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

Running title: Tat induces CD4$^+$ T cell activation

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Abstract

Objective: HIV infection is characterized by several immune dysfunctions, such as chronic activation of the immune system, premature ageing and loss of $CD4^+$ T cell, 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 dysfunctions 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 TCR engagement in the presence or absence of Tat. Cytokine production, surface phenotype and expression of transcription factors important for T cell programming were measured. Purified Naïve $CD4^+$ T cells were cultured in non-polarizing conditions in the presence or absence of Tat and their proliferation and differentiation was evaluated.

Results: Tat favors the secretion of IL-2, IFNγ and TNFα in $CD4^+$ T cells, as well as the up-regulation 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 the recruitment into the effector memory T cell pool.

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

Keywords: HIV, Tat, CD4, immune activation, T cell programming

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 presently 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 towards 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, that 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 minutes 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, Bergish Gladbach, Germany) according to manufacturer's instructions and cultured, as detailed in supplemental information, in the absence or presence of the Tat protein in 24-well flat bottomed polystyrene plates pre-coated overnight at 4 °C with PBS or anti-CD3 mAb (0.5 μg/ml; R&D Systems, MN, USA). Naïve $CD4^+$ T cells were cultured in non-polarizing condition as previously described [22] and detailed in supplemental information.

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.

Gene expression analysis

Gene expression was evaluated by qPCR as detailed in supplemental information.

Results

Tat enhances $CD4^+$ T cell activation

The HIV-1 Tat protein, which is released by infected cells, enhances the production of pro-inflammatory 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 hours 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 (Figure S1). This result was confirmed by cytokine intracellular staining of the cells that demonstrated increased production of IL2, IFNγ and TNFα (Fig. 1b) after 18 hours of treatment with Tat when compared to 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). Since 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, whereas it increased significantly the expression of T-bet and Eomes transcription factors in CD3/CD28 activated $CD4^+$ T cells as compared to $CD4^+$ 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 pro-inflammatory cytokines in activated $CD4^+$ T cells, and influences the expression of transcription factors crucial for T cell programming 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 programming, 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 non-polarizing (NP) conditions 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, NP conditions induced 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 till day 18. To determine whether Tat affected the differentiation of naïve CD4+ T cells cultured in NP conditions, the phenotype of T helper lymphocytes was assessed. Overall, NP conditions prompted the loss of CD45RA expression (Fig. 2b), suggesting a shift towards a non-naïve phenotype that had started by day 12, with a more pronounced downregulation by day 18. Interestingly, this phenomenon was more pronounced

in the presence of Tat. In fact, higher numbers of central memory (CM, CD45RA$^-$, CCR7$^+$, CD27$^+$), transitional memory (TM, CD45RA$^-$, CCR7$^-$, CD27$^+$) and effector memory (EM, CD45RA$^-$, CCR7$^-$, CD27$^-$) CD4$^+$ T cells were generated in the presence of Tat as compared to NP conditions alone (Fig. 2c). It is noteworthy that EM CD4$^+$ T cells were almost absent in cultures derived from naïve CD4$^+$ T cells activated under NP conditions, 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 pro-inflammatory cytokines and 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 non-polarizing conditions. 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 lines 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 antiretroviral therapy [36], whose success is dependent by 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 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 anti-apoptotic 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], induces 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]. Interestingly, loss of naïve T cells, accumulation of differentiated lymphocytes and increased level of pro-inflammatory 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 pro-inflammatory cytokines and differentiation of TCR-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 conclusions, 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 ageing. Therefore, the induction of anti-Tat immune responses by Tat administration can be an effective strategy for restoration of the immune system.

Acknowledgments

FN and RG conceived and designed the experiments and analyzed the data. FN, FS, EG, VF and MC performed the experiments. FN, A. Cafaro, A. Caputo, CG, BE and RG wrote the manuscript.

Legends

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 hours, IL-2, IFNγ, TNFα (a), T-bet and Eomes (c) mRNA levels were quantified by qPCR and normalized to untreated cells. (b) PBLs 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 hours, 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 to Tat-untreated activated control cells.

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 7 (left) and means +/- SEM of data normalized to day 0 (right) 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 7 (left, expressed as histogram plot) and means +/- SEM of data normalized to baseline levels (right) are shown (n=7). (c) Percentages of different CD4$^+$ T cell subpopulations were calculated at 18 days of culture. Data from 7 healthy donors are presented. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to Tat-untreated control cells.

References

1. Di Mascio M, Sereti I, Matthews LT, Natarajan V, Adelsberger J, Lempicki R*, et al.* **Naive T-cell dynamics in human immunodeficiency virus type 1 infection: effects of highly active antiretroviral therapy provide insights into the mechanisms of naive T-cell depletion.** *J Virol* 2006; **80**:2665-2674.
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. 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.

7. 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.

8. 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.

9. 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.

10. 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.

11. 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.

12. 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.

13. Debaisieux S, Rayne F, Yezid H, Beaumelle B. **The ins and outs of HIV-1 Tat.** *Traffic* 2012; **13**:355-363.

14. 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.

15. Sforza F, Nicoli F, Gallerani E, Finessi V, Reali E, Cafaro A*, et al.* **HIV-1 Tat affects the programming and functionality of human CD8(+) T cells by modulating the expression of T-box transcription factors.** *AIDS* 2014; **28**:1729-1738.

16. 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.

17. Nicoli F, Chachage M, Clowes P, Bauer A, Kowour D, Ensoli B*, et al.* **Association between different anti-Tat antibody isotypes and HIV disease progression: data from an African cohort.** *Bmc Infectious Diseases* 2016; **16:**344.

18. Ensoli F, Cafaro A, Casabianca A, Tripiciano A, Bellino S, Longo O*, et al.* **HIV-1 Tat immunization restores immune homeostasis and attacks the HAART-resistant blood HIV DNA: results of a randomized phase II exploratory clinical trial.** *Retrovirology* 2015; **12**:33.

19. 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.

20. Bellino S, Tripiciano A, Picconi O, Francavilla V, Longo O, Sgadari C*, et al.* **The presence of anti-Tat antibodies in HIV-infected individuals is associated with containment of CD4+ T-cell decay and viral load, and with delay of disease progression: results of a 3-year cohort study.** *Retrovirology* 2014; **11**:49.

21. Zauli G, La Placa M, Vignoli M, Re MC, Gibellini D, Furlini G*, et al.* **An autocrine loop of HIV type-1 Tat protein responsible for the improved survival/proliferation capacity of permanently Tat-transfected cells and required for optimal HIV-1 LTR transactivating activity.** *J Acquir Immune Defic Syndr Hum Retrovirol* 1995; **10**:306-316.

22. Bosque A, Planelles V. **Studies of HIV-1 latency in an ex vivo model that uses primary central memory T cells.** *Methods* 2011; **53**:54-61.

23. 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.

24. 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.

25. Stienne C, Michieletto MF, Benamar M, Carrie N, Bernard I, Nguyen XH*, et al.* **Foxo3 Transcription Factor Drives Pathogenic T Helper 1 Differentiation by Inducing the Expression of Eomes.** *Immunity* 2016; **45**:774-787.

26. Szabo SJ, Sullivan BM, Stemmann C, Satoskar AR, Sleckman BP, Glimcher LH. **Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells.** *Science* 2002; **295**:338-342.

27. Hazenberg MD, Hamann D, Schuitemaker H, Miedema F. **T cell depletion in HIV-1 infection: how CD4+ T cells go out of stock.** *Nat Immunol* 2000; **1**:285-289.

28. Sicurella M, Nicoli F, Gallerani E, Volpi I, Berto E, Finessi V*, et al.* **An attenuated Herpes Simplex Virus type 1 (HSV1) encoding the HIV-1 Tat protein protects mice from a deadly mucosal HSV1 challenge.** *PLoS One* 2014; **9**:e100844.

29. Robbins GK, Spritzler JG, Chan ES, Asmuth DM, Gandhi RT, Rodriguez BA*, et al.* **Incomplete reconstitution of T cell subsets on combination antiretroviral therapy in the AIDS Clinical Trials Group protocol 384.** *Clin Infect Dis* 2009; **48**:350-361.

30. d'Ettorre G, Paiardini M, Ceccarelli G, Silvestri G, Vullo V. **HIV-associated immune activation: from bench to bedside.** *AIDS Res Hum Retroviruses* 2011; **27**:355-364.

31. Kamata M, Nagaoka Y, Chen IS. **Reassessing the role of APOBEC3G in human immunodeficiency virus type 1 infection of quiescent CD4+ T-cells.** *PLoS Pathog* 2009; **5**:e1000342.

32. Zack JA, Kim SG, Vatakis DN. **HIV restriction in quiescent CD4(+) T cells.** *Retrovirology* 2013; **10**:37.

33. Alanio C, Nicoli F, Sultanik P, Flecken T, Perot B, Duffy D*, et al.* **Bystander hyperactivation of preimmune CD8+ T cells in chronic HCV patients.** *Elife* 2015; **4:**e07916.

34. Li CJ, Ueda Y, Shi B, Borodyansky L, Huang L, Li YZ*, et al.* **Tat protein induces self-perpetuating permissivity for productive HIV-1 infection.** *Proc Natl Acad Sci U S A* 1997; **94**:8116-8120.

35. Secchiero P, Zella D, Capitani S, Gallo RC, Zauli G. **Extracellular HIV-1 tat protein up-regulates the expression of surface CXC-chemokine receptor 4 in resting CD4+ T cells.** *J Immunol* 1999; **162**:2427-2431.

36. Johnson TP, Patel K, Johnson KR, Maric D, Calabresi PA, Hasbun R*, et al.* **Induction of IL-17 and nonclassical T-cell activation by HIV-Tat protein.** *Proc Natl Acad Sci U S A* 2013; **110**:13588-13593.

37. Schacker TW, Bosch RJ, Bennett K, Pollard R, Robbins GK, Collier AC*, et al.* **Measurement of naive CD4 cells reliably predicts potential for immune reconstitution in HIV.** *J Acquir Immune Defic Syndr* 2010; **54**:59-62.

38. Gibellini D, Caputo A, Celeghini C, Bassini A, La Placa M, Capitani S*, et al.* **Tat-expressing Jurkat cells show an increased resistance to different apoptotic stimuli, including acute human immunodeficiency virus-type 1 (HIV-1) infection.** *Br J Haematol* 1995; **89**:24-33.

39. Chen D, Wang M, Zhou S, Zhou Q. **HIV-1 Tat targets microtubules to induce apoptosis, a process promoted by the pro-apoptotic Bcl-2 relative Bim.** *EMBO J* 2002; **21**:6801-6810.

40. Gibellini D, Bassini A, Pierpaoli S, Bertolaso L, Milani D, Capitani S*, et al.* **Extracellular HIV-1 Tat protein induces the rapid Ser133 phosphorylation and activation of CREB transcription factor in both Jurkat lymphoblastoid T cells and primary peripheral blood mononuclear cells.** *J Immunol* 1998; **160**:3891-3898.

41. 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.

42. 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.

43. 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.

44. Nicoli F, Sforza F, Gavioli R. **Different expression of Blimp-1 in HIV infection may be used to monitor disease progression and provide a clue to reduce immune activation and viral reservoirs.** *AIDS* 2015; **29**:133-134.

45. Haissman JM, Vestergaard LS, Sembuche S, Erikstrup C, Mmbando B, Mtullu S*, et al.* **Plasma cytokine levels in Tanzanian HIV-1-infected adults and the effect of antiretroviral treatment.** *J Acquir Immune Defic Syndr* 2009; **52**:493-497.

46. Cervia JS, Chantry CJ, Hughes MD, Alvero C, Meyer WA, 3rd, Hodge J*, et al.* **Associations of proinflammatory cytokine levels with lipid profiles, growth, and body composition in HIV-infected children initiating or changing antiretroviral therapy.** *Pediatr Infect Dis J* 2010; **29**:1118-1122.

47. Appay V, Fastenackels S, Katlama C, Ait-Mohand H, Schneider L, Guihot A*, et al.* **Old age and anti-cytomegalovirus immunity are associated with altered T-cell reconstitution in HIV-1-infected patients.** *AIDS* 2011; **25**:1813-1822.

48. Appay V, Almeida JR, Sauce D, Autran B, Papagno L. **Accelerated immune senescence and HIV-1 infection.** *Exp Gerontol* 2007; **42**:432-437.

49. Appay V, Kelleher AD. **Immune activation and immune aging in HIV infection.** *Curr Opin HIV AIDS* 2016; **11**:242-249.

50. Zauli G, Furlini G, Re MC, Milani D, Capitani S, La Placa M. **Human immunodeficiency virus type 1 (HIV-1) tat-protein stimulates the production of interleukin-6 (IL-6) by peripheral blood monocytes.** *New Microbiol* 1993; **16**:115-120.

51. Franceschi C, Bonafe M, Valensin S, Olivieri F, De Luca M, Ottaviani E*, et al.* **Inflamm-aging. An evolutionary perspective on immunosenescence.** *Ann N Y Acad Sci* 2000; **908**:244-254.

52. Comandini A, Naro C, Adamo R, Akbar AN, Lanna A, Bonmassar E*, et al.* **Molecular mechanisms involved in HIV-1-Tat mediated inhibition of telomerase activity in human CD4(+) T lymphocytes.** *Mol Immunol* 2013; **54**:181-192.

53. Beaupere C, Garcia M, Larghero J, Feve B, Capeau J, Lagathu C. **The HIV proteins Tat and Nef promote human bone marrow mesenchymal stem cell senescence and alter osteoblastic differentiation.** *Aging Cell* 2015; **14**:534-546.