Immunomodulatory properties of the HIV-1 Tat protein

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Key Words

Tat, HIV, AIDS, CD8$^+$ T cell, immune activation, immune dysfunction, T cell activation, T-bet, Eomes.

Abstract

Background: T cells are functionally compromised during HIV infection despite their increased activation and proliferation. Although T cells hyperactivation is a predictive marker for disease progression, its causes are not completely understood. Tat is a regulatory protein of HIV, necessary for viral gene expression, that can be released extracellularly and increases activation and cytokines release in uninfected CD4$^+$ T lymphocytes and APCs. However, whether Tat can modulate also CD8$^+$ T cell functions is not clear.

Methods: We examined the effect of Tat from different HIV clades on CD8$^+$ T cell responses and antiviral immunity in different *in vitro* and *in vivo* models of T cell activation by TCR engagement, including HSV infection. Moreover, a cross-sectional study on HIV-infected subjects was conducted to determine the association between anti-Tat antibodies and immune impairment.

Results: The presence of both clade B and clade C Tat during priming of CD8$^+$ T cells favors the activation of antigen-specific CTLs. CD8$^+$ T cells activated in the presence of Tat show an increased expression of T-bet and Eomes, two “master regulators” of T cell functionality and development. Overstimulation of effector CD8$^+$ T cells generated in the presence of Tat turns to a partial dysfunctionality at the peak of the response, and worsens HSV acute infection. Moreover, Tat favors the development of effector memory CD8$^+$ T cells and a transient loss of B cells, two hallmarks of the chronic immune activation observed in HIV-infected patients. In accordance with a Tat-mediated impairment of the immune system, anti-Tat IgM are found preferentially in patients with high CD4$^+$counts, suggesting a protective role of anti-Tat immunity.

Conclusion: Our data provide evidence that Tat, irrespectively to the HIV clade, contributes to CD8$^+$ T cell dysfunctions and support a role of Tat in deleterious immune activation in HIV-infected individuals.

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List of Abbreviations

aa	amino acid
Ab	antiboidy
ABTS	2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt
ADCC	antibody-dependent cellular cytotoxicity
Ag	antigen
AICD	activation-induced cell death
AIDS	acquired immunodeficiency syndrome
APC	allophycocyanin
APC	antigen presenting cell
APC-Cy 7	allophycocyanin covalently bound to Cyanin 7
BCR	B cell receptor
CD	cluster of differentiation
CEF	Cytomegalo virus, Eppstein Barr virus, Influenza virus
CFSE	5(6)-carboxy-fluorescein diacetate succinimidyl ester
CNS	central nervous system
CRF	circulating recombinant form
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DMSO	DMSO Dimethylsulfoxid
dNTP	dNTP desoxy-nucleoside triphosphate
EBV	Eppstein Barr virus
EC	elite controller
ELISA	ELISA enzyme-linked immunosorbent assay
Elispot	ELISPOT enzyme-linked immunospot assay
ERK	extracellular receptor-activated kinase
FACS	FACS "fluorescence activated cell sorting"
FCS	FCS fetal calf serum
FITC	FITC Fluoresceinisothiocyanat
gp	glycoprotein
HAART	highly Active Anti-Retroviral Therapy
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
HSV	Herpes Simplex virus
ICS	intracellular cytokine staining
IFN	interferon

Ig	immunoglobulin
IL	interleukin
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
LTNP	long term non progressors
LTR	long terminal repeat
mAb	monoclonal antibody
MHC	major histocompatibility complex
MPEC	memory precursor effector cells
MTT	3-(4,5-dimethylthiozol-2-yl)2,5-diphenyltetrazolium bromide
nAb	neutralizing antibody
p.i.	post-infection
PAMP	pathogen-associated molecular pattern
PBL	peripheral plood lympohocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PC	plasma cell
PCR	polymerase chain reaction
PE	Phycoerythrin
PerCP-Cy5	Peridinin Clorophyll protein covalently bound to Cyanin 5
PHA	Phytohämagglutinin
PMA	phorbol 12-myristate 13-acetate
PrEP	pre-exposure profilaxis
PRR	pathogen recognition receptors
PTD	protein transduction domain
qPCR	quantitative polymerase chain reaction
SEM	standard error of the mean
SFU	spot forming units
SIV	simian immunodeficiency virus
SLEC	short lived effector cells
TAR	trans-activation response element
T_{CM}	central memory T cells

1 Introduction

Since its isolation in 1983 [1], the human immunodeficiency virus (HIV) is still one of the major plagues worldwide with about 34 million of infected individuals, 2.5 million of new infections and 1.7 million of deaths per year [2]. HIV is the causative agent of AIDS (acquired immunodeficiency syndrome), a condition characterized by loss over time of immune cell functions which allows intrusion by several different infectious agents. Thus, HIV infection results in a complex impairment of the immune system.

1.1 The Immune system

The human immune system is composed by two major branches, the innate immunity and the adaptive immunity, and both of them are involved in the response against HIV.

The innate immunity is the first and nonspecific reaction initiated after every infection, with the aim to block the spread of the pathogen and to induce the inflammatory process. Moreover, innate immune cells activated by the interaction between PAMPs (pathogen-associated molecular patterns, molecular structures belonging to microorganisms) and PRRs (pathogen recognition receptors, expressed on the surface of innate immune cells) favor the onset of the adaptive immunity. This cross talking among the two major branches of the immune system happens mostly through two mechanisms:

1. activation of pro-inflammatory signaling pathways, resulting in the production of antimicrobial molecules, of pro-inflammatory cytokines and chemokines and of co-stimulatory molecules that, soluble or expressed on the cell surface, activate adaptive immune cells;
2. phagocytosis of the pathogen by macrophages, neutrophils and dendritic cells (DCs). This is followed by the processing of the antigen and their presentation, through MHC molecules, to T lymphocytes (see below).

The adaptive (or acquired) immunity is composed by cell-mediated and humoral responses, mediated respectively by the T and the B lymphocytes.

1.1.1 Cellular response

The worst damage caused by the HIV infection to the human immune system is at the level of the cellular immunity. The pathogen-specific cellular immunity is mediated by $CD4^+$ and $CD8^+$ T lymphocytes; $CD4^+$ T cells

are the preferential target of the virus and they massively dye during the course of the infection, but also $CD8^+$ T cells experience important dysfunctions. Moreover, because $CD4^+$ T cells provide help for both B and $CD8^+$ T lymphocyte responses, their loss during HIV infection leads to impairment both the two arms of the adaptive immunity.

T lymphocytes mount specific responses against antigens (Ags) that are presented through major histocompatibility complex (MHC) molecules; indeed, intracellular or internalized antigens are processed by the cellular proteolytic systems ("antigen processing") that generates small peptides (epitopes); these small peptides, associated to the MHC molecules, migrate to the cell surface to be recognized by the specific T cell receptor. Intracellular Ags are presented through MHC class I molecules, present on all nucleate cells, and activate $CD8^+$ T cells, while internalized antigens are presented through MHC class II molecules, present on "professional" APCs like DCs, macrophages and B cells, and activate $CD4^+$ T cells. However, professional APCs can also present exogenous antigens to $CD8^+$ T cells through a mechanism called "cross presentation". T lymphocytes recognize the presented antigen through the TCR, a complex of proteins that includes a dimer (usually formed by α and β subunits, but in some cases γ and δ subunits can be found), responsible for the binding with the epitope, and the CD3 chains (consisting in other three protein dimers), responsible for the signal transduction. Signal transduction begins with the activation of protein tyrosine kinases, serine/threonine kinases and the mTOR kinase, continues with the involvement of several second messenger cascades and end with the entering into the nucleus of some transcription factors (TFs), like NFAT, AP-1 and NFκB, that stimulate the expression of key T cell associated genes. Moreover, TCR stimulation leads to increased avidity of integrins for their ligands, thus favoring the adhesion of T cells to APCs.

Further to TCR stimulation, also defined as "signal 1", T cells activation requires costimulatory signals ("signal 2") and other additional signals from the environment, like the presence of pro-inflammatory cytokines ("signal 3") [3]. Several co-stimulatory receptors are exposed on T cell surface; among them, CD28 seems to be the most relevant. CD28 binds to ligands (CD80, CD86) present on APCs. Co-stimulation through CD28 results in an enhanced and prolonged activation of signals downstream TCR, in the transcription of the *il-2* gene through the engagement of the CD28 RE (response element), a sequence within the IL-2 promoter, and in the enhancement of the mRNA half-life of multiple cytokine genes [4]. CD28 co-stimulation is responsible for the activation of c-Jun kinase and PI3K/AKT/mTOR axis, thus contributing to anti-apoptotic effects due to the up-regulation of Bcl-2. Physiologically, a balance among MAP kinase family enzymes, in particular between ERK and c-Jun, is important for T cell survival, and while ERK is activated through TCR stimulation, c-Jun depends mostly by CD28 signalling

[5]. Type 2 signals are provided by different kind of co-stimulation which include CD28, tumor necrosis factor receptors (TNFR), CD40-CD154 interaction and IL-2. The IL-2 receptor is composed by three chains, the α chain (CD25), the β chain (CD122, common with IL-15 receptor) and the γ chain, common to other cytokines receptors defined as "common γ chain" cytokines (IL-4, IL-7, IL-9, IL-15 and IL-21), and induce the proliferation of the stimulated T cell [6]. A third signal is required for the "expansion" of antigen stimulated T cells, and it consists in the pro-inflammatory milieu due to the secretion by APCs or by neighboring cells of IFNα and γ, IL-12, IL-23 or other pro-inflammatory cytokines [3].

The modulation of these 3 signals during the priming of naïve T lymphocytes is of great importance to generate a productive response leading to strong effector functions, cell survival and memory generation. Following antigen stimulation, that can be represented by any infections, antigen-specific T cells undergo a remarkable phase of "expansion": naïve precursors massively proliferate and differentiate into "effectors", encharged of the clearance of the pathogen [7]. Effector $CD4^+$ T cells may accomplish their duty of "orchestrating" the immune response both remaining in lymph nodes to help B cells or migrating into the site of infection, thus supporting the activities of innate immunity cells or cytotoxic T lymphocytes (CTLs), directly involved in the clearance of the pathogen through the secretion cytotoxic proteins. T helper lymphocytes that recognize the epitope bound to MHC class II molecules on the surface of the same DC presenting the antigen to the $CD8^+$ T cell can help the activation of the $CD8^+$ T cell itself through the secretion of IL-2 (beneficial for CTLs expansion) or enhancing, on DCs, the expression of co-stimulatory molecules and the production of cytokines in favour of the $CD8^+$ T cell. The ability of T helper cells to sustain (or depress) a certain kind of immunity depends by the lineage that they acquire: Th1, Th2, Th17 and Treg. The acquisition of a certain lineage strongly depend by the cytokine milieu (Table 1.1) that determines the expression of specific TFs. In particular, Th1/Th2 balance is regulated by T-bet and Gata-3, both induced by TCR stimulation and, in a different way, by several cytokines [8]. T-bet enhances responsiveness to IL-12 up-regulating IL-12Rβ2 and promotes IFNγ expression, thus favoring a Th1 phenotype, while Gata-3 is the TF responsible for the acquisition of a Th2 lineage [9]. T-bet and Gata-3 expression is regulated, in turn, by the different activation of the two complexes of the intracellular kinase mTOR, mTORC1 and mTORC2, that also control Th17 development [10, 11].

IL-12 plays a fundamental role in this process since it promotes Th1 development in addition to ensures a robust expansion and the acquisition of effector functions by $CD8^+$ T cells favoring the secretion of IFNγ and granzime B through the activation of the PI3K-Akt pathway that leads to mTOR phosphorylation and T-bet expression [12, 13].

T helper lineage	Principal inductive cytokines	Role of T helper cells subpopulations
Th1	IL-12, IFNγ, IFNα	Defense vs intracellular organisms: NK, macrophages and CTLs activation and IFNγ secretion.
Th2	IL-4	Mucosal and epithelial defense, promotion of IgE and eosinophil/basophil/mast cell-mediated immune reaction, response to parasites
Th17	TGFβ, IL-6, IL-23	Defense vs extracellular organisms, release of pro-inflammatory cytokines, activation of T cells, NK and neutrophils.
Treg	TGFβ, IL-10	Suppression and control of the immune response

Table 1.1 T helper lineage commitment

Remarkably, expression of T-bet in $CD4^+$ and $CD8^+$ T cells is induced downstream of TCR, IFNγ and IL-12 signaling, and promotes the transcription of effector genes [14]. The effector functions of $CD4^+$ and $CD8^+$ T cells is dependent also by Eomes, belonging to the T-box TFs family, like T-bet [15]. However, T-bet and Eomes display an opposite function in the memory development (see below) and, moreover, their expression seems inversely related; for instance, IL-12 induces T-bet and represses Eomes [12], while IFNα increases Eomes expression and IFNγ production in effector $CD8^+$ T cells but has no effect on T-bet expression [13].

After the antigen is cleared (or the pathogens go into latency), the vast majority of effector T cells die during the so called "contraction phase", and the survivors differentiate into memory cells that, in case of secondary infection, will be ready to give birth to a new immune response [16]. The death of effector cells once the antigenic challenge has been met, is of essential importance to avoid tissue damages and enlargement of secondary lymphoid organs, and usually involves the 90% of the clonal population [17]. Their elimination occurs by apoptosis and AICD (activation-induced cell death), displayed by several mechanism like privation of IL-2 or stimulation of CD95 (also known as Fas). CD95 is a surface receptor up-regulated after TCR stimulation that, when triggered by its ligand (present as soluble or on the surface of other activated cells), initiates a signalling pathway that brings to caspase activation and, so, to apoptosis. At the same time, cells can prevent this process by the up-regulation of some anti-apoptotic genes like Bcl-2 [18].

High amounts of antigens and a prolonged stimulation can increase the size of the expansion of both $CD4^+$ and $CD8^+$ T cells [17, 19]. However, the fraction of effectors undergoing the contraction phase does not seem to

depend on the magnitude of the expansion phase [17], while the kinetic of expansion and contraction are strictly dependent, as the onset of $CD8^+$ T cell contraction has been shown to initiate about 5 days after the last interaction with Ag-loaded DC [20]. The amount of effector cells undergoing contraction is mostly regulated by events occurring in the early expansion. Higher number of cells surviving the contraction phase correlates with low levels of inflammation [21], increased interaction among CD80 or CD86 expressed on DCs with CD28 expressed on the T cells [22] and IL-2 co-stimulation [22, 23].

The pool of effector T cells surviving the contraction phase will generate the memory T cells. Memory T cells do not require antigen stimulation for their maintenance, but are dependent on IL-7 and IL-15 signalling that mediates homeostasis and survival, up-regulating anti-apoptotic molecules such as Bcl-2 [24]. Bcl-2 down-regulation seems related to the modification of the T cell subsets occurring during HIV infection [25]. Memory cells can be categorized into two broad subpopulations: "central memory" (T_{CM}) and "effector memory" (T_{EM}), although the development of multiparametric flow cytometry allowed the characterization of other T cell subsets. T_{CM} reside mostly in lymph nodes and are responsible for the clonal expansion after re-exposure to antigen, while T_{EM} are disseminated within peripheral tissues where they display immediate effector functions. Their different role and anatomical distribution determine their phenotype; for example, lymph node homing receptors like CD62L and CCR7 are expressed on T_{CM} but not on T_{EM}. However, this differentiation seems more a simplistic way of describing the T cell memory organization than a clear picture of the reality, because several experiments have demonstrated that T_{CM} can display secretory capacity and T_{EM} may proliferate [26]. Nevertheless, it is well established that the secondary response, mediated by memory cells, is faster and more intense than the primary response [27, 28].

In the case of chronic or latent infections, and particularly during the HIV infection, the persistence of the pathogen misleads the physiological development of the cellular response, affecting the functionality of memory cells, that can exhibit poor recall proliferation, exhausted phenotype, loss of effector functions and a skewed composition of T cell memory subpopulations [29].

1.1.2 $CD8^+$ T cell programming

Long lived memory $CD8^+$ T cells seem to originate from a subset of effector cells called MPEC, memory precursor effector cells, in contrast to SLEC, short lived effector cells. The balance of MPEC/SLEC among a population of effectors depend by the overall amount and duration of the three above mentioned signals, thus the first 48-96 hours of stimulation determine the fate of the future memory population. In particular, the development of T_{CM}

cells is dictated by a short and reduced antigenic stimulation [30, 31]. The composition of the effector T cell subsets and, as a consequence, the generation of long lived memory T cells, is related by the balance of the two TFs T-bet and Eomes; indeed, T-bet is important for the generation of SLECs, and its expression has to decrease for the development of a functional memory population, while Eomes is crucial for the ability of memory T cells to respond to IL-7 signaling, important for memory homeostasis [13, 32]. The expression of T-bet and Eomes is regulated by the mTOR kinase, whose activity promote T-bet at the expense of Eomes; thus, mTOR inhibition promote the accumulation of memory precursors $CD8^+$ T cells, the increased and sustained expression of prosurvival genes (Bcl-2 and Bcl-3) and the development of fully competent memory $CD8^+$ T cells [13], as well as the maintenance and the antigen-recall responses of competent memory $CD8^+$ T cells [33].

Other TFs are involved in the development of memory T cells like the Foxo family, Bcl-6 and Blimp-1. The forkhead box O1 (FOXO1) is downstream to mTORc2 and under the stringent control of AKT-mediated phosphorylation and nuclear exclusion, induced by IL-12 and IFNα signalling [34]. FOXO1 targets IL-7 receptor subunit-α, CD62L, Bcl-2 and Eomes, thus promoting memory differentiation [34]. FOXO3 too needs phosphorylation, induced by IL-7 and IL-15 [35], for nuclear exclusion, and its inhibition leads to an enhanced expansion protecting effector cells from apoptosis; this results in an increased accumulation of memory $CD8^+$ T cells, without effects on the phenotype [36].

Bcl-6, the transcriptional repressor of Granzyme B, is important for $CD4^+$ and $CD8^+$ memory T cells formation [37] and interacts with T-bet for Th1 development [38]. Bcl-6 is negatively regulated by Blimp-1, a TF required for T cell homeostasis, cytokine secretion [39] and more expressed in SLEC than MEPC and T_{CM} [40]; of note, Blimp-1/Bcl-6 balance is involved also in the formation and maintenance of memory B cells.

The interaction with $CD4^+$ T cells and DCs, both targets of HIV infection, play a fundamental role in determining the $CD8^+$ T cells programming. Indeed an increased interaction between DCs and T cells due to a higher epitope density does not affect the expansion but enhances the survival of $CD8^+$ T cells during the contraction phase through an increased expression of both Bcl-6 and Eomes [41], the TFs required for long lived memory cells development. Moreover, DCs interact with $CD4^+$ T cells to promote the development of $CD8^+$ T_{EM} cells [42]. Together, DCs and $CD4^+$ T cells may influence $CD8^+$ T cells programming through the cytokines they secrete. For instance, IL-2 promotes the expansion of SLECs and the accumulation of T_{EM} [42], IL-15 favors the formations of MPECs [19] while pro-inflammatory cytokines such as IL-12 and IFNγ enhance the contraction phase [21] and the formation of SLECs through the induction of T-bet [43].

1.1.3 Humoral response

The humoral immunity is mediated by immunoglobulins (Ig), secreted by B lymphocytes. Ig molecules are constituted by four chains, each of those formed by a variable and a constant region. While the variable region of Igs determines the antigen specificity, the constant region contributes to the creation of the antigen binding domain and determines the effector functions of antibodies (Abs) and their fate of being secreted or remaining on the cell surface. Abs anchored to the cell membrane constitute the B cell receptor (BCR) that induces the antigen recognition by B cells, while secreted Abs traffic into the tissues and across mucosal surface. Moreover, the constant regions determine the subdivision of Abs into five different types: IgA, IgD, IgE, IgG, IgM, each with different properties and functions (Table 1.2).

Immunoglobulin	**Principal features**
IgA	IgA is the predominant isotype in the mucosal secretions, but can be found in the blood too. IgA are present as monomer, dimers, trimers and tetramers. The IgA-mediated effects are different from the immune mechanisms mediated by other Abs, and include the blocking of microbial receptors, mucus trapping and induction of phagocytosis.
IgD	They are principally found on the cell surface of naive and mature B cells.
IgE	They are mostly associated with allergy, hypersensitivity reactions and immunity against parasites.
IgG	IgG constitute approximately the ¾ of total Ig in the blood; they characterize the B cell memory response, and usually arose after a switch of B cell secretion from IgM to IgG. They can mediate complement activation or cellular cytotoxicity (ADCC), but they can also directly neutralize the pathogen (neutralizing antibodies). They are subdivided in four subtypes: IgG1, IgG2, IgG3 and IgG4, that display some different effector functions.
IgM	Constituting the primary antibody response, IgM can be found both anchored on the cell membrane and in the soluble form, where they are organized in pentamer or hexamer. IgM are very effective in activating the classical pathways of the complement and in the opsonization of pathogens.

Table 1.2 Antibodies isotypes

The interaction between the BCR and the antigen induces the activation of multiple signaling reactions that involve kinase pathways (JNK, PI3K-Akt, ERK) thus leading to the activation of several TFs like NF-κB and NFAT, close resembling the signal mediated by TCR. The cellular responses elicited by BCR stimulation are different (survival or apoptosis, change in location, proliferation), depending by the developmental stage of the B cell, the presence of other soluble or cellular signals and the interaction with $CD4^+$ T lymphocytes. BCR allows also the endocytosis of the antigen and its presentation, through class II MHC molecules, to $CD4^+$ T cells, thus inducing T-dependent antibody responses. Indeed, B cells can secrete Igs with and without T helper cells. In the latter case, these responses are usually fast and dominated by IgM, while T-dependent antibody responses promote isotype switching (from IgM to IgG) and the generation of B memory cells and long lived plasmacells (PC). T cell help to antibody responses is provided by specific T helper lymphocytes previously primed by DCs; these $CD4^+$ T cells, according to the kind of cytokines produced, control the isotype of IgG secreted; in particular, IL-4 induces IgG1 production while IFN-γ (secreted by both $CD4^+$ and $CD8^+$ T cells) and IL-6 induce IgG2a production. Class switching to IgG2a and survival of memory B cells secreting IgG2a require T-bet expression [44].

Antibody may "neutralize" the antigen blocking its interaction with host receptors, or they may eliminate microbes and toxins through different mechanisms that include the involvement of some innate mechanisms as complement activation, phagocytosis or antibody-dependent cellular cytotoxicity by NK cells. The involvement of phagocytes or NK cells requires the interaction of the heavy constant chain of Ig with the leukocyte Fc receptor that, after having been triggered, will deliver signals to activate the cell and stimulate its microbicidal or phagocytic activity (opsonization).

1.2 The HIV infection: epidemiology and virology

HIV-1 and HIV-2 belongs to the family of Retroviridae, genus of Lentiviruses, and are the disease causing agents of AIDS. HIV is thought to be derived by its simian version, SIV, in west central African Countries [45], and after a slow and focal spread, probably yet in the early 20^{th} century [46], rapid urbanisation and immigration led to a worldwide spread probably at the end of '70s, until the first recognized case of AIDS was reported in 1981[47]. Nowadays, the region most hit by this plague is sub-Saharian Africa, that accounts for 23.5 million of infected individuals, followed by Asia (4.8 million). HIV-2 shows a reduced pathogenicity and a longer clinical course compared to HIV-1, and is predominantly found in West Africa [48]; indeed most of the global AIDS pandemic is due to HIV-1.

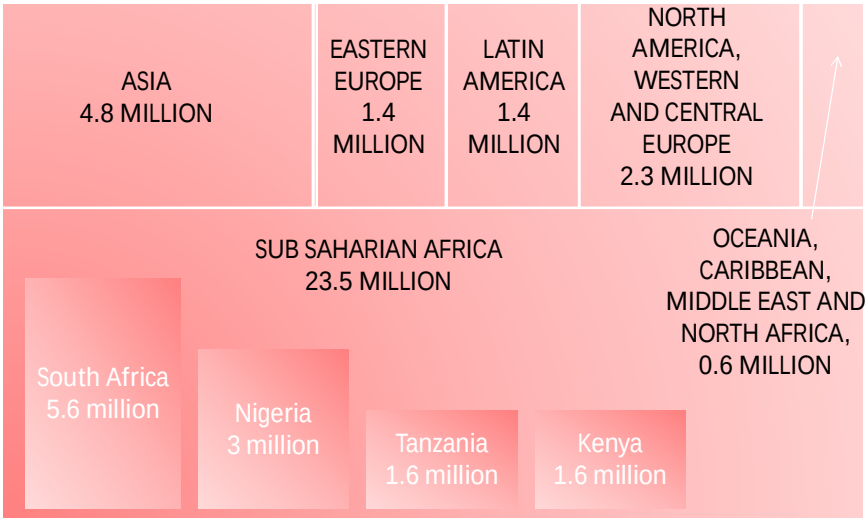

Figure 1.1 HIV-infected people. *Regional overview adapted by [2]*

Based on their genetic differences, four major groups of HIV-1 (M, N, O, P) have been recognized [49]; the M group is responsible for about the 90% of HIV-1 infections, and at least 9 subtypes, or clades, can be distinguished (A, B, C, D, F, G, H, J, K), in addition to others recombinants forms (CRFs) [50]. The clade C virus is responsible for about the half of all HIV infections, followed by subtype A and B [51]. HIV-1 subtypes are characterized by some differences in progression rate and virulence [52, 53], and show a peculiar geographical distribution; in particular, subtype C viruses are predominant in developing countries accounting for the vast majority of all global HIV-1 infections (southern Africa, India and Brazil), subtype B viruses in Europe, America, Australia but also southeast Asia, northern Africa and the Middle East, subtype A is the main genetic form in central/east Africa and in east Europe and subtype D is mostly present in East Africa [54].

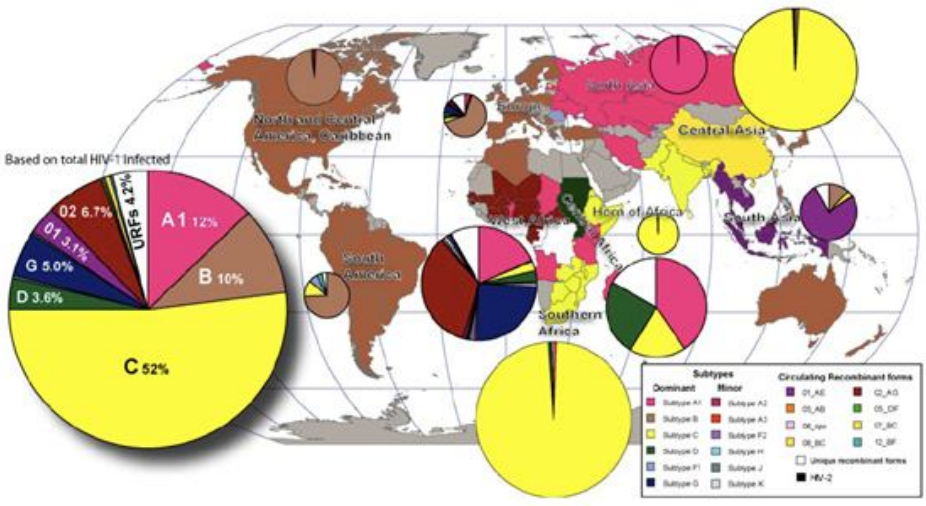

Figure 1.2 HIV-1 subtypes distribution. *Adapted by [55]*

1.2.1 Virus structure and cycle

HIV viral particles have a diameter of about 100 nm and are built in a concentric structure consisting in an envelope, a matrix and a capsid. The capsid contains the genome, composed by two identical copies of single-stranded RNA that codifies for three major genes (gag, pol, and env) and six accessory genes (tat, rev, nef, vpr, vif, and vpu). Structural proteins composing the viral particles are encoded by env and gag, while the pol gene encodes for enzymes crucial for viral replication (reverse transcriptase, integrase, protease). The regulatory proteins deriving from accessory genes exert functions important for viral infectivity and replication, as well as for the immune system impairment.

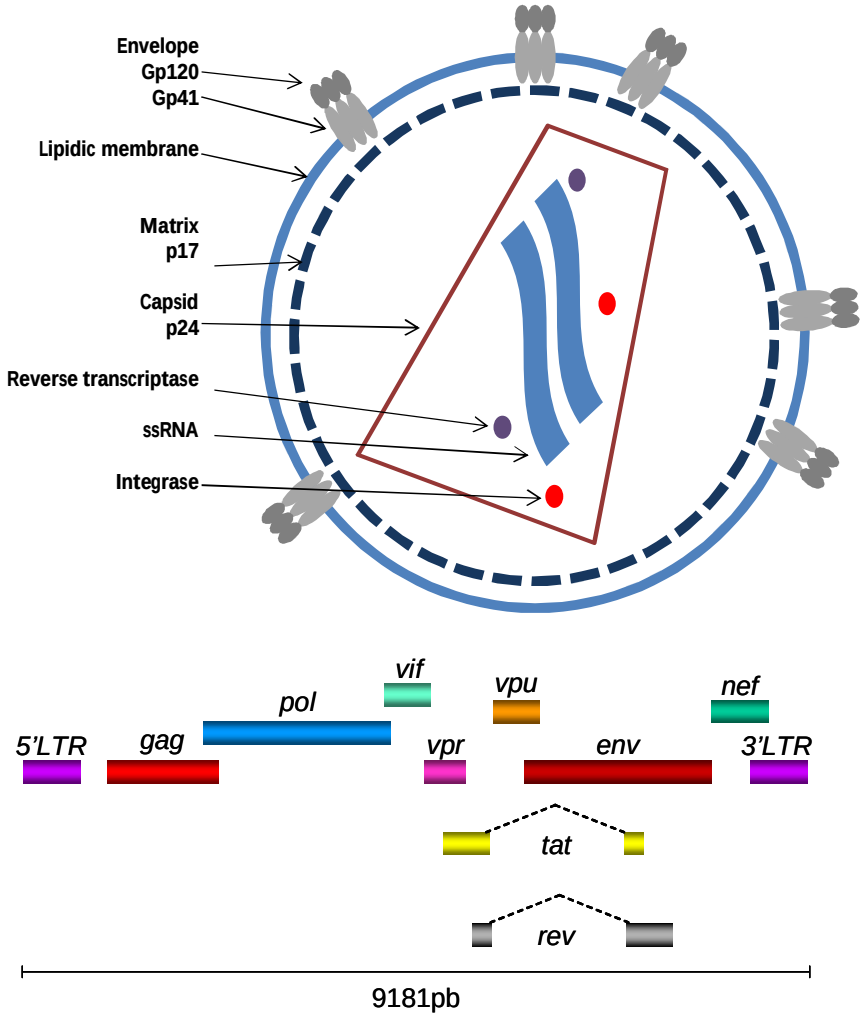

Figure 1.3 HIV-1 virion and genome

The replication cycle of HIV is a multistep process that involves many virus-cell interactions; it starts with the binding of the envelope protein gp120 with the CD4 molecule, mostly expressed on T lymphocytes, macrophages and DCs; the following interaction with chemokine receptors expressed on the cell surface (commonly CXCR4 and CCR5) permits the fusion of the virus with the cell membrane and the subsequent entry of the viral capsid.

The next step is the uncoating of the core freeing the viral RNA into the cytoplasm of the target cell where, through the reverse transcriptase (also present in the HIV virion), is transcribed into double-stranded DNA and subsequently integrated into the host genome.

Once integrated, the proviral DNA may remain latent until the host cell will be activated. When this happens, cellular RNA polymerase II complex will transcript viral genome. The first proteins produced are Tat, Rev and Nef. The proviral DNA is enclosed by two long terminal repeats (LTR); the 5' LTR includes the promoter, an "enhancer" sequence and a region called "TAR" (Trans-Activation Responsive). Tat binds to the TAR site of the nascent RNA to stimulate the transcription and the formation of longer RNA transcripts, that will be translated in protein precursors. These, after cleavage, will compose the new viral particles. This final step begins with the assembly of the core; the core then migrates towards the cell surface and buds through plasma membrane, that contains viral structural proteins.

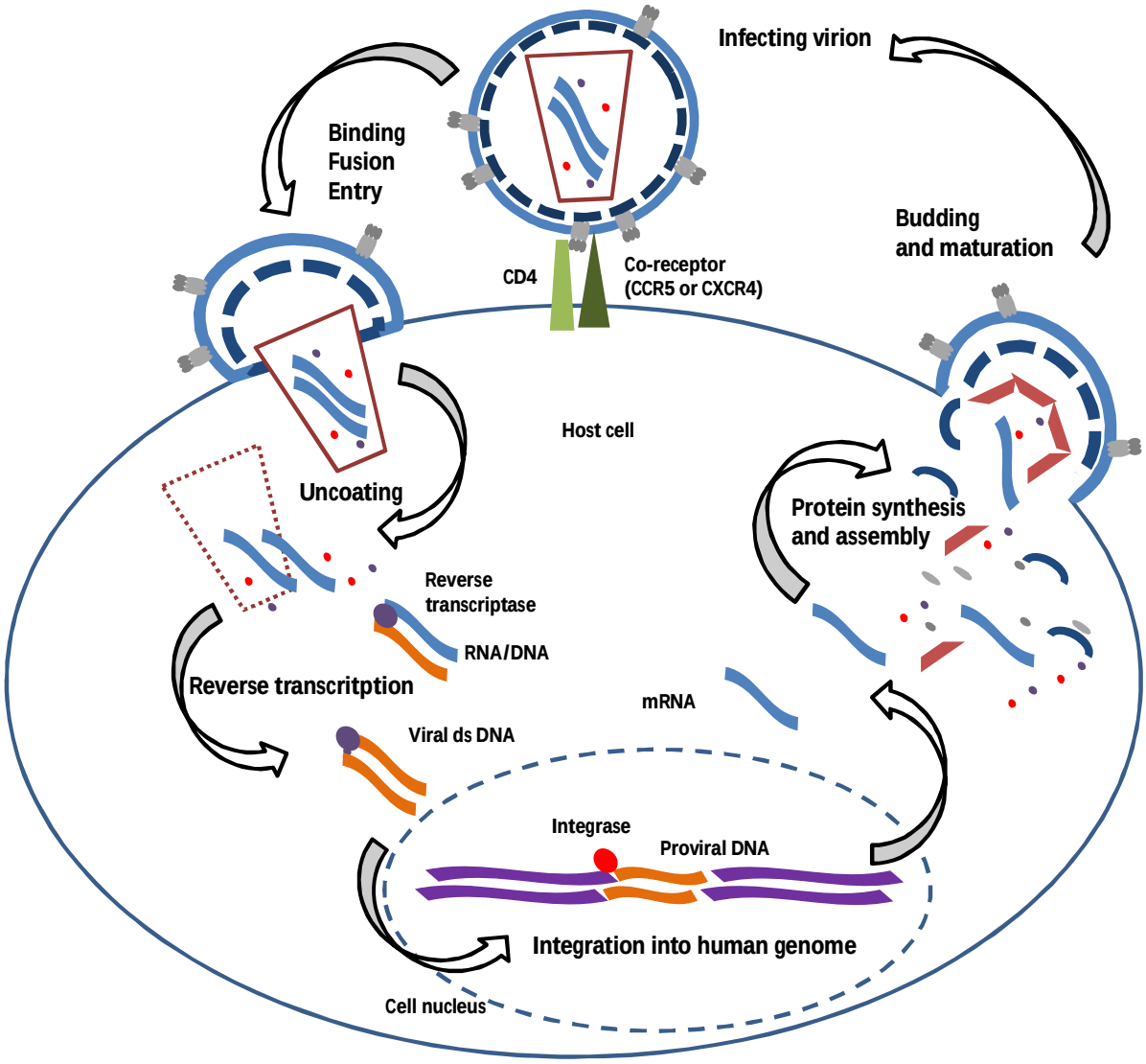

Figure 1.4 HIV-1 replication cycle

The replication cycle utilizes several host transcription factors and requires the cell to be activated, thus the immune response arisen to eliminate the virus can trigger further virus production.

1.3 The HIV infection: pathogenesis

HIV infects preferentially $CD4^+$ T lymphocytes, macrophages and DCs, even if other cell types have been described as target of the infection [51]. Virus transmission requires the direct exposure to infected blood or secretions, and is highly dependent on the virus concentration, viral isolate and host susceptibility. The most common routes of transmission include sexual contact, vertical transmission (mother to child transmission) and needles exchanges, thus populations most at risk for becoming infected include sex workers, men who have sex with men and people who inject drugs [56]. The contribution of the different routes of infection to the global epidemiology varies across the regions of the world.

The transmitting virus usually utilizes the CCR5 chemokine receptor for viral entry (R5 viruses) [57] and establishes a localized infection determining a severe loss of $CD4^+$ T cells, especially in the gut [58]. Infected $CD4^+$ T cells expand locally and start to produce pro-inflammatory chemokines and cytokines, recruiting to the site of infection plasmacitoid dendritic cells (pDC) and new targets of infection thus determining the dissemination to lymph nodes; from the lymph nodes, HIV spreads systemically via the blood stream. Several flu-like or mononucleosis-like symptoms may characterize the acute phase (first 6 weeks), and the infection is countered only by innate mechanisms of immunity and by intracellular resistance factors like APOBEC3, TRIM5α and thetherin [59]. This phase is followed by the development of B and T cell responses that determine the decline of the viral load to a "set point" and the raise of $CD4^+$ count, and symptoms begin to disappear. Phenotypic changes in viral isolates determine the appearance of X4 viruses, characterized by the usage of CXCR4 co-receptor, or of dual tropic (X4R5) viruses; these viral strains show some difference in tropism, infectivity and disease progression [57, 60]. The situation can remain asymptomatic, in absence of therapy, up to 10 years; however, in this time frame, a slow loss of $CD4^+$ T cells and immune impairment occur, and the virus continues to replicate in some body compartments. $CD4^+$ T cells death does not involve only infected cells and is not exclusively due to direct cytopathic effects of HIV, but can be caused by several mechanisms including apoptosis induction and anti-$CD4^+$ T cells cytotoxic activity [61]. This "clinical latency" phase is accompanied by the establishment of cellular reservoirs, resting memory $CD4^+$ T cells that host the integrated virus with low expression of viral antigens, not to be eliminated by the adaptive immune system [62].

A “chronic immune activation” status that characterizes the whole T cell compartment (see below), as well as a continuous viral replication, contribute to the destruction of the lymphoid tissue. These detrimental effects, and the $CD4^+$ count drop to 200 cells/µl, favors the onset of opportunistic infections (eg. Pneumocystis carinii, toxoplasmosis, Candida, CMV, Mycobacterium Tuberculosis) and tumors [63, 64], as well as of sever problems at the central nervous system (CNS), leading the patient to death.

1.3.1 Immune activation and other immune dysfunctions

T helper cells are known to interact with other many cell types like APCs, B cells and $CD8^+$ T lymphocytes; so, HIV infection can, directly or through the loss of $CD4^+$ T cells, influence other compartments of the adaptive immunity. Several damages at the level of lymphoid organs have been extensively demonstrated [65], and defects in T cells maturation and differentiation [66] and in the composition of the T cell subsets are quite common in AIDS patients; indeed, the HIV infection is characterized by decrease of naïve and expansion of memory T cells, in particular of the effector memory subset [56, 67, 68].

$CD4^+$ and $CD8^+$ T cells show several dysfunctions like impairment of cytolitic functions [69], loss of polyfunctionality [70, 71], exhaustion [72, 73], increased T cell proliferation [74, 75] and susceptibility to apoptosis [76, 77]. T lymphocytes from HIV-positive subjects are also characterized by a deep modification of the intracellular signaling [78], as demonstrated by the impaired response to TCR- and IL-2- mediated stimulation [56, 79] and by the skewed expression of some important TFs, including T-bet and Eomes [80, 81]. These detrimental effects of the HIV infection on the whole T cell compartment, regardless antigen-specificity, and that involve uninfected cells too [82], are a direct consequence of the chronic and systemic immune activation characterizing the immune system of HIV-infected individuals, that is one of major determinants of pathogenesis [83]. In fact, T cell hyperactivation contributes to immune impairment and creates new target of infections, since HIV infects preferentially activated $CD4^+$ T cells.

The causes of chronic immune activation are still poorly known. HIV infected subjects are characterized by high levels of pro-inflammatory cytokines and chemokines [84] that contribute to T cells hyperactivation and increase HIV-1 replication [85]. At the same time, T cells activation is induced by the reactivation of some co-infections (HCV, HSV-2, CMV, EBV) and by the disruption of the mucosal barrier that results in microbial translocation from the gut to the systemic immune system [82, 86, 87]. In addition, some viral structural proteins, like the gp120 [88], and some regulatory proteins (Tat, Nef and Vpr) known to be secreted by infected cells [89-91], have been shown to favor T lymphocytes activation. Immune activation involves also other cell types, like B cells, NK and

DCs [92-94]. In fact DCs show, during chronic HIV infection, an activated phenotype, and increase their spontaneous production of pro-inflammatory cytokines and chemokines, thus contributing to the overall hyperactivation of the immune system [95, 96].

1.3.2 HIV persistence in cellular reservoirs

One of the most important barriers to the elimination of HIV is its persistence in cellular reservoirs [97], that consist in latently infected central memory (T_{CM}) and transitional memory (T_{TM}) $CD4^+$ T cells [98]. Their homeostatic proliferation, low proliferation rates and long term maintenance sustained by IL7 and IL15 make them a very stable viral reservoirs [98]. Latently infected $CD4^+$ T cells may persist in the body for many years and are supposed to derive from infected activated $CD4^+$ T cells that switch to a resting memory phenotype and reduce the transcription factors required for HIV replication. Thus, the infected host cells persist for years until they receive a stimulatory signal that induces their activation and, concomitantly, induces viral production. The lack of viral reactivation in absence of T cell stimulation seems to depend by multiple mechanisms that involve modifications to the chromatin environment, low levels of host transcription factors (like NF-kB, NFAT or p-TEFb) important for HIV expression [99] and mutations of Tat and its RNA target, TAR [100].

1.4 HIV control and cure

The early detection of the infection is the first step to try to cure the disease as it is estimated that only half of the population living with HIV know their HIV status [2], and untreated HIV infection leads the patient to death in about 10 years. Since the approval of Azidothymidine in the USA in 1987, more than 20 antiretroviral agents, targeting almost completely the virus cycle, were introduced. The combination of different antiretroviral drugs (HAART: highly active antiretroviral therapy) allowed the evolution of HIV infection into a chronic condition, were fatality may be avoided [101]. HAART initiation results in an increase of the $CD4^+$ count and in a suppression of the viral load [102], although HIV-positive treated patients show shorter life expectancy than uninfected peers, especially when the therapy is initiated in advanced stages of the disease [103].

Despite the important results reached through the introduction of antiretroviral drugs (halving of AIDS-related deaths between 2005 and 2011 [2]), several problems regarding HAART remain unresolved like the side effects, the right time to start, the lifelong and daily adherence required, the unavailability of pediatric formulations and the issue of drug resistance [104]. Moreover, long-term side effects are not known, and HIV infected subjects

show, even in the presence of HAART, an acceleration of ageing [105]. Treated patients show variable responses to therapy in term of viral suppression, clinical response and, most of all, immunological responses [106, 107], as it has been widely demonstrated that HAART does not restore completely immune functions [56, 70, 71].

However, one of the biggest problems of HAART remains its cost, that severely affects the coverage. Even if the coverage of antiretroviral therapy has increased dramatically in the last 2 years (60% more of people accessing to treatments), mostly thanks to Indian generic producers [108], 6-8 of the 15 million of people eligible for treatments are not receiving antiretroviral drugs [2]. TRIPS agreements (trade-related aspects of intellectual property rights), introduced by the World Trade Organization (WTO) to uniform intellectual property rules of the WTO members through a 20-years patent, constituted a big limitation to universal antiretroviral access. Recently, UNITAID launched the Medicines Patent Pool, that permits to produce low-cost generic formulations of molecules made available by the Patent Holder in exchange of royalties [109]; since its introduction several multinational pharmaceutical companies have joined it, and the dialogue among patent holders and generic manufacturers is still ongoing to increase antiretroviral coverage.

Nevertheless, the most important unresolved problem to achieve HIV cure is the issue of eradication, as current antiretroviral drugs cannot eliminate cellular reservoirs [110]. Thus, growing efforts aimed at the reservoirs elimination have identified some approaches, still under study, to integrate pharmacological therapy with the re-activation of HIV from $CD4^+$ latently infected T cells directly through the activation of the DNA transcription [111] or indirectly through the activation of the T cell compartment [112]. However, many steps forward have still to be done to achieve a final cure for AIDS, and current pharmacological approaches need to be implemented and integrated with other strategies.

1.4.1 HIV prevention

Antiretroviral drugs have demonstrated their importance not only at the individual level, but also from the community point of view. Recent studies underline the importance of HAART as a prevention method: indeed, HIV suppression prevents virus transmission from HIV infected women to the newborns (409000 infected-children avoided from 2009 to 2011 [2]) and in serodiscordant couples [113]. Some new approaches that include the administration of antiretroviral drugs to healthy people at high risk of infection have also been proposed (pre-exposure prophylaxis, PrEP), even if with discordant results [114, 115]. The implementation of HAART and the introduction of PrEP to prevent new infections are issues strongly debated nowadays, because arise some ethical problems such as the exposure of patients to longer treatment periods, with the increase risk of side

effects, and the high cost carried by the option of early treatment of HIV positive patients with CD4 count above the threshold of 350 cells/µl.

In 2011, 700000 fewer new infections per year were registered than in 2001 and, in this decade, several countries decreased HIV incidence also more than 60% [2]. Despite this trend, some regions (Middle East, north Africa, eastern Europe and central Asia) still suffer for an increase of people newly infected. The best results in the field of incidence reduction were observed in those countries putting much efforts in prevention and treatment programs [2]. Indeed, the best way for preventing HIV infections is, nowadays, through the education. Factors like gender inequality or social and economic underdevelopment may have detrimental effects on sexual education or on a safe sexual behavior [116]. Moreover, stigma and discrimination of people living with HIV and of "populations most at risk" for HIV can be a deterrent for both prevention methods usage and diagnostic testing. So, if the key role of safe sex and of practices like circumcision have been already demonstrated, many efforts have been put to develop other complementary prevention approaches, like vaccination.

1.4.2 Searching for a vaccine

Some HIV infected patients naturally control HIV infection in absence of any therapy. They are called "long term non progressors" (LTNP), for the low viral load exhibited and the slow $CD4^+$ count decline; among them, the ones maintaining undetectable viremia for years are called "elite controllers"(EC) [117]. This group of subjects has been extensively studied to determine correlates of protection that can be important for vaccination strategies, although genetic host factors are often the causes of the viral control [118, 119].

$CD8^+$ T cells seems to play a key role in the control of HIV infection, as the detection of HIV-specific CTLs coincides with a viral load decline [120], and some HLA class I alleles are associated with the control of the infection [121]. $CD8^+$ T cell responses quality, more than magnitude, contribute to viral control: indeed, HIV-specific CTLs from viral controller display increased polyfunctionality [69], proliferative capacity and antiviral activity [122]. Interestingly, protective alleles target epitopes showing CTL escape mutations with high cost for the viral fitness [123]. In fact, HIV mutates the dominant epitopes [124] to avoid cellular immunity, and protective CTL responses put pressure on mutations that result in a loss of replication competence. This dynamic process also leads to continues changes in the virus and, thus, to the development of genetically diverse viruses in the single individual [123, 125]. Regarding the antigen targeted, anti-Gag responses correlate with low viremia, while anti-Env responses are more frequent in patients with high viral load [126]. Moreover, the

breadth of anti-Gag responses, especially if directed to two regions of Gag (Gag 1-75 and Gag 248-500), seems to be a protective correlate [121, 126].

Also humoral responses have been shown to induce escape mutants [127]. However, if the CTL response seems protective for the control of HIV infection, neutralizing antibodies (nAbs) production needs high viral loads, and their potency and breadth do not seem the correlate of protection in EC [128]. The recent discovery of broadly nAbs able to confer a wide response against global circulating viruses [129] strengthened the concept of the induction of humoral response more for the prevention of HIV infection than for therapeutic vaccines. However, Abs may act through different mechanisms, as antibody-dependent cellular cytotoxicity (ADCC), shown to be important for the viral control [130, 131]. First vaccine trials were based on this concept, with the aim to induce Abs against envelope proteins. However, they failed in inducing protection against acquisition [132]. So, scientific world moved the attention to vaccine candidates able to induce cellular response but, again, the results were not promising, and the trial was stopped for an increased risk of HIV acquisition in vaccinees [133], due to some pre-existent immunity to the viral vector used, and to an inappropriate bias of CTL responses towards less-conserved epitopes [134].

The third vaccine candidate finishing phase 3 trial was the RV144, that showed about a 30% of protection against HIV acquisition but no effects on viral control after infection [135]. The trial, conducted in Thailand, consisted in a prime with a canarypox expressing Gag, Pro and gp120 and a gp120 boost [135]. The studies on correlates of analysis show that, while anti-V1V2 (variable regions of the gp120) plasmatic IgG correlated with decreased risk of infection among vaccinated and non-vaccinated, ant-Env plasmatic IgA correlated with a minor control of the infection interfering with the ability of the vaccine to decrease HIV infection. These data arose the question if different antibody isotypes influence the level of vaccine efficacy [136]. The mechanism behind the interference of IgA with protective immunity has not been elucidated; however, some correlate analysis suggest that IgA can interfere with different protective mechanisms provided by IgG, through the binding to the same site used by IgG [136].

The development of vaccines against HIV has been explored, in addition to prevent HIV acquisition, also to control viral replication and disease progression in HIV-infected individuals, with the aim to discover therapeutic vaccines that may substitute HAART. However, nowadays no candidates have been shown to be highly immunogenic and, at the same time, confer protection from viral rebound after HAART interruption [137, 138].

Thus, the research on preventive and therapeutic vaccines against HIV is still ongoing, and accounts for many candidates undergoing the different trial phases.

1.5 The Tat protein of HIV: role in viral fitness

Tat is a regulatory protein of HIV fundamental for viral cycle and is involved in many aspects of immunopathogenesis. The main role of Tat in the viral fitness is to trans activate the transcription of the HIV genome. Indeed the first round of HIV-trascritpion is inefficient and leads to the production only of some regulatory proteins [139]. Among these, Tat is synthetized and removes the obstacles to the elongation binding to the TAR sequence on the nascent viral RNA [140], thus promoting the transcription of the full HIV genome. The transcription process involves several host TFs such as NFAT-1 and NF-κB. Some studies have also explored a possible role of Tat in reverse transcription, discovering that this process may be stimulated by low concentrations of Tat and inhibited by high amounts [141].

Beside these fundamental activities, Tat plays other roles important for HIV replication and spread. In fact, Tat can be expressed prior to viral integration [142], leading to the activation of the infected T cell, thus promoting viral cycle and replication [143, 144]; in addition, Tat can be released extracellularly by a leaderless secretory pathway [145, 146]. Upon release, Tat binds heparan sulphate proteoglycans of the extracellular-matrix and is detected in the tissues of infected individuals [145, 147] and can target immune cells expressing RGD-binding integrin receptors via its RGD-binding site, thus inducing integrin-mediated signals and entering the cells [147-149]. In this way Tat can enter and activate uninfected cells, rendering them more prone to be infected [150, 151], and induces HIV co-receptors expression [152, 153], thus favouring HIV spread. To this regard, a recent study suggests that Tat interacts with Env forming a novel virus entry complex favoring R5 or X4 virus entry and productive infection of DCs via an integrin-mediated pathway that can be blocked only in the presence of both anti-Tat and anti-Env antibodies [154].

Extracellular Tat can also enter infected cells leading to HIV genome transactivation [155] and rescuing viral expression from latently infected cells [156], thus promoting reactivation of latent reservoirs [157]. Indeed, low concentrations of Tat have been shown to be important mechanisms of HIV latency [158], and latently-infected $CD4^+$ T cells are enriched for Tat variants carrying domains with impaired transactivation activity [100, 159] but possibly competent for those pleiotropic functions exerted on human cells. Among these effects, it is important to underline the activation of Akt and the induction of the anti-apoptotic protein Bcl-2 that could be involved in reservoirs maintenance [160-162].

Tat is composed by two exons, and can be found in two forms, generated by translation from multiple spliced viral transcripts. One form is 72 amino acids in length and encoded by a one-exon transcript. The other form is

encoded by two exons and has an additional C-terminal domain, and can be 86 or 101 amino acids long depending by differences in the position of translational stop codons in the second coding exon [163]. While the two-exon Tat is generated early in infection, the one-exon Tat appears in the late phase [164]. Both forms are able to activate HIV-1 gene expression but only the two-exon form mediates the immune hyper activation of infected cells [165], suggesting that, in later phases, the one-exon Tat that prevails does not activate infected cells.

Tat protein is formed by multiple domains [163, 166], able to interact with different receptors [150, 167-169]:

Sequence	Function
aa 1-20	Acidic/proline-rich domain that binds bivalent ions able to mediate interactions among Tat monomers.
aa 21-37	Cysteine rich domain, highly conserved and necessary for LTR transactivation; cysteines are involved in intramolecular disulphide bonds formation.
aa 41-48	Core region important for LTR transactivation.
aa 49-57	Basic domain, highly conserved, contains the protein transduction domain (PTD) that allows the binding of the TAR sequence and with heparan sulphates. This domain allows nuclear transportation and uptake of Tat by cells, and seems responsible of neurotoxic effects.
aa 58-72	Glutamine rich region.
aa 73-86/101	C-terminal domain, contains the RGD motif that mediates the binding with integrins. It is codified by the second exon.

Table 1.3 Tat domains

1.5.1 Inter-clade differences

Infection by different HIV-1 subtypes could result in a different progression to AIDS and response to HAART [170, 171]. Variability among HIV-1 clades in cell tropism and co-receptors usage can account for these differences in virulence [170, 171]. To this regard, it has been shown that clade B Tat, but not C Tat, has the capacity to render $CD4^+$ T cells more susceptible to X4 HIV-1 infection increasing CXCR4 expression [172].

Subtype differences at the level of Tat sequence impact also the viral replication: clade C and E Tat display a higher transactivation potential than B Tat due to a better affinity to the TAR element and to a longer half-life [173]. Moreover, subtype C isolates possess QGD in place of the RGD domain in the second exon of Tat gene, and

this confers to clade C Tat a greater capacity in activating LTRs [174]. It has been reported that Tat can tolerate about 40% of sequence variation and, while mutations at the level of the first 21 amino acids are well tolerated, changes in residues from 22 to 40 can have deleterious effects, at least respect to transactivation [175, 176].

Tat displays several immunomodulatory properties (see section below) that may be affected by sequence variations. The replacement of RGD domain with QGD confers to C Tat a lower capacity in inducing apoptosis of activated macrophages [174]. Moreover, the replacement of a Cysteine (B Tat) by a Serine (C Tat) that occurs in position 31 seems responsible for opposite effects on cytokine secretion and [Ca^{2+}] flux in monocytes [177, 178]. Indeed, it has been shown that B Tat favors the secretion of pro-inflammatory cytokines while clade C Tat induces the secretion of anti-inflammatory molecules [179], although the opposite effects of B and C Tat on cytokines secretion are still controversial [177]. Clade B Tat, in addition to favor the secretion of pro-inflammatory cytokines, would also induce an increased release of neuropathogenic agents compared to C Tat [52, 180], and this would be the cause of the higher neurotoxicity of subtype B compared to subtype C HIV [181].

Modifications at the level of the primary and tertiary structures of Tat can also impact its immunogenicity [176], although B and T cell immunogenic regions of Tat are conserved among the HIV-1 M group [182, 183]. In fact, usually anti-Tat antibodies elicited against one Tat clade are able to recognize other Tat variants [176], and an effective cross-recognition of a B-clade strain-derived Tat protein by individuals infected with different local viruses has been demonstrated [178, 184].

1.6 The Tat protein of HIV: immunomodulatory properties

Along with the several roles played by Tat in infection acquisition and viral fitness, other functions have been attributed to this protein regard different aspects of disease progression. In fact, Tat interacts with various co-infecting opportunistic pathogens during AIDS progressions [185], is directly implicated in the pathogenesis of AIDS-related Kaposi's sarcoma [147, 167, 186] and some vasculopathic conditions in AIDS patients [187, 188], and causes several damages in CNS [52, 180], thus leading to HIV-associated dementia, a pathology present in one-third of adults infected with HIV [189]. Moreover, several immunomodulatory properties have been attributed to Tat, suggesting its contribution to $CD4^+$ T cells loss, chronic immune activation and T cell dysfunctions. Indeed, as previously mentioned, Tat can be released [145, 146, 155, 186] and enter uninfected cells, inducing integrin-mediated signals [147-149] or directly binding with the human genome to up-regulate genes linked to immune response, cell adhesion, cell activation and cell death [190].

1.6.1 Effects of Tat on antigen presentation and antigen presenting cells

It has been shown that Tat modulates antigen presentation at different levels. Indeed, Tat modifies the composition and the activity of the proteasome, affecting the generation and recognition of CTL peptide epitopes [191, 192]. In particular, Tat increases the presentation of subdominants epitopes at the expense of the immunodominant ones [192, 193]. Some reports also describe a modulation exerted by Tat on HLA class I molecules, even if results are not concordant [194, 195]. Inconsistencies among the studies may derive by the observation that only certain epitope/MHC class I complexes are enhanced by Tat [196]. However, recently, a Tat-mediated enhancement of HLA-ABC and HLA-DR expression on DCs has been demonstrated [148, 149].

Further to modulated antigen presentation, Tat induces the maturation and activation of APCs, as demonstrated in macrophages [197], monocytes [198] and DCs [148, 149]. In particular Tat has been shown to enhance release of several cytokines in monocytes, macrophages and DCs [148, 149, 198-201], as well as to up regulate co-stimulatory molecules such as CD40, CD80, CD83 and CD86 [148, 149]. As during chronic HIV-1 infection DCs are reported to acquire an activated phenotype [95, 96] and spontaneously produce of pro-inflammatory cytokines/chemokines [94], Tat-mediated effects on APCs might contribute to immune activation.

Finally, Tat activates the adhesion of monocytes to endothelial cells and their transmigration through endothelial monolayer, thus contributing to vascular and tissues damages and cardiovascular diseases [202, 203].

1.6.2 Effects of Tat on B and T lymphocytes

Further to provide a stimulatory signal through APCs, Tat directly activates $CD4^+$ T cells that undergo anti-CD3/CD28 stimulation, in a mechanism dependent by CD28 co-stimulation that enhances IL-2 secretion [165, 204, 205] and that may result in an increased susceptibility to HIV1 infection [205]. Moreover, it has been shown that Tat induces release of other pro-inflammatory cytokines involved in T cell activation and differentiation, such as IL-8, IL-12 and $TNF\alpha$ [206-208]. Thus, Tat contributes to $CD4^+$ T cells hyperactivation. This would be confirmed by the observation that Tat, expressed on the surface of heterologous cells, activates and induces proliferation of human PBMCs, in a mechanisms dependent by CD3 stimulation that involves the up regulation of $IFN\gamma$ and T-bet [209].

Tat-mediated modulation of B cell lymphocytes has not been yet completely characterized. However, it has been reported that Tat up-regulates the expression of Fas, an activation marker that mediates for apoptotic signals [210], and modifies B cells cytokines release [207], proposing a role of Tat in B cell hyperactivation. Moreover, it has been shown that Tat promoted contemporarily proliferation and apoptosis of B cells [211] depending by the

kind of stimulus used and by the B cell subtype [212]. Regarding to a Tat-modulation of the humoral response, it has been observed that Tat decreases IgM, IgG, and IgA production *in vitro* [212], although *in vivo* models showed an adjuvant property of Tat related to humoral responses [213].

1.6.3 Dual role of Tat on cell viability and proliferation

The HIV infection is characterized by extensive loss of B and $CD4^+$ T cells and by the simultaneous hyperproliferation of lymphocytes. As Tat may exit infected cells and target uninfected T cells and APCs, its role on cell cycle and cell survival has been extensively investigated. However, reports show conflicting results, and Tat seems to both promote and inhibit cell proliferation and to display at the same time anti- and pro-apoptotic effects.

$CD4^+$ and $CD8^+$ T cells hyper activation is a feature of chronic immune activation, and Tat-mediated enhanced proliferation of $CD4^+$ T lymphocytes has been proposed as a mechanism of pathogenesis [151, 155] and associated to tumorigenic potential [147, 155, 167, 186]. On the contrary, suppressive effects have been ascribed to Tat and taken as explanation of the immune impairment occurring during HIV-infection. Tat-mediated inhibition of proliferation [201, 214] would be due to the inhibition of CD26 activity (marker involved in cell growth) [215] and the enhanced release of the suppressive cytokine IL-10 [177, 179].

Further to suppress proliferation, some reports have shown that Tat mediates apoptosis of bystander and activated T cells, thus contributing to $CD4^+$ loss during HIV infection. This effect would be displayed through different mechanisms like modulation of cell cycle regulators [216], increased expression of pro-apoptotic molecules [217] or enhancement of microtubule polymerization [218]. Moreover, Tat favours oxidative stress [219], which may induce cells to divide but, at higher levels, causes apoptosis or necrosis [220], and activates the pro-apoptotic pathway Egr1-PTEN-Akt-FOXO3a [221]. These mechanisms lead to the Tat-mediated up-regulation of the TNF-related apoptosis inducing ligand (TRAIL) [197, 222] and of the "death receptor" Fas (CD95) in different cell types [210, 223].

On the contrary, many reports show that cell lines expressing Tat are protected by serum starvation-, TRAIL- and Fas- mediated apoptosis [160, 224-226] through the up regulation of NF-κB dependent anti-apoptotic molecules, in particular Bcl-2 [160, 225, 227]. Same phenomenon occurs when co-culturing cells with low doses of Tat protein (nM) or with immobilized Tat, while higher concentrations of soluble Tat seem to induce apoptosis [150, 197, 224, 227]. Tat-mediated enhancement of NF-κB and NFAT and the activation of the anti-apoptotic pathway

PI3K/Akt/GSK-3 [161] are also usually observed at nanomolar concentrations [161, 228], confirming that these anti-apoptotic effects are preferentially activated by low amounts of Tat.

1.6.4 Effects of Tat on intracellular signalling

Tat displays different properties depending by the concentration and localization (intra or extracellular), suggesting that it may signal through different pathways. Extracellular receptor-activated kinase (ERK) and c-Jun N-terminal kinase (JNK) are two kinases lying downstream to TCR and CD28 and triggered by Tat. The two-exon form of Tat is required to activate JNK, and this effect follows a dose-dependent fashion. Conversely, both the one and two exon forms of Tat activate ERK/MAPK, not following a clear dose-dependence as the maximal activation occurs at very low Tat amounts (1nM) [91, 229]. The dose of Tat required for JNK activation (micromolar range) is the same shown to inhibit cell growth, while ERK/MAPK activation occurs at lower concentrations (nanomolar range), like the Tat-mediated proliferation [91]. Thus, it has been proposed that Tat may induce pro-apoptotic signals through JNK (sustained JNK activation is associated with apoptosis), while mediates cell proliferation through the ERK pathway, whose activation is required for cell growth [230]. The pleiotropic effects of Tat on these two pathways are confirmed by the observation that the rapamycin, which inhibits mTORC1 activity, abolished Tat-mediated activation of JNK while enhanced Tat-mediated activation of ERK/MAPK, suggesting the involvement of mTOR in Tat-induced signalling [91].

The activation by extracellular Tat of JNK and ERK occurs through two NADPH oxidases, respectively Nox2 and Nox4 [187, 231, 232], which are in turn activated by the GTPase Rac, downstream the interaction between integrins and the RGD domain of Tat [231]. Nox4 mediates Tat-dependent proliferation, whereas Nox2 mediates cytoskeletal rearrangements and oxidative stress (Fig. 1.5) [231, 233].

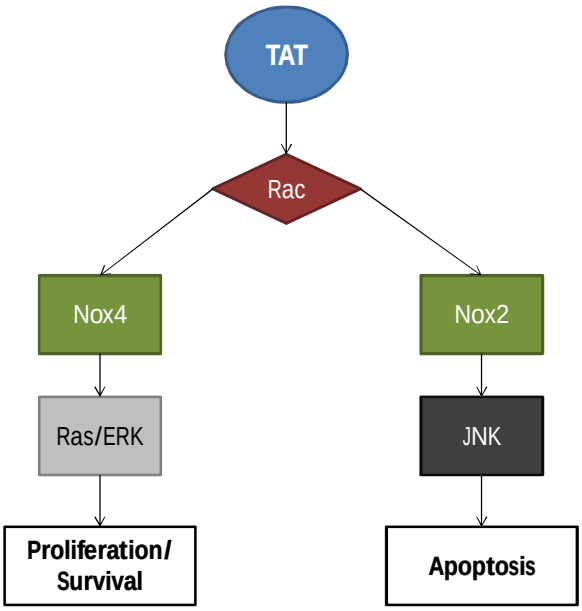

Figure 1.5 Tat intracellular signalling. *Adpated by [231].*

In addition to mediate effects on T cell proliferation, the ERK pathway is also involved in Tat-mediated increase of IL-2 production. Indeed, through ERK, Tat activates c-fos which constitutes, in association with c-jun, the transcription factor AP-1 [234]. AP-1, cooperating with NFAT, also activated by Tat, binds to IL-2 promoter to induce IL-2 production [235].

As previously mentioned, Tat-mediated anti-apoptotic effects are also due to the activation of PI3K/Akt, a pathway crucial for cell survival, proliferation, gene expression and cell migration and activated by nano/picomolar concentrations of Tat through its basic and RGD domains [162, 228, 236]. Tat induces the degradation of PTEN, a negative regulator of Akt [236]; once activated, Akt triggers, through Nox2, NF-κB [233]. Tat has been shown to activate NF-κB through different signalling cascades, indirectly modulating or directly interacting with NF-kB regulators [204, 237]. The induction of NF-kB has been proposed to be one of the fundamental mechanisms that mediates Tat-induced T cell hyperactivation [165, 185, 204, 238].

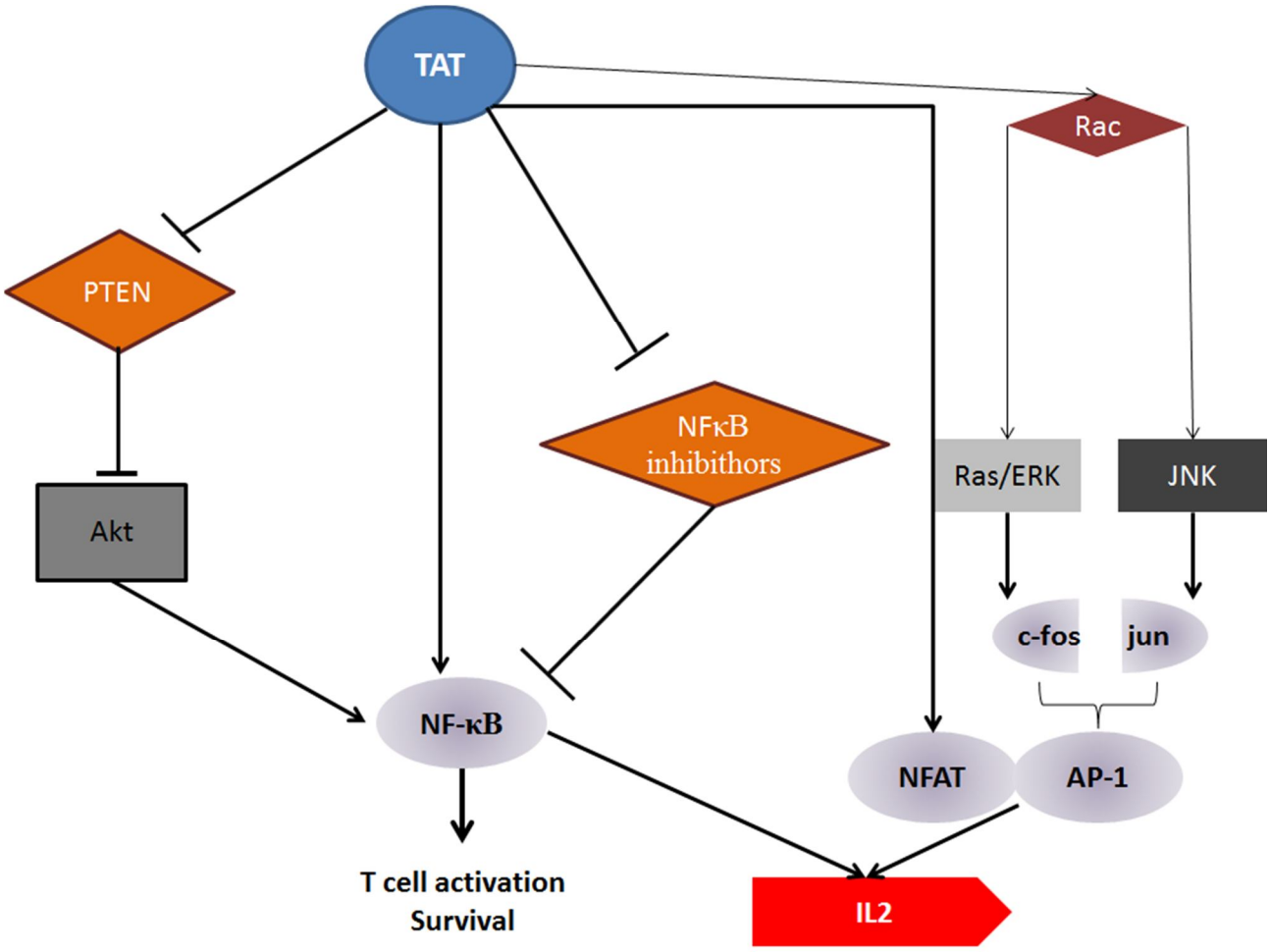

Figure 1.6 Tat-mediated NF-κB activation and IL-2 induction

1.7 The Tat protein of HIV: protective immunity

The CTL responses against HIV proteins that are produced in the first phases of the infection are of particular importance for viral control, and this is highlighted by the detection of Tat-CTL escape mutants early after infection [239, 240]. Moreover, there is evidence that the presence of anti-Tat CTLs correlates with non-progression in HIV-positive individuals [241]. The N-terminus region, the core region and the basic domain of Tat contain the majority of T cell [242, 243], as well of B cell [184, 244], epitopes.

Consistent with the roles of Tat in HIV pathogenesis and it's early production after infection, anti-Tat IgM and IgG are more frequent in the asymptomatic stage of the infection [245, 246]. Anti anti-Tat IgG are present in a small proportion of HIV-infected individuals, but are more frequently found in nonprogressors [247]. Indeed, several works describe the protection conferred by anti-Tat IgG from $CD4^+$ decline [248, 249], high viral load or p24 antigenemia [250, 251] and disease progression [247, 250, 251].

1.7.1 Tat-based vaccine

Tat plays a key role in HIV life cycle and progression to AIDS and anti-Tat humoral and cellular immunities constitute a correlate of protection. Thus, the inclusion of Tat as antigen in preventive and therapeutic vaccines against HIV has been pursued by several groups.

Immunization with Tat in different animals models elicited antibody responses able to block Tat-entry and Tat effects on gene expression and replication [Rewied in 252]. Moreover, Tat-vaccinated monkeys kept viremia at undetectable levels and prevented $CD4^+$ decline after challenge with SHIV viruses, suggesting an abortive infection [253, 254]. In addition, in Tat-vaccinated and SHIV-challenged macaques was observed long-term protection, absence of viral reservoirs and of virus replication, and both anti-Tat humoral and cellular responses were responsible for this effect [255, 256]. These results, along with safety and immunogenicity data collected in several animals models [245, 252], supported the development of a Tat-based vaccine. A preventive and a therapeutic phase I trial were conducted in parallel with the recombinant biologically active HIV-1 Tat (86aa) to evaluate safety and immunogenicity [257, 258]. After the successful achievement of the end-points, the Tat vaccine advanced in clinical phase II therapeutic trials in Italy and South Africa. Results from an *ad hoc* exploratory interim analysis on 87 HAART-treated HIV-positive individuals receiving the Tat vaccine during phase II trial was published in 2010 [259]. Tat-injection reversed immune activation signs decreasing levels of cellular and soluble markers of immune activation and hypergammaglobulinemia. Of note, restoration of immune functions after Tat-injection was confirmed by the reversion of $CD4^+$ T cells and B lymphocytes loss in Tat-immunized individuals, as well as by the increased cellular responses to HIV and heterologous recall antigens [259].

A second Tat-based vaccine that utilizes a Tat variant isolated from a group of HIV controllers African patients (Tat Oyi)[260] entered in phase I clinical trial in France in 2013.

1.7.2 Tat as adjuvant

The immunomodulatory properties displayed by Tat render this molecule an attractive adjuvant for other antigens. Indeed, as previously mentioned, Tat induces DCs maturation and activation, thus favoring co-stimulation of T cells and potentiating T cell responses [148, 149]. In addition, the Tat-mediated modification of proteasome composition and antigen presentation [191, 192] increases T cell responses to subdominant epitopes. Indeed, it has been demonstrated that co-immunization of mice with OVA and Tat protein induces CTL responses against subdominant and cryptic OVA-derived epitopes which were not detected in mice vaccinated

with OVA alone. Similarly, mice vaccinated with the HIV-1 Gag, Env or V2-deleted Env antigens in combination with Tat showed Th-1 type and CTL responses directed to a larger number of T cell epitopes, as compared to mice vaccinated with Gag, Env or V2-deleted Env proteins alone [193]. Interestingly, the adjuvant properties mediated by Tat did not affect Th2-type responses [193, 261], consistent with in vitro studies that demonstrate that Tat induces a predominant Th1-type adaptive immune response [148, 149].

A short sequence of the Tat protein, the PTD (aa 47-59), is already used for its property to enable conjugated proteins to enter the cells [262]. Thus, the fusion of heterologous antigen with Tat or PTD has been used to increase MHC class I antigen presentation and increase $CD8^+$ T cell responses [263]. In addition, some studies suggest that PTD itself increases the expression of certain MHC-I/peptide complexes [196]. Finally, some works have demonstrated that Tat possesses auto-adjuvanticity and adjuvanticity to unrelated antigens with respect to humoral responses [264].

Considering all these features possessed by Tat, its use in combination with other antigens to develop a vaccine against HIV has been proposed. Thus, combination of Tat with Gag or Env antigens has been explored in different formulation and has been shown to be safe and immunogenic in mice [193, 261, 265] and to protect SHIV challenged monkeys from $CD4^+$ T cells decline, high viremia and, in some cases, from infection acquisition [266, 267]. Given the promising results collected during the pre-clinical phases, the Italian National Institute of Health (ISS), in collaboration with Novartis, is now enrolling volunteers for a phase I clinical trial based on Tat and Env proteins combination (study ISS P-002).

In parallel, also the possibility of associating Tat with other regulatory proteins has been explored in mice and monkeys [Reviewed in 252]; in particular, the combination of Tat and Rev seems to protect macaques in a preventative approach [268, 269]. More in general, several multi-component vaccines containing Tat have been tested in animals, and different approaches that include the combination of structural and regulatory HIV antigens, as well as strategies aiming at generating immune responses against multiple early and late antigens of different HIV clades are now at the clinical stage [Reviewed in 252].

2 Objective

The overall objective of this study is the characterization of the immunomodulatory properties of the Tat protein of HIV-1 on $CD8^+$ T cells, to understand the role of this protein in T cell hyperactivation and dysfunctions as well as to consider its use in vaccination approaches.

It is known that extracellular Tat induces integrin-mediated signals and efficiently enters cells, resulting in the activation and modulation of several cellular functions in $CD4^+$ T cells and professional APCs, suggesting that Tat may play an important role in the chronic immune activation described during the HIV infection. However, whether Tat can, directly or indirectly, affect $CD8^+$ T cell functions is not clear.

The first part of the study was aimed to investigate whether Tat (clade B and C) may modulate the transcriptional profile and the functionality of $CD8^+$ T cells. *In vitro* experiments were performed on human resting and activated $CD8^+$ T cells, in the presence or absence of Tat, alone or together with $CD4^+$ T cells. Data collected allow to better understand the role of extracellular Tat on uninfected $CD4^+$ and $CD8^+$ T lymphocytes and their hyperactivation during HIV infection.

The second part of the study was aimed to understand how the immunomodulatory properties displayed by Tat on APCs and T lymphocytes affect, *in vivo*, the overall immune responses against a viral infection and the generation and maintenance of effector and memory $CD8^+$ T lymphocytes. The obtained results provide new insights about the role of Tat on the immune activation and immune impairment occurring during HIV infection.

Finally, the third part of the study consisted in a comprehensive analysis regarding the interplay of the different anti-Tat antibody isotypes in HIV control. Indeed, as immune activation is a marker of disease progression, and as Tat affects T cell activation and functionality, we sought to determine if anti-Tat immunity may play a protective role. Moreover, cross-clade reactivity of anti-Tat antibodies was assessed to better understand the potential use of clade B Tat in vaccination strategies against subtype C HIV-1.

Data collected in this study provide important evidence about the role of Tat on immune activation and immune impairment and further confirm the importance of anti-Tat immunity. Moreover, the comparison between clade B and C Tat effects provide new insights in the disease mechanisms of the different HIV clades. Furthermore, the capacity of Tat to activate T cells, if finely regulated, may be beneficial for the development of new subunit-combined vaccines in which B or C Tat may play at the same time the role of antigen and adjuvant.

3 Methods

3.1 *In vitro* activities

3.1.1 Human cells

Buffy coats from healthy volunteers that provided consent were obtained from the university hospital in Ferrara. Peripheral blood lymphocytes (PBLs) were separated by use of Ficoll–Hypaque density gradient centrifugation followed by 90 minutes of adhesion on a plastic support at 37°C to eliminate monocytes.

$CD4^+$ and $CD8^+$ T cells were sorted by MACS magnetic negative selection (Miltenyi Biotec) according to manufacturer instructions (purity > 95% assessed by FACS). Cell sorting occurred before or after the treatment, as specified in the experiments.

PHA-activated blasts were obtained by stimulation of PBLs with 1 µg/ml purified PHA for 3 days and expanded in medium supplemented with human rIL-2 (1000 U/ml).

T2 cells were cultured overnight at 26°C in 1 ml of serum-free AIM-V medium. Cells were washed, treated with mitomycin C, and pulsed with 10^{-5}M of the different peptides for 3 hours at 37°C in AIM-V medium.

3.1.2 Tat and Gag proteins

The HIV-1 Tat proteins from the human immunedeficiency virus type 1 (HIV-1) clade B or clade C were produced in *E. coli* and purified by diethylaminoethyl (DEAE) and heparin sepharose chromatography by DIATHEVA [270]. The Tat protein was resuspended in PBS containing 0.1% of BSA for in *vitro* experiments or in saline buffer containing 1% sucrose and 1% human serum albumin for *in vivo* experiments. The purified Tat protein was fully biologically active, as determined by the rescue assay on HLM-1 cell line carrying a Tat-defective HIV provirus [145, 155], by the induction of transcription in TZM-bl cells, which contain a luciferase reporter gene under the transcriptional control of the HIV LTR and are commonly used to assess HIV infectivity, as well as by Tat uptake by monocyte-derived dendritic cells (MDDCs) evaluated by intracellular staining for Tat in flow cytometry [16]. This assay constitutes the potency test for HIV-1 Tat protein, since it is highly specific for the reduced form of Tat (uptake does not occur with the oxidized form), and is strictly dose-dependent, allowing a precise determination of the content of active protein in the preparation (National AIDS Center, ISS, Rome). LPS presence was assessed and endotoxin concentration was below the detection limit (0.05 EU/µg), as determined by the Limulus

Amoebocyte Lysate analysis (Pyrochrome, Associates of Cape Cod, Falmouth, MA). The HIV-1SF162 Gag (502 aa) protein was obtained from Novartis.

3.1.3 Culture conditions and anti-CD3/CD28 stimulation

For "resting conditions", $3x10^6$ PBLs, $CD4^+$ or $CD8^+$ T cells were cultured in 2 ml of RPM containing 10% FCS ("complete medium") in 24-well flat bottomed polystyrene plates, in the presence or absence of Tat.

For surface phenotype analysis, proliferation assessment and analysis of transcriptional profile, cells activation occurred through anti-CD3 and anti-CD28 mAbs. 24-well flat bottomed polystyrene plates were coated overnight at 4°C with PBS and anti-CD3 mAb (0.5 µg/ml). Soluble anti-CD28 mAb (0.1 µg/ml) and, when required, Tat, were added after cells seeding ($3x10^6$ cells per well in 2ml of complete medium).

For intracellular staining, cells were activated with anti-CD3 and anti-CD28 mAbs. 96-well flat bottomed polystyrene plates were coated overnight at 4°C with PBS and anti-CD3 mAb (1.5 µg/ml). Soluble anti-CD28 mAb (1 µg/ml), anti-CD49d (1 µg/ml), and, when required, Tat, were added after cells seeding ($1x10^6$ cells per well in 0.2 ml of complete medium).

3.1.4 Generation of CTLs culture

HLA A11-restricted EBV-specific CTL cultures reacting against the EBV-encoded nuclear Ag 4 (EBNA4)-derived IVTDFSVIK (IVT, aa 416–424) and HLA A2-restricted EBV-specific CTL cultures reacting against the EBV-encoded LMP2-derived CLGGLLTMV (CLG, aa 426–434) epitope and the LMP1-derived YLQQNWWTL (YLQ, aa 159–167) epitope were obtained by stimulation, in the presence or absence of Tat (0.1 µg/ml), of lymphocytes from HLA-A2-positive and HLA-A11-positive EBV-seropositive volunteers with peptide-pulsed T2 cells. HLA A2-restricted survivin-specific CTL cultures reacting against the survivin tumor antigen ELT (ELTLGEFLKL) were obtained by stimulation, in the presence or absence of Tat (0.1 µg/ml), of lymphocytes from HLA-A2-positive healthy volunteers with peptide-pulsed T2 cells.

Briefly, PBLs were plated at $3x10^6$ cells per well in 24-well plates in complete medium and stimulated with peptide-pulsed T2 cells at a stimulator-responder ratio of 1:20, in the presence or absence of Tat. Cultures were re-stimulated after 7 and 14 days, and the medium was supplemented from day 8 with 1000 U/ml rIL-2. On days 21, CTLs cultures were tested for CTL activity using cytotoxicity assay.

Alternatively, PBLs from healthy volunteers were plated at $3x10^6$ cells per well in 24-well plates in complete medium and stimulated with 32µg/ml of CEF peptide pool (Anaspec), in the presence or absence of Tat. Cultures

were re-stimulated after 7 days, and the medium was supplemented from day 8 with 1000 U/ml rIL-2. On day 14, CTLs cultures were tested for IFNγ release using Elispot assay.

Peptides were synthesized by solid phase method and purified by HPLC to >98% purity (UF Peptides, University of Ferrara).

3.1.5 Cytotoxicity assay

The cytotoxic activity was assayed in standard 5 hours ^{51}Cr release assays. Target cells (PHA blasts) were labeled with $Na_2{}^{51}CrO_4$ (3.5 MBq/10^6 cells, Perkin Elmer) for 60 minutes at 37°C and, where indicated, pulsed for 45 minutes with 10^{-6} M of the different peptides at 37°C. Subsequently, cells were washed three times and incubated with effector cells. ^{51}Cr release was measured after 5 hours at 37°C through the use of a γ-counter. Maximum ^{51}Cr release was evaluated treating target cells with Triton X-100, while spontaneous ^{51}Cr release was evaluated in target cells incubated alone in complete medium. Cytotoxicity tests were routinely run at different E:T ratios in triplicate. Percentage of specific lysis was calculated as 100 x (cpm sample - cpm medium)/(cpm Triton X-100 - cpm medium). Spontaneous release was always < 10%. None of the tested peptides affected spontaneous release.

3.1.6 Elispot assay

CTLs (4 x 10^4) were seeded in triplicate on microplate 96-well unifilter (Whatman.) coated with an anti IFNγ mAb (Pierce, USA). CTLs were stimulated with CEF peptide pool. CTLs incubated with medium alone were used as negative control, whereas CTLs stimulated with PHA (Wellcome Diagnostics.) represented the positive control. Plates were incubated for 24 hours and washed, and then a biotinylated anti-IFNγ mAb (1 µg/ml; Pierce) was added to the wells. After 60 min, the plates were washed again and HRP-conjugated streptavidin (Pierce) was added at room temperature for 45 minutes. Individual IFNγ producing cells were detected using 3-amino-9-ethylcarbazole cromogen kit (Sigma-Aldrich) and counted by ELISPOT reader (AELVIS). The number of specific IFNγ secreting T cells, expressed as spot-forming units per 10^6 cells, was calculated by subtracting the negative control values.

3.1.7 Intracellular Staining

PBLs were stimulated with anti-CD3, anti-CD28 and anti-CD49d as described above in the presence of Monensin (1 µg per well) and CD107b-FITC (bioscience product). Duration of stimulation varied in different assays. PBLs

were stimulated for 2 h before adding Brefeldin A (BFA; Sigma-Aldrich). After stimulation, PBLs were washed once with PBS and incubated with EDTA for 15 minutes at room temperature. Subsequently, surface proteins were stained for 20 minutes and cells were washed twice with FACS buffer (PBS, 1% FCS). The cells were permeabilized using the Cytofix/Cytoperm kit (BD), after which they were stained with anti-CD3 APC-Cy7, anti-IFNγ V450, anti-IL-2 APC and anti-TNFα PE Cy7 (BD). Cells were then washed twice and fixed in CellFix (BD). Cells were analyzed with a FACScanto II. Electronic compensation was conducted with antibody capture beads (BD) stained separately with the individual antibodies used in the test samples. Flow cytometry data were analyzed using FlowJo (version 8.8.3; Tree Star, Inc.).

3.1.8 Flow Cytometry

All stainings were carried out in FACS buffer for 30 min at 4°C with the following antibodies: HLA-DR FITC, CD38 PE/Dy 747, CD25 FITC, CD4 PE, CD8 PE (Immunotool); CD95 FITC (BD); CD4 Qdot 705, CD8 Qdot 705 (Life Technologies). Proliferation assessment was carried out through CFSE staining. PBLs were labeled with a solution containing 5 µM 5(6)-carboxy-fluorescein diacetate succinimidyl ester (CFSE, eBioscience) for 10 minutes at 37°C and then complete medium was added. Cells were washed twice before culturing. Data were acquired on a BD FACScan and analyzed using BD Cell Quest Pro software.

3.1.9 MTT assay

MTT test was used to study T cells survival. PBLs were seeded in triplicate in 96-well plates at a density of $1x10^5$ in a final volume 130 µl of complete medium and Tat was added at the concentration of 0.1 or 1 µg/ml. For stimulation with anti-CD3/CD28, plates were pre-coated overnight with 0.3 µg of anti-CD3 mAb/well at 4°C, and 0.2 µg of anti-CD28 mAb was added at the time of cells seeding. For stimulation with PHA or PMA, 0.2µg of PHA or 1ng of PMA and 125ng of Ionomycin were added at the time of cells seeding. For stimulation of LPS, 1µg of LPS was added at the time of cells seeding. Cells were cultured for 6 days, and then 25 µl of a 3-(4,5-dimethylthiozol-2-yl)2,5-diphenyltetrazolium bromide solution (MTT, Sigma-Aldrich) (12 mM) were added. After two hours of incubation, 100 µl of lysing buffer (50% DMF + 20% SDS, pH 4.7) were added to convert the MTT solution into a violet colored formazane. After additional 18 hours the solution absorbance, proportional to the number of live cells, was measured by spectrophotometer at 570 nm.

3.1.10 Reverse transcription (RT) and quantitative real time PCR

DNase-treated total RNA was isolated from cells using Trizol reagent (Life Technologies) according to the manufacturer's instructions and used to perform cDNA synthesis (High Capacity cDNA Reverse Trnscription Kit, Applied Biosystems). cDNA was PCR-amplified with a Chromo4 real-time PCR Detection System using Kapa SYBR Green Fast qPCR Kit (Kapabioststems) according to the manufacturer's recommendations with the following cycle conditions: 3 minutes at 95°C, then 40 cycles of 15 seconds at 95°C, and 20 seconds at 60°C. Quantitative PCR was performed using the pairs of primers shown in Table 3.1. The relative levels for each RNA were calculated by the $2^{-\Delta\Delta CT}$ method using human 18s as housekeeping gene. Each CT value is the mean of two biological replicates and each assay was performed a minimum of two times.

Gene	Forward 5'-3'	Reverse 5'-3'
T-bet	GCGCCAGGAAGTTTCATTTG	GGAAAGTAAAGATATGCGTGTTGG
Eomes	TCATTACGAAACAGGGCAGG	TGCATGTTATTGTCGGCTTTG
IL-2	AAGAATCCCAAACTCACCAGG	ATTGCTGATTAAGTCCCTGGG
18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Table 3.1 Primers used for qPCR

3.2 *In vivo* activities

3.2.1 Viruses

The wild-type Herpes Simplex virus type 1 (HSV1), LV strain [271], and a replication-incompetent HSV1 virus, named S0ZgJGFP, were used in this study to assess immunomodulation by Tat protein.

All viruses were generated and provided by the laboratory of Dr. Peggy Marconi, Department of Life Sciences and Biotechnology, University of Ferrara.

3.2.2 Peptides

HSV1 K^b-restricted peptides SSIEFARL (SSI), derived from glycoprotein B (gB), and QTFDFGRL (QTF), derived from ribonucleotide reductase 1 (RR1), were used to evaluate anti-HSV1 T cell responses in C57BL/6 mice.

Gag K^d-restricted peptides (Table 3.2) [261] were used to evaluate anti-Gag T cell responses in Balb/c mice and were provided by the NIH AIDS Repository Reagents and References Program. Peptide stocks were prepared in DMSO at 10^{-2} M concentration, stored at -20 °C, and diluted in complete medium before use.

Peptide Sequence	Gag aa	Code	T cell response
TVATLYCVHQRIEVK	Gag 81-95	TVA	CD8
SPEVIPMFSALSEGA	Gag 165-179	SPE	CD8
AMQMLKET	Gag 197-205	AMQ	CD8
AAEWDRLHPVHAGPI	Gag 209-223	AAE	CD8
IYKRWIILGL	Gag 261-270	IYK	CD8
IVRMYSPTSILDIRQ	Gag 273-287	IVR	CD8
VDRFYKTLRAEQASQ	Gag 297-311	VDR	CD4
YKTLRAEQASQEVKN	Gag 301-315	YKT	Not identified
MTETLLVQNANPDCK	Gag 317-331	MTE	Not identified

Table 3.2 Gag peptide epitopes

3.2.3 Mice immunization and infection and samples collection

BALB/c female mice (Charles River Laboratories) were immunized with 5 µg of HIV-1 Gag protein alone or in combination with 5 µg of Tat protein. Immunogens (100 µl) were given subcutaneously at a single site in the back at day 0, and mice were sacrificed at day 10. C57BL/6 female mice (Charles River Laboratories) were pre-treated, one week before infection, with 2 µg/100 µl of Depo-Provera® (Depo-medroxy-progesterone acetate; Pharmacia & Upjohn) subcutaneously in the neck, to bring the mice at the same estrous stage and render them more susceptible to HSV1 infection. C57BL/6 mice were inoculated intravaginally with 1 x 10^4 of wild-type HSV1 (strain LV) or 1 x 10^8 of replication-defective HSV1 (S0ZgJGFP). Before injection, the virus was thawed on ice, sonicated for 5 seconds, and stored on ice. Mice were anaesthetized with 5% isofluorane to allow scraping of the vagina with a pipe scraper (in order to remove the mucus that could trap the virus) and then inoculated with the purified virus (in 10 µl of total volume for each mouse) using a pipette-tip. Part of mice were injected, at the time of infection, with Tat protein (5µg) given subcutaneously (Tat-treated mice), or with only Tat-suspension buffer (control mice). After infection, mice were observed daily to monitor the appearance of local and/or systemic clinical signs of infection including death. Disease severity was measured using the following scores: 0 (no signs

of infection), 1 (appearance of ruffled hair), 2 (appearance of cold sores on and around the vagina), 3 (appearance of paralysis of the back limbs) and 4 (mouse death). Mice were sacrificed at different time points to analyse anti-HSV1 immune responses on fresh splenocytes cultures (individual mice) by means of IFN-γ Elispot assays or dextramer staining. At sacrifice, mice were anesthetized intraperitoneally with 100 µl of isotonic solution containing 1 mg of Zoletil (Virbac) and 200 µg Rompun (Bayer) to collect blood and spleens.

The presence of HSV1-specific IgM and IgG in sera was evaluated by enzyme-linked immunoassays (ELISA). Blood samples were collected from retro-orbital plexus, incubated for 16 hours at 4°C, centrifuged for 10 minutes at 10000g to obtain sera and stored at -80°C until analysis. Each group was composed of three/five animals. Each experiment was repeated three times.

Mice experiments were conducted according to European and Institutional guidelines for housing and care of laboratory animals and performed under protocols approved by the Italian Ministry for Health.

3.2.4 Elispot assay

IFN-γ Elispot assays were carried out using the murine kits provided by Becton Dickinson, according to the manufacturer's instructions. Briefly, nitrocellulose plates were coated with 5 µg/ml of anti-IFN-γ mAb for 16 hours at 4°C. Plates were then washed with PBS and blocked with RPMI 1640 supplemented with 10% FCS for 2 hours at 37°C. Total splenocytes from individual mice (1-5 x 10^5 cells) were added to duplicate wells and incubated with HSV1- or Gag-derived peptides (10^{-6} M) for 24 hours at 37°C. Controls were represented by cells incubated with 5 µg/ml of Concanavaline A (GE Healthcare) as positive control or with medium alone (negative control). Spots were quantified using an AELVIS 4-Plate Elispot Reader. The number of spots counted in the peptide-treated cultures minus the number of spots counted in the untreated cultures was the specific response. Results are expressed as number of spot forming units (SFU)/10^6 cells. Values at least 3-fold higher than the mean number of spots in the control wells (untreated cells) and ≥ 50 SFU/10^6 cells were considered positive.

3.2.5 Flow Cytometry

All stainings were carried out in FACS buffer for 30 minutes at 4°C. The following antibodies were utilized: CD8 FITC (Immunotools); CD8 PE, CD4 FITC, B220 FITC, CD62L PE (eBioscience); CD8 PerCP, CD4 PerCP, CD95 PeCy7 (BD Biosciences); PD1 FITC (Biologend).

For dextramer staining, spleen cells (1 × 10^6) were incubated for 10 minutes at room temperature with PE labeled SSI dextramer (Immudex) and washed prior staining with surface antigen antibodies.

Data were acquired on a BD FACScan or a FACS Canto II and analyzed using BD Cell Quest Pro or Diva software.

3.2.6 Enzyme-linked immunosorbent assay (ELISA)

Anti-HSV1 specific antibodies in sera (IgM, IgG, IgG1 and IgG2a titers) were measured on samples collected from individual mice using 96-well immunoplates (Nunc Max Sorp) previously coated with 100 ng/well of HSV1 viral lysate (Herpes Simplex Type 1 Purified Viral Lysate, Tebu-bio), resuspended in PBS containing 0.05% NaN_3, for 16 hours at 4°C. Plates were washed five times with PBS (pH 7.0) containing 0.05% Tween 20 (Sigma) (washing buffer) using an automatic washer (BioRad Model 1575 ImmunoWash) and then blocked for 90 minutes at 37°C by the addition of 200 µl/well of PBS containing 0.5% milk and 0.05% NaN_3. After extensive washes, 100 µl/well of appropriate dilutions of each serum were dispensed in duplicate wells and then incubated for 90 minutes at 37°C. Plates were washed again before the addition of 100 µl/well of HRP-conjugated goat anti-mouse IgG (Sigma), diluted 1:1000, or HRP-conjugated goat anti-mouse IgM (Sigma), diluited 1:7500, in PBS containing 0.05% Tween 20 and 1% BSA, and incubated at 37°C for 90 minutes. In each plate, two wells were incubated with PBS containing 0.5% milk and 0.05% NaN_3 and the secondary antibodies (blank). Analysis of anti-HSV1 IgG isotype was determined using a goat anti-mouse antibody directed against IgG1 or IgG2a (Sigma), diluted 1:30,000 in PBS containing 0.05% Tween 20 and 1% BSA. After incubation, plates were washed five times and subsequently a solution of 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) substrate (Roche) was added. The absorbance values were measured after 50 minutes of incubation at 405 nm with an automatic plate reader (SUNRISE TECAN). The cut-off value was estimated as the mean OD of 3 negative control sera plus 0.05. Each OD value was subtracted of the blank and cut-off values to obtain a net OD value. IgG titers were calculated by intercept function using the Excel program.

3.3 Cross-sectional study

3.3.1 Study design

Human serum samples from 96 HAART-naïve HIV-1-positive subjects were obtained from the WHIS cohort, Mbeya Medical Reserch Center, Mbeya, Tanzania. The WHIS cohort contains information about viral load, $CD4^+$ counts, phenotype of T cells and Elispot data for the majority of enrolled individuals. Thus, our study was a cross-sectional study nested in the WHIS study. This study was approved by the ethics committee of the Tanzanian

National Institute of Medical Research and conducted according to the principles expressed in the Declaration of Helsinki. All participants provided written informed consent before enrolment into the study.

3.3.2 Enzyme-linked immunosorbent assay (ELISA)

Human anti-Tat IgA, IgM and IgG were measured in human sera using 96-well immunoplates (Nunc Max Sorp) previously coated with 100 ng/well of clade B or clade C Tat re-suspended in carbonate buffer, for 16 hours at 4°C. Plates were washed five times with PBS (pH 7.0) containing 0.05% Tween 20 (Sigma) and then blocked with PBS containing 0.05% Tween 20 and 1% BSA for 90 minutes at 37°C (IgG) or 60 minutes at room temperature (IgA) or with PBS containing 5% milk for 60 minutes at 37°C (IgM). After extensive washes, 100 µl/well of appropriate dilutions of each serum diluted in PBS containing 0.05% tween and 1% BSA ("block buffer" IgG and IgA) or PBS containing 5% milk ("block buffer" IgM) were dispensed in duplicate wells and then incubated for 90 minutes at 37°C. Plates were washed again before the addition of 100 µl/well of HRP-conjugated anti-human IgG (Sigma), diluted 1:1000, or HRP-conjugated anti-human IgA (Sigma) diluted 1:3000, or HRP-conjugated anti-human IgM (Sigma), diluted 1:1000, in block buffer and incubated at 37°C for 90 minutes (IgG) or for 60 minutes (IgA and IgM). In each plate, two wells were incubated with block buffer and the secondary antibodies (blank). After incubation, plates were washed five times and incubated with block buffer for 15 minutes at 37°C (IgG and IgM). Plates were washed five times and subsequently a solution of ABTS was added. The absorbance values were measured after 50 minutes of incubation at 405 nm with an automatic plate reader (SUNRISE TECAN). The cut-off value was estimated as the mean OD of 3 negative control sera plus 0.05. Each OD value was subtracted of the blank and cut-off values to obtain a net OD value. Titers were calculated by intercept function using the Excel program. As control sera were considered samples from HIV-negative subjects from the same cohort.

4 Results

4.1 Effects of the HIV-1 Tat protein on CD8+ and CD4+ T cell programming

4.1.1 Tat contributes to the activation of CD8+ T cells

Several studies have reported the capacity of the HIV-1 Tat protein to activate CD4+ T cells and increase IL-2 production in both HIV-infected and uninfected cells exposed to different stimuli [165, 205, 238]. Conversely, the role of Tat on CD8+ T cells, that are also substantially affected by HIV infection, is still unknown. To determine whether Tat could modulate CD8+ T cell responses, we first assessed the capacity of Tat to contribute to the activation of memory CD8+ T cells. To this purpose, PBLs from healthy volunteers (Methods section 3.1.1) were stimulated, in the presence or absence of Tat, with CEF peptide pool, a group of 32 peptides, 8-12 amino acids in length, with sequences derived from the human Cytomegalovirus, Epstein-Barr Virus and Influenza Virus (Methods section 3.1.4). After two rounds of stimulation, PBLs were tested for IFNγ release in Elispot assay against CEF peptides (Methods section 3.1.6). As shown in Fig. 4.1, the presence of Tat induced higher numbers of IFNγ-secreting epitope-specific CD8+ T cells suggesting that Tat favors the activation of memory CD8+ T cells.

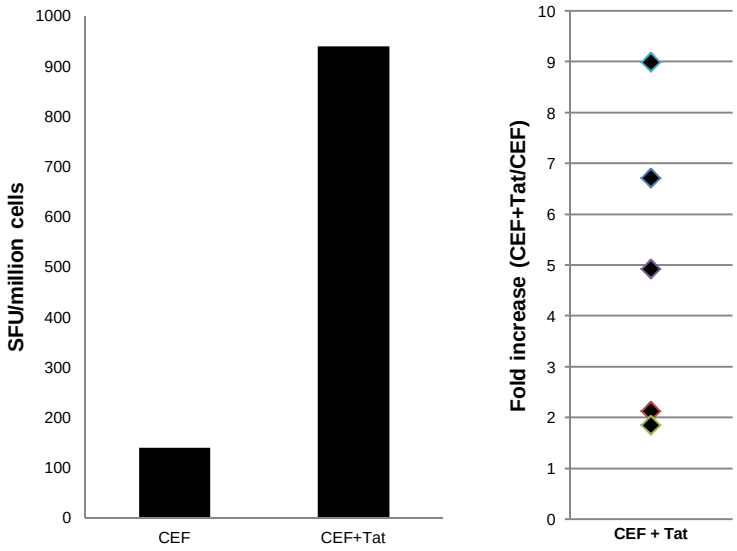

Figure 4.1 Tat favors the activation of antigen-specific memory CD8+ T cells. *PBLs from healthy volunteers (n=5) were stimulated with CEF peptide pool in the presence or absence of 0.1 µg/ml of Tat protein. After two weeks, cells were tested in IFNγ Elispot against CEF peptide pool. Bars show SFU/million cells after the subtraction of the background of one representative donor out of five. Dots show all the donors represented as fold increase of Tat-treated cells compared to cells stimulated in the absence of Tat (normalized to 1).*

In addition, PBLs obtained from healthy HLA class I-typed EBV-seropositive volunteers were stimulated three times with cells pulsed with EBV-derived CTL peptide epitopes in the presence or absence of Tat (0.1 μg/ml). Specifically, PBLs were stimulated with the subdominant HLA-A2-presented CLGGLLTMV (CLG) or YLQQNWWTL (YLQ) epitopes [272, 273] or the immunodominant HLA-A11-presented IVTDFSVIK (IVT) epitope [274] (Methods section 3.1.4). The cytotoxic activity of CTL cultures generated in the presence or absence of Tat, was tested using standard ^{51}Cr-release assays against autologous PHA-blasts, pulsed or not with the relevant synthetic peptide (Methods section 3.1.5). All three CTL cultures generated in the presence of Tat clearly exhibited higher percentages of specific lysis compared to CTL cultures generated in the absence of Tat (Fig. 4.2). These observations suggest that Tat favors the activation of memory CD8$^+$ T cells; however, they do not clarify whether Tat must be present at time of the stimulation or may enhance the cytotoxic activity of activated CD8$^+$ T cells. Thus, to address this issue, CTL cultures specific for the HLA-A2-presented CLG epitope were generated in the absence of Tat. Subsequently, CTL cultures untreated or treated for 1 or 2 days with the Tat protein at the concentration of 0.1 μg/ml were tested for their cytotoxic activity throughout ^{51}Cr-release assays against autologous PHA-blasts, pulsed or not with the CLG peptide. As shown in Fig. 4.2 D, CTL cultures, treated or not with Tat, lysed target cells at similar levels, suggesting that Tat enhances CD8$^+$ T cells activation only if present contemporarily with the stimulus, but does not enhance effector functions of CTLs that have been already generated.

To determine whether the Tat protein favors also the activation of naïve T cells, PBLs from HLA-A2 healthy volunteers were stimulated with the synthetic ELT peptide in presence or absence of the Tat protein. ELT (ELTLGEFLKL) peptide is a CTL epitope, presented by HLA-A2 [275, 276], belonging to the anti-apoptotic protein survivin that is overexpressed in tumor cells [277]. No T-cell reactivity against this epitope is normally detected in healthy patients [277]. The specificity of cultures, obtained in the presence or absence of Tat, was tested by 5 h ^{51}Cr-release assays against PHA-blasts pulsed with the ELT-peptide (Fig. 4.2 E). HLA-A2 positive PHA-blasts pulsed with the ELT-peptide were efficiently lysed by CTLs generated in the presence of Tat, while unpulsed HLA-A2 positive blasts or HLA-A2 positive blasts pulsed with the ELT peptide were not lysed by CTLs generated in the absence of Tat, clearly demonstrating a role of Tat in the enhancement of the activation of naïve CD8$^+$ T cells.

Taken together, these results suggest that Tat contributes to the priming and the activation of antigen-specific naïve and memory CD8$^+$ T cells.

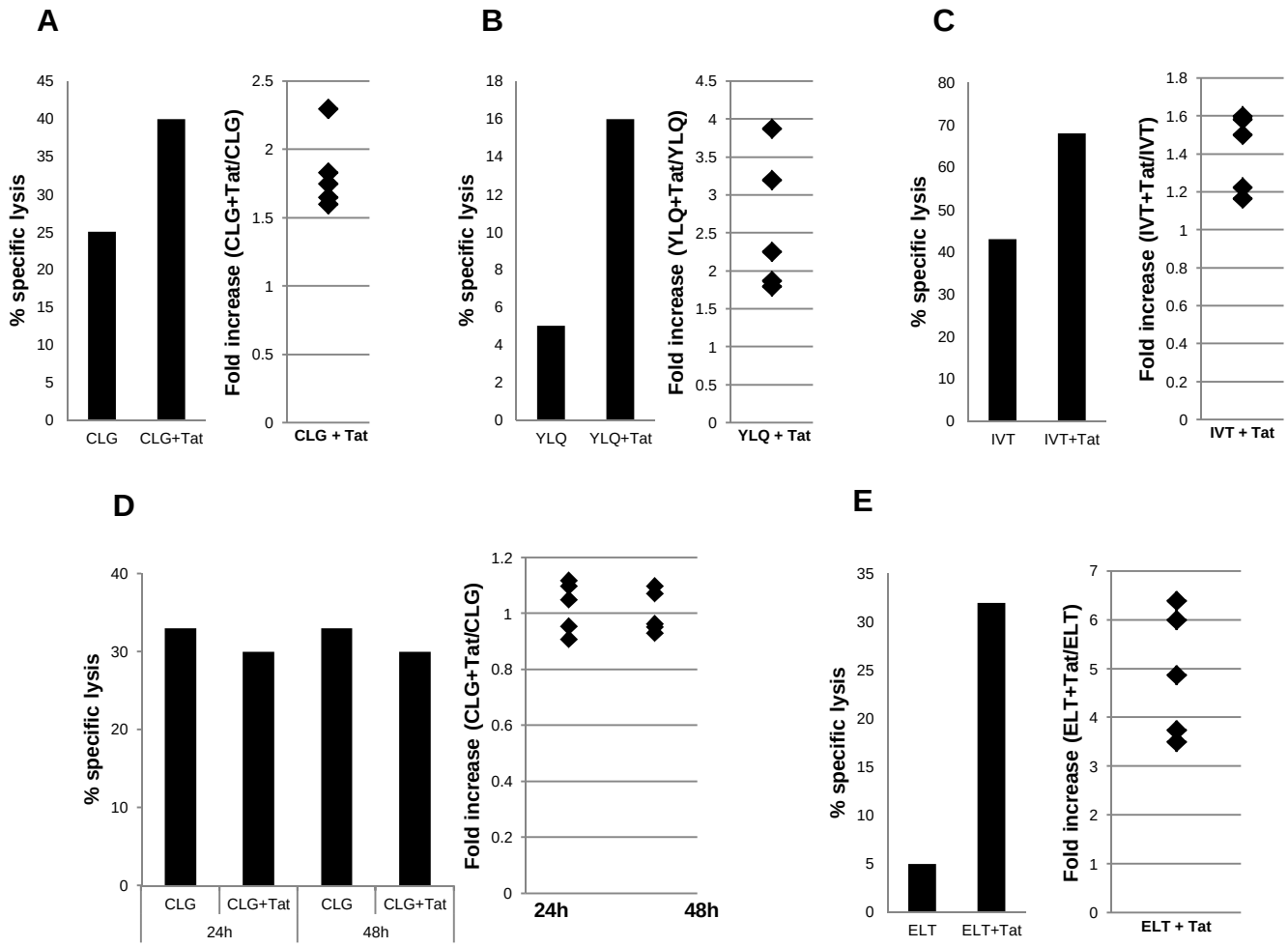

Figure 4.2 Tat favors the activation of antigen-specific naïve and memory CTLs. *CTLs cultures specific for CLG (A), YLQ (B) or IVT (C) EBV-derived epitopes were generated, in the presence or absence of Tat (0.1µg/ml), from lymphocytes purified from EBV-positive volunteers (n=5) and tested for their cytotoxic activity throughout ^{51}Cr-release assay against autologous peptide-pulsed PHA-blasts. (D) CTLs cultures specific for CLG peptide epitope were generated from lymphocytes purified from EBV-positive volunteers (n=5). 48 or 24 hours prior to ^{51}Cr-release assay, CTL cultures were treated with the Tat protein (0.1µg/ml). (E) CTLs cultures specific for ELT survivin-derived epitope were generated, in the presence or absence of Tat (0.1µg/ml), from lymphocytes purified from healthy volunteers (n=5) and tested for their specificity throughout ^{51}Cr-release assay against autologous peptide-pulsed PHA-blasts. Bars show percentages of specific lysis of one representative donor out of five. Dots show all the donors represented as fold increase of Tat-treated cells compared to cells stimulated in the absence of Tat (normalized to 1).*

4.1.2 Tat enhances IL-2 expression in CD8$^+$ and CD4$^+$ T cells

It has been demonstrated that Tat favors CD4$^+$ T cell activation and IL-2 secretion after stimulation with antibodies specific for the CD3 and CD28 receptors that mimic physiological T cell activation [165, 204, 205]. To confirm these results, PBLs from healthy volunteers were stimulated with anti-CD3/CD28 (Methods section 3.1.3) in the presence of different doses of Tat (from 0.001 µg/ml to 1 µg/ml), and IL-2 mRNA levels were measured after 4 hours (Methods section 3.1.10). At all Tat doses, except the lowest (0.001µg/ml), a 200-fold

induction of IL-2 mRNA was detected by real-time PCR in response to anti-CD3/CD28 treatment, while only a 75-fold induction was observed in the absence of Tat (Fig. 4.3 A).

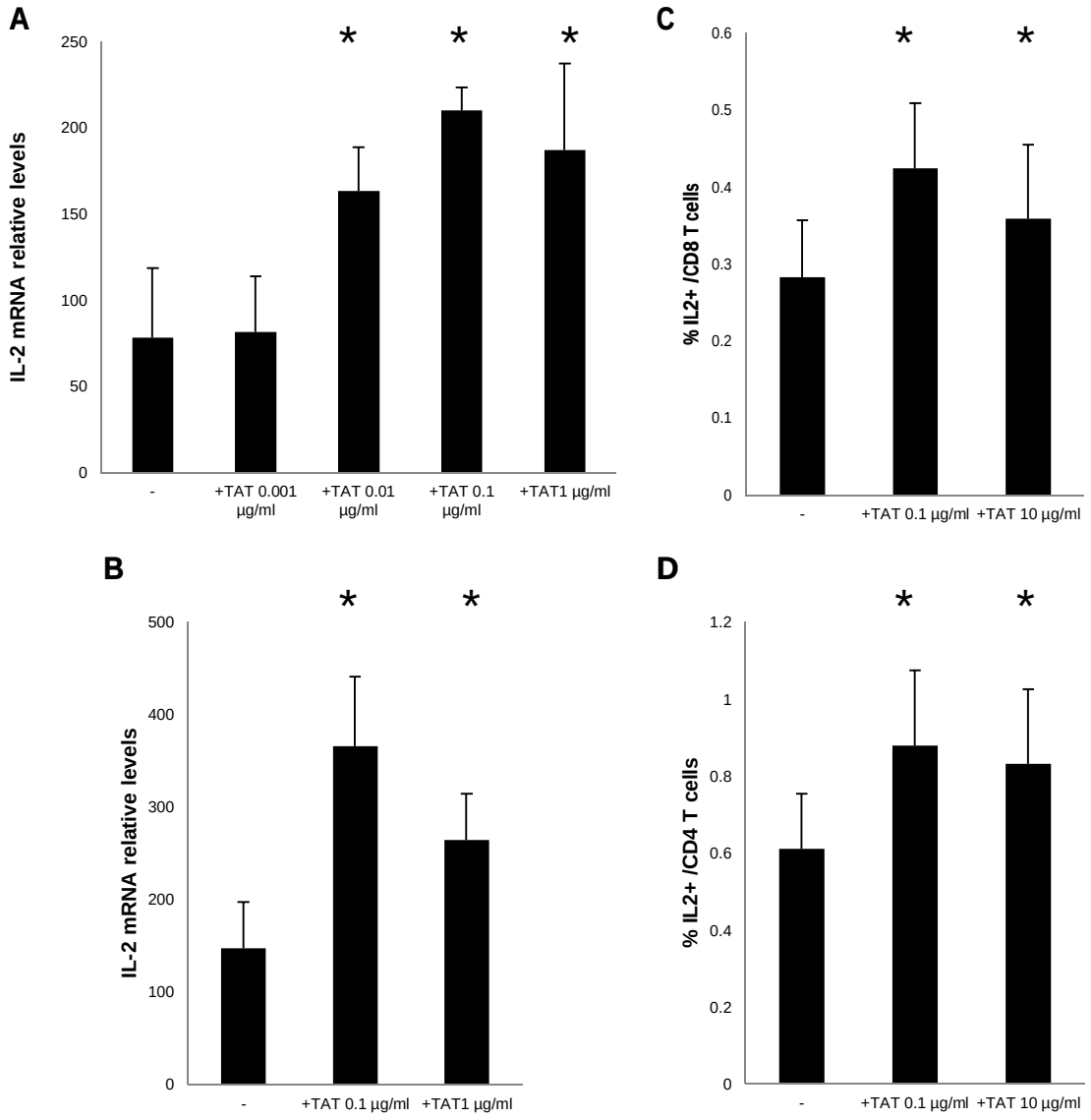

Figure 4.3 Tat enhances IL-2 production. *PBLs from healthy volunteers (n=6) were activated with anti-CD3/CD28 in the presence or absence of different concentrations of Tat for 4 (A) or 24 (B) hours. IL-2 mRNA levels were quantified by qPCR and normalized to untreated PBLs. (C-D) PBLs from healthy volunteers (n=8) were activated with anti-CD3/CD28 in the presence or absence of different concentrations of Tat for 18 hours. Percentages of $CD8^+$ (C) or $CD4^+$ (D) T cells secreting IL-2 were determined by ICS. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to control cells (-).*

Similar results were obtained at 24 hours after stimulation (Fig. 4.3 B), demonstrating that the effect is long lasting. Tat-mediated enhancement of IL-2 production was also evaluated by intracellular cytokine staining (ICS, Methods section 3.1.7). To this aim, PBLs from healthy subjects were activated with anti-CD3/CD28 in presence or absence of Tat (0.1 and 10 µg/ml), and the secretion of IL-2 was measured in $CD8^+$ and $CD4^+$ T cells at 6 and 18

hours after treatment. No effect of Tat on IL-2 production by $CD8^+$ and $CD4^+$ T cells was observed at 6 hours after stimulation (not shown). However, an average 1.5-time fold increase of IL-2 secretion was observed after 18 hours of stimulation in both $CD8^+$ and $CD4^+$ T cells activated in the presence of 0.1 µg/ml of Tat compared with $CD8^+$ and $CD4^+$ T cells activated without Tat (from 0.28% to 0.43% $IL\text{-}2^+CD8^+$ in the absence or presence of Tat respectively, $p<0.05$, Fig. 4.3 C, and from 0.60% to 0.90% $IL\text{-}2^+CD4^+$ in the absence or presence of Tat respectively, $p<0.05$, Fig. 4.3 D).

These data further confirm that Tat enhances $CD8^+$ and $CD4^+$ T cells activation and demonstrate for the first time that Tat increases IL-2 secretion in $CD8^+$ T cells.

4.1.3 Transcriptional profile of $CD8^+$ T cells activated in the presence of Tat

As Tat enhances activation of $CD8^+$ and $CD4^+$ T cells stimulated by TCR engagement (different CD8 peptide epitopes and anti-CD3/CD28 stimulation), we sought to determine whether this effect was linked to a modulation of the expression of some TFs important for the activation and effectors functions of T cells. To this aim, mRNA levels of T-bet were measured in PBLs activated in the presence of different Tat doses. T-bet is known to be up-regulated after T cell activation and to promote the transcription of effector genes [14].

Consistently with the results observed measuring IL-2 mRNA, Tat enhanced T-bet expression of more than two times at 4 and 24 hours after stimulation (Fig. 4.4 A-B). The reported results demonstrate that the effect was absent at the lowest dose of Tat (0.001 µg/ml), while was almost equivalent when Tat was present at concentrations ranging between 0.01 and 1 µg/ml, although the highest fold increase was observed at the 0.1 µg/ml dose. This concentration was chosen to perform the subsequent experiments.

It is known that extracellular Tat activates $CD4^+$ T cells binding, via its RGD region, to the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins expressed on the cell surface [147-150]. To understand whether the Tat-mediated enhancement of T-bet expression was due to the same mechanism, PBLs were preincubated with Abs directed against $\alpha_v\beta_3$ and $\alpha_5\beta_1$ and subsequently stimulated with anti-CD3/CD28. The reported results demonstrated that the effect of Tat was significantly inhibited by blocking integrins (Fig. 4.4 C), indicating that Tat enhanced T-bet expression through its binding with $\alpha_v\beta_3$ and $\alpha_5\beta_1$. Blocking experiments with anti-Tat immune sera demonstrated that this effect was specifically mediated by Tat (Fig. 4.4 C).

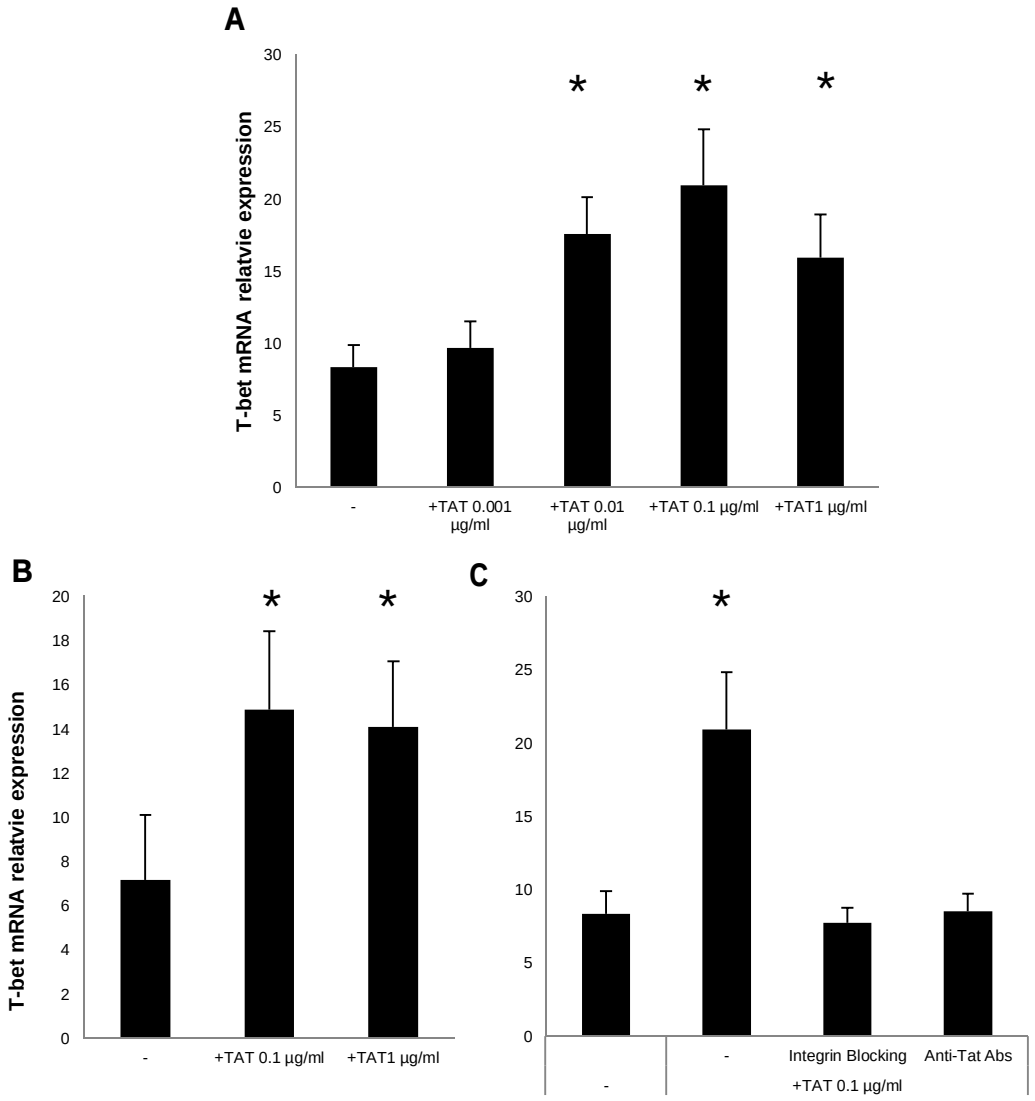

Figure 4.4 Tat increases T-bet expression. *PBLs from healthy volunteers (n=6) were activated with anti-CD3/CD28 in the presence or absence of different concentrations of Tat for 4 (A) or 24 (B) hours. T-bet mRNA levels were quantified by qPCR and normalized to untreated PBLs. (C) PBLs were pre-incubated with anti-integrins monoclonal antibodies or with anti-Tat immune sera for 1 hour at room temperature before activation and Tat treatment. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to control cells (-).*

To characterize the effects of Tat on $CD8^+$ T cells transcriptional profile, the expression of IL-2, T-bet and Eomes mRNA levels was evaluated in purified $CD8^+$ T cells activated, alone or in the presence of $CD4^+$ T cells, with anti-CD3/CD28. Eomes expression was assessed, along with T-bet, as it also controls effector functions of $CD4^+$ and $CD8^+$ T cells [11].

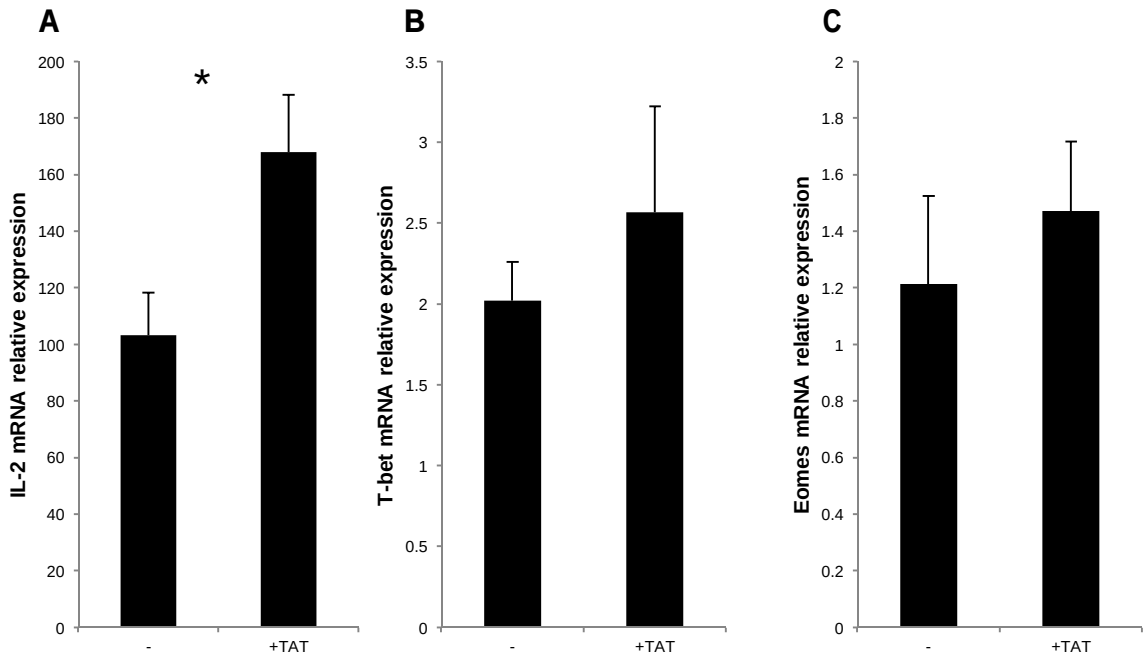

Figure 4.5 Tat-effects on transcriptional profile of CD8⁺ T cells activated without CD4⁺ T cells help. *CD8⁺ T cells were purified from PBLs from healthy volunteers (n=6) and activated with anti-CD3/CD28 in the presence or absence of 0.1µg/ml of Tat for 4 hours. IL-2 (A), T-bet (B) and Eomes (C) mRNA levels were quantified by qPCR and normalized to untreated CD8⁺ T cells. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test test was used. *P<0.05: Tat-treated cells compared to control cells (-).*

The expression of T-bet and Eomes in CD8⁺ T lymphocytes stimulated in the absence of CD4⁺ T cells was not modified by the presence of Tat (Fig. 4.5 B-C). However, Tat significantly increased IL-2 mRNA of CD8⁺ T cells stimulated without CD4⁺ T cell help (from 100 to 170-fold induction in CD8⁺ T cells stimulated in the absence or presence Tat respectively, $p<0.05$, Fig. 4.5 A).

As CD4⁺ T cells provide help for CD8⁺ T cells stimulation , we sought to determine the transcriptional profile of CD8⁺ T cells activated in the presence of CD4⁺ T lymphocytes. Interestingly, under these experimental conditions, a significant up-regulation of all the three genes taken into consideration was observed in CD8⁺ T cells stimulated in the presence of Tat compared with CD8⁺ T cells activated without Tat (Fig. 4.6). In fact, CD8⁺ T cells stimulated in the presence of Tat exhibited a 65-fold increase of IL-2 mRNA, compared to a 30-fold increase observed in CD8⁺ T lymphocytes stimulated without Tat ($p<0.05$, Fig. 4.6 A). Moreover, T-bet and Eomes relative levels were up-regulated from 0.93 and 0.87 respectively in CD8⁺ T cells activated in the absence of Tat to 1.8 and 1.53 in CD8⁺ T cells activated in the presence of Tat (Fig. 4.6 B-C).

Taken together, these data demonstrate that Tat up-regulates IL-2, T-bet and Eomes expression in activated CD8⁺ T cells. Notably, T-bet and Eomes up-regulation required the help by CD4⁺ T cells.

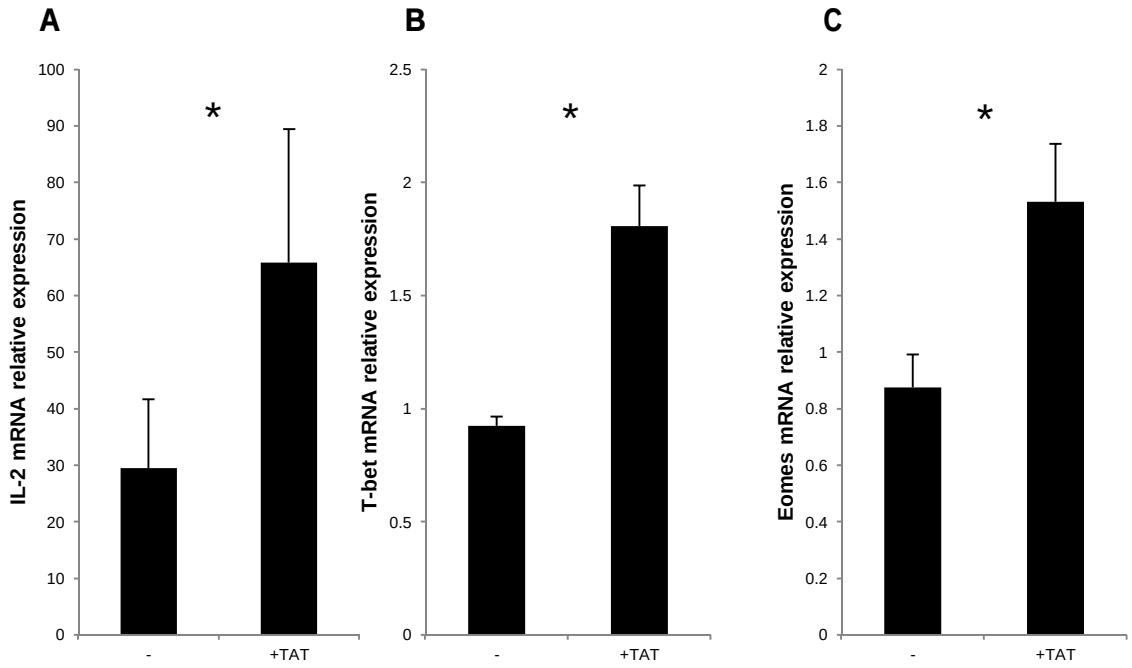

Figure 4.6 Tat-effects on transcriptional profile of CD8⁺ T cells activated with CD4⁺ T cells help. *PBLs from healthy volunteers (n=6) were activated with anti-CD3/CD28 in the presence or absence of 0.1µg/ml of Tat. After 4 hours of stimulation, CD8⁺ T cells were purified. IL-2 (A), T-bet (B) and Eomes (C) mRNA levels were quantified by qPCR and normalized to untreated CD8⁺ T cells. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to control cells (-).*

4.1.4 Transcriptional profile of CD4⁺ T cells activated in the presence of Tat

As we have shown that the Tat-mediated up-regulation of T-bet and Eomes in CD8⁺ T cells requires the presence of CD4⁺ T cells, we sought to determine whether Tat could also modulate the transcriptional profile of activated CD4⁺ T cells. To this aim, purified CD4⁺ T cells were activated, in the presence or absence of Tat, with anti-CD3/CD28, and the expression of IL-2, T-bet and Eomes mRNA was evaluated 4 hours after stimulation. As previously demonstrated [205], the presence of Tat during the stimulation induced a significant increase in IL-2 mRNA expression (from 190 to 470-fold induction in CD4⁺ T cells stimulated in the absence or presence of Tat respectively, $p<0.05$, Fig. 4.7 A). Notably, a 15.5-fold increase of T-bet mRNA was observed in CD4⁺ T cells activated in the presence of Tat as compared to a 7-fold increase in CD4⁺ T cells activated without Tat ($p<0.05$, Fig. 4.7 B). However, no significant differences were observed in Eomes expression in CD4⁺ T cells activated in the presence or absence of Tat (Fig. 4.7 C).

Thus, these data provide evidence that the activation of CD4⁺ T cells in the presence of Tat enhances T-bet expression.

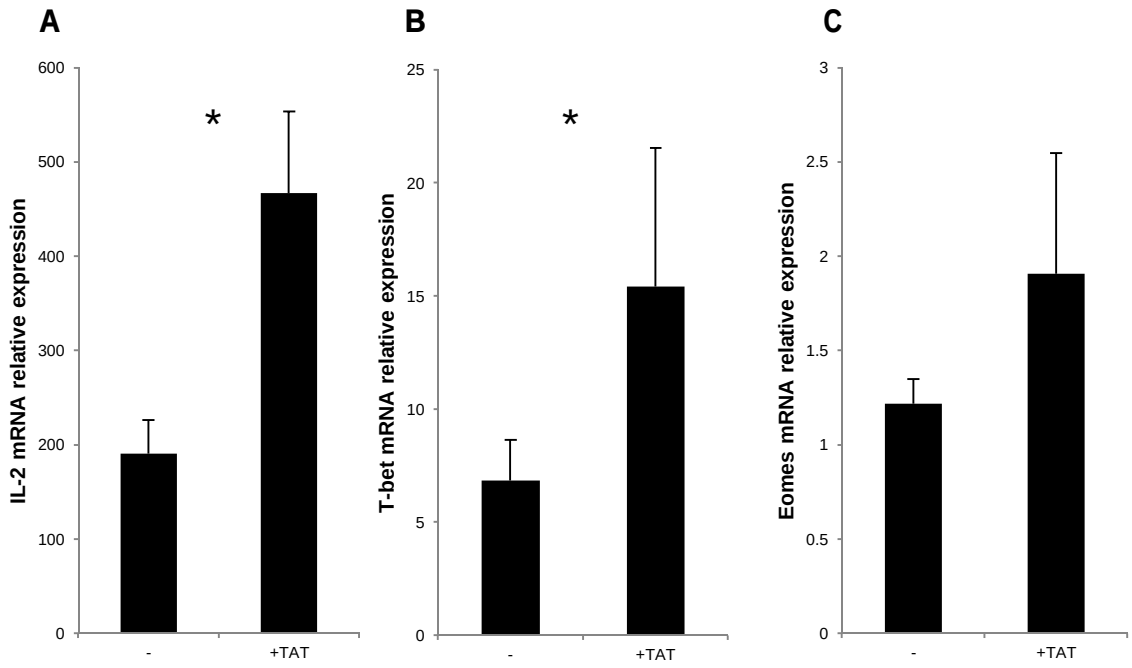

Figure 4.7 Tat-effects on transcriptional profile of activated CD4+ T cells. CD4+ T cells were purified from *PBLs from healthy volunteers (n=6) and activated with anti-CD3/CD28 in the presence or absence of 0.1µg/ml of Tat for 4 hours. IL-2 (A), T-bet (B) and Eomes (C) mRNA levels were quantified by qPCR and normalized to untreated CD4+ T cells. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to control cells (-).*

4.1.5 Tat effects on the basal transcriptional profile of CD8+ and CD4+ T cells

We next investigated whether Tat could also modulate the basal transcriptional prolife of resting CD8+ and CD4+ T cells. To this aim, T-bet and Eomes mRNA levels were measured in purified CD8+ and CD4+ T cells cultured for 4 hours in the presence or absence of Tat. As previous results demonstrate that Tat modulates T-bet and Eomes transcription in CD8+ T cells only if CD8+ T cells are co-cultured with CD4+ T cells, we maintained the same conditions to evaluate the effects of Tat on basal transcription of resting CD8+ T cells. T-bet mRNA expression was not modified by the presence of Tat in both CD8+ and CD4+ T cells (fold increase of T-bet mRNA levels in CD8+ and CD4+ T cells cultured in the presence of Tat not significantly different from 1, taken as reference value of CD8+ and CD4+ T cells cultured in the absence of Tat, Fig. 4.8 A). Interestingly, Eomes expression was up-regulated of 1.7 folds in CD8+ T cells cultured in the presence of Tat compared to CD8+ T cells cultured in the absence of Tat. No Tat-mediated effects on Eomes expression were observed in CD4+ T cells (Fig. 4.8 B).

Thus, these results suggest that Tat induces T-bet and Eomes transcription by two independent mechanisms, up-regulating the basal transcription of Eomes in CD8+ T cells and acting in synergy with stimulation to enhance T-bet mRNA levels in both CD8+ and CD4+ T cells.

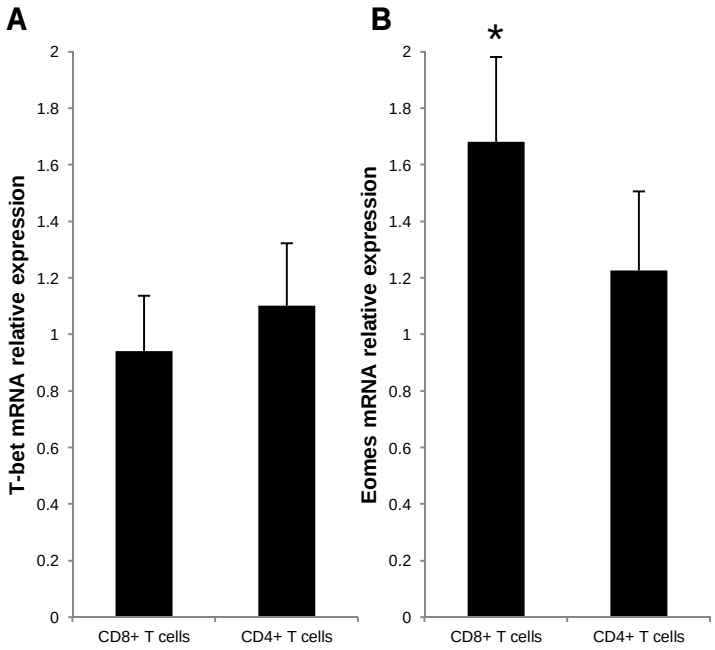

Figure 4.8 Tat-effects on transcriptional profile of resting CD8⁺ and CD4⁺ T cells. *PBLs from healthy volunteers (n=6) were cultured in the presence of 0.1 µg/ml of Tat. After 4 hours, CD8⁺ T cells were purified. CD4⁺ T cells were purified from PBLs from healthy volunteers (n=6) and cultured in the presence of 0.1 µg/ml of Tat for 4 hours. T-bet (A) and Eomes (B) mRNA levels were quantified by qPCR and normalized to untreated CD8⁺ and CD4⁺ T cells. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to control cells (1).*

4.1.6 Tat does not directly increase proliferation nor modifies the phenotype of activated CD8⁺ and CD4⁺ T cells

Our results demonstrate that Tat enhances effector functions and modifies the transcriptional profile of stimulated CD8⁺ and CD4⁺ T cells favoring their activation; thus, we first sought to determine whether these Tat-mediated effects resulted in an enhanced T cells proliferation. PBLs stimulated with anti-CD3/CD28 were cultured up to six days in the presence or absence of Tat, and CD8⁺ and CD4⁺ T cells proliferation was measured by CFSE staining (Methods section 3.1.8). As shown in Fig. 4.9, no differences in T cells proliferation were observed in the presence or absence of Tat. Moreover Tat, even at different doses, did not enhance CD8⁺ or CD4⁺ T lymphocytes proliferation at longer or shorter stimulation time or when stimulation was performed with different amount of anti-CD3/CD28 (not shown).

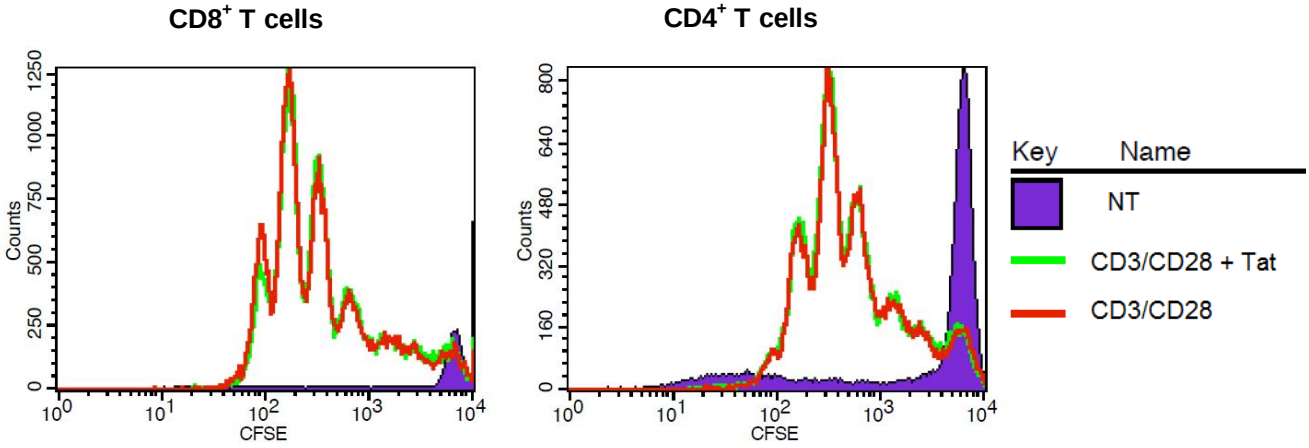

Figure 4.9 Tat does not enhance T cells proliferation. *PBLs from healthy volunteers were activated with anti-CD3/CD28 in the presence or absence of 0.1 µg/ml of Tat and cultured up to six days. Proliferation of CD8+ and CD4+ T cells was assessed by CFSE staining. One representative experiment out of five is shown.*

This is consistent with previous reports showing that soluble Tat was ineffective in enhancing anti-CD3/CD28 induced proliferation [150, 205]. Furthermore, the same reports demonstrated that higher doses of Tat could mediate apoptosis. To assess this, PBLs were stimulated with different stimuli (anti-CD3/CD28, PMA, PHA, LPS, Methods section 3.1.9) in the presence of different Tat doses (0.1 and 1 µg/ml). Our results demonstrated that PBLs stimulated in the presence of Tat did not display any significant death during the six days of culture compared to PBLs activated without Tat (Fig. 4.10). The same experiments were repeated with increasing amounts of Tat and varying the doses of stimuli, but no Tat-mediated effect on cell viability was observed (not shown).

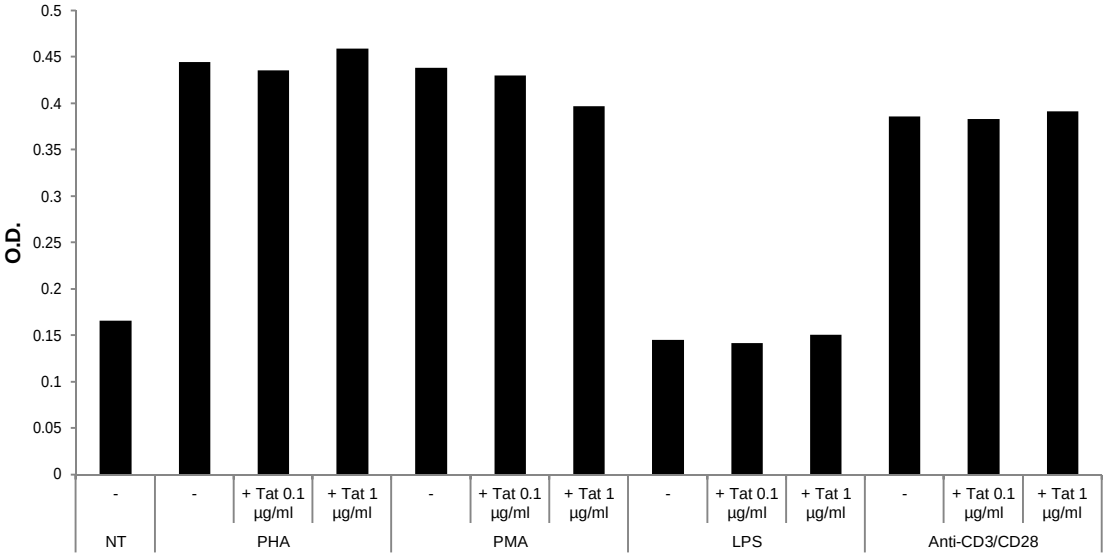

Figure 4.10 Tat does not affect T cells viability. *PBLs from healthy volunteers were activated with different kind of stimuli: PHA, PMA + Ionomycin, LPS and anti-CD3/CD28, in the presence or absence of different concentrations of Tat, and cultured up to six days. PBLs viability was measured by MTT assay. One representative experiment out of five is shown.*

Finally, to assess if Tat-mediated modulation of the transcriptional profile of CD4+ and CD8+ T cells impacted their surface phenotype, the expression of different activation markers (HLA-DR, CD38, CD95, CD25) was assessed on CD4+ and CD8+ T cells activated by anti-CD3/CD28 in the presence or absence of Tat (Methods section 3.1.8). The presence of Tat did not modify the phenotype of activated CD4+ and CD8+ T cells characterized at 10 and 24 hours after stimulation (Fig. 4.11). The measurement of the expression of the same markers performed at earlier (6 hours) and later (48 hours) time points confirmed these results (not shown).

Thus, we can conclude that Tat increases the functionality of CD8+ and CD4+ T cells without affecting their proliferation and phenotype.

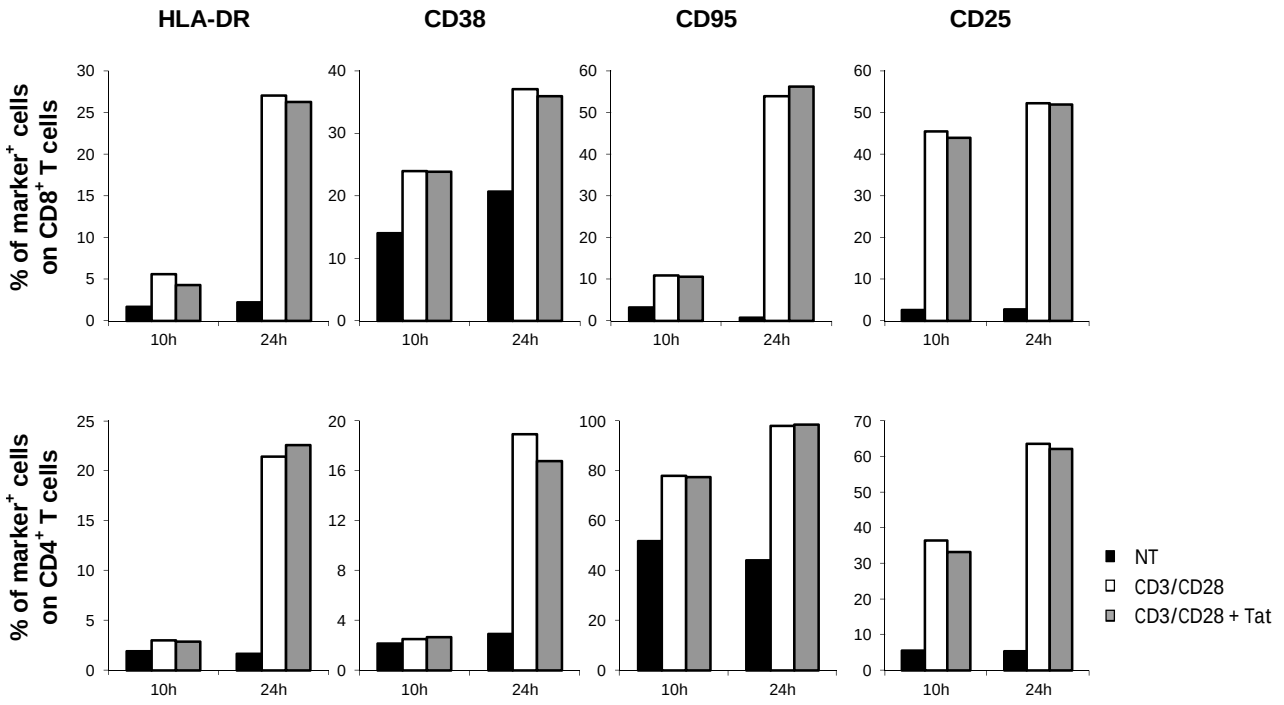

Figure 4.11 Tat does not affect T cells phenotype. *PBLs from healthy volunteers were activated with anti-CD3/CD28, in the presence or absence of 0.1 µg/ml of Tat. After 10 or 24 hours of stimulation the expression of HLA-DR, CD38, CD95 and CD25 was assessed by flow cytometry on CD8+ and CD4+ T cells. One representative experiment out of five is shown.*

4.1.7 Comparison between clade B and clade C Tat effects on T cell activation

Different HIV-1 subtypes display some differences in progression rate and virulence [52, 53]. As Tat is necessary for the viral cycle and contributes to progression to AIDS, differences at the level of its sequence may be responsible for the higher or lower virulence of a particular HIV subtype. In the majority of the studies, the immunomodulatory properties ascribed to Tat have been characterized using clade B Tat. However, clade C HIV-1 is responsible of the half of infections worldwide [51]. Thus, the comparison between clade B and clade C Tat immunomodulatory properties may be of interest to understand differences between subtype B and C HIV-1

pathogenicity. In particular, we compared clade B and clade C Tat for their effects on $CD8^+$ and $CD4^+$ T cell activation.

To address this issue, PBLs from healthy subjects were activated with anti-CD3/CD28 in presence or absence of clade B and clade C Tat (0.1 and 10 μg/ml), and IL-2, TNFα and IFNγ secretion was measured by ICS after 6 and 18 hours of stimulation. Results were normalized considering the percentage of cytokine-secreting cells after anti-CD3/CD28 stimulation as "1". Thus, the percentage of cytokine-secreting cells after anti-CD3/CD28 in the presence of Tat is reported as fold increase compared to the stimulation without Tat. This allows to better compare clade B and clade C Tat effects avoiding individual variability of volunteers to anti-CD3/CD28 activation.

The results demonstrated that clade B Tat enhanced IL-2 production in $CD4^+$ and $CD8^+$ T cells at 18, but not 6 hours after stimulation. Moreover, the effects of clade B Tat on IL-2 secretion were observed at concentrations ranging from 0.1 to 10 μg/ml. On the contrary, clade C Tat enhanced IL-2 production by $CD4^+$ and $CD8^+$ T cells yet at 6 hours after stimulation, while at 18 hours the effect reached statistical significance only in $CD4^+$ T cells. Moreover, the C Tat-mediated effects were more pronounced at the highest dose (10μg/ml) while 0.1 μg/ml of C Tat only modestly enhanced IL-2 production (Fig. 4.12).

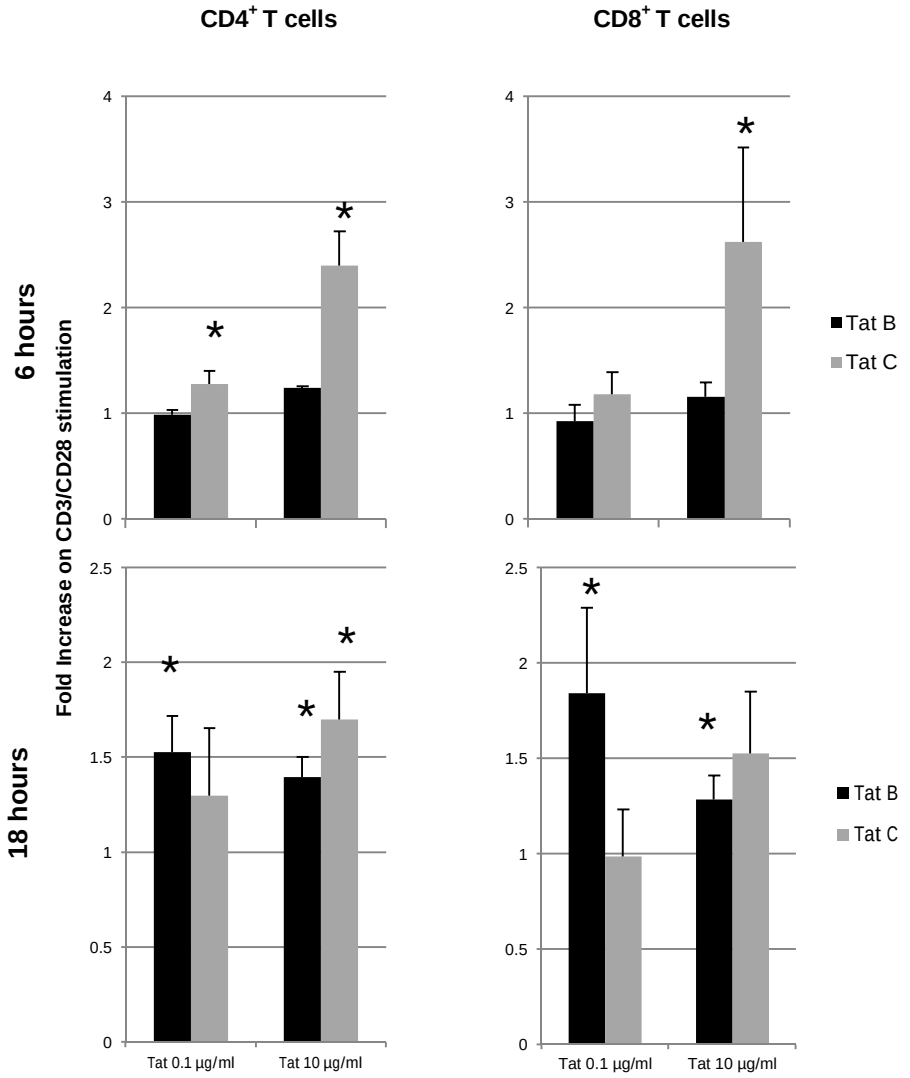

Figure 4.12 Comparison between clade B and C Tat on IL-2 production. *PBLs from healthy volunteers were activated with anti-CD3/CD28, in the presence or absence of different doses of clade B (8 donors) or clade C (6 donors) Tat. After 6 or 18 hours of stimulation, the IL-2 release by CD4+ and CD8+ T cells was assessed by ICS. Fold increase of the number of IL-2 secreting cells compared to PBLs stimulated in the absence of Tat is shown. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to control cells (1).*

When assessing TNFα production, we observed a similar phenomenon, as clade B Tat significantly enhanced TNFα secretion by CD4+ T cells only after 18 hours of stimulation, while clade C Tat significantly enhanced TNFα production in both CD4+ and CD8+ T cells at 6, but not at 18 hours post activation (Fig. 4.13).

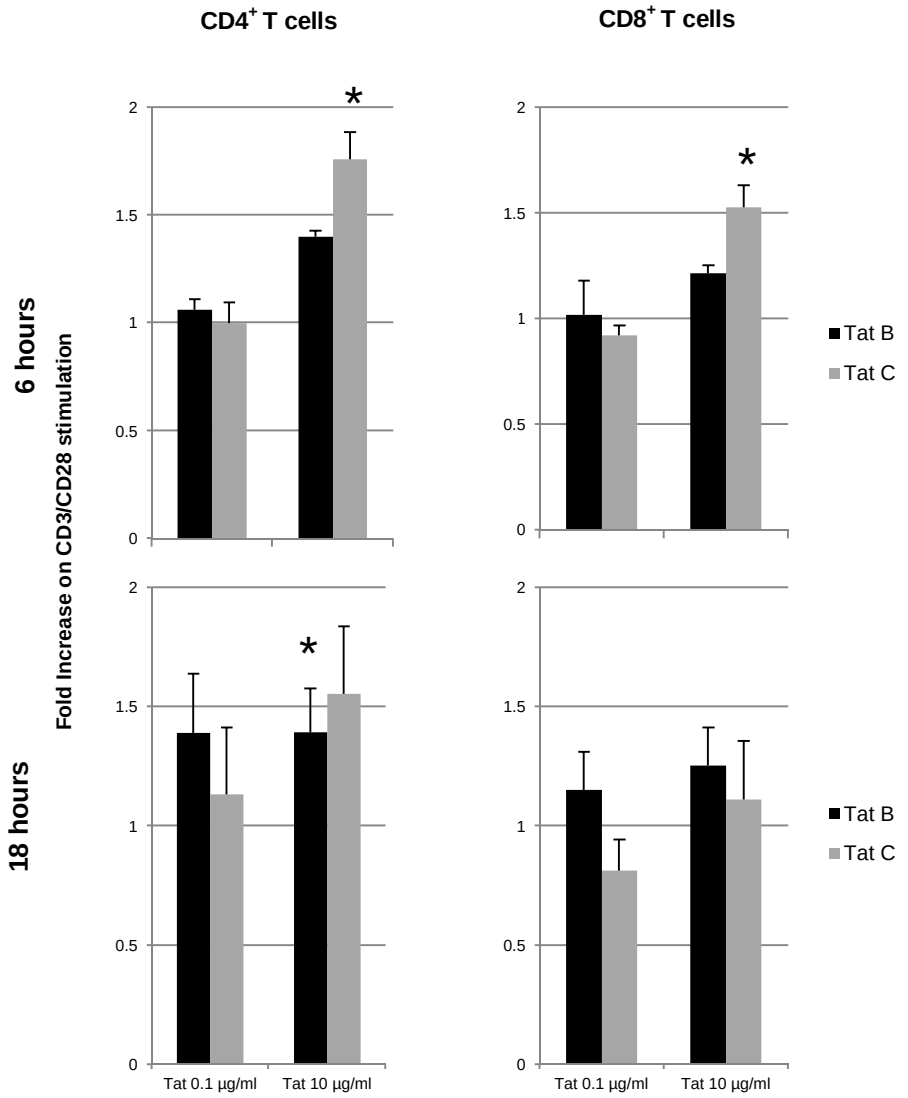

Figure 4.13Comparison between clade B and C Tat on TNFα production. *PBLs from healthy volunteers were activated with anti-CD3/CD28, in the presence or absence of different doses of clade B (8 donors) or clade C (6 donors) Tat. After 6 or 18 hours of stimulation, the TNFα release by CD4+ and CD8+ T cells was assessed by ICS. Fold increase of the number of TNFα secreting cells compared to PBLs stimulated in the absence of Tat is shown. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to control cells (1).*

Finally, we measured the production of IFNγ by CD4+ and CD8+ T cells activated in the presence of clade B or C Tat. Consistently with previous results, clade B Tat did not modulate cytokine production at 6 hours after stimulation, while both doses of B Tat significantly enhanced IFNγ secretion by $CD4^+$ T cells at 18 hours after stimulation (Fig. 4.14). On the contrary, clade C Tat increased IFNγ production exclusively at the highest dose, exerting its effects in both $CD4^+$ and $CD8^+$ T cells at 6 hours after stimulation and on $CD4^+$ T cells at 18 hours after stimulation (Fig. 4.14).

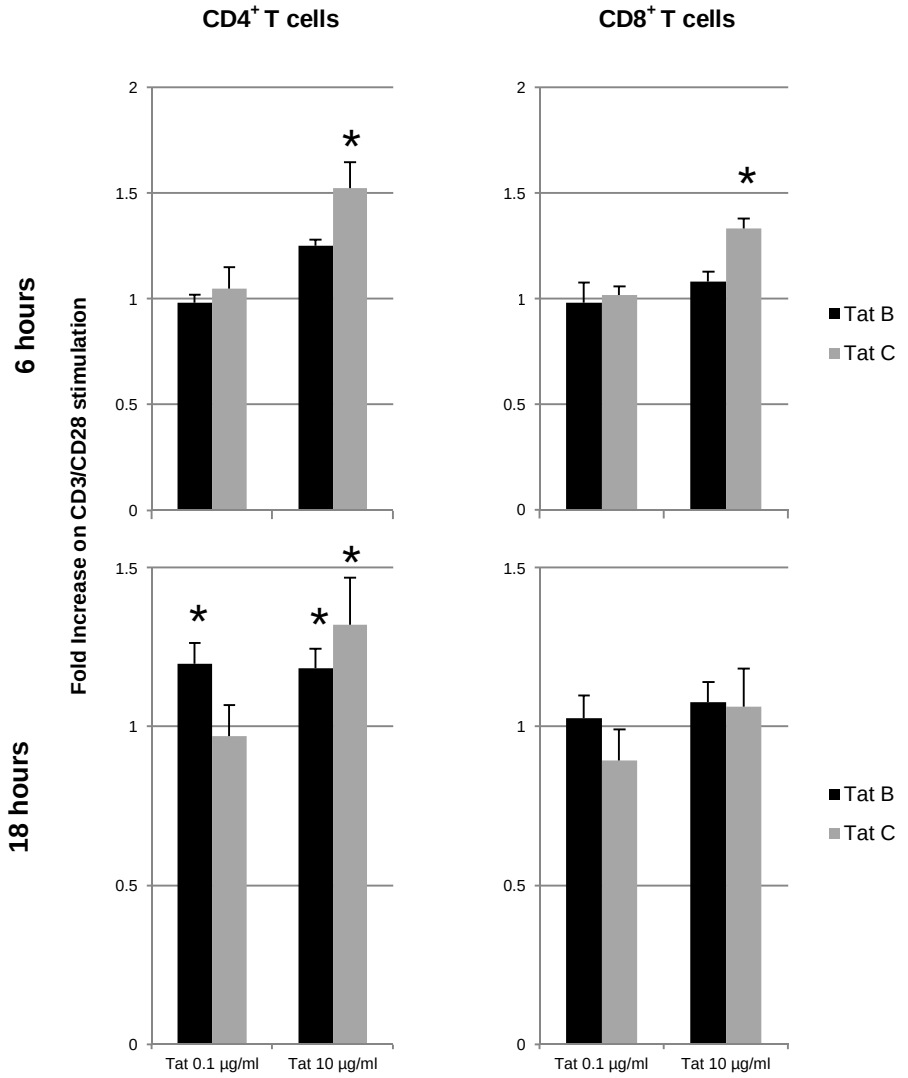

Figure 4.14 Comparison between clade B and C Tat on IFNγ production. *PBLs from healthy volunteers were activated with anti-CD3/CD28, in the presence or absence of different doses of clade B (8 donors) or clade C (6 donors) Tat. After 6 or 18 hours of stimulation, the IFNγ release by CD4+ and CD8+ T cells was assessed by ICS. Fold increase of the number of IFNγ secreting cells compared to PBLs stimulated in the absence of Tat is shown. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to control cells (1).*

Taken together, these results show that both clade B and clade C Tat activate CD8+ and CD4+ T cells but in different dose-dependent and time-dependent fashions. Indeed, the effects of clade B Tat were observed at low concentrations and at 18 hours after stimulation, while the effects of clade C Tat, although more pronounced, were observed only at the highest concentration and yet at 6 hours after stimulation.

To understand whether these two different Tat forms also differently modulated the transcriptional profile of CD8+ and CD4+ T cells, we compared clade B and clade C Tat for their capacity to up-regulate T-bet and Eomes expression in activated CD8+ and CD4+ T cells. Thus, CD8+ and CD4+ T cells from healthy volunteers were stimulated with anti-CD3/CD28 in the presence of B or C Tat. Results are expressed as fold increase of mRNA

levels comparing T cells stimulated in the presence of Tat with T cells activated in the absence of Tat (considered as a reference value of "1").

Results demonstrated that clade C Tat mediated the same effect as subtype B enhancing T-bet and Eomes expression of about 2 times in activated CD8+ T cells (Fig. 4.15), although the number of performed experiments was not enough to determine statistical significance (n=3).

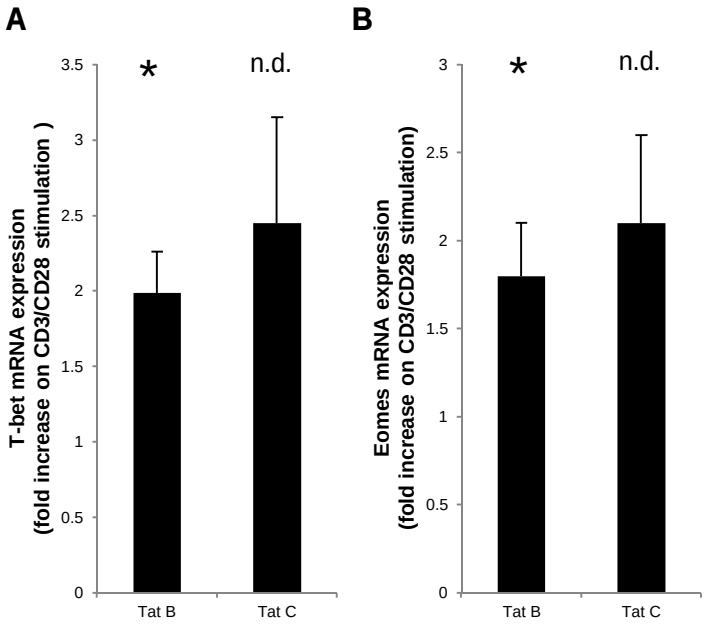

Figure 4.15 Comparison between clade B and C Tat on transcriptional profile of activated CD8+ T cells. *PBLs from healthy volunteers were activated with anti-CD3/CD28 in the presence of 0.1 µg/ml of clade B (6 donors) or C (3 donors) Tat. After 4 hours, CD8+ T cells were purified. Fold increase of T-bet (A) and Eomes (B) mRNA expression compared to CD8+ T cells stimulated in the absence of Tat is shown. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to control cells (1); n.d.: not determined.*

The assessment of the transcriptional profile of CD4+ T cells activated in the presence of clade B Tat showed a significant Tat-mediated up-regulation of T-bet but not of Eomes (Fig. 4.16). However, when CD4+ T cells where activated in the presence of clade C Tat, an up-regulation of both T-bet and Eomes was observed (Fig 4.16).

Taken together, these data show that both clade B and clade C Tat enhanced the activation of CD8+ and CD4+ T cells increasing T-bet expression. Interestingly, while B Tat increased Eomes mRNA levels exclusively in CD8+ T cells, clade C Tat up-regulated Eomes in both CD4+ and CD8+ T cells.

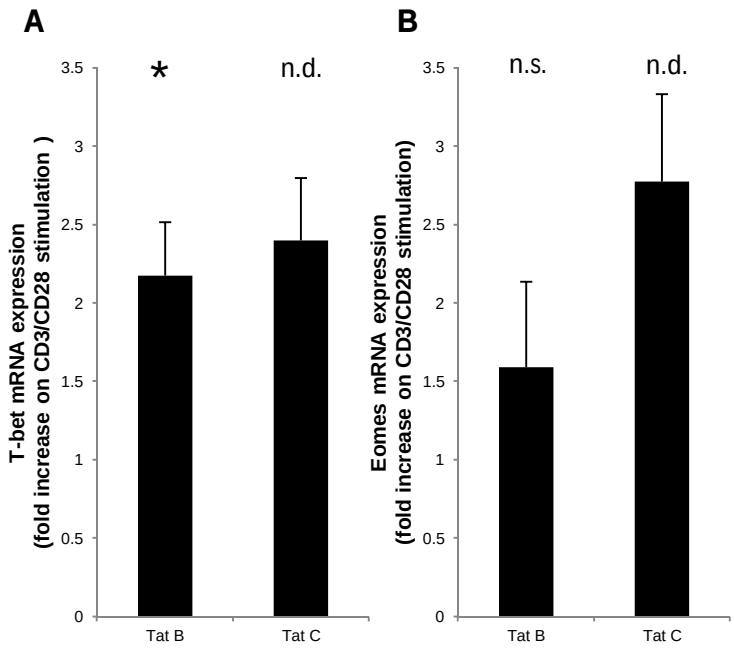

Figure 4.16 Comparison between clade B and C Tat on transcriptional profile of activated CD4⁺ T cells. *CD4⁺ T cells were purified from PBLs from healthy volunteers and activated with anti-CD3/CD28 in the presence of 0.1μg/ml of clade B (6 donors) or clade C (3 donors) Tat for 4 hours. Fold increase of T-bet (A) and Eomes (B) mRNA expression compared to CD8⁺ T cells stimulated in the absence of Tat is shown. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to control cells (1); n.d.: not determined; n.s.: not significant.*

4.1.8 Comparison between clade B and clade C Tat-effects on the basal transcriptional profile of CD8⁺ and CD4⁺ T cells

We have previously demonstrated that clade B Tat did not affect the basal transcriptional profile of resting CD4⁺ T cells, while it significantly increased Eomes (but not T-bet) expression in resting CD8⁺ T cells (Fig. 4.8). Similarly, when we evaluated clade C Tat effects on the transcriptional profile of resting CD4⁺ T cells, we did not observe any modulation of T-bet and Eomes expression (Fig 4.17). As previously mentioned, number of samples has to be enlarged to allow any statistical testing. We next evaluated clade C Tat effects on T-bet and Eomes expression of resting CD8⁺ T cells. Neither clade B nor clade C Tat modulated the transcription of T-bet, while both of them up-regulated Eomes expression in resting CD8⁺ T cells (Fig. 4.18). These results further demonstrate that Tat, irrespectively to the subtype, induces T cell activation by affecting CD8⁺ and CD4⁺ T cells transcriptional profile.

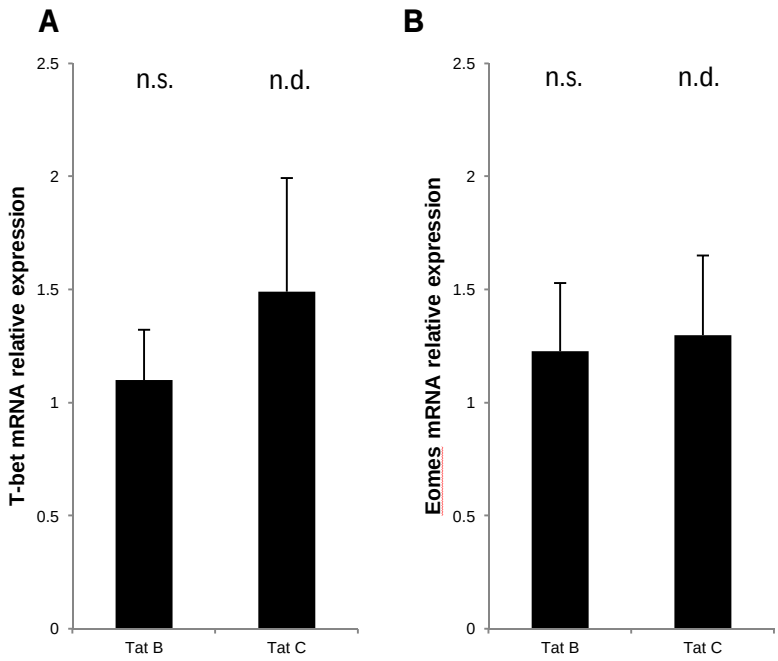

Figure 4.17 Comparison between clade B and C Tat on transcriptional profile of resting CD4⁺ T cells. *CD4⁺ T cells were purified from PBLs from healthy volunteers and cultured in the presence of 0.1µg/ml of clade B (6 donors) or clade C (3 donors) Tat for 4 hours. T-bet (A) and Eomes (B) mRNA levels were quantified by qPCR and normalized to untreated CD4⁺ T cells. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to control cells (1); n.d.: not determined; n.s.: not significant.*

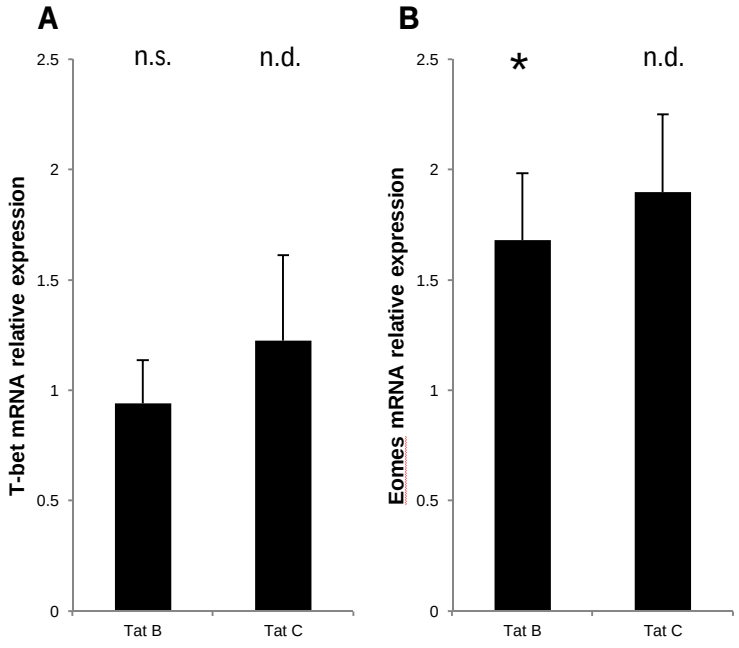

Figure 4.18 Comparison between clade B and C Tat on transcriptional profile of resting CD8⁺ T cells. *PBLs from healthy volunteers were cultured in the presence of 0.1µg/ml of clade B (6 donors) or clade C (3 donors) Tat. After 4 hours, CD8⁺ T cells were purified. T-bet (A) and Eomes (B) mRNA levels were quantified by qPCR and normalized to untreated CD4⁺ T cells. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to control cells (1); n.d.: not determined; n.s.: not significant.*

4.2 Tat-mediated modulation of viral-specific cellular and humoral responses

4.2.1 Tat enhances T cell responses against co-administered antigens

Our data suggest that Tat enhances the activation of naïve and memory $CD4^+$ and $CD8^+$ T cells if stimulated in its presence. To confirm these *in vitro* observations, the effect of Tat on the expansion of naïve $CD8^+$ T cells was investigated using an *in vivo* model of protein vaccination previously characterized in our laboratory [193, 261]. Specifically, Balb/c mice were immunized with the HIV-1 Gag protein alone or in combination with Tat (Methods section 3.2.3) and, ten days after immunization, fresh splenocytes from immunized mice were assayed by IFNγ Elispot (Methods section 3.2.4) to evaluate T cell responses directed against previously identified Gag-derived peptides [261] containing one CD4 and eight CD8 epitopes (Table 3.2).

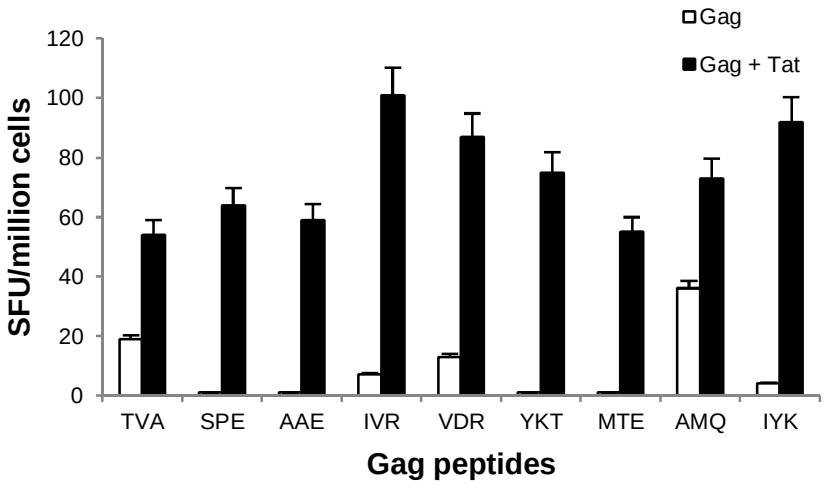

Figure 4.19 Tat-mediated T cell activation. *Balb/c mice (3 per groups) were injected with 5 µg of Gag alone or in combination with 5 µg of Tat. Ten days after vaccination mice were sacrificed and fresh splenocytes assayed in IFNγ Elispot against the indicated Gag-derived T cell peptide epitopes (see also Table 3.2). One representative experiment out of three is shown.*

As shown in Fig. 4.19, immunization with Gag alone did not induce detectable Gag-specific T cell responses, whereas immunization with the Gag protein in the presence of Tat elicited significant Gag-specific responses directed against all tested CD4 and CD8 epitopes. These data confirm that Tat activates $CD4^+$ T cells and demonstrate that the Tat protein induces the expansion of $CD8^+$ T cells.

4.2.2 Tat increases the duration and decreases the magnitude of antiviral CD8+ T cell responses

The stimulatory effect of Tat on CD8+ T cells (see section above) and its capacity to increase antigen presentation, as reported in previous studies [148, 149, 192, 193], suggest that Tat may play a role in the hyperactivation of CD8+ T cells observed during HIV infection. To understand how the immunomodulatory properties displayed by Tat on APCs and T lymphocytes affect the overall immune responses against a viral infection, C57BL/6 mice were infected intravaginally (i.v.) with wild type HSV1 (strain LV) with or without the Tat protein administered at the time of infection by the subcute route (Methods section 3.2.3). At days 5, 8 and 13 post-infection (p.i.), the presence of HSV1-specific CD8+ T cells was evaluated on splenocytes by staining with MHC-peptide dextramers specific for the immunodominant SSI CTL epitope of glycoprotein B (Methods section 3.2.5). As shown in Fig. 4.20 A, at day 5 p.i. low numbers of SSI-specific T cells were detected in both groups of mice. However, at day 8 p.i., when CD8+ T cell response reached the peak of expansion, and at day 13 p.i., corresponding to the contraction phase of the T cell response, the percentage of SSI-specific CD8+ T cells was significantly higher in mice treated with the Tat protein than that measured in the control group inoculated with HSV1 alone.

Since the presence of antigen-specific CD8+ T cells does not necessarily correlate to a functional cytotoxic phenotype [278, 279], the activity of SSI-specific CD8+ T cells isolated from mice infected with HSV1, with and without Tat, was further investigated. Moreover, to better characterize the population of HSV1-specific CD8+ T cells, CTL responses against the subdominant QTFDFGRL (QTF) epitope of ribonucleotide reductase 1 (RR1) were also analysed. To this purpose, fresh splenocytes purified from mice infected with HSV1, in the presence or absence of Tat, were tested at different time points p.i. by evaluating IFNγ release against the immunodominant SSI and the subdominant QTF epitopes (Methods section 3.2.4). As shown in Fig. 4.20 B, the expansion phase of HSV1-specific CD8+ T cell responses against both epitopes was more robust in mice treated with Tat as compared to that observed in the control group. Indeed, significantly higher numbers of SSI- and QTF-specific CD8+ T cells secreting IFNγ were detected in the Tat-treated group at days 5 and 6 p.i., respectively (Fig. 4.20 C).

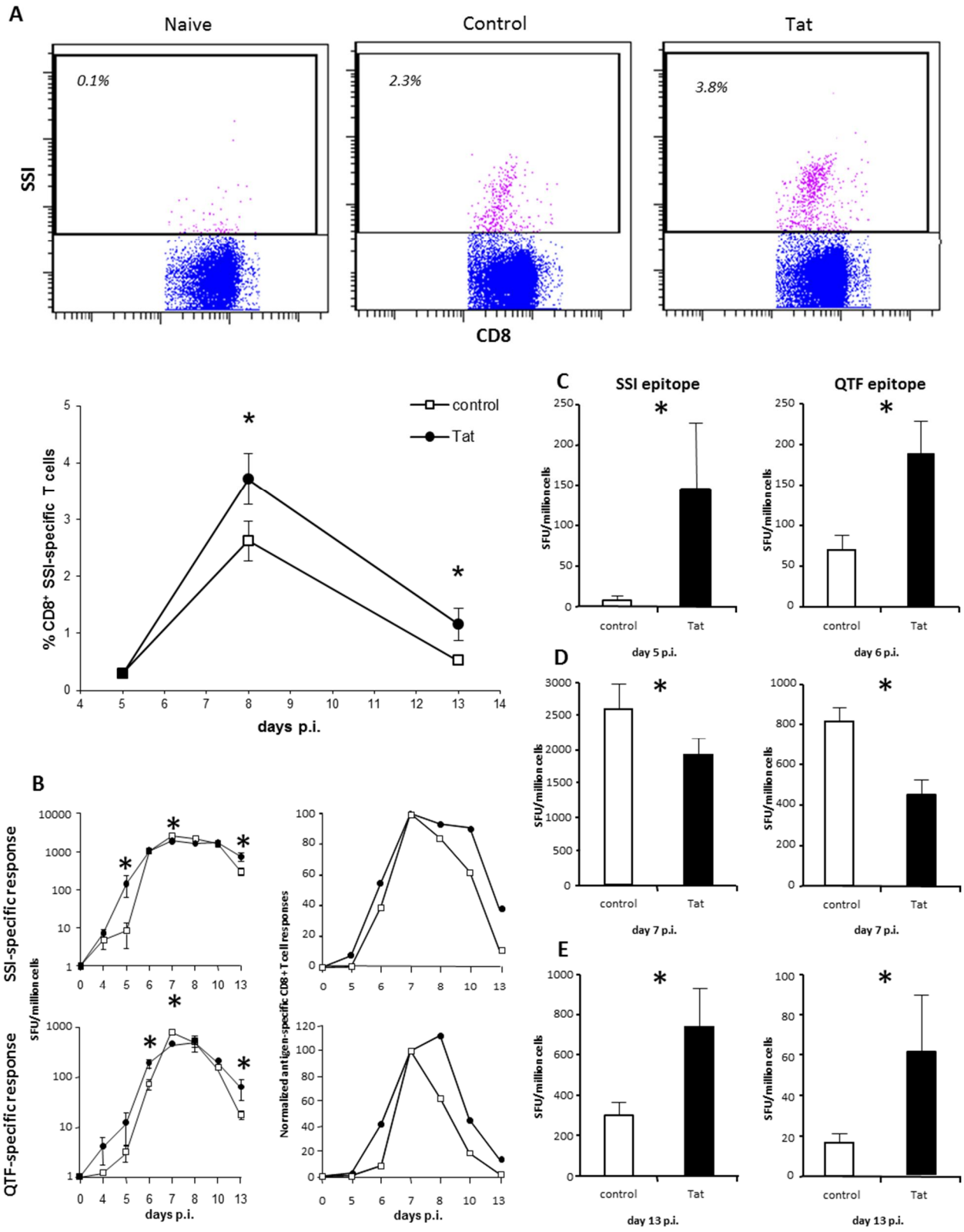

Figure 4.20 Tat modulates the kinetics and the magnitude of CTL responses. *Splenocytes were purified from control and Tat-treated HSV1-infected C57/BL6 mice at days 4, 5, 6, 7, 8, 10 and 13 post-infection. (A) Percentage of SSI-specific CD8⁺ T cells detected by dextramer staining. Dot plots show dextramer positive CD8⁺ T cells of one representative mice per group (including a naïve uninfected mice) at day 8 p.i.. Graph presents mean ± SEM of 5 mice per group. One representative experiment out of three is shown. (B) Kinetics of SSI- and QTF-specific cellular responses detected by IFNγ Elispot on fresh splenocytes. Data are presented as mean ± SEM of 5 mice per group (left panel). Expansion and contraction were normalized, for each group, to values detected at day 7 (right panel). One representative experiment out of three is shown. (C) SSI- and QTF- specific IFNγ responses at days 5 and 6 post-infection. (D) SSI- and QTF- specific IFNγ responses at day 7 post-infection. (E) SSI- and QTF-specific IFNγ responses at day 13 post-infection. For statistical analysis two-tailed Mann Whitney test was used. *P<0.05.*

Conversely, and despite the higher percentage of SSI-specific $CD8^+$ T cells measured by dextramer staining (Fig. 4.20 A), at day 7 p.i., corresponding to the peak of the expansion phase of CTL responses, a significant lower number of SSI- and QTF-specific IFNγ-secreting cells was observed in mice treated with Tat compared to the control group (Fig. 4.20 B-D). To try to solve this apparent contradiction, the expression of CD62L and IL-7 receptor (CD127) was evaluated on SSI-specific effector T cells, since these markers define specific $CD8^+$ subpopulations. In particular, during the expansion of CTL responses, the majority of antigen-specific $CD8^+$ T cells are usually within the $CD62L^-CD127^-$ subset, consistent with the effector phenotype, while a small subset of T cells retains the expression of CD62L and CD127 molecules resulting in a different pattern of cytokine secretion [280]. The results of these analyses showed that the largest population of SSI-specific CTLs consisted in $CD62L^-CD127^-$ cells (not shown) in both groups, indicating that the lower number of IFNγ-secreting T cells in Tat-treated mice was not due to the unbalancement of effector cells subpopulations. It is likely that the robust expansion of HSV1-specific $CD8^+$ T cells induced by Tat damps the cytokine release at the peak of the response, resembling the impaired functionality that follows hyperactivation of $CD8^+$ T cells during chronic immune activation [71, 119, 281].

Finally, we analysed SSI- and QTF-specific CTL responses during the contraction phase. As shown in Fig. 4.20 B and E, a significant higher number of SSI- and QTF-specific $CD8^+$ T cells secreting IFNγ was detected in the Tat-treated group at day 13 p.i., demonstrating that Tat delays the contraction phase of the antiviral CTL response. Taken together, these data suggest that the presence of Tat at the time of priming results in primary $CD8^+$ T cell responses that start earlier and last longer but have a lower intensity at the peak.

4.2.3 Tat treatment does not contribute to the control of acute HSV1 infection

Several studies demonstrate that helper and cytotoxic T lymphocytes are functionally important in response to infection with HSV [282]. In particular, CTLs are critical in limiting the number and severity of herpes lesions, promoting recovery from primary and recurrent infections [283, 284]. To determine whether the different CTL responses against HSV1 elicited in the presence or absence of Tat have an impact on the outcome of viral disease, HSV1-infected, control and Tat-treated, mice were monitored daily for the appearance of typical HSV1 clinical manifestations. Disease severity was measured using scores starting from no signs of infection (score 0), appearance of ruffled hair (score 1), appearance of cold sores on and around the vagina (score 2), appearance of paralysis of the back limbs (score 3) and mouse death (score 4). As shown in Fig. 4.21 A, the clinical manifestations were significantly more intense in Tat-treated mice between days 6 and 8 p.i., at the peak of the

expansion of the cellular responses, when the animals infected in the presence of Tat showed a lower magnitude of the antiviral responses. However, mice from the two groups showed the same probability of developing signs of disease (Fig. 4.21 B), suggesting that the prolonged expansion phase detected in mice treated with Tat can worsen acute HSV1 infection.

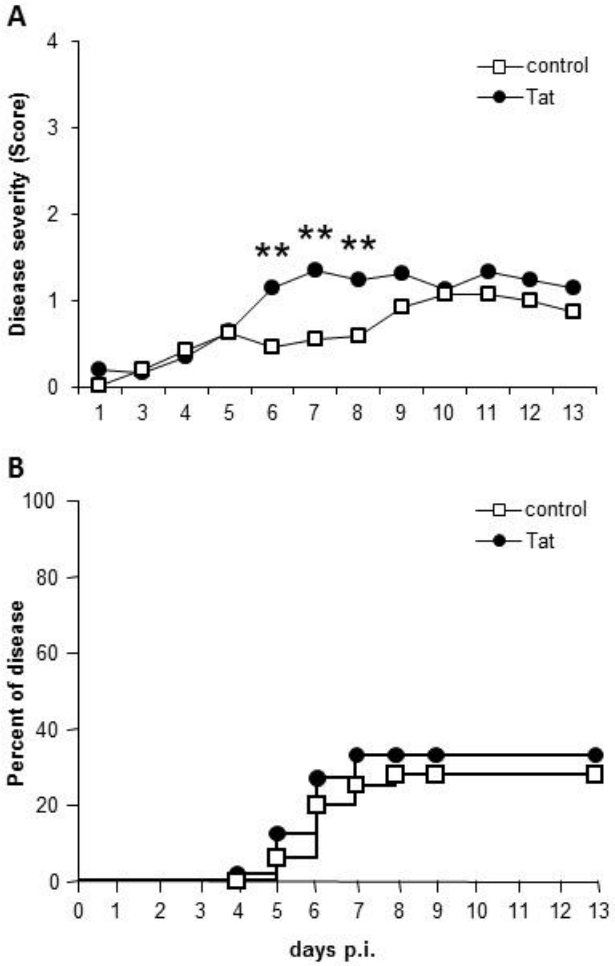

Figure 4.21 Tat does not contribute to the control of HSV1 acute infection. *Control and Tat-treated HSV1-infected C57/BL6 mice were checked daily for the appearance of disease signs. (A) Mean of disease scores of 20 mice per group is shown. For statistical analysis two-tailed Mann Whitney test was used. **P<0.01. (B) Probability of developing disease signs is shown for each group. Figure represents Kaplan-Meier estimation of the probability of clinical manifestations. For statistical analysis Log rank test was used. One representative experiment out of three is shown.*

4.2.4 Tat-mediated stimulatory effects involve only antigen-specific CD8⁺ T cells

To investigate whether Tat treatment affects the whole CD8⁺ T cell compartment or only HSV1-specific CD8⁺ T lymphocytes, the number of the different lymphocytes subpopulations was evaluated in spleens of control and Tat-treated HSV1-infected mice at different time points after infection (Methods section 3.2.5). As shown in Fig. 4.22 A, the number of CD8⁺ T cells was similar in the two groups during the whole course of the experiment. To exclude pro-apoptotic effects due to the Tat treatment that may hide proliferation of CD8⁺ T cells, the expression

of the pro-apoptotic Fas receptor (CD95) was measured on $CD8^+$ T cells derived from control and Tat-treated mice, and no differences were detected between the two groups (Fig. 4.22 B). These data indicate that Tat favors the activation of antigen-primed $CD8^+$ T cells, and exclude pro-apoptotic effects exerted by Tat on bystander $CD8^+$ T cells. Some studies suggest that Tat is able to induce apoptosis in bystander B and $CD4^+$ T cells [210, 211, 217, 221, 285]. However, conflicting results have been reported by several groups, and both proliferative and pro-apoptotic effects have been ascribed to Tat (see section 1.6.3). Assessment of spleen composition revealed no differences at the level of $CD4^+$ T cell numbers between the two groups (Fig. 4.22 C). Moreover, the $CD4^+/CD8^+$ratio was not modified by Tat treatment (data not shown). Of note, when measuring the numbers of B lymphocytes, a mild but significant loss of B cells in Tat-treated mice was observed as compared to the control group (Fig. 4.22 D). Interestingly, the effect was transient, lasted few days after Tat injection (day 4 and 6 p.i.) and quickly disappeared. However, reduction in the number of B cells is present early in HIV infection and persists in HAART [82, 286]. Altogether, these data demonstrate that Tat does not induce proliferation or apoptosis of bystander T lymphocytes but transiently affects B cell numbers.

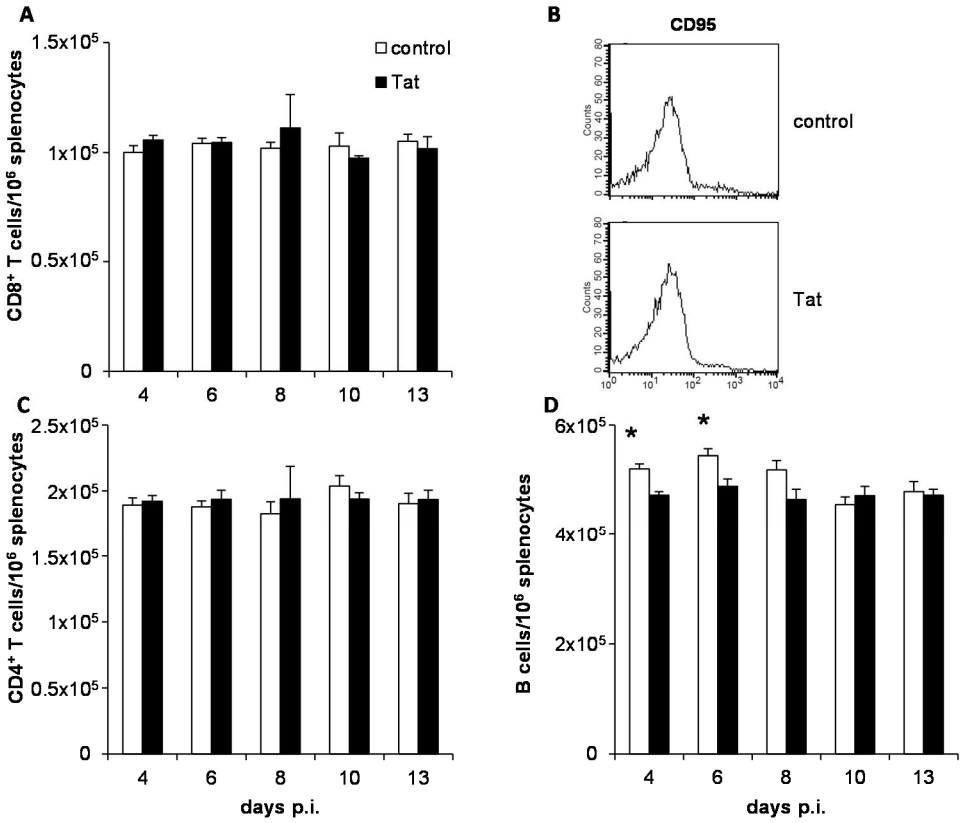

Figure 4.22 Tat does not activate bystander T cells. *Control and Tat-treated HSV1-infected C57/BL6 mice were sacrificed at days 4, 6, 8, 10 and 13 post-infection. $CD8^+$ (A), $CD4^+$ (C) and B (D) lymphocytes numbers were measured by flow cytometry. Data are presented as mean ± SEM of 5 mice per group. For statistical analysis two-tailed Mann Whitney test was used. *P<0.05. One representative experiment out of three is shown. (B) CD95 expression was measured by flow cytometry on $CD8^+$ T cells. One representative experiment out of five is shown.*

4.2.5 Tat modulates the composition of the antigen-specific CD8+ T cell memory pool

The differentiation of memory T cells is programmed during the early phases of the immune response [19]. Since our results indicate that Tat modulates the expansion and contraction phases of antigen-specific CTL responses, we evaluated whether Tat modifies the development of the memory CD8+ T cell pool. To this end, HSV1-infected control and Tat-treated mice were analysed 70 days p.i. for the presence of SSI-specific memory T cells. Data from dextramer staining (Fig. 4.23 A) and IFN-γ Elispot (not shown) showed that the numbers of epitope-specific CD8+ memory T cells were comparable among the two groups. The analysis of the phenotype of SSI-specific CD8+ T cells revealed a significant lower expression of CD62L (Fig. 4.23 B) in Tat-treated mice, indicating a larger population of HSV1-specific effector memory CD8+ T cells.

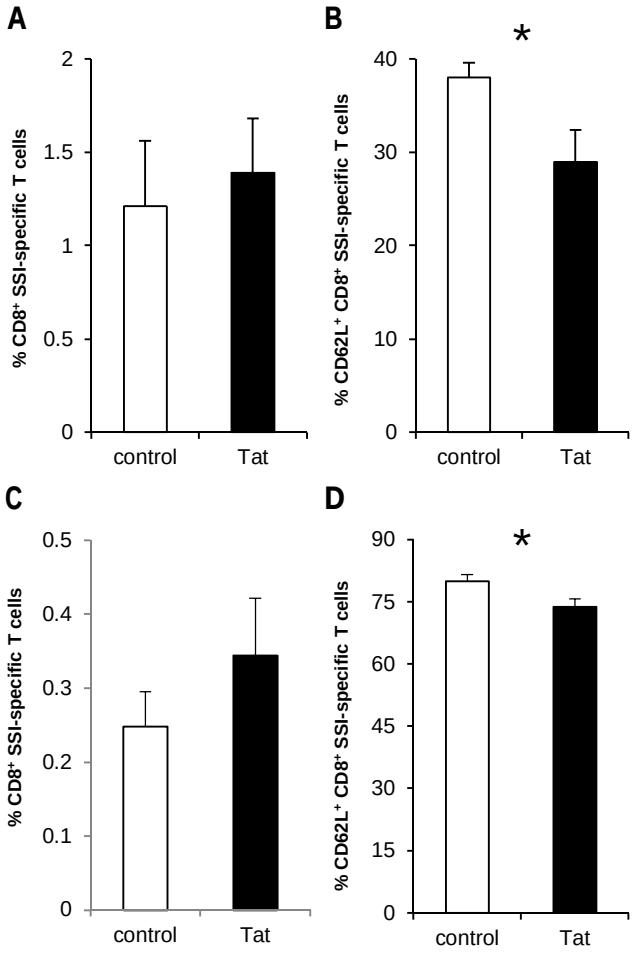

Figure 4.23 Tat administered at the time of antigen-priming favors an effector memory phenotype. *Control and Tat-treated mice were infected with HSV1 wt (A and B) or with replicative-defective HSV1 (C and D) and sacrificed at days 70 post-infection. (A and C) Percentage of SSI-specific CD8+ T cells detected by dextramer staining. (B and D) CD62L expression was measured by flow cytometry on SSI-specific CD8+ T cells. Data are presented as mean ± SEM of 5 mice per group. For statistical analysis two-tailed Mann Whitney test was used. *P<0.05. One representative experiment out of three is shown.*

To exclude that the effect of Tat on T cell memory phenotype was due to HSV1 reactivation, C57BL/6 mice were infected intravaginally with a replication-defective strain of HSV (S0ZgJGFP), that primes immune responses

without establishing latency, in the presence or absence of Tat. At day 70 p.i., the presence and the phenotype of SSI-specific memory T cells were determined in control and Tat-treated mice. As shown in Fig. 4.23 C, the percentage of SSI-specific $CD8^+$ T cells was similar between the two groups, and SSI-specific $CD8^+$ T cells from Tat-treated mice exhibited a significantly lower expression of CD62L (Fig. 4.23 D), indicating that the presence of Tat during priming favors the increase of $CD8^+$ effector memory T cells.

Taken together, these data show that the prolonged duration and the diminished magnitude of the effector phase observed when Tat is present at the time of priming favor an effector memory phenotype.

4.2.6 Tat induces IgG class-switching in B cells without affecting the magnitude of antigen-specific humoral responses

IFNγ secreted by T cells is known to skew switching patterns from IgG1 to IgG2a in responding B cells [287]. Thus, as Tat prolongs IFNγ secretion and up-regulates T-bet expression, also involved in Th1 lineage commitment, we analysed whether the presence of Tat at the time of priming induces changes in the Th1/Th2 IgG balance (Methods section 3.2.6). As reported in Fig. 4.24 A, at day 20 p.i., Tat-treated animals showed a prevalent anti-HSV1 IgG2a response with titers higher than IgG1 suggesting a Th1 pattern of response, whereas the control group showed a balanced anti-HSV1 Th1/Th2 pattern (similar levels of anti-HSV1 IgG1 and IgG2a). The predominant induction of antibodies associated to a Th1 response in Tat-treated mice was still evident 70 days after the infection (Fig. 4.24 A), while control mice showed a prevalence of a Th2 pattern (higher titers of IgG1). These data further demonstrate that the presence of Tat at the time of priming induces a Th1-type response [149, 193, 288].

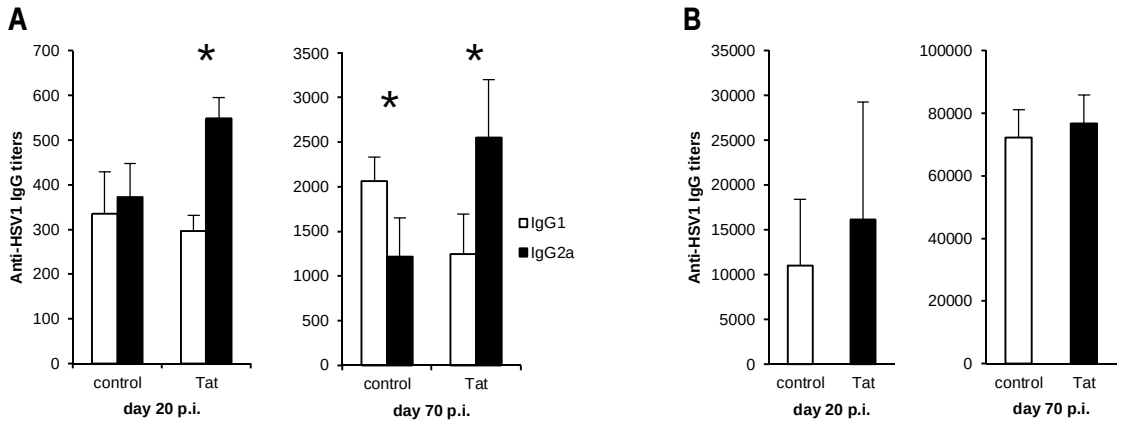

Figure 4.24 Tat administered at the time of antigen-priming favors a Th1 profile of the humoral response. *Blood samples from control and Tat-treated HSV1-infected mice were collected and the presence of anti-HSV1 antibodies was detected by ELISA assay. (A) Anti-HSV1 IgG1 and IgG2a were measured at days 20 (left) and 70 (right) post-infection. (B) Total anti-HSV1 IgG were measured at days 20 (left) and 70 (right) post-infection. Data are presented as mean ± SEM of 5 mice per group. For statistical analysis two-tailed Mann Whitney test was used. *P<0.05. One representative experiment out of three is shown.*

To investigate whether Tat modifies the magnitude of the antiviral humoral response, we determined anti-HSV1 antibody titers in sera from Tat-treated and control HSV1-infected mice. Specifically, mice sera collected at 20 and 70 days p.i. were tested for the presence of anti-HSV1 specific IgM (day 20 p.i.) and IgG (day 20 and 70 p.i.). No significant differences were detected among the two groups at both time points, for IgG (Fig. 4.24 B) nor for IgM (not shown).

Thus, Tat modulates the quality of the anti-viral humoral response without affecting its magnitude.

4.3 Role of anti-Tat humoral immunity

4.3.1 Frequency of anti-Tat humoral responses and correlation with $CD4^+$ count

The results presented in chapters 4.1 and 4.2 demonstrate a key role of Tat in contributing to immune activation and immune dysfunctions, suggesting that anti-Tat immunity should protect from deleterious effects exerted by Tat. Consistently, a protective role exerted by anti-Tat humoral immunity has been demonstrated. In particular, anti-Tat antibodies are more frequently found in nonprogressors [247] and protect HIV-infected subjects from $CD4^+$ decline [248, 249].

Anti-Tat natural immunity is present only in a fraction of HIV-positive subjects, although frequencies of anti-Tat IgG and IgM vary depending on the cohort [249, 250]. As the majority of these studies were usually performed in subjects infected with clade B HIV, we sought to determine the role of anti-Tat antibodies in an African cohort composed of individuals infected with non-clade B forms of HIV. To this aim, sera were collected from HIV-positive volunteers enrolled in Mbeya, Tanzania, a region where subtype C appears to be the prevalent subtype (prevalence > 40%), followed by a high proportion of intersubtype recombinants, while type B infections are almost absent; among recombinants, more than 85% contained subtype C sequences in their genome [289-291].

The study population consisted of 96 HAART-naïve chronically HIV-1-infected subjects (Table 4.1), and sera collection occurred at least 14 months after the infection.

	Median	Range
Age (year)	36	20-61
$CD4^+$ count (cells/µl)	402	64-1301
Female (%)	61%	-
Male (%)	39%	-

Table 4.1Characteristics of study participants

Sera were tested in ELISA assays to assess the presence and the titer of IgA, IgG, IgM against Tat B and C (Methods section 3.3.2). As shown in Fig. 4.25, of the 96 subjects, 15 (16%) were anti-Tat IgA (B and/or C) positive, 49 (51%) anti-Tat IgM (B and/or C) positive and 44 (46%) anti-Tat IgG (B and/or C) positive.

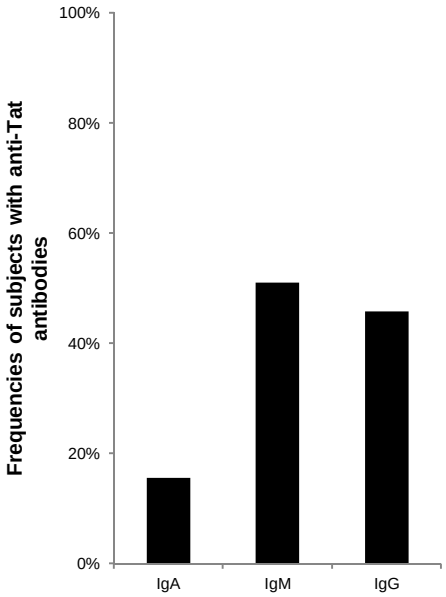

Figure 4.25 Frequencies of anti-Tat humoral responses. *Sera from study participants were tested in ELISA assay to look for anti-Tat IgA, IgM or IgG.*

To assess the impact of the presence of anti-Tat antibodies on $CD4^+$ count, we then evaluated the frequencies of the different anti-Tat antibodies in subjects with high or low $CD4^+$ counts (less or more than 350 $CD4^+$ T cells/µl). Anti-Tat IgG were present almost at the same frequency in the two groups, while a significant higher prevalence of anti-Tat IgM was detected in individuals with $CD4^+$ counts greater than 350 cells/µl compared to subjects with low $CD4^+$ counts (p=0.02). Unexpectedly, subjects with low $CD4^+$ counts exhibited a higher frequency of anti-Tat IgA compared to patients with counts > 350, although the result did not reached statistical significance (Table 4.2).

$CD4^+$ count (cells/µl)	Anti-Tat IgA (percentage)	p value	Anti-Tat IgM (percentage)	p value	Anti-Tat IgG (percentage)	p value
<350	22%	0.16	37%	0.02	49%	0.68
>350	11%		61%		43%	

Table 4.2 Association between anti-Tat immunity and $CD4^+$counts. *Cross-sectional assessment of serum IgA, IgM and IgG anti-Tat antibodies in HIV-infected patients with different $CD4^+$counts. For statistical analysis Fisher's exact test was used.*

To better understand the association of the different anti-Tat antibodies classes with $CD4^+$counts, individuals were stratified according to the presence of one (IgA, IgM or IgG) or multiple anti-Tat antibody classes.

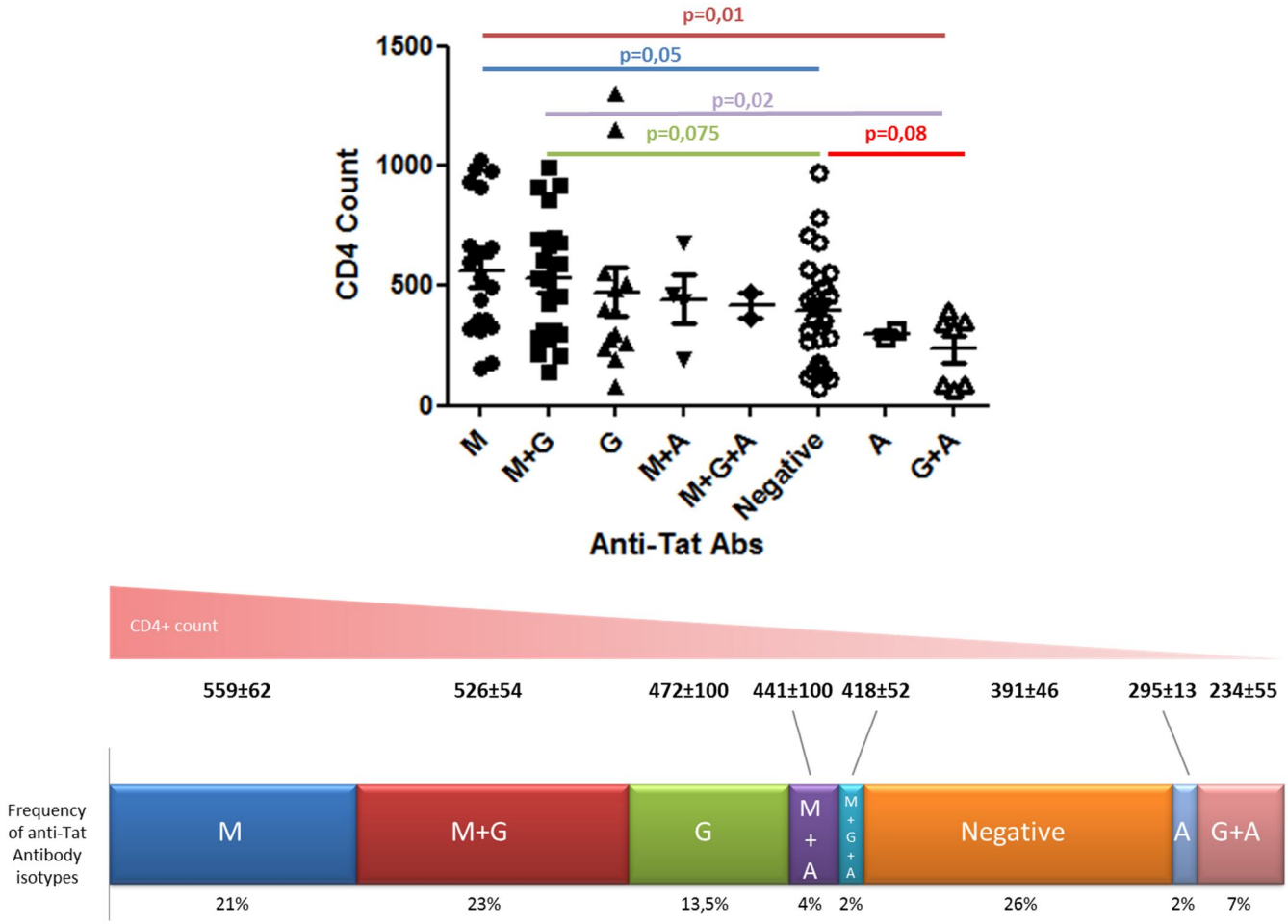

Figure 4.26 Anti-Tat humoral responses and CD4⁺counts. *HIV-infected patients were divided according the quality of anti-Tat antibodies. For every group, the mean CD4⁺count ± SEM was calculated. For statistical analysis two-tailed Mann Whitney test was used.*

As shown in Fig. 4.26, subjects with only anti-Tat IgM (21% of the total) displayed a significant higher CD4⁺ count (559 cells/µl) compared to anti-Tat naïve individuals (26% of the total, 391 cells/µl, p=0.05). The simultaneous presence of anti-Tat IgM and IgG was also associated with high CD4⁺ counts (526 cells/µl), although the comparison with the anti-Tat naïve group did not reach statistical significance (p=0.075). Furthermore, subjects with only IgM or with IgM and IgG exhibited CD4⁺ counts significantly higher than individuals possessing anti-Tat IgA and IgG (234 cells/µl), suggesting that the onset of anti-Tat IgA correlates with low CD4⁺counts.

These results suggest that anti-Tat IgM may protect from CD4⁺ T cells loss.

4.3.2 Antibody cross-recognition of clade B and C Tat protein in HIV-1–infected Tanzanian subjects

The IgG epitopes of the Tat protein are highly conserved among different HIV clades [175, 176], as demonstrated by the cross-recognition of a clade B Tat protein by individuals infected with different viral strains [178, 184].

However, these analysis are often based exclusively on IgG, and do not take into consideration other antibody isotypes. Thus, to assess the ability of anti-Tat IgA, IgM and IgG from HIV-infected patients to cross-react with different Tat variants, anti-Tat B and C humoral immunity was evaluated in every subject. Thus, subjects with anti-Tat Abs were stratified according to their ability to recognize exclusively Tat B, exclusively Tat C or both clade B and C Tat. As shown in Fig. 4.27, 54% of individuals with anti-Tat IgA was able to cross-recognize both clade B and clade C Tat, while 33% and 13% of subjects recognized only clade C and B Tat respectively. Similar proportions were observed among individuals with anti-Tat IgG. Interestingly, 76% of individuals with anti-Tat IgM was able to cross-recognize both clade B and clade C Tat, while 20% and 4% of subjects recognized only clade C and B Tat respectively, indicating that IgM display the highest capacity to cross-recognize Tat of different clades. Taken together, these data demonstrate that a high proportion of anti-Tat responders may recognize different Tat forms, suggesting that Tat B cell-epitopes are conserved among different HIV clades. The contribution of the different anti-Tat antibody classes to the protection from immune activation and unbalancement of $CD8^+$ and $CD4^+$ T cell subsets is now under investigation.

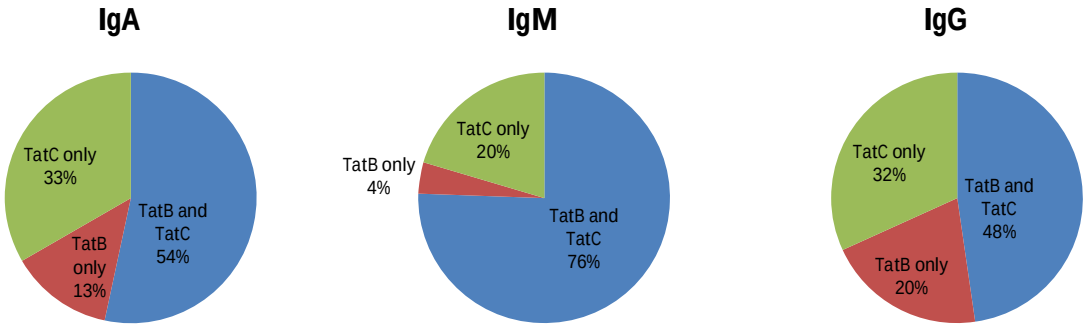

Figure 4.27 Cross-clade recognition capacity of the different anti-Tat antibodies. *Sera from HIV-infected individuals were assessed for their capacity to recognize clade B or clade C Tat. The percentage of individuals able to recognize only B Tat, only C Tat or both B and C Tat is displayed for every antibody class.*

5 Discussion

The HIV-1 Tat protein is fundamental for viral fitness [151, 165] and contributes on different levels to disease progression [82, 147, 165, 204]. Anti-Tat antibodies are more frequent in the asymptomatic stage of the infection and in nonprogressors [245, 247, 249], and their induction by Tat immunization in HIV-infected patients reverses signs of immune activation and T cell dysfunctions [259]. Tat, released by infected cells, efficiently enters uninfected cells and induces integrin-mediated signals [147-149], resulting in the activation and modulation of several cellular functions in $CD4^+$ T lymphocytes [151, 160, 165, 192, 204, 205, 208, 292], including the induction of IL-2 secretion [165, 205, 238], suggesting that Tat may play an important role in the chronic immune activation present during the HIV infection. However, whether Tat can, directly or indirectly, modulate the $CD8^+$ T cell response is unclear.

5.1 Effects of the HIV-1 Tat protein on $CD8^+$ and $CD4^+$ T cell programming

In this part of the study we examined the effects of Tat on $CD8^+$ T cells. We demonstrated that $CD8^+$ T cells activated in the presence of Tat exhibited an increased IL-2 release (Fig. 4.3). Several mechanisms may account for this effect as it has been reported that Tat favors the activation of some TFs required for IL-2 transcription like NF-κB, recruited by Akt [165, 204], NFAT [235, 293] and AP-1, activated by Tat through the the ERK pathway [234, 294]. Moreover, Tat superinduces factors binding to the CD28-responsive element (CD28RE), a DNA element mediating IL-2 gene activation by CD28 costimulation [165, 204].

Naïve and memory $CD8^+$ T cells activated *in vitro* by TCR engagement (different CD8 peptide epitopes and anti-CD3/CD28 stimulation) in the presence of Tat exhibited an increased activation as detected by cytokine release and cytotoxic assay (Figures 4.1-4.2), however the proliferation and phenotype of $CD8^+$ T cells were not affected by the presence of Tat (Figures 4.9-4.11). Notably, $CD8^+$ T cells activated in the presence of Tat showed an increased mRNA level of T-bet and Eomes (Figures 4.4-4.6). The modulation of the transcriptional profile of $CD8^+$ T cells by Tat requires the help of $CD4^+$ T cells, which also exhibited, when activated in the presence of Tat, an increased level of T-bet transcription, but not of Eomes (Fig. 4.7). Moreover, Tat-mediated enhancement of T-bet mRNA levels in $CD8^+$ T cells was observed only after activation, while Eomes up-regulation by Tat occurred also

in resting CD8+ T cells (Fig. 4.8), suggesting two different mechanisms of action exerted by Tat to modulate the expression of these TFs (Fig. 5.1).

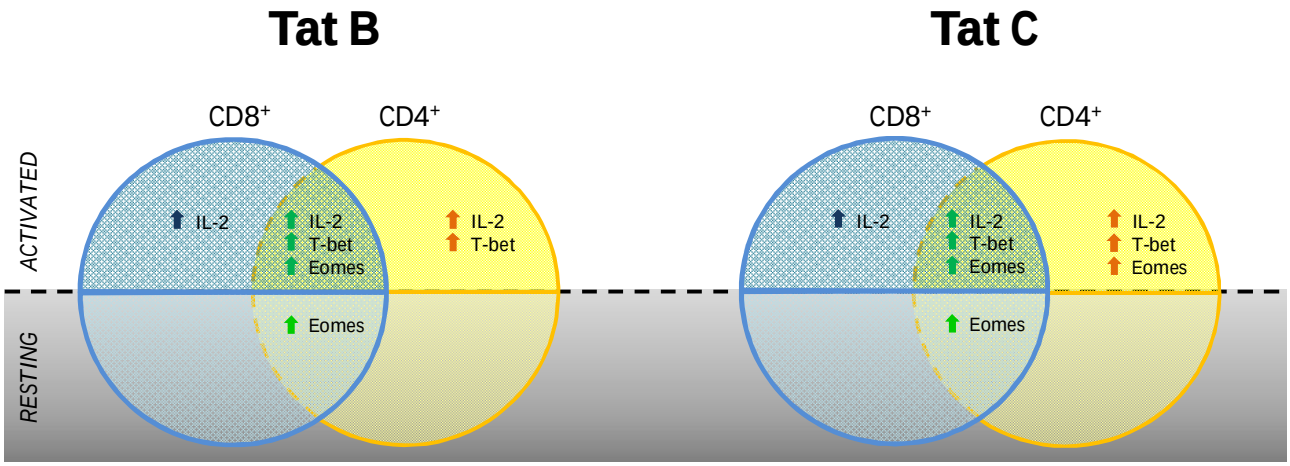

Figure 5.1 Clade B and C Tat-mediated modulation of CD8+ and CD4+ T cells transcriptional profile

We observed that the Tat-mediated up-regulation of T-bet was abolished by integrin blocking, suggesting that the RGD domain of Tat and its interaction with $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins are necessary for this effect. Interestingly, it is known that Tat mediates the activation of the ERK/MAPK and PI3K/Akt pathways through its RGD domain [236, 295]. As both ERK and Akt are involved in T-bet induction [30, 296, 297], it plausible to think that Tat triggers these pathways to mediate T-bet up-regulation. Moreover, the ERK pathway is also involved in Eomes up-regulation [298].

Naïve and memory CD8+ T cells activated by peptide epitopes in the presence of Tat exhibited a greater IFNγ release and lysis of target cells. Of note, the effect was abolished when Tat was added after the stimulation (Fig. 4.2), suggesting that Tat favors the expansion and the functionality of effectors cells only if present at the beginning of the stimulation. It is tempting to speculate that Tat potentiates the production of cytokines and cytolytic molecules through the induction of T-bet and Eomes (Fig. 4.6). Indeed, T-bet and Eomes control the transcription of IFNγ, perforins and granzymes in CD8+ T cells, and the cytotoxic potential of CTLs lacking one or both these TFs is greatly reduced [32, 299, 300]. In contrast to the Tat-mediated enhancement of IFNγ release after CEF stimulation, CD8+ T cells activated by anti-CD3/CD28 in the presence of Tat did not exhibited an increased IFNγ production, proliferation, nor an up-regulation of surface activation markers. This is consistent with literature data showing that T-bet deficiency does not affect secretory capacity, proliferation and phenotype of CD8+ T cells activated with anti-CD3/CD28, while T-bet-deficient CD8+ T cells fail to acquire effector phenotype and functionality after peptide stimulation [301]. Thus, the control of T-bet and Eomes on T cell functions is strictly dependent on the quality

of the stimulus. Moreover, Eomes up-regulation cannot mediate IFNγ release after anti-CD3/CD28 stimulation as it is known to induce IFNγ only late after clonal expansion and not at short time (i.e. 18 hours) after T cell activation [32, 299, 300].

In contrast to $CD8^+$ T cells, $CD4^+$ T cells activated by anti-CD3/CD28 in the presence of Tat exhibited an enhanced secretion of IFNγ (Fig. 4.14). Several reports show that T-bet controls in a different way IFNγ production by $CD4^+$ and $CD8^+$ T cells [32, 301, 302], and this may explain the different association between T-bet expression and IFNγ secretion in $CD4^+$ and $CD8^+$ T cells activated by anti-CD3/CD28 in the presence of Tat.

Thus, our data provide evidence that Tat favors the activation of $CD4^+$ T cells by the up-regulation of T-bet and of $CD8^+$ T cells through the cooperation of T-bet and Eomes, suggesting its potential contribution to immune activation during the course of HIV infection. Moreover, as T-bet is also involved in Th1 development, these data further demonstrate that the presence of Tat favors Th1-type responses [149, 193, 288].

To understand whether clade B and clade C Tat exerted the same effects on T cell activation, these two forms of Tat were compared for their capacity to modulate cytokines secretion and transcriptional profile in $CD8^+$ and $CD4^+$ T cells. Our results show that both clade C and clade B Tat enhance T cell activation. However, clade C Tat-mediated effects occurred earlier but at doses 100 times higher than clade B Tat-mediated effects. Moreover the analysis of the T-bet and Eomes expression suggested that clade C Tat altered more deeply the transcriptional profile of $CD4^+$ T cells, while B and C Tat exerted the same effects on $CD8^+$ T cells. Indeed, clade C Tat up-regulated both T-bet and Eomes in activated $CD4^+$ T cells, while clade B Tat only increased T-bet expression in activated $CD4^+$ T cells (Fig. 5.1). This suggests that clade C Tat contributes, more than clade B Tat, to the hyperactivation of $CD4^+$ T cells. Whether B and C Tat trigger the same intercellular pathways has still to be elucidated, although it is known that differences between the two Tat forms at the level of the cysteine rich domain and of the RGD domain (necessary for integrin binding) result in a different modulation of monocytes functionality [174, 179, 303]. However, our results show that clade B and C Tat act in a different dose-dependent and time-dependent manners, suggesting the involvement of alternative cellular signalling. Further studies are currently on-going to enlarge the number of samples and to investigate the mechanisms of action.

Studies describing the modulation of T-bet and Eomes expression on the whole T cells compartment during the course of HIV infection are missing, as the few reports available focus on HIV-specific $CD8^+$ T cells [80, 81]. As our model takes into consideration the effects of Tat on the transcriptional profile of $CD8^+$ and $CD4^+$ T cells irrespectively to their antigen-specificity, results reported here suggest that the HIV infection may, through the release of Tat [145, 146, 155, 186], affect T-bet and Eomes expression in the whole T cell compartment. The

alteration of T cell transcriptional profile, in the context of a chronic infection as HIV, may result in a deep and long-lasting modulation of $CD8^+$ and $CD4^+$ T cells responses. Indeed, it is has been reported that Eomes is usually up-regulated in exhausted $CD8^+$ T cells during chronic infections [304], suggesting also a Tat-mediated contribution to the exhaustion of $CD8^+$ T cells during HIV infection [305].

5.2 Tat-mediated modulation of viral-specific cellular and humoral responses

Our results demonstrate that Tat activates $CD8^+$ T cells and affects the transcriptional profile of resting and activated T lymphocytes. Moreover, it is known that Tat modulates the functionality of $CD4^+$ T lymphocytes [151, 160, 165, 192, 204, 205, 208, 292] and professional APCs [148, 149]. Thus, it is reasonable to think that Tat may affect *in vivo* the development of cellular responses, thus contributing to the immune impairment observed during HIV infection. To characterize the role of Tat in T cell hyperactivation and dysfunction, we examined whether the presence of Tat could affect $CD8^+$ T cell responses and antiviral immunity in different *in vivo* models of antigenic stimulation including a viral antigen and a viral infection i.e. HSV that is common in HIV infection. The presence of Tat during *in vivo* priming by the HIV-1 Gag protein and by infection with HSV1, favored the activation of antigen-specific CTLs (Figures 4.19-4.20), but no effect was detected on bystander resting T cells (Fig. 4.22). Effector $CD8^+$ T cells generated *in vivo* in the presence of Tat underwent an enhanced and prolonged expansion that turned to a partial dysfunctionality at the peak of the response (Fig. 4.20), that worsened acute infection (Fig. 4.21). Moreover, Tat-mediated T cell activation favored the development of effector memory $CD8^+$ T cells (Fig. 4.23) and a Th1 pattern of humoral response (Fig. 4.24).

The Tat-mediated enhanced expansion of CTLs (Figures. 4.19-4.20) may be due to several mechanisms. It has been demonstrated that the magnitude of the expansion of $CD8^+$ T cells is related to events occurring in the first 24 hours after antigenic stimulation [21, 306], and expansion requires TCR engagement (signal 1), co-stimulation (signal 2) and a pro-inflammatory environment (signal 3) [307]. Tat is known to affect all these signalling pathways. Indeed, Tat modulates antigen processing and improves the expression of certain epitope/MHC-I complexes on the surface of APCs leading to an increased stimulation of epitope-specific $CD8^+$ T cells (Signal 1) [148, 149, 192, 193, 196]. Moreover, it has been demonstrated that Tat activates NF-κB [204, 237, 308], T-bet and Eomes (Figures 4.4, 4.6 and 4.7), three transcription factors critical for the stimulation and the expansion of T lymphocytes [309]. In addition, Tat modulates signal 2 enhancing CD28-mediated stimulation [165], CD40 expression [148, 149], IL-2 production [165, 204, 205] and DC maturation and activation [148, 149].

Finally, Tat is known to increase secretion of IL-12 by DCs [148, 149], IFNα by macrophages [201] and of other pro-inflammatory cytokines [208, 310] contributing to the type 3 signal.

The results also demonstrate that antigen-specifc CTLs primed in the presence of Tat began secreting IFNγ at earlier time after HSV1 infection (Fig. 4.20 C). However, at the peak of the response, while dextramer staining indicated the presence of more effector cells in Tat-treated mice (Fig. 4.20 A), IFNγ Elispot analysis revealed the presence of a lower fraction of functional antigen-specific $CD8^+$ T cells (Fig. 4.20 B and D). Nevertheless, epitope-specific T cells were not completely exhausted, as demonstrated by the lack of PD1 up-regulation (not shown) and their recovery during the contraction and memory phases (Figures 4.20 E and 4.23 A). This dysfunctional status, defined as "stunning" [311], has been described in the presence of excessive stimulation [279, 311-313] and coincided with a transient lower control of infection (Fig. 4.21 A). The presence of a high percentage of antigen-specific T cells not secreting IFNγ has been described in several infections [69, 278, 279, 314-319]. In the case of HIV, the majority of reports show that the IFNγ secreting CTLs are about the 10-30% of tetramers-specific $CD8^+$ T cells [315, 317-319]. The different sensitivity of the techniques cannot explain completely the discordance between IFNγ and tetramers analysis, that has been described as an impaired functionality of $CD8^+$ T cells during HIV infection [69, 71, 320, 321]. Indeed, in HIV-positive patients the loss of IFNγ secretion can be observed also in CTLs specific for other pathogens [69, 316], as immune activation leads the whole T cell compartment to dysfunction, irrespectively to antigen-specificity [82]. Our data indicated that only about 30% of epitope-specific $CD8^+$ T cells secretes IFNγ at the peak of the response in Tat-treated mice, while almost all antigen-specific CTLs are fully functional in the control groups (Table 5.1), reflecting the impairment of functionality observed in $CD8^+$ T cells from HIV-infected subjects.

Percentage of Dextramer$^+$ Cells Detected by Elispot

Group	Peak (%)		Contraction (%)	
control	92.7	± 13.2	50.9	± 8.5
Tat	33.3	± 12.5	37.1	± 12.7

Table 5.1 Percentage of Dextramer$^+$ Cells Detected by Elispot. *Proportion of SSI-specific cells/million splenocytes, as detected by dextramer staining, secreting IFNγ in response to SSI stimulation, as detected by Elispot, at days 8 (peak) and 13 (contraction) post-infection*

Although the stunned phenotype of HSV1-specifc CTLs was still evident in Tat-treated mice at day 13 p.i. (Table 5.1), the kinetics of antigen specific $CD8^+$ T cell responses (Fig. 4.20 A) revealed that Tat-treated mice exhibited a

delayed contraction of IFNγ-secreting cells as compared to the control group. It is thus plausible that IFNγ-secreting cells developed in the presence of Tat are less susceptible to death during the contraction phase. An increased survival of effectors T cells during the contraction phase has been attributed to the interaction among CD80 or CD86 expressed on DCs with CD28 expressed on the T cells [22], to Bcl-2 expression [21] and to IL-2 co-stimulation [22, 23]. As Tat is known to induce DC activation and expression of CD80 and CD86 [148, 149], to directly enhance CD28 co-stimulation [165], to up-regulate Bcl-2 expression [160, 322] and to promote IL-2 secretion [150, 165, 205, 323], it is tempting to speculate that these different Tat-mediated mechanisms are involved in the delayed contraction phase occurring in Tat-treated mice. Moreover, as IL-2 favors the generation of $CD62L^{low}$ effector memory cells [324], Tat-mediated IL-2 secretion can account for the accumulation of effector memory $CD8^+$ T cells observed in Tat-treated mice (Fig. 4.23). Thus, the study of the kinetics of CTL responses in Tat-treated mice reveals that Tat prolongs the activation of $CD8^+$ T cells, and this further supports a role of Tat in immune activation that turns to be deleterious for HIV-infected individuals. Indeed, despite the longer expansion phase and the delayed contraction, Tat-treated mice were unable to control the acute HSV1 infection better than control mice. Moreover, the Tat–mediated hyperactivation of CTLs promoted the accumulation of effector memory $CD8^+$ T cells, a phenotype found at high frequencies in HIV-infected individuals [68, 325, 326], and reverted in HAART-treated patients by immunization with Tat [259].

Finally, we observed, for the first time in an *in vivo* model, a Tat-mediated transient loss of B cells. A role of Tat in B cell apoptosis has already been proposed [210-212], and B cell loss is present in HIV-infected subjects even under a suppressive HAART [82, 286], and it is reverted by induction of anti-Tat antibodies in HAART-immunized subjects [259]. This suggests that Tat may, directly or indirectly, contribute to the death of B lymphocytes in HIV-positive patients. Characterization of HSV1-specific humoral responses revealed also a Tat-mediated modulation of Th1/Th2 antibody pattern in absence of any effect on the magnitude of IgG and IgM responses (Fig. 4.24), maybe due to the prolonged IFNγ release observed in Tat-treated mice and to the T-bet up-regulation [287]. These observations further confirm that Tat induces a predominant Th1-type adaptive immune response [149, 193, 288].

In conclusion, the results of this study indicate that Tat modulates $CD8^+$ T cells activation and functionality resulting in a CTL response that starts earlier and lasts longer, but with a lower intensity at its peak. We propose a model by which a Tat-mediated enhancement of CTL activation and proliferation turns to be loss of functionality and accumulation of effector memory $CD8^+$ T cells. The Tat protein of HIV is known to exit infected cells [145, 146, 155] and exert immunomodulatory effects in both non-infected and non HIV-specific T cells

[147-149, 160, 192, 193, 208, 292]. It is interesting to note that several Tat-mediated effects like the activation of DCs [148, 149], $CD4^+$ [165, 204] and $CD8^+$ T cells, the induction of an effector memory phenotype and the loss of B cells, as reported here, are hallmarks of the chronic immune activation observed in HIV-infected patients [68, 82, 96, 286, 321, 325-327]. Although further studies are needed to better characterize these effects and the molecular pathways involved, we propose a key role of Tat in progression to AIDS contributing to immune activation and T cell dysfunctions.

5.3 Role of anti-Tat humoral immunity

The Tat protein of HIV-1, in addition to play a key role in the life cycle of the virus, is involved in several aspects of pathogenesis like $CD4^+$T cell loss, dementia and Kaposi Sarcoma, as discussed elsewhere in this thesis. Furthermore, the results presented in this study suggest that Tat may play a key role in immune activation and immune dysfunctions during HIV infection. Consistent with this, it has been demonstrated that anti-Tat humoral and cellular responses protect HIV-infected patients from progression to AIDS and restore immune functions [247, 250, 251, 259, 328]. Anti-Tat antibodies are present only in a fraction of HIV positive subjects [249, 250], but they are more frequently found in nonprogressors [197, 247], suggesting that the induction of anti-Tat humoral response may be a suitable strategy for preventive and therapeutic vaccines against HIV. Recent data from other vaccine trials (RV144) have enlightened the concept that different antibody isotypes may influence the level of vaccine efficacy and interfere with vaccination protective mechanisms [136]. As studies correlating anti-Tat humoral immunity and disease progression usually focus on IgG, and little is known about the prevalence of anti-Tat IgA and IgM and their interplay in HIV control, we sought to determine the frequency of anti-Tat IgA, IgM and IgG through a cross-sectional study performed in a cohort of HAART-naïve HIV-infected subjects in south east Tanzania. Anti-Tat IgM and IgG showed a similar prevalence (around 50%), while anti-Tat IgA were present in about 15% of subjects (Fig. 4.25). Data from other cohorts show conflicting results about the frequencies of anti-Tat IgG responses, as their prevalence varies in a range between 10 % [184, 245, 249] up to 80% or more [250, 329]. These variations may reflect differences both at the level of the cohort or of the assays sensitivity. However, prevalence data about anti-Tat IgA and IgM are more rare in literature and determined for the first time in an African cohort by this study.

Our results demonstrate a higher prevalence of anti-Tat IgM in HIV-positive individuals with $CD4^+$counts > 350 cells/µl (Table 4.2 and Fig.4.26), suggesting a protective role of anti-Tat IgM. Consistently, data from European

and American cohorts show higher frequencies of anti-Tat IgM in individuals with high $CD4^+$ counts or in the asymptomatic stage of the disease [245, 246]. Thus it is tempting to speculate that the association among the presence of anti-Tat IgM and high $CD4^+$ counts is constant among subjects infected with different HIV strains. Of note, anti-Tat IgM displayed a very high cross-clade recognition capacity (Fig. 4.27).

No significant differences regarding anti-Tat IgG prevalence were detected among patients with high or low $CD4^+$ counts, although it has been shown that anti-Tat IgG protect from $CD4^+$ decline in African [248] and European [249] cohorts. However, HIV-positive patients which possessed both anti-Tat IgG and IgM showed $CD4^+$ counts significantly higher than individuals with both anti-Tat IgG and IgA. This suggests a complex interplay among different antibodies isotypes, as anti-Tat IgA seem to appear in late stages of disease and to interfere with the protective capacity of the other anti-Tat antibodies, a phenomenon already observed in other contexts [136].

The development of a vaccine against HIV should face the problem of sequence differences among the several HIV-subtypes, in order to induce antibodies that react with a broad range of HIV strains. The Tat protein has been shown to conserve B cell immunogenic regions among different HIV clades [182, 183, 252] and anti-Tat antibodies elicited against one particular Tat clade may recognize Tat from different HIV subtypes [176, 330]. In accordance, our data demonstrate that about the half of patients with anti-Tat IgG or IgA were able to recognize both clade B anc C Tat, and this percentage reached 75% for anti-Tat IgM.

Although not all Tat variants are equally recognized by sera collected from people infected with different HIV strains [176, 330], it has been reported that clade B Tat may be efficiently cross-recognized by anti-Tat antibodies from African individuals infected with different HIV subtypes [176, 178, 184]. Our data show a high proportion of individuals with anti-Tat antibodies were able to recognize clade B Tat, with percentages ranging from 67% to 80% depending by the antibodies isotypes. Moreover, 13% of anti-Tat IgA positive subjects, 4% of IgM positive subjects and 20% of IgG positive subjects recognized clade B but not clade C Tat. Although we did not characterize the HIV subtypes in our cohort, it is known that, in the Mbeya District, the vast majority of individuals are infected with clade C HIV-1 or with recombinants forms containing type C sequences, while subtype B is almost absent, suggesting that a clade B Tat-based vaccine could induce cross-clade reactive antibodies in non type B HIV-1-infected individuals. Clade B Tat is now the antigen of a vaccine undergoing phase two clinical trial in Italy and South Africa and of a phase one study based on the co-administration of Tat and Env.

6 Conclusions

6.1 Implications for HIV pathogenesis

In this study we demonstrate, through the use of different *in vitro* and *in vivo* models, that the Tat protein of HIV-1 deeply alters the $CD8^+$ T cell response and the antiviral immunity. Indeed, Tat affects the transcriptional profile and functionality of $CD8^+$ T cells and hyperactivates T lymphocytes with deleterious effects on the control of acute infections. T cell hyperactivation is an hallmark of HIV infection whose causes are still unknown. Our results suggest a model by which Tat, released by HIV-infected cells, contributes to immune activation and immune dysfunctions during the course of HIV infection, affecting the functionality and the transcriptional profile of uninfected cells. Notably, these effects are mediated by Tat from different HIV subtypes, although our data suggest differences in the mechanism of action that have still to be elucidated but may account for the different pathogenicity among HIV clades. Consistently with a deleterious effect of Tat on the immune system of HIV-infected individuals, a cross-sectional study conducted on HIV-infected subjects revealed that anti-Tat IgM were more frequent in patients with high $CD4^+$counts suggesting, along with several other reports, that anti-Tat immunity is important to control disease progression. Interestingly, the Tat T cell and B cell epitopes have been shown to be conserved among different HIV subtypes, and our data demonstrated that clade B Tat may be efficiently recognized by antibodies from non-B HIV infected individuals, suggesting its use to induce anti-Tat immune responses.

Thus, our data provide new insights regarding the causes of immune activation and underscore the importance of addressing anti-Tat immunity in future preventive and therapeutic approaches aimed at HIV control and cure.

6.2 Implications for vaccines design

The immunomodulatory capacity displayed by Tat may be exploited to increase immune responses directed to poorly immunogenic vaccine antigens. Indeed, we demonstrated here that the co-administration of Tat with the Gag protein of HIV-1 dramatically increased Gag immunogenicity (Fig. 4.19) favoring $CD8^+$ and $CD4^+$ T cells responses not elicited by immunization with Gag alone. Moreover, it has been demonstrated that Tat broadens T cell responses to co-antigens [192, 193] and favors the induction of Th1-type responses [148, 149], suggesting its use as adjuvant in vaccination strategies. During last years this hypothesis has been explored through a model

of low antigenic stimulation (to avoid the Tat-mediated overstimulation observed with wild-type HSV1 infection) and constant Tat expression using a recombinant attenuated replication-competent Herpes simplex virus type 1 expressing the HIV1 Tat protein (HSV1-Tat). Assessment of HSV1-specific T cell responses in C57BL/6 mice immunized intravaginally with HSV1-Tat revealed significant higher IFN-γ responses compared to mice immunized with a control virus (HSV1-LacZ). Analysis of HSV1-specific humoral responses in sera from HSV1-Tat or HSV1-LacZ-immunized mice revealed that HSV1-specific IgG titers were detected in few mice infected intravaginally with HSV1-Tat but never in mice infected with HSV1-LacZ. Furthermore, the IgG isotype was analyzed and the reported results demonstrate the presence of IgG2a but not of IgG1 antibodies indicating a Th1-type immune response. Finally, mice were challenged with a lethal dose of wild-type HSV1. All animals treated intravaginally with HSV1-LacZ progressively developed severe HSV1 disease and died, while mice treated with HSV1-Tat presented a mild and transient disease and all of them survived to the viral challenge. Taken together, these results demonstrate that the intravaginal immunization of mice with a recombinant HSV1 vector expressing Tat induces stronger HSV1-specific cellular and humoral responses compared to the infection with a recombinant HSV1 control vector and protect from challenge with lethal doses of wt HSV1 (Sicurella et al, submitted). Thus, Tat effects on T cell activation may be finely modulated to enhance vaccines efficacy.

7 References

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