HIV-1 Tat affects the programming and functionality of human CD8$^+$ T cells by modulating the expression of T-box transcription factors

Fabio Sforza[a,*], Francesco Nicoli[a,*], Eleonora Gallerani[a], Valentina Finessi[a], Eva Reali[b], Aurelio Cafaro[c], Antonella Caputo[d], Barbara Ensoli[c] and Riccardo Gavioli[a]

Objective: HIV infection is characterized by several immune dysfunctions of both CD8$^+$ and CD4$^+$ T cells as hyperactivation, impairment of functionality and expansion of memory T cells. CD8$^+$ T-cell dysfunctions have been associated with increased expression of T-bet, Eomesdermin and pro-inflammatory cytokines, and with down-regulation of CD127. The HIV-1 Tat protein, which is released by infected cells and detected in tissues of HIV-positive individuals, is known to contribute to the dysregulation of CD4$^+$ T cells; however, its effects on CD8$^+$ T cells have not been investigated. Thus, in this study, we sought to address whether Tat may affect CD8$^+$ T-cell functionality and programming.

Methods: CD8$^+$ T cells were activated by TCR engagement in the presence or absence of Tat. Cytokine production, killing capacity, surface phenotype and expression of transcription factors important for T-cell programming were evaluated.

Results: *Tat* favors the secretion of interleukin-2, interferon-γ and granzyme B in CD8$^+$ T cells. Behind this functional modulation we observed that Tat increases the expression of T-bet, Eomesdermin, Blimp-1, Bcl-6 and Bcl-2 in activated but not in unstimulated CD8$^+$ T lymphocytes. This effect is associated with the down-regulation of CD127 and the up-regulation of CD27.

Conclusion: Tat deeply alters the programming and functionality of CD8$^+$ T lymphocytes.

AIDS 2014, **28**:000–000

Keywords: CD8$^+$, Eomes, HIV, immune activation, T-cell programming, Tat, T-bet

Introduction

HIV is one of the major plagues in the world for the number of people infected and deaths per year [1]. The most devastating damages caused by HIV infection are observed at the level of cellular immunity, and include the depletion of CD4$^+$ T cells and important dysfunctions of both CD8$^+$ and CD4$^+$ T cells as impairment of functionality [2,3], exhaustion [4], increased T-cell proliferation [5], susceptibility to apoptosis [6,7] and expansion of memory T cells [8–10]. This status of chronic immune activation and immune senescence involves the whole T-cell compartment, including uninfected and non-HIV-specific T cells [11], is also present during antiretroviral therapy (ART) and contributes to the appearance of AIDS-defining and

[a]Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, [b]Laboratory of Translational Immunology, I.R.C.C.S. Istituto Ortopedico Galeazzi, Milan, [c]National AIDS Center, Istituto Superiore di Sanità, Rome, and [d]Department of Molecular Medicine, University of Padova, Padova, Italy.

Correspondence to Professor Riccardo Gavioli, PhD, Department of Life Sciences and Biotechnology, University of Ferrara, via Fossato di Mortara 74, 44121 Ferrara, Italy.

E-mail: r.gavioli@unife.it

* Dr Fabio Sforza and DrFrancesco Nicoli contributed equally to the writing of this article.

Received: 29 January 2014; revised: 20 April 2014; accepted: 22 April 2014.

DOI:10.1097/QAD.0000000000000315

ISSN 0269-9370 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins

nondefining diseases [12]. Different mechanisms contribute to these phenomena, including $CD4^+$ T-cell loss, viral replication and effects of HIV proteins such as gp120, Nef and Tat [5,11].

Several studies have reported that the HIV-1 Tat protein activates $CD4^+$ T cells and increases pro-inflammatory cytokine production in both HIV-infected and uninfected cells [13–15]. In fact, Tat, in addition to be required for viral replication and infectivity [16,17], is released extracellularly [18], even during ART [19], and enters neighboring cells, affecting their functionality [20–24]. Moreover, it has been demonstrated that anti-Tat immunity is important for disease control and restoration of immune functions [25,26], suggesting that Tat may contribute to immune activation. In accordance with this hypothesis, we have recently shown in murine models that Tat favors the activation and the expansion of antigen-specific CTLs and modulates antiviral responses causing dysfunctions similar to those observed in HIV-infected individuals [22,27].

Dysfunctionality of T lymphocytes in HIV-positive patients has been linked to the deep modification of their transcriptional profile [28,29]. In particular, it has been shown that $CD8^+$ T cells from HIV-positive patients display an effector phenotype and a simultaneous increased expression of two T-box transcription factors, T-bet and Eomesdermin (Eomes), which correlate with viral load and decrease after ART [29]. T-bet and Eomes are the master regulators of effector and memory functions in $CD8^+$ T cells [30]. Indeed, it has been shown that, although both transcription factors are important for the generation of different memory T-cell subsets, higher T-bet expression favors a short-lived effector phenotype, whereas higher Eomes expression is important for development of central memory T cells [31,32]. Moreover, T-bet and Eomes expression is modulated by Bcl-6 and Blimp-1 [33]. Bcl-6 plays a central role in survival and proliferation of T cells, whereas Blimp-1 is required for the development of CTLs and terminal effector cells [33–35]. These transcription factors are regulated by or regulate interferon (IFN)-γ and interleukin (IL)-2 signaling [34–36]. As we have shown in murine models that Tat enhances TCR stimulation favoring IFN-γ production [27], and several works describe that Tat increases IL-2 release in $CD4^+$ T cells [13,15], we sought to determine whether Tat may favor the activation of human $CD8^+$ T cells affecting the expression of these transcription factors. We demonstrate here for the first time that $CD8^+$ T cells activated in the presence of Tat enhance their effector functions and display an increased expression of those transcription factors important for T-cell programming.

Thus, this study provides evidence that Tat modulates the functionality and the fate of human $CD8^+$ T lymphocytes, suggesting that this viral protein contributes to T-cell dysfunctions during HIV infection.

Materials and methods

Human cells and culture conditions

Buffy coats from healthy volunteers who provided consent were obtained from the University Hospital of Ferrara. Peripheral blood lymphocytes (PBLs) were separated by use of Ficoll–Hypaque (Lonza, Basel, Switzerland) density gradient centrifugation followed by 90 min of adhesion on a plastic support at 37°C to remove monocytes.

Peripheral blood lymphocytes (3×10^6) were cultured in 2 ml of RPMI (Gibco, Life Technologies, Carlsbad, California, USA) containing 10% FCS (complete medium) in the absence or presence of the Tat protein in 24-well flat bottomed polystyrene plates coated overnight at 4°C with PBS or anti-CD3 mAb (0.5 μg/ml; R&D Systems, Minneapolis, Minnesota, USA). Soluble anti-CD28 mAb (0.1 μg/ml; R&D Systems), Tat and anti-Tat immune sera were added, when indicated, after cell seeding. In the blocking experiments with anti-integrin antibodies, cells were preincubated with 10 μg/ml of anti-$\alpha_5\beta_1$ and anti-$\alpha_v\beta_3$ antibodies (Merck Millipore, Billerica, Massachusetts, USA) on rotation in RPMI + 0.05% BSA for 1 h at room temperature.

$CD8^+$ T cells were sorted by MACS magnetic negative selection (Miltenyi Biotec, Bergish Gladbach, Germany) according to manufacturer's instructions (purity >95% assessed by FACS).

Tat protein

HIV-1 Tat from the human T-lymphotropic virus type IIIB isolate (BH10 clone) was expressed in *Escherichia coli* and purified by heparin-affinity chromatography and HPLC, as described previously [20]. The lyophilized Tat protein was stored at −80°C to prevent oxidation, reconstituted in degassed buffer before use, and handled, as described [20]. Endotoxin concentration was undetectable (detection threshold: 0.05 EU/μg).

Generation of CTL cultures

EBV-specific and survivin-specific CTL cultures were obtained by stimulation of PBLs with peptide-pulsed T2 cells [37], in the absence or presence of Tat (see supplementary materials and methods, http://links.lww.com/QAD/A525).

Cytotoxicity and Elispot assays

The cytotoxic activity of CTL cultures was assayed against peptide-pulsed target cells in standard 5-h ^{51}Cr-release assays [38]. For Elispot assays, CTLs were seeded on 96-well Elispot plates precoated with an anti-IFN-γ or

antigranzyme B mAb and stimulated with EBV-derived CD8$^+$ peptides (see supplementary materials and methods, http://links.lww.com/QAD/A525).

Reverse transcription and quantitative real-time PCR

DNase-treated total RNA was isolated from cells and cDNA was PCR-amplified. For each RNA, the relative levels were calculated by the $2^{-\Delta\Delta CT}$ method using human 18S as housekeeping gene (see supplementary materials and methods, http://links.lww.com/QAD/A525).

Results

Tat enhances interleukin-2 and interferon-γ production in CD8$^+$ T cells

Tat is released by infected cells and is detected in the tissues and in the sera of HIV-infected individuals at concentrations within the nanomolar range [18,39–41]. As some reports showed that Tat enhances the release of IL-2 and pro-inflammatory cytokines from activated PBLs [13,42], we first sought to determine whether the amounts of secreted Tat usually found *in vivo* may account for this effect. To this aim, PBLs from healthy donors were activated with anti-CD3/CD28 in the absence or presence of different doses of Tat (from 0.001 to 1 μg/ml), and IL-2 mRNA levels were measured after 4 h by quantitative PCR (qPCR). As shown in Fig. 1a, a 75-fold increase of IL-2 mRNA was observed in PBLs activated in the absence of Tat compared to untreated PBLs, whereas the presence of Tat induced a 150–200-fold increase of IL-2 mRNA expression. This effect was observed at similar levels for all Tat doses except at 0.001 μg/ml, and it was abolished after incubation with anti-Tat-positive sera (Fig. S1, http://links.lww.com/QAD/A525). Similar results were obtained at 24 h after activation (Fig. 1b), demonstrating that this effect is long-lasting. As the highest fold increase was observed at 0.1 μg/ml of Tat, this dose was chosen to perform the subsequent experiments.

It is already known that Tat favors the release of IL-2 from CD4$^+$ T cells [15]; however, the effect of Tat on CD8$^+$ T lymphocytes has never been investigated. Thus, we evaluated whether Tat affects the expression of IL-2 mRNA in CD8$^+$ T cells purified from unstimulated or activated PBLs cultured in the absence or presence of Tat. Interestingly, Tat significantly increased the expression of IL-2 mRNA in CD8$^+$ T cells purified from activated PBLs (Fig. 1c), and this effect was further confirmed by intracellular cytokine staining (Fig. S2, http://links.lww.com/QAD/A525).

We next examined whether the presence of Tat could also modulate IFN-γ production. Consistent to what was observed for IL-2 production, Tat dramatically enhanced IFN-γ mRNA expression in CD8$^+$ T cells purified from activated PBLs (Fig. 1d). Of note, Tat did not induce IL-2 or IFN-γ production in CD8$^+$ T cells purified from unstimulated PBLs (Fig. 1c and d).

These results demonstrate that physiological concentrations of Tat enhance the production of IL-2 and IFN-γ in CD8$^+$ T cells activated with anti-CD3/CD28.

Tat affects the expression of T-bet, Eomesdermin and other key transcription factors in activated CD8$^+$ T cells

T-bet and Eomes are transcription factors that are up-regulated during HIV infection [29] and that control IFN-γ production [30,43]. Since we have shown here that Tat enhances IFN-γ production in human CD8$^+$ T cells stimulated by TCR engagement, we next characterized the expression of T-bet and Eomes in CD8$^+$ T cells purified from unstimulated or activated PBLs cultured in the absence or presence of Tat. Moreover, the expression of other transcription factors important for T-cell functionality, survival and programming, such as Blimp-1, Bcl-6 and Bcl-2, was analyzed.

As shown in Fig. 2, mRNA levels of all five transcription factors measured were significantly increased in CD8$^+$ T cells purified from PBLs activated in the presence of Tat. Notably, Tat up-regulated not only genes required for effector functions (as T-bet, Eomes and Blimp-1), but also transcription factors important for memory development (Bcl-6 and Eomes) and T-cell survival (Bcl-2). Tat did not significantly increase transcription factor expression in CD8$^+$ T cells purified from unstimulated PBLs, although the results obtained show a tendency of a Tat-mediated enhancement of the two memory-related transcription factors Eomes and Bcl-6.

To assess whether the increased mRNA levels resulted in increased protein expression, T-bet and Eomes proteins were evaluated by western blotting in CD8$^+$ T cells at 24 and 48 h after activation. As shown in Fig. 3a, CD8$^+$ T cells purified from PBLs activated in the presence of Tat exhibited an increase of T-bet and Eomes expression 48 h after the activation.

Extracellular Tat is known to activate CD4$^+$ T cells by binding with its RGD region the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins [44]. To understand whether the enhancement of transcription factor expression induced by Tat was integrin-mediated, PBLs were preincubated with Abs directed against $\alpha_v\beta_3$ and $\alpha_5\beta_1$ and subsequently activated with anti-CD3/CD28 in the absence or presence of Tat. As shown in Fig. 3b, transcription factor expression was not up-regulated by Tat in CD8$^+$ T cells purified from PBLs activated in the presence of anti-integrin Abs, suggesting that the binding of Tat with αvβ3 and α5β1 may be required for the enhancement of transcription factor expression. However, we have to point out that

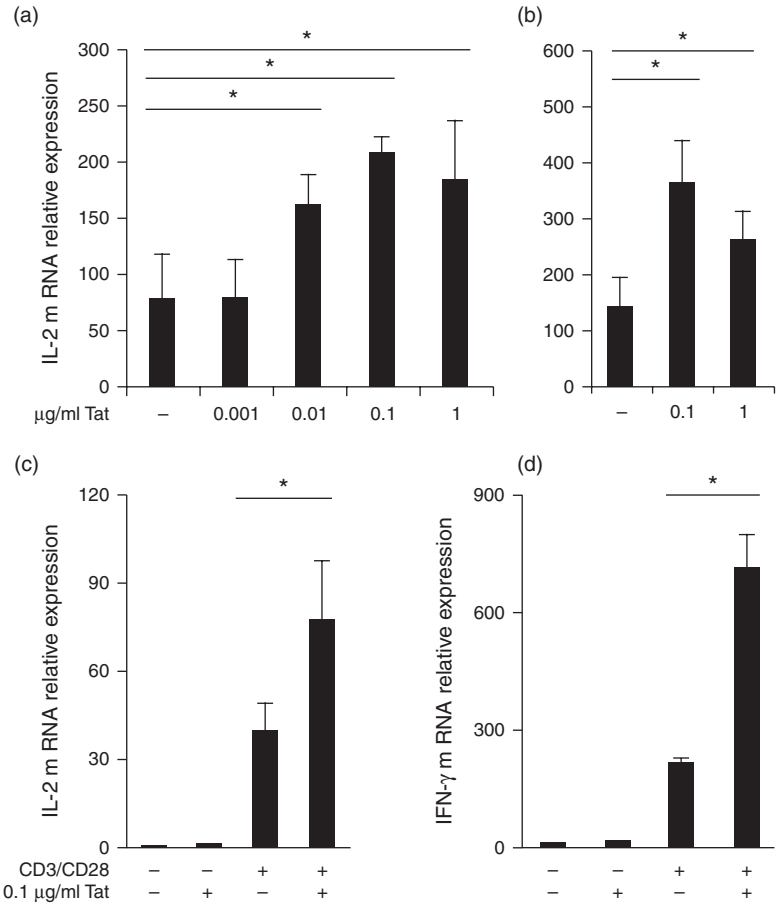

Fig. 1. Tat enhances interleukin-2 and interferon-γ production. (a, b) PBLs from healthy donors ($n = 6$) activated with anti-CD3/CD28 were cultured in the absence or presence of the indicated concentrations of Tat for (a) 4 h or (b) 24 h and IL-2 mRNA levels were quantified by qPCR and normalized to untreated cells. (c, d) PBLs from healthy donors ($n = 6$) unstimulated or activated with anti-CD3/CD28 were cultured in the absence or presence of Tat (0.1 µg/ml). After 4 h, $CD8^+$ T cells were purified and (c) IL-2 and (d) IFN-γ mRNA levels were quantified by qPCR and normalized to untreated cells. Data are presented as mean ± SEM. For statistical analysis, two-tailed Wilcoxon signed-rank test was used. $^*P < 0.05$: Tat-treated cells compared to Tat-untreated control cells. IFN-γ, interferon-γ; IL, interleukin; PBL, peripheral blood lymphocyte; qPCR, quantitative PCR.

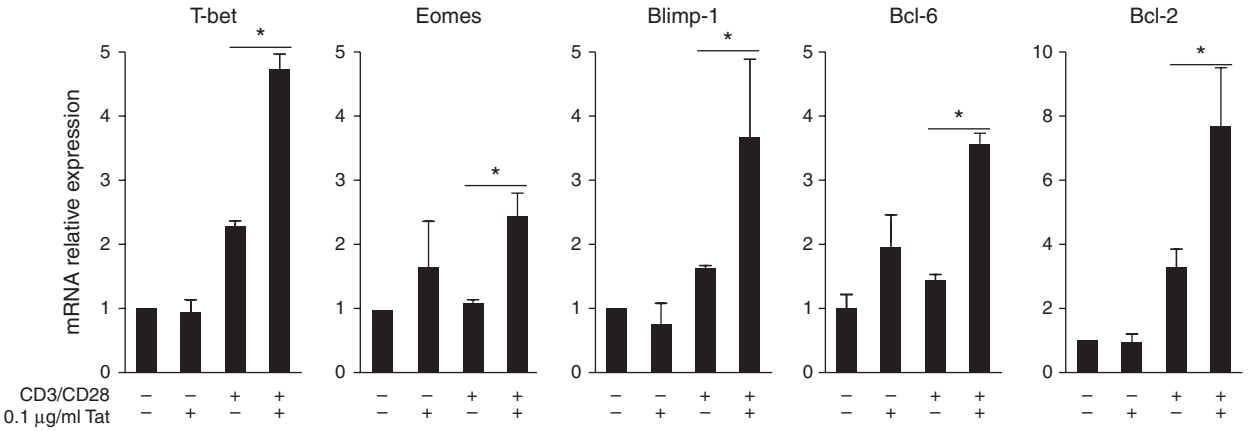

Fig. 2. The effect of Tat on transcriptional profile of $CD8^+$ T cells. The PBLs from healthy donors ($n = 8$) unstimulated or activated with anti-CD3/CD28 were cultured in the absence or presence of Tat (0.1 µg/ml). After 4 h, $CD8^+$ T cells were purified and mRNA levels of the indicated molecules were quantified by qPCR and normalized to untreated cells. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed-rank test was used. $^*P < 0.05$: Tat-treated cells compared to Tat-untreated control cells. PBL, peripheral blood lymphocyte; qPCR, quantitative PCR.

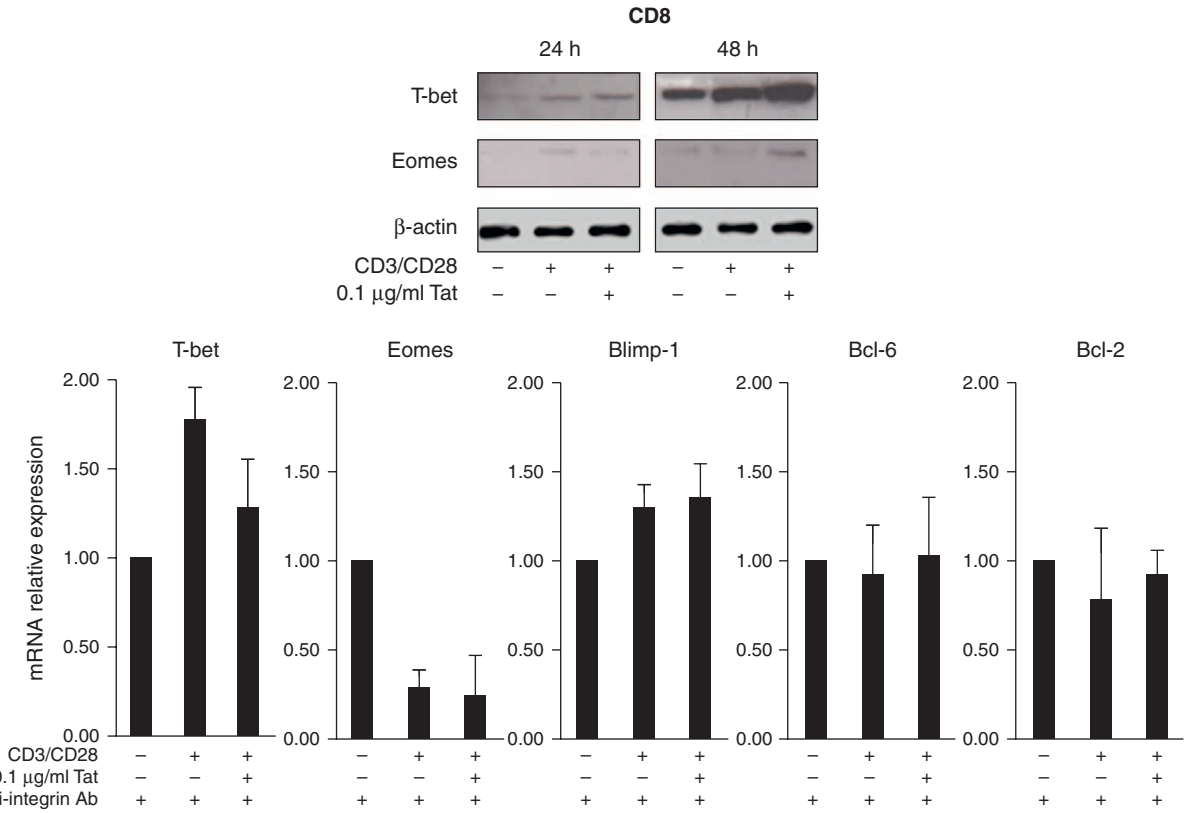

Fig. 3. The effect of Tat is inhibited by integrins blocking. The PBLs from healthy donors ($n = 6$) unstimulated or activated with anti-CD3/CD28 were cultured in the absence or presence of Tat (0.1 μg/ml). (a) CD8$^+$ T cells were purified after 24 and 48 h, and expression of T-bet and Eomes proteins was assessed by western blotting. The results of one representative experiment out of six are shown. (b) PBLs were preincubated with anti-integrins monoclonal antibodies for 1 h at room temperature before activation with anti-CD3/CD28 and Tat treatment. After 4 h, CD8$^+$ T cells were purified and mRNA levels of the indicated molecules were quantified by qPCR and normalized to untreated cells. Eomes, Eomesdermin; PBL, peripheral blood lymphocyte; qPCR, quantitative PCR.

preincubation with anti-integrin antibodies affected CD8$^+$ T-cell activation induced by anti-CD3/CD28. In particular, integrin blocking prevented the mRNA level increase of Bcl-6 and Bcl-2 and down-modulated Eomes (compare Fig. 2 and Fig. 3b), but did not affect the increase of T-bet and Blimp-1 in activated CD8$^+$ T cells. Thus, our results indicate that the Tat-mediated increase of T-bet and Blimp-1 is abolished by integrins blocking, whereas the role of the binding of Tat to integrins on the expression of Eomes, Bcl-6 and Bcl-2 remains to be elucidated.

Taken together, these data demonstrate that Tat favors the activation of CD8$^+$ T cells affecting the expression of transcription factors crucial for T-cell programming and functionality.

Tat down-regulates CD127 expression and modulates T-cell fate

It has been recently shown that the expression of T-bet and Eomes in memory CD8$^+$ T cells from HIV-infected individuals is associated with decreased expression of the IL-7 receptor CD127, and increased IFN-γ and granzyme B levels [29]. As Tat up-regulates T-bet and Eomes (Figs. 2 and 3), as well as IFN-γ (Fig. 1) and granzyme B production (Fig. 4), we then assessed whether Tat could affect CD127 expression in activated CD8$^+$ T cells. Moreover, we also measured the expression of CD25, the alpha chain of the receptor for IL-2, whose production is modulated by Tat. As shown in Fig. 5a, activation of PBLs with anti-CD3/CD28 increased the expression of CD25 and decreased the expression of CD127 on CD8$^+$ T cells. The presence of Tat did not affect the percentage of CD8$^+$ T cells expressing CD25, whereas it decreased the fraction of CD8$^+$ T lymphocytes expressing CD127. Interestingly, this effect was mediated by Tat in both unstimulated and activated CD8$^+$ T cells.

As T-bet, Eomes, Bcl-6, Blimp-1 and Bcl-2 regulate at different extent the T-cell programming and memory development, we next sought to determine the fate of CD8$^+$ T cells exposed for a longer time to Tat. To this aim, we evaluated the expression of the memory markers CD45RO and CD27 and of the exhaustion marker PD-1 in unstimulated or activated PBLs cultured for up to

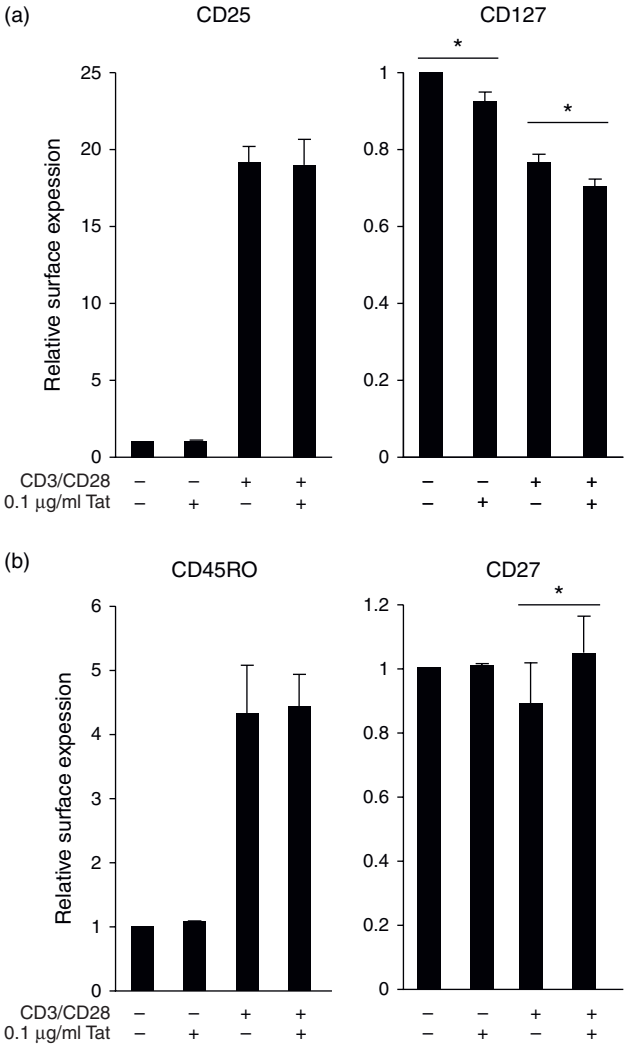

Fig. 4. Tat favors the activation of antigen-specific memory and naïve CTLs. (a–c) CTL cultures specific for (a) YLQ, (b) CLG, or (c) IVT EBV-derived epitopes were generated, in the absence or presence of Tat (0.1 μg/ml), from lymphocytes purified from EBV-positive donors and tested for their cytotoxic activity by ^{51}Cr-release assay against autologous unpulsed or peptide-pulsed PHA blasts (E : T ratio 10 : 1). (d) CTL cultures specific for the ELT survivin-derived epitope were generated, in the absence or presence of Tat (0.1 μg/ml), from lymphocytes purified from healthy donors and tested for their cytotoxic activity by ^{51}Cr-release assay against autologous unpulsed or peptide-pulsed PHA-blasts (E : T ratio 10 : 1). (e) CTL cultures specific for the CLG peptide epitope were generated from lymphocytes purified from EBV-positive donors. 24 or 48 h before the ^{51}Cr-release assay (E : T ratio 10 : 1), CTL cultures were treated with the Tat protein (0.1 μg/ml). (f) CTL cultures specific for YLQ and CLG EBV-derived epitopes were generated, in the absence or presence of Tat (0.1 μg/ml), from lymphocytes purified from EBV-positive donors, and tested for their IFN-γ and granzyme B release by Elispot assay. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed-rank test was used. $*P < 0.05$: Tat-treated cells compared to Tat-untreated control cells. IFN-γ, interferon-γ.

8 days in the absence or presence of Tat. In long-term cell cultures, the presence of Tat did not modulate the expression of CD45RO (Fig. 5b) and PD-1 (Fig. S3, http://links.lww.com/QAD/A525), whereas it increased the expression of CD27 in activated but not in unstimulated CD8$^+$ T cells (Fig. 5b). Interestingly, CD27 expression was not affected by Tat after 24 or 48 h of culture (not shown). These results suggest that the Tat-mediated modulation of T-bet, Eomes and the other transcription factors may be associated with the CD127 down-regulation and the accumulation of CD27$^+$CD8$^+$ T cells.

Tat favors the activation of antigen-specific naïve and memory CD8$^+$ T cells

We next assessed whether the presence of Tat in long-term cell cultures could also affect activation and functionality of antigen-specific memory and naïve CD8$^+$ T cells. To this aim, PBLs obtained from healthy HLA class I-typed EBV-seropositive donors were stimulated *ex vivo* with cells pulsed with EBV-derived CTL peptide epitopes in the absence or presence of Tat. Specifically, PBLs were stimulated with the subdominant HLA-A2-restricted CLGGLLTMV (CLG) or YLQQNWWTL (YLQ) epitope [45,46], or with the immunodominant HLA-A11-restricted IVTDFSVIK (IVT) epitope [38]. The cytotoxic activity of each CTL culture generated in the absence or presence of Tat was tested against autologous PHA blasts, pulsed or not with the relevant synthetic peptide, in a standard ^{51}Cr-release assay. As shown in Fig. 4a–c, all the three CTL cultures generated in the presence of Tat exhibited higher percentages of specific lysis compared to those generated in the absence of Tat.

To determine whether the Tat protein also favors the activation of naïve T cells, PBLs from HLA-A2 healthy donors were stimulated with the synthetic ELT peptide in the absence or presence of the Tat protein. The ELT (ELTLGEFLKL) peptide is a CTL epitope, presented by HLA-A2 [47,48], belonging to the surviving antiapoptotic protein, which is overexpressed in tumor cells [49]. No T-cell reactivity against this epitope is normally detected in healthy individuals [49]. The specificity of CTL cultures was tested against PHA blasts, pulsed or not with the ELT peptide, by ^{51}Cr-release assays (Fig. 4d). HLA-A2-positive PHA blasts pulsed with the ELT peptide were efficiently lyzed only by CTL cultures generated in the presence of Tat, demonstrating that Tat favors the priming of naive CD8$^+$ T cells.

These observations suggest that Tat favors the activation of CD8$^+$ T cells, but do not clarify whether the increased cytotoxic activity observed in CTL cultures generated in the presence of Tat depends on a higher number or a higher functionality of epitope-specific CD8$^+$ T cells. To address this issue, CTL cultures specific for the HLA-A2-restricted CLG epitope were generated in the absence of Tat and were then left untreated or preincubated with the

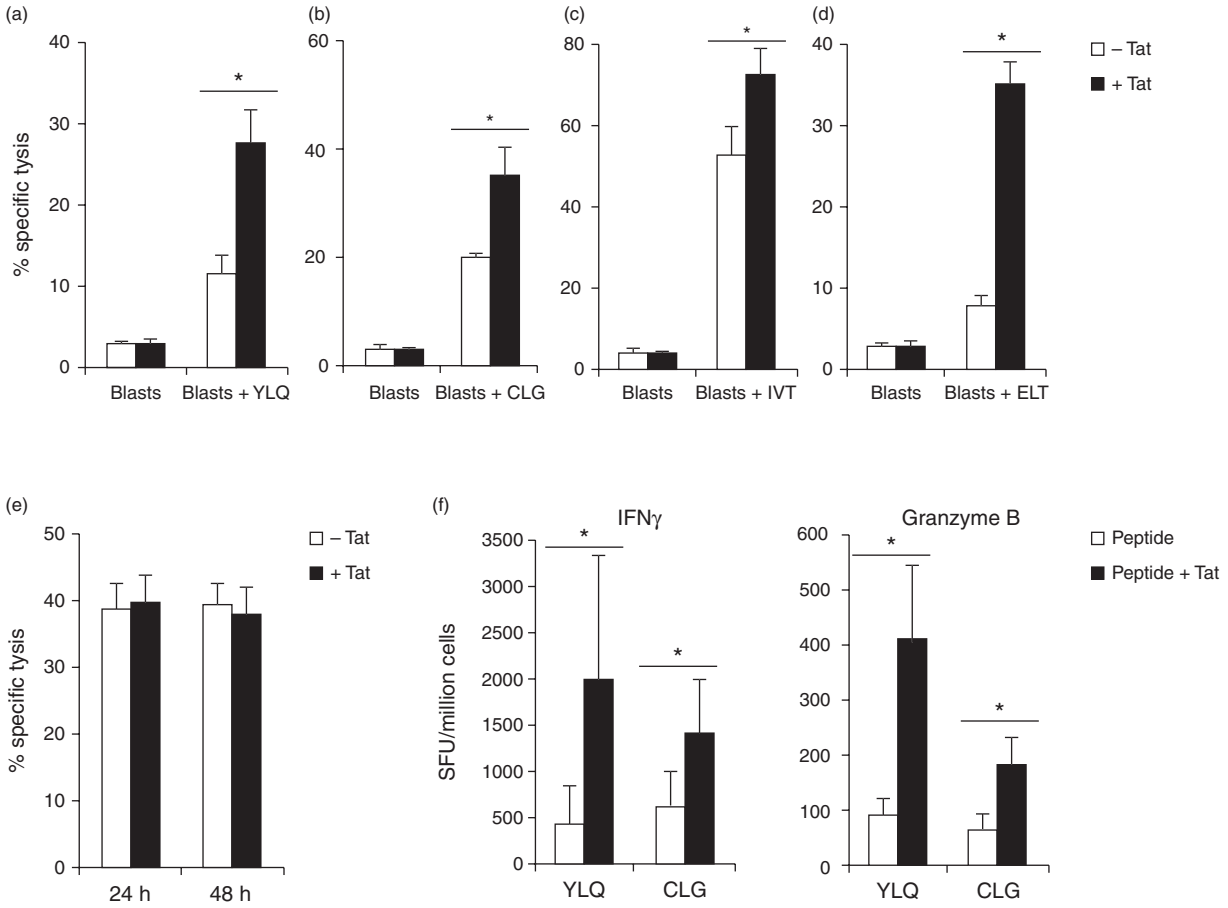

Fig. 5. Tat modulates the phenotype of CD8$^+$ T cells. PBLs from healthy donors ($n = 6$) unstimulated or activated with anti-CD3/CD28 were cultured in the absence or presence of Tat (0.1 μg/ml). (a) After 24 h, the percentage of CD8$^+$ T cells expressing CD25 and CD127 was measured. (b) After 8 days, the percentage of CD8$^+$ T cells expressing CD45RO and CD27 was measured. Results are presented after normalization to untreated cells. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed-rank test was used. $*P < 0.05$: Tat-treated cells compared to Tat-untreated control cells. PBL, peripheral blood lymphocyte.

Tat protein 24/48 h before the cytotoxic activity. As shown in Fig. 4e, CTL cultures lyzed target cells at similar levels, suggesting that Tat does not enhance effector functions, but rather must be present at the time of the priming, thus favoring CTL expansion. To confirm this hypothesis, CLG and YLQ-specific CTLs generated in the absence or presence of Tat were assayed in IFN-γ and granzyme B Elispot assays to evaluate differences in the number of antigen-specific T cells. As shown in Fig. 4f, CTL cultures generated in the presence of Tat exhibited higher numbers of both IFN-γ and granzyme B CLG and YLQ-specific CTLs, suggesting that Tat favors the expansion of epitope-specific and actively secreting CD8$^+$ T cells.

Discussion

We demonstrate here that the HIV-1 Tat protein, which is released by infected cells and found extracellularly in HIV-positive individuals [18,39,40], favors the activation and effector functions of CD8$^+$ T cells (Figs. 1 and 4). Interestingly, behind this functional modulation we observed that Tat increases the expression of T-bet, Eomes, Blimp-1, Bcl-6 and Bcl-2 in activated but not in unstimulated CD8$^+$ T lymphocytes (Fig. 2), leading to the down-regulation of CD127 and the up-regulation of CD27 (Fig. 5). The Tat-mediated increase of T-bet and Blimp-1 require the binding of Tat to integrins (Fig. 3). Thus, these results are indicating that the programming and functionality of CD8$^+$ T cells are deeply altered by the amount of Tat close to the concentrations measured in HIV-infected individuals. Indeed, it has been demonstrated that the plasma of HIV-positive patients may contain up to 40 ng/ml of soluble Tat [39,41], value which probably reaches higher concentrations in tissues where Tat is sequestered by glycosaminoglycans and heparan sulphate proteoglycans of the extracellular matrix [18,41]. Moreover, it has been proposed that Tat continues to be secreted even during HAART [19], as confirmed by the immune restoration observed after the induction of anti-Tat immunity in HIV-infected HAART-treated individuals [26].

It is known that Tat favors IL-2 secretion in $CD4^+$ T cells [15]. Here we show for the first time that $CD8^+$ T cells activated in the presence of Tat also exhibited increased production of IL-2 (Fig. 1). Several mechanisms may account for this effect, as it has been reported that Tat favors the activation of transcription factors required for IL-2 transcription, like NF-κB [13,14], NFAT [50] and AP-1 [51]. Moreover, Tat superinduces factors binding to the CD28-responsive element (CD28RE), which mediates IL-2 gene activation by CD28 costimulation [13,14].

The results also demonstrate that naïve and memory $CD8^+$ T cells activated *in vitro* in the presence of Tat exhibit an increased IFN-γ production and cytotoxic activity (Figs. 1 and 4). The effect was abolished when Tat was added after the stimulation, suggesting that Tat favors the expansion and the functionality of effector cells only if present at the beginning of the stimulation. It is likely that Tat potentiates the production of cytokines and cytolytic molecules through the induction of T-bet, Eomes and Blimp-1 (Fig. 2), which control at different levels the transcription of IFN-γ, perforins and granzymes [30,43,52]. The interaction of the RGD domain of Tat with $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins seems to be necessary, as we observed that the Tat-mediated up-regulation of the T-bet and Blimp-1 was abolished by integrin blocking. It is known that Tat mediates the activation of the ERK/MAPK and PI3K/Akt pathways through its RGD domain [53,54], and both ERK and Akt are involved in T-bet induction [55,56]. Moreover, the ERK pathway also favors Eomes and Blimp-1 up-regulation [57,58].

Of note, an increased production of effector molecules like IFN-γ and granzymes, as well as an enhancement of T-bet and Eomes in $CD8^+$ T cells, is observed in HIV-infected individuals [29,59–62]. Thus, our in-vitro observations suggest that Tat may be responsible for, or contribute to, all these effects *in vivo*.

The role of Tat on T-cell survival is highly debated [63–66]. We report that Tat enhances the expression of the antiapoptotic marker Bcl-2 in activated $CD8^+$ T cells. However, the up-regulation of Bcl-2 does not appear to be due to a direct effect of Tat on Bcl-2 expression, as instead demonstrated in $CD4^+$ T cells [63], since it was observed after activation with anti-CD3/CD28 and Tat further increased it. Thus, our results indicate that Tat may differently affect Bcl-2 expression in $CD4^+$ and $CD8^+$ T cells. Interestingly, the presence of Tat did not modulate PD-1 expression (Fig. S3, http://links.lww.com/QAD/A525), a marker of exhaustion up-regulated in HIV-specific $CD8^+$ T cells which poorly control the infection [67,68].

We found that $CD8^+$ T cells activated in the presence of Tat exhibited increased levels of Blimp-1, which favors the development of effector memory T cells [34,52]. Intriguingly, we also observed the up-regulation of Bcl-6, which promotes the development of a central memory phenotype and is repressed by Blimp-1 [34], suggesting a Tat-mediated mechanism that deserves further investigations. Moreover, the presence of Tat favors the expression of CD27, a hallmark of incomplete differentiation to effector cells [10,69,70], and causes CD127 down-regulation not only in unstimulated $CD8^+$ T cells, as previously demonstrated [71,72], but also in activated $CD8^+$ T lymphocytes. Interestingly, CD127 down-regulation is observed in HIV-infected individuals in association with immune activation [73,74], higher levels of T-bet and Eomes, and increased granzyme B and IFN-γ release [29]. In conclusion, our results indicate that Tat modulates programming and secretory capacity of $CD8^+$ T cells, suggesting that it may be involved in the development of $CD8^+$ T lymphocytes with an effector profile as observed during HIV infection [2,29]. We propose a model by which HIV, through the release of Tat, may affect T-bet and Eomes expression, thus contributing to immune activation and to a profound and long-lasting modulation of $CD8^+$ T-cell responses. Thus, our observations provide new hints on the role that Tat may play in $CD8^+$ T-cell dysfunctionalities during HIV infection, suggesting that the induction anti-Tat immune responses may be a valuable tool to protect HIV-infected individuals from immune dysfunctions.

Acknowledgements

F.S., F.N., and R.G. conceived and designed the experiments and analyzed the data. F.S., F.N., E.G., V.F., and E.R. performed the experiments. F.S., F.N., A.C., A.C., B.E., and R.G. wrote the manuscript.

We would like to thank Mirko Pinotti, Silvia Cellini, Matteo Rosa and Nicola Cavallari for technical assistance.

Funding: This work was supported by grants from the University of Ferrara and by the Gilead Fellowship Program.

Conflicts of interest

F.S. was partially supported by the 'Carlo Fornasini' Foundation. F.N. was partially supported by the Italian Center of Biotechnology (CIB). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. UNAIDS report on the global AIDS epidemic 2012. In Joint United Nations Programme on HIV/AIDS. UNAIDS.
2. Harari A, Petitpierre S, Vallelian F, Pantaleo G. **Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy.** *Blood* 2004; **103**: 966–972.

3. Migueles SA, Weeks KA, Nou E, Berkley AM, Rood JE, Osborne CM, *et al*. **Defective human immunodeficiency virus-specific CD8+ T-cell polyfunctionality, proliferation, and cytotoxicity are not restored by antiretroviral therapy.** *J Virol* 2009; **83**: 11876–11889.
4. Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, *et al*. **Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction.** *Nat Med* 2006; **12**:1198–1202.
5. Catalfamo M, Wilhelm C, Tcheung L, Proschan M, Friesen T, Park JH, *et al*. **CD4 and CD8 T cell immune activation during chronic HIV infection: roles of homeostasis, HIV, type I IFN, and IL-7.** *J Immunol* 2011; **186**:2106–2116.
6. Finkel TH, Tudor-Williams G, Banda NK, Cotton MF, Curiel T, Monks C, *et al*. **Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes.** *Nat Med* 1995; **1**:129–134.
7. Groux H, Torpier G, Monte D, Mouton Y, Capron A, Ameisen JC. **Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals.** *J Exp Med* 1992; **175**:331–340.
8. Champagne P, Ogg GS, King AS, Knabenhans C, Ellefsen K, Nobile M, *et al*. **Skewed maturation of memory HIV-specific CD8 T lymphocytes.** *Nature* 2001; **410**:106–111.
9. van Baarle D, Kostense S, van Oers MH, Hamann D, Miedema F. **Failing immune control as a result of impaired CD8+ T-cell maturation: CD27 might provide a clue.** *Trends Immunol* 2002; **23**:586–591.
10. Roos MT, van Lier RA, Hamann D, Knol GJ, Verhoofstad I, van Baarle D, *et al*. **Changes in the composition of circulating CD8+ T cell subsets during acute Epstein-Barr and human immunodeficiency virus infections in humans.** *J Infect Dis* 2000; **182**:451–458.
11. Haas A, Zimmermann K, Oxenius A. **Antigen-dependent and -independent mechanisms of T and B cell hyperactivation during chronic HIV-1 infection.** *J Virol* 2011; **85**:12102–12113.
12. Papagno L, Spina CA, Marchant A, Salio M, Rufer N, Little S, *et al*. **Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection.** *PLoS Biol* 2004; **2**:E20.
13. Ott M, Emiliani S, Van Lint C, Herbein G, Lovett J, Chirmule N, *et al*. **Immune hyperactivation of HIV-1-infected T cells mediated by Tat and the CD28 pathway.** *Science* 1997; **275**:1481–1485.
14. Kwon HS, Brent MM, Getachew R, Jayakumar P, Chen LF, Schnolzer M, *et al*. **Human immunodeficiency virus type 1 Tat protein inhibits the SIRT1 deacetylase and induces T cell hyperactivation.** *Cell Host Microbe* 2008; **3**:158–167.
15. Secchiero P, Zella D, Curreli S, Mirandola P, Capitani S, Gallo RC, *et al*. **Pivotal role of cyclic nucleoside phosphodiesterase 4 in Tat-mediated CD4+ T cell hyperactivation and HIV type 1 replication.** *Proc Natl Acad Sci U S A* 2000; **97**:14620–14625.
16. Monini P, Cafaro A, Srivastava IK, Moretti S, Sharma VA, Andreini C, *et al*. **HIV-1 Tat promotes integrin-mediated HIV transmission to dendritic cells by binding Env spikes and competes neutralization by anti-HIV antibodies.** *PLoS One* 2012; **7**:e48781.
17. Fisher AG, Feinberg MB, Josephs SF, Harper ME, Marselle LM, Reyes G, *et al*. **The trans-activator gene of HTLV-III is essential for virus replication.** *Nature* 1986; **320**:367–371.
18. Chang HC, Samaniego F, Nair BC, Buonaguro L, Ensoli B. **HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region.** *AIDS* 1997; **11**:1421–1431.
19. Mediouni S, Darque A, Baillat G, Ravaux I, Dhiver C, Tissot-Dupont H, *et al*. **Antiretroviral therapy does not block the secretion of the human immunodeficiency virus Tat protein.** *Infect Disord Drug Targets* 2012; **12**:81–86.
20. Fanales-Belasio E, Moretti S, Nappi F, Barillari G, Micheletti F, Cafaro A, *et al*. **Native HIV-1 Tat protein targets monocyte-derived dendritic cells and enhances their maturation, function, and antigen-specific T cell responses.** *J Immunol* 2002; **168**:197–206.
21. Gavioli R, Gallerani E, Fortini C, Fabris M, Bottoni A, Canella A, *et al*. **HIV-1 Tat protein modulates the generation of cytotoxic T cell epitopes by modifying proteasome composition and enzymatic activity.** *J Immunol* 2004; **173**:3838–3843.
22. Gavioli R, Cellini S, Castaldello A, Voltan R, Gallerani E, Gagliardoni F, *et al*. **The Tat protein broadens T cell responses directed to the HIV-1 antigens Gag and Env: implications for the design of new vaccination strategies against AIDS.** *Vaccine* 2008; **26**:727–737.
23. Debaisieux S, Rayne F, Yezid H, Beaumelle B. **The ins and outs of HIV-1 Tat.** *Traffic* 2012; **13**:355–363.
24. Huigen MC, Kamp W, Nottet HS. **Multiple effects of HIV-1 trans-activator protein on the pathogenesis of HIV-1 infection.** *Eur J Clin Invest* 2004; **34**:57–66.
25. Rezza G, Fiorelli V, Dorrucci M, Ciccozzi M, Tripiciano A, Scoglio A, *et al*. **The presence of anti-Tat antibodies is predictive of long-term nonprogression to AIDS or severe immunodeficiency: findings in a cohort of HIV-1 seroconverters.** *J Infect Dis* 2005; **191**:1321–1324.
26. Ensoli B, Bellino S, Tripiciano A, Longo O, Francavilla V, Marcotullio S, *et al*. **Therapeutic immunization with HIV-1 Tat reduces immune activation and loss of regulatory T-cells and improves immune function in subjects on HAART.** *PLoS One* 2010; **5**:e13540.
27. Nicoli F, Finessi V, Sicurella M, Rizzotto L, Gallerani E, Destro F, *et al*. **The HIV-1 Tat protein induces the activation of CD8+ T cells and affects in vivo the magnitude and kinetics of antiviral responses.** *PLoS One* 2013; **8**:e77746.
28. Schweneker M, Favre D, Martin JN, Deeks SG, McCune JM. **HIV-induced changes in T cell signaling pathways.** *J Immunol* 2008; **180**:6490–6500.
29. Hasley RB, Hong C, Li W, Friesen T, Nakamura Y, Kim GY, *et al*. **HIV immune activation drives increased Eomes expression in memory CD8 T cells in association with transcriptional down-regulation of CD127.** *AIDS* 2013; **27**:1867–1877.
30. Intlekofer AM, Takemoto N, Wherry EJ, Longworth SA, Northrup JT, Palanivel VR, *et al*. **Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin.** *Nat Immunol* 2005; **6**:1236–1244.
31. Takemoto N, Intlekofer AM, Northrup JT, Wherry EJ, Reiner SL. **IL-12 inversely regulates T-bet and eomesodermin expression during pathogen-induced CD8+ T cell differentiation.** *J Immunol* 2006; **177**:7515–7519.
32. McLane LM, Banerjee PP, Cosma GL, Makedonas G, Wherry EJ, Orange JS, *et al*. **Differential localization of T-bet and Eomes in CD8 T cell memory populations.** *J Immunol* 2013; **190**:3207–3215.
33. Martins G, Calame K. **Regulation and functions of Blimp-1 in T and B lymphocytes.** *Annu Rev Immunol* 2008; **26**:133–169.
34. Crotty S, Johnston RJ, Schoenberger SP. **Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation.** *Nat Immunol* 2010; **11**:114–120.
35. Martins GA, Cimmino L, Liao J, Magnusdottir E, Calame K. **Blimp-1 directly represses Il2 and the Il2 activator Fos, attenuating T cell proliferation and survival.** *J Exp Med* 2008; **205**:1959–1965.
36. Rao RR, Li Q, Odunsi K, Shrikant PA. **The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin.** *Immunity* 2010; **32**:67–78.
37. Micheletti F, Guerrini R, Formentin A, Canella A, Marastoni M, Bazzaro M, *et al*. **Selective amino acid substitutions of a subdominant Epstein-Barr virus LMP2-derived epitope increase HLA/peptide complex stability and immunogenicity: implications for immunotherapy of Epstein-Barr virus-associated malignancies.** *Eur J Immunol* 1999; **29**:2579–2589.
38. Gavioli R, Kurilla MG, de Campos-Lima PO, Wallace LE, Dolcetti R, Murray RJ, *et al*. **Multiple HLA A11-restricted cytotoxic T-lymphocyte epitopes of different immunogenicities in the Epstein-Barr virus-encoded nuclear antigen 4.** *J Virol* 1993; **67**:1572–1578.
39. Westendorp MO, Frank R, Ochsenbauer C, Stricker K, Dhein J, Walczak H, *et al*. **Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120.** *Nature* 1995; **375**:497–500.
40. Ensoli B, Buonaguro L, Barillari G, Fiorelli V, Gendelman R, Morgan RA, *et al*. **Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation.** *J Virol* 1993; **67**:277–287.
41. Xiao H, Neuveut C, Tiffany HL, Benkirane M, Rich EA, Murphy PM, *et al*. **Selective CXCR4 antagonism by Tat: implications for in vivo expansion of coreceptor use by HIV-1.** *Proc Natl Acad Sci U S A* 2000; **97**:11466–11471.

42. Ott M, Lovett JL, Mueller L, Verdin E. **Superinduction of IL-8 in T cells by HIV-1 Tat protein is mediated through NF-kappaB factors.** *J Immunol* 1998; **160**:2872–2880.
43. Pearce EL, Mullen AC, Martins GA, Krawczyk CM, Hutchins AS, Zediak VP, *et al*. **Control of effector CD8+ T cell function by the transcription factor Eomesodermin.** *Science* 2003; **302**: 1041–1043.
44. Zauli G, Gibellini D, Celeghini C, Mischiati C, Bassini A, La Placa M, *et al*. **Pleiotropic effects of immobilized versus soluble recombinant HIV-1 Tat protein on CD3-mediated activation, induction of apoptosis, and HIV-1 long terminal repeat transactivation in purified CD4+ T lymphocytes.** *J Immunol* 1996; **157**:2216–2224.
45. Lee SP, Thomas WA, Murray RJ, Khanim F, Kaur S, Young LS, *et al*. **HLA A2.1-restricted cytotoxic T cells recognizing a range of Epstein-Barr virus isolates through a defined epitope in latent membrane protein LMP2.** *J Virol* 1993; **67**:7428–7435.
46. Khanna R, Burrows SR, Nicholls J, Poulsen LM. **Identification of cytotoxic T cell epitopes within Epstein-Barr virus (EBV) oncogene latent membrane protein 1 (LMP1): evidence for HLA A2 supertype-restricted immune recognition of EBV-infected cells by LMP1-specific cytotoxic T lymphocytes.** *Eur J Immunol* 1998; **28**:451–458.
47. Casati C, Dalerba P, Rivoltini L, Gallino G, Deho P, Rini F, *et al*. **The apoptosis inhibitor protein survivin induces tumor-specific CD8+ and CD4+ T cells in colorectal cancer patients.** *Cancer Res* 2003; **63**:4507–4515.
48. Schmitz M, Diestelkoetter P, Weigle B, Schmachtenberg F, Stevanovic S, Ockert D, *et al*. **Generation of survivin-specific CD8+ T effector cells by dendritic cells pulsed with protein or selected peptides.** *Cancer Res* 2000; **60**:4845–4849.
49. Andersen MH, Pedersen LO, Becker JC, Straten PT. **Identification of a cytotoxic T lymphocyte response to the apoptosis inhibitor protein survivin in cancer patients.** *Cancer Res* 2001; **61**:869–872.
50. Hidalgo-Estevez AM, Gonzalez E, Punzon C, Fresno M. **Human immunodeficiency virus type 1 Tat increases cooperation between AP-1 and NFAT transcription factors in T cells.** *J Gen Virol* 2006; **87**:1603–1612.
51. Gibellini D, Re MC, Ponti C, Celeghini C, Melloni E, La Placa M, *et al*. **Extracellular Tat activates c-fos promoter in low serum-starved CD4+ T cells.** *Br J Haematol* 2001; **112**:663–670.
52. Kallies A, Xin A, Belz GT, Nutt SL. **Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses.** *Immunity* 2009; **31**:283–295.
53. Chugh P, Bradel-Tretheway B, Monteiro-Filho CM, Planelles V, Maggirwar SB, Dewhurst S, *et al*. **Akt inhibitors as an HIV-1 infected macrophage-specific antiviral therapy.** *Retrovirology* 2008; **5**:11.
54. Toschi E, Bacigalupo I, Strippoli R, Chiozzini C, Cereseto A, Falchi M, *et al*. **HIV-1 Tat regulates endothelial cell cycle progression via activation of the Ras/ERK MAPK signaling pathway.** *Mol Biol Cell* 2006; **17**:1985–1994.
55. Lee K, Gudapati P, Dragovic S, Spencer C, Joyce S, Killeen N, *et al*. **Mammalian target of rapamycin protein complex 2 regulates differentiation of Th1 and Th2 cell subsets via distinct signaling pathways.** *Immunity* 2010; **32**:743–753.
56. Chang CF, D'Souza WN, Ch'en IL, Pages G, Pouyssegur J, Hedrick SM. **Polar opposites: Erk direction of CD4 T cell subsets.** *J Immunol* 2012; **189**:721–731.
57. Yasuda T, Kometani K, Takahashi N, Imai Y, Aiba Y, Kurosaki T. **ERKs induce expression of the transcriptional repressor Blimp-1 and subsequent plasma cell differentiation.** *Sci Signal* 2011; **4**:ra25.
58. Rafei M, Rouette A, Brochu S, Vanegas JR, Perreault C. **Differential effects of (c cytokines on postselection differentiation of CD8 thymocytes.** *Blood* 2013; **121**:107–117.
59. Rehr M, Cahenzli J, Haas A, Price DA, Gostick E, Huber M, *et al*. **Emergence of polyfunctional CD8+ T cells after prolonged suppression of human immunodeficiency virus replication by antiretroviral therapy.** *J Virol* 2008; **82**:3391–3404.
60. Sousa AE, Chaves AF, Doroana M, Antunes F, Victorino RM. **Kinetics of the changes of lymphocyte subsets defined by cytokine production at single cell level during highly active antiretroviral therapy for HIV-1 infection.** *J Immunol* 1999; **162**:3718–3726.
61. Eylar EH, Lefranc C, Baez I, Colon-Martinez SL, Yamamura Y, Rodriguez N, *et al*. **Enhanced interferon-gamma by CD8+ CD28- lymphocytes from HIV+ patients.** *J Clin Immunol* 2001; **21**:135–144.
62. Pae Y, Minagawa H, Hayashi J, Kashiwagi S, Yanagi Y. **Enhanced IFN-gamma production in vitro by CD8+ T cells in hemophiliacs with AIDS as demonstrated on the single-cell level.** *Clin Immunol* 1999; **92**:111–117.
63. Zauli G, Gibellini D, Caputo A, Bassini A, Negrini M, Monne M, *et al*. **The human immunodeficiency virus type-1 Tat protein upregulates Bcl-2 gene expression in Jurkat T-cell lines and primary peripheral blood mononuclear cells.** *Blood* 1995; **86**:3823–3834.
64. Gulow K, Kaminski M, Darvas K, Suss D, Li-Weber M, Krammer PH. **HIV-1 trans-activator of transcription substitutes for oxidative signaling in activation-induced T cell death.** *J Immunol* 2005; **174**:5249–5260.
65. Zhang M, Li X, Pang X, Ding L, Wood O, Clouse K, *et al*. **Identification of a potential HIV-induced source of bystander-mediated apoptosis in T cells: upregulation of trail in primary human macrophages by HIV-1 Tat.** *J Biomed Sci* 2001; **8**:290–296.
66. Gibellini D, Re MC, Ponti C, Maldini C, Celeghini C, Cappellini A, *et al*. **HIV-1 Tat protects CD4+ Jurkat T lymphoblastoid cells from apoptosis mediated by TNF-related apoptosis-inducing ligand.** *Cell Immunol* 2001; **207**:89–99.
67. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, *et al*. **PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression.** *Nature* 2006; **443**:350–354.
68. Petrovas C, Casazza JP, Brenchley JM, Price DA, Gostick E, Adams WC, *et al*. **PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection.** *J Exp Med* 2006; **203**:2281–2292.
69. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, *et al*. **Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections.** *Nat Med* 2002; **8**:379–385.
70. van Baarle D, Kostense S, Hovenkamp E, Ogg G, Nanlohy N, Callan MF, *et al*. **Lack of Epstein-Barr virus- and HIV-specific CD27- CD8+ T cells is associated with progression to viral disease in HIV-infection.** *AIDS* 2002; **16**:2001–2011.
71. Faller EM, Sugden SM, McVey MJ, Kakal JA, MacPherson PA. **Soluble HIV Tat protein removes the IL-7 receptor alpha-chain from the surface of resting CD8 T cells and targets it for degradation.** *J Immunol* 2010; **185**:2854–2866.
72. Faller E, Kakal J, Kumar R, Macpherson P. **IL-7 and the HIV Tat protein act synergistically to down-regulate CD127 expression on CD8 T cells.** *Int Immunol* 2009; **21**:203–216.
73. Paiardini M, Cervasi B, Albrecht H, Muthukumar A, Dunham R, Gordon S, *et al*. **Loss of CD127 expression defines an expansion of effector CD8+ T cells in HIV-infected individuals.** *J Immunol* 2005; **174**:2900–2909.
74. Benito JM, Lopez M, Lozano S, Gonzalez-Lahoz J, Soriano V. **Down-regulation of interleukin-7 receptor (CD127) in HIV infection is associated with T cell activation and is a main factor influencing restoration of CD4(+) cells after antiretroviral therapy.** *J Infect Dis* 2008; **198**:1466–1473.