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Longitudinal comparison of virus load parameters and CD8 T-cell suppressive capacity in two SIVcpz-infected chimpanzees

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Abstract: In a longitudinal study we address the hypothesis that resistance to disease progression in lentivirus-infected chimpanzees is related to potent non-cytotoxic suppression of virus replication. In a long-term follow-up, the viral suppressive capacity in two simian immunodeficiency virus (SIV)cpz-infected chimpanzees was correlated with two polymerase chain reaction (PCR)- and two culture-based virus load measurements. In both animals, quantitative virus isolation (QVI) tended to decline slowly, whereas *in vitro* virus suppression was sustained or increased over time. In general, plasma virus loads in SIVcpz-infected animals were maintained for extended periods of time. Based on current assays that measure virus suppressive capacity in peripheral blood, it was not possible to conclude that virus suppression played a major role in the maintenance of the disease-free state in lentivirus-infected chimpanzees.

Key words: animal models of lentivirus infection – cell-mediated immunity

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Introduction

Of the simian immunodeficiency virus (SIV)cpz-infected chimpanzees identified to date, none has had any evidence of AIDS-related disease [39, 47]. Similarly, of the more than 100 chimpanzees infected with HIV-1, only one has developed hematological changes compatible with AIDS [22, 23, 38, 46, 62]. Long-term non-progressive HIV infections have also been detailed to occur in the infected human population, but in contrast to chimpanzees, occur very rarely [15]. In HIV-1-infected chimpanzees and humans, the absence of disease progression is associated with low virus load [6, 10, 61]. Mechanisms of resistance to the HIV-1-induced disease in infected humans or chimpanzees are commonly believed to be host factors. They may involve: (a) strong anti-viral CTL responses [52]; (b) a high level of circulating β-chemokines secreted by natural killer (NK) and CD8 + T cells [9, 17, 41]; (c) other soluble factors such as CD8-activated suppressive factor (CAF), macrophage-derived chemokine (MDC), or IL-16 [2, 32, 44], capable of suppressing virus replication.

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Determination of the *in vitro* suppressive capacity of the peripheral blood mononuclear cells (PBMC) on virus replication in HIV-1-infected individuals can be considered a broad measure of anti-viral suppressive activity of the immune system (cytotoxicity in combination with suppressive soluble factors). Cross-sectional and longitudinal studies in HIV-1-infected individuals have shown that *in vitro* CD8 + T cell suppressive activity on virus replication decreases with the onset of AIDS [31, 35, 36]. This CD8 + T cell-mediated suppression of virus replication may contribute to the maintenance of a low viral load and prevent *in vivo* disease progression.

We have previously shown that a naturally SIVcpz-infected chimpanzee (Ch-No) has detectable PBMC and plasma viremia [46, 47] and may be a relevant animal model to investigate fluctuations of cellular immune responses in relation to viremia. To date, data have been lacking on the longitudinal evolution of viral plasma RNA levels and cell-associated virus load in relation to *in vitro* CD8 + T cell suppressive capacity on virus replication in SIVcpz-infected chimpanzees.

We undertook a longitudinal follow-up of the *in vitro* suppressive capacity of PBMCs and *in vivo* virus replication as measured by plasma and cell-associated virus load in two SIVcpz-ant-infected chimpanzees. To gain further insight into the mechanism of *in vitro* suppression of virus replication, we examined the level of β-chemokine secretion and the MHC class I and II restriction associated with *in vitro* suppression, as well as the expression of CCR5 by chimpanzees' CD4 + T memory cells.

Materials and methods

Animals

Nine chimpanzees (*Pan troglodytes*) housed at the Biomedical Primate Research Center (BPRC) in Rijswijk, The Netherlands, were included in the study: (a) three previously described chimpanzees [22] infected with HIV-1. Two of these (Ch-Bu and Ch-Ma) were inoculated twice (12 and 30 October 1984) with bone-marrow cells infected with the prototype laboratory strain of HIV-1 (LAI strain). The third animal (Ch-Co) was inoculated twice (December 1982) with uncultured blood from an Amsterdam AIDS patient [22, 56]. (b) Two chimpanzees between 11 and 15 years of age (Ch-No and Ch-Ni) were infected with SIVcpz-ant. Ch-No was naturally infected in the wild and was identified by serological tests in 1989 [46]. Ch-Ni was experimentally infected with uncultured PBMCs from Ch-No in September 1995 [24]. (c) Four healthy uninfected chimpanzees that have been previously described acted as controls.

All nine animals were housed at the BPRC for the duration of the study. Both ethylenediaminetetraacetic acid (EDTA; used for virus titration in PBMCs, cell phenotyping, and suppression experiments) and heparinized (used for virus titration in plasma) peripheral blood samples were obtained consecutively every 3–4 months for infected and control animals. For Ch-Ni, extra bleedings were performed weekly immediately after infection.

Quantification of cell-associated and cell-free plasma viremia end-point dilution

Virus was isolated by co-culturing chimpanzee PBMCs or plasma with PBMCs from a healthy (HIV-negative) human blood donor that had been transformed 72 hours previously with phytohemagglutinin (PHA) and subsequently transferred to IL-2-supplemented medium (R&D Systems; 10 U/ml) as previously described [47]. SIVcpz infectious virus titers were determined by assessing plasma viremia and the number of infected PBMCs in circulation using the method described by Ho et al. [25]. The release of viral antigen in culture supernatants was examined by an in-house HIV antigen capture assay [3]. Results were reported as tissue culture infectious dose (TCID) per ml of plasma or per million PBMCs as previously described [46].

Determination of plasma viral RNA loads

EDTA plasma samples from Ch-No (n = 24) and Ch-Ni (n = 24) were further subjected to two different quantitative plasma virus load assays. The first plasma RNA assay was developed at the AIDS Reference Laboratory, Vrije Universiteit Brussel (VUB), Belgium. The detailed protocol will be described elsewhere. Subsequently, this viral load assay will be referred to as VL-ITM. Briefly, the viral load RNA test is based on a competitive reverse transcriptase-ploymerase chain reaction (RT-PCR) in the *pol* region, which is conserved between SIVcpz-ant and HIV-1. The competitor (or quantification standard; QS) is a synthetic RNA with the same primer binding sites as the HIV-1 or SIVcpz target, but with a 40-base pair insertion that allows the QS and the wild-type amplicons to be distinguished. Known amounts of QS RNA (1000, 500, 50, 5 copies) are added to the different aliquots of the sample before extraction. PCR products are run on an agarose gel and the amount of SIVcpz-ant RNA copies is determined by computerized scanning of the gel bands.

A second plasma RNA assay was a highly sensitive quantitative competitive (QC) RT-PCR based on an internal standard to compensate for sample degradation during purification and amplification, as well as for variation in amplification efficiency due to co-purified PCR inhibitory agents. A calibrated amount of internal standard was added before RNA purification and was co-amplified within the same reaction mixture as previously described [56, 57], with modifications [58]. Subsequently, this second method will be referred to as VL-BPRC.

Suppressive activity of chimpanzees' PBMCs on SIVcpz replication

Suppression of virus replication was monitored every 3–4 months on fresh cells beginning at month 44 of follow-up for Ch-No and month 2 for Ch-Ni. Fresh PBMCs were purified by a Ficoll-Hypaque gradient separation technique. Two million PBMCs were retained and the remainder (30–80 million) were used for CD4+ T- and CD8+ T-lymphocyte enrichment by a positive immunomagnetic selection technique (Dynabeads M-450 CD8 and CD4 and DETACHaBEAD, Dynal, Oslo). The composition of PBMC, CD4+T and CD8+T cell subsets was monitored by FACscan analysis. After 72 hours of PHA stimulation (1 μg/ml), cells were cultured on 24-well plates in 2 ml of RPMI 1640, 10% bovine calf serum (BCS), 100 U/ml penicillin, 100 μg streptomycin, 2 mM glutamine, 10 U/ml of IL-2. Experimental conditions were as follows: (a) PBMCs (2×10^6 per well); (b) CD4+ cells alone (0.5×10^6 per well); and (c) CD4+T plus CD8+T cells (ratio 1:1, 0.5×10^6 of each cell subset per well). Fifty percent of the medium was replaced twice a week. Cell cultures were maintained for 4 weeks and monitored for viral antigen release by an in-house HIV antigen capture assay [3]. Enriched CD4+T cell cultures acted as positive control for viral antigen production. A valid experiment required antigen to be released into the medium of the enriched CD4+T cell culture. Absence of virus replication in the PBMC and/or in the reconstituted CD4/CD8+T cell cultures until the end of the experiment indicated successful suppression.

Quantification of RANTES, MIP-1α, and MIP-1β in culture supernatants

CD4+T, CD4/CD8+T, and PBMC culture supernatants, collected at 3–4-day intervals, were stored at −20°C and thawed for the determination of RANTES, MIP-1α, and MIP-1β concentrations by an ELISA technique (Quantikine EIA-kit; R&D System, Minneapolis, MN) according to the instructions of the manufacturer.

Neutralization of virus suppression by anti-class I and class II antibodies

Human anti class I (clone W6/32) and class II (clone 9.3F10) [5, 60] monoclonal antibodies were added to PBMC and reconstituted CD4/CD8+ T-cell cultures in order to block possible MHC class I and II restricted pathways for virus suppression. Specific binding of these antibodies to chimpanzees' cells was tested by adding goat anti-mouse fluoro-iso-thiocyanate (GAM-FITC; DAKO, Glostrup, Denmark), to lymphocytes and monocytes from Ch-No and an HIV-1-infected person and analyzed by FACScan. Briefly, PBMC and CD4/CD8+ T cell cultures were supplemented at the initiation of the culture with antibodies singly or in combination at a final concentration of 10 μg/ml. Antibodies were replenished every 3 days upon medium exchange. Viral antigen release was monitored by an in-house antigen capture assay [3].

Determination of cellular expression of CCR5 and CXCR4

Expression of CCR5 and CXCR4 on CD4+T cells of the two SIVcpz-infected, the three HIV-1-infected and the four negative control chimpanzees was determined in three separate experiments using either indirect or direct staining with specific antibodies and flow cytometry analysis.

Indirect staining. Fifty microliters of fresh heparinized whole blood was sequentially incubated with (a) unconjugated monoclonal antibodies: 5 μl of anti CXCR4 (clone 12G5, NIH) or 2.5 μl of anti-CCR5 (clone 2D7, NIH) followed by a washing step with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA); (b) 5 μl of GAM-FITC (DAKO) diluted 1/5 in PBS with 1% BSA followed by a washing step; (c) 10 μl of mouse serum to block free binding sites on the GAM-FITC; (d) 5 μl CD45RO-FITC and 5 μl of CD4-PerCP; (e) 1 ml of lyzing solution (Becton Dickinson, 1/10 in distilled water). Cells were then washed and resuspended in 0.3 ml PBS 1% paraformaldehyde (PFA).

Direct staining. Fifty microliters of fresh heparinized whole blood were simultaneously incubated with (a) 5 μl of CD45-RO FITC (DAKO); (b) 5 μl of CXCR4-PE or CCR5-PE (Pharmingen, San Diego, CA); and (c) 5 μl of CD4-PerCP

(Becton Dickinson). Cells were then incubated with lyzing solution, washed in PBS 1% BSA, and resuspended in 0.3 PBS 1% PFA.

Statistical analyses

Comparisons between the two groups were made using the Mann–Whitney U-test. Correlation between the two sets of data (e.g., infectious virus titers and plasma RNA copy number) was evaluated by the Spearman rank-order correlation. $P < 0.05$ was taken as the level of statistical significance.

Results

PBMC and plasma infectious virus titers tend to decrease over time in the SIVcpz-ant-infected chimpanzees

Virus isolation and titration were performed on consecutive plasma and PBMC samples covering 11 and 4 years of follow-up of Ch-No (n = 29 PBMC and 28 plasma samples) and Ch-Ni (n = 19 PBMC and 19 plasma samples), respectively.

Virus was isolated in 96% (28/29) and 89% (17/19) of the PBMC samples from Ch-No and Ch-Ni, respectively. SIVcpz-ant could be isolated seven times from the plasma of Ch-No (25%) and nine times (47.3%) in Ch-Ni. In Fig. 1, negative plasma and PBMC virus titers are given values of 0.1 (less than 1 infectious particle per ml of plasma or per 4 million PBMCs). PBMC infectious virus titer medians were higher in Ch-No than in Ch-Ni (5 versus 0.5 TCID, $P = 0.022$). Plasma infectious virus titer medians were 0 and 0.5 in Ch-No and Ch-Ni, respectively.

Evolution of plasma and cell associated viremia did not follow the same pattern in the two SIVcpz-ant-infected chimpanzees (see Fig. 1). In Ch-No, peaks of infectious viremia alternated with low or undetectable virus titers over a period of 93 months, without any synchrony between the plasma and PBMC values. Infectivity tended to significantly decrease over time in both the cells (Spearman rank-order correlation: $R = -0.407$, $P = 0.029$) and plasma ($R = -0.432$, $P = 0.022$). After month 93, PBMC and plasma infectious virus titers both remained low or undetectable ($P > 0.05$) until the end of the observation period.

In Ch-Ni, the elevated plasma and PBMC viremia observed immediately after infection drastically decreased and kept a descending slope over time until the end of the observation period ($R = -0.821$, $P < 0.0001$ for PBMC titers; $R = -0.811$, $P < 0.0001$ for plasma titers). At month 19, plasma and cell infectivity stabilized at low or undetectable levels and remained significantly lower as compared with the first period of observation ($P = 0.004$ for PBMC titers; $P = 0.0004$ for plasma titers).

Plasma virus load does not correlate with cell-associated or cell-free titers in the productively infected chimpanzees

With the VL-ITM technique in Ch-Ni, all of the 12 samples taken before the time of infection were negative, while virus was detected in all the samples collected after the time of infection (n = 11) ranging between $\log_{10} 3.65$ and $\log_{10} 5.75$ virus copies per ml (median = $\log_{10} 4.27$, Fig. 1B). In Ch-No, all 24 plasma samples tested had detectable SIVcpz-ant RNA ranging from $\log_{10} 3.93$ to $\log_{10} 5.80$ virus copies per ml (median = $\log_{10} 4.86$, Fig. 1A).

With the VL-BPRC technique plasma, RNA copies were systematically lower in Ch-Ni compared as with the VL-ITM technique and ranged from $\log_{10} 4.70$ to $\log_{10} 2.71$ virus copies per ml (median = $\log_{10} 3.66$). In Ch-No, RNA copy number ranged from $\log_{10} 5.18$ to $\log_{10} 3.40$ virus copies per ml (median = $\log_{10} 4.49$) with VL from the two techniques differing by more than 1 $\log_{10}$ at three time points (months 99, 102, and 108).

There was no significant correlation between plasma VL and infectious virus titers in the plasma (VL-ITM: $R = -0.086$, $P = 0.697$ and $R = 0.284$, $P = 0.397$; VL-BPRC: $R = -0.059$, $P = 0.817$ and $R = 0.523$, $P = 0.098$ for Ch-No and Ch-Ni, respectively) nor in the PBMCs (VL-ITM $R = -0.058$, $P = 0.794$ and $R = 0.559$, $P = 0.074$; VL-BPRC: $R = 0.434$, $P = 0.072$ and $R = 0.187$, $P = 0.582$ for Ch-No and Ch-Ni, respectively).

Capacity of PBMC to suppress virus replication increases over time

The purity of the CD4 + T and CD8 + T cell fractions was always greater than 90%, with less than 8% contaminating CD8 + T in the enriched CD4 + T. Virus replication in PBMC and/or CD4/CD8 + T cell cultures during the experiment was considered as a lack of suppression. Results were reported as qualitative data (presence or absence of suppression) since the release of viral antigen was not quantified as viral antigen units/ml but as optical density.

The suppressive capacity of PBMC on SIVcpz-ant replication fluctuated in Ch-No during the first 93 months of follow-up and stabilized at month 96. This fluctuation was not related to the CD4/CD8 + T cell ratio in the PBMC (data not shown).

In Ch-Ni, suppression was observed from month 2 of follow-up (Fig. 1B). In both chimpanzees, there were occasional discrepancies between suppressive capacity of the CD8 + T and unfractionated PBMCs: at months 66, 80, 102, and 108 for Ch-No and 9 and 25 for Ch-Ni (Fig. 1A and B). Virus did not grow out of the enriched CD4 + T cells on two occasions for Ch-No (month 105 and 114, CD4 + T cell purity = 95 and 98%, respectively) and on three occasions in Ch-Ni (months 16, 40, 43; CD4 + T cell purity = 92, 91, and 90%, respectively).

In three trans-well suppression experiments (Ch-No month 80, Ch-Ni months 2 and 9, data not shown), we were unable to suppress virus replication by autologous CD8 + T cells when they were separated from the CD4 + T cells by a semi-permeable membrane. In the same experiments, virus was suppressed in CD4 + T in direct contact with CD8 + T cells as well as in unfractionated PBMCs, indicating that efficient suppression required cell-to-cell contact. For this reason, we decided to follow-up suppression involving direct cell-to-cell contact.

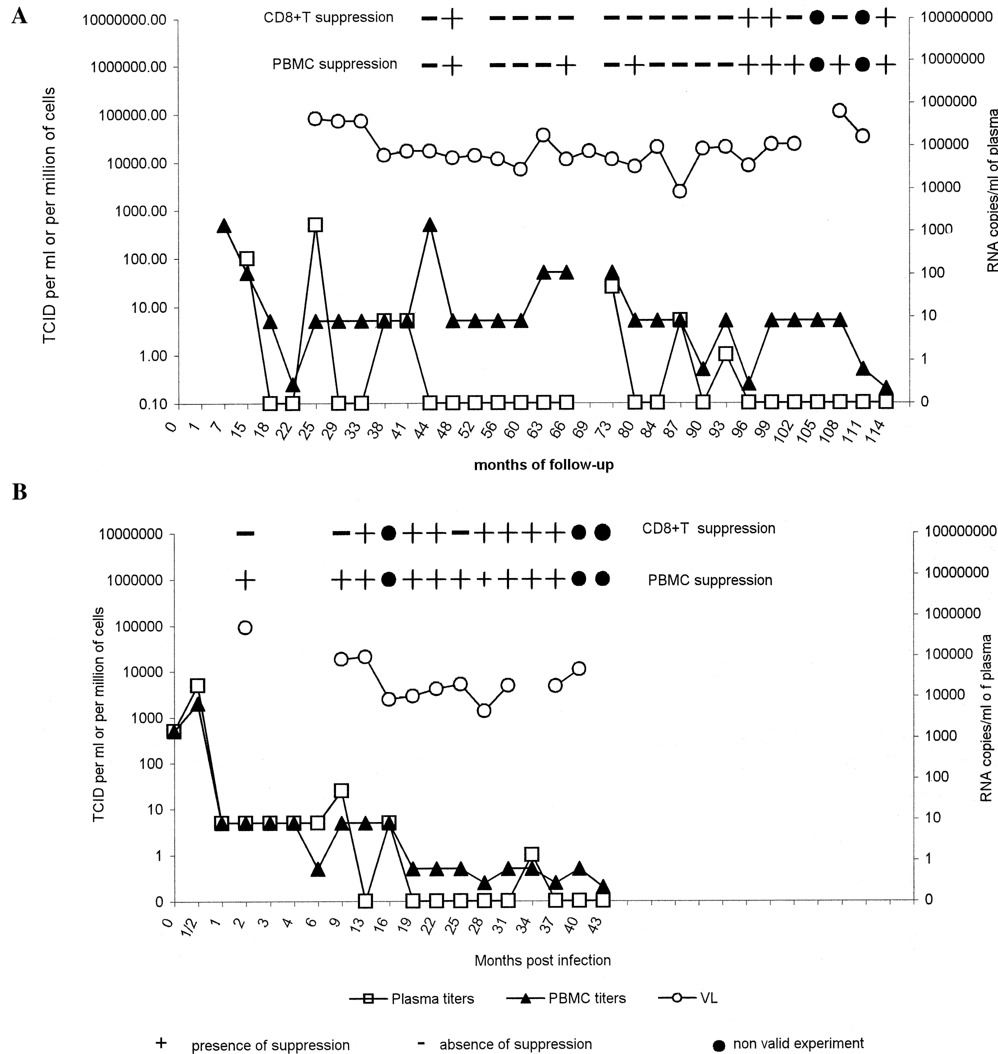

Fig. 1. Unfractionated and CD8 + T cell *in vitro* suppression capacity on virus replication and infectious virus titers and VL over time in Ch-No (**A**) and Ch-Ni (**B**). *In vitro* virus suppression was first monitored (+) at month 44 and month 2 of follow-up for Ch-No and Ch-Ni, respectively. Experiments were not valid (●) at months 105 and 114 for Ch-No and 16, 40 and 43 for Ch-Ni. Virus suppression was not monitored (—) at months 52 and 69 for Ch-No and months 3, 4 and 6 for Ch-Ni. *In vitro* suppression of virus replication by unfractionated PBMCs stabilizes at month 96 for Ch-No and is stable throughout the observation period for Ch-Ni. Infectious virus titers and plasma RNA level (VL) are plotted on logarithmic scales. Virus titers were first quantified at month 7 in the cells and month 15 in the plasma for Ch-No, and from the beginning of infection in Ch-Ni. Virus titration was not done at month 69 in Ch-No. Some plasma samples were not available for VL measurement in Ch-No and Ch-Ni. Virus titers of 0.1 in the plasma and 0.2 in the PBMC correspond to the absence of virus isolation from 1 ml of plasma and 4 million PBMCs, respectively. Plasma and PBMC infectious virus titers stabilize at low levels at months 96 in Ch-No and 19 in Ch-Ni.

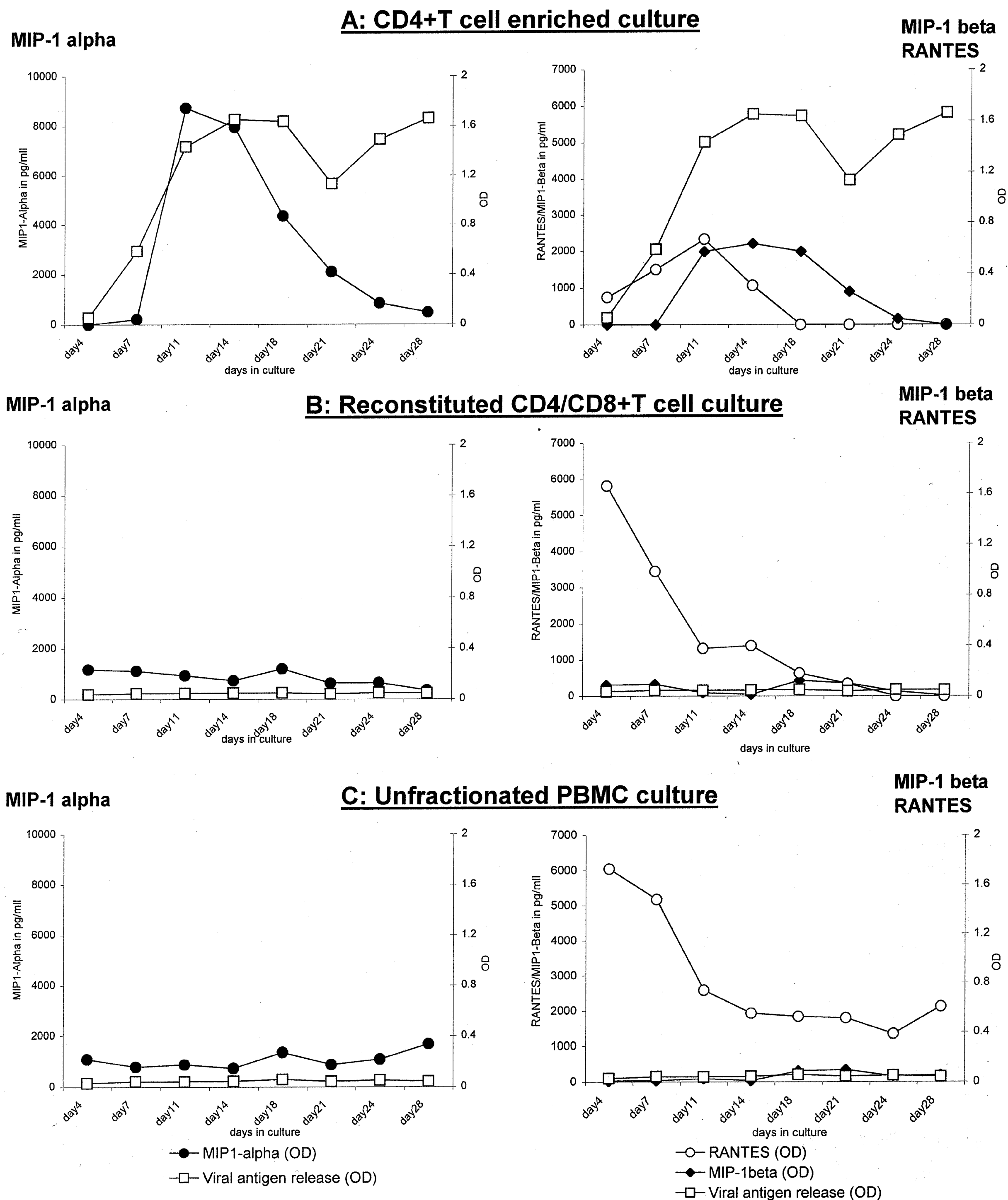

Fig. 2. RANTES, MIP1-α, and MIP1-β secretion by PHA-stimulated cells during a suppression experiment in Ch-No. ELISA tests were done on culture SN stored at −20°C (experiment from month 114). In CD4 + T cell culture (**A**), secretion of the 3 β-chemokines follows the production of viral antigen. In reconstituted CD4/CD8 + T (**B**) and unfractionated PBMC (**C**), RANTES levels are high at the beginning of the experiment and associated with absence of viral antigen release whereas levels of MIP1-α and MIP1-β remain low.

Capacity of PBMCs to suppress SIVcpz-ant replication does not correlate with *in vitro* concentration of RANTES, MIP-1α, or MIP1

Dosages of RANTES, MIP1-α, and MIP1-β indicated that the presence of virus in PHA-stimulated CD4 + T cells was positively correlated with

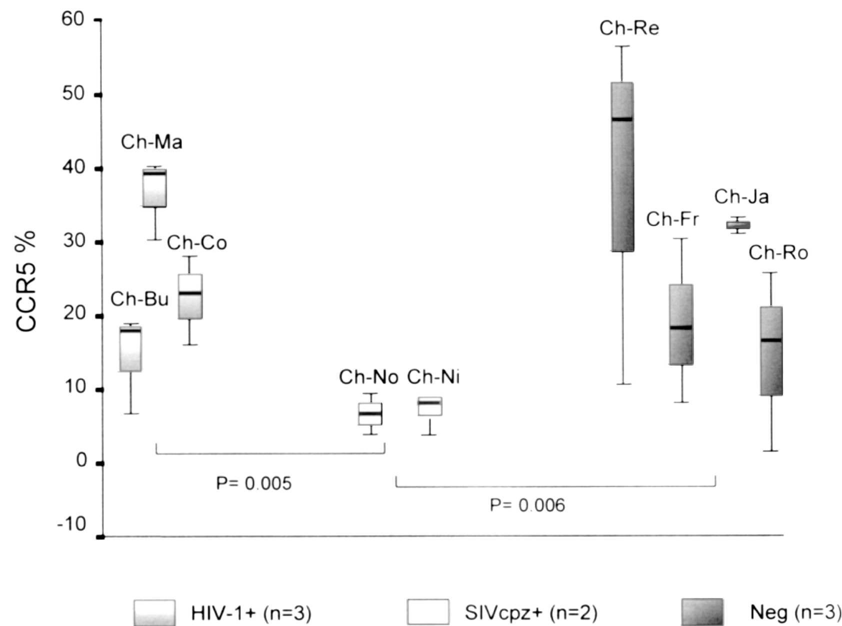

Fig. 3. CCR5 expression on CD4 + T memory cells (CD45RO + , CD4 +) of two SIVcpz-infected, three HIV-1-infected and three negative control chimpanzees. The box plots enclose the 1st and 3rd quartiles (lower and upper edge) and the median (bold line). The whisker caps represent the maximum and minimum values. CCR5 expression in SIVcpz-infected chimpanzees' cells is significantly lower than in the HIV-1-infected and the negative control chimpanzees' cells (Mann–Whitney U-test). CCR5 expression was comparable on HIV-1-infected and negative chimpanzees' cells.

concentrations of β-chemokines (Fig. 2). In contrast to MIP-1α and MIP1-β, a high concentration of RANTES at the beginning of an experiment was associated with the absence of virus replication in the reconstituted CD4/CD8 + T cells and unfractionated PBMC cultures. Eventually, RANTES levels decreased without any release of SIVcpz antigen in culture supernatants.

Anti-class I and -class II antibodies do not abrogate SIVcpz-ant replication *in vitro*

Addition of anti-class I and II antibodies separately or combined did not prevent virus suppression in CD4/CD8 + T and PBMC cultures (data not shown). FACscan analysis confirmed that the specific binding of W6/32 and 9.3F10 monoclonal antibodies on lymphocytes from chimpanzees was comparable with the binding to lymphocytes from an HIV-1-infected individual (data not shown).

Expression of cellular CCR5 and CXCR4 on CD4 + T cells

CCR5 expression was highly variable among chimpanzees (mean = 20.8%, ranging from 0.8 to 54.8%) but appeared to be stable within the same animal (data not shown). CCR5 expression was significantly lower in the SIVcpz-ant-infected chimpanzees compared with the HIV-1 infected chimpanzees and the uninfected control group ($P = 0.005$ and $P = 0.006$, respectively; Fig. 3). CXCR4 expression was high (mean = 71.3%, ranging from 70 to 88%) and comparable among all animals. The type of coloration did not influence results of cell phenotyping.

Discussion

In this study we set out to determine if CD8 + T cells mediated suppression and if this inversely correlated with any of two PCR or two culture-based protocols of virus load determination over time in SIVcpz-infected chimpanzees. Despite significant fluctuations in these animals, the various virus load parameters tended to decrease with time. This is in contrast to the majority of HIV-1-infected individuals in which viremia increases within 10 years of infection unless therapeutic intervention is undertaken. According to criteria previously established for humans [16, 45], HIV-1- and SIVcpz-infected chimpanzees can be classified as LTNP. Ch-No and Ch-Ni remain asymptomatic with stable CD4 + T cell count (data not shown) and maintain immune functions [28, 29]. Contrary to HIV-1-infected chimpanzees (data not shown) they have a productive SIVcpz infection with a considerable plasma RNA VL. Median values of plasma VL and the absence of any significant correlation with infectious virus titers are comparable with HIV-1 asymptomatic patients who

have CD4 + T cell counts > 200/mm^3 as well as LTNP [10, 11, 14, 19, 48]. This discrepancy can be explained by the higher sensitivity of VL compared with virus titration by end point co-culture to measure cell-free virus in the plasma. VL can measure the defective virus in the plasma that will be missed by a conventional method of virus titration [1, 55]. Differences in RNA copy number obtained with the VL-ITM and VL-BPRC techniques may be related to the different procedures used to quantify the PCR products.

Our results indicate that decreasing infectious virus titers in SIVcpz-infected chimpanzees were associated with maintenance or improvement of *in vitro* suppression of virus replication over time. In Ch-No, establishment of a stable *in vitro* suppression of virus replication corresponded to the onset of a prolonged period of low or undetectable infectious virus titers. PBMCs were only able to fully control viral replication subsequent to month 96, whereas Ch-Ni's PBMCs suppressed virus replication soon after infection. Although we cannot exclude the influence of the human donor CD8 + T and NK cells on the outcome of virus titration [54], this suggests that fluctuations of plasma and cell-associated virus titers were related to the actual number of infectious virus circulating in the host.

Since there was no overall correlation between cell infectious virus titers and *in vitro* suppression, there was no evidence that isolation of SIVcpz-ant from autologous PBMCs (which measures virus suppression) was dependent on PBMC infectious virus titers. Two samples with PBMC virus titers of 50 TCID showed absence of virus replication in autologous PBMCs (presence of suppression), while virus replication (absence of suppression) was observed in samples with PBMC titers ranging from less than 0.20 to 0.5 $TCID_{50}$. Conversely, it remains to be determined whether *in vitro* virus suppression reflects a parameter that plays a role in the maintenance of low infectious virus titers or if it is just a surrogate marker of conserved immune functions in the chimpanzees. Plasma viral RNA load, which presumably evaluates viral replication, which also takes place in the lymphoid compartment, did not correlate with *in vitro* suppression, which measures a parameter associated with peripheral blood cells.

Efforts were made to further unravel the *in vitro* mechanism of virus suppression by chimpanzee PBMCs. The capacity of CD8 + T cells to control virus replication *in vitro* has been reported to be a correlate of protective immunity [8]. In HIV-1-infected humans and SIVmac-infected macaques, this suppression is ascribed to CD8 + T cells [18, 52, 63]. Discordant observations between the suppressive capacity of the CD8 + T and PBMCs in Ch-No (months 66, 84, 105, and 111) and Ch-Ni (months 2, 9, and 28) raised the possibility that another cell subset might be involved in suppressing SIVcpz-ant replication. NK cells, which have been described as being capable of suppressing HIV-1 replication in human [17], were not shown to contribute to *in vitro* suppression of viral replication [27] under our experimental conditions. Based on our observations that an absence of physical contact between producing and effector cells considerably reduces virus suppression, we tentatively propose that suppression of SIVcpz replication is mediated by a factor associated with the cell membrane or by a soluble substance that requires cell-to-cell contact for optimal secretion. Our hypothesis is unlikely to include CAF since its activity is known to be contact-independent [32, 35, 36]. Other factors might be involved that have an MHC-independent effector phase, where secretion is more efficiently triggered in an antigen-specific and/or MHC-mediated fashion [21, 63–65]. Ohashi et al. also described some non-cytolytic inhibitory factors acting through a contact-dependent, MHC-independent mechanism [40], suggesting their possible association with the cell membrane.

We did not obtain evidence for MHC-restricted suppression by using human anti-class I and II antibodies, in accordance with our previous observation that the naturally SIVcpz-infected chimpanzee does not have detectable specific SIVcpz-ant CTL [29]. This finding also suggests that contact-dependent suppression of virus replication by the CD8 + T cell is not MHC-restricted. Nonetheless, we cannot exclude that the human antibodies W6/32 and 9.3F10 may fail to abrogate a possible MHC-restricted CD8 + T cell mechanism of virus suppression in chimpanzees.

We obtained further evidence that CD8 + T suppression was mediated by factors different from β-chemokines. SIVcpz uses CCR5 as its principal co-receptor for cell entry, which can be blocked by recombinant β-chemokines [7, 12, 27, 29; M. Dittmar, personal communication]. In our study, measurement of β-chemokines in culture supernatants revealed that RANTES, MIP1-α, and MIP1-β secretion by PHA-stimulated cells was contemporary with virus production and did not correlate with the PBMCs and CD8 + T cell suppressive capacity on *in vitro* virus replication. Greco and colleagues obtained similar results with HIV-1-infected human cells [20]. It has been previously reported that the *in vivo* and *in vitro* level of secretion of β-chemokines does not substantially influence HIV-1/SIVcpz infection and pathogenesis

[26, 27, 37, 49, 66]. In addition to the CD8 + T cell-mediated anti-viral activity, CD4 + lymphocytes may influence viral replication by pre- or post-entry barriers, such as CTL activity [30, 33, 42], increased secretion of β-chemokines, or down-regulation of cell surface CCR5 [50]. Low expression of CCR5 on CD4 + T lymphocytes of SIVcpz-infected chimpanzees was different from the increase described for HIV-1-infected patients relative to negative controls [43]. In Ch-Ni, additional phenotyping analysis revealed a decline in CCR5 expression immediately following the experimental SIVcpz-ant infection (data not shown). In chimpanzees, HIV-1 generally evolves toward a dormant infection with undetectable viral loads, whereas SIVcpz provokes a productive infection, although this is not associated with escalating immune activation [28]. A higher rate of virus replication in Ch-No and Ch-Ni may result in a more important selective destruction of infected CCR5 positive CD4 + T memory cells compared with the HIV-1-infected chimpanzees. Since CCR5 is the main co-receptor used by SIVcpz for cell entry [7, 12, 27; M. Dittmar, personal communication], a decline in CCR5 expressing CD4 + lymphocytes could lead to a lower availability of target cells for SIVcpz infection. Binding of anti-CCR5 antibodies on the chimpanzees' cells supports our previous sequence analysis that none of the animals bears the 32-base pair deletion in the CCR5 gene [34, 59] associated with resistance to infection and disease progression [51, 53].

In conclusion, our results reveal that *in vitro* control of SIVcpz in the PBMCs from naturally and experimentally infected chimpanzees improved or was maintained over time given 11 and 4 years of follow-up, respectively. This control corresponded with the maintenance of relatively low infectious VLs over time. The mechanism by which virus was controlled in the PBMCs *in vitro* involved CD8 + T cells, was not mediated by secretion of β-chemokines. It was more efficient upon cell-to-cell contact but was not MHC-restricted. This suppressive substance may be another specific soluble factor in addition to those already described [4] or a membrane-associated factor. It remains unclear whether the capacity of the PBMCs to suppress SIVcpz *in vitro* reflects a parameter that plays a role in the control of infection *in vivo*. In contrast to HIV-1-infected individuals, where cellular activation is associated with an increase in the number of CCR5 expressing CD4 + T cells [13], SIVcpz infection seems to result in a decline of target cell availability. Further investigations on the mechanism of chimpanzees' CD8 + T-cell suppression as well as their CD4 + T cell capacity to support infection and virus replication are in progress.

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References

1. Andreoni M, Sarmati L, Ercoli L et al.: Correlation between changes in plasma HIV RNA levels and in plasma infectivity in response to antiretroviral therapy. AIDS Res Human Retroviruses 13:555–561, 1997.
2. Baier M, Werner A, Bannert N, Metzer K, Kurth R: HIV suppression by interleukine-16. Nature 378:563, 1995.
3. 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 capturing assay. J Virol Methods 73(1):65–70, 1998.
4. Bogers WMJM, Koornstra WH, Dubbes RH, Nara P, Buijs L, Heeney JL: Potent HIV-1 inhibiting soluble factor from chimpanzee peripheral blood cells. In: Guenounou M (ed). HIV and Cytokines. Inserm: Paris 211–215, 1997.
5. Brodsky FM, Parham P: Monomorphic anti-HLA-A, B, C monoclonal antibodies detecting molecular subunits and combinatorial determinants. J Immunol 128(1):129–135, 1982.
6. Broström C, Visco-Comandini U, Yun Z, Sönneborg A: Longitudinal quantification of human immunodeficiency virus type 1 DNA and RNA in long-term nonprogressors. J Infect Dis 179:1542–1548, 1999.
7. Chen Z, Zhou P, Ho DD, Landau N, Marx PA: Genetically divergent strains of simian immunodeficiency virus use CCR5 as a coreceptor for entry. J Virol 71:2705–2710, 1997.
8. Clerici M, Shearer GM: Correlates of protection in HIV infection and the protection of HIV infection to AIDS. Immunol Lett 51:63–73, 1996.
9. Cocchi F, DeVico L, Garzino-demo A, Arya SK, Gallo RC, Lusso P: Identification of RANTES, MIP-1α and MIP-1β as the major HIV-suppressive Factors produced byCD8 + T cells. Science 270:1811–1815, 1995.
10. Comar M, Simonelli C, Zanussi S et al.: Dynamics of HIV-1 mRNA expression in patients with long term nonprogressive HIV-1 infection. J Clin Invest 100(4):893–903, 1997.
11. Delamare C, Burgard M, Deveau C et al.: Longitudinal study of plasma HIV infection measured using AMPLICOR HIV monitor and NASBA HIV-1 RNA QT tests. J Med Virol 54:60–68, 1998.
12. Deng H, Liu R, Ellmer W et al.: Identification of a major co-receptor for primary isolates of HIV-1. Nature 381:661–666, 1996.
13. De Roda Husman AM, Blaak H, Brouwer M, Schuitmaker H: CC chemokine receptor 5 cell-surface expression in relation to CC chemokine receptor 5 genetyope and the clinical course of HIV-1 infection. J Immunol 163(8):4597–4603, 1999.
14. Ercoli L, Sarmati L, El-Sawaf G et al.: Plasma viremia titration and RNA quantitation in ICD-p24 negative HIV type-1-infected patient. AIDS Res Human Retroviruses 11(10):1203–1207, 1995.
15. Easterbrook PJ: Non-Progression in HIV-1 infection. AIDS 8:1179–1182, 1994.

16. Easterbrook PJ, Schrager LK: Long term non-progression in HIV infection: methodological issues and scientific priorities. Report of an international European community – National Institutes of Health Workshop, The Royal Society, London, England, November 27–29, 1995. Scientific Coordinating Committee. AIDS Res Human Retroviruses 14:1211–1228, 1998.
17. Fehniger TA, Herbein G, Yu H et al.: Natural killer cells from HIV-1 + patients produce C-C chemokines and inhibit HIV-1 infection. J Immunol 161(11):6433–6438, 1998.
18. Ferbas J: Perspective on the role of CD8 + cells suppressor factors and cytotoxic T lymphocytes during HIV infection. AIDS Res Human Retroviruses 14(Suppl 2): S153–S160, 1998.
19. Garcia F, Vidal C, Gatell JM, Miró JM, Soriano A, Pumarola T: Viral loads in asymptomatic patients with CD4 + lymphocyte count above 500×10^6/l. AIDS 11:53–57, 1997.
20. Greco G, Berker E, Levy J: Differences in HIV replication in CD4 + lymphocytes are not related to β-chemokines production. AIDS Res Human Retroviruses 14(16):1407–1411, 1998.
21. Gauduin M-C, Glickman RL, Means R, Johnson P: Inhibition of human immunodeficiency virus (HIV) replication by CD8 + T lymphocytes from macaques immunized with live attenuated SIV. J Virol 72:6315–6324, 1998.
22. Heeney J, Jonker R, Koomstra W et al.: The resistance of HIV-infected chimpanzees to progression to AIDS correlates with absence of HIV-related T-cell dysfunction. J Med Primatol 22:194–200, 1993.
23. Heeney JL: AIDS: A disease of impaired T-helper (Th)-cell renewal? Immunol Today 16:515–520, 1995.
24. Heeney JL, Niphuis H, Ten Haaft PJF et al.: Transmission of SIVcpz and kinetics of infection in the presence and absence of concurrent HIV-1 infection in chimpanzees (in preparation).
25. Ho D, Moudgil T, Alam M: Quantification of human immunodeficiency virus type 1 in the blood of infected persons. N Engl J Med 321:1621–1623, 1989.
26. Kakkanaiah VN, Ojo-Amaize EA, Peter JB: Concentrations of circulating β-chemokines do not correlate with viral loads in human immunodeficiency virus-infected individuals. Clin Diagn Lab Immunol 5(4):499–502, 1998.
27. Ondoa P, Vingerhoets J, Vereecken C et al.: *In vitro* suppression of SIVcpz is suppressed by CD8 + T cells but not by NK cells of chimpanzees (submitted).
28. Kestens L, Ondoa P, Vereecken K et al.: SIVcpz-ant causes a non-escalating immune activation and uses multiple co-receptors in chimpanzees (abstract No. 11210). In: 12th World AIDS Conference Geneva. June 28–July 3, 1998.
29. 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 172:957–963, 1995.
30. Kundu SK, Merigan TC: Equivalent recognition of HIV protein, Env, gag and pol by CD4 + and CD8 + cytotoxic T-lymphocytes. AIDS 6:643–649, 1992.
31. Landay AL, Mackewicz C, Levy JA: An activated CD8 + T cell phenotype correlates with anti HIV activity and asymptomatic clinical status. Clin Immunol Immunopathol 69:106–116, 1993.
32. Levy JA, Mackewicz CE, Barker E: Controlling HIV pathogenesis: the role of the non-cytotoxic anti-HIV response of CD8 + T cells. Immunol Today 17(5):217–224, 1996.
33. Littaua RA, Oldstone MBA, Takeda A, Ennis FA: A CD4 + cytotoxic T-lymphocyte clone to a conserved epitope on human immunodeficiency virus type 1 p24.: Cytotoxic activity and secretion of IL-2 and IL-3. J Virol 66:608–611, 1992.
34. Liu R, Paxton WA, Choe S et al.: Homozygous defect in HIV-1 co-receptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell 272:1955–1958, 1996.
35. Mackewicz C, Levy JA: CD8 + cell anti HIV activity: Non-lytic suppression of virus replication. AIDS Res Human Retroviruses 8:1039–1050, 1992.
36. Mackewicz C, Ortega HW, Levy JA: CD8 + cells anti-HIV activity correlates with the clinical state of the infected individuals. J Clin Invest 87:1462–1466, 1991.
37. McKenzie SW, Dallalio G, North M, Frame P, Means R Jr: Serum chemokine levels in patients with non-progressing HIV infection. AIDS 10:F29–F33, 1996.
38. Novembre FJ, Saucier M, Anderson DC et al.: Development of AIDS in a chimpanzee infected with human immunodeficiency virus type 1. J Virol 71:4086–4091, 1997.
39. Nyambi P, Lewi P, Peeters M et al.: Study of the dynamics of neutralization escape mutants in a chimpanzee naturally infected with the simian immunodeficiency virus SIVcpz-ant. J Virol 71(3):2320–2330, 1997.
40. Ohashi T, Kubo M, Kato H et al.: Role of class I major histocompatibility complex-restricted and -unrestricted suppression of human immunodeficiency virus type 1 replication by CD8 + T lymphocytes. J Gen Virol 80:209–216, 1999.
41. 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 vivo. J Clin Invest 102:223–231, 1998.
42. Orentas RJ, Hildreth JEK, Obah E et al.: Induction of CD4 + human cytolytic T cells specific for HIV-infected cells by a gp 160 subunit vaccine. Science 241:1232–1237, 1990.
43. Ostrowski MA, Justement SJ, Catanzaro A et al.: Expression of chemokine receptors CXCR4 and CCR5 in HIV-1-infected and uninfected individuals. J Immunol 161(6):3195–3201, 1998.
44. Pal R, Garzino-Demo A, Markham PD et al.: Inhibition of HIV-1 infection by the β-chemokine MDC. Science 278:695–698, 1997.
45. Pantaleo G, Menzo S, Vaccarezza M et al.: Studies in long-term nonprogressive human immunodeficiency virus infection. N Engl J Med 332:209–216, 1995.
46. 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 6:447–451, 1992.
47. Peeters M, Janssens W, Vanden Haesevelde M et al.: Virologic and serologic characteristics of a natural chimpanzee lentivirus infection. Virology 211:312–315, 1995.
48. Phillips AN, Eron JJ, Bartlett JA, the North American Lamivudine HIV Working Group et al.: HIV-1 RNA levels and the development of clinical disease. AIDS 10:859–865, 1996.
49. Polo S, Veglia F, Malnati MS et al.: Longitudinal analysis of serum chemokine levels in the course of HIV-1 infection. AIDS 13(4):447–454, 1999.
50. Robbins PA, Roderiquez GL, Peden KWC, Norcross M: Human immunodeficiency virus type 1 infection of antigen-specific CD4 cytotoxic T lymphocytes. AIDS Res Human Retroviruses 16:1397–1406, 1998.

51. Samson M, Libert F, Doranz BJ et al.: Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature 382:722–725, 1996.
52. Schmitz J, Kuroda MJ, Santra S et al.: Control of viremia in simian immunodeficiency virus infection by CD8 + lymphocytes. Science 283:857–860, 1999.
53. Siciliano SJ, Kuhman SE, Weng Y et al.: A critical site in the core of the CCR5 chemokine receptor required for binding and and infectivity of human immunodeficiency virus type 1. J Biol Chem 274:1905–1913, 1999.
54. Spira A, Ho D: Effect of different donor cells on human immunodeficiency virus type-1 replication and selection in vitro. J Virol 69:422–442, 1995.
55. Tedder RS, Kaye S, Loveday C et al.: Comparison of culture and non culture-based methods for quantification of viral load and resistance to antiretroviral drugs in patients given zidovudine monotherapy. J Clin Microbiol 36(4):1056–1063, 1998.
56. Ten Haaft PJF, Cornelissen M, Goudsmit J et al.: Virus load in chimpanzees infected with human immunodeficiency virus type 1: Effect of pre-exposure vaccination. J Gen Virol 76:1015–1020, 1995.
57. Ten Haaft PJF, Verstrepen B, Uberla K, Rosenwirth B, Heeney JL: A pathogenic threshold of virus load defined in simian immunodeficiency virus- or simian-human immunodeficiency virus-infected macaques. J Virol 72:10281–10285, 1998.
58. Ten Haaft PJF, Murthy K, Salas et al.: Differences in early virus loads with different phenotypic variants of HIV-1 and SIVcpz in chimpanzees. AIDS 15:1–8, 2001.
59. Ten Haaft PJF, Murthy KK, Verstrepen BE, Eichberg JW, Heeney JL: Intact CCR5 co-receptors in HIV-1 infected chimpanzees. AIDS 11:1291–1304, 1997.
60. Van Voorhis WC, Steiman RM, Hair LS et al.: Specific antimononuclear phagocyte monoclonals antibodies. Application to the purification of dendritic cells and the tissue localization of macrophages. J Exp Med 158(1):126–145, 1983.
61. Vesanen M, Stevens CE, Taylor P, Rubinstein P, Saksela K: Stability in controlling viral replication identifies Long-Term Nonprogressors as a distinct subgroup among human immunodeficiency virus type 1-infected persons. J Virol 70(12):9035–9040, 1996.
62. Villinger F, Brara SS, Brice GT et al.: Immune and hematopoietic parameters in HIV-1 infected chimpanzees during clinical progression toward AIDS. J Med Primatol 26(1–2):11–18, 1997.
63. Walker C, Moody DJ, Stites DP, Levy JA: CD8 + lymphocytes can control HIV infection in vitro by suppressing virus replication. Science 234:1563–1566, 1986.
64. Walker CM, Erickson AL, Hsueh F, Levy J: Inhibition of human immunodeficiency virus replication in acutely infected CD4 + cells by CD8 + cells involves a non-cytolytic mechanism. J Virol 65:5921–5927, 1991.
65. Yang OO, Kalams S, Trocha A et al.: Suppression of human immunodeficiency type 1 replication by CD8 + cells: evidence for HLA class I restricted triggering of cytolytic and non-cytolytic mechanisms. J Virol 71:3120–3128, 1997.
66. Zanussi S, D'Andrea M, Simonelli C, Battiston V, Tirelli U, De Paoli P: CD8 + cells in HIV infection produce macrophage inflammatory protein-1 alpha and RANTES: a comparative study in long-term survivors and progressor patients. Immunol Lett 53(2–3):105–108, 1996.