**REVIEW**

# New insights into pathogenesis point to HIV-1 Tat as a key vaccine target

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## Abstract

Despite over 30 years of enormous effort and progress in the field, no preventative and/or therapeutic vaccines against human immunodeficiency virus (HIV) are available. Here, we briefly summarize the vaccine strategies and vaccine candidates that in recent years advanced to efficacy trials with mostly unsatisfactory results. Next, we discuss a novel and somewhat contrarian approach based on biological and epidemiological evidence, which led us to choose the HIV protein Tat for the development of preventive and therapeutic HIV vaccines. Toward this goal, we review here the role of Tat in the virus life cycle as well as experimental and epidemiological evidence supporting its key role in the natural history of HIV infection and comorbidities. We then discuss the preclinical and clinical development of a Tat therapeutic vaccine, which, by improving the functionality and homeostasis of the immune system and by reducing the viral reservoir in virologically suppressed vaccinees, helps to establish key determinants for intensification of combination antiretroviral therapy (cART) and a functional cure. Future developments and potential applications of the Tat therapeutic vaccine are also discussed, as well as the rationale for its use in preventative strategies. We hope this contribution will lead to a reconsideration of the current paradigms for the development of HIV/AIDS vaccines, with a focus on targeting of viral proteins with key roles in HIV pathogenesis.

## Introduction

### Exploring vaccine development strategies and predicting vaccine efficacy

The human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) epidemic is still a major global health challenge, with 38.0 million people living with HIV (PLWH) in 2019, and 1.7 million newly infected (https://www.unaids.org/en/resources/presscentre/pressreleaseandstatementarchive/2020/july/20200706_global-aids-report). Thus, there is an urgent need to implement effective strategies to prevent and cure HIV infection and reduce virus transmission [1].

Although combination antiretroviral therapy (cART) is effective in suppressing the virus and reducing mortality, it does not eradicate HIV, which invariably rebounds upon suspension of treatment due to virus release by a small pool of

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long-lived cells harboring latent provirus, which are insensitive to cART and invisible to the immune system [2]. This hampers cART effectiveness in containing HIV transmission and spread [3].

Furthermore, despite great efforts and major advancements in our understanding of HIV pathogenesis and in vaccine development, effective vaccines against HIV/AIDS are still lacking.

The challenges for developing a vaccine against HIV stem from (i) the high rate of intra- and inter-individual HIV genetic evolution, leading to the unceasing emergence of new (escape) variants, (ii) the failure to identify strong and unequivocal correlates of protection in studies of natural infection as well as in vaccine studies, and (iii) the knowledge gap regarding the degree of protection conferred by the immune response to antigenic epitopes present in the transmitted/founder viruses versus viral variants emerging during chronic infection.

The first vaccine candidates largely focused on the glycoproteins of the virus envelope, which mediate the attachment and entry of HIV into the cell (gp120 or gp160) [4, 5]. AIDSVAX was the first of the Env-based vaccines to be tested in efficacy trials (VAX 003, NCT 00006327 and VAX 004, NCT 00002441). However, AIDSVAX failed to induce a protective, neutralizing humoral response, most likely due to the high variability of Env in the circulating virus variants [6, 7]. These failures shifted the focus to cell-mediated immunity, owing to an accumulating body of evidence indicating a major role for CD8$^+$ T cells in controlling HIV infection [8–12]. Vaccines aimed at inducing effective cellular responses were based on live recombinant viral vectors, mainly pox and adenovirus vectors, and/or DNA consisting of the *gag, pol*, and *nef* genes from clade B or clade C HIV, with or without Env [13, 14]. Although all approaches induced measurable cell-mediated immune responses, they had to be halted due to the greater number of HIV-1 infections occurring in vaccinees as compared to placebo, as observed in the STEP (HVTN 502, NCT00095576) and Phambili (HVTN 503, NCT 00413725) trials, or they did not show efficacy (HVTN 505, NCT00865566) [4, 15–19]. The vectors used to deliver the HIV proteins are believed to have contributed to vaccine failure [20].

Expectations were then raised by the RV144 (NCT00223080) trial utilizing a recombinant canarypox vector expressing Env from clade E and Gag and the HIV protease from clade B HIV (the ALVAC-HIV vCP1521 vaccine antigen component), followed by a boost with an alum-adjuvanted subunit HIV gp120 from clades B and E (the AIDSVAX B/E vaccine antigen component). The vaccine induced a humoral immune response against gp120 and antibody-dependent cellular cytotoxicity that was able to confer protection from infection in 31.2% of the vaccinated volunteers [21], leading to the design of the HVTN 702

trial (NCT 02968849) conducted in South Africa, which, however, did not show efficacy (https://www.niaid.nih.gov/news-events/experimental-hiv-vaccine-regimen-ineffective-preventing-hiv).

Concurrently, major efforts and resources were devoted to the isolation and characterization of broadly neutralizing antibodies (bNAbs) from infected patients [22]. It turned out that, in general, bNAbs have a very uncommon structure, are often polyreactive and autoreactive, possibly the result of escape from immune selection of the B cell repertoire, as also suggested by the lack of germline precursors in the B cell repertoire [23]. Accordingly, immunogens structurally designed to optimally bind bNAbs failed to induce them *in vivo* [24–26]. These obstacles are so difficult to overcome [27] that the efficacy of bNAbs is presently being evaluated in passive immunization strategies [28].

More recently, a computational methodology was developed to generate polyvalent mosaic immunogens starting from the sequences of naturally occurring variants of common T cell epitopes that may ensure coverage of HIV diversity [29]. An example of this "mosaic" approach is the Ad26/Ad26 plus gp140 HIV-1 vaccine, which was recently shown to induce robust humoral and cellular immune responses in a phase 1/2a clinical trial, and it is being evaluated in a phase 2b efficacy study in sub-Saharan Africa (NCT03060629).

Efforts have also been directed to optimization of analysis of vaccine trial results with the aim of identifying correlates of protection. Among these, the COMPASS (Computerized Optimization Model for Predicting and Analyzing Support Scenarios) analysis, a bioinformatics tool to identify antigen-specific T cell responses and subsets, was recently used to investigate vaccine-induced immune responses leading to the identification of new "correlates of protection/immunity" previously missed in the RV144 HIV vaccine efficacy trial [30, 31].

Failures in HIV preventative and therapeutic vaccine strategies have led us to focus on alternative strategies using a "pathogenetic" approach aimed at targeting the HIV Tat protein, alone or combined with other HIV antigens, for eliciting protective immunity for preventative and therapeutic vaccination of untreated or cART-treated patients.

## Tat and HIV pathogenesis

### Role of Tat in the virus life cycle

#### Tat-mediated effects on HIV-1 infectivity, replication, and transmission

The HIV Tat protein is a potent transcriptional transactivator of virus gene expression that is essential for virus infectivity, replication, and transmission (Fig. 1). Tat is incorporated
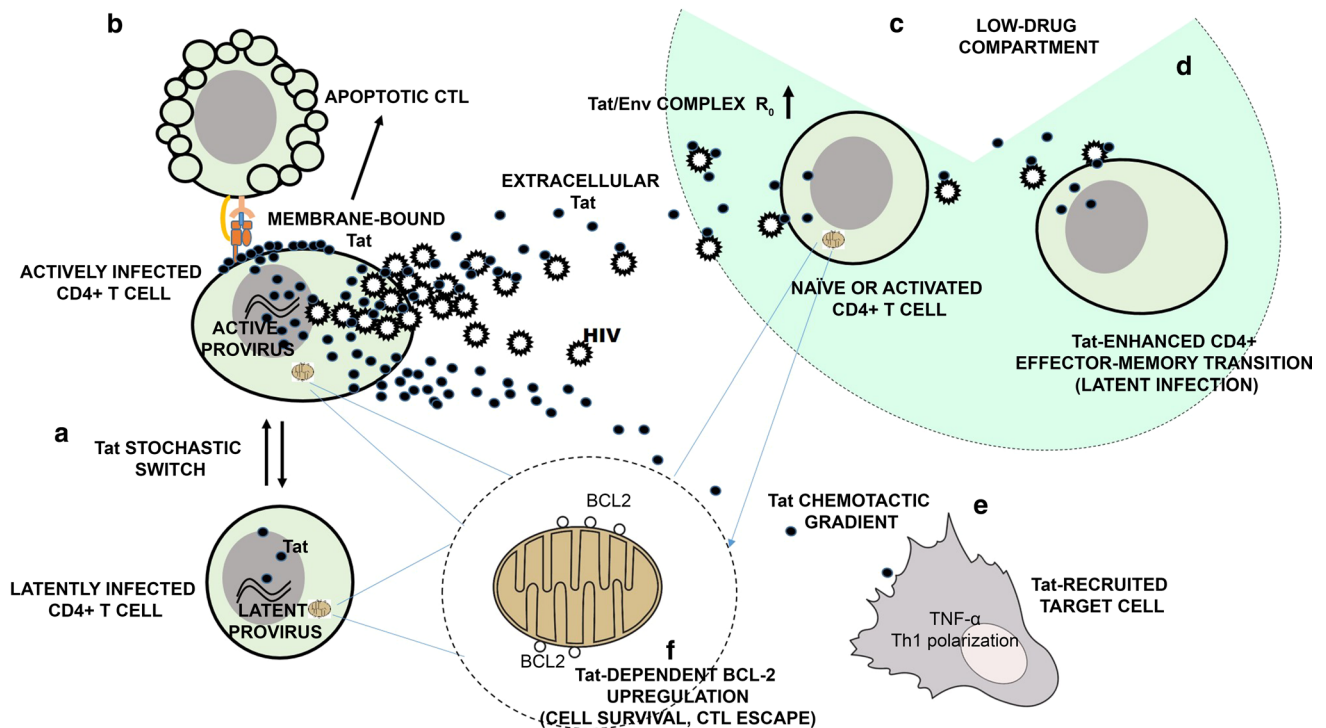
**Fig. 1** Roles of extracellular Tat in HIV in virus life cycle and in the latent virus reservoir. **A** Stochastic oscillations of HIV-1 Tat protein expression (the Tat stochastic switch) determine the fate of HIV infection [40]; **B** Membrane-bond extracellular Tat induces CTL apoptosis [64]; **C** Extracellular Tat binds Env to form a HIV entry complex [35] that increases virus infectivity and the HIV basic reproduction number ($R_0$), thus increasing the chances of infection in tissue compartments with low-drug penetration; at the same time, extracellular Tat induces in naïve CD4+ T cells a non-classical activation pathway rendering these cells susceptible to HIV infection [54];

**D** Extracellular Tat enhances the expansion and differentiation of naïve CD4+ T cells into effector-memory cells [75], thus increasing the number of cells transitioning from the activated to the resting state, a cell state that favors latent infection [94]; **E** Extracellular Tat forms chemotactic gradients [46; 48, 49] recruiting monocytes/macrophages and dendritic cells to the site of infection (51), induces the release of pro-inflammatory cytokines [59–63] and the maturation of dendritic cells with a Th-1 polarization [52]; **F** Tat upregulates BCL2 in CD4+ T cells, leading to increased reservoir cell survival [93] while rendering these cells resistant to CTL killing [94]

into HIV-1 virions [32] to prime both endogenous (intra-virion) and post-entry reverse transcription [33], to activate virus gene expression, and to form a complex with HIV-1 Env to enhance virus entry and spread [34, 35].

Tat is expressed very early upon infection, even prior to virus integration [36], most likely to ensure that the transcriptional activity of the incoming virus can cross the extinction threshold. Tat expression, in fact, enhances both transcription initiation, which is mediated by its interaction with SP1 elements in the HIV-1 transcriptional promoter [37, 38], and transcription elongation, which involves Tat-mediated recruitment of positive transcription elongation factor b (P-TEFb) to the transactivation responsive region (TAR) of the nascent RNA [39]. By these means, Tat increases viral transcription by 100-fold and establishes a strong positive transcriptional feedback loop at the HIV-1 long terminal repeat (LTR) [40, 41]. Notably, the early expression of Tat appears to enhance, rather than stabilize,

basal transcriptional fluctuations that occur naturally at the HIV LTR. As a consequence, Tat expression itself is driven into amplified stochastic oscillations around the extinction threshold [40, 41]. As described in the next section, this pattern of Tat expression plays a major role in determining whether infected cells enter a state of active replication or latency [40, 41]. In addition to these activities, Tat expression downregulates cell membrane expression of major histocompatibility complex (MHC) class I [42] and II [43] surface molecules, damping cell-mediated adaptive immunity against HIV. Moreover, in infected dendritic cells (DCs), Tat induces the expression of several chemokines, including interferon-gamma-induced protein 10 (IP-10, CXCL10), human monokine induced by interferon gamma (huMIG, CXCL9), and monocyte chemotactic protein 2 (MCP-2, CCL8), attracting activated T cells and macrophages to the sites of infection, thus facilitating the dissemination of HIV infection [44].

## Role of extracellular Tat in HIV-1 infectivity and virus spread

A large proportion of the Tat protein produced by the infected cell is released in the absence of cell death or cell permeability changes in the extracellular milieu [45–47] to generate a local reservoir of extracellular Tat, which plays a key role in the establishment and propagation of HIV infection. In fact, once released, Tat binds through its basic region to heparan sulfate proteoglycans (HSPG) present in the extracellular matrix [45, 46, 48, 49], generating long-lasting chemotactic gradients that attract HIV-1 target cells, particularly, activated endothelial cells and monocytes/macrophages, to the sites of infection [50, 51]. In addition, extracellular Tat enters dendritic cells [52] and activated endothelial cells [53] very efficiently and, to a lesser extent, CD4+ T cells [54], activating the expression of genes favoring HIV dissemination. In particular, in naïve CD4+ T cells, Tat upregulates the expression of the HIV-1 co-receptors C-X-C motif chemokine receptor 4 (CXCR4) and C-C chemokine receptor type 5 (CCR5) [55], while antagonizing CXCR4 [56], thus increasing the susceptibility of resting CD4+ T cells to infection by R5-tropic HIV-1 strains. Furthermore, extracellular Tat induces a non-classical activation pathway in naïve CD4+ T cells, rendering them susceptible to productive infection [54, 57]. Notably, extracellular Tat binds trimeric HIV-1 Env, forming a cell entry complex (the Tat/Env complex) that targets the RGD-binding integrins expressed at high levels by dendritic cells, enhancing the infection of these cells [34, 35]. Furthermore, extracellular Tat induces dendritic cells to mature to a T helper (Th)-1-polarizing phenotype [58] and stimulates the production and release of inflammatory cytokines by various cell types [59–63]. Of note, inflammatory cytokines activate endothelial cells and render them susceptible to Tat entry and permissive to productive HIV infection [53]. In addition, extracellular Tat bound to the HSPG of the infected cell membrane triggers the apoptotic death of engaged CD8+ T cell effectors [64]. Thus, extracellular Tat contributes to modeling a tissue microenvironment favoring the recruitment, infection, and immune escape of HIV-1 target cells. All of these activities of extracellular Tat contribute to ensuring that the basic reproduction number ($R_0$) of HIV remains > 1 both upon virus acquisition at the portal of entry and during chronic infection.

## Role of the Tat feedback transcriptional loop in the "choice" between latent and active infection

HIV-1 infection is characterized by the early establishment of a self-renewing, large pool of short-lived, actively infected cells producing viral progeny. However, concomitant latent infection occurs in a minority of cells that show prolonged longevity and are maintained for a long period of time. As described previously, the choice between productive and latent infection of CD4+ T cells appears to be determined by stochastic oscillations in the expression levels of Tat around the threshold of viral transcriptional extinction [41]. This modality of Tat expression (the "Tat stochastic switch") (Fig. 1) appears to operate irrespectively of the state of cell activation [41]. Nevertheless, the activation or deactivation of the HIV-1 transcriptional promoter by environmental or epigenetic stimuli can increase or decrease the probability ratio between latent and active infection [41]. The Tat stochastic switch likely explains several characteristic features of HIV infection both *in vitro* and in clinical studies. In particular: (i) distinct pools of latently and productively infected cells are simultaneously established upon experimental infection of activated or resting CD4+ T cells [65–67]; (ii) clonal CD4+ T cell populations harboring the same provirus at the same integration site show a 'bifurcating' expression pattern, where HIV expression is high in some cells and low in others [40]; (iii) polyclonal TCR stimulators stochastically reactivate HIV in only a small number of cells in the latently infected pool present in cultures of CD4+ T cells isolated from HIV-infected patients [68, 69]; (iv) a spontaneous fluctuation between a latent and productive infection phenotype is observed in clonal cultures of infected CD4+ T cell lines [40]; (v) virus rebound in patients undergoing analytical treatment interruption follows a stochastic, exponential distribution [70]; (vi) the "shock and kill" strategy to cure HIV, based on the use of HIV latency-reversing agents believed to deterministically reactivate latent HIV, has failed to reduce the HIV reservoirs [71]; and (vii) HIV reactivation in latently infected CD4+ T cells is virtually abolished by treatment with compounds that block the interaction of Tat with TAR, a finding that has led to the development of strategies to permanently shut off HIV transcription (the "block and lock" strategy) [72].

## Role of extracellular Tat in the dynamics of latent HIV reservoirs

The small pool of long-lived cells harboring latent HIV persists indefinitely in infected individuals despite cART. Sporadic reactivation of latent provirus results in waves of virus production that, although it may not succeed in infecting other cells due to antiretroviral treatment, can give rise to intermittent low-level viremia that persists even after years of treatment [73]; if cART is suspended, low-level viremia leads to virus rebound [74]. Although several cell types can harbor latent or silent HIV, resting naïve and memory CD4+ T cells constitute the most prominent HIV reservoir [73]. Considering the capability of extracellular Tat to target and enter CD4+ T cells [54], to stimulate the expression of host cell genes [54, 55, 75], and to reactivate latent provirus [40, 45, 76], it

can be speculated that extracellular Tat plays a prominent role in the dynamics of HIV reservoirs. This conclusion is supported by the faster kinetics of proviral DNA decay observed in individuals on long-term cART immunized with a Tat vaccine [77, 78], suggesting that immunity against extracellular Tat, the vaccine target, may destabilize the HIV reservoirs. The following lines of evidence point to the possible mechanism(s) underlying this effect of Tat immunization.

Despite lack of evidence of virus evolution during long-term cART [79–84], indirect evidence indicates that ongoing cycles of virus replication may indeed occur in low-drug lymphoid tissue compartments, replenishing the HIV reservoirs [85–89]. However, HIV reproduction in these infection niches would be prone to extinction due to the reduction of $R_0$ caused by the suboptimal drug levels of the microenvironment. Hence, the enhancement of infectivity conferred to HIV by the Tat/Env complex, which appears to be particularly effective at a low multiplicity of infection [35], would conceivably be of key importance for virus reproduction in these tissues. Since anti-Tat Abs produced during natural infection or induced by Tat immunization neutralize the Tat/Env complex [35], these data suggest that immunization with Tat might blunt this potential pathway of reservoir replenishment. $CD4^+$ T cells undergoing the effector-to-memory transition may be pivotal in this context. These cells have been shown to be particularly susceptible to latent HIV infection due to the transient upregulation of the HIV co-receptor CCR5 and the concomitant downregulation of cellular and proviral gene expression [90]. Hence, activated $CD4^+$ T cells transitioning to the resting state represent a key target to enrich latent-HIV reproduction in compartments with suboptimal drug levels. Since extracellular Tat is known to enhance the expansion and differentiation of naïve $CD4^+$ T cells towards the effector-memory phenotype [75], we speculate that immunization with Tat may decrease the density of transitioning cells in these tissue niches. Moreover, extracellular Tat delays Fas-mediated apoptosis in infected $CD4^+$ T cells [91] and upregulates the anti-apoptotic gene B-cell lymphoma 2 (Bcl-2) in peripheral blood mononuclear cells (PBMCs) and $CD4^+$ T cells, as well as endothelial cells [92], promoting their survival [93]. Notably, recent findings indicate that Bcl-2 upregulation renders reservoir $CD4^+$ T cells resistant to cytotoxic T lymphocyte (CTL) killing [94]. Thus, extracellular Tat may prolong the half-life of latently infected $CD4^+$ T cells, which, conversely, would be decreased by anti-Tat Abs induced by Tat immunization. Finally, anti-Tat Abs would abrogate the capacity of cell-membrane-bound Tat to induce the apoptosis of $CD8^+$ T cell effectors [64], restoring, at least in part, the effectiveness of cell-mediated immunity against the virus reservoirs.

Figure 1 summarizes the main pathways through which HIV-1 Tat regulates the virus life cycle and the genesis and maintenance of the virus reservoirs.

## Tat and comorbidities

The experimental and epidemiological data reported above indicate that Tat is an optimal target for HIV cure and eradication strategies. However, a Tat-based therapeutic immunization may have other beneficial effects against HIV/AIDS, since *in vitro* and *in vivo* evidence indicates that HIV-1 Tat plays a role in the increased incidence and aggressiveness of several AIDS- and non-AIDS-defining comorbidities, including tumors and cardiovascular, renal, liver and neurological diseases. The development and progression of these comorbidities are attributed to the combined effects of reduced immune surveillance, immune activation, immune dysregulation, and viral coinfections (Epstein-Barr virus [EBV], human herpesvirus 8 [HHV-8], human papillomavirus [HPV], hepatitis B virus, hepatitis C virus) characterizing HIV infection [95].

Of note, the introduction of highly effective new antiretrovirals and new drug combinations capable of suppressing viral replication has greatly reduced the burden of most AIDS-related complications by restoring and preserving immune function from the decline associated with HIV infection, with a general increase in quality and expectancy of life [96]. However, since cART does not eliminate non-replicating virus, life-long cART is essential for insuring viral suppression. Moreover, even under fully successful therapy, residual viral replication and/or gene transcription persist [97, 98], particularly in lymphoid tissues with suboptimal antiviral drug penetration [89, 99]. As a result, chronic inflammation and immune dysregulation persist, leading to premature aging and a much higher incidence and mortality rate for chronic noninfectious comorbidities as compared to the general population, particularly in patients starting cART with very low $CD4^+$ T cell counts or in those who are poorly compliant with therapy [96]. An increased life expectancy, longer exposure to risk factors, and drug toxicity also correlate with the appearance of non AIDS-related comorbidities. However, although cART reduces the risk for AIDS-defining comorbidities, including tumors and opportunistic infections, their incidence is still higher than in the general population and can also occur in patients responding to therapy [96, 100].

In this context, experimental evidence indicates that HIV-1 Tat may play a direct pathogenetic role in HIV-associated comorbidities by modulating the cellular pathways that lead to their development and/or progression. Although a comprehensive examination of these data is outside of the scope of this review, some of these direct effects are briefly described below and summarized in Table 1.

**Table 1** Evidence indicating that the HIV-1 Tat protein is directly involved in the increased incidence and severity of comorbidities and coinfections affecting PLWH

| | Direct effects of Tat | References |
|---|---|---|
| **AIDS-associated tumors** | | |
| Kaposi's sarcoma | Proliferation, migration and invasion of KS cells | [48, 49, 92, 101–110] |
| | Recruitment of HHV-8-infected circulating KS cells; HHV-8 reactivation from latency; increase of tumor progression induced by HHV-8-encoded oncoproteins | |
| | Induction of inflammatory cytokines/adhesion marker expression in endothelial cells and KS cells | |
| | Induction of inflammatory cell adhesion and extravasation | |
| | Induction of angiogenesis and vascular permeability in synergy with bFGF or inflammatory cytokines, by activating MMPs and cell-cycle progression in endothelial cells | |
| | Increased survival by promoting Bcl-2 expression in endothelial cells | |
| Cervical carcinoma | Disruption of epithelial tight junctions and HPV entry into epithelium by activation of integrin and MAPK signaling | [111–116] |
| | Transactivation of the HPV long control region and increase of HPV-E7 expression | |
| | Upregulation of HPV-E6 expression and decrease of p53 protein levels | |
| Lymphoproliferative disorders | Enhancement of EBV-driven B cell proliferation | [117–122] |
| | Induction of mutagenic DNA repair h-polymerase | |
| | Repositioning of the MYC locus near the immunoglobulin heavy chain gene locus in B cells with increased risk of chromosomal translocation | |
| | High rate of mutation leading to B cell malignancies | |
| **Comorbidities** | | |
| Cardiovascular diseases | Modulation of adhesion molecules expression, with increased vascular adhesion of monocytes and endothelial dysfunction associated with atherosclerosis, coronary artery disease, myocarditis, and hypertrophic cardiomyopathy | [123–126] |
| | Decrease of endothelium-dependent vasorelaxation and endothelial NOS production in coronary arteries | |
| Nephropathy | Increase of glomerular permeability by altering cytoskeleton organization and nephrin distribution in human podocytes | [127, 128] |
| | Renal expression of HIV genes, bFGF and VEGF-A | |
| Osteoporosis | Reduced differentiation of bone marrow osteoblast precursor stem cells into osteoblastic cells | [129–131] |
| | Upregulation of osteoclast bone resorption | |
| | Enhanced osteoclast differentiation | |
| Enteropathy | Pro-oxidant and pro-apoptotic effects on colonic epithelial cells and consequent disruption of intestinal barrier integrity | [132–134] |
| | Direct anti-proliferative effects on enterocytes, with intestinal mucosal atrophy | |
| | Increased excitability of the enteric nervous system | |
| Pulmonary disease | Suppression of CFTR with alteration of mucociliary clearance | [135–137] |
| | Release of reactive oxygen species and activation of platelet-derived growth factor with development of pulmonary hypertension | |
| CNS neuropathy | Disruption of the blood-brain barrier and neuronal synapses | [126, 128, 138–141] |
| | Increased expression of inducible NOS and NO release in astrocytes, microglia, and brain endothelial cells, with induction of oxidative stress, mitochondrial injury, and inflammation | |
| | Involvement in the development of HIV-1-associated neurocognitive disorders | |

**Table 1** (continued)

| | Direct effects of Tat | References |
|---|---|---|
| Chronic immune activation and aging | Involvement in production of proinflammatory cytokines by dendritic cells and monocytes/macrophages | [62, 63, 75, 142–144] |
| | Activation of CD4$^+$ T cells, inducing the release of proinflammatory cytokines and the expression of transcription factors such as T-bet and Eomes | |
| | Alteration of functionality of CD8$^+$ T cells through the induction of T-bet, Eomes and Blimp-1 expression | |
| | Immune senescence by impairment of hTERT levels and telomerase activity in CD4$^+$ T cells | |
| Opportunistic infections | | |
| Viruses | Transactivation of heterologous viral promoters (JCV, CMV, HHV-6) directly or indirectly through TNFα-induced NF-κB signaling | [145–151] |
| | Interaction with HSV-1 ICP0 to induce HIV-1 transcription and translation in HIV-1/HSV-1-coinfected cells | |
| | Increased viral replication and/or protein translation in HHV-8, HHV-6, or BKV-infected cells | |
| Bacteria | Increased *Mycobacterium avium* replication efficiency in macrophages through integrin binding and increased production of TGF- β1 | [152–160] |
| | Synergy with *Mycobacterium smegmatis*, BCG, *Mycobacterium avium*, and *Mycobacterium tuberculosis* to enhance HIV-LTR transcription | |
| | Enhanced bacterial proliferation through binding the promoters of genes regulating cell division (*Escherichia coli* and *Pseudomonas aeruginosa*) | |
| Protozoa and fungi | Increased uptake and growth of *Leishmania* and other intracellular parasites by inducing TGF- β1 or IL-10 expression | |
| | Reduced clearance of *Cryptosporidium parvum* by modulation of TLR translation in infected cholangiocytes | |
| | Binding to *Candida albicans* and accelerated germination and elongation | |
| | Increased phagocytosis of Tat-bound and serum-opsonised *Candida albicans* by monocytes | |

Abbreviations: KS, Kaposi's sarcoma; HHV, human herpes virus; bFGF, basic fibroblast growth factor; MMPs, matrix metalloproteinases; bcl-2, B-cell lymphoma 2; HPV, human papillomavirus; MAPK, mitogen-activated protein kinases; EBV, Epstein-Barr virus; JCV, John Cunningham virus; CMV, cytomegalovirus; TNFα, tumor necrosis factor alpha; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; HSV-1, herpes simplex virus 1; BCG, bacillus Calmette–Guérin; TGF- β1, transforming growth factor β1; IL-10, interleukin 10; TLR, Toll-like receptor; VEGF-A, vascular endothelial growth factor A; NOS, nitric oxide synthase; NO, nitric oxide; HIV, human immunodeficiency virus; CFTR, cystic fibrosis transmembrane conductance regulator; T-bet, T-box expressed on T cells; Eomes, eomesodermin; hTERT, human telomerase reverse transcriptase

HIV-1 Tat has been implicated directly in the pathogenesis of AIDS-associated tumors, such as Kaposi's sarcoma (KS), cervical cancer, and lymphoproliferative disorders, while only indirect evidence is available for those tumors whose risk is only modestly increased during HIV infection, such as lung and liver cancer (non-AIDS-defining cancers). In particular, Tat promotes the migration, invasion, and proliferation of KS cells and activated endothelial cells [48, 49, 92, 101–106] (Table 1). This is due to the ability of Tat to mimic and/or enhance the effects of extracellular matrix molecules, which regulate and enhance the effects of angiogenic growth factors on endothelial cell growth and locomotion through RGD-binding integrins that function as Tat receptors [50, 101–103]. Tat also induces angiogenesis and vascular permeability in synergy with angiogenic factors or inflammatory cytokines by activating matrix metalloproteinases (MMPs) [92] and promotes the recruitment of HHV-8-infected circulating KS cells into tissues [103], triggers HHV-8 reactivation from latency, and accelerates tumor progression induced by HHV-8-encoded oncoproteins [107–110] (Table 1).

Moreover, among the various effects in the other AIDS-associated tumors, Tat can also interact with several oncogenes. For example, it upregulates HPV-E6 and -E7 oncoprotein activity in cervical cancer, thus increasing cell proliferation and survival and angiogenesis [111–116], and it promotes Myc repositioning near the immunoglobulin (Ig) heavy chain locus in normal B cells, thus increasing the risk of specific chromosomal translocations during lymphomagenesis [117–122] (Table 1).

Individuals with HIV also have a disproportionate risk of various comorbidities to which the Tat protein appears to contribute. In particular, Tat plays an important role in HIV-related cardiac dysfunction by modulating the expression of adhesion molecules on the endothelium and facilitating the adhesion of monocytes [123]. This leads to vascular inflammation and endothelial dysfunction, which is associated with the pathogenesis of cardiovascular complications affecting PLWH, including atherosclerosis, coronary artery disease, myocarditis, and hypertrophic cardiomyopathy [124–126] (Table 1). Tat may also contribute to functional alterations occurring in HIV-associated nephropathy by altering glomerular permeability and by inducing the redistribution and loss of nephrin in podocytes [127, 128] (Table 1). By interfering with osteoblastic differentiation and enhancing osteoclast differentiation, Tat also contributes to increased osteopenia/osteoporosis affecting PLWH [129–131] (Table 1). Tat also plays a key role in HIV-1-associated intestinal disorders, as it alters the expression of many genes regulating survival and growth of enterocytes and the excitability of the enteric nervous system, contributing to the gut dysfunction that has been described in HIV patients [132–134] (Table 1). Moreover, Tat may be implicated in pulmonary complications in PLWH by altering mucociliary clearance and contributing to the development of pulmonary hypertension [135–137] (Table 1). Tat is also known to induce neurotoxicity by disrupting the blood-brain barrier and neuronal synapses, promoting oxidative stress in astrocytes, microglia, and brain endothelial cells, and activating inflammation, thus contributing to the development of HIV-1-associated neurocognitive disorders [126, 128, 138–141] (Table 1). Finally, Tat is involved in overstimulation of the immune system and production of the high level of inflammatory responses seen in HIV-infected patients, contributing to the exacerbation of several immune dysfunctions observed during disease progression, such as chronic immune activation and premature aging [62, 63, 75, 142–144] (Table 1).

HIV-1 Tat has also been shown to interact with various opportunistic pathogens during AIDS progression, increasing the incidence and severity of infections. In this context, Tat can increase the transcription of the genomes of other viruses, such as John Cunningham virus (JCV) [145], cytomegalovirus (CMV) [145], and HHV-6 [146], either directly, by binding heterologous viral promoters, or indirectly, by activating the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway through its binding to the tumor necrosis factor alpha (TNF-α) promoter (Table 1). Tat also modulates the transcription and translation of herpes simplex viruses, and of HHV-6 and HHV-8 [146–151] (Table 1), which are considered cofactors for the development of several tumors, including nodular sclerosis Hodgkin lymphoma, gastrointestinal cancer, glial tumors, and/or KS.

Moreover, via its RGD motif, Tat can bind integrins present on the surface of pathogens such as mycobacteria and fungi [152, 153] (Table 1). This binding increases mycobacterial replication efficiency and accelerates the germination and elongation of *Candida albicans* to form pseudohyphae, contributing to the increased severity of these opportunistic infections in HIV-infected patients [154–156]. In addition, HIV-1 Tat can cooperate with bacteria to synergistically enhance transcription from the HIV LTR and can regulate some bacterial gene expression and proliferation [157, 158] (Table 1). HIV-1 Tat has also been shown to interact with protozoa of the family Trypanosomatidae [159, 160]. It was reported to induce the uptake of *Leishmania* parasites and to increase their intracellular growth (Table 1). Thus, in HIV coinfections, Tat enhances infection and replication of both pathogens and HIV-1, further worsening the infectious burden in the host.

Taken together, all of these findings provide a strong rationale for targeting Tat in intervention strategies aimed at improving the clinical management and the quality of life of PLWH, even in the cART era.

## The Tat-based concept for HIV vaccine development

### Regulation of immune responses by HIV Tat

The biologically active Tat protein displays immunomodulatory features that make it an attractive antigen, alone or combined with other HIV proteins, for the design of new HIV vaccines [161–165]. In particular, native Tat protein is very efficiently taken up by monocyte-derived dendritic cells and, upon cellular entry, promotes DC maturation and activation, leading to a more efficient presentation of both allogeneic and exogenous antigens and resulting in an increased antigen-specific T cell response [52]. By modifying the catalytic subunit composition of the immunoproteasome, the Tat protein broadens epitope-specific CTL and Th-1 immune responses to heterologous antigens [161–166]. Moreover, through its basic domain, Tat increases the surface expression of peptides complexed with MHC class I molecules [167], whereas dimerization promoted by the cysteine-rich region confers on Tat the capability to trigger strong immune response against itself as well as other antigens [168].

Taken together, these findings clearly demonstrate that Tat is not only an antigen but also a novel and potent immunoregulatory protein capable of promoting Th-1 responses and broadening the spectrum of epitopes recognized by T cells. This property may be important for the design of adjuvant-free Tat-based vaccines, or for using Tat as an immunoregulator to improve the efficacy of other vaccines. It is noteworthy that co-immunization with Env and Gag proteins

together with Tat induces T-cell responses to all of these antigens, ruling out potential interference and providing evidence that a vaccine formulation containing Tat and other relevant HIV target proteins is feasible, and it may have the advantage of controlling virus replication at both the early and late stages of the infection [165]. The inherent ability of Tat to broaden the T cell repertoire may indeed be particularly relevant in vaccination strategies against other viral infections or tumors. In fact, it is widely held that increasing the size and broadening the diversity of T cell responses to a given antigen would increase the efficacy of a vaccine and reduce epitope mutation and CTL escape [165, 166]. In this regard, vaccination with a live attenuated herpes simplex virus (HSV) vector expressing Tat increased memory CD8$^+$ T cell responses against epitopes present in subjects without recurrences, who were therefore called "asymptomatic", and it was considered protective, inducing anti-HSV IgG2a (an indicator of a Th1-driven B cell response) and protecting mice from death after a lethal challenge with wild-type HSV [142, 169–171]. In particular, Tat increases *in vitro* and *in vivo* IFN-γ, granzyme B, and interleukin-2 (IL-2) release [142, 144], and in mice, injection with Tat increases the frequency of HSV1-specific effector memory CD8$^+$ T cells [142], which are associated with asymptomatic HSV infection in humans [172]. Finally, Tat upregulates expression of T-box in T cells (T-bet), Eomes, B lymphocyte-induced maturation protein 1 (Blimp-1), and Bcl-2 in activated CD8$^+$ T cells [144], factors that are upregulated in HSV1-specific CD8$^+$ T cells of asymptomatic but not symptomatic individuals [173]. Thus, inclusion of the tat gene in attenuated or replication-defective HSV1-based vectors is an innovative strategy for development of effective preventive and therapeutic HSV vaccines. Furthermore, administration of Tat to mice acutely infected with *Mycobacterium tuberculosis* (Mtb) significantly reduced leucocyte infiltration and tissue damage in the lung, suggesting a modulatory role for Tat, also in the early response to Mtb, affecting tuberculosis pathogenesis [174].

## Role of the immune response to Tat

### Epidemiological evidence

Epidemiological studies have shown that asymptomatic patients and non-progressors have a higher prevalence of anti-Tat Abs than progressors [175], as shown by a slower and milder evolution of the disease and a better responsiveness to antiretroviral therapy. In particular, the presence of anti-Tat Abs correlates with a significant containment of CD4$^+$ T cell loss and plasma viral load (VL) increases [175–183]. Regarding cell-mediated immunity, CTLs to Tat are frequently detected in asymptomatic HIV-1-infected individuals and have been shown to inversely correlate with

progression to AIDS [184, 185]. Because HIV-infected cells express Tat very early after infection, vaccine-induced anti-Tat CTLs may eliminate infected cells and block HIV infection at an early stage. Furthermore, the detection of anti-Tat CTL escape mutants early after infection in humans highlights the selective pressure that the immune system exerts on Tat and the importance of anti-Tat CTLs early after infection [186, 187]. Thus, the control of HIV replication by CTLs targeted to early proteins, and in particular to Tat, is relevant for the containment of the infection and progression to disease. Indeed, we have very recently reported [188] that, in individuals on long-term ART, anti-Tat immunity was associated with higher nadir CD4$^+$ T-cell counts, control of low-level viremia, and long-lasting CD4$^+$ T cell recovery, but not with decreased immune activation. In particular, individuals with both humoral responses and a high frequency of cell-mediated responses experienced a progressive increase in CD4$^+$ T cells and the CD4$^+$/CD8$^+$ T-cell ratio. These changes were not observed in individuals lacking these responses. Furthermore, the cell-mediated response to Tat, alone or associated with the anti-Tat Ab response, was also found to be predictive of a significant increase in NK or B cells, respectively. Notably, the anti-Tat antibody level was associated with control of very-low-level viremia, suggesting a block of HIV reactivation from virus reservoirs by Abs targeting extracellular Tat [188].

Altogether, these studies indicate that both the cellular and humoral Tat-specific immune response contribute to the control of infection and/or disease progression.

### Preclinical studies with a Tat-based vaccine

Based on the above epidemiological evidence and its key role in the HIV life cycle and disease pathogenesis, the Tat protein was chosen as a vaccine candidate for preclinical and clinical development for the prevention and treatment of HIV-1 infection. In preclinical studies in nonhuman primates, the Tat protein or tat DNA were found to be safe and to elicit a broad and specific immune response, which protected macaques against challenges with the X4-tropic SHIV89.6P [189–194]. In particular, Tat-specific Ab levels and CD4$^+$ and CD8$^+$ T cell responses were high and stable only in the animals that controlled primary infection at its early stages. Of interest, vaccinated and protected macaques did not experience virus rebound upon administration of tetanus toxoid, a stimulus known to activate the immune system and to trigger HIV replication [192]. In addition, vaccinees, but not control animals, were able to contain a second, fivefold higher, intravenous challenge with the same virus upon initial overt infection [193]. Overall, these results indicate that vaccination with Tat had induced long-term memory Tat-specific immune responses, allowing long-term

containment of virus replication and spread in blood and tissues.

Co-immunization with Tat and other HIV antigens, particularly with Env, underscores the contribution of anti-Tat immunity to vaccine efficacy. In particular, no infection or a statistically significant reduction of viral load and proviral DNA was observed in cynomolgus macaques that had been co-immunized with HIV-1 Tat and Env-ΔV2 (an oligomeric gp140 protein deleted of the V2 region) proteins in a complex and challenged intrarectally with a high dose (70 $MID_{50}$) of the R5-tropic SHIV$_{SF162P4cy}$ [35]. Rhesus macaques were immunized mucosally with replicating adenovirus vectors carrying the HIV-1 clade B tat and env transgenes and then boosted at the systemic level with the Tat and Env proteins. All macaques became infected following intravenous challenge with a high dose of homologous SHIV-89.6P. However, macaques vaccinated with Tat/Env displayed a 4-log lower chronic viremia and experienced a mild CD4$^+$ T cell loss as compared to control animals [195]. Furthermore, sterilizing immunity or control of infection was observed in rhesus macaques immunized with a multicomponent vaccine (multimeric HIV-1 clade C gp160, HIV-1 clade B Tat, and SIV Gag-Pol particles) delivered either systemically or mucosally and then challenged with the heterologous HIV-C env SHIV-1157ip [196]. The search for humoral correlates of protection identified anti-Tat Abs against the N-terminus of Tat as a relevant one, a finding corroborated by binding and functional assays [197]. Thus, in monkey models of efficacy, both humoral and cellular anti-Tat immunity plays a role in preventing infection and disease progression. Furthermore, Tat/Env co-immunization studies also indicate a role of Tat in modulating the immunogenicity of Env towards potentially protective responses.

## Clinical development: preventative and therapeutic phase I trials

Based on these results, preventative and therapeutic, double blind, placebo-controlled phase I trials with the biologically active Tat (ISS P-001, ClinicalTrials.gov Identifier: NCT00529698; ISS T-001, ClinicalTrials.gov Identifier: NCT00505401) were conducted in Italy, meeting both primary (safety) and secondary (immunogenicity) endpoints [198–200]. The Tat vaccine was safe and, in the HIV-infected volunteers, did not induce virus replication, as indicated by preservation the of CD4$^+$ T cell counts and by the absence of significant plasma viremia rebounds. The long-term follow-up (ISS OBS P-001, ClinicalTrials.gov Identifier: NCT01024764) showed the persistence of anti-Tat NAbs up to five years after the first immunization [199, 200].

A phase I preventive trial was also conducted with the combination of Tat and V2-loop-deleted Env (ΔV2-Env) in Italy (ISS P-002, ClinicalTrials.gov Identifier:

NCT01441193). This was a multicentric, open-label, phase I trial conducted in healthy volunteers to evaluate the safety and the immunogenicity of the vaccine based on the association of HIV-1 Tat and ΔV2-Env proteins, as compared to vaccination with single proteins. Tat and ΔV2-Env proteins, either in association or as single components, were administered by a prime-boost regimen consisting of three intradermal priming doses followed by two intramuscular boosting injections. The Tat/ΔV2-Env vaccination was safe and immunogenic, as indicated by the development of Ab responses to the vaccine antigen(s) in all participants [Ensoli, unpublished data]. Importantly, while the highest anti-Tat Ab response was detected in the subjects vaccinated with Tat alone, the highest anti-Env Ab responses were recorded in the volunteers co-immunized with Tat/ΔV2-Env, which is consistent with previous studies showing better priming provided by Tat-mediated entry of Env in dendritic cells [165].

## Clinical development: therapeutic phase II trials

Moving forward, the therapeutic vaccination was prioritized over the preventive one as a shorter and more cost-effective route to proof of efficacy [201]. Therapeutic phase II trials for cART intensification were conducted in Italy and South Africa in patients on successful cART. The Italian phase II study (ClinicalTrials.gov Identifier: NCT00751595) was an exploratory phase II open-label therapeutic trial, randomized on the different regimens utilized [77, 78]. It enrolled 168 HIV-infected (B clade) anti-Tat Ab-negative adults on long-term (mean >6 years) cART who were virologically suppressed, with CD4$^+$ T cell counts ≥200 cells/mmc, and were vaccinated with 7.5 or 30 µg of the Tat protein (clade B) without adjuvant, administered intradermally 3 or 5 times, one month apart. Both primary (immunogenicity) and secondary (safety) endpoints were met. No increase in virological biomarkers was observed. The results also showed a reduction in immune activation and durable increases in CD4$^+$ T cells, B cells, NK cells, and CD4$^+$ and CD8$^+$ central memory T cell subsets, with a reduction in effector memory cells, indicating a shift of the immune response towards homeostasis [77]. None of these changes were observed in anti-Tat-Ab-negative subjects on effective cART enrolled in a parallel observational study at the same clinical centers (ISS OBS T-002) (ClinicalTrials.gov Identifier: NCT01024556) [77, 78]. Of note, Tat immunization induced a reduction of HIV-1 DNA load in blood, especially in volunteers receiving 30 µg of Tat, given three times, that continued throughout the 8-year follow-up (ClinicalTrials.gov Identifier: NCT02118168) [78, 202]. Tat-specific cellular responses also contributed to HIV proviral DNA reduction. Furthermore, the induction, upon vaccination, of CD38$^+$/HLA-DR$^+$ CD8$^+$ T cells and natural killer (NK) cells

endowed with killer activity against virus-infected cells may have also contributed to the reduction of the virus reservoir. cART intensification by the Tat vaccine was durable, as the increase in CD4$^+$ T cells persisted during the 8-year follow-up and was accompanied by a progressive, slow decrease in HIV proviral DNA in the peripheral blood, which became undetectable in 34% of all vaccinees and in 48% of volunteers in the group receiving 30 µg of Tat three times [202]. These results indicate that the induction of anti-Tat immune responses intensifies cART efficacy and attacks the cART-resistant virus reservoir. A confirmatory randomized, double-blind, placebo-controlled (randomization ratio, 1:1), safety and immunogenicity phase II therapeutic trial (ISS T-003, ClinicalTrials.gov Identifier: NCT01513135) was then conducted in South Africa in 200 HIV-infected (C clade) anti-Tat Ab-negative adults who were virologically suppressed, with CD4$^+$ T cell counts ≥200 cells/mmc [203]. The clade-B Tat vaccine (30 µg) was administered intradermally three times at monthly intervals. The vaccine was safe and induced durable and high titers of anti-Tat Abs that were capable of cross-recognizing the Tat protein from different HIV clades and cross-neutralizing both clade B and C HIV viruses. Cross-recognition and cross-neutralization correlated with an increase in CD4$^+$ T cell counts, a key target for cART intensification [203]. Of note, vaccination contained the VL rebound and maintained CD4$^+$ T cell counts above the baseline levels in subjects who were noncompliant with therapy as compared to (noncompliant) placebo, suggesting that Tat vaccine intensification of cART may counterbalance incomplete adherence to treatment [203]. An extended follow-up study of this trial (ISS T-003 EF-UP) is underway. Overall, the Tat vaccine study shows for the first time that cART can be intensified by therapeutic immunization and that the proviral DNA load can be progressively lowered.

## Conclusions

So far, HIV vaccine design based on structural proteins has not been successful, nor have empirical vaccines, reinforcing the concept that a pathogenetic approach must be undertaken to identify key virulence factors to target with a vaccine. In particular, a "pathogenesis-driven" approach should be aimed at targeting key viral products responsible of virus transmission and activation and maintenance of virus reservoirs. For this, evidence provided by the natural history of infection and lessons from earlier trials are key.

Tat vaccination represents an example of a "pathogenic-driven" intervention that is potentially effective for both preventative and therapeutic strategies, since it is aimed at blocking virus transmission and spread. The rationale is based on the evidence that HIV-1 Tat, which is necessary for HIV gene expression, replication, and cell-to cell transmission, appears also to be critical in the initial steps of virus acquisition. In fact, it has been shown that Tat, which is present on virus particles, binds to Env spikes, promoting HIV infection of dendritic cells and spreading to T lymphocytes, even in the presence of anti-Env NAbs, and that anti-Tat Abs are necessary to restore neutralization and prevent HIV dissemination, as demonstrated by virus containment at the site of inoculation in macaques vaccinated with Tat and Env-ΔV2 proteins and challenged intrarectally with the SHIV$_{SF162P4cy}$, but not in control animals [35].

In a therapeutic setting, cART intensification by a Tat vaccine has proven effective at promoting immune system restoration by improving CD4$^+$ T cell recovery and immune system functions while reducing virus reservoirs and immune activation/dysregulation [77, 78, 202]. These combined effects may reduce the negative effects of non-adherence to therapy on virus transmission, and hence global community VL, new infections, and drug resistance. Thus, an intervention that restores immune responses may allow periodic drug-free time.

The different therapeutic uses of Tat vaccination in HIV infection must be confirmed in dedicated trials to determine whether administration of Tat in patients receiving long-term (late) cART can provide prolonged post-treatment control (extended drug-free periods with low or undetectable VL) in an analytic treatment interruption (ATI) trial, opening new perspectives for functional cure and eradication strategies. In this regard, the durability of the increase in CD4$^+$ T cell count and the progressive decrease in HIV proviral DNA to undetectable levels observed in vaccinees over the 8-year follow-up [202] are promising, as a reduced proviral HIV-1 DNA load at study entry has been reported to be independently associated with a delayed and milder HIV-1 RNA rebound after ATI [204] and post-treatment control [205].

Moreover, in volunteers who were vaccinated with Tat toxoid and developed a high titer of Abs that neutralized Tat bioactivity, a prolonged off-therapy time was recorded in the 2 years of follow-up [206]. Similarly, a delayed and weaker HIV RNA rebound was observed upon ATI in the volunteers immunized with Tat Oyi, who mounted strong immune responses against Tat [207]. Of note, the 33-µg dose was the most immunogenic and efficacious of the three doses tested (11, 33, and 99 µg) [207], which is in substantial agreement with our data from the ISS T-002 trial, in which the 30-µg dose of recombinant Tat protein gave the best results [78].

The Tat vaccine should also be evaluated in poor immunological responders to determine whether it ameliorates the response to cART at the beginning of therapy, with the aim of reducing the time to a virological and immunological response. It is also important to evaluate the Tat vaccine in HIV-infected cART-treated adolescents and children, as they face the longest time on antiretroviral treatment and

therefore are the ones most needing approaches ensuring virus control despite poor adherence, adoption of therapy simplification regimens, or prolonged time off-therapy. Furthermore, Tat could be used as a co-treatment to improve the efficacy of pre-exposure prophylaxis. It will be very important to evaluate the effects of the Tat vaccine in these contexts on HIV DNA in lymphoid tissues and other compartments, as cART does not attack the latent HIV reservoir, to determine the impact of vaccination on solid-tissue HIV reservoirs and residual disease. If proven effective in phase III efficacy trials, cART intensification by the Tat vaccine will allow the public health system to better target resources towards more-advanced HIV care strategies. In fact, cART intensification by the Tat vaccine is expected to reduce the rate of treatment failure and the prevalence of AIDS and non-AIDS comorbidities and allow periodic drug-free time, particularly in infants, children, and adolescents facing life-long cART and its severe side-effects, which can lead to low therapy adherence. Moreover, further depletion of the reservoirs of cART-resistant latent HIV resulting from Tat vaccination promises to blunt HIV rebound upon low adherence, which is one of the main causes of the development of drug resistance and virus transmission. Notably, vaccination with Tat in "very early" treatment of acute infection could stall the expansion of HIV reservoirs, opening new perspectives for a functional cure for HIV infection.

The generation of anti-Tat monoclonal Abs (mAbs) for immunotherapy should also be considered, as major advancements have been made in the generation and use of mAbs for immunotherapy [208]. In the few studies that have been conducted so far, mAbs generated in murine models and in humans upon vaccination with the Tat protein and directed against the amino terminus of Tat were effective at blocking Tat-induced transactivation and viral replication [209, 210], even across different Tat clades [211]. Thus, studies thoroughly addressing the protective effects of anti-Tat mAbs, alone or in association with other therapeutic interventions (cART, broadly neutralizing Abs, latency-reversing agents, etc.) in preclinical models and then in humans, are warranted.

Finally, the inherent Th-1- and CTL-promoting properties of Tat indicate the Tat may represent a new weapon that can be used as a vaccine against HIV/AIDS and as a potent Th-1 regulatory protein for increasing the efficacy of vaccines against other intracellular pathogens, such as HSV and Mtb.

## Declarations

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