useful indicator of cell activation status (12). Thus, a Coulter Z2

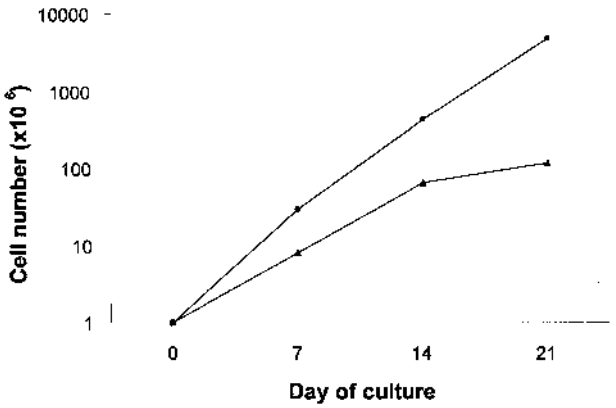

FIG. 2. Increased proliferation rate of macaque primary CD4-enriched peripheral blood mononuclear cells (PBMCs) by the addition of feeder cells in vitro. A total of 1×10^6 macaque CD4-enriched PBMCs were cultured in AIM-V medium containing 10% FBS and IL-2 (100 U/mL). Cells were activated by 5 μg/mL Con A. To the cultures, irradiated B-lymphoblastoid cell line and human PBMC feeder cells (*closed circles*) were added every 7 days at a rate of 10 feeder cells per macaque cell. Cultures in the absence (*closed triangles*) of feeder cells were used as a control. Cells were restimulated at day 7 and day 14 with the same stimulants. Fresh medium was added to the culture as required, and excess cells were discarded. Cell number was determined on a Z2 particle analyzer. The total number of cells that would be expected to accumulate is displayed, taking into account discarded cells. Data represent two different experiments using $CD4^+$-enriched PBMCs from different rhesus macaques.

particle analyzer was used to measure MCV after stimulation of macaque CD4-enriched PBMCs by various stimulants. As shown in Figure 3, resting cells have an MCV of circa 240 fL. CD4 T cells stimulated with PHA increased in volume from 240 fL to nearly 634 fL by 4 days. The cell volume declined over the course of 3 days to 400 fL. At this point, the cells were restimulated by addition of the same stimulant, and the cell volume again returned to near 657 fL by day 9. Similar results were observed in Con A–stimulated cells. The cells stimulated with anti-CD3/CD28 immunobeads reached a peak MCV by day 4 (727 fL) but took 7 days to return to a volume of 399 fL. Therefore, the cell cultures were restimulated at day 11 with the immunobeads, and the cell volume again returned to near 764 fL by day 14. These data reveal that the cyclic changes in cell volume are dependent, in part, on the type of stimulus. The maximum MCV and time required to return to baseline (350–400 fL) vary with the stimulant type, suggesting that the various types of stimulants activate cells through distinctive mechanisms. The MCV of anti-CD3/CD28-stimulated cells declined more slowly than was seen with cells stimulated with either PHA or Con A. These data also demonstrate that a decrease in cell volume serves as an indication that cells have become responsive to further restimulation and do not have to rest before restimulation. Furthermore, if cells were allowed to grow in culture until their size returned to resting cell volumes, they become unresponsive to restimulation, and cell death, likely due to apoptosis, began to occur (data not show). Our results show that macaque primary CD4-enriched PBMCs are best restimulated at 350 to 450 fL, which is similar to that observed with human CD4 T cells (12).

Effect of Different Stimulants on the Cell Expansion

The above data also suggested that macaque CD4-enriched PBMC cells appear to require additional

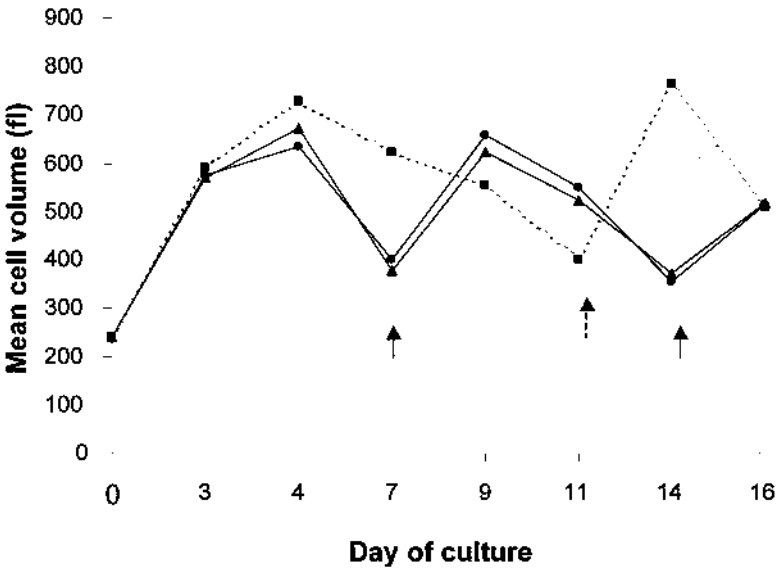

FIG. 3. Cyclic changes in cell volume are dependent, in part, on the type of stimulants used. A total of 1×10^6 macaque CD4-enriched peripheral blood mononuclear cells (PBMCs) were cultured in AIM-V medium containing 10% FBS and IL-2 (100 U/mL). Cells were activated on day 0 by 5 μg/mL Con A (*solid triangles*), 2 μg/mL phytohemagglutinin (PHA) (*solid circles*), and anti-CD3/CD28 immunobeads (*solid squares*), respectively. At the initiation of the culture, macaque primary CD4-enriched PBMCs had a mean cell volume (MCV) of 240 fL. Repeated additions of the same stimulants were performed with the cultures activated with Con A and PHA at day 7 and day 14 (*arrow with solid line*) and the cultures activated with anti-CD3/CD28 immunobeads at day 11 (*arrow with dotted line*). The interval between stimulations was determined by the MCV measured with a Coulter Z2 particle analyzer. Cells were restimulated when the MCV fell below 400 fL. The data shown are from one macaque and are representative of similar results for cell expansion and cell cycle changes seen with different stimuli using cells from several different macaques.

"growth factors" for optimal growth ex vivo. Although these factors are not well characterized, they are supplied by cocultivation with irradiated hPBMC and BCLC feeder cells. In addition, various types of stimulants activate cells in a distinctive way, which may affect their proliferation rate. Therefore, several commonly used T-cell stimulants, including PHA, Con A, anti-CD3/CD28, and PMA/ionomycin, were chosen to characterize the proliferation potential of macaque primary CD4-enriched PBMCs ex vivo. In this study, CD4-enriched PBMCs were incubated in complete medium containing 100 U/mL IL-2 and stimulated with PHA, Con A, PMA/ionomycin, and anti-CD3/CD28 immunobeads, respectively, in the presence of irradiated BLCL cells and hPBMCs at the rate of 10 feeder cells per macaque cell. Restimulation was performed as described in the Materials and Methods section. Among these stimulants, Con A activation resulted in the highest cell expansion (4693-fold) (Fig. 4) over 21 days, and the lowest cell expansion (100-fold) was observed in the cells stimulated with PMA/ionomycin. PHA stimulation induced cell growth at a rate of about 1.8-fold lower than did Con A at day 21. Anti-CD3/CD28-activated cells displayed a higher expansion fold than PMA/ionomycin but lower than that of Con A or PHA.

The next question that was addressed is whether hPBMC and BLCL feeder cells make an equal contribution to the increased cell expansion or whether both hPBMC and BLCL feeder cells are required for optimal cell expansion. To address this question, CD4-enriched PBMCs were incubated with Con A or anti-CD3/CD28 beads in the presence of irradiated BLCL cells and/or hPBMCs. We found that BLCL feeder cells were more effective than hPBMC feeder cells in promoting proliferation of macaque CD4-enriched PBMCs (data not shown). As shown in Figure 5, a higher proliferation rate was achieved with Con A–stimulated cells in the presence of BLCL feeder cells (9648-fold) compared with the use of both BLCL and hPBMC feeder cells (4693-fold) at day 21. Similar results were observed in anti-CD3/CD28- or PHA-activated cells. These data indicate that the combined use of both BLCL and hPBMC feeder cells cannot further increase the fold of cell expansion and that the addition of hPBMCs does not synergize with a BLCL to further promote CD4 T-cell proliferation.

Conditioned Media Augment CD4-Enriched Peripheral Blood Mononuclear Cell Proliferation

To determine whether the improved cell proliferation is induced by soluble factors secreted from the feeder cells or/and by intercellular interaction through surface molecules on feeder cells, we evaluated the effect of CM on the proliferation of macaque primary CD4-enriched PBMCs. Cells were cultured in 50:50 fresh complete AIM-V medium/BLCL-CM or hPBMC-CM containing 100 U/mL IL-2. Figure 6 reveals that CM can promote cell proliferation, because a higher fold expansion is achieved in either Con A– or anti-CD3/CD28-stimulated cells in the presence of BLCL-CM or hPBMC-CM compared with the cultures without the addition of CM. In Con A–activated cells, the use of BLCL-CM resulted in a higher cell proliferation rate over 21 days (1124-fold) than did the use of hPBMC-CM (575-fold), which is consistent with the results observed in the feeder cell experiments described previously (Fig. 5). Similarly, a higher fold cell expansion of 927 was seen with the cells activated by anti-CD3/CD28 plus the addition of BLCL-

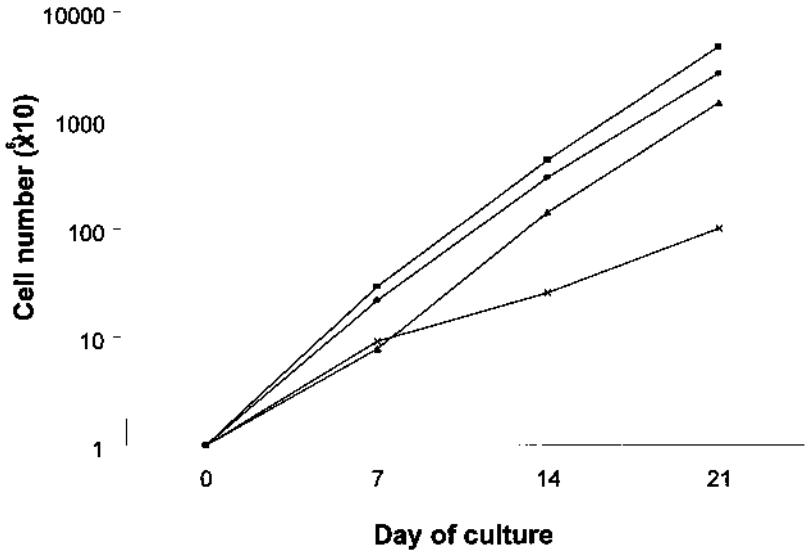

FIG. 4. Effect of various stimulants on proliferation rate of macaque CD4-enriched peripheral blood mononuclear cells (PBMCs). A total of 1×10^6 macaque CD4-enriched PBMCs were cultured in AIM-V medium containing 10% FBS and IL-2 (100 U/mL). Cells were activated by 5 μg/mL Con A (*solid squares*), 2 μg/mL phytohemagglutinin (PHA) (*solid circles*), 10 ng/mL PMA and 200 ng/mL ionomycin (*cross symbol*), and anti-CD3/CD28 immunobeads at a rate of three beads per cell (*solid triangle*), respectively. To the cultures, irradiated B-lymphoblastoid cell line and human PBMC feeder cells were added every 7 days at a rate of 10 feeder cells per macaque cell. The cells were restimulated with the same stimulants at days 7 and 14 (for PHA and Con A activation) or at day 11 (for anti-CD3/CD28 activation). Cells were maintained and counted as described in the legend for Figure 2. Data represent two different experiments using cells from different rhesus macaques.

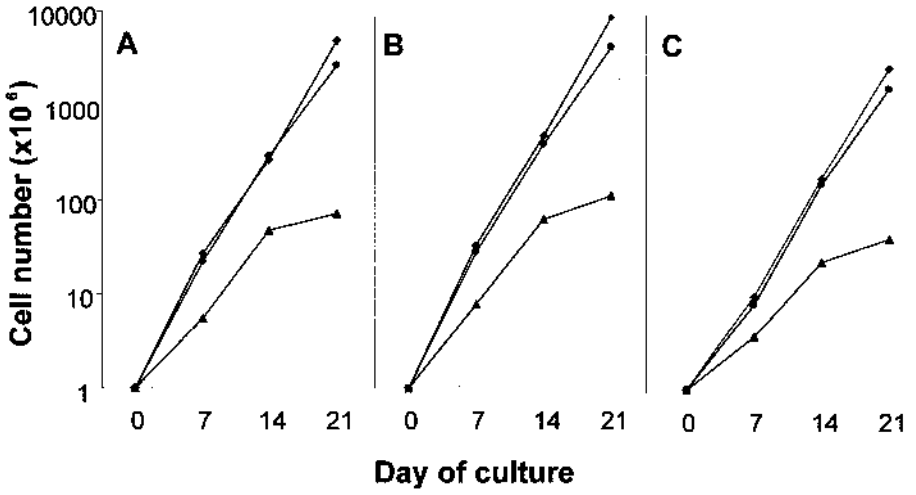

FIG. 5. Comparative efficacy of various feeder cells in promoting proliferation of macaque CD4-enriched peripheral blood mononuclear cells (PBMCs). A total of 1 × 10^6 macaque CD4-enriched PBMCs were cultured in AIM-V containing 10% FBS and IL-2 (100 U/mL). Cells were activated by 2 μg/ml phytohemagglutinin (PHA) (**A**), 5 μg/mL Con A (**B**), and anti-CD3/CD28 immunobeads at a rate of three beads per cell (**C**). To the cultures, B-lymphoblastoid cell line (BLCL) feeder cells (*solid squares*), BLCL plus human PBMC feeder cells (*solid circles*), or no feeder cells (*solid triangle*) were added every 7 days. Cells were maintained, restimulated, and counted as described in the legends for Figures 2 and 3. Data represent two different experiments using different rhesus macaques.

CM compared with the use of hPBMC-CM (302-fold) over 21 days. Finally, the combined use of BLCL-CM and hPBMC-CM did not further improve the proliferation rate of the cells activated by Con A or anti-CD3/CD28 beads (data not shown). A higher rate of cell growth was seen with Con A– or anti-CD3/CD28-activated cells in the presence of BLCL feeder cells than with the addition of BLCL-CM (Figs. 5,6), suggesting that feeder cells can provide better conditions than their CM for proliferation of macaque CD4-enriched PBMCs.

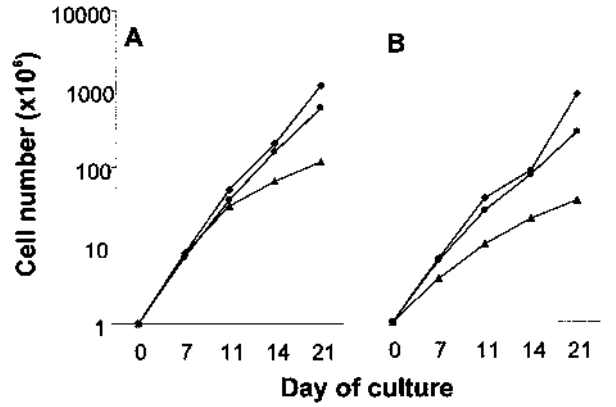

FIG. 6. Effect of conditioned medium (CM) on the expansion of macaque CD4-enriched peripheral blood mononuclear cells (PBMCs). A total of 1 × 10^6 macaque CD4-enriched PBMCs were cultured with no CM (*solid triangles*) or in 50:50 AIM-V medium/CM from a B-lymphoblastoid cell line (*solid diamond*) or human PBMCs (*solid circle*) containing 10% FBS and IL-2 (100 U/mL). The cells were activated by 5 μg/mL Con A (**A**) or anti-CD3/CD28 immunobeads (**B**) at a rate of three beads per cell. Cells were maintained, restimulated, and counted as described in the legends for Figures 2 and 3. Data represent two different experiments using different rhesus macaques.

Effect of Activation by Different Stimulants on CCR5 and CXCR4 Expression

CCR5 and CXCR4 on CD4 T cells are two major coreceptors for HIV-1 entry. Other studies have shown that CCR5 surface expression is downregulated in both macaque and human CD4 T cells after anti-CD3/CD28 stimulation and that the level of CCR5 on the cultured CD4 T cells appears to be associated with their resistance to HIV-1 infection in vitro and in vivo (13,14). Therefore, we examined levels of CCR5 and CXCR4 expression on the cultured macaque CD4-enriched PBMCs activated by PHA, Con A, and anti-CD3/CD28 immunobeads, respectively. Cells were cultured in 50:50 complete AIM-V/BLCL-CM supplemented with 100 U/mL IL-2. A decreased level of CXCR4 expression was seen in all the cultures activated by PHA, Con A, or anti-CD3/CD28 (Fig. 7). CXCR4 expression on Con A–activated CD4-enriched cells decreased the least and retained a plateau level of >85% $CXCR4^+$ cells over 17 days. A dramatic decline in CXCR4 level was seen with cells stimulated by anti-CD3/CD28 (66% decrease at day 17) and by PHA (50% decrease at day 17). Similarly, early and sustained CCR5 downregulation was observed with the CD4-enriched PBMCs activated by each of the three stimulants to a prestimulation level of 39% (Con A), 16% (PHA), and 37% (anti-CD3/28) at day 17 (Fig. 8). Like CXCR4 expression described previously, CCR5 level declined relatively slowly in Con A–activated cells compared with anti-CD3/CD28-activated cells. Among these stimulants, it appears that Con-A–activated cells maintained relatively higher expression of CXCR4 and CCR5 over the 17 days.

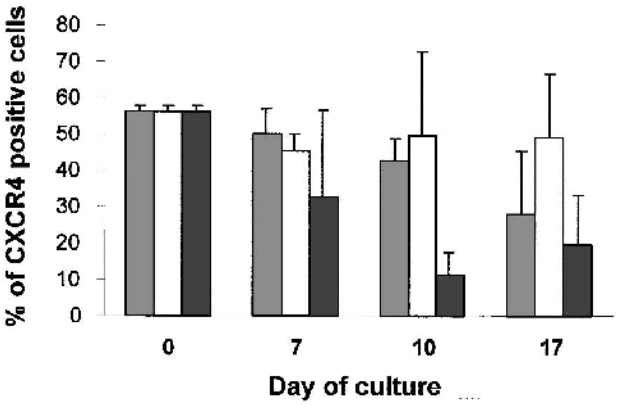

FIG. 7. CXCR4 expression in macaque CD4-enriched peripheral blood mononuclear cells (PBMCs) activated by various stimulants. A total of 2×10^6 macaque CD4-enriched PBMCs were activated with 5 μg/mL Con A (*white bar*), 1 μg/mL phytohemagglutinin (*black bar*), or anti-CD3/CD28-coated beads (*gray bar*) at a rate of three beads per cell, respectively. The cells were cultured in 50:50 AIM-V medium/CM from a B-lymphoblastoid cell line containing 10% FBS and IL-2 (100 U/mL). The cells were stimulated every 7 days and maintained as described in Figure 2. FACS analysis for CXCR4 was performed on the indicated days. Values are the means ± SD of two experiments using cells from two different macaques.

Effect of Activation by Different Stimulants on Transduction Efficiency with Retroviral Vectors

One of limiting factors in gene therapy strategies using primary $CD4^+$ T cells has been a relatively low level of transduction with MoLV-based retroviral vectors. In this study, we examined the transduction rate of two retroviral vectors, pLNCΔNGFRsFvhutat2 and pLNCΔNGFR (a mock vector), on macaque CD4-enriched PBMCs activated by various stimulants. A transduction protocol using centrifugation and low temperature was used in this experiment, because Bunnell and colleagues (10) reported that this method can improve transduction efficiency of retroviral vectors with rhesus lymphocytes. Twenty-four hours after stimulation with PHA, Con A, or anti-CD3/CD28 immunobeads, cells in a six-well plate were incubated with retroviral vectors in the presence of 8 μg/mL protamine sulfate and 200 μg/mL IL-2. The plates were then centrifuged at 2500 rpm at 32°C for 1 to 2 hours. This process was repeated one more time at the 24-hour interval. The gene transfer was determined by FACS analysis for NGFR expression on the transduced cells. As shown in Table 1, all three stimulants supported comparable levels of transduction of activated CD4-enriched PBMCs for both the LNCΔNGFR and pLNCΔNGFRsFvhutat2 vectors. In general, the transduction efficiency was not as high as reported by other investigators, which might be related to limiting MOI (10). In addition, incorporation of fibronectin, poly-L-lysine, and phosphate depletion procedures into the protocol did not improve transduction efficiency in these studies (data not shown).

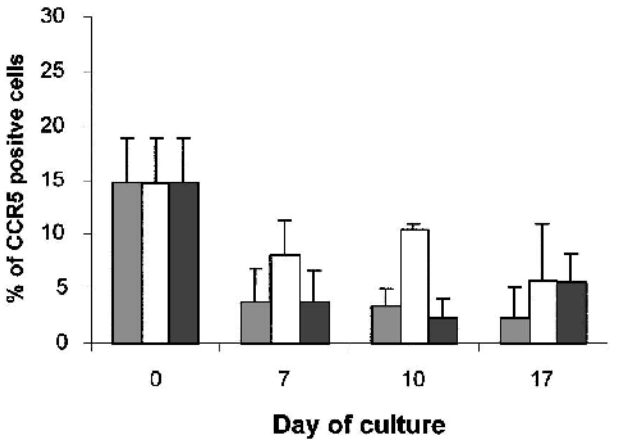

FIG. 8. CCR5 expression on macaque CD4-enriched peripheral blood mononuclear cells (PBMCs) activated by various stimulants. A total of 2×10^6 macaque CD4-enriched PBMCs were activated with 5 μg/mL Con A (*white bar*), 1 μg/mL phytohemagglutinin (*black bar*), or anti-CD3/CD28 immunobeads (*gray bar*) at a rate of three beads per cell, respectively. The cells were cultured in 50:50 AIM-V medium/CM from a B-lymphoblastoid cell line containing 10% FBS and IL-2 (100 U/mL). The cells were stimulated every 7 days and maintained as described in Figure 2. FACS analysis for CXCR4 was performed on the indicated days. Values are the means ± SD of two experiments using cells from two different macaques.

DISCUSSION

The use of peripheral lymphocytes for autotransfusion therapy and gene therapy for HIV-1 infection and AIDS has been in development for several years. These T-cell–based immunotherapy strategies generally require ex

TABLE 1. *Percent transduction of retroviral-mediated gene transfer into CD4-enriched PBMC activated by various stimulants*

	Vector	
Stimulant	pLNΔNGFR	pLNΔNGFRsFvhutat2
PHA	8, 8	5, 5
Con A	11, 13	7, 7
Anti-CD3/28	3, 13	7

FACS analysis of transduction efficiency of CD4-enriched PBMC 24 h after activation 5 μg/ml Con A, 1 μg/ml PHA, and anti-CD3/CD28 beads (3:1, beads:cell), respectively, cells were transduced with retroviral supernatants containing pLNCΔNGFR or pLNCΔNGFR- sFvhutat2 using an moi of 0.25 as described in Materials and Methods. 7 days posttransduction, cells were collected for FACS analysis for NGFR expression in the transduced cells. The percentages of duplicate samples are shown and are representative of at least 4 different experiments using cells from different macaques. In this study, the expression levels of the anti-tat intrabody in the transduced macaque PBMCs was not tested.

vivo cell proliferation to a large number for autotransfusion (10,14). Because of the value of the rhesus macaque model in anti-HIV-1 gene therapy and the necessity for both safety and efficacy testing of gene therapeutics prior to human clinical trials, we conducted studies to optimize the ex vivo manipulation of macaque CD4 T lymphocytes. We first compared the effects of activation by PHA, Con A, PMA/ionomycin, and anti-CD3/CD28-coated beads on in vitro proliferation of macaque primary CD4-enriched PBMCs. It is known that the addition of certain exogenous feeder cells can improve conditions for long-term culture of T lymphocytes in vitro (15,16). Therefore, in this present work, we assessed the potential of two feeder cells, hPBMCs and a rhesus macaque BLCL, for promotion of CD4-enriched PBMC proliferation. Our findings demonstrated that the presence of feeder cells can enhance cell expansion (Figs. 2,4). Brice et al. (13) observed a maximum 30-fold expansion of $CD4^+$ T cells from rhesus macaques after 2 weeks of feeder cell–free culture with anti-CD3/CD28 immunobeads. In this study, a cell expansion of 163-fold was achieved with CD4-enriched PBMCs activated by anti-CD3/CD28 immunobeads over 14 days in the presence of BLCL feeder cells (Fig. 5). When added with BLCL feeder cells, Con A–activated cells showed the highest cell expansion of 9647-fold over 21 days, in turn, from high to low, PHA (4855-fold), and anti-CD3/CD28 beads (2367-fold) (Fig. 5). BLCL feeder cells appear superior to hPBMCs in promoting proliferation of macaque CD4-enriched PBMCs. The potent proliferation-supporting efficacy of BLCL cells is independent of the type of stimulant, because a higher fold cell expansion was achieved with both Con A– and anti-CD3/CD28-activated cells using BLCL feeders compared with hPBMC feeders (data not shown). The combined use of both feeder cells did not further improve the conditions for their proliferation, however (Fig. 5). A lower fold cell expansion was seen with cells activated by PMA/ionomycin compared with anti-CD3/CD28 immunobeads (Fig. 4), which is similar to the results observed by Brice et al. (13) with human CD4 T cells. The difference among the various stimulants in their ability to effect proliferation of CD4-enriched PBMCs appears to be associated with their different/distinctive mechanisms of action. Both PHA and Con A belong to polyclonal mitogenic lectins, which activate the entire T-cell repertoire (15). Henkart and Fisher (17) found that Con A was able to bind to about twice the amount of labeled surface protein as PHA, although the pattern of Con A receptors on human lymphocytes was similar to the pattern of PHA receptors. This might be an explanation for a higher efficacy of Con A compared with PHA as measured by the proliferation rate of macaque CD4 cells (Figs. 4,5). Anti-CD3/CD28 activates lymphocytes in a way different from PHA and Con A by interaction with CD3 and CD28 molecules on T cells. It has also been reported that anti-CD3/CD28 activation is able to maintain a polyclonal population of human CD4 T cells for at least 8 weeks in culture (12). The adaptive transfer of autologous CD4 T cells stimulated with anti-CD3/CD28-coated beads and expanded ex vivo for 6 to 8 weeks induces T-cell proliferation and retains T-cell effector functions as assessed by the levels of cytokines in HIV-infected patients, and no severe adverse effects are observed (14). In our study, the maximum size of cells activated by the anti-CD3/CD28 immunobeads was larger and the time required to return to baseline was longer than for cells stimulated by Con A or PHA (Fig. 3). Similar findings have been reported by other investigators (12). In addition, other reports have demonstrated that both the quantity and ratio of cytokines and chemokines in the supernatant of lymphocytes activated by various stimulants are different, which may influence cell proliferation function (13,18).

T lymphocytes under stimulation exhibit periodic changes in cell volume, and restimulation is required for maintaining cell growth. The cyclic changes in cell size are dependent in part on the type of stimulant, and the maximum size of the cells and the time course on their volume changes appear to vary with stimulant type (Fig. 3). The basis for the cyclic changes in cell size remains unknown but may be related to differences of cell cycle distribution. Cell sizing is useful for determining when to restimulate the cultures. Macaque CD4-enriched PBMCs activated by Con A at day 0 and restimulated at day 11 could not sustain exponential expansion in either the presence or the absence of feeder cells (data not shown); however, cells sustained exponential proliferation over 21 days when restimulation was performed before cell volume returned to the baseline volume (Fig. 3). Our experience has shown that macaque CD4-enriched PBMCs are best restimulated at around 350 to 400 fL, which is similar to that observed with human CD4 T cells (12). It is also noteworthy that the more the culture is restimulated, the shorter is the time interval between restimulation. In addition, it is too late to restimulate the cells when they have returned to a resting T-cell volume. Therefore, it is important to monitor changes in cell volume to determine the restimulation interval, which provides the cells with optimal proliferation conditions.

It appears that the feeder cells affect proliferation of macaque CD4-enriched PBMCs, at least partially, through paracrine growth factors secreted from the feeders, because an increased cell proliferation was seen with

Con A– or anti-CD3/CD28-activated cells in the presence of BLCL-CM compared with cells cultured in the complete medium alone. Similar observations were reported by Barillari et al. (19); however, the composition of the growth factors in the CM is not known. The major advantage of using CM is its ease of use in terms of avoiding procedures for irradiation and removal of cell debris present in the culture prior to infusion into subjects for gene therapy and autotransfusion therapy. The CM can be stored at −80°C and used within 1 month, which avoids the continuous culturing of feeder cells. Like their feeder cells, a higher fold cell expansion was observed with cells activated by Con A or anti-CD3/CD28 plus BLCL-CM compared with hPBMC-CM (Fig. 6). Thus, BLCL-CM can be considered an alternative exogenous source of growth factors for long-term expansion of primary macaque CD4 T cells.

It is known that chemokine receptor expression on T lymphocytes depends on their states of activation and differentiation (20). Brice et al. (13) reported that macaque CD4 lymphocytes stimulated ex vivo with anti-CD3/CD28 beads displayed clear downregulation of CCR5 on day 6 as measured by PCR of CCR5 cDNA from the cultured cells. Similarly, a decline of CCR5 expression was observed in human $CD4^+$ T cells from HIV-1–infected patients when stimulated by anti-CD3/CD28 beads and analyzed by FACS on day 8. These data are in agreement with our findings that CCR5 expression was downregulated with macaque CD4-enriched PBMCs stimulated by anti-CD3/CD28 beads. We also found that expression of CCR5 was decreased on these macaque cells stimulated by Con A and PHA. Interestingly, these expanded macaque and human $CD4^+$ T cells with decreased levels of CCR5 were refractory to infection with SIVmac251 and CCR5 tropic HIV_{us1}, respectively (13,14). It was reported that stimulation with PHA, PMA, and anti-CD3 strongly downregulates CXCR4 expression in hPBMCs as measured by FACS analysis for surface CXCR4 (20). As shown here, CXCR4 expression on primary macaque CD4-enriched PBMCs was downregulated by stimulation with PHA, Con A, and anti-CD3/CD28 (Fig. 7). The low level of CXCR4 expression on the stimulated cells has been explained in part by receptor internalization, which appears to be associated with activation of PKC, because CXCR4 downregulation by PHA, PMA, and anti-CD3 was inhibited by a specific PKC inhibitor (21,22). The internalization of CXCR4 is dependent on cell activation (21). Further investigations by Bermejo et al. (21) indicated that human $CD4^+$ T lymphocytes showing activation-induced CXCR4 downregulation were refractory to infection of X4 tropic HIV strain NL4.3 in vitro. The degree of CXCR4 downregulation on macaque primary CD4-enriched PBMCs was dependent on the stimulant type. Stimulation by anti-CD3/CD28 led to a more marked downregulation of CXCR4 compared with PHA or Con A (Fig. 7). Among these three stimulants, Con A–stimulated cells maintained the highest levels of CXCR4 expression on day 17.

In summary, the data presented in this report demonstrate that substantial ex vivo expansion of macaque primary CD4-enriched PBMCs is possible if feeder cells or their CM is used and cyclic restimulation is performed based on the changes in cell volume. Cells activated by various stimulants showed different proliferation rates and levels of CCR5 and CXCR4 coreceptors but similar transduction efficiencies. These results provide useful information on the macaque model for designing CD4 T-cell–based adoptive autotransfusion therapy and gene therapy strategies for HIV infection/AIDS.

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