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Characterization of $\gamma\delta$ T cells in naïve and HIV-infected chimpanzees and their responses to T cell activators *in vitro*

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Abstract

Background— $\gamma\delta$ T cells are effector cells that eliminate cancer and virus infected cells. Chimpanzees are an endangered species that can naturally and experimentally infected with SIV and HIV, respectively, but no information about the functionality of $\gamma\delta$ T cells during chronic lentiviral infection is currently available.

Methods—Healthy and HIV-infected chimpanzee $\gamma\delta$ T cells were characterized by flow cytometry. $\gamma\delta$ T subsets were studied after stimulation with T cell activators and the release of cytokines was analyzed by Luminex assay.

Results— $\gamma\delta$ T cell subsets, V δ 1 and V δ 2V γ 9 showed different patterns in the expression of CD4, CD195, CD159a and CD159c. Stimulation of $\gamma\delta$ T cells resulted in increased levels of CD4 and HLA-DR, more pronounced in V δ 1 T cells. Distinct cytokine patterns were found between healthy and HIV-infected chimpanzees.

Conclusions—Analyses of major chimpanzee $\gamma\delta$ T subsets show similarities to human $\gamma\delta$ T cells, and suggest different functionality and roles in their immune response against HIV infection.

Keywords

gamma/delta; HIV/AIDS; Chimpanzee

Introduction

T cells represent the effector cell type of the adaptive immune system. T cell populations harbor a myriad of lymphocytes carrying clonally variable antigen receptors, mostly $\alpha\beta$ T-cell receptor ($\alpha\beta$ TCR) type, destined to recognize antigens with incredible precision. A small subset of CD3+ T cells carries an alternative TCR heterodimer composed of γ and δ chains, the $\gamma\delta$ T cell subset, which displays characteristics of both the innate and the adaptive immune system. $\gamma\delta$ T cells are distinguished from other innate cells in that they express a TCR that make them produce unique responses and distinctive functions [6, 7, 24]. The $\gamma\delta$ T

cells recognize nonpeptide and nonprocessed bacterial and environmental antigens [10, 14], as well as stress-associated antigens expressed on epithelial cells and on certain tumor lines and primary carcinomas [22, 23]. Recognition of self-antigens and production of cytokines early in immune responses may position $\gamma\delta$ T cells as unique effectors or regulators of autoimmunity. By releasing MCP-2, one of the monocyte chemotactic proteins, $\gamma\delta$ T cells may contribute to recruiting and activation of neutrophils that can release antimicrobial factors such as α -defensins activating innate immunity mechanisms to combat infections, especially at mucosa sites [1, 2]. $\gamma\delta$ T cells can lyse infected macrophages and thereby limit the spread of infectious microorganisms [41], but also kill a broad variety of tumor cells [31]. Because of the expression of Toll-like receptors (TLRs) they can initiate rapid signals to activate dendritic cells and modulate the suppressive activity of regulatory T cells, therefore linking the innate and acquired immune systems [26]. The $\gamma\delta$ T cells are an “activated yet resting” circulating lymphocyte population, able to quickly and effectively respond to danger signals without the need for antigen processing and presentation.

In the peripheral blood of healthy adult human individuals, $\gamma\delta$ T cells account for 1%–5% of circulating T cells, whereas they comprise a much higher proportion of T cells in other anatomical localizations, notably in the epithelia of the small intestine and in the skin [25]. In the small intestine 10%-15% of intraepithelial lymphocytes can be found whereas in the colon the proportion can be as high as 20%-40% [20, 21]. In contrast to $\alpha\beta$ T cells, the available germ line repertoire of $V\gamma$ and $V\delta$ genes is rather small. In the peripheral blood of healthy individuals, there is a marked predominance within the $\gamma\delta$ T cell population of cells expressing the $V\gamma 9$ together with the $V\delta 2$ (50%-95% of the $\gamma\delta$ TCR) [29, 44]. The distribution is different in other localizations; among the intraepithelial $\gamma\delta$ T cells in the human small intestine and the skin, T cells expressing $V\delta 1$ in association with various $V\gamma$ elements are more frequent [13, 27]. Similar to $\alpha\beta$ T cells, $\gamma\delta$ T cells are targeted to different organs guided by homing receptors, local induction and/or selection processes by local antigens, cytokines, chemokines and cell-matrix interactions [8, 12].

As the major component in the mucosal immune system, $\gamma\delta$ T cells may have a decisive role in early phases of viral infection as they produce antiviral factors such as MIP-1 α , MIP-1 β and RANTES (CCL3, CCL4, and CCL5, respectively) competing for CCR5, one of the co-receptors of HIV [48, 54]. $\gamma\delta$ T cells can also kill virus-infected cells using both cytolytic and noncytolytic mechanisms after they interact with molecules expressed under stress (MICA, MICB) [38, 46, 47]. Some observations suggest an important role of $\gamma\delta$ T cells in the early control of virus infection. For instance, in human HIV, HTLV, EBV, and HSV-1 virus infections, the levels of $\gamma\delta$ T cells are increased and they are localized at infection sites [5, 11, 17–19, 46]. A role in protection was suggested by experimental intrarectal SIV infection in macaques [52]; however, since $\gamma\delta$ T cells can also be infected [55], their activation and subsequent malfunction may also contribute to the immunopathogenesis, allowing viral spread and persistence.

Chimpanzees are the only nonhuman primates that are susceptible to HIV infection, which very rarely leads to disease development in these primates [33, 40]. In the wild, chimpanzees are also susceptible to infection with SIVcpz, and there is evidence that this infection leads

to CD4 T cell depletion and pathogenesis [32]. Immunological mechanisms of HIV or SIV disease resistance in the chimpanzee are unknown, including the role of $\gamma\delta$ T cells.

Chimpanzees at the Southwest National Primate Center (SNPRC) that have been infected with HIV for many years, do not show any sign of CD4 depletion have shown persistent viremia. In the present study, and in order to gain a better understanding of their biology, $\gamma\delta$ T cells in naïve and HIV-infected chimpanzees were studied for *in vivo* phenotyping and biological responses *in vitro*. We found that the two major $\gamma\delta$ T cell subsets (V δ 1 and V δ 2/V γ 9 T cell) in the blood differ in their phenotypes and the ability to be activated by exogenous T cell activators.

Materials and methods

Specimens and cell separation

Heparinized blood from 2 naïve (Chimp-1 and Chimp-2) and 4 HIV- infected (Chimp-3, Chimp-4, Chimp-5 and Chimp-6) chimpanzees was obtained at SNPRC, at Texas Biomedical Research Institute, San Antonio, TX. Animal care and treatment were all in accordance with Institute's Institutional Animal Care and Use Committee (IACUC) policies and approved protocols. Peripheral mononuclear cells (PBMCs) were obtained by density centrifugation over cell separation media (CellGro, Manassas, VA) and were washed twice with phosphate-buffered saline (PBS) prior to phenotyping, $\alpha\beta$ T cell depletion and culturing. Two of the HIV-infected and 1 of the naïve chimpanzee PBMCs were further depleted of $\alpha\beta$ T cells, B cells and monocytes by positive selection using mouse antibodies antihuman TCR $\alpha\beta$ (clone IP26), CD14 (clone M5E2) and CD20 (clone 2H7) (all from Biolegend, San Diego, CA) and magnetic beads coated with anti-mouse antibody (DynaClection™ Pan Mouse IgG kit, Invitrogen, Oslo, Norway according to manufacturers' instructions. After magnetic separation the cells were washed twice in PBS, counted, phenotyped by flow cytometry, and cultured.

Flow cytometry analysis

Cell phenotyping was performed by multicolor flow cytometry using a Cyan ADP with 3 lasers and analyzed with Summit software (Beckman-Coulter, Indianapolis, IN). PBMCs and purified cells were stained with a six tube panel of antibodies labeled with 8 different fluorescent color dyes (FITC or Alexa Fluor 488, PE, PerCP-Cy5.5, PE-Cy7, APC, APC-H7 or Alexa Fluor 700, Pacific Blue and V500) for 30 minutes at 4°C. After incubation cells were washed in cold PBS and fixed with 1.6% methanol-free formaldehyde-PBS. Cells were analyzed in the cytometer within 0.5 hr – 4 hr. Monoclonal antibodies directed against V δ 1 (TS8.2) and V δ 2 (15D) were purchased from Endogen (Woburn, MA); CD3 (SP34.2), CD4 (L200), CD8 (SK1), CD20 (2H7), HLA-DR (G46-6/L243), CD16 (3G8), TCR $\alpha\beta$ (T10B9.1A-31) and CD195 (3A9) from BD-biosciences (San Jose, CA); V γ 9 (B3), CD69 (FN50), V δ 2 (B6), $\gamma\delta$ TCR (B1), $\gamma\delta$ TCR (5A6-E9) and CD25 (BC96) from Biolegend (San Diego, CA); CD159a/NKG2A (Z199) from Beckman-Coulter (Indianapolis, IN); and CD159c (134522) from A&D Systems (Minneapolis, MN).

Stimulation assays

PBMCs and $\alpha\beta$ TCR-depleted cells were cultured at a concentration of 2×10^6 cells/ml in T25 flasks under different conditions: 1) *RPMI-10*: RPMI (Cellgro, Manassas, VA) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 1% L-glutamin (Cellgro), 1% penicillin-streptomycin (Biowhitaker, Walkersville, MD), 1% Non-essential amino acids (Cellgro) and 25mM Hepes (Cellgro); 2) *IPP*: RPMI-10 plus 15 μ M Isopentenyl-Pyrophosphate (SIGMA-Aldrich, St Louis MO); 3) *PHA*: RPMI-10 plus 5 μ M Phytohemagglutinin-L (SIGMA-Aldrich, St Louis MO). And 4) *CD3/CD81*: nontissue culture treated plates (NUNC, Thermo Fisher Scientific, Waltham, MA) were incubated in sterile conditions with anti-CD3 (clone HIT3a, Biolegend) and anti-CD81 (clone JS-81, Biolegend) at a concentration of 0.5 μ g/ml; after 2 h at 37°C plates were saturated with 2% BSA for 20 minutes at 37°C and rinsed once with PBS following a modified protocol from Lamers et al [34]. Plates were prepared fresh prior to use.

After 48 hours incubation at 37°C, 5% CO₂, cells were washed and resuspended in RPMI-10 containing 40U/ml IL-2 (Advanced Biotech, Totowa, NJ) at a concentration of 2×10^6 cells/ml and transferred to new T25 flasks. IPP was added to a final concentration of 15 μ M. After seven days of culture, supernatants were harvested and frozen at 80°C for Luminex assays and viral testing. Cells were stained for flow cytometry analyses with the panel of antibodies mentioned above.

HIV testing and viral loads in plasma and supernatants

Quantitative RT-PCR (C-TaqMan) was performed to quantify HIV in plasma and supernatants. RNA was purified from 100 μ l of plasma/supt using RNA-Bee (Tel-Test Inc, Friendswood, TX), following manufacturer's protocol. The equivalent of 10ul plasma/supt RNA was amplified by qRT-PCR using the RNA UltraSense™ One-Step Quantitative RT-PCR System from Invitrogen (Life Technologies, Carlsbad, CA). Primers and probes were designed for the amplification of the Gag gene using the Primer Express Software from Applied Biosystems (Life Technologies): 5'-acccatgttttcagcattatcaga-3' (Forward) and 5'-tgcttgatgtccccctactgt-3' (Reverse) both made by Invitrogen (Life Technologies); probe 5'-ccaccccccaagatttaaacaccatgct-3' labeled 5'-/56-FAM//36-TAMsp/3' was made by Integrated DNA Technologies (IDT, Coralville, IA). Samples were run in a 7500 Real-Time PCR system from Applied Biosystems for 45 cycles (48°C for 30 min, 95°C 10 min, 45 cycles at 95°C 15 sec and 60°C 1 min). Analysis was done with the TaqMan analysis software for 7500 System SDS (sequence detection software) version 1.4.

We also performed intracellular staining using an anti-core antibody (clone FH190-1-1) labeled with Phycoerythrin (RD-1) or FITC purchased at Beckman-Coulter (Indianapolis, IN). A quantity of $0.5-0.8 \times 10^6$ PBMC/tube were first stained for surface antigens with CD3, V δ 1, V γ 9, V δ 2, V γ δ TCR, CD8, CD16 and CD4 (clones and companies are the same as above) for 30 minutes at 4°C. Cells were washed and fixed using BD FACS Lysing solution (BD-Biosciences) for 20 minutes at room temperature, washed by centrifugation and permeabilized using BD lysis II for 15 min at room temperature, washed twice with 0.5% FBS-PBS. Intracellular antibody or isotype control was added to the tubes and incubated for 30 min at room temperature. Finally, after two washes by centrifugation, cells

were fixed in 1.6% methanol-free formaldehyde and run in a Cyan ADP cytofluorometer (Beckman-Coulter).

Immunological profiling of soluble proteins

Cell culture supernatants were tested for forty eight different soluble proteins by Luminex assays with Bio-Plex Pro Assays kits (#M50-OKCAFOY and #MF0-005KMII, Bio-Rad laboratories, Inc, Hercules, CA) following the manufacturer's instructions. Analytes included: MIP-1 β , MIP-1 α , TNF- α RANTES, MCP-1, IP-10, IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-12(p40), IL-13, IL-15, IL-16, IL-17, IL-18, IFN- γ , IL-1R α , GM-CSF, Eotaxin, Basic FGF, VEGF, PDGF-BB, G-CSF, IL-2R α , MIG, IFN- α 2, SDF-1 α , MCP-3, LIF, TNF- β , MIF, β -NGF, GRO- α , HGF, SCF, TRAIL, M-CSF, CTACK and SCGF- β . The plates were measured on the Luminex xMAP 100 system (Luminex Corp.), and analyzed using Masterplex QT 2010 software (Miraibio Corp., South San Francisco, CA).

Results

Phenotyping of PBMCs from naïve and HIV infected chimpanzees

All antibodies used for these studies cross-reacted with chimpanzee's lymphocyte antigens (data not shown); with the exception of anti-human $\gamma\delta$ TCR clone B1, which did not recognize chimpanzee proteins. Table 1 shows the percentile for lymphocyte populations analyzed by flow cytometry in PBMCs of 2 naïve (Chimp-1 and Chimp-2) and 4 HIV infected chimpanzees (Chimp-3, Chimp-4, Chimp-5 and Chimp-6). All chimpanzees showed similar phenotypes to the ones seen in healthy humans, including normal amounts of CD4 cells. Only Chimp-6 had a decrease of CD4+ T cells compared to tests done in previous years (from 33.40% to 19.94%), with Chimp-4 having a high percent of CD4 T cells and low amounts of V $\gamma\delta$ T cells in blood and V δ 1 numbers slightly higher than V δ 2. Figure 1A demonstrates the strategy for flow cytometry analyses of $\gamma\delta$ T cells, with the majority of V γ 9 cells simultaneously expressing the V δ 2 receptor, as it occurs in human blood, and very rarely sharing expression of V δ 1.

The presence of HIV receptor and co-receptor differed in subsets of $\gamma\delta$ T cells (Figure 1B). V δ 1 T cells expressed more CD4 than V δ 2/V γ 9 cells, and V δ 2/V γ 9 cells expressed more CCR5 than V δ 1 cells; interestingly, CCR5 expression was higher in HIV infected animals. The activation marker CD69 was generally expressed at higher levels in V δ 1 cells; two of the infected chimpanzees showed very high values of this molecule (30% in Chimp-5 and 20% in Chimp 6). The two subsets $\gamma\delta$ T cells expressed the CD8 marker in the range of 20–60%, but with a trend to be at higher values in V δ 2/V γ 9 T cells. More than 50% of V δ 1 T cells expressed CD16, while the expression of this marker in V δ 2/V γ 9 T cells was variable and always lower than in V δ 1 T cells.

In summary, our analysis of $\gamma\delta$ T cells in circulation demonstrates clear differences in the level of expression of several relevant markers between naïve and HIV-infected chimpanzees.

$\gamma\delta$ T cell changes in response to in vitro stimulation

We analyzed the response of $\gamma\delta$ T cells to different stimuli, either in bulk PBMC cultures or in cell preparation enriched for $\gamma\delta$ T cells. Enrichment for $\gamma\delta$ T cells was done by negative selection to avoid direct $\gamma\delta$ TCR activation; because $\gamma\delta$ T cells uniformly express CD3, and may also express CD16, CD4, and CD8, we could not use such antibodies for depletion, and we were not able to eliminate NK and NKT cells. PBMCs and $\alpha\beta$ TCR-depleted PBMCs were cultured for additional 5 days after first 48 hours of activation with Isopentenyl pyrophosphate (*IPP*, potent nonpeptide stimulator of $\gamma\delta$ T cells [57]), anti-*CD3/CD81* antibodies (combination of antibodies stimulates both $\alpha\beta$ T and $\gamma\delta$ T cells) and *PHA* (a lectin that stimulates all lymphocytes); control consisted of cells cultured for 48 hours in RPMI-10 with no supplements.

In general, all the stimulation conditions resulted in an increase of T cell numbers on detriment of B and NK cells, with the CD4/CD8 ratio maintained after one week culture (data not shown). Regarding $\gamma\delta$ T cells, changes in the proportion of $\gamma\delta$ T cell subsets were calculated as a ratio considering the percent of the T cell subset V δ 1 or V δ 2/V γ 9 before and after stimulation (Figure2). In vitro stimulation produced an increment in V δ 1 T cells, either in PBMC or in $\alpha\beta$ TCR-depleted PBMCs; however, V δ 2/V γ 9 T cells had variable responses and mostly decreased, even for *IPP* activation. The highest rate of V δ 1 increase was observed after *CD3/CD81* activation, which was very variable for the naïve chimpanzees. In the $\alpha\beta$ T cell-depleted cultures, the same increase in numbers of V δ 1 T cells was observed, but the changes in V δ 2/V γ 9 were variable from animal to animal.

In summary, these experiments demonstrate a differential response by chimpanzee $\gamma\delta$ T cell subsets to T cell stimulation.

Effect of activation of $\gamma\delta$ T cells and expression of HIV receptors

Even though seven days of culture did not show a high rate of proliferation (as seen in the percentage of different subsets of lymphocytes in the cultures), activation is clearly seen through the expression of CD69 (Figure 3A). High expression of CD69 demonstrated that cells were activated in all conditions, even when cells were only exposed to IL-2, since unstimulated cells expressed low levels of CD69. In general, the level of activation of V δ 2/V γ 9 T cells was higher than the V δ 1 subset. HLA-DR was also increased in all conditions but only in the infected chimpanzees (Figure 3B), especially in V δ 1 T cells where HLA-DR reached higher levels compared to V δ 2 T cells.

CCR5 expression varied considerably after seven days of activation (Figure 3C). In naïve chimpanzees, the activation with *IL-2* or *IPP* increased CCR5 in V δ 1 T cells, but decreased it in V δ 2/V γ 9 T cells. By contrast, V δ 1 T cells of HIV-infected chimpanzees increased CCR5 expression while expression on V δ 2/V γ 9 T cells, which was already higher than in cells from naïve animals, did not change. Anti-*CD3/CD81* stimulation induced CCR5 expression on V δ 1 in almost all individuals.

CD4 expression in V δ 1 cells was increased by all the different activation conditions, including unstimulated cells cultured in IL-2 (Figure 3D); the best stimulant for CD4 expression was Anti-*CD3/CD81*. For example, in naïve specimens (Chimp-1 and Chimp-2)

CD4 expression was increased from 16% to 19% and from 26% to 46% respectively, with only *IL-2* supplemented media but to 31% and 76% with *CD3/CD81* respectively. In V δ 2/V γ 9 T cells, CD4 expression did not change significantly after stimulation.

Expression of activating or inhibiting NK receptors in stimulated $\gamma\delta$ T cells

We used flow cytometry for the measurement of activating (NKG2A, CD159a) and inhibitory (NKG2C, CD159c) NK markers on $\gamma\delta$ T cells (Figure 4). Fresh V δ 1 T cells expressed from 12% to 47% NKG2A and from 3% to 14% NKG2C. On the other hand, fresh V δ 2 T cells expressed from 35% to 60% NKG2A, but very low levels of NKG2C. The highest change in NKG2A expression was seen for V δ 2 T cells stimulated with anti-*CD3/CD81*. Activation with *IL-2* and *IPP* worked similarly in both subsets, but was effective at increasing NKG2C expression to almost double the original percent in the V δ 1 subset.

Release of cytokines in PBMC cultures

The characterization of cytokines released in cultures of stimulated chimpanzee cells was performed by Luminex assays. In general, the anti-*CD3/CD81* antibody stimulation promoted the release of cytokines in consistently high levels compared to the other activating conditions, whereas stimulation with *IPP* did not significantly increase cytokine values compared to the concentrations seen in supernatants of unstimulated cells cultured with *IL-2* alone (*IL-2*). The effect of stimulation with anti-*CD3/CD81*, and to a lesser extent for *PHA*, could clearly be seen in the elevated concentrations of the soluble *IL-2* receptor alpha chain (*IL-2R α* , sCD25), and in the low values of *IL-2*, even though this cytokine was exogenously added to all conditions after 48 hours of stimulation (Figure 5A). Similarly, anti-*CD3/CD81* and *PHA* elevated the expression of G-CSF and GRO- α in PBMC from HIV-infected chimpanzees, but only anti-*CD3/CD81* had the same effect for the naïve animal (Figure 5). When comparing naïve and HIV-infected animals, stimulation with the mitogen *PHA* had opposite effects for cytokines such as IP-10, TNF- α , and TNF- β , since expression of these cytokines was downregulated in PBMC from HIV-infected chimpanzees, whereas it did not change (IP-10) or increased (TNF- α and TNF- β) in the naïve animal Chimp-1 (Figure 5B).

The effect of stimulation of chimpanzee PBMCs on the expression of chemokines known to bind to HIV coreceptors was very interesting. For example, naïve Chimp-1 had consistent high levels of the CCR5-binding proteins MIP-1 α , MIP-1 β , and RANTES, which only slightly increased after stimulation with anti-*CD3/CD81* and *PHA*; however, PBMC from all the HIV-infected chimpanzees released much lower levels of these chemokines, and there was only significant upregulation after stimulation with anti-*CD3/CD81*. The CXCR4-binding protein SDF-1, on the other hand, was released at similar concentrations by PBMC from all chimpanzees (Figure 5C).

When PBMC were depleted of $\alpha\beta$ TCR⁺ cells, B cells, and monocytes, and the remaining cells were stimulated in the same conditions, patterns of cytokine expression for the two HIV-infected chimpanzees were similar to the ones seen for the parallel bulk PBMC cultures were observed, although the concentration of several cytokines (MCP3, SCF, IP-10, MIG, and M-CSF) were considerably reduced (data not shown). When activated by anti-*CD3/*

CD81, exclusively, cultures without $\alpha\beta$ T cells, B or Monocytes produced high amounts of certain cytokines in HIV-infected animals but not in the uninfected Chimp-1. This happened for IL-1 β , IL-4, IL5, IL-6, IL9, IFN γ , IL-10, IL-13, IL-17, IL-18, GM-CSF and Eotaxin (data not shown). Moreover, same cytokines were not produced in high concentration similarly to the uninfected cells in any of the other conditions including *PHA*. The supernatants of the uninfected chimpanzee, however, have shown that $\alpha\beta$ -depletion promoted a high release of RANTES and IFN γ in all conditions of activation; that was not seen in the bulk PBMC cultures of the same chimpanzee (Figure 6).

Viral loads in plasma and PBMC cultures

In order to identify whether chimpanzee $\gamma\delta$ T cells harbored infectious HIV, we performed intracellular p24 flow cytometry assays in fresh and stimulated PBMC. However, the total events on the lymphocyte gate never surpassed the 5×10^5 events, and this assay was not sensitive enough to detect p24 in the $\gamma\delta$ T cell populations, even after the $\alpha\beta$ TCR depletion, which increased the proportion of NK, NKT and $\gamma\delta$ T cells. Nonetheless, viral loads in plasma and in supernatants of activated cells showed active replication in PBMCs of the infected chimpanzees even when CD4 $\alpha\beta$ T cells were highly depleted (Table 2, see Chimp-6). Chimp-5 had undetectable virus in plasma and virus replication was only induced at very low levels in *PHA*-stimulated cells. Interestingly, viral replication in the different stimulation conditions showed the lowest viral production in the *PHA* activated cultures, which is the standard procedure for activating HIV replication in latently infected cells.

Discussion

$\gamma\delta$ T cells were associated to HIV/AIDS very early during the first decade in HIV history, with the first noted association published as the increased levels of V δ 1 cells in patients in 1989 [5]. Corroborated in 1991, high levels of $\gamma\delta$ T cells in peripheral blood of HIV-infected patients were found again to be V δ 1 [18]. As V δ 1 increased in circulation, the major subset V δ 2 was decreasing and an inverted ratio was noticeable in those patients. Significant alterations of peripheral $\gamma\delta$ T cell distribution and function have been previously reported in HIV-infected persons [5, 9, 17, 18, 49, 56]. The major $\gamma\delta$ T cell subset showing the TCR V δ 2V γ 9 decreases in the blood of HIV infected individuals, more evidently in HIV-1 patients with opportunistic or other co-infections [39] or they are frequently unable to proliferate [49, 55, 56]. In this report we show that chimpanzees present a similar pattern to circulating human $\gamma\delta$ T cells with V δ 2V γ 9 being the major subset in peripheral blood although HIV-infected chimpanzees did not show the expected inversion V δ 2/V δ 1. The fact that chimpanzees do not show an inversion in the $\gamma\delta$ T cell subsets together with the different susceptibility to HIV infection [43, 45] and to cell activation [51] compared to humans, suggests that the role of $\gamma\delta$ T cells, and probably other NK cells, during HIV infection may be different in chimpanzees. Probably, high amounts of anti-viral cytokines are released at the time of primary infection, leading to lower the load set point so chimpanzees become infected but they do not progress to disease, similar to human long term survivors.

Low levels of CD4, undetectable by flow cytometry may be sufficient for productive HIV infection [28]. $\gamma\delta$ T cells express CD4, CCR5 and CXCR4 [30, 58], they are susceptible to

infection with HIV [55] and so, possible reservoirs of the AIDS virus. By testing the chimpanzee blood we found that high levels (20 to 60%) of V δ 1 cells express CD4 and V δ 2, instead, express high levels of CCR5 (40 to 80%) making them different in suitability to infection with HIV. Although V δ 2/V γ 9 T cells have been demonstrated to have direct or bystander anti-HIV activity [2, 46], no reports have been made on that V δ 1 T cells have killing activity against the AIDS virus. On the other hand, the natural killer cell receptor family is another important group of co-stimulators for regulating the activity of $\gamma\delta$ T cells. NKG2A transfer inhibitory signals while NKG2C promote the activation to kill [4]. In this report we show that V δ 1 T cells expressed higher amounts of NKG2C than V δ 2 and that would be in accordance with the statement above. Moreover, the Fc receptor CD16 has been shown to improve the antiviral activity of V δ 2 negative T cells by inducing the production of IFN γ [16]. This supports our hypothesis that V δ 1 T cells are antiviral surveillants and are present in the peripheral blood to defend the host against HIV spread. Unfortunately, we could not detect infected cells by flow cytometry to corroborate this hypothesis. On the other hand, CD4 positive $\gamma\delta$ T cells can be increased after culture with IL-2 or after viral infection [55]. We recovered virus in supernatant $\alpha\beta$ T cell-depleted cultures from chimpanzee Chimp-6 at one week of incubation with IL-2, IPP, anti-CD3/CD81 or PHA. Depletion of $\alpha\beta$ T cells, monocytes and B cells resulted in cell cultures with high amounts of NK and increased amounts of $\gamma\delta$ T cells. This suggests that other sources of HIV replication may account, but whether those are $\gamma\delta$ T cells or NK cells (that are more than 60% of the culture in this chimpanzee) is not possible to discriminate.

$\gamma\delta$ T cells which express CD16, the NK cell marker, together with NK cells can be a major source of anti-viral cytokines such as IFN γ , TNF α , SDF-1 α and β -chemokines (MIP-1 α , MIP-1 β and RANTES) and they may play a very important role in HIV-infection [15, 49]. Li et al suggested that V δ 2 cells working together with NK cells, both at high levels and activated, can interact with each other and control inflammation [37], they may be suitable targets for immunotherapeutic in AIDS. The fact that NK cells are the most potent producers of β -chemokines combined with the observation that chimpanzees have constitutively three to 10 times more NK cells than humans [42, 50], make this cell subset an important source of β -chemokines in vivo. The CD16+ T cells (that include $\gamma\delta$ T and other NKT cells) may also account for secreting antiviral factors in chimpanzees more than NK cells themselves. The amount of NK cells can account for influencing the responses to activation in cultures other than the *CD3/CD81* culture since CD81 inhibit NK cell activation promoting $\gamma\delta$ T cell proliferation and activation [53]. When we depleted the $\alpha\beta$ T cells, only the uninfected chimpanzee produced high amounts of RANTES, this strongly suggests that NK cells and, probably also the $\gamma\delta$ T cells are defective in the HIV-infected chimpanzees.

δ 2T cells are activated early during the acute phase of viral infections and display potent antiviral responses producing high levels of IFN γ and other cytokines and chemokines: TNF α , IL-1 α , IL-6, G-MCSF, TPO, OSM, MIP-1 α , MIP-1 β , RANTES, SDF-1, MCP-2, MDC and GRO. [46, 47]. Some of these chemokines directly influence the rate of HIV or SIV replication through binding to the CCR5 co-receptor [36, 47]. PBMCs from chimpanzees chronically infected with HIV were found to respond to general activators as seen by elevation of CD69, CD25 and HLA-DR, however, the PBMCs produced lower

levels of the antiviral chemokines than the uninfected chimpanzee, probably due to their antiviral immune system being exhausted.

The scarcity of peripheral $\gamma\delta$ T cells makes them difficult to monitor in vivo. In small non-human primates infected with SIV, $\gamma\delta$ T cells were associated with protection, showing not only increased amounts of these cells in the mucosa of protected monkeys, but more $\gamma\delta$ T cells with the expression of CD69, CD44, and CD45RO activation markers in the infected animals [35]. However, in those studies, there was no discrimination on the different subpopulations of $\gamma\delta$ T cells and, probably, it will be difficult to isolate enough amounts of cells to perform significant cultures and discriminate the function of rare subsets, specially V δ 1 population that is so small in blood. In our chimpanzee studies, we were able to characterize V δ 1 and V δ 2V γ 9 populations based on different surface markers associated to HIV infection and activation. On the other hand, since Chimp-5, with undetectable viremia, had very high content of V δ 1 T cells activated (30%) and, when in vitro cultured in the presence of anti-CD3/anti-CD81 antibodies Chimp-5 and also Chimp-3 (with low viremia), V δ 1 cells responded proliferating showing increased expression of activation marker, it is likely to preclude that this subset of cells is still functional and may be important in keeping down the virus.

By Luminex assays, we also found a different pattern of cytokines for HIV-infected and uninfected chimpanzees after activating cultures were performed, and especially when we depleted the $\alpha\beta$ T cells, B cells and monocytes, indicating that infected individuals have their innate immune system disturbed as well. This will corroborate the hypothesis by Altfeld et al, suggesting that there is an incomplete activation of NK cells in these individuals, and this may be due to chronic stimulation resulting in cell exhaustion and anergy [3]. This is also corroborated by that the uninfected chimpanzee sowed to release more antiviral factors after stimulation.

The failing control of early HIV infection by the innate immune system as well as in chronic infection is likely to be of major importance and needs further investigation. Studying the different subpopulations of innate cells can help for future development of new treatments and vaccination strategies to benefit patients not only for HIV but for other virus infections and emergent diseases in humans and why not to say in the chimpanzee at risk of new infections in nature. In the case of HIV/SIV, the chimpanzee immune system presents a real incognita since they can control the virus and progressing to disease is not occurring compared to humans thus studying their immune system could help to understand the human immune system. Current investigations on chimpanzees are being stopped and our results may be one of the last data in characterizing their immune cells.

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Table 1

Lymphocyte phenotypes in normal and HIV-infected chimpanzees.

	Chimp-1 ^a	Chimp-2	Chimp-3	Chimp-4	Chimp-5	Chimp-6
T cells	58.91 ^b	81.84	80.88	79.44	66.47	57.94
CD4 T cells	34.40	30.98	49.71	61.57	40.65	19.94
CD8 T cells	24.28	51.53	32.07	20.30	28.24	36.71
NK8 cells	31.58	1.74	10.24	9.83	16.01	32.50
NK16 cells	32.12	7.90	7.74	11.98	26.50	35.52
NK16 T cells	2.61	43.08	13.49	14.20	16.98	22.72
B cells	6.07	8.01	12.55	11.66	4.02	7.04
V δ 2 T cells	0.58	2.03	4.58	0.31	3.97	2.45
V δ 1 T cells	0.67	0.81	0.88	0.83	0.27	0.70
V γ 9 T cells	0.42	3.46	4.67	0.32	5.24	2.38
V γ 8T cells	0.93	2.80	4.77	0.42	4.13	3.33

^a Peripheral blood from naive (chimp-1 and -2), and HIV-infected chimpanzees (chimp-3 to -6)

^b Data are provided as percentage of cells in the lymphocyte gate.

Table 2

HIV viral concentration in plasma and supernatants after 7 day culture

	Plasma	IL-2	IPP	CD3/CD81	PHA
Chimp-3	551	28,500	35,900	330,000	12,500
Chimp-3 DEP		ND	ND	ND	ND
Chimp-4	4,870	7,280	5,610	2,890	213
Chimp-4 DEP		ND	ND	ND	ND
Chimp-5	Und	Und	Und	Und	Und
Chimp-5 DEP		Und	Und	Und	93
Chimp-6	2,480	43,300	42,700	94,900	8,280
Chimp-6 DEP		5,770	11,100	17,300	621

Supernatants were from cultured PBMCs or $\alpha\beta$ - CD14- and CD20-depleted PBMCs (DEP).

Values represent copies/ μ l