AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 18, Number 5, 2002, pp. 373–382
© Mary Ann Liebert, Inc.

In Vitro Replication of SIVcpz Is Suppressed by β-Chemokines and CD8$^+$ T Cells But Not by Natural Killer Cells of Infected Chimpanzees

PASCALE ONDOA,[1] JOHAN VINGERHOETS,[1,2] CHRIS VEREECKEN,[1] GUIDO VAN DER GROEN,[1] JONATHAN L. HEENEY,[3] and LUC KESTENS[1]

ABSTRACT

Unlike humans, chimpanzees are relatively resistant to AIDS after infection with HIV-1 or simian immunodeficiency virus of chimpanzee (SIVcpz). We hypothesized that resistance to disease progression is associated with efficient suppression of virus replication possibly by β-chemokines secreted by CD8$^+$ lymphocytes and especially natural killer (NK) cells. *In vitro* suppression of virus replication can be easily studied in SIVcpz-infected chimpanzees because they produce high infectious virus titers in their peripheral blood. A study was undertaken to assess the sensitivity of SIVcpz to β-chemokines *in vitro* and to investigate the role of endogenous β-chemokines in relation to the *in vitro* capacity of CD8$^+$ lymphocytes and NK cells of chimpanzees to suppress SIVcpz replication. Our results show that SIVcpz uses CCR5 as a coreceptor to gain cell entry and is sensitive to recombinant β-chemokines *in vitro*. Here we report that despite their potent capacity to produce RANTES, NK cells of infected chimpanzees do not suppress SIVcpz replication *in vitro*, in contrast to CD8$^+$ lymphocytes. We also show that endogenous β-chemokines are not the predominant factors mediating *in vitro* suppression.

INTRODUCTION

HIV-1- AND SIVcpz-INFECTED CHIMPANZEES are relatively resistant to AIDS and only rarely develop disease.[1–6] A possible explanation may be that peripheral blood mononuclear cells (PBMCs) from chimpanzees have a potent capacity to suppress virus replication *in vivo*, resulting in the maintenance of a low viral load.[7] In HIV-infected humans, *in vitro* virus replication can be suppressed in a noncytolytic way by autologous CD8$^+$ T cells[8–10] and through various mechanisms. One of these involves soluble substances that are potent suppressor factors of virus replication *in vitro* and include the CD8$^+$ T cell antiviral factor (CAF), and β-chemokines such as "regulated on activation normal T expressed and secreted" (RANTES), macrophage inflammatory protein 1α (MIP-1α) and MIP-1β.[8,11] β-Chemokines are the natural ligands of the CC chemokine receptor 5 (CCR5), a cell surface molecule that is used by non-syncytium-inducing (NSI) macrophage-tropic strains of HIV-1 as a coreceptor to gain cell entry. These substances can inhibit NSI strain replication by early postbinding blocks in fusion and virus entry, which involve the β-chemokine receptor CCR5 (R5) on monocytes and CD4$^+$ lymphocytes.[6,12,–15]

In HIV-infected humans, *in vitro* CD8$^+$ T cell-mediated suppression of virus replication is more pronounced in asymptomatic patients or in long-term nonprogressors (LTNPs)[8,16,17] and declines with progression to AIDS,[18] suggesting that efficient virus suppression is important in disease resistance.[19] More specifically, a higher level of β-chemokine secretion by CD8$^+$ T cells has been observed in asymptomatic patients, as well as in macaques immunized with attenuated strains of SIVmac,[20] and may be a possible correlate of resistance to disease in infected humans and nonhuman primates.[21,22] *In vitro* suppression of virus replication has also been observed in chimpanzees infected with SIVcpz-ant, a lentivirus closely related to HIV-1.[23–26] Because SIVcpz infection results in high infectious virus titers in chimpanzees,[27] in contrast to HIV-1, it may be a more suitable model with which to study host–virus interaction during chronic infections in chimpanzees. In a previ-

[1]Departments of Microbiology and Clinical Sciences, Institute of Tropical Medicine, 2000 Antwerp, Belgium.
[2]Present address: VIRCO Group of Companies, B-2800 Mechelen, Belgium.
[3]Department of Virology, Biomedical Primate Research Center, Rijswijk 2280 GH, The Netherlands.

ous study, we observed that although CD8$^+$ T cells directly exert a potent suppressive effect on virus replication, we cannot rule out that other cell subsets, such as natural killer (NK) cells, may be implicated in the *in vitro* suppression.[24] We also reported that in a naturally SIVcpz-ant-infected chimpanzee, the decline in PBMC and plasma-associated infectious virus titers coincided with a significant increase in the absolute number of NK cells, which could suggest that these cells contribute to the immune control of SIVcpz infection in this species.[23]

Several studies have shown that NK cells are potent producers of β-chemokines and can suppress virus replication in infected humans.[28,29] We hypothesized that resistance to disease progression of SIVcpz-infected chimpanzees was associated with efficient suppression of virus replication by NK cell-secreted β-chemokines.

To investigate this we measured the tropism, coreceptor usage, and sensitivity to β-chemokines of SIVcpz *in vitro*. We evaluated the contribution of NK cells in the production of endogenous β-chemokines by chimpanzee PBMCs. Finally, we analyzed the role of these chemokines, in relation to the *in vitro* suppressive capacity of CD8$^+$ T and NK cells, in SIVcpz replication in chimpanzee PBMCs.

MATERIALS AND METHODS

Chimpanzees

Two chimpanzees, one (Ch-No) naturally infected with SIVcpz-ant since 1989[27] and another (Ch-Ni) experimentally infected with peripheral blood mononuclear cells (PBMCs) from Ch-No in October 1995, were included in this study. Three chimpanzees (Ch-Bu, Ch-Ma, and Ch-Co) have been experimentally infected with HIV-1 between 1982 and 1984. In addition, four healthy uninfected animals served as controls. Fresh EDTA (ethylenediaminetetraacetic acid)–peripheral blood was obtained from these nine chimpanzees during the entire study period.

All protocols were approved by the Ethical Review Boards of the Institute of Tropical Medicine (Antwerp, Belgium) and the Biomedical Primate Research Center (BPRC, Rijswijk, The Netherlands) and were implemented according to international guidelines.

Coreceptor usage studies of SIVcpz-ant

Characterization of coreceptor usage of SIVcpz-ant was performed by using GHOST cells transfected with CD4, long terminal repeat (LTR)-driven enhanced green fluorescent protein (GFP), and either CC chemokine receptor 5 (CCR5) or CXC chemokine receptor 4 (CXCR4).[30] The GHOST cells are human osteosarcoma (HOS) cells transfected with the human CD4 gene, a coreceptor (either CXCR4 or CCR5), and the indicator gene, green fluorescent protein (GFP), which is under the control of an HIV-2 promotor, inducible by Tat. The GHOST cells expressing CXCR4 and CCR5 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% glutamine, 2% penicillin plus streptomycin, geneticin (200 μg/ml), hygromycin (25 μg/ml), and puromycin (1 μg/ml). The cultures were maintained at 37°C in a 5% CO_2 humidified incubator. Cell monolayers, when confluent, were resuspended by using 0.25% trypsin. GHOST cells were seeded in 24-well plates at a concentration of 35,000 cells/well/0.5 ml. On the following day, the cell monolayers were incubated with 500 μl of undiluted SIVcpz-ant stock (SIVcpz antigen positive) from cocultures of phytohemagglutinin (PHA)-stimulated human PBMCs and PBMCs from the naturally SIVcpz-ant-infected chimpanzee. Virus was allowed to adsorb overnight, after which the virus-containing medium was washed once with phosphate-buffered saline (PBS). Subsequently, 1 ml of complete medium, as described above, was added. The cells were harvested on day 6 postinfection. On the day of harvest, cell monolayers were washed again with PBS, resuspended in 300 μl of 1 mM EDTA in PBS, and fixed in formaldehyde at a final concentration of 2%. *In vitro* infection of GHOST cells was monitored by determining the percentage of GFP-positive cells by flow cytometry. GHOST cells transfected with CD4 and LTR-driven enhanced GFP only (parental cell line) served as coreceptor-negative GHOST cell controls. HIV-1BaL and HIV-1Mn primary isolates were used as positive virus controls for CCR5 and CXCR4 usage, respectively.

SIVcpz sensitivity to recombinant β-chemokines in vitro

Recombinant human RANTES, recombinant macrophage inhibitory protein 1α (rMIP1α), and rMIP-1β were obtained from Peprotech (London, UK). To examine their effect on virus production, 1 μg of each recombinant β-chemokine was added at the initiation of a culture of enriched CD4$^+$ T cells from the naturally infected chimpanzee in a final volume of 2 ml. CD4$^+$ T cell enrichment was done by an immunomagnetic technique (Dynal, Oslo, Norway). These β-chemokine concentrations are 3 to 10 times higher than the concentration required to inhibit β-chemokine-sensitive HIV-1 strains.[11,13] Cultures of purified CD4$^+$ T cells in the absence of β-chemokines acted as positive controls. Recombinant β-chemokines were replenished every 3 to 4 days on the exchange of 50% of culture medium (0.5 μg of each β-chemokine in 1 ml of medium).

Detection of intracellular RANTES expression in chimpanzee lymphocytes

For the intracellular detection of RANTES, freshly obtained EDTA–whole blood from infected (SIVcpz-ant and HIV-1) and noninfected chimpanzees was washed twice with Hanks' buffered salt solution (HBSS; GIBCO-BRL, Life Technologies, Paisley, UK) and a third time with RPMI 1640 (GIBCO-BRL) containing 10% bovine calf serum (BCS; HyClone, Logan, UT) and reconstituted to its original volume. Briefly, 100 μl of washed blood was diluted 1:5 with RPMI 1640 and 10% BCS. Subsequently, 2.5 μl monensin (2 μM/ml, final concentration; Sigma, St. Louis, MO) was added and the cells were left overnight at 37°C and 5% CO_2 in an incubator.

Cell surface staining was performed on whole blood by incubating the cells with 5 μl of monoclonal anti-CD3–peridinin chlorophyll protein (PerCP) and CD8–fluorescein isothiocyanate (FITC) (Becton Dickinson, Erembodegem, Belgium) for 15 min at room temperature. The whole blood specimen was treated with 2 ml of lysing solution from Becton Dickinson for 10 min in the dark to remove the red blood cells, centrifuged, and washed with PBS–1% bovine serum albumin (BSA). Lysed whole blood was fixed with 100 μl of Cytoperm (Serotec, Raleigh, NC) reagent A for 15 min at room temperature,

washed, and permeabilized with 100 μl of reagent B for 30 min at room temperature. Subsequently, permeabilized cell aliquots were washed and incubated for 15 min at room temperature with phycoerythrin (PE)-conjugated monoclonal antibodies to RANTES (PharMingen, Carlsbad, CA). Finally, cells were washed in PBS–1% BSA, fixed with 1% paraformaldehyde in PBS, and analyzed by FACScan (Becton Dickinson).

CD8 expression on natural killer cells of humans and chimpanzees

The expression levels of CD8 on human NK cells were measured in samples from 21 healthy human blood donors and in 287 consecutive routine blood samples from HIV-seropositive patients of the outpatient clinic of the Institute of Tropical Medicine in Antwerp. Staining of chimpanzee NK cells was performed on four samples per animal obtained at 3-month intervals and was used to calculate the mean value per chimpanzee.

EDTA–whole blood samples were stained with anti-CD3–FITC and anti-CD16/56–PE to count all NK cells ($CD3^-CD16^+CD57^+$ cells) and with anti-CD3–FITC and CD8–PE to count the $CD8^+$ NK cells ($CD3^-CD8^+$). All reagents were from Becton Dickinson. The cell surface staining was performed as described in the previous section.

In vitro *suppression studies of SIVcpz-ant replication by autologous cell subsets*

Purified $CD4^+$ and $CD8^+$ T cells were obtained from fresh PBMCs by positive immunomagnetic separation (CD4 and CD8 positive isolation kit; Dynal).

NK cells were purified from PBMCs by negative immuno-

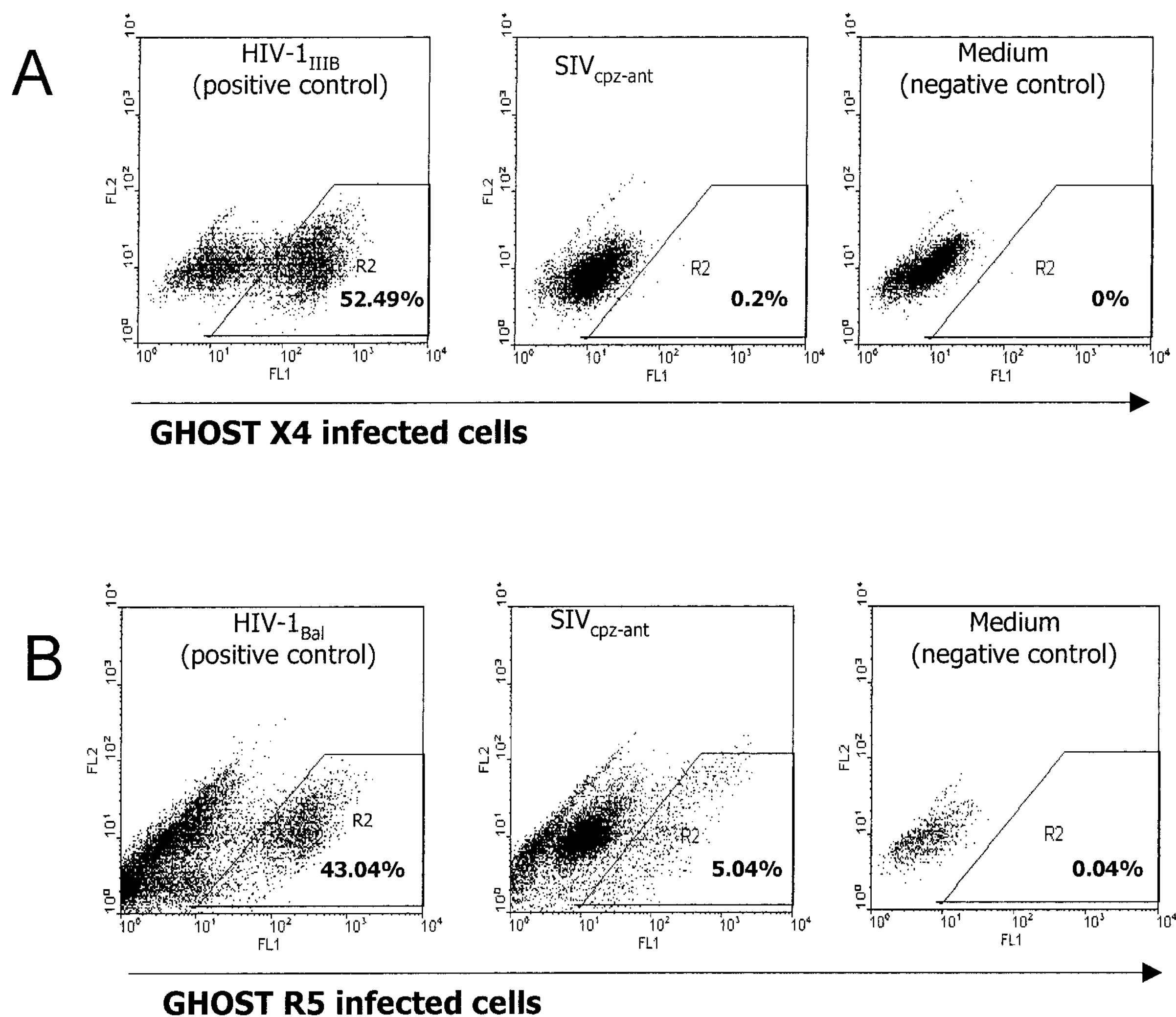


FIG. 1. SIVcpz-ant coreceptor usage analysis. GHOST cell lines expressing the CXCR4 (**A**) and CCR5 (**B**) coreceptor were infected with a positive control isolate (HIV-1IIIB and HIV-1Bal, respectively, dot plots on the left) or with SIVcpz-ant (dot plots in the middle). The background level of fluorescence was determined by incubating the cells with culture medium (dot plots on the right). The results were determined by FACScan analysis. Cells were first gated on the basis of forward and side scatter (data not shown). The number of infected cells was determined by the scattergram of fluorescence versus forward scatter after setting the gates of uninfected cells. More than 99 of 15,000 cells had to be fluorescent for a virus to be considered positive for infectivity in each of the GHOST cell lines. It is shown that SIVcpz-ant infects GHOST cell lines expressing CCR5 (5% positive cells) but not GHOST cell lines expressing CXCR4 (0.04% positive cells).

magnetic separation according to the instructions of the manufacturer (StemSep NK cell enrichment; StemCell Technologies, Vancouver, BC, Canada). The purification of NK cells is based on removal of T cells, B cells, and monocytes from PBMCs, using a cocktail of monoclonal antibodies directed against CD3, CD4, CD19, CD14, CD66b, and glycophorin. The purity of each subset was verified by flow cytometry after staining of PBMCs, purified CD4$^+$ T cells, CD8$^+$ T cells, and NK cells with fluorochrome-conjugated anti-CD3, anti-CD4, and anti-CD8 monoclonal antibodies. Subsequently cells were stimulated with PHA (0.5 μg/ml; Wellcome, Dartford, UK) in complete medium (RPMI 1640, 10% BCS, penicillin [100 U/ml], streptomycin [100 μg/ml], 2 mM glutamine). After 3 days, the cells were washed and cultured as PBMCs, purified CD4$^+$ T cells alone, purified CD4$^+$ T cells together with purified CD8$^+$ T cells, or purified CD4$^+$ T cells together with purified NK cells. Recombinant interleukin 2 (IL-2, 10 U/ml; R&D Systems, Minneapolis, MN) was added to all cultures. Every 3 days, 50% of each culture supernatants was replaced with fresh IL-2-supplemented medium. Culture supernatants were frozen at −20°C until HIV antigen detection by an in-house HIV antigen capture enzyme-linked immunosorbent assay (ELISA)[31] and RANTES dosage by commercial test.

To verify that optimal levels of β-chemokine secretion by the NK cells was achieved in an *in vitro* suppression experiment using standard PHA/IL-2 stimulation, we performed suppression experiments with differential stimulation of the NK cells. In the first experiment, 0.5×10^6 NK cells from Ch-No were stimulated in parallel with PHA (0.5 μg/ml) and with a combination of IL-12/IL-15 (10 μg/ml of each cytokine) for 3 days before they were put in contact with PHA-stimulated CD4$^+$ T cells in IL-2-supplemented medium for 4 weeks as described in the previous section. Cultures of enriched CD4$^+$ T and reconstituted CD4$^+$/CD8$^+$ T cells acted as control for viral antigen release. Culture supernatants were frozen at −20°C until the dosage of viral antigen (in-house)[31] and RANTES (R&D Systems) was administered.

Anti-β-chemokine-neutralizing antibodies

Neutralizing polyclonal goat antiserum specific for human RANTES, MIP-lα, or MIP-1β was obtained from R&D Systems. The lyophilized preparation of each anti-chemokine antibody was reconstituted in PBS and a stock mixture was prepared containing anti-RANTES (1 mg/ml), anti-MIP-1α (1 mg/ml), anti-MIP-lβ (1 mg/ml) antibodies. The final concentration of antibodies to RANTES, MIP-1α, and MIP-1β used in experiments was 20 μg/ml, sufficient to neutralize, respectively, 0.02 μg/ml of rRANTES, 0.05 μg/ml of rMIP-1α, and 0.1 μg/ml of rMIP-1β, according to the manufacturer and as previously described.[11,13] In the neutralization experiments, the antibody mixture was added at the initiation of the culture of reconstituted CD4$^+$/CD8$^+$ T cells. Cultures of purified CD4$^+$ T cells and reconstituted CD4$^+$/CD8$^+$ T cells in the absence of antibodies acted as positive controls for virus replication and suppression, respectively. Antibodies were replenished every 3 days on medium exchange.

Viral antigen released in culture supernatant was monitored by an in-house HIV antigen capture assay.[31]

The concentration of RANTES in the supernatants of the suppression experiments (enriched CD4$^+$ T cells, reconstituted CD4$^+$/CD8$^+$ T and CD4$^+$ T/NK cells, and unfractionated PBMCs) was measured by commercial enzyme-linked immunosorbent assay according to the manufacturer instructions (R&D Systems). Every 3 to 4 days, 50% of the culture medium was collected in aliquots and kept frozen at −20°C without being thawed, except immediately before testing.

Statistical analysis

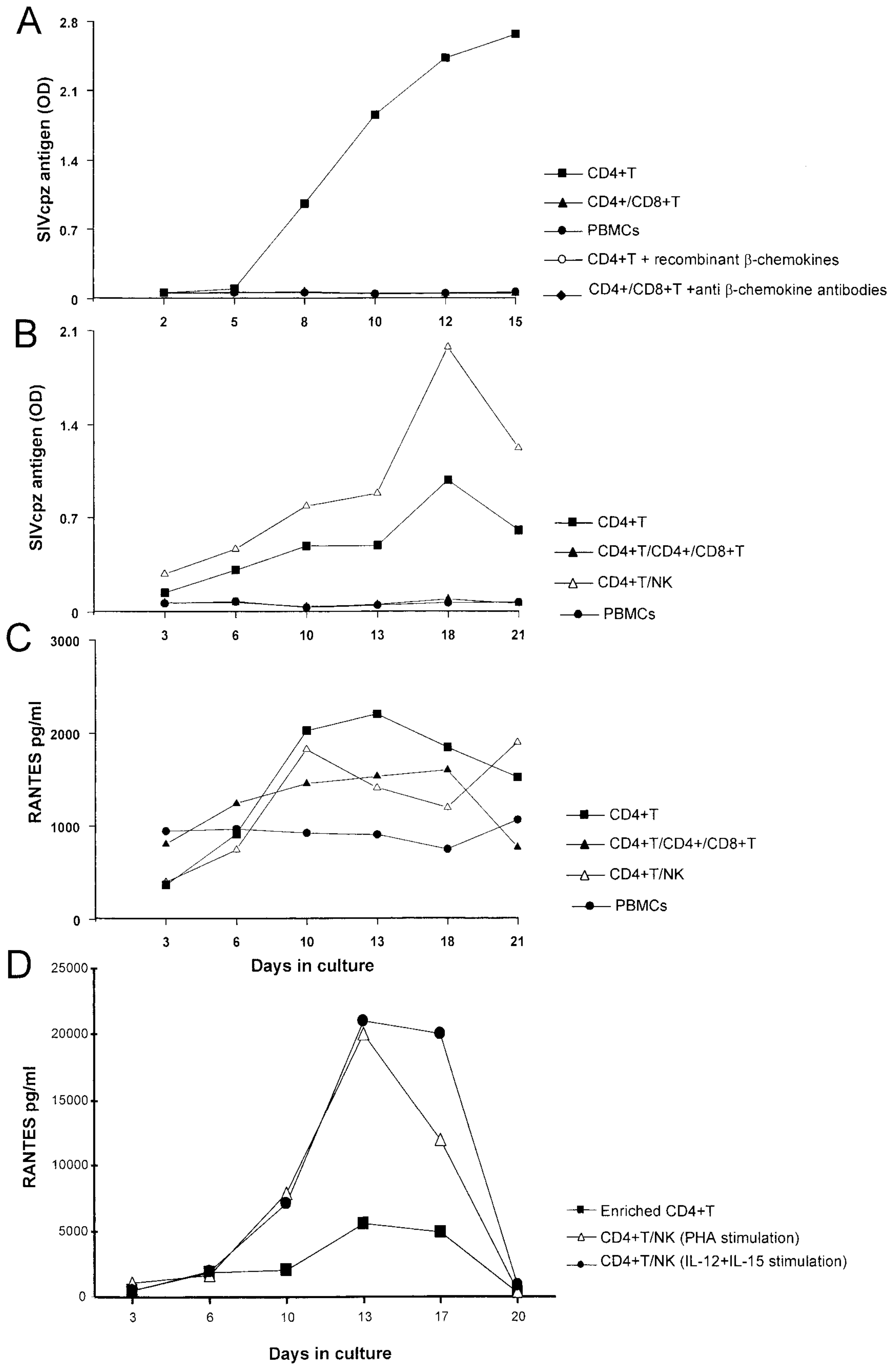
Differences between two groups (e.g., comparison of NK cell percentages and absolute numbers between chimpanzees and humans) were tested for statistical significance by the nonparametric Mann–Whitney U test. α was set at 0.05.

RESULTS

SIVcpz-ant uses CCR5 as coreceptor

To investigate the coreceptor usage of SIVcpz-ant we incubated CCR5- and CXCR4-transfected GHOST cells with SIVcpz-ant. HIV-1BaL and HIV-1IIIB acted as positive controls for CCR5 and CXCR4 usage, respectively. The level of

FIG. 2. *In vitro* suppression SIVcpz-ant replication. (**A**) CD8$^+$ T cell-mediated *in vitro* suppression of SIVcpz-ant replication was measured in cocultures of purified CD4$^+$ and CD8$^+$ T cells from the SIVcpz-ant-infected chimpanzee Ch-No. The cell subsets were positively purified, and then stimulated with PHA for 3 days. Subsequently, as a measure of virus replication, virus antigen was monitored in cultures of PBMCs (●), purified CD4$^+$ T cells (■), and purified CD4$^+$ T cells plus CD8$^+$ T cells (▲) (concentration, 1:1) by ELISA. The *in vitro* suppressive effect of exogenous β-chemokines on virus production was measured in purified CD4$^+$ T cells supplemented with MIP-1α, MIP-1β, and RANTES (○). The effect of a mixture of blocking antibodies to these chemokines on virus suppression was tested in a culture of reconstituted CD4$^+$/CD8$^+$ T cells (◆). (**B**) The effect of NK cells on *in vitro* suppression of SIVcpz-ant replication was assessed with PBMCs from the SIVcpz-ant-infected chimpanzee Ch-Ni. NK cells were highly purified by negative separation, using immunomagnetic beads. All cell subsets were stimulated with PHA, and IL-2 was supplemented every 3 days. The concentration of virus antigen in culture supernatants was used as a measure of virus replication. PBMCs (●); purified CD4$^+$ T cells (■); CD4$^+$ T cells plus CD8$^+$ T cells (▲); CD4$^+$ T cells [plus] NK cells (△). (**C**) The extracellular concentration of RANTES was measured in culture supernatants of the suppression experiments presented in (**B**). PBMCs (○); purified CD4$^+$ T cells (■); CD4$^+$ T cells plus CD8$^+$ T cells (▲); CD4$^+$ T cells plus NK cells (△). (**D**) The extracellular concentration of RANTES was measured in the supernatant of enriched CD4$^+$ T (■) and reconstituted CD4$^+$ T/NK cultures, wherein NK cells had been stimulated with PHA (△) and a combination of IL-12 and IL-15 (●) for 3 days. In this experiment, NK cells did not show any capacity to suppress virus replication despite high levels of RANTES secretion, irrespective of the type of stimulation.


A
SIVcpz antigen (OD)
2.8
2.1
1.4
0.7
0
2
5
8
10
12
15
CD4+T
CD4+/CD8+T
PBMCs
CD4+T + recombinant β-chemokines
CD4+/CD8+T +anti β-chemokine antibodies
B
SIVcpz antigen (OD)
2.1
1.4
0.7
0
3
6
10
13
18
21
CD4+T
CD4+T/CD4+/CD8+T
CD4+T/NK
PBMCs
C
RANTES pg/ml
3000
2000
1000
0
3
6
10
13
18
21
Days in culture
CD4+T
CD4+T/CD4+/CD8+T
CD4+T/NK
PBMCs
D
RANTES pg/ml
25000
20000
15000
10000
5000
0
3
6
10
13
17
20
Days in culture
Enriched CD4+T
CD4+T/NK (PHA stimulation)
CD4+T/NK (IL-12+IL-15 stimulation)

background fluorescence was determined by incubating the GHOST cells with culture medium. More than 99 cells per 15,000 cells had to be fluorescent for a virus to be considered positive for infectivity in each of the GHOST cell lines. Infection of CXCR4-transfected GHOST cells with SIVcpz-ant and HIV-1IIIB resulted in 0.2 and 52.49% positive cells, respectively, with a null background fluorescence (Fig. 1A). Infection of CCR5-expressing GHOST cells with SIVcpz-ant and HIV-1Bal resulted in 5.04 and 43.04% positive cells, respectively, with a background of 0.04% fluorescing cells (Fig. 1B). Less than 0.25% of the negative control cells (parental GHOST cell line) were fluorescent after incubation with the same dose of viruses. These results indicated that SIVcpz-ant utilizes the CCR5 coreceptor.

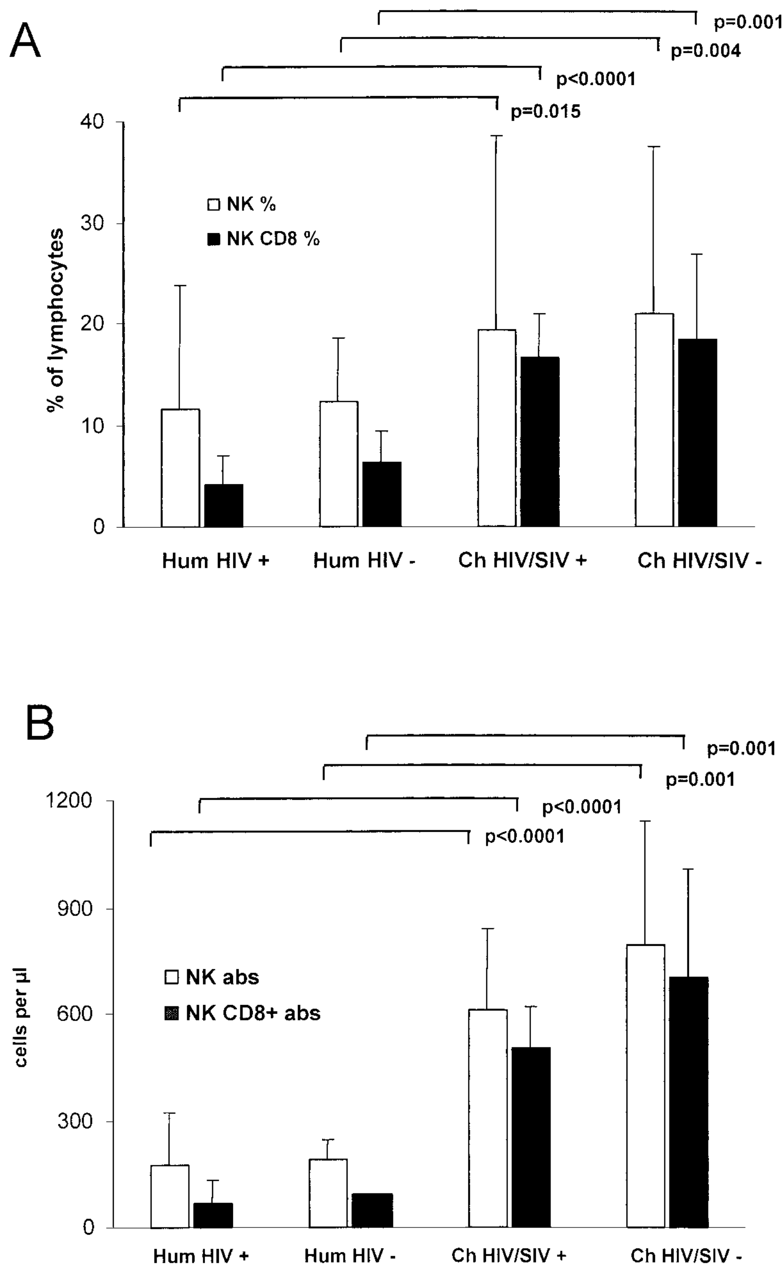


FIG. 3. Comparison of percentage and absolute number of NK cells between humans and chimpanzees. SIVcpz-ant infection is not associated with significant changes in percentage (**A**) and absolute number (**B**) of NK cells in chimpanzees. Results represent the mean + standard deviation of 21 healthy human blood donors (Hum HIV^-), of 287 HIV-seropositive patients (Hum HIV^+). The mean of four samples per animal obtained at 3-month intervals was used to calculate the mean value per chimpanzee. These mean values were used to calculate the mean and standard deviation of four HIV-1/SIVcpz-ant seronegative control chimpanzees (Ch HIV/SIV^-), and of two SIVcpz-ant- and three HIV-1-infected chimpanzees (Ch HIV/SIV^+).

β-Chemokines suppress SIVcpz-ant replication in vitro

We investigated whether addition of exogenous β-chemokines was capable of suppressing SIVcpz-ant virus antigen production in PHA-stimulated purified $CD4^+$ T cell cultures from SIVcpz-ant-infected chimpanzees. The results of a representative experiment are given in Fig. 2A. When a mixture of rRANTES, rMIP-1α, and rMIP-1β was added to a culture of purified PHA-stimulated $CD4^+$ T cells, no virus antigen was detected in the culture supernatants, whereas virus replication was detected in $CD4^+$ T cells cultured in the absence of recombinant β-chemokines. This clearly indicated that the β-chemokines are able to block SIVcpz-ant replication in chimpanzee cells *in vitro.*

Chimpanzees have a higher percentage of NK cells than humans and these cells express relatively more RANTES than do T cells

Figure 3 shows that chimpanzees have significantly more NK cells than humans do. The measure of the intracellular expression of β-chemokines in $CD4^+$ and $CD8^+$ T cells, and in an NK cell subset ($CD8^+CD3^-$ lymphocytes), also shows that NK and $CD8^+$ T cells of chimpanzees express relatively more RANTES than do $CD4^+$ lymphocytes. In uninfected chimpanzees, intracellular RANTES expression was significantly higher in $CD8^+$ T cells (median, 80%) and $CD8^+CD3^-$ NK cells (median, 91%) than in $CD4^+$ T cells (median, <10%) (Fig. 4). Intracellular RANTES expression in $CD4^+$ T cells, $CD8^+$ T cells, and $CD8^+$ NK cells did not significantly differ between HIV-1- or SIVcpz-ant-infected chimpanzees and uninfected control chimpanzees (Fig. 4).

Chimpanzee $CD8^+$ T cells but not NK cells suppress SIVcpz-ant replication in vitro

PHA-stimulated PBMCs from SIVcpz-ant-infected chimpanzees do not consistently produce virus *in vitro.* This was repeatedly shown with at least five consecutive blood samples from two SIVcpz-ant-infected chimpanzees (results not shown). In contrast, when PHA-stimulated purified $CD4^+$ T cells were used, cultures consistently produced high levels of viral antigens during 2 weeks of culture. To investigate whether the *in vitro* suppression of SIVcpz replication in PHA-stimulated PBMCs was due to both $CD8^+$ T cells and NK cells or only to $CD8^+$ T cells we performed various subset purifications. When purified autologous $CD8^+$ T cells were added to purified PHA-stimulated $CD4^+$ T cells, viral antigen production was blocked or significantly reduced. A representative example of these experiments, using cells from the naturally and experimentally infected chimpanzees, is shown in Fig. 2A and B. In these experiments purity of the $CD4^+$ T cells was 94 and 98% respectively, with less than 5% contamination by $CD8^+$ T cells. Purity of the $CD8^+$ T cells was 96 and 98%, respectively, with less than 3% contamination by $CD8^+$ NK cells (data not shown). The data show that chimpanzee $CD8^+$ T cells have a potent capacity to suppress SIVcpz-ant replication *in vitro.*

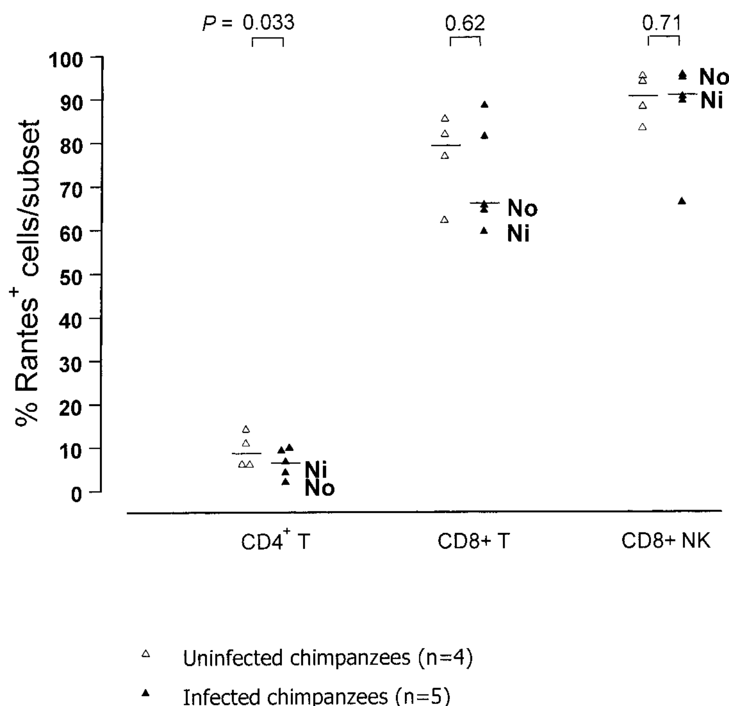


FIG. 4. Constitutive intracellular levels of RANTES were measured on nonstimulated $CD4^+$ T, $CD8^+$ T, and NK cell subsets of HIV-1-infected ($n = 3$) and SIVcpz-ant-infected ($n = 2$) (▲) and noninfected (△) control ($n = 4$) chimpanzees. Results are presented as the percentage of positive cells in each subset and represent individual data points and medians of chimpanzee data. Comparison between groups of data was done by the nonparametric Mann–Whitney U test. NK and $CD8^+$ T cells of both infected and uninfected chimpanzees express relatively more RANTES than did $CD4^+$ lymphocytes.

Because PBMCs of chimpanzees contain a relatively high percentage of $CD8^+$ NK cells (Fig. 3), and because this cell subset produces high levels of RANTES (Fig. 4), we also examined whether NK cells contribute significantly to the "blocking" of viral antigen production in our PHA-stimulated PBMC cultures. To test this we purified $CD4^+$ T cells, $CD8^+$ T cells, and NK cells (thus including $CD8^+$ and $CD8^-$ NK cells) from both SIVcpz-ant-infected chimpanzees. Subsequently, we measured virus antigen production in PHA-stimulated PBMCs and $CD4^+$ T cell cultures, and in cocultures of $CD4^+$ T cells and NK cells on one hand and in cocultures of $CD4^+$ T cells and $CD8^+$ T cells on the other hand. In these experiments, the purity of the NK cells was greater than 99% (data not shown). PHA-stimulated PBMCs and cocultures of PHA-stimulated $CD4^+$ plus $CD8^+$ T cells at a ratio of 1:1 did not produce detectable levels of viral antigen in culture supernatants. In contrast, virus antigen production was not suppressed in cocultures of $CD4^+$ T cells plus NK cells at the same ratio (1:1). Figure 2B shows the results of a representative experiment using cells from Ch-Ni. In this experiment the purity of $CD4^+$ T, $CD8^+$ T, and NK cells was 94, 96, and 99, respectively. These experiments show that NK cells, as compared with $CD8^+$ T cells, do not contribute significantly to *in vitro* suppression of virus antigen production in PHA-stimulated PBMC cultures from SIVcpz-ant-infected chimpanzees. Differential stimulation of NK cells with PHA or IL-12/IL-15 resulted in the absence of virus suppression (in contrast to *in vitro* virus suppression by $CD8^+$ T cells) and in comparable levels of RANTES in the supernatant of reconstituted $CD4^+$ T/NK cultures (Fig. 2D).

β-Chemokines are not the only factors involved in in vitro *SIVcpz-ant suppression*

When a combination of β-chemokine-blocking antibodies (anti-RANTES, anti-MIP-1α, and anti-MIP-1β) was added to cocultures of purified $CD4^+$ plus $CD8^+$ T cells, the suppressive effect of $CD8^+$ T cells on virus antigen production was not neutralized. In these experiments, the blocking antibodies were used at a final concentration of 20 μg/ml, sufficient to block, respectively, rRANTES (0.2 μg/ml), rMIP-1α (0.05 μg/ml), and rMIP-1β (0.1 μg/ml) according to the manufacturer. These values are on average 100-fold greater than the average concentration of β-chemokines produced by cultured PBMCs of chimpanzees (Fig. 2C). Furthermore, extracellular concentrations of endogenously produced RANTES did not correlate with $CD8^+$ T cell-mediated suppression of SIVcpz-ant antigen production *in vitro* (Fig. 2B and C).

DISCUSSION

In previous reports, we have shown that $CD8^+$ lymphocytes from SIVcpz-ant-infected chimpanzees could suppress endogenous SIVcpz-ant replication *in vitro*.[23,24] This phenomenon was found to be comparable to the suppression of HIV-1 replication by human and chimpanzee $CD8^+$ T cells.[7,32–35] *In vitro* suppression of virus replication in SIVcpz-infected chimpanzees improves over time in association with decreasing infectious virus titers.[24] In HIV-1-infected humans, the suppressive capacity of PBMCs is more pronounced in LTNPs as compared with rapid progressors,[21] suggesting that a noncytolytic antiviral mechanism may play a major role in resistance to disease progression.

Because we were not able to detect virus-specific cytotoxicity in SIVcpz-infected chimpanzees,[23] we hypothesized that resistance of SIVcpz-infected chimpanzees to AIDS might correlate with potent suppression of virus replication. We argued that chimpanzees have a significantly larger population of NK cells as compared with humans. We reasoned that these cells could still play a significant role in the inhibition of virus replication by secreting β-chemokines.

In the present study we demonstrate that SIVcpz-ant, isolated from a naturally infected chimpanzee, utilizes the R5 coreceptor in accordance with its NSI phenotype,[36] a criterion associated with HIV-1 suppression by β-chemokines. Specifically, R5-dependent macrophage (M)-tropic HIV-1 strains have been shown to be susceptible to β-chemokines such as RANTES, MIP-1α, and MIP-1β, predominantly secreted by $CD8^+$ T cells.[8] Our data confirm that in *vitro* SIVcpz-ant replication can indeed be suppressed by addition of exogenous β-chemokines, similar to what has been observed for R5 HIV-1 isolates.

Here we report that NK cell levels are 3 to 10 times higher in chimpanzees compared with humans. We also demonstrate that NK cells are potent producers of RANTES, just like $CD8^+$ T lymphocytes in both infected and uninfected chimpanzees. However, in the present study using NK cells as effector cells, we could not demonstrate a consistent suppression of virus replication by NK cells. This was despite the fact that the levels of RANTES secreted by NK cells and $CD8^+$ T cells were found to be comparable. The levels of RANTES, produced *in vitro* by $CD8^+$ T cells showing a suppressive capacity, were lower than the 3 to 5 ng/ml described to be the minimum concentrations of the recombinant human β-chemokine needed to obtain a significant reduction in the replication of sensitive viruses.[10,12,37] In contrast, levels of RANTES secretion higher than 20 ng/ml were associated with an inability of NK cells to suppress virus replication *in vitro*. The addition of neutralizing antibodies to β-chemokines in culture supernatant did not reverse the suppressive activity of $CD8^+$ T cells *in vitro*, suggesting that β-chemokines are not the principal suppressor factors in these experiments. This conclusion was further substantiated by the observation that effective *in vitro* SIVcpz-ant suppression by $CD8^+$ T cells did not coincide with increased RANTES concentrations in the culture supernatants.

The fact that β-chemokines are not the main factor involved in suppression may explain why NK cells do not suppress virus replication i*n vitro*. In conclusion, although in vitro replication of SIVcpz-ant can be suppressed by exogenous β-chemokines, they do not appear to be to be the predominant $CD8^+$ T cell suppressor factor responsible for *in vitro* suppression of SIVcpz-ant. Second, with regard to NK cells, we have no evidence that the high resistance of chimpanzees to SIVcpz-ant-induced disease is mediated by endogenous β-chemokines or is associated with the ability of these cells to suppress virus replication *in vitro*.

ACKNOWLEDGMENTS

This study was supported by the Belgian Fonds voor Wetenschappelijk Onderzoek (F.W.O.) Vlaanderen, grant nos. 3.036498 and G.0396.99, and EU grants BMH4-CT97-2005, BMH1-CT95-0206, and BMH4-CT97-2067.

REFERENCES

1. Villinger F, Brar SS, Brice GT, *et al*: Immune and heamatopoietic parameters in HIV-1 infected chimpanzees during clinical progression towards AIDS. J Med Primatol 1997;26:11–18.
2. Heeney JL: AIDS: A disease of impaired Th-cell renewal. Immunol Today 1995;16:515–520.
3. Koopman G, Haaksma AG, ten-Velden J, Hack CE, and Heeney JL: The relative resistance of HIV type 1-infected chimpanzees to AIDS correlates with the maintenance of follicular architecture and the absence of infiltration by $CD8^+$ cytotoxic T lymphocytes. AIDS Res Hum Retroviruses 1999;15:365–373.
4. Novembre FJ, Saucier M, Anderson DC, *et al.*: Development of AIDS in a chimpanzee infected with human immunodeficiency virus type 1. J Virol 1997;71:4086–4091.
5. O'Neil SP, Novembre FJ, Hill AB, *et al.*: Progressive infection in a subset of HIV-1 positive chimpanzees. J Infect Dis 2000;182: 1051–1062.
6. Davis IC, Girard M, and Fultz PN: Loss of $CD4^+$ T cells in human immunodeficiency virus type 1-infected chimpanzees is associated with increased lymphocyte apoptosis. J Virol 1998;72: 4623–4632.
7. Heeney JL, Bruck C, Goudsmit J, *et al.*: Immune correlates of protection from HIV infection and AIDS. Immunol Today 1997; 18:4–8.
8. Walker CM, Moody DJ, Stites DP, and Levy JA: $CD8^+$ lymphocytes can control HIV infection in vitro by suppressing virus replication. Science 1986;234:1563–1566.
9. Tsubota H, Lord CI, Watkin DI, Morimoto C, and Letvin NL: A cytotoxic T lymphocyte inhibits acquired immunodeficiency syndrome virus replication in peripheral blood lymphocytes. J Exp Med 1989;169:1421–1434.
10. Yang O, Kalams SA, Trocha A, *et al.*: Suppression of human immunodeficiency virus type 1 replication by $CD8^+$ T cells: Evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms. J Virol 1997;71:3120–3128.
11. Cocchi F, Devico AL, Garzinodemo A, *et al.*: Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by $CD8^+$ T cells. Science 1995;270: 1811–1815
12. Jansson M, Popovic M, Karlsson A, *et al.*: Sensitivity to inhibition by beta-chemokines correlates with biological phenotypes of primary HIV-1 isolates. Proc Natl Acad Sci USA 1996;93:15382–15387.
13. Mackewicz CE, Barker E, Greco G, Reyesteran G, and Levy JA: Do β-chemokines have clinical relevance in HIV infection? J Clin Invest 1997;100:921–930.
14. Leith JG, Copeland KFT, McKay PJ, Richards CD, and Rosenthal KL: $CD8^+$ T-cell-mediated suppression of HIV-1 long terminal repeat-driven gene expression is not modulated by the CC chemokines RANTES, macrophage inflammatory protein (MIP)-1 alpha and MIP-1 beta. AIDS 1997;11:575–580.
15. Oravecz T, Pall M, and Norcross MA: Beta-chemokine inhibition of monocytotropic HIV-1 infection. Interference with a postbinding fusion step. J Immunol 1996;157:1329–1332.
16. Kannagi M, Masuda T, Hattori T, *et al.*: Interference with human immunodeficiency virus (HIV) replication by $CD8^+$ T cells in peripheral blood leukocytes of asymptomatic HIV carriers in vitro. J Virol 1990;64:3399–3406.
17. Mackewicz, CE, Ortega HW, and Levy JA: $CD8^+$ cell anti-HIV activity correlates with the clinical state of the infected individual. J Clin Invest 1991;87:1462–1466.
18. Landay AL, Mackewicz CE, and Levy AJ: An activated $CD8^+$ T cell phenotype correlates with ant-HIV activity and asymptomatic clinical status. Clin Immunol Immunopathol 1993;69:106–116.
19. Levy JA, Mackewicz CE, and Barker E: Controlling HIV pathogenesis: The role of the noncytotoxic anti-HIV response of $CD8^+$ T cells. Immunol Today 1996;17:217–224.
20. Gauduin MC, Glickman RL, Means R, and Johnson P: Inhibition of simian immunodeficiency virus (SIV) replication by $CD8^+$ T lymphocytes from macaques immunized with live attenuated SIV. J Virol 1998;72:6315–6324.
21. Ullum H, Cozzi Lepri A, Vistor J, *et al.*: Production of β-chemokines in human immunodeficiency virus (HIV) infection: Evidence that high levels of macrophage inflammatory protein-1B are associated with a decreased risk of HIV disease progression. J Infect Dis 1998;177:331–336.
22. Garzino-Demo A, DeVico AL, Cocchi F, *et al.*: β-Chemokines and protection from HIV type 1 disease. AIDS Res Hum Retroviruses 1998;14(Suppl 2):S177–S184.
23. Kestens L, Vingerhoets J, Peeters M, *et al.*: Phenotypic and functional parameters of cellular immunity in a chimpanzee with a naturally acquired simian immunodeficiency virus infection. J Infect Dis 1995;172:957–963.
24. Ondoa P, Kestens L, Davis D, *et al.*: Longitudinal comparison of virus load parameters and CD8 T-cell suppressive capacity in two SIVcpz infected chimpanzees. J Med Primatol 2001;30:243–253.
25. Vanden Haesevelde MM, Peeters M, Jannes G, *et al.*: Sequence analysis of a highly divergent HIV-1–related lentivirus isolated from a wild captured chimpanzee. Virology 1996;221:346–350.
26. Gao F, Bailes E, Robertson DL, *et al.*: Origin of HIV-1 in the chimpanzee *Pan troglodytes troglodytes*. Nature 1999;397:436-441.
27. Peeters M, Fransen K, Delaporte E, *et al.*: Isolation and characterization of a new chimpanzee lentivirus (simian immunodeficiency virus isolate cpz-ant) from a wild- captured chimpanzee. AIDS 1992;6:447–451.
28. Fehniger TA, Herbein G, Yu HX, *et al.*: Natural killer cells from HIV-1^+ patients produce C-C chemokines and inhibit HIV-1 infection. J Immunol 1998;161:6433–6438.
29. Oliva A, Kinter AL, Vaccarezza M, *et al.*: Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication in vitro. J Clin Invest 1998;102:223–231.
30. Cecilia D, Kewal Ramani VN, O'Leary J, *et al.*: Neutralization profiles of primary human immunodeficiency virus type 1 isolates in the context of coreceptor usage. J Virol 1998;72:6988–6996.
31. Beirnaert E, Willems B, Peeters M, *et al.*: Design and evaluation of an in-house HIV-1 (group M and O), SIVmnd and SIVcpz antigen capture assay. J Virol Methods 1998;73:65–70.
32. Bogers WM, Koornstra WH, Dubbes RH, *et al.*: Characteristics of primary infection of a European human immunodeficiency virus type 1 clade B isolate in chimpanzees. J Gen Virol 1998;79: 2895–2903.
33. Castro BA, Walker CM, Eichberg JW, and Levy JA: Suppression of human immunodeficiency virus replication by $CD8^+$ cells from infected and uninfected chimpanzees. Cell Immunol 1991;132: 246–255.
34. Husch B, Eibl MM, and Mannhalter JW: CD3, CD8 double-positive cells from HIV-1-infected chimpanzees show group-specific inhibition of HIV-1 replication. AIDS Res Hum Retroviruses 1993; 9:405–413.

35. Buijs L, Bogers WM, Eichberg JW, and Heeney JL: $CD8^+$ cell-mediated immune responses: relation to disease resistance and susceptibility in lentivirus-infected primates. J Med Primatol 1997;26:129–138.
36. Peeters M, Janssens W, Vanden Haesevelde M, *et al.*: Virologic and serologic characteristics of a natural chimpanzee lentivirus infection. Virology 1995;211:312–315.
37. Mackewicz CE, Barker E, Levy JA, *et al.*: Role of β-chemokines suppressing HIV replication. Science 1996;274:1393–1395.

Address reprint requests to:
Luc Kestens
Laboratory of Immunology, Department of Microbiology
Institute of Tropical Medicine
Nationalestraat 155
B-2000 Antwerp, Belgium

E-mail: lkestens@itg.be