PD-1 expression on human CD8 T cells depends on both state of differentiation and activation status

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Objective and design: PD-1 expression on HIV-specific CD8 T cells was recently reported to reflect functional exhaustion, resulting in uncontrolled HIV-1 replication. Assessing PD-1 expression on T cells may be highly relevant in T-cell immunology and vaccine monitoring. However, this requires us to gain further insights into the significance of PD-1 expression on CD8 T cells in humans.

Methods: We performed a detailed analysis of PD-1 expression pattern on various CD8 T cell subsets from healthy or HIV infected donors.

Results: PD-1 expression has two facets in vivo. On the one hand, it is linked to T-cell differentiation: PD-1 is up-regulated on early/intermediate differentiated subsets, which include HIV and Epstein–Barr virus-specific CD8 T-cell populations, but is down-regulated during late stages of differentiation. On the other hand, it is linked to T-cell activation: on PD-1 positive cells, PD-1 over-expression occurs along with the up-regulation of activation markers such as CD38 or HLA-DR.

Conclusions: PD-1 expression on CD8 T cells, including those specific for HIV, can be related both to their differentiation stage and their activation status. It is important to consider these findings when assessing the expression of PD-1 on T cells.

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Keywords: activation, CD8 T cells, differentiation, HIV, PD-1

Introduction

The balance between positive and negative signals delivered by costimulatory molecules to T cells is critical for the ultimate fate of cellular immune responses [1]. Programmed death 1 (PD-1 or CD279) is a member of the CD28 family, reported to be expressed on CD4 and CD8 T cells, NKT cells, B cells and monocytes upon activation [2–5]. The majority of the data points to negative regulation of T cell activation, proliferation and cytokines production by the ligands of PD-1 (PD-L1 and PD-L2) [6–8], although there is some evidence showing delivery of positive signals to T cells [9]. The PD-1/PD-L pathway is thought to play a significant role in the regulation of the immune response in both lymphoid and non-lymphoid organs [10].

Recent work has emphasised the inhibitory effect which the PD-1/PD-L pathway may have on virus-specific CD8 T cell responses. Data from the lymphocytic choriomeningitis virus infection model suggests that continued high levels of PD-1 expression on virus-specific CD8 T cells reflects a state of cellular exhaustion, which prevents optimal antiviral function in persistently infected mice [11]. Blockade of this pathway using anti-PD-L1 antibodies resulted in improved T-cell function (better proliferation, cytokine production and cytotoxic activity) and more efficient viral control (i.e. reduced viral load) [11]. This was followed by reports [12,13] in humans suggesting that the immunoregulatory PD-1/PD-L1 pathway had a role in the previously described functional impairments of HIV-specific T cells [14,15] and their incapacity to control HIV-1 replication. HIV-specific CD8

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T cells were found to express higher levels of PD-1 compared with cytomegalovirus (CMV)-specific CD8 T cells; and blocking PD-1 engagement to PD-L1 resulted in boosting functional capacities of HIV-specific CD8 T cells (cytokine production and proliferation in response to cognate peptides) in *in vitro* experiments. Most importantly, the expression level of PD-1 on both CD4 and CD8 T cells was positively correlated with patient viral load, leading to the conclusion that PD-1 high HIV-specific T cells were exhausted and unable to control viral replication, resulting in HIV disease progression.

The association between PD-1 expression on HIV-specific T cells, cellular exhaustion and disease progression may represent a major advance in our understanding of HIV pathogenesis and may have a major impact in the field of HIV research, in relation to diagnosis of HIV disease progression and development of immunotherapies. A good interpretation of PD-1 assessment therefore necessitates an in depth understanding of the detailed expression pattern of PD-1 on CD8 T cells. In order to provide such understanding, we assessed PD-1 expression in relation to cellular activation and differentiation, on distinct human CD8 T-cell subsets, including virus-specific cells. We report here that PD-1 expression on CD8 T cells is related both to their stage of differentiation and their activation status.

Materials and methods

Study subjects and samples

Blood samples were taken from HIV-infected patients attending the clinic, as well as from healthy donors, with known HLA type, and Epstein–Barr virus (EBV) and CMV serologies. HIV-infected patients included donors treated with antiretroviral agents or naive of treatment (viral loads ranging from 200 to 340 000 copies/ml). Mononuclear cells were isolated over a Lymphoprep gradient and then either directly stained or cryopreserved until use. These assays were approved by the Local Research Ethics Committee.

Reagents

Directly conjugated and unconjugated antibodies were obtained from the following vendors: BD Biosciences (San Jose, California, USA): CD8 (FITC), CD45RA (FITC), CCR7 (PE-Cy7), CD38 (APC), CD107a (Cy5-PE), IFNγ (Alexa700), and TNFα (PE-Cy7); Beckman Coulter, Marseilles, France: HLA-DR (ECD), CD45RA (ECD); Caltag (Burlingame, California, USA): CD8 (Alexa405); eBioscience (San Diego, California, USA): CD27 (APC-cyanin7); Dako (Glostrup, Denmark): CD3 (Cascade Yellow); R&D systems (Minneapolis, California, USA): CCR7 (FITC). PE conjugated PD-1 were produced as previously described [16]. HLA-A3 HIV Nef-QK10, HLA-A3 Gag-RY10, HLA-B7 Gag-GL9 and HLA-B27 Gag-KK10 pentamers were purchased from ProImmune (Abingdon, Oxfordshire, UK). Tetramers were produced as previously described [17] and included the following epitopes: HLA-A2 CMV pp65-NV9 and IE1-VL9; HLA-A2 EBV BMLF1-GL9, LMP2-CV9 and BMRF1-YV9; HLA-B7 CMV pp65-TM10 and pp65-RL11; HLA-B8 CMV IE1-EM9, IE1-EM10, IE1-QV9; HLA-B8 EBV BZLF1-RL8, EBNA-3A-FL9 and EBNA-3A-QL9; HLA-B35 CMV pp65-IY11; HLA-B35 EBV EBNA-1-YM9, EBNA-3A-HY11 and BZLF1-EY11.

Flow cytometry and polyfunctional assessment

Cell surface marker stainings were performed as previously described [18]. Briefly, titrated tetramers or pentamers (tricolor or PE conjugated) were added to 1×10^6 peripheral blood mononuclear cells (PBMC) or 200 μl whole blood, followed by addition of a panel of titrated antibodies and incubated for 15 min at room temperature. Red blood cells were then lysed using FACSlyse^{TM} buffer (BD Biosciences), lymphocytes washed and stored in Cell Fix^{TM} buffer (BD Biosciences) at 4°C until analysis. Stainings were analysed on a LSR2 flow cytometer (Becton Dickinson) or on an Epics flow cytometer (Beckman Coulter) with appropriate isotype controls and colour compensation. Data were analysed using FlowJo v8.2 (Tree Star, Inc., Schnelldorf, Germany) and DIVA softwares.

To assess functional capacity, 1×10^6 pentamer/tetramer prestained PBMC were incubated in the presence of specific peptide (5 μM) and anti-CD107a antibodies (10 μl) for 1h at 37°C in a 5% CO_2 incubator, followed by an additional 5 h in the presence of the secretion inhibitors monensin (2.5 μg/ml; Sigma-Aldrich, Ashland, Oregon, USA) and Brefeldin A (5 μg/mL; Sigma-Aldrich). Negative controls were obtained in absence of peptide and prestaining with pentamer/tetramer. Cytofix/Cytoperm (BD Biosciences) was used for permeabilisation of the cells prior to staining for intracellular cytokines. Percentage frequencies of multifunctional cells were calculated within the total population of detectable antigen-specific CD8 T cells.

Statistical analysis

Statistical analysis was performed using GraphPad prism software. Data were compared using the Mann–Whitney U test or Student's t test. P values above 0.05 were considered not significant.

Results

CD8 T-cell differentiation and PD-1 expression

The CD8 T-cell population can be divided into several subsets based on the surface expression of CD45RA, CCR7 and CD27, positioned along a linear pathway of postthymic differentiation in humans: CD45RA+/CCR7+/CD27+ (naive) → CD45RA−/CCR7+/CD27+ → CD45RA−/CCR7−/CD27+ → CD45

RA−/CCR7−/CD27− → CD45RA+/CCR7−/CD27− (terminally differentiated) [19,20]. Since the expression of a range of receptors involved in the regulation of T-cell activation (e.g., CD28, 4-1BB) is known to be differentially expressed according to the stage of T-cell differentiation, we wondered if a similar phenomenon may occur in the case of PD-1. PD-1 expression (as percentage of PD-1 positive cells) was therefore assessed on these subsets by flow cytometry (Fig. 1a). We observed significant differences in PD-1 expression between the distinct CD8 T-cell subsets along the pathway of CD8 T-cell differentiation (Fig. 1b and c). While naive CD8 T cells exhibit no or little PD-1, it is up-regulated after antigenic priming, and expressed particularly on CD8 T cells at an early/intermediate stage of differentiation (i.e., CCR7−/CD27+). However, its expression decreases as cells reach late stages of differentiation. This pattern of expression was observed both in healthy and HIV-infected donors and was independent of antiretroviral therapy. This suggests a direct link between the level of differentiation and the expression of PD-1 on CD8 T cells.

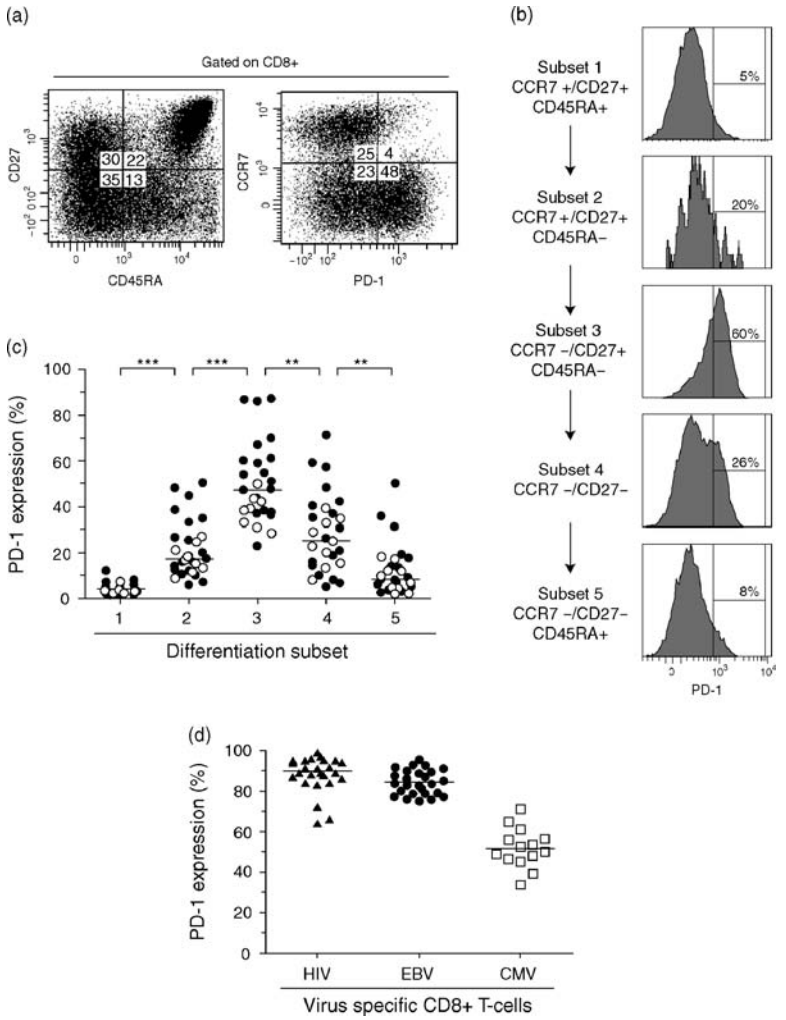

Fig. 1. PD-1 expression on CD8 T-cell differentiation subsets. (a) Representative FACS profile of CD45RA, CCR7 and CD27 on CD8 T cells from one donor to assess PD-1 expression on CD8 T-cell differentiation subsets (defined according to CD45RA, CCR7 and CD27 expression). Percentages of cells in the different quadrants are shown. (b) Representative histograms showing PD-1 expression on different CD8 T-cell differentiation subsets. The arrow depicts the differentiation pathway of CD8 T cells based on the phenotypic markers (subset 1 corresponds to naive cells, subset 5 to highly differentiated cells). Percentages of PD-1 positive cells within each subset are indicated. (c) PD-1 expression on differentiation subsets from 29 donors (10 healthy, ○; 19 HIV infected, ●). The horizontal bars indicate the median. $^{**}P < 0003$ and $^{***}P < 0\,0001$ (Mann–Whitney test). (d) PD-1 expression on HIV, EBV or CMV-specific CD8 T cells. Percentages of PD-1 positive cells are shown within virus-specific CD8 T cells from healthy or HIV infected donors. Virus-specific CD8 T cells were identified in PBMC using peptide/MHC class I complex, covering a range of HIV (n = 4), CMV (n = 8) or EBV (n = 9) epitopes restricted through HLA-A2, A3, B7, B8, B27 and B35 molecules (eiptopes are described in the method section). The horizontal bars indicate the median.

This expression pattern is important for the assessment of PD-1 on virus-specific CD8 T cells, as these cells are known to accumulate at certain stages of differentiation according to their specificity. For instance, HIV or EBV-specific CD8 T cells are usually found in the CCR7−/CD27+ subset of differentiation (mostly PD-1 positive), in contrast, CMV-specific CD8 T cells are usually highly differentiated (mostly PD-1 negative) [19–21]. In keeping with recently published work [12,22], we found that the vast majority of HIV or EBV-specific CD8 T cells were indeed PD-1 positive, in contrast with CMV-specific populations (Fig. 1d). This disparity is very likely to be related to the state of differentiation of the distinct populations of virus-specific CD8 T cells.

CD8 T-cell activation and PD-1 expression

Similar to T-cell differentiation, up-regulation or down-regulation of a variety of receptors can occur as T cells become activated. An up-regulation of PD-1 on activated T cells was previously suggested [5], and is also observed in the context of human infections, for instance during the acute phase of infectious mononucleosis (Sauce *et al.*, unpublished data) and of hepatitis C infection [23]. We next wanted to investigate the potential relationship between PD-1 expression and CD8 T-cell activation, which is particularly significant in HIV infection. We therefore looked at PD-1 in relation to the expression of CD38 and HLA-DR in both healthy and HIV-infected donors. These cell surface molecules are known to be up-regulated *in vivo* on CD8 T cells upon antigenic activation and are commonly used as markers of T-cell activation. We observed that activated CD8 T cells (i.e., $CD38^{high}$ or $HLA\text{-}DR^{high}$) express higher levels of PD-1, compared to resting cells (Fig. 2a). This was also evident looking only at antigen-experienced cells, by excluding the naive cell population (CD45RA+/CCR7+/CD27+ subset) from the analysis (Fig. 2b). In order to refine the analysis and avoid a possible bias due to the differential PD-1 expression among distinct subsets of differentiation, we concentrated our analysis exclusively on PD-1 positive cells (which include the HIV-specific CD8 T cells). PD-1 up-regulation was assessed by measuring its mean fluorescence intensity (MFI). The MFI provides a mean to assess differential expression levels of a receptor within a population that expresses this receptor. Within the PD-1 positive cell population, a consistent up-regulation of PD-1 was observed on the cell surface of $CD38^{high}$ or $HLA\text{-}DR^{high}$ CD8 T cells (Fig. 2c). These data therefore show that the activation status does influence PD-1 expression on CD8 T cells, and that antigen-experienced cells, already PD-1 positive, can further up-regulate PD-1 as they are activated. In order to verify that the association between PD-1 expression and T-cell differentiation was not related to prevalent activated profiles in differentiated subsets (in particular the CD27+/CCR7− subset, which exhibits the strongest expression PD-1), we analysed the expression of CD38 or HLA-DR on the same five previously studied subsets. We did not find an analogous association between activation status to T-cell differentiation like the one observed for PD-1 expression and T-cell differentiation (data not shown). Overall, the expression of PD-1 on CD8 T cells presents two distinct facets: it is related first to the differentiation stage (negative versus positive expression) and also to the activation status (further up-regulation) of the CD8 T cells.

PD-1 expression on CD8 T cells in HIV infection

In HIV infection, viral replication can induce a significant activation of CD8 T cells; up-regulation of CD38 on HIV-specific CD8 T cells has been commonly observed in the presence of high viral load or viral rebound [24–26]. In order to see if an increase in viral replication has a direct effect on PD-1 expression in relation to activation, we assessed CD38 and PD-1 expression on CD8 T cells from antiretroviral-treated HIV-infected patients who had a treatment interruption resulting in viral load rise. A concomitant up-regulation of CD38 and PD-1 on antigen-experienced CD8 T cells was observed as the HIV infected donors experienced a viral rebound (Fig. 3).

Taking into account the two facets of PD-1 expression on CD8 T cells, HIV-specific CD8 T cells express PD-1 (due to their differentiated phenotype), which may be further up-regulated with antigenic activation of the cells due to viral replication. We provide examples of HIV tetramer gated CD8 T cells from two HIV infected individuals with either a low or a high viral load during the chronic phase of their infection. In keeping with recent studies, we observed a higher expression of PD-1 (in MFI) in the patient with high viral load, compared to the patient with low viral load. However, this occurred together with increased levels of CD38 expression on these cells (Fig. 4a). This observation was not HIV epitope dependent as other HIV-specific CD8 T-cell populations detected in these donors behaved similarly (Fig. 4b).

Since it has been suggested that the functional capacity of CD8 T cells is directly related to their expression of PD-1, functional properties of a series of HIV-specific CD8 T cells from HIV progressors or non-progressors was also assessed. For this purpose, we analysed their capacity to produce cytokines [such as interferon (IFN)γ and tumour necrosis factor (TNF)α], or to upregulate CD107a (as a marker of degranulation) upon stimulation with a cognate peptide. Despite clear PD-1 expression, some HIV-specific populations could show strong immediate functional capacity as exemplified in Fig. 5a. Overall, we observed some variability in terms of cytokine secretion and/or degranulation capacities between populations, but there was no direct association between the functional capacity assessed here and PD-1 expression, either considering a single function such as IFNγ production (Fig. 5b), or the polyfunctional capacity (i.e., three functions simultaneously) (Fig. 5c).

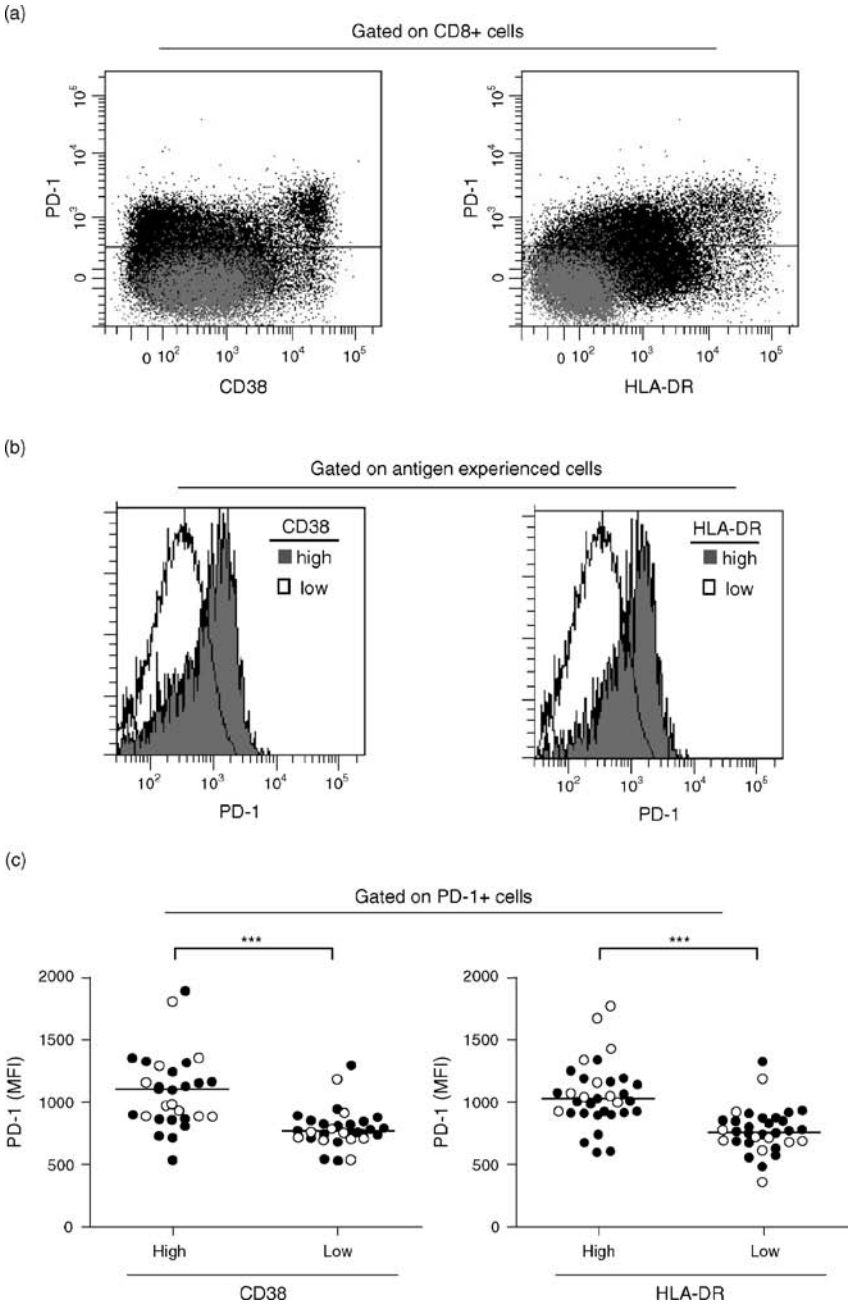

Fig. 2. PD-1 over-expression in relation to CD8 T-cell activation. (a) Representative staining for PD-1 together with the activation markers CD38 or HLA-DR on whole CD8 T cells. PD-1 negative naive CD8 T cells (CD45RA+/CCR7+/CD27+) are highlighted in grey. (b) Representative example for PD-1 expression on antigen-experienced CD8 T cells (i.e., excluding CD45RA/CCR7/CD27 cells) either activated ($CD38^{high}$ or $HLA\text{-}DR^{high}$) or resting. (c) PD-1 staining intensity within the PD-1 positive cell population according to CD38 or HLA-DR expression. The PD-1 MFI is shown on PD-1 positive gated cells, comparing activated versus resting cells from 29 donors (10 healthy, ○; 19 HIV infected, ●). The horizontal bars indicate the median. $^{***}P < 0.0001$ with the paired t test.

Discussion

The clear association between CD8 T-cell differentiation and PD-1 expression suggests that the role played by the PD-1/PD-L pathway in regulating T-cell activation can vary according to the stage of differentiation. Importantly it may the reason for the differences in PD-1 expression observed between CD8 T cells specific for different viruses. Virus-specific CD8 T-cell populations may express different levels of PD-1 according to their differentiation stage, and this may be independent of their capacity to control their respective virus. In addition, our data show that CD8 T cells can further up-regulate PD-1 when they are activated (e.g. express activation markers

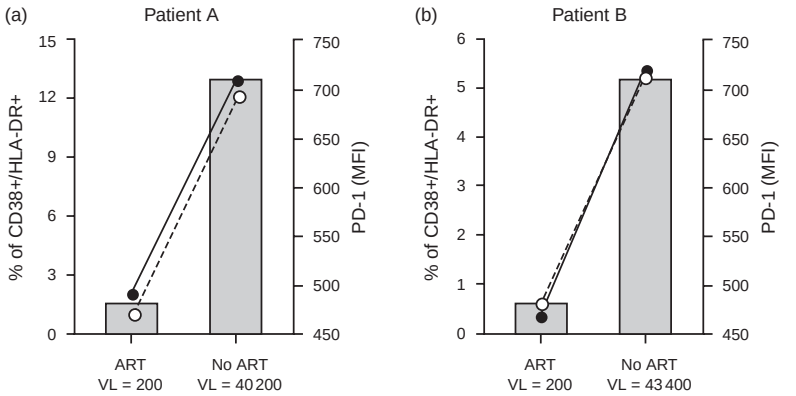

Fig. 3. PD-1 and CD38 expression with treatment interruption. Percentages of activated cells (i.e. $CD38^{high}$/$HLADR^{high}$ cells, open symbols) and PD-1 expression (MFI, closed symbols) were assessed longitudinally on antigen-experienced CD8 T cells from HIV infected patients who displayed an increase in viral load (VL, copies/ml, grey bars) after stopping antiretroviral treatment. Two patients with antiretroviral treatment interruption are shown. The 'no ART' time point is 4 weeks after treatment interruption.

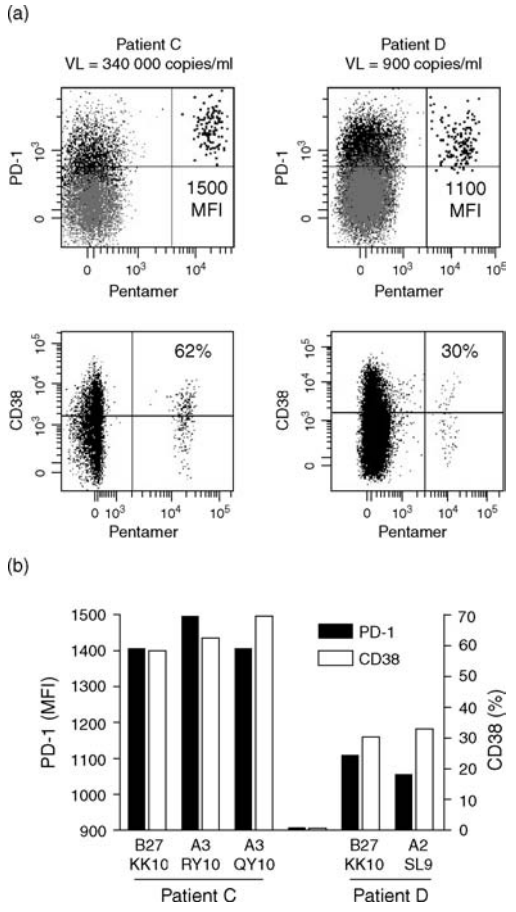

Fig. 4. PD-1 and CD38 expression on HIV-specific CD8 T cells. (a) Representative staining of PD-1 and CD38 expression on HIV-specific CD8 T cells. Results are showm for a patient with high viral load (cells specific for the HLA-A3 restricted RY10 epitope) and from a patient with low viral load (cells specific for the HLA-B27 restricted KK10 epitope) (both patients are antiretroviral treatment naive).

like CD38 or HLA-DR). In HIV infection, the persistence of viral replication can sustain a constant activation of the responder cells. The analysis of CD38 is particularly relevant in the context of HIV infection as a clear correlation has been demonstrated between the level of CD38 expression on CD8 T cells and HIV viral load [27–30]. This is important because the correlation observed between PD-1 expression on CD8 T cells and HIV viral load [12,13] may be related to the correlation between CD38 expression on CD8 T cells and HIV viral load, due to the relationship between PD-1 up-regulation and CD8 T-cell activation. These findings need to be taken into account when interpreting the expression of PD-1 on CD8 T cells. While on the one hand, high PD-1 expression on HIV-specific CD8 T cells may reflect cellular exhaustion and result in uncontrolled high viral load, on the other hand, it is also possible that HIV-specific CD8 T cells express PD-1 as a result of their differentiation, and that PD-1 is up-regulated further due to T-cell activation in the presence of high viral load.

In the latter case, PD-1 expression may then be independent from T-cell exhaustion. Of note, we found no association between PD-1 expression and direct functional capacity (i.e., secretion of cytokines and degranulation). Instead, PD-1 expressing HIV-specific CD8 T cells displayed a clearly multifunctional profile. This data is in agreement with the findings by Petrovas *et al.*, who reported robust cytokine secretion by PD-1 positive cells in both humans and primates [22,31], and indicates that PD-1 expression does not necessary mean

Fig. 4. (*Continued*).
PD-1 MFI and the percentage of CD38 positive for the HIV-specific CD8 T cells are indicated. (b) PD-1 (MFI) and CD38 (%) expression on other HIV-specific CD8 T-cell populations (different epitope and/or HLA restriction) detected in the same two patients.

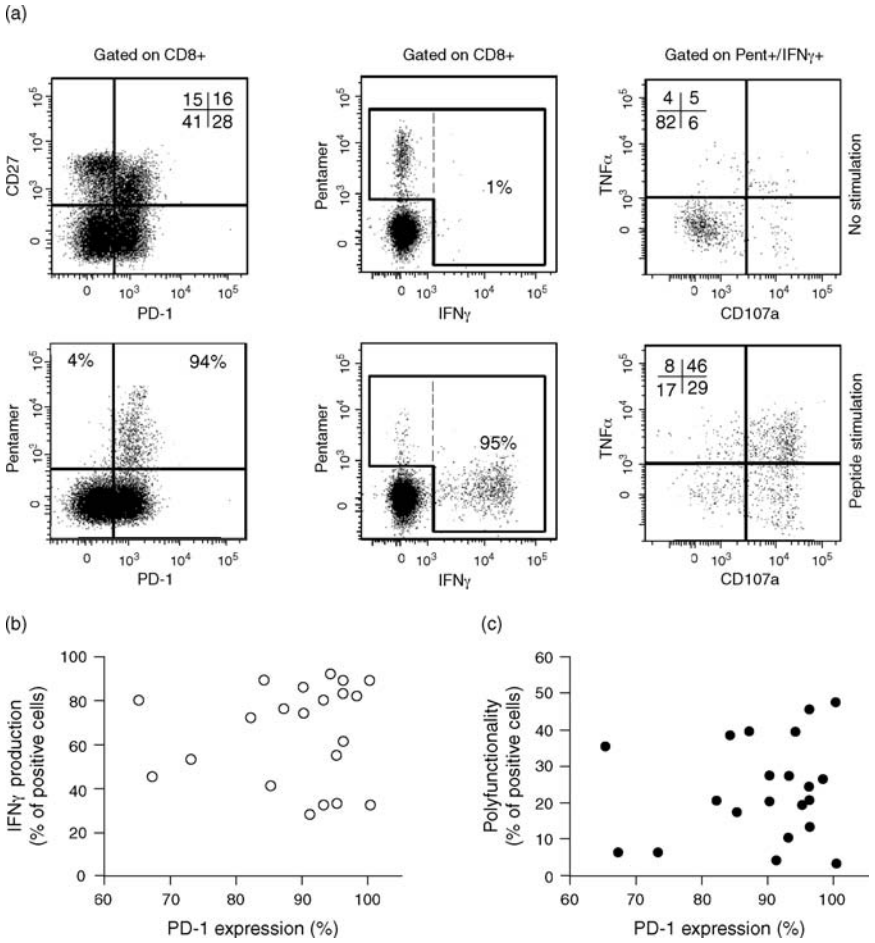

Fig. 5. PD-1 expression and HIV-specific CD8 T-cell polyfunctionality. (a) Representative PD-1 staining (left panel) and functional profile (middle and right panels) for the same HIV-specific CD8 T-cells population (specific for the HLA-B27 restricted KK10 epitope). For functional assessment, cells were stimulated for 6 h in the presence of cognate peptide prior to intracellular staining for the cytokines IFNγ and TNFα as well as the degranulation marker CD107a. Percentages of cells in the different quadrants or IFNγ positive are indicated. (b,c) PD-1 expression (i.e., percentage of PD-1 positive cells within tetramer positive populations) is plotted as a function of (b) IFNγ production capacity (i.e. background substracted percentage of IFNγ positive cells within antigen-specific populations upon stimulation) or (c) polyfunctional capacity (i.e., background substracted percentage of IFNγ/TNFα/CD107a triple-positive cells within antigen-specific populations upon stimulation). A series of HIV-specific CD8 T cells (n = 21, including responses to HLA-A3 Nef-QK10 or Gag-RY10, HLA-B7 Gag-GL9 and HLA-B27 Gag-KK10) from treatment naive HIV infected donors (n = 15) was analysed for direct functional capacity.

dysfunction or exhaustion. The literature has portrayed PD-1 as an inhibitory receptor with an important role in regulating the immune response. Depending on their activation status and their differentiation level, T cells are likely to switch on self regulating mechanisms. As an element of such regulatory systems, PD-1 may be naturally expressed on cells that require regulation of their activity. For instance, it was shown that PD-1 inhibits adverse immune responses to prevent hyperresponsiveness (including autoimmunity) [32,33]. The upregulation of PD-1 on activated CD8 T cells may be part of the homeostatic mechanism to control proliferation and apoptosis (as shown by Petrovas *et al.* [22,31]), in order to avoid over growth and over reactivity. PD-1 expression seems strongest on early/intermediate differentiated CD8 T cells (CD27+/CCR7−), subsets that include a majority of antigen-experienced committed cells, of which tight regulation may be essential. In contrast, 'very early differentiated/central memory' cells (CD27+/CCR7+/CD45RA−) may only express low PD-1 levels as suppressing the growth or activation of such cells, often described as 'memory progenitors', may not be advantageous. Regarding highly differentiated CD8 T cells (CD27−/CCR7−/CD45RA+) (which express also lower PD-1 levels), recent data from our group show that modulation of several factors (i.e., significant down-regulation of gene expression) that influence proliferation potency (e.g., fosB, junB and RASA1) or apoptosis (e.g., Bcl-2, IPLA2 and TNFR1) occurs in this subset, (manuscript submitted) indicating that the regulation of this subset activity can at least occur at a different level (independently from PD-1).

Due to PD-1 regulatory properties, blockage of the PD-1/PD-L pathway at the cellular level should indeed result in the increased capacity of virus-specific T cells to respond to antigenic stimulation, as observed [12,13]. This may not be dissimilar to the effect of blocking CTLA-4, (another inhibitory receptor that shares 23% amino acid sequence homology with PD-1), which leads to the enhancement of T-cell reactivity and function [34,35]. In the same line, stimulating CD28 (using anti-CD28 antibodies), a co-stimulatory receptor from the same family as PD-1 enhances the responsiveness of T cells to activation. This is often used in *in vitro* assays to increase the sensitivity of the assays, with no relation to cellular exhaustion [36,37].

To conclude, we have shown that the expression of the inhibitory receptor PD-1 on CD8 T cells can be related both to their differentiation stage and their activation status. PD-1 is expressed by subsets of antigen-experienced CD8 T cells, and predominantly on early/intermediate differentiated populations (including the majority of HIV and EBV-specific CD8 T cells), and can be further up-regulated as these cells become activated (e.g., in the presence of high viral load). This suggests a differential regulation of CD8 T-cell proliferation and apoptosis via the PD-1/PD-L pathway according to the status of the cells. The significance of PD-1 expression on CD8 T cells needs to be interpreted in light of these findings.

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