

RESEARCH ARTICLE

Subset- and Antigen-Specific Effects of Treg on CD8+ T Cell Responses in Chronic HIV Infection

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Abstract

We, and others, have reported that in the HIV-negative settings, regulatory CD4+ CD25^{high}FoxP3+ T cells (Treg) exert differential effects on CD8 subsets, and maintain the memory / effector CD8+ T cells balance, at least in part through the PD-1/PD-L1 pathway. Here we investigated Treg-mediated effects on CD8 responses in chronic HIV infection. As compared to Treg from HIV negative controls (Treg/HIV-), we show that Treg from HIV infected patients (Treg/HIV+) did not significantly inhibit polyclonal autologous CD8+ T cell function indicating either a defect in the suppressive capacity of Treg/HIV+ or a lack of sensitivity of effector T cells in HIV infection. Results showed that Treg/HIV+ inhibited significantly the IFN-γ expression of autologous CD8+ T cells stimulated with recall CMV/EBV/Flu (CEF) antigens, but did not inhibit HIV-Gag-specific CD8+ T cells. In cross-over cultures, we show that Treg/HIV- inhibited significantly the differentiation of either CEF- or Gag-specific CD8+ T cells from HIV infected patients. The expression of PD-1 and PD-L1 was higher on Gag-specific CD8+ T cells as compared to CEF-specific CD8+ T cells, and the expression of these markers did not change significantly after Treg depletion or co-culture with Treg/HIV-, unlike on CEF-specific CD8+ T cells. In summary, we show a defect of Treg/HIV+ in modulating both the differentiation and the expression of PD-1/PD-L1 molecules on HIV-specific CD8 T cells. Our results strongly suggest that this particular defect of Treg might contribute to the exhaustion of HIV-specific T cell responses.

Author Summary

Protective anti-viral response requires balanced differentiation of effector and memory CD8 T cells. Regulatory T cells with suppressive activity (Treg) provide a protective

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immune response by differential effects on CD8 T cell subsets. In healthy donors, Treg inhibit the differentiation and proliferation of effectors while preventing the apoptosis of memory cells. These effects are achieved in part by regulating the levels of PD-1/PD-L1 apoptosis-inducing molecules on activated T cells. Despite an expansion of Treg, HIV-1 infection is characterized by a chronic immune activation and “exhausted” HIV-specific effectors expressing high levels of PD-1 and PD-L1. This raised the question about the role of Treg in the HIV+ settings, and the possible therapeutic effect of their depletion. According to our data, the suppressive effect of Treg is highly dependent on the stage of CD8 T cell differentiation, and therefore, is subset- and antigen-specific. Thus, Treg from HIV+ donors inhibit common antigen-specific CD8 effectors, while “exhausted” HIV-specific clones appear resistant to Treg effects. In addition, we show that the subset composition and inhibitory potential of Treg may evolve in the course of infection. These data suggest that the ultimate failure to contain HIV infection results from both an intrinsic impairment of Treg function and a decreased responsiveness of effector clones, associated with high levels of PD-1/PD-L1 expression. Our data also indicate that a simple modulation of PD1/PD-L1 expression on CD8 T effector clones may not restore the protective T cell response.

Introduction

CD8 T cells play a crucial role in the control of viral infection. While several lines of evidence indicate that HIV-specific CD8 T cells are involved in the response to HIV infection [1,2], their failure to clear the virus in the vast majority of infected individuals has not yet been elucidated [3–5]. Chronic viral infection with ongoing antigenic stimulation may result in exhaustion of virus-specific T cells due to the engagement of down-regulatory mechanisms. Negative pathways involving the programmed cell death-1 (PD-1) and other inhibitory receptors such as CTLA-4, Tim-3 and LAG-3 can be critical in limiting the magnitude or duration of the antiviral CD8 T-cell response [6–8]. In human, the phenotype and functional potential of chronically stimulated CD8 T cells differ among viral infections (9). HIV-specific response is characterized by a decreased proliferative capacity of the central memory CD8 T cell pool, incomplete differentiation of effectors with reduced cytotoxic ability, and increased sensitivity to apoptosis as compared to CD8 T cells specific for non-progressive persistent infections, such as EBV or cytomegalovirus (CMV) [10,11]. Several studies have suggested a particular role for the PD-1/PD-L1 pathway in the exhaustion of HIV-specific CD8 T cells. PD-1 is upregulated on HIV-specific [12] and simian immunodeficiency virus (SIV)-specific CD8 T cells [13], and the level of PD-1 expression is associated with decreased HIV-specific CD8 T-cell proliferation [14]. The frequency of PD-1 expression on HIV-specific CD8+ T cells was shown to parallel the levels of plasma HIV viral load, while negatively correlating with CD4+ T cell counts [12]. Furthermore, in non-human primate model, in vitro blockade of PD-1 on CD8 T cells enhanced the proliferation and cytokine production of antiviral CD8 T cells, suggesting that immune exhaustion was reversible [15]. Finally, a recovery of antigen-specific CD8 T cells from exhaustion has been demonstrated in vivo after blocking the PD-1/PD-L1 interaction in a murine model of LCMV infection, as well as in a SIV-macaque model. [13,16].

In parallel to the “exhausted” CD8 T cell response, an expansion of regulatory CD4+FoxP3+ T cells with suppressive activity (Treg) is well established in the chronic phase of HIV infection as well as in other chronic infections [17–23], Treg have a key role in maintaining peripheral tolerance, and limiting chronic inflammatory responses. In the healthy immune system, we and

others have shown that Treg are responsible for balanced differentiation of memory and effector CD8 T cells in case of strong stimulation, thus providing a protective immune response [24,25]. We have shown that Treg may regulate differentially the apoptosis of memory and effector CD8 T cells through the PD-1/PD-L1 pathway, by regulating the levels of PD-L1 expression [25]. Gal-9, another inhibitory receptor constitutively expressed by Treg may provide the ligand for selective inducing of tolerance in Tim-3-expressing effectors [26].

In the settings of chronic infection, Treg may be either beneficial through limiting non-specific immune activation, or have detrimental effect by suppressing effective antigen-specific immune responses [27]. It seems that disease outcome depends on the equilibrium between a balanced Treg to effector T-cell response and immune activation [28]. Indeed, the increased level of Treg in HIV+ patients has been linked to a reduced immune activation and low viral load [29,30] but the accumulation of Treg in lymphoid tissues was also found to be associated with a high viremia, both in infected patients and SIV-infected macaques [31,32]. In addition, high Treg levels have been associated with poor immune restoration under ART [33].

Thus, the role of Treg in chronic HIV infection remains an open question. Moreover, the subset-specific effects of Treg on CD8 responses of HIV+ patients and the underlying mechanisms have not been evoked yet. Logically, in conditions of prolonged stimulation and effector T cell exhaustion, the mechanisms and molecules implicated in Treg-mediated inhibition would also evolve, or be perturbed. Recent data have challenged the notion of Treg as a homogeneous, and stable population, suggesting that, under inflammatory conditions, they may acquire different phenotype and functions [34–36]. As a consequence, the therapeutic potential of Treg depletion, whether combined or not with blocking of PD-1/PD-L1 or other signaling checkpoints to activate a potent anti-viral immune response, is still an unresolved issue of utmost interest.

In this paper, we studied the inhibitory effects of Treg on CD45RA/CCR7-defined naïve, memory and effector CD8 T subsets in HIV+ subjects after polyclonal and antigen-specific stimulation. Our results provide evidence that, in conditions of chronic HIV infection, Treg may exert subset- and clonally- specific inhibitory effects on CD8 T cells. These results indicate that the ultimate failure to contain generalized HIV-1-associated immune activation is a joint result of intrinsic impairment of Treg function and decreased responsiveness of the effector clones, at least in part associated with the high levels of PD-1/PD-L1 expression.

Results

Treg inhibit the proliferation and differentiation of effector CD8 T cells in chronic HIV infection

First of all, using PBMC from $n = 14$ HIV-1-positive subjects under ART we confirmed the inhibitory effect of Treg on the proliferation of global CD8 T cells after polyclonal stimulation (coated anti-CD3 for 5 days). The percentage (mean \pm SD) of proliferating (CFSE^{low}) CD8 T cells on D5 was $65 \pm 12\%$ in the presence of Treg (total PBMC) vs. $82 \pm 8.9\%$ after Treg depletion, $P < 0.01$ (Fig 1A). Next, we compared the proliferation of CCR7/CD45RA-defined CD8 T subsets: naïve (N, CD45RA+CCR7+), central memory (CM, CD45RA-CCR7+), effector memory+effector (EM+E, CD45RA-CCR7-) and terminal effector (TE, CD45RA+CCR7-) (Fig 1B). The CD8 TCM subset contained (mean \pm SD) $57 \pm 20\%$ CFSE^{low} cells in the presence of Treg vs. $74 \pm 12\%$ in their absence ($P < 0.01$), and the CD8 EM+ E subset, $69 \pm 14\%$ vs. $79 \pm 6\%$, respectively, $P < 0.001$. At the same time, the share of CFSE^{low} cells within the N and TE pools was not affected significantly by the presence or absence of Treg (Fig 1B and 1C). These data showed that the inhibitory effect of Treg on CD8 T cells was subset-specific.

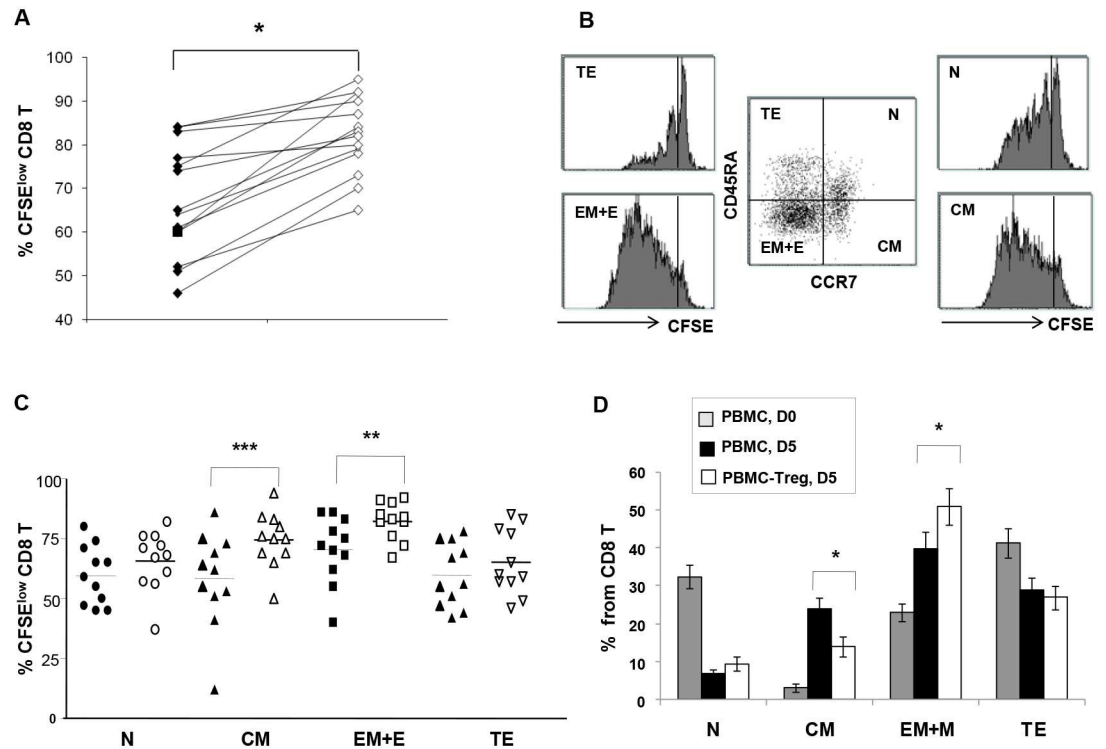


Fig 1. Treg inhibit the proliferation of CD8 T cells from HIV+ patients in a subset-specific manner. **A.** Individual data (n = 14) of proliferation rates of CD8 T cells after polyclonal stimulation (5d, anti-CD3) in the presence (black) or in the absence (white) of Treg. **B.** Representative dot-plot of CD8 T cell-gated PBMC showing the CCR7/CD45RA defined subsets: naïve (N), central memory (CM), effector-memory and effector (EM+E), terminal effector (TE), at D5 of anti-CD3 stimulation, and their further analysis according to CFSE intensity. **C.** Individual data of proliferation rates of CCR7/CD45RA-defined CD8 T (CD8 N, CM, EM+E, and TE) subsets (**P<0.01, ***P<0.001, n = 11, Student's T-test). **D.** Proportions of CCR7/CD45RA-defined CD8 T subsets before (grey) and after polyclonal stimulation, in the presence (black) or in the absence (white) of Treg. Bars correspond to mean \pm SD, n = 11, * P<0.05, Student's T-test.

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Since polyclonal proliferation may be accompanied by differentiation, CFSE^{low} CD8 T cells detected on D5 may have originated from N, M or E subsets. To clarify this issue we compared the proportions of CD8 subsets before and after stimulation, in the presence or in the absence of Treg. Polyclonal stimulation increased the proportions (mean%) of CM and EM+E subsets at the expense of N and TE CD8 T subsets. More detailed analysis showed that the increase of CM subset was more important in the presence of Treg (from 4% to 22%) as compared to their absence (14%) (P<0.05). The increase of the EM+E subset was less significant in the presence of Treg (from 22% to 40%) than in their absence (51%) (P<0.05 (Fig 1D)). These results suggest that in chronic HIV infection Treg exert differential effects on the CD8+ T cell subsets and prevent the differentiation of CM cells towards the effector-memory stage.

Treg-mediated inhibition of CD8+ T cell responses in ART- HIV+ patients differs at the clonal level

Next, we studied Treg-mediated effects on the production of IFN- γ by CD8 T cells after polyclonal stimulation of PBMC from ART-naïve HIV+ patients, or HIV- controls. As expected, overnight anti-CD3 stimulation of total PBMC from HIV+ subjects resulted in lower differentiation of IFN γ + producing cells in PBMC from HIV+ subjects as compared to PBMC from HIV- controls (mean \pm SD % of CD8+CD69+IFN γ + cells of 2.1 \pm 0.95 vs. 5.9 \pm 0.92, P <0.05;

n = 10). Treg depletion increased the percentage of IFN γ -secreting CD8 T cells in HIV-negative controls (mean $9.8 \pm 0.9\%$), ($P < 0.01$ for comparison with cultures with Treg) but not in HIV+ patients (mean $3.9 \pm 0.9\%$), ($P = \text{NS}$) (Fig 2A). In the settings of non-ART-treated patients, IFN γ expression in total PBMC was mostly confined to the EM+E subset (S1B Fig, embedded), while in healthy controls, IFN γ was mostly expressed by CM CD8 T cells (S1A Fig, embedded). Treg inhibitory effect was observed for all subsets in HIV-negative settings, reaching significance for CM and EM+E subsets, while in HIV+ donors it was observed only at the level of CM IFN γ -producing cells, confirming our hypothesis about differential effects of Treg in HIV settings (S1 Fig). In fact, these results might suggest that in conditions of HIV infection, CD8 T cells were defective, not only in terms of IFN γ production but also in sensitivity to Treg-mediated suppression, and/or Treg could be less suppressive.

It is well known that memory CD8 T cells differ in phenotype depending on their specificity. Therefore, we further compared the inhibitory effect of Treg from ART naive-HIV+ patients on autologous CD8 T cells having different antigen specificity. To this end, PBMC were stimulated overnight with HIV Gag or CEF peptides, either in the presence or in the absence of Treg. Treg depletion had a non-significant effect on the frequency of HIV Gag-specific CD8+IFN γ + T (mean% 0.3 vs. 0.45 with or without Treg, respectively; $P = 0.05$) while the frequency of CEF-specific CD8+IFN γ + T from the same patients increased significantly (0.38 vs. 0.87%, $P < 0.001$), (Fig 2B). We concluded that Treg inhibitory effects observed in HIV infection differed depending on the CD8 T cell specificity.

Treg from HIV infected patients do not modulate the expression of pro-apoptotic molecules on autologous CD8 T cells

Activated CD8 T cells express increasing levels of pro-apoptotic molecules, such as PD-1 and its ligand, PD-L1. We have previously shown that, in healthy donors, Treg-mediated inhibition

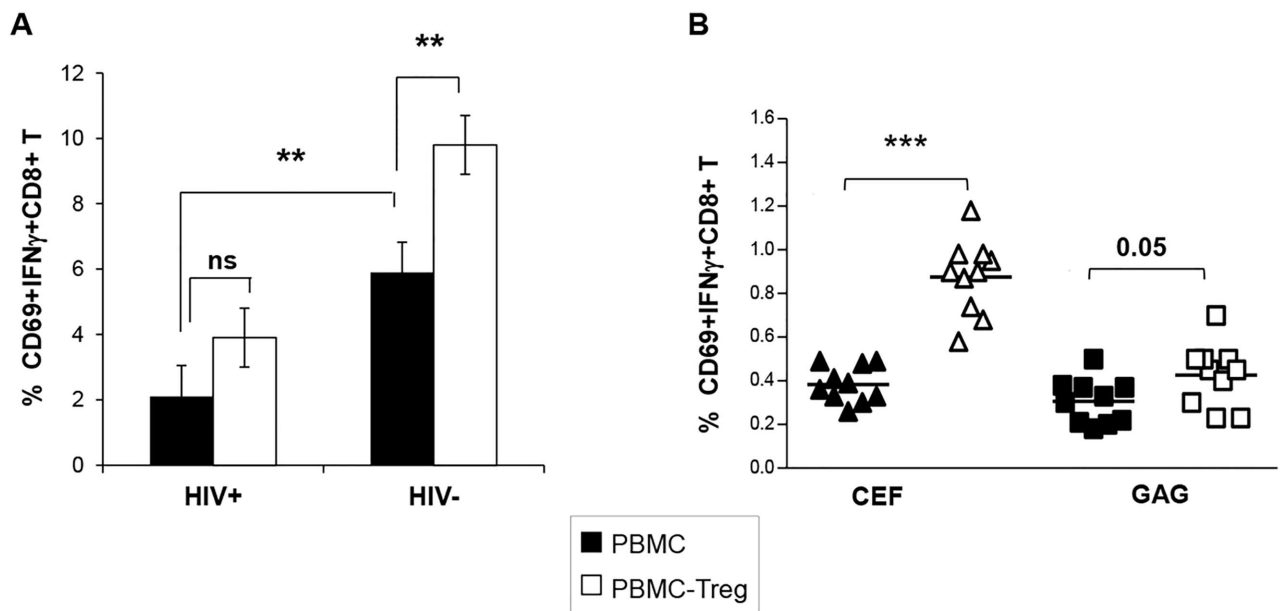


Fig 2. CD8 T cell sensitivity to Treg-mediated inhibition differs at the clonal level. **A.** Pooled data showing the effect of Treg on the IFN- γ secretion of polyclonally stimulated CD8 T cells in 10HIV-1+ and in 10HIV- subjects. Bars represent mean \pm SD. **B.** Individual data showing the percentage of IFN- γ secreting CD8 T cells after peptide-specific (CEF or Gag) stimulation of total and Treg-depleted PBMC from HIV+ ART- patients. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n = 10, paired Student's T-test).

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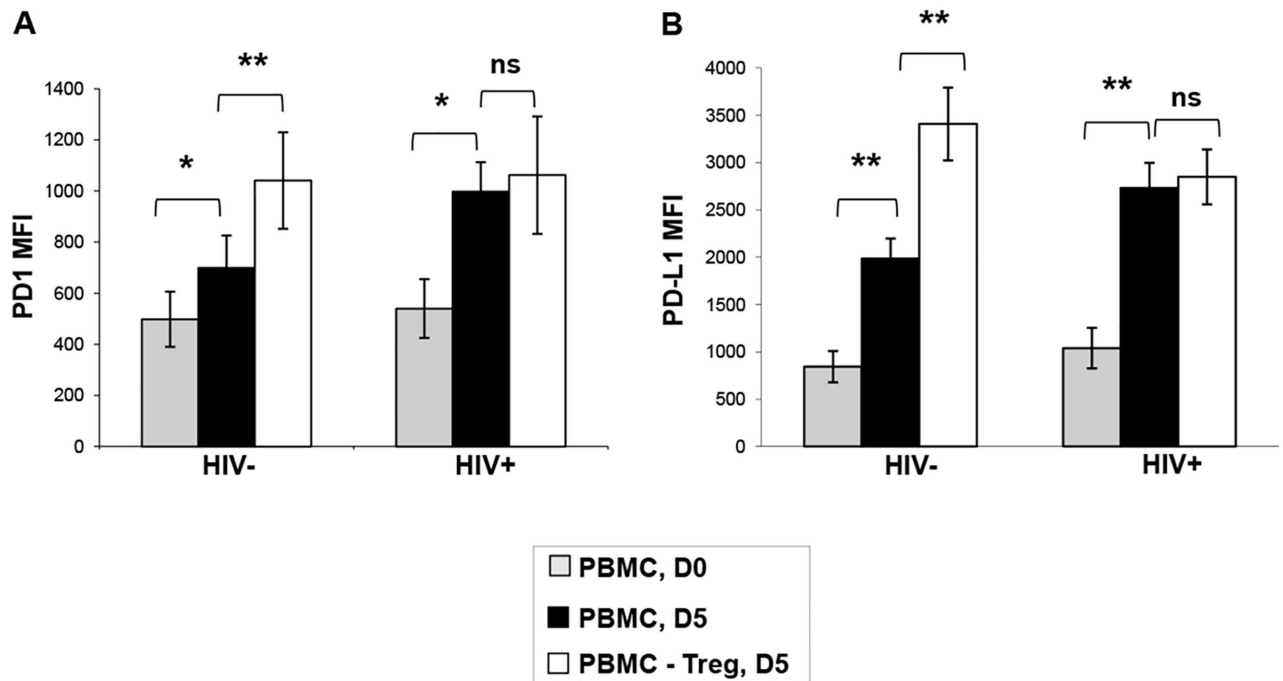


Fig 3. Treg from HIV infected patients do not modulate the expression of pro-apoptotic molecules on autologous CD8 T cells. Pooled data about the expression levels of PD-1 (A) and PD-L1 (B) on CD8 T cells from HIV+ or HIV- subjects, before (grey) and after 5 days of polyclonal stimulation in the presence (black) or in the absence (white) of Treg. (n = 6, ** P<0.01, Student's T-test).

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of PD-L1 expression rescued preferentially the PD-1^{high} CM CD8 subset and impacted the balance M/E cells [25]. To check whether this regulatory mechanism operated in HIV infection, we compared the expression of PD-1 and PD-L1 on total CD8 T from HIV+ART- patients, and from HIV-controls, before and after 5 days of polyclonal stimulation, in the presence or in the absence of Treg. The baseline expression levels (mean MFI) of PD-1 and PD-L1 on the global CD8 T population from HIV-negative and HIV+ donors were not significantly different 498±108 vs. 540±115 for PD-1 and 844±176 vs. 1040±202 for PD-L1, respectively (Fig 3A and 3B). After polyclonal stimulation, the expression levels of PD-1 on CD8 T cells rose significantly to 699±127 and 998±115 in HIV- and HIV+ donors, respectively (P<0.05 for both comparisons to unstimulated conditions). Similarly, these increases were 1985±186 and 2730±305 for PD-L1 expression (P<0.01 for both comparisons to unstimulated conditions). In the HIV-negative settings, stimulation in the absence of Treg resulted in significantly higher levels of PD-1 and PD-L1 on the activated CD8 T cells, as compared to stimulation in the presence of Treg: 1041±189 vs. 699±127 for PD-1 and 3407±116 vs. 1985±176 for PD-L1 (P<0.01 for both comparisons). Interestingly, Treg depletion had no such effect in HIV+ donors: 1062±230 vs. 998±115 for PD-1, and 2848±320 vs. 2730±305 for PD-L1 respectively, (P>0.05 for both comparisons), (Fig 3A and 3B). In addition, we studied the levels of Annexin V+ cells within N, CM, EM+E and TE CD8 subsets from HIV+ donors (n = 6) after 48h anti-CD3 stimulation, either in the presence of Treg or after their depletion. No significant difference was detected, indicating that CD8+ T cells from HIV-infected patients are not influenced by the suppressive effect of Treg in terms of expression of PD-1/PDL-1 and activation-induced apoptosis (S2 Fig).

Next, we investigated whether Treg effects on the expression of PD-1 and PD-L1 were dependant on the antigen specificity of effector cells. To this end, the expression of PD-1 and

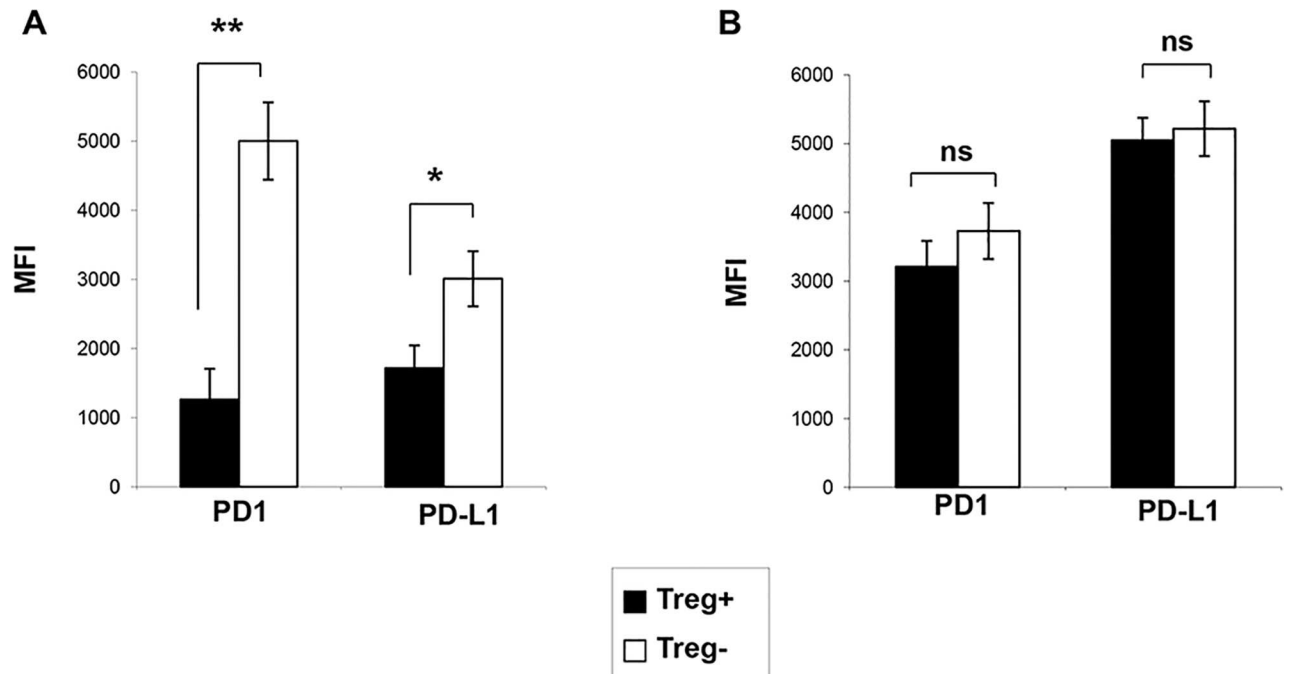


Fig 4. Treg-mediated regulation of PD-1/PD-L1 expression on CD8 T cells from HIV+ patients is clonally-specific. Pooled data on the levels of PD-1 and PD-L1 expression on CEF-specific (A) and Gag-specific (B) CD8 T cell effectors stimulated overnight in the presence (black) or in the absence (white) of Treg. (* P <0.05, *** P <0.001, n = 10, paired Student t-test).

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PD-L1 on the IFN γ + CD69+CD8 T cells of HIV+ ART- donors was studied after 18h stimulation with CEF or HIV Gag peptides, as described in M&M section. CEF-specific CD8 T effectors expressed moderate levels of PD-1 and PD-L1, that increased significantly after Treg depletion (MFI 1263 vs. 5003, P<0.01 for PD-1, and 1720 vs. 3060 for PD-L1, P<0.05 for both comparisons), (Fig 4A). A similar effect was noted on CEF-specific CD4 T cells following Treg depletion with an increase from 891 to 1918 for PD-1MFI and 1449 to 2862 for PD-L1 MFI, (P<0.05 for both comparisons, S3A Fig).

As expected, HIV Gag-specific CD8 T cells expressed significantly more pro-apoptotic molecules as compared to the CEF specific CD8 T cell clones at baseline: 3208 vs. 1263 for PD-1 and 5049 vs. 1720 for PDL1 (P<0.05 for both comparisons) (Fig 4B). Noteworthy, this expression did not change significantly after Treg depletion: 3727 and 5216 respectively for PD-1 and PDL1 (Fig 4B). Similarly, HIV Gag-specific CD4 T cells expressed comparable levels of PD-1 and PD-L1, regardless of the presence or absence of Treg: 2920 vs. 3037, and 4734 vs. 4851, respectively (S3B Fig). Altogether, these data showed that the functional inhibitory effects of Treg on T cells, as well as their regulatory effects on the expression of PD-1 and PD-L1 differ at the specificity level.

Treg/HIV+ lack suppressive capacity as compared to Treg/HIV- and fail to modulate PD-1/PD-L1 expression on HIV-specific CD8 T cells

To explain the observed antigen-specific effects, we wondered whether Treg from HIV+ patients (Treg/HIV+) were functionally deficient, therefore failing to regulate the excessive differentiation, and the expression of proapoptotic molecules by HIV-specific clones. To this end, we set co-cultures with purified CD8 T cells and Treg from HIV+ART- patients, in the

presence or in the absence of HIV Gag, or CEF antigens. In cross-over cultures, either the CD8 T cells or Treg were replaced with those from HIV- controls (Treg/HIV-). IFN γ expression by CD8 effectors as well as PD-1 and PD-L1 expression levels on the effector and on the regulatory T cells were measured 18h later. According to our results, Treg/HIV- suppressed the expression of IFN γ by Gag-specific CD8 T better than patients' Treg (mean IFN γ inhibition% of 53 \pm 12 vs. 27 \pm 22; P<0.05). Although the suppressive effect of Treg/HIV+ varied considerably between patients, replacement with Treg/HIV- invariably resulted in a more important inhibition, indicating a decreased inhibitory potential of Treg in the settings of chronic HIV infection (Fig 5A). In a series of experiments we demonstrated the direct effect of HIV viral

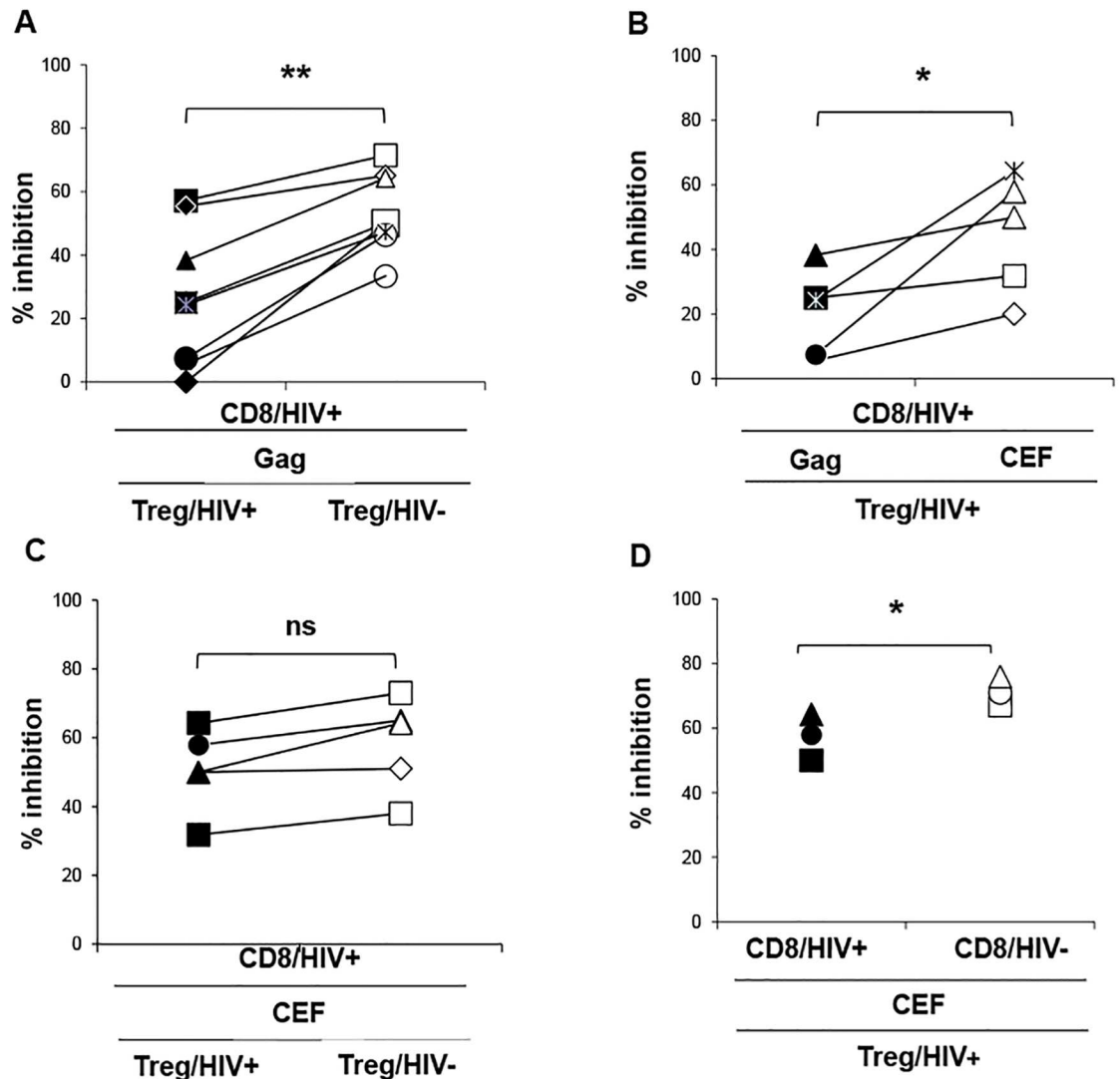


Fig 5. Treg/HIV+ lack suppressive capacity as compared to Treg/HIV- and fail to modulate PD-1/PD-L1 expression on HIV-specific CD8 T cells. Individual data from co-culture and cross-culture studies comparing the inhibition of IFN γ expression by **A.** Gag-stimulated HIV+ CD8 T cells in the presence of autologous (left) or HIV- (right) CD4+CD25^{high} T cells, (n = 8). **B.** Gag-stimulated (left) and CEF-stimulated (right) HIV+ CD8 T cells in the presence of autologous CD4+CD25^{high} T cells, (n = 5). **C.** CEF-stimulated HIV+ CD8 T cells in the presence of autologous (left) or HIV- (right) CD4+CD25^{high} T cells, (n = 5). **D.** CEF-stimulated autologous co-cultures and cross-cultures in which HIV + CD8 T have been replaced with CD8 T from HIV- donors, (n = 3; * p<0.05, Student's T-test).

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load on Treg function. Treg from patients receiving ART, and with confirmed viral suppression, had a significantly increased inhibitory potential on Gag-specific CD8 T response as compared to Treg isolated from patients with active viral replication. This was observed either in autologous conditions (co-cultures of CD8 T cells from untreated patients with Treg from same patients after ART treatment) or in allogenic conditions (Treg from other patients under ART) (mean IFN γ inhibition % of 34 vs. 15, respectively, MW $p < 0.01$), (S5 Fig).

Next we investigated whether this defect of Treg/HIV+ concerned also non HIV-specific T cells.

Results showed that Treg/HIV+ inhibited the autologous CEF-specific CD8 T much better than Gag-specific ones (average inhibition %, [min–max]: 45, [18–64]) vs. 21 [7–38], $P < 0.05$ (Fig 5B) and similarly to Treg/HIV- in cross-over cultures (51 [32–64] vs. 58, [38–73], $P > 0.05$), (Fig 5C). Further on, the PD-1 MFI on the CEF-specific CD8 T cells decreased significantly from 3504 to 2465 and 2595 in the presence of Treg/HIV+ and Treg/HIV-, respectively ($P < 0.05$ for both comparisons, (S4A Fig). Similarly, PD-L1 MFI decreased from 8900 to 6155 and 5748, respectively, ($P < 0.01$ for both comparisons, (S4B Fig). At the same time, no significant difference was noted between the expression levels of PD-1 and PD-L1 on Gag-specific effectors, whether in the presence of Treg /HIV+ or Treg/HIV- (MFI for PD-1: 4642 without Treg vs. 3535 and 3595, in the presence of Treg/HIV+ and Treg/HIV-, respectively; MFI for PD-L1: 11047 without Treg vs. 9425 and 8931, in the presence of Treg/HIV+ and Treg/HIV-, respectively ($P > 0.05$ for all comparisons, S4 Fig). Finally, Treg isolated from three HIV+ patients suppressed CEF-specific CD8 T-cells from HIV- controls much better than CD8 T-cells from HIV+ patients (mean inhibition % of IFN γ expression 71 ± 5 vs. 57 ± 7 respectively; $P < 0.05$), (Fig 5D).

In summary, these results showed a defect in the regulation of CD8 T cell proliferation, differentiation and function in HIV infected individuals. We show that the suppressive capacity of Treg is impaired in HIV-infected patients as compared to those from non HIV-infected individuals. Moreover, this impairment is dependent on the specificity of CD8 T cells. The lack of Treg-mediated effects on the expression of PD-1/PD-L1 molecules on HIV-specific CD8 T cells, in contrast to other recall antigen-specific clones from the same patients, strongly suggest that this particular defect of Treg might contribute to the exhaustion of HIV-specific T cell responses.

Discussion

In this study we explored the suppressive effects of Treg on CD8 T cell responses in chronic HIV infection. The present data extend significantly our previous results in non-infectious context [25] showing that the suppressive effect of Treg is highly dependent on the stage of CD8 T cell differentiation. Likely, these data contribute to explain that the ultimate failure to contain generalized HIV-1-associated immune activation in HIV+ infection results from both an intrinsic impairment of Treg function and a decreased responsiveness of effector clones, at least in part associated with the high levels of PD-1/PD-L1 expression.

First, we demonstrate that Treg from HIV+ individuals display lower suppressive effects as compared to those from HIV- controls. Second, regarding the effects of Treg on particular CD8 T cell populations, we show clearly that Treg suppress mostly the differentiation of effector-memory and effector CD8+ T cells in HIV-infected patients. Third, HIV-specific CD8+ T cells were less sensitive to the suppressive effects of Treg as compared to other recall antigen-specific CD8+ T cells. Finally, HIV-specific effector CD8 T cells are resistant to Treg-mediated modulation of the “exhaustion” molecules PD-1/PDL-1.

Numerous studies have shown that Treg expand during acute and chronic HIV-1 infection, and inhibit effector CD8 T cell responses in vitro [17,21,37]. Recent data about the

heterogeneous nature of Treg and their plasticity indicate that Treg effects may differ depending on the phase of immune response, the nature of stimulus and the differentiation state of the target subsets.

In the HIV-negative settings, Treg exert differential effects on the proliferation, maturation and apoptosis of CD8 subsets and in this way maintain the memory/effector cell balance in case of strong stimulation [25]. Globally, we show here that Treg from HIV+ donors with controlled viral load inhibited the differentiation of naïve CD8 T cells into CCR7⁻ CD45RA⁻ EM+E cells, and favored the accumulation of cells with CM (CD45RA⁺ CCR7⁺) phenotype. The memory-like phenotype of HIV-specific CD8 cells combined with decreased proliferative and cytokine-secreting capacity, has been well documented [3,5,11]. We show for the first time that these characteristics of the HIV-specific pool may result from the subset-specific homeostatic effects of Treg that have become irrelevant in conditions of generalized and lasting immune activation.

In addition to the subset-specific effects of Treg, we demonstrate that Treg-mediated inhibition differs depending on the antigen specificity of CD8 response. In our hands, the effect of HIV+ Treg on HIV Gag-specific clones was significantly inferior to the inhibitory effect on CEF-specific cells, whether from HIV+ or HIV- donors. One logical explanation is the differing sensitivity of the responding cells to Treg-mediated inhibition. In healthy donors, CD8 cells specific for common viral infections as CMV, EBV, and flu are composed mostly of non-activated memory cells [9,38]. In the HIV+ settings, virus-specific cells are either in a state of immune senescence as a result of increased susceptibility to co-infections [39,40] combined with frequent by-stander activation [41], or—exhaustion resulting from continuous stimulation in the settings of high antigen load, and characterized by progressive loss of effector functions [42]. Therefore “exhausted” HIV-specific CD8 would be less capable to produce IFN γ as compared to the “senescent” non-HIV specific ones, and Treg depletion may not restore their functionality.

Increased expression of the co-inhibitory programmed cell death-1 (PD-1) receptor is a hallmark of exhausted T cells [12,14,43]. Studies in animal models and in humans have pinpointed PD-1/PD-L1 pathway as crucial for the outcome of a virus-specific response, independently of CD4⁺ T cell help [14,44–46]. The levels of PD-1 and its ligand correlate with the antigen burden and the duration of stimulation as shown in HIV, HCV, and HBV chronically infected patients [14,18,45,46]. Activated CD8 T cells engaged towards CM differentiation express the highest levels of PD-1 and are therefore much prone to “exhaustion” [14]. In the HIV-negative settings, CM CD8 could be rescued from activation-induced apoptosis through Treg-mediated regulation of PD-L1 expression, both on by-stander CD8 and on Treg themselves [25]. Here, we show that in HIV-infected donors this regulatory mechanism is operating only with respect to non-HIV specific clones. No such effect was observed with Gag-specific CD8, implying that Treg are losing their ability to regulate CD8 response through PD-1/PD-L1 pathway in case of immune exhaustion. Further, ART-mediated viral suppression may not readily revert high PD1 and PD-L1 expression on CD8 T cells. In our hands, PD1 and PD-L1 expression was not significantly diminished when replacing autologous Treg with Treg from settings with undetectable VL (S5 Fig). In line with our observations, it was shown that the reversible regulation of PD-1 expression may be eventually lost, as in HIV infected subjects, PD-1 levels on CD4 and CD8T cells remain high following anti-retroviral therapy [43].

In addition to a differential sensitivity of the regulated clones, we have demonstrated that the suppressive potential of Treg changes in the HIV+ settings. Treg are directly affected by HIV, and may experience variable extent of impairment. In different models of chronic stimulation, Treg survival and functions closely depended on PD-1 [47,48], as well as on PD-L1 expression [47,49]. In our hands, autologous Treg inhibited HIV-specific responses less well

than did Treg from HIV-negative donors. The correlation between the viral load and Treg inhibitory effect in the studied group of HIV+ART- patients did not reach significant values. However, ART-mediated suppression of HIV VL significantly restored Treg function, which might be in part explained by a significantly decreased expression of apoptosis-inducing receptors on Treg themselves (S6 Fig).

Data on the quality and evolution of Treg-mediated inhibition in chronic infection are limited and contradictory. Studies on peripheral Treg have found a decreased suppressive capacity of Treg from patients with progressive HIV infection [21,37], while two recent studies reported preserved suppressive capacity of peripheral blood and mucosal Treg in non-controlled HIV-1 infection [31,50]. However, both studies have assessed Treg inhibitory effect on the proliferation of anti-CD3 stimulated non-Treg CD4+ T cells, and not on antigen-specific CD8 T cell responses. Even more so, Shaw et al. [31] observed a trend toward increased suppression by mucosal Treg from noncontrollers regarding one particular effect: OX40 co-expression by non-Treg CD4 T. This was associated with significantly increased CD38 expression by mucosal Treg. Globally, these results may indicate that Treg can modulate effector functions to different degrees depending on the tissue microenvironment and specific pathways engaged. The genuine heterogeneity of Treg has just started to be elucidated. Three phenotypically and functionally distinct Treg subpopulations have been defined: CD45RA+FoxP3^{low} naïve, CD45RA-FoxP3^{high} effector, and a CD45RA-FoxP3^{low} non suppressive subset [51]. A recent study showed that it was the effector Treg that were consistently affected during HIV infection, and were negatively correlated with the magnitude of HIV-specific CD8 T-cell responses [52]. According to our depletion protocol [27], we have characterized mostly the function of the effector Treg subset. The observed higher inhibitory effect of Treg from HIV-negative donors on Gag-specific cells (S5 Fig) was probably due to a higher effector/naïve Treg subset ratio as compared to HIV+ Treg. In line with this hypothesis, preincubation with Gag peptides enhanced the suppressive effect of HIV+ Treg by inducing the differentiation of naïve Treg, or of non-Treg CM or EM cells [53,54] or either by inducing the apoptosis of PD-1^{high} non-functional Treg. Similar effects of low-dose antigen stimulation on the generation of Ag-specific adaptive Treg have been reported in other *in vitro* models [55,56].

In line with the present results, we have previously reported that in ART- chronic HIV+ patients, the suppressive activity of CD4+CD25+ T on the proliferation response to tuberculin did not vary over time while the suppressive activity in response to p24 decreased significantly in the course of 24 months [37]. Globally, these data consistently demonstrate that the suppressive efficiency of Treg decreases in the settings of chronic infection in a clonally-specific manner.

Therefore, we support the hypothesis that the ultimate failure to contain generalized HIV-1-associated immune activation, even in ART treated HIV+ patients, is a joint result of intrinsic impairment of Treg function and decreased responsiveness of the effector clones, at least in part associated with the high levels of PD-1/PD-L1 expression.

Elimination of Treg in the settings of chronic infection has been considered as an attractive approach to restore virus-specific CD8 responses [57]. We have recently shown that responses to the administration of a therapeutic vaccine in HIV+ infected patients are influenced by the frequency of HIV-specific Treg at baseline, and vaccinees who displayed lower levels of HIV-specific Treg responded better to the therapeutic vaccine [58]. Yet, a simple elimination of Treg would also abrogate their homeostatic effects leading to autoimmune phenomena.

Blockade of inhibitory receptors also results in functional recovery of exhausted T cells; the most convincing examples including PD-1 blockade in HIV, HBV, and HCV infection [13,47,59–61]. However, repeated anti-PD-1 treatment may increase the likelihood of autoimmune events just as Treg depletion does. PD-1/PD-L1 blockade might reactivate Treg as well,

and compromise the reactivation of antigen-specific response. Even if viral load suppression, followed by PD1/PD-L1 blockade could restore virus-specific response, the containment of low-level immune activation may prevent the complete restoration of Treg proliferation or suppression function [62]. Here, we prompt the possibility to improve the composition and functionality of the Treg pool through selective modulation of Treg subsets. Treg subsets could respond differently to external signals, and therefore—be modulated, using blocking Abs, chemical products or cytokines [34,35,58]. We have recently shown a differential in vitro effect of IL-7 on purified Treg subsets from HIV-negative individuals, and confirmed that IL-7 therapy in HIV+ infected subjects impacted also Treg function [34].

In summary, our present results demonstrate subset- and antigen-specific Treg effects in the settings of chronic HIV infection. These observations point out also a defect in the suppressive capacity of Treg which may explain the persistence of immune activation in HIV+ treated patients. Finally, these data may help the further precision of immune-intervention therapies.

Materials and Methods

Patients and cell populations

Blood samples from HIV-1-positive subjects, either naive from treatment (ART-, $n = 10$, mean CD4+ T cells counts 545 ± 158 / μ l; mean viral load 3.9 ± 1.1 log HIV RNA copies /ml), or under ART ($n = 14$, mean CD4+ T cells counts 675 ± 242 cells; viral load < 1.6 log HIV RNA copies /ml), were collected at the Clinical Immunology Department of Henri Mondor Hospital Créteil, France and the National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria, in the course of routine immune monitoring. Blood samples from HIV-negative healthy donors were obtained at the Regional Blood Transfusion Centre, Creteil, France. The research was conducted in accordance with the Declaration of Helsinki. The study was approved by the respective ethical committees, and written informed consent from all subjects were obtained before study initiation. Peripheral blood mononuclear cells (PBMC) were isolated using standard gradient separation technique. Half of them were Treg-depleted, using anti-CD25 Dynabeads and a Dynall magnetic separation system, after the manufacturer's protocol (Invitrogen Dynal AS, Oslo, Norway). The Treg-depleted fraction contained $< 0.1\%$ CD4 T expressing high levels of CD25 and FoxP3 transcription factor as verified by flow cytometry (S8 Fig). In a limited number of experiments total CD8 T cells, and CD4+CD25^{high} Treg fractions were obtained from frozen or fresh PBMC, using negative isolation kits from Miltenyi Biotec (Bergisch-Gladbach, Germany) according to the manufacturer's instructions, as previously described. The purity of purified populations was $>95\%$. Treg cells were defined as CD4+CD25^{high}FoxP3+CD127^{low}T cells as we have previously reported [25].

Proliferation assays

PBMC/Treg+ and PBMC depleted in Treg (Treg- fraction) from HIV-1+ART+ patients were stained with CFSE (Molecular probes, Eugene OR, US) at final concentration 0.5μ M and cultivated in 96-well U-bottom plates, coated with 5μ g/mL anti-CD3 mAb (UCHT1; Beckman Coulter, Villepinte, France); (total cell concentration 1.25×10^5 /ml and final volume 200μ l; Treg/effector ratio 1:4 as previously described [25]). On day 5 (D5) cells were washed and surface-stained with a combination of CD27/CD8/CD45RA/CCR7/CD3 mAbs. CD8 T cell subsets were defined as follows: CCR7+CD45RA+ (naïve, N), CCR7+CD45RA- (central-memory, CM), CCR7-CD45RA- (effector-memory + effector, EM+E), and CCR7-CD45RA+ (terminal effector, TE). Proliferation rates were assessed according to the percentage of CFSE^{low} cells at D5 by multicolor flow cytometry (FACSCanto, BD).

Intracellular cytokine production and apoptosis studies

For intracellular cytokine staining (ICS), overnight stimulation of PBMC (either Treg-depleted or not) was carried out in plates coated with anti-CD3 (5 µg/ml) or with a pool of whole Gag 15-mer peptides (2 µg/ml) supplemented with anti-CD28 and anti-CD49d antibodies (1 µg/ml of each), or control CEF (CMV, EBV and influenza virus) peptides (5µg/ml each). Brefeldin A (10 µg/ml) was added 1h after the peptide stimulation. Cells were surface stained with anti-CD8, anti-CD4, anti-CD69, anti-PD-1 and anti-PD-L1 mAb and ICS was performed with PE-Cy7- or FITC-conjugated IFN γ antibody. For co-culture studies CD8⁺ T cells (5×10^5 cells/well) were cultivated in 48-well plates, in the presence or absence of autologous or allogeneic CD4⁺CD25⁺ T cell fractions at a ratio 4:1, and autologous monocytes (10^4 /well.) In a limited number of experiments, the CD4⁺CD25⁺ T cell fraction was stimulated with Gag 15-mer peptides (2 µg/ml) in the presence of autologous monocytes (10^4 /well.) for 18h before the setting of co-cultures. For apoptosis studies, PBMC Treg⁺ and PBMC Treg⁻ fractions from HIV-1+ART+ patients and from HIV- controls were cultivated in 96-well round-bottom plates coated with anti-CD3 at 5 µg/mL. Forty-eight hours later, cells were harvested, washed with cold PBS, suspended in binding buffer (containing 2.5 mM CaCl₂), and stained with annexin V-FITC (apoptosis kit; BD Pharmingen) and a combination of CD27-PE/CD8-PerCP/CD45RA-APC mAbs for 20 minutes on ice. At the end of the culture, 300 µL of binding buffer was added, and the samples were analyzed within 30 minutes by flow cytometry while kept on ice.

Immunofluorescence and flow cytometry

Anti-CD25-APC-Cy5, anti-CD127-Alexa-Fluor700, anti-CD4-FITC or Pacific blue, anti-CD45RA-FITC or PE-Cy5, anti-CCR7-PE, or PE-Cy7, anti-CD8-PerCP, anti-CD3-AmCyan, anti-IFN-gamma-FITC or APC-Cy7, anti-CD69-APC, anti-PD-1-PE and anti-PD-L1-PECy7, anti-FoxP3-PE were all products of BD Biosciences. Cells were analysed by FACSCanto II, and LSR II (BD Immunocytometry systems). At least 20 000 CD4 or CD8-gated events, and at least 100000 CD4 or CD8-gated events were collected for cell surface and intracellular studies, respectively.

Statistical analysis

Flow cytometry data were analyzed by FACSDiva v.6.1.2. and FlowJo software. Statistically significant differences were assessed by one-way ANOVA, followed by paired T-test, or by unpaired T-test assuming independent samples where appropriate. Correlations were assessed using Spearman's rank order test (GraphPad[®] Prism 5.0 statistical software).

Supporting Information

S1 Fig. Effects of Treg on the expression of IFN γ by CD8 T cells at the subset level. Individual data showing the effect of Treg on IFN γ secretion within CCR7/CD45RA-defined CD8 T subsets, after 18-hour stimulation with anti-CD3 antibodies in the presence (black) or in the absence (white) of Treg, in HIV- negative (A) and in HIV+ART-naïve (B) subjects. Embedded: pooled data showing the distribution of IFN γ + CD8 T cells within the CD45RA/CCR7 defined subsets in HIV-(left) and in HIV+ settings (right). Bars represent mean \pm SD. (* P <0.05, ** P <0.01, *** P <0.001, n = 10, paired Student's T-test). (TIF)

S2 Fig. Activation-induced apoptosis escapes from Treg control in HIV+ settings. Pooled data about the percentage of AnnexinV+ cells within CD27/CD45RA-defined CD8 T subsets,

after 48 hour stimulation with anti-CD3 antibodies in the presence (black) or in the absence (white) of Treg. (ns $P > 0.05$, (n = 6, Student's T-test).

(TIF)

S3 Fig. Treg-mediated regulation of PD-1/PD-L1 expression on CD4 T cells from HIV+ patients is clonally-specific. Pooled data on the levels of PD-1 and PD-L1 expression on CEF-specific (A) and Gag-specific (B) CD4 T cells stimulated overnight in the presence (black) or in the absence (white) of Treg. (* $P < 0.05$, *** $P < 0.001$, n = 10, paired Student t-test).

(TIF)

S4 Fig. The failure of Treg/HIV+ to modulate PD-1/PD-L1 expression depends on the antigen specificity of CD8 T cells. Individual data from co-culture and cross-culture studies comparing the expression of PD1 (A) and PD-L1 (B) on HIV+ CD8 T cells, stimulated with CEF (left) or Gag (right) peptides, in the absence (grey) or in the presence of autologous, HIV+ (black) or of HIV- (right) CD4+CD25^{high} T cells, (n = 8), (* $p < 0.05$, Student's T-test).

(TIF)

S5 Fig. Treg inhibitory potential changes depending on HIV viral load. A representative experiment in which non-stimulated (a) or Gag-stimulated (b-d) HIV+ CD8 T cells from ART-naïve patient were cultured either in the absence of Treg, in the presence of autologous Treg, or in the presence of allogenic Treg from an ART+ patient with undetectable HIV VL (A) Individual data from co-culture and cross-culture studies comparing the inhibition of IFN γ expression by Gag-stimulated HIV+ CD8 T cells from ART-naïve patients in the presence of Treg from the same time point (left) or Treg from a different blood draw/or patient, after HIV VL suppression (right)(B).

(TIF)

S6 Fig. PD1 and PD-L1 expression on CD8 T cells and Treg depending on HIV viral load. Individual data from co-culture and cross-culture studies comparing PD1 and PD-L1 expression by Gag-stimulated HIV+ CD8 T cells from ART-naïve patients in the presence of Treg from the same time point or Treg from a different blood draw/or patient, after HIV VL suppression (left panel). PD1 and PD-L1 expression by Treg from ART-naïve patients and Treg from a different time point/or patient, after HIV VL suppression (right panel).

(TIF)

S7 Fig. The composition and inhibitory effect of Treg in HIV+ settings can be modulated. A. Inhibition of IFN γ expression by Gag-stimulated HIV+ CD8 T cells in the presence of autologous CD4+CD25^{high} T cells, set in co-culture *ex vivo* or after 18 hour preincubation of Treg with Gag peptides. Proportions of effector (CD25+FoxP3^{high}CD45RA-) and naïve (FoxP3^{low}CD45RA+) Treg before (B) and after (C) 18h preincubation with Gag peptides (a representative example of 4 separate experiments).

(TIF)

S8 Fig. Flow cytometry analysis of PBMC before and after Treg depletion. PBMC before (upper panel) and after Treg-depletion with anti-CD25 Dynabeads as specified in Material and methods section (lower panel) were permeabilized and stained with a combination of FoxP3/CD25/CD127/CD4 mAbs to verify the efficiency of depletion. A representative example is presented; cells were gated on CD4 expression.

(TIF)

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Author Contributions

Conceived and designed the experiments: YL CL MN.

Performed the experiments: MN MM DA AW.

Analyzed the data: MN CL AW MM.

Wrote the paper: MN AW CL YL.

References

- Musey L, Hughes J, Schacker T, Shea T, Corey L, McElrath MJ. Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. *N Engl J Med* 1997; 337: 1267–1274. doi: [10.1056/NEJM199710303371803](https://doi.org/10.1056/NEJM199710303371803) PMID: [9345075](https://pubmed.ncbi.nlm.nih.gov/9345075/)
- Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD. Temporal association of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 1994; 68: 4650–4655. PMID: [8207839](https://pubmed.ncbi.nlm.nih.gov/8207839/)
- Appay V, Nixon DF, Donahoe SM, Gillespie GM, Dong T, King A, Ogg GS, Spiegel HM, Conlon C, Spina CA, Havlir DV, Richman DD, Waters A, Easterbrook P, McMichael AJ, Rowland-Jones SL. HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. *J Exp Med* 2000; 192: 63–75. PMID: [10880527](https://pubmed.ncbi.nlm.nih.gov/10880527/)
- Champagne P, Ogg GS, King AS, Knabenhans C, Ellefsen K, Nobile M, Appay V, Rizzardi GP, Fleury S, Lipp M, Forster R, Rowland-Jones S, Sekaly RP, McMichael AJ, Pantaleo G. Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature* 2001; 410: 106–111. doi: [10.1038/35065118](https://doi.org/10.1038/35065118) PMID: [11242051](https://pubmed.ncbi.nlm.nih.gov/11242051/)
- Kostense S, Ogg GS, Manting EH, Gillespie G, Joling J, Vandenberghe K, Veenhof EZ, van BD, Jurriaans S, Klein MR, Miedema F. High viral burden in the presence of major HIV-specific CD8(+) T cell expansions: evidence for impaired CTL effector function. *Eur J Immunol* 2001; 31: 677–686. doi: [10.1002/1521-4141\(200103\)31:3<677::AID-IMMU677>3.0.CO;2-M](https://doi.org/10.1002/1521-4141(200103)31:3<677::AID-IMMU677>3.0.CO;2-M) PMID: [11241270](https://pubmed.ncbi.nlm.nih.gov/11241270/)
- Jin HT, Anderson AC, Tan WG, West EE, Ha SJ, Araki K, Freeman GJ, Kuchroo VK, Ahmed R. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A* 2010; 107: 14733–14738. doi: [10.1073/pnas.1009731107](https://doi.org/10.1073/pnas.1009731107) PMID: [20679213](https://pubmed.ncbi.nlm.nih.gov/20679213/)
- Goldberg MV, Maris CH, Hipkiss EL, Flies AS, Zhen L, Tudor RM, Grosso JF, Harris TJ, Getnet D, Whartenby KA, Brockstedt DG, Dubensky TW Jr., Chen L, Pardoll DM, Drake CG. Role of PD-1 and its ligand, B7-H1, in early fate decisions of CD8 T cells. *Blood* 2007; 110: 186–192. doi: [10.1182/blood-2006-12-062422](https://doi.org/10.1182/blood-2006-12-062422) PMID: [17392506](https://pubmed.ncbi.nlm.nih.gov/17392506/)
- Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, Betts MR, Freeman GJ, Vignali DA, Wherry EJ. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 2009; 10: 29–37. doi: [10.1038/ni.1679](https://doi.org/10.1038/ni.1679) PMID: [19043418](https://pubmed.ncbi.nlm.nih.gov/19043418/)
- Tussey LG, Nair US, Bachinsky M, Edwards BH, Bakari J, Grimm K, Joyce J, Vessey R, Steigbigel R, Robertson MN, Shiver JW, Goepfert PA. Antigen burden is major determinant of human immunodeficiency virus-specific CD8+ T cell maturation state: potential implications for therapeutic immunization. *J Infect Dis* 2003; 187: 364–374. doi: [10.1086/367707](https://doi.org/10.1086/367707) PMID: [12552420](https://pubmed.ncbi.nlm.nih.gov/12552420/)
- Sacre K, Carcelain G, Cassoux N, Fillet AM, Costagliola D, Vittecoq D, Salmon D, Amoura Z, Katlama C, Autran B. Repertoire, diversity, and differentiation of specific CD8 T cells are associated with immune protection against human cytomegalovirus disease. *J Exp Med* 2005; 201: 1999–2010. doi: [10.1084/jem.20042408](https://doi.org/10.1084/jem.20042408) PMID: [15967826](https://pubmed.ncbi.nlm.nih.gov/15967826/)
- Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, Ogg GS, King A, Lechner F, Spina CA, Little S, Havlir DV, Richman DD, Gruener N, Pape G, Waters A, Easterbrook P, Salio M, Cerundolo V, McMichael AJ, Rowland-Jones SL. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med* 2002; 8: 379–385. doi: [10.1038/nm0402-379](https://doi.org/10.1038/nm0402-379) PMID: [11927944](https://pubmed.ncbi.nlm.nih.gov/11927944/)
- Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C, Mncube Z, Duraiswamy J, Zhu B, Eichbaum Q, Altfeld M, Wherry EJ, Coovadia HM,

- Goulder PJ, Klenerman P, Ahmed R, Freeman GJ, Walker BD. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006; 443: 350–354. doi: [10.1038/nature05115](https://doi.org/10.1038/nature05115) PMID: [16921384](https://pubmed.ncbi.nlm.nih.gov/16921384/)
13. Velu V, Titanji K, Zhu B, Husain S, Pladevega A, Lai L, Vanderford TH, Chennareddi L, Silvestri G, Freeman GJ, Ahmed R, Amara RR. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature* 2009; 458: 206–210. doi: [10.1038/nature07662](https://doi.org/10.1038/nature07662) PMID: [19078956](https://pubmed.ncbi.nlm.nih.gov/19078956/)
 14. Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, Boulassel MR, Delwart E, Sepulveda H, Balderas RS, Routy JP, Haddad EK, Sekaly RP. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med* 2006; 12: 1198–1202. doi: [10.1038/nm1482](https://doi.org/10.1038/nm1482) PMID: [16917489](https://pubmed.ncbi.nlm.nih.gov/16917489/)
 15. Petrovas C, Price DA, Mattapallil J, Ambrozak DR, Geldmacher C, Cecchinato V, Vaccari M, Tryniszewska E, Gostick E, Roederer M, Douek DC, Morgan SH, Davis SJ, Franchini G, Koup RA. SIV-specific CD8+ T cells express high levels of PD1 and cytokines but have impaired proliferative capacity in acute and chronic SIVmac251 infection. *Blood* 2007; 110: 928–936. doi: [10.1182/blood-2007-01-069112](https://doi.org/10.1182/blood-2007-01-069112) PMID: [17440051](https://pubmed.ncbi.nlm.nih.gov/17440051/)
 16. West EE, Jin HT, Rasheed AU, Penalzo-MacMaster P, Ha SJ, Tan WG, Youngblood B, Freeman GJ, Smith KA, Ahmed R. PD-L1 blockade synergizes with IL-2 therapy in reinvigorating exhausted T cells. *J Clin Invest* 2013; 123: 2604–2615. doi: [10.1172/JCI67008](https://doi.org/10.1172/JCI67008) PMID: [23676462](https://pubmed.ncbi.nlm.nih.gov/23676462/)
 17. Weiss L, Donkova-Petrini V, Caccavelli L, Balbo M, Carbonneil C, Levy Y. Human immunodeficiency virus-driven expansion of CD4+CD25+ regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood* 2004; 104: 3249–3256. doi: [10.1182/blood-2004-01-0365](https://doi.org/10.1182/blood-2004-01-0365) PMID: [15271794](https://pubmed.ncbi.nlm.nih.gov/15271794/)
 18. Trehanpati N, Kotillil S, Hissar SS, Shrivastava S, Khanam A, Sukriti S, Mishra SK, Sarin SK. Circulating Tregs correlate with viral load reduction in chronic HBV-treated patients with tenofovir disoproxil fumarate. *J Clin Immunol* 2011; 31: 509–520. doi: [10.1007/s10875-011-9509-7](https://doi.org/10.1007/s10875-011-9509-7) PMID: [21305387](https://pubmed.ncbi.nlm.nih.gov/21305387/)
 19. Scholzen A, Minigo G, Plebanski M. Heroes or villains? T regulatory cells in malaria infection. *Trends Parasitol* 2010; 26: 16–25. doi: [10.1016/j.pt.2009.10.004](https://doi.org/10.1016/j.pt.2009.10.004) PMID: [19914134](https://pubmed.ncbi.nlm.nih.gov/19914134/)
 20. Lundgren A, Suri-Payer E, Enarsson K, Svennerholm AM, Lundin BS. Helicobacter pylori-specific CD4+ CD25high regulatory T cells suppress memory T-cell responses to H. pylori in infected individuals. *Infect Immun* 2003; 71: 1755–1762. doi: [10.1128/IAI.71.4.1755-1762.2003](https://doi.org/10.1128/IAI.71.4.1755-1762.2003) PMID: [12654789](https://pubmed.ncbi.nlm.nih.gov/12654789/)
 21. Kinter AL, Hennessey M, Bell A, Kern S, Lin Y, Daucher M, Planta M, McGlaughlin M, Jackson R, Ziegler SF, Fauci AS. CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. *J Exp Med* 2004; 200: 331–343. doi: [10.1084/jem.20032069](https://doi.org/10.1084/jem.20032069) PMID: [15280419](https://pubmed.ncbi.nlm.nih.gov/15280419/)
 22. Boettler T, Spangenberg HC, Neumann-Haefelin C, Panther E, Urbani S, Ferrari C, Blum HE, von WF, Thimme R. T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. *J Virol* 2005; 79: 7860–7867. doi: [10.1128/JVI.79.12.7860-7867.2005](https://doi.org/10.1128/JVI.79.12.7860-7867.2005) PMID: [15919940](https://pubmed.ncbi.nlm.nih.gov/15919940/)
 23. Aandahl EM, Michaelsson J, Moretto WJ, Hecht FM, Nixon DF. Human CD4+ CD25+ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *J Virol* 2004; 78: 2454–2459. doi: [10.1128/JVI.78.5.2454-2459.2004](https://doi.org/10.1128/JVI.78.5.2454-2459.2004) PMID: [14963140](https://pubmed.ncbi.nlm.nih.gov/14963140/)
 24. Suvas S, Kumaraguru U, Pack CD, Lee S, Rouse BT. CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. *J Exp Med* 2003; 198: 889–901. doi: [10.1084/jem.20030171](https://doi.org/10.1084/jem.20030171) PMID: [12975455](https://pubmed.ncbi.nlm.nih.gov/12975455/)
 25. Nikolova M, Lelievre JD, Carriere M, Bensussan A, Levy Y. Regulatory T cells modulate differentially the maturation and apoptosis of human CD8-T cell subsets. *Blood* 2009.
 26. Elahi S, Niki T, Hirashima M, Horton H. Galectin-9 binding to Tim-3 renders activated human CD4+ T cells less susceptible to HIV-1 infection. *Blood* 2012; 119: 4192–4204. doi: [10.1182/blood-2011-11-389585](https://doi.org/10.1182/blood-2011-11-389585) PMID: [22438246](https://pubmed.ncbi.nlm.nih.gov/22438246/)
 27. Chevalier MF, Weiss L. The split personality of regulatory T cells in HIV infection. *Blood* 2013; 121: 29–37. doi: [10.1182/blood-2012-07-409755](https://doi.org/10.1182/blood-2012-07-409755) PMID: [23043072](https://pubmed.ncbi.nlm.nih.gov/23043072/)
 28. Simonetta F, Bourgeois C. CD4+FOXP3+ Regulatory T-Cell Subsets in Human Immunodeficiency Virus Infection. *Front Immunol* 2013; 4: 215. doi: [10.3389/fimmu.2013.00215](https://doi.org/10.3389/fimmu.2013.00215) PMID: [23908654](https://pubmed.ncbi.nlm.nih.gov/23908654/)
 29. Eggena MP, Barugahare B, Jones N, Okello M, Mutalya S, Kityo C, Mugenyi P, Cao H. Depletion of regulatory T cells in HIV infection is associated with immune activation. *J Immunol* 2005; 174: 4407–4414. PMID: [15778406](https://pubmed.ncbi.nlm.nih.gov/15778406/)
 30. Prendergast A, Prado JG, Kang YH, Chen F, Riddell LA, Luzzi G, Goulder P, Klenerman P. HIV-1 infection is characterized by profound depletion of CD161+ Th17 cells and gradual decline in regulatory T cells. *AIDS* 2010; 24: 491–502. doi: [10.1097/QAD.0b013e3283344895](https://doi.org/10.1097/QAD.0b013e3283344895) PMID: [20071976](https://pubmed.ncbi.nlm.nih.gov/20071976/)

31. Shaw JM, Hunt PW, Critchfield JW, McConnell DH, Garcia JC, Pollard RB, Somsouk M, Deeks SG, Shacklett BL. Increased frequency of regulatory T cells accompanies increased immune activation in rectal mucosae of HIV-positive noncontrollers. *J Virol* 2011; 85: 11422–11434. doi: [10.1128/JVI.05608-11](https://doi.org/10.1128/JVI.05608-11) PMID: [21880771](https://pubmed.ncbi.nlm.nih.gov/21880771/)
32. Pereira LE, Villinger F, Onlamoon N, Bryan P, Cardona A, Pattanapanyasat K, Mori K, Hagen S, Picker L, Ansari AA. Simian immunodeficiency virus (SIV) infection influences the level and function of regulatory T cells in SIV-infected rhesus macaques but not SIV-infected sooty mangabeys. *J Virol* 2007; 81: 4445–4456. doi: [10.1128/JVI.00026-07](https://doi.org/10.1128/JVI.00026-07) PMID: [17314162](https://pubmed.ncbi.nlm.nih.gov/17314162/)
33. Saison J, Maucort BD, Chidiac C, Demaret J, Malcus C, Cotte L, Poitevin-Later F, Miallhes P, Venet F, Trabaud MA, Monneret G, Ferry T. Increased Regulatory T-Cell Percentage Contributes to Poor CD4 (+) Lymphocytes Recovery: A 2-Year Prospective Study After Introduction of Antiretroviral Therapy. *Open Forum Infect Dis* 2015; 2: ofv063. doi: [10.1093/ofid/ofv063](https://doi.org/10.1093/ofid/ofv063) PMID: [26110165](https://pubmed.ncbi.nlm.nih.gov/26110165/)
34. Younas M, Hue S, Lacabaratz C, Guguin A, Wiedemann A, Surenaud M, Beq S, Croughs T, Lelievre JD, Levy Y. IL-7 modulates in vitro and in vivo human memory T regulatory cell functions through the CD39/ATP axis. *J Immunol* 2013; 191: 3161–3168. doi: [10.4049/jimmunol.1203547](https://doi.org/10.4049/jimmunol.1203547) PMID: [23966629](https://pubmed.ncbi.nlm.nih.gov/23966629/)
35. Nikolova M, Carriere M, Jenabian MA, Limou S, Younas M, Kok A, Hue S, Seddiki N, Hulin A, Delauneau O, Schuitemaker H, Herbeck JT, Mullins JI, Muhtarova M, Bensussan A, Zagury JF, Lelievre JD, Levy Y. CD39/adenosine pathway is involved in AIDS progression. *PLoS Pathog* 2011; 7: e1002110. doi: [10.1371/journal.ppat.1002110](https://doi.org/10.1371/journal.ppat.1002110) PMID: [21750674](https://pubmed.ncbi.nlm.nih.gov/21750674/)
36. da Silva MM, Piccirillo CA. Functional stability of Foxp3+ regulatory T cells. *Trends Mol Med* 2012; 18: 454–462. doi: [10.1016/j.molmed.2012.06.001](https://doi.org/10.1016/j.molmed.2012.06.001) PMID: [22771168](https://pubmed.ncbi.nlm.nih.gov/22771168/)
37. Kared H, Lelievre JD, Donkova-Petrini V, Aouba A, Melica G, Balbo M, Weiss L, Levy Y. HIV-specific regulatory T cells are associated with higher CD4 cell counts in primary infection. *AIDS* 2008; 22: 2451–2460. doi: [10.1097/QAD.0b013e328319edc0](https://doi.org/10.1097/QAD.0b013e328319edc0) PMID: [19005268](https://pubmed.ncbi.nlm.nih.gov/19005268/)
38. Chen G, Shankar P, Lange C, Valdez H, Skolnik PR, Wu L, Manjunath N, Lieberman J. CD8 T cells specific for human immunodeficiency virus, Epstein-Barr virus, and cytomegalovirus lack molecules for homing to lymphoid sites of infection. *Blood* 2001; 98: 156–164. PMID: [11418475](https://pubmed.ncbi.nlm.nih.gov/11418475/)
39. Appay V, Almeida JR, Sauce D, Autran B, Papagno L. Accelerated immune senescence and HIV-1 infection. *Exp Gerontol* 2007; 42: 432–437. doi: [10.1016/j.exger.2006.12.003](https://doi.org/10.1016/j.exger.2006.12.003) PMID: [17307327](https://pubmed.ncbi.nlm.nih.gov/17307327/)
40. Papagno L, Spina CA, Marchant A, Salio M, Rufer N, Little S, Dong T, Chesney G, Waters A, Easterbrook P, Dunbar PR, Shepherd D, Cerundolo V, Emery V, Griffiths P, Conlon C, McMichael AJ, Richman DD, Rowland-Jones SL, Appay V. Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection. *PLoS Biol* 2004; 2: E20. doi: [10.1371/journal.pbio.0020020](https://doi.org/10.1371/journal.pbio.0020020) PMID: [14966528](https://pubmed.ncbi.nlm.nih.gov/14966528/)
41. Schurich A, Henson SM. The Many Unknowns Concerning the Bioenergetics of Exhaustion and Senescence during Chronic Viral Infection. *Front Immunol* 2014; 5: 468. doi: [10.3389/fimmu.2014.00468](https://doi.org/10.3389/fimmu.2014.00468) PMID: [25309548](https://pubmed.ncbi.nlm.nih.gov/25309548/)
42. Wherry EJ, Blattman JN, Murali-Krishna K, van der MR, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 2003; 77: 4911–4927. doi: [10.1128/JVI.77.8.4911-4927.2003](https://doi.org/10.1128/JVI.77.8.4911-4927.2003) PMID: [12663797](https://pubmed.ncbi.nlm.nih.gov/12663797/)
43. Kulpa DA, Lawani M, Cooper A, Peretz Y, Ahlers J, Sekaly RP. PD-1 coinhibitory signals: the link between pathogenesis and protection. *Semin Immunol* 2013; 25: 219–227. doi: [10.1016/j.smim.2013.02.002](https://doi.org/10.1016/j.smim.2013.02.002) PMID: [23548749](https://pubmed.ncbi.nlm.nih.gov/23548749/)
44. Maier H, Isogawa M, Freeman GJ, Chisari FV. PD-1:PD-L1 interactions contribute to the functional suppression of virus-specific CD8+ T lymphocytes in the liver. *J Immunol* 2007; 178: 2714–2720. PMID: [17312113](https://pubmed.ncbi.nlm.nih.gov/17312113/)
45. Larrubia JR, Ito-Martinez S, Miquel J, Calvino M, Sanz-de-Villalobos E, Gonzalez-Praetorius A, Albertos S, Garcia-Garzon S, Lokhande M, Parra-Cid T. Bim-mediated apoptosis and PD-1/PD-L1 pathway impair reactivity of PD1(+)/CD127(-) HCV-specific CD8(+) cells targeting the virus in chronic hepatitis C virus infection. *Cell Immunol* 2011; 269: 104–114. doi: [10.1016/j.cellimm.2011.03.011](https://doi.org/10.1016/j.cellimm.2011.03.011) PMID: [21481848](https://pubmed.ncbi.nlm.nih.gov/21481848/)
46. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006; 439: 682–687. doi: [10.1038/nature04444](https://doi.org/10.1038/nature04444) PMID: [16382236](https://pubmed.ncbi.nlm.nih.gov/16382236/)
47. Wang W, Lau R, Yu D, Zhu W, Korman A, Weber J. PD1 blockade reverses the suppression of melanoma antigen-specific CTL by CD4+ CD25(Hi) regulatory T cells. *Int Immunol* 2009; 21: 1065–1077. doi: [10.1093/intimm/dxp072](https://doi.org/10.1093/intimm/dxp072) PMID: [19651643](https://pubmed.ncbi.nlm.nih.gov/19651643/)
48. Wong M, La CA, Hahn BH. Blockade of programmed death-1 in young (New Zealand Black x New Zealand White)F1 mice promotes the suppressive capacity of CD4+ regulatory T cells protecting from

- lupus-like disease. *J Immunol* 2013; 190: 5402–5410. doi: [10.4049/jimmunol.1202382](https://doi.org/10.4049/jimmunol.1202382) PMID: [23636058](https://pubmed.ncbi.nlm.nih.gov/23636058/)
49. Sherman AC, Trehanpati N, Daucher M, Davey RT, Masur H, Sarin SK, Kottlilil S, Kohli A. Augmentation of hepatitis B virus-specific cellular immunity with programmed death receptor-1/programmed death receptor-L1 blockade in hepatitis B virus and HIV/hepatitis B virus coinfecting patients treated with adefovir. *AIDS Res Hum Retroviruses* 2013; 29: 665–672. doi: [10.1089/AID.2012.0320](https://doi.org/10.1089/AID.2012.0320) PMID: [23259453](https://pubmed.ncbi.nlm.nih.gov/23259453/)
 50. Angin M, Kwon DS, Streeck H, Wen F, King M, Rezai A, Law K, Hongo TC, Pyo A, Piechocka-Trocha A, Toth I, Pereyra F, Ghebremichael M, Rodig SJ, Milner DA Jr., Richter JM, Altfield M, Kaufmann DE, Walker BD, Addo MM. Preserved function of regulatory T cells in chronic HIV-1 infection despite decreased numbers in blood and tissue. *J Infect Dis* 2012; 205: 1495–1500. doi: [10.1093/infdis/jis236](https://doi.org/10.1093/infdis/jis236) PMID: [22427677](https://pubmed.ncbi.nlm.nih.gov/22427677/)
 51. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D, Mathian A, Nakahata T, Yamaguchi T, Nomura T, Ono M, Amoura Z, Gorochov G, Sakaguchi S. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* 2009; 30: 899–911. doi: [10.1016/j.immuni.2009.03.019](https://doi.org/10.1016/j.immuni.2009.03.019) PMID: [19464196](https://pubmed.ncbi.nlm.nih.gov/19464196/)
 52. Simonetta F, Lecuroux C, Girault I, Goujard C, Sinet M, Lambotte O, Venet A, Bourgeois C. Early and long-lasting alteration of effector CD45RA(-)Foxp3(high) regulatory T-cell homeostasis during HIV infection. *J Infect Dis* 2012; 205: 1510–1519. doi: [10.1093/infdis/jis235](https://doi.org/10.1093/infdis/jis235) PMID: [22457280](https://pubmed.ncbi.nlm.nih.gov/22457280/)
 53. Finney OC, Riley EM, Walther M. Phenotypic analysis of human peripheral blood regulatory T cells (CD4+FOXP3+CD127lo/-) ex vivo and after in vitro restimulation with malaria antigens. *Eur J Immunol* 2010; 40: 47–60. doi: [10.1002/eji.200939708](https://doi.org/10.1002/eji.200939708) PMID: [19877016](https://pubmed.ncbi.nlm.nih.gov/19877016/)
 54. Zhang X, Chang L, X, Xiao X, Sun R, Tian Z, Wei H. CD4(+)CD62L(+) central memory T cells can be converted to Foxp3(+) T cells. *PLoS One* 2013; 8: e77322. doi: [10.1371/journal.pone.0077322](https://doi.org/10.1371/journal.pone.0077322) PMID: [24155942](https://pubmed.ncbi.nlm.nih.gov/24155942/)
 55. Long SA, Rieck M, Tatum M, Bollyky PL, Wu RP, Muller I, Ho JC, Shilling HG, Buckner JH. Low-dose antigen promotes induction of FOXP3 in human CD4+ T cells. *J Immunol* 2011; 187: 3511–3520. doi: [10.4049/jimmunol.1003880](https://doi.org/10.4049/jimmunol.1003880) PMID: [21865550](https://pubmed.ncbi.nlm.nih.gov/21865550/)
 56. Nilsson J, Boasso A, Velilla PA, Zhang R, Vaccari M, Franchini G, Shearer GM, Andersson J, Chougnet C. HIV-1-driven regulatory T-cell accumulation in lymphoid tissues is associated with disease progression in HIV/AIDS. *Blood* 2006; 108: 3808–3817. doi: [10.1182/blood-2006-05-021576](https://doi.org/10.1182/blood-2006-05-021576) PMID: [16902147](https://pubmed.ncbi.nlm.nih.gov/16902147/)
 57. Penalzoza-MacMaster P, Kamphorst AO, Wieland A, Araki K, Iyer SS, West EE, O'Mara L, Yang S, Konieczny BT, Sharpe AH, Freeman GJ, Rudensky AY, Ahmed R. Interplay between regulatory T cells and PD-1 in modulating T cell exhaustion and viral control during chronic LCMV infection. *J Exp Med* 2014; 211: 1905–1918. doi: [10.1084/jem.20132577](https://doi.org/10.1084/jem.20132577) PMID: [25113973](https://pubmed.ncbi.nlm.nih.gov/25113973/)
 58. Brezar V, Ruffin N, Richert L, Surenaud M, Lacabartz C, Palucka K, Thiebaut R, Banchereau J, Levy Y, Seddiki N. Decreased HIV-specific T-regulatory responses are associated with effective DC-vaccine induced immunity. *PLoS Pathog* 2015; 11: e1004752. doi: [10.1371/journal.ppat.1004752](https://doi.org/10.1371/journal.ppat.1004752) PMID: [25816350](https://pubmed.ncbi.nlm.nih.gov/25816350/)
 59. Wang F, Wan L, Zhang C, Zheng X, Li J, Chen ZK. Tim-3-Galectin-9 pathway involves the suppression induced by CD4+CD25+ regulatory T cells. *Immunobiology* 2009; 214: 342–349. doi: [10.1016/j.imbio.2008.10.007](https://doi.org/10.1016/j.imbio.2008.10.007) PMID: [19362679](https://pubmed.ncbi.nlm.nih.gov/19362679/)
 60. Sakthivel P, Gereke M, Bruder D. Therapeutic intervention in cancer and chronic viral infections: antibody mediated manipulation of PD-1/PD-L1 interaction. *Rev Recent Clin Trials* 2012; 7: 10–23. PMID: [22023178](https://pubmed.ncbi.nlm.nih.gov/22023178/)
 61. Eichbaum Q. PD-1 signaling in HIV and chronic viral infection—potential for therapeutic intervention? *Curr Med Chem* 2011; 18: 3971–3980. PMID: [21824094](https://pubmed.ncbi.nlm.nih.gov/21824094/)
 62. Peligero C, Argilagué J, Guerri-Fernandez R, Torres B, Ligeró C, Colomer P, Plana M, Knobel H, Garcia F, Meyerhans A. PD-L1 Blockade Differentially Impacts Regulatory T Cells from HIV-Infected Individuals Depending on Plasma Viremia. *PLoS Pathog* 2015; 11: e1005270. doi: [10.1371/journal.ppat.1005270](https://doi.org/10.1371/journal.ppat.1005270) PMID: [26633181](https://pubmed.ncbi.nlm.nih.gov/26633181/)