Chimpanzee CD4+ T cells are relatively insensitive to HIV-1 envelope-mediated inhibition of CD154 up-regulation

Abstract
CD40-CD154 interaction forms a key event in regulation of crosstalk between dendritic cells and CD4 T cells. In human immunodeficiency virus (HIV)-1 infected patients CD154 expression is impaired, and the resulting loss of immune responsiveness by CD4+ T cells contributes to a progressive state of immunodeficiency in humans. Although chimpanzees are susceptible to chronic HIV-1/SIVcpz infection, they are relatively resistant to the onset of AIDS. This relative resistance is characterized by maintenance of CD4+ T cell populations and function, which is highly compromised in human patients. In our cohort of chronically HIV-1- and SIVcpz-infected chimpanzees, we demonstrated the capacity to produce IL-2, following CD3/CD28 stimulation, as well as preserved CD154 up-regulation. Cross-linking of CD4 with mAb was found to inhibit CD3/CD28-induced up-regulation of CD154 equally in chimpanzees and humans. However, specific cross-linking with trimeric recombinant HIV-1 gp140 revealed reduced sensitivity for inhibition of CD154 up-regulation in chimpanzees, requiring fourfold higher concentrations of viral protein. Chimpanzee CD4+ T cells are thus less sensitive to the immune-suppressive effect of low-dose HIV-1 envelope protein than human CD4+ T cells.
Introduction

Human immunodeficiency virus type 1 (HIV-1) infection in humans is characterized by a precipitous loss of immune competence, eventually allowing the establishment of opportunistic infections and cancers [1, 2]. This loss of immune function is ultimately observed at later stages as a peripheral decrease in the number of CD4+ T cells [3, 4]. However, before the numerical loss of CD4+ T cells is apparent, HIV-1-infected individuals already have reduced antigen-specific and mitogen-stimulated CD4+ T cell responses [5–12]. These defects include diminished proliferative capacity and a functional loss in the ability to produce key cytokines. Induction of these important immuno-modulatory cytokines is strongly dependent on dendritic cell (DC)-T cell interaction. Exposure to antigen will lead to DC activation [13–15], induce migration to lymphoid tissues and result in antigen presentation to T cells via MHC-TCR/CD3 interaction [16]. Subsequent activation of antigen-specific T cells leads to CD154 up-regulation which, via binding to CD40 on the DC, induces DC maturation, which is accompanied by up-regulation of the costimulatory molecules CD80 and CD86 and by IL-12 secretion [17–20]. Subsequent binding of these costimulatory molecules to CD28 on the T cell in combination with IL-12 can then induce a Th1 response against the antigen, mediated by IL-2, TNF-α and IFN-γ secretion [21–26]. Independently, several groups have confirmed that the induction of CD154 is decreased in HIV-1-infected persons [21, 27–32]. In addition, reduced capacity to produce IL-2 and consequential reduced antigen-specific T cell proliferation were reported [8, 26], which can be restored via addition of IL-2 or via CD28 co-stimulation [5, 33]. Cross-linking of CD4 with mAb or with HIV-1 envelope protein results in an inhibition of the CD3-mediated up-regulation of CD154 via an impairment of the normal CD3 signal transduction cascade [34, 35]. This perturbation of the normal cell activation and CD154 upregulatory pathway is thought to form a major contribution to immune suppression [36], prior to the wholesale loss of CD4+ T-cells, thus facilitating HIV evasion and ultimately immunodeficiency in chronically infected humans.

Chimpanzees, humankind’s closest living relatives, are susceptible to chronic infection by HIV-1 and closely related SIVcpz infections. However, despite 98% genetic similarity, this species is relatively resistant to disease caused by lentiviruses. During more than 20 years of observation of HIV-1 infection in this species, relatively few animals have developed an AIDS-like disease, providing unique evolutionary
insight and the opportunity to investigate the key features in HIV-1 infection and (the absence of) subsequent disease progression [37–40]. In most HIV-1-infected chimpanzees, no reduction in CD4+ T-cell numbers, no decrease in response to recall antigens, and no overt and sustained increase in expression of activation markers or apoptosis is seen [40–43]. Furthermore, high proliferative responses against HIV as well as other pathogens, like cytomegalovirus, were noted [44, 45]. This study addressed the hypothesis that CD4+ T cell function is maintained in this species in the face of persistent HIV-1 infection. Specifically, we examined levels of CD154 expression and its regulation in relation to binding of the HIV-1 envelope to the CD4 receptor. Our findings reveal reduced expression levels of CD4 in chimpanzees as well as a reduced sensitivity for suppression of CD154 up-regulation if the natural, trimeric HIV-1 gp140, the primary viral ligand for CD4, is used as a CD4 cross-linking agent.

**Materials and Methods**

*Animal subjects and human donors*

Peripheral blood mononuclear cells (PBMC) were obtained from adult HIV-1/SIVcpz-infected as well as from uninfected chimpanzees housed at the BPRC in Rijswijk, The Netherlands. All HIV-1/SIVcpz-infected animals belonged to a cohort of animals that were exposed to various HIV-1 strains in several studies in the past, as previously described in detail [38, 39, 43, 73]. Animals were housed in groups according to the international guidelines for non-human primate care and use. PBMC from fresh blood were isolated using density gradient centrifugation (lymphocyte separation medium, ICN Biochemicals, Aurora, OH) and cryopreserved in FCS with 10% DMSO and stored at –135°C before use. Human peripheral blood was obtained from informed healthy volunteer donors via The Netherlands Red Cross Blood Bank, and, after obtaining informed consent, from anti-retroviral treatment naïve HIV-1-infected patients (median CD4+ T cell count 380 X 10⁶ cells/L (range 230–470) and median plasma HIV-1 RNA 4.7 log10 copies/mL (3.4–5.2)). All human blood samples were processed similar to the chimpanzee PBMC.
**CD154 and intracellular cytokine staining on PBMC of HIV-1 (un)infected subjects**

PBMC were incubated with anti-CD3/CD28 antibody-coated microbeads (Dynal, Oslo, Norway) added at a concentration of 2.5 µL/1 X 10⁶ cells for 2 h, followed by the addition of Golgiplug (BD Pharmingen, San Diego, CA) and overnight culture. After culturing, cells were stained for CD3-PE (clone SK7), CD4-PERCP (clone L200), CD8-APC-CY7 (clone SK1) and CD45RA-PE-CY7 (clone 5H9). After permeabilization and fixation, using BD Pharmingen Cytofix/Cytoperm solution according to the protocol, cells were stained for CD154-APC (clone TRAP1; all BD Pharmingen) in the presence of 10% human AB serum (Sanquin reagents, Amsterdam, The Netherlands). For the intracellular cytokine expression experiments, cells were first surface-stained for CD3-APC (clone SK7), CD4-PERCP, CD8-APC-CY7, CD45RAPE-CY7, followed by permeabilization and fixation and incubation with IL-2-PE (clone MQ1–17H12) in the presence of 10% human AB serum. Cells were fixed overnight with 2% paraformaldehyde in PBS and all samples were analyzed on a flow cytometer (FACSaria; Beckton Dickinson, Mountainview, CA).

**Inhibition of CD154 expression**

In order to evaluate the inhibitory capacities of CD4 ligation, CD4+ T cells were isolated from PBMC using a CD4 negative selection kit (BD Pharmingen). Purified CD4 cells were incubated with the following CD4-binding agents; anti-CD4 domain 1 antibody (clone QS4120; Ancell, Bayport, MN), anti-CD4 domain 2 anti-body (clone M-T411; Ancell; negative control), recombinant gp120 and trimeric O-gp140 of HIV-1 SF162 (Chiron, Emeryville, CA). After 1 h of incubation at 4°C, anti-CD3/CD28 antibody coated microbeads were added to the cells at a concentration of 2.5 µl/1 X 10⁶ cells. The cultures were incubated overnight at 37°C. After stimulation, cells were harvested and stained for CD154 (clone TRAP1) and CD69 (clone FN50; BD Pharmingen) and fixed using 2% paraformaldehyde. CD154 expression was determined on a flow cytofluorometer (FACSaria; Beckton Dickinson).
In order to evaluate expression of CD4 on the surface of human and chimpanzee T cells, PBMC were stained with a panel of antibodies directed against several epitopes of the CD4 receptor, used in a concentration range made to reach saturation levels (SK3-APC, L200-PE, QS4120 and M-T441). For the non-labeled antibodies, the cells were further incubated with goat anti-mouse APC-conjugated secondary antibodies, used at saturating levels. For assessing gp140 binding, CD4+ T cells were incubated for 1 h at 4°C with increasing doses of O-gp140, followed by staining with a FITC-conjugated anti-HIV-1gp120 mAb (clone BO1188; AALTO, Dublin, Ireland). Samples were recorded using either a FACScan or FACSaria flow cytometer, both from Becton Dickinson. All data were analyzed with Diva software (Becton Dickinson).
Results

IL-2 cytokine production and CD154 expression is not altered in chronically HIV-infected chimpanzees

Previous reports have demonstrated reduced expression of IL-2 as well as reduced CD154 up-regulation in HIV-1-infected subjects following PMA/ionomycin stimulation [34, 46]. Subsequently, a similarly impaired IL-2 expression was shown following stimulation through the physiologically more relevant CD3/CD28 pathway [10]. Previously, we have shown that PMA/ionomycin induced IL-2 expression was maintained in a cohort of HIV-1- and SIVcpz-infected chimpanzees [41]. Here, we applied stimulation through the CD3/CD28 pathway to investigate IL-2 as well as CD154 expression, as a measure of T helper (Th) function, in uninfected versus HIV-1- or SIVcpzANT-infected chimpanzees and compared this directly to uninfected versus HIV-1-infected human subjects. As shown in Fig. 1A, CD3/CD28-triggered production of IL-2 was highly reduced in CD4 T cells in the HIV-1-infected patients.

![Flow cytometry graph showing IL-2 and CD154 expression in CD4 T cells](image.png)

Figure 1. IL-2 and CD154 expression in CD4 T cells after stimulation with anti-CD3/CD28 antibodies in healthy humans, HIV-1-infected persons, healthy chimpanzees and HIV-1/SIVcpz-infected chimpanzees as measured by flow cytometry. Expression on all CD4 T cells, CD4CD45RA− and CD4CD45RA+ cells is shown as percentage of the respective (sub)populations. Data represent averages of at least five individuals per group with their standard deviation; * represents significant difference by Student's t-test (p<0.005).
but maintained in HIV-1-infected chimpanzees. Further subdivision in CD45RA–memory cells and CD45RA+ naive cells showed preferential expression of IL-2 in the memory cell subset. However, the decrease in IL-2 production in HIV-infected humans was seen to a similar extent in both the memory and naive subset, while high levels of IL-2 production were maintained in both subsets in the HIV-1-infected chimpanzees (Fig. 1A). Following CD3/CD28 ligation, only very low levels of CD154 induction could be reached in human HIV-infected subjects (3%; Fig. 1B), whereas in HIV-1-infected chimpanzees the expression of CD154 was induced in 29% of the CD4 Tcells, which is found to be in the same range as in uninfected chimpanzees and humans (25 and 33%, respectively). No difference in CD154 expression was found between CD45RA– and CD45RA+ cells (Fig. 1B).

*CD154 expression is inhibited by CD4 ligation.*

As an upstream mechanism for the impaired CD154 expression in HIV-1-infected humans, it was shown that cross-linking CD4 can inhibit CD3/CD28-triggered expression of CD154 in humans. We therefore investigated whether the same effect could be observed in chimpanzees. CD4+ T cells from healthy chimpanzees and human donors were pre-incubated for 1 h with an anti-CD4 antibody directed against domain 1 (functional part of the receptor) or domain 2 (non-functional). Subsequently, cells were activated by culturing them in the presence of anti-CD3/CD28 antibody-coated microbeads. Both in human and chimpanzee CD4+ Tcells, the CD4 domain 1-preligated group showed reduced expression of CD154 (Fig. 2), whereas the domain 2-ligated (data not shown) groups showed strong induction of CD154 by the CD3/CD28 stimulus. In order to evaluate the effect of cross-linking of CD4 with the HIV-1 envelope, the experiment was repeated with recombinant monomeric gp120 as well as trimeric gp140 at a relatively high concentration (10 µg/ml).
As shown in Fig. 2, both chimpanzee and human CD4+ T cell activation was reduced by trimeric gp140 binding to CD4, resulting in a strong decrease of CD154 expression, which was of similar magnitude as ligation of CD4 with the domain 1-specific antibody. Monomeric gp120 did not result in a significant decrease of CD154 expression (data not shown), indicating that the trimeric envelope complex is required for functional consequences of cross-linking of the CD4 receptor. As shown in Fig. 2B, expression of the activation marker CD69 was not affected by cross-linking of CD4, either by the anti-CD4 domain 1 mAb or the trimeric gp140.

**CD4 expression differs between species**

While chimpanzee and human CD4+ T cells seemed to be equally susceptible to the immune-suppressive effect of CD4 cross-linking, subsequent mAb titration studies with a library of CD4 antibodies to several epitopes of the receptor showed a 30% lower expression of CD4 on chimpanzee cells at saturation level (Fig. 3A).
Further analysis of the expression of CD4 in at least four different humans and chimpanzees showed similar results (Fig. 3B), leading to the conclusion that the differences were not due to a different binding capacity of the antibody, but to the expression of CD4 on the membrane of chimpanzee T cells. Chimpanzee CD4+ T cells are less sensitive to gp140 mediated suppression of CD154 up-regulation.

In order to evaluate the functional relevance of this difference in CD4 expression, a CD154 induction experiment was performed in the presence of decreasing concentrations of anti-CD4 domain 1 antibody. This titration did not expose any differences between humans and chimpanzees with respect to the inhibitory capacity of the CD4 antibody, i.e. both human and chimpanzee CD4+ T cells showed impairment at a concentration of approximately 0.01 µg/mL of anti-CD4 antibody (Fig. 4A), which is far below the saturation level of the CD4 receptor (Fig. 3).
The dose-dependent effects of viral envelope protein were tested in a similar manner as the anti-CD4 antibody. The results of this experiment revealed that in human CD4+ T cells 0.2 µg/ml gp140 sufficed to inhibit CD154 up-regulation, whereas in chimpanzees 0.8 µg/ml was required (Fig. 4B). These results seem to indicate that in an environment with relatively low concentrations of viral envelope protein, chimpanzee CD4+ T cells can function normally, in contrast to human CD4+ T cells. The concentration of trimeric gp140 at which these divergent effects were detected was far below the saturation level (Fig. 5). However, it must be noted that at these low gp140 concentrations, a slightly reduced binding to chimpanzee CD4+ T cells could be seen in comparison to human CD4+ T cells.

Figure 4. Dose-response curves of the inhibitory effects of CD4-binding agents on CD3/CD28-induced CD154 expression on CD4+ T cells of healthy humans and in chimpanzees. Percentage of inhibition of expression is plotted against the concentration of anti-CD4 antibody (A) or recombinant HIV-1 gp140 (B). Data are represented as averages with SEM calculated from experiments on four (A) or five (B) separate individuals; * represents significant difference by Student's t-test (p<0.005).

Figure 5. O-gp140 binding to chimpanzee and human CD4+ T cells. Cells were incubated with increasing concentrations of O-gp140 and subsequently stained for bound protein using FITC-conjugated anti-HIV-1 gp120 mAb. Shown is the MFI average ± standard deviation of two chimpanzees and two human donors.
Discussion

Generation of effective immune responses to pathogens or cancer cells requires closely coordinated crosstalk between Th cells and DC, as one of the first steps in orchestrating a strong cellular immune response. CD4+ T cells that have been activated via their T-cell receptor express CD154, which via binding to its ligand CD40 leads to the necessary subsequent activation of DC. Subsequently, up-regulation of CD80 and CD86 provides additional T cell stimulation signals via CD28 binding, inducing T cell proliferation and IL-2 production. Both CD154 expression on CD4+ T cells and IL-2 production have been reported to be reduced in HIV-1-infected persons [8, 10, 30, 47–52]. Here, we showed that, in addition to the previously published maintenance of PMA/ionomycin-induced IL-2 expression [37, 40, 42, 53, 54], stimulation with a more physiological CD3/CD28 cross-linking agent similarly generated high IL-2 expression in CD4+ T cells of HIV-1-and an SIVcpzANT-infected chimpanzee. This preservation was seen in the IL-2 high-expressing CD45RA– memory cell subset as well as in CD45RA+ cells, while IL-2 expression was lost in both subsets in HIV-1-infected humans.

In view of the central regulatory role of CD154, we then proceeded to study its expression in HIV-1-infected chimpanzees.

In contrast to its low expression in HIV-1-infected humans, we found strong expression of CD154 following CD3/CD28 stimulation in HIV-1-infected chimpanzees. There was no difference observed between CD45RA– and CD45RA+ cells, which is probably due to the overnight stimulation period, which has been reported to result in similar levels of induction on both subsets after PMA/ionomycin stimulation [55].

Several groups have found evidence that the binding of HIV-1 envelope protein to the CD4 receptor can lead to reduced CD154 expression [34, 35]. Cross-linking of CD4 was shown to interfere with signal transduction cascades initiated by CD3/CD28 stimulation [56], resulting in inhibition of up-regulation of CD154. Using CD4 blocking agents we showed that, as in humans, chimpanzee CD4+ T cells are susceptible to CD4-induced inhibition of CD3/CD28-induced CD154 expression. Both an antibody to domain 1 of the CD4 receptor as well as recombinant trimeric gp140 were able to inhibit CD154 up-regulation. Importantly, when we used trimeric
gp140 for cross-linking of CD4, a reduced sensitivity for inhibition of CD154 expression was found on chimpanzee compared to human CD4 T cells. These differences were masked when trimeric gp140 was used at a high concentration or when a relatively strong cross-linking agent like anti-domain 1 mAb was used. Strikingly, we observed that chimpanzee CD4+ T cells express 30% lower amounts of the CD4 receptor on their membrane. This cannot be explained by reduced binding capacity of the antibodies, since we have observed this effect with four different anti-CD4 mAb and know that at least the SK3 binding site is identical between human and chimpanzee CD4 [57]. An explanation for this lower expression of CD4 is at present unknown. HIV-1 Nef was reported to induce similar cell surface down-regulation of CD4 on human and chimpanzee CD4+ T cells [58]. While it could be speculated that the lower expression of CD4 on chimpanzee cells is responsible for their reduced sensitivity to CD4-mediated CD154 down-modulation, it must be stressed that these inhibitory effects have been observed below receptor saturation levels (Fig. 3, 5). Also at these lower antibody orgp140 concentrations, a slight difference in fluorescence signal is seen between human and chimpanzee cells. On the other hand, the data do imply that the reduced sensitivity of chimpanzee CD4 T cells to the CD154 down-modulating effect of gp140 cross-linking is not an indirect consequence of the number of CD4 molecules that need to be cross-linked, as chimpanzees have less CD4 but require more gp140 for CD154 inhibition. Importantly, Fomsgaard et al. [57] have identified four amino acid changes in the V1J1 loop of chimpanzee CD4 that are all part of the HIV-1 binding site, which could possibly explain why we observed a reduced sensitivity of chimpanzee cells to cross-linking with gp140, but not with anti-CD4 mAb. Especially, a difference in net charge induced by the change from glutamic acid to glycine at position 77 could affect CD4 conformation and binding of gp120. While it has been described that chimpanzee T cells express high levels of sialic acid-binding immunoglobulin-like lectin (SIGLEC) 5 and respond poorly to TCR/CD3 stimulation [59], we obtained similar levels of CD69 expression, varying between 80 and 90%, in both species. Thus, an intrinsic difference in susceptibility to CD3/CD28 stimulation does not seem to play a role. Although the full implications of this difference in sensitivity to gp140-mediated down-modulation of CD154 between chimpanzees and humans are not yet clear, it could imply that low plasma virus levels would contribute to the relative resistance of chimpanzees to immunosuppressive effects of HIV-1. This should become especially apparent several months after
infection, when virus replication reaches relatively low steady-state levels. It is striking that initially in most infected humans strong CTL responses are formed, which are known to precede control of virus replication. However, subsequently, Th responses become impaired, leading to the formation of a relatively dysfunctional CTL response characterized by elevated PD-1 expression, decreased IFN-γ and perforin expression, resulting in a poor control of virus replication [60–71]. Maintenance the CD154-CD40 costimulatory pathway may be an important factor in the preservation of adequate CD4+ T cell help during this critical phase of the infection. Besides preservation of Th function, nonpathogenic infection of HIV-1 or SIVcpz in chimpanzees is also accompanied by low levels of activation marker expression [41]. However, since cross-linking of CD4 was found to have no effect on CD69 expression, other mechanisms may be involved in preserving this low state of cell activation. Additionally, the low-level expression of viral receptors on target cells in the chimpanzee host, especially in active sites of viral replication such as the intestinal mucosa [72], may provide this host with a selective advantage.

**Statistical analysis**

Statistical analysis was performed on the data as indicated (*) in the figures by Student's t-test. A non-parametric Mann-Whitney test was used to compare CD4 expression levels between human and chimpanzee peripheral blood T cells shown in Fig. 3B.

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