The HIV-1 Tat protein affects human CD4$^+$ T-cell programing and activation, and favors the differentiation of naïve CD4$^+$ T cells

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Objective: HIV infection is characterized by several immune dysfunctions, such as chronic activation of the immune system, premature aging and loss of CD4$^+$ T cells, in particular within the naïve compartment. The Tat protein of HIV is released extracellularly and enters neighboring cells affecting their functionality, for instance impacting on CD8$^+$ T-cell programs and activity. As the presence and/or induction of anti-Tat immune responses is associated with reduced T-cell dysfunction and CD4$^+$ T-cell loss, we investigated whether Tat impacts human resting or activated CD4$^+$ T cells.

Methods: Purified CD4$^+$ T cells were activated by T cell receptor engagement in the presence or absence of Tat. Cytokine production, surface phenotype and expression of transcription factors important for T-cell programing were measured. Purified naïve CD4$^+$ T cells were cultured in nonpolarizing conditions in the presence or absence of Tat and their proliferation and differentiation was evaluated.

Results: Tat favors the secretion of IL2, IFNγ and TNFα in CD4$^+$ T cells, as well as the upregulation of T-bet and Eomes expression. Naïve CD4$^+$ T cells cultured in the presence of Tat showed enhanced expansion and differentiation toward memory phenotype, showing in particular recruitment into the effector memory T-cell pool.

Conclusion: Tat affects the programing and functionality of CD4$^+$ T lymphocytes favoring the differentiation of naïve CD4$^+$ T cells.

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Introduction

HIV infection strongly affects cellular immunity, causing the depletion of $CD4^+$ T cells, in particular within the naïve compartment [1], and dysfunction of both $CD8^+$ and $CD4^+$ T lymphocytes [2–6]. This status of chronic immune dysregulation involves the whole T-cell compartment, including uninfected T cells [7], and is not completely restored during effective antiretroviral therapy (ART). There is a general consensus on the complexity of these phenomena which seem to be due not only to viral replication and $CD4^+$ T cells loss, but also to the immunomodulatory activity of HIV products, including Tat [5,7]. Indeed, the HIV-1 Tat protein is released extracellularly [8], even during ART [9], and enters neighboring cells affecting their functionality [10–15]. In this context, it has been shown that Tat has a strong impact on $CD8^+$ T-cell programs and activity [15] and, in murine models, favors the activation of $CD8^+$ T cells and the modulation of antiviral responses [16], causing dysfunctions similar to those observed in HIV-infected individuals. It is also noteworthy that naturally acquired or vaccine-induced anti-Tat immunity limits T-cell dysfunction, $CD4^+$ T-cell loss and viral load, and is associated with the reduction of proviral DNA, resulting in the delay of disease progression [17–21]. However, whether Tat has a direct or indirect effect upon the $CD4^+$ T-cell compartment is currently unknown. To shed light on this issue, we have determined whether extracellular bioactive Tat impacts human resting or activated $CD4^+$ T cells. Our results show that Tat promotes the activation of $CD4^+$ T cells as well as differentiation of naïve $CD4^+$ T cells toward memory subtypes that may result in the generation of new targets of 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.

Total and naïve $CD4^+$ T cells were sorted by MACS magnetic selection (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions and cultured, as detailed in Supplemental information, http://links.lww.com/QAD/B210, in the absence or presence of the Tat protein in 24-well flat-bottomed polystyrene plates precoated overnight at 4 °C with PBS or anti-CD3 mAb (0.5 μg/ml; R&D Systems, Minneapolis, Minnesota, USA). Naïve $CD4^+$ T cells were cultured in nonpolarizing condition as previously described [22] and detailed in Supplemental information, http://links.lww.com/QAD/B210.

Tat protein

HIV-1 Tat from 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 [10]. The lyophilized Tat protein was then stored at −80 °C and handled as described [10]. Endotoxin concentration was below the detection limit (0.05 EU/μg).

Flow cytometry

Surface and intracellular staining were performed as detailed in Supplemental information, http://links.lww.com/QAD/B210.

Gene-expression analysis

Gene expression was evaluated by quantitative as detailed in Supplemental information, http://links.lww.com/QAD/B210.

Results

Tat enhances $CD4^+$ T-cell activation

The HIV-1 Tat protein, which is released by infected cells, enhances the production of proinflammatory cytokines from activated PBLs and $CD8^+$ T cells [15,23,24]. To understand whether soluble Tat, at physiological concentration within a nanomolar range, may induce cytokine production in $CD4^+$ T cells, resting or anti-CD3/CD28-stimulated T helper lymphocytes from healthy donors were cultured for 4 h in the absence or presence of 0.1 μg/ml of Tat protein. As shown in Fig. 1a, Tat significantly increased the expression of IL2, IFNγ and TNFα mRNAs in anti-CD3/CD28-stimulated $CD4^+$ T cells, but not in resting lymphocytes. This effect was observed at similar levels for Tat doses ranging from 0.01 to 1 μg/ml, and it was abolished after incubation with anti-Tat positive sera (Fig. S1, http://links.lww.com/QAD/B210). This result was confirmed by cytokine intracellular staining of the cells that demonstrated increased production of IL2, IFNγ and TNFα (Fig. 1b) after 18 h of treatment with Tat when compared with untreated cells. However, the expression of early (CD69) and late (CD25, CD38, HLA-DR) activation markers was not affected by the presence of Tat (Fig. S2, http://links.lww.com/QAD/B210). As these results indicate that, in human-activated $CD4^+$ T cells, Tat enhances the production of Th1-type cytokines, which are under the control of T-box transcription factors [25,26], we characterized the expression of T-bet and Eomes in resting and activated $CD4^+$ T cells cultured in the absence or in the presence of Tat. As shown in Fig. 1c, Tat did not induce the mRNA expression of T-box transcription factors in unstimulated $CD4^+$ T cells,

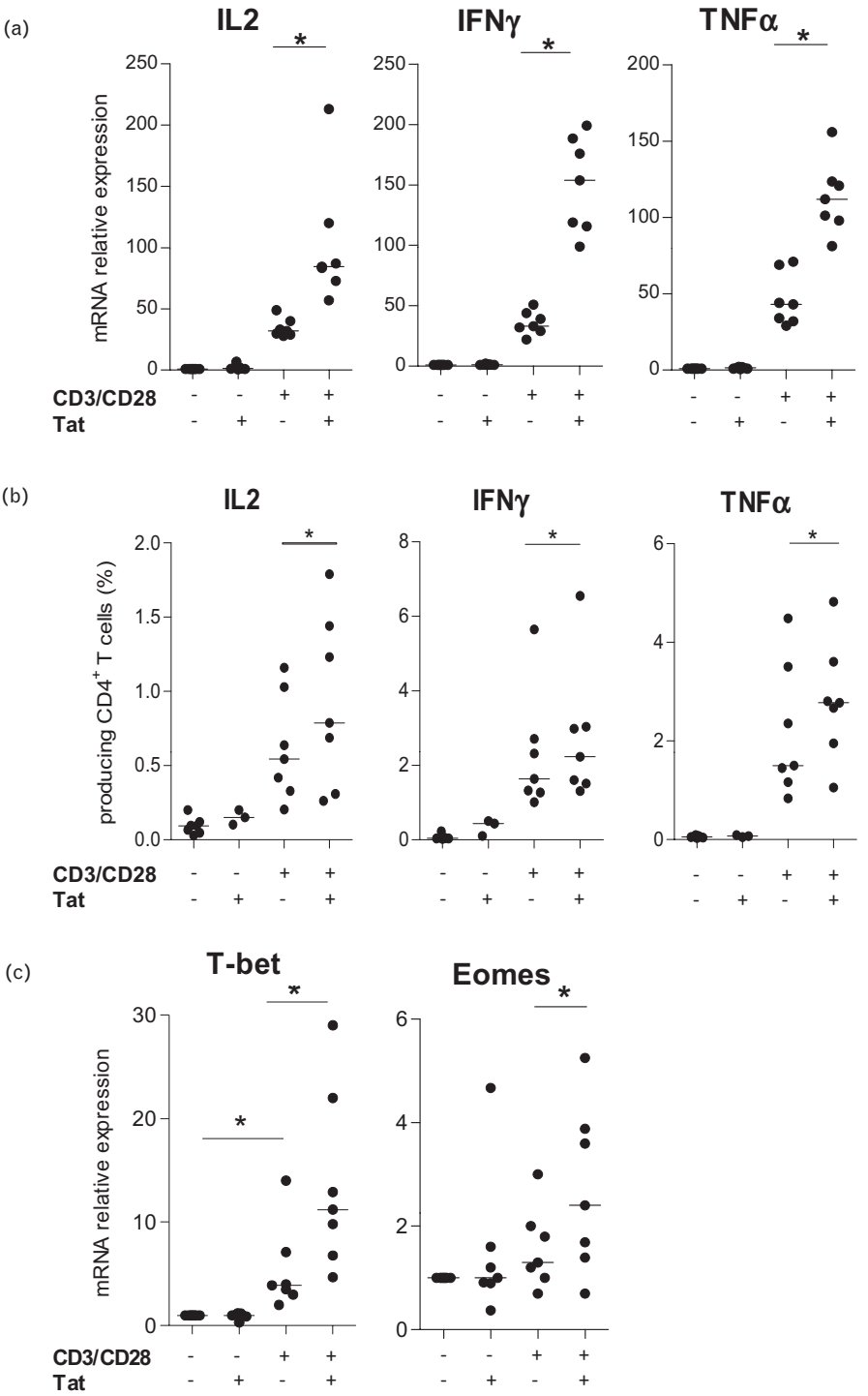

Fig. 1. Tat favors CD4$^+$ T-cell activation. CD4$^+$ T cells purified from healthy donors ($n = 7$) unstimulated or activated with anti-CD3/CD28 were cultured in the absence or presence of soluble Tat (0.1 μg/ml). After 4 h, IL2, IFNγ, TNFα (a), T-bet and Eomes (c) mRNA levels were quantified by qPCR and normalized to untreated cells. (b) Peripheral blood lymphocytes from healthy donors ($n = 3$–7) unstimulated or activated with anti-CD3/CD28 were cultured in the absence or presence of 0.1 μg/ml of Tat. After 18 h, IL2, IFNγ and TNFα production was measured by intracellular cytokine staining in CD4$^+$ T cells. Dots represent single donors and lines represent the median. For statistical analysis, two-tailed Wilcoxon signed-rank test was used. $^*P < 0.05$: Tat-treated activated cells compared with Tat-untreated activated control cells. qPCR, quantitative

whereas it increased significantly the expression of T-bet and Eomes transcription factors in CD3/CD28-activated $CD4^+$ T cells as compared with $CD4^+$ T cells activated with CD3/CD28 and cultured in the absence of Tat. Thus, at a physiological concentration, soluble Tat protein enhances the production of proinflammatory cytokines in activated $CD4^+$ T cells and influences the expression of transcription factors crucial for T-cell programing and functionality.

Tat favors the expansion and the differentiation of naïve $CD4^+$ T cells

The HIV-related chronic immune activation plays a major role in the increased proliferation and differentiation of naïve T cells into memory cells [1,27] leading to a decline of naïve T cells. As our data clearly indicate that Tat favors the activation of $CD4^+$ T cells and the expression of transcription factors controlling T-cell programing, we wondered whether Tat had also an effect upon proliferation and differentiation of naïve lymphocytes, thus participating in immune activation and pathogenesis of HIV infection. To address this, purified naïve $CD4^+$ T cells were cultured, in the presence or absence of Tat, in nonpolarizing conditions (NPC) to induce their activation and differentiation toward a memory phenotype avoiding potential biases due to polarization toward some specific T helper cell subpopulations [22]. As shown in Fig. 2a, NPC induced

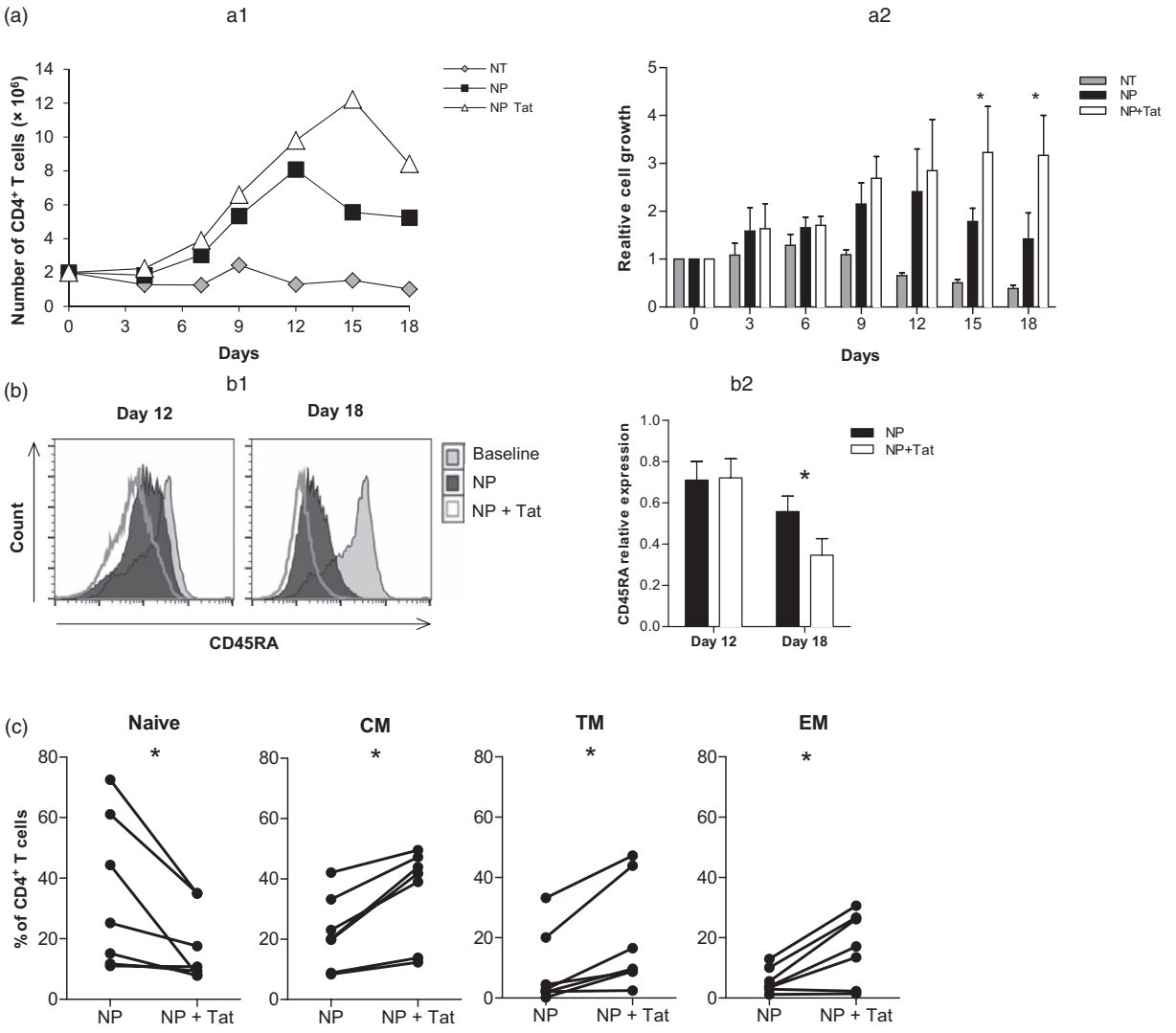

Fig. 2. Tat affects homeostasis of naïve $CD4^+$ T cells. Purified naïve $CD4^+$ T cells from healthy donors were cultured in NPC in the absence or presence of 0.1 μg/ml of soluble Tat. (a) Cell number was evaluated along the course of the cell culture. One representative donor out of seven (a1) and means ± SEM of data normalized to day 0 (a2) are shown ($n = 7$). (b) Expression of CD45RA was evaluated by flow cytometry at 12 and 18 days of culture. One representative donor out of seven (b1, expressed as histogram plot) and means ± SEM of data normalized to baseline levels (b2) are shown ($n = 7$). (c) Percentages of different $CD4^+$ T-cell subpopulations were calculated at 18 days of culture. Data from seven healthy donors are presented. For statistical analysis, two-tailed Wilcoxon signed-rank test was used. $^*P < 0.05$: Tat-treated cells compared with Tat-untreated control cells. NPC, nonpolarizing conditions.

the proliferation of naïve CD4$^+$ T cells starting from day 7 and reaching the peak at day 12. The addition of Tat enhanced duration and magnitude of naïve T helper cell expansion which peaked at day 15 and remained higher until day 18. To determine whether Tat affected the differentiation of naïve CD4$^+$ T cells cultured in NPC, the phenotype of T helper lymphocytes was assessed. Overall, NPC prompted the loss of CD45RA expression (Fig. 2b), suggesting a shift toward a nonnaïve phenotype that had started by day 12, with a more pronounced downregulation by day 18. Significantly, this phenomenon was more pronounced in the presence of Tat. In fact, higher numbers of central memory (CD45RA$^-$, CCR7$^+$, CD27$^+$), transitional memory (CD45RA$^-$, CCR7$^-$, CD27$^+$) and effector memory (CD45RA$^-$, CCR7$^-$, CD27$^-$) CD4$^+$ T cells were generated in the presence of Tat as compared with NPC alone (Fig. 2c). It is noteworthy that effector memory CD4$^+$ T cells were almost absent in cultures derived from naïve CD4$^+$ T cells activated under NPC, whereas they were strongly induced in the presence of Tat (Fig. 2c). Taken together, these data suggest that Tat supports the activation of naïve CD4$^+$ T cells promoting their transition toward more differentiated phenotypes.

Discussion

The Tat protein of HIV is released by infected cells [8] and interacts with neighboring cells [10–15]. We showed here that soluble Tat favors the activation of CD4$^+$ T cells inducing the release of proinflammatory cytokines and the expression of transcription factors such as T-bet and Eomes which are crucial for T-cell activation and differentiation. In addition, Tat increased the expansion and differentiation of naïve CD4$^+$ T cells activated in NPC. These findings, together with the observations made in CD8$^+$ T cells [15,16,28], confirm that Tat plays an important role in the hyperactivation of the T-cell compartment, a phenomenon characterizing the progression to AIDS and possibly the residual disease observed in successfully ART-treated individuals [29,30].

Naïve CD4$^+$ T cells are resistant to productive HIV infection due to their quiescent state [31,32]. However, their number dramatically decreases during AIDS [1], in part due to the status of chronic immune activation which favors their differentiation into memory and effector cells [27,33]. Tat, by favoring naïve T-cell activation, promotes their recruitment into the memory compartment and, fostering the exit from a quiescent state, might also contribute to the generation of new potential targets of infection, in line with previous observations showing higher susceptibly to HIV infection by CD4$^+$ T cells exposed to Tat [34,35]. Tat expression has been detected in tissues from patients on ART [36], whose success is dependent on the levels of naïve CD4$^+$ T cells [37], a compartment not always fully reconstituted by ART [29]. Therefore, our data suggest that blocking Tat effects may favor therapy efficacy, as indeed observed in ART-treated individuals vaccinated with the Tat protein that showed restored T-cell responses against heterologous antigens and a rise in CD4$^+$ T-cell count [18,19].

In previous works conducted with cell lines, Tat was alternatively shown to promote apoptosis or to have antiapoptotic effects, for instance promoting the release of IL2 [38–40]. On primary human CD4$^+$ T cells, Tat immobilized on solid support, but not high concentrations of soluble Tat, was shown to mediate IL2 production [41,42]. In contrast, we showed here that soluble Tat, used at physiological concentrations [43], induced IL2 production in primary human CD4$^+$ T cells. Thus, our data would argue against a direct effect of Tat on T-cell death as the main mechanism of CD4$^+$ T-cell depletion.

Tat does not promote the exit from a quiescent state of resting lymphocytes, thus probably not affecting viral reservoirs [44]. However, in activated T helper lymphocytes, it favors the production of IL2, IFNγ and of TNFα, whose plasmatic levels are increased in HIV-infected individuals [45,46]. Significantly, loss of naïve T cells, accumulation of differentiated lymphocytes and an increased level of proinflammatory cytokines are hallmarks of the accelerated immunosenescence characterizing HIV-infected individuals [47–49]. Our data suggest that Tat may support this phenomenon through the induction of proinflammatory cytokines and differentiation of T cell receptor-stimulated naïve CD4$^+$ T cells toward late stages of differentiation, such as effector memory T cells. Accordingly, Tat has been shown to induce production of IL6 [50], which is associated with immunosenescence [51], reduction of telomerase activity in CD4$^+$ T cells [52] and senescence of bone marrow mesenchymal stem cells [53].

In conclusion, our data suggest that Tat may contribute to the exacerbation of several immune dysfunctions observed during AIDS progression, such as chronic immune activation and premature aging. Therefore, the induction of anti-Tat immune responses by Tat administration can be an effective strategy for restoration of the immune system.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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