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The HIV-Tat protein favors the activation of T lymphocytes. Implications for new therapeutic strategies against HIV and other viral infections

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“We'll find a way,
when all hope is gone”
Slipknot

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LIST OF ABBREVIATIONS

aa	amino acid
Ab	antibody
Ag	antigen
AIDS	acquired immunodeficiency syndrome
AP-1	activating protein 1
APC	allophycocyanin
APC	antigen presenting cell
APC-Cy 7	allophycocyanin covalently bound to Cyanin 7
BSA	bovine serum albumine
CD	cluster of differentiation
CFSE	5(6)-carboxy-fluorescein diacetate succinimidyl ester
CT	cycle threshold
CTL	cytotoxic T lymphocyte
DAMP	damage-associated molecular pattern
DC	dendritic cell
DMSO	dimethylsulfoxid
DMF	dimethylformamide
dNTP	desoxy-nucleoside triphosphate
EBV	Epstein-Barr virus
ELISPOT	enzyme-linked immunospot assay
ERK	extracellular receptor-activated kinase
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluoresceinisothiocyanat
gp	glycoprotein
ART	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
KLRG1	killer cell lectin-like receptor subfamily G member 1
LCL	lymphoblastoid cell line
LPS	lipopolysaccharide
LTR	long terminal repeat
LvTat	HSV1 attenuated replication-competent vector expressing Tat
LvLacZ	HSV1 attenuated replication-competent vector expressing LacZ
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
NFAT	nuclear factor of activated T cells
NF-kB	Nuclear factor-kappaB
NK	natural killer cells
PAMP	pathogen-associated molecular pattern
PBL	peripheral plood lympohocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP-Cy5	peridinin Clorophyll protein covalently bound to Cyanin 5
PHA	phytohämagglutinin
PMA	phorbol 12-myristate 13-acetate
PRR	pathogen recognition receptors
PTD	protein transduction domain
qPCR	quantitative polymerase chain reaction
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SFU	spot forming units

SIV	simian immunodeficiency virus
SLEC	short lived effector cells
STAT	signal transducers and activators of transcription
TAR	trans-activation response element
Th	T helper
T_{CM}	central memory T cell
T_{EM}	effector memory T cell

1. INTRODUCTION

The human immunodeficiency virus (HIV) is one of the major plague in the world for new cases of infection, number of people infected and deaths.

In 2012, 2.3 million of new HIV infections (1.9 million–2.7 million) and 35.3 million of HIV-positive people have been reported all over the world [1].

Since its isolation, in the 1983, HIV has represented a social, economic and sanitary issue.

Despite the massive scale up of antiretroviral therapy (ART) has dramatically prolong the life of HIV-infected individuals and the number of new infections is decreasing, for every 10 people starting antiretroviral therapy 16 people are newly infected [2]. Today, a winning HIV/AIDS strategy does not exist, and the escalating cost for treatment will become increasingly difficult for undeveloped countries to meet. Strategies for either eradicating the virus from infected individuals or boosting their immune response so that antiviral drugs can be discontinued are urgently needed.

The most devastating damages caused by HIV infection are observed at level of cellular immunity and include the depletion of $CD4^+$ T cells and important dysfunctions of both $CD8^+$ and $CD4^+$ T cells as impairment of functionality [3, 4], exhaustion [5], increased T cell proliferation [6], susceptibility to apoptosis [7, 8] and expansion of memory T cells [9-11]. This status of chronic immune activation and immune senescence involves the whole T cell compartment, including uninfected and non-HIV specific T cells [12], is also present during ART and contributes to the appearance of AIDS-defining and non-defining diseases [13]. Different mechanisms contribute to these phenomena including $CD4^+$ T cells loss, viral replication and effects of HIV proteins such as gp120, Nef and Tat [6, 12]. However, how HIV can modulate the $CD8^+$ and $CD4^+$ T cells responses is not completely clear.

1.1 IMMUNE SYSTEM

The immune system is a rapid, specific and powerful mechanism of defense against viruses, bacteria and other potential dangerous microorganisms.

It is divided in two major sectors, the innate immunity and the adaptive immunity (Figure 1.1).

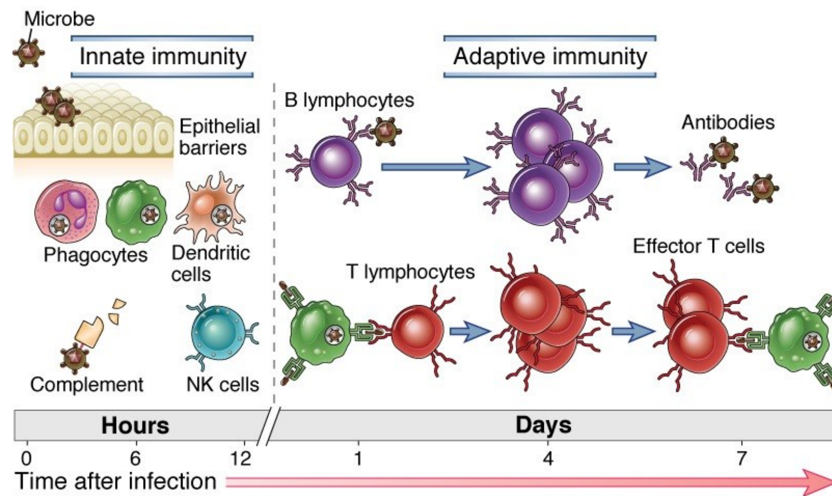


Figure 1.1. The innate and the adaptive immunity. Adapted by [14]

1.1.1 Innate immunity

The innate immunity system consists of multiple distinct subsystems that perform different functions in host defense.

The first defense consists in preformed soluble molecules like complement and cytokines, present in blood, extracellular fluid, and epithelial secretions that can kill or inhibit the proliferation of some classes of pathogens. The second defense is mediated by cells like Natural Killers (NKs), eosinophils, basophils, and mast cells specialized in the recognition of specific microbial structures and molecules such as PAMPs (pathogen associated molecular patterns) and DAMPs (damage associated molecular patterns) [14, 15], identified by cellular pattern recognition receptors (PRRs).

PRRs recognition of microbial structures causes a cascade of intracellular events that leads to pro-inflammatory cytokines and chemokines release, and to phagocytosis mediated by macrophages and dentritic cells (DC). On the whole these responses cooperate with co-stimulatory molecules, soluble or expressed on the cells surface, to activate adaptive immune cells.

1.1.2 Adaptive immunity

Adaptive immunity is carried out by cells called lymphocytes which act through two classes of responses: antibody response and cell-mediated immune response.
The first is mediated by B lymphocytes (B cells) which are responsible of the release of antibodies such as IgG, IgM, IgA, IgE. The latter is triggered by T lymphocytes (T cells), which are divided in two subpopulations: $CD4^+$ T cells, also known as T helper lymphocytes (Th), and $CD8^+$ T cells, also known as cytotoxic T lymphocytes (CTLs).

1.1.3 $CD4^+$ T cells

$CD4^+$ T cells are the main orchestrators of immune responses to infections. This subset of T cells is extremely important for the optimal activation and development of memory $CD8^+$ T cells [16], for the secretory activity of B and NK cells and for the antigen metabolism of professional antigen presenting cells (APC).
$CD4^+$ T helper subsets include Th1, Th2, Th17 and Treg cells [15, 17] (Figure 1.2 and Table 1.1). There are evidences that each of these subsets is involved in the defense against specific microorganisms.

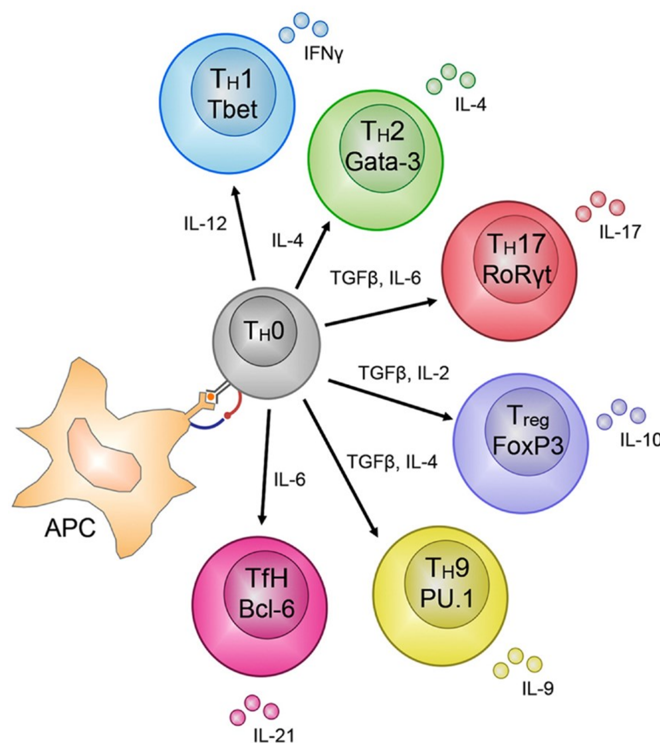


Figure 1.2. $CD4^+$ T cells generation. Adapted by [18]

Th1 cells mediate immune responses against intracellular pathogens [19]. Concerted with antigen recognition, interleukin-12 (IL-12) is the cytokine that drives naïve $CD4^+$ T cells into Th1 subset type. IL-12Rβ1 is constitutively expressed on naïve $CD4^+$ T cells and its expression is increased in Th1 cells. The most important cytokine products of Th1 cells are interferon-γ (IFNγ), lymphotoxin α (LTα), and IL-2. IFNγ is important to activate macrophages and $CD8^+$ T cells, and increases antigen processing and presentation to T cells [20]. IL-2 production is critical for $CD4^+$ and $CD8^+$ T-cell memory maintenance since it promotes cell survival, inducing the expression of the anti-apoptotic protein Bcl-2 [19, 21]. IL-2 can also stimulate the activation and proliferation of $CD8^+$ T cells and enhances the expression of co-stimulatory molecules on APCs surface and the production of other cytokines in favor of $CD8^+$ T cells. Moreover, IL-2 induces the release of other cytokines like IL-4, important for the development of other Th subsets and stimulates the proliferation of NK and B cells. LTα activates neutrophils and lymphoid organogenesis.

Th2 cells mediate host defense against extracellular parasites including helminthes [22-24]. The induction of Th2 subset is regulated by IL-4 that acts with a mechanism of feedback to produce itself and IL-5. Those cytokines produced by Th2 cells, promote the production of IgE and the differentiation and activation of eosinophils [20]. Th2 cells also support B lymphocytes stimulating the production of large amount of IgM. IL-4 antagonizes the macrophage-activating actions of IFNγ and, in association with IL-5, can inhibit acute and chronic inflammation, limiting consequences due to persistent Th1 immune responses.

Th17 cells mediate immune responses against extracellular bacteria and fungi [25]. Their cytokine products are IL-17, IL-21, and IL-22. IL-17 recruits and activates neutrophils during immune responses [26], while IL-21 is a stimulatory factor for Th17 differentiation and serves as positive feedback, as does IL-2 for Th1 and IL-4 for Th2 cells. In addition, IL-21 acts on $CD8^+$ T cells, B cells, NK cells, and dendritic cells [27]. IL-22 mediates host defense against bacterial pathogens, and these functions may largely depend upon IL-23 stimulation of innate cells.

Treg cells play a critical role in maintaining self-tolerance as well as in regulating immune responses [28]. Treg cells exert their suppressive functions through several mechanisms,

some of which require cell-to-cell contact or the production of cytokines, including TGF-β and IL-10. High levels of CD25 and IL-2Rα expression on Treg cells suggest the importance of IL-2 to regulate proliferation and induction of Treg suppressor activity.

T helper lineage	Cytokines production	Inductive cytokines	Role
Th1	IFNγ IL-2	IL-12 IL-2 IFNγ	Mediate immune responses against intracellular pathogens: NK, macrophages, CTLs activation and IFNγ secretion
Th2	IL-4 IL-5 IL-6	IL-4 IL-2	Mediate defense against extracellular parasites; Promotion of IgE and eosinophil/basophil/mast cell-mediated immune reaction
Th17	IL-17 IL-21 IL-22	IL-6 TGF-β	Mediate immune responses against extracellular bacteria and fungi; Release of proinflammatory cytokines, activation of T cells, NK and neutrophils
Treg	TGF-β IL-10	TGF-β IL-10 IL-2	Regulation and suppression of immune response

Table 1.1. The $CD4^+$ T cells subsets

1.1.4 $CD8^+$ T cells

$CD8^+$ T cells are critical to protect the organism against intracellular pathogens and tumor cells. In contrast to $CD4^+$ T cells, $CD8^+$ T cells are the direct executioners of pathogen-infected cells.

During the immune response to viruses and intracellular microbes, naïve $CD8^+$ T cells are activated in cytotoxic T lymphocytes (CTL) to undergo a massive clonal expansion, with acquisition of new effector functions.

After antigen recognition, CTLs proliferate and kill the cellular target mainly with three mechanisms:

- Exocytosis of granules containing perforin that polymerize on target cell membrane causing osmotic cell lysis.
- Secretion of granzymes (serine proteases) that induce apoptosis in target cells [29].
- Expression of Fas ligand (FasL) that induces apoptosis by a specific and direct interaction with Fas molecules present on target cells [30]. Target killing by CTLs is antigen specific and contact dependent.

1.2 CD4$^+$ AND CD8$^+$ T CELLS ACTIVATION

T lymphocytes mount specific responses against antigens (Ags) which are peptides generated by the cellular proteolytic system ("antigen processing") and are presented through MHC molecules to the TCR. MHC class I and the MHC class II molecules, also known as Human Leukocyte Antigen (HLA), are controlled by genes located on chromosome 6, that encodes cell surface molecules specialized to present antigenic peptides to T cells. All MHC-I molecules consist of two polypeptide chains, a heavy chain α and a monomorphic light chain β2-microglobulin (β2m).

The two chains are linked non-covalently via interaction of β2m and the α3 domain. Like MHC class I molecules, MHC-II molecules are also heterodimers, but in this case consist of two homologus peptides, an α and β chains, both of which are encoded in the MHC (Figure 1.3).

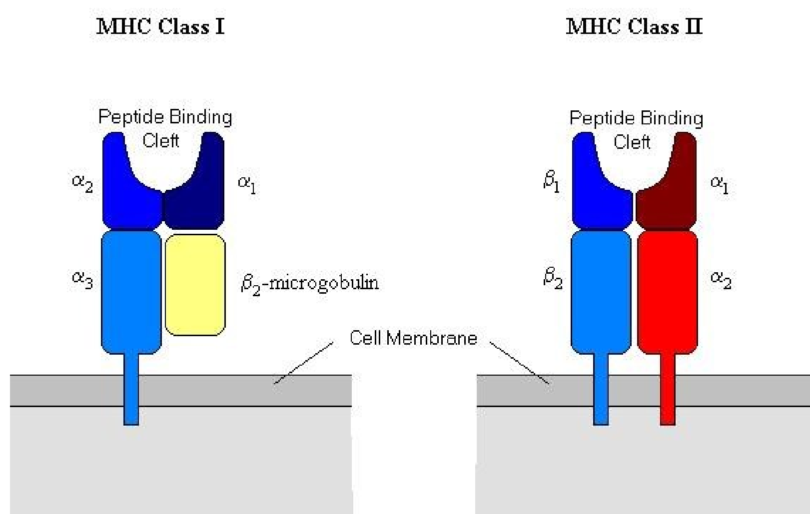


Figure 1.3. MHC classes I and II molecule structure

The MHC-I system draws its spectrum of peptides from proteins in the cytosol of all nucleated cells. In contrast to MHC-I, MHC-II molecules are expressed on a more limited set of cells called APC cells such as somatic cells, B cells, macrophages and dendritic cells. Thereby, MHC molecules are a molecular reflection of the health of cells that synthesize them (for MHC-I molecules) or of the local environment in which cells reside (for MHC-II molecules).

MHC-I and MHC-II molecules also show a different interaction with T cells. In particular, the nonpolymorphic α3 domain of MHC-I binds the CD8 co-receptor of T cells while the membrane proximal domains of MHC-II interacts exclusively with the CD4 molecule. CD8 and CD4 molecules serve as co-receptors on the surface of T lymphocytes, providing both adhesion and specific activation signals that modulate T cells. Epitope associated to the MHC molecules migrate to the cell surface to be recognized by the specific TCR.

The TCR is a disulfide-linked membrane-anchored heterodimer normally consisting of the highly variable α and β chains expressed as part of complex with the invariant CD3 chain molecules [31] (Figure 1.4).

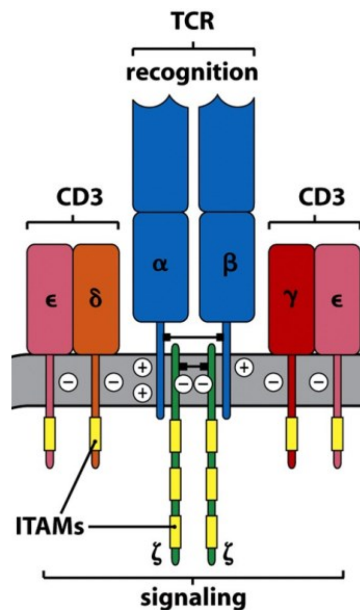


Figure 1.4. T cell Receptor

The generation of TCR diversity is based on somatic recombination of the DNA encoded segment in individual T cells.

The combinatorial rearrangement of multiple gene segments, the error-prone process of joining these different fragments, and the various combinations of α with β chains or the

less represented γ with δ chains lead to big TCR diversity and, thereby, enables receptors to respond to a broad spectrum of antigens that pathogens show.

It is known that activation of T cells requires multiple signals: signal 1, antigen-specific delivered via TCR/MHC interaction, signal 2, delivered by co-stimulatory molecules (including IL-2), and signal 3, delivered by pro-inflammatory cytokines and chemokines [32].

Several co-stimulatory receptors are exposed on T cells surface; among them, CD28 and CTLA-4 co-stimulatory molecules bind B7 receptor on APCs surface. CTLA-4-mediated inhibitory signals dominate when T cells encounter antigen on APCs expressing low levels of B7, while CD28 activating signals dominate when antigen is presented by APCs with high B7 levels [33-35]. 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 [36].

In addition, CD3 seems to be responsible for signal transduction following the antigen recognition events. This idea was suggested by the observation that antibodies directed against CD3 proteins generate the same T cell activation events as stimulation of T cells with peptide antigen plus MHC [37, 38].

Co-stimulators are required for the full maturation of responses in T cells. Furthermore, the combination of these signals induces cytokines secretion, clonal expansion and differentiation of precursor $CD4^+$ and $CD8^+$ T cells into effector (Figure 1.5).

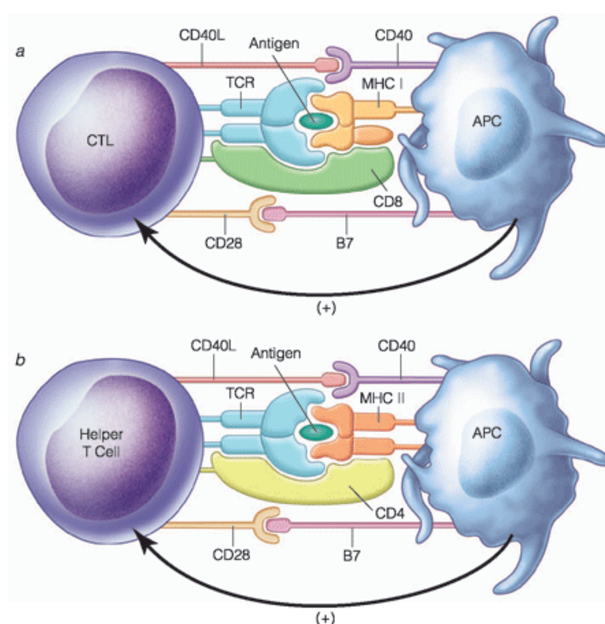


Figure 1.5. Immune $CD8^+$ (a) and $CD4^+$ (b) synapsis

In concert with antigen recognition, the cytokine environment modulates the polarization of T-cell homeostasis. In particular, IFNγ, IL-2, IL-7, IL-4, IL-9, IL-12 and IL-15 supply survival and expansion of $CD4^+$ and $CD8^+$ T cells.
The T cell activation provided by TCR, co-stimulatory molecules and cytokines determine the change of transcription factors (TFs) expression that act as master regulators by controlling the expression of a panel of genes and conferring a specific phenotype. A large number of TFs participate in initial T cell activation, such as nuclear factor of activated T cells (NFAT), activating protein-1 (AP-l), and nuclear factor-kappaB (NF-kB), that interact with transcriptional partners determining the activation of T cells.
NFAT is required for the expression of IL-2, IL-4, TNF, and other cytokine genes. Interestingly, productive T cells activation not only requires prolonged nuclear NFAT, but also depends on physical interactions between NFAT and AP-1 on composite promoter elements. Activation of AP-1 typically involves synthesis of Fos protein and phosphorylation of c-Jun protein.
NF-kB is a transcription factor that is activated in response to TCR signals and is essential for cytokine synthesis. NF-kB fulfills a central role in the cellular stress response and in inflammation by controlling the expression of a network of inducers and effectors that define responses to pathogens and other classes of danger signals [39].
Following NFAT, AP-1 and NF-kB dependent T cell activation, a subsequent phase of clonal expansion ensues.

1.2.1 $CD4^+$ and $CD8^+$ T cells programming

$CD4^+$ Th1/Th2 balance is regulated by T-bet and GATA3, both induced by TCR stimulation and by several cytokines. T-bet enhances responsiveness to IL-12 favoring IL-12Rβ2 expression and is regulate by or regulate IFNγ production, thus supporting a Th1 phenotype; while GATA3 is the TF responsible for the acquisition of a Th2 lineage promoting IL-4 secretion [40, 41]. T-bet and GATA3 are regulated by mTOR kinase through activation of the PI3K-Akt pathway [42]. STAT proteins also regulate the expression of many genes required for the differentiation of various $CD4^+$ T helper cell lineages.
Therefore, STAT1 and STAT5 contribute to Th1 differentiation by enhancing T-bet and IFNγ expression, respectively, while STAT6 mediates IL-4 production and Th2 differentiation [41, 43].

During $CD8^+$ T cell-mediated immune response, Eomesdermin (Eomes), another T-box transcription factor like T-bet, is essential for the development of effector and memory phenotype.

Eomes is crucial in first phase of $CD8^+$ effector development and along with T-bet it regulates $CD8^+$ cytotoxic functions [44]. Otherwise, Eomes and T-bet expression are inversely related in central memory (T_{CM}) cells [45]; while T-bet decreases in the contraction phase, Eomes expression is necessary. The level of inflammation and the availability of certain cytokines (e.g. IL-12) influence the levels of T-bet and Eomes available to the effector cell and that this in turn influences which cells will remain $CD8^+$ effector T cells and which will transition to the memory $CD8^+$ T cell pool [46].

Other two transcription factors crucial for the activation and the development of $CD4^+$ and $CD8^+$ T cells are Blimp-1 and Bcl-6, that are reciprocally antagonist. Blimp-1 is a transcriptional factor encoded by Prdm1 and correlated to effector and memory functions of both $CD8^+$ and $CD4^+$ T cells. Blimp-1 in $CD8^+$ T cells negatively regulates Bcl-6 and, as in $CD4^+$ T cells, it is activated by IL-2 driving $CD8^+$ T cells to CTLs differentiation. Bcl6 is a transcriptional repressor of granzyme B and it is involved in $CD4^+$ and $CD8^+$ memory T cells formation [47]. Although many aspects of Blimp-1 and Bcl-6 regulation in $CD4^+$ T cells are still unclear, some studies suggest a key role of Bcl-6 in $CD4^+$ T cells survival and proliferation as well as Tfh formation. In addition, it has been demonstrated the significant influence of Blimp-1 in terminal effector cells differentiation and in Th1 maturation where it interacts with T-bet [48] (Table 1.2 and Figure 1.6).

	Activated $CD8^+$ T cells	**Activated $CD4^+$ T cells**			
		Th1	**Th2**	**Th17**	**Treg**
TFs expression	Blimp-1 T-bet Eomes	T-bet Blimp-1 STAT1 STAT5	Gata3 STAT6	Bcl-6 RORγt	Foxp3

Table 1.2. Transcription factor expressing in activated T lymphocytes

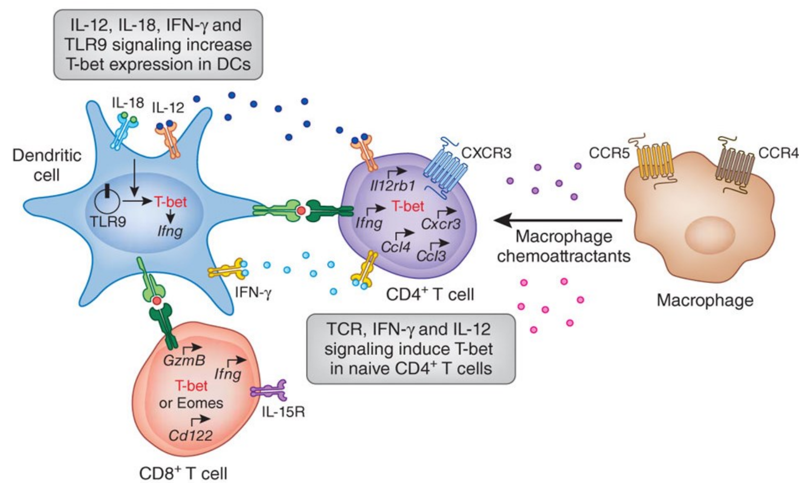


Figure 1.6. Transcriptional signaling after activation in CD4+ (a) and CD8+ (b) T cells. Adapted by [49]

Intensity of stimuli through TCR-MHC signaling, cytokines production and presence of different subsets of immune cells can deeply modulate transcription factors programming in both CD4+ and CD8+ T cells, driving the immune response to different fates.

The modulation of T cell responses to antigen and the improvement of activation are goals extremely important for the development of new therapies and vaccines against tumors and pathogens.

1.2.2 Memory CD4+ and CD8+ T cells

During a response to infection, there are three characteristic phases: a period of initial cell activation and expansion, a contraction or death phase, and the establishment and maintenance of immunological memory (Figure 1.7).

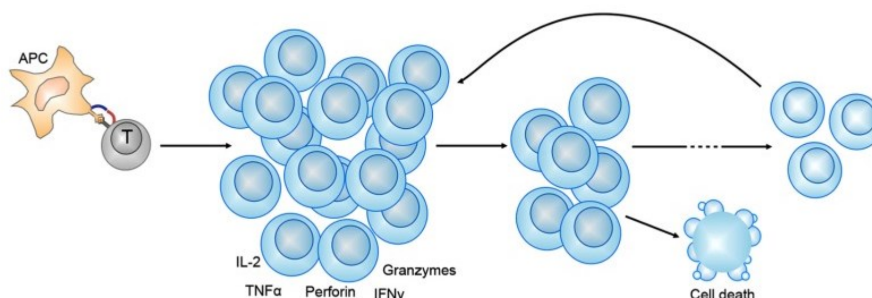


Figure 1.7. T cells differentiation and fate. Adapted by [18]

There are two competing models of memory T cell development: linear and not-linear divergent differentiation. In the linear model, activated naïve T cells develop initially into effector cells and, a small fraction of these cells, survives at contraction phase and becomes memory cells. In the not-linear divergent model, a fraction of activated naïve T cells differentiates directly into memory cells. Both these theories are supported by different reports however, recently some studies indicate the linear model as the most consistent [50, 51].

Memory cells are divided into two different subpopulations: "central memory" (T_{CM}) and "effector memory" (T_{EM}) (Table 1.2).

	Phenotype	**Molecules secreted**
Central memory (T_{CM})	CCR7 High CD62L High CD44 IL-7 Receptor (CD127) IL-15 Receptor (CD122) CXCR5	IFNγ low IL-2 high IL-4 low CD40 ligand IL-17A
Effector memory (T_{EM})	CD62L Low CD44 IL-7 Receptor (CD127) IL-15 Receptor (CD122) CD45RA	IFNγ IL-4 IL-5

Table 1.3. Memory phenotype and secretion capacity

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. The role and the homing capacity of memory cells is determined by their phenotype. For instance, the expression of some cellular markers like CCR7 and CD62L on memory cells surface regulate their susceptibility to antigen and consequently cells proliferation and cytokines release. Cells proliferation and cytokines production represent the two main aspects of this population. Indeed, while T_{CM} are extremely proliferative but have a low cytokine secretion capacity after antigen recognition, T_{EM} are not so efficient in proliferation but display high secretory potential.

During acute viral infection, antigen-driven differentiation of naive $CD8^+$ T cells leads to the expression of cytolytic molecules and cytokines that facilitate the control of infections.

After the expansion and contraction phases, during which the infection is cleared, some antigen-specific $CD8^+$ T cells survive and gain the capacity of self-renewal in lymphoid and non-lymphoid tissues, and a heightened ability to recall effector functions relative to their naïve precursors. Molecular studies of $CD8^+$ T cells differentiation during acute viral infection have demonstrated that at the effector stage, memory precursor cells destinated to survive into the memory phase, can be identified by some transcriptional factors such as Eomes, Blimp-1 and cellular marker like CD127 (IL-7 receptor), CD62L (homing in lymphoid organs) and CD122 (IL-15 receptor) [52, 53]. The long term maintenance of memory $CD8^+$ T cells does not require the antigen stimulation, but it is dependent on IL-7 and IL-15 signalling that mediates homeostasis and survival, up-regulating anti-apoptotic molecules such as Bcl-2 [54]. T_{CM} $CD8^+$ 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 activation 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 [55, 56].

Naïve CD4 T cells must usually interact with mature antigen-loaded dendritic cells to be successfully activated. This interaction takes place in the T-cell areas of secondary lymphoid organs, locations that have evolved to facilitate contact between antigen-specific T cells and DCs. $CD4^+$ T cells require prolonged and high TCR signaling stimulation in order to differentiate into effectors and subsequently form the memory pool [57]. Memory $CD4^+$ T cells express much lower levels of CD122, that correlates with a lower dependence on IL-15 compared to $CD8^+$ T cells. However, IL-7 appears to be the main survival cytokine for $CD4^+$ memory cells, as cells cannot survive in its absence. Moreover, memory $CD4^+$ T cells life tends to be shorter than that of memory $CD8^+$ T cells [58].
Memory $CD4^+$ T cells are important during the “clinical latency” phase of HIV which 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 [59]. 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 [60].

1.3 THE HIV INFECTION

In 1981 appeared, in Unites States, a new disease, characterized by a deficiency in the immune system [61] which consists in a marked reduction in CD4$^+$ cell numbers and enhanced B-cell proliferation and hypergammaglobulinemia. After two years of research, this disease known as acquired immune deficiency syndrome (AIDS), was connected to a new human retrovirus named HIV [62]. The AIDS viruses, HIV-1 and HIV-2, consist of different virus groups and a lot of different clades subtype for every virus group. Based on their genetic differences, four groups of HIV-1 (M, N, O, P) have been identified and inside these groups it is possible to distinguish 9 subpopulations or clades (A, B, C, D, F, G, H, J, K). The four groups of HIV-1 isolated differ from each other by the chemokine receptor they use for cell entry. HIV viruses that use to enter cells the chemokine receptor 4 (CXCR4) are termed X4, the ones that use chemokine receptor 5 (CCR5) are termed R5, and the others that use both CXCR4 and CCR5 are termed R5X4 [63].

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) (Figure 1.8).

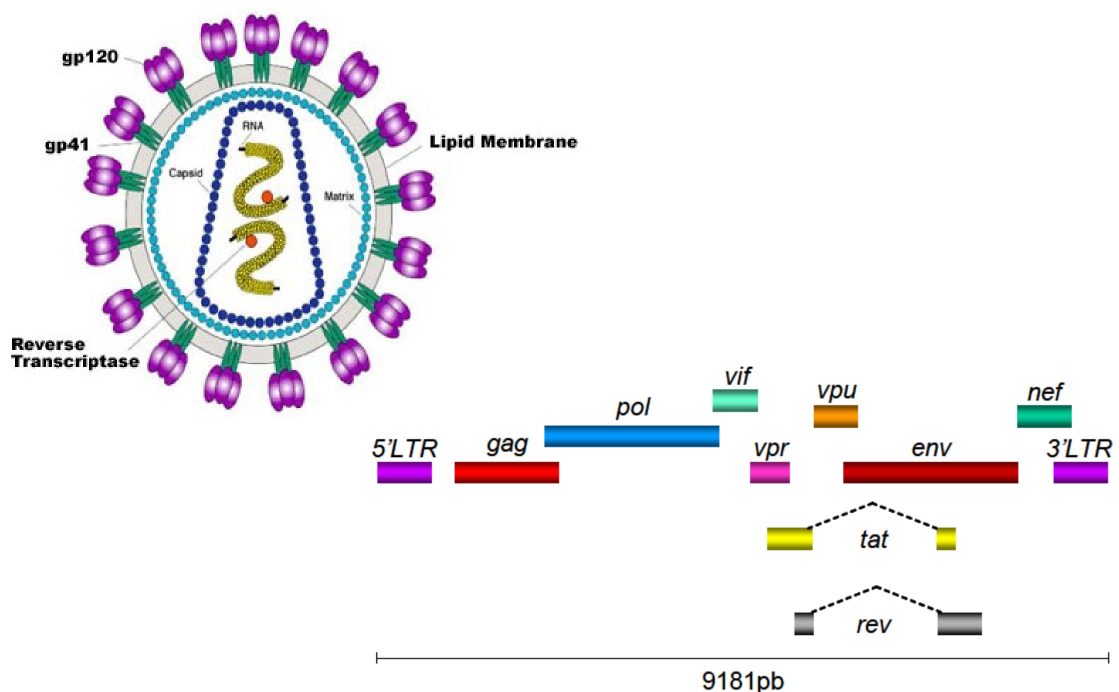


Figure 1.8. The HIV virus and genome

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.

HIV-1 is one of the most polymorphic viruses known and exists as a swarm of genetically related variants. The polymorphic nature of HIV-1 can be directly attributed to its error prone reverse transcriptase and complexity of its cDNA formation. Together with other host factors, the evolution of the viral genome underlies all of the changes in the biological characteristics of HIV-1 including cytopathic ability, immune evasion, co-receptor usage and tropism.

$CD8^+$ 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 [64], and some HLA class I alleles are associated with the control of the infection [65]. HIV mutates the dominant epitopes 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 [66]. This great variability makes extremely difficult the search for a vaccine that can induce protective antibodies and both innate and adaptive cellular immune anti-HIV responses capable of controlling the disease. Up to now, 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 ART.

Vaccine candidates able to induce cellular response were developed but the results were not promising, and the trial was stopped for an increased risk of HIV acquisition in vaccines [67], due to some pre-existent immunity to the viral vector used, and to an inappropriate bias of CTL responses towards less conserved epitopes.

However, nowadays no candidates have been shown to be highly immunogenic and, at the same time, confer protection from viral rebound after ART interruption [68, 69]. Thus, the research on preventive and therapeutic vaccines against HIV is still ongoing.

One of the most important barriers to the elimination of HIV is its persistence in cellular reservoirs [70], that consist in latently infected central memory and transitional memory $CD4^+$ T cells [71]. Their homeostatic proliferation, low proliferation rates and long term maintenance sustained by IL7 and IL15 make them a very stable viral reservoirs [71]. 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.

1.3.1 EFFECTS OF HIV ON CD4+ AND CD8+ T CELLS

As mentioned above, the most devastating damages caused by HIV infection are observed at level of cellular immunity.

Depletion of CD4+ T cells is a primary reason for the opportunistic infections and cancers associated with HIV infection. Several factors can be involved in this CD4+ T cells loss: effects on permeability of cell membrane due to virus replication, induction of apoptosis via immune activation or viral replication, direct cytopathic effects of HIV, anti-CD4+ autoantibodies, anti-CD4+ T cells cytotoxic activity [72, 73] and destruction of bone marrow [74]. CD4+ T cells depletion can be also increased by cytokines, like TNF-α and several HIV protein such as Nef, gp120, Vpr and Tat may also affect CD4+ T cells viability. Moreover, autophagy has been noted as a possible cause of bystander CD4+ T cells death following HIV infection [73].

CD8+ T cells play a pivotal role in the suppression of viral replication and in the clearance of virally infected cells during HIV infection. Besides, CTLs from HIV-infected patients appear to degenerate into a functionally impaired state, irrespective to ART treatment [75]. HIV down-regulates surface MHC-I expression from infected cells, by altering the pattern of cytokine production, T cell signaling and engagement of cellular receptors [76] and the down-regulation of CD3 and CD28 has been associated with defects in TCR stimulation. Indeed, the HIV accessory genes Vpu and Tat play an important role in MHC-I down-regulation [77].

Dysfunctionality of T lymphocytes in HIV-positive patients has been linked to the deep modification of their transcriptional profile [78, 79]. In particular, it has been shown that CD8+ T cells from HIV-positive patients display an effector phenotype and a simultaneous increased expression of two T-box transcription factors, T-bet and Eomes [79].

High HIV virus levels correlate with higher level of Eomes in CD8+ T cells memory. Also T-bet may play a role in increasing the cytotoxic potential observed in HIV-specific CD8+ T cells. Besides, it has been found that a high level of T-bet correlates with a better control of HIV virus replication. T-bet expression is strongly correlated with perforin and granzyme B production and may influence the effector status of HIV-specific CD8+ T cells [80, 81].

HIV also enhances the secretion of the anti-inflammatory cytokine transforming growth factor (TGF-β) by CD8$^+$ T cells, thus inhibiting the IFNγ response to HIV antigens. In CD8$^+$ T cells from HIV-positive patients, activation of the mitogen activated protein kinase (MAPK) results in the activation of transcription factor cascade that regulates such cellular processes as cell cycle, stress response, apoptosis, differentiation and proliferation [76] (Figure 1.9).

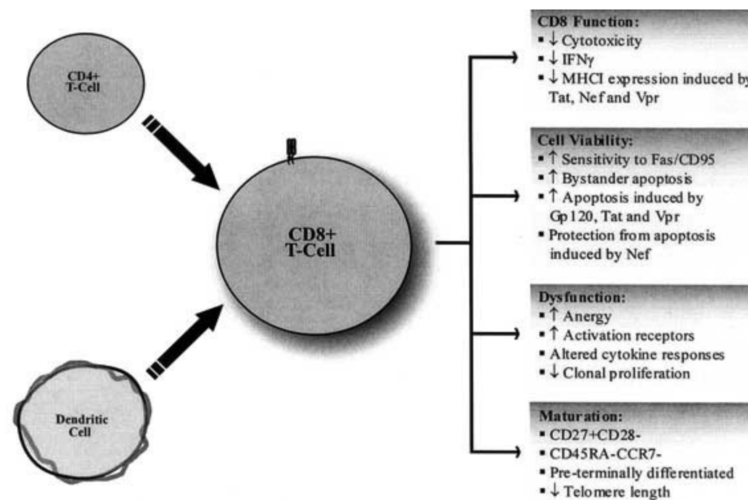


Figure 1.9. HIV effects on CD8+ T cells. Adapted by [76]

1.4 HIV-1 TAT PROTEIN

HIV-Tat is a small protein whose length varies between 80 and 103 residues (14 KDa) depending on the different position of transcriptional stop codons in the second coding exon [82]. The predominant and most common form is 101 residues long (Figure 1.10).

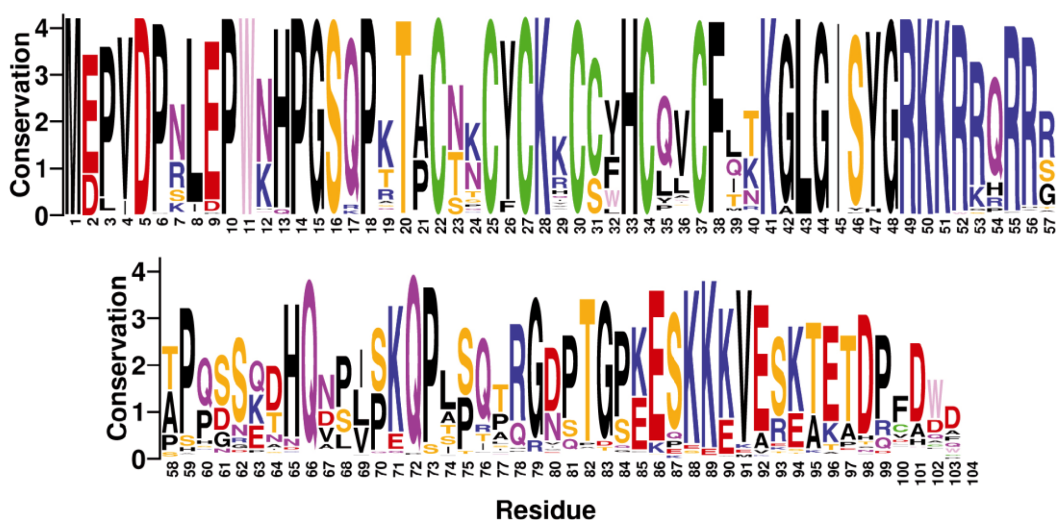


Figure 1.10. HIV-1 Tat sequence conservation. Letter size is proportional to residue conservation [83]

Tat is required for HIV-1 transcription and is therefore able to reach the nucleus. During the earlier (Tat-independent) initiation phase of HIV replication, a short RNA, trans-activation response element (TAR), is produced from the HIV-1 long terminal repeat (LTR).

In the elongation phase, Tat, cyclin T1 and CDK9 form a ternary complex that binds TAR and then induces the phosphorylation of RNA polymerase II, enabling the production of full-length HIV-1 transcripts and their release.

When Tat is present, 99% of the transcripts are transcribed to their full length. However, in the absence of Tat, 87% of the initiated transcripts terminate prematurely at positions +55 to +59 [84].

Besides its involvement in viral replication, Tat is implicated in HIV spread. Indeed, its expression prior viral integration, can strongly increase the number of the HIV-1 co-receptors CXCR4 and CCR5 at the surface of $CD4^+$ T cells, favoring viral infection [85].

After HIV infection, Tat is early produced and secreted by infected T cells and in the absence of cell lysis and can alter functions of both infected and uninfected T cells. Tat reaches the cytosol using a pathway that relies on endocytosis, low pH-driven membrane insertion and Hsp90-catalysed translocation. Intriguingly, the Tat concentration detected in supernatant during *in vitro* experiments of infection reaches the same levels observed in sera of HIV-1-infected individuals.

After the release in sera, Tat can bind heparan sulphate proteoglycans (HSPGs) [86] and the integrin receptors $\alpha_5\beta_1$ and $\alpha_V\beta_3$ through its Arg-Gly-Asp (RGD) motif (residues 78–80) and enters in uninfected cells [87-89].

Six distinct functional domains have been characterized in the Tat protein:

1. N-terminal (amino acids 1–21),
2. Cysteine-rich (amino acids 22–37)
3. Core (amino acids 38–48)
4. Basic (amino acids 49–57)
5. Glutamine rich region (58-72)
6. C-terminal (amino acids 73–86/101)

The N-terminal domain binds bivalent ions able to mediate interactions among Tat monomers; the cysteine-rich and core domains are highly conserved and are necessary for LTR transactivation (the absence cause the loss of Tat transactivation activity); the basic domain consists of a stretch of basic amino acids necessary for nuclear localization,

binding to TAR-RNA and to with heparan sulfate; the C-terminal domain of Tat is encoded by the second exon and contains an RGD motif typical of extracellular matrix proteins, which mediates the binding with integrins [90, 91].

Several studies have reported that HIV-1 Tat protein activates $CD4^+$ T cells and increases pro-inflammatory cytokine production in both HIV-infected and uninfected cells [92].

Tat protein, possesses several immunomodulatory features making it an attractive molecule to be exploited for vaccination strategies and therapeutic interventions aimed at modulating antigen-specific immune responses in different types of human diseases [92]. In particular, the biologically active clade B Tat protein targets very actively immature dendritic cells, induces their maturation and polarizes the immune response to the Th1 pattern through transcriptional activation of TNFα gene expression, leading to a more efficient presentation of both allogeneic and heterologous antigens [93]. In addition, Tat possesses intrinsic adjuvanticity attributed to its capacity to dimerize [169], increases the number of Treg cells [67] and induces protective immunity against Leishmania major [94].

Moreover, it has been demonstrated that anti-Tat immunity is important to control the disease and to restore immune functions [82, 95], suggesting that Tat may contribute to immune activation.

1.4.1 Effects of HIV-Tat protein on $CD4^+$, $CD8^+$ T cells and APCs

As mentioned before, Tat can be released by infected cells and be internalized by other cells through binding heparan sulfate [86], integrin receptors [96] and via endocytosis [97]. Tat displays several effects on T cells, including activation, proliferation [92, 98], apoptosis [98, 99] and also favors the expression of chemokines and cytokines [100, 101].

Tat activates $CD4^+$ T cells after anti-CD3/CD28 stimulation, in a mechanism dependent by CD28 co-stimulation that enhances IL-2 secretion [92] and may result in an increased susceptibility to HIV-1 infection [102]. Moreover, Tat inhibits the NAD-dependent deacetylase sirtuin-1 (SIRT1) activity [103] potentiating NF-κB transcriptional activity and unveils a molecular mechanism by which hyperactivation of immune cells is promoted during HIV infection. In addition, it has been shown that Tat induces the release of other pro-inflammatory cytokines involved in T cells activation and differentiation, such as IL-8, IL-12 and TNFα [104, 105]. Along with IFNγ secretion induced by $CD4^+$ T cells, it has been observed a strong up-regulation of T-bet expression which is mainly implicated in generating Th1 type of immune response [105].

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 [106] and associated to tumorigenic potential [107, 108]. 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 [109, 110] would be due to the inhibition of CD26 activity [111] and the enhanced release of the suppressive cytokine IL-10 [112]. Thus, it has been proposed that Tat may induce pro-apoptotic signals through JNK and it mediates cell proliferation through the ERK pathway, whose activation is required for cell growth [113, 114]. The pleiotropic effects of Tat on these two pathways are confirmed by the observation that rapamycin, which inhibits mTor activity, abolished Tat-mediated activation of JNK, while it enhanced Tat-mediated activation of ERK/MAPK, suggesting the involvement of mTOR in Tat-induced signalling [91] (Figure 1.11).

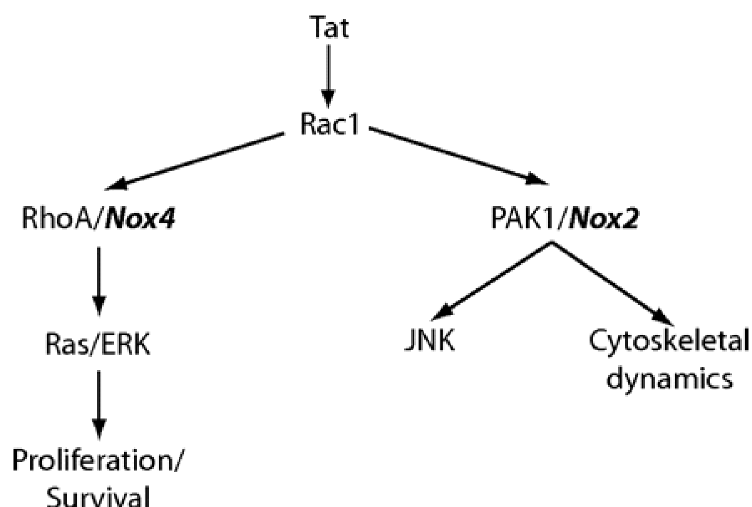


Figure 1.11. Differential activation of MAP kinases by distinct Nox pathways. Adapted by [114]

While Tat inhibits cells proliferation and induces apoptosis at high concentrations [98, 99, 115, 116], at lower physiological concentrations (nM) Tat promotes CD4$^+$ T cells survival [117, 118].

This is confirmed by the observation that Tat, expressed on the surface of heterologous cells, activates and induces proliferation of human peripheral blood mononuclear cells (PBMCs), in a mechanism dependent by CD3 stimulation [105].

Several authors have suggested that the generalized decrease in CD127 expression on CD8$^+$ T cells in HIV-infected patients is the result of chronic antigen stimulation and immune activation [119, 120]. Indeed, HIV-Tat protein and IL-7 seem to act

synergistically to down-regulate CD127 on $CD8^+$ T cells isolated from healthy volunteers. This synergism appears to be mediated, at least in part, by JAKs as addition of JAK inhibitor 1 completely blocked IL-7 ability to down-regulate CD127 and abolished synergy with Tat. Importantly, Tat has no effect on $CD8^+$ T cells viability [121].

In addition to these effects on $CD4^+$ and $CD8^+$ T cells programming and homeostasis, Tat can modulate 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 [122, 123]. In particular, Tat increases the presentation of subdominants epitopes at the expense of the immunodominant ones [123]. Furthermore, some studies demonstrate that Tat enhances the release of several cytokines in monocytes, macrophages and DCs [124-127], as well as to up regulate costimulatory molecules such as CD40, CD80, CD83 and CD86 [124]. However, recently, a Tat-mediated enhancement of HLA-ABC and HLA-DR expression on DCs has been demonstrated [124].

DC are professional APCs actively involved in CTLs development, and $CD4^+$ T cells support $CD8^+$ T lymphocytes in their generation and maintenance of effector and memory subsets; thus, it is reasonable to think that Tat-mediated effects on these cell types could also affect the $CD8^+$ T cell response and, thus, the control of infection.

The immunomodulatory properties displayed by Tat make this molecule an attractive adjuvant for other antigens. Indeed, since Tat plays a key role in HIV life and its progression, it can be considered a perfect candidate to preventive and therapeutic AIDS vaccine.

Safety and immunogenicity data collected in several animals models [128, 129], in addition with results obtained in Tat-vaccinated monkeys, that showed a viremia at undetectable levels and absence of $CD4^+$ decline after challenge with SHIV viruses [130, 131], supported the development of a Tat-based vaccine.

A preventive and a therapeutic phase I trials have already been performed in parallel with the recombinant biologically active HIV-1 Tat (86aa) to evaluate safety and immunogenicity [132]. After the successful achievement of the end-points, Tat vaccine was administered to 87 ART-treated HIV-positive individuals, both in Italy and South Africa, during phase II clinical trial [133]. Promising results are obtained such as a restoration of immune functions by the reversion of $CD4^+$ T cells and B lymphocytes loss in Tat-immunized individuals. In 2013, a second Tat-based vaccine that utilizes a Tat variant isolated from a group of HIV controllers African patients (Tat Oyi) has entered in phase I clinical trial in France [134].

2. AIMS

The HIV infection is associated with a state of chronic immune activation that drives the general de-regulation of the immune system. T cells of HIV positive people are indeed characterized by an active proliferation, loss of functionality and susceptibility to apoptosis. These phenomena do not involve only HIV-infected or HIV-specific T cells, but are generalized to the all T lymphocyte compartment. The causes of this phenomena are not yet understood, although the contribution of HIV viral proteins (alone or in combination with other cellular factors) may play a key role in the modulation of T cell activities. It is known that the HIV-Tat protein, the transactivator of HIV gene expression, is essential for viral replication [135-137] and, therefore, for establishment of infection and virus reactivation [138-140]. Upon virus entry into cells, Tat is expressed by proviral DNA prior to virus integration [141], and it is released extracellularly by a leaderless secretory pathway [138, 139]. Upon release, Tat binds heparan sulphate proteoglycans of the extracellular matrix and integrins, and via this receptors binding, could enter cells very efficiently.

In previous studies it has been demonstrated that the Tat protein modulates cellular responses to heterologous antigens [142] suggesting that Tat displays immunomodulatory features, only partially characterized at molecular level, affecting CD4- and CD8-mediated cellular responses. In addition, it has been found that Tat increases the activation and maintenance of antigen specific T cells in mice [143]. These preliminary studies suggest that Tat may affect $CD4^+$ and $CD8^+$ T cells. Thus, the identification of the role of Tat on signals and transcriptional programs of T cells differentiation, survival and proliferation, is a key to clarify the role of this HIV protein on disease progression and immune dysfunctions caused by HIV infection. In addition, immunomodulatory proprieties of Tat may be used to enhance vaccines efficacy.

The specific aims of my study are:

1. The evaluation of the effects of Tat on cytokines production by T cells.
2. The evaluation of the effects of Tat on proliferation and viability of T cells.
3. The evaluation of the effects of Tat on transcriptional profile of T cells.
4. The evaluation of the effects of Tat on phenotype of T cells.
5. The evaluation of the effects of Tat on the expansion of antigen-specific T cells.
6. The evaluation of the effects of Tat on the expansion of HSV1-specific $CD8^+$ T cells after immunization with recombinant Herpes Simplex vector.

7. The evaluation of the effects of Tat on HSV1 specific $CD8^+$ T cells memory subpopulations after immunization with recombinant Herpes Simplex vector.

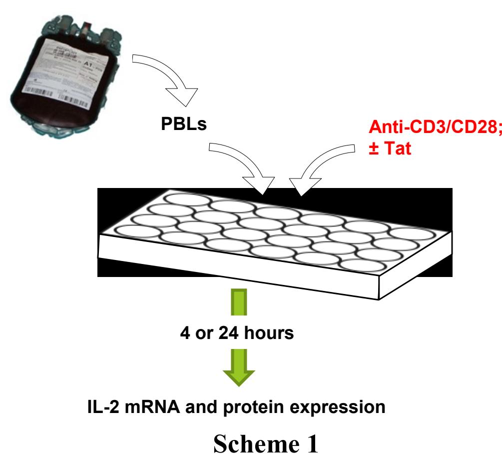
In the table below are summarized the methods for each specific aim; details in “Material and Methods” section.

Effect of Tat on T cells	**Methods**	**Specific aims**
Cytokines release	Elispot - Bio-Plex	1
Protein expression	Western Blotting - Intracellular Staining	1-3
CTLs cytotoxicity	^{51}Cr-release assay	5
Phenotype	FACS	4-6-7
mRNA expression	SYBR green quantitative PCR	3
Proliferative capacity	MTT assay - CFSE assay	2

3. RESULTS

3.1 Tat enhances the production of IL-2 in PBLs

Several studies have reported the capacity of the HIV-1 Tat protein to activate PBLs and $CD4^+$ T cells resulting in an increase of IL-2 production when cells were exposed to different stimuli, including antibodies specific for the CD3 and CD28 (anti-CD3/CD28) receptors that mimic physiological T cell activation [92, 102-104, 144]. To confirm these results, we first sought to determine whether the amounts of secreted Tat usually found *in vivo* may account for this effect. To this aim, PBLs from healthy donors were activated with anti-CD3/CD28 in the absence or presence of different doses of Tat (from 0.001 µg/ml to 1 µg/ml), and IL-2 mRNA levels were measured after 4 hours by qPCR (Scheme 1).



Scheme 1

As shown in Figure 3.1a, a 75 fold-increase of IL-2 mRNA was observed in PBLs activated in the absence of Tat compared to untreated PBLs, while the presence of Tat induced a 150-200 fold-increase of IL-2 mRNA expression. This effect was observed at similar levels for all Tat doses except at 0.001 µg/ml. Similar results were obtained at 24 hours after activation (Figure 3.1b), demonstrating that this effect is long lasting. As the highest fold-increase was observed at 0.1 µg/ml of Tat, this dose was chosen to perform the subsequent experiments. Of note, this concentration is within the range of the physiological concentration of Tat found in sera of HIV-positive individuals. Moreover, to assess whether the increased mRNA levels resulted in increased cytokine release, IL-2 secretion from PBLs was evaluated by Bio-Plex at 24 hours after activation.

As shown in Figure 3.1c, a 2 fold increase of the production of IL-2 was detected in PBLs activated in the presence of Tat.

To further confirm that the increase of the production of IL-2 was mediated by the presence of Tat, similar experiments were performed in the presence of anti-Tat antibodies obtained from sera of immunized mice. As shown in Figure 3.2, the incubation with anti-Tat antibodies during the treatment, abolished the Tat-mediated effect.

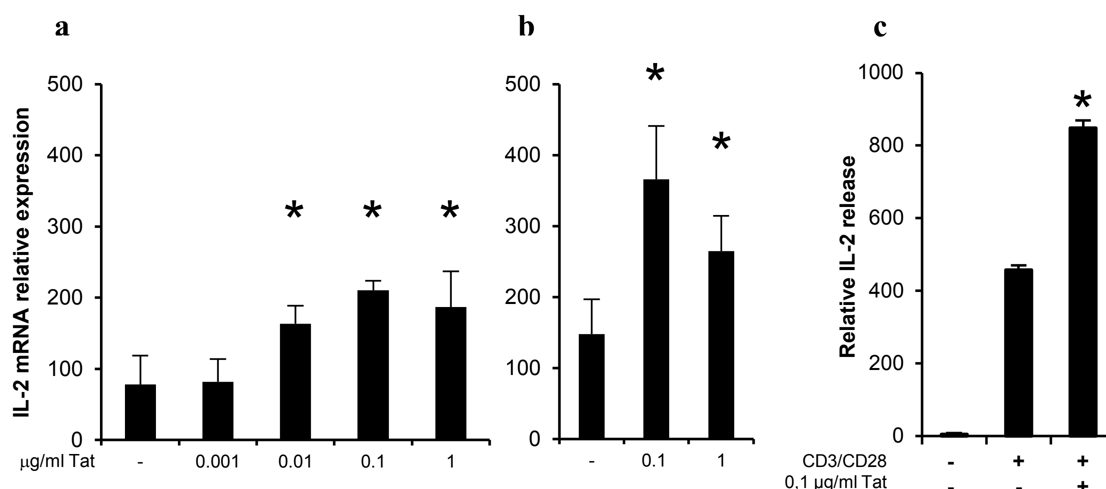


Figure 3.1. Tat enhances IL-2 production in activated PBLs. (a, b) PBLs from healthy donors (n=6) activated with anti-CD3/CD28 were cultured in the absence or presence of the indicated concentrations of Tat for 4 (a) or 24 (b) hours and IL-2 mRNA levels were quantified by qPCR and normalized to untreated cells. (c) PBLs from healthy donors (n=6) unstimulated or activated with anti-CD3/CD28 were cultured in the absence or presence of Tat (0.1 µg/ml). After 24 hours IL-2 release was quantified by Bio-Plex and normalized to untreated cells. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to Tat-untreated control cells.

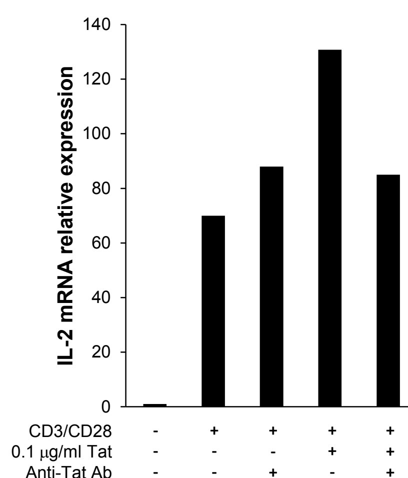
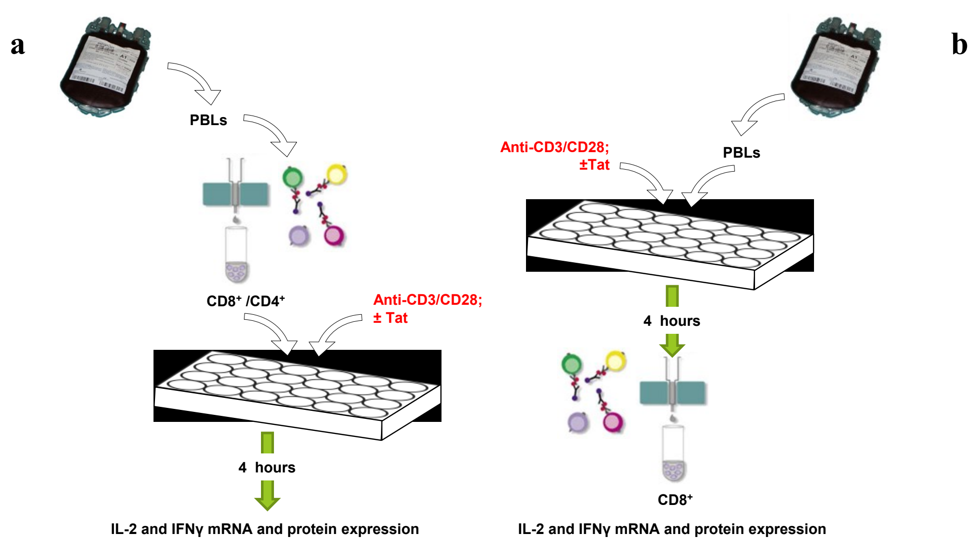


Figure 3.2. Tat-mediated increase of IL-2 production in activated PBLs is abolished by anti-Tat antibodies. PBLs from healthy donors unstimulated or activated with anti-CD3/CD28 were cultured in the absence or presence of Tat (0.1 µg/ml) and anti-Tat immune antibodies. IL-2 mRNA levels were quantified by qPCR and normalized to untreated cells. The results of one representative experiment out of three are shown.

3.2 Tat enhances IL-2 and IFNγ production in $CD8^+$ and $CD4^+$ T cells

We sought to determine whether Tat specifically affects cytokine production in activated $CD4^+$ and $CD8^+$ T cells. To this aim, first the expression of IL-2 mRNA was evaluated in activated $CD4^+$ and in $CD8^+$ T cells activated alone or purified from activated PBLs (Scheme 2a and 2b).



Scheme 2

Interestingly, Tat significantly increased IL-2 mRNA in both $CD8^+$ T cells activated alone (Figure 3.3a) or purified from activated PBLs (Figure 3.3b). Moreover, consistently with literature results [102], the presence of Tat during the stimulation induced a significant increase of IL-2 mRNA expression in $CD4^+$ T cells (Figure 3.3c).

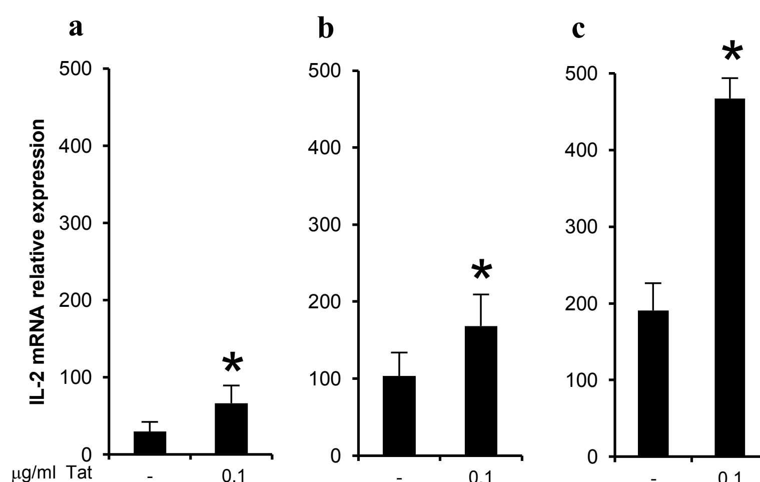


Figure 3.3. Tat enhances IL-2 production in CD8$^+$ and CD4$^+$ T cells. (a) CD8$^+$ T cells were purified from PBLs from healthy donors (n=6) and activated with anti-CD3/CD28 in the presence or absence of 0.1µg/ml of Tat for 4 hours. (b) PBLs from healthy donors (n=6) were activated with anti-CD3/CD28 in the presence or absence of 0.1µg/ml of Tat. After 4 hours of stimulation, CD8$^+$ T cells were purified. (c) CD4$^+$ T cells were purified from PBLs from healthy donors (n=6) and activated with anti-CD3/CD28 in the presence or absence of 0.1µg/ml of Tat for 4 hours. IL-2 mRNA levels were quantified by qPCR and normalized to untreated cells. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to control cells.

To confirm these results, the IL-2 production was also evaluated by intracellular cytokine staining (ICS). 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 (Figure 3.4a and 3.4b). There was no effect of Tat on IL-2 production by CD8$^+$ and CD4$^+$ T cells at 6 hours after stimulation (data not shown). However, an average of 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 and 10 µg/ml of Tat compared to CD8$^+$ and CD4$^+$ T cells activated without Tat.

Taken together, these data demonstrate for the first time that Tat increases the production of IL-2 in CD8$^+$ T cells.

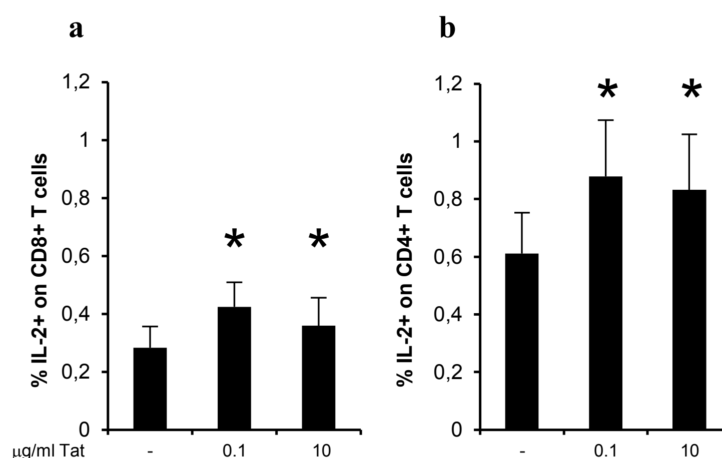


Figure 3.4. Tat enhances percentage of IL-2$^+$ CD8$^+$ and IL-2$^+$ CD4$^+$ T cells. PBLs from healthy donors (n=7) were activated with anti-CD3/CD28 in the presence or absence of different concentrations of Tat for 18 hours. Percentages of CD8$^+$ (a) or CD4$^+$ (b) T cells secreting IL-2 were determined 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.

Thus, we next investigated whether the presence of Tat during the activation of T cells could also modulate IFNγ production. To this aim, first the expression of IFNγ mRNA was evaluated in activated CD4$^+$ and in CD8$^+$ T cells activated alone or purified from activated PBLs. The presence of Tat during the stimulation dramatically enhanced IFNγ production in CD8$^+$ T purified from activated PBLs and in CD4$^+$ T cells activated alone but not in CD8$^+$ T cells activated alone (Figure 3.5 a-c).

Taken together, these results demonstrate that physiological concentrations of Tat enhance the production of IL-2 and IFNγ in both CD8$^+$ and CD4$^+$ T cells activated with anti-CD3/CD28.

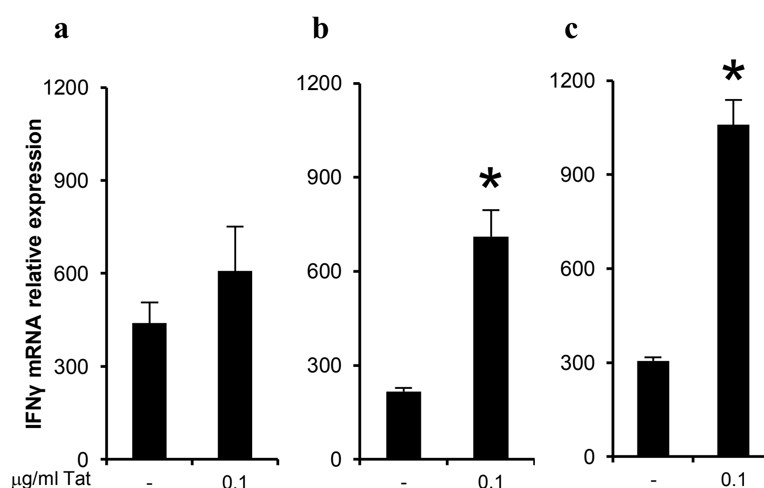
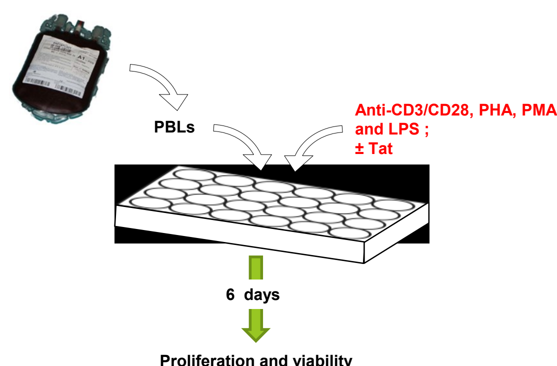


Figure 3.5. Tat enhances IFNγ production in $CD8^+$ and $CD4^+$ T cells. (a) $CD8^+$ T cells were purified from PBLs from healthy donors (n=6) and activated with anti-CD3/CD28 in the presence or absence of 0.1µg/ml of Tat for 4 hours. (b) PBLs from healthy donors (n=6) were activated with anti-CD3/CD28 in the presence or absence of 0.1µg/ml of Tat. After 4 hours of stimulation, $CD8^+$ T cells were purified. (c) $CD4^+$ T cells were purified from PBLs from healthy donors (n=6) and activated with anti-CD3/CD28 in the presence or absence of 0.1µg/ml of Tat for 4 hours. IFNγ mRNA levels were quantified by qPCR and normalized to untreated cells. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *$P<0.05$: Tat-treated cells compared to control cells.

3.3 Tat does not affect the proliferation of activated $CD8^+$ and $CD4^+$ T cells

We next evaluated whether the presence of Tat during the activation of T cells resulted in an enhanced proliferation. To this purpose, PBLs activated with different stimuli (anti-CD3/CD28, PHA, PMA and LPS) in the absence or presence of Tat were cultured up to six days, and PBLs, $CD8^+$ and $CD4^+$ T cell proliferation was measured by CFSE staining and MTT assay (Scheme 3).



Scheme 3

As shown in Figure 3.6, the presence of Tat did not affect the proliferation of PBLs activated with the indicated stimuli. In addition, Tat did not affect proliferation of $CD4^+$ and $CD8^+$ T cells activated with CD3/CD28 (Figure 3.7). This is consistent with previous reports showing that soluble Tat was ineffective in enhancing anti-CD3/CD28 induced proliferation [102, 115].

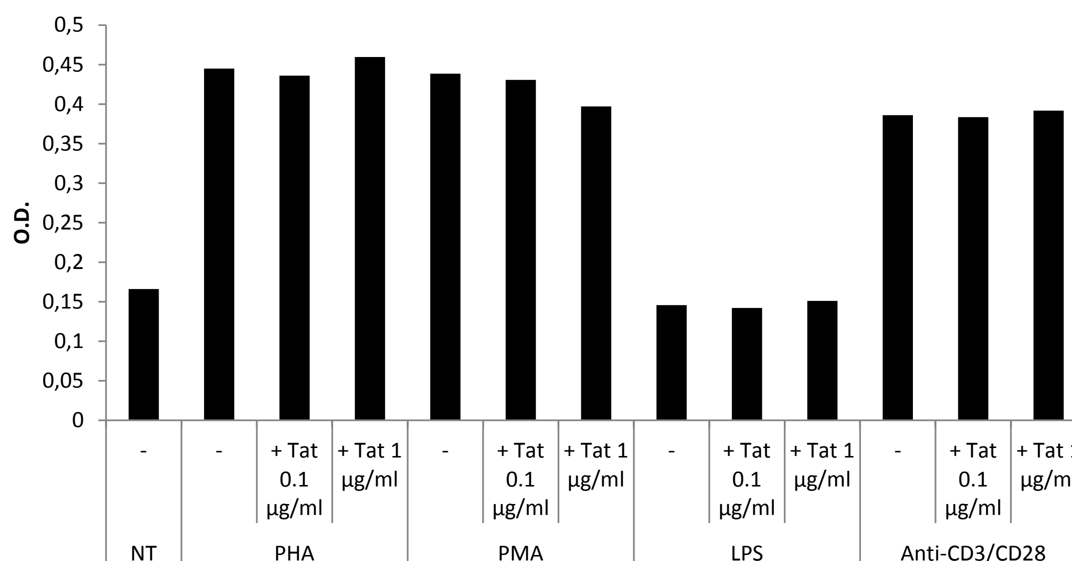


Figure 3.6. Tat does not affect T cell proliferation. PBLs from healthy donors were activated with different kind of stimuli: PHA, PMA + Ionomycin, LPS and anti-CD3/CD28, in the presence or absence of different concentrations of Tat, and cultured up to six days. Cell proliferation was measured by MTT assay. One representative experiment out of three is shown.

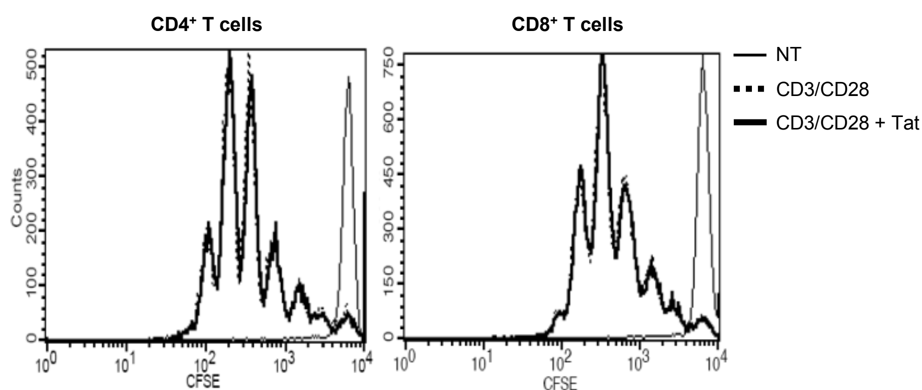
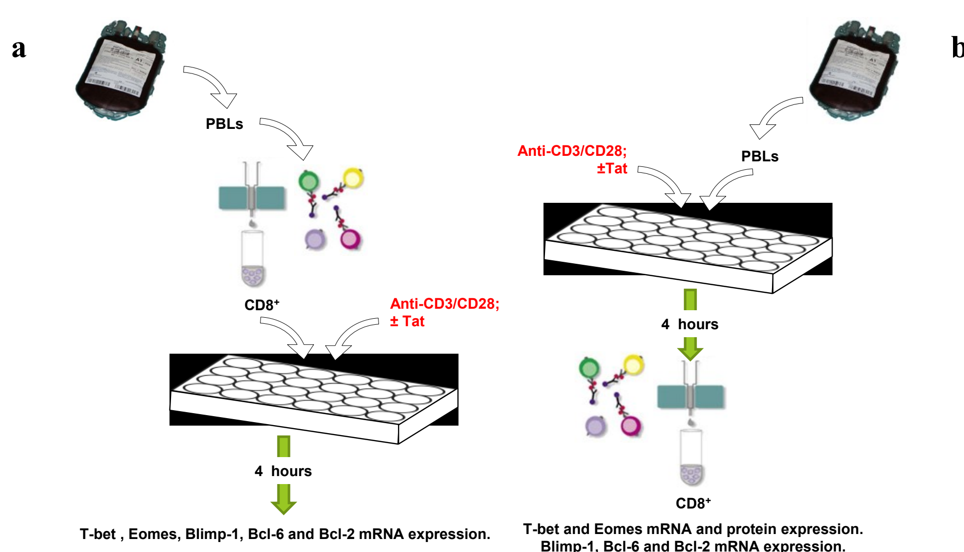


Figure 3.7. Tat does not affect CD4$^+$ and CD8$^+$ T cells proliferation. PBLs from healthy donors were activated with anti-CD3/CD28 in the absence or presence of 0.1 µg/ml of Tat and cultured up to six days. Proliferation of CD4$^+$ and CD8$^+$T cells was assessed by CFSE staining. One representative experiment out of three is shown.

3.4 Tat affects the expression of key transcription factors in activated CD8$^+$ T cells

T-bet and Eomes are transcription factors (TFs) that are up-regulated during HIV infection [79] and that control IFNγ production [45, 145]. Since we have shown here that Tat enhances IFNγ production in human CD8$^+$ T cells stimulated by TCR engagement, we next characterized the expression of T-bet and Eomes in CD8$^+$ T cells activated alone (Scheme 4a) or purified from activated PBLs (Scheme 4b) cultured in the absence or presence of Tat. Moreover, the expression of other TFs important for T cell functionality, survival and programming, as Blimp-1, Bcl-6 and Bcl-2, was analyzed.



Scheme 4

As shown in Figure 3.8a the presence of Tat during the stimulation did not change at a significant level the transcriptional profile of $CD8^+$ T cells activated alone, as the expression of all the TFs investigated was not affected by Tat-treatment. Conversely, mRNA levels of all the five TFs taken into consideration were significantly increased in $CD8^+$ T cells purified from PBLs activated in the presence of Tat (Figure 3.8b). Notably, Tat up-regulated not only genes required for effector functions (as T-bet, Eomes, Blimp-1), but also TFs important for memory development (Bcl-6 and Eomes) and T cell survival (Bcl-2). Moreover, these results demonstrate that Tat does not directly affect $CD8^+$ T cells, but requires other lymphocyte subpopulations to modulate the TFs expression in $CD8^+$ T cells. Finally, to assess whether the increased mRNA expression correlated with an increased protein expression, T-bet and Eomes proteins were evaluated by western blotting in $CD8^+$ T cells at 24 and 48 hours after activation. Notably, $CD8^+$ T cells purified from PBLs activated in the presence of Tat exhibited an increase of T-bet and Eomes expression 48 hours after the activation (Figure 3.9).

Taken together, these data demonstrate that Tat favors the activation of $CD8^+$ T cells affecting the expression of TFs crucial for T cell programming and functionality. Of note, this Tat-mediated effect required the help of other lymphocyte subpopulations.

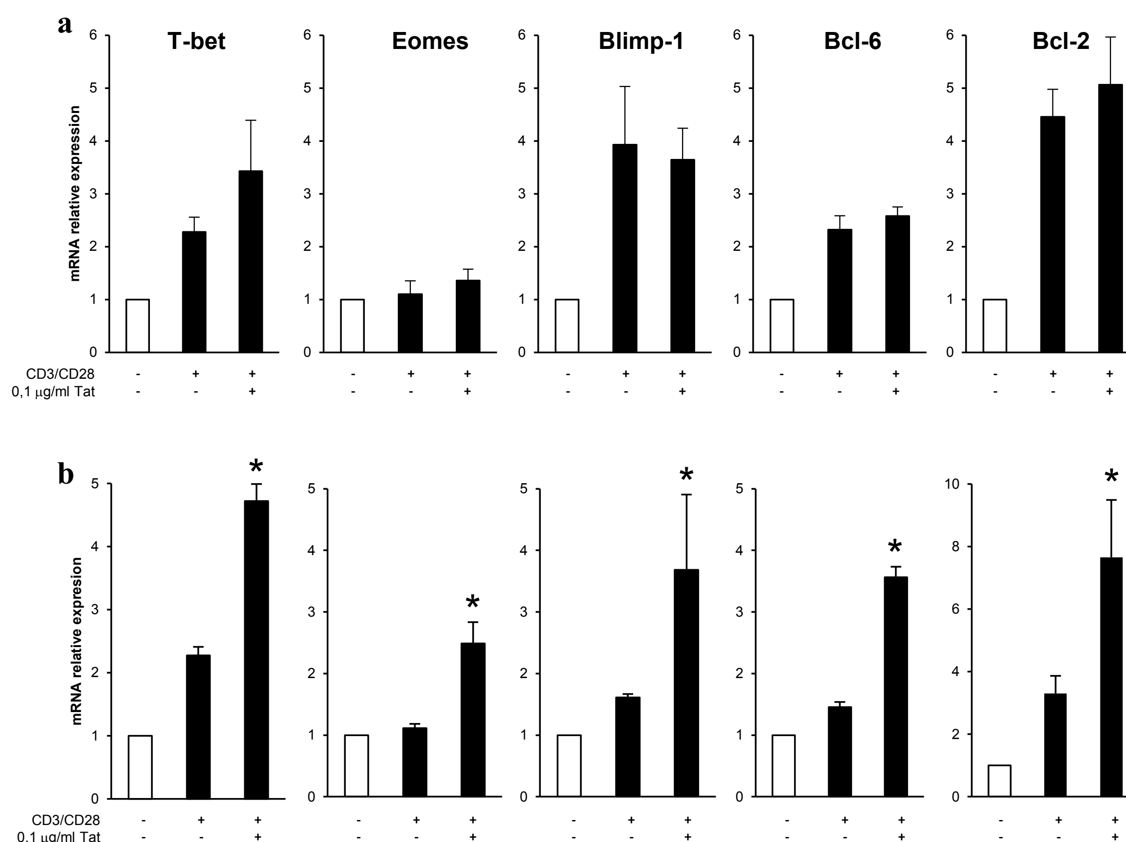


Figure 3.8. The effect of Tat on the transcriptional profile of CD8$^+$ T cells. (a) CD8$^+$ T cells were purified from PBLs of healthy donors (n=8), activated with anti-CD3/CD28 and cultured in the absence or presence of 0.1µg/ml of Tat. After 4 hours, mRNA levels of the indicated molecules were quantified by qPCR and normalized to untreated cells. (b) PBLs from healthy donors (n=8) activated with anti-CD3/CD28 were cultured in the absence or presence of Tat (0.1 µg/ml). After 4 hours, CD8$^+$ T cells were purified and mRNA levels of the indicated molecules were quantified by qPCR and normalized to untreated cells. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to Tat-untreated control cells.

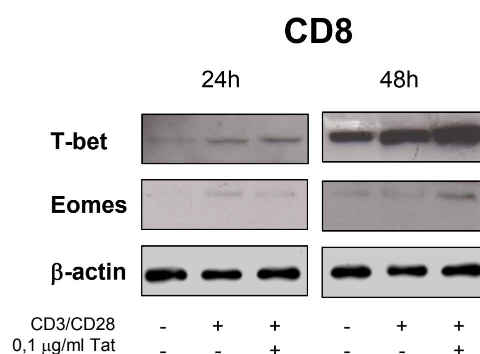
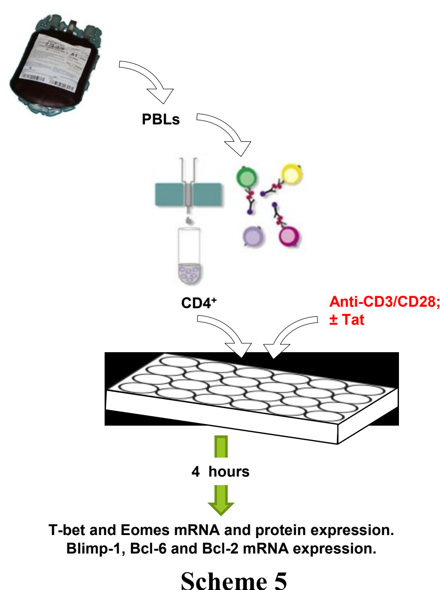


Figure 3.9. Tat enhances T-bet and Eomes protein expression in CD8$^+$ T cells. PBLs from healthy donors unstimulated or activated with anti-CD3/CD28 were cultured in the absence or presence of Tat (0.1 µg/ml). (a) CD8$^+$ T cells were purified after 24 and 48 hours, and expression of T-bet and Eomes proteins was assessed by western blotting. The results of one representative experiment out of three are shown.

3.5 Tat affects the expression of key transcription factors in activated $CD4^+$ T cells

As we have shown that the Tat-mediated up-regulation of T-bet, Eomes, Blimp-1, Bcl-6, Bcl-2 in $CD8^+$ T cells requires the presence of other T lymphocyte subpopulations, we sought to determine whether Tat could also modulate the transcriptional profile of $CD4^+$ T lymphocytes which are known to contribute to the activation of $CD8^+$ T cells. To this aim, purified $CD4^+$ T cells were activated, in the absence or presence of Tat, with anti-CD3/CD28, and the expression of the above mentioned TFs was evaluated 4 hours after activation (Scheme 5).



Scheme 5

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. Moreover, Tat-treatment significantly increased the expression of Blimp-1, Bcl-6 and Bcl-2 but not of Eomes (Figure 3.10). Finally, to assess whether the increased mRNA expression correlated with an increased protein expression, T-bet protein was evaluated by western blotting in $CD4^+$ T cells at 24 and 48 hours after activation. Notably, $CD4^+$ T cells activated in the presence of Tat exhibited an increase of T-bet expression after 48 hours after the stimulation (Figure 3.11).

Thus, these data provide evidence that Tat favors the activation of $CD4^+$ T cells up-regulating the expression of key TFs.

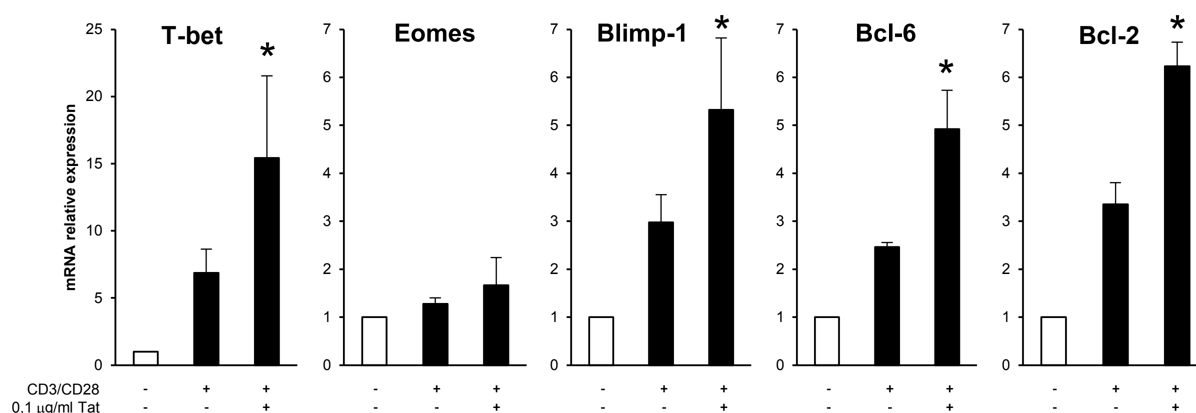


Figure 3.10. The effect of Tat on the transcriptional profile of CD4$^+$ T cells. CD4$^+$ T cells were purified from PBLs of healthy donors (n=8), activated with anti-CD3/CD28 and cultured in the absence or presence of 0.1μg/ml of Tat. After 4 hours, mRNA levels of the indicated molecules were quantified by qPCR and normalized to untreated cells. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to Tat-untreated control cells.

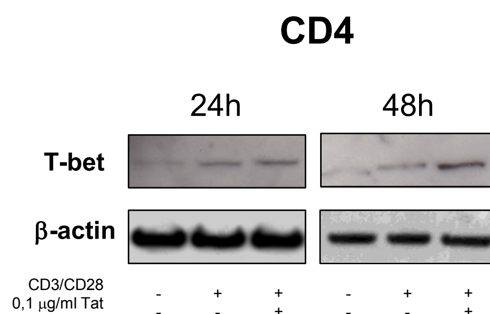


Figure 3.11. Tat enhances T-bet and Eomes protein expression in CD4$^+$ T cells. CD4$^+$ T cells were purified from PBLs of healthy donors and were activated with anti-CD3/CD28 were cultured in the absence or presence of Tat (0.1 μg/ml). After 24 and 48 hours, the expression of T-bet protein was assessed by western blotting. The results of one representative experiment out of three are shown.

3.6 $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins blocking affects Tat-mediated effect on the transcriptional profile in activated PBLs

Extracellular Tat is known to activate $CD4^+$ T cells by binding with its RGD region the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins [115]. To understand whether the enhancement of TFs expression induced by Tat was integrin-mediated, PBLs were pre-incubated with Abs directed against $\alpha_v\beta_3$ and $\alpha_5\beta_1$ and subsequently activated with anti-CD3/CD28 in the absence or presence of Tat. The effect of Tat was significantly inhibited by integrins blocking (Figure 3.12), indicating that the binding of Tat to $\alpha_v\beta_3$ and $\alpha_5\beta_1$ is required for the enhancement of TFs expression.

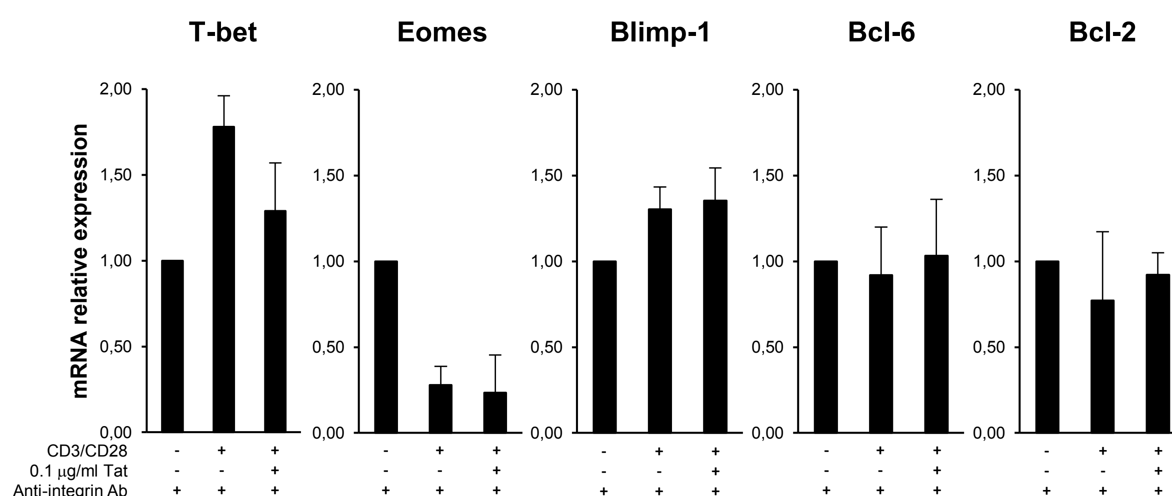


Figure 3.12. The effect of Tat is inhibited by integrins blocking. PBLs from healthy donors (n=3) unstimulated or activated with anti-CD3/CD28 were cultured in the absence or presence of Tat (0.1 µg/ml). PBLs were pre-incubated with anti-integrins monoclonal antibodies for 1 hour at room temperature before activation with anti-CD3/CD28 and Tat treatment. After 4 hours, CD8+ T cells were purified and mRNA levels of the indicated molecules were quantified by qPCR and normalized to untreated cells. Data are presented as mean ± SEM.

Thus we next investigated whether the Tat effects on $CD8^+$ T cells were mediated by cell-to-cell contact or by soluble molecules released by PBLs activated in the presence of Tat. To this aim, purified $CD8^+$ T cells were stimulated with anti-CD3/CD28 and co-cultured with supernatant derived from PBLs activated in the absence or presence of Tat. As it is also possible that Tat might directly act on $CD8^+$ T cells in synergy with other soluble molecules, we included a condition where Tat was added together with supernatant derived from PBLs activated in the absence of Tat. As shown in Figure 3.13, the supernatant

derived from PBLs activated in the presence of Tat was unable to increase IL-2, IFNγ and T-bet expression.

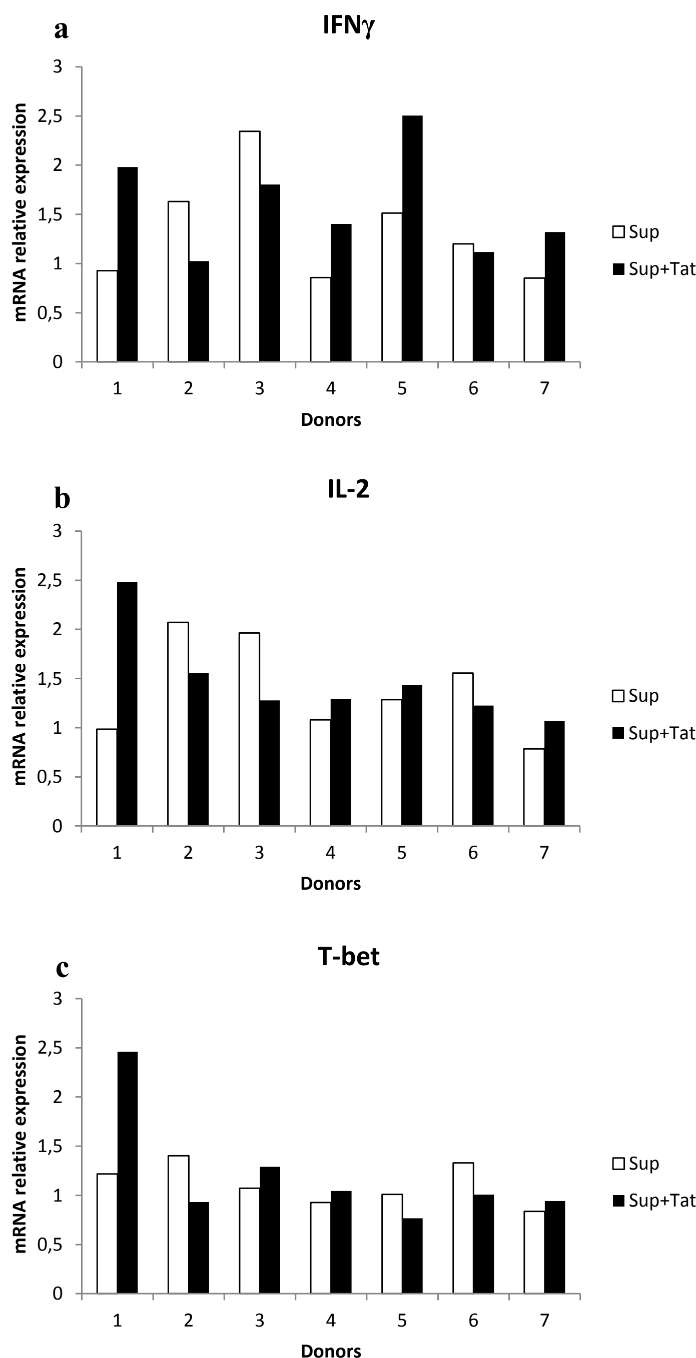


Figure 3.13. Supernatants from PBLs treated with Tat did not enhance IFNγ, IL-2 and T-bet expression in $CD8^+$ T cells. $CD8^+$ T cells were purified from PBLs of healthy donors (n=7), activated with anti-CD3/CD28 and cultured with the supernatant from PBLs activated with anti-CD3/CD28 in the absence or presence of 0.1µg/ml of Tat for 4 hours., mRNA levels of IFNγ (a), IL-2 (b) and T-bet (c) were quantified by qPCR and normalized to untreated cells.

However we can not exclude that these negative results may be due to the experimental conditions. Thus, further experiments are required to clarify the role of other immune cell subpopulations on the activation of $CD8^+$ T cells.

3.7 Tat down regulates CD127 expression and modulates the fate of T cells

It has been recently shown that the expression of T-bet and Eomes in memory $CD8^+$ T cells from HIV-infected individuals is associated with decreased expression of the IL-7 receptor CD127, and increased IFNγ and granzyme B levels [29]. As Tat up-regulates T-bet and Eomes (Figures 3.8 and 3.9) as well as IFNγ (Figure 3.5) and granzyme B production (Figure 3.15), we then assessed whether Tat could affect CD127 expression in activated $CD8^+$ T cells. Moreover, we also measured the expression of CD25, the alpha chain of the receptor for IL-2, whose production is modulated by Tat. As shown in Figure 3.14a, activation of PBLs with anti-CD3/CD28 increased the expression of CD25 and decreased the expression of CD127 on $CD8^+$ T cells. The presence of Tat did not affect the percentage of $CD8^+$ T cells expressing CD25, while it decreased the fraction of $CD8^+$ T lymphocytes expressing CD127. Interestingly, this effect was mediated by Tat in both unstimulated and activated $CD8^+$ T cells.
As T-bet, Eomes, Bcl-6, Blimp-1 and Bcl-2 regulate at different extent the T cell programming and memory development, we next sought to determine the fate of $CD8^+$ T cells exposed for longer time to Tat. To this aim, we evaluated the expression of CD45RO and CD27, two markers of memory cells, in unstimulated or activated PBLs cultured for up to 8 days in the absence or presence of Tat. In long-term cell cultures the presence of Tat did not modulate the expression of CD45RO, while it increased the expression of CD27 in activated but not in unstimulated $CD8^+$ T cells (Figure 3.14b). Interestingly, CD27 expression was not affected by Tat after 24 or 48 hours of culture (not shown).

These results suggest that the Tat-mediated modulation of T-bet, Eomes and the other TFs may be associated with the CD127 down-regulation and the accumulation of $CD27^+CD8^+$ T cells.

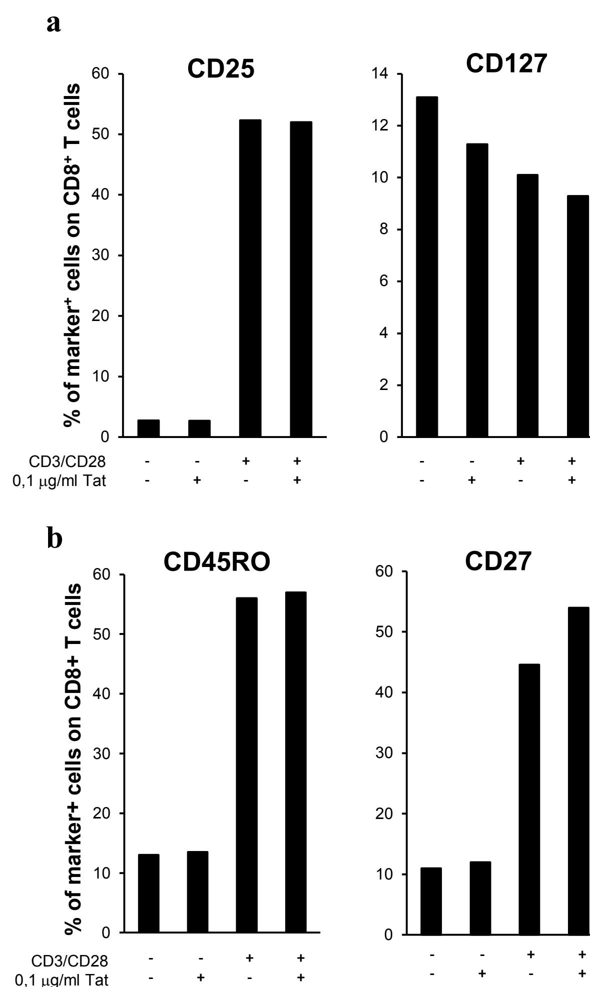


Figure 3.14. Tat modulates the phenotype of CD8$^+$ T cells. PBLs from healthy donors (n=6) unstimulated or activated with anti-CD3/CD28 were cultured in the absence or presence of Tat (0.1 µg/ml). (a) After 24 hours, the percentage of CD8$^+$ T cells expressing CD25 and CD127 was measured. (b) After 8 days, the percentage of CD8$^+$ T cells expressing CD45RO and CD27 was measured. The results of one representative experiment out of six are shown.

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

We next assessed whether the presence of Tat in long-term cell cultures could also affect activation and functionality of antigen-specific memory and naïve CD8$^+$ T cells. To this aim, PBLs obtained from healthy HLA class I-typed EBV-seropositive donors were stimulated ex-vivo with cells pulsed with EBV-derived CTL peptide epitopes in the absence or presence of Tat. Specifically, PBLs were stimulated with the subdominant HLA-A2-restricted CLGGLLTMV (CLG) or YLQQNWWTL (YLQ) epitope [146, 147] or

with the immunodominant HLA-A11-restricted IVTDFSVIK (IVT) epitope [148]. The cytotoxic activity of each CTL culture generated in the absence or presence of Tat was tested against autologous PHA-blasts, pulsed or not with the relevant synthetic peptide, in a standard ^{51}Cr-release assay. As shown in Figure 3.15a-c, all the three CTL cultures generated in the presence of Tat exhibited higher percentages of specific lysis compared to those generated in the absence of Tat.

To determine whether the Tat protein favors also the activation of naïve T cells, PBLs from HLA-A2 healthy donors were stimulated with the synthetic ELT peptide in the absence or presence of the Tat protein. The ELT (ELTLGEFLKL) peptide is a CTL epitope, presented by HLA-A2 [149, 150], belonging to the anti-apoptotic protein survivin that is overexpressed in tumor cells [151]. No T-cell reactivity against this epitope is normally detected in healthy patients [151]. The specificity of CTL cultures was tested against PHA-blasts, pulsed or not with the ELT-peptide, by ^{51}Cr-release assays (Figure 3.15d). HLA-A2 positive PHA-blasts pulsed with the ELT-peptide were efficiently lysed only by CTL cultures generated in the presence of Tat, demonstrating that Tat favors the priming of naïve $CD8^+$ T cells.

These observations suggest that Tat favors the activation of $CD8^+$ T cells, but do not clarify whether the increased cytotoxic activity observed in CTL cultures generated in the presence of Tat depends on a higher number or a higher functionality of epitope-specific $CD8^+$ T cells. To address this issue, CTL cultures specific for the HLA-A2-restricted CLG epitope were generated in the absence of Tat and then were left untreated or pre-incubated with the Tat protein 24/48 hours before the cytotoxic activity. As shown in Figure 3.15e, CTL cultures lysed target cells at similar levels, suggesting that Tat does not enhance effector functions, but rather must be present at the time of the priming thus favouring CTL expansion. To confirm this hypothesis, CLG- and YLQ-specific CTLs generated in the absence or presence of Tat were assayed in IFNγ and granzyme B Elispot assays to evaluate differences in the number of antigen specific T cells. As shown in Figure 3.15f, CTL cultures generated in the presence of Tat exhibited higher numbers of both IFNγ and granzyme B CLG- and YLQ-specific CTLs, suggesting that Tat favors the expansion of epitope-specific and actively secreting $CD8^+$ T cells.

Taken together, these data demonstrate that Tat favors the priming of naïve $CD8^+$ T cells and the expansion of memory $CD8^+$ T cells.

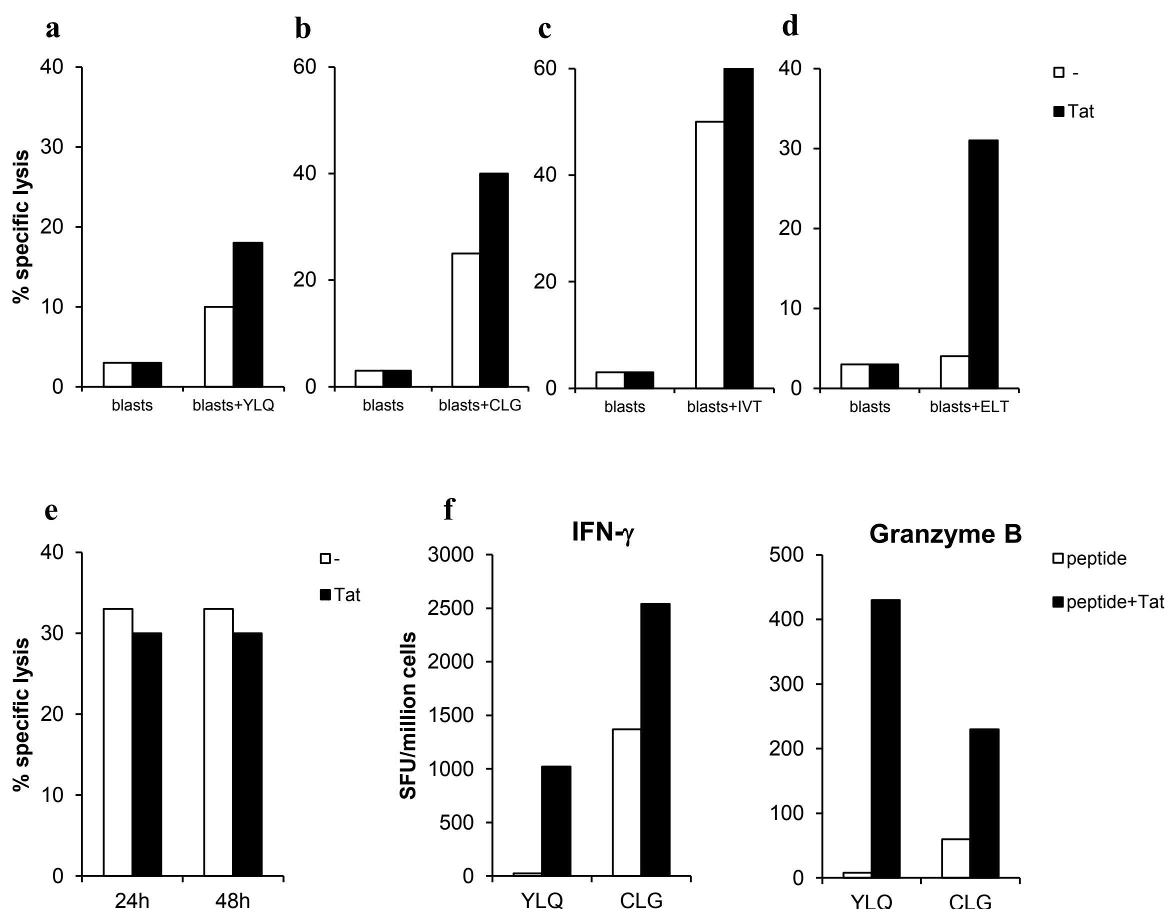


Figure 3.15. Tat favors the activation of antigen-specific memory and naïve CTLs. (a-c) CTL cultures specific for YLQ (a), CLG (b), or IVT (c) EBV-derived epitopes were generated, in the absence or presence of Tat (0.1 µg/ml), from lymphocytes purified from EBV-positive donors and tested for their cytotoxic activity by 51Cr-release assay against autologous unpulsed or peptide-pulsed PHA-blasts (E:T ratio 10:1). (d) CTL cultures specific for the ELT survivin-derived epitope were generated, in the absence or presence of Tat (0.1 µg/ml), from lymphocytes purified from healthy donors and tested for their cytotoxic activity by 51Cr-release assay against autologous unpulsed or peptide-pulsed PHA-blasts (E:T ratio 10:1). (e) CTL cultures specific for the CLG peptide epitope were generated from lymphocytes purified from EBV-positive donors. 24 or 48 hours before the 51Cr-release assay (E:T ratio 10:1), CTL cultures were treated with the Tat protein (0.1 µg/ml). (f) CTL cultures specific for YLQ and CLG EBV-derived epitopes were generated, in the absence or presence of Tat (0.1 µg/ml), from lymphocytes purified from EBV-positive donors and tested for their IFNγ and granzyme B release by Elispot assay. The results of one representative experiment out of five are shown.

3.9 Tat does not affect the expression of IL-2 and IFNγ in unstimulated $CD8^+$ and $CD4^+$ T cells

We demonstrate that Tat changes some specific phenotype markers and enhances the activation and functionality of $CD8^+$ and $CD4^+$ T cells stimulated by TCR engagement (different CD8 peptide epitopes and anti-CD3/CD28 stimulation). Thus, we sought to determine whether Tat could also modulate unstimulated T cells.

To this aim, IFNγ and IL-2 mRNA levels were measured in unstimulated $CD8^+$ and $CD4^+$ T cells cultured in the absence or presence of Tat without any activation. After 4 hours of treatment, the expression of IFNγ and IL-2 mRNA levels was not significantly affected by the presence of Tat (Figure 3.16 a-c).

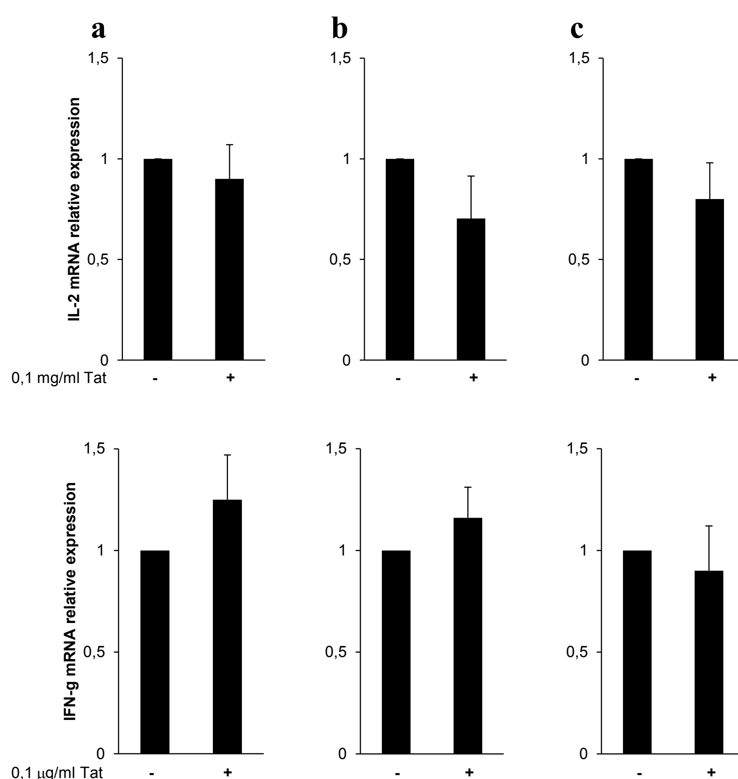


Figure 3.16. Tat does not enhance IFNγ and IL-2 production in unstimulated $CD8^+$ and $CD4^+$ T cells. (a) $CD8^+$ T cells were purified from PBLs of healthy donors (n=6), and cultured in the absence or presence of 0.1µg/ml of Tat. After 4 hours, IL-2 and IFNγ mRNA levels were quantified by qPCR and normalized to untreated cells. (b) PBLs from healthy donors (n=6) were cultured in the absence or presence of Tat (0.1 µg/ml). After 4 hours, $CD8^+$ T cells were purified and IL-2 and IFNγ mRNA levels were quantified by qPCR and normalized to untreated cells. (c) $CD4^+$ T cells were purified from PBLs from healthy donors (n=6) and cultured in the absence or presence of 0.1µg/ml of Tat. After 4 hours, IL-2 and IFNγ mRNA levels were quantified by qPCR and normalized to untreated cells. Data are presented as mean ± SEM.

3.10 Tat does not modulate the transcriptional profile of unstimulated CD8+ and CD4+ T cells

We next investigated whether Tat could also modulate the basal transcriptional prolife of unstimulated CD8+ and CD4+ T cells. To this aim, T-bet, Eomes, Blimp-1, Bcl-6 and Bcl-2 mRNA levels were measured in purified CD8+ (treated alone or sorted from treated PBLs) and CD4+ T cells cultured in the absence or presence of Tat for 4 hours. After 4 hours of treatment, the expression of the analyzed TFs was not significantly affected by the presence of Tat (Figure 3.17), although the obtained results show a tendency of a Tat-mediated enhancement of the two memory-related TFs Eomes and Bcl-6 in CD8+ T cells purified from Tat-treated PBLs (Figure 3.17b).

Thus, these results suggest that Tat alone does not modulate the transcriptional profile of CD8+ and CD4+ T cells after four hours of treatment.

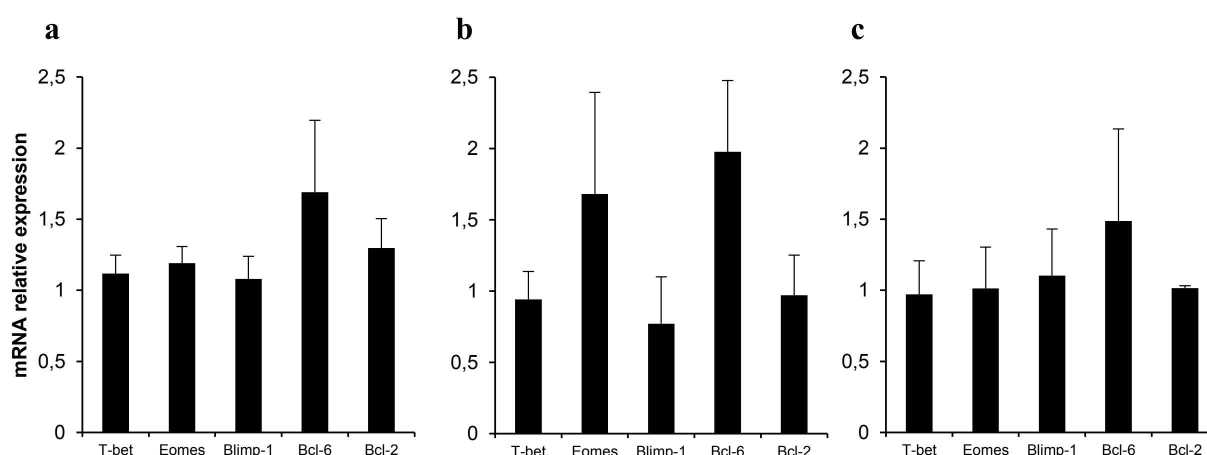


Figure 3.17. Tat does not modulate the transcriptional profile of unstimulated CD8+ and CD4+ T cells. (a) CD8+ T cells were purified from PBLs of healthy donors (n=8) and cultured in the absence or presence of 0.1µg/ml of Tat. After 4 hours, mRNA levels of the indicated molecules were quantified by qPCR and normalized to untreated cells. (b) PBLs from healthy donors (n=8) were cultured in the absence or presence of Tat (0.1 µg/ml). After 4 hours, CD8+ T cells were purified and mRNA levels of the indicated molecules were quantified by qPCR and normalized to untreated cells. (c) CD4+ T cells were purified from PBLs of healthy donors (n=8) and cultured in the absence or presence of 0.1µg/ml of Tat. After 4 hours, mRNA levels of the indicated molecules were quantified by qPCR and normalized to untreated cells. Data are presented as mean ± SEM.

3.11 Tat modulates T-bet and Eomes mRNA expression in unstimulated CD8$^+$ and CD4$^+$ T cells after 24 hours of treatment (Preliminary data)

It has been show that after 4 hours of treatment, Tat could not modulate, in unstimulated T cells, any of the TFs taken into consideration. However, the expression of these TFs in unstimulated T cells may require longer exposition to Tat. To this aim, T-bet and Eomes mRNA levels were measured in purified CD8$^+$ (treated alone or sorted from treated PBLs) and CD4$^+$ T cells cultured in the absence or presence of Tat for 24 hours. Interestingly, after 24 hours of stimulation, CD8$^+$ T cells purified from PBLs cultured with Tat showed a significant up-regulation of T-bet and Eomes compared to CD8$^+$ T cells cultured without Tat (Figure 3.18b), however, CD8$^+$ T cells cultured alone were not affected by treatment with Tat (Figure 3.18a). Furthermore, T-bet was also significantly up-regulated by Tat in CD4$^+$ T cells (Figure 3.18c). Finally, our results show a tendency for the down-regulation of Eomes expression in CD4$^+$ T cells (fold change = 0.5, p=0.06) that however did not reach statistical significance.

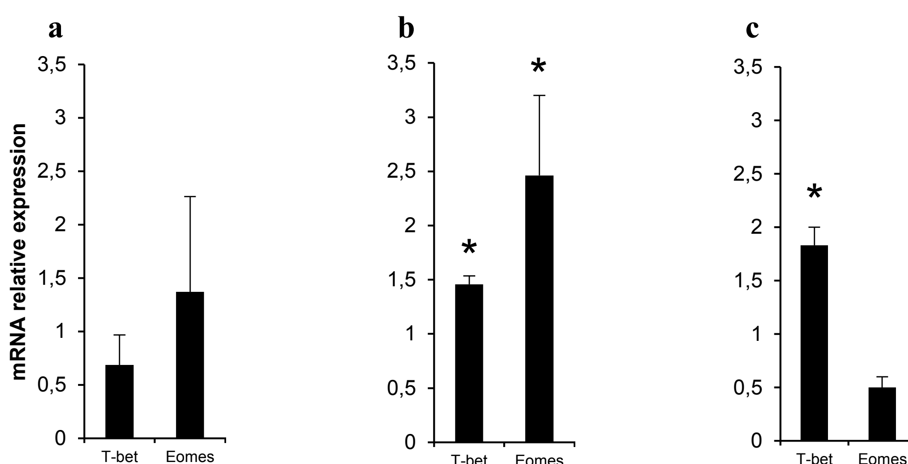
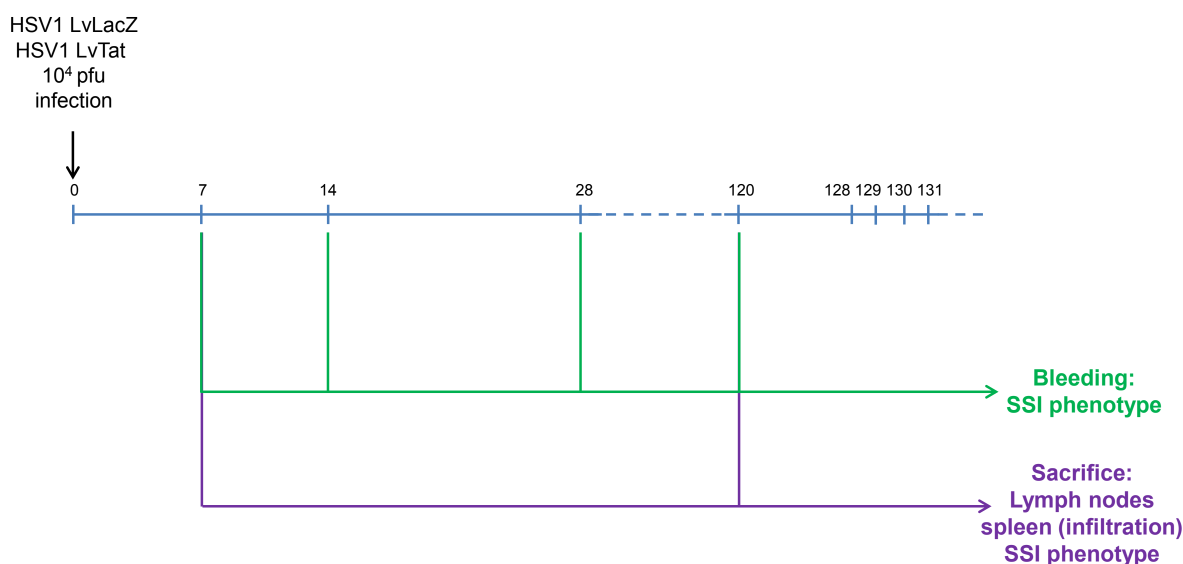


Figure 3.18. Tat modulates T-bet and Eomes mRNA expression in unstimulated CD8$^+$ and CD4$^+$ T cells after 24 hours of treatment. (a) CD8$^+$ T cells were purified from PBLs of healthy donors (n=7) and cultured in the absence or presence of 0.1µg/ml of Tat. After 24 hours, mRNA levels of the indicated molecules were quantified by qPCR and normalized to untreated cells. (b) PBLs from healthy donors (n=8) were cultured in the absence or presence of Tat (0.1 µg/ml). After 24 hours, CD8$^+$ T cells were purified and mRNA levels of the indicated molecules were quantified by qPCR and normalized to untreated cells. (c) CD4$^+$ T cells were purified from PBLs of healthy donors (n=8) and cultured in the absence or presence of 0.1µg/ml of Tat. After 24 hours, mRNA levels of the indicated molecules were quantified by qPCR and normalized to untreated cells. Data are presented as mean ± SEM. Data are presented as mean ± SEM. For statistical analysis two-tailed Wilcoxon signed rank test was used. *P<0.05: Tat-treated cells compared to Tat-untreated control cells.

3.12 Immunization with an attenuated HSV1 vector expressing Tat increases the number of HSV1-specific $CD8^+$ T cells and favors the development of effector memory T cells

The above reported results indicate that Tat favors the activation of $CD8^+$ T cells. Moreover, it has been previously demonstrated that Tat broadens T cell responses to co-antigens [142]. Thus, these observations suggest that Tat may be used as an adjuvant in vaccination strategies to favor the induction of $CD8^+$ T cell responses. This hypothesis was investigated using, as a model, a vaccine candidate against HSV1 infection. Specifically, we explored the use of a live-attenuated HSV1 vector expressing Tat (LvTat, see "Material and Methods" section for details) compared to a control HSV1 vector expressing LacZ (LvLacZ). To this aim, C57BL/6 mice were immunized intravaginally (i.vag.) with LvTat or LvLacZ vectors and the percentage of $CD8^+$ T cells specific for an immunodominant HSV-derived K^b-restricted epitope $CD8^+$ (SSI) was measured by dextramer staining at days 7, 14, 28 and 120 post immunization (Scheme 7).



Scheme 7

As shown in Figure 3.19, the percentage of SSI-specific $CD8^+$ T cells in blood was significantly higher during the expansion (from day 7 to 14 p.i.), contraction (day 28 p.i)

and memory phases (day 120 p.i.) in mice immunized with LvTat compared to the control group inoculated with LvLacZ.

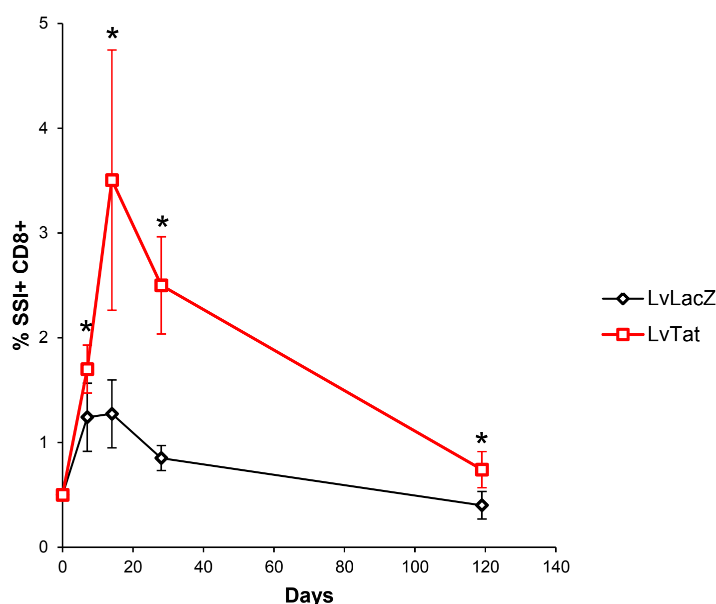


Figure 3.19. Tat increases the percentage of SSI-specific CD8$^+$ T cells. Blood samples were collected at days 7, 14, 28 and 120 post-immunization from LvLacZ and LVTat immunized C57/BL6 mice. The percentage of SSI-specific CD8$^+$ T cells detected by dextramer staining in whole blood are shown. Data are presented as mean ± SEM of 5 mice per group. For statistical analysis two-tailed Mann Whitney test was used. *P<0.05.

Furthermore, the number of SSI-specific CD8$^+$ T cells was also evaluated in spleens and lymph nodes at day 7 (expansion phase) and at day 120 (memory phase). As shown in Figures 3.20 and 3.21, significantly higher numbers of HSV-specific CD8$^+$ T cells were present in the spleens but not in lymph nodes of mice immunized with LvTat compared to mice immunized with LvLacZ.

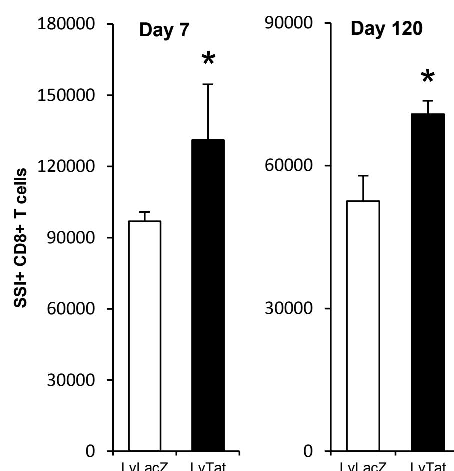


Figure 3.20. Tat increases the number of SSI-specific $CD8^+$ T cells in spleens. At days 7 and 120 post-infection splenocytes were harvested from LVLacZ and LVTat immunized C57/BL6 mice. Total number of SSI-specific $CD8^+$ T cells was detected by dextramer staining. Data are presented as mean ± SEM of 5 mice per group. For statistical analysis two-tailed Mann Whitney test was used. *P<0.05.

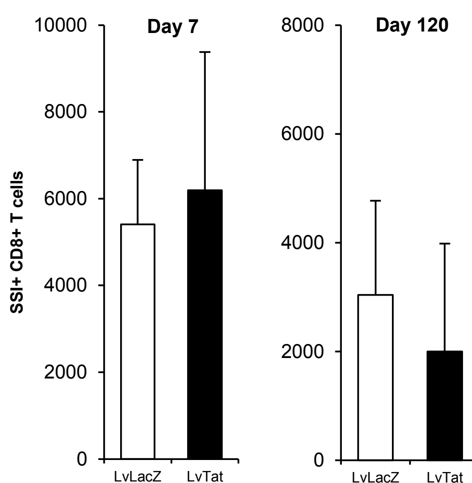


Figure 3.21. Tat does not increase the number of SSI-specific $CD8^+$ T cells in lymph nodes. At days 7 and 120 post-infection lymph nodes were harvested from HSV1-LacZ and HSV1-Tat HSV1-infected C57/BL6 mice. Percentage of SSI-specific $CD8^+$ T cells was detected by dextramer staining. Data are presented as mean ± SEM of 5 mice per group.

To assess whether Tat affects the phenotype of SSI-specific CD8+ T cells, we evaluated the expression of KLRG and CD127, since the expression of these markers define two distinct populations of effector $CD8^+$ T cells: $CD127^-KLRG^+$ short lived effector cells (SLECs) which confer immediate protection, but contribute little to the long-lived memory repertoire and $CD127^+KLRG1^-$ memory precursor effector cells (MPECs) which have the ability to respond to survival signals and develop into long-lived memory cells.

Thus, we evaluated, at day 7 post-immunization, the percentage of SSI-specific $CD8^+$ MPECs and SLECs in blood, spleens and lymph nodes of C57/BL6 mice immunized with LvLacZ or LVTat vectors. Similar percentage of SSI-specific $CD8^+$ MPECs and SLECs were detected at day 7 in blood, spleens and lymph nodes and no significant differences were detected among mice immunized with LvTat and LvLacZ (Figure 3.22 a-c).

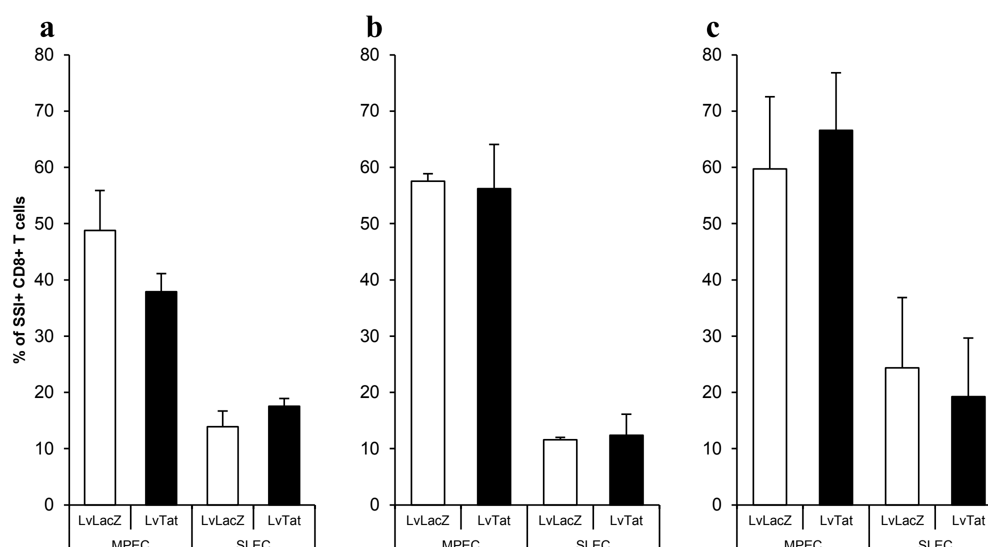


Figure 3.22. Tat effect on the phenotype of effector SSI-specific $CD8^+$ T cells. Blood samples (a), spleen (b) and lymph nodes (c) were collected at day 7 post immunization from LvLacZ and LVTat immunized C57/BL6 mice. Proportion of MPEC ($CD127^+KLRG1^-$) and SLEC ($CD127^-KLRG^+$) is shown as percentage of SSI-specific $CD8^+$ T cells. Data are presented as mean ± SEM of 5 mice per group.

Since antigen-experienced T cells can be phenotypically classified into effector memory T cells (T_{EM}) and central memory T cells (T_{CM}) by CD62L expression (T_{EM} are $CD62L^-$), we then evaluated the phenotype of SSI-specific memory $CD8^+$ T cells. To this aim, the expression of CD62L was investigated on SSI-specific $CD8^+$ T cells in blood, spleen, and lymph nodes after immunization with LvTat and LvLacZ. As shown in Figure 3.23, the analysis of the phenotype of SSI-specific $CD8^+$ T cells in blood at day 14, 28 and 120

revealed a significant lower expression of CD62L in LvTat immunized mice, indicating a larger population of HSV1-specific effector memory $CD8^+$ T cells.

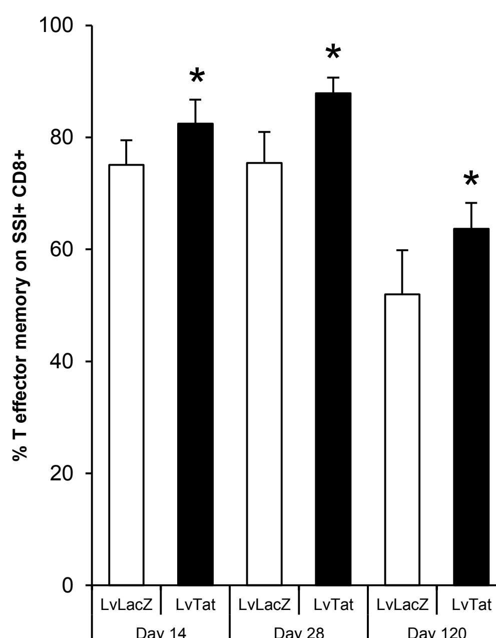


Figure 3.23. Tat effect on the phenotype of memory SSI-specific $CD8^+$ T cells. Blood samples were collected at days 14, 28 and 120 post-immunization from LvLacZ and LVTat immunized C57/BL6 mice. Proportion of effector memory T cells ($CD62L^-$) is shown as percentage of SSI-specific $CD8^+$ T cells. Data are presented as mean ± SEM of 5 mice per group. For statistical analysis two-tailed Mann Whitney test was used. *$P<0.05$.

Furthermore, the analysis was performed in spleens and lymph node at day 120 post immunization. As show in Figure 3.24, LvTat-immunized mice displayed, compared to control mice, a significant lower expression of CD62L in SSI-specific $CD8^+$ T cells in spleens, while in lymph nodes this difference is less marked but still present.

Thus, these data show that the immunization with LvTat is capable of inducing a larger number of HSV1-specific $CD8^+$ T cells and increases the number of effector memory T cells. Of note, some recent studies indicate that an effector memory phenotype is associated to a better protection induced by vaccination [152, 153].

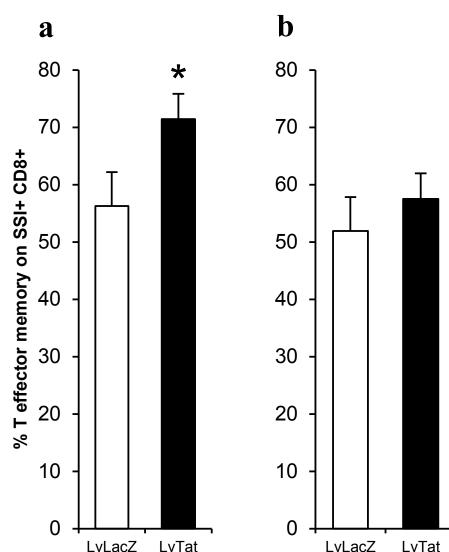
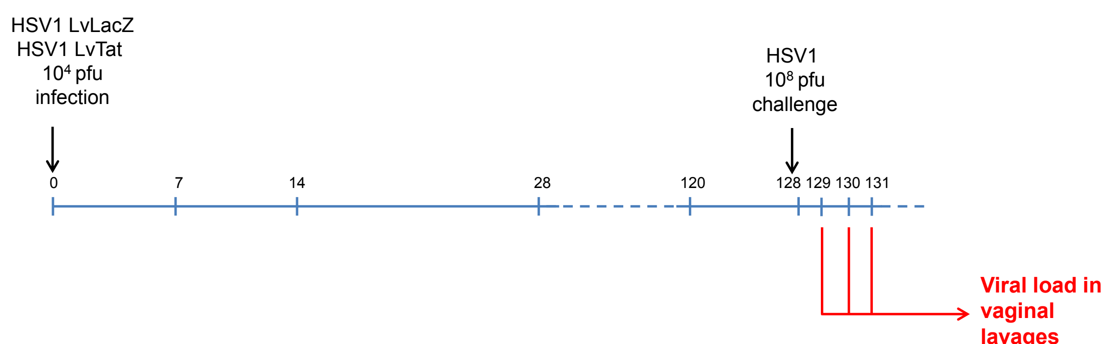


Figure 3.24. Tat effect on the phenotype of memory SSI-specific CD8$^+$ T cells. Spleen (a) and lymph nodes (b) were collected at day 120 post immunization from LvLacZ and LVTat immunized C57/BL6 mice. Proportion of effector memory T cells (CD62L$^-$) is shown as percentage of SSI-specific CD8$^+$ T cells. Data are presented as mean ± SEM of 5 mice per group. For statistical analysis two-tailed Mann Whitney test was used. *$P<0.05$.

3.13 Immunization with an attenuated HSV1 vector expressing Tat favors the control of HSV1 infection

We demonstrated that immunization with LvTat increases the number of SSI-specific CD8$^+$ T cells and favors an effector memory phenotype. We next tried to understand whether this Tat-mediated modulation of the pool of HSV-specific T cells may provide protection against genital HSV infection. To this aim, mice were challenged intravaginally with a lethal dose of wild type HSV at 128 days after immunization and viral titers were measured in vaginal lavages at days 1, 2, 3 post-challenge (Scheme 8).



Scheme 8

Notably, at day 1, 2 and 3 post challenge, mice immunized with LvTat manifested significantly less viral titers in the vagina compared to mice immunized with LvLacZ (Figure 3.25) suggesting a better control of HSV1 infection.

These results demonstrate that LvTat immunization could enhance protective immunity against HSV infection decreasing viral replication in vagina.

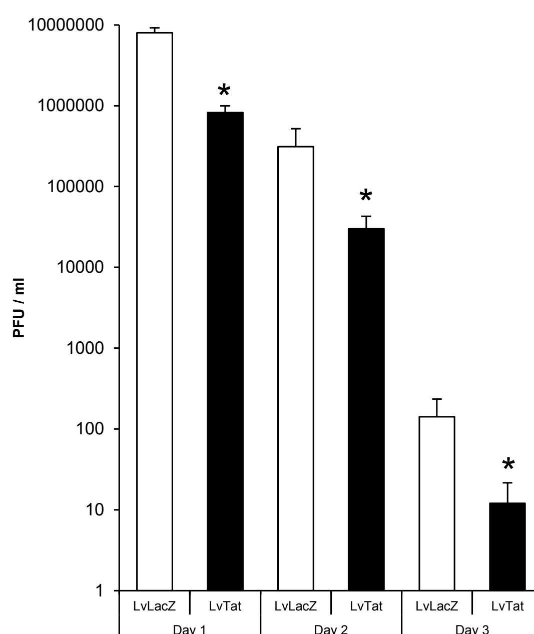


Figure 3.25. Tat decreases viral titers after challenge. Viral titers were measured in vaginal lavages at days 1, 2, 3 post-challenge. Data are presented as mean ± SEM of 5 mice per group. For statistical analysis two-tailed Mann Whitney test was used. *P<0.05.

4. DISCUSSION

We demonstrate here that the HIV-1 Tat protein, which is released by infected cells and found extracellularly in HIV-positive individuals [88, 99, 107], favors the activation and effector functions of $CD8^+$ and $CD4^+$ T cells (Figures 3.3, 3.4,3.5 and 3.15). Interestingly, behind this functional modulation we observed that Tat increases the expression of T-bet, Eomes, Blimp-1, Bcl-6 and Bcl-2 in $CD8^+$ T lymphocytes purified from activated PBLs (Figures 3.8 and 3.9) and in $CD4^+$ T lymphocytes activated alone (Figures 3.10 and 3.11) but not in unstimulated $CD8^+$ and $CD4^+$ T cells (Figures 3.17). The Tat-mediated increase of the expression of T-bet in $CD8^+$ and $CD4^+$ T cells and of Eomes only in $CD8^+$ T cells, suggests that Tat may impact the T cell programming of $CD8^+$ and $CD4^+$ T lymphocytes. This effect requires the interaction of Tat with integrins (Figure 3.12) and leads to the down-regulation of CD127 and the up-regulation of CD27 (Figure 3.14). Interestingly, the effect of Tat on activated $CD8^+$ T cells was abolished only when CD8+ T cells were activated in the presence of other cells type, suggest the requirement of cell-to-cell contact or the release of soluble factors. Other studies are required to clarify this observation.
It is known that Tat favors IL-2 secretion in $CD4^+$ T cells [102]. Here we show for the first time that also $CD8^+$ T cells activated in the presence of Tat exhibited increased production of IL-2 (Figures 3.3 and 3.4). Several mechanisms may account for this effect, as it has been reported that Tat favors the activation of TFs required for IL-2 transcription, like NF-κB [92, 103], NFAT [154] and AP-1 [155]. Moreover, Tat superinduces factors binding to the CD28-responsive element (CD28RE) which mediates IL-2 gene activation by CD28 co-stimulation [92, 103].
The results also demonstrate that naïve and memory $CD8^+$ T cells activated *in vitro* in the presence of Tat exhibit an increased IFNγ production and cytotoxic activity (Figures 3.5 and 3.15). The effect was abolished when Tat was added after the stimulation, suggesting that Tat favors the expansion and the functionality of effector cells only if present at the beginning of the stimulation. It is likely that Tat potentiates the production of cytokines and cytolytic molecules through the induction of T-bet, Eomes and Blimp-1 (Figures 3.8 and 3.9), which control at different levels the transcription of IFNγ, perforins and granzymes [45, 145, 156]. The interaction of the RGD domain of Tat with $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins seems to be necessary, as we observed that the Tat-mediated up-regulation of the TFs was abolished by integrins blocking. It is known that Tat mediates the activation of the ERK/MAPK and PI3K/Akt pathways through its RGD domain [157, 158], and both ERK

and Akt are involved in T-bet induction [159, 160]. Moreover, the ERK pathway also favors Eomes and Blimp-1 up-regulation [161, 162].

Of note, an increased production of effector molecules like IFNγ and granzymes, as well as an enhancement of T-bet and Eomes in $CD8^+$ T cells, are observed in HIV-infected individuals [79, 163-166]. Thus, our *in vitro* observations suggest that Tat may be responsible of, or contribute to all these *in vivo* effects.

However, PBLs, $CD8^+$ and $CD4^+$ T cells activated by anti-CD3/CD28 in the presence of Tat did not exhibited an increased proliferation (Figures 3.6 and 3.7). 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 [167]. Thus, the control of T-bet on T cell functions is strictly dependent on the quality of the stimulus. Moreover these results suggest that hyperproliferation of T cells observed during immune activation in HIV-infected individuals may not be due directly to Tat effects. The lack of proliferation even in the presence of IL-2 up-regulation suggest an intriguingly mechanism that needs further elucidation. As discussed below, it is possible that the Tat-mediated IL-2 increase is limited to the initial hours after stimulation and then repressed by Tat-mediated Blimp-1 and T-bet up-regulation [167] in both activated $CD8^+$ and $CD4^+$ T cells, and this would explain the lack of increased proliferation.

The role of Tat on T cell survival is highly debated [118, 168-170]. We report that Tat enhances the expression of the anti-apoptotic marker Bcl-2 in activated $CD8^+$ and $CD4^+$ T cells. However, the up-regulation of Bcl-2 does not appear to be due to a direct effect of Tat on Bcl-2 expression, as instead demonstrated in $CD4^+$ T cells [118], since it was observed after activation with anti-CD3/CD28 and Tat further increased it. Thus, our results indicate that Tat may differently affect Bcl-2 expression in $CD4^+$ and $CD8^+$ T cells.

We found that $CD8^+$ and $CD4^+$ T cells activated in the presence of Tat exhibited increased levels of Blimp-1, which favors the development of effector memory T cells [156, 171], a phenomenon that we have also observed in vivo by immunization of mice with a recombinant HSV1 vector expressing Tat. Intriguingly, we also observed the up-regulation of Bcl-6, which is repressed by Blimp-1 [171], suggesting a Tat-mediated mechanism that deserves further investigations. Moreover, the presence of Tat favors the expression of CD27, a hallmark of incomplete differentiation to effector cells [11, 172, 173], and causes CD127 down-regulation not only in unstimulated $CD8^+$ T cells, as previously demonstrated [121, 174], but also in activated $CD8^+$ T lymphocytes. Interestingly, CD127

down-regulation is observed in HIV-infected individuals in association with immune activation [119, 175], higher levels of T-bet and Eomes and increased granzyme B and IFNγ release [79].

In conclusion, our results indicate that Tat modulates programming and secretory capacity of both $CD8^+$ and $CD4^+$ T cells, suggesting that it may be involved in the development of T lymphocytes with an effector profile as observed during HIV infection [3, 79]. We propose a model by which HIV, through the release of Tat, may affect T-bet and Eomes expression thus contributing to immune activation and to a profound and long-lasting modulation of T cell responses.

Thus, our observations provide new hints on the role that Tat may play in $CD8^+$ and $CD4^+$ T cell dysfunctionalities during HIV infection, suggesting that the induction anti-Tat immune responses may be a valuable tool to protect HIV-infected individuals from immune dysfunctions.

The modulation of the pathways that lead to optimal generation and maintenance of T cells is of considerable importance for the development of both prophylactic and therapeutic vaccines able to induce broad, strong, long lasting and protective immune responses. Thus, the characterization of molecules able to increase the immunogenicity of a vaccine may be a valuable tool for the identification of new strategies for vaccine development. We believe that the capacity of Tat to activate T cells, may be beneficial for the development of new vaccines in which Tat may play the role of adjuvant. Indeed, our in vitro results demonstrate that Tat affects the transcriptional profile of T lymphocytes and increases T cell functions. Moreover, it is known that Tat modulates the functionality of $CD4^+$ T lymphocytes [92, 102, 103, 106, 118, 123] and professional APCs [176] and affects *in vivo* the magnitude and kinetics of antiviral responses [177]. Tat also induces changes in the subunit composition of the immune proteasome, which correlate to altered enzymatic activities and modulation of CTL epitope generation in virally-infected cells and broadens in vivo T cell responses against subdominat epitopes of a co-antigen (otherwise not expressed, or only poorly expressed) [123, 142, 178].

In this study we have characterized $CD8^+$ T cell responses in an in vivo model of immunization with a HSV vector expressing Tat.

We demonstrate that the presence of Tat within a vector favored the expansion and mantainence of epitope-specific $CD8^+$ T cells (Figures 3.19 and 3.20). We also observe

that Tat favors the development of effector memory T cells (Figures 3.23 and 3.24), which have been recently demonstrated to be optimal for protective immunity against certain pathogens [152]. Importantly, we show that immunization with a viral vector expressing Tat confers protection to mice challenged with a lethal dose of HSV1 (Figure 3.25).
These observations, in addition to our previous in vivo findings showing that Tat induces epitope-specific T cell responses directed against subdominant and cryptic epitopes of heterologous antigens which are not detected in mice vaccinated in the absence of Tat [142], suggest that this protein may be used as powerful adiuvant in vaccination strategies.

Taken together these results indicate that Tat modulates $CD8^+$ T cellular responses *in vivo* and that these responses are associated with a better protection against a reinfection. Thus, the immunomodulatory proprieties of Tat may be exploited for vaccination strategies and therapeutic interventions aimed at modulating antigen-specific immune responses in different types of human diseases.

5. MATERIALS AND METHODS

5.1 Human cells and culture conditions

Buffy coats from healthy volunteers, that provided consent, were obtained from the University Hospital of Ferrara. Peripheral blood lymphocytes (PBLs) were separated by use of Ficoll–Hypaque (Lonza, Basel, Switzerland) density gradient centrifugation followed by 90 minutes of adhesion on a plastic support at 37°C to remove monocytes.
PBLs ($3x10^6$) were cultured in 2 ml of RPMI (Gibco, Life Technologies, Carlsbad, CA, USA) containing 10% FCS (complete medium) in the absence or presence of the Tat protein in 24-well flat bottomed polystyrene plates coated overnight at 4°C with PBS or anti-CD3 mAb (0.5 μg/ml; R&D Systems, Minneapolis, MN, USA). Soluble anti-CD28 mAb (0.1 μg/ml; R&D Systems), Tat and anti-Tat immune sera were added, when indicated, after cells seeding. In the blocking experiments with anti-integrins antibodies cells were preincubated on rotation in RPMI + 0.05% BSA containing the competitor antibodies at the concentrations of 10 μg/ml for one hour at room temperature. MoAbs raised against the whole α5β1 or αvβ3 integrins were purchased from Merck Millipore (Billerica, MA, USA).
$CD4^+$ and $CD8^+$ T cells were sorted by MACS magnetic negative selection (Miltenyi Biotec, Bergish Gladbach, Germany) according to manufacturer's instructions (purity > 95% assessed by FACS).
PHA-activated blasts were obtained by stimulation of PBLs with 1 μg/ml purified PHA (Sigma-Aldrich, St. Louis, MO, USA) for 3 days at 37°C and expanded in complete medium supplemented with human rIL-2 (10 U/ml; R&D Systems).
The 174/T2 cell line (T2) was obtained by fusion of the peptide transporter mutant 174 LCL with the T cell line CEM. Cells were cultured overnight at 26°C in 1 ml of serum-free AIM-V medium (Gibco). Cells were washed, treated with mitomycin C (Sigma-Aldrich), and pulsed with 10^{-5} M of the different peptides for 3 hours at 37°C in AIM-V 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).

5.2 Tat protein

HIV-1 Tat (sequence: MEPVDPRLEPWKHPGSQPKT ACTNCYCKKCCFHCQVCFIT KALGIS YG RK KRRQRRR PPQGSQTHQVSLSKQ PTSQSRGDPTGPKE) from the human T lymphotropic virus type IIIB isolate (BH10 clone) was expressed in Escherichia coli and purified by heparin-affinity chromatography and HPLC, as described previously [176]. The lyophilized Tat protein was stored at -80°C to prevent oxidation, reconstituted in degassed buffer before use, and handled, as described [176]. Endotoxin concentration was undetectable (detection threshold: 0.05 EU/µg).

5.3 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 Transcription Kit, Applied Biosystems, Life Technologies). cDNA was PCR-amplified with a Chromo4 real-time PCR Detection System using Kapa SYBR Green Fast qPCR Kit (Kapabiosystems, Wilmington, MA, USA) 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 (TEMA ricerca, Bologna, Italy) shown in Table 5.1. The relative levels for each RNA were calculated by the $2^{-\Delta\Delta CT}$ method using human 18S as housekeeping gene. CT values are 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
IFNγ	TGACCAGAGCATCCAAAAGAG	CGACAGTTCAGCCATCACTTG
Bcl-2	GATCCAGGATAACGGAGGC	GGCATGTTGACTTCACTTGTG
Bcl-6	CCCTATCCCTGTGAAATCTGTG	CTGGCTTTTGTGACGGAAATG
Blimp-1	ACTGGGTAGAGATGAACGAGAC	CCACAGAGTCATATCCGCATC
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Table 5.1. Primers used for qPCR.

5.4 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 and anti-IL-2 APC (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.).

5.5 Bio-Plex assay

Cytokines in cells culture supernatants were measured by Bio-Plex cytokine assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's recommendations. Briefly, 50 µl of cytokine standards or samples (supernatants recovered from treated cells) were incubated with 50 µl of anti-cytokine conjugated beads in 96-well filter plates for 30 min at room temperature under shaking. Plates were then washed by vacuum filtration

three times with 100 µl of Bio-Plex wash buffer, 25 µl of diluted detection antibody was added and plates were incubated for 30 min at room temperature under shaking. After 3 filter washes, 50 µl of streptavidin–phycoerythrin was added, and the plates were incubated for 10 min at room temperature under shaking. Finally, plates were washed by vacuum filtration three times, beads were suspended in Bio-Plex assay buffer and samples were analyzed on a Bio-Rad 96-well plate reader using the Bio-Plex Suspension Array System and Bio-Plex Manager software (Bio-Rad).

5.6 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 100 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, 0.2 µg of PHA or 1 ng of PMA and 125 ng 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 2 hours of incubation, 100 µl of lysing buffer (50% dimethylformamide (DMF) + 20% sodium dodecyl sulfate (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.

5.7 CFSE assay

$CD4^+$ and $CD8^+$ T cells proliferation was measured using CFSE, a cell division tracking dye (Molecular Probes, Eugene, OR). The cells were incubated at 37˚C for 10 min and then the reaction was stopped by adding 4–5 volumes of cold RPMI 1640 medium containing 10% FCS. After washing and re-suspending the cells into the warm RPMI 1640 medium with 10% FCS, they were then plated in anti-CD3/CD28 Ab coated plates. After 6 days of incubation, the cells were stained with CD4-PerCP-Cy5.5 and CD8-PE Ab (ImmunoTools, Friesoythe, Germany) and acquired for FACS analysis. Unstimulated CFSE-labeled cells served as a nondividing control.

5.8 Western blotting

Cell extracts, corresponding to 10 μg of total proteins (quantified by BCA protein assay kit, Pierce, Waltham, MA, USA), were loaded onto 12% SDS-polyacrylamide gels and analyzed by Western Blotting procedure.

Blots were probed with antibodies specific for T-bet (Millipore, Billerica, MA, USA) at 1:1000 dilution, Eomes (Millipore) at 1:200 dilution and β-actin (Sigma-Aldrich) at 1:10000. Immunocomplexes were detected by means of the ECL Western Blot detection kit (Amersham Pharmacia, GE Healthcare, Little Chalfont, U.K.).

5.9 Flow cytometry

All stainings were carried out in PBS containing 1% FCS for 30 min at 4°C. For staining of human samples the following antibodies were utilized: CD45RO-FITC, CD25-FITC, CD8-PE, CD27-PE, CD8-PE-Dy647 (ImmunoTools); CD127-PerCP-Cy 5.5 (BD Pharmingen, San Diego, CA, USA). Data were acquired on a BD FACScan and analyzed using BD Cell Quest Pro software.

For staining of mice samples the following antibodies were utilized: CD8 APC-H7 (Immunotools); CD127 PE-Cy7, KLRG1 APC, CD62L PE, CD62L FITC (eBioscience); CD3 PerCP-Cy5.5 (BD Biosciences). For dextramer staining, cells (1×10^6) were incubated for 10 minutes at room temperature with PE labeled HSV1 K^b-restricted peptides SSIEFARL (SSI) dextramer (Immudex) and washed prior staining with surface antigen antibodies for 30 minutes at 4°C.

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

5.10 Generation of CTL cultures

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 absence or presence 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 absence or presence of Tat (0.1 μg/ml), of lymphocytes from HLA-A2-positive healthy volunteers with peptide-pulsed T2 cells [179].
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 absence or presence of Tat. Alternatively, cells were stimulated directly with peptides (10^{-5} M) in the absence or presence of Tat. Cultures were re-stimulated after 7 and 14 days, and the medium was supplemented from day 8 with 10 U/ml rIL-2. On days 21, CTL cultures were tested for activity using cytotoxicity or Elispot assays.
Peptides were synthetized by solid phase method and purified by HPLC to >98% purity (UF Peptides, University of Ferrara).

5.11 Cytotoxicity assay

The cytotoxic activity was assayed in standard 5-hours ^{51}Cr-release assays [148]. Target cells (PHA blasts) were labeled with $Na_2{}^{51}CrO_4$ (3.5 MBq/10^6 cells; PerkinElmer, Waltham, MA, USA) 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 3 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.

5.12 Elispot assay

CTLs (4 x 10^4) were seeded in triplicate on microplate 96-well unifilter (Whatman, GE Healthcare, Little Chalfont, U.K.) pre-coated with an anti-IFNγ and granzyme B mAb (Pierce). CTLs were stimulated with CLG and YLQ EBV-derived CD8 peptides. CTLs incubated with medium alone were used as negative control, whereas CTLs stimulated

with PHA represented the positive control. Plates were incubated for 24 hours and washed, and then a biotinylated anti-IFNγ and granzyme B mAb (1 μg/ml) 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 and granzyme B producing cells were detected using 3-amino-9-ethylcarbazole cromogen kit (Sigma-Aldrich) and counted by ELISPOT reader (AELVIS, Hannover, Germany). The number of specific IFNγ and granzyme B secreting T cells, expressed as spot-forming units per 10^6 cells, was calculated by subtracting the negative control values.

5.13 Viruses

Mice were immunized using HSV1 attenuated replication-competent vectors expressing Tat (LvTat) or LacZ as control gene (LvLacZ).
The recombinant attenuated replication-competent HSV1LacZ (LvLacZ), where the UL41 gene is deleted by the insertion of the lacZ gene under the control of the HSV ICP0pr, and the recombinant live attenuated LTat vector (LvTat), constructed by means of homologous recombination between UL41 sequences of the pB41-tat plasmid and the HSV1-LacZ vector, were generated and provided by the laboratory of Dr. Peggy Marconi, Department of Life Science and Biotechnology, University of Ferrara.
The wild-type Herpes Simplex virus type 1 (HSV1), LV strain [180], was used for challenge experiments.

5.14 Mice immunization, challenge and samples collection

C57BL/6 female mice (Charles River Laboratories) were pretreated, 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 LvLacZ and LvTat. 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.

After infection, mice were observed daily to monitor the appearance of local and/or systemic clinical signs of infection including death. Mice were sacrificed at different time points to analyse anti-HSV1 immune responses in spleen and lymph nodes by dextramer staining. Alternatively, blood samples were collected in heparinized tubes from retro-orbital plexus. Red blood cells were lysed twice using 3 ml of red blood cell lysing buffer (Sigma-Aldrich) at different time points to measure HSV-specific cellular responses by 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.

At day 128, mice (n=5 per experimental group) were challenged intravaginally with a lethal dose of wild-type HSV1 (strain LV; 1.5 x 10^8 pfu per mice). Viral stocks were titered in vitro, according to standard procedures [181], and stored at -80 °C. Titers of viral stocks ranged between 7 x 10^9 to 2 x 10^{11} plaque forming units (pfu)/ml. After challenge, vaginal washes were collected in PBS and store at -20° C to perform virus titrations.

Mice were observed daily to monitor their health and the appearance of clinical signs.

Each group was composed of three/five animals. 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.

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Full Paper

Modified *N,O*-Nucleosides: Design, Synthesis, and Anti-tumour Activity

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A preliminary library of modified *N,O*-nucleosides was prepared and tested on a selected number of human cancer lines that include SKOV3, SW480, and K562. Thymine, *N*-benzyl substituents, and aromatic rings contribute to an increase of the biological activity, up to 10–25 μM, that appeared also reliant on the calculated lipophilicity of the nucleosides, expressed as cLogP, where P represents the partition coefficient of a solute between n-octanol and water.

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PROOF ONLY

Introduction

In recent years, a large number of nucleoside analogues with antiviral and/or antitumour properties have been designed and synthesized.[1,2] Nucleosides, in fact, comprise the largest class of clinically useful antiviral agents and they continue to be excellent candidates as anticancer drugs.[3]

In the search for effective, selective, and non-toxic agents, a variety of modifications of the naturally occurring structure have been devised on both the sugar and the nucleobase. Of particular interest are nucleoside analogues in which the furanose ring has been replaced by *N,O*-heterocyclic systems, as in isoxazolidine **1** and isoxazoline **2** derivatives, in which B represents pyrimidine or purine nucleobases (Fig. 1).[4–6]

Most of the *N,O*-containing nucleoside analogues described in the literature possess remarkable antiviral activity but a modest potential activity towards human cancer cell lines,[7,8] contrary to what is observed with substituted nucleosides and thionucleosides, in particular 4′-thio-β-D-arabinofuranosylcytosine (4′-Thio-Ara-C).[3,9–11]

In a previous study, we reported the efficient synthesis of several *N,O*-nucleosides by direct 1,3-dipolar cyclization methodology.[12,13] The cyclization was carried out between selected nitrones **3** and a set of unprotected vinyl nucleobases **4** under microwave (MW) irradiation, in the absence of solvent or catalyst (Scheme 1). The cycloadducts are formed in good yield, with complete regioselectivity and notable *cis* diastereoselectivity, up to 98 %, resulting from a dominant (*Z*)-*exo* nitrone–alkene approach.[13]

The *N,O*-nucleosides of the previous study were evaluated by in vitro assays for their antiproliferative activity on LCL_s, JiJoye, and Jurkat cell lines, and were found particularly promising.[13]

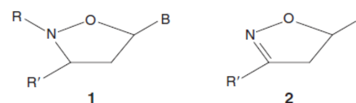
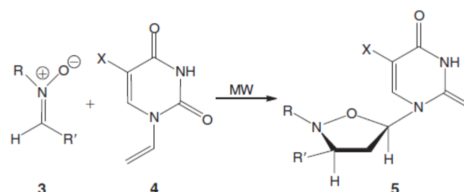


Fig. 1. General structure of *N,O*-nucleosides.



Scheme 1. Synthesis of *N,O*-nucleosides via 1,3-dipolar cycloaddition of nitrones **3** with vinyl nucleobases **4** by microwave (MW) irradiation.

Results and Discussion

In analogy with specific absorption rate (SAR) tests designed to prove the minimal structural requirements for antiproliferative activity in the NCI 60 panel of human cancers,[14] we investigated variations in the four canonical quadrants of our compounds **5** (Fig. 2), and some of these were submitted to growth inhibition assays on SKOV3 (cisplatin-resistant) and SW480 cell lines established for ovarian and colon cancers respectively (Table 1). It was found that compound **5d** exhibited greater potency (25–50 μM) than either compounds **5a** or **5e** on these

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Synthesis and biological evaluation of diastereoisomerically pure *N,O*-nucleosides

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ABSTRACT

Several *N,O*-nucleosides have been synthesized in good yields by direct 1,3-dipolar cyclization methodology, in the absence of solvent. A remarkable *cis* stereoselectivity (de 98%) was observed by tuning the substituents on the nitrone moiety. A good number of these *N,O*-nucleosides have been evaluated for cytotoxic activity against selected cellular lines. Some of the tested compounds have proven to be potential antiproliferative drugs.

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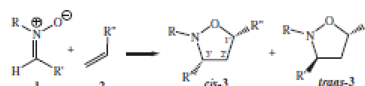
1. Introduction

Many strategies for the preparation of modified nucleosides have been reported in last decades in response to the pressing need of new treatments against virus infections. In a recent approach to these derivatives the ribose unit has been replaced by either carbo- or heterocyclic rings, being *N,O*-containing moieties, that is, isoxazolidine and isoxazoline nucleosides, particularly promising.[1] This was the case of the substituted isoxazolidine pyrinodemin-A which displayed cytotoxicity, of isoxazolidinium salts used in tumor therapy[2] and of isoxazolidines bearing an allylic oxygen active against a number of human cancer cell lines.[3] Furthermore, phosphonated carbocyclic 2′-oxa-3′-aza nucleosides showed powerful inhibiting activity toward the reverse transcriptase of the human retrovirus T-cell leukemia/lymphotropic virus type 1.[4,5]

The synthesis of isoxazolidinyl nucleosides is usually carried out using a classical 1,3-dipolar cycloaddition that represents the most successful protocol for the construction of biologically active derivatives as the already mentioned modified isoxazolidines, amino acids, β-lactams, amino carbohydrates, and alkaloids.[6] In 1,3-dipolar cycloadditions to isoxazolidines, the dipolarophiles are usually alkenes, whereas dipoles are represented by suitable nitrones. The regioselectivity and stereoselectivity of this reaction has been explained by frontier molecular orbital theory. In particular, the cycloaddition of nitrones with mono-substituted electron-rich alkenes involves a dominant $HOMO_{alkene}$–$LUMO_{nitrone}$ interaction, as a consequence of an inverse electron demand (IED).[7,8] Several investigations, including theoretical studies, confirmed that this reaction is characterized by almost exclusive *ortho* regioselectivity i.e. formation of 1′-substituted isoxazolidines **3**-type only, while the *cis–trans* selectivity depends on different factors as the possible interconversion between *E/Z* isomers of **1**[9,10] and the bulk of the substituents present on both nitrone and alkene (Scheme 1).

Our contribution to this field[11,12] has been devoted toward the synthesis of modified nucleosides based on the *N,O*-heterocyclic ring **6**, obtained through microwave irradiated[13–15] direct cyclization of suitable nitrones **4** and unprotected vinylated nucleobases **5** (Scheme 2). The 1,3-dipolar cycloadditions were conveniently carried out in environmentally acceptable conditions as the absence of solvent and the quantitative recover/recycle of unreacted **4**, present in stoichiometric excess.

In this paper we describe the synthesis of a new class of *N,O*-nucleosides obtained in high diastereoisomeric *cis–trans* excess,



Scheme 1. Synthesis of *N,O*-nucleosides via 1,3-dipolar cycloaddition of nitrones and substituted alkenes.

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Synthesis and proteasome inhibition of *N*-allyl vinyl ester-based peptides

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Inhibition of the proteasome, the multicatalytic protease complex responsible for the turnover of many cellular proteins, represents an attractive target in the development of new drug therapies, proteasome inhibitors being potentially useful tools for the treatment of pathologies such as cancer, as well as inflammatory, immune and neurodegenerative diseases. Based on our previous development of a new class of inhibitors bearing a *C*-terminal VE cluster able to interact with catalytic threonine, we report herein the synthesis and activity of new VE-based peptide analogs bearing an additional allyl pharmacophore unit at the *C*- or *N*-terminal position of the pseudotripeptide sequence. In the new series, the structural modification carried out to the prototype determine a decrease of proteasome inhibition. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: N-allyl derivatives; inhibitors; proteasome; pseudopeptides

Introduction

The proteasome, a multicatalytic protease complex [1], is an essential component of the ubiquitin–proteasome system (UPS) which is involved in prokaryotic and eukaryotic intracellular protein degradation pathways. The UPS is implicated in many biological processes, such as stress response, cell cycle control and differentiation, apoptosis and the generation of peptide antigens [2–5]. These cellular functions are linked to an ubiquitin- and ATP-dependent protein degradation pathway involving the 26S proteasome, which contains a central barrel-like core and a 20S proteolytic chamber composed of four stacked rings. These inner rings are made up of seven different β subunits, and each β-ring contains three different active sites. In particular, the $\beta 1$ subunit contains a post-acidic (PGPH) active site, the $\beta 2$ subunit expresses T-L activity, and a ChT-L proteolytic function is carried out by the $\beta 5$ subunit [6–13].

Considering the implication of the multicatalytic complex in various cellular processes, modulation of proteasomal activities is extremely interesting from a therapeutic perspective. Indeed, a variety of natural and synthetic products have been tested as inhibitors of the different proteasomal enzymatic subunits [14–26]. In particular, the boron derivative PS341 (Bortezomib) has been used in the treatment of multiple myeloma [27,28], and other such molecules have been evaluated for their effect on many disease states, including inflammation and cancer, as well as on modulation of immune responses.

Our previous studies report the design and development of a new class of peptide-based proteasome inhibitors containing a VE moiety as a potential substrate for Michael addition with the catalytic threonine through a mechanism similar to that of the well-known vinyl sulfone inhibitors. As recently reported [29–31], also the natural pseudopeptidic product Syringolin A and, even if in minor way, the analogous Syringolin B irreversibly inhibit with the same catalytic mechanism the $\beta 2$ and $\beta 5$ subunits of the proteasome. As some derivatives with the basic general structure HMB-Xaa-Xbb-Leu-VE have shown favorable pharmacokinetic properties, potent inhibition and general selectivity for the $\beta 2$ proteasome subunit [32,33], a further series of VE derivatives were synthesized substantially modifying the molecular level with respect to the basic structure. These cyclic analogs or *N*-terminal-prolonged pseudotripeptides showed differing biological profiles [34,35]. DFT calculations and docking simulations carried out on HMB-Val-Ser-Leu-VE proved that the *C*-terminal pharmacophore is surrounded by several residues; in particular, the OH group of the active threonine Thr1 interacts with the carbonyl of the VE group with a O···O distance of 2.74 Å [36].

Herein we describe the synthesis and proteasome inhibition of novel *N*-allyl, VE-based peptide derivatives (Figure 1), further to an interesting study by Prof. Norm Radin which showed that anticancer and other drugs possessing potent biological activity contain polar allyl groups [37]. Our series possessed an *N*-allylic function at the *C*- or *N*-terminal position of the pseudotripeptide prototype H-Leu-Leu-Leu-VE. The aim of our work was to evaluate

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Abbreviations used: *ChT-L, chymotrypsin-like; HATU, O-(7-azabenzotriazolyl)-tetramethyl uronium hexafluorophosphate; PGPH, peptidyl-glutamyl peptide hydrolyzing; T-L, trypsin-like; WSC, 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide; VE, vinyl ester; Z, benzyloxy carbonyl-N-hydroxysuccinimide..*

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IMMUNOLOGY ORIGINAL ARTICLE

Proteasome inhibitors induce the presentation of an Epstein–Barr virus nuclear antigen 1-derived cytotoxic T lymphocyte epitope in Burkitt's lymphoma cells

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Summary

The Epstein–Barr virus (EBV) nuclear antigen 1 (EBNA1) is generally expressed in all EBV-associated tumours and is therefore an interesting target for immunotherapy. However, evidence for the recognition and elimination of EBV-transformed and Burkitt's lymphoma (BL) cells by cytotoxic T lymphocytes (CTLs) specific for endogenously presented EBNA1-derived epitopes remains elusive. We confirm here that CTLs specific for the HLA-B35/B53-presented EBNA1-derived HPVGEADYFEY (HPV) epitope are detectable in the majority of HLA-B35 individuals, and recognize EBV-transformed B lymphocytes, thereby demonstrating that the GAr domain does not fully inhibit the class I presentation of the HPV epitope. In contrast, BL cells are not recognized by HPV-specific CTLs, suggesting that other mechanisms contribute to providing a full protection from EBNA1-specific CTL-mediated lysis. One of the major differences between BL cells and lymphoplastoid cell lines (LCLs) is the proteasome; indeed, proteasomes from BL cells demonstrate far lower chymotryptic and tryptic-like activities compared with proteasomes from LCLs. Hence, inefficient proteasomal processing is likely to be the main reason for the poor presentation of this epitope in BL cells. Interestingly, we show that treatments with proteasome inhibitors partially restore the capacity of BL cells to present the HPV epitope. This indicates that proteasomes from BL cells, although less efficient in degrading reference substrates than proteasomes from LCLs, are able to destroy the HPV epitope, which can, however, be generated and presented after partial inhibition of the proteasome. These findings suggest the use of proteasome inhibitors, alone or in combination with other drugs, as a strategy for the treatment of EBNA1-carrying tumours.

Keywords: Burkitt's lymphoma; cytotoxic T lymphocytes; Epstein–Barr virus nuclear antigen 1; Epstein–Barr virus; proteasome inhibitors

Introduction

The Epstein–Barr virus (EBV) is a widespread virus that establishes life-long persistent infections in B lymphocytes in the vast majority of human adults. These EBV-infected B cells can proliferate *in vitro*, giving rise to lymphoblastoid cell lines (LCLs) that express at least nine latency-associated viral antigens: the nuclear antigens EBNA1 to EBNA6 and the membrane proteins LMP1, LMP2A and LMP2B.[1]

The proliferation of EBV-infected cells is monitored *in vivo* by T lymphocytes that specifically recognize viral antigens as peptides derived from the processing of endogenously expressed viral proteins presented on the surface of the target cell as a complex with MHC class I molecules.[2] In particular, EBNA3, EBNA4 and EBNA6 (also known as EBNA3A, 3B and 3C) contain immunodominant epitopes for cytotoxic T lymphocyte (CTL) responses over a wide range of HLA backgrounds. In contrast, EBNA2, EBNA5, LMP1 and LMP2 are subdominant targets that are presented in the context of a limited number of HLA restrictions.[3–7] Conflicting with previous observations,[4,5,8] CTL responses against EBNA1 have also

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Unexpected One-Pot Synthesis of Highly Conjugated Pentacyclic Diquinoid Compounds

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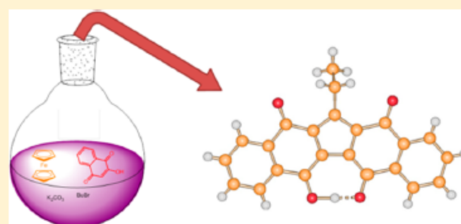
Supporting Information

ABSTRACT: A new class of pentacyclic diquinoid compounds has been synthesized with a facile one-pot reaction of two molecules of 2-hydroxynaphthoquinone and 1-bromoalkanes in the presence of ferrocene. These molecules were isolated as enol tautomers that exhibit intramolecular hydrogen bond and extended electronic conjugation as proved by the intense absorption spectrum with a broad band between 400 and 600 nm. The spectroscopic and electrochemical characterization of this new class of compounds has been performed. One of the synthesized diquinoid derivatives showed a significant cytotoxicity with IC_{50} values of 25−50 μM against Cisplatin-Resistant SKOV3 and colon carcinoma SW480 cell lines. The results of our study provide a valuable tool to a one-pot synthesis of highly conjugated polyquinones, analogous to important biological systems, with significant antitumoral activity.



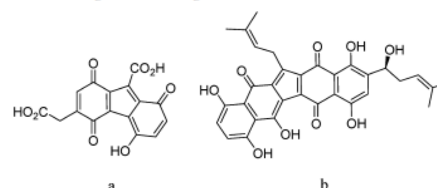
INTRODUCTION

Naphthoquinone-based molecules are ubiquitous in plants, having key roles in several transformations.[1] Vitamin K derivatives, for example, are naphthoquinones involved in biological processes such as the catalysis of the cyclic photophosphorylation pathways[2] and the decarboxylation of glutamic acid.[3] Moreover, in the photosynthetic system, quinones act as the final electron acceptors in reaction centers after sunlight harvesting process. The understanding of the photosynthetic mechanism has stimulated the synthesis of artificial mimicking models, based on electron transfer processes. Accordingly, the donor and acceptor moieties used in such models are mainly inspired by the pigments found in photosynthetic reaction centers.[4] Therefore, quinones have often been used as acceptor moieties together with easily oxidizable compounds, such as porphyrin or ferrocene derivatives, in donor−acceptor dyads mimicking natural photosynthesis.[5]

In this paper we described a new and unexpected synthesis of highly conjugated pentacyclic diquinoid compounds. Although our initial aim was not the diquinones formation, we studied these products because of their interesting absorption properties and in view of their relevance in many fields, and in particular in medical applications.[6]

For example, a quinoid tricyclic derivative with antibiotic properties, the hipposudoric acid (Chart 1a), was isolated from the skin secretion of hippopotamus after a sequence of extraction processes.[7] The chemical synthesis of hypposudoric acid is possible, but only with a difficult, time-consuming multistep route,[7b] as usually occurs for the synthesis of natural poliquinonoid compounds.[8] Due to its absorption spectrum in the visible region, hipposudoric acid appears to be produced to

Chart 1. Diquinoid Compounds in Nature[a]



[a](a) Structure of hipposudoric acid. (b) Structure of one of the shikonin derivatives after microbial transformation.

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Platinum and ruthenium complexes of new long-tail derivatives of PTA (1,3,5-triaza-7-phosphaadamantane): Synthesis, characterization and antiproliferative activity on human tumoral cell lines

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ABSTRACT

The alkylation of PTA, (1,3,5-triaza-7-phosphaadamantane), with long chain alkyl iodides produced the novel ionic derivatives $(PTAC_{12}H_{25})I$, $(PTAC_{16}H_{33})I$ and $(PTAC_{18}H_{37})I$. Anion exchange gave the PF_6^- analogs $(PTAC_{12}H_{25})PF_6$, $(PTAC_{16}H_{33})PF_6$ and $(PTAC_{18}H_{37})PF_6$. The new phosphines were characterized by ESI-MS, elemental analysis, ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy and their coordination ability was investigated towards Pt(II) and Ru(II) cores. Complexes cis-$[PtCl_2(PTAR)](PF_6)_2$ (**1–3**) and $[CpRuCl(PPh_3)(PTAR)]I$ (**5–7**) have been isolated and characterized. Antiproliferative activity, given as estimated IC_{50}, was tested for each complex against appropriate human tumoral lines (T2, SKOV3 and SW480) in comparison with cisplatin, with the parent complexes $[CpRuCl(PPh_3)(PTA)]$ and $[CpRuCl(PPh_3)(PTAMe)]I$ and with the free ligands.

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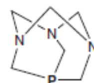
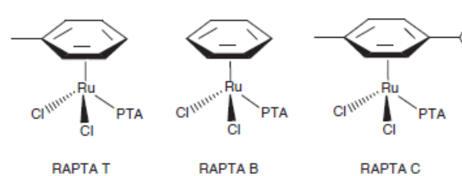
1. Introduction

The phosphine PTA (1,3,5-triaza-7-phosphaadamantane), first prepared by Daigle in 1974 [1], has been recently rediscovered and much exploited in coordination chemistry because of its valuable properties as a ligand [2]: (i) its coordination to a wide range of metals occurs in most cases via phosphorus, frequently showing regioselectivity of this donor-atom, although some examples of *N*-coordination have been recently reported [3,4]; (ii) it is remarkably soluble in water and stable to oxidation in comparison with other non-aromatic phosphines; (iii) it has a low steric hindrance and a narrow cone angle; (iv) it is non-toxic and biocompatible.



PTA = 1,3,5-triaza-7-phosphaadamantane

All of these characteristics are at the root of the extensive research relating to PTA coordination compounds and their application in catalysis [5,6] and in medicine (Pt, Ru, Au) [7–10]. In particular, Ru–PTA complexes called RAPTA-type, studied by Dyson [11–13], showed a pH dependent action which makes them selective towards cancer cell.



Another important feature of PTA is that all the positions of the cage structure are reactive in specific conditions, allowing researchers to design and prepare families of PTA derivatives presenting some of the parent compound properties, amplified and improved. The three carbon atoms bonded to phosphorus are known as *upper rim* and their functionalization is particularly relevant for catalysis allowing e.g., the introduction of chiral centers or of other coordinating groups in proximity to phosphorus, the

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RESEARCH ARTICLE

C-terminal *trans,trans*-muconic acid ethyl ester partial retro-inverso pseudopeptides as proteasome inhibitors

Christian Franceschini[1], Claudio Trapella[1], Rosaria Calia[1], Alessandra Scotti[1], Fabio Sforza[2], Riccardo Gavioli[2], and Mauro Marastoni[1]

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Abstract

The development of specific inhibitors of the proteasome represents an important opportunity for new drug therapies. The central role of the multicatalytic complex in the intracellular proteolysis mediated by ubiquitin-proteasome pathway goes to discovery many molecules able to selectively inhibits enzymatic active subsites. Now, we report synthesis and activity of a new partial retro-inverso oligopseudopeptide derivatives bearing a *trans,trans*-muconic acid ethyl ester pharmacophoric unit at the C-terminal. Some analogues selectively inhibited in μM range the caspase-like (C-L) activity in the β1 subunit of the proteasome.

Keywords: Retro-inverso peptides, muconic acid, caspase-like inhibition, proteasome

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Introduction

The degradation of intracellular proteins plays a central role in regulating cellular function and maintaining homeostasis. Most of the cellular proteins are degraded through the ubiquitin proteasome pathway (UPP) including those involved in main processes such as apoptosis, cell cycle, transcription, antigen presentation by Major histocompatibility complex (MHC) and others[1]. Defects within this system are associated with several diseases, including cancer. Proteins are first tagged by a polyubiquitin chain in a complex enzymatic process and then recognized and degraded by the proteasome 26S[2].

This 26S multicatalytic complex consists of a 20S proteolytic core particle, which has a cylindrical shape, with the seven different α and seven different β subunits forming four stacked rings, and two 19S regulatory caps which recognize polyubiquitinated protein substrates and promote their entry into the central catalytic chamber[3–5]. Three major proteolytic activities of proteasome can be distinguished as trypsin-like (T-L) located in β2, chymotrypsin-like (ChT-L) in β5 and peptidyl-glutamyl peptide hydrolase (PGPH) or caspase-like (C-L) activities in β1 subunits[6]. The catalytic residue of the 20S proteasome is a threonine, responsible for the cleavage of substrates through nucleophilic attack[7]. Inhibition of enzymatic activity with specific proteasome inhibitors may provide an anti-tumoral and other therapeutic effects[8,9].

Several classes of synthetic and natural compounds which inhibit the proteolytic activities of the multicatalytic complex have been developed, and have contributed significantly to the identification of the essential functions of the 26S proteasome in various processes and pathways in eukaryotic cells[10].

Most proteasome inhibitors are short synthetic peptides bearing a C-terminal pharmacophoric groups, such as aldehyde (e.g. MG132), boronic acid (Bortezomib) applied in multiple myeloma therapy, epoxyketone (Carfilzomib) and vinyl sulphone, or natural molecules (epoxomicin, lactacystin, salinosporamide, syringolins A and B) that form covalent adducts with catalytic threonine[11–19]. Non-covalent inhibitors, such as TMC-95A (a naturally-constrained cyclic tripeptide) have been

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Synthesis and *in vitro* cytotoxicity of deoxyadenosine–bile acid conjugates linked with 1,2,3-triazole

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We report herein the synthesis and biological evaluation of novel deoxynucleoside–bile acid conjugates linked through a 1,2,3-triazole ring. The conjugates were synthesized *via* Cu(I) mediated 1,3-dipolar cycloaddition reaction ('click' chemistry) of 3-azidobile acid derivatives and terminal alkyne moieties linked to the C-8 position of deoxyadenosine. All novel molecules were evaluated *in vitro* for their anti-proliferative activity against four human cell lines (*i.e.*, leukemic T Jurkat and K562; colon carcinoma HCT116; and ovarian cancer A2780) and their cytotoxicity toward human fibroblast cells. Several conjugates exhibited strong anti-proliferative activity against human leukemia T cells. The best cytotoxicity was observed for **HdA-CDC** on both leukemia cell lines with IC_{50} up to 8.51 μM. The apoptotic activity of several conjugates was also established.

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Introduction

Among the variety of molecular scaffolds employed as building blocks for the design and synthesis of novel conjugates, bile acids and their derivatives have received a great deal of attention in many fields.[1–6] Bile acids are a class of biogenetic compounds involved in different biological processes.[7] The interest in this class of natural compounds is explained by the peculiar combination of features such as rigidity, chirality, amphiphilicity and modularity, together with their availability and low cost.

Moreover, lipophilicity, another well-known feature of bile acid structure, is believed to be correlated with induction of cytotoxicity and apoptosis in several human cancer cells.[8–11] Synthetic derivatives of hydrophobic bile acids, especially chenodeoxycholic acid and ursodeoxycholic acid conjugates, significantly inhibit cell growth and induce apoptosis in various human cancer cells, including breast carcinoma,[12] leukemic T cells,[13] prostate cancer cells,[14] stomach cancer cells,[15] colon cancer,[16] cervical carcinoma,[17] glioblastoma multiforme,[18] and hepatocellular carcinoma.[19]

The literature also describes a vast amount of bile acid conjugates with improved pharmacological profiles in terms of bioavailability and biostability, which demonstrate the further potential of modified bile acids to act as hybrid molecules and prodrug moieties in drug discovery.[3,20]

We recently synthesized a new prodrug obtained by the conjugation of zidovudine (AZT) with ursodeoxycholic acid, aimed at obviating the poor ability of AZT to permeate in the CNS or intracellular compartments.[21] At the same time, Bortolini *et al.* reported the efficient synthesis of a new class of bile acid-based hydroxyl-bisphosphonates with the aim of improving the bioavailability of bone disorder drugs.[22]

The expanding number of applications employing bile acid templates has stimulated the development of new conjugates with enhanced properties, obtained using specific and in some cases orthogonal reactions. The copper(I)-catalyzed Huisgen–Sharpless–Meldal 1,3-dipolar cycloaddition between alkynes and azides (CuAAC or 'click' chemistry) to form 1,4-disubstituted 1,2,3-triazole linkers is a well-established reaction for the preparation of bioconjugates.[23–25] 1,2,3-Triazole moieties are attractive linkers, because they are stable under typical physiological conditions and form hydrogen bonds, which can be suitable for solubility enhancement and for binding of biomolecular targets.[26–28]

Few examples of bile acid–nucleoside conjugates have been reported in the literature,[21,29–32] and to the best of our knowledge there are no synthetic approaches with 1,2,3-triazoles as connecting units, nor about the cytotoxic evaluation of bile acid–nucleoside conjugates as new potential anticancer drugs.

Purine nucleoside analogues are a pharmacologically interesting class of compounds with a long history for treatment of various viral and tumor diseases.[33–35] Particularly, several

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RESEARCH ARTICLE

Synthesis and biological properties of C-terminal vinyl ketone pseudotripeptides

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[2]*Department of Biochemistry and Molecular Biology, University of Ferrara, Ferrara, Italy*

Abstract
The ubiquitin-proteasome pathway responsible for the turnover of many cellular proteins represents an attractive target in the development of new drug therapies: In particular, modulation of the proteasome activity by specific inhibitors may represent a useful tool for the treatment of tumours. Here, we report synthesis and activity of a new series of oligopseudopeptide analogues bearing a vinyl ketone pharmacophoric unit at the C-terminal position. Some derivatives showed inhibition in the μM range of the trypsin-like (T-L) active site of the proteasome.

Keywords: Vinyl ketone derivatives, inhibitors, proteasome, pseudopeptides

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Introduction

The proteasome 26S, a multi-catalytic protease[1], is an essential component of the ubiquitin-proteasome pathway (UPP) that degrades many proteins in eukaryotic cells. Fundamental cellular functions are linked to an ubiquitin- and ATP-dependent degradation of proteins involved in different pathways such as stress response, cell cycle control and differentiation, apoptosis and the regulation of transcription factors generation[2]. Proteins destined to degradation are tagged by a covalently linked polyubiquitin chain in a process involving three enzymes in a successive action E1 (Ubiquitin-activating enzyme), E2 (Ubiquitin-conjugating enzyme) and E3 (Ubiquitin-ligase)[3,4]. Poliubiquitin chain linked to proteins represents the signal for degradation by multi-catalytic complex that contains a central barrel-like core and a 20S proteolytic chamber composed of four stacked rings capped by two 19S structures[5,6]. The two outer rings of the 20S are composed of seven α-subunits, whereas the two inner rings are made up by seven different β-subunits, and each β-ring contains three different active sites. In particular, the β1 subunit contains a post-acidic (PGPH) active site, the β2 subunit expresses trypsin-like (T-L) activity, and a chymotrypsin-like (ChT-L) proteolytic function is carried out by the β5 subunit. All the proteolytic cavities utilize the γ-hydroxyl function as a nucleophile and the α-amine as a proton donor-acceptor of the N-terminal threonine residue in the catalytic cycle[7,8].

A proteasome isoform can be formed in response to cytokine signalling that induces the expression of different β-subunits and regulatory cap to constitute immunoproteasome capable to generate epitopes for presentation by MHC class I molecules[9].

Considering the crucial implication of the proteasome in various cellular processes, modulation of enzymatic activities is extremely interesting from a therapeutic perspective[10–12]. Natural and synthetic products have been tested as inhibitors of the different multi-catalytic complex subunits[13–21]. *In vitro* and *in vivo* studies demonstrated that proteasome inhibitors showed anti-proliferative and pro-apoptotic activities against solid and haematologic tumours. In particular, the boron derivative PS341 (Bortezomib) was used in the treatment of multiple myeloma[22,23]. Other molecules were evaluated for their effect on many disease states, including inflammation and cancer, as well as on modulation of immune responses[24].

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