Nef promotes evasion of human immunodeficiency virus type 1-infected cells from the CTLA-4-mediated inhibition of T-cell activation

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CTLA-4 is a negative regulator of T-cell receptor-mediated CD4⁺ T-cell activation and function. Upregulation of CTLA-4 during human immunodeficiency virus type 1 (HIV-1) infection on activated T cells, particularly on HIV-specific CD4⁺ T cells, correlates with immune dysfunction and disease progression. As HIV-1 infects and replicates in activated CD4⁺ T cells, we investigated mechanisms by which HIV-1 modulates CTLA-4 expression to establish productive viral infection in these cells. Here, we demonstrate that HIV-1 infection in activated CD4⁺ T cells was followed by Nef-mediated downregulation of CTLA-4. This was associated with a decreased T-cell activation threshold and significant resistance to CTLA-4 triggering. In line with these *in vitro* results, quantification of pro-viral HIV DNA from treatment-naïve HIV-infected subjects demonstrated a preferential infection of memory CD4⁺CTLA-4⁺ T cells, thus identifying CTLA-4 as a biomarker for HIV-infected cells *in vivo*. As transcriptionally active HIV-1 and Nef expression *in vivo* were previously shown to take place mainly in the CD3⁺CD4⁺CD8[−] [double-negative (DN)] cells, we further quantified HIV DNA in the CTLA-4⁺ and CTLA-4[−] subpopulations of these cells. Our results showed that DN T cells lacking CTLA-4 expression were enriched in HIV DNA compared with DN CTLA-4⁺ cells. Together, these results suggested that HIV-1 preferential infection of CD4⁺CTLA-4⁺ T cells *in vivo* was followed by Nef-mediated concomitant downregulation of both CD4 and CTLA-4 upon transition to productive infection. This also highlights the propensity of HIV-1 to evade restriction of the key negative immune regulator CTLA-4 on cell activation and viral replication, and therefore contributes to the overall HIV-1 pathogenesis.

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INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) infection is characterized by the progressive decline in the absolute numbers of CD4⁺ T cells and by an elevated state of

immune activation (Brenchley *et al.*, 2006; Grossman *et al.*, 2006; Mellors *et al.*, 1997). As a counter-mechanism to chronic T-cell activation, a mounting number of immune checkpoint receptors including the immunoglobulin superfamily member CTLA-4 are specifically upregulated on HIV-specific T cells (Kaufmann *et al.*, 2007; Larsson *et al.*, 2013; Leng *et al.*, 2002; Rueda *et al.*, 2012). The upregulation and engagement of these immune checkpoint receptors leads to a hierarchical but reversible functional impairment of T cells (El-Far *et al.*, 2008; Kaufmann & Walker, 2008; Khaitan & Unutmaz, 2011; Trautmann *et al.*, 2007). Expression and engagement of CTLA-4 by its ligands CD80 and CD86 results in inhibition of T-cell activation (Kroczek *et al.*, 2004) through mechanisms involving intracellular phosphatases, i.e. SHP-1, that inhibit T-cell receptor (TCR) downstream signals, including Akt phosphorylation (Kroczek *et al.*, 2004; Parry *et al.*, 2005). This results in the arrest of T-cell glucose consumption, proliferation and IL-2 secretion (Guntermann & Alexander, 2002; Harlin *et al.*, 2002; Krummel & Allison, 1996). Engagement of CTLA-4 also increases T-cell motility by decreasing the contact time between T cells and antigen-presenting cells, thereby preventing responses to low-affinity sub-threshold self-MHC agonists (Schneider *et al.*, 2006).

Considering the heightened expression of CTLA-4 on CD4⁺ T cells, the major targets of HIV-1, it is important to determine whether CTLA-4 restricts HIV replication in CD4⁺ T cells and whether HIV-1 has evolved to overcome this potential immune restriction mechanism. The viral protein Nef is known to play an important role in modulating the cellular microenvironment required for efficient viral replication, by downregulating multiple cell surface molecules through its interference with the intracellular sorting machinery (Aiken *et al.*, 1994; Arold & Baur, 2001). Expression of Nef leads to the early activation of infected cells and promotes secretion of the major T-cell growth factor IL-2 (Schrager & Marsh, 1999; Wu & Marsh, 2001). In this regard, we have previously demonstrated that CTLA-4 is downmodulated by HIV-1 Nef in co-transfection systems *in vitro* (El-Far *et al.*, 2013). This novel mechanism involves Nef-mediated CTLA-4 degradation in lysosomal compartments and requires Nef motifs involved in CD4 downregulation.

Here, we assessed the permissiveness of CTLA-4⁺CD4⁺ T cells to HIV-1 infection both *in vitro* and *in vivo*. We also investigated the mechanisms by which HIV-1 interfered with the role of CTLA-4 in regulating CD4⁺ T-cell activation and function to enhance its own replication.

RESULTS

Preferential infection of CTLA-4⁺CD4⁺ T cells *in vitro*

To assess the permissiveness of CTLA-4⁺CD4⁺ T cells to HIV-1 infection, we first enriched for these cells by

stimulating purified primary CD4⁺ T cells via the TCR for 72 h. As a strategy to avoid antibody-mediated engagement of the CTLA-4 pathway, we monitored the expression of a panel of activation markers that positively correlated with CTLA-4 expression to identify surface marker candidates that could be used to sort CTLA-4⁺ T cells. TCR-stimulated CD4⁺ T cells upregulated the expression of surface and intracellular CTLA-4 together with other activation markers, including CD25, CD154, CD38, CD62L and CD71 (Figs 1a and S1a, available in the online Supplementary Material). Amongst these markers, the expression of CD25 and CTLA-4 largely overlapped, with up to 90 % of CD25⁺ cells expressing CTLA-4. CD25⁺CTLA-4^{hi} and CD25⁺CTLA-4^{lo} subsets sorted by flow cytometry (Fig. 1b) were infected with the HXB2 HIV-1 strain expressing the WT *nef* gene (HIV-1^{Nef+}) or with the same strain harbouring a frameshift mutation in the *nef* coding sequence (HIV-1^{ΔNef}). Following 48 h infection with HIV-1^{Nef+} or HIV-1^{ΔNef}, the CD25⁺CTLA-4^{hi}-infected subset was enriched in HIV p24⁺ cells compared with CD25⁺CTLA-4^{lo} cells ($n=3$; $P=0.002$ for HIV-1^{Nef+}, $P=0.0004$ for HIV-1^{ΔNef}) (Fig. 1c, d). Together, these results demonstrated that CD4⁺ T cells expressing high levels of CTLA-4 following TCR stimulation were highly permissive to HIV-1 in the presence or absence of the regulatory protein Nef.

Nef downregulates CTLA-4 expression in infected primary CD4⁺ T cells

To assess the ability of Nef to modulate CTLA-4, CD4 and CD3 in primary CD4⁺ T cells, we measured the expression of these molecules by flow cytometry on productively infected cells (HIV p24⁺ cells) following 48 h infection with HIV-1^{Nef+} or HIV-1^{ΔNef}. Infection with HIV-1^{Nef+} resulted in a significantly reduced frequency of CTLA-4 expression on p24⁺ cells (mean decrease of 65 %, $P=0.006$ for surface expression and 52 %, $P<0.0001$ for intracellular expression) when compared with infection with HIV-1^{ΔNef} (Fig. 2a, b). Similarly, the mean fluorescence intensity (MFI) of CTLA-4 was significantly decreased upon HIV-1^{Nef+} infection (Fig. 2b). As expected, cells infected with HIV-1^{Nef+} virus downregulated the expression of CD4 (Fig. 2a, b), whereas CD3 expression was not affected. The Nef-mediated impact on CD4 was markedly and significantly observed on the p24⁺ cells as early as 24 h post-infection compared with the modest impact on CTLA-4 expression (Fig. S1b, c), which became significant by 48 h (Fig. 2a, b). This was likely due to the contribution of other viral proteins such as Env and Vpu, known to be involved in CD4 downregulation to prevent superinfection. Of note, the impact of Nef on the expression of both CTLA-4 and CD4 was specific for p24⁺ cells (Fig. S2a, b).

To investigate the capacity of Nef from other lentiviruses to downregulate CTLA-4 expression, we infected primary CD4⁺ T cells with GFP-reporter viruses expressing Nef from simian immunodeficiency virus (SIV; cpz and mac239),

Fig. 1. Phenotypic characterization and permissiveness to HIV-1 infection of CD4⁺CTLA-4⁺ T cells. Total CD4⁺ T cells were isolated by negative selection using magnetic beads, and stimulated with immobilized CD3 (1 $\mu\text{g ml}^{-1}$) and soluble CD28 antibodies (0.5 $\mu\text{g ml}^{-1}$) for 72 h. (a) Representative surface staining for surrogate activation markers co-expressed with CTLA-4 (intracellular) on activated CD4⁺ T cells. Analysis was performed on gated CD3⁺CD4⁺ T cells that excluded the viability dye LIVE/DEAD. (b) Enrichment of CTLA-4^{high} T cells by sorting CD25⁺ T cells using flow cytometry. Left panel: CTLA-4 and CD25 levels on activated T cells prior to sorting. Right panels: purity of the two populations CTLA-4^{hi} and CTLA-4^{lo} after cytometry sorting. (c) Representative HIV p24 intracellular staining in CD25⁺CTLA-4^{hi} (upper panels) and CD25⁺CTLA-4^{lo} (lower panels) T cells infected with HIV-1^{Nef+} or HIV-1^{ΔNef} at 48 h post-infection. SSC, side scatter. (d) Mean $\pm$ SD frequency of HIV-infected cells in CD25⁺CTLA-4^{hi} and CD25⁺CTLA-4^{lo} T cells when cells were infected with HIV-1^{Nef+} or HIV-1^{ΔNef} ($n=3$). Paired t -test P values are indicated.

as well as HIV-1. Our data demonstrated that Nef proteins from both SIVcpz and SIVmac239 were able to downregulate CTLA-4 expression levels, similar to HIV Nef (Fig. 2c). In agreement with previous reports (Schindler *et al.*, 2006), SIVmac239 Nef, and to a lesser extent SIVcpz Nef,

downregulated CD3 expression (Fig. 2c). As expected, and in contrast to non-human primate lentiviruses, HIV Nef did not downregulate CD3. Thus, we identified the downmodulation of CTLA-4 as a conserved function of Nef from human and non-human primate lentiviruses.

Fig. 2. HIV-1 Nef downregulates CTLA-4 expression in HIV-infected primary CD4⁺ T cells. CD4⁺ T cells were activated for 48 h as in Fig. 1 and exposed to HIV for an additional 48 h. (a) Representative dot-plots showing the percentage of HIV p24⁺ cells (left panels) expressing CTLA-4 (intracellular and extracellular), CD4 and CD3 after infection with HIV-1 Δ Nef (upper panels) or HIV-1Nef⁺ (lower panels). Numbers in each quadrant refer to the percentage of HIV p24⁺ T cells expressing CTLA-4, CD4 and CD3. (b) Percentages (upper panels) and MFI (lower panels) of CTLA-4, CD4 and CD3 following infection with HIV-1Nef⁺ or HIV-1 Δ Nef ($n=22$). Paired t -test P values are indicated. (c) Left panels: representative FACS data for CTLA-4

(upper panels) and CD3 expression (lower panels) on total GFP⁺ and GFP⁻ cells after infection with GFP-HIV-1^{ΔNef}, GFP-HIV-1^{Nef+}, GFP-SIVcpz^{Nef+} and GFP-SIVmac239^{Nef+} (representative of $n=3$). Right panels: histograms showing the frequency of CTLA-4⁺ and CD3⁺ cells from the GFP⁺ subpopulations (infected cells) for the four different viruses at 48 h post-infection ($n=3$). Paired t -test P values are indicated; NS, not significant.

CTLA-4 downregulation by Nef coincides with enhanced IL-2 production

Given the fact that CTLA-4 triggering inhibits IL-2 production in uninfected T cells (Guntermann & Alexander, 2002; Harlin *et al.*, 2002; Krummel & Allison, 1996), we first examined whether Nef-mediated CTLA-4 downregulation was associated with enhanced IL-2 production in HIV-infected T cells. TCR-activated CD4⁺ T cells were infected with HIV-1^{Nef+} or HIV-1^{ΔNef} for 2 days, and IL-2 production was measured by intracellular cytokine staining in response to a second TCR triggering. Of note, the first round of TCR triggering led to the upregulation of the CTLA-4 ligand CD86 (Fig. S3) on activated T cells, in agreement with earlier reports (Azuma *et al.*, 1993; Podojil & Miller, 2009). T cells infected with HIV-1^{Nef+} or HIV-1^{ΔNef} produced higher levels of IL-2 upon TCR stimulation as compared with non-infected controls (Fig. 3a) ($n=5$; $P<0.0001$ and $P=0.002$, respectively, for frequency; $P=0.0002$ and $P=0.0002$, respectively, for MFI); this effect was significantly more pronounced in T cells infected with HIV-1^{Nef+} compared with HIV-1^{ΔNef} ($n=5$; $P=0.002$ and $P=0.003$ for frequency and MFI, respectively). These results were attributed to productively infected cells, as the frequency and MFI of IL-2⁺ cells within the HIV p24⁺, but not HIV p24⁻, subset were significantly higher in cells infected with HIV-1^{Nef+} compared with HIV-1^{ΔNef} viruses ($P=0.0002$ for frequency, $P=0.01$ for MFI; Fig. 3b, c). Of note, there was a tendency for a negative correlation between the level of expression of CTLA-4 (MFI) on the p24⁺ subpopulations from both HIV-1^{Nef+}- and HIV-1^{ΔNef}-infected cells and the frequency of IL-2⁺ cells. However, this correlation did not reach statistical significance, likely due to the limited number of measures ($n=5$) (data not shown). These observations were further confirmed by measuring IL-2 production by ELISA. CD4⁺ T cells infected with HIV-1^{Nef+} compared with HIV-1^{ΔNef} produced higher levels of IL-2 (mean 1.5-fold) (Fig. 3d; $n=3$; $P=0.04$).

To test whether Nef-induced downregulation of CTLA-4 expression (Fig. 2) was associated with decreased cellular responsiveness to CTLA-4-mediated negative regulation of T-cell activation, CD3, CD28 and CTLA-4 were simultaneously cross-linked on T cells infected with HIV-1^{ΔNef} or HIV-1^{Nef+}. As shown in Fig. 3(e), the magnitude of inhibition of IL-2 production in T cells infected with HIV-1^{ΔNef} was significantly higher (47 %) than in cells infected with HIV-1^{Nef+} (24 %) ($n=8$; $P=0.01$). These results supported a model in which HIV Nef abrogated the ability of CTLA-4 to negatively regulate T-cell activation, reflected here by IL-2 production.

Nef-mediated CTLA-4 downregulation is associated with decreased TCR activation threshold and abrogates the negative impact of CTLA-4 on viral replication

We next performed experiments to determine whether Nef-induced downregulation of CTLA-4 was associated with a decreased TCR activation threshold (Schrager & Marsh, 1999). We measured the levels of IL-2 production in T cells infected with HIV-1^{Nef+} compared with HIV-1^{ΔNef} upon TCR/CTLA-4 co-cross-linking with limiting concentrations of CD3/CD28 antibodies. The results (Fig. 4a, b) demonstrated that HIV-1^{Nef+}-infected cells produced significantly higher levels of IL-2 (up to twofold) compared with HIV-1^{ΔNef}-infected cells ($n=4$; $P=0.02$) under suboptimal TCR activation and CTLA-4 triggering conditions (35/7 ng CD3/CD28 antibodies). Differences were not statistically significant when high CD3/CD28 concentrations were used (280/56 ng CD3/CD28 antibodies) (Fig. 4a, b).

The impact of CTLA-4 cross-linking on viral replication was further assessed in T cells infected with the replication-competent viruses HIV-1^{Nef+} or HIV-1^{ΔNef}. Levels of total HIV DNA were significantly decreased (25–55 %) following 4 days of CTLA-4 cross-linking in T cells infected with HIV-1^{ΔNef} compared with HIV-1^{Nef+} in the absence of any viral inhibitors that could prevent new cycles of infection ($n=4$; $P=0.04$) (Fig. 4c). In line with these results, CTLA-4 triggering led to decreased levels of viral production, measured by p24 release in the culture supernatant, in HIV-1^{ΔNef}-infected T cells compared with Nef-competent viruses (Fig. 4d). Of note, as shown in Fig. 2, CTLA-4 expression was significantly lower on HIV-1^{Nef+} compared with HIV-1^{ΔNef}-infected cells prior to CTLA-4 triggering (data not shown).

Together, these results demonstrated that Nef enhanced T cell activation and viral replication under suboptimal TCR triggering conditions, consistent with its ability to downregulate CTLA-4 on infected T cells.

Memory CD4⁺CTLA-4⁺ T cells are preferentially infected in HIV⁺ subjects

CTLA-4 is upregulated on total and HIV-specific CD4⁺ T cells (Kaufmann *et al.*, 2007; Leng *et al.*, 2002; Rueda *et al.*, 2012). However, the selective viral permissiveness of CTLA-4⁺ T cells in HIV-infected subjects remains unknown. Consistent with previous reports (Kaufmann *et al.*, 2007; Leng *et al.*, 2002), our results demonstrated that CTLA-4 expression was significantly higher on total CD4⁺ but not CD8⁺ T cells from treatment-naïve HIV-infected compared

Fig. 3. CTLA-4 downregulation by Nef coincides with enhanced IL-2 production in primary CD4⁺ T cells. CD4⁺ T cells were activated for 48 h as in Fig. 1, exposed to HIV-1 for an additional 48 h and restimulated via CD3/CD28 antibodies

(plate-coated CD3 antibodies, $0.1 \mu\text{g ml}^{-1}$; soluble CD28 antibodies, $0.1 \mu\text{g ml}^{-1}$). (a) Left panel: frequency of CTLA-4⁺ cells on the HIV p24⁺ subpopulations from HIV-1^{ΔNef} compared with HIV-1^{Nef+}-infected cells at 48 h post-infection ($n=5$). Percentages (middle panel) and MFI (right panel) of IL-2-expressing T cells measured by intracellular staining in the total CD4⁺ T cells (including both HIV p24⁺ and p24⁻) infected with HIV-1^{Nef+} or HIV-1^{ΔNef} upon 15 h of TCR restimulation (the same donors as in the left panel). (b) Representative dot-plots showing the frequency of IL-2-expressing cells in the HIV p24⁺ and p24⁻ populations from cells infected with HIV-1^{Nef+} and HIV-1^{ΔNef} compared with non-infected control cells. (c) Percentages (left panel) and MFI (right panel) of IL-2⁺ cells within the p24⁺ or p24⁻ populations in cells infected with HIV-1^{Nef+} or HIV-1^{ΔNef}. (d) IL-2 production was measured by ELISA at 24 h following TCR restimulation (plate-coated CD3 antibodies, $0.1 \mu\text{g ml}^{-1}$; soluble CD28 antibodies, $0.1 \mu\text{g ml}^{-1}$) of cells infected with HIV-1^{Nef+} or HIV-1^{ΔNef} and uninfected cells (Mock) from three different donors. (e) Levels of IL-2 production measured by ELISA after TCR and CTLA-4 simultaneous cross-linking on CD4⁺ T cells infected with either HIV-1^{Nef+} or HIV-1^{ΔNef} in eight donors. Simultaneous TCR and CTLA-4 cross-linking was carried out using beads coated with CD3 and CD28 antibodies (35 and $7 \text{ ng}/20 \times 10^6$ beads, respectively; four beads per cell) and a fixed concentration ($1 \mu\text{g}/20 \times 10^6$ beads) of CTLA-4 antibodies. Relative IL-2 values were calculated by dividing the concentration of IL-2 produced upon CTLA-4 and TCR simultaneous cross-linking by the concentration of IL-2 produced after TCR stimulation alone. Nef-mediated CTLA-4 downregulation in cells infected with HIV-1^{Nef+} (as shown in Fig. 2) was confirmed in each experiment prior to CTLA-4 triggering. Paired *t*-test *P* values are indicated; NS, not significant.

with uninfected subjects in terms of frequency (8.3 ± 1.3 and 5 ± 1.5 , respectively; $P=0.02$) and MFI (7180 versus 6226, respectively; $P=0.02$) (Fig. S4a). As expected, CTLA-4 was mainly expressed by the memory (CD45RA⁻) fraction of CD4⁺ T cells compared with the naive (CD45RA⁺) subset ($P=0.008$ and $P=0.006$ for viraemic subjects and HIV-negative donors, respectively) (Fig. S4b).

To assess the permissiveness of memory CTLA-4⁺ T cells to HIV-1 infection *in vivo*, we sorted three subsets of CD4⁺ T cells based on the levels of CTLA-4 expression: CTLA-4^{hi} (~1–2 %), CTLA-4^{med} (~10–15 %) and CTLA-4⁻ (~60–85 %) (Fig. 5a). Results from three different treatment-naive viraemic HIV-infected subjects showed that the frequency of T cells carrying integrated HIV DNA was two- to fourfold higher in CTLA-4^{hi} cells compared with CTLA-4^{med} cells and six- to 18-fold higher compared with CTLA-4⁻ cells (Fig. 5b). Together, these data indicated that CD4⁺CTLA-4⁺ T cells were preferentially infected in treatment-naive HIV-infected subjects.

CD3⁺CD4⁻CD8⁻ double-negative (DN) T cells lacking CTLA-4 are infected in HIV⁺ subjects

Results in Fig. 2 demonstrated that infection of primary CD4⁺ T cells with Nef-competent HIV-1 *in vitro* led to downregulation of both CTLA-4 and CD4. By gating on HIV p24-expressing cells (infected with HIV-1^{Nef+} *in vitro* for 2 days) we observed that these cells were divided into two subpopulations: p24⁺CD4⁻ T cells (indicative of Nef expression and downregulation of CD4) and p24⁺CD4⁺ T cells (infected cells that retained the expression of CD4). The expression of CD4 surface receptor on these latter cells may either indicate a recent infection or a defective infection with no expression of Nef. By further measuring the total levels of CTLA-4 and CD4 expression on these two subpopulations, we observed that the MFI of CTLA-4 was significantly lower in p24⁺CD4⁻ cells compared with p24⁺CD4⁺ cells ($P=0.0004$) and also compared with the p24⁻CD4⁺ cells from the same infection ($P<0.0001$) (Fig. S5a, b). These results suggested a direct relationship

between the productive HIV-1^{Nef+} infection, Nef expression and CTLA-4/CD4 simultaneous downregulation. To determine whether CTLA-4/CD4 downregulation occurred in infected T cells *in vivo*, we first measured the frequency of CD4⁻ T cells in PBMCs from HIV-infected and uninfected subjects by gating on CD3⁺CD4⁻CD8⁻ DN T cells. The results (Fig. 5c) clearly showed that the frequency of DN T cells was significantly increased in treatment-naive HIV-infected viraemic subjects compared with virally suppressed subjects receiving combination antiretroviral therapy (cART) (Table 1) and HIV-negative controls ($n=13$ per group; $P=0.03$ and $P=0.005$, respectively).

We further monitored CTLA-4 expression on DN T cells. Results (Fig. 5d) showed that CTLA-4 expression was significantly upregulated on total DN cells from HIV-infected compared with uninfected subjects ($n=5$ per group) (13 versus 3 %, $P=0.002$). The use of CD45RA as a marker to distinguish between naive and memory T cells (Riou *et al.*, 2007; Sallusto *et al.*, 1999) demonstrated that CTLA-4 was exclusively expressed by naive DN CD45RA⁺ T cells, whereas memory DN CD45RA⁻ T cells lacked CTLA-4 (Fig. 5d). Of note, in addition to γ/δ T cells, the memory DN CD45RA⁻ (CTLA-4⁻) subset included a significant fraction of α/β T cells (Fig. S6). As the majority of γ/δ T cells lack the expression of CD4 and CD8 surface markers (Girardi, 2006), accumulation of α/β T cells within the DN subset in treatment-naive HIV viraemic subjects may be the result of an ongoing viral infection leading to the downregulation of the CD4 receptor together with CTLA-4. To determine whether DN cells lacking CTLA-4 were preferentially infected with HIV *in vivo*, we further quantified the proviral HIV DNA load in DN T cells expressing (or not) CTLA-4 in HIV-infected subjects. To avoid the permeabilization step that would have been required to stain CTLA-4 intracellularly, we used the surface marker CD45RA as a surrogate for CTLA-4 intracellular expression, and sorted CD45RA⁺ (CTLA-4⁺) and CD45RA⁻ (CTLA-4⁻) DN T cells from HIV-infected viraemic subjects [viral load: 4.0 – $4.8 \log$ (copies ml^{-1})];

Fig. 4. CTLA-4 downregulation is associated with decreased T-cell activation threshold and enhanced HIV-1 replication upon CTLA-4 triggering. CD4⁺ T cells were activated for 48 h as in Fig. 1, exposed to HIV for an additional 24 h, and restimulated via beads coated with CD3/CD28 and CTLA-4 antibodies or matched isotype control for an additional 24 h (for IL-2 quantification), 4 days (for HIV DNA quantification) or 9 days (for HIV p24 quantification). (a) Levels of IL-2 production measured by ELISA in a representative subject. Simultaneous TCR and CTLA-4 cross-linking was carried out using decreasing concentrations of CD3 and CD28 antibodies (CD3: 280–35 ng/20×10⁶ beads; CD28: 56–7 ng/20×10⁶ beads; four beads per cell) and a fixed concentration (1 µg/20×10⁶ beads) of CTLA-4 antibodies on cells infected with HIV-1^{Nef+} or HIV-1^{ΔNef}. (b) Fold increase in IL-2 production from HIV-1^{Nef+} over HIV-1^{ΔNef} under simultaneous TCR and CTLA-4 cross-linking (mean ± SD from four subjects). (c) CTLA-4-dependent inhibition of viral replication as monitored by quantification of total HIV DNA at day 4 following TCR (CD3 and CD28 antibodies 35/7 ng/20×10⁶ beads, respectively; four beads per cell) and CTLA-4 (1 µg/20×10⁶ beads) cross-linking (or isotype control: Iso-IgG) on HIV-1^{Nef+} and HIV-1^{ΔNef}-infected cells (*n*=4). (d) HIV p24 production measured by ELISA at day 6 following simultaneous TCR and CTLA-4 cross-linking on cells infected with eGFP-HIV-1 virus expressing Nef compared with the ΔNef virus. Shown are the p24 production levels from both viral infections under simultaneous TCR and CTLA-4 cross-linking relative to TCR⁺ Isotype activation (*n*=3). Prior to TCR/CTLA-4 triggering, CD4⁺ T cells infected for 24 h with eGFP viruses were treated with and maintained in 5 µM azidothymidine-containing medium to prevent reinfection. Paired *t*-test *P* values are indicated.

n=4] and measured levels of integrated HIV DNA by real-time PCR. The data (Fig. 5e) showed that memory CD45RA⁻ DN T cells lacking CTLA-4 expression were

highly infected with HIV-1 (400–2500 copies of integrated HIV DNA/10⁶ cells). Interestingly, integrated HIV DNA was exclusively detected in the CD45RA⁻ (CTLA-4⁻) but

Fig. 5. Enrichment in pro-viral HIV DNA in memory CD4⁺CTLA-4⁺ and memory DN CTLA-4⁻ T cells from treatment-naive subjects. (a) Gating strategy used to sort CTLA-4^{hi}, CTLA-4^{med} and CTLA-4⁻ subsets within memory CD45RA⁻CD4⁺ T cells from PBMCs of treatment-naive HIV-infected subjects. SSC, side-scatter. (b) Frequency of cells harbouring integrated HIV DNA in each sorted cell subset. CD4, CD4 count (mm⁻³); VL, viral load [log (copies ml⁻¹)]. (c) Frequency of CD3⁺CD4⁻CD8⁻ DN T cells from treatment-naive HIV-infected subjects compared with HIV-negative and cART-treated subjects (n=13 in each group). Non-parametric Mann-Whitney two-tailed test P values are indicated. (d) Left panel: CTLA-4 levels on total DN T cells from HIV-negative (n=3) and HIV-1 viraemic subjects (n=3). Right panel: CTLA-4 expression on DN T cells expressing (or not) CD45RA in a representative HIV-infected subject. (e) Quantification of integrated HIV DNA in memory (CD45RA⁻) and naive (CD45RA⁺) subsets of both CD4⁺ and DN T cells in four HIV-infected viraemic subjects. CD4, CD4 count (mm⁻³); VL, viral load [log (copies ml⁻¹)].

Table 1. Clinical characteristics of the study population

Category	No. of Subjects	Median (IQR*) CD4 count (mm ⁻³)	Median (IQR*) CD8 count (mm ⁻³)	cART	Median viral load [log (copies ml ⁻¹)]
Treatment-naïve	22	378 (338–499)	736 (515–1002)	No	4.8
Virally suppressed	15	675 (606–773)	598 (405–996)	Yes	<1.7
Elite controllers	15	641 (505–819)	740 (483–1139)	No	<1.7

*Interquartile range: Q1–Q3.

not the CD45RA⁺ (CTLA-4⁺) fraction of the DN T cells. In contrast, in cells that were still expressing the CD4 marker, CD4⁺ T cells, the memory CTLA-4-enriched CD45RA⁻ subset harboured most of the integrated HIV DNA (300–4000 HIV DNA copies/10⁶ cells), whereas the CD45RA⁺ subset (CTLA-4^{lo}) contained low but detectable levels of HIV DNA (50–500 HIV DNA copies/10⁶ cells). Altogether, these observations indicate that memory CTLA-4⁺CD4⁺ T cells and memory DN T cells lacking CTLA-4 were preferentially infected *in vivo*.

High expression of activation markers on T cells enriched with HIV-1 infection

As Nef-mediated downregulation of CTLA-4 enhanced T cell activation (Figs 3 and 4), we investigated whether the memory DN CTLA-4⁻ T-cell subset, harbouring HIV DNA *in vivo*, expressed activation markers such as Ki67 and HLA-DR. The results (Fig. 6a, b) demonstrated that Ki67 and HLA-DR were highly expressed on CD45RA⁻ (CTLA-4⁻) DN T cells from treatment-naïve HIV-infected subjects compared with uninfected controls (11 versus 2.5% for Ki67, $P=0.04$; 21 versus 5% for HLA-DR, $P=0.004$; $n=6$ per group), suggesting that infection of this subset *in vivo* was associated with high levels of cell activation and cycling potential. Higher frequencies of Ki67⁺ and HLA-DR⁺ T cells were also observed within the CD4⁺CTLA-4⁺ memory subset from treatment-naïve HIV-infected subjects compared with uninfected controls, although not to the extent of DN CTLA-4⁻ T cells (3 versus 1.2% for Ki67, $P=0.004$ and 2.6 versus 1.4% for HLA-DR, $P=0.1$). Moreover, higher frequencies of Ki67⁺ T cells within memory DN T cells (CD45RA⁻CTLA-4⁻) were observed in treatment-naïve compared with cART-treated, elite controllers (Table 1) and uninfected subjects (3.5-, 2.5- and threefold increase, respectively) (Fig. 6c). Importantly, the frequency of activated memory DN CTLA-4⁻ T cells positively correlated with plasma viral load in viraemic subjects ($P=0.0278$) (Fig. 6d). Together, these data indicated that memory DN CTLA-4⁻ T cells exhibited a superior state of activation that was compatible with enhanced capacity for HIV-1 infection and replication.

DISCUSSION

In this study, we demonstrated that HIV-1 preferentially infects CD4⁺CTLA-4⁺ T cells and that Nef downregulates

CTLA-4 expression in these infected cells *in vitro*. Together with the Nef-mediated decrease in the TCR activation threshold, CTLA-4 downregulation and the subsequent resistance to CTLA-4 triggering are consistent with enhanced cell activation and viral replication.

Following infection of primary CD4⁺ T cells with HIV-1^{Nef+} and HIV-1^{ΔNef} viruses, we observed that HIV-1^{ΔNef} was readily detectable in cells expressing high levels of CTLA-4 compared with HIV-1^{Nef+} or non-infected cells. Although this may suggest that infection with HIV-1^{ΔNef} increases CTLA-4 expression or that the HIV-1^{Nef+} virus infects cells expressing lower levels of CTLA-4, our data strongly suggest that this is due to Nef-mediated downregulation of CTLA-4 upon infection in activated cells. This is supported by our earlier observations on the ability of HIV Nef to downregulate CTLA-4 upon the co-expression of these two proteins *in vitro* (El-Far *et al.*, 2013). Moreover, in the current study we showed that HIV-1^{Nef+} and HIV-1^{ΔNef} viruses exclusively infected sorted cells expressing high levels of CTLA-4. Furthermore, both viruses were readily detectable in cells expressing high levels of CTLA-4 at an earlier time point post-infection, 24 h, with moderate CTLA-4 downregulation followed by significant Nef-mediated CTLA-4 downregulation by 48 h post-infection.

Our results further indicated that Nef-mediated CTLA-4 downregulation is a conserved function between human and non-human primate lentiviruses as both HIV-1 and SIV (cpz and mac239) demonstrated a significant impact on CTLA-4 expression. This new function of Nef, together with the established ability to downregulate CD4 and MHC-I (Benson *et al.*, 1993; Cohen *et al.*, 1999; Collins *et al.*, 1998; Little *et al.*, 1994; Lubben *et al.*, 2007; Piguet *et al.*, 1999; Schwartz *et al.*, 1996; Williams *et al.*, 2002), highlights its unique role in the evasion of HIV from the immune restriction on viral replication to exacerbate viral pathogenesis. Nef-mediated downregulation of CTLA-4 confirms the multipronged nature of Nef interference with cellular signalling to promote optimal activation, whilst protecting against cell killing (Benson *et al.*, 1993; Cohen *et al.*, 1999; Collins *et al.*, 1998; Little *et al.*, 1994; Lubben *et al.*, 2007; Piguet *et al.*, 1999; Schwartz *et al.*, 1996; Williams *et al.*, 2002).

Immune activation during HIV infection leads to the upregulation of CTLA-4 at the surface of activated T cells

Fig. 6. Expression of activation markers on memory CD4⁺CTLA-4⁺ and memory DN CTLA-4⁻ T cells. PBMCs from HIV-infected and uninfected subjects were analysed for the surface expression of HLA-DR and the intracellular expression of Ki67 on gated CD3⁺ T-cell subsets *ex vivo*. (a) Expression of Ki67 (upper panels) and HLA-DR (lower panels) on memory CD4⁺ T-cells (CTLA-4^{hi}) and memory DN T cells (CTLA-4⁻) from a representative treatment-naïve HIV-infected subject and a representative HIV-negative control. (b) HLA-DR and Ki67 expression in these subsets (six subjects per group). (c) Expression of the activation marker Ki67 on the memory fraction of DN T cells (CTLA-4⁻) from viraemic HIV-infected subjects ($n=17$) compared with aviraemic cART-treated subjects ($n=15$), elite controllers (EC, $n=15$) and HIV-negative subjects ($n=15$).

Non-parametric Mann–Whitney two-tailed test *P* values are shown in (b) and (c). (d) Correlation between the expression of Ki67 on the memory fraction of DN T-cells (CTLA-4⁻) and plasma viral load in HIV-infected treatment naive subjects (*n*=17). Spearman correlation *r* (rho) and *P* values are indicated in (d).

(Leng *et al.*, 2002). The upregulation of CTLA-4 and other immune checkpoint receptors represents an important physiological mechanism that limits T-cell activation (Guntermann & Alexander, 2002; Harlin *et al.*, 2002; Krummel & Allison, 1996). Indeed, treatment of SIV-infected macaques with anti-CTLA-4 blocking antibodies increased T-cell activation and viral replication at the systemic and mucosal level, and exacerbated the loss of CCR5⁺CD4⁺ T cells in tissues (Cecchinato *et al.*, 2008). These observations indicated that CTLA-4 plays a potent role in the negative regulation of T-cell activation, which limits SIV and most likely HIV replication and dissemination. Our data strongly support this hypothesis as primary CD4⁺ T cells infected with Nef-competent HIV down-regulated CTLA-4 expression and consequently became refractory to CTLA-4-mediated inhibition of T-cell activation, accompanied by higher IL-2 production and viral replication.

The role of Nef in enhancing T-cell activation and cytokine production in HIV-infected cells is complex. Whilst certain reports describe an inhibitory role, others support a direct implication of Nef in T-cell activation (reviewed by Markle *et al.*, 2013). In this regard, it was shown that HIV disrupts the immunological synapse and decreases the TCR signalosome, including Lck, at the surface of HIV-infected T cells by Nef-mediated internalization of Lck (Thoulouze *et al.*, 2006), likely to protect against activation-induced cell death. The fact that Nef alters the TCR signalosome at the immunological synapse, meanwhile lowering the activation threshold of infected T cells (Schrager & Marsh, 1999; Wu & Marsh, 2001), suggests that other compensatory mechanisms could be involved in mediating the enhanced T-cell activation that we and others (Schrager & Marsh, 1999) have observed. In this regard, it was recently shown that Nef reroutes kinase-active pools of Lck away from the plasma membrane and directs them to the trans-Golgi network (TGN) for selective targeting of T-cell downstream signalling pathways (Pan *et al.*, 2012). This leads to enhanced IL-2 production by TGN-associated Ras/Erk activation (Pan *et al.*, 2012). In line with these observations, our current work identifies a novel mechanism by which Nef compensates for the previously described low levels of TCR signalosome at the plasma membrane (Thoulouze *et al.*, 2006) by decreasing the TCR activation threshold of infected T cells, meanwhile downregulating CTLA-4 to evade potential negative regulation upon suboptimal stimulation and engagement of CTLA-4 that is known to inhibit proximal TCR stimulation by dissociating Lck from ZAP-70 (Guntermann & Alexander, 2002; Harlin *et al.*, 2002; Krummel & Allison, 1996). Indeed, under such suboptimal stimulations, Nef also associates with the cellular kinase PAK2 and mediates enhancement

of T-cell activation in both infected and bystander cells (Olivieri *et al.*, 2011). Thus, it remains important to measure precisely the exact contribution of each of these mechanisms, particularly CTLA-4 downregulation, to the overall Nef-mediated enhancement of T-cell activation. This could be done, in part, by using Nef mutants that are defective in CTLA-4 downregulation. Nonetheless, the Nef mutants diminishing CTLA-4 downregulation by Nef that we have identified to date (El-Far *et al.*, 2013) overlap with other functions of Nef, such as CD4 downregulation (Fauré *et al.*, 2004; Lindwasser *et al.*, 2008). Therefore, further studies are needed for detailed mapping of Nef to dissect and separate these functions.

Earlier reports by Kaufmann *et al.* (2007) showed that HIV-specific CD4⁺ T cells express relatively high levels of CTLA-4, an indicator of T-cell exhaustion, and that blockade of CTLA-4 restores T-cell functions. Our current study reveals that CD4⁺CTLA-4⁺ memory T cells are preferential targets for HIV infection *in vitro* and in treatment-naïve subjects. This is consistent with findings by Douek *et al.* (2002) that HIV-1 preferentially infects HIV-specific CD4⁺ T cells. The enrichment of HIV DNA in CD4⁺CTLA-4⁺ T cells *in vivo* may seem to contrast with our results generated *in vitro* where HIV-1 infection was associated with CTLA-4 downregulation. However, it was previously shown that active viral transcription and productive HIV infection *in vivo* take place in cells losing the expression of the CD4 marker and thus becoming CD4⁻ (CD3⁺CD4⁻CD8⁻ DN cells). As higher ratios of viral RNA/DNA were detected in these DN cells compared with the CD4⁺ T cells (Kaiser *et al.*, 2007). Thus, the CD4⁺CTLA-4⁺-infected cells *in vivo* may then represent a recent infection and/or harbour latent HIV. Indeed, our *in vivo* data revealed that proviral HIV DNA was exclusively detected in the DN T cells expressing a memory phenotype and lacking the expression of CTLA-4 from treatment-naïve HIV-infected subjects (subjects with high viral loads). Together, these observations are consistent with our *in vitro* data showing simultaneous downregulation of CD4 and CTLA-4 markers and the accumulation of CD4⁻CTLA-4⁻ cells following productive HIV infection (Fig. S4). Although we did not directly assess the levels of viral transcription in this minor fraction of DN cells *in vivo*, others studies showed that these cells contained the majority of unspliced and multiply spliced viral transcripts, including Nef (Kaiser *et al.*, 2007). DN T cells are highly heterogeneous and include γ/δ T cells. Nevertheless, we demonstrated that DN CTLA-4⁻ T cells in HIV-infected and uninfected subjects include a significant fraction of α/β T cells. As HIV-1 infects mostly CD4⁺ T cells, one can assume that DN CTLA-4⁻ T cells carrying HIV DNA in infected subjects originate from T cells expressing CD4.

However, further studies are needed to directly assess HIV infection within α/β DN CTLA-4⁺ T cells. Finally, the expression of proliferation and activation markers on DN CTLA-4⁺ T cells, together with the finding that the frequency of these cells positively correlates with plasma viral load in treatment-naïve HIV-infected subjects, further suggest that DN CTLA-4⁺ T cells are major sites of HIV replication *in vivo* and play a role in HIV pathogenesis.

In conclusion, our findings reveal a novel mechanism for HIV-1 pathogenesis in which the coupled functions of the Nef-mediated decrease of the TCR activation threshold and CTLA-4 downregulation are likely to sensitize HIV-1-infected cells to supoptimal stimulations, whilst protecting against the CTLA-4 negative regulation to achieve optimal viral replication. In addition, the new function of Nef in CTLA-4 downregulation that we identified in the current study, together with the well-described functions of Nef in downmodulating CD4 and MHC-I, are likely to facilitate the escape of HIV-infected T cells from attack and restriction by the immune system, thus promoting productive viral replication and persistence. Finally, further studies are required to determine whether, on the one hand, all the CD4⁺CTLA-4⁺ HIV-infected cells *in vivo* will undergo CTLA-4 downregulation, and subsequently be subject to vigorous viral replication and elimination, or, on the other hand, high levels of CTLA-4 on certain subsets of HIV-infected CD4⁺ T cells would increase the activation threshold of these cells and/or restrict viral replication, leading to their persistence and contribution to the long-lived HIV reservoir. These studies are urgently needed for the development of novel treatment strategies targeting the HIV reservoir to complement the current classes of viral inhibitors towards a cure for HIV infection.

METHODS

Study population. HIV-negative controls and HIV-infected subjects (all participants were adults) signed written informed consent approved by the Institutional Review Boards of the Royal Victoria Hospital and the Centre de Recherche du Centre Hospitalier de l'Université de Montréal. Research conformed to ethical guidelines established by the Ethics Committee of the University of Montreal Health Center. All subjects underwent leukapheresis to collect large number of PBMCs. The characteristics of the study population of HIV-infected subjects are shown in Table 1.

Antibodies and polychromatic flow cytometry analysis.

Fluorochrome-conjugated antibodies used for polychromatic flow cytometry analysis were CD3-Pacific Blue (UCHT1), CD4-Alexa Fluor 700 (RPA-T4), CD45RA-allophycocyanin (APC)-Cy7 (H1100), CTLA-4-phycoerythrin (PE) and CTLA-4-APC (BN13), IL-2-PE (5344.111), CD8-PerCpCy5.5 (SK1), γ/δ -1 TCR-PE (11F2), α/β -1 TCR-FITC (WT31), CD154-PE (TRAP1), HLA-DR-FITC (G-466), HLA-DR-APC-Cy7 (L243), Ki67-FITC (B56), CD38-PE (HIT2), CD62L-PE, CD71-PE-Cy5 (M-A712), CD25-PE (2A3) (BD Pharmingen) and HIV p24-FITC (KC57) (Beckman Coulter). The viability dye LIVE/DEAD (Invitrogen) was used to exclude dead cells from our analysis. Cells were analysed by FACS using a BD LSR II cytometer (BD), and FACSDiva (BD) and FlowJo (Tree Star) software. Positivity gates were placed using 'fluorescence minus one

(FMO)', as described previously (Roederer, 2002). At least 2×10^5 events were acquired and analysed.

Magnetic cell sorting. Total CD4⁺ T cells were isolated from total PBMCs of HIV-infected and uninfected subjects by negative selection using magnetic beads (Miltenyi). Cell purity was typically >95 % as determined by flow cytometry analysis (BD LSR II).

Virus preparation and infection of primary CD4⁺ T cells. The following infectious molecular clones were used: (i) HXB2-based virions expressing (pHXBnPLAP-IRES-N⁺, HIV-1^{Nef+}) or lacking HIV Nef (pHXBnPLAP-IRES-N⁻, HIV-1^{ΔNef}) (Chen *et al.*, 1996) and (ii) NL4.3-based pBR-NL4.3-IRES-eGFP constructs including (or not) species-specific Nef variants from HIV-1 (pBR43IeG-nef⁺, GFP-HIV-1^{Nef+} and pBR43IeG-nef⁻, GFP-HIV-1^{ΔNef}), SIVcpz (pBR43IeG-cpzTAN3nef, GFP-SIVcpz^{Nef+}) or SIVmac239 (pBR43IeG-mac239nef, GFP-SIVmac239^{Nef+}) (Schindler *et al.*, 2005). Plasmids were obtained through the National Institutes of Health AIDS Reagent Program. Viral stocks were produced by calcium phosphate transfection of 293T cells, as described previously (Jordan *et al.*, 1996). Infection of TCR-stimulated primary CD4⁺ T cells was carried out using 50 ng HIV p24 for each batch of 5×10^5 cells by spinoculation (O'Doherty *et al.*, 2000). Detection of HIV-infected cells was carried out by intracellular staining using anti-p24 antibody. In our initial experiments we observed that the signal-to-background ratio was higher when HIV-infected cells were stained intracellularly with fluorescence-conjugated HIV p24 mAbs compared with indirect staining with antibodies against the marker gene PLAP (human placental alkaline phosphatase) expressed by both HIV-1^{Nef+} and HIV-1^{ΔNef}.

TCR and CTLA-4 triggering. For infection experiments, CD4⁺ T cells were stimulated using immobilized CD3 ($1 \mu\text{g ml}^{-1}$) and soluble CD28 antibodies ($0.5 \mu\text{g ml}^{-1}$) in RPMI 1640/10 % FBS for 48–72 h. In other sets of experiments, cells were activated using magnetic beads (CELLlection Pan Mouse IgG kit; Dynal Biotech) coated with CD3, CD28 (BD) and CTLA-4 (CTLA-4.38 mAbs from G. J. F.) antibodies or matched isotype controls (four beads per cell).

Quantification of HIV DNA. Total and integrated HIV DNA was quantified by nested real-time PCR, as described previously (Brussel & Sonigo, 2003; Chomont *et al.*, 2009), in CD4⁺ T cells infected *in vitro* and isolated from HIV-infected subjects.

ELISA. Viral replication was quantified using an in-house HIV p24 ELISA, as described previously (Chomont *et al.*, 2009). IL-2 levels were quantified using an ELISA kit (BD) according to the manufacturer's protocol.

Statistical analysis. All statistical analyses were performed using the GraphPad Prism 5 software.

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